Safety of antibiotic and probiotic feed additives for *Gallus gallus domesticus*

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Declaration

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Summary

The inclusion of antibiotics in broiler feed is of great concern, as many resistant pathogenic bacteria may spread to other farming animals and humans. Alternative methods are thus required to improve broiler health and performance without detrimental consequences. The objective of this study was to evaluate the effect of a multi-species probiotic on the health and growth performance of *Gallus gallus domesticus.* Bacteria from different segments of the gastrointestinal tract (GIT) of healthy free-range broilers were isolated, identified to species level by amplifying the genes encoding 16S rDNA, *recA* and *gyrB,* and comparing the sequences with those listed in GenBank. A select few isolates were screened for probiotic characteristics. Among the 609 isolates sampled from the GIT, *Lactobacillus johnsonii* DPN184, *Lactobacillus salivarius* DPN164, *Lactobacillus crispatus* DPN167, *Lactobacillus gallinarum* DPN164, *Enterococcus faecalis* DPN94 and *Bacillus amyloliquefaciens* DPN123 tolerated acidic conditions (pH 2 to 3), were resistant to bile salts (0.2 to 2.0 % w/v) and produced exopolysaccharides. *Bacillus amyloliquefaciens* DPN123, isolated from the duodenum, produced extracellular amylase, phytase and antimicrobial lipopeptides (surfactin and iturinA1). *Enterococcus faecalis* DPN94, isolated from the jejunum and ileum, produced phytase and bile salt hydrolase. The genome of *E. faecalis* DPN94 contained several genes that may encode virulence, but not the production of cytolysin. Differences in opinion exist regarding the role virulence genes may play in the colonisation of epithelial cells. *Lactobacillus johnsonii* DPN184, isolated from the cecum, produced hydrogen peroxide. *Lactobacillus salivarius* DPN181, isolated from the colon, produced hydrogen peroxide and high levels of lactic acid. *Lactobacillus crispatus* DPN167 was isolated from the crop, proventriculus and ventriculus, and produced hydrogen peroxide and bile salt hydrolase. *Lactobacillus gallinarum* DPN164 was isolated from the jejunum and ileum.

A multi-species probiotic, consisting of *L. johnsonii* DPN184, *L. salivarius* DPN164, *L. crispatus* DPN167, *L. gallinarum* DPN164, *E. faecalis* DPN94 and *B. amyloliquefaciens* DPN123, was added to the feed of broilers and its effect on growth performance, size of the lymphoid organs, gizzard mass, mineral content of the tibia bones and red blood cell parameters determined. A separate group of broilers was administered a combination of sulphadiazine, colistin and trimethoprim through their feed and subjected to the same tests. A third group of birds received standard feed without additives and served as control. On day 19, the villi of broilers on antibiotics had larger surface areas, and higher lymphocyte and basophil counts compared to broilers from probiotic and control treatment groups. On day 29, the cecal microbiome of broilers from the control and probiotic treatment groups were similar but differed significantly from broilers that received antibiotics. Probiotic administration did not alter homeostasis of the normal GIT microbiome, suggesting that probiotics rather modulate the microbiome by preventing dysbiosis induced by pathogenic microorganisms. Birds on antibiotics had lower levels of *Enterobacteriaceae* and higher levels of unidentified Clostridiales, *Brucellaceae*, *Synergistaceae*, *Erysipelotrichaceae* and *Coriobacteriaceae* in their ceca. The multi-species probiotic repressed the growth of *Listeria monocytogenes* EDGE *in vivo*, most probably by lowering the cell's metabolic activity, by competing with *Listeria* for receptor sites on the gut wall or mucosa, or by production of antimicrobial compounds such as short-chain fatty acids, hydrogen peroxide and lipopeptides.

Salmonella Enteritidis 147 invaded Caco-2 cells and altered claudin-3 tight junctions between the cells, leading to monolayer disruption. *Salmonella* decreased tight junctions by invading eukaryotic cells which led to cell death. Interaction of *S.* Enteritidis with broiler epithelial cells led to the up-regulation of lysozyme C and G, cathelicidin 2 and 3, myeloid protein 1, trypsin inhibitor CITI-1, gallinacin-2 and ubiquitin-fold modifier 1, and the down-regulation of glutaredoxin-1, gallicin-7 and vigilin. Up-regulated proteins acted as chemotactic compounds, inhibitors of microbial enzymes, and played critical roles during stress. Down-regulated proteins activated natural killer cells, and regulated apoptosis and antimicrobial defence systems. The multi-species probiotic was not cytotoxic, but the metabolic end products were. The probiotic bacteria adhered to Caco-2 cells but did not invade them, and decreased claudin-3 tight junctions but did not disrupt the monolayer. Probiotics decreased claudin-3 tight junctions by producing short-chain fatty acids, hydrogen peroxide and antimicrobial lipopeptides. In broilers administered with the multi-species probiotic, transgelin 2/3, elongation factor-1 beta and anterior gradient 2 were up-regulated, but carnitine O-acetyltransferase, adenylate kinase 2, superoxide dismutase Cu-Zn and protein SET down-regulated. Upregulated proteins were involved in the proliferation, migration and healing of cells and regulation of the cytoskeleton, whereas down-regulated proteins were important in fatty acid transport, energy homeostasis, nucleotide metabolisms, free-radical elimination and signal transduction. Concluded from these studies, the multi-species probiotic was non-toxic and interacted with epithelial cells in a symbiotic manner.

Feeding of *Salmonella enterica* serovar Enteritidis A9 to broilers had no effect on body mass, and no significant differences were observed with respect to immune organ weights, haematological parameters and serum interferon gamma levels. Colonisation of *Salmonella* in the cecum of broilers that received oxytetracycline was, however, lower on days 11 (one day post infection, dpi 1) and 14 (dpi 4), but then increased to levels corresponding to those of birds in the control and probiotic groups. At first, the antibiotics decreased the cell numbers of *Salmonella* in the cecum, but higher levels were recorded with continuous administration. The increase in cell numbers may be due to antibiotics disturbing the microbiome in the GIT, indirectly favouring the colonisation of *Salmonella*. On day 29 (dpi 19), the cell numbers of *Salmonella* in the cecum of broilers administered with the multi-species probiotic were similar to those of infected and uninfected birds. Broilers that received oxytetracycline displayed higher serum bactericidal activity against *Salmonella* on day 11 (dpi 1) compared to birds from the probiotic and control groups. In addition, on day 29 (dpi 19) birds on probiotics had higher serum bactericidal activity against *Salmonella* than birds in the control group. Broilers receiving the multi-species probiotic had higher levels of lysozyme in their serum on day 11 (dpi 1) compared to uninfected broilers. Broilers receiving the antibiotic and probiotic had higher T lymphocyte responses compared to broilers from the control treatment groups on day 17 (dpi 7). These results suggested that antibiotic and probiotic feed additives stimulated the immune response of broilers infected with *Salmonella.*

The designed multi-species probiotic possessed numerous beneficial characteristics and its daily use as a feed additive was deemed safe, as probiotic use did not negatively affect the performance of healthy birds. The probiotic strains adhered to intestinal epithelial cells and crosstalk between these cells did not induce negative proteomic changes. The multi-species probiotic also increased broiler

immune responses during *Salmonella* infection, which suggests that the strains may be used as an alternative feed additive to improve broiler health and performance.

Opsomming

Toevoeging van braaikuiken voer met antibiotika is 'n groot bedreiging vir die mensdom, omdat weerstandbiedende patogeniese bakterieë na ander plaasdiere of mense kan versprei. Alternatiewe metodes word benodig om braaikuiken gesondheid en groei prestasie te verbeter, sonder enige nadelige uitwerking. Die doelstelling van hierdie studie was om die effek van 'n multi-stam probiotikum op die gesondheid en groei prestasie van *Gallus gallus domesticus* te bestudeer. Bakterieë is vanuit verskillende segmente in die spysveteringskanaal (SVK) van gesonde vrylopende braaikuikens geïsoleer, en is tot op spesievlak geïdentifiseer deur die gene wat vir 16S rDNA, *recA* en *gyrB* kodeer, te amplifiseer en met DNA volgordes in GenBank te vergelyk. Die multi-stam probiotikum verteenwoordig isolate uit elke segment in die SVK. Van die 609 bakterieë geïsoleer uit die SVK, het *Lactobacillus johnsonii* DPN184, *Lactobacillus salivarius* DPN164, *Lactobacillus crispatus* DPN167, *Lactobacillus gallinarum* DPN164, *Enterococcus faecalis* DPN94 en *Bacillus amyloliquefaciens* DPN123 hoë verdraagsaamheid getoon vir suurtoestande (pH 2 to 3) en gal soute (0.2 tot 2.0 % m/v), en het so ook hetero-eksopolisakkariede geproduseer. *B*acillus *amyloliquefaciens* DPN123, wat uit die duodenum geïsoleer is, het ekstrasellulêre amilase, fitase en antimikrobiese lipopeptiede (surfactin en iturin A1) geproduseer. *Enterococcus faecalis* DPN94, geïsoleer uit die jejunum en ileum, het fitase en galsout hidrolase geproduseer. Die genoom van *E. faecalis* DPN94 het vir verskeie virulensie gene gekodeer, maar nie vir die produksie van sitolisien nie. 'n Mengingsverskil betaan oor die rol wat virulensie gene in kolonisasie van epiteelselle speel. *Lactobacillus johnsonii* DPN184, geïsoleer uit die sekum, het waterstofperoksied geproduseer. *Lactobacillus salivarius* DPN181, geïsoleer uit die kolon, het waterstofperoksied en hoë vlakke melksuur geproduseer. *Lactobacillus crispatus* DPN167 is uit die krop en maag geïsoleer, en het waterstofperoksied en galsout hidrolase geproduseer. *Lactobacillus gallinarum* DPN164 is uit die jejunum en ileum geïsoleer.

Die multi-stam probiotikum, bestaande uit *L. johnsonii* DPN184, *L. salivarius* DPN164, *L. crispatus* DPN167, *L. gallinarum* DPN164, *E. faecalis* DPN94 and *B. amyloliquefaciens* DPN123, is by die voer van braaikuikens gevoeg en die uitwerking daarvan op groeiprestasie, grootte van limfoïede organe, spiermaag massa, mineraal inhoud van die tibia bene en rooibloedsel parameters bepaal. Braaikuikens van 'n aparte groep het 'n kombinasie van sulfadiasien, kolistien en trimetoprim deur middel van hul voer ontvang en is aan dieselfde toetse onderwerp. 'n Derde groep voëls het normale voer, sonder bymiddels, ontvang en het as kontrole gedien. Op dag 19 het braaikuikens wat antibiotika ontvang het 'n groter villi oppervlak getoon, asook hoër limfosiet- en basofiel getalle in vergelyking met braaikuikens uit die probiotika en kontrole groepe. Op dag 29 was die sekum mikrobioom van braaikuikens in die kontrole- en probiotika groepe soortgelyk, maar aansienlik verskillend van die mikrobioom in die antibiotika groep. Die sekum mikrobioom van braaikuikens wat antibiotika ontvang het, het laer vlakke *Enterobacteriaceae* en hoër vlakke onbekende Clostridiales, *Brucellaceae*, *Synergistaceae*, *Erysipelotrichaceae* en *Coriobacteriaceae* bevat. Die multi-stam probiotikum het die groei van *Listeria monocytogenes* EDGE *in vivo* onderdruk, waarskynlik deur die metaboliese aktiwiteit van die selle te verlaag, met *Listeria* mee te ding vir vashegting aan reseptore op die epiteelselle of mukosa, deur antimikrobiese komponente soos kortketting vetsure, waterstofperoksied en lipopeptiede te produseer.

Salmonella Enteritidis 147 het Caco-2-selle binnegedring en die claudin-3-digte kruisings tussen die selle, asook die monolaag, ontwrig. Die interaksie van *S*. Enteritidis met braaikuiken epiteel selle het tot die verhoging in lisosiem C en G, katelicidin 2 en 3, myeloïde proteïen 1, trypsien inhibeerder CITI-1, gallisien -2 en ubiquitin-vou modifikator 1 vlakke aanleiding gegee, maar 'n verlaaging in gluteredoksien-1, gallisien-7 en vigilien teweeg gebring. Die proteïene wat verhoog is speel 'n rol in chemotaktiese verbindings, inhibeer ensieme en beheer stres. Die proteïene wat verlaag is speel 'n belangrike rol in aktivering van natuurlike moordenaarselle, regulering van apoptose en antimikrobiese verdedigingstelsels. Die multi-stam probiotika was nie sitotoksies nie, maar hul metaboliese eindprodukte was wel. Probiotiese bakterieë het aan Caco-2 selle gebind, maar het hulle nie binnegedring nie, en het claudin-3 stywe kruisings veminder, maar het nie die monolaag ontwrig nie. Epiteel selle van braaikuikens wat blootgestel is aan die multi-stam probiotikum het tot die verhoging van transgelin 2/3, verlengings faktor-1 beta en anterior gradiënt 2, en verlaaging van karnitien O-asetieltransferase, adenylaatkinase 2, superoksied dismutase [Cu-Zn] en proteïen SET aanleiding gegee. Die proteïene wat verhoog is, is betrokke by sel proliferasie, sel migrasie, genesing en sitoskelet regulering. Die proteïene wat verlaag is speel 'n belangrike rol in die vervoer van vetsure, handhawing van energievlakke, nukleotied metabolisme, die eliminasie van vryradikale en seintransduksie. Hierdie resultate het getoon dat die probiotiese bakterieë nie toksies is nie en in 'n simbiotiese verhouding met epiteelselle is.

Salmonella enterica serovar Enteritidis A9 toediening het geen effek op liggaamsmassa gehad nie en geen beduidende verskille is waargeneem met betrekking tot immuun orgaangewigte, hematologiese parameters en serum interferon gamma vlakke nie. Kolonisasie van *Salmonella* in die sekum van braaikuikens wat oksitetrasiklien ontvang het, was egter laer op dag 11 (1 dag na infeksie, dni 1) en dag 14 (dni 4), maar het daarna toegeneem tot vlakke wat ooreenstem met dié van voëls in die beheer- en probiotikum groepe. Aanvanklik het antibiotika die selgetalle van *Salmonella* in die sekum verlaag, maar hoër vlakke is aangeteken met aaneenlopende toediening. Die toename in selgetalle mag die gevolg wees van mikrobioom versteuring in die SVK, wat indirek die kolonisasie van *Salmonella* bevoordeel. Op dag 29 (dni 19) was die selgetalle van *Salmonella* in die sekum van braaikuikens wat die multi-stam probiotikum toegedien is, soortgelyk aan dié van besmette en onbesmette voëls. Braaikuikens wat oksitetrasiklien ontvang het, het op dag 11 (dni 1) hoër serum bakteriedodende aktiwiteit teen *Salmonella* getoon as voëls van die probiotikum- en kontrole groepe. Daarbenewens het voëls op probiotika teen dag 29 (dni 19) hoër serum bakteriedodende aktiwiteit teen *Salmonella* getoon as voëls in die kontrole groep. Braaikuikens op probiotika het op dag 11 (dni 1) hoër vlakke lisosiem in hul serum gehad, in vergelyking met onbesmette braaikuikens. Braaikuikens wat antibiotika en probiotika ontvang het, het hoër T-limfosiet reaksies gehad in vergelyking met braaikuikens van die kontrole behandelingsgroepe op dag 17 (dni 7). Hierdie resultate dui daarop dat die toediening van antibiotika en probiotika die immuunrespons van braaikuikens wat met *Salmonella* besmet is, gestimuleer het.

Die multi-stam probiotikum het talle voordelige eienskappe getoon en die insluiting daarvan in daaglikse voer is as veilig beskou, aangesien dit nie die ontwikkeling van gesonde voëls negatief beïnvloed het nie. Die aanhegting van probiotiese bakterieë aan epiteelselle het nie negatiewe

proteomiese veranderinge tot gevolg gehad nie. Die multi-stam probiotikum het ook die immuunrespons van braaikuikens tydens *Salmonella* infeksie verhoog, wat daarop dui dat die multi-stam probiotikum gebruik kan word as 'n alternatiewe toevoeging tot die voer van braaikuikens.

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Notes

The language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Microbiology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between the chapters has, therefore, been unavoidable. Results from chapter 4 have been published in *Scientific Reports* (Neveling, D.P., van Emmenes, L., Ahire, J.J., Pieterse, E., Smith, C. and Dicks, L.M.T., 2017. Safety assessment of antibiotic and probiotic feed additives for *Gallus gallus domesticus*. *Scientific Reports* 7, e12767, DOI: 10.1038/s41598-017-12866-7).

Contents

List of abbreviations

AFM	Atomic force microscopy
BASO	Basophils
BLAST	Basic local alignment search tool
BWG	Body weight gain
CFU	Colony forming units
DPI	Days post infection
EOS	Eosinophils
EPS	Exopolysaccharides
ESI-MS	Electron spray ionisation mass spectroscopy
FCR	Feed conversion ratio
FI	Feed intake
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography–mass spectrometry
GIT	Gastrointestinal tract
HBSS	Hank's balanced salt solution
HCT	Haematocrit value
HET	Heterophils
HGB	Haemoglobin content
HPLC	High-performance liquid chromatography
IVIS	*In vitro* imaging system
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LFQ	Label-free quantification
LYM	Lymphocytes
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MIC	Minimal inhibitory concentration
MONO	Monocytes
MS/MS	Tandem mass spectrometry
NMDS	Non-metric multidimensional scaling
PBS	Phosphate buffered saline
PCA	Principal component analysis
RBC	Red blood cells
RDW	Red cell distribution width
SCFA	Short chain fatty acids
STRING	Search tool for the retrieval of interacting genes/proteins
TJ	Tight junctions
WBC	White blood cells

Chapter 1

General Introduction

The commercial poultry industry is worldwide ranked amongst the highest sources of animal protein. Due to intensive research and refined genetic selection programmes, a broiler nowadays requires only one third of the feed consumed in the 1950's (Havenstein *et al*., 2003). Despite the vast improvements made in housing and feeding, bacterial and fungal infections are still causing substantial financial losses (Hughes, 2001). One of the reasons is that farming has become so quality controlled that newly hatched chicks do not spend enough time in the presence of mature birds, leaving little opportunity for exposure to beneficial (probiotic) bacteria from the gastrointestinal tract (GIT) of hens. A few gut microorganisms on the surface of the egg may find their way to the GIT of the hatchling, but the contact time is far too little. Separated from the hens, the broilers are confined to a clean and well-controlled environment, and the only microorganisms that invade and colonise the GIT are those present in the feed. A stable and balanced gut microbiome develops 2 to 4 weeks after hatching (Kabir, 2009). Slow colonisation of the consortium renders broilers more susceptible to pathogens (Ahmad, 2006; Kabir, 2009). In addition, the immune system of four-week old broilers is under-developed, making them more susceptible to infections (Callaway *et al*., 2008). These infections result in weight loss, poor meat quality and often death. Of all bacterial pathogens, *Salmonella*, *Campylobacter* and *Clostridium* spp. are responsible for most of the financial losses and, if not controlled, may pose a health risk to other farm animals and humans (Kabir, 2009).

Salmonella epidemics in the USA have been directly linked to outbreaks in poultry (Guo *et al*., 2011; Pui *et al*., 2011; Scallan *et al*., 2011). The genus *Salmonella* consists of two species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies with more than 2600 serovars (Foley *et al*., 2008). *Salmonella* serovars frequently associated with broilers are *S*. Enteritidis, *S*. Typhimurium, *S*. Infantis and *S*. Kentucky (Rodriguez *et al*., 2006). Infections caused by the serovars are difficult to treat and lead to increased morbidity and mortality amongst broilers (Martin *et al*., 2004; Chang *et al*., 2015).

Conventionally, antibiotics were used as feed additives to improve growth performance and prevent disease. In intensive poultry farming antibiotics such as tetracycline, bacitracin, tylosin, salinomycin, virginiamycin, bambermycin, amoxicillin, colistin, tilmicosin are commonly used as feed additives to promote broiler health (Diarra and Malouin, 2014; Gonzalez Ronquillo and Angeles Hernandez, 2017; Mehdi *et al*., 2018; Wongsuvan *et al*., 2018). However, the inclusion of antibiotics in animal feed has been banned in Europe and many other parts of the world to control the spreading of antibiotic-resistant pathogens (Alloui *et al*., 2013; Gupta and Das, 2013). In fact, the use of antibiotics as feed additives in animal production has been implicated as a major contributing factor to the emergence of multi-drug resistant bacteria (Chattopadhyay, 2014; Economou and Gousia, 2015). The emergence and rapid spread of multiple drug-resistant *Salmonella* Typhimurium phage-type DT104 and ceftriaxone-resistant *S. enterica* serovars such as Heidelberg, Newport, and Typhimurium is of great threat to animal and human health (Folster *et al*., 2012; Liljebjelke *et al*., 2017). Resistance to colistin,

an antibiotic that was in clinical use in the 1950's but discontinued in the 1980's due to nephro-and-neurotoxicity (Falagas and Kasiakou, 2005), has now re-emerged (Gupta *et al*., 2009; Dhariwal and Tullu, 2013). Resistance to colistin is no longer confined to genes on the genome. Recently, genes encoding colistin resistance (*mcr-1)* have also been found on conjugative plasmids (Olaitan *et al*., 2014; Hasman *et al*., 2015; Osei Sekyere *et al*., 2016). This is distressing, as colistin is still regarded as critically important last resort antibiotic. Alternatives to antibiotics, at least in the animal feed industry, is an absolute necessity (Medina and Pieper, 2016). Probiotics, prebiotics, synbiotics, enzymes, organic acids and herbs have been considered as alternatives (Huyghebaert *et al*., 2011).

Lactobacillus, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* spp. are generally used as probiotic feed additives for broilers (Saint-Cyr *et al*., 2017; Manafi *et al*., 2018; Wang *et al*., 2018). Some of the beneficial effects reported for probiotics are improved growth performance (Wang *et al*., 2017a; Yun *et al*., 2017 Manafi *et al*., 2018), modulation of intestinal microbiota (Higgins *et al*., 2006; Cao *et al*., 2018), inhibition of pathogen colonisation (Kabir *et al*., 2005; Mountzouris *et al*., 2007), intestinal histomorphological changes (Samanya and Yamauchi, 2002; Chichlowski *et al*., 2007), immunomodulation (Haghighi *et al*., 2005; Stringfellow *et al*., 2011; Wang *et al*., 2017b), modulation of haemato-biochemical parameters (Islam *et al*., 2004; Ashayerizadeh *et al*., 2009) and improved sensory characteristics of the meat (Pelicano *et al*., 2003; Abdulla *et al*., 2017). Inconsistent results have been reported on probiotics developed for chickens, including broilers. This may be due to differences in microbial species and strains, incorrect preparation of the strains, poor adherence to the gut wall and mucosa, environmental factors, or poor management (Patterson and Burkholder, 2003; Kabir, 2009). Most commercially available probiotics for broilers contain one or two strains, most of which have not been isolated from the GIT of chickens.

The objective of this study was to develop a multi-species probiotic for *Gallus gallus domesticus*, aimed at improving the health and growth performances, and prevent colonisation by *Salmonella enterica*. Bacteria were isolated from different sections of the gastrointestinal tract (GIT) of healthy free-range broilers and identified to species level using 16S rDNA, *recA* and *gyrB* gene sequences. Isolates from each section of the GIT were screened for probiotic properties such as the production of digestive enzymes, hydrogen peroxide, exopolysaccharides and antimicrobial compounds, tolerance to acid and bile salt, ability to aggregate and form biofilms, and cell hydrophobicity. In addition, identified probiotic strains were screened for the presence of virulence factors. The safety of the multi-species probiotic (*L*. *crispatus* DPN167, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94 and *B*. *amyloliquefaciens* DPN123) versus an antibiotic combination (sulphadiazine, colistin and trimethoprim in combination) as feed additives were assessed. The safety of these additives was evaluated based on growth performance, immune organ weight, gizzard weight, histomorphology of the small intestine, haematology, tibia bone mineral weight, inhibition of *L*. *monocytogenes* EGDe *in vivo* and changes in the cecal microbiome composition. The effect of the multi-species probiotic on Caco-2 epithelial cells was studied by monitoring cytotoxicity, adhesion and invasion and effect on claudin-3 tight junctions between the cells. Changes in the proteome of ileum epithelial cells when in contact with the multi-species probiotic and *S*. Enteritidis 147 were recorded. The ability of the multi-species probiotic and antibiotic (oxytetracycline) in inhibiting *Salmonella enterica* subsp. *enterica* serovar

Enteritidis A9 colonisation was assessed *in vivo*. The ability of treatments in inhibiting *Salmonella* colonisation was assessed by following *Salmonella* colonisation in the cecum, determining the effect on growth performance, haematological parameters, serum bactericidal and lysozyme activity, immune organ weights and T-lymphocyte response rates. The development of a multi-species probiotic capable of improving broiler health, performance and inhibit pathogen colonisation, would decrease the need for use of antibiotic feed additives, decreasing the selection of antibiotic-resistant bacteria.

References

Abdulla, N.R., Zamri, A.N.M., Sabow, A.B., Kareem, K.Y., Nurhazirah, S., Ling, F.H., Sazili, A.Q., Loh, T.C. (2017). Physico-chemical properties of breast muscle in broiler chickens fed probiotics, antibiotics or antibiotic-probiotic mix. *J. Appl. Anim. Res.* 45(1), p 64-70, DOI: 10.1080/09712119.2015.1124330.

Ahmad, I. (2006). Effect of probiotics on broilers performance. *Int. J. Poult. Sci.* 5(6), p 593-597, DOI: 10.3923/ijps.2006.593.597.

Alloui, M.N., Szczurek, W., Świątkiewicz, S. (2013). The usefulness of prebiotics and probiotics in modern poultry nutrition: a review. *Ann. Anim. Sci.* 13(1), p 17-32, DOI: https://doi.org/10.2478/v10220-012-0055-x.

Ashayerizadeh, A., Dabiri, N., Ashayerizadeh, O., Mirzadeh, K.H., Roshanfekr, H., Mamooee, M. (2009). Effect of dietary antibiotic, probiotic and prebiotic as growth promoters, on growth performance, carcass characteristics and hematological indices of broiler chickens. *Pakis. J. Biol. Sci.* 12(1), p 52–57.

Callaway, T.R., Edrington, T.S., Anderson, R.C., Harvey, R.B., Genovese, K.J., Kennedy, C.N., Venn, D.W., Nisbet, D.J. (2008). Probiotics, prebiotics and competitive exclusion for prophylaxis against bacterial disease. *Anim. Health Res. Rev.* 9(2), p 217-225, DOI: 10.1017/S1466252308001540.

Cao, G.T., Zhan, X.A., Zhang, L.L., Zeng, X.F., Chen, A.G., Yang, C.M. (2018). Modulation of broilers' caecal microflora and metabolites in response to a potential probiotic *Bacillus amyloliquefaciens*. *J. Anim. Physiol. Anim. Nutr.* In Press, DOI: 10.1111/jpn.12856.

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M., Hanage, W.P. (2015). Antibiotics in agriculture and the risk to human: how worried should we be? *Evol. Appl.* 8(3), p 240-247, DOI: 10.1111/eva.12185.

Chattopadhyay, M.K. (2014). Use of antibiotics as feed additives: a burning question. *Front. Microbiol.* 5, Article ID: 334, DOI: 10.3389/fmicb.2014.00334.

Chichlowski, M., Croom, W.J., Edens, F.W., McBride, B.W., Qiu, R., Chiang, C.C., Daniel, L.R., Havenstein, G.B., Koci, M.D. (2007). Microarchitecture and spatial relationship between bacteria and ileal, cecal, and colonic epithelium in chicks fed a direct-fed microbial, PrimaLac, and salinomycin. *Poult. Sci.* 86(6), p 1121–1132, DOI: 10.1093/ps/86.6.1121.

Dhariwal, A.K., Tullu, M.S. (2013). Colistin: re-emergence of the 'forgotten' antimicrobial agent. *J. Postgrad. Med.* 59(3), p 208-215, DOI: 10.4103/0022-3859.118040.

Economou, V., Gousia, P. (2015). Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infect*. *Drug Resist*. 8, p 49-61, DOI: 10.2147/IDR.S55778.

Falagas, M.E., Kasiakou, S.K. (2005). Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin*. *Infect*. *Dis*. 40(9), p 1333–1341, DOI: 10.1086/429323.

Foley, S.L., Lynne, A.M., Nayak, R. (2008). *Salmonella* challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. *J*. *Anim*. *Sci*. 86(14), E149–E162, DOI: 10.2527/jas.2007-0464.

Folster, J.P., Pecic, G., Singh, A., Duval, B., Rickert, R., Ayers, S., Abbott, J., McGlinchey, B., Bauer-Turpin, J., Haro, J., Hise, K., Zhao, S., Fedorka-Cray, P.J., Whichard, J., McDermott, P.F. (2012). Characterization of extended-spectrum cephalosporin-resistant *Salmonella enterica* serovar Heidelberg isolated from humans in the United States 2009. *Foodborne Pathog*. *Dis*. 9(7), p 638-645, DOI: 10.1089/fpd.2012.1130.

Guo, C., Hoekstra, R.M., Schroeder, C.M., Pires, S.M., Ong, K.L., Hartnett, E., Naugle, A., Harman, J., Bennett, P., Cieslak, P., Scallen, E., Rose, B., Holt, K.G., Kissler, B., Mbandi, E., Roodsari, R., Angulo, F.J., Cole, J.D. (2011). Application of Bayesian techniques to model the burden of human salmonellosis attributable to U.S. food commodities at the point of processing: adaptation of a Danish model. *Foodborne Pathog*. *Dis*. 8(4), p 509–516, DOI:10.1089/fpd.2010.0714.

Gupta, A.R., Das, S. (2013). The benefits of probiotics in poultry production: an overview. *Int*. *J*. *Livest*. *Res*. 3(1), p 18-22, DOI: 10.5455/ijlr.20130213091011.

Gupta, S., Govil, D., Kakar, P.N., Prakash, O., Arora, D., Das, S., Govil, P., Malhotra, A. (2009). Colistin and polymyxin B: A re-emergence. *Indian J*. *Crit*. *Care Med*. 13(2), p 49-53, DOI: 10.4103/0972-5229.56048.

Haghighi, H.R., Gong, J., Gyles, C.L., Hayes, M.A., Sanei, B., Parvizi, P., Gisavi, H., Chambers, J.R., Sharif, S. (2005). Modulation of antibody-mediated immune response by probiotics in chickens. *Clin*. *Diagn*. *Lab*. *Immunol*. 12(12), p 1387-1392, DOI: 10.1128/CDLI.12.12.1387-1392.2005.

Hasman, H., Hammerum, A.M., Hansen, F., Hendriksen, R.S., Olesen, B., Agersø, Y., Zankari, E., Leekitcharoenphon, P., Steggerm M., Kaas, R.S., Cavaco, L.M., Hansen, D.S., Aarestrup, F.M., Skov, R.L. (2015). Detection of mcr-1 encoding plasmid-mediated colistin-resistant *Escherichia coli* isolates from human bloodstream infection and imported chicken meat, Denmark 2015. *Euro*. *Surveill*. 20(49), Article ID: 30085, DOI: 10.2807/1560-7917.ES.2015.20.49.30085.

Havenstein, G.B., Ferket, P.R., Qureshi, M. (2003). Growth, livability, and feed conversion of 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. *Poult Sci* 8, p 1500–1508, DOI: 10.1093/ps/82.10.1500.

Higgins, J.P., Higgins, S.E., Vicente, J.L., Wolfenden, A.D., Tellez, G., Hargis, B.M. (2007). Temporal effects of lactic acid bacteria probiotic culture on *Salmonella* in neonatal broilers. *Poult*. *Sci*. 86(8), p 1662–1666, DOI: 10.1093/ps/86.8.1662.

Hughes, R.J. (2001). Variation in the digestive capacity of the broiler chicken. *Rec*. *Adv*. *Anim*. *Nutr*. *Aust*. 13, p 153–161.

Huyghebaert, G., Ducatelle, R., Van Immerseel, F. (2011). An update on alternatives to antimicrobial growth promoters for broilers. *Vet*. *J*. 187, p 182-188, DOI: 10.1016/j.tvjl.2010.03.003.

Islam, M.W., Rahman, M.M., Kabir, S.M.L., Kamruzzaman, S.M., Islam, M.N. (2004). Effects of probiotics supplementation on growth performance and certain haemato-biochemical parameters in broiler chickens. *Bangl*. *J*. *Vet*. *Med*. 2, p 39–43, DOI: 10.3329/bjvm.v2i1.1933.

Kabir, S.M.L. (2009). The role of probiotics in the poultry industry. *Int*. *J*. *Mol*. *Sci*. 10(8), p 3531-3546, DOI: 10.3390/ijms10083531.

Kabir, S.M.L., Rahman, M.M., Rahman, M.B., Hosain, M.Z., Akand, M.S.I., Das, S.K. (2005). Viability of probiotics in balancing intestinal flora and effecting histological changes of crop and caecal tissues of broilers. *Biotech*. 4(4), p 325–330, DOI: 10.3923/biotech.2005.325.330.

Liljebjelke, K.A., Hofacre, C.L., White, D.G., Ayers, S., Lee, M.D., Maurer, J.J. (2017). Diversity of antimicrobial resistance phenotypes in *Salmonella* isolated from commercial poultry farms. *Front*. *Vet*. *Sci*. 4, Article ID: 96, DOI: 10.3389/fvets.2017.00096.

Manafi, M., Hedayati, M., Mirzale, S. (2018). Probiotic *Bacillus* species and *Saccharomyces boulardii* improves performance, gut histology and immunity in broiler chickens. *S.A*. *J*. *Anim*. *Sci*. 48(2), p 379-389, DOI: 10.4314/sajas.v48i2.19.

Martin, J.L., Fyfe, M., Doré, K., Buxton, J.A., Pollari, F., Henry, B., Middleton, D., Ahmed, R., Jamieson, F., Ciebin, B., McEwen, S.A., Wilson, J.B., Multi-Provincial *Salmonella* Typhimurium Case-Control Study Steering Committee. (2004). Increased burden of illness associated with antimicrobial resistant *Salmonella enterica* serotype Typhimurium infections. J. Infect. Dis. 189(3), p 377–384., DOI:10.1086/381270.

Medina, E., Pieper, D.H. (2016). Tackling threats and future problems of multidrug-resistant bacteria. In: Stadler, M., Dersch, P. (Eds.), How to Overcome the Antibiotic Crisis. *Curr*. *Top*. *Microbiol*. *Immunol*. 398, p 3-33, Springer, Cham, DOI: 10.1007/82_2016_492.

Mehdi, Y., Létourneau-Montminy, M-P., Gaucher, M-L., Chorfi, Y., Suresh, G., Rouissi, T., Brar, S.K., Côté, C., Ramirez, A.A., Godbout, S. (2018). Use of antibiotics in broiler production: Global impacts and alternatives. *Anim*. *Nutr*. 4, p 170-178, DOI: 10.1016/j.aninu.2018.03.002.

Mountzouris, K.C., Tsirtsikos, P., Kalamara, E., Nitsch, S., Schatzmayr, G., Fegeros, K. (2007). Evaluation of the efficacy of probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. *Poult*. *Sci*. 86(2), p 309–317, DOI: 10.1093/ps/86.2.309.

Olaitan, A.O., Morand, S., Rolain, J-M. (2014). Mechanisms of polymyxin resistance: acquired and intrinsic resistance in bacteria. *Front*. *Microbiol*. 5, Article ID: 643, DOI: 10.3389/fmicb.2014.00643.

Osei Sekyere, J., Govinden, U., Bester, L.A., Essack, S.Y. (2016). Colistin and tigecycline resistance in carbapenemase-producing Gram negative bacteria: emerging resistance mechanisms and detection methods. *J*. *Appl*. *Microbiol*. 121(3), p 601-617, DOI:10.1111/jam.13169.

Patterson, J.A., Burkholder, K.M. (2003). Application of prebiotics and probiotics in poultry production. *Poult*. *Sci*. 82(4), p 627-631, DOI: 10.1093/ps/82.4.627.

Pelicano, E.R.L., Souza, P.A., Souza, H.B.A., Oba, A., Norkus, E.A., Kodawara, L.M., Lima, T.M.A. (2003). Effect of different probiotics on broiler carcass and meat quality. Rev. Bras. Cienc. Avic. 5(3), p 207–214, DOI: 10.1590/S1516-635X2003000300009.

Pui, C.F., Wong, W.C., Chai, L.C., Tunung, R., Jeyaletchumi, P., Noor Hidayah, M.S., Ubong, A., Farinazleen, M.G., Cheah, Y.K., Son, R. (2011). *Salmonella*: a foodborne pathogen. *Int*. *Food Res*. *J*. 18, 465–473.

Rodriguez, A., Pangloli, P., Richards, H.A., Mount, J.R., Draughon, F.A. (2006). Prevalence of *Salmonella* in diverse environmental farm samples. *J*. *Food Prot*. 69(11), p 2576-2580, DOI: 10.4315/0362-028X-69.11.2576.

Saint-Cyr, M.J., Haddad, N., Taminiau, B., Poezevara, T., Quesne, S., Amelot, M., Daube, G., Chemaly, M., Dousset, X., Guyard-Nicodème, M. (2017). Use of the potential probiotic strain *Lactobacillus salivarius* SMXD51 to control *Campylobacter jejuni* in broilers. *Int*. *J*. *Food Microbiol*. 247, p 9-17, DOI: 10.1016/j.ijfoodmicro.2016.07.003.

Samanya, M., Yamauchi, K. (2002). Histological alterations of intestinal villi in chickens fed dried *Bacillus subtilis* var. natto. *Comp*. *Biochem*. *Physiol*. *Physiol*. 133(1), p 95–104, DOI: 10.1016/S1095-6433(02)00121-6.

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R.V., Widdowson, M-A., Roy, S.L., Jones, J.L., Griffin, P.M. (2011). Foodborne illness acquired in the United States – major pathogens. *Emerg*. *Infect*. *Dis*. 17(1), p 7–15, DOI: 10.3201/eid1701.P11101.

Stringfellow, K., Caldwell, D., Lee, J., Mohnl, M., Beltran, R., Schatzmayr, G., Schatzmayr, G., Fitz-Coy, S., Broussard, C., Farnell, M. (2011). Evaluation of probiotic administration on the immune response of coccidiosis-vaccinated broilers. *Poult*. *Sci*. 90(8), p 1652-1658, DOI: 10.3382/ps.2010-01026.

Wang, H., Ni, X., Qing, X., Liu, L., Lai, J., Khalique, A., Li, G., Pan, K., Jing, B., Zheng, D. (2017b). Probiotic enhanced intestinal immunity in broilers against subclinical necrotic enteritis. *Front*. *Immunol*. 8, Article ID 1592, DOI: 10.3389/fimmu.2017.01592.

Wang, H., Ni, X., Qing, X., Liu, L., Xin, J., Luo, M., Khalique, A., Dan, Y., Pan, K., Jing, B., Zeng, D. (2018). Probiotic *Lactobacillus johnsonii* BS15 improves blood parameters related to immunity in broiler experimentally infected with subclinical necrotic enteritis. *Front*. *Microbiol*. 9, e49, DOI: 10.3389/fmicb.2018.00049.

Wang, H., Ni, X., Qing, X., Zeng, D., Luo, M., Liu, L., Li, G., Pan, K., Jing, B. (2017a). Live probiotic *Lactobacillus johnsonii* BS15 promotes growth performance and lowers fat deposition by improving lipid metabolism, intestinal development, and gut microflora in broilers. *Front*. *Microbiol*. 8, Article ID: 1073, DOI: 10.3389/fmicb.2017.01073.

Wongsuvan, G., Wuthiekanun, V., Hinjoy, S., Day, N.P.J., Limmathurotsakul, D. (2018). Antibiotic use in poultry: a survey of eight farms in Thailand. *Bull*. *World Health Organ*. 96, p 94-100, DOI: 10.2471/BLT.17.195834.

Yun, W., Lee, D.H., Choi, Y.I., Kim, I.H., Cho, J.H. (2017). Effects of supplementation of probiotics and prebiotics on growth performance, nutrient digestibility, organ weight, fecal microbiota, blood

profile, and excreta noxious gas emissions in broilers. *J*. *Appl*. *Poult*. *Res*. 26(4), p 584-592, DOI: 10.3382/japr/pfx033.

Chapter 2

Understanding the role probiotics play in broiler health and performance

Abstract

Poultry meat is an important protein source, as evident from the rapid growth in the industry over the last three decades. The poultry industry is thus under immense pressure to rear healthy birds in the shortest possible time, and without the addition of growth hormones or antibiotics to the feed. Organic acids, probiotics, prebiotics, synbiotics, and more complex nutritional compositions are some of the natural feed additives experimented with. The exact benefit probiotics and synbiotics have is difficult to prove, especially with so many variations on the market. Several studies have shown that probiotics may improve broiler health and performance by modulating nutrition and digestion, changing the microbial composition in the gastrointestinal tract (GIT) and stimulating the immune system. Apart from producing digestive enzymes or stimulating host enzyme production, some probiotic strains produce vitamins, regulate cholesterol blood levels, produce antioxidants, and regulate energy production in the mitochondria. Pathogen overgrowth is prevented by competitive exclusion, competition for nutrients, production of antimicrobial compounds and metabolites that enhances the growth of beneficial bacteria. Probiotics developed for broilers are aimed at regulating the integrity of the gut wall, control cell proliferation and apoptosis, improve bone health, regulate brain signalling via the brain-gut-microbiome axis, and improve meat characteristics. Probiotics modulate the immune system by inhibiting pathogen-induced immune activation, regulating pro-inflammatory and anti-inflammatory reactions and dictating adaptive immune system development. Future scientific endeavours are required to fully understand the role probiotics play in improving broiler health and growth, i.e. defining the composition of a healthy microbiome, determine the role each member has in host physiology and determine the molecular crosstalk which occurs between probiotic and epithelial cells in the GIT. Such studies would provide researchers with greater insight into the molecular mechanisms behind the therapeutic benefits of probiotics and synbiotics. This review provides insight into probiotics as feed additives and focuses on the various mechanisms probiotics use to modulate the physiology of broilers to improve health and growth.

Introduction

Broilers today require one third of the feed that birds consumed in the 1950's, due to intensive research and genetic selection (Havenstein *et al*., 2003). Despite all these improvements, the performance of flocks varies due to infections caused by pathogenic bacteria. In addition, pathogenic bacteria contaminate the carcasses, and this may lead to food-borne diseases in humans (Baurhoo *et al*., 2009). Microbial infections caused by *Salmonella*, *Campylobacter* and *Clostridium* continue to challenge the poultry industry (Kabir, 2009). Newly-hatched broilers acquire their initial GIT microbiome from eggshells during hatching, feed and the husbandry environment (Rinttilä and Apajalahti, 2013). Through continuous feeding, microbes gradually colonise the GIT to form a stable microbial consortium within 2 to 4 weeks (Kabir, 2009). Broilers with delayed maturation of the microbiome are more susceptible to infections (Ahmad, 2006; Kabir, 2009). In addition, the immune system of broilers within the first 4 weeks are under-developed, making them more susceptible to infections (Callaway *et al*., 2008). Bacteria that initially colonise the GIT are important as they direct the development of the immune system and the microbiome composition, which ultimately affects the growth of broilers (Apajalahti *et al*., 2004).

Conventionally, antibiotics were widely used as feed additives to inhibit pathogen colonisation. However, the European Union banned the use of antibiotics due to natural selection of resistant bacteria (Hashemi and Davoodi, 2010). Antimicrobial resistance genes are widely distributed in bacteria in the environment (Goldstein *et al*., 2001), and these bacteria can confer antibiotic resistance to other bacteria without continues pressure (Salyers and Amabile-Cuevas, 1997). Animal farming will increase rapidly in the future, creating the need for responsible use of antibiotics and the discovery of novel alternatives (Boland *et al*., 2013). The occurrence of multiple antibiotic-resistant bacteria will increase in the future if no restrictions are put in place with regards to the responsible use of antibiotics as feed additives.

Alternative prophylactics are thus sought to improve broiler growth performance and prevent pathogenic infections. One such approach involves the use of probiotics to exclude, or inhibit, the growth of pathogens in the GIT. In broilers, microbial species belonging to the genera *Lactobacillus*, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida* and *Saccharomyces* are generally used as probiotics (Kabir, 2009). Despite evidence supporting the benefits of adding probiotics to the feed of broilers, the precise mechanisms by which these microorganisms influence the host biology have not been studied in depth (Sherman *et al*., 2009). This is due to the highly complex, not well defined, multifactor interactions which occur between the host and probiotic cells (Ducatelle *et al*., 2015). This review assesses the benefits of probiotic use as feed additives for broilers and discusses the mechanisms by which probiotics improve health and growth performance.

Multifactorial role in health and growth

Numerous studies have shown that probiotics can improve broiler growth and decrease mortalities, which is important for large-scale farming (Alloui *et al*., 2013, Amerah *et al*., 2013; Pourakbari *et al*., 2016). Probiotics regulate broiler health and growth by modulating nutrition and digestion, GIT

microbiome composition, host physiology and the immune system (Figure 1). Benefits of probiotic use are due to the combined multi-factorial interaction (Ajuwon, 2016). Contradictory information regarding the role probiotics play in improving growth has been reported which may be due to differences in rearing systems, bacterial strain compositions and probiotic dosing concentrations (Olnood *et al*., 2015; Peng *et al*., 2016; Salehimanesh *et al*., 2016). Rearing systems and environmental factors affects broiler growth performance (Santos *et al*., 2008). Probiotic characteristics are strain dependent; thus, significant differences exist with respect to therapeutic capabilities (Patterson and Burkholder, 2003). In addition, dose variations may also alter the efficacy of the probiotic (Mountzouris *et al*., 2010). It is thus difficult to directly assess different studies using probiotics because the efficacy of probiotics depends on various factors such as species composition and viability, administration level, application method, frequency of application, overall diet, bird age, overall farm hygiene, and environmental stress factors (Patterson and Burkholder, 2003).

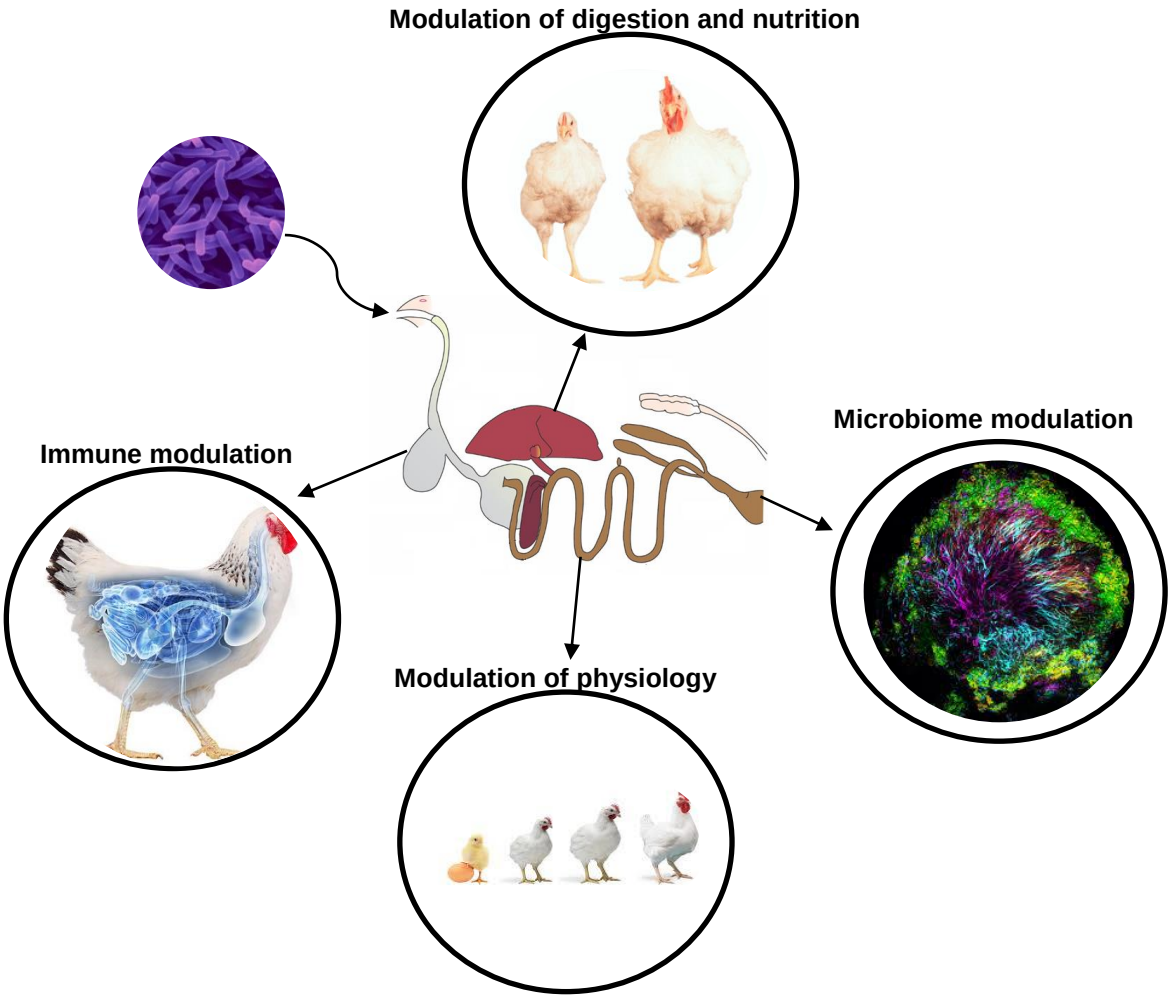

Figure 1: General modes of action by which probiotics improve broiler health and performance.

Modulation of nutrition and digestion

Probiotics improve broiler nutrition and digestion by various mechanisms such as the production of digestive enzymes, vitamins, exopolysaccharides, antioxidants, regulate cholesterol metabolism and provides energy through fermentative metabolites (Figure 2) (Zhang and Kim, 2014; Zhang *et al*., 2016; Wealleans *et al*., 2017). Probiotic bacteria can produce different digestive enzymes (i.e. phytases, lipases, amylases and proteases), which increases digestion in the GIT, and subsequently increases nutrient uptake (Jin *et al*., 2000; Wang and Gu, 2010; Flint *et al*., 2012). Nutrients made available by the host enzymes are mainly absorbed in the stomach and small intestine, whereas in the ileum bacteria utilise simple carbohydrates (Krajmalnik-Brown *et al*., 2012). In the colon, indigestible carbohydrates and proteins are converted to nutrients by bacterial enzymes (Sonnenburg *et al*., 2005; Flint *et al*., 2012). Improved nutrient digestibility also decreases the development of enteric diseases, as undigested nutrients are a predisposing factor for the development of microbiome dysbiosis (Carding *et al*., 2015). Members of the phyla *Firmicutes*, *Actinobacteria* and *Verrucomicrobium* produce different types of digestive enzymes (Sonnenburg *et al*., 2005; Flint *et al*., 2012), but in the broader context, bacterial enzymes form a small part of the total digestive capacity of the host (Bedford and Schulze, 1998). In addition to producing digestive enzymes, probiotics can also stimulate the expression and translation of host-related digestive enzymes, increasing digestive capacity and improving growth (Sun *et al*., 2016; Hmani *et al*., 2017).

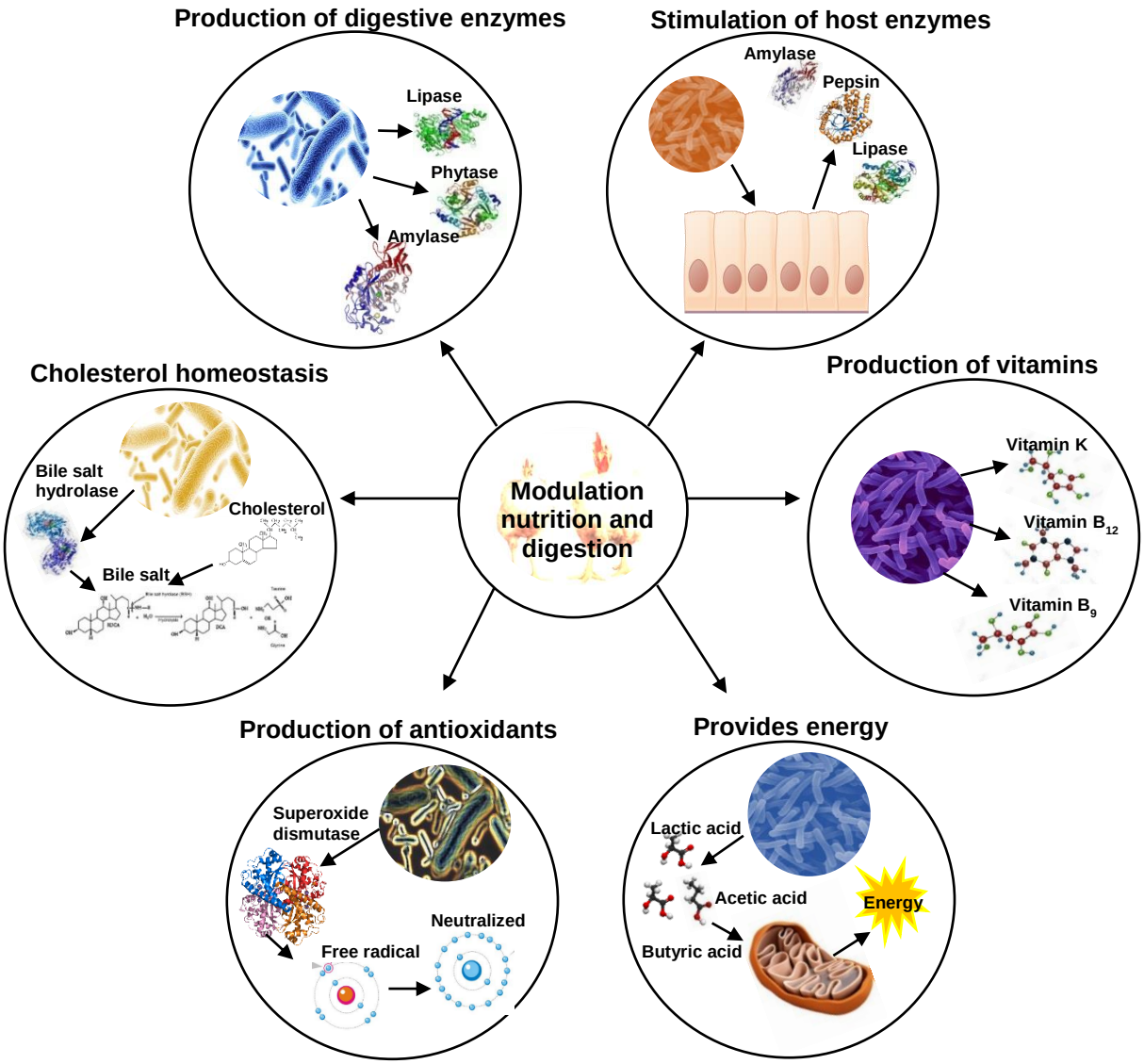

Figure 2: Modes of action by which probiotics modulate broiler nutrition and digestion.

Probiotic bacteria may provide energy from indigestible compounds in the form of fermentative end-products such as lactate, acetate, propionate and butyrate (den Besten *et al*., 2013; Ríos-Covián *et al*., 2016). These metabolites are transported from the GIT to various organs where they are used as energy sources, act as substrates, or signal molecules, in the metabolism of lipids, glucose and cholesterol (Hedge *et al*., 1982; Fushimi *et al*., 2006; Gao *et al*., 2009). Although short-chain fatty acids (SCFAs) serve as additional energy, it is believed that only a small proportion (up to 25%) is recovered (Bolton and Dewar, 1965; Annison *et al*., 1968).

Probiotic bacteria from the genera *Bifidobacterium* and *Lactobacillus* synthesise vitamin K and B vitamins, i.e. biotin, cobalamin, folates, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine (Kleerebezem and Vaughan, 2009; LeBlanc *et al*., 2013). Vitamins are essential micronutrients for eukaryotes, as they are required in numerous vital biochemical processes such as bodily functions, growth and reproduction. Deficiencies in vitamins lead to the development of several diseases (LeBlanc *et al*., 2013). Compared to prokaryotes, eukaryotes are unable to synthesise

vitamins and must acquire these from exogenous sources (LeBlanc *et al*., 2013). Microbially produced vitamins are thought to be absorbed in the colon, as colonocytes absorb biotin, thiamine, folates, riboflavin, pantothenic acid, and menaquinones (Ichihashi *et al*., 1997; Said and Mohammed, 2006; Pompei *et al*., 2007).

Probiotic bacteria synthesise a wide variety of glycan structures, such as exopolysaccharides (EPS), microbial glycans; and capsular polysaccharides (Porter and Martens, 2017). Therapeutic benefits of glycans remain to be elucidated; however, they protect microbial cells against adverse environmental conditions and facilitate adhesion and biofilm formation (Ruas-Madiedo *et al*., 2006; Patel and Prajapati, 2013). Exopolysaccharides protect bacteria from dehydration, phagocytosis, predation, bacteriophage attack, and from the adversary effects of antibiotics and toxic compounds (Roberts, 1996). Probiotic bacteria produce exopolysaccharides with different chemical subunits, and structural variations determine the prebiotic potential (Korakli and Vogel, 2006). Probiotic-produced EPS influences the host by modulating the innate and adaptive immune systems (Hidalgo-Cantabrana *et al*., 2012; Matsuzaki *et al*., 2014), poses antitumor properties (Nishimura, 2014), lower cholesterol levels (Maeda *et al*., 2004; Ryan *et al*., 2015), inhibit pathogens and promote growth of beneficial bacteria, and act as antioxidants (Fernandez *et al*., 2002; Liu *et al*., 2011a; Hongpattarakere *et al*., 2012).

Metabolites originating from probiotics have antioxidative effects by scavenging for reactive oxygen species (ROS) or preventing the formation thereof (Kim *et al*., 2006; Azcárate-Peril *et al*., 2011). Oxidative stress caused by ROS are detrimental to the health and performance of broilers (Douglas *et al*., 2016). Reactive oxygen species have unpaired electrons which carry out rapid chain reactions, which destabilises other molecules and lead to the generation of free radicals, such as superoxide anions, hydroxyl radicals and transition metals (Mishra *et al*., 2015). Antioxidants terminate the chain reactions by removing free radical intermediates and inhibit other oxidation reactions by neutralising free radicals (Halliwell and Gutteridge, 1990). Bacterial enzymes with antioxidative properties include glutathione S-transferase, glutathione, glutathione reductase, glutathione peroxidase, superoxide dismutase, and catalase (Kumar *et al*., 2010). Antioxidative enzymes modulate circulatory oxidative stress and protect cells against carcinogen-induced damage (Wang *et al*., 2017). The presence of ROS may lead to the development of numerous chronic diseases and lipid peroxidation, which contributes to hyperlipidaemia and hyperglycaemia. This results in an increase in the level of pro-atherogenic lipoproteins and a decrease in the level of HDL (high-density lipoproteins) cholesterol (Tsimikas and Miller, 2011; Stancu *et al*., 2012).

Some probiotic bacteria regulate cholesterol homeostasis by hydrolysing bile acids, which prevents their reabsorption in the intestine, indirectly lowering cholesterol levels (Yalçin *et al*., 2016; Shokryazdan *et al*., 2017). The exact mechanism by which cholesterol is utilised is poorly understood. However, it is thought that bile acids are formed from cholesterol in the liver, and hydrolysis of bile acids by bacterial enzymes increases the rate of cholesterol to bile acid conversion, indirectly leading to a decrease in cholesterol levels (Jadhav *et al*., 2015). Probiotics administered to broilers decrease cholesterol levels in meat and blood (Yalçin *et al*., 2016; Shokryazdan *et al*., 2017). Several mechanisms have been proposed by which probiotic bacteria decrease cholesterol levels and include

assimilation of cholesterol by the growing cell, binding of cholesterol to the cellular surface, incorporation of cholesterol into the cellular membrane, deconjugation of bile via bile salt hydrolase and co-precipitation of cholesterol with deconjugated bile (Ooi and Liong, 2010; Kumar *et al*., 2012).

Microbiome modulation

The GIT contains complex microbial ecosystems, consisting of trillions of commensal bacteria living in symbiosis with the host. Interactions between the host and microbiome are important for development, health, nutrition and digestion, and food safety (Pflughoeft and Versalovic, 2012; Oakley *et al*., 2013). Members of the GIT microbiome have evolved symbiotic relationships, providing biochemical and metabolic pathways not present in the host (Egert *et al*., 2006). Detrimental changes in the microbiome composition (dysbiosis) disrupts mutualistic interactions and lead to disease (Frank *et al*. 2011). Disease development is believed to be due to changes in the core microbiome metabolic function, rather than changes in specific microbial members (Hemarajata and Versalovic, 2013). Probiotics restore microbiome homeostasis by inhibiting pathogens and by promoting the growth of beneficial bacteria (Hemarajata and Versalovic, 2013). Immune activation or chronic inflammation induced by pathogens contributes to decreased health and growth in poultry. Probiotics and their metabolites induce shifts in the microbiome composition, from a predominant pathogenic microbiome to a more beneficial microbiome (Howarth and Wang, 2013). Probiotic bacteria modulate the GIT microbiome composition by competitively excluding bacteria from the mucosal surface, competing for nutrients, and by producing exopolysaccharides and antimicrobial compounds (SCFA, H_2O_2 and antimicrobial peptides; Figure 3).

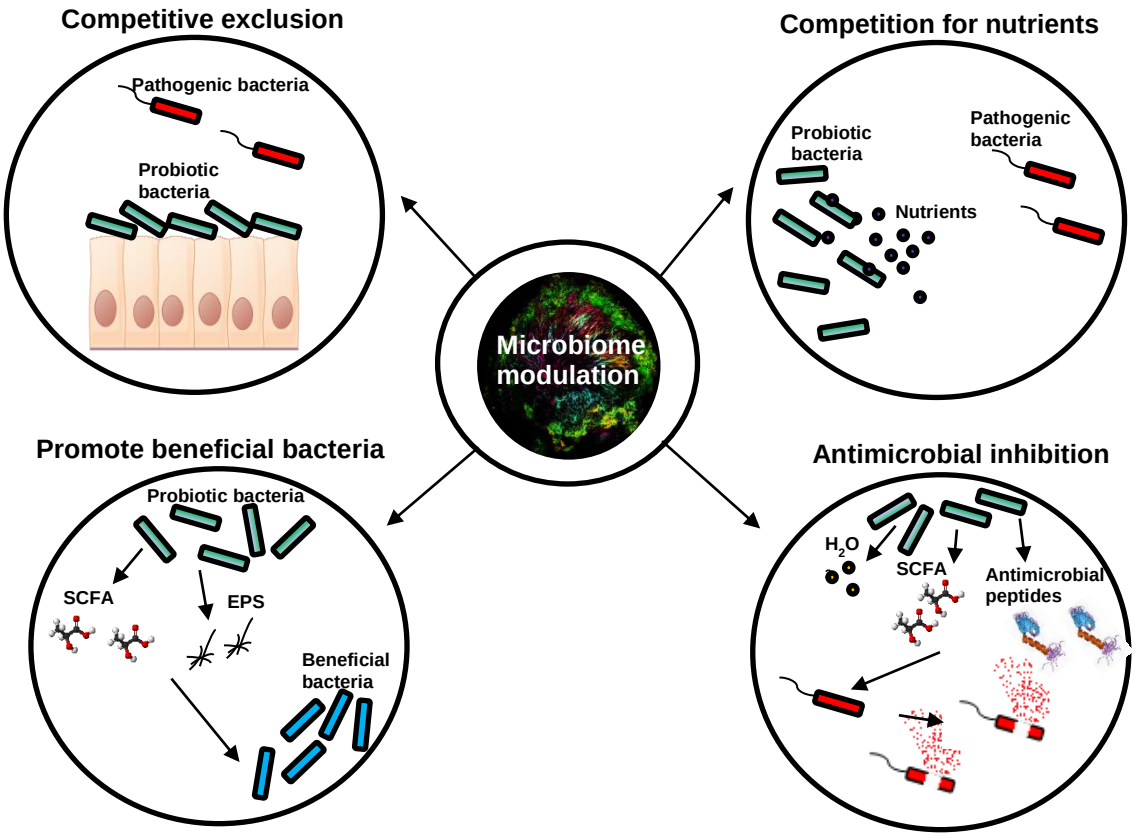

Figure 3: Modes of action by which probiotics modulate the GIT microbiome composition.

Probiotic bacteria inhibit pathogen colonisation in the GIT by competing for binding sites on the gut mucosa surface (Patterson and Burkholder, 2003; Collado *et al*. 2007). In addition, probiotics compete for nutrients in the gut environment, inhibiting pathogen colonisation (Schneitz, 2000; O'Toole and Cooney, 2008). Probiotic bacteria from the lactic acid bacteria (LAB) group produce numerous metabolites such as lactate, acetate, isovalerate, butyrate and propionate. These metabolites penetrate the cell wall of susceptible bacteria, leading to disruption of normal cell physiology that results in death (Jadhav *et al*., 2015; Ríos-Covián *et al*., 2016). SCFA decreases the pH of the cytoplasm (Mani-López *et al*., 2012). This induces uncoupling reactions which result in denaturation of proteins, enzymes, and nucleic acids (Russell, 1992; Alakomi *et al*., 2000 and 2007).

Probiotic bacteria can also modulate the microbiome composition by producing hydrogen peroxide (H_2O_2) (Linley *et al*., 2012). Hydrogen peroxide passes through the cell walls and membranes of susceptible bacteria, after which it reacts in the cytoplasm with internal cellular components, leading to cell apoptosis and necrosis (Denyer and Stewart, 1998). In addition, H_2O_2 damage the membrane of susceptible bacteria, inducing the release of intracellular components which leads to cell death (Linley *et al*., 2012). Hydrogen peroxide removes electrons from susceptible chemical groups, oxidising them, and becoming reduced in the process (Neyens and Baeyens 2003). Transition metal salts such as iron salts, ozone and UV light activate H_2O_2 to form hydroxyl radicals (OH^-) which are strong oxidants capable of destroying microbial cells (Neyens and Baeyens 2003; Moore and Payne 2004). The reaction with metal salts is known as the Fenton reaction, and reaction with H_2O_2 leads to the production of hydroxyl radicals, ferric iron (Fe^{3+}), hydroperoxyl radicals (HO^{2}) and/or superoxide radicals (O^{2})

which oxidises DNA, proteins and membrane lipids (Mortazavi *et al*. 2005). At low levels, bacteria have evolved numerous mechanisms to prevent the detrimental effects of H_2O_2, however, at high concentrations, these defence mechanisms become overwhelmed which leads to extracellular and intracellular damage (Russell, 2003). Gram-negative bacteria are generally more resistant to hydrogen peroxide than Gram-positive bacteria, with bacterial spores and mycobacteria being resistant (McDonnell and Russell 1999; Maillard, 2002).

Some probiotic bacteria produce antimicrobial peptides which modulate the composition of the microbiome, by killing pathogenic and sensitive bacteria, thereby promoting colonisation of the producing strain and non-susceptible bacteria (Eijsink *et al*., 2002; Spinler *et al*. 2008; O'Shea *et al*. 2011; Zheng *et al*., 2015). Bacteriocins are small, bacterially produced peptides that possess antimicrobial activity (Klaenhammer, 1993; Cotter *et al*., 2005). The ability of bacteriocin-producing microorganisms to inhibit pathogens *in vitro* has been well documented, however, studies involving *in vivo* protection are scarce (Dobson *et al*., 2012). Bacteriocins may function as colonizing peptides, facilitating the introduction and/or dominance of the producing strain into an already occupied niche (Riley and Wertz, 2002), or may act directly as an antimicrobial inhibiting pathogenic strains (Majeed *et al*., 2011), or function as signalling molecules, either signalling other bacteria, or signalling cells of the host immune system (Gobbetti *et al*., 2007; Meijerink *et al*., 2010). Bacterial communication occurs via extracellular diffusible signalling molecules (metabolites, i.e. SCFA, H_2O_2 and antimicrobial peptides) which allows the bacterial population to synchronise grouped behaviour facilitating coordinated multicellular functionality of the GIT microbiome (Gillor and Ghazaryan, 2007). In Gram-negative bacteria, (*N*-acyl) homoserine lactone typically serves as a signal molecule, while in Gram-positive bacteria, peptides, including some bacteriocins, frequently serve as signalling molecules (Sturme *et al*., 2002). Some bacteriocins thus have a dual role, acting as both inhibitors of susceptible bacteria and as signalling molecules (Fajardo and Martinez, 2008).

Probiotic bacteria also modulate the GIT microbiome composition by producing exopolysaccharides (EPS) (Salazar *et al*., 2009). In addition to being critical for survival, EPS are also important molecules in host–microbe interactions (Conover *et al*., 2010). Probiotic-derived EPS can indirectly modulate the microbiome composition by selectively enhancing the growth of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* by serving as nutrient sources for bacterial growth (Salazar *et al*., 2009; Harutoshi, 2013).

The question that remains unanswered is the role probiotics play in modulating the host microbiome. Due to the lack in understanding the role different members of the microbiome play in host physiology, and since the composition of a healthy microbiome is unknown, understanding the induced microbiome changes and what effect it has on broiler health and performance are not well understood (Bäckhed *et al*., 2012). In addition, it is difficult to compare microbiome studies as the GIT microbiome is affected by numerous host and environmental factors (Sanders, 2016). Currently, little is known about how the avian microbiome develops or the factors that affect its composition, and how changes in taxonomic composition relate to changes in metabolic functioning and morphological development (Sanders, 2016). Large taxonomic variability exists in the microbiome of individuals, despite conservation of metabolic function (Van der Wielen *et al*., 2002; Danzeisen *et al*., 2011). Although the

composition of a healthy microbiome remains to be elucidated; it appears that the establishment of a mature microbiome by probiotics positively impacts the host (Patterson, 2011). With the advancement in technologies, future research will elucidate the precise role probiotics play in modulating the microbiome and what effects it has on broiler health and growth performance (Oakley *et al*., 2014).

Modulation of broiler physiology

Probiotic bacteria modulate broiler physiology (Figure 4) by regulating GIT barrier integrity, modulating cell proliferation and apoptosis, regulating bone health, influencing brain signalling via the brain-gut-microbiome axis and improving carcass meat characteristics (Howarth and Wang, 2013; Lambert *et al*., 2017; Yue *et al*., 2017).

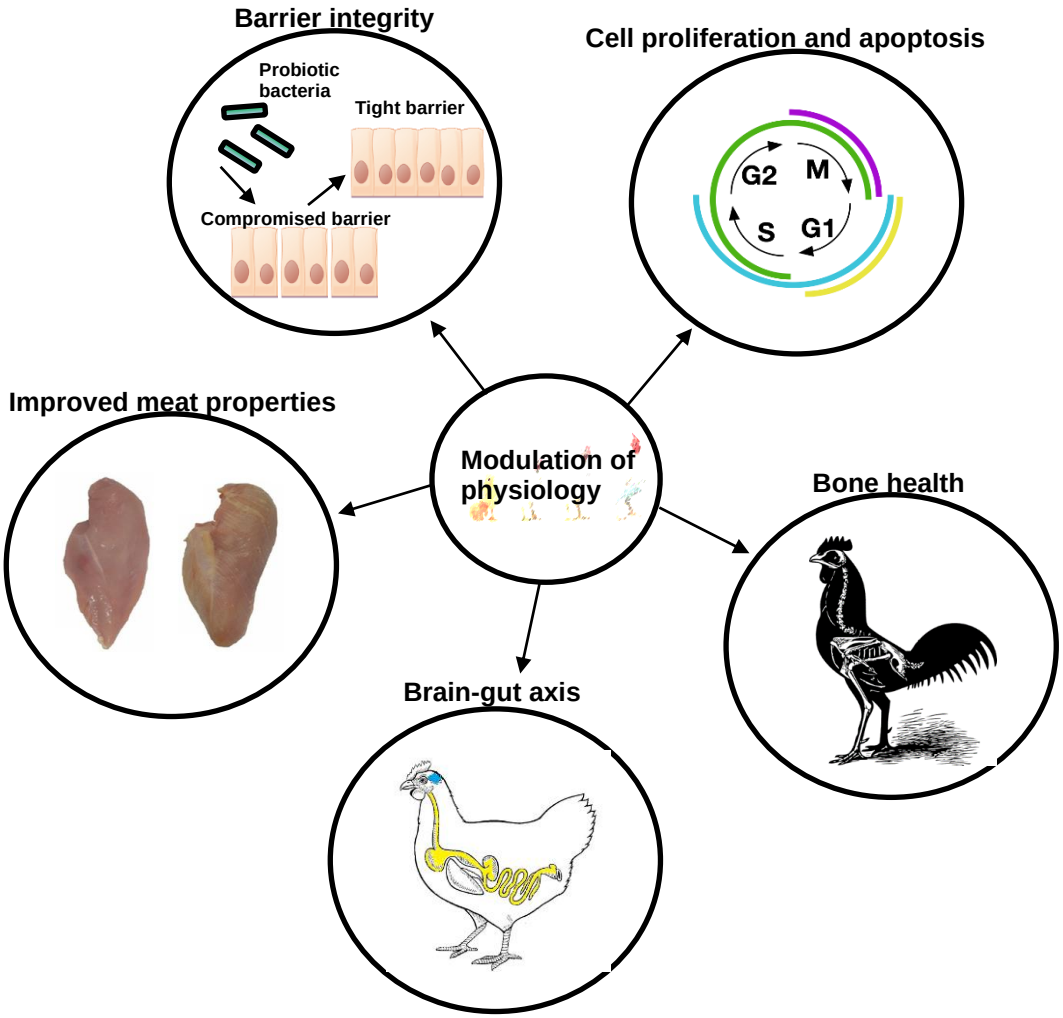

Figure 4: Modes of action by which probiotics modulate broiler physiology.

The epithelial monolayer forms a barrier between the luminal content and the interstitial tissue, which prevents diffusion of harmful content from the lumen into the tissue which eventually ends up in the systemic circulatory system (Anderson and Van Itallie, 1995). Maintenance of the GIT barrier

integrity is critical in preventing translocation of pathogenic bacteria into the intestinal lumen, which leads to immunological stress and disease (Dignass, 2001; Rao and Samak, 2013). Probiotic bacteria regulate epithelial permeability by modulating tight junction (TJ) proteins, inhibiting pathogen colonisation, modulating cell proliferation and apoptosis, and mucin production (Corridoni *et al*., 2012; Howarth and Wang, 2013). Gut permeability is controlled via regulation TJ protein (occludin, zonula occludens, claudins and junction adhesion molecules) expression (Harhaj and Antonetti, 2004; Howarth, 2010; Zhou *et al*., 2012). These proteins are located at the sub-apical aspect of the lateral membranes and build the physical connections between cells (Zhou *et al*., 2012). Increased permeability leads to mucosal barrier dysfunction; however, probiotics can normalise TJ protein expression and/or localisation restoring barrier integrity (Anderson *et al*., 2010; Corridoni *et al*., 2012). Tight junction assembly is dependent on the activation of MAPK signalling pathways (Fanning *et al*., 1998). Intestinal permeability is modulated by probiotics by stimulating Toll-like receptors on epithelial surfaces, leading to the activation of important signalling cascades such as mitogen-activated protein kinases (MAPK) and NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) pathways (Zhang *et al*., 2005; Ewaschuk *et al*., 2008; Karczewski *et al*., 2010; Llewellyn and Foey, 2017). Mitogen-activated protein kinases consist of serine-threonine protein kinases such as extracellular signal-related kinases (ERK), c-Jun amino-terminal kinases (JNK) and p38 (Dent *et al*., 2003). Activation of ERK and JNK results in activation and nuclear translocation of the transcription factor activator protein 1 (AP-1) which induces transcription of pro-inflammatory genes (Llewellyn and Foey, 2017). Probiotic bacteria indirectly enhance epithelial barrier integrity by preventing pathogen-induced barrier damage (Bron *et al*., 2017). Metabolites produced by probiotics such as lactic acid, acetic acid and butyric acid may enhance barrier integrity by regulating the expression and assembly of TJ proteins (Putaala *et al*., 2008; Peng *et al*., 2009). Probiotics also produce polyphosphates and cytoprotective molecules (heat-shock proteins) which enhance barrier integrity by suppressing oxidative stress-induced barrier damage by MPK dependent mechanisms (Zhang *et al*., 2005; Karczewski *et al*., 2010; Segawa *et al*., 2011). Additionally, probiotic-derived proteins p40 and p75 protect barrier integrity from oxidative damage by inhibiting hydrogen peroxide-induced redistribution of TJ and adherence junction proteins by activating protein kinase C (PKC) and MAPK signalling pathways (Seth *et al*., 2008).

A balance exists between epithelial cell proliferation, differentiation and apoptosis (Llewellyn and Foey, 2017). Probiotics can modulate cell kinetics by regulating homeostasis of cell proliferation and apoptosis (Howarth and Wang, 2013). Apoptosis is a cell suicide mechanism to control cell numbers and to eliminate old cells, however, unscheduled and uncontrolled apoptosis is detrimental to the host (Ashkenazi and Dixit, 1998). In addition, an increase in apoptosis over cell proliferation leads to increased susceptibility to pathogenic infections (Prisciandaro *et al*., 2011). Numerous researchers have shown that probiotics regulate transcription of genes related to cell proliferation and apoptosis (Zhang *et al*., 2005; Ko *et al*., 2007; Howarth and Wang, 2013). In the GIT of broilers, probiotic administration has been shown to regulate villus cell proliferation (Sun *et al*., 2016). The structure and architecture of villi are important as their morphology regulates the capacity of nutrients absorption and defence responses against pathogens (Sun *et al*., 2016). Probiotics regulate cytokine and oxidant-induced epithelial apoptosis by proteins p75 and p40 which activates anti-apoptotic Akt in a

phosphatidylinositol-3'-kinase (PI3K)-dependent manner and inhibiting pro-apoptotic p38/MAPK activation (Yan and Polk, 2002; Yan *et al*., 2007). Reduced apoptosis improves barrier integrity and increases resistance to bacterial invasion (Hausmann, 2010). In addition, numerous other studies have shown that probiotics decrease cell apoptosis by differentially expressing apoptosis inhibitor-related proteins, i.e. HIAP2/cIAP, TLR-2, COX2, and PGE2 proteins (Khailova *et al*., 2010; Dykstra *et al*., 2011).

Probiotic bacteria also modulate intestinal barrier integrity by modulating mucin production (Tsirtsikos *et al*., 2012; Howarth and Wang, 2013). Mucins are the major protein component coating the GIT. Mucins are highly glycosylated macromolecules excreted by goblet cells (Stringer *et al*., 2009). Mucins form a protective layer covering the epithelial surface, protecting against bacterial overgrowth and acting as binding sites for microorganisms (Specian and Oliver, 1991; Robbe *et al*., 2004). Probiotic bacteria degrade glycoprotein linkages within mucin to facilitate contact with epithelial cells (González-Rodríguez *et al*., 2012). Probiotics normalise intestinal integrity through the restoration of the mucus layer by adjusting the mucin monosaccharide composition, mucus layer thickness, and mucin gene expression (O'Callaghan *et al*., 2012; Tsirtsikos *et al*., 2012; Howarth and Wang, 2013). The structural and functional properties of mucins influence bacterial adhesion to the mucosal surface. In broilers, probiotics modulate intestinal mucin monosaccharide compositions, subsequently influencing the GIT microbiome composition (Tsirtsikos *et al*., 2012).

Until recently, the role probiotics play in bone health remained largely unknown (Lambert *et al*., 2017; Quach and Britton, 2017). Detrimental microbiota shifts in the GIT microbiome leads to dysbiosis which decreases bone density (Sylvester, 2017). Probiotics are capable of modulating bone mineralisation and development by impacting multiple aspects (Nahashon *et al*., 1994; Mutuş *et al*., 2006). Probiotics modulate bone health by impacting nutrient acquisition important for bone growth (Ca^{2+} and P^{3-}), modulate barrier integrity and immune responses, and by the production of serotonin or estrogen-like molecules (Lavoie *et al*., 2017; Ramsey and Isales, 2017). Probiotics indirectly modulate bone health by regulating microbiome health leading to increased intestinal absorption of minerals (Ca^{2+} and P^{3-}) important for bone health (Christakos *et al*., 2017). In addition, probiotics can also regulate bone health by neuroendocrine signalling pathways inducing intestinal cells to produces endocrine factors such as incretins, estrogen-like molecules and serotonin which acts as signals for bone cells (Lavoie *et al*., 2017; Ramsey and Isales, 2017). In broilers, probiotics increase bone thicknesses and improve the mineral content and bone breakage strength (Plavnik and Scott, 1980; Mutuş *et al*., 2006).

Extensive communication occurs between the brain and the GIT microbiome via the brain-gut-microbiome axis which consists of the central nervous system (CNS), neuroendocrine and neuroimmune systems, sympathetic and parasympathetic branches of the autonomic nervous system, the enteric nervous system (ENS), and the intestinal microbiome (Zhou & Foster, 2015; Sherwin *et al*., 2016; Dinan and Cryan, 2017). This axis has been extensively studied in mammals, but rarely in birds. Due to the high similarities between mammals and birds, it is highly likely that an avian gut-brain axis exists. Communication along the axis is bidirectional, and signals from the brain regulate the motor, sensory, and secretory functions of the intestine, and visceral messages from the gut influences brain function (Dinan *et al*., 2015). Disruption of homeostasis in the gut–brain axis leads to GIT and psychiatric disorders (Neufeld and Foster, 2009). Probiotic bacteria can secrete numerous

neurotransmitters and modulators (short-chain fatty acids, γ-aminobutyric acid, serotonin, catecholamines, dopamine, noradrenaline and acetylcholine) which induce epithelial cells to release molecules that modulate neural signalling within the ENS and, consequently, impact brain function and behaviour, and the feedback from the brain to ENS regulates the microbiome composition and metabolic function (Cryan and O'Mahony, 2011; Lyte, 2011; Furness *et al*., 2014). Probiotic bacteria may also influence the brain-gut-microbiome axis by regulating tryptophan metabolism (Yue *et al*., 2017). Tryptophan is the sole precursor of serotonin which regulates GIT motility and intestinal secretions, and aggressive behaviour and stress response (O'Mahony *et al*. 2015; Puglisi-Allegra and Andolina 2015). Probiotics stimulate enterochromaffin cells to express tryptophan hydroxylase leading to increased serotonin synthesis (Reigstad *et al*., 2015; Yano *et al*., 2015). In broilers, probiotics alleviate heat stress by inhibiting both systemic and brain inflammation, decrease detrimental social behaviours (cannibalism, aggression, and feather pecking) and modulate emotional reactivity and memory via the brain-gut-microbiome axis (Parois *et al*., 2017).

The role probiotics play in improving broiler carcass meat characteristics is highly debated. Some studies have reported improved meat attributes such as water-holding capacity, tenderness, lipid oxidation stability, sensory properties, and microbial safety (Aksu *et al*., 2005; Yang *et al*., 2010). On the contrary, others have noted that there are no synergistic effects of probiotics on meat quality (Zhang *et al*., 2012; Kim *et al*., 2016). Disagreements might be due to the differences in experimental conditions or probiotic compositions used. Probiotic administration in broilers improves the chemical, nutritional and sensorial characteristics of meat by increasing protein and free amino acid content, decreasing fat content and improving sensory properties (Liu *et al*., 2012; Abdulla *et al*., 2017). Pathogenic bacteria negatively affect meat quality by negatively impacting growth (Wang *et al*., 2017). In broilers infected with pathogens, probiotic administration improved meat quality by decreasing pathogen-induced gut permeability (Wang *et al*., 2017). A proteomic study found that probiotics affect meat quality by improving meat colour, water holding capacity, pH and decreasing abdominal fat content (Zheng *et al*., 2014). Altered expression of cytoskeletal and chaperone proteins contributed to the improved water holding capacity and meat colour, while increased pH was due to down-regulation of β-enolase and pyruvate kinase muscle isozymes (Zheng *et al*., 2014).

Immune modulation

The avian immune system is similar to the mammalian system consisting of the innate and adaptive immune responses. The innate immune response is the first line of defence and acts via non-specific defence mechanisms, whereas the adaptive immune system consists of specific defence mechanism acting via humoral and cell-mediated responses. The innate immune system protects the host by inducing inflammation in the presence of foreign compounds and primes the adaptive immune system to respond to these antigens (Yan and Polk, 2011). Probiotics regulate immune responses by interacting as immune activators or suppressors within the GIT and gut-associated lymphoid tissue (GALT) (Hardy *et al*., 2013; Llewellyn and Foey, 2017). They regulate the innate and adaptive immune system by secreting metabolites which act as signalling molecules (Delcenserie *et al*. 2008), inhibit

pathogens (Hardy *et al*., 2013), and regulate the functions of dendritic cells, macrophages, and T and B lymphocytes (Cosseau *et al*., 2008; Hooper and Macpherson, 2010; Santos Rocha *et al*., 2012).

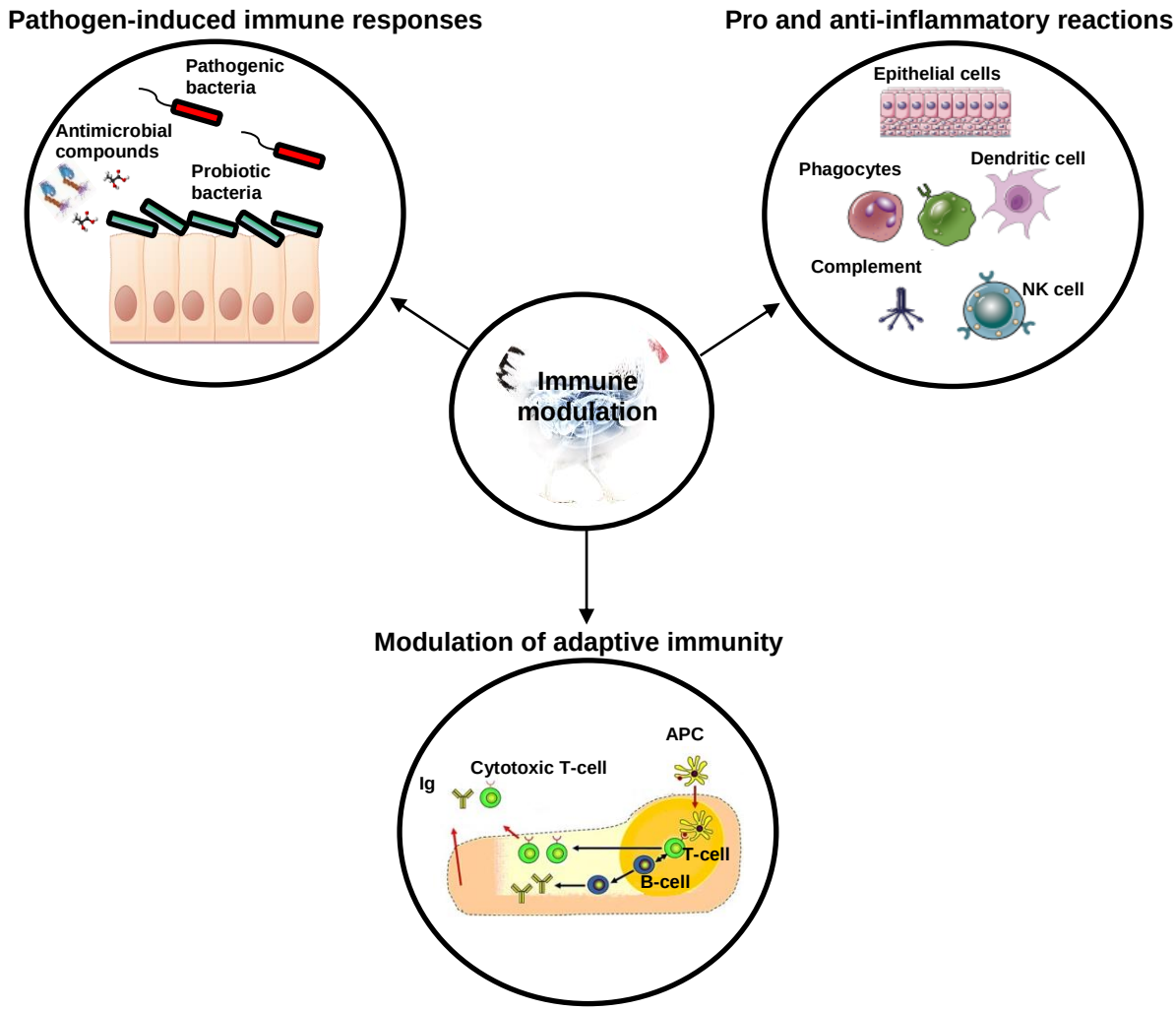

Figure 5: Modes of action by which probiotics modulate the immune system.

Probiotics stimulate various aspects of the innate immune system by up-regulating innate immune responses and enhancing antimicrobial defences and phagocytosis (Delcenserie *et al*. 2008; Adhikari and Kim, 2017; Llewellyn and Foey, 2017). Cells of the innate immune system produce cytokines in response to antigens, essential for inducing inflammation and critical for initiation of adaptive immune responses (Llewellyn and Foey, 2017). Probiotics can indirectly regulate the immune system by inhibiting pathogen colonisation (Hardy *et al*., 2013). Immune cells sense antigens (bacteria and their metabolites) via pattern recognition receptors (PRRs) on their cell surface (Akira and Hemmi, 2003). Intestinal epithelial cells such as T cells, monocytes, and antigen presenting cells (dendritic cells, B cells, and macrophages) contain PRRs to sense foreign antigens (Fong *et al*., 2016). Numerous PRRs have been identified which include toll-like receptors (TLR), membrane-bound C-type lectin receptors (CLRs), cytosolic proteins like NOD-like receptors (NLRs) and RIG-1–like receptors (RIRs) (Elinav *et al*., 2011; Osorio *et al*., 2011; Kang and Im, 2015; Jiménez-Dalmaroni *et al*., 2016). Probiotics

may bind to these PRRs and stimulate immune cells to secrete various cytokines such as interleukin-1 (IL-1), IL- 6 and tumour necrosis factor alpha (TNFα) (Manicassamy and Pulendran, 2009).

Antigen-presenting cells (APC) in the Payers patches of the GIT sense foreign antigens leading to cytokine release initiating the inflammatory response (Miettinen *et al*., 1996; Veckman *et al*., 2004). In addition, internalisation of antigens in APC induces cell maturation were antigens are degraded into peptides and loaded onto major histocompatibility complex (MHC) intracellularly and expressed together with lymphocyte costimulatory molecules (CD80 and CD 86) on the cell surfaces (Banchereau and Steinman, 1998). Mature APC migrates to lymphoid organs to secrete cytokines modulating the activity of T-cell adaptive immunity (Schnare *et al*., 2001; Ma *et al*., 2009). In addition, probiotics crosstalk with the immune system via Microfold cells (M-cells) in the GALT of the Peyer's patches and in the mucosa-associated lymphoid tissue (MALT) (Llewellyn and Foey, 2017). These cells transport antigens from the lumen to the lymphoid tissues within the mucosa, initiating mucosal immune responses (Neutra, 1998; Corr *et al*., 2008). Phagocytosis by phagocytes (heterophils, monocytes, macrophages, mast cells, and dendritic cells) are responsible for early activation of the inflammatory responses which releases toxic compounds such as ROS and lytic enzymes, and further recruitment of immunocompetent cells (Rabson *et al*., 2005).

Once the innate immune cells have sensed the presence of microbes via microbe-associated molecular pattern (MAMPs) binding to PRRs, activation of NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling pathway occurs leading to the expression of pro-inflammatory cytokines, chemokines and antimicrobial peptides (Kawai and Akira, 2007). Antigen binding to the TLRs leads to the recruitment of intracellular adaptor proteins MyD88 (myeloid differentiation primary response gene 88), TIRAP/MAL (TIR domain containing adaptor protein) and TRIF (toll receptor—IL-1 receptor factor) which contain a toll-IL-1 receptor (TIR) domain (Muzio *et al*., 1998; Akira *et al*., 2006; Muzio *et al*., 2013). These adaptor proteins interact with the receptor through TIR-TIR binding, recruiting interleukin receptor-associated kinases (IRAK-1,2 and -4) and TNF receptor-associated factor 6 (TRAF6) (Akira *et al*., 2006). Recruitment of these factors leads to the activation of the mitogen-activated protein kinases (MAPK) such as extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinases (JNK) and p38 protein kinase, and transcription factors NFκB and AP-1 (activator protein 1) (Manicassamy and Pulendran, 2009). Activation of transcription factors NFκB and AP-1 lead to the transcription of pro-inflammatory (IL1β, IL-6, IL-8, TNFα) and anti-inflammatory (IL-10) cytokines and anti-viral type 1 interferons (IFNα, IFNβ) (Manicassamy and Pulendran, 2009; Kawai and Akira, 2011). Depending on the probiotic strain, the strain can either induce immune activation by signalling for the transcription of IL-12, IL-1β, and TNF-α or trigger tolerance signalling by stimulating anti-inflammatory cytokine transcription, i.e. IL-10 and TGF-β levels (Llewellyn and Foey, 2017).

The NFκB pathway is an important signalling cascade for the activation of numerous immune responses, which regulate transcription of pro-inflammatory cytokine TNFα and the heterophil chemokine IL-8 (Karin and Ben-Neriah, 2000). Probiotics regulate pro-inflammatory responses by modulating various aspects of the TLR4/ NFκB signalling pathways (Donato *et al*., 2010; Liu *et al*., 2011b). In immune cells, probiotics increase TLR expression, leading to higher NFκB responses, increasing cytokine production, up-regulating co-stimulatory molecules on APC, and activating T cells

(Medzhitov and Janeway, 2000; Akira and Hemmi, 2003). Probiotics inhibit inflammation by negatively modulating regulators of TLR4 (A20 and Bcl-3) and upregulating B-cell lymphoma 3 (Bcl-3) protein which stabilises the repressive NFκB homodimers (Xiao *et al*., 2007; Takanashi *et al*., 2013). Probiotics can also attenuate pro-inflammatory responses by inhibiting NFκB and p38 MAPK signalling pathways, resulting in reduced expression of IL-6, IL-8, IL-1β and MCP-1 (monocyte chemoattractant protein-1) (Takanashi *et al*., 2013). Probiotics can also decrease inflammation by negatively regulating TLR4 signalling via up-regulation of Toll-interacting protein (Tollip) which inhibits TLR adaptor proteins, down-regulating expression of NFκB (Takanashi *et al*., 2013). Probiotics can also reduce inflammation by preventing pathogen-induced activation of TLR4 and MyD88, and the phosphorylation of IKKα, IKKβ, IκBα and NFκB subunit p65, inhibiting inflammatory cytokines production (Finamore *et al*., 2014). Probiotics can also modulate proinflammatory responses by suppressing NFκB inhibitor IκB (inhibitor of kappa B) degradation by bacterial reactive oxygen species (ROS) (Kumar *et al*., 2007). Reactive oxygen species oxidatively inactivates the Ubc12 enzyme responsible for the ubiquitination of the inhibitory molecule IκB. Therefore, IκBα is not targeted for proteasomal degradation suppressing NFκB activation (Kumar *et al*., 2007; Lin *et al*., 2009). Probiotics can stimulate the production of the anti-inflammatory cytokine IL-10 initiating anti-inflammatory responses. Probiotics bind to receptors and activates the JAK1/STAT3 (Janus kinase-signal transducers and activators of transcription) pathway, where STAT3 (Signal transducer and activator of transcription 3) is activated by phosphorylation, inhibiting the expression of pro-inflammatory genes and up-regulating genes from the suppressor of cytokine synthesis (SOCS) family (Chau *et al*., 2009; Hutchins *et al*., 2013).

Probiotics also modulate the activity of natural killer (NK) and mast cells (Llewellyn and Foey, 2017). Natural killer cells are cytotoxic lymphocytes which release granules consisting of perforin and proteases upon antigen stimulation, triggering both the innate and adaptive immune responses (Juul-Madsen *et al*., 2014). Following activation and phagocytosis, cytotoxic lymphocytes present antigen processed fragment to B and T lymphocytes thereby directing the development of the adaptive immune system (Juul-Madsen *et al*., 2014). Mast cells are important for mucosal surveillance of foreign compounds and release histamine and heparin which control intestinal functions such as epithelial secretion, integrity and function and may act as inflammatory cells expressing IL-13 and CD40 which regulates B-lymphocyte function (Lorentz *et al*., 2000; Bischoff and Krämer, 2007; da Silva *et al*., 2014).

Adaptive immune responses are the specific defence mechanism of the immune system which acts via humoral and cell-mediated responses. In broilers, the primary lymphoid organs (thymus and bursa of Fabricius) are responsible for lymphocyte production and development, and once developed these cells travel to secondary lymphoid organs (spleen and mucosal-associated lymphoid tissues) where they encounter antigens and differentiate into effector cells (Fong *et al*., 2016). Probiotics modulate the adaptive immune system by stimulating the innate immune system which dictates the development of the humoral and cell-mediated systems such as B and T lymphocytes development (Nayebpor *et al*., 2007; Fong *et al*., 2016).

The adaptive immune system is primarily mediated by B lymphocytes which produce immunoglobulins that disable antigens by opsonisation for phagocytosis, neutralisation, and complement activation (Juul-Madsen *et al*., 2014). Avian antibody arsenal consists of immunoglobulin

M (IgM), IgG, and IgA (Davison *et al*., 2008). In the GIT IgA plays a critical role in mucosal immunity (Fagarasan and Honjo, 2003; Macpherson and Uhr, 2004). Probiotics modulate antibody production in antigen-primed dendritic cells which migrate to mesenteric lymph nodes (MLN) to differentiate naïve CD4+ Th0 cells into various Th subpopulations depending on the cytokine secretion pattern (Martin-Fontecha *et al*., 2003; Macpherson and Uhr, 2004). In addition, specialised dendritic cells in the Peyer's patches extend their protrusions into the intestinal lumen to capture microbes by endocytosis, phagocytosis, and micropinocytosis (Niess *et al*., 2005; Ohnmacht *et al*., 2009). Upon capturing, cells transport these antigens across the epithelium were cells mature and derive B cells into plasma cells, leading to immunoglobulin production (Niess *et al*., 2005; Ohnmacht *et al*., 2009). Probiotics induce maturation and cytokine expression in dendritic cells which leads to activation of T helper 1 (Th1), Th2 or Th3 (Christensen *et al*., 2002; Hart *et al*., 2004). Furthermore, probiotics modulate the adaptive immune system by M-cell uptake, where probiotics interact with APC in MLN to activate naive plasma cells into becoming IgA-producing B cells. These cells coat the mucosal surface with antibodies to control microbial penetration (Macpherson and Uhr, 2004).

Cell-mediated immunity is controlled by T lymphocytes such as CD4+ (helper T cells) and CD8+ cells (cytotoxic T cells) (Smith and Göbel, 2014). Helper T cells are activated by recognition of a class II MHC coupled with processed antigens on APC (Janeway *et al*., 2001). Once activated, CD4+ cells divide and produce a variety of cytokines that activate B and T lymphocytes, as well as other immune cells (Janeway *et al*., 2001). Proliferating CD4+ cells differentiate into one of two major subtypes, i.e. Type 1 and Type 2 helper T cells (TH1 and TH2, respectively) (Kaiko *et al*., 2008). The TH1 cells produce cytokines which encourage inflammation and activation of macrophages, and B and T lymphocytes (Kaiko *et al*., 2008). Cytokines also inhibit the function of TH2 cells, to elicit immune responses to intracellular pathogens (Kaiko *et al*., 2008). Cytokines secreted by TH2 cells stimulate B lymphocyte proliferation and antibody production, while inhibiting the activity of TH1 cells enhances the immune response to extracellular pathogens (Kaiser and Stäheli, 2014). Cytotoxic T cells are responsible for the recognition and lysis of cells infected with pathogens in association with a class I MHC molecule (Fong *et al*., 2016). Activated cytotoxic T lymphocytes promote cell-mediated immune response and induce phagocytosis together with TH1 cells (Fong *et al*., 2016).

Conclusions

The role probiotics play in improving broiler health and performance has thus far remained speculative as showing the exact function in a complex host which is affected by numerous environmental factors remains difficult. Although probiotics have been recognised to possess numerous therapeutic properties and participate in various host functions, the precise molecular mechanisms behind positive effects remain largely unknown. With the advent of high-throughput analysis techniques such as metagenomics, proteomics and metabolomics insight into the role probiotics play in modulating broiler health and growth performance will be gained. Nevertheless, researchers have suggested that probiotics can influence broilers performance by modulating nutrition and digestion, GIT microbiome composition, host physiology and the immune system. Probiotics modulate nutrition and digestion by

producing digestive enzymes or stimulating host enzyme production, by producing vitamins critical for various biochemical pathways, regulate cholesterol levels, prevent oxidative damage by producing antioxidants, and assists in mitochondrial energy production. Probiotics modulate the GIT microbiome by competitively excluding pathogens for the GIT surface, compete for nutrients, produce antimicrobial compounds inhibiting pathogen growth, and produce metabolites which selectively enhance the growth of beneficial bacteria. Probiotics modulate broiler physiology by regulating GIT barrier integrity, homeostasis of cell proliferation and apoptosis, enhance bone health, regulate brain signalling via the brain-gut-microbiome axis, and improve carcass meat characteristics. Probiotics modulate the immune system by inhibiting pathogen-induced immune responses, control proinflammatory and anti-inflammatory reactions, and modulate the adaptive immune system. Although there is suggestive evidence for each of these therapeutic benefits, the exact molecular mechanism remains poorly understood. Future research is required to better understand the role probiotics play in improving broiler health and growth performance. These include defining the composition of a healthy broiler microbiome, determining the role members of the microbiome play in host physiology, elucidating the molecular crosstalk which occurs between probiotics and epithelial cells, and determine under which physiological circumstances probiotic consumption become beneficial for the host. Elucidating these unknowns will provide researchers with greater insight into the molecular mechanisms behind probiotic therapeutic benefits.

References

Abdulla, N.R., Zamri, A.N.M., Sabow, A.B., Kareem, K.Y., Nurhazirah, S., Ling, F.H., Sazili, A.Q., Loh, T.C. (2017). Physico-chemical properties of breast muscle in broiler chickens fed probiotics, antibiotics or antibiotic-probiotic mix. *J. Appl. Anim. Res.* 45(1), p 64-70, DOI: 10.1080/09712119.2015.1124330.

Adhikari, P.A., Kim, W.K. (2017). Overview of prebiotics and probiotics: Focus on performance, gut health and immunity- A review. *Ann. Anim. Sci.* 17(4), p 949-966, DOI: 10.1515/aoas-2016-0092.

Ahmad, I. (2006). Effect of probiotics on broilers performance. *Int. J. Poult. Sci.* 5(6), p 593-597, DOI: 10.3923/ijps.2006.593.597.

Ajuwon, K.M. (2016). Toward a better understanding of mechanisms of probiotics and prebiotics action in poultry species. *J. Appl. Poult. Res.* 25(2), p 277-283, DOI: 10.3382/japr/pfv074.

Akira, S., Hemmi, H. (2003). Recognition of pathogen-associated molecular patterns by TLR family. *Immunol. Lett.* 85(2), p 85-95, DOI: 10.1016/S0165-2478(02)00228-6.

Akira, S., Uematsu, S., Takeuchi, O. (2006). Pathogen recognition and innate Immunity. *Cell* 124(4), p 783–801, DOI: 10.1016/j.cell.2006.02.015.

Aksu, M.İ., Karaŏlu, M., Esenbŭa, N., Kaya, M., Macit, M., Ockerman, H.W. (2005). Effect of a dietary probiotic on some quality characteristics of raw broiler drumsticks and breast meat. *J. Muscle Foods* 16(4), p 306-317, DOI: 10.1111/j.1745-4573.2005.00023.x.

Alakomi, H-L., Puupponen-Pimiä, R., Aura, A-M., Helander, I.M., Nohynek, L., Oksman-Caldentey K-M., Saarela, M. (2007). Weakening of *Salmonella* with selected microbial metabolites of berry-

derived phenolic compounds and organic acids. *J*. *Agric*. *Food Chem*. (2007) 55(10), p 3905–3912, DOI:10.1021/jf070190y.

Alakomi, H-L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., Helander, I.M. (2000). Lactic acid permeabilizes Gram-negative bacteria by disrupting the outer membrane. *Appl*. *Environ*. *Microbiol*. 66(5), p 2001–2005, DOI:10.1128/ AEM.66.5.2001-2005.2000.

Alloui, M.N., Szczurek, W., Świątkiewicz, S. (2013). The usefulness of prebiotics and probiotics in modern poultry nutrition: a review. Ann. Anim. Sci. 13(1), p 17-32, DOI: 10.2478/v10220-012-0055-x.

Amerah, A.M., Jansen van Rensburg, C., Plumstead, P.W., Kromm, C., Dunham, S. (2013). Effect of feeding diets containing a probiotic or antibiotic on broiler performance, intestinal mucosa-associated avian pathogenic *E*. *coli* and litter water-soluble phosphorus. *J*. *Appl*. *Anim*. *Nutr*. 1(7), p 1-7, DOI: 10.1017/jan.2013.4.

Anderson, J.M., Van Itallie, C.M. (1995). Tight junctions and the molecular basis for regulation of paracellular permeability. *Am*. *J*. *Physiol*. 269(4 Pt 1), G467–75, DOI: 10.1152/ajpgi.1995.269.4.G467.

Anderson, R.C., Cookson, A.L., McNabb, W.C., Park, Z., McCann, M.J., Kelly, W.J., Roy, N.C. (2010). *Lactobacillus plantarum* MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. *BMC Microbiol*. 10, Article ID: 316, DOI: 10.1186/1471-2180-10-316.

Annison, E.F., Hill, K.J., Kenworthy, R. (1968). Volatile fatty acids in the digestive tract of the fowl. *Br*. *J*. *Nutr*. 22(2), p 207–216, DOI:10.1079/BJN19680026.

Apajalahti, J., Kettunen, A., Graham, H. (2004). Characteristics of the gastrointestinal microbial communities, with special reference to chicken. *Worlds Poult*. *Sci*. *J*. 60(2), p 223–32, DOI:10.1079/WPS200415.

Ashkenazi, A.; Dixit, V.M. (1998). Death receptors: Signalling and modulation. *Science* 281(5381), p 1305–1308, DOI: 10.1126/science.281.5381.1305.

Azcárate-Peril, M. A.; Sikes, M.; Bruno-Bárcena, J. M. (2011). The intestinal microbiota, gastrointestinal environment and colorectal cancer: a putative role for probiotics in prevention of colorectal cancer? *Am*. *J*. *Physiol*. *Gastrointest*. *Liver Physiol*. 301(3), G401−G424, DOI: 10.1152/ajpgi.00110.2011.

Bäckhed, F., Fraser, C.M., Ringel, Y., Sanders, M.E., Sartor, R.B., Sherman, P.M., Versalovic, J., Young, V., Finlay, B.B. (2012). Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host Microbe* 12(5), p 611–622, DOI: 10.1016/j.chom.2012.10.012.

Banchereau, J., Steinman, R.M. (1998). Dendritic cells and the control of immunity. *Nature* 392(6673), p 245–252, DOI: 10.1038/32588.

Baurhoo, B., Ferket, P.R., Zhao, X. (2009). Effects of diets containing different concentrations of mannanoligosaccharide or antibiotics on growth performance, intestinal development, cecal and litter microbial populations, and carcass parameters of broilers. *Poult*. *Sci*. 88(11), p 2262-2272, DOI: 10.3382/ps.2008-00562.

Bedford, M.R., Schulze, H. (1998). Exogenous enzymes for pigs and poultry. *Nutr*. *Res*. *Rev*. 11(1), p 91–114, DOI: 10.1079/NRR19980007.

Bischoff, S.C., Krämer, S. (2007). Human mast cells, bacteria, and intestinal immunity. *Immunol*. *Rev*. 217(1), p 329–337, DOI: 10.1111/j.1600-065X.2007.00523.x

Boland, M.J., Rae, A.N., Vereijken, J.M., Meuwissen, M.P.M., Fischer, A.R.H., van Boekel, M.A.J.S., Rutherfurd, S.M., Gruppen, H., Moughan, P.J., Hendriks, W.H. (2013). The future supply of animal-derived protein for human consumption. *Trends Food Sci*. *Technol*. 29(1), p 62-73, DOI: 10.1016/j.tifs.2012.07.002.

Bolton, W., Dewar, W.A. (1965). The digestibility of acetic, propionic and butyric acids by the fowl. *Br*. *Poult*. *Sci*. 6(2), p 103–105, DOI:10.1080/00071666508415562.

Bron, P.A., Kleerebezem, M., Brummer, R-J., Cani, P.D., Mercenier, A., MacDonald, T.T., Garcia-Ródenas, C.L., Wells, J.M. (2017). Can probiotics modulate human disease by impacting intestinal barrier function? *Br*. *J*. *Nutr*. 117(1), p 93-107, DOI: 10.1017/S0007114516004037.

Callaway T. R. Edrington T. S. Anderson R. C. Byrd J. A. Nisbet D. J. (2008). Gastrointestinal microbial ecology and the safety of our food supply as related to *Salmonella*. *J*. *Anim*. *Sci*. 86(14), p E163–E172, DOI: 10.2527/jas.2007-0457.

Carding, S., Verbeke, K., Vipond, D.T., Corfe, B.M., Owen, L.J. (2015). Dysbiosis of the gut microbiota in disease. *Microb*. *Ecol*. *Health Dis*. 26, Article ID: 26191, DOI: 10.3402/mehd.v26.26191.

Chau, T.A., McCully, M.L., Brintnell, W., An, G., Kasper, K.J., Vinés, E.D., Kubes, P., Haeryfar, S.M., MacCormick, J.K., Cairns, E., Heinrichs, D.E., Madrenas, J. (2009). Toll-like receptor 2 ligands on the staphylococcal cell wall downregulate superantigen-induced T cell activation and prevent toxic shock syndrome. *Nat*. *Med*. 15(6), p 641–648, DOI: 10.1038/nm.1965.

Christakos, S., Veldurthy, V., Patel, N., Wei, R. (2017). Intestinal regulation of calcium: vitamin D and bone physiology. *Adv*. *Exp*. *Med*. *Biol*. 1033, p 3–12, DOI: 10.1007/978-3-319-66653-2_1.

Christensen, H.R., Frokiaer, H., Pestka, J.J. (2002). Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. *J*. *Immunol*. 168(1), p 171-178, DOI: 10.4049/jimmunol.168.1.171.

Collado, M.C., Meriluoto, J., Salminen, S. (2007). Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. *Lett*. *Appl*. *Microbiol*. 45(4), p 454–460, DOI: 10.1111/j.1472-765X.2007.02212.x.

Conover, M.S., Sloan, G.P., Love, C.F., Sukumar, N., Deora, R. (2010). The Bps polysaccharide of *Bordetella pertussis* promotes colonisation and biofilm formation in the nose by functioning as an adhesin. *Mol*. *Microbiol*. 77(6), p 1439–1455, DOI: 10.1111/j.1365-2958.2010.07297.x.

Corr, S.C., Gahan, C.C.G.M., Hill, C. (2008). M-cells: origin, morphology and role in mucosal immunity and microbial pathogenesis. *FEMS Immunol*. *Med*. *Microbiol*. 52(1), p 2-12, DOI: 10.1111/j.1574-695X.2007.00359.x.

Corridoni, D., Pastorelli, L., Mattioli, B., Locovei, S., Ishikawa, D., Arseneau, K.O., Chieppa, M., Cominelli, F., Pizarro, T.T. (2012). Probiotic bacteria regulate intestinal epithelial permeability in experimental ileitis by a TNF-dependent mechanism. *PLoS ONE* 7, Article ID: e42067, DOI: 10.1371/journal.pone.0042067.

Cosseau, C., Devine, D.A., Dullaghan, E., Gardy, J.L., Chikatamarla, A., Gellatly, S., Yu, L.L., Pistolic, J., Falsafi, R., Tagg, J., Hancock, R.E.W. (2008). The commensal *Streptococcus salivarius* K12 downregulates the innate immune responses of human epithelial cells and promotes host microbe homeostasis. *Infect*. *Immun*. 76(9), p 4163-4175, DOI: 10.1128/IAI.00188-08.

Cotter, P.D., Hill, C., Ross, R.P. (2005). Bacteriocins: developing innate immunity for food. *Nat*. *Rev*. *Microbiol*. 3(10), p 777–788, DOI: 10.1038/nrmicro1273.

Cryan, J.F., O'Mahony, S.M. (2011). The microbiome-gut-brain axis: from bowel to behavior. *Neurogastroenterol*. *Motil*. 23(3), p 187–192, DOI: 10.1111/j.1365-2982.2010.01664.x.

Da Silva, E.Z., Jamur, M.C., Oliver, C. (2014). Mast cell function a new vision of an old cell. *J*. *Histochem*. *Cytochem*. 62(10), p 698-738, DOI: 10.1369/0022155414545334.

Danzeisen, J.L., Kim, H.B., Isaacson, R.E., Tu, Z.J., Johnson, T.J. (2011). Modulations of the chicken cecal microbiome and metagenome in response to anticoccidial and growth promoter treatment. *PLoS ONE* 6, Article ID: e27949, DOI: 10.1371/journal.pone.0027949.

Davison, F., Magor, K.E., Kaspers, B. (2008). Structure and evolution of avian immunoglobulins. In: Davison, F., Kaspers, B., Schat, K.A. (Eds). Avian Immunology. Elsevier Ltd., London, UK, p 107-127.

Delcenserie, V., Martel, D., Lamoureux, M., Amiot, J., Boutin, Y., Roy, D. (2008). Immunomodulatory effects of probiotics in the intestinal tract. *Curr*. *Issues Mol*. *Boil*. 10(1/2), p 37-54.

den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D-J., Bakker, B.M. (2013). The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. *J*. *Lipid Res*. 54(9), p 2325–2340, DOI: 10.1194/jlr.R036012.

Dent, P., Yacoub, A., Fisher, P.B., Hagan, M.P., Grant, S. (2003). MAPK pathways in radiation responses. *Oncogene* 22, p 5885–5896, DOI: 10.1038/sj.onc.1206701.

Denyer, S.P., Stewart, G.S.A.B. (1998). Mechanisms of action of disinfectants. *Int*. *Biodeter*. *Biodegrad*. 41(3-4), p 261-268, DOI: 10.1016/S0964-8305(98)00023-7.

Dignass, A.U. (2001). Mechanisms and modulation of intestinal epithelial repair. *Inflamm*. *Bowel Dis*. 7(1), p 68–77, DOI: 10.1097/00054725-200102000-00014.

Dinan, T.G., Cryan, J.F. (2017). Gut instincts: Microbiota as a key regulator of brain development, ageing and neurodegeneration. *J*. *Physiol*. 595(2), p 489–503, DOI: 10.1113/JP273106.

Dinan, T.G., Stilling, R.M., Stanton, C., Cryan, J.F. (2015). Collective unconscious: how gut microbes shape human behavior. *J*. *Psychiatr*. *Res*. 63, p 1–9, DOI: 10.1016/j.jpsychires.2015.02.021.

Dobson, A., Cotter, P.D., Ross, R.P., Hill, C. (2012). Bacteriocin production: a probiotic trait? *Appl*. *Environ*. *Microbiol*. 78(1), p 1-6, DOI: 10.1128/AEM.05576-11.

Donato, K.A., Gareau, M.G., Wang, Y.J., Sherman, P.M. (2010). *Lactobacillus rhamnosus* GG attenuates interferon-gamma and tumour necrosis factor-alpha-induced barrier dysfunction and pro-inflammatory signalling. *Microbiology* 156(11), p 3288–3297, DOI: 10.1099/mic.0.040139-0.

Douglas, D.N., Pu, C.H., Lewis, J.T., Bhat, R., Anwar-Mohamed, A., Logan, M., Lund, G., Addison, W.R., Lehner, R., Kneteman, N.M. (2016). Oxidative stress attenuates lipid synthesis and increases mitochondrial fatty acid oxidation in hepatoma cells infected with hepatitis C virus. *J*. *Biol*. *Chem*. 291(4), p 1974-1990, DOI: 10.1074/jbc.M115.674861.

Ducatelle, R., Eeckhaut, V., Haesebrouck, F., Van Immerseel, F. (2015). A review on prebiotics and probiotics for the control of dysbiosis: present status and future perspectives. *Animal* 9(1), p 43-48, DOI: 10.1017/S1751731114002584.

Dykstra, N.S., Hyde, L., Adawi, D., Kulik, D., Ahme, S., Molin, G., Jeppsson, B., Mackenzie, A., Mack, D.R. (2011). Pulse probiotic administration induces repeated small intestinal *MUC3* expression in rats. *Pediatr*. *Res*. 69(3), p 206-211, DOI: 10.1203/PDR.0b013e3182096ff0.

Egert, M., De Graaf, A.A., Smidt, H., De Vos, W.M., Venema, K. (2006). Beyond diversity: functional microbiomics of the human colon. *Trends Microbiol*. 14(2), p 86–91, DOI: 10.1016/j.tim.2005.12.007.

Eijsink, V.G.H., Axelsson, L., Diep, D.B., Havarstein, L.S., Holo, H., Nes, I.F. (2002). Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie Van Leeuwenhoek* 81(1-4), p 639–654, DOI: 10.1023/A:1020582211262.

Elinav, E., Strowig, T., Henao-Mejia, J., Flavell, R.A. (2011). Regulation of the antimicrobial response by NLR proteins. *Immunity* 34(5), p 665–679, DOI: 10.1016/j.immuni.2011.05.007.

Ewaschuk, J.B, Diaz, H., Meddings, L., Diederichs, B., Dmytrash, A., Backer, J., Looijer-van Langen, M., Madsen, K.L. (2008). Secreted bioactive factors from *Bifidobacterium infantis* enhance epithelial cell barrier function. *Am*. *J*. *Physiol*. *Gastrointest*. *Liver Physiol*. 295(5), G1025–1034, DOI: 10.1152/ajpgi.90227.2008.

Fagarasan, S., Honjo, T. (2003). Intestinal IgA synthesis: regulation of front-line body defences. *Nat*. *Rev*. *Immunol*. 3(1), p 63–72, DOI: 10.1038/nri982.

Fajardo, A., Martínez, J.L. (2008). Antibiotics as signals that trigger specific bacterial responses. *Curr*. *Opin*. *Microbiol*. 11(2), p 161–167, DOI: 10.1016/j.mib.2008.02.006.

Fanning, A.S., Jameson, B.J., Jesaitis, L.A., Anderson, J.M. (1998). The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. *J*. *Biol*. *Chem*. 273(45), p 29745–29753, DOI: 10.1074/jbc.273.45.29745.

Fernandez, F., Hinton, M., Van Gils, B. (2002). Dietary mannan-oligosaccharides and their effect on chicken caecal microflora in relation to *Salmonella* Enteritidis colonisation. *Avian*. *Pathol*. 31(1), p 49–58, DOI: 10.1080/03079450120106000.

Finamore, A., Roselli, M., Imbinto, A., Seeboth, J., Oswald, I.P., Mengheri, E. (2014). *Lactobacillus amylovorus* inhibits the TLR4 inflammatory signalling triggered by enterotoxigenic *Escherichia coli* via modulation of the negative regulators and involvement of TLR2 in intestinal Caco-2 cells and pig explants. *PLoS ONE* 9(4), e94891, DOI: 10.1371/journal.pone.0094891.

Flint, H.J., Scott, K.P., Duncan, S.H., Louis, P., Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. Gut *Microbes* 3(4), p 289–306, DOI: 10.4161/gmic.19897.

Fong, F.L.Y., Shah, N.P., Kirjavainen, P., El-Nezami, H. (2016). Mechanism of action of probiotic bacteria on intestinal and systemic immunities and antigen-presenting cells. *Int*. *Rev*. *Immunol*. 35(3), p 179-188, DOI: 10.3109/08830185.2015.1096937.

Frank, D., Zhu, W., Sartor, R.B., Li, E. (2011). Investigating the biological and clinical significance of human dysbioses. *Trends Microbiol*. 19(9), p 427–434, DOI: 10.1016/j.tim.2011.06.005.

Furness, J.B., Callaghan, B.P., Rivera, L.R., Cho, H.J. (2014). The enteric nervous system and gastrointestinal innervation: integrated local and central control. *Adv*. *Exp*. *Med*. *Biol*. 817, p 39–71, DOI: 10.1007/978-1-4939-0897-4_3.

Fushimi, T., Suruga, K., Oshima, Y., Fukiharu, M., Tsukamoto, Y., Goda, T. (2006). Dietary acetic acid reduces serum cholesterol and triacylglycerols in rats fed a cholesterol-rich diet. *Br*. *J*. *Nutr*. 95(5), p 916–924, DOI: 10.1079/BJN20061740.

Gao, Z., Yin, J., Zhang, J., Ward, R.E., Martin, R.J., Lefevre, M. *et al*. (2009). Butyrate improves insulin sensitivity and increases energy expenditure in mice. *Diabetes* 58(7), p 1509–1517, DOI: 10.2337/db08-1637.

Gillor, O., Ghazaryan, L. (2007). Recent advances in bacteriocin application as antimicrobials. *Recent Pat*. *Antiinfect*. *Drug Discov*. 2, p 115–122, DOI: 10.2174/157489107780832613.

Gobbetti, M., De Angelis, M., Di Cagno, R., Minervini, F., Limitone, A. (2007). Cell-cell communication in food related bacteria. *Int*. *J*. *Food Microbiol*. 120(1-2), p 34–45, DOI: 10.1016/j.ijfoodmicro.2007.06.012.

Goldstein, C., Lee, M.D., Sanchez, S., Hudson, C., Phillips, B., Register, B., Grady, M., Liebert, C., Summers, A.O., White, D.G., Maurer, J.J. (2001). Incidence of class 1 and 2 integrases in clinical and commensal bacteria from livestock, companion, animals, and exotics. *Antimicrob*. *Agents Chemother*. 45(3), p 723-726, DOI: 10.1128/AAC.45.3.723-726.2001.

González-Rodríguez, I., Sánchez, B., Ruiz, L., Turroni, F., Ventura, M., Ruas-Madiedo, P., Gueimonde, M., Margolles, A. (2012). Role of extracellular transaldolase from *Bifidobacterium bifidum* in mucin adhesion and aggregation. *Appl*. *Environ*. *Microbiol*. 78(11), p 3992–3998, DOI: 10.1128/AEM.08024-11.

Halliwell, B., Gutteridge, J.M. (1990). The antioxidants of human extracellular fluids. *Arch*. *Biochem*. *Biophys*. 280(1), p 1−8, DOI: 10.1016/0003-9861(90)90510-6.

Hardy, H., Harris, J., Lyon, E., Beal, J., Foey, A.D. (2013). Probiotics, prebiotics and immunomodulation of gut mucosal defences: homeostasis and immunopathology. *Nutrients* 5(6), p 1869-1912, DOI: 10.3390/nu5061869.

Harhaj, N.S.; Antonetti, D.A. (2004). Regulation of tight junctions and loss of barrier function in pathophysiology. *Int*. *J*. *Biochem*. *Cell Biol*. 36(7), p 1206–1237, DOI: 10.1016/j.biocel.2003.08.007.

Hart, A.L., Lammers, K., Brigidi, P., Vitali, B., Rizzello, F., Gionchetti, P., Campieri, M., Kamm, M.A., Knight, S.C., Stagg, A.J. (2004). Modulation of human dendritic cell phenotype and function by probiotic bacteria. *Gut* 53(11), p 1602-1609, DOI: 10.1136/gut.2003.037325.

Harutoshi, T. (2013). Exopolysaccharides of lactic acid for food and colon health applications. In: Kongo M (Ed), Lactic acid bacteria: R & D for Food, Health and Livestock Purposes, p 222- 38, DOI: 10.5772/50839, INTECH, London, UK.

Hashemi, S.R, Davoodi, H. (2010). Phytogenics as new class of feed additive in poultry industry. *J*. *Anim*. *Vet*. *Adv*. 9(17), p 2295-2304, DOI: 10.3923/javaa.2010.2295.2304.

Hausmann, M. (2010). How bacteria-induced apoptosis of intestinal epithelial cells contributes to mucosal inflammation. *Int*. *J*. *Inflam*. 2010, Article ID: 574568, DOI: 10.4061/2010/574568.

Havenstein, G.B., Ferket, P.R., Qureshi, M.A. (2003). Growth, livability, and feed conversion 1957 versus 2001 broilers when fed representative 1957 and 2001 broiler diets. *Poult*. *Sci*. 82(10), p 1500-1508, DOI: 10.1093/ps/82.10.1500.

Hedge, S.N., Roll, B.A., Coates, M.E. (1982). The effects of gut microflora and dietary fibre on energy utilization by the chick. *Br*. *J*. *Nutr*. 48(1), p 73–80, DOI:10.1079/ BJN19820089.

Hemarajata, P., Versalovic, J. (2013). Effects of probiotics on gut microbiota: mechanisms of intestinal immunomodulation and neuromodulation. *Therap*. *Adv*. *Gastroenterol*. 6(1), p 39.51, DOI: 10.1177/1756283X12459294.

Hidalgo-Cantabrana, C., López, P., Gueimonde, M., de los Reyes-Gavilán, C.G., Suñarez, A., Margolles, A., Ruas-Madiedo, P. (2012). Immune modulation capability of exopolysaccharides synthesised by lactic acid bacteria and bifidobacteria. *Probiotics Antimicrob*. *Proteins* 4(4), p 227–237, DOI: 10.1007/s12602-012-9110-2.

Hmani, H., Daoud, L., Jlidi, M., Jalleli, K., Ali, M.B., Brahim, A.H., Bargui, M., Dammak, A., Ali, M.B. (2017). A *Bacillus subtilis* strain as probiotic in poultry: selection based on *in vitro* functional properties and enzymatic potentialities. *J*. *Ind*. *Microbiol*. *Biotechnol*. 44(8), p 1157-1166, DOI: 10.1007/s10295-017-1944-x.

Hongpattarakere, T., Cherntong, N., Wichienchot, S., Kolida, S., Rastall, R.A. (2012). *In vitro* prebiotic evaluation of exopolysaccharides produced by marine isolated lactic acid bacteria. *Carbohydr*. *Polym*. 87(1), p 846–852, DOI: 10.1016/j.carbpol.2011.08.085.

Hooper, L.V., Macpherson, A.J. (2010). Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nat*. *Rev*. *Immunol* 10(3), p 159–169, DOI: 10.1038/nri2710.

Howarth, G.S. (2010). Probiotic-derived factors: probiotaceuticals? *J*. *Nutr*. 140(2), p 229–230, DOI: 10.3945/jn.109.118844.

Howarth, G.S., Wang, H. (2013). Role of endogenous microbiota, probiotics and their biological products in human health. *Nutrients* 5(1), p 58-81, DOI: 10.3390/nu5010058.

Hutchins, A.P., Diez, D., Miranda-Saavedra, D. (2013). The IL-10/STAT3-mediated anti-inflammatory response: recent developments and future challenges. *Brief*. *Funct*. *Genom*. 12(6), p 489–498, DOI: 10.1093/bfgp/elt028.

Ichihashi, T., Takagishi, Y., Uchida, K., Yamada, H. (1992). Colonic absorption of menaquinone-4 and menaquinone-9 in rats. *J*. *Nutr*. 122(3), p 506–512, DOI: 10.1093/jn/122.3.506.

Jadhav, K., Sharma, K.S., Katoch, S., Sharma, V.K., Mane, B.G. (2015). Probiotics in broiler poultry feeds: a review. *J*. *Anim*. *Nutr*. *Physiol*. 1, p 4-16.

Janeway, C.A., Travers, P., Walport, M., Schlomchik, M.J. (2001). Immunobiology: The Immune System in Health and Disease (5th edition). Garland Science., New York, U.S.A.

Jiménez-Dalmaroni, M.J., Gerswhin, M.E., Adamopoulos, I.E. (2016). The critical role of toll-like receptors- From microbial recognition to autoimmunity: a comprehensive review. *Autoimmun*. *Rev*. 15(1), p 1-8, DOI: 10.1016/j.autrev.2015.08.009.

Jin, L.Z., Ho, Y.W., Abdullah, N., Jalaludin, S. (2000). Digestive and bacterial enzyme activities in broilers fed diets supplemented with *Lactobacillus* cultures. *Poult*. *Sci*. 79(6), p 886-891, DOI: 10.1093/ps/79.6.886.

Juul-Madsen, H.R., Viertlboeck, B., Smith, A.L., Göbel, T.W.F. (2014). Avian innate immune responses. In: Davison, F., Kaspers, B., Schat, K.A. (Eds.). Avian Immunology (2nd edition). Elsevier Ltd., London, UK, p 129-158.

Kabir, S.M.L. (2009). The role of probiotics in the poultry industry. *Int*. *J*. *Mol*. *Sci*. 10(8), p 3531-3546, DOI: 10.3390/ijms10083531.

Kaiko, G.E., Horvat, J.C., Beagley, K.W., Hansbro, P.M. (2008). Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology* 123(3), p 326-338, DOI: 10.1111/j.1365-2567.2007.02719.x.

Kaiser, P., Stäheli, P. (2014). Avian cytokines and chemokines. In: Davison, F., Kaspers, B., Schat, K.A. (Eds.). Avian Immunology (2nd edition). Elsevier Ltd., London, UK, p 203-222.

Kang, H-J., Im, S-H. (2015). Probiotics as an immune modulator. *J*. *Nutr*. *Sci*. *Vitaminol*. 61, S103-S105, DOI: 10.3177/jnsv.61.S103.

Karczewski, J., Troost, F.J., Konings, I., Dekker, J., Kleerebezem, M., Brummer, R.J, Wells, J.M. (2010). Regulation of human epithelial tight junction proteins by *Lactobacillus plantarum in vivo* and protective effects on the epithelial barrier. *Am*. *J*. *Physiol*. *Gastrointest*. *Liver Physiol*. 298(6), G851–G859, DOI: 10.1152/ajpgi.00327.2009.

Karin, M., Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: The control of NF-[kappa]B activity. *Ann*. *Rev*. *Immunol*. 18, p 621–663, DOI: 10.1146/annurev.immunol.18.1.621.

Kawai, T., Akira, S. (2007). Signalling to NF-kappaB by Toll-like receptors. *Trends Mol*. *Med*. 13(11), p 460–469, DOI: 10.1016/j.molmed.2007.09.002.

Kawai, T., Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34(5), p 637–650, DOI: 10.1016/j.immuni.2011.05.006.

Khailova, L., Mount Patrick, S.K., Arganbright, K.M., Halpern, M.D., Kinouchi, T., Dvorak, B. (2010). *Bifidobacterium bifidum* reduces apoptosis in the intestinal epithelium in necrotizing enterocolitis. Am. *J*. *Physiol*. *Gastrointest*. *Liver Physiol*. 299(5), G1118-1127, DOI: 10.1152/ajpgi.00131.2010.

Kim, H.S., Jeong, S.G., Ham, J.S., Chae, H.S., Lee, J.M., Ahn, C.N. (2006). Antioxidative and probiotic properties of *Lactobacillus gasseri* NLRI-312 isolated from Korean infant feces. *Asian-Australas*. *J*. *Anim*. *Sci*. 19(9), p 1335−1341, DOI: 10.5713/ajas.2006.1335.

Kim, H.W., Yan, F.F., Hu, J.Y., Cheng, H.W., Kim, Y.H.B. (2016). Effects of probiotics feeding on meat quality of chicken breast during postmortem storage. *Poult*. *Sci*. 95(6), p 1457-1464, DOI: 10.3382/ps/pew055.

Klaenhammer TR. (1993). Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol*. *Rev*. 12(1-3), p 39–85, DOI: 10.1016/0168-6445(93)90057-G.

Kleerebezem, M., Vaughan, E.E. (2009). Probiotic and gut lactobacilli and bifidobacteria: molecular approaches to study diversity and activity. *Ann*. *Rev*. *Microbiol*. 63, p 269–290, DOI: 10.1146/annurev.micro.091208.073341.

Ko, J.S., Yang, H.R., Chang, J.Y., Seo, J.K. (2007). *Lactobacillus plantarum* inhibits epithelial barrier dysfunction and interleukin-8 secretion induced by tumor necrosis factor-alpha. *World J*. *Gastroenterol*. 13(13), p 1962–1965, DOI: 10.3748/wjg.v13.i13.1962.

Korakli, M., Vogel, R.F. (2006). Structures/function relationship of homopolysaccharide producing glycansucrases and therapeutic potential of their synthesised glycans. *Appl*. *Microbiol*. *Biotechnol*. 71(6), p 790-803, DOI: 10.1007/s00253-006-0469-4.

Krajmalnik-Brown, R., Ilhan, Z.E., Kang, D.W., DiBaise, J.K. (2012). Effects of gut microbes on nutrient absorption and energy regulation. *Nutr*. *Clin*. *Pract*. 27(2), p 201–214, DOI: 10.1177/0884533611436116.

Kumar, A., Wu, H., Collier-Hyams, L.S., Hansen, J.M., Li, T., Yamoah, K., Pan, Z.Q., Jones, D.P., Neish, A.S. (2007). Commensal bacteria modulate cullin-dependent signalling via generation of reactive oxygen species. *EMBO J*. 26(21), p 4457–4466, DOI: 10.1038/sj.emboj.7601867.

Kumar, M., Kumar, A., Nagpal, R., Mohania, D., Behare, P., Verma, V., Kumar, P., Poddar, D., Aggarwal, P.K., Henry, C.J., Jain, S., Yadav, H. (2010). Cancer-preventing attributes of probiotics: an update. *Int*. *J*. *Food Sci*. *Nutr*. 61(5), p 473−496, DOI: 10.3109/09637480903455971.

Kumar, M., Nagpal, R., Kumar, R., Hemalatha, R., Verma, V., Kumar, A., Chakraborty, C., Singh, B., Marotta, F., Jain, S., Yadav, H. (2012). Cholesterol-lowering probiotics as potential biotherapeutics for metabolic diseases. *Exp*. *Diabetes Res*. 2012, Article ID: 902917, DOI: 10.1155/2012/902917.

Lambert, M.N.T., Thybo, C.B., Lykkeboe, S., Rasmussen, L.M., Frette, X., Christensen, L.P., Jeppesen, P.B. (2017). Combined bioavailable isoflavones and probiotics improve bone status and estrogen metabolism in postmenopausal osteopenic women: a randomized controlled trial. *Am*. *J*. *Clin*. *Nutr*. 106(3), p 909–920, DOI: 10.3945/ajcn.117.153353.

Lavoie, B., Lian, J.B., Mawe, G.M. (2017). Regulation of bone metabolism by serotonin. *Adv*. *Exp*. *Med*. *Biol*. 1033, p 35–46, DOI: 10.1007/978-3-319-66653-2_3.

LeBlanc, J.G., Milani, C., de Giori, G.S., Sesma, F., van Sinderen, D., Ventura, M. (2013). Bacteria as vitamin suppliers to their host: a gut microbiota perspective. *Curr*. *Opin*. *Biotechnol*. 24(2), p 160-168, DOI: 10.1016/j.copbio.2012.08.005.

Lin, P.W., Myers, L.E.S., Ray, L., Song, S.C., Nasr, T.R., Berardinelli, A.J., Kundu, K., Murthy, N., Hansen, J.M., Neish, A.S. (2009). *Lactobacillus rhamnosus* blocks inflammatory signalling in vivo via reactive oxygen species generation. *Free Radic*. *Biol*. *Med*. 47(8), p 1205-1211, DOI: 10.1016/j.freeradbiomed.2009.07.033.

Linley, E., Denyer, S.P., McDonnell, G., Simons, C., Maillard, J.Y. (2012). Use of hydrogen peroxide as a biocide: a new consideration of its mechanisms of biocidal action. *J*. *Antimicrob*. *Chemother*. 67(7), p 1589-1596, DOI: 10.1093/jac/dks129.

Liu, C.F., Tseng, K.C., Chiang, S.S., Lee, B.H., Hsu, W.H., Pan, T.M. (2011a). Immunomodulatory and antioxidant potential of *Lactobacillus* exopolysaccharides. *J*. *Sci*. *Food Agric*. 91(12), p 2284–2291, DOI: 10.1002/jsfa.4456.

Liu, X., Yan, H., Xu, Q., Yin, C., Zhang, K., Wang, P., Hu, J. (2012). Growth performance and meat quality of broiler chickens supplemented with *Bacillus licheniformis* in drinking water. *Asian-Australas*. *J*. *Anim*. *Sci*. 25(5), p 682-689, DOI: 10.5713/ajas.2011.11334.

Liu, Y., Fatheree, N.Y., Mangalat, N., Rhoads, J.M. (2011b). *Lactobacillus reuteri* strains reduce incidence and severity of experimental necrotizing enterocolitis via modulation of TLR4 and NF-κB signalling in the intestine. *Am*. *J*. *Physiol*. *Gastrointest*. *Liver Physiol*. 302(6), p G608–G617, DOI: 10.1152/ajpgi.00266.2011.

Llewellyn, A., Foey, A. (2017). Probiotic modulation of innate cell pathogen sensing and signalling events. *Nutrients* 9(10), Article ID: E1156, DOI: 10.3390/nu9101156.

Lorentz, A., Schwengberg, S., Sellge, G., Manns, M.P., Bischoff, S.C. (2000). Human intestinal mast cells are capable of producing different cytokine profiles: role of IgE receptor cross-linking and IL-4. *J*. *Immunol*. 164(1), p 43–48, DOI: 10.4049/jimmunol.164.1.43.

Lyte, M. (2011). Probiotics function mechanistically as delivery vehicles for neuroactive compounds: microbial endocrinology in the design and use of probiotics. *Bioessays* 33(8), p 574–581, DOI: 10.1002/bies.201100024.

Ma, X.J., Pan, X.L., Lv, Z.H., Xu, F.L., Liu, D.Y., Lei, D.P., Xia, M., Luan, X.Y. (2009). Therapeutic influence on circulating and monocyte-derived dendritic cells in laryngeal squamous cell carcinoma patients. *Acta Otolaryngol*. 129(1), p 84–91, DOI: 10.1080/00016480802020459.

Macpherson, A.J., Uhr, T. (2004). Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria. *Science* 303(5664), p 1662–1665, DOI: 10.1126/science.1091334.

Maeda, H., Zhu, X., Suzuki, S., Suzuki, K., Kitamura, S. (2004). Structural characterization and biological activities of an exopolysaccharide kefiran produced by *Lactobacillus kefiranofaciens* WT-2B(T). *J*. *Agri*. *Food Chem*. 52(17), p 5533-5538, DOI: 10.1021/jf049617g.

Maillard, J-Y. (2002). Bacterial target sites for biocide action. *J*. *Appl*. *Microbiol*. 92(s1), p 16S-27S, DOI: 10.1046/j.1365-2672.92.5s1.3.x

Majeed, H., Gillor, O., Kerr, B., Riley, M.A. (2011). Competitive interactions in *Escherichia coli* populations: the role of bacteriocins. *ISME J*. 5(1), p 71–81, DOI: 10.1038/ismej.2010.90.

Manicassamy, S., Pulendran, B. (2009). Modulation of adaptive immunity with Toll-like receptors. *Semin*. *Immunol*. 21(4), p 185–193, DOI: 10.1016/j.smim.2009.05.005.

Mani-López, E., García, H.S., López-Malo, A. (2012). Organic acids as antimicrobials to control *Salmonella* in meat and poultry products. *Food Res*. *Int*. 45(2), p 713–21. DOI: 10.1016/j.foodres.2011.04.043.

Martin-Fontecha, A., Sebastiani, S., Höpken, U.E., Uguccioni, M., Lipp, M., Lanzavecchia, A., Sallusto, F. (2003). Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J*. *Exp*. *Med*. 198(4), p 615–621, DOI: 10.1084/jem.20030448.

Matsuzaki, C., Kamishima, K., Matsumoto, K., Koga, H., Katayama, T., Yamamoto, K., Hisa, K. (2014). Immunomodulating activity of exopolysaccharide-producing *Leuconostoc mesenteroides* strain NTM048 from green peas. *J*. *Appl*. *Microbiol*. 116(4), p 980–989, DOI: 10.1111/jam.12411.

McDonnell, G., Russell, A.D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clin*. *Microbiol*. *Rev*. 12(1), p 147–179.

Medzhitov, R., Janeway, C. (2000). Innate immune recognition: mechanisms and pathways. *Immunol*. *Rev*. 173(1), p 89-97, DOI: 10.1034/j.1600-065X.2000.917309.x.

Meijerink, M., van Hemert, S., Taverne, N., Wels, M., de Vos, P., Bron, P.A., Savelkoul, H.F., van Bilsen, J., Kleerebezem, M., Wells, J.M. (2010). Identification of genetic loci in *Lactobacillus plantarum* that modulate the immune response of dendritic cells using comparative genome hybridisation. *PLoS ONE* 5, Article ID: e10632, DOI: 10.1371/journal.pone.0010632

Miettinen, M., Vuopio-Varkila, J., Varkila, K. (1996). Production of human tumor necrosis factor alpha, interleukin-6, and interleukin-10 is induced by lactic acid bacteria. *Infect*. *Immun*. 64(12), p 5403–5405.

Mishra, V., Shah, C., Mokashe, N., Chavan, R., Yadav, H., Prajapati, J. (2015). Probiotics as potential antioxidants: a systematic review. *J*. *Agric*. *Food Chem*. 63(14), p 3615-3626, DOI: 10.1021/jf506326t.

Moore, S.L., Payne, D.N. (2004). Types of antimicrobial agents. In Fraise, A.P., Lambert, P.A. and Mailard, J.-Y. (Eds), Principles and Practice of Disinfection, Preservation and Sterilisation, p 10–77, Blackwell Publishing Inc, Oxford, UK.

Mortazavi, S.B., Sabzali, A., Rezaee, A. (2005). Sequence Fenton reaction for decreasing phenol formation during benzene chemical conversion in aqueous solutions. *Iran*. *J*. *Env*. *Health Sci*. *Eng*. 2(2), p 62–71.

Mountzouris, K.C., Tsitrsikos, P., Palamidi, I., Arvaniti, A., Mohnl, M., Schatzmayr, G., Fegeros, K. (2010). Effects of probiotic inclusion levels in broiler nutrition on growth performance, nutrient digestibility, plasma immunoglobulins, and cecal microflora composition. Poult. Sci. 89(1), p 58-67, DOI: 10.3382/ps.2009-00308.

Mutuş, R., Kocabağli, N., Alp, M., Acar N., Eren, M., Gezen, Ş.Ş. (2006). The effect of dietary probiotic supplementation on tibial bone characteristics and strength in broilers. *Poult*. *Sci*. 85(9), p 1621-1625, DOI: 10.1093/ps/85.9.1621.

Muzio, M., Natoli, G., Saccani, S., Levrero, M., Mantovani, A. (1998). The human toll signalling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J*. *Exp*. *Med*. 187(12), p 2097–2101, DOI: 10.1084/jem.187.12.2097.

Muzio, M., Ni, J., Feng, P., Dixit, V.M. (2013). Pillars article: IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signalling. *Science*. 278(5343), p 1612–1615, DOI: 10.1126/science.278.5343.1612.

Nahashon, S.N., Nakaue, H.S., Mirosh, L.W. (1994). Production variable and nutrient retention in single comb White Leghorn laying pullets fed diets supplemented with direct-fed microbials. *Poult*. *Sci*. 73(11), p 1699–1711, DOI: 10.3382/ps.0731699.

Nayebpor, M., Farhoman, P., Hashemi, A. (2007). Effects of different levels of direct fed microbials (*Primalac*) on growth performance and humoral immune response in broiler chickens. *J*. *Anim*. *Vet*. *Adv*. 6(11), p 1308-1313.

Neufeld, K., Foster, J. (2009). Effects of gut microbiota on the brain: implications for psychiatry. *J*. *Psychiatry Neurosci*. 34(3), p 230–231.

Neutra, M.R. (1998). Current concepts in mucosal immunity. V Role of M cells in transepithelial transport of antigens and pathogens to the mucosal immune system. *Am*. *J*. *Physiol*. 274(5 Pt1), G785-G791.

Neyens, E., Baeyens, J. (2003). A review of classic Fenton's peroxidation as an advanced oxidation technique. *J*. *Hazard*. *Mater*. 98(1-3), p 33–50, DOI: 10.1016/S0304-3894(02)00282-0.

Niess, J.H., Brand, S., Gu, X., Landsman, L., Jung, S., McCormick, B.A., Vyas, J.M., Boes, M., Ploegh, H.L., Fox, J.G., Littman, D.R., Reinecker, H.C. (2005). CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science* 307(5707), p 254–258, DOI: 10.1126/science.1102901.

Nishimura, J. (2014). Exopolysaccharides produced from *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Adv*. *Microbiol*. 4(14), p 1017–1020, DOI: 10.4236/aim.2014.414112.

O'Callaghan, J., Butto, L.F., Macsharry, J., Nally, K., O'Toole, P.W. (2012). Influence of adhesion and bacteriocin production by *Lactobacillus salivarius* on the intestinal epithelial cell transcriptional response. *Appl*. *Environ*. *Microbiol*. 78(15), p 5196–5203, DOI: 10.1128/AEM.00507-12.

O'Mahony, S.M., Clarke, G., Borre, Y.E., Dinan, T.G., Cryan, J.F. (2015). Serotonin, tryptophan metabolism and the brain-gut-microbiome axis. *Behav*. *Brain Res*. 277, p 32–48, DOI: 10.1016/j.bbr.2014.07.027.

O'Shea, E.F., Cotter, P.D., Stanton, C., Ross, R.P., Hill, C. (2011). Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanisms: bacteriocins and conjugated linoleic acid. *Int*. *J*. *Food Microbiol*. 152(3), p 189–205, DOI: 10.1016/j.ijfoodmicro.2011.05.025.

O'Toole, P., Cooney, J. (2008). Probiotic bacteria influence the composition and function of the intestinal microbiota. *Interdiscip*. *Perspect*. *Infect*. *Dis*. 2008, Article ID: 17525, DOI: 10.1155/2008/175285.

Oakley, B.B., Lillehoj, H.S., Kogut, M.H., Kim, W.L., Maurer, J.J., Pedroso, A., Lee, M.D., Collett, S.R., Johnson, T.J., Cox, N.A. (2014). The chicken gastrointestinal microbiome. *FEMS Microbiol*. *Lett*. 360(2), p 100-112, DOI: 10.1111/1574-6968.12608.

Oakley, B.B., Morales, C.A., Line, J., Berrang, M.E., Meinersmann, R.J., Tillman, G.E., Wise, M.G., Siragusa, G.R., Hiett, K.L., Seal, B.S. (2013). The poultry-associated microbiome: network analysis and farm-to-fork characterizations. *PLoS ONE* 8, Article ID: e57190, DOI: 10.1371/journal.pone.0057190.

Ohnmacht, C., Pullner, A., King, S.B., Drexler, I., Meier, S., Brocker, T., Voehringer, D. (2009). Constitutive ablation of dendritic cells breaks self-tolerance of CD4 T cells and results in spontaneous fatal autoimmunity. *J*. *Exp*. *Med*. 206(3), p 549–559, DOI: 10.1084/jem.20082394.

Olnood, C.G., Beski, S.S.M., Choct, M., Iji, P.A. (2015). Novel probiotics: Their effects on growth performance, gut development, microbial community and activity of broiler chickens. *Anim*. *Nutr*. 1(3), p 184-191, DOI: 10.1016/j.aninu.2015.07.003.

Ooi, L-G., Liong, M-T. (2010). Cholesterol-lowering effects of probiotics and prebiotics: A review of in vivo and in vitro findings. *Int*. *J*. *Sci*. 11(6), p 2499-2522, DOI: 10.3390/ijms11062499.

Osorio, F., Reis e Sousa, C. (2011). Myeloid C-type lectin receptors in pathogen recognition and host defense. *Immunity* 34(5), p 651–664, DOI: 10.1016/j.immuni.2011.05.001.

Parois, S., Calandreau, L., Kraimi, N., Gabriel, I., Leterrier, C. (2017). The influence of a probiotic supplementation on memory in quail suggests a role of gut microbiota on cognitive abilities in birds. *Behav*. *Brain Res*. 331, p 47-53, DOI: 10.1016/j.bbr.2017.05.022.

Patel, A., Prajapati, J.B. (2013). Food and health applications of exopolysaccharides produced by lactic acid bacteria. *Adv*. *Dairy Res*. 1(2), Article ID: 1000107, DOI: 10.4172/2329-888X.1000107.

Patterson, J.A. (2011). The commensal microbiota. In: Callaway, T.R., Ricke, S.C. (Eds), Direct-Fed Microbials and Prebiotics for Animals. Springer, New York, U.S.A, p. 3–11.

Patterson, J.A., Burkholder, K.M. (2003). Application of prebiotics and probiotics in poultry production. Poult. Sci., 82(4), p 627–631, DOI: 10.1093/ps/82.4.627.

Peng, L., Li, Z.R., Green, R.S., Holzman, I.R., Lin, J. (2009). Butyrate enhances the intestinal barrier by facilitating tight junction assembly via activation of AMP-activated protein kinase in Caco-2 cell monolayers. *J*. *Nutr*. 139(9), p 1619–1625, DOI: 10.3945/jn.109.104638.

Peng, Q., Zeng, X.F., Zhu, J.L., Wang, X., Liu, T., Hou, C.L., Thacker, P.A., Qiao, S.Y. (2016). Effects of dietary Lactobacillus plantarum B1 on growth performance, intestinal microbiota, and short chain fatty acid profiles in broiler chickens. Poult. Sci. 95(4), p 893-900, DOI: 10.3382/ps/pev435.

Pflughoeft, K., Versalovic, J. (2012). Human microbiome in health and disease. *Annu*. *Rev*. *Pathol*. 7, p 99–122, DOI: 10.1146/annurev-pathol-011811-132421.

Plavnik, I., Scott, M.L. (1980). Effects of additional vitamins, minerals or brewers yeast upon leg weaknesses in broiler chickens. *Poult*. *Sci*. 59(2), p 459–464, DOI: 10.3382/ps.0590459.

Pompei, A., Cordisco, L., Amaretti, A., Zanoni, S., Raimondi, S., Matteuzzi, D., Rossi, M. (2007). Administration of folate-producing bifidobacteria enhances folate status in Wistar rats. *J*. *Nutr*. 137(12), p 2742–2746, DOI: 10.1093/jn/137.12.2742.

Porter, N.T., Martens, E.C. (2017). Polysaccharides in gut microbial ecology and physiology. *Annu*. *Rev*. *Microbiol*. 71, p 349-369, DOI: 10.1146/annurev-micro-102215-095316.

Pourakbari, M., Seidavi, A., Asadpour, L., Martínez, A. (2016). Probiotic level effects on growth performance, carcass traits, blood parameters, cecal microbiota, and immune response of broilers. *An*. *Acad*. *Bras*. *Ciênc*. 88(2), p 1011-1021, http://dx.doi.org/10.1590/0001-3765201620150071.

Prisciandaro, L.D., Geier, M.S., Butler, R.N., Cummins, A.G., Howarth, G.S. (2011). Evidence supporting the use of probiotics for the prevention and treatment of chemotherapy-induced intestinal mucositis. *Crit*. *Rev*. *Food Sci*. *Nutr*. 51(3), p 239–247, DOI: 10.1080/10408390903551747.

Puglisi-Allegra, S., Andolina, D. (2015). Serotonin and stress coping. *Behav*. *Brain Res*. 277, p 58–67, DOI: 10.1016/j.bbr.2014.07.052.

Putaala, H., Salusjärvi, T., Nordström, M., Saarinen, M., Quwehand, A.C., Bech Hansen, E., Rautonen, N. (2008). Effect of four probiotic strains and *Escherichia coli* O157:H7 on tight junction integrity and cyclo-oxygenase expression. *Res*. *Microbiol*. 159(9-10), p 692–698, DOI: 10.1016/j.resmic.2008.08.002.

Quach, D., Britton, R.A. (2017). Gut microbiota and bone health. *Adv*. *Exp*. *Med*. *Biol*. 1033, p 47–58, DOI: 10.1007/978-3-319-66653-2_4.

Rabson, A., Roitt, I.M., Delves, P.J. (2005). Really essential medical immunology 2nd edition. Blackwell Publishing Ltd, Oxford, UK.

Ramsey, W., Isales, C.M. (2017). Intestinal incretins and the regulation of bone physiology. *Adv*. *Exp*. *Med*. *Biol*. 1033, p 13–33, DOI: 10.1007/978-3-319-66653-2_2.

Rao, R.K., Samak, G. (2013). Protection and restitution of gut barrier by probiotics: nutritional and clinical implications. *Curr*. *Nutr*. *Food Sci*. 9(2), p 99-107, DOI: 10.2174/1573401311309020004.

Reigstad, C.S., Salmonson, C.E., Rainey, J.F., Szurszewski, J.H., Linden, D.R., Sonnenburg, J.L., Farrugia, G., Kashyap, P.C. (2015). Gut microbes promote colonic serotonin production through an effect of short-chain fatty acids on enterochromaffin cells. *FASEB J*. 29(4), p 1395–1403, DOI: 10.1096/fj.14-259598.

Riley, M.A., Wertz, J.E. (2002). Bacteriocin diversity: ecological and evolutionary perspectives. *Biochimie* 84(5-6), p 357–364, DOI: 10.1016/S0300-9084(02)01421-9.

Rinttilä, T., Apajalahti, J. (2013). Intestinal microbiota and metabolites- Implications for broiler chicken health and performance. *J*. *Appl*. *Poult*. *Res*. 22(3), p 647-658, DOI: 10.3382/japr.2013-00742.

Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., de los Reyes-Gavilán, C.G., Salazar, N. (2016). Intestinal short chain fatty acids and their link with diet and human health. *Front*. *Microbiol*. 7, Article ID: 185, DOI: 10.3389/fmicb.2016.00185.

Robbe, C., Capon, C., Coddeville, B., Michalski, J.C. (2004). Structural diversity and specific distribution of O-glycans in normal human mucins along the intestinal tract. *Biochem*. *J*. 384(2), p 307–316, DOI: 10.1042/BJ20040605.

Roberts, I.S. (1996). The biochemistry and genetics of capsular polysaccharide production in bacteria. *Annu*. *Rev*. *Microbiol*. 50, p 285–315, DOI: 10.1146/annurev.micro.50.1.285.

Ruas-Madiedo, P., Gueimonde, M., Margolles, A., de los Reyes-Gavilán, C.G., Salminen, S. (2006). Exopolysaccharides produced by probiotic strains modify the adhesion of probiotics and enteropathogens to human intestinal mucus. *J*. *Food Prot*. 69(8), p 2011-2015, DOI: 10.4315/0362-028X-69.8.2011.

Russell, A.D. (2003). Similarities and differences in the responses of microorganisms to biocides. *J*. *Antimicrob*. *Chemother*. 52(5), p 750-763, DOI: 10.1093/jac/dkg422.

Russell, J.B. (1992). Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. *J*. *Appl*. *Bacteriol*. 73(5), p 363–370, DOI: 10.1111/j.1365-2672.1992.tb04990.x.

Ryan, P.M., Ross, R.P., Fitzgerald, G.F., Caplice, N.M., Stanton, C. (2015). Sugar-coated: exopolysaccharide producing lactic acid bacteria for food and human health applications. *Food Funct*. 6(3), p 679–693, DOI: 10.1039/C4fo00529e.

Said, H.M.; Mohammed, Z.M. (2006). Intestinal absorption of water-soluble vitamins: an update. *Curr*. *Opin*. *Gastroenterol*. 22(2), p 140–146, DOI: 10.1097/01.mog.0000203870.22706.52.

Salazar, N., Ruas-Madiedo, P., Kolida, S., Collins, M., Rastall, R., Gibson, G., de Los Reyes-Gavilán, C.G. (2009). Exopolysaccharides produced by *Bifidobacterium longum* IPLA E44 and *Bifidobacterium animalis* subsp. *lactis* IPLA R1 modify the composition and metabolic activity of

human faecal microbiota in pH-controlled batch cultures. *Int*. *J*. *Food Microbiol*. 135(3), p 260–267, DOI: 10.1016/j.ijfoodmicro.2009.08.017.

Salehimanesh, A., Mohammadi, M., Roostaei-Ali Mehr, M. (2016). Effect of dietary probiotic, prebiotic and symbiotic supplementation on performance, immune responses, intestinal morphology and bacterial populations in broilers. J. Anim. Physiol. Anim. Nutr. 100(4), p 694-700, DOI: 10.1111/jpn.12431.

Salyers, A.A., Amábile-Cuevas, C.F. (1997). Why are antibiotic resistance genes so resistant to elimination? *Antimicrob*. *Agents Chemother*. 41(11), p 2321-2325.

Sanders, M.E. (2016). Probiotics and microbiota composition. *BMC Med*. 14(1), Article ID: 82, DOI: 10.1186/s12916-016-0629-z.

Santos Rocha, C., Lakhdari, O., Blottiere, H.M., Blugeon, S., Sokol, H., Bermúdez-Humarán, L.G., Azevedo, V., Miyoshi, A., Doré, J., Langella, P., Maguin, E., van de Guchte, M. (2012). Anti-inflammatory properties of dairy lactobacilli. *Inflamm*. *Bowel Dis*. 18(4), p 657-666, DOI: 10.1002/ibd.21834.

Santos, F.B.O., Sheldon, B.W., Santos, A.A., Ferket, P.R. (2008). Influence of housing system, grain type, and particle size on Salmonella colonisation and shedding of broilers fed triticale or corn-soybean meal diets. Poult. Sci 87(3), p 405-420, DOI: https://doi.org/10.3382/ps.2006-00417.

Schnare, M., Barton, G.M., Holt, A.C., Takeda, K., Akira, S., Medzhitov, R. (2001). Toll-like receptors control activation of adaptive immune responses. *Nat*. *Immunol*. 2(10), p 947–950, DOI: 10.1038/ni712

Schneitz, C. (2005). Competitive exclusion in poultry-30 years of research. *Food Control* 16(8), p 657–667, DOI: 10.1016/j.foodcont.2004.06.002.

Segawa, S., Fujiya, M., Konishi, H., Ueno, N., Kobayashi, N., Shigyo, T., Kohgo, Y. (2011). Probiotic-derived polyphosphate enhances the epithelial barrier function and maintains intestinal homeostasis through integrin-p38 MAPK pathway. *PLoS ONE* 6(8), e23278, DOI: 10.1371/journal.pone.0023278.

Seth, A., Yan, F., Polk, D.B., Rao, R.K. (2008). Probiotics ameliorate the hydrogen peroxide-induced epithelial barrier disruption by a PKC- and MAP kinase-dependent mechanism. *Am*. *J*. *Physiol*. *Gastrointest*. *Liver Physiol*. 294(4), p G1060–1069, DOI: 10.1152/ajpgi.00202.2007.

Sherman, P.M., Ossa, J.C., Johnson-Henry, K. (2009). Unraveling mechanisms of action of probiotics. Nutr. Clin. Pract. 24(1), p 10-14, DOI: 10.1177/0884533608329231.

Sherwin, E., Sandhu, K.V., Dinan, T.G., Cryan, J.F. (2016). May the force be with you: the light and dark sides of the microbiota-gut-brain axis in neuropsychiatry. *CNS Drugs* 30(11), p 1019–1041, DOI: 10.1007/s40263-016-0370-3.

Shokryazdan, P., Jahromi, M.F., Liang, J.B., Ramasamy, K., Sieo, C.C., Ho, Y.W. (2017). Effects of a *Lactobacillus salivarius* mixture on performance, intestinal health and serum lipids of broiler chickens. *PLoS ONE* 12(5), Article ID: e0175959, DOI: 10.1371/journal.pone.0175959 M.

Smith, A.L., Göbel, T.W. (2014). Avian T cells: antigen recognition and lineages. In: Davison, F., Kaspers, B., Schat, K.A. (Eds.). Avian Immunology (2nd edition). Elsevier Ltd., London, UK, p 91-102.

Sonnenburg, J.L., Xu, J., Leip, D.D., Chen, C.H., Westover, B.P., Weatherford, J., Buhler, J.D., Gordon, J.I. (2005). Glycan foraging in vivo by an intestine-adapted bacterial symbiont. *Science* 307(5717), p 1955–1959, DOI: 10.1126/science.1109051.

Specian R.D., Oliver M.G. (1991). Functional biology of intestinal goblet cells. *Am*. *J*. *Physiol*. 260, p C183–C193, DOI: 10.1152/ajpcell.1991.260.2.C183.

Spinler, J.K., Taweechotipatr, M., Rognerud, C.L., Ou, C.N., Tumwasorn, S., Versalovic, J. (2008). Human derived probiotic *Lactobacillus reuteri* demonstrate antimicrobial activities targeting diverse enteric bacterial pathogens. *Anaerobe* 14(3), p 166–171, DOI: 10.1016/j.anaerobe.2008.02.001.

Stancu, C.S., Toma, L., Sima, A.V. (2012). Dual role of lipoproteins in endothelial cell dysfunction in atherosclerosis. *Cell Tissue*. *Res*. 349(2), p 433–446, DOI: 10.1007/s00441-012-1437-1.

Stringer, A.M.; Gibson, R.J.; Logan, R.M.; Bowen, J.M.; Yeoh, A.S.; Hamilton, J.; Keefe, D.M. (2009). Gastrointestinal microflora and mucins may play a critical role in the development of 5-fluorouracil-induced gastrointestinal mucositis. *Exp*. *Biol*. *Med*. 234(4), p 430–441, DOI: 10.3181/0810-RM-301.

Sturme, M.H., Kleerebezem, M., Nakayama, J., Akkermans, A.D., Vaugha, E.E., de Vos, W.M. (2002). Cell to cell communication by autoinducing peptides in gram-positive bacteria. *Antonie Van Leeuwenhoek* 81(1-4), p 233–243, DOI: 10.1023/A:1020522919555.

Sun, Y., Rajput, I.R., Arain, M.A., Li, Y., Baloch, D.M. (2016). Oral administration of *Saccharomyces boulardii* alters duodenal morphology, enzymatic activity and cytokine production response in broiler chickens. *Anim*. *Sci*. *J*. 88(8), p 1204-1211, DOI: 10.1111/asj.12757.

Sylvester, F.A. (2017). Inflammatory bowel disease: effects on bone and mechanisms. *Adv*. *Exp*. *Med*. *Biol*. 1033, p 133–150, DOI: 10.1007/978-3-319-66653-2_7.

Takanashi, N., Tomosada, Y., Villena, J., Murata, K., Takahashi, T., Chiba, E., Tohno, M., Shimazu, T., Aso, H., Suda, Y.; Ikegami, S., Itoh, H., Kawai, Y., Saito, T., Alvarez, S., Kitazawa, H. (2013). Advanced application of bovine intestinal epithelial cell line for evaluating regulatory effect of lactobacilli against heat-killed enterotoxigenic *Escherichia coli*-mediated inflammation. *BMC Microbiol*. 13, e54, DOI: 10.1186/1471-2180-13-54.

Tsimikas, S., Miller, Y.I. (2011). Oxidative modification of lipoproteins: mechanisms, role in inflammation and potential clinical applications in cardiovascular disease. *Curr*. *Pharm*. *Des*. 17(1), p 27–37, DOI: 10.2174/138161211795049831.

Tsirtsikos, P., Fegeros, K., Balaskas, C., Kominakis, A., Mountzouris, K.C. (2012). Dietary probiotic inclusion level modulates intestinal mucin composition and mucosal morphology in broilers. *Poult*. *Sci*. 91(8), p 1860–1868, DOI: 10.3382/ps.2011-02005.

Van der Wielen, P.W., Keuzenkamp, D.A., Lipman, L.J.A., van Knapen, F., Biesterveld, S. (2002). Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. *Microb*. *Ecol*. 44(3), p 286–93, DOI: 10.1007/s00248-002-2015-y.

Veckman, V., Miettinen, M., Pirhonen, J., Siren, J., Matikainen, S., Julkunen, I. (2004). *Streptococcus pyogenes* and *Lactobacillus rhamnosus* differentially induce maturation and production of Th1-

type cytokines and chemokines in human monocyte-derived dendritic cells. *J*. *Leukoc*. *Biol*. 75(5), p 764–771, DOI: 10.1189/jlb.1003461.

Wang, H., Ni, X., Liu, L., Zheng, D., Lai, J., Qing, X., Li, G., Pan, K., Jing, B. (2017). Controlling of growth performance, lipid deposits and fatty acid composition of chicken meat through a probiotic, *Lactobacillus johnsonii* during subclinical *Clostridium perfringens* infection. *Lipids Health Dis*. 16, Article ID: 38, DOI: 10.1186/s12944-017-0408-7.

Wang, Y., Gu, Q. (2010). Effect of probiotic on growth performance and digestive enzyme activity of Arbor Acres broilers. *Res*. *Vet*. *Sci*. 89(2), p 163-167, DOI: 10.1016/j.rvsc.2010.03.009.

Wealleans, A.L., Walsh, M.C., Romero, L.F., Ravindran, V. (2017). Comparative effects of two multi-enzyme combinations and a *Bacillus* probiotic on growth performance, digestibility of energy and nutrients, disappearance of non-starch polysaccharides, and gut microflora in broiler chickens. *Poult*. *Sci*. 93(12), p 4287-4297, DOI: 10.3382/ps/pex226.

Xiao, H., Gulen, M.F., Qin, J., Yao, J., Bulek, K., Kish, D., Altuntas, C.Z., Wald, D., Ma, C., Zhou, H., Tuohy, V.K., Fairchild, R.L., de la Motte, C., Cua, D., Vallance, B.A., Li, X. (2007). The Toll-interleukin-1 receptor member SIGIRR regulates colonic epithelial homeostasis, inflammation, and tumorigenesis. *Immunity* 26(4), p 461–475, DOI: 10.1016/j.immuni.2007.02.012.

Yalçin, S., Eser, H., Yalçin, S., Yalçin, S.S. (2016). Effects of probiotics on some blood parameters and serum IgG in broilers. *J*. *Pediatr*. *Gastr*. *Nutr*. 63(1S), Article ID: S57, DOI: 10.1097/01.mpg.0000489636.98750.ba.

Yan, F., Cao, H., Cover, T.L., Whitehead, R., Washington, M.K., Polk, D.B. (2007). Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. *Gastroenterology* 132(2), p 562–575, DOI: 10.1053/j.gastro.2006.11.022.

Yan, F., Polk, D.B. (2002). Probiotic bacterium prevents cytokine-induced apoptosis in intestinal epithelial cells. *J*. *Biol*. *Chem*. 277(52), p 50959–50965, DOI: 10.1074/jbc.M207050200.

Yan, F., Polk, D.B. (2011). Probiotics and immune health. *Curr*. *Opin*. *Gastroenterol*. 27(6), p 496-501, DOI: 10.1097/MOG.0b013e32834baa4d.

Yang, X., Zhang, B., Guo, Y., Jiao, P., Long, F. (2010). Effects of dietary lipids and *Clostridium butyricum* on fat deposition and meat quality of broiler chickens. *Poult*. *Sci*. 89(2), p 254-260, DOI: 10.3382/ps.2009-00234.

Yano, J.M., Yu, K., Donaldson, G.P., Shastri, G.G., Ann, P., Ma, L., Nagler, C.R., Ismagilov, R.F., Mazmanian, S.K., Hsiao, E.Y. (2015). Indigenous bacteria from the gut microbiota regulate host serotonin biosynthesis. *Cell* 161(2), p 264–276. DOI: 10.1016/j.cell.2015.02.047.

Yue, Y., Guo, Y., Yang, Y. (2017). Effects of dietary L-tryptophan supplementation on intestinal response to chronic unpredictable stress in broilers. *Amino Acids* 49(7), p 1227-1236, DOI: 10.1007/s00726-017-2424-3.

Zhang, L., Li, N., Caicedo, R., Neu, J. (2005). Alive and dead *Lactobacillus rhamnosus* GG decrease tumor necrosis factor-alpha-induced interleukin-8 production in Caco-2 cells. *J*. *Nutr*. 135(7), p 1752–1756, DOI: 10.1093/jn/135.7.1752.

Zhang, L., Zhang, L., Zhan, X., Zeng, X., Zhou, L., Cao, G., Chen, A., Yang, C. (2016). Effects of dietary supplementation of probiotic, *Clostridium butyricum*, on growth performance, immune response,

intestinal barrier function, and digestive enzyme activity in broiler chickens challenged with *Escherichia coli* K88. *J. Anim. Sci. Biotechnol*. 7, Article ID: 3, DOI: 10.1186/s40104-016-0061-4.

Zhang, Z.F., Kim, I.H. (2014). Effects of multistrain probiotics on growth performance, apparent ileal nutrient digestibility, blood characteristics, cecal microbial shedding, and excreta odor contents in broilers. *Poult. Sci*. 93(2), p 364-370, DOI: 10.3382/ps.2013-03314.

Zhang, Z.F., Zhou, T.X., Ao, X., Kim, I.H. (2012). Effects of β-glucan and *Bacillus subtilis* on growth performance, blood profiles, relative organ weight and meat quality in broilers fed maize-soybean meal based diets. *Livest. Sci*. 150(1-3), p419-424, DOI: 10.1016/j.livsci.2012.10.003.

Zheng, A., Luo, J., Meng, K., Li, J., Zhang, S., Li, K., Liu, G., Cai, H., Bryden, W.L., Yao, B. (2014). Proteome changes underpin improved meat quality and yield of chickens (*Gallus gallus*) fed the probiotic *Enterococcus faecium*. *BMC Genomics* 15, Article 1167, DOI: 10.1186/1471-2164-15-1167.

Zheng, J., Gänzle, M.G., Lin, X.B., Ruan, L., Sun, M. (2015). Diversity and dynamics of bacteriocins from human microbiome. *Environ. Microbiol*. 17(6), p 2133–2143, DOI: 10.1111/1462-2920.12662.

Zhou, L., Foster, J.A. (2015). Psychobiotics and the gut-brain axis: In the pursuit of happiness. *Neuropsych. Dis. Treat*. 11, p 715-723, DOI: 10.2147/NDT.S61997.

Zhou, Y.K., Qin, H.L., Zhang, M., Shen, T.Y., Chen, H.Q., Ma, Y.L., Chu, Z.X., Zhang, P., Liu, Z.H. (2012). Effects of *Lactobacillus plantarum* on gut barrier function in experimental obstructive jaundice. *World J. Gastroenterol*. 18(30), p 3977–3991, DOI: 10.3748/wjg.v18.i30.3977.

Chapter 3

Characterisation of *L. johnsonii* DPN184, *L. salivarius* DPN164, *L. crispatus* DPN167, *L. gallinarum* DPN164, *E. faecalis* DPN94 and *B. amyloliquefaciens* DPN123 isolated from healthy free-range broilers

Abstract

A total of 609 bacteria were isolated from the gastrointestinal tract (GIT) of healthy free-range broilers and identified to species level using 16S rDNA, *gyrB* and *recA* gene sequence similarity. Six isolates (*Lactobacillus johnsonii* DPN184, *Lactobacillus salivarius* DPN164, *Lactobacillus crispatus* DPN167, *Lactobacillus gallinarum* DPN164, *Enterococcus faecalis* DPN94 and *Bacillus amyloliquefaciens* DPN123) had *in vitro* tolerance towards acid conditions and bile salts. All isolates produced hetero-exopolysaccharides and formed biofilms. *Bacillus amyloliquefaciens* DPN123 was isolated from the duodenum and produced extracellular amylase, phytase, and antimicrobial compounds (surfactin and iturin A1) which had activity towards *Micrococcus luteus*, *Listeria monocytogenes*, Entero-invasive *Escherichia coli* and *Salmonella enterica* serovar Enteritidis. *Enterococcus faecalis* DPN94 was isolated from the jejunum and ileum and produced phytase and bile salt hydrolase. In addition, the strain's genome coded for virulence genes such as *cad*, *ace*, *slyA*, *asa1*, *EF3314*, *EF0109*, *cob*, *asp1*, *efaA*, *gelE* and *cpd*, however, not for cytolysin (*cylA*, *cylB* and *cylM*). *Lactobacillus johnsonii* DPN184 was isolated from the cecum and produce hydrogen peroxide. *Lactobacillus salivarius* DPN181 was isolated from the colon and produce hydrogen peroxide and high levels of lactic acid. *Lactobacillus crispatus* DPN167 was isolated from the crop, proventriculus and ventriculus, and produced hydrogen peroxide and bile salt hydrolase. *Lactobacillus gallinarum* DPN164 was isolated from the jejunum and ileum. In conclusion, strains isolated from healthy free-range broilers showed tolerance towards simulated GIT conditions and possessed numerous beneficial characteristics such as the production of amylase, phytase, bile salt hydrolase, hydrogen peroxide and antimicrobial compounds.

Introduction

Lactobacillus species dominate the anterior small intestine, crop, duodenal and jejunal epithelial cells, and digesta of chicken (Watkins and Kratzer, 1983). Early microbiota studies found that *L*. *salivarius*, *L*. *reuteri*, *L*. *acidophilus* and *L*. *crispatus* inhabited the entire digestive tract (Mead, 1997; Sarra *et al*., 1992; Smith, 1965). Through phylogenetic analysis of 16S rRNA gene sequences, a global bacterial census was created for poultry (Wei *et al*., 2013). Although the census is not complete, 13 phyla of bacteria were found, of which Firmicutes, Bacteroidetes, and Proteobacteria were the most dominant. More than 900 species were identified, which represented 117 genera which were dominated by *Clostridium*, *Ruminococcus*, *Lactobacillus* and *Bacteroides* (Wei *et al*., 2013; Pan and Yu, 2014; Yan *et al*., 2017; Borda-Molina *et al*., 2018).

Probiotic bacteria need to be tolerant towards acidic conditions and bile salts to survive transit in the gastrointestinal tract. Probiotics producing digestive enzymes such as amylase, phytase and bile salt hydrolase can improve broiler growth performance (Onderci *et al*., 2006; Tang *et al*., 2013). Bile salt hydrolase provides strains with resistance to bile salts, and hydrolyse bile salts to form glycine or taurine, and a steroid core (Liong and Shah 2005). Hydrolysed bile salts compared to non-hydrolysed is less absorbed in the intestine, leaving more free bile acids to be excreted via the faeces (Begley *et al*. 2006). Increased excretions of bile salts decrease the total amount of available bile salts available. Lost bile salts can be replenished via the synthesis from cholesterol, which subsequently leads to a reduction in cholesterol serum levels (Begley *et al*. 2006). Amylase is capable of hydrolysing α- 1,4- glycosidic linkages in polysaccharides containing three or more 1,4-α-linked glucose units and has specificity towards starches, glycogen and oligosaccharides. Amylase supplementation to feed has been shown to significantly improve digestibility of nutrients and improve broiler growth performance (Onderci *et al*., 2006; Tang *et al*., 2013). Phytase hydrolyses phytic acid (*myo*-inositol hexaphosphate) to *myo*-inositol and inorganic phosphate (Mitchell *et al*., 1997). Phytic acid is a plant phosphorus storage form and accounts for 50-80 % of total phosphorus present in cereal grains and legumes (Dalal, 1977). Phytic acid has a low bioavailability due to the lack of endogenous phytate-degrading enzymes (Selle *et al*., 2006). In addition, phytic acid exerts antinutritive effects (Adeola and Cowieson, 2011), sequestering essential cations, i.e. Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+}, reducing their bioavailability (Graf and Eaton, 1990).

Another important characteristic which should be considered for probiotics is the inhibition of pathogens such as *Clostridium perfringens*, *Salmonella enterica* and *Campylobacter jejeni* (Kabir, 2009). Infection of poultry with *C*. *perfringens* may cause acute clinical or subclinical diseases (Van Immerseel *et al*., 2004). In acute form, *C*. *perfringens* increases mortality rates, whereas the subclinical form damages the intestinal mucosa (necrotic enteritis) leading to decreased nutrient absorption and subsequently decreased growth performance (Kaldusdal and Løvland, 2000; Hofacre *et al*., 2003). *Clostridium perfringens* transmission from poultry to human's lead to food-borne diseases (Regan *et al*., 1995; Hook *et al*., 1996). Infections by *S*. *enterica* and C. *jejeni* cause diarrhoea decreasing feed intake subsequently decreasing growth performance, and transmission to humans lead to food-borne diseases such as salmonellosis and campylobacteriosis (Marcq *et al*., 2011; Antunes *et al*., 2016; Skarp *et al*., 2016; Awad *et al*., 2017). It is imperative that the presence of pathogenic bacteria in broilers are

controlled, to ultimately improve bird health and decrease the risk of transmission of food-borne pathogens to humans.

Commercially available probiotics include PoultryStar® (*Enterococcus faecium*, *P. acidilactici*, *Bacillus animalis*, *Lactobacillus salivarius*, *Lactobacillus reuteri* and prebiotic fructooligosaccharides), CLOSTAT™ (*Bacillus subtilis*) and Floramax® (*Lactobacillus salivarius* and *P. parvulus*). PoultryStar® administration decrease colonisation of *C. jejuni* and *Salmonella* Enteritidis (Sterzo *et al*., 2007; Ghareeb *et al*., 2012). CLOSTAT™ improved feed conversion, intestinal morphology, enhanced immune responses, and inhibited GIT colonisation by *C. jejuni*, *Escherichia coli* and *Salmonella* Minnesota (Teo and Tan, 2007; Melegy *et al*., 2011; Lourenco *et al*., 2012; Abudabos *et al*., 2013). Floramax® B11 improved weight gain, bone characteristics, intestinal morphology, immune responses and decreased *S*. Enteritidis colonisation (Gutierrez-Fuentes *et al*., 2013; Prado-Rebolledo *et al*., 2017).

The objective of this study was to screen the GIT of healthy free-range chickens for microorganisms with beneficial characteristics. Isolates were phylogenetically identified using 16S rDNA, *recA* and *gyrB* gene sequence similarity. Beneficial characteristics assessed included; acid and bile salt tolerance; production of enzymes, i.e. amylase, phytase and bile salt hydrolase; production of hydrogen peroxide; aggregative and biofilm formation ability, hydrophobic properties, production of exopolysaccharides and antimicrobial peptides. Enterococcal isolates were screened for the presence of virulence factors.

Materials and Methods

Isolation of bacteria

Twenty-five free-range broilers (Cobb 500) from Hermanus, Graafwater, Fisantekraal and Grabouw in the Western Cape, South Africa, were slaughtered at one of the commercial abattoirs. The GIT of each bird was aseptically removed, the crop, proventriculus, ventriculus, duodenum, small intestine, cecum and large intestine separated, and placed in a Sterilin™ homogenizing bag with 100 mL sterile phosphate-buffered saline (PBS; 0.8 %, w/v, NaCl; 0.02 %, w/v, KCl; 0.142 %, w/v, Na_2HPO_4; 0.024 %, w/v, KH_2PO_4; pH 7.5). The samples were homogenised for 3 min in a stomacher (Interscience, St Nom la Bretêche, France), serially diluted and plated onto LBS (*Lactobacillus* selective media) agar, M17 agar, BHI (brain-heart infusion) agar, CA (columbia agar), MRS (de Man, Rogosa and Sharpe) agar, MRS-B agar (MRS supplemented with 1 %, w/v, ox bile), NA (nutrient agar), BSM (bifidus selective media) agar, VRBA (violet red bile agar), BEA (bile esculin agar) and DRCM (differential reinforced clostridial media) agar. All media were purchased from Merck (Darmstadt, Germany) unless otherwise stated. Plates were incubated at 37 °C under aerobic and anaerobic conditions for 48 h and colonies with different morphology selected. Anaerobic conditions were achieved using anaerobic flasks with gas-generating kits (Anaeropack, MGC, Tokyo, Japan).

Biochemical characterisation

Pure cultures were Gram stained. Catalase activity was determined using 3 % (v/v) hydrogen peroxide (H_2O_2). Oxidase activity was determined using 1 % (w/v) TMPD (N,N,N′,N′-tetramethyl-p-phenylenediamine). Oxygen requirements for growth of isolates were determined by inoculating isolates in thioglycolate broth (Merck), as described by Tille and Forbes (2014). Carbohydrate fermentation reactions were recorded using API CHL galleries (Bio Merieux, Maray l'Etoile, France), as per manufacturer's instructions. Production of lactic acid, acetic acid and ethanol from D-glucose was determined using HPLC. Isolates were grown in either MRS or BHI broth under aerobic and anaerobic conditions at 37 °C for 7 d. After incubation, cell-free supernatants were collected by centrifugation (10 000 × *g*, 10 min, 4 °C) and sterilised using a 0.22 µm Millex-HV Millipore filter (Merck Millipore, Billerica, Massachusetts). Perchloric acid (2 %, v/v) was added to supernatants and incubated at 4 °C for 10 min, followed by the addition of 7 N KOH and 24 h of incubation at 4 °C. Samples were centrifuged (12,4709 × *g*, 10 min, 4 °C), passed through a 0.22 µm membrane filter and analysed by HPLC (Waters Corporation, Massachusetts, USA), equipped with a Biorad Aminex HPX 87 H column (Biorad Laboratories, Hercules, California) and a refractive index detector. The mobile phase was 5 mM H_2SO_4 (0.6 mL/min) at 55 °C. Fractions were collected with a Waters 717 autosampler (Waters Corporation, Milford, Massachusetts).

Acid and bile tolerance

Cells were collected from an overnight culture by centrifugation (8000 × *g*, 5 min, 4 °C), washed with sterile PBS (pH 7.5) solution, and cell numbers determined. Sterile PBS solution, adjusted to pH 2 and 3, respectively, were inoculated with log 7 CFU/ml and incubated at 37 °C for 3 h. Changes in viable cells were determined by plating onto either MRS or BHI agar after each hour and incubating under aerobic and anaerobic conditions at 37 °C for 24 h. Viable cells were expressed as percentage viable cells. Bile salt tolerance was determined by growing isolates (log 7 CFU/ml) in MRS or BHI broth, supplemented with 0, 0.2, 0.5, 1 and 2 % (w/v) ox bile salt, under aerobic and anaerobic conditions (depending on isolate growth requirements) at 37 °C for 24 h. Viability after 24 h was determined by plating out on MRS or BHI agar and incubating under aerobic and anaerobic conditions at 37 °C for 24 h. Cell viability was expressed as percentage viable cells.

Hydrogen peroxide production

Isolates producing H_2O_2 were identified using an agar plate assay (Müller, 1984; Rabe and Hillier, 2003), utilising ABTS [2,2'-azino-di-(3-ethyl benzothiazoline-6-sulphonic acid)] and horseradish peroxidase. Isolates were streaked out on hydrogen peroxide agar (1.8 %, w/v, MRS; 1 %, w/v, peptone; 0.2 %, w/v, glucose; 0.5 %, w/v, sodium chloride; 0.25 %, w/v, disodium phosphate; 0.02 mg/ml, 3000 U horseradish peroxidase; 0.30 mg/ml ABTS and 1.2 %, w/v, agar) and incubated at 37 °C for 48 h under anaerobic conditions. After incubation, plates were exposed to oxygen for 30 min, and areas surrounding the colonies inspected for the formation of a dark blue-violet colour, which indicates the presence of hydrogen peroxide.

Antimicrobial activity

Isolates which produced antimicrobial compounds were identified using the agar-well diffusion assay (van Staden, 2015). Supernatant of overnight grown cultures were collected by centrifugation (20 000 × *g*, 10 min, 4 °C), and filter-sterilised using a 0.22 µm membrane filter. Panel microorganisms consisted of *Lactococcus lactis* QU2, *Micrococcus luteus* ATCC 4698, *Listeria monocytogenes* EDGE, Enterohaemorrhagic *Escherichia coli* (EHEC) O157:H7, Enteropathogenic *Escherichia coli* (EPEC) B170, Enteroaggregative *Escherichia coli* (EAEC) 3591-87, Enterotoxigenic *Escherichia coli* (ETEC) H10407, Enteroinvasive *Escherichia coli* (EIEC) ATCC 43892, *Salmonella typhimurium* Xen26, *Salmonella enterica* serovar Enteritidis A9, *Klebsiella pneumoniae* P3 and *Pseudomonas aeromonas* PA-01. Overnight grown indicator microorganisms (10^8 CFU/ml) were inoculated (1 %, v/v) into unsolidified semi-solid agar (0.8 %, m/v) [BHI, MRS or Luria-Bertani (LB, Merck) depending on the species], poured in Petri-dishes and allowed to solidify. A 5 mm-wide well was cut in the agar. The untreated supernatant, supernatant adjusted to pH 7, and pH-neutralised supernatant treated with 0.1 mg/ml proteinase K and 0.1 mg/ml pepsin, respectively, for 1 h at 37 °C, were pipetted (50 µl) into the wells. The plates were incubated at 37 °C for 18 h and after that examined for zones of inhibition.

Antimicrobial compounds were purified by methods previously described by van Staden (2015). BHI was clarified with activated-XAD-16 beads before autoclaving. XAD-16 beads were activated using 80 % (v/v) isopropanol containing 0.1% (v/v) trifluoroacetic acid (TFA). Activated beads were rinsed with distilled water before use. A single colony was inoculated into 10 ml of clarified BHI broth and incubated aerobically on an orbital shaker (100 rpm) at 37 ˚C for 24 h. The culture was subsequently reinoculated (1 %, v/v) into clarified BHI broth and incubated on an orbital shaker (100 rpm) at 37 ˚C for 48 h. Cells were removed by centrifugation (10,000 × g for 25 min at 4 ˚C), and the cell-free supernatant added to activated-XAD-16 beads (2 %, m/v) and placed on an orbital shaker (50 rpm) at 4 ˚C for 4 h. Beads were collected and washed with 30 % (v/v) ethanol, followed by three double-distilled water washes. Peptides bound to beads were eluted using 80 % (v/v) isopropanol (containing 0.1% TFA, v/v) and filtered through a 0.2 µm cellulose acetate filter (Sartorius AG, Göttingen, Germany). Isopropanol was removed using rotary evaporation (RotaVapor®, Buchi). Isopropanol free samples were separated by reverse phase chromatography using Sep-Pak C18 column (Waters, Massachusetts, USA). The column was washed with double-distilled water and manually eluted using a stepwise gradient from 40 to 80 % (10 % increments) with isopropanol (containing 0.1 % TFA, v/v). Fractions were tested for activity against *S. enterica* serovar Enteritidis A9 or *L. monocytogenes* EDGE using the agar-well diffusion assay as described above.

The molecular mass of the partially purified antimicrobial compounds was determined in a Waters Quadrupole Time-of-Flight (Q-TOF) Synapt G2 equipped with an Electrospray ionisation (ESI) source operated in a positive ion mode and was coupled to an Acquity UPLC for the UPLC–MS analysis. Samples (3 µl, 1 mg/ml) were directly injected into a Z spray electrospray ionisation source for direct mass analysis (3 Kv capillary voltage, 15 V cone voltage and a source temperature of 120 °C). The identities of compounds were confirmed with high-resolution MS by comparing it with the mass/charge ratio (m/z) of standards (bacillomycin, fengycin, mycosubtilin, iturin A and surfactin). Data acquisition in the positive mode was performed by MS scanning a second analyser through the m/z range of 200–

3000 Da and the data analysed using Masslynx software version 4.1 (Waters Corporation, Milford, USA).

Phylogenetic relatedness

Genomic DNA was isolated using the Zymo DNA extraction kit (Zymo Research, Irvine, California), according to the manufacturer's instructions. The 16S rDNA gene was amplified by PCR using DNA primers 8F and 1512R (Table 1), according to the method used by Neveling *et al*. (2012). Universal primers recAF and recAR (Tabel 1) were used to amplify *recA* genes (Sarmiento-Rubiano *et al*., 2010). Primers for *gyrB* genes were designed specifically for each species based on results obtained from 16S rDNA and *recA* gene sequence similarity (Table 1). The 16S rDNA, *gyrB* and *recA* amplicons were purified using the Zymoclean™ gel DNA recovery kit (Zymo Research, Irvine, California), according to the manufacturer's instructions. Amplicons were cloned into pGEM®-T Easy vector (Promega, Wisconsin, USA) and transformed into *E*. *coli* DH5α. Competent *E*. *coli* DH5α cells were prepared and transformed as previously described (Neveling *et al*., 2012).

Plasmids were isolated from transformed cells using the PureYield™ plasmid isolation kit (Promega). The 16S rDNA, *gyrB* and *recA* genes were sequenced using the BigDye Terminator V3.1 sequencing kit (Applied Biosystems, Foster City, California). All genes ligated into plasmids were sequenced using the universal primer M13, forward and reverse. Primers 8F, 1512R, 1100R, 520R and 930F were used to sequence plasmids containing the 16S rDNA fragment (Table 1). The *recA* gene was sequenced using primers recAF and recAR. The *gyrB* genes were sequenced using species-specific primers to confirm strain identification (Table 1). Blast analysis was performed, and sequences aligned using MEGA version 6 (Tamura *et al*., 2013). A phylogenetic tree was constructed using sequence lengths of approximately 1500 bp for 16S rDNA, 500-1000 bp for *gyrB* and 600 bp for *recA* genes. Phylogenetic analyses were done using the neighbour-joining method (Saitou and Nei, 1987), maximum-likelihood (Cavalli-Sforza and Edwards, 1967) and maximum parsimony algorithms (Kluge and Farris, 1969). Bootstrapping was performed according to methods described by Felsenstein (1985).

Table 1: PCR primers used to amplify 16S rDNA, *recA* and *gyrB* genes.

Gene	Primer	Primer Sequence	Specificity
16S rDNA	8F	5′-CACGGATCCAGACTTTGATYMTGGCTCAG-3′	Universal
	1512R	5′-GTGAAGCTTACGGYTAGCTTGTTACGACTT-3′	Universal
	930F	5′-GCACAAGCGGTGGAGCATGTGG-3′	Universal
	1100R	5′-AGGGTTGCGCTCGTTG-3′	Universal
	520R	5′-ACCGCGGCTGCTGGC-3′	Universal
recA	recAF	5′-GAAAARRAYTTYGGWAARGGYKCDRTBATGCG-3′	Universal
	recAR	5′-TACATRATRTCDACTTCWSMNMSYTTRAATGG-3′	Universal
gyrB	gyrBAF	5′-CCTGTGGCCGATCTTGAAGT-3′	*B. amyloliquefaciens*
	gyrBAR	5′-AGGAATGTTTCCAGCGCAGA-3′	*B. amyloliquefaciens*
	gyrLJF	5′-GGCGGTGGCGGATATAAAGT-3′	*L. johnsonii*
	gyrLJR	5′-GATCCACCGGCAGAGTTACC-3′	*L. johnsonii*
	gyrLCF	5′-AGCGTAAAGACACTGCCGGAA-3′	*L. crispatus*
	gyrLCR	5′-AGCAGGGTTCTAATGTGGGC-3′	*L. crispatus*
	gyrEFF	5′-GTGGTGCGGTTGTTGATGAC-3′	*E. faecalis*
	gyrEFR	5′-CGACATCGGATCGGTCATA-3′	*E. faecalis*
	gyrLSF	5′-CAGTCTTACATGCCGGTGGT-3′	*L. salivarius*
	gyrLSR	5′-CGTGTTACCTCACGTGCTCT-3′	*L. salivarius*
	gyrLGF	5′-ACGGCCGTGTTAAGGATGAT-3′	*L. gallinarum*
	gyrLGR	5′-CTCGTGCAATCCCACCTTCAT-3′	*L. gallinarum*

Phytase activity

Strains producing phytase were determined using an agar plate assay as previously described by Bae *et al*. (1999). Strains were streaked onto either MRS or BHI agar (each supplemented with 2 %, w/v, sodium-phytate), and incubated under aerobic and anaerobic conditions at 37 °C for 4 d. After incubation, plates were flooded with 2 % (w/v) aqueous cobalt chloride for 5 min. The solution was removed, and plates flooded with a solution consisting of equal volumes of 6.25 % (w/v) ammonium molybdate in 5.5 % (v/v) sulphuric acid and 0.42 % (w/v) ammonium vanadate for 5 min. The solution was removed, and plates were examined for clear zones, indicative of phytic acid hydrolysis.

Phytase enzymes were purified using an ethanol precipitation method as described by Askelson *et al*. (2014). An overnight culture was inoculated at 1 % (v/v) in either MRS or BHI broth supplemented with 0.1 % (w/v) sodium phytate and 0.2 % (w/v) glucose and incubated under aerobic and anaerobic conditions at 37 °C for 24 h. Supernatant and cells were separated by centrifugation (9000 × *g*, 10 min, 4 °C), filter sterilised using a 0.22 µm membrane filter, and chilled ethanol (-80 °C) which constituted 70 % of the total volume was added and incubated at -20 °C for 24 h. After precipitation, the solution was separated into extracellular enzyme and alcohol by centrifugation (12 000 × *g*, 10 min, 4 °C). Extracellular enzymes were resuspended in 0.1 M sodium acetate-acetic acid buffer (pH 5.5), and stored at -20 °C.

Phytase enzyme activity was quantified by measuring the amount of liberated phosphate from sodium phytate, using methods described by Tungala *et al*. (2013). One unit of phytase produces 1 µmol of inorganic phosphate per minute at 50 °C. The reaction mixture contained 100 µl of isolated extracellular enzyme solution and 900 µl of 100 mM sodium acetate-acetic acid buffer (pH 5.5),

supplemented with 2 mM sodium phytate, and incubated at 50 °C for 15 min. The reaction was stopped with the addition of 500 µl of 10 % (w/v) trichloroacetic acid (TCA). The released inorganic phosphate was measured by adding 1 ml of colour reagent (4 volumes of 2.5 %, w/v, ammonium molybdate in 5.5 %, v/v, sulphuric acid and 1 volume of 2.5 %, w/v, ferrous sulphate). The mixture was centrifuged (10 000 × *g*, 5 min, 4 °C), and incubated at room temperature for 15 min, and absorbance measured at 700 nm. Results were compared to a standard curve prepared with K_2HPO_4 ranging from 1-500 µmol. The amount of protein present in the enzyme extract was determined using the Pierce® BCA protein assay kit (Thermo Fisher Scientific), as per manufacturer's instruction.

Amylase activity

Amylase activity was determined using a starch hydrolysis agar plate assay as previously described by Deb *et al*. (2013). Isolates were streaked onto starch agar (0.3 %, w/v, beef extract; 1 %, w/v, starch; 1.2 %, w/v, agar; pH 7.5) and incubated under aerobic and anaerobic conditions at 37 °C 48 h. Starch hydrolysis was revealed by flooding plates with 1 % (w/v) iodine solution, a blue/purple colour indicated the presence of starch and clear zones hydrolysis.

Amylase enzymes were purified using an approach as described by Kaur *et al*. (2012). Basal medium (0.1 %, w/v, KH_2PO_4; 0.25 %, w/v, Na_2HPO_4; 0.1 %, w/v, NaCl; 0.2 %, w/v $(NH_4)_2SO_4$; 0.005 %, w/v, $MgSO_4$; 0.005 %, w/v, $CaCl_2$; 0.2 %, w/v, tryptone and 1 %, w/v, soluble starch) was inoculated at 1 % (v/v) with an overnight culture, and incubated under aerobic and anaerobic conditions at 37 °C for 48 h. Cell-free supernatants were obtained by centrifugation (10 000 × *g*, 15 min, 4 °C). Amylase activity was quantified using methods previously described by Kaur *et al*. (2012). The reaction mixture consisted of 1.8 ml substrate solution (1 %, w/v, soluble starch in 0.05 M sodium phosphate buffer, pH 6.5) and 0.2 ml isolated cell-free supernatant, and incubated at 50 °C for 20 min. The reaction was stopped by the addition of 3 ml dinitrisalicylic acid solution (1 %, w/v, dinitrisalicylic acid; 0.2 %, w/v, phenol; 0.05 %, w/v, sodium sulphite; 1 %, w/v, sodium hydroxide). The mixture was then heated at 90 °C for 15 min to develop a red-brown colour. After that, 1 ml of 40 % (w/v) potassium sodium tartrate solution was added to stabilise the colour. Samples were cooled on ice and absorbance recorded at 595 nm. One unit amylase was defined as the activity required to release 1 µmol of reducing sugar (glucose or maltose) per minute. A standard curve was constructed with D-glucose, ranging from 1-500 µmol. The protein concentration present in the cell-free supernatants were determined using the Pierce® BCA protein assay kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

Bile salt hydrolase and haemolytic activity

Strains were screened for bile salt hydrolase (BSH) production according to the method used by Franz *et al*. (2001a). Cultures were streaked onto MRS or BHI agar supplemented with 0.5 % (w/v) sodium salt of taurodeoxycholic acid and 0.037 % (w/v) $CaCl_2$. Plates were incubated aerobically or anaerobically (depending on the strains growth requirements) at 37 °C for 48 h. After incubation, surrounding areas were inspected for precipitation, indicative of BSH activity. Strain haemolytic activity was determined by streaking onto blood agar (7% v/v sheep blood), and incubating under aerobic and

anaerobic conditions at 37 °C for 48 h. Strains that produced green-hued zones (α-haemolysis) or did not produce any effect (γ-haemolysis) were considered non-haemolytic, whereas strains displaying blood lysis were classified as haemolytic (β-haemolysis).

Exopolysaccharide production

Exopolysaccharide (EPS) producers were identified using an agar plate assay as previously described by Stingele *et al*. (1996). Cultures were streaked onto ruthenium red milk agar (10 %, w/v, skim milk; 1 %, w/v, sucrose; 0.0004 %, w/v, ruthenium red and 1.5 %, w/v, agar), and incubated under aerobic and anaerobic conditions at 37 °C for 48 h. Cultures producing whitish colonies indicated EPS producers and pinkish colonies non-producers.

Exopolysaccharides were purified using an ethanol precipitation method as described by Notararigo *et al*. (2013). Strains were inoculated at 1 % (v/v) in either MRS or BHI broth and incubated under aerobic and anaerobic conditions at 37 °C for 48 h. Cells were harvested by centrifugation (8000 × *g*, 10 min, 4 °C). The supernatant was collected, and EPS precipitated by the addition of double-volume chilled absolute ethanol (-80 °C). The mixture was stored at 4 °C for 48 h and thereafter centrifuged (5000 × *g*, 10 min, 4 °C). The pellet was collected and dissolved in dH_2O supplemented with 8 % (w/v) trichloroacetic acid (TCA) and incubated overnight at 4 °C under continuous stirring. The precipitated protein solution was centrifuged (5000 × *g*, 10 min, 4 °C). The supernatant was collected, and double volume chilled absolute ethanol (-80 °C) was added and incubated at 4 °C for 24 h. The precipitate was collected by centrifugation (10 000 × *g*, 10 min, 4 °C). Precipitate was dissolved in dH_2O and dialysed using a Pierce Slide-A-Lyzer® 10 kDa dialysis cassette (Thermo Fisher Scientific) against dH_2O at 4 °C for 48 h. Purified EPS was lyophilised and stored at 4 °C. Total amount of EPS produced was determined, and their carbohydrate, protein and phosphate composition were determined. Total carbohydrate content was determined using the sulphuric acid method as previously described by Ahire at al. (2015). Briefly, 200 µl of sample (2 mg/ml) was mixed with 700 µl of 77 % (v/v) sulphuric acid and incubated at 4 °C for 10 min. Thereafter, 100 µl of cold 1 % (w/v) tryptophan was added and incubated at 100 °C for 30 min. Samples were cooled to 4 °C, and optical absorbance measured at 490 nm. Pure dextran (MW 71.4 kDa) was used to construct a standard curve ranging from 1-1000 µg/ml. Protein content was determined using the Pierce® BCA protein assay kit (Thermo Fisher Scientific), as per manufacturer's instructions.

Sugar composition was determined by GC-MS as previously described (Neveling *et al*., 2012). Exopolysaccharides (2 mg/ml) were hydrolysed in 2 M trifluoroacetic acid (TFA) at 80 °C for 12 h. Samples were then dried under vacuum (Speed Vac RC 10.09, Jouan Inc., Virginia, USA), washed twice with 100 % (v/v) methanol and dried again. Dried samples were resuspended in 30 mg/mL methoxyamine (dissolved in pyridine), incubated at 30 °C for 90 min, and thereafter N,O-bistrifluoroacetamide and trimethylsilyl chloride (BSTFA + TMCS; 99:1) added and incubated at 30 °C for 1 h. Derivatised samples were resolved on a 15 m Thermo 2607-1300 TG-SQC column (Thermo Fisher Scientific; 0.25 mm x 0.25 µm) in an Agilent 6890 N GC (Agilent Technologies, Wilmington, Delaware) coupled to an Agilent 5975 MSD GC–MS system. The following conditions were used: an initial temperature of 100 °C, increased to 180 °C at 20 °C/min and kept at this temperature for 2 min,

then increased to 330 °C at 8 °C/min and kept at this temperature for 3 min. Sugar standards (2 mg/ml) were derivatised with 30 mg/mL methoxyamine (dissolved in pyridine) and BSTFA + TCMS, as described before, and analysed in parallel. Percentage carbohydrates were calculated by measuring the peak areas of the sugars against a standard. Retention times for sugars were examined by extracted ion chromatography, 307 m/z fragment for internal standard ribitol, D-fructose, D-ribose, D- xylose and L-arabinose, 117 m/z for L-rhamnose, 333 m/z for N-acetyl-glucosamine, 203 m/z for glucosamine, 361 m/z for trehalose and maltose, 437 m/z for sucrose and 319 m/z for D-galactose, D- glucose, D-mannose and D-mannitol. Infrared spectra of EPS were collected using Fourier transform infrared (FTIR) spectroscopy. Attenuated total reflectance-Fourier-transform infrared (ATR-FTIR) spectra were recorded in the range of 600 to 3500 cm^{-1} using a Thermo Scientific Nicolet iS10 FTIR (Thermo Fisher Scientific) spectrometer. ATR-FTIR spectra were collected using 500 scans with a 4 cm^{-1} resolution. Background and atmospheric suppression were subtracted using the OMNIC software (Thermo Fisher Scientific).

Aggregation and biofilm formation

Cells from an overnight culture were harvested (8000 × *g*, 5 min, 37 °C) and washed twice with sterile PBS solution. Cell densities of each bacterial strain were adjusted to an optical density ($OD_{600\ nm}$) of 0.5 in PBS. The culture (4 ml) was dispensed in sterile tubes and vortexed gently for 10 s and incubated at 37 °C for 1 h. The optical density (OD) of upper 1 ml suspension was measured at 600 nm. Aggregation was expressed as 1.00 − (O.D. upper suspension/O.D. total bacterial suspension) × 100 (Del Re *et al*. 2000).

The ability of strains to aggregate and form biofilms on glass disks was determined by atomic force microscopy (AFM) and confocal microscopy. Sterile glass disks (radius 0.4 cm) were placed in a 48-well polystyrene flat-bottom microtiter plate. Overnight-grown cells were diluted in either MRS or BHI to 10^5 CFU/ml. One ml cell suspension was added to each well. Plates were incubated either aerobically or anaerobically at 37 °C for 48 h. Surface topology of biofilms was assessed by AFM using the Easyscan 2 AFM (Nanosurf Inc., California, USA). Images were acquired in static force mode at a scan rate of 2 Hz using a ContAl-G (Innovative solutions Bulgaria, Sofia, Bulgaria) silicon cantilever (resonant frequency of 13kHz and force constant of 0.2 N/m), operated at an I-Gain of 2400, P-Gain of 2800 and a set-point of 30 nN. For confocal microscopy, live and dead cells in biofilms were stained with LIVE/DEAD® BacLight™ bacterial viability kit (Thermo Fisher Scientific, Massachusetts, USA) and EPS with FilmTracer™ SYPRO® ruby biofilm matrix stain (Thermo Fisher Scientific), as per manufacturer's instructions. Confocal images were recorded using a Carl Zeiss LSM 780 confocal microscope (Carl Zeiss, Oberkochen, Germany), fitted with an external Olympus digital camera (15 MP, Olympus Corp., Japan).

Microbial adhesion to solvents (MATS)

Cells from an overnight culture were suspended in 0.1 M KNO_3 followed by centrifugation (8000 × *g*, 5 min, 37 °C) and washing with PBS solution. Optical density (OD) of suspensions were measured at 600 nm (A_0). Three milliliters of cell suspension was mixed with 1 ml solvent (ethyl acetate, chloroform,

or xylene) by gentle vortexing for 10 s and incubated at room temperature for 20 min. After incubation, the aqueous layer was removed carefully, and OD was measured at 600 nm (A_t).

Hydrophobicity percentage was expressed as ($A_0 – A_t / A_0$) × 100 (Bellon-Fontaine *et al*., 1996). Three different solvents were tested in this study; xylene which is an apolar solvent, chloroform a monopolar and acidic solvent; and ethyl acetate a monopolar and basic solvent. Only bacterial adhesion to xylene reflects cell surface hydrophobicity or hydrophilicity. The values of MATS obtained with the two other solvents, chloroform and ethyl acetate, were regarded as a measure of electron donor (basic) and electron acceptor (acidic) capacity, respectively (Bellon-Fontaine *et al*., 1996).

Antibiotic susceptibility

Minimum inhibitory concentrations (MICs) of different antimicrobial agents were determined using the broth microdilution method according to guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015) and the technical guidance prepared by the European Food Safety Authority Panel on additives and products or substances used in animal feed (FEEDAP, 2012). ISO-Sensitest broth (Thermo Fisher Scientific) was inoculated (log 4 CFU/ml) and incubated under aerobic and anaerobic conditions at 37 °C for 24 h. Resistance to antimicrobial agent's penicillin, ampicillin, vancomycin, erythromycin, tetracycline, ciprofloxacin, rifampin, chloramphenicol, gentamicin, kanamycin, trimethoprim and cefoperazone was determined (concentration ranging from 0.125 to 128 mg/l). After incubation, optical density was determined and compared to controls (no antibiotic and inoculated media).

The MICs were defined as the lowest concentration that inhibited visible growth. MIC interpretive breakpoints for *Lactobacillus* (intrinsic resistance) were as follows: penicillin (1 mg/l), ampicillin (1 mg/l), erythromycin (1 mg/l), tetracycline (8 mg/l), chloramphenicol (4 mg/l), kanamycin (16 mg/l), gentamycin (16 mg/l), trimethoprim (512 mg/l), rifampin (1 mg/l), cefoperazone (8 mg/l) and vancomycin (2 mg/l) (FEEDAP, 2012). MIC interpretive breakpoints for *Enterococcus* were as follows: penicillin (16 mg/l), ampicillin (4 mg/l), erythromycin (4 mg/l), tetracycline (2 mg/l), ciprofloxacin (4 mg/l), chloramphenicol (8 mg/l), kanamycin (512 mg/l), gentamycin (32 mg/l), trimethoprim (> 1 mg/l), rifampin (1 mg/l), cefoperazone (8 mg/l) and vancomycin (4 mg/l) (EUCAST, 2018). MIC interpretive breakpoints for *Bacillus* were as follows: penicillin (2 mg/l), ampicillin (2 mg/l), erythromycin (4 mg/l), tetracycline (8 mg/l), ciprofloxacin (0.5 mg/l), chloramphenicol (8 mg/l), kanamycin (8 mg/l), gentamycin (4 mg/l), trimethoprim (24 mg/l), rifampin (0.5 mg/l), cefoperazone (8 mg/l) and vancomycin (4 mg/l) (FEEDAP, 2012).

Enterococcus virulence factors

The presence of *cylA*, *cylB* and *cylM* (Gilmore *et al*., 1994), *gelE* (Qin *et al*., 2000), *cpd* (Clewell *et al*., 2000), *asp1* (Galli *et al*., 1990), *cob* (Clewell *et al*., 2000), *cad* (An and Clewell, 2002), *EF3314* (Creti *et al*., 2009), *asa1* (Vankerckhoven *et al*., 2004), *efaA* (Templer *et al*., 2008) and *ace* (Duprè *et al*., 2003) in the genome of *Enterococcus* strains were determined by PCR and Southern hybridisation (Table 2). In addition, gelatinase production was determined by stab inoculating cultures in either MRS or BHI

broth (supplemented with 3 %, w/v, gelatin) and incubating under aerobic and anaerobic conditions at 37 °C for 5 d. After incubation, cultures were cooled to 4 °C for 5 h, and thereafter inspected for fluidity, indicative of gelatin hydrolysis.

Southern hybridisation confirmed the absence of the genes. Genes not amplified by PCR were amplified from clinical isolates *E. faecalis* DPNS1 and *E. faecalis* DPNS2. PCR amplicons were gel excised using the Zymo gel extraction kit (Zymo Research, Irvine, California). PCR fragments were ligated into pGEM®-T Easy vector (Promega, Wisconsin, USA) and transformed in *E. coli* DH5α by electroporation, described above. Plasmids were isolated using QIAprep plasmid isolation kit (Qiagen, Hilden, Germany), as per manufacturer's instructions. DNA amplicons were sequenced as previously described. DIG-labelled DNA probes were constructed from these amplicons using the DIG DNA labelling kit (Roche, Basel, Switzerland), as per manufacturer's instructions. Total genomic DNA was isolated from *Enterococcus* strains using methods previously described and digested using enzymes *BamHI*, *EcoRV* and *HindIII* at 37 °C for 5 h (1 U enzyme per 1 µg DNA). Digested gDNA was separated on a 0.8 % (w/v) agarose TBE gel at 100 V for 2 h.

Southern blotting was conducted by alkaline transfer of DNA onto a positively charged Amersham Hybond-N+ (GE Healthcare Life Sciences, Chicago, USA) nylon membrane by capillary transfer. Firstly, gels were agitated in 0.25 M HCl for 15 min at room temperature and washed with 0.4 N NaOH for 30 min. A capillary blot was done overnight using a Hyband-N+ nylon membrane and 0.4 N NaOH. After blotting the membrane was rinsed in 2 x SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7) for 1 min. The membrane was then air dried and exposed to 254 nm wavelength for 5 min. Membranes were pre-hybridized in DIG Easy Hyb™ buffer (Roche) for 1 h at 42 °C. The DIG-probe was added to the solution and incubated overnight at 40 °C, and thereafter membranes washed twice with 2 x SSC supplemented with 0.1 % (w/v) SDS for 15 min at room temperature. Membranes were then washed twice with 0.1 % SSC (0.015 M NaCl, 0.0015 M sodium citrate, pH 7) supplemented with 0.1 % SDS, at 65 °C for 20 min. Membranes were then rinsed in wash buffer (0.1 M maleic acid; 0.15 M NaCl; 0.5 %, v/v, tween 20; pH 7.5) for 5 min at room temperature. Membranes were equilibrated in blocking solution (0.1 M maleic acid; 0.15 M NaCl; 4 %, w/v, skim milk) for 30 min, anti-DIG Fab fragment added, and membranes rinsed twice in wash buffer for 15 min. Membranes were soaked in detection buffer (0.1 M Tris base and 0.1 M NaCl, pH 9.5) for 5 min, and thereafter incubated for 15 min with CDP-Star® (Sigma Aldrich, Missouri, USA) at 37 °C. The membrane was then exposed to X-ray film for 1 h and the autoradiograph developed.

Statistical analyses

GraphPad Prism 6 (GraphPad Software Inc., California, USA) was used to perform statistical analyses. Data of acid, bile salt tolerance and microbial adhesion to solvents were analysed by one-way ANOVA to determine the significance of the main effects and interactions. The mean variances were compared using the Tukey post-hoc test. Differences were considered significant if p-values were less than 0.05.

Table 2: Primers used for the amplification of *Enterococcus* virulence factors, and their role in virulence.

Gene	Forward primer	Reverse primer	Size	Role in virulence
asa1	5′-CCAGCCAACTATGGCGGAATC-3′	5′-CCTGTCGCAAGATCGACTGTA-3′	529 bp	aggregation substance
ace	5′-GGAATGACCGAGAACGATGGC-3′	5′-GCTTGATGTTGGCCTGCTTCCG-3′	616 bp	collagen adhesin precursor
cad	5′-ACGAACTCTTCTAGCGCAGC-3′	5′-AGGTGATCCGTCTTCTTCGC-3′	705 bp	sex pheromones cAD1 precursor
slyA	5′-AAATTGGCATGATTGCGCGG-3′	5′-GCCTTGTTCTTCCAAACGCT-3′	197 bp	transcriptional regulator MarR family
EF_3314	5′-AGAGGGACGATCAGATGAAAAA-3′	5′-ATTCCAATTGACGATTCACTTC-3′	566 bp	putative surface antigen
cylA	5′-TGGATGATAGTGATAGGAAGT-3′	5′-TCTACAGTAAATCTTTCGTCA-3′	517 bp	activation of cytolysin
cylM	5′-CTGATGGAAAGAAGATAGTAT-3′	5′-TGAGTTGGTCTGATTACATTT-3′	742 bp	posttranslational modification of cytolysin
cylB	5′-ATTCCTACCTATGTTCTGTTA-3′	5′-AATAAACTCTTCTTTTCCAAC-3′	843 bp	cytolysin B transport protein
asp1	5′-AAGAAAAAGAAGTAGACCAAC-3′	5′-AAACGGCAAGACAAGTAAATA-3′	1,553 bp	aggregation protein
efaA	5′-GACAGACCCTCACGAATA-3′	5′-AGTTCATCATGCTGTAGTA-3′	705 bp	endocarditis specific antigen
cob	5′-AACATTCAGCAAACAAAGC-3′	5′-TTGTCATAAAGAGTGGTCAT-3′	1405 bp	Pheromone cOB1 precursor/lipoprotein
cpd	5′-TGGTGGGTTATTTTTCAATTC-3′	5′-TACGGCTCTGGCTTACTA-3′	782 bp	pheromone cPD1 lipoprotein
gelE	5′-ACCCCGTATCATTGGTTT-3′	5′-ACGCATTGCTTTTCCATC-3′	419 bp	gelatinase

Results and Discussion

Biochemical characterisation

Bacterial isolates were screened for resistance to acidic conditions and bile salts, and the production of hydrogen peroxide and antimicrobial compounds. From 609 isolates, six (DPN184, DPN181, DPN164, DPN167, DPN123 and DPN94) showed resistance to acidic conditions and bile salts, and displayed antimicrobial activity against pathogenic bacteria (*Listeria*, *Escherichia* and *Salmonella*). Isolate DPN123 was aerobic and DPN94 facultatively anaerobic. Microaerophilic or anaerobic growth was required by isolates DPN184, DPN181, DPN164 and DPN167. Isolate DPN167 was isolated from the crop, proventriculus and gizzard area and DPN123 from the duodenum. Isolate DPN94 and DPN164 was isolated from the small intestine i.e. jejunum and ileum. Isolate DPN164 was isolated from the cecum and DPN181 isolated from the large intestine. Fermentation products produced by isolates after 7 d were determined by HPLC (Table 3). None of the isolates produced ethanol. Fermentation by isolates DPN167, DPN164, DPN181 and DPN184 resulted in low pH (pH 3). This is due to the production of high levels of lactic acid (Table 3), compared to DPN94. All isolates produced relative amounts of acetic acid as a by-product. All isolates fermented D-glucose and esculin (Table 4). Most isolates fermented D-fructose and D-mannose, except DPN167. Only isolate DPN123 fermented methyl-αD-glucopyranoside, L-arabinose D-turanose. Most isolates fermented maltose, except DPN181. Most isolates fermented raffinose, except for DPN94. Only isolate DPN94 fermented gluconate, 2-keto-gluconate and D-melezitose. Most isolates fermented N-acetyl-glucosamine, except DPN164. Fermentation results provided insight into the physiology and nutrition characteristics of each isolate.

Table 3: Fermentation by-products produced by isolates after 7 days fermentation. Isolate DPN123 was grown in BHI aerobically and isolates DPN184, DPN164, DPN167, DPN181, DPN94 were grown in MRS under anaerobic conditions.

	Fermentation product (g/L)		
Isolate	Lactic acid	Acetic acid	Ratio (lactic: acetic acid)
DPN184	15.44	1.74	9:1
DPN164	25.42	2.86	9:1
DPN167	19.92	1.92	10:1
DPN181	26.95	1.98	14:1
DPN94	11.71	2.22	5:1
DPN123	1.98	0.48	4:1

Table 4: Carbohydrate fermentation profile of isolates.

Acid from	DPN184	DPN164	DPN167	DPN181	DPN94	DPN123
Glycerol	-	-	-	-	+	+
L-Arabinose	-	-	-	-	-	+
D-Ribose	-	w	w	w	+	+
D-Xylose	w	-	-	-	-	+
D-Galactose	w	+	-	+	+	+
D-Glucose	+	+	+	+	+	+
D-Fructose	+	+	-	+	+	+
D-Mannose	+	+	-	+	+	+
Inositol	-	-	-	-	w	+
D-Mannitol	-	-	-	+	+	+
D-Sorbitol	-	-	-	-	+	+
Methyl-αD-Glucopyranoside	-	-	-	-	-	+
N-Acetyl-Glucosamine	+	-	+	+	+	+
Amygdalin	-	-	+	-	+	w
Arbutin	-	-	-	-	+	+
Esculin	+	+	+	+	+	+
Salicin	-	-	-	-	+	+
D-Cellobiose	+	+	-	-	+	+
D-Maltose	+	+	+	-	+	+
D-Lactose	-	-	-	+	w	+
D-Melibiose	-	-	-	+	-	+
Sucrose	+	+	+	+	w	+
D-Trehalose	-	-	+	+	+	+
D-Melezitose	-	-	-	-	+	-
D-Raffinose	+	+	+	+	-	+
Starch	w	-	-	-	w	+
Glycogen	-	-	-	-	-	+
Xylitol	-	-	-	-	-	+
Gentobiose	w	-	-	-	+	+
D-Turanose	-	-	-	-	-	+
D-Tagatose	-	-	+	-	+	-
Gluconate	-	-	-	-	+	-
2-Keto-Gluconate	-	-	-	-	w	-

[+] Indicates fermentation and [−] indicates no fermentation, [w] indicates weak fermentation. Carbohydrates not listed were not fermented by any of the strains.

Acid and bile tolerance

Most isolates showed good tolerance towards acidic conditions after 3 h exposure (Figure 1). All isolates showed high cell viability at pH 3 after 3 h of exposure (62–95 %). Isolate DPN94 was most tolerant, followed by DPN164, DPN181, DPN167, DPN184 and DPN123. Isolates DPN181 and DPN94 did not survive pH 2 after 3 h exposure, however, low cell numbers were detected after 2 h (25–29 %). Mean retention time in the proventriculus and gizzard vary between 30 min to 1 h (Shires *et al*., 1987; Dänicke *et al*., 1999). Most tolerant towards pH 2 after 3 h exposure was DPN123, followed by DPN167, DPN164 and DPN184. Isolate DPN123 showed the highest tolerance due to the inherent ability to form endospores. The gastric juice secreted from the proventriculus has a pH of approximately 2 to 3 (Riley and Austic, 1984; Mahagna *et al*., 1995). However, the amount of feed, retention time and chemical characteristics of the feed in the gizzard or proventriculus area usually results in higher pH values. The pH of gizzard content varies from 1.9 to 4.5, with an average of 3.5 (Svihus, 2014). It is of importance that probiotic cells survive passing through the proventriculus and gizzard, so to ultimately colonisation the gastrointestinal tract. Results suggest that all probiotic strains could survive transit through the acidic environments in the proventriculus and gizzard.

All isolates showed high tolerance towards bile salts after 24 h exposure (Figure 2). The most tolerant to least tolerant towards 2 % bile-salt after 24 h exposure was DPN123, DPN184, DPN164, DPN181, DPN167 and DPN94. Bile is produced in the liver, stored in the gallbladder, and released into the small intestine for digestion of fats. Bile contains a group of detergent-like bile salts which not only play a role in fat digestion and absorption but also displays bactericidal activity (Hofmann and Mysels, 1992; Gunn, 2000). Thus, resistance to bile salts is essential to allow bacteria to survive in the GIT. The total bile salt concentrations in the chicken duodenum, jejunum, and cecum were determined to be 1.75, 7, and 0.085 mg/ml, respectively (Lin *et al*., 2003). Retention time of feed in the duodenum was determined to be 5 min (Noy and Sklan, 1995), jejunum 40-60 min (Rougière and Carré, 2010) and the retention in the ileum 20-30 min (Weurding *et al*., 2001). Results show that the identified probiotic strains can survive the bile-salt concentrations in the duodenum, jejunum and cecum and thus could survive and colonise the environment.

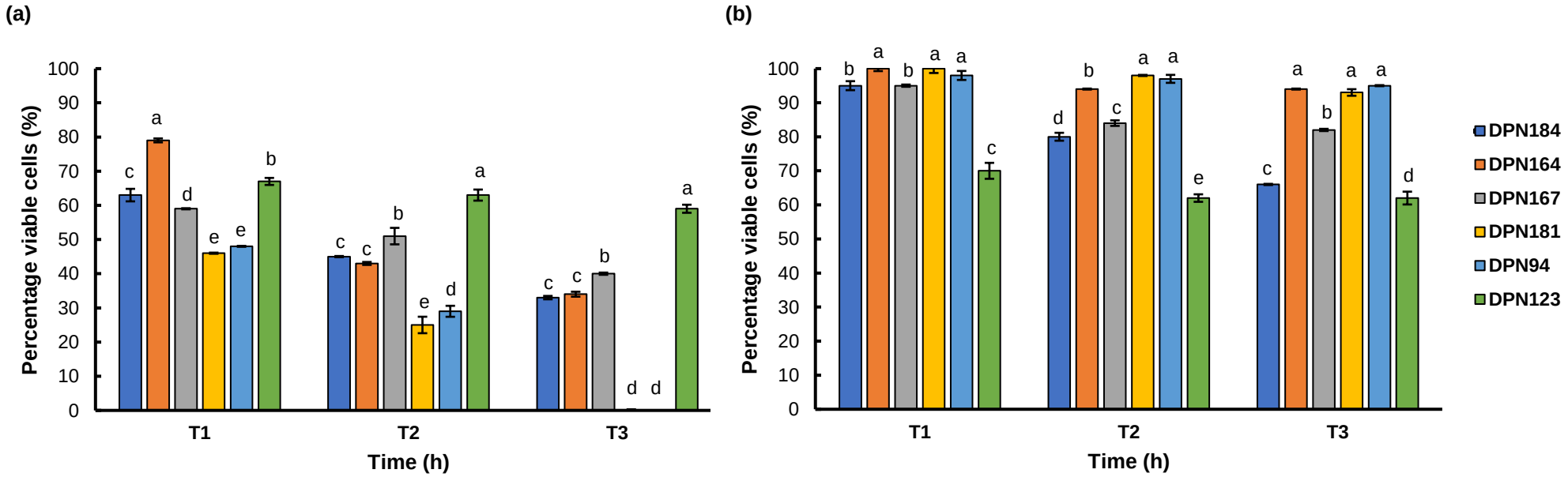

Figure 1: Viability of isolates after exposure to acidic conditions i.e. (a) pH 2 and (b) pH 3 for 1-3 hours. Data is expressed as mean percentage cell viability and error bars indicate standard deviations (n= 3). Bars with different superscripts (a, b, c) differ significantly ($p < 0.05$).

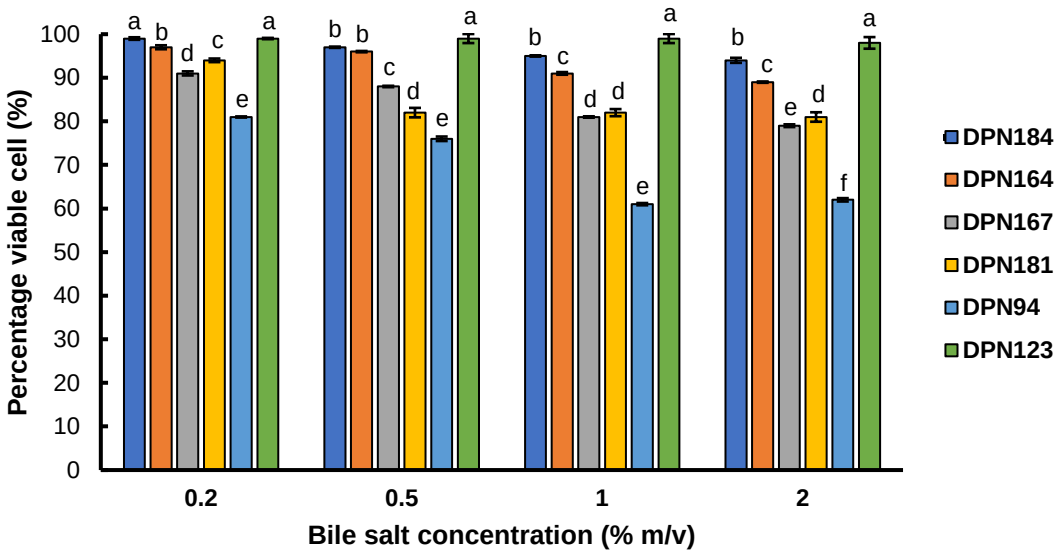

Figure 2: Viability of isolates after exposure to 0.2, 0.5, 1 and 2 % (m/v) bile salt for 24 hours. Data is expressed as mean percentage viable cells and error bars indicate standard deviations (n= 3). Bars with different superscripts (a, b, c) differ significantly ($p < 0.05$).

Hydrogen peroxide production

Hydrogen peroxide was produced by isolates DPN184, DPN181 and DPN167. Hydrogen peroxide has an inhibitory effect and has been shown to inhibit the growth of pathogens such as *Staphylococcus aureus* (Dahiya and Speck, 1968), *Salmonella* Typhimurium (Watson and Schubert, 1969), and *Listeria monocytogenes* (Siragusa and Johnson, 1989).

Antimicrobial activity

Untreated supernatant of isolates DPN181, DPN164, DPN184, DPN94 and DPN167 inhibited *L. monocytogenes* QU2 and *Salmonella enterica* serovar Enteritidis A9, *K. pneumoniae* P3, Enterohaemorrhagic *E. coli* O157:H7 (EHEC), Enteroinvasive *E. coli* ATCC 43892 (EIEC), Enteroaggregative *E. coli* 3591-87 (EAEC), *S. typhimurium* Xen26, Enteropathogenic *E. coli* B170 (EPEC) and Enterotoxigenic *E. coli* H10407 (ETEC). After pH adjustment (pH 7), none of the isolates retained their antimicrobial activity, indicating inhibition was thus due to lactic and acetic acid, which caused acidification of the cytoplasm and disruption of the proton motive force. A decrease in pH leads to trans-membrane gradients resulting in decrease energy production (Wee *et al*., 2006). The supernatant of DPN123 inhibited *M. luteus* ATCC 4698, *L. monocytogenes* EDGE, Enteroinvasive *E. coli* ATCC 43892 and *Salmonella enterica* serovar Enteritidis A9. After the supernatant was pH adjusted (pH 7), proteinase K and pepsin treated the supernatant retained its antimicrobial activity indicating that the isolate produced non-proteinase antimicrobial compounds.

Reverse phase chromatography of supernatant from isolate DPN123 yielded two fractions, 50 % isopropanol and 70 % isopropanol, with antimicrobial activity towards *L. monocytogenes* EDGE and *Salmonella enterica* serovar Enteritidis A9. Fractions were subjected to direct infusion using positive mode electrospray ionisation mass spectrometry (ESI–MS) to determine molecular mass and compound identity. The spectra of the possible biosurfactant compounds produced were compared to the surfactin, mycosubtilin, bacitracin, iturin A and fengycin standards. In the ESI–MS spectrum of the 50 % fraction, a cluster of m/z peaks with a difference of approximately 14 or 22 or 28 atomic mass units (amu) in their molecular ion species were detected, revealing five groups of analogue molecules (Figure 3). The spectra in positive mode showed the main groups of molecular ions at m/z 994.65, 1008.66, 1022.68, and 1036.69 which corresponded to the protonated singly charged species $[M+ H]^+$ (Figure 3; Table 5). Their corresponding sodium adducts $[M+Na]^+$ were also detected at m/z 1016.63, 1030.64, 1044.65 and 1058.66 (Figure 3a; Table 5). The singly charged protonated molecular species $[M+H]^+$ at m/z 994.65, 1008.66, 1022.68 and 1036.66 and their corresponding singly charged sodiated molecules $[M+Na]^+$ (1016.6, 1030.6, 1044.66 and 1058.68) all differed by 14 or 28 amu (Table 5). The detected high-resolution Mr values (ppm < 10) of the possible surfactin analogues in the 50 % fraction corresponded to that of the C13, C14, C15 and C16 surfactin analogues (Srf1-5) (Figure 3a; Table 5). In the ESI–MS spectrum of the 70 % fraction, a cluster of m/z peaks with a difference of approximately 14 or 22 or 28 atomic mass units (amu) in their molecular ion species were detected, revealing five groups of analogue molecules (Figure 3b). The spectra in positive mode showed the main groups of molecular ions at m/z 1017.5, 1031.6 and 1045.6 which corresponded to the deaminated charged species $[M+3H_2O-3NH_3]^+$ (Figure 3b; Table 6). Their corresponding potassium adducts $[M+K]^+$ at m/z

1053.5 and 1067.5 and hydrogenated adducts [M+OH] at m/z 1083.5 were also detected (Figure 3b; Table 6). The singly charged protonated molecular species and their corresponding singly charged potassium molecules all differed by 14 or 28 amu (Table 6). The detected high-resolution Mr values (ppm < 10) of the possible iturin A1 analogues in the 50 % fraction corresponded to that of the C12, C14, C15 and C16 iturin A1 analogues (Figure 3b; Table 6). Production of lipopeptides surfactin and iturin A1 by *B. amyloliquefaciens* strains have been well documented (Yu *et al*., 2002; Sun *et al*., 2006; Alvarez *et al*., 2011).

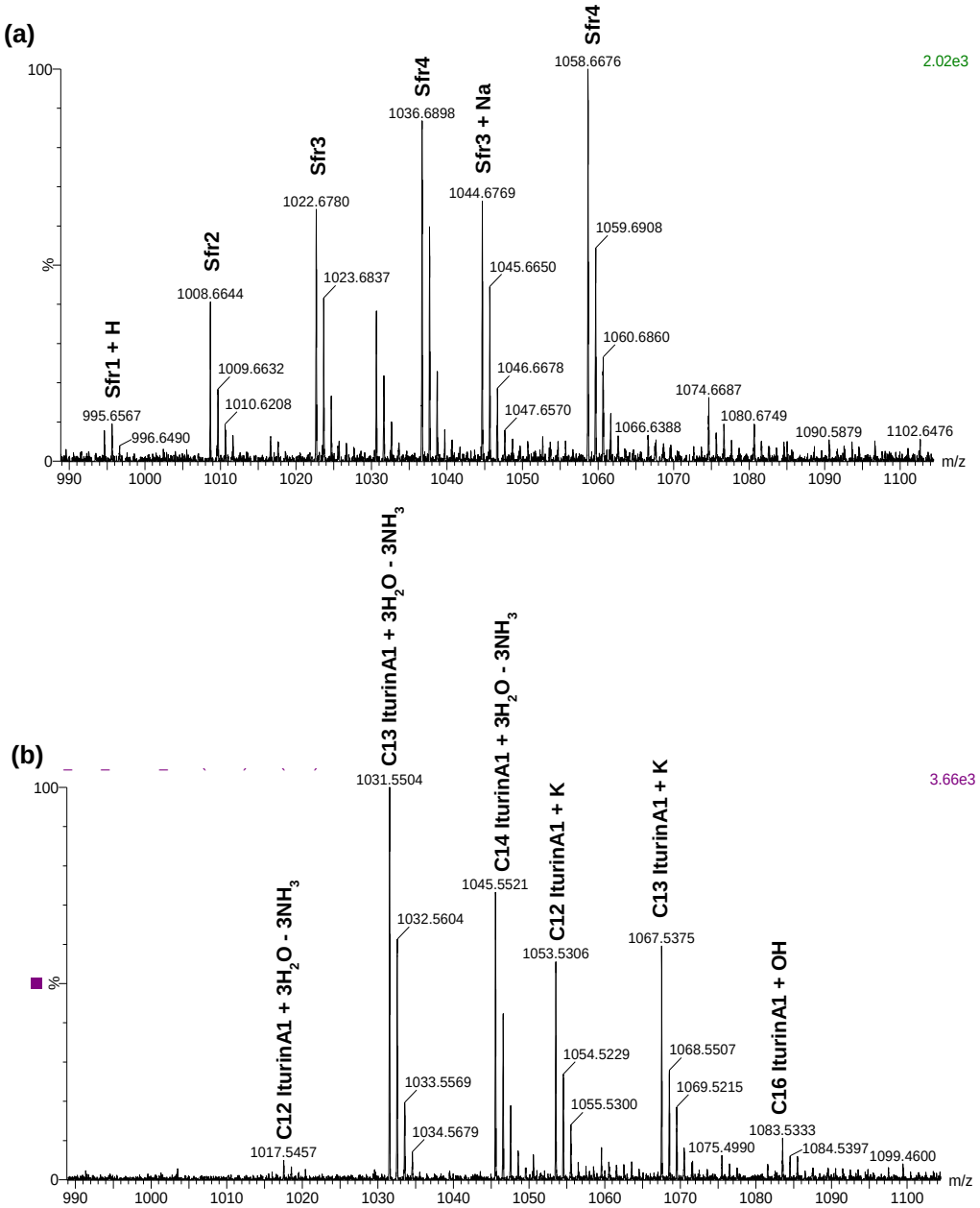

Figure 3: ESI–MS analysis of 50 % isopropanol fraction (a) and 70 % isopropanol fraction (b). The positive mass spectrum generated with MaxEnt 3 is shown. Refer to Table 9 and 10 for identities and expected m/z and Mr values.

Table 5: Summary of the detected surfactin lipopeptides extracted from DPN123, as detected using high resolution mass spectrometry (<10 ppm).

Peptide	Abbr.	Structure	Mr	m/z $[M+H]^+$	m/z $[M+Na]^+$	m/z $[M+2Na-H]^+$	m/z $[M+K]^+$
Surfactin 1	Srf1	cyclo[($C_{13}H_{24}O_2$)-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-L-Leu-L-Val]	993.6	994.6	1016.6	1038.6	1032.6
		cyclo[($C_{13}H_{24}O_2$)-L-Glu-L-Ile-D-Leu-L-Val-L-Asp-L-Leu-L-Val]					
Surfactin 2	Srf2	cyclo[($C_{14}H_{26}O_2$)-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-L-Leu-L-Val]	1007.7	1008.7	1030.6	1052.6	1046.6
		cyclo[($C_{14}H_{26}O_2$)-L-Glu-L-Ile-D-Leu-L-Val-L-Asp-L-Leu-L-Val]					
		cyclo-[($C_{13}H_{24}O_2$)-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-L-Leu-L-Leu]					
		cyclo[($C_{13}H_{24}O_2$)-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-L-Leu-L-Ile]					
Surfactin 3	Srf3	cyclo[($C_{15}H_{28}O_2$)-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-L-Leu-L-Val]	1021.7	1022.7	1044.7	1066.6	1060.6
		cyclo[($C_{15}H_{28}O_2$)-L-Glu-L-Ile-D-Leu-L-Val-L-Asp-L-Leu-L-Val]					
		cyclo[($C_{14}H_{26}O_2$)-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-L-Leu-L-Leu]					
		cyclo[($C_{14}H_{26}O_2$)-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-L-Leu-L-Ile]					
Surfactin 4	Srf4	cyclo[($C_{15}H_{28}O_2$)-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-L-Leu-L-Leu]	1035.7	1036.7	1058.7	1080.7	1074.6
		cyclo[($C_{15}H_{28}O_2$)-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-L-Leu-L-Ile]					
		cyclo[($C_{15}H_{28}O_2$)-L-Glu-L-Ile-D-Leu-L-Val-L-Asp-L-Leu-L-Ile]					
Surfactin 5	Srf5	cyclo[($C_{16}H_{30}O_2$)-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-L-Leu-L-Leu]	1049.70	1050.71	1072.69	1094.67	1088.66

Table 6: Summary of the detected isoforms of iturin A1 lipopeptide extracted DPN123, as detected using high resolution mass spectrometry (<10 ppm).

Structure	Mr	m/z $[M+H]^+$	m/z $[M+Na]^+$	m/z $[M+K]^+$	m/z [M+OH]	m/z [M+OH+ H]	m/z [M + H_2O - NH_3]	m/z [M + $2H_2O$ - $2NH_3$]	m/z [M + $3H_2O$ - $3NH_3$]
cyclo[(Beta-N-C16)-L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	1068.7	1069.6	1091.6	1107.5	1083.5	1084.6	1069.5	1070.5	1071.5
cyclo[(Beta-N-C15)-L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	1054.6	1055.6	1077.5	1093.5	1069.5	1070.5	1055.5	1056.5	1057.5
cyclo[(Beta-N-C14)-L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	1042.6	1043.6	1065.5	1081.5	1057.5	1058.5	1043.5	1044.5	1045.5
cyclo[(Beta-N-C13)-L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	1028.5	1029.5	1051.5	1067.5	1043.5	1044.5	1029.5	1030.5	1031.5
cyclo[(Beta-N-C12)-L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	1014.5	1015.5	1037.5	1053.5	1029.5	1030.5	1015.5	1016.5	1017.5

Phylogenetic relatedness

BLAST analysis of 16S rDNA (Figure 4), *recA* (Figure 5) and *gyrB* (Figure 6) gene sequences were used to assign species identify to isolates. Isolate DPN184 was identified as *L*. *johnsonii* (16S rDNA 100 % sequence similarity to *L*. *johnsonii* N6.2, *recA* gene 98.1 % sequence similarity to *L*. *johnsonii* FI9785 and *gyrB* gene 99.5% similarity to *L*. *johnsonii* FI9785). Isolate DPN181 was identified as *L*. *salivarius* (16S rDNA 99.7 % sequence similarity to *L*. *salivarius* JCM1046, *recA* gene 99.4 % sequence similarity to *L*. *salivarius* UCC118 and *gyrB* gene 98.1% similarity to *L*. *salivarius* Ren). Sequence similarity identified isolate DPN164 as *L*. *gallinarum* (16S rDNA 99.7 % sequence similarity to *L*. *gallinarum* I26, *recA* gene 100 % sequence similarity to *L*. *gallinarum* LMG 9435T and *gyrB* gene 99% similarity to *L*. *gallinarum* BCRC 17266). Isolate DPN167 was identified as *L*. *crispatus* (16S rDNA 99.9 % sequence similarity to *L*. *crispatus* ST1, *recA* gene 99.6 % sequence similarity to *L*. *crispatus* LMG 11415 and *gyrB* gene 99.7 % similarity to *L*. *crispatus* ST1). Isolate DPN123 was identified as *B*. *amyloliquefaciens* (16S rDNA 99.7 % sequence similarity to *B*. *amyloliquefaciens* L-S60, *recA* gene 99.2 % sequence similarity to *B*. *amyloliquefaciens* BCRC 17266 and *gyrB* gene 99.7 % similarity to *B*. *amyloliquefaciens* chenj) and DPN94 as *E*. *faecalis* (16S rDNA 99.7 % sequence similarity to *E*. *faecalis* ATCC 29212, *recA* gene 99.8 % sequence similarity to *E*. *faecalis* ATCC 29212 and *gyrB* gene 100 % similarity to *E*. *faecalis* DENG1).

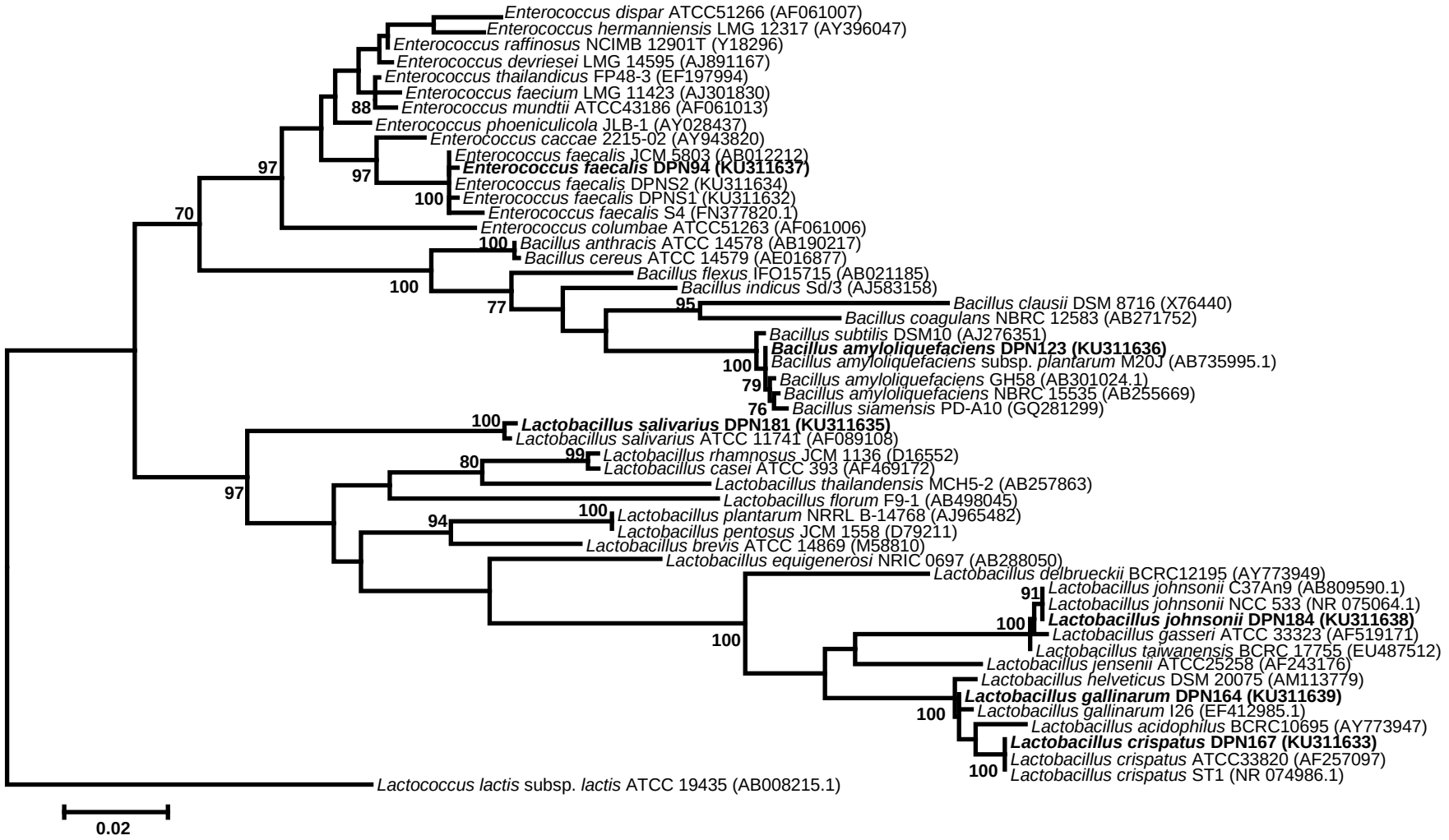

Figure 4: Phylogenetic relatedness of isolates (printed in bold), with other taxa, based on partial 16S rRNA sequences. The phylogenetic tree was constructed by the maximum-likelihood method. *Lactococcus lactis* subsp. lactis was used as an outgroup.

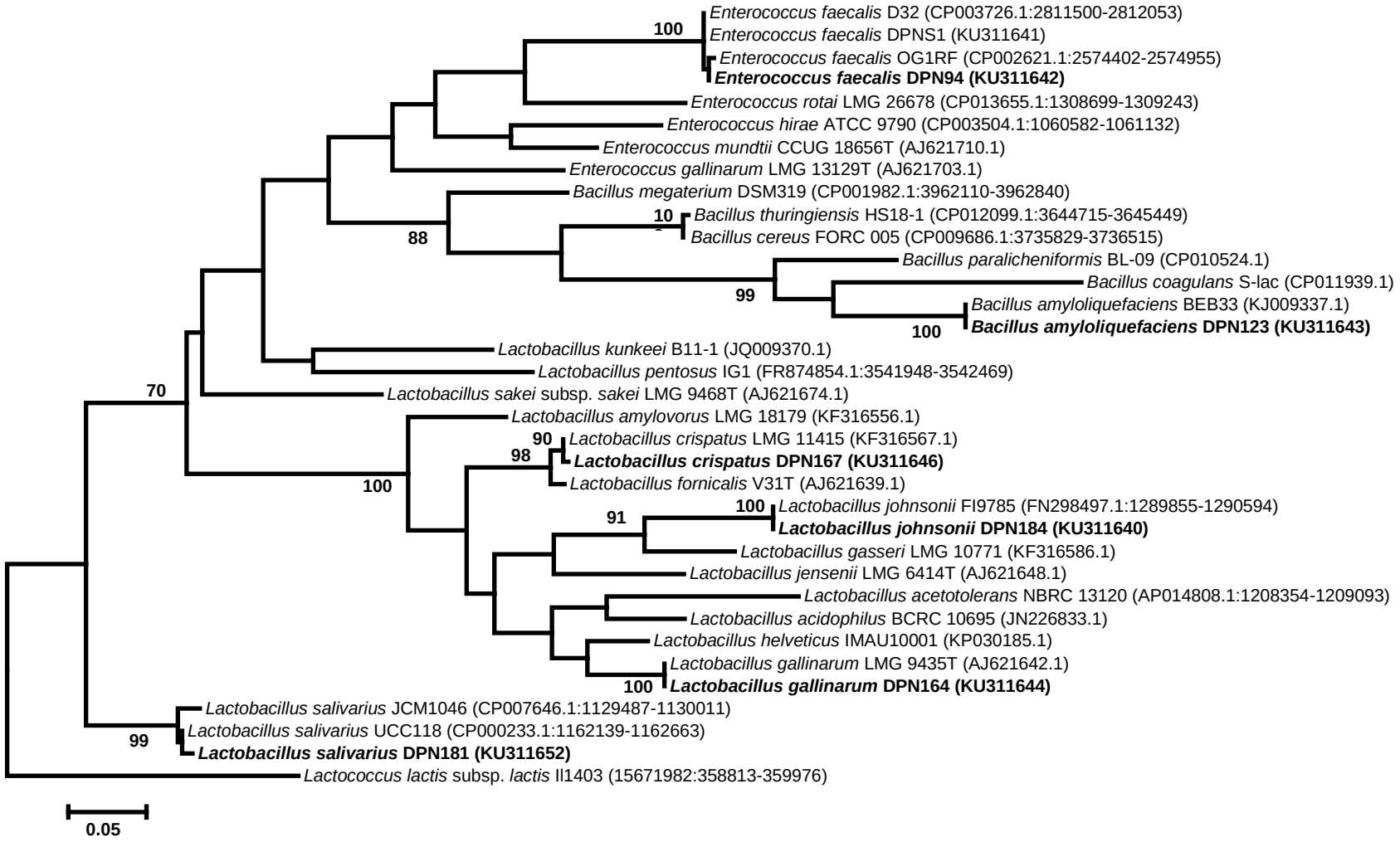

Figure 5: Phylogenetic relatedness of isolates (printed in bold), with other taxa, based on partial recombinase A sequences. The phylogenetic tree was constructed by the maximum-likelihood method. Lactococcus lactis subsp. lactis was used as an outgroup.

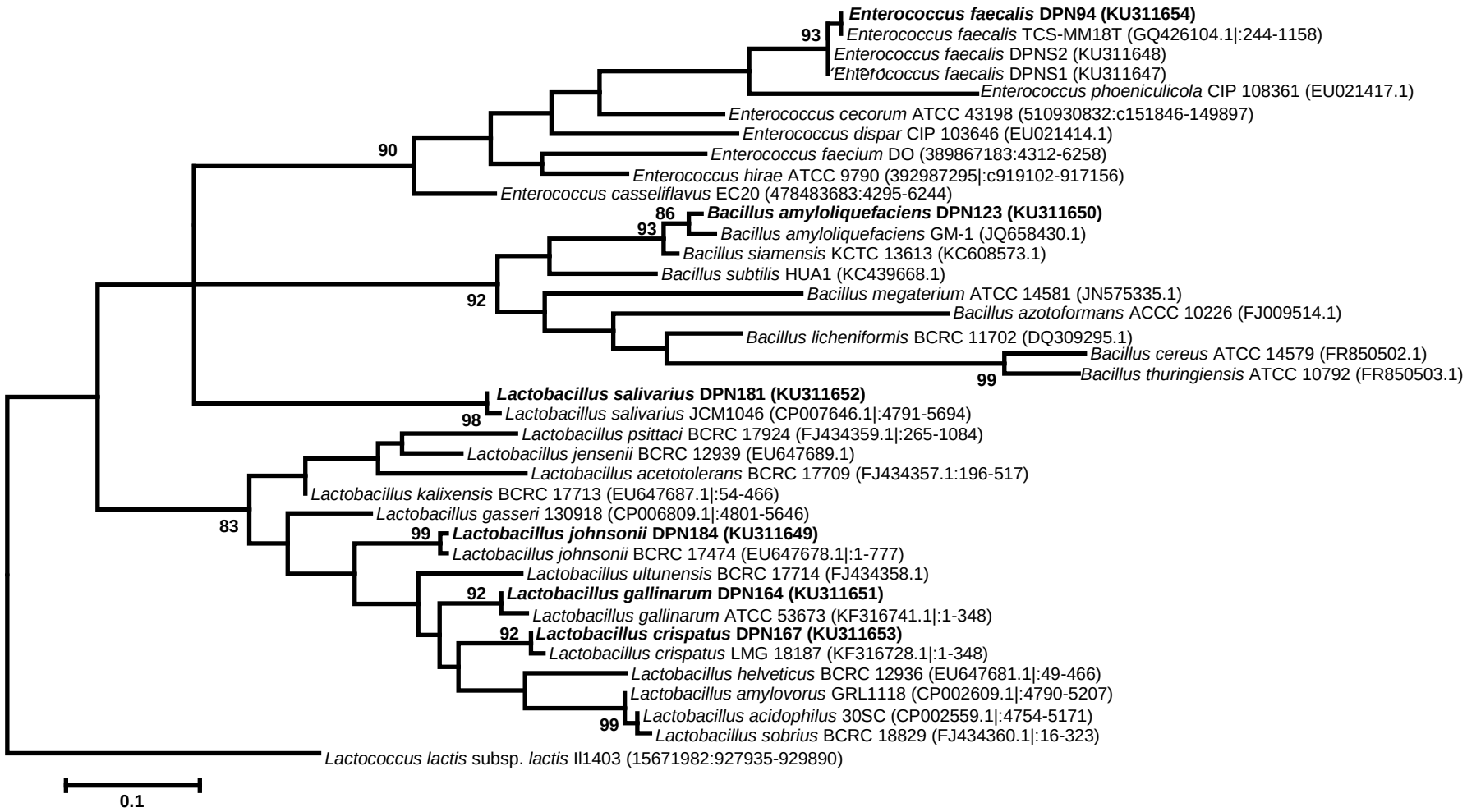

Figure 6: Phylogenetic relatedness of isolates (printed in bold), with other taxa, based on partial gyrase B subunit sequences. The phylogenetic tree was constructed by the maximum-likelihood method. *Lactococcus lactis* subsp. *lactis* was used as an outgroup.

Haemolytic activity and phytase, amylase and bile salt hydrolase production

None of the strains exhibited an effect on red blood cells and was identified as γ-haemolytic. *E. faecalis* DPN94, *L. crispatus* DPN167, *L. johnsonii* DPN184, *L. gallinarum* DPN164 and *L. salivarius* DPN181 showed partial haemolysis due to acid production and were identified as α-haemolytic. Haemolytic activity is an important aspect to consider when selecting probiotic strains (FAO/WHO, 2002). None of the strains had β-haemolytic activity.

Bile salt hydrolase (BSH) was produced by *L. crispatus* DPN167 and *E. faecalis* DPN94. Bile salt hydrolases have been identified in bacterial strains belonging to the genera *Lactobacillus* (Sridevi *et al*., 2009), *Bifidobacterium* (Liong and Shah, 2005) and *Enterococcus* (Franz *et al*., 2001b). Bile salt hydrolase production has previously been identified in *L. crispatus* (Tanaka *et al*., 1999; Mosser and Savage, 2001) and *E. faecalis* strains (Franz *et al*., 2001b; Chand *et al*., 2016).

Bacillus amyloliquefaciens DPN123 produced amylase. Spectrophotometric quantification showed that the strain produced 42.5 U/mg. Amylase production by *B. amyloliquefaciens* has been well documented (Deb *et al*., 2013; Saha *et al*., 2014).

Phytases hydrolyse phytic acid (*myo*-inositol hexaphosphate) to *myo*-inositol and inorganic phosphate (Mitchell *et al*., 1997). Phytic acid is a plant phosphorus storage form and accounts for 50- 80% of total phosphorus present in cereal grains and legumes (Dalai, 1977). Phytic acid has a low bioavailability due to the lack of endogenous phytate-degrading enzymes (Selle *et al*., 2006). In addition, phytic acid exerts antinutritive effects (Adeola and Cowieson, 2011), sequestering essential cations, i.e. Ca^{2+}, Mg^{2+}, Fe^{2+}, and Zn^{2+}, reducing their bioavailability (Graf and Eaton, 1990). Spectrophotometric analysis showed that strains *L. salivarius* DPN181 (2.99 U/mg), *L. johnsonii* DPN184 (15.12 U/mg), *E. faecalis* DPN94 (18.89 U/mg) and *B. amyloliquefaciens* DPN123 (157.47 U/mg) produced enzymes capable of degrading phytic acid. Administration of microbial phytases to improve digestibility of phytic acid is common in the poultry industry (Selle *et al*., 2007). Increase in phytate phosphorus bioavailability due to microbial phytases have been shown to improve in bone mineralisation (Francesch *et al*., 2009), increase body weight (Cowieson and Adeola, 2007) and reduce antinutritive effects (Ravindran *et al*., 1999). Phytase production by *B. amyloliquefaciens* has been well documented (Idriss *et al*., 2002; Boukhris *et al*., 2015). No phytase genes have yet been identified for *Lactobacillus* spp. (Askelson *et al*., 2014). Previously a suspected phytase enzyme was isolated from *L. sanfranciscensis* (De Angelis *et al*., 2003); however, the enzyme had greater substrate specificity toward p-nitrophenyl phosphate over phytate, suggesting that the enzyme was a non-phytate-specific acid phosphatase (Zamudio *et al*., 2001; Palacios *et al*., 2005). Phytate degradation by *L. salivarius* DPN181 and *L. johnsonii* DPN184 could be due to nonspecific acid phosphatases. Phytase activity has been suggested for *Enterococcus* sp. (Marounek *et al*., 2009; Anastasio *et al*., 2010). However, no phytase gene for species belonging to this genus have been reported.

Exopolysaccharide production

All strains produced exopolysaccharides (EPS), with the highest to lowest producers being *L. johnsonii* DPN184 (128 mg/l), *L. gallinarum* DPN164 (104 mg/l), *E. faecalis* DPN94 (81 mg/ml), *L. crispatus*

DPN167 (81 mg/l), *L*. *salivarius* (81 mg/l) and *B*. *amyloliquefaciens* DPN123 (69 mg/l), respectively. Exopolysaccharides fills intracellular spaces between bacteria and together with proteins, nucleic acids and lipids compose the structure of the biofilm matrix. Exopolysaccharides protects bacterial cells against detrimental conditions (Vuong *et al*., 2004; Herasimenka *et al*., 2007). Membrane-associated EPS promotes adherence, facilitating attachment to the mucosal lining (Yadav *et al*., 2011). Exopolysaccharides possesses numerous health benefits such as immune activation (Vinderola *et al*., 2006), antitumor effects (Maalouf *et al*., 2011), blood pressure and cholesterol-lowering properties (Madhuri and Prabhakar, 2014), reduction in lactose intolerance and prevention of diarrhoea (Grandy *et al*., 2010), antimicrobial properties and wound healing properties (Wu *et al*., 2010), activation of cytokine production (Bleau *et al*., 2010), and attenuation of colitis (Lebeer *et al*., 2007). In addition, EPS acts as a prebiotic, stimulating the growth of beneficial bacteria belonging to the genera *Bifidobacterium* and *Lactobacillus* (Harutoshi, 2013).

Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra of partially purified EPS was determined (Figure 7). The absorption bands at 2937 and 2853 cm^{-1} were present which represents the C-H stretching of methyl and methylene groups (Wang *et al*. 2010). The absorption band in the region 1650-1540 cm^{-1} represents the stretching vibrations of enol and amide groups (Singh *et al*., 2011). The peaks between 1440-1382 cm^{-1} were due to the >C=O stretch of the COO^- groups and C-O bond from COO^- groups (Wang *et al*. 2010). The region between 800 and 1200 cm^{-1} is recognised as a fingerprint region and is used to characterise different polysaccharides (Sekkal and Legrand, 1993). The absorption band at 809 cm^{-1} indicates the presence of α-glycosidic linkages (Sekkal and Legrand, 1993). The bands at 870-890 cm^{-1} indicate the presence of β-glycosidic linkages (Tao *et al*. 2008). The band at 734 cm^{-1} is due to C-O-C bending vibration (Sekkal and Legrand, 1993). Broad bands at 1027 and 1128 cm^{-1} are due to C-O-C glycosidic band vibration and indicate the presence of pyranose configurations (Wang *et al*. 2010). The strong absorption at 1649 cm^{-1} and 1560 cm^{-1} is attributed to C≡O stretching and N-H bending vibration of amide groups (Wang *et al*. 2010). The weak band at 3288 cm^{-1} is assigned to N-H stretching vibration (Wang *et al*. 2010).

Exopolysaccharides isolated from *L*. *crispatus* DPN167, *B*. *amyloliquefaciens* DPN123, *L*. *gallinarum* DPN164, *L*. *johnsonii* DPN184, *L*. *salivarius* DPN181 and *E*. *faecalis* DPN94 contained 82.44 %, 124.73 %, 124.61 %, 124,61 %, 102.80 %, 118.24 % and 126.87 % (w/w) sugar. These crude extracts contained 0.099 %, 0.063 %, 0.098 %, 0.210 %, 0.338 % and 0.237 % (w/w) DNA, respectively. And 6.7 %, 4.4 %, 8.3 %, 9.3 %, 9.8 % and 7.1 % (w/w) protein, respectively. Only EPS isolated from *L*. *salivarius* DPN181 contained 0.237 % (w/w) RNA. Sugar composition of the partially purified EPS was determined by GC-MS (Table 7). Most EPS contained L-arabinose, except for EPS produced by *B*. *amyloliquefaciens* DPN123. All EPS contained D-glucose, D-galactose and D-mannose. Only EPS produced by *L*. *gallinarum* DPN164 contained D-fructose. Glucosamine, N-acetyl-glucosamine, L-rhamnose, D-ribose, D-mannose, sucrose, D-xylose and galacturonic acid were not detected. Exopolysaccharide production have been well documented in strains *L*. *crispatus* (Donnarumma *et al*., 2014), *L*. *johnsonii* (Dertli *et al*., 2013 and 2016), *L*. *salivarius* (Liu *et al*., 2009; Sanhueza *et al*., 2015) and *E*. *faecalis* (Venkatesh *et al*., 2016) and *B*. *amyloliquefaciens* (Malick *et al*., 2017). Production of exopolysaccharides by *L*. *gallinarum* have not been reported.

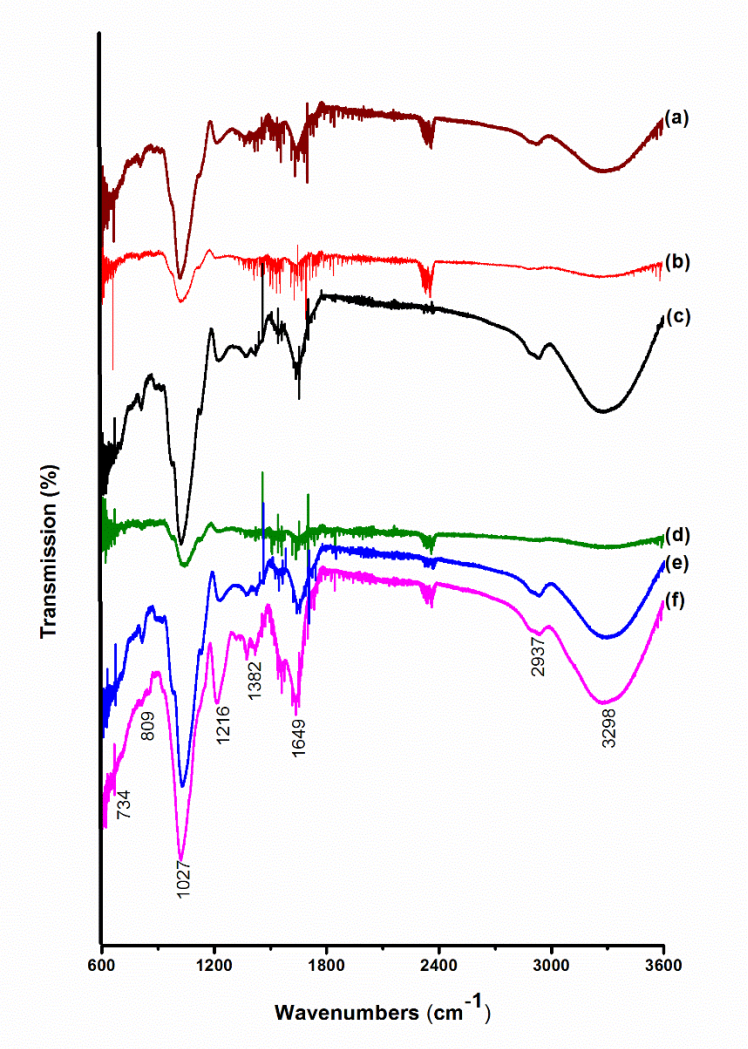

Figure 7: FTIR spectra of partially purified EPS isolated from (a) *L. johnsonii* DPN184, (b) *L. gallinarum* DPN164, (c) *L. crispatus* DPN167, (d) *L. salivarius* DPN181, (e) *E. faecalis* DPN94 and (f) *B. amyloliquefaciens* DPN123.

Table 7: Percentage sugar composition of partially purified exopolysaccharides.

	Sugars (expressed as percentage)				
EPS from strain	L-Arabinose	D-Glucose	D-Fructose	D-Galactose	D-Mannose
L. johnsonii DPN184	14	35	-	30	21
L. gallinarum DPN164	11	25	14	19	27
L. crispatus DPN167	12	35	-	28	25
L. salivarius DPN181	15	29	-	32	24
E. faecalis DPN94	11	11	-	27	26
B. amyloliquefaciens DPN123	-	38	-	38	24

Hydrophobicity, aggregation and biofilm formation

All strains except *L*. *gallinarum* DPN164 showed a stronger affinity for chloroform than for ethyl acetate (Figure 8). These strains possessed more electron donor groups on their cell surface, *L*. *gallinarum* DPN164 possessed more electron acceptor groups. Bacteria showing affinity to xylene above 40 % present high hydrophobic characteristics (Giarous *et al*., 2009). Probiotic bacteria should have a hydrophobic surface for high adhesion capabilities to GIT (Del Re *et al*., 2000). A high percentage of *L*. *crispatus* DPN167, *L*. *salivarius* DPN18 and *L*. *johnsonii* DPN184 cells adhered to xylene (Figure 8), indicating that these strains possess hydrophobic cell surfaces. Strains *L*. *gallinarum* DPN164, *E*. *faecalis* DPN94 and *B*. *amyloliquefaciens* DPN123 showed low levels of adhesion to xylene and possessed hydrophilic cell surfaces. The presence of glyco-proteinaceous material at the cell surface results in higher hydrophobicity, whereas hydrophilic surfaces are due to polysaccharides (Green and Klaenhammer 1994; Rojas and Conway 1996).

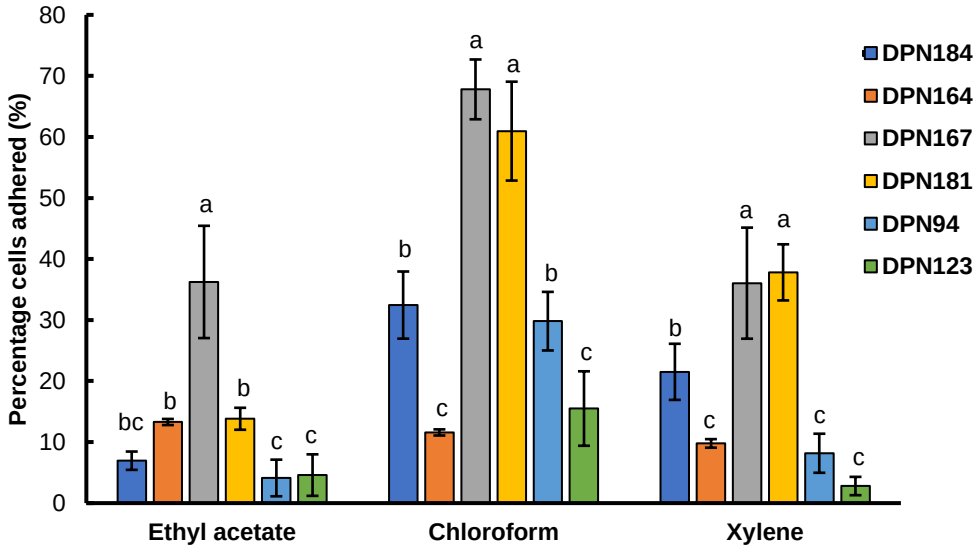

Figure 8: Adhesion of strains to solvent ethyl acetate, chloroform and xylene. DPN184- *L*. *johnsonii*, DPN164- *L*. *gallinarum*, DPN167- *L*. *crispatus*, DPN181- *L*. *salivarius*, DPN94- *E*. *faecalis* and DPN123- *B*. *amyloliquefaciens*. Bars with different superscripts (a, b, c) differ significantly ($p < 0.05$). Error bars indicate standard deviations (n= 3).

Most aggregative to least aggregative were *B*. *amyloliquefaciens* DPN123 (20.2 % ± 0.5), *L*. *gallinarum* DPN164 (11.6 % ± 0.5), *L*. *salivarius* DPN181 (11.5 % ± 1.0), *E*. *faecalis* DPN94 (11.5 % ± 0.2), *L*. *johnsonii* DPN184 (10.0 % ± 3.1) and *L*. *crispatus* DPN167 (6.5% ± 0.1). According to Del Re *et al*. 2000, strains with values lower than 10 %, are considered to have weak aggregative abilities. Strains *B*. *amyloliquefaciens* DPN123, *L*. *gallinarum* DPN164, *L*. *salivarius* DPN181, *E*. *faecalis* DPN94 and *L*. *johnsonii* DPN184 possessed high aggregative abilities and *L*. *crispatus* DPN167 weak aggregative abilities. All strains aggregated after 48 h to form biofilms on the surface of glass disks (Figure 9). The mean square root surface roughness was determined by AFM, the more aggregative the cells, the rougher the surface. The highest to lowest root mean square surface roughness for were

as follows; *B*. *amyloliquefaciens* DPN123 (0.212 µm), *L*. *gallinarum* DPN164 (0.208 µm), *L*. *salivarius* DPN181 (0.208 µm), *L*. *crispatus* DPN167 (0.207 µm), *L*. *johnsonii* DPN184 (0.174 µm) and *E*. *faecalis* DPN94 (0.164 µm). Aggregation and biofilm formation by strains were also assessed by confocal microscopy (Figure 10). Strains *L*. *salivarius* DPN181, *B*. *amyloliquefaciens* DPN123, *L*. *gallinarum* DPN164 and *L*. *johnsonii* DPN184 formed complex biofilm matrixes due to the increased staining by SYPRO® ruby. Aggregative ability correlates with the ability to adhere to epithelial cells (Kos *et al*., 2003), an important property for the selection of probiotic strains (Bao *et al*., 2010; Kotzamanidis *et al*., 2010).

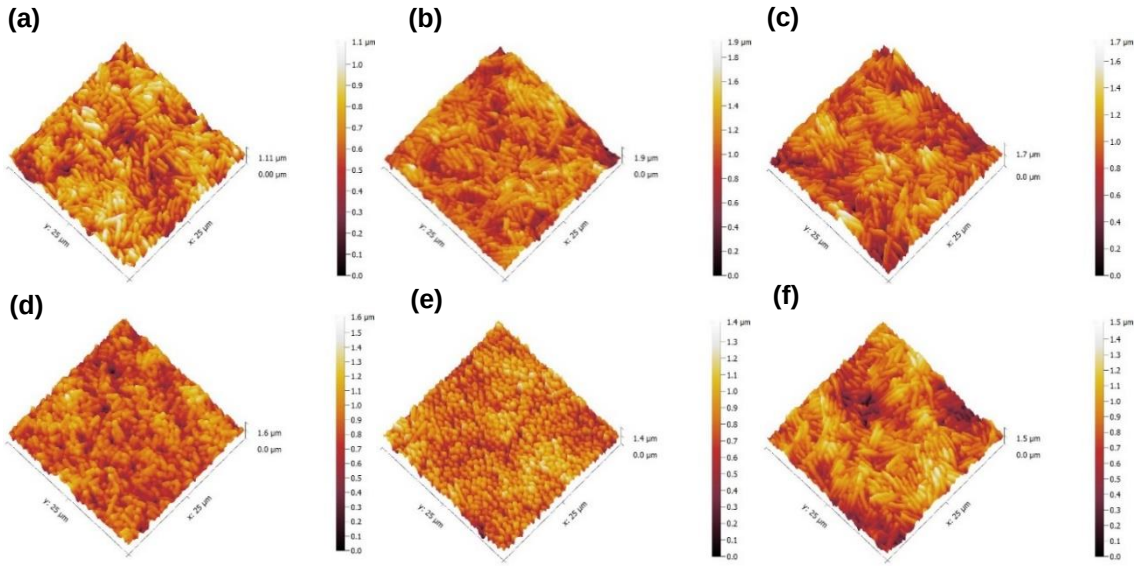

Figure 9: Three-dimensional topographical AFM images of aggregated cells after 24 h of (a) *L*. *johnsonii* DPN184, (b) *L*. *gallinarum* DPN164, (c) *L*. *crispatus* DPN167, (d) *L*. *salivarius* DPN181, (e) *E*. *faecalis* DPN94 and (f) *B*. *amyloliquefaciens* DPN123.

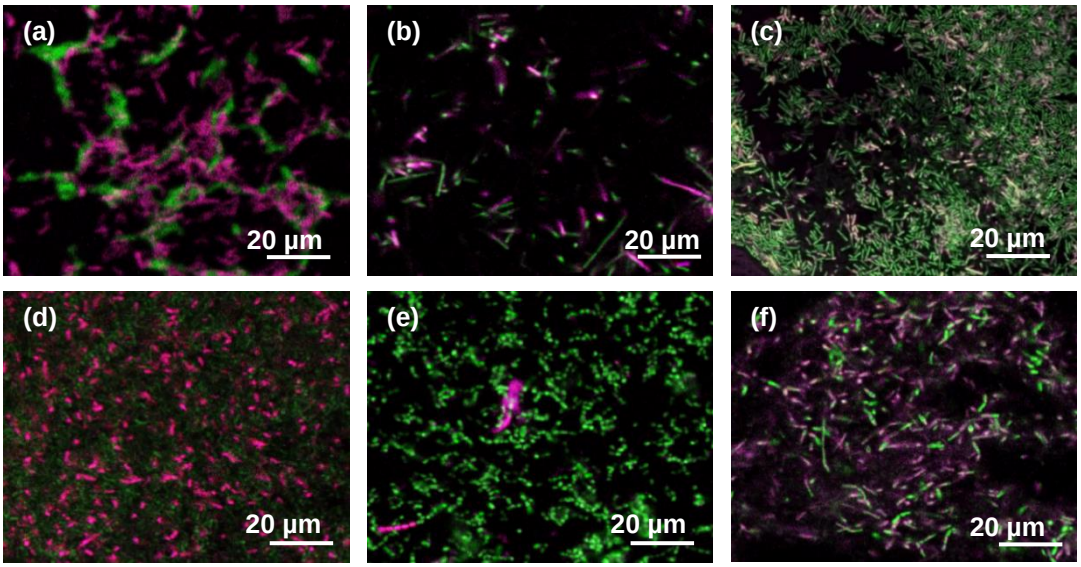

Figure 10: Confocal images of aggregated cells after 24 h of (a) *L*. *johnsonii* DPN184, (b) *L*. *gallinarum* DPN164, (c) *L*. *crispatus* DPN167, (d) *L*. *salivarius* DPN181, (e) *E*. *faecalis* DPN94 and (f) *B*. *amyloliquefaciens* DPN123. Cells were stained with stained cells with LIVE/DEAD® BacLight™ bacterial viability kit and EPS with FilmTracer™ SYPRO® Ruby Biofilm Matrix Stain.

Antibiotic susceptibility

Minimal inhibitory concentration (MIC) of strains towards different antibiotics are shown (Table 8). The MIC interpretative criteria used were those published by the CLSI (CLSI, 2015) and those by the FEEDAP (FEEDAP, 2012). For safety reasons, transferable antibiotic resistance of probiotics should be determined (De Vuyst *et al*., 2003).

All *Lactobacillus* strains (*L*. *crispatus* DPN167, *L*. *salivarius* DPN181, *L*. *johnsonii* DPN184, and *L*. *gallinarum* DPN164) were sensitive to penicillin, ampicillin, gentamycin and trimethoprim. All lactobacilli strains showed resistance to erythromycin, tetracycline, ciprofloxacin, chloramphenicol and kanamycin. Only *L*. *salivarius* DPN181 was resistant to vancomycin and cefoperazone. Members of the genera *Lactobacillus* are intrinsically resistant to vancomycin, trimethoprim, kanamycin and ciprofloxacin (Hummel *et al*., 2007; Ammor *et al*., 2008a). Vancomycin resistance occurs due to the replacement of terminal D-alanine residues by D-lactate or D-serine in muramyl pentapeptide, preventing binding (Ammor *et al*., 2008a; Sharma *et al*., 2015). Intrinsic ciprofloxacin resistance is due to an unknown mechanism (Hummel *et al*., 2007). Resistance to kanamycin is due to the absence of cytochrome-mediated electron transporter, disabling uptake (Charteris *et al*., 2001). Acquired resistance for tetracycline, erythromycin, chloramphenicol and cefoperazone have been identified in the genera *Lactobacillus* (Chopra and Roberts, 2001; Roberts, 2005; Ammor *et al*., 2007; Gueimonde *et al*., 2013). The most common resistance found in lactobacilli is tetracycline (Ammor *et al*., 2008b). At least 11 different tetracycline resistance genes have been classified for members of *Lactobacillus* (Lahtinen *et al*., 2009). Chloramphenicol resistance genes have been identified for members of *Lactobacillus* (Klare *et al*., 2007; Mayrhofer *et al*., 2010). Erythromycin resistance genes have been identified in several *Lactobacillus* spp. (van Hoek *et al*., 2008). A previous study assessing the occurrence of antibiotic resistance in *Lactobacillus* spp. found low occurrences of cefoperazone resistance (Gad *et al*., 2014).

Table 8: Minimal inhibitory concentration (MIC) of strains towards different antibiotics.

	MIC (mg/l)					
Antibiotic	*L. johnsonii* DPN184	*L. gallinarum* DPN164	*L. crispatus* DPN167	*L. salivarius* DPN181	*E. faecalis* DPN94	*B. amyloliquefaciens* DPN123
Penicillin G	<0.125	<0.125	<0.125	<0.125	0.5	<0.125
Ampicillin	1	<0.125	<0.125	<0.125	0.25	<0.125
Vancomycin	2	0.25	0.25	>128	1	1
Erythromycin	>128	>128	>128	>128	>128	<0.125
Tetracycline	128	128	128	128	64	16
Ciprofloxacin	>128	>128	>128	>128	8	0,5
Rifampin	0.5	0.25	0.25	0.25	0.5	<0.125
Chloramphenicol	16	8	16	8	8	2
Gentamicin	<0.125	<0.125	<0.125	<0.125	16	0.5
Kanamycin	>128	>128	>128	>128	>128	6
Trimethoprim	>128	>128	0.25	>128	<0.125	8
Cefoperazone	1	0.5	2	16	16	0.5

Enterococcus faecalis DPN94 was susceptible to penicillin, ampicillin, vancomycin, chloramphenicol and gentamycin, however, were resistant towards erythromycin, tetracycline, ciprofloxacin, kanamycin and trimethoprim. Members of the genera *Enterococcus* have intrinsic resistance to trimethoprim. Trimethoprim binds to dihydrofolate reductase and inhibits reduction of dihydrofolic acid to tetrahydrofolic acid essential for thymidine synthesis (Brogden *et al*., 1982). *Enterococcus* can absorb folic acid from the environment, bypassing the effects of trimethoprim (Bushby and Hitchings, 1968). *In vitro* tests yield a susceptible result; however, trimethoprim is ineffective in inhibiting *Enterococcus* (Chenoweth *et al*., 1990). Resistance towards kanamycin is by acquired aminoglycoside-modifying enzymes or nucleotidyltransferases (Carlier and Courvalin, 1990; Hollenbeck and Rice, 2012). Tetracycline resistance genes (*tetM*, *tetO*, *tetS*, *tetK*, *tetL* and *tetU*) confer resistance to members *Enterococcus* genera (Chopra and Roberts, 2001; Huys *et al*., 2004). Two major resistance mechanisms allow erythromycin resistance in *Enterococcus*, i.e. ribosomal methylases and efflux pump systems (Singh *et al*., 2001). Enterococci have acquired resistance to ciprofloxacin by mutations in the *parC* and *gyrA* regions (Leavis *et al*., 2006).

Bacillus amyloliquefaciens DPN123 was susceptible to penicillin, ampicillin, vancomycin, gentamycin, erythromycin, ciprofloxacin, kanamycin and trimethoprim, however, were resistant to tetracycline. Tetracycline resistance in the genera *Bacillus* is due *tetL* (Phelan *et al*., 2011), *tetM* (Roberts *et al*., 1999), and *tetK* (Neela *et al*., 2009) genes.

Enterococcal virulence factors

Enterococcus faecalis DPN94 was confirmed to be gelatinase negative by the gelatinase hydrolysis test. PCR confirmed that the genome of *E. faecalis* DPN94 encoded for *cob* (NCBI ID KU311661), *cpd* (KU311666), *efaA* (KU311663), *gelE* (KU311665), *cad* (KU311655), *ace* (KU311656), *slyA*

(KU311657), *asa1* (KU311658), *EF3314* (KU311660) and *asp1* (KU311662) genes. Southern hybridisation showed that the genome of *E*. *faecalis* DPN94 did not encode for *cylA*, *cylM* and *cylB* genes (Figure 11).

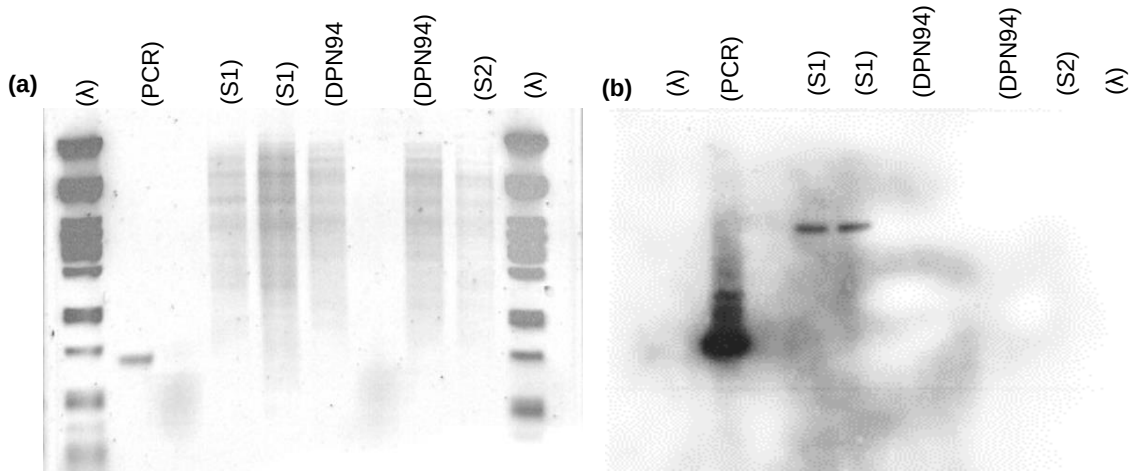

Figure 11: Gel electrophoresis separation of (a) PCR amplicon of *cylB* gene and fragmented gDNA from *E*. *faecalis* DPN94, *cylB* negative *E*. *faecalis* S2 and *cylB* positive *E*. *faecalis* S1. Autoradiograph (b) of corresponding southern blot hybridized with DIG-labelled *cylB* probe.

The *cad* gene encodes the sex pheromone cAD1 precursor, which acts as a signal for conjugation (An and Clewell, 2002). The *ace* gene codes for a collagen adhesin precursor which mediates association of bacteria to host proteins, i.e. collagen I, IV and laminin (Lebreton *et al*., 2009). The *sly*A gene is a repressor for the expression of virulence factors (Michaux *et al*., 2011a and b). The *asa1* gene codes for an aggregation substance, allowing aggregation to facilitate conjugation (Galli *et al*., 1990). The *EF3314* gene codes for a cell-anchor surface protein implicated in biofilm formation (Creti *et al*., 2009). The *cob* gene codes for a sex pheromone cOB1 precursor/lipoprotein involved during conjugation (Clewell, 2011). The *asp1* gene codes for an aggregation substance required for cell-to-cell contact during conjugation and adhesion to eukaryotic cells (Cariolato *et al*., 2008). The *efaA* gene codes for cell wall adhesion (Lowe *et al*., 1995). The *gelE* gene codes for gelatinase which is a zinc metalloprotease which hydrolyses gelatin, casein, haemoglobin and other bioactive compounds (Walters *et al*., 2003). *Enterococcus faecalis* DPN94 encoded for the *gelE* gene; however, no gelatinase activity was detected by the gelatine hydrolysis test. It has been shown that the presence of the *gelE* gene does not correlate with its expression (Medeiros *et al*., 2014). The *cpd1* gene codes for pheromone cPD1 lipoprotein involved in conjugation (Clewell *et al*., 2000). The genome of *E*. *faecalis* DPN123 did not encode for *cylA*, *cylB* and *cylM* genes. Cytolysin is a bacterial toxin with haemolytic and antibacterial activity against Gram-positive bacteria (Gilmore *et al*., 1994). The incidence of virulence genes in *E*. *faecalis* strains from environmental and clinical sources is high (Creti *et al*., 2004; Martín-Platero *et al*., 2009). It has been suggested that virulence factors should not be considered true virulence determinants, but rather auxiliary factors associated to the colonisation of the gastrointestinal tract (Toledo-Arana *et al*., 2001; Pillar and Gilmore, 2004). Genes which were present

in most environmental isolates are *ace*, *efaA* and *EF3314*, *asa1*, *asp1* and the *gelE* gene (Creti *et al*., 2004; Trivedi *et al*., 2011). Clinical strains have a higher prevalence of *asp1*, *esp* and *cylA* genes (Creti *et al*., 2004). The presence of these virulence genes in the genome of *E. faecalis* DPN94 could rather be beneficial attributes as it allows the strain to have greater adhesion and aggregation abilities.

Conclusions

From 609 bacteria isolated from healthy broilers, six strains (*L. crispatus* DPN167, *L. salivarius* DPN181, *L. johnsonii* DPN184, *L. gallinarum* DPN164, *E. faecalis* DPN94 and *B. amyloliquefaciens* DPN123) showed high tolerance towards acidic conditions and bile salt. All strains produced hetero-exopolysaccharides which aided their tolerance towards these harsh conditions. All strains could form biofilms as confirmed by AFM and confocal microscopy. *Bacillus amyloliquefaciens* DPN123 produced extracellular amylase which could increase in starch degradation, improving growth performance. Hydrogen peroxide was produced by *L. johnsonii* DPN184, *L. salivarius* DPN181 and *L. crispatus* DPN167 which could assist cells in inhibiting colonisation of sensitive pathogenic bacteria. Phytase enzymes were produced by *B. amyloliquefaciens* DPN123 and *E. faecalis* DPN94, which could increase nutrient availability in feed, improving growth performance. Antimicrobial lipopeptides surfactin and iturin A1 were produced by *B. amyloliquefaciens* DPN123 and showed antimicrobial activity towards *M. luteus* ATCC 4698, *L. monocytogenes* EDGE, Enteroinvasive *E. coli* ATCC 43892 and *S. enterica* serovar Enteritidis A9. Bile salt hydrolase was produced by *E. faecalis* DPN94 and *L. crispatus* DPN167, which could indirectly lead to lowering of cholesterol effects. The genome of *E. faecalis* DPN94 encoded for virulence genes *cad*, *ace*, *slyA*, *asa1*, *EF3314*, *EF0109*, *cob*, *asp1*, *efaA*, *gelE* and *cpd*, however, did not encode for cytolysin (*cylA*, *cylB* and *cylM*). In conclusion, strains isolated from healthy broilers showed *in vitro* tolerance towards simulated gastrointestinal tract conditions and possessed numerous beneficial characteristics.

References

Abudabos, A.M., Alyemni, A.H., Al Marshad, M.B.A. (2013). *Bacillus subtilis* PB6 based probiotic (CloSTAT™) improves intestinal morphological and microbiological status of broiler chickens under *Clostridium perfringens* challenge. *Int. J. Agri. Biol.* 15(5), p 978-982.

Adeola, O., Cowieson, A.J. (2011). Board-invited review: Opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. *J. Anim. Sci.* 89(10), p 3189-3218, DOI:10.2527/jas.2010-3715.

Ahire, J.J., Neveling, D.P., Hattingh, M., Dicks, L.M.T. (2015). Ciprofloxacin-eluting nanofibers inhibits biofilm formation by *Pseudomonas aeruginosa* and a methicillin-resistant *Staphylococcus aureus*. *PLoS ONE* 10(4), e0123648, DOI:10.1371/journal.pone.0123648.

Alvarez, F., Castro, M., Príncipe, A., Borioli, G., Fischer, S., Mori, G., Jofré, E. (2011). The plant-associated *Bacillus amyloliquefaciens* strains MEP218 and ARP23 capable of producing the

cyclic lipopeptides iturin or surfactin and fengycin are effective in biocontrol of sclerotinia stem rot disease. *J. Appl. Microbiol*. 112(1), p 159-174, DOI: 10.1111/j.1365-2672.2011.05182.x.

Ammor, M.S., Florez, A.B., Mayo, B. (2007). Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiol*. 24(6), p 559-570, DOI: 10.1016/j.fm.2006.11.001.

Ammor, M.S., Flórez, A.B., van Hoek, A.H., de los Reyes-Gavilán, C.G., Aarts, H.J., Margolles, A., Mayo, B. (2008a). Molecular characterization of intrinsic and acquired antibiotic resistance in lactic acid bacteria and bifidobacteria. *J. Mol. Microbiol. Biotechnol*. 14(1-3), p 6–15, DOI: 10.1159/000106077.

Ammor, M.S., Gueimonde, M., Danielsen, M., Zagorec, M., van Hoek A.H.A.M., de los Reyes-Gavilán, C.G., Mayo, B., Margolles, A. (2008b). Two different tetracycline resistance mechanisms, plasmid-carried tet(L) and chromosomally located transposon-associated tet(M), coexist in *Lactobacillus sakei* Rits 9. *Appl. Environ. Microbiol*. 74(5), p 1394–1401, DOI: 10.1128/AEM.01463-07.

An, F.Y., Clewell, D.B. (2002). Indentification of the cAD1 sex pheromone precursor in *Enterococcus faecalis*. *J. Bacteriol*. 184(7), p 1880-1887, DOI: 10.1128/JB.184.7.1880-1887.2002.

Anastasio, M., Pepe, O., Cirillo, T., Palomba, S., Blaiotta, G., Villani, F. (2010). Selection and use of phytate-degrading LAB to improve cereal-based products by mineral solubilization during dough fermentation. *J. Food Sci*. 75(1), M28-35, DOI: 10.1111/j.1750-3841.2009.01402.x.

Antunes, P., Mourão, J., Campos, J., Peixe, L. (2016). Salmonellosis: the role of poultry meat. *Clin. Microbiol. Infect*. 22(2), p 110-121, DOI: 10.1016/j.cmi.2015.12.004.

Askelson, T.E., Campasino, A., Lee, J.T., Duong, T. (2014). Evaluation of phytate-degrading *Lactobacillus* culture administration to broiler chickens. *Appl. Environ. Microbiol*. 80(3), 943-950, DOI: 10.1128/AEM.03155-13.

Awad, W.A., Hess, C., Hess, M. (2017). Enteric pathogens and their toxin-induced disruption of the intestinal barrier through alteration of tight junctions in chickens. *Toxins* 9(2), e60, DOI: 10.3390/toxins9020060.

Bae, H.D., Yanke, L.J., Cheng, K.J., Selinger, L.B. (1999). A novel staining method for detecting phytase activity. *J. Microbiol. Methods* 39(1), p 17-22, DOI: 10.1016/S0167-7012(99)00096-2

Bao, Y., Zhang, Y., Zhang, Y., Liu, Y., Wang, S., Dong, X., Wang, Y., Zhang, H. (2010). Screening of potential probiotic properties of *Lactobacillus fermentum* isolated from traditional dairy products. *Food Control* 21(5), p 695-701, DOI: 10.1016/j.foodcont.2009.10.010.

Begley, M., Hill, C., Gahan, C.G.M. (2006). Bile salt hydrolase activity in probiotics. *Appl. Environ. Microbiol*. 72(3), p 1729–1738, DOI: 10.1128/AEM.72.3.1729-1738.2006.

Bellon-Fontaine, M.N., Rault, J., van Oss, C.J. (1996). Microbial adhesion to solvents: a novel method to determine the electron-donor/electron-acceptor or Lewis acid-base properties of microbial cells. *Colloids and Surfaces B: Biointerfaces* 7(1-2), p 47–53, DOI: 10.1016/0927-7765(96)01272-6.

Bleau, C., Monges, A., Rashidan, K., Laverdure, J.P., Lacroix, M., Van Calsteren, M.R., Millette, M., Savard, R., Lamontagne, L. (2010). Intermediate chains of exopolysaccharides from

Lactobacillus rhamnosus RW- 9595M increase IL-10 production by macrophages. *J*. *Appl*. *Microbiol*. 108(2), p 666-675, DOI: 10.1111/j.1365-2672.2009.04450.x.

Borda-Molina, D., Seifert, J., Camarinha-Silva, A. (2018). Current perspectives of the chicken gastrointestinal tract and its microbiome. *Comput*. *Struct*. *Biotechnol*. *J*. 16, p 131-139, DOI: 10.1016/j.csbj.2018.03.002.

Boukhris, I., Farhat-Khemakhem, A., Blibech, M., Bouchaala, K., Chouayekh, H. (2015). Characterization of an extremely salt-tolerant and thermostable phytase from *Bacillus amyloliquefaciens* US573. *Int*. *J*. *Biol*. *Macromol*. 80, p 581-587, DOI: 10.1016/j.ijbiomac.2015.07.014.

Brogden, R.N., Carmine, A.A., Heel, R.C., Speight, T.M., Avery, G.S. (1982). Trimethoprim: a review of its antibacterial activity, pharmacokinetics and therapeutic use in urinary tract infections. *Drugs* 23(6), p 405–30, DOI: 10.2165/00003495-198223060-00001.

Bushby, S.R., Hitchings, G.H. (1968). Trimethoprim, a sulphonamide potentiator. *Br*. *J*. *Pharmacol*. *Chemother*. 33(1), p 72-90, DOI: 10.1111/j.1476-5381.1968.tb00475.x.

Cariolato, D., Andrighetto, C., Lombardi, A. (2008). Occurrence of virulence factors and antibiotic resistance in *Enterococcus faecalis* and *Enterococcus faecium* collected from dairy and human samples in North Italy. *Food Control* 19(9), p 886-892, DOI: 10.1016/j.foodcont.2007.08.019.

Carlier, C., Courvalin, P. (1990). Emergence of 4',4''-aminoglycoside nucleotidyltransferase in enterococci. *Antimicrob*. *Agents Chemother*. 34(8), p 1565-1569, DOI: 0066-4804/90/081565-05$02.00/0.

Cavalli-Sforza, L.L., Edwards, A.W.F. (1967). Phylogenetic analysis models and estimation procedures. *Am*. *J*. *Hum*. *Genet*. 19(3), p 233–257.

Chand, D., Ramasamy, S., Suresh, C.G. (2016). A highly active bile salt hydrolase from *Enterococcus faecalis* shows positive cooperative kinetics. *Process Biochem*. 51(2), p 263-269, DOI: 10.1016/j.procbio.2015.12.006.

Charteris, W.P., Kelly, P.M., Morelli, L., Collins, J.K. (2001). Gradient diffusion antibiotic susceptibility testing of potentially probiotic lactobacilli. *J*. *Food Prot*. 64(12), p 2007-2014.

Chenoweth, C.E., Robinson, K.A., Schaberg, D.R. (1990). Efficacy of ampicillin versus trimethoprim-sulfamethoxazole in a mouse model of lethal enterococcal peritonitis. *Antimicrob*. *Agents Chemother*. 34(9), p 1800-1802, DOI: 10.1128/AAC.34.9.1800.

Chopra, I., Roberts, M. (2001). Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol*. *Mol*. *Biol*. *Rev*. 65(2), p 232-260, DOI: 10.1128/MMBR.65.2.232-260.2001.

Clewell, D.B. (2011). Tales of conjugation and sex pheromones. *Mob*. *Genet*. *Elements*. 1(1), p 38-54, doi: 10.4161/mge.1.1.15409.

Clewell, D.B., An, F.Y., Flannagan, S.E., Antiporta, M., Dunny, G.M. (2000). Enterococcal sex pheromone precursors are part of signal sequences for surface lipoproteins. *Mol*. *Microbiol*. 35(1), p 246-247, DOI: 10.1046/j.1365-2958.2000.01687.x.

CLSI (2015). Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Four Informational Supplement. CLSI document M100-S24

Cowieson, A.J., Adeola, O. (2005). Carbohydrases, protease, and phytase have an additive beneficial effect in nutritionally marginal diets for broiler chicks. *Poult*. *Sci*. 84(12), p 1860–1867.

Creti, R., Fabretti, F., Koch, S., Huebner, J., Garsin, D.A., Baldassarri, L., Montanaro, L., Arciola, C.R. (2009). Surface protein EF3314 contributes to virulence properties of *Enterococcus faecalis*. *Int*. *J*. *Artif*. *Organs* 32(9), p 611-620.

Creti, R., Imperi, M., Bertuccini, L., Fabretti, F., Orefici, G., Di Rosa, R., Baldassarri, L. (2004). Survey for virulence determinants among *Enterococcus faecalis* isolated from different sources. *J*. *Med*. *Microbiol*. 53(1), p 13-20, DOI: 10.1099/jmm.0.05353-0.

Dahiya, R.S., Speck, M.L. (1968). Hydrogen peroxide formation by lactobacilli and its effect on *Staphylococcus aureus*. *J*. *Dairy Sci*. 51(10), p 1568-1572, DOI: 10.3168/jds.S0022-0302(68)87232-7.

Dalal, R.C. (1977). Soil organic phosphorus. *Adv*. *Agron*. 29, p 83-117, DOI: 10.1016/S0065-2113(08)60216-3.

Dänicke, S., Vahjen, W., Simon, O., Jeroch, H. (1999). Effects of dietary fat type and xylanase supplementation to rye-based broiler diets on selected bacterial groups adhering to the intestinal epithelium, on transit time of feed, and on nutrient digestibility. *Poult*. *Sci*. 78(9), p 1292-1299.

De Angelis, M., Gallo, G., Corbo, M.R., McSweeney, P.L.H., Faccia, M., Giovine, M., Gobbetti, M. (2003). Phytase activity in sourdough lactic acid bacteria: purification and characterization of a phytase from *Lactobacillus sanfranciscensis* CB1. *Int*. *J*. *Food Microbiol*. 87(3), p 259-270, DOI: 10.1016/S0168-1605(03)00072-2.

De Vuyst, L., Foulquié-Moreno, M.R., Revets, H. (2003). Screening for enterocins and detection of hemolysin and vancomycin resistance in enterococci of different origins. *Int*. *J*. *Food Microbiol*. 84(3), p 299-318, DOI: 10.1016/S0168-1605(02)00425-7.

Deb, P., Talukdar, S.A., Mohsina, K., Sarker, P.K., Sayen, S.M.A. (2013). Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. *SpringerPlus* 2(154), p 1-12, DOI: 10.1186/2193-1801-2-154.

Del Re, B., Sgorbati, B., Miglioli, M., Palenzona, D. (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Lett*. *Appl*. *Microbiol*. 31(6), p 438–442, DOI: 10.1046/j.1365-2672.2000.00845.x.

Dertli, E., Colguhoun, I.J., Gunning, A.P., Bongaerts, R.J., Le Gall, G., Bonev, B.B., Mayer, M.J., Narbad, A. (2013). Structure and biosynthesis of two exopolysaccharides produced by *Lactobacillus johnsonii* FI9785. *J*. *Biol*. *Chem*. 288(44), p 31938-31951, DOI: 10.1074/jbc.M113.507418.

Dertli, E., Mayer, M.J., Colguhoun, I.J., Narbad, A. (2016). EpsA is an essential gene in exopolysaccharide production *Lactobacillus johnsonii* FI9785. *Microb*. *Biotechnol*. 9(4), p 496-501, DOI: 10.1111/1751-7915.12314.

Donnarumma, G., Molinaro, A., Cimini, D., De Castro, C., Valli, V., De Gregorio, V., De Rosa, M., Schiraldi, C. (2014). *Lactobacillus crispatus* L1: high cell density cultivation and exopolysaccharide structure characterization to highlight potentially beneficial effects against vaginal pathogens. *BMC Microbiol*. 14, 137, DOI: 10.1186/1471-2180-14-137.

Duprè, I., Zanetti, S., Schito, A.M., Fadda, G., Sechi, L.A. (2003). Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). *J*. *Med*. *Microbiol*. 52(6), p 491-498, DOI: 10.1099/jmm.0.05038-0.

EUCAST: The European Committee on Antimicrobial Susceptibility Testing. (2018). Breakpoint tables for interpretation of MICs and zone diameters. Version 8.0, http://www.eucast.org.

FAO/WHO joint working group report on drafting guidelines for the evaluation of probiotics in food, Canada, London, Ontario (April 30 and May 1, 2002), http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf

FEEDAP: EFSA Panel on Additives and Products or Substances used in Animal Feed. (2012). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA J*. 10(6), 2740, DOI: 10.2903/j.efsa.2012.2740.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evol*. 39(4), p 783–791, DOI: 10.2307/2408678.

Francesch, M., Geraert, P.A. (2009). Enzyme complex containing carbohydrases and phytase improves growth performance and bone mineralisation of broilers fed reduced nutrient corn–soybean-based diets. *Poult*. *Sci*. 88 (9), p 1915–1924, DOI: 10.3382/ps.2009-00073.

Franz, C.M., Specht, I., Haberer, P., Holzapfel, W.H. (2001a). Bile salt hydrolase activity of Enterococci isolated from food: screening and quantitative determination. *J*. *Food*. *Prot*. 64(5), p 725-729, DOI; 10.4315/0362-028X-64.5.725.

Franz, C.M.A.P., Specht, I., Haberer, P., Holzapfel, W.H. (2001b). Bile salt hydrolase activity of enterococci isolated from food: screening and quantitative determination. *J*. *Food Prot*. 64(5), p 725-729.

Gad, G.F.M., Abdel-Hamid, A.M., Farag, Z.S.H. (2014). Antibiotic resistance in lactic acid bacteria isolated from some pharmaceutical and dairy products. *Braz*. *J*. *Microbiol*. 45(1), p 25-33, DOI: 10.1590/S1517-83822014000100005.

Galli, D., Lottspeich, F., Wirth, R. (1990). Sequence analysis of *Enterococcus faecalis* aggregation substance encoded by the sex pheromone plasmid pAD1. *Mol*. *Microbiol*. 4(6), p 895-904, DOI: 10.1111/j.1365-2958.1990.tb00662.x.

Ghareeb, K., Awad, W.A., Mohnl, M., Porta, R., Biarnés, M., Böhm, J., Schatzmayr, G. (2012). Evaluating the efficacy of an avian-specific probiotic to reduce the colonisation of *Campylobacter* jejuni in broiler chickens. *Poult*. *Sci*. 91(8), p 1825-1832, DOI: 10.3382/ps.2012-02168.

Giaouris, E., Chapot-Chartier, M.P., Briandet, R. (2009). Surface physicochemical analysis of natural *Lactococcus lactis* strains reveals the existence of hydrophobic and low charged strains with altered adhesive properties. *Int*. *J*. *Food Microbiol*., 131(1), p 2–9.

Gilmore, M.S., Segarra, R.A., Booth, M.C., Bogie, C.P., Hall, L.R., Clewell, D.B. (1994). Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. *J*. *Bacteriol*. 176(23), p 7335-7344, DOI: 10.1128/jb.176.23.7335-7344.1994.

Graf, E., Eaton, J.W. (1990). Antioxidant functions of phytic acid. *Free Radic*. *Biol*. *Med*. 8(1), p 61-69, DOI: 10.1016/0891-5849(90)90146-A.

Grandy, G., Medina, M., Soria, R., Terán, C.G., Araya, M. (2010). Probiotics in the treatment of acute rotavirus diarrhoea. A randomized, double-blind, controlled trial using two different probiotic preparations in Bolivian children. *BMC Infect*. *Dis*. 10, 253, DOI: 10.1186/1471-2334-10-253.

Green, J.D., Klaenhammer, T.R. (1994). Factors involved in adherence of lactobacilli to human Caco-2 cells. *Appl*. *Environ*. *Microbiol*. 60(12), p 4487–4494.

Gueimonde, M., Sánchez, B., de los Reyes-Gavilán, C.G., Margolles, A. (2013). Antibiotic resistance in probiotic bacteria. *Front*. *Microbiol*. 4, 202, DOI: 10.3389/fmicb.2013.00202.

Gunn, J.S. (2000). Mechanisms of bacterial resistance and response to bile. *Microbes Infect*. 2(8), p 907-913, DOI: 10.1016/S1286-4579(00)00392-0.

Gutierrez-Fuentes, C.G., Zuñiga-Orozco, L.A., Vicente, J.L., Hernandez-Velasco, X., Menconi, A., Kuttappan, V.A., Kallapura, G., Latorre, J., Layton, S., Hargis, B.M., Téllez, G. (2013). Effect of a lactic acid bacteria based probiotic, Floramax-B11®, on performance, bone qualities, and morphometric analysis of broiler chickens: An economic analysis. *Biol*. *Syst*. 2(3), 113, DOI: 10.4172/2329-6577.1000113.

Harutoshi, T. (2013). Exopolysaccharides of lactic acid bacteria for food and colon health applications In Kongo, M. (Ed.), Biochemistry, genetics and molecular biology. In: Lactic acid bacteria: R&D for food, health and livestock purposes (p 222- 238). Rijeka, Croatia: InTech Publishing.

Herasimenka, Y., Cescutti, P., Impallomeni, G., Rizzo, R. (2007). Exopolysaccharides produced by *Inquilinus limosus*, a new pathogen of cystic fibrosis patients: novel structures with usual components. *Carbohydr*. *Res*. 342(16), p 2404-2415, DOI: 10.1016/j.carres.2007.07.012.

Hofacre, C.L., Beacorn, T., Collett, S., Mathis, G. (2003). Using competitive exclusion, mannan-oligosaccharide and other intestinal products to control necrotic enteritis. *J*. *Appl*. *Poult*. *Res*. 12, p 60-64, DOI: 10.1093/japr/12.1.60.

Hofmann, A.F., Mysels, K.J. (1992). Bile acid solubility and precipitation *in vitro* and *in vivo*: the role of conjugation, pH, and Ca^{2+} ions. *J*. *Lipid Res*. 33(5), p 617-626.

Hollenbeck, B.L., Rice, L.B. (2012). Intrinsic and acquired resistance mechanisms in *enterococcus*. *Virulence* 3(5), p 421-433, DOI: 10.4161/viru.21282.

Hook, D., Jalaludin, B., Fitzsimmons, G. (1996). *Clostridium perfringens* food-borne-outbreak: an epidemiological investigation. *Aust*. *N*. *Z*. *J*. *Public Health* 20(2), p 119-122.

Hummel, A.S., Hertel, C., Holzapfel, W.H., Franz, C.M.A.P. (2007). Antibiotic resistances of starter and probiotic strains of lactic acid bacteria. *Appl*. *Environ*. *Microbiol*. 73(3), p 730-739, DOI: 10.1128/AEM.02105-06.

Huys, G., D'Haene, K., Collard, J., Swings, J. (2004). Prevalence and molecular characterization of tetracycline resistance in *Enterococcus* isolates from food. *Appl*. *Environ*. *Microbiol*. 70(3), p 1555-1562, DOI: 10.1128/AEM.70.3.1555-1562.2004.

Idriss, E.E., Makarewicz, O., Farouk, A., Rosner, K., Greiner, R., Bochow, H., Richter, T., Borriss, R. (2002). Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. *Microbiol*. 148(7), p 2097-2109, DOI: 10.1099/00221287-148-7-2097.

Kabir, S.M.L. (2009). The role of probiotics in the poultry industry. *Int*. *J*. *Mol*. *Sci*. 10(8), p 3531-3546, DOI: 10.3390/ijms10083531.

Kaldhusdal, M., Løvland, A. (2000). The economical impact of *Clostridium perfringens* is greater than anticipated. *World Poult*. 16, p 50-51.

Kaur, A., Kaur, M., Samyal, M.L., Ahmed, Z. (2012). Isolation, characterization and identification of bacterial strain producing amylase. *J*. *Microbiol*. *Biotech*. *Res*. 2(4), p 573-579.

Klare, I., Konstabel, C., Werner, G., Huys, G., Vankerckhoven, V., Kahlmeter, G., Hildebrandt, B., Müller-Bertling, S., Witte, W., Goossens, H. (2007). Antimicrobial susceptibilities of *Lactobacillus*, *Pediococcus* and *Lactococcus* human isolates and cultures intended for probiotic or nutritional use. *J*. *Antimicrob*. *Chemother*. 59(5), p 900-912, DOI: 10.1093/jac/dkm035.

Kluge, A.G., Farris, J.S. (1969). Quantitative phyletics and the evolution of the anurans. *Syst*. *Zool*. 18(1), p 1–32.

Kos, B., Šušković, J., Vuković, S., Šimpraga, M., Frece, J., Matošić, S. (2003). Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *J*. *Appl*. *Microbiol*. 94(6), p 981–987, DOI: 10.1046/j.1365-2672.2003.01915.x.

Kotzamanidis, C., Kourelis, A., Litopoulou-Tzanetaki, E., Tzanetakis, N., Yiangou, M. (2010). Evaluation of adhesion capacity, cell surface traits and immunomodulatory activity of presumptive probiotic *Lactobacillus* strains. *Int*. *J*. *Food Microbiol*. 140(2-3), p 154-163, DOI: 10.1016/j.ijfoodmicro.2010.04.004.

Lahtinen, S.J., Boyle, R.J., Margolles, A., Frías, R., Gueimonde, M. (2009). Safety assessment of probiotics In Charalampopoulos D., Rastall R.A (Eds), Prebiotics and Probiotics Science and Technology (p 1193–1225). Berlin, Germany, Springer-Verlag Publishing.

Leavis, H.L., Willems, R.J.L., Top, J., Bonten, M.J.M. (2006). High-level ciprofloxacin resistance from point mutations in *gyrA* and *parC* confined to global hospital-adapted clonal lineage CC17 of *Enterococcus faecium*. *J*. *Clin*. *Microbiol*. 44(3), p 1059-1064, DOI:10.1128/JCM.44.3.1059–1064.2006.

Lebeer, S., Ceuppens, J., Geboes, K., Van Assche, G., Rutgeerts, P., Vanderleyden, J., De Keersmaecker, S. (2007). Mechanisms of probiotic-host interaction with IBD as a case study: a role for exopolysaccharides? *Commun*. *Agric*. *Appl*. *Biol*. *Sci*. 72(1), p 41-45.

Lebreton, F., Riboulet-Bisson, E., Serror, P., Sanguinete, M., Posteraro, B., Torelli, R., Hartke, A., Auffray, Y., Giard, J. (2009). *Ace*, which encodes an adhesin in *Enterococcus faecalis*, is regulated by Ers and is involved in virulence. *Infect*. *Immun*. 77(7), p 2832-2839, DOI: 10.1128/IAI.01218-08.

Lin, J., Sahin, O., Michel, L.O., Zhang, Q. (2003). Critical role of multidrug efflux pump CmeABC in bile resistance and *in vivo* colonisation of *Campylobacter jejuni*. *Infect*. *Immun*. 71(8), p 4250-4259, DOI: 10.1128/IAI.71.8.4250-4259.2003.

Liong, M.T., Shah, N.P. (2005). Bile salt deconjugation and BSH activity of five bifidobacterial strains and their cholesterol co-precipitating properties. *Food Res*. *Int*. 38(2), p 135–142, DOI: 10.1016/j.foodres.2004.08.003.

Liu, C-T., Hsu, I-T., Chou, C-C., Lo, P-R., Yu, R-C. (2009). Exopolysaccharide production of *Lactobacillus salivarius* BCRC 14759 and *Bifidobacterium bifidum* BCRC 14615. *World J*. *Microbiol*. *Biotechnol*. 25, p 883-890, DOI: 10.1007/s11274-009-9965-x.

Lourenco, M.C., Kuritza, L.N., Westphal, P., Muniz, E., Pickler, L., Santin, E. (2012). Effects of *Bacillus* subtilis in the dynamics of infiltration of immunological cells in the intestinal mucosa of chickens challenged with *Salmonella* Minnesota. *Int*. *J*. *Poult*. *Sci*. 11(10), p 630-634, DOI: 10.3923/ijps.2012.630.634.

Lowe, A.M., Lambert, P.A., Smith, A.W. (1995). Cloning of an *Enterococcus faecalis* endocarditis antigen: homology with adhesins from some oral streptococci. *Infect*. *Immun*. 63(2), p 703-706.

Maalouf, K., Baydoun, E., Rizk, S. (2011). Kefir induces cell-cycle and apoptosis in HTLV-1-negative malignant T-lymphocytes. *Cancer Manag*. *Res*. 3, p 39-47, DOI: 10.2147/CMR.S15109.

Madhuri, K.V., Prabhakar, K.V. (2014). Microbial exopolysaccharides: Biosynthesis and potential applications. *Orient*. *J*. *Chem*. 30(3), p 1401-1410, DOI: 10.13005/ojc/300362.

Mahagna, M., Nir, I., Larbier, M., Nitsan, Z. (1995). Effect of age and exogenous amylase and protease on development of the digestive tract, pancreatic enzyme activities and digestibility of nutrients in young meat-type chicks. *Reprod*. *Nutr*. *Dev*. 35(2), p 201-212.

Malick, A., Khodaei, N., Benkerroum, N., Karboune, S. (2017). Production of exopolysaccharides by selected *Bacillus* strains: Optimization of media composition to maximize the yield and structural characterization. *Int*. *J*. *Macromol*. 102, p 539-549, DOI: 10.1016/j.ijbiomac.2017.03.

Marcq, C., Cox, E., Szalo, I.M., Théwis, A., Beckers, Y. (2011). *Salmonella* Typhimurium oral challenge in mature broilers: bacteriological, immunological, and growth performance aspects. *Poult*. *Sci*. 90, p 59-67, DOI: 10.3382/ps.2010-01017.

Marounek, M., Břeňová, N., Suchorská, O., Mrázek, J. (2009). Phytase activity in rabbit cecal bacteria. *Folia Microbiol*. 54(2), p 111-114, DOI: 10.1007/s12223-009-0016-7.

Martín-Platero, A.M., Valdivia, E., Maqueda, M., Martínez-Bueno, M. (2009). Characterization and safety evaluation of enterococci isolated from Spanish goats' milk cheeses. *Int*. *J*. *Food Microbiol*. 132(1), p 24-32, DOI: 10.1016/j.ijfoodmicro.2009.03.010.

Mayrhofer, S., Mair, C., Kneifel, W., Domig, K.J. (2011). Susceptibility of bifidobacteria of animal origin to selected antimicrobial agents. *Chemother*. *Res*. *Pract*. 2011, 989520, DOI: 10.1155/2011/989520.

Mead, G.C. (1997). Bacteria in the gastrointestinal tract of birds. In Mackie, R.I., White, B.A., Isaacson R.E. (eds), Gastrointestinal microbiology, vol. 2. Gastrointestinal microbes and host interactions p. 216-240. Chapman and Hall, New York, NY.

Medeiros, A.W., Pereira, R.I., Oliveira, D.V., Martins, P.D., d'Azevedo, P.A., Van der Sand, S., Frazzon, J., Frazzon, A.P.G. (2014). Molecular detection of virulence factors among food and clinical *Enterococcus faecalis* strains in South Brazil. *Braz*. *J*. *Microbiol*. 45(1), p 327-332, DOI: 10.1590/S1517-83822014005000031.

Melegy, T., Khaled, N.F., El-Bana, R., Abdellatif, H. (2011). Effect of dietary supplementation of *Bacillus subtilis* PB6 (CLOSTAT™) on performance, immunity, gut health and carcass traits in broilers. *J*. *Am*. *Sci*. 7(12), p 891-898.

Michaux, C., Martini, C., Hanin, A., Auffray, Y., Hartke, A., Giard, J.C. (2011b). SlyA regulator is involved in bile salts stress response of *Enterococcus faecalis*. *FEMS Microbiol*. *Lett*. 324(2), p 142-146, DOI: 10.1111/j.1574-6968.2011.02390.x.

Michaux, C., Sanguinetti, M., Reffuveille, F., Auffray, Y., Posteraro, B., Gilmore, M.S., Hartke, A., Giard, J. (2011a). SlyA is a transcriptional regulator involved in the virulence of *Enterococcus faecalis*. *Infect*. *Immun*. 79(7), p 2638-2645, DOI: 10.1128/IAI.01132-10.

Mitchell, D.B., Vogel, K., Weimann, B.J., Pasamontes, L., van Loon, A.P.G.M. (1997). The phytase subfamily of histidine acid phosphatases: isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*. *Microbiol*. 143, p 245-252, DOI: 10.1099/00221287-143-1-245.

Mosser, S.A., Savage, D.C. (2001). Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in Lactobacilli. *Appl*. *Environ*. *Microbiol*. 67(8), p 3476-3480, DOI: 10.1128/AEM.67.8.3476-3480.2001.

Müller, H.E. (1984). ABTS peroxidase medium as a highly sensitive plate assay for detection of hydrogen peroxide production in bacteria. *J. Microbiological Methods* 2(2), p 101-102, DOI: 10.1016/0167-7012(84)90035-6.

Neela, F.A., Nonaka, L., Rahman, M.H., Suzuki, S. (2009). Transfer of the chromosomally encoded tetracycline resistance gene tet(M) from marine bacteria to *Escherichia coli* and *Enterococcus faecalis*. *World J*. *Microbiol*. *Biotechnol*. 25(6), p 1095–1101, DOI: 10.1007/s11274-009-0004-8.

Neveling, D.P., Endo, A., Dicks, L.M.T. (2012). Fructophilic *Lactobacillus kunkeei* and *Lactobacillus brevis* isolated from fresh flowers, bees and bee-hive. *Curr*. *Microbiol*. 65(5), p 507-515, DOI 10.1007/s00284-012-0186-4.

Notararigo, S., Nácher-Vázquez, M., Ibarburu, I., Werning, M.L., de Palencia, P.F., Dueñas, M.T., Aznar, R., López, P., Prieto, A. (2013). Comparative analysis of production and purification of homo- and hetero-polysaccharides produced by lactic acid bacteria. *Carbohydrate Polymers* 93(1), p 57-64, DOI: 10.1016/j.carbpol.2012.05.016.

Noy, Y., Sklan, D. (1995). Digestion and absorption in the young chick. *Poult*. *Sci*. 74(2), p 366-373, DOI: 10.3382/ps.0740366.

Onderci, M., Sahin, N., Sahin, K., Cikim, G., Aydín, A., Ozercan, I., Aydín, S. (2006). Efficacy of supplementation of α-amylase-producing bacterial culture on the performance, nutrient use, and gut morphology of broiler chickens fed a corn-based diet. *Poult*. *Sci*. 85(3), p 505-510.

Palacios, M.C., Haros, M., Rosell, C.M., Sanz, Y. (2005). Characterization of an acid phosphatase from *Lactobacillus pentosus*: regulation and biochemical properties. *J*. *Appl*. *Microbiol*. 98(1), p 229-237, DOI: 10.1111/j.1365-2672.2004.02447.x.

Pan, D., Yu, Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes* 5(1), p 108-119, DOI: 10.4161/gmic.26945.

Phelan, R.W., Clarke, C., Morrissey, J.P., Dobson, A.D.W., O'Gara, F., Barbosa, T.M. (2011). Tetracycline resistance-encoding plasmid from *Bacillus* sp. strain #24, isolated from the marine sponge *Haliclona simulans*. *Appl*. *Environ*. *Microbiol*. 77(1), p 327–329, DOI: 10.1128/AEM.01239-10.

Pillar, C.M., Gilmore, M.S. (2004). Enterococcal virulence-pathogenicity islands of *E*. *faecalis*. *Front*. *Biosci*. 9, p 2335-2346, DOI: 10.2741/1400.

Prado-Rebolledo, O.F., de Jesus Delgado-Machuca, J., Macedo-Barragan, R.J., Garcia-Márquez, L.J., Morales-Barrera, J.E., Latorre, J.D., Hernandez-Velasco, X., Tellez, G. (2017). Evaluation of a selected lactic acid bacteria-based probiotic on *Salmonella enterica* serovar Enteritidis colonisation and intestinal permeability in broiler chickens. *Avian Pathol*. 46(1), p 90-94, DOI: 10.1080/03079457.2016.1222808.

Qin, X., Singh, K.V., Weinstock, G.M., Murray, B.E. (2000). Effects of *Enterococcus faecalis fsr* genes on production of gelatinase and serine protease in virulence. *Infect*. *Immun*. 68(5), p 2579-2586.

Rabe, L.K., Hillier, S.L. (2003). Optimization of media for detection of hydrogen peroxide production by *Lactobacillus* species. *J*. *Clin*. *Microbiol*. 41(7), p 3260-3264, DOI: 10.1128/JCM.41.7.3260-3264.2003.

Ravindran, V., Selle, P.H., Bryden, W.L. (1999). Effects of phytase supplementation, individually and in combination, with glycanase, on the nutritive value of wheat and barley. *Poult*. *Sci*. 78(11), p 1588-1595, DOI: 10.1093/ps/78.11.1588.

Regan, C.M., Syed, Q., Tunstall, P.J. (1995). A hospital outbreak of *Clostridium perfringens* food poisoning implications for food hygiene review in hospitals. *J*. *Hosp*. *Infect*. 29(1), p 69-73, DOI: 10.1016/0195-6707(95)90295-3.

Riley W.W., Austic, R.E. (1984). Influence of dietary electrolytes on digestive tract pH and acid-base status of chicks. *Poult*. *Sci*. 63(11), p 2247-2251.

Roberts, A.P., Pratten, J., Wilson, M., Mullany, P. (1999). Transfer of a conjugative transposon, Tn5397 in a model oral biofilm. *FEMS Microbiol*. *Lett*. 177(1), p 63–66, DOI: 10.1111/j.1574-6968.1999.tb13714.x.

Roberts, M.C. (2005). Update on acquired tetracycline resistance genes. *FEMS Microbiol*. *Lett*. 245(2), p 195-203, DOI: 10.1016/j.femsle.2005.02.034.

Rojas, M., Conway, P.L. (1996). Colonisation by lactobacilli of piglet small intestinal mucus. *J*. *Appl*. *Bacteriol*. 81(5), p 474–480.

Rougière, N., Carré, B. (2010). Comparison of gastrointestinal transit times between chickens from D^+ and D^- genetic lines selected for divergent digestion efficiency. *Animal* 4(11), p 1861-1872, DOI: 10.1017/S1751731110001266.

Saha, K., Maity, S., Roy, S., Pahan, K., Pathak, R., Majumdar, S., Gupta, S. (2014). Optimization of amylase production from *B*. *amyloliquefaciens* (MTCC 1270) using solid state fermentation. *Int*. *J*. *Microbiol*. (2014), Article ID 764046, DOI: 10.1155/2014/764046.

Saitou, N., Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol*. *Biol*. *Evol*. 4(4), p 406-425, DOI: 10.1093/oxfordjournals.molbev.a040454.

Sanhueza, E., Paredes-Osses, E., González, C.L., García, G.A. (2015). Effect of pH in the survival of *Lactobacillus salivarius* strain UCO_979C wild type and the pH acid acclimated variant. *Electron*. *J*. *Biotechnol*. 18(5), p 343-346, DOI: 10.1016/j.ejbt.2015.06.005.

Sarmiento-Rubiano, L., Berger, B., Moine, D., Zúñiga, M., Pérez-Martínez, G., Yebra, M.J. (2010). Characterization of a novel *Lactobacillus* species closely related to *Lactobacillus johnsonii* using a combination of molecular and comparative genomics methods. *BMC Genomics* 11, 504, DOI: 10.1186/1471-2164-11-504

Sarra, P.G., Morelli, L., Bottazzi, V. (1992). The lactic microflora of fowl. In Wood B.J.B. (ed.). The lactic acid bacteria in health and disease, vol. 1. p. 3-19. Elsevier Applied Science, London, United Kingdom.

Sekkal, M., Legrand, P. (1993). A spectroscopic investigation of the carrageenans and agar in the 1500-100 cm^{-1} spectral range. *Spectrochimica Acta* 49(2), p 209-221, DOI: 10.1016/0584-8539(93)80176-B.

Selle, P.H., Ravindran, V., Bryden, W.L., Scott, T. (2006). Influence of dietary phytate and exogenous phytase on amino acid digestibility in poultry: a review. *J. Poult. Sci*. 43(2), p 89-103, DOI: 10.2141/jpsa.43.89.

Selle, P.H., Ravindran, V., Ravindran, G., Bryden, W.L. (2007). Effects of dietary lysine and microbial phytase on growth performance and nutrient utilisation of broiler chickens. *Asian-Australas. J. Anim. Sci*. 20(7), p 1100-1107, DOI: 10.5713/ajas.2007.1100.

Sharma, P., Tomar, S.K., Sangwan, V., Goswami, P., Singh, R. (2015). Antibiotic resistance of *Lactobacillus* sp. isolated from commercial probiotic preparations. *J. Food Safety* 36, p 38-51, DOI: 10.1111/jfs.12211.

Shires, A., Thompson, J.R., Turner, B.V., Kennedy, P.M., Goh, Y.K. (1987). Rate of passage of canola meal and corn-soybean meal diets through the gastrointestinal tract of broiler and white leghorn chickens. *Poult. Sci*. 66(2), p 289-298.

Singh, R.P., Shukla, M.K., Mishra, A., Kumari, P., Reddy, C.P.K., Jha, B. (2011). Isolation and characterization of exopolysaccharides from seaweed associated bacteria *Bacillus licheniformis*. *Carbohydr. Polym*. 84(3), p 1019-1026, DOI: 10.1016/j.carbpol.2010.12.061.

Siragusa, G.R., Johnson, M.G. (1989). Inhibition of Listeria monocytogenes growth by the lactoperoxidase-thiocyanate-H_2O_2 antimicrobial system. *Appl. Environ. Microbiol*. 55(11), p 2802-2805.

Skarp, C.P.A., Hänninen, M-L., Rautelin, H.I.K. (2016). Campylobacteriosis: the role of poultry meat. *Clin. Microbiol. Infect*. 22(2), p 103-109, DOI: 10.1016/j.cmi.2015.11.019.

Smith, H.W. (1965). The development of the flora of the alimentary tract in young animals. *J. Pathol. Bacteriol*. 90, p 495-513, DOI: 10.1002/path.1700900218.

Sridevi, N., Vishwe, P., Prabhune, A. (2009). Hypocholesteremic effect of bile salt hydrolase from *Lactobacillus buchneri* ATCC 4005. *Food Res. Int*. 42(4), p 516–520, DOI: 10.1016/j.foodres.2009.02.016.

Sterzo, E.V., Paiva, J.B., Mesquita, A.L., Freitas Neto, O.C., Berchieri, S. (2007). Organic acids and/or compound with defined microorganisms to control *Salmonella enterica* serovar Enteritidis experimental infection in chickens. *Rev. Bras. Cienc. Avic*. 9(1), p 69-73, DOI: 10.1590/S1516-635X2007000100010.

Stingele, F., Neeser, J.R., Mollet, B. (1996). Identification and characterization of the eps (Exopolysaccharide) gene cluster from *Streptococcus thermophilus* Sfi6. *J. Bacteriol*. 178(6), p 1680-1690.

Sun, L., Lu, Z., Bie, X., Lu, F., Yang, S. (2006). Isolation and characterization of a co-producer of fengycins and surfactins, endophytic *Bacillus amyloliquefaciens* ES-2, from *Scutellaria*

baicalensis Georgi. *Worlds J. Microbiol. Biotechnol*. 22(12), p 1259-1266, DOI: 10.1007/s11274-006-9170-0.

Svihus, B. (2014). Function of the digestive system. *J. Appl. Poult. Res*. 23(2), p 306-314, DOI: 10.3382/japr.2014-00937.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol*. 30(12), p 2725-2729, DOI: 10.1093/molbev/mst197.

Tanaka, H., Doesburg, K., Iwasaki, T., Mierau, I. (1999). Screening of lactic acid bacteria for bile salt hydrolase activity. *J. Dairy Sci*. 82(12), p 2530-2535, DOI: 10.3168/jds.S0022-0302(99)75506-2.

Tang, D.F., Iji, P., Choct, M., Ru, Y.J. (2013). Effect of α-amylase on performance and nutrient utilization of broilers fed diets based on cassava and soybean meal. 24th Annual Australian Poultry Science Symposium, 17-20 February 2013, p 88-91, Sydney, New South Wales, Australia.

Tao, F., Biao, G.Z., Yu, J.Z., Ning, Z.H. (2008). Isolation and characterization of an acidic polysaccharide from *Mesona Blumes* gum. *Carbohydr. Polym*. 71(2), p 159-169, DOI: 10.1016/j.carbpol.2007.05.017.

Templer, S.P., Rohner, P., Baumgartner, A. (2008). Relation of *Enterococcus faecalis* and *Enterococcus faecium* isolates from food and clinical specimens. *J. Food Prot*. 71(10), p 2100-2104.

Teo, A.Y., Tan, H.M. (2007). Evaluation of the performance and intestinal gut microflora of broilers fed on corn-soy diets supplemented with *Bacillus subtilis* PB6 (CloSTAT). *J. Appl. Poult. Res*. 16(3), 296–303, DOI: 10.1093/japr/16.3.296.

Tille, P.M., Forbes, B.A. (2014). Bailey & Scott's diagnostic microbiology (Thirteenth edition.). St. Louis, Missouri: Elsevier.

Toledo-Arana, A., Valle, J., Solano, C., Arrizubieta, M.J., Cucarella, C., Lamata, M., Amorena, B., Leiva, J., Penadés, J.R., Lasa, I. (2001). The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. *Appl. Environ. Microbiol*. 67(10), p 4538-4545, DOI: 10.1128/AEM.67.10.4538-4545.2001.

Trivedi, K., Cupakova, S., Kappiskova, R. (2011). Virulence factors and antibiotic resistance in enterococci isolated from food-stuffs. *Vet. Med*. (Praha) 56(7), p 352-357.

Tungala, A., Narayanan, K.A., Muthuraman, M.S. (2013). Isolation of phytase producing bacteria from poultry faeces and optimization of culture conditions for enhanced phytase production. *Int. J. Pharm. Pharm. Sci*. 5(4), p 264-269.

van Hoek, A.H.A.M., Margolles, A., Damig, K.J., Korhonen, J.M., Życka-Krzesińka, J., Bardowsky J.K., Danielsen, M., Huys, G., Morelli, L., Aarts, H.J.M. (2008). Molecular assessment of erythromycin and tetracycline resistance genes in lactic acid bacteria and bifidobacteria and their relation to the phenotypic resistance. *Int. J. Probiotics Prebiotics* 3(4), p 3271–280.

Van Immerseel, F., De Buck, J., Pasmans, F., Huyghebaert, G., Haesebrouck, F., Ducatelle, R. (2004). *Clostridium perfringens* in poultry: an emerging threat for animal and public health. *Avian Pathol*. 33(6), p 537-549, DOI: 10.1080/03079450400013162.

Van Staden, A.D. (2015). *In vitro* and *in vivo* characterization of amyloliquecidin, a novel two-component lantibiotic produced by *Bacillus amyloliquefaciens*. PhD Microbiology, Stellenbosch University, Stellenbosch, South Africa.

Vankerckhoven, V., Van Autgaerden, T., Vael, C., Lammens, C., Chapelle, S., Rossi, R., Jabes, D., Goossens, H. (2004). Development of a multiplex PCR for the detection of *asa1*, *gelE*, *cylA*, *esp*, and *hyl* genes in Enterococci and survey for virulence determinants among European hospital isolates of *Enterococcus faecium*. *J*. *Clin*. *Microbiol*. 42(10), p 4473-4479, DOI: 10.1128/JCM.42.10.4473-4479.2004.

Venkatesh, P., Balraj, M., Ayyanna, R., Ankaiah, D., Arul, V. (2016). Physicochemical and biosorption properties of novel exopolysaccharides produced by *Enterococcus faecalis*. *LWT Food Sci*. *Technol*. 68, p 606-614, DOI: 10.1016/j.lwt.2016.01.005.

Vinderola, G., Perdigón, G., Duarte, J., Famworth, E., Matar, C. (2006). Effects of the oral administration of the exopolysaccharide produced by *Lactobacillus kefiranofaciens* on the gut mucosal immunity. *Cytokine* 36(5-6), p 254-60, DOI: 10.1016/j.cyto.2007.01.003.

Vuong, C., Kocianova, S., Voyich, J.M., Yao, Y., Fischer, E.R., DeLeo, F.R., Otto, M. (2004). A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J*. *Biol*. *Chem*. 279(52), p 54881-54886, DOI: 10.1074/jbc.M411374200.

Walters, C.M., Antiporta, M.H., Murray, B.E., Dunny, G.M. (2003). Role of the *Enterococcus faecalis* GelE protease in determination of cellular chain length, supernatant pheromone levels, and degradation of fibrin and misfolded surface proteins. *J*. *Bacteriol*. 185(12), p 3613-3623, DOI: 10.1128/JB.185.12.3613-3623.2003.

Wang, Y., Li, C., Liu, P., Ahmed, Z., Xiao, P., Bai, X. (2010). Physical characterization of exopolysaccharide produced by *Lactobacillus plantarum* KF5 isolated from Tibet Kefir. *Carbohydrate Polymers* 82(3), p 895-903, DOI: 10.1016/j.carbpol.2010.06.013.

Watkins, B.A., Kratzer, F.H. (1983). Effect of oral dosing of *Lactobacillus* strains on gut colonisation and liver biotin in broiler chicks. *Poult*. *Sci*. 62(10), p 2088-2094.

Watson, J.A., Schubert, J. (1969). Action of hydrogen peroxide on growth inhibition of *Salmonella typhimurium*. *J*. *Gen*. *Microbiol*. 57(1), p 25-34, DOI: 10.1099/00221287-57-1-25.

Wee, Y., Kimm, J., Ryu, H. (2006). Biotechnological production of lactic acid and its recent applications. *Food Technol*. *Biotechnol*. 44(2), p 163–172.

Wei, S., Morrison, M., Yu, Z. (2013). Bacterial census of poultry intestinal microbiome. *Poult*. *Sci*. 92(3), p 671-683; DOI: 10.3382/ps.2012-02822.

Weurding, R.E., Veldman, A., Veen, W.A.G., van der Aar, P.J., Verstegen, M.W.A. (2001). Starch digestion rate in the small intestine of broiler chickens differs among feedstuffs. *J*. *Nutr*. 131(9), p 2329-2335.

Wu, M.H., Pan, T.M., Wu, Y.J., Chang, S.J., Chang, M.S., Hu, C.Y. (2010). Exopolysaccharide activities from probiotic bifidobacterium: Immunomodulatory effects (on J774A.1 macrophages) and antimicrobial properties. *Int*. *J*. *Food Microbiol*. 144(1), p 104-110, DOI: 10.1016/j.ijfoodmicro.2010.09.003.

Yadav, V., Prappulla, S.G., Jha, A., Poonia, A. (2011). A novel exopolysaccharide from probiotic *Lactobacillus fermentum* CFR 2195: Production, purification and characterization. *Biotechnol*. *Bioinf*. *Bioeng*. 1(4), p 415-421.

Yan, W., Sun, C., Yuan, J., Yang, N. (2017). Gut metagenomic analysis reveals prominent roles of *Lactobacillus* and cecal microbiota in chicken feed efficiency. *Sci*. *Rep*. 7, e45308, DOI: 10.1038/srep45308.

Yu, G.Y., Sinclair, J.B., Hartman, G.L., Bertagnolli, B.L. (2002). Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. *Soil Biol*. *Biochem*. 34(7), p 955-963, DOI: 10.1016/S0038-0717(02)00027-5.

Zamudio, M., González, A., Medina, JA. (2001). *Lactobacillus plantarum* phytase activity is due to non-specific acid phosphatase. *Lett*. *Appl*. *Microbiol*. 32(3), p 181–184, DOI: 10.1046/j.1472-765x.2001.00890.x.

Chapter 4

Safety assessment of antibiotic and probiotic feed additives for *Gallus gallus domesticus*

Abstract

This study assessed the safety of antibiotic and probiotic use as feed additives in broilers. An alternative to antibiotics is desperately needed as their use possess an imminent threat to human health. Hatched broilers were randomly divided into three groups with 100 birds per group. Group 1 received sulphadiazine, colistin and trimethoprim as antibiotic feed additive. Group 2 received a multi-species probiotic consisting of *Lactobacillus crispatus* DPN167, *Lactobacillus salivarius* DPN181, *Lactobacillus gallinarum* DPN164, *Lactobacillus johnsonii* DPN184, *Enterococcus faecalis* DPN94 and *Bacillus amyloliquefaciens* DPN123. Group 3 (control) received standard feed with no additives. The effect of feed additives on broiler health and performance were assessed on days 19 and 29, to follow the effect during and after development. Growth performance, size of the lymphoid organs, gizzard mass, mineral content of the tibia bones and red blood cell parameters were similar for broilers from all treatment groups. On day 19, broilers on antibiotics had a larger surface villi area and higher lymphocyte and basophil counts compared to broilers from the probiotic and control groups. On day 29 the cecal microbiomes of broilers from the control and probiotic treatment groups were similar. Cecal microbiomes of broilers administered antibiotics significantly differed from the control and probiotic treatment groups, having lower levels of *Enterobacteriaceae* and higher levels of unidentified Clostridiales, *Brucellaceae*, *Synergistaceae*, *Erysipelotrichaceae* and *Coriobacteriaceae*. Broilers receiving probiotics showed a decrease in bioluminescence when administered bioluminescent *Listeria monocytogenes*, indicating that the probiotic can decrease pathogen metabolic activity. This study provides valuable information on the health and performance of broilers when administered antibiotics and probiotics as feed additives.

Introduction

Poultry reared on a large scale in intensive production systems are more prone to develop microbial infections (Kabir, 2009). Necrotic enteritis, caused by *Clostridium perfringens* and coccidiosis, caused by *Eimeria* spp., are the most challenging of all poultry diseases and are difficult to control (Kabir *et al*., 2004). The use of antibiotics as growth promoter in animal feed has been banned by the European Union to control natural selection for antibiotic-resistant pathogens (Montagne *et al*., 2003) and ensure that currently available antibiotics remain effective in the treatment of animal and human infections.

The general assumption is that chickens intensively reared do not acquire beneficial microbiota from the environment (Čermák and Skřivanová, 2006). Furthermore, the immune system of broilers, especially in the first month, is not well developed and they are susceptible to bacterial infections caused by *Campylobacter jejuni*, *C*. *perfringens*, *Salmonella enterica* and *Escherichia coli* (Ohimain and Ofongo, 2012). It is thus not surprising that broilers reared intensively are more susceptible to microbial infections (Patterson and Burkholder, 2003). Those that do survive the first two weeks have a good chance to develop a stable consortium of intestinal microbiota during the following two weeks (Lee *et al*., 2010).

Alternative supplements that enhance growth and protect broilers from pathogenic infections is desperately needed. Numerous beneficial effects of probiotics administered to broilers have been reported, e.g. improvement in growth performance (Sun *et al*., 2005; Mountzouris *et al*., 2007), increased digestion of nutrients (Li *et al*., 2008), modulation of intestinal microflora (Yu *et al*., 2008), inhibition of pathogens (Higgins *et al*., 2008; Mountzouris *et al*., 2009), competitive exclusion of pathogens and antagonism (Kabir *et al*., 2005), and modulation of gut mucosal immunity (Chichlowski *et al*., 2007). However, the addition of probiotics to broiler feed is still far from being implemented on a regular basis (Ducatelle *et al*., 2015), mainly due to a lack in in-depth knowledge about the complex dynamics of the poultry gut (Rehman *et al*., 2007) and the multitude of parameters that influences the efficacy of probiotics. Differences in microbial species and strains, methods used to propagate probiotic strains, differences in the ability of the strains to adhere to the gastrointestinal tract (GIT), number of evidence-based clinical trials (Klaenhammer and Kullen, 1999), production standards (Fasoli *et al*., 2003), environmental factors and management6 are a few of the variabilities. More research on the intestinal ecosystem, inter-microbial and microbiota-host interactions are required (Ducatelle *et al*., 2015).

In this study, we evaluated the effect of antibiotics (sulphadiazine, colistin and trimethoprim in combination) and a multi-species probiotic (*L*. *crispatus*, *L*. *salivarius*, *L*. *gallinarum*, *L*. *johnsonii*, *E*. *faecalis* and *B*. *amyloliquefaciens*) on the performance of healthy broilers. Parameters assessed included growth performance, immune organ weight, gizzard weight, histomorphology of the small intestine, haematology, tibia bone mineral weight, inhibition of *L*. *monocytogenes* EGDe *in vivo* and changes in cell numbers of cecal microorganisms. Understanding what physiological changes these feed additives induce in healthy broilers is important to assess their safety with long-term use.

Materials and Methods

Birds and housing

The study was approved by the Research Ethics Committee: Animal Care and Use of Stellenbosch University, Stellenbosch (registration number SU-ACUD15-00016). All experiments were performed in accordance with relevant guidelines and regulations. Three-hundred day old as-hatched Cobb 500 broiler chicks were divided into 30 cages of 2 m^2 each (10 birds per cage) and housed in a temperature-controlled poultry rearing house at Mariendahl experimental farm, Stellenbosch University. Each treatment group consisted of 10 cages (100 broilers). Each cage was equipped with feeders and automatic water dispensers. The humidity, temperature and light were controlled according to the Cobb Broiler Management Standards (Cobb Vantress, Colchester, UK) and the South African Animal Welfare Act.

Bacterial strains and preparation of the probiotic

The multi-species probiotic consisted of *L. crispatus* DPN167, *L. salivarius* DPN181, *L. gallinarum* DPN164, *L. johnsonii* DPN184, *E. faecalis* DPN94 and *B. amyloliquefaciens* DPN123. Of all bacteria isolated from healthy free-range broilers, strains from these six species were the most resistant to gastric acids and bile, adhered the best to gut epithelial cells and inhibited the growth of *Listeria monocytogenes* and *Salmonella enterica* serovar Typhimurium *in vitro*. The strains were cultured in molasses medium, which consisted of 5.0 % (w/v) molasses, 0.3 % (w/v) yeast extract, 0.2 % (w/v) peptone, 0.004 % (w/v) $MnSO_4$, 0.001 % (w/v) Na-citrate, 0.4 % (w/v) K_2HPO_4 and 0.02 % (v/v) Tween80. The medium was sterilised at 121 °C for 15 min, cooled to 25 °C, the upper phase removed from the sediment and again autoclaved. Thioglycolate (0.15 %, w/v) was added to the growth medium of *L. crispatus* DPN167 and *L. johnsonii* DPN184 to create an anaerobic environment. Incubation was for 3 to 4 days at 37 °C. Cells were harvested (8000 × g, 10 min, 4 °C), washed with sterile PBS (0.8 %, w/v, NaCl; 0.02 %, w/v, KCl; 0.142 %, w/v, Na_2HPO_4; 0.024 %, w/v, KH_2PO_4; pH 7.5) and resuspended in sterile cryoprotectant (10 %, w/v, lactose and 10.0 %, w/v, sucrose, autoclaved at 121 °C for 10 min and cooled to 4 °C). The number of viable cells per gram freeze-dried culture was determined by plating onto MRS Agar (Biolab) or BHI Agar (Biolab). Plates were incubated at 37 °C for 24 h under aerobic and anaerobic conditions. The strains were combined to yield a total cell count of 2.8×10^8 CFU/g freeze-dried powder, consisting of 2.6×10^7 CFU *L. crispatus* DPN167, 3.6×10^7 CFU *L. salivarius* DPN181, 1.3×10^8 CFU *L. gallinarum* DPN164, 1.9×10^7 CFU *L. johnsonii* DPN184, 5.1×10^7 CFU *E. faecalis* DPN94 and 1.9×10^7 CFU *B. amyloliquefaciens* DPN123.

Feeding trials

The feed contained maize, soya oilcake, sunflower oilcake, canola oilcake, wheat, bran, Ca-phosphate, limestone, salt, lysine, methionine and threonine. The pre-starter was supplied at 178 g per bird (over 7 days). The starter diet was supplied at 354 g per bird (over 7 days), grower diet at 1596 g per bird (over 7 days) and a finisher diet at 1883 g per bird (over 11 days). Feed of broilers from the probiotic treatment group was supplemented with the multi-species probiotic as follows: pre-starter was

supplemented with 24 mg dried probiotic cells per gram feed to yield 6.7 × 10^6 CFU/gram feed, consisting of 6.1 × 10^5 CFU *L*. *crispatus* DPN167, 8.4 × 10^5 CFU *L*. *salivarius* DPN181, 3.1 × 10^6 CFU *L*. *gallinarum* DPN164, 4.4 × 10^5 CFU *L*. *johnsonii* DPN184, 1.2 × 10^6 CFU *E*. *faecalis* DPN94 and 4.4 × 10^5 CFU *B*. *amyloliquefaciens* DPN123. The starter feed was supplemented with 12 mg probiotic powder per gram feed (3.3 × 10^6 CFU/gram feed, consisting of 3.1 × 10^5 CFU *L*. *crispatus* DPN167, 4.2 × 10^5 CFU *L*. *salivarius* DPN181, 1.6 × 10^6 CFU *L*. *gallinarum* DPN164, 2.2 × 10^5 CFU *L*. *johnsonii* DPN184, 6.1 × 10^6 CFU *E*. *faecalis* DPN94 and 2.2 × 10^5 CFU *B*. *amyloliquefaciens* DPN123). Grower was supplemented with 5.4 mg probiotic powder per gram feed (1.5 × 10^6 CFU/gram feed, consisting of 1.4 × 10^5 CFU *L*. *crispatus* DPN167, 1.9 × 10^5 CFU *L*. *salivarius* DPN181, 7.0 × 10^5 CFU *L*. *gallinarum* DPN164, 1.0 × 10^5 CFU *L*. *johnsonii* DPN184, 2.8 × 10^5 CFU *E*. *faecalis* DPN94 and 1.0 × 10^5 CFU *B*. *amyloliquefaciens* DPN123). The finisher was supplemented with 3.5 mg probiotic powder per gram feed (9.9 × 10^5 CFU/g feed, consisting of 9.0 × 10^4 CFU *L*. *crispatus* DPN167, 1.3 × 10^5 CFU *L*. *salivarius* DPN181, 4.4 × 10^5 CFU *L*. *gallinarum* DPN164, 6.2 × 10^4 CFU *L*. *johnsonii* DPN184, 1.8 × 10^5 CFU *E*. *faecalis* DPN94 and 6.5 × 10^4 CFU *B*. *amyloliquefaciens* DPN123). Average daily intake of the multi-species probiotic from day 1 to 29 during the different feeding stages is listed in Table 1. Broilers from the probiotic treatment group received between 1.0 and 4.1 × 10^8 CFU daily of the multi-species probiotic consisting of *Lactobacillus crispatus* DPN167 (9.3 × 10^6 to 3.8 × 10^7 CFU), *Lactobacillus salivarius* DPN181 (1.3 × 10^7 to 5.3 × 10^7 CFU), *Lactobacillus gallinarum* DPN164 (4.6 × 10^7 to 1.9 × 10^8 CFU), *Lactobacillus johnsonii* DPN184 (6.8 × 10^6 to 2.8 × 10^7 CFU), *Enterococcus faecalis* DPN94 (1.8 × 10^7 to 7.5 × 10^7 CFU) and *Bacillus amyloliquefaciens* DPN123 (6.8 × 10^6 to 2.8 × 10^7 CFU).

Broilers in the antibiotic treatment group (10 cages) received the same ration in the four feeding cycles, but the feed was supplemented with a combination of sulphadiazine (0.375 ppm/gram feed), colistin (0.128 ppm/gram feed) and trimethoprim (0.075 ppm/gram feed) and contained no probiotics. Broilers from the antibiotic treatment group received on average between 7.5 to 61.1 ppm sulphadiazine, 2.6 to 20.9 ppm colistin and 1.5 to 12.2 ppm trimethoprim daily for 29 days (Table 1). The three antibiotics were selected, as they are often included as feed additives (Robert *et al*., 2016). Broilers in the untreated group (10 cages) served as the control and received feed without antibiotics and probiotics. Lactose and sucrose were added to the feed used in each feeding cycle of the antibiotic and control treatment groups to yield concentrations identical to the feed administered to the probiotic treatment group.

Health and growth performance

Visual health and growth performance of the birds were evaluated based on daily feed consumption and changes in body mass. Weekly weight and feed intake per pen were recorded, and individual weights were calculated as an average of the pen weight. Average feed conversion ratio (FCR) calculated from the feed intake (FI) and body weight gain (BWG). All the birds were weighed, and the change in body mass of each cage calculated relevant to the mass recorded on day 1.

Haematology, organ weight and histology

On days 19 and 29, twenty birds per treatment were randomly selected, euthanised by cervical dislocation and blood collected into K_2-EDTA tubes by exsanguination. These two days were selected based on the developing stage of the GIT. Previous studies (Leeson and Summers, 2009) have shown that on day 19 the GIT is not fully developed, whereas 10 days later, on day 29, the GIT is considered mature. Automated full blood counts were performed using the Celldyne 3700CS haematology analyser (Abbott Diagnostics, Illinois, USA). The number of erythrocytes and their parameters, i.e. haemoglobin content, haematocrit value, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and erythrocyte cell distribution width were determined. The total number of leukocytes was recorded as well as subpopulation counts for heterophils, lymphocytes, monocytes, eosinophils and basophils. Blood platelet (thrombocyte) counts were also recorded.

The spleen and bursa Fabricius of twenty birds per treatment on days 19 and 29, and the gizzards on day 29 were excised and weighed. The gizzards were dissected longitudinally and rinsed under running water before being weighed. The duodenum of 20 broilers per treatment on days 19 and 29 were collected, longitudinally dissected and carefully washed with sterile PBS. The samples were preserved in 10 % (v/v) formaldehyde-saline for 30 days, cut to size, placed into embedding cassettes, and processed and impregnated in paraffin wax, using an automated tissue processor TISSUE TEK II 4640B (Miles Laboratories Inc., Naperville, IL). Sections (5 µm in thickness) were prepared with a rotary microtome (Reichert Jung, Heidelberg, Austria), deparaffinised and rehydrated, before staining with haematoxylin and eosin. These sections, prepared as described by Presnell and Schreibman (1997), were examined using a Nikon SMZ800 (Nikon Corporation, Tokyo, Japan) stereomicroscope, equipped with a 2.5 × magnification objective lens and a Nikon DS-Fi1 digital camera (Nikon Corporation). Images were analysed using ImageJ software (National Institutes of Health, Maryland, USA). Villi height and area were measured from the tip of the villi to the villous-crypt junction for 10 consecutive intact villi. Crypt depth was estimated by measuring 10 crypts per section. Crypt depth was the vertical distance from the villous-crypt junction to the lower limit of the crypt.

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Table 1: Average daily feed, probiotic and antibiotic consumption rate, by broilers from the probiotic and antibiotic treatment groups.

Feed	Probiotic concentration (CFU/g feed)	Age	Daily average feed consumption (g)	Daily average probiotic consumption (CFU)	Daily average antibiotic consumption (ppm)		
					Sulpha diazine	Colistin	Trimethoprim
Pre-starter	6.7×10^6	0	140	9.4×10^8	52.5	17.9	10.5
		1					
		2					
		3					
		4					
		5					
		6					
		7					
Starter	3.3×10^6	8	30	2.0×10^8	11.3	3.8	2.3
		9	35	2.3×10^8	13.1	4.5	2.6
		10	39	2.6×10^8	14.6	5.0	2.9
		11	44	2.9×10^8	16.5	5.6	3.3
		12	50	3.4×10^8	18.6	6.4	3.8
		13	55	3.7×10^8	20.6	7.0	4.1
		14	61	4.1×10^8	22.9	7.8	4.6
Grower	1.5×10^6	15	67	1.0×10^8	25.1	8.6	5.0
		16	73	1.1×10^8	27.4	9.3	5.5
		17	80	1.2×10^8	30.0	10.2	6.0
		18	86	1.3×10^8	2.3	11.0	6.5
		19	93	1.4×10^8	34.9	11.9	7.0
		20	100	1.5×10^8	37.5	12.8	7.5
		21	107	1.6×10^8	40.1	13.7	8.0
Finisher	9.9×10^5	22	115	1.1×10^8	43.1	14.7	8.6
		23	122	1.2×10^8	45.8	15.6	9.2
		24	130	1.3×10^8	48.8	16.6	9.8
		25	137	1.4×10^8	51.4	17.5	10.3
		26	144	1.4×10^8	54.0	18.4	10.8
		27	151	1.5×10^8	56.6	19.3	11.3
		28	157	1.6×10^8	58.9	20.1	11.8
		29	163	1.6×10^8	61.1	20.9	12.2

Mineralisation of the tibia

The right tibia from the carcasses of twenty birds per treatment on day 29 were cleaned from tissue and cartilage and the dry matter of each determined according to the method described by the Association of Official Analytical Chemists (Horwitz, 2001). In short, tibias placed in porcelain cubicles were, dried at 100 ºC for 24 h, cooled down for 30 min in a desiccator and then weighed. The tibia was then broken in half, defatted in petroleum for 48 h (Rama Rao and Reddy, 2001), dried at 100 ºC for 24 h and weighed. Lastly, the tibias were exposed to 600 ºC for 24 h, and the ash weighed.

In vivo inhibition of *L. monocytogenes*

The ability of the antibiotic and probiotic feed additives in inhibiting colonisation and proliferation of *L. monocytogenes in vivo* was assessed. On day 14, twelve broilers per treatment group were relocated to the animal housing unit of the Department of Animal Science, Stellenbosch University and each placed in separate cages. Water and feed were supplied ad libitum. On day 15, the feed was withdrawn 2 h before the administration of *L. monocytogenes* EGDe, a bioluminescent strain obtained from Caliper Life Sciences (Massachusetts, USA). Strain EGDe contains plasmid PL2lux with the *luxABCDE* operon of *Photorhabdus luminescence*. Each of the birds was administered 100 µl (4.28×10^8 CFU) *L. monocytogenes* EGDe by intragastric gavage. Broilers from the probiotic treatment group were administered 100 µl of the multi-species probiotic (8.34×10^8 CFU) by intragastric gavage, 2 h before the administration of *L. monocytogenes* EGDe.

The probiotic preparation was prepared as follows: *L. crispatus* DPN167, *L. salivarius* DPN181, *L. gallinarum* DPN164, *L. johnsonii* DPN184 and *E. faecalis* DPN94 were cultured in MRS broth for 12 h at 37 °C under anaerobic conditions. *Bacillus amyloliquefaciens* DPN123 was cultured in BHI broth for 12 h at 37 °C under aerobic conditions using an orbital shaker at 100 rpm. Cells were harvested (8000 × g, 3 min, 25 °C), washed with two volumes of sterile PBS and resuspended in 100 µl gavage buffer (0.2 M $NaHCO_3$ buffer containing 1 %, w/v, glucose, pH 8) to yield 8.3×10^8 CFU (5.2×10^7 CFU *L. crispatus* DPN167, 6.2×10^7 CFU *L. salivarius* DPN181, 1.2×10^8 CFU *L. gallinarum* DPN164, 1.3×10^8 CFU *L. johnsonii* DPN184, 2.3×10^8 CFU *E. faecalis* DPN94 and 2.4×10^8 CFU *B. amyloliquefaciens* DPN123). *Listeria monocytogenes* EGDe was cultured in BHI broth (supplemented with 7.5 µg/ml chloramphenicol) under aerobic conditions using an orbital shaker at 100 rpm for 6 h at 37 °C. Cells were harvested (8000 × g, 3 min, 25 °C), washed with two volumes of sterile PBS and resuspended in gavage buffer to yield 4.2×10^8 CFU per 100 µl.

After 2 h, and again 3.5 h, of administering *L. monocytogenes* EGDe, six broilers per treatment group were euthanised by cervical dislocation. The gastrointestinal tract (GIT) of each bird was dissected longitudinally and screened for the emission of bioluminescence from cells of *L. monocytogenes* EGDe by using the Caliper *in vivo* imaging system (IVIS® 100, Caliper Life Sciences). The IVIS was equipped with a cooled charge-coupled-device camera mounted on a light-tight specimen chamber. Exposure was 3 min. Photons emitted at 620 nm were calculated using the software version 3 of Caliper Life Sciences. The values obtained were expressed as photons per second per cm^2 per steradian (p. S^{-1}. cm^{-1}. sr^{-1}). Regions of interest (ROI) were selected manually. Background bioluminescence was corrected for by overlaying images from intestines with non-

bioluminescent bacteria. The GIT of each bird was then dissected to separate the duodenum, jejunum, ileum, ceca and colon. Each section was weighed, homogenised in sterile PBS, serially diluted and plated on BHI agar supplemented with 7.5 µg/ml chloramphenicol. Plates were incubated at 37 °C for 24 h and the number of viable cells expressed as CFU/gram gut.

Cecal microbiota composition

On day 29 cecal digesta content was collected from six broilers per treatment group and stored at – 20 °C. Metagenomic DNA was isolated using the iPrep ChargeSwitch gDNA kit (ThermoFisher, Massachusetts, USA), with a few modifications. One millilitre Tris-HCl buffer (pH 8.0) was added to 200 mg cecal digesta and incubated overnight at 37 °C, in the presence of 50 µl lysozyme (100 mg/ml). Cells were collected (10 000 × g, 10 min, 4 °C), suspended in 1 ml ChargeSwitch Lysis Buffer and incubated overnight at 56 °C in the presence of 20 µl of proteinase K (20 mg/ml). DNA was then purified using the iPrep gDNA isolation protocol for AB Library Builder (ThermoFisher) and concentrations assessed using Nanodrop (ThermoFisher) and Qubit readings (ThermoFisher), as per manufacturers' instructions.

Sequencing of the hypervariable region of the 16S rDNA gene was performed using the Ion Torrent 16S™ Metagenomics Kit (ThermoFisher). DNA (10 ng) was amplified using 16S primer sets 1 (V2-4-8) and 2 (V3-6, 7-9), and 15 µl Ion Environmental Master Mix in a final volume of 30 µl. Amplification was carried out for 18 cycles, with a 10 min initial denaturation at 95 °C, followed by denaturation at 95 °C for 30 secs, annealing at 58 °C for 30 secs, and elongation at 72 °C for 20 sec. Equal volumes of PCR products were then pooled and purified. Pooled purified amplicons were used to create sequence libraries via the Ion Plus Fragment Library Kit (ThermoFisher) with sample indexing using the Ion Xpress™ Barcode Adapters 1-96 Kit (ThermoFisher). Template preparation was performed using the Ion OneTouch™ 2 System and the Ion S5 OT2 Kit (ThermoFisher). Sequencing was conducted using the Ion S5™ Sequencing reagents on the Ion S5™ system using the Ion 530™ chip. Primary data analysis was performed with Torrent Suite™ Software v4.0 with automated secondary analysis using Ion Reporter™ Software v4.0 (ThermoFisher) and Calypso software (Zakrzewski *et al*., 2016). The sequences were deposited on the NCBI SRA databank under Bioproject ID PRJNA352351. The accession numbers are SAMN05971353 (Control 1), SAMN05971354 (Control 2), SAMN05971355 (Control 3), SAMN05971356 (Control 4), SAMN05971357 (Control 5), SAMN05971358 (Control 6), SAMN05971359 (Antibiotic 1), SAMN05971360 (Antibiotic 2), SAMN05971361 (Antibiotic 3), SAMN05971362 (Antibiotic 4), SAMN05971363 (Antibiotic 5), SAMN05971364 (Antibiotic 6), SAMN05971365 (Probiotic 1), SAMN05971366 (Probiotic 2), SAMN05971367 (Probiotic 3), SAMN05971368 (Probiotic 4), SAMN05971369 (Probiotic 5) and SAMN05971370 (Probiotic 6).

Statistical analyses

GraphPad Prism 6 (GraphPad Software Inc., California, USA) was used to perform statistical analyses. Data of growth performance, gizzard and lymphoid organ weight, histomorphological and haematological parameters, tibia bone weights, viable cell counts, and bioluminescent counts were

analysed by one-way ANOVA to determine the significance of the main effects and interactions. The mean variances were compared using the Fisher's LSD test. Differences were considered significant if p values were less than 0.05.

Multidimensionality of biodiversity, various indices of diversity and community composition were calculated and compared using the Calypso software (Zakrzewski, *et al*., 2016). Alpha diversity was calculated using the Shannon index, chao1 index, evenness index and richness index. Alpha diversities were compared using ANOVA analysis. Species diversity was analysed by mcpHill analysis and significant differences determined by the Turkey test (Pallmann *et al*., 2012). Data were filtered by removing taxa with less than 0.01 % abundance, and data were normalised by total sum scaling (TSS). Normalization method was applied for downstream analyses, i.e. taxa relative abundance, β-diversity, and group significance. Beta diversity was analysed using Bray-Curtis dissimilarity and visualised by nonmetric multidimensional scaling (NMDS) and significant differences between treatment groups determined by Anosim (Anderson, 2011). ANOVA analysis was performed to compare diversity between treatment groups, and pairwise comparison assessed using the student t-test.

Results and Discussion

Health and growth performance

The average feed conversion ratios (FCRs) of broilers in the probiotic and antibiotic treatment groups were not significantly different from that recorded for broilers in the untreated group on days 7, 14, 21 and 28 (Table 2), suggesting that neither the multi-species probiotic nor the antibiotics influenced growth performance. No improvement in growth performance was observed for broilers receiving antibiotics because broilers were grown in hygienic conditions and were disease free. Similar results with regards to probiotics were published by Fasoli and co-workers (Fasoli *et al*., 2003). The authors reported an increase in body weight of broilers that received *Enterococcus faecium* M74, but their FCRs were not significantly different from broilers in control groups on a probiotic-free diet (Fasoli *et al*., 2003). However, studies conducted by Shim *et al*. 2010, Sen *et al*. 2012 and Chen *et al*. 2013, using different probiotic compositions, showed an improvement in growth performance. The authors attributed the enhanced growth to an increase in digestive enzyme activity, coupled with additional changes, such as a decrease in ammonia production and maintenance of beneficial microbiota in the GIT. In a recent study where broilers were fed a multi-species probiotic consisting of *L*. *johnsonii*, *L*. *crispatus*, *L*. *salivarius* and an unidentified *Lactobacillus* sp., no changes in body weight gain (BWG), feed intake (FI) and FCR were observed (Olnood *et al*., 2015). Similar conclusions were drawn when a multi-species probiotic, consisting of hetero- and homofermentative *Lactobacillus* spp., were administered to broilers (Awad *et al*., 2009).

The inconsistency in reports regarding the effect of probiotics on growth performance may be due to differences in rearing conditions, strain compositions, the number of viable cells administered and frequency of administration. Santos and co-workers (Santos *et al*., 2008) have shown differences in growth performance when broilers were reared free-range, on an open floor, and in cages. Cage rearing is considered more hygienic, as broilers are not in direct contact with faeces (Reece *et al*.,

1971). However, cage rearing could also lead to food-safety concerns due to the inadequate transfer of beneficial microbiota from the environment (Santos *et al*., 2008). Rearing conditions should thus always be considered when the effect of additives on growth performance is studied (Pourakbari w*et al*., 2016). In the present study, broilers were reared in cages elevated from the floor.

Probiotic characteristics are strain dependent, and the combination of strains may influence the efficacy of a multi-species probiotic (Ramos *et al*., 2013). The number of viable cells administered, and the dose frequency are equally important. Most probiotics are administered at 10^7 to 10^9 CFU per day (Mountzouris *et al*., 2010). In the present study, broilers received between 1.0×10^8 and 4.1×10^8 CFU of the multi-species probiotic per day. Antibiotics and probiotics act as prophylactics that inhibit the development of pathogenic bacteria and improves growth. However, our results indicated that the daily administration of a multi-species probiotic (10^8 CFU) did not have a positive, nor negative, effect on growth performance.

Haematology, organ weight and histology

Several factors, such as physiological and environmental conditions (Graczyk *et al*., 2003), diet (Kurtoğlu *et al*., 2005), water and feed restriction (Lamošová *et al*., 2004), age (Seiser *et al*., 2000) and administration of drugs (Zaman *et al*., 1995) affect the blood parameters of healthy broilers. On day 19 the white blood cell (WBC), heterophil (HET), monocyte (MONO) and eosinophil (EOS) counts were not significantly different for broilers from the different treatment groups (Table 3). Lymphocyte (LYM) and basophil (BASO) counts, on the other hand, were significantly different on day 19. Broilers from the antibiotic treatment group had a higher mean LYM ($p= 0.012$) and BASO (0.018) count compared to the probiotic treatment group. LYM and BASO counts were not significantly different between probiotic and control, and antibiotic and control treatment groups on day 19 (Table 3). Lymphocytes include natural killer cells, T-cells and B-cells (Rabson *et al*., 2005). T cells (thymus cells) and B-cells (bone marrow- or bursa-derived cells) are the major cellular components of the adaptive immune response. T-cells are involved in cell-mediated immunity, whereas B-cells are primarily responsible for humoral immunity (Rabson *et al*., 2005).

Table 2: Mean (± standard deviation) body weight gain (BWG), feed intake (FI), feed conversion ratio (FCR) of broilers (n= 10) from day of hatch to day 28, receiving no treatment, the antibiotic combination and the multi-species probiotic.

	Day 0-7			Day 0-14			Day 0-21			Day 0-28		
Treatment	**BWG**	**FI**	**FCR**	**BWG**	**FI**	**FCR**	**BWG**	**FI**	**FCR**	**BWG**	**FI**	**FCR**
Control	152 ± 12.0	243 ± 13.2	1.61 ± 0.13	438 ± 23.1	579 ± 8.2	1.33 ± 0.08	978 ± 54.4	1566 ± 42.9	1.60 ± 0.06	1675 ± 97.8	2861 ± 169.5	1.72 ± 0.09
Antibiotic	158 ± 20.4	244 ± 14.1	1.58 ± 0.28	437 ± 31.9	576 ± 4.5	1.33 ± 0.10	994 ± 54.4	1570 ± 64.5	1.58 ± 0.07	1766 ± 95.8	2923 ± 62.6	1.67 ± 0.07
Probiotic	160 ± 9.40	245 ± 17.9	1.54 ± 0.13	428 ± 15.6	576 ± 5.0	1.35 ± 0.05	961 ± 59.1	1526 ± 53.3	1.59 ± 0.07	1712 ± 78.6	2935 ± 84.7	1.70 ± 0.04
p value	0.509	0.957	0.749	0.644	0.463	0.806	0.443	0.163	0.792	0.112	0.326	0.141

Table 3: Mean (± standard deviation) total leukocyte count (WBC) on days 19 and 29, and composition of heterophils (HET), lymphocytes (LYM), monocytes (MONO), eosinophils (EOS) and basophils (BASO) of broilers (n= 20) receiving different treatments.

	WBC (10^6/µl)		HET (10^3/µl)		LYM (10^3/µl)		MONO (10^3/µl)		EOS (10^3/µl)		BASO (10^3/µl)	
Treatment	19 D	29 D	19 D	29 D	19 D	29 D	19 D	29 D	19 D	29 D	19 D	29 D
Control	34.5 ± 26.5	27.9 ± 21.3	5.8 ± 2.5	7.2 ± 2.4	23.9^{ab} ± 20.8	18.1 ± 18.5	1.9 ± 1.9	0.75 ± 0.74	0.12 ± 0.04	0.17 ± 0.12	1.44^{ab} ± 1.53	0.49 ± 0.48
Antibiotic	49.5 ± 33.5	29.5 ± 27.3	6.3 ± 2.5	5.9 ± 3.5	38.7^{a} ± 27.8	17.6 ± 18.9	3.1 ± 2.6	0.55 ± 0.60	0.18 ± 0.11	0.16 ± 0.10	2.74^{a} ± 1.65	0.64 ± 0.97
Probiotic	21.2 ± 11.9	32.6 ± 21.9	4.7 ± 2.6	7.3 ± 2.8	13.9^{b} ± 8.0	23.3 ± 20.3	1.4 ± 1.3	0.68 ± 0.65	0.10 ± 0.06	0.16 ± 0.12	1.09^{b} ± 1.04	0.64 ± 0.67
p value	0.07	0.832	0.414	0.281	**0.040***	0.621	0.168	0.698	0.708	0.950	**0.046***	0.820

* $p < 0.05$, $^{a, b}$ Means within columns with different superscripts differ significantly ($p < 0.05$)

Table 4: Mean (± standard deviation) erythrocyte count and haemoglobin content (HGB), haematocrit value (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and erythrocytes cell distribution width (RDW) of broilers (n= 20) on days 19 and 29, receiving different treatments.

	RBC (10^6/µl)		**HGB (g/dl)**		**HCT (%)**		**MCV (f)**		**MCH (pg)**		**MCHC (g/dl)**		**RDW (%)**	
Treatment	19 D	29 D	19 D	29 D	19 D	29 D	19 D	29 D	19 D	29 D	19 D	29 D	19 D	29 D
Control	2.22 ± 0.26	2.59 ± 0.34	13.24 ± 1.71	14.75 ± 1.83	18.33 ± 2.12	20.49 ± 2.85	82.56 ± 2.85	81.17 ± 2.84	59.62 ± 1.86	58.31 ± 2.17	72.24 ± 2.02	71.88 ± 2.84	12.70^{bc} ± 0.85	12.56 ± 0.63
Antibiotic	2.19 ± 0.28	2.27 ± 0.74	13.22 ± 1.83	12.56 ± 5.11	18.39 ± 2.16	19.10 ± 4.73	84.06 ± 2.71	81.33 ± 3.47	60.24 ± 1.33	58.08 ± 1.33	71.72 ± 2.57	71.44 ± 2.70	12.93^{b} ± 1.08	12.67 ± 1.26
Probiotic	2.17 ± 0.45	2.46 ± 0.30	10.88 ± 3.21	14.24 ± 2.12	17.76 ± 3.78	19.41 ± 2.33	81.64 ± 2.40	79.26 ± 2.39	52.43 ± 20.55	57.04 ± 2.42	64.57 ± 25.86	72.01 ± 3.48	13.76^{a} ± 0.41	12.81 ± 0.97
p value	0.960	0.174	0.056	0.135	0.861	0.549	0.140	0.106	0.301	0.223	0.475	0.888	**0.022***	0.794

* $p < 0.05$, $^{a, b\ c}$ Means within columns with different superscripts differ significantly ($p < 0.05$)

Natural killer cells are part of the innate immune system and play a major role in defending the host from tumours and virus-infected cells (Rabson *et al*., 2005). Basophils are granulocytes responsible for inflammatory responses and production of heparin and histamine (Rabson *et al*., 2005). A higher BASO count is characteristic of a pro-inflammatory response and may be the result of sensitivity to antibiotics or the presence of bacteria that elicits an immune response. Transiently higher LYM counts on day 19 were indicative of a response to the presence of specific immune provoking bacteria. These counts usually normalise when bacterial cell numbers are brought under control, or after a few days when the body develops tolerance to the antibiotics. Both these responses are undesired. As energy for growth is relayed to elicit an immune response, probiotics showed the opposite and did not elicited an immune response, which is desirable. On day 29, no significant differences were recorded in WBC, HET, LYM, MONO, EOS and BASO counts for broilers from the different treatment groups (Table 3).

Lymphocytes are the major circulating immune cells in birds and HET are functionally equivalent to neutrophils that participate in inflammation and phagocytosis. A high HET/LYM ratio and high glucocorticoid level is an indication of stress (Zulkifli *et al*., 2000; Davis *et al*., 2008). High HET/LYM ratios have also been associated with increased mortality (Sepp *et al*., 2010). No significant differences were recorded between the mean HET/LYM ratios of broilers from the different treatment groups on days 19 ($p = 0.737$) and 29 ($p = 0.357$) (Table 4). Thrombocytes stop bleeding by clumping and plugging injured blood vessels. No significant differences were recorded in thrombocyte counts for the different treatment groups on days 19 ($p = 0.121$) and day 29 ($p = 0.350$) (Table 4).

On day 19, broilers from the different treatment groups had no significant differences in total red blood cell (RBC), haemoglobin content (HGB), haematocrit content (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin counts (MCHC); listed in Table 4. The erythrocyte cell distribution (RDW) of broilers from different treatment groups on day 19 were significantly different ($p = 0.022$). Broilers receiving the multi-species probiotic had a higher mean RDW on day 19, compared to the antibiotic ($p= 0.033$) and control ($p= 0.009$) groups. No significant differences were recorded between the antibiotic and control treatment groups. Higher RDW levels may be due to older age of RBC, mixed deficiency (iron, B12 or folate), recent haemorrhage and false positive results from EDTA anticoagulated blood (Evans and Jehle, 1991; Bowen and Remaley, 2014). Results suggest that probiotic-treated broilers may in some way allow for RBC to age further before being recycled, as RDW increases with cell age. The relatively small RDW changes supports this interpretation, as the differences would have been larger in the event of deficiency or haemorrhage. Broilers receiving different treatments on day 29 had no significant differences in their RBC, HCT, MCV, MCH, and MCHC and RDW counts.

Bursa of Fabricius and the spleen are lymphoid organs which forms part of the avian immune system (Yegani and Korver, 2008). The spleen filters and regenerates antibodies, whereas the bursa of Fabricius is the site of haematopoiesis responsible for B-cell production. Immune organ weights are weighed to evaluate the immune status of broilers (Chen *et al*., 2013). The bursa of Fabricius is the primary lymphoid and probiotic administration can lead to an increase in weight (Willis *et al*., 2007), which may be considered an improvement of the immune system (Nourmohammadi *et al*., 2011),

however, excessive responses depress growth performance (Collett, 2005). Administration of either the multi-species probiotic or antibiotics did not alter the relative weights of the spleen, bursa of Fabricius and the spleen: bursa of Fabricius ratio on days 19 and 29 (Table 5). Conflicting results were reported for the probiotic Protexin® (*Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Bifidobacterium bifidum*, *Streptococcus thermophilus*, *Enterococcus faecium*, *Aspergillus oryzae* and *Candida pintolopesii*) (Dizaji *et al*., 2012; Pourakbari *et al*., 2016). Pourakbari *et al*. 2016 observed no differences in spleen or bursa weights of broilers raised in cages, but Dizaji *et al*. 2012 reported an increase in spleen weights when broilers were raised on the floor. Concluded from these studies, differences in rearing conditions, i.e. housing, feed composition and environmental factors, probably played an important role for the observed discrepancies. In our study the multi-species probiotic (*L*. *crispatus* DPN167, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94 and *B*. *amyloliquefaciens* DPN123) was administered to broilers reared in cages, which could be the reason why no differences in immune organ weights were observed. It may thus be more applicable to study the effect of the multi-species probiotics on broilers raised under less hygienic conditions, as cage rearing is considered more hygienic than pen rearing (Reece *et al*., 1971).

Table 5: Relative lymphoid organ weight (± standard deviation) and ratio obtained from broilers (n= 20) slaughtered on days 19 and 29.

	Spleen %		**Bursa %**		**Spleen/Bursa ratio**	
Treatment	19 D	29 D	19 D	29 D	19 D	29 D
Control	0.068 ± 0.018	0.08 ± 0.01	0.247 ± 0.068	0.18 ± 0.03	0.276 ± 0.053	0.49 ± 0.11
Antibiotic	0.069 ± 0.016	0.09 ± 0.02	0.267 ± 0.063	0.16 ± 0.05	0.260 ± 0.046	0.61 ± 0.31
Probiotic	0.062 ± 0.017	0.09 ± 0.02	0.276 ± 0.064	0.17 ± 0.04	0.268 ± 0.058	0.63 ± 0.24
p value	0.694	0.559	0.675	0.246	0.853	0.166

Relative gizzard to body weight ratio is used to assess the efficiency of mechanical feed digestion. Supplementation of feed with either the multi-species probiotic or antibiotics had no significant effect on the relative gizzard weights on days 19 and 29 (Table 6). Researchers using a multi-species probiotic which consisted of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Pediococcus acidilactici*, *Bacillus subtilis* and *Saccharomyces boulardii* (Saiyed *et al*., 2015) and a single strain probiotic *Eubacterium* sp. (Awad *et al*., 2006) similarly reported no significant differences in the relative gizzard weights.

Table 6: Mean gizzard weight relative to body weight (± standard deviation) obtained from broilers (n= 20) slaughtered on day 29.

Treatment	Body weight (g)	Gizzard weight (g)	Gizzard: Body weight %
Control	1869 (± 149.60)	31.97 (± 4.48)	1.61 (± 0.21)
Antibiotic	1893 (± 158.31)	29.52 (± 2.87)	1.66 (± 0.21)
Probiotic	1873 (± 158.52)	29.39 (± 3.75)	1.58 (± 0.17)
p-value	0.882	0.067	0.424

The surface of the small intestine contains villi that increases the surface area and leads to increased absorption. At the base of the villi, tubular invaginations (crypts) extend into connective tissue to form enterocytes (absorptive cells). A decrease in villi height leads to a reduction in surface area and reduces the absorption of nutrients (Pluske *et al*., 1996). The ratio between villi height and crypt depth is used as an indicator of digestive capacity. A low ratio correlates to decreased digestion and absorption (Montagne *et al*., 2003). Deeper crypt depths correlate with increased absorption (Hedemann *et al*., 2003). However, shorter villi and deeper crypts may decrease absorption and increase endogenous losses through loss of enterocytes, thus leading to a decrease in absorption (Xu *et al*., 2003). The villus height, crypt depth and villus to crypt depth ratios of broilers from the different treatment groups were not significantly different on day 19 (Table 7). However, the mean villi area on day 19 for the different treatments were significantly different ($p = 0.042$). Broilers from the untreated group had larger villi area compared to the antibiotic ($p= 0.029$) and probiotic ($p= 0.026$) treatment groups. No significant differences were recorded between the probiotic and antibiotic treatment groups. Larger villi areas lead to larger surface areas and increased absorption of nutrients (Matur and Eraslan, 2012). However, increased villi area could also be considered detrimental for nutrient absorption. If villi height is the same for two treatment groups (as in the current study), but the area per villus is larger in the one treatment group, the number of villi per cm^2 are less and so also the total surface area for nutrient absorption. Numerous anatomical characteristics affect the absorptive capacity, i.e. tract length, villus height and width, and the number of villi per unit area all contribute to absorptive capacity (Miles *et al*., 2006). The chicks in our study were day old as-hatched and from the same genetic lineage, representing a homogenous collection of broilers with similar anatomical characteristics. Taken together, this suggests that both the antibiotic and multi-species probiotic treatment groups had better absorption capacity when compared to the control group at this time point. As observed for haematological parameters, this difference was also transient, as the villus height and area, crypt depth and villus to crypt depth ratios were similar for the three groups on day 29 (Table 7). The effects of probiotics on villus surface area seems to depend on the segment in which the bacteria colonise (Matur and Eraslan, 2012). For example, researchers assessing *B. subtilis* LS 1-254 and GalliPro® - which consist of *B. subtilis* DSM 1729955 - found an increase in the villus height, surface area and villus height-to-crypt depth ratio. This highlights the requirement for probiotic-specific assessments, as well as comprehensive analyses of various segments of the GIT before firm conclusions can be made.

Table 7: Mean (± standard deviation) villi height, villi area, crypt depth and villus height: crypt depth ratio of the duodenum of broilers (n= 20) slaughtered on day 29.

	Villi Height (µm)		Villi Area (µm)		Crypt depth (µm)		Villi height: crypt depth	
Treatment	19 D	29 D	19 D	29 D	19 D	29 D	19 D	29 D
Control	275 ± 21	1522 ± 90	8332[a] ± 1448	215439 ± 55696	45 ± 10	240 ± 23	6.30 ± 1.20	6.40 ± 0.69
Antibiotic	275 ± 39	1496 ± 153	6810[b] ± 1333	214644 ± 49877	42 ± 10	232 ± 27	6.87 ± 1.78	6.53 ± 0.95
Probiotic	260 ± 20	1515 ± 170	6573[b] ± 1073	216848 ± 48577	40 ± 8	219 ± 21	7.32 ± 1.75	6.99 ± 0.98
p value	0.556	0.949	**0.042***	0.952	0.298	0.213	0.659	0.449

* $p < 0.05$, [a, b] Means within columns with different superscripts differ significantly ($p < 0.05$)

Mineralisation of the tibia

The degree of bone mineralisation affects bone strength, phosphorus and/or calcium deficiencies and lead to an increase in bone breakage and defects (Brenes *et al*., 2003). This influences animal welfare, growth performance and meat quality (Driver *et al*., 2006). Tibia bone weight and ash weight is used to evaluate bone mineralisation (Onyango *et al*., 2003). Probiotics support calcium absorption primarily by the production of metabolites, enzymes and vitamins, some of which participate in the metabolism of calcium (Scholz-Ahrens *et al*., 2007). Broilers from the different treatment groups showed no significant differences in their tibia bone weights, or bone ash percentages on day 29 (Table 8) and administration of either the multi-species probiotic or antibiotic combination did not alter bone mineralisation efficiency.

Table 8: Mean (± standard deviation) fat free dry bone, ash and the percentage bone ash of tibia obtained from broilers (n= 20) slaughtered at 29 days.

Treatment	Fat free dry bone weight (g)	Fat free bone ash weight (g)	Fat free bone ash percentage (%)
Control	3.57 ± 0.40	1.79 ± 0.19	50.27 ± 2.73
Antibiotic	3.52 ± 0.40	1.78 ± 0.19	50.62 ± 3.09
Probiotic	3.41 ± 0.37	1.74 ± 0.21	50.86 ± 1.89
p value	0.496	0.721	0.799

Inhibition of *L. monocytogenes in vivo*

Bioluminescent *L. monocytogenes* was administered to broilers to determine whether the antibiotic and probiotic feed additives could inhibit colonisation and proliferation of the pathogenic bacterium *in vivo*. Transition of bioluminescent *L. monocytogenes* EGDe in the gastrointestinal tract of broilers from the different treatment groups, after 2.0 and 3.5 h, is shown in Fig. 1. Lower levels of bioluminescence were observed in the GIT of broilers from the probiotic treatment group after 3.5 h, compared to broilers

from the control and antibiotic groups. High levels of bioluminescence were observed in the ileum and colon, and low levels in the duodenum and cecum (Fig. 1). Broilers from the control group showed high bioluminescent readings (mean $p.S^{-1}.cm^{-1}.sr^{-1}$) in the ileum (3.17×10^4) and low readings in the duodenum (3.46×10^3), cecum (9.48×10^3) and colon (9.71×10^3) after 2 h (Fig. 2). After 3.5 h, high readings were observed in the ileum (5.5×10^4) and low readings in the duodenum (3.19×10^3), jejunum (3.2×10^3) and cecum (3.52×10^3). Broilers from the antibiotic treatment group had high bioluminescence readings in the colon (1.69×10^4), and low levels in the duodenum (3.28×10^3), jejunum (2.07×10^3), ileum (9.45×10^3) and cecum (3.82×10^3) after 2 h (Fig. 2). After 3.5 h, high bioluminescence readings were observed in the ileum (1.01×10^5) and colon (4.69×10^4), and low readings in the duodenum (2.21×10^3), jejunum (3.21×10^3) and cecum (6.93×10^3). For probiotic-treated broilers, high readings were observed in the ileum (3.13×10^4) and colon (3.43×10^4), and low readings in the duodenum (2.32×10^3) and cecum (2.84×10^3) at 2 h. After 3.5 h, low levels were observed in the duodenum (2.79×10^3), cecum (5.14×10^3) and colon (3.62×10^3). Decrease in bioluminescence observed in the probiotic treatment group after 3.5 h suggests that the multi-species probiotic inhibits growth of *L*. *monocytogenes in vivo*. Bioluminescent readings in the ileum after 3.5 h were significantly different for treatment groups ($p = 0.0001$). Readings recorded for the probiotic treatment group were significantly lower compared to the antibiotic ($p = 0.0002$) and control ($p = 0.0201$) groups, but the control and antibiotic treatment groups were similar. The cell numbers of *L*. *monocytogenes* per gram intestine for the duodenum, jejunum, ileum, cecum and colon, 2 and 3.5 h after administration of *L*. *monocytogenes*, is shown in Fig. 3. The ileum harboured the highest number of *L*. *monocytogenes* (5-7 log CFU/ gram ileum; Fig. 3). No significant differences for the log CFU/g intestine were observed for the different GIT sections from the different treatment groups (Fig. 3). The multi-species probiotic inhibited colonisation and growth of *L*. *monocytogenes in vivo*, as determined by the Caliper *in vivo* imaging system (IVIS®). However, no cell death of *L*. *monocytogenes* was recorded in the GIT, as determined by standard culturing and plating onto BHI agar (Biolab, Biolab Diagnostics, Midrand, SA) supplemented with 7.5 µg/ml chloramphenicol. Growth inhibition could be due to the production of organic acids, diacetyl, acetoin, hydrogen peroxide and bacteriocins, or through competitive exclusion from the GIT (Höltzel *et al*., 2000; Magnusson and Schnürer, 2001).

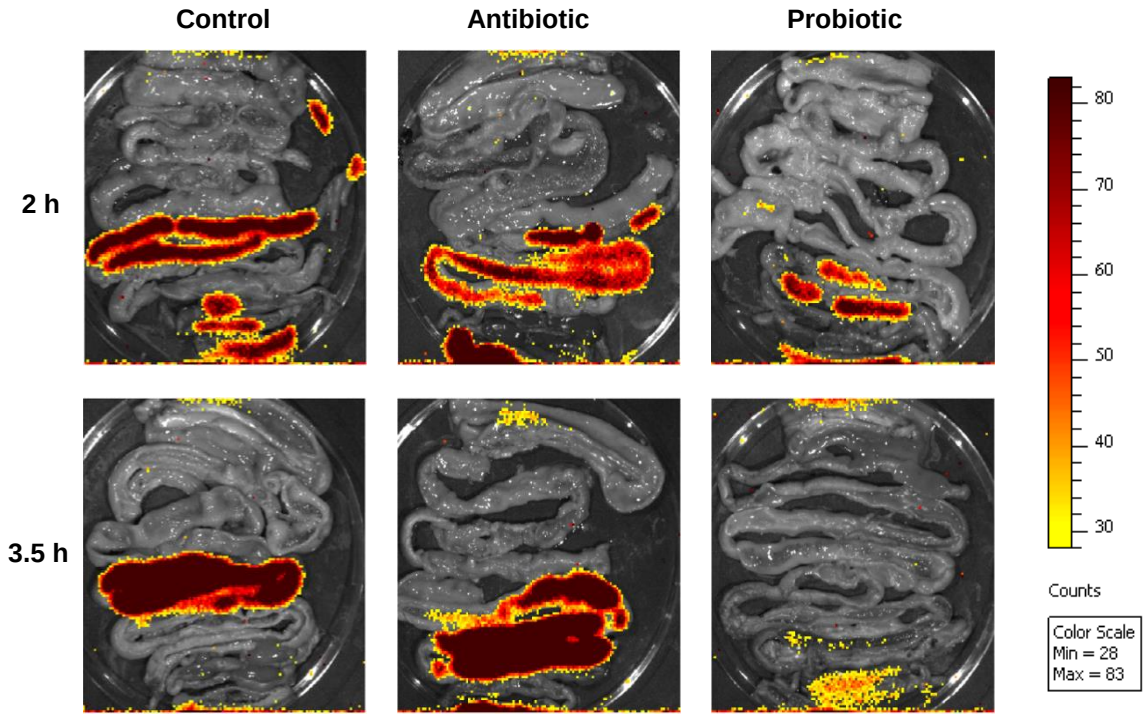

Figure 1: Bioluminescent images of isolated gastrointestinal tracts of broilers from the different treatments groups (i.e. multi-species probiotic, antibiotic combination and control) at 2 and 3.5 h after administration of bioluminescent *L*. *monocytogenes* EGDe (4.2×10^8 CFU).

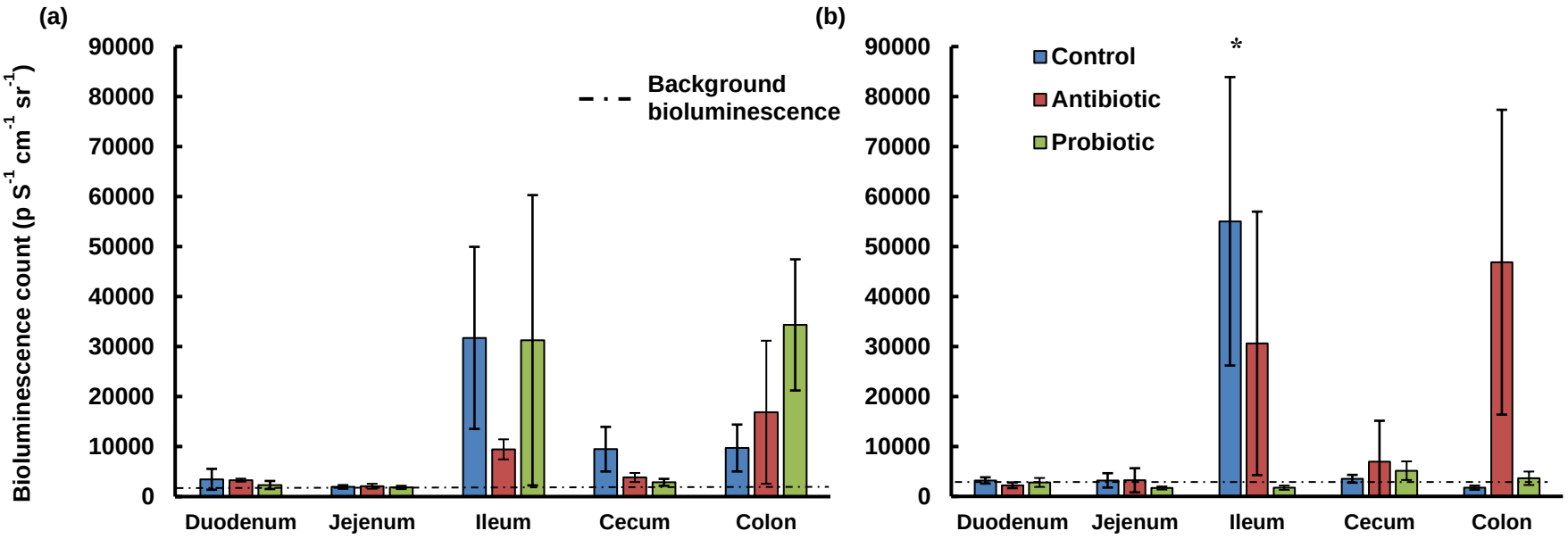

Figure 2: Bioluminescence counts (p S^{-1} cm^{-1} sr^{-1}) for the different gastrointestinal compartments (duodenum, jejunum, ileum, cecum and colon) of broilers from the different treatment groups (multi-species probiotic, antibiotic combination and control) at (a) 2 and (b) 3.5 h after administration of *L*. *monocytogenes* EGDe. * Indicates significant differences ($p < 0.05$; Kruskal-Wallis nonparametric test). Error bars indicate standard deviations (n= 12).

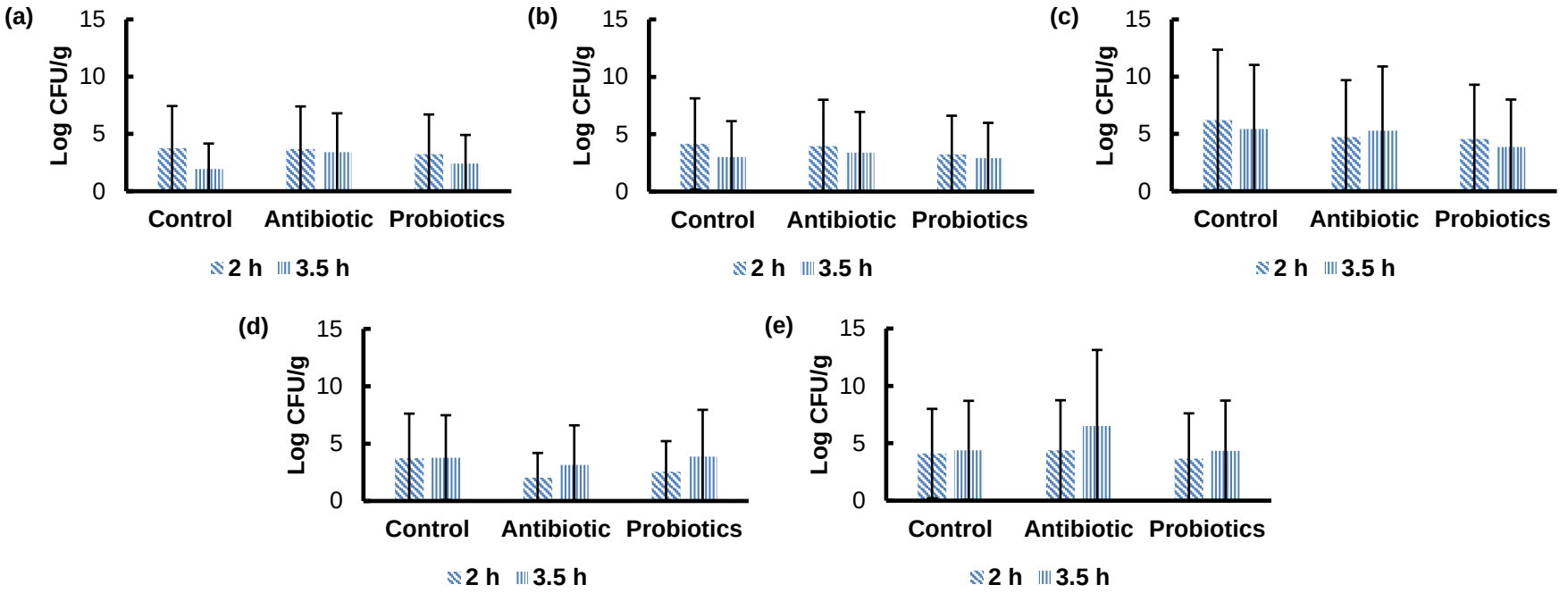

Figure 3: Cell numbers (log CFU/ g intestine) of *L. monocytogenes* EGDe recorded in the (a) duodenum, (b) jejunum, (c) ileum, (d) ceca and (e) colon at 2 and 3.5 h after administration of 4.2×10^8 CFU of *L. monocytogenes* EGDe. The $\log_{10}$ averages of the CFU/g intestine were plotted (± standard deviation). Error bars indicate standard deviations (n= 12).

Cecum microbiome

The cecum microbiome grouped into 13 operational taxonomic units (OTU's), representing the phyla Actinobacteria, Armatimonadetes, Acidobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Fusobacteria, Geminatimonadetes, Proteobacteria, Synergistetes, Spirochaetes, and Tenericutes (Fig. 4). Only two phyla were present at a mean relative abundance of $\geq$ 1% and belonged to Proteobacteria (33-72 %) and Firmicutes (26-66 %). The majority of the Proteobacteria sequences corresponded to sequences recorded for *Enterobacteriaceae* (19-64 %) and *Hyphomicrobiaceae* (2-7 %). The majority of the Firmicutes sequences correlated with sequences of *Enterococcaceae* (2-6 %), *Lactobacillaceae* (4-11 %), *Clostridiaceae* (6-13 %), *Eubacteriaceae* (1-5 %), *Lachnospiraceae* (6-9 %), *Ruminococcaceae* (9-21 %) and *Erysipelotrichaceae* (2-8 %), as shown in Fig. 5.

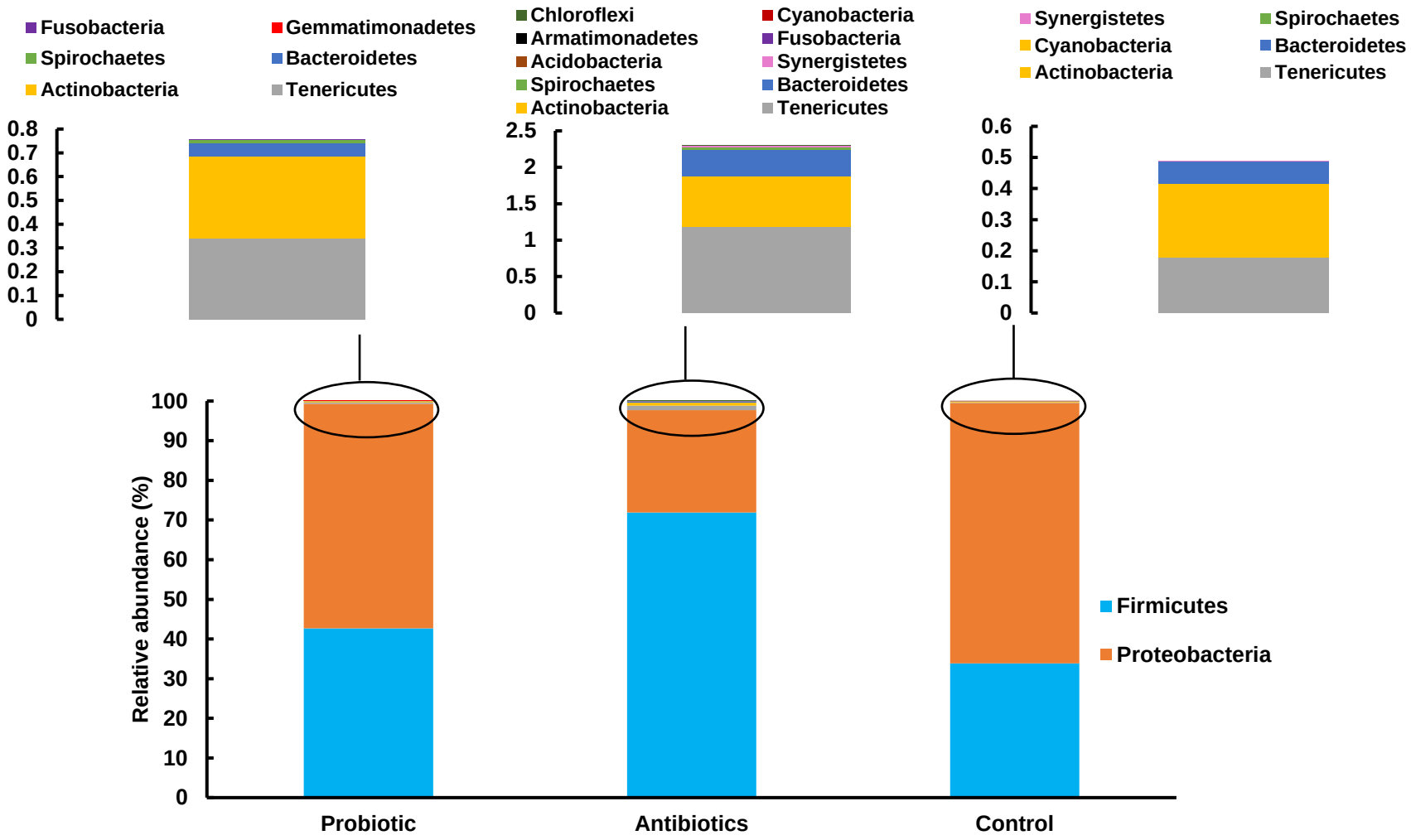

Figure 4: Phyla present in the cecum microbiome of broilers from the different treatment groups i.e. multi-species probiotic, antibiotic combination and untreated.

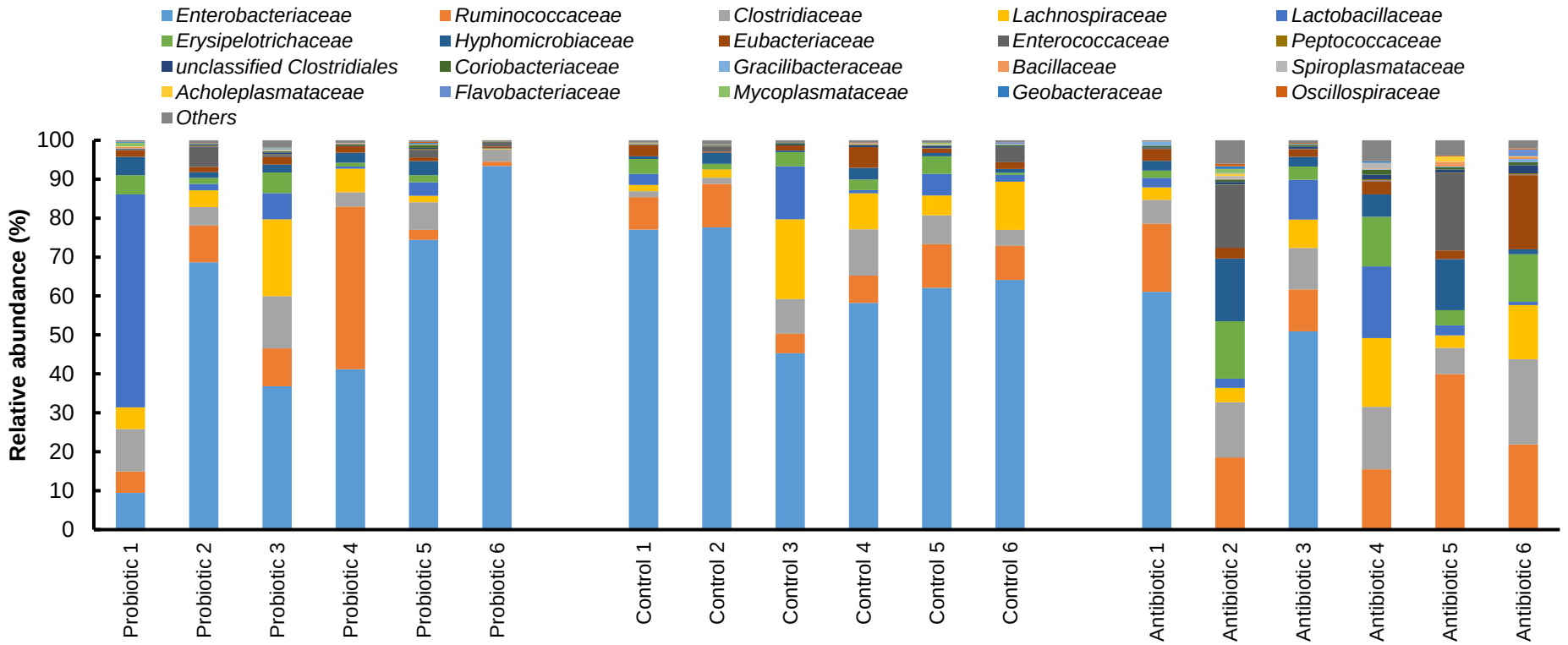

Figure 5: Abundant bacterial families present in the cecum microbiota of broilers from the different treatment groups.

Chao1 and richness indexes for broilers from the different treatment groups did not differ significantly (Fig. 6). However, the Shannon diversity index (p= 0.019) and evenness index (p= 0.021) differed significantly between the treatment groups. Microbiomes from the antibiotic treatment group were more diverse and OTU's were, compared to the control and probiotic treatment groups, more evenly distributed. Analysis by mcpHill (Pallmann *et al*., 2012) revealed that the microbiomes of the control and probiotic treatment groups were similar with respect to rare, average and high abundant species diversity (q = -1, 1, 3; p > 0.05), as shown in Table 9. The antibiotic and control treatment groups differed significantly with respect to average and high abundant species diversity (q = 1, 3; p = 0.028 and p = 0.041 respectively) but did not differ in rare species diversity (q= -1; p > 0.05). The antibiotic and probiotic treatment groups did not differ significantly with respect to rare and high abundant species diversity (q = -1, 3; p > 0.05), but differed significantly with respect to average abundant species diversity (q = 1; p = 0.041). The NMDS plot revealed that microbiomes from the antibiotic treated group formed a cluster separate from the control and probiotic treatment groups (Fig. 7). Adonis analysis revealed significant differences between community composition and treatment (p= 0.029).

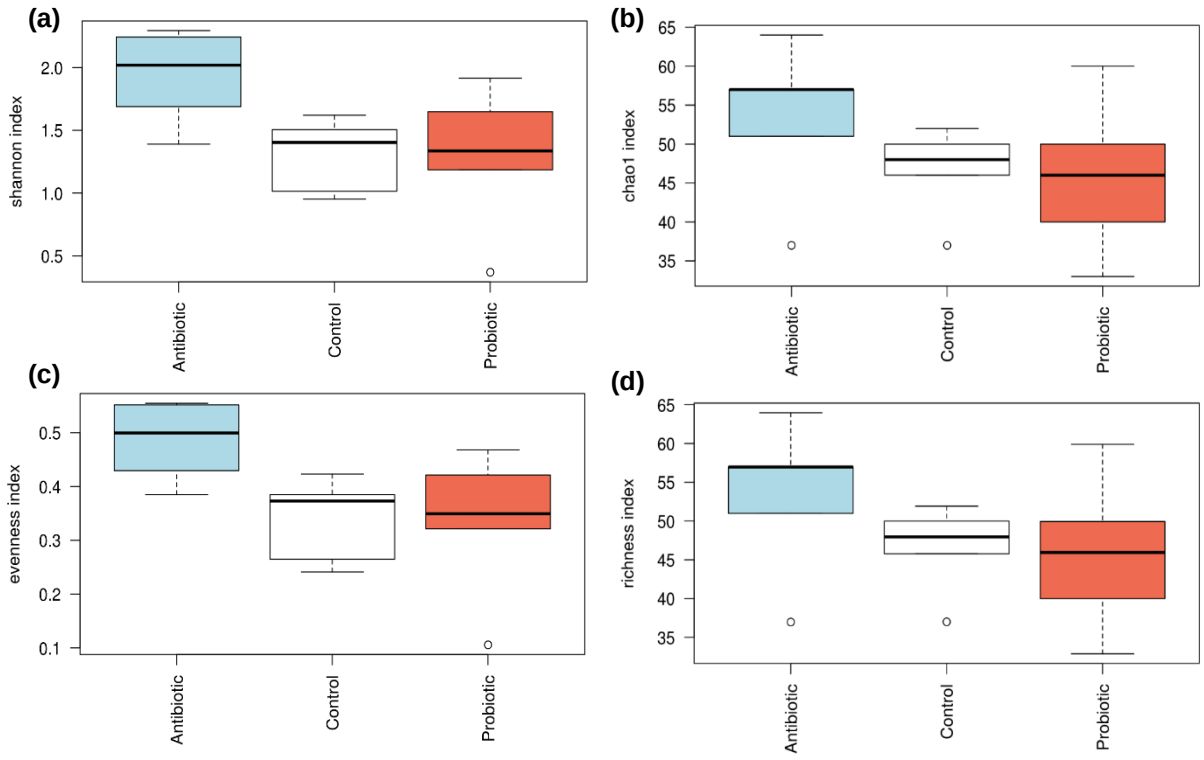

Figure 6: Total species richness obtained by the (a) Shannon's diversity index (ANOVA significance of p = 0.02), (b) Chao 1 index (ANOVA significance of p = 0.22), (c) evenness index (ANOVA significance of p= 0.021) and (d) richness index (ANOVA significance of p = 0.216) for cecal bacterial communities of broilers from the different treatment group (i.e. multi-species probiotic, antibiotic combination and untreated). Error bars indicate standard deviations (n= 6).

Table 9: mcpHill diversity analysis of group differences in biodiversity.

Comparison	q (Hill number)	p-value
Control - Antibiotic	-1	0.969
Control - Antibiotic	0	0.639
Control - Antibiotic	1	**0.028***
Control - Antibiotic	2	**0.035***
Control - Antibiotic	3	**0.041***
Probiotic - Antibiotic	-1	0.961
Probiotic - Antibiotic	0	0.521
Probiotic - Antibiotic	1	**0.041***
Probiotic - Antibiotic	2	0.055
Probiotic - Antibiotic	3	0.065
Probiotic - Control	-1	1
Probiotic - Control	0	1
Probiotic - Control	1	1
Probiotic - Control	2	1
Probiotic - Control	3	1

* $p < 0.05$

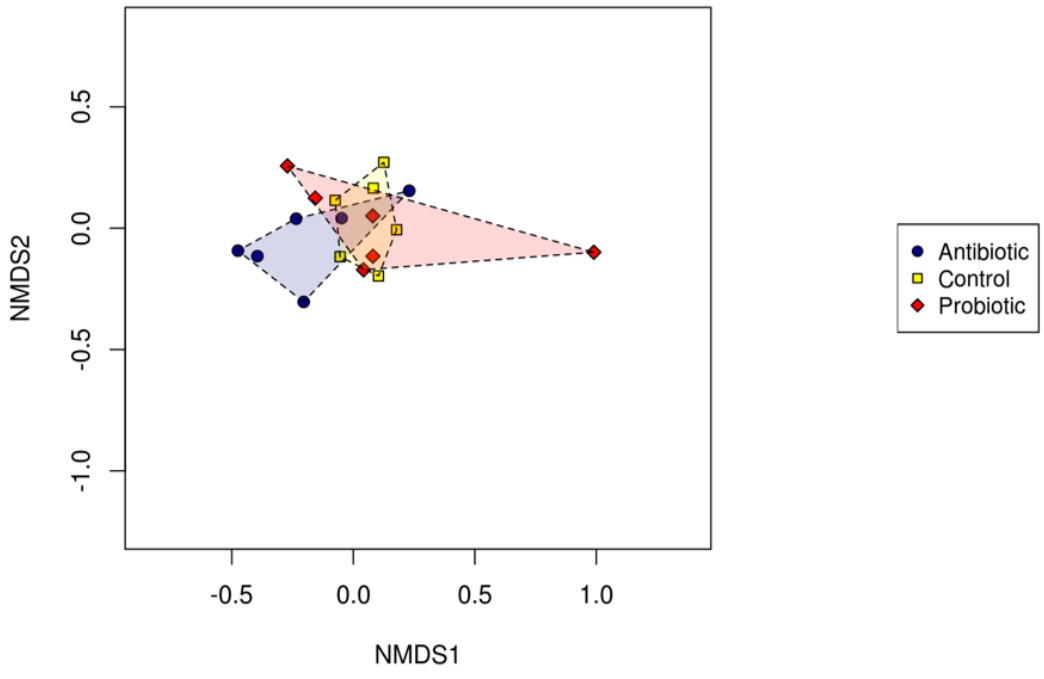

Figure 7: Non-metric multidimensional scaling (NMDS) ordination plot of bacterial communities of the different treatment groups (i.e. multi-species probiotic, antibiotic combination and control) based on the Bray-Curtis distance.

Families present in more than 50 % of broilers from a specific treatment were considered part of the microbiome. Microbiomes of broilers from the different treatment groups shared 26 families, i.e.

Geobacteraceae, *Acholeplasmataceae*, unclassified Clostridiales, *Bacillaceae*, *Clostridiaceae*, Clostridiales Family XI, XIII and XIX Incertae Sedis, *Spiroplasmataceae*, *Ruminococcaceae*, *Planococcaceae*, *Peptostreptococcaceae*, *Peptococcaceae*, *Paenibacillaceae*, *Oscillospiraceae*, *Coriobacteriaceae*, *Mycoplasmataceae*, *Enterobacteriaceae*, *Lactobacillaceae*, *Lachnospiraceae*, *Hyphomicrobiaceae*, *Gracilibacteraceae*, *Veillonellaceae*, *Enterococcaceae*, *Eubacteriaceae* and *Erysipelotrichaceae* (Fig. 8).

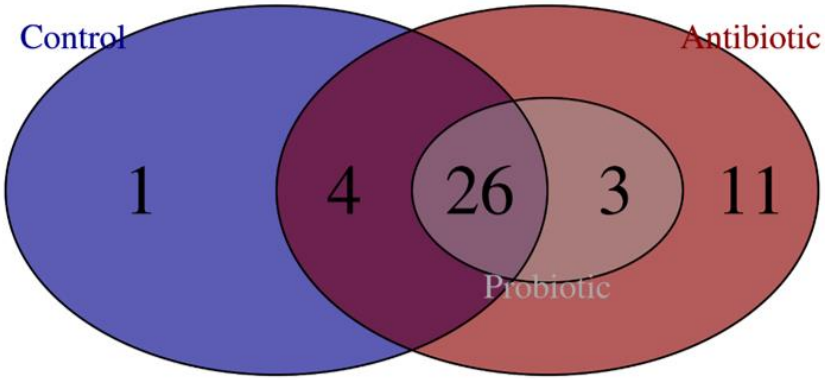

Figure 8: Venn diagram of the core shared bacterial families and unique families present in cecal microbiome of broilers from the different treatment groups (i.e. multi-species probiotic, antibiotic combination and untreated).

Microbiomes from the antibiotic and control treatment groups had four families in common, i.e. *Streptococcaceae* (0.03-0.05 %), *Aerococcaceae* (0.007-0.01 %), *Anaeroplasmataceae* (0.008-0.03 %) and *Xanthomonadaceae* (0.005-0.008 %). Genera of *Streptococcaceae* are found in environmental habitats and mammalian hosts, and consists of genera *Streptococcus*, *Lactococcus*, and *Lactovum* (Lory, 2014). The family *Aerococcaceae* consists of the genera *Aerococcus*, *Abiotrophia*, *Dolosicoccus*, *Eremococcus*, *Facklamia*, *Globicatella*, and *Ignavigranum* (Stackebrandt, 2014). Members of this family are present in environmental and clinical habitats (Stackebrandt, 2014). The family *Anaeroplasmataceae* comprises of anaerobic mycoplasmas *Anaeroplasma* and *Asteroleplasma*, commensals of the rumen, with no reported pathogenicity (Brown *et al*., 2015). The family *Xanthomonadaceae* consists of the genera *Xanthomonas*, *Frateuria*, *Fulvimonas*, *Luteimonas*, *Lysobacter*, *Nevskia*, *Pseudoxanthomonas*, *Rhodanobacter*, *Schineria*, *Stenotrophomonas*, *Thermomonas*, and *Xylella* (Saddler and Bradbury, 2015). Members are typically characterised as environmental microorganisms, apart from *Stenotrophomonas* which is infrequently implicated in infections (Saddler and Bradbury, 2015).

The microbiomes of the antibiotic and probiotic treatment groups shared three families, i.e. *Entomoplasmataceae* (0.05-0.26 %), *Syntrophomonadaceae* (0.02-0.2 %) and *Oceanospirillaceae* (0.005-0.01 %). The family *Entomoplasmataceae* comprises the genera *Entomoplasma* and *Mesoplasma* (Gasparich, 2014). Members of *Entomoplasmataceae* have no pathogenicity to their insect or plant host (Gasparich, 2014). The family *Syntrophomonadaceae* includes the genera *Candidatus Contubernalis*, *Carboxydocella*, *Dethiobacter*, *Pelospora*, *Syntrophomonas*,

Syntrophothermus, *Thermohydrogenium* and *Thermosyntropha* (Sobieraj and Boone, 2006). Members are present in anaerobic environments where organic matter is degraded to methane and carbon dioxide (Sobieraj and Boone, 2006). The family *Oceanospirillaceae* consists of 17 genera, all halotolerant/halophilic, except for *Balneatrix* which has been isolated from freshwater and clinical samples (Satomi and Fujii, 2014).

The antibiotic treatment group had 11 unique families, i.e. *Pseudomonadaceae* (0.008 %), *Staphylococcaceae* (0.008 %), *Flavobacteriaceae* (0.3 %), Clostridiales Family XIV. Incertae Sedis (0.008 %), *Brachyspiraceae* (0.04 %), *Demequinaceae* (0.007 %), *Desulfuromonadaceae* (0.01 %), *Alicyclobacillaceae* (0.06 %), *Microbacteriaceae* (0.01 %), *Synergistaceae* (0.015 %) and *Brucellaceae* (0.008 %). The family *Pseudomonadaceae* consists of the genera *Azomonas*, *Azomonotrichon*, *Azorhizophilus*, *Azotobacter*, *Cellvibrio*, *Mesophilobacter*, *Pseudomonas*, *Rhizobacter*, *Rugamonas*, and *Serpens* (Garrity *et al*., 2015). Infection by *P*. *aeruginosa* in broilers is associated with respiratory infections, diarrhoea and septicaemia (Shukla and Mishra, 2015). The family *Staphylococcaceae* consists of genera *Jeotgalicoccus*, *Macrococcus*, *Nosocomiicoccus*, *Salinicoccus*, *Gemella* and *Staphylococcus* (Lory, 2014). *Staphylococcus* members are commensal microorganisms, occasionally causing mastitis in cattle. Major infections associated with genus are due to *S*. *aureus* infections in humans (Gordon and Lowy, 2008). The family *Flavobacteriaceae* contains more than 90 genera present in a wide variety of habits i.e. water, soil, animals and plants (McBride, 2014). Many members of the family are capable of digesting macromolecules and polysaccharides (McBride, 2014). Majority of clostridia present in the GIT of broilers belongs to the family Clostridiales Family XIV Incertae Sedis, with positive traits such as production of butyric acid that promotes a healthy intestinal epithelium (Apajalahti and Kettunen, 2006). *Brachyspiraceae* has been elevated to the order Brachyspiriales ord. nov. (Gupta *et al*., 2013). The family consists of the genera *Brachyspira*, *Exilispira* and *Brevinema* (Rosenberg, 2014a). Broilers harbour pathogenic *B*. *hyodysenteriae*, *B*. *intermedia*, *B*. *pilosicoli* and *B*. *alvinipulli* and non-pathogenic species *B*. *innocence*, *B*. *murdochii*, and *B*. *pulli* (Feberwee *et al*., 2008; Medhanie and Mcewen, 2013). *Brachyspira* colonises the large intestine and causes intestinal disease and mortality (Ue *et al*., 2011). The precise significance of *Brachyspira* spp. in birds, species involved, and the epidemiology is not fully understood (Rosenberg, 2014a). The family *Demequinaceae* consists of the genus *Demequina* and is present in soil and marine environments (Ue *et al*., 2011). The family *Desulfuromonadaceae* contains the genera *Desulfuromonas*, *Desulfuromusa*, *Pelobacter*, *Malonomonas*, and *Geoalkalibacter* (Greene, 2014). Members are strictly anaerobic and are found in anoxic environments where they play an important role in the degradation of organic matter and syntrophic associations (Greene, 2014). None of the members are considered pathogenic (Greene, 2014). The *Alicyclobacillaceae* family consists of the genera *Alicyclobacillus*, *Kyrpidia*, and *Tumebacillus* (Stackebrandt, 2014). The family *Microbacteriaceae* consists of numerous genera present in several different environments, i.e. terrestrial and aquatic ecosystems, associations with plants, fungi, animals and clinical specimens (Glöckner *et al*., 2000; Evtushenko and Takeuchi, 2006). Several species and subspecies of the family include either plant pathogens, or organisms for which plant pathogenicity has been suggested (Evtushenko and Takeuchi, 2006). The majority of OTU's were classified to family level, however, members of the genera *Microbacterium* and *Leucobacter* were

present. Members of the genus *Microbacterium* are widely distributed in various environments and are associated with plants, insects and clinical specimens (Evtushenko and Takeuchi, 2006). However, little is known about the natural habitats of members of the genus *Leucobacter* (Evtushenko and Takeuchi, 2006).

The microbiomes of the control treatment group contained one unique family, *Chitinophagaceae* (0.01 %). The family *Chitinophagaceae* consists of the genera *Balneola*, *Filimonas*, *Flavisolibacter*, *Gracilimonas*, *Lacibacter*, *Niastella*, *Terrimonas*, *Asinibacterium* and *Chitinophaga* (Rosenberg, 2014b). Members of this family are found in a range of environments, with some species capable of cellulose hydrolysis (Rosenberg, 2014b).

The following families were significantly different (Fig. 9) for the different treatment groups: unclassified Clostridiales ($p= 0.011$), *Coriobacteriaceae* ($p= 0.012$), *Synergistaceae* ($p= 0.013$), *Enterobacteriaceae* ($p= 0.018$), *Erysipelotrichaceae* ($p= 0.026$) and *Brucellaceae* ($p= 0.033$). The antibiotic treatment group had higher levels of unclassified Clostridiales (3.4-fold increase), *Coriobacteriaceae* (2.9-fold increase), *Synergistaceae* (unique family of antibiotic group), *Erysipelotrichaceae* (3.3-fold increase), and *Brucellaceae* (unique family of antibiotic group) and were significantly different from the probiotic ($p < 0.05$) and control groups ($p < 0.05$). The families from probiotic and control treatment groups did not differ significantly. The antibiotic group had lower levels of *Enterobacteriaceae* (3.5-fold decrease) and were significantly different ($p < 0.05$) than the control group. No significant differences were recorded between the antibiotic and probiotic treatment groups, and between the control and probiotic treatment group. Reduction in the levels of *Enterobacteriaceae* is due to the presence of sulphadiazine, trimethoprim and colistin. Sulphadiazine is bacteriostatic with a wide spectrum against Gram-positive and Gram-negative bacteria (Carr *et al*., 1973). Trimethoprim is active against aerobic Gram-positive bacteria (*Staphylococcus*) and aerobic Gram-negative bacteria (*Enterobacter*, *Escherichia*, *Klebsiella* and *Proteus*) (Gleckman *et al*., 1981). Colistin has bactericidal activity against most Gram-negative aerobic bacilli, i.e. *Acinetobacter*, *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Escherichia*, *Salmonella*, *Shigella* and *Citrobacter* spp. (Falagas *et al*., 2005).

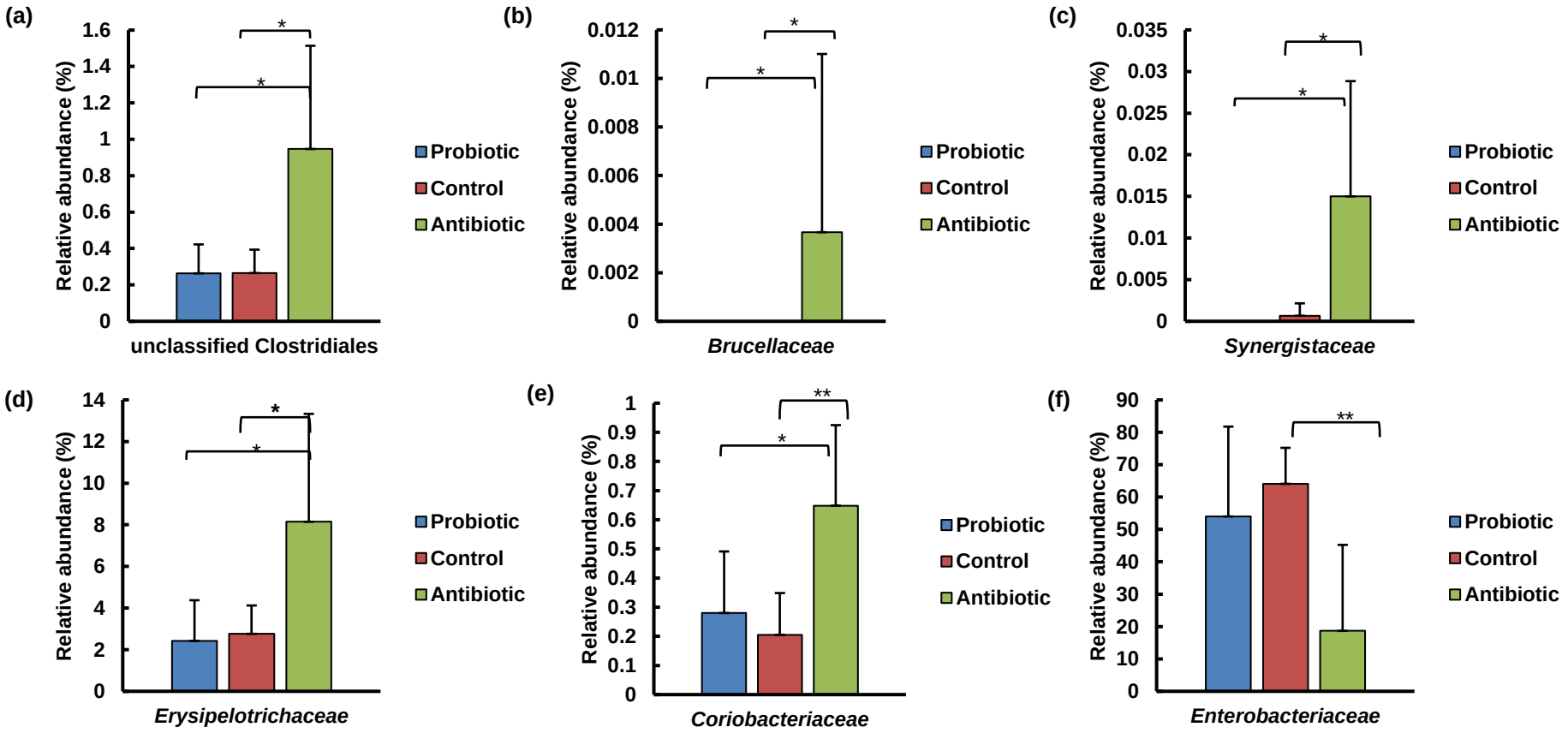

Figure 9: Cecal bacterial families, i.e. (a) unclassified Clostridiales, (b) *Brucellaceae*, (c) *Synergistaceae*, (d) *Erysipelotrichaceae*, (e) *Coriobacteriaceae* and (f) *Enterobacteriaceae* whose abundance significantly differs between the different treatments groups' i.e. multi-species probiotic, antibiotic combination and untreated (ANOVA significance, * indicates $p < 0.05$ and ** $p < 0.001$). Error bars indicate standard deviations (n= 6).

The family *Erysipelotrichaceae* comprises the genera *Allobaculum*, *Bulleidia*, *Catenibacterium*, *Coprobacillus*, *Eggerthia*, *Erysipelothrix*, *Holdemania*, *Kandleria*, *Solobacterium* and *Turicibacter* (Verbarg *et al*., 2014). Members are highly immunogenic and flourish during post-treatment with broad-spectrum antibiotics (Palm *et al*., 2014; Dinh *et al*., 2015). *Erysipelotrichaceae* has been correlated to inflammation (Dinh *et al*., 2015). Evidence associating members of this family to disease is correlative, and studies examining the impact abundance has on the host is required (Kaakoush, 2015). The family *Coriobacteriaceae* consists of genera *Adlercreutzia*, *Asaccharobacter*, *Atopobium*, *Collinsella*, *Coriobacterium*, *Cryptobacterium*, *Denitrobacterium*, *Eggerthella*, *Enterorhabdus*, *Gordonibacter*, *Olsenella*, *Paraeggerthella*, *Parvibacter*, and *Slackia* (Clavel *et al*., 2014). They are normal habitants of the mammalian GIT. Members can modulate host metabolism by increased cholesterol absorption (Martínez *et al*., 2009), energy metabolism via glycogenesis and enhanced triglycerides synthesis as well as hepatic detoxification pathways (Claus *et al*., 2011), and activation of the isoflavone daidzein a dietary phytoestrogen abundant in soybean (Clavel and Mapesa, 2014). However, several members of *Atopobium*, *Eggerthella*, *Gordonibacter*, *Olsenella*, and *Paraeggerthella* have been implicated in the development of infections, abscesses, intestinal diseases, tumours, periodontitis, vaginosis, and bacteraemia (Thota *et al*., 2011). The majority of OTU's were identified to family level. However, the genera *Eggerthella*, *Enterorhabdus* and *Gordonibacter* were identified. A decrease in *Coriobacteriaceae* numbers has been correlated to reduced plasma interleukin-6 concentrations and chronic inflammation (Martínez *et al*., 2013). The lymphocyte and basophil concentrations for broilers from the antibiotic treatment group were higher on day 19. This could be due to the increase in abundance of *Coriobacteriaceae*. However, knowledge on how and when members of *Coriobacteriaceae* start to become detrimental to the hosts is unknown (Clavel and Lepage, 2014).

The family *Brucellaceae* (0.008 %) was only found in broilers from the antibiotic treatment group. The family consists of the genera *Brucella*, *Crabtreella*, *Daeguia*, *Mycoplana*, *Ochrobactrum*, *Paenochrobactrum*, and *Pseudochrobactrum* (Kämpfer *et al*., 2014). The majority OTU's were identified to family level. However, species from the genus *Ochrobactrum* were present. Several *Ochrobactrum* spp. are opportunistic microorganisms and cycle from soil-rhizoplane to immunocompromised individuals (Kämpfer *et al*., 2014). The family *Enterobacteriaceae* consists of 51 genera which includes commensal and pathogenic microorganisms (Janda, 2006). The majority of sequences could only be classified to family level. However, low levels (0.01-0.4 %) of the following genera were present: *Citrobacter*, *Cronobacter*, *Enterobacter*, *Escherichia*, *Shigella*, *Klebsiella*, *Mangrovibacter*, *Pluralibacter*, *Raoutella*, *Salmonella*, *Edwardi*, *Hafnia*, *Trabulsiela* and *Serratia*. *Escherichia*, *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter* and *Proteus* are opportunistic pathogens and have been associated with diarrhoea, urinary tract infections, mastitis, arthritis and meningitis (Fairbrother *et al*., 2005; Nagy and Fekete, 2015). Members are generally considered enteric pathogens of animals and some species are associated with a range of diseases (Janda, 2006). Most of sequences from the unclassified Clostridiales group were identified to family level. However, the genera *Flavonifractor* and *Pseudoflavonifactor* were identified. The broiler cecum and its mucosal tissue are dominated by Clostridiales (Gong *et al*., 2007; Lund *et al*., 2010). Members are known for their conversions of complex polysaccharides to short chain fatty acids such as butyrate which has

significant positive effects on growth (Biddle *et al*., 2013). However, members are more prominent in inflamed colons, indicating that they may accumulate during the development of colitis (Zhang *et al*., 2015). On the contrary, most evidence suggests that the majority of Clostridiales are non-pathogenic and are beneficial to the host (Rinttilä and Apajalahti, 2013).

The family *Synergistaceae* (0.015 %) was only found in broilers from the antibiotic group. Low levels of this family have been reported in the cecum of broilers (Wei *et al*., 2016). *Synergistaceae* inhabit anaerobic environments, i.e. animal gastrointestinal tracts, soil, oil wells, and wastewater treatment plants. In addition, members are present in sites of diseases i.e. cysts, abscesses, gastrointestinal infections and soft tissue infections and are considered opportunistic pathogens (Vartoukian *et al*., 2007). *Fusobacteriaceae*, *Flavobacteriaceae*, *Rhizobiaceae*, *Vibrionaceae*, *Xanthomonadaceae*, *Comamonadaceae*, *Campylobacteraceae* and Clostridiales Incertae Sedis XIII are associated with high feed conversion ratios (Singh *et al*., 2012). *Victivallaceae*, *Synergistaceae*, *Prevotellaceae*, *Rikenellaceae*, *Enterobacteriaceae* and *Ruminococcaceae* are associated with low feed conversion ratios (Singh *et al*., 2012).

A better understanding of the bacterial composition and activity, and the underlying mechanisms by which they modulate the GIT environment, is required to improve the understanding of the role specific bacteria have on the host health and feed utilization (Wei *et al*., 2016). Several studies have investigated the influence that dietary changes have on microbial community structure (Deplancke *et al*., 2002; Collier *et al*., 2003). However, understanding how these changes in bacterial composition relate to metabolic changes, which ultimately relate to improved health and performance needs to be elucidated (Wei *et al*., 2016).

Conclusions

Supplementation of broiler feed with the antibiotic combination (sulphadiazine, colistin and trimethoprim) or multi-species probiotic (*L*. *crispatus* DPN167, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94 and *B*. *amyloliquefaciens* DPN123) had no effect on the weight gain, feed intake, feed conversion ratio's, relative lymphoid organ weights, relative gizzard weights, tibia bone parameters and haematological parameters. Broilers from the antibiotic treatment group had higher levels of lymphocytes and basophils counts, and the control group had larger villi area, but these effects were transient and only statistically significant on day 19. Reduced *L*. *monocytogenes* bioluminescence was observed in the ileum of broilers receiving the multi-species probiotic at 3.5 h after administration of the pathogen. The microbiome of broilers from the antibiotic treatment group had significant lower levels of *Enterobacteriaceae*, and higher levels of unclassified Clostridiales, *Brucellaceae*, *Synergistaceae*, *Erysipelotrichaceae* and *Coriobacteriaceae* in their cecum on day 29. Understanding how these microbiota changes relate to metabolic changes in the host, and the role they play in GIT health and disease needs to be elucidated. While there have been many similar studies, information on feed additives is scarce. This study provides basic knowledge required to investigate potential alternatives to antibiotics.

References

Al-Baadani, H.H., Abudabos, A.M., Al-Mufarrej, S.I., Alzawqari, M. (2016). Effects of dietary inclusion of probiotics, prebiotics and synbiotics on intestinal histological changes in challenged broiler chickens. *S*. *Afr*. *J*. *Anim*. *Sci*. 46, p 157-165, DOI: 10.4314/sajas.v46i2.6.

Anderson, M.J. (2011). A new method for non-parametric multivariate analysis of variance. *Austral*. *Ecol*. 26, p 32-46, DOI: 10.1111/j.1442-9993.2001.01070.pp.x.

Apajalahti, J., Kettunen, A. (2006). Microbes of the chicken gastrointestinal tract. In: Perry, G.A. (Ed.). Avian gut function in health and disease. CAB International, Oxfordshire, UK, p 124-135.

Awad, W.A., Böhm, J., Razzazi-Fazeli, E., Ghareeb, K., Zentek, J. (2006). Effect of addition of a probiotic microorganism to broiler diets contaminated with deoxynivalenol on performance and histological alterations of intestinal villi of broiler chickens. *Poult*. *Sci*. 85, p 974-979, DOI: 10.1093/ps/85.6.974.

Awad, W.A., Ghareeb, K., Abdel-Raheem, S., Böhm, J. (2009). Effects of dietary inclusion of probiotic and synbiotic on growth performance, organ weights, and intestinal histomorphology of broiler chickens. *Poult*. *Sci*. 88, p 49-56, DOI: 10.3382/ps.2008-00244.

Biddle, A., Stewart, L., Blanchard, J., Leschine, S. (2013). Untangling the genetic basis of fibrolytic specialization by *Lachnospiraceae* and *Ruminococcaceae* in diverse gut communities. *Diversity* 5, p 627–640, DOI:10.3390/d5030627.

Bowen, R.A.R., Remaley, A.T. (2014). Interferences from blood collection tube components on clinical chemistry assays. *Biochem*. *Med*. (Zagreb) 24, p 31-44, DOI: 10.11613/BM.2014.006.

Brenes, A., Viveros, A., Arija, I., Centeno, C., Pizarro, M., Bravo, C. (2003). The effect of citric acid and microbial phytase on mineral utilization in broiler chicks. *Anim*. *Feed Sci*. *Technol*. 110(1-4), p 201-219, DOI: 10.1016/S0377-8401(03)00207-4 (2003).

Brown, D.R., Bradbury, J.M., Johansson, K. (2015). *Anaeroplasmataceae*. In: Whitman, W.B. (Ed.). Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley & Sons in association with Bergey's Manual Trust, New Jersey, USA, DOI: 10.1002/9781118960608.fbm00245.

Carr, H.S., Wlodkowski, T.J., Rosenkranz, H.S. (1973). Silver sulfadiazine: *In vitro* antibacterial activity. *Antimicrob*. *Agents Chemother*. 4, p 585-587.

Čermák, L., Skřivanová, E. (2016). Influence of pasture rearing on the cecal bacterial microbiota in broiler chickens. *Scientia Agriculturae Bohemica* 47, p 124-128, DOI: 10.1515/sab-2016-0018.

Chen, W., Wang, J.P., Yan, L., Huang, Y.Q. (2013). Evaluation of probiotics in diets with different nutrient densities on growth performance, blood characteristics, relative organ weight and breast meat characteristics in broilers. *Br*. *Poult*. *Sci*. 54, p 635–641, DOI: 10.1080/00071668.2013.825369.

Chichlowski, M., Croom, W.J., Edens, F.W., McBride, B.W., Qiu, R., Chiang, C.C., Daniel, L.R., Havenstein, G.B., Koci, M.D. (2007). Microarchitecture and spatial relationship between bacteria and ileal, cecal, and colonic epithelium in chicks fed a direct-fed microbial, PrimaLac, and salinomycin. *Poult*. *Sci*. 86(6), p 1121-1132, DOI: 10.1093/ps/86.6.1121.

Claus, S.P., Ellero, S.L., Berger, B., Krause, L., Bruttin, A., Molina, J., Paris, A., Want, E.J., de Waziers, I., Cloarec, O., Richards, S.E., Wang, Y., Dumas, M.E., Ross, A., Rezzi, S., Kochhar, S., Van

Bladeren, P., Lindon, J.C., Holmes, E., Nicholson, J.K. (2011). Colonisation-induced host-gut microbial metabolic interaction. *MBio* 2(2), e00271–e00210, DOI: 10.1128/mBio.00271-10.

Clavel, T., Mapesa, J.O. (2013). Phenolics in human nutrition: importance of the intestinal microbiome for isoflavone and lignan bioavailability. In: Ramawat, K.G. and Merillon, J.M. (Eds). Phytochemistry, Botany and Metabolism of Alkaloids, Phenolics and Terpenes. Sringer Publishing, Berlin, Germany, p 2433-2463, DOI: 10.1007/978-3-642-22144-6_94.

Clavel, T., Lepage, P., Charrier, C. (2014). The family *Coriobacteriaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.). The prokaryotes: Actinobacteria. Springer, Berlin, Germany, p 201-238, DOI: 10.1007/978-3-642-30138-4_343.

Collett, S. (2005). Strategies for improving gut health in commercial broiler operations. In: Lyons, T. and Jacques K.A. (Eds.). Nutritional biotechnology in the feed and food industries. Proceedings of Alltech's 21st Annual Symposium, p 17-30.

Collier, C.T., van der Klis, J.D., Deplancke, B., Anderson, D.B., Gaskins, H.R. (2003). Effects of tylosin on bacterial mucolysis, *Clostridium perfringens* colonisation, and intestinal barrier function in a chick model of necrotic enteritis. *Antimicrob*. *Agents Chemother*. 47, p 3311–3317, DOI: 10.1128/AAC.47.10.3311-3317.2003.

Davis, A.K., Maney, D.L., Maerz, J.C. (2008). The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Funct*. *Ecol*. 22, p 760–772, DOI: 10.1111/j.1365-2435.2008.01467.x.

Deplancke, B., Vidal, O., Ganessunker, D., Donovan, S.M., Mackie, R.I., Gaskins, H.R. (2002). Selective growth of mucolytic bacteria including *Clostridium perfringens* in a neonatal piglet model of total parenteral nutrition. Am. J. Clin. Nutr. 76(5), p 1117–1125, DOI: 10.1093/ajcn/76.5.1117.

Dinh, D.M., Volpe, G.E., Duffalo, C., Bhalchandra, S., Tai, A.K., Kane, A.V., Wanke, C.A., Ward, H.D. (2015). Intestinal microbiota, microbial translocation, and systemic inflammation in chronic HIV infection. *J*. *Infect*. *Dis*. 211(1), p 19–27, DOI: 10.1093/infdis/jiu409.

Dizaji, B.R., Hejazi, S., Zakeri, A. (2012). Effects of dietary supplementations of prebiotics, synbiotics and acidifiers on growth performance and organ weights of broiler chicken. *Eur*. *J*. *Exp*. *Biol*. 2, p 2125-2129.

Driver, J.P., Pesti, G.M., Bakalli, R.I., Edwards, H.M (2006). The effect of feeding calcium-and phosphorus-deficient diets to broiler chickens during the starting and growing-finishing phases on carcass quality. *Poult*. *Sci*. 85, p 1939-1946, DOI: 10.1093/ps/85.11.1939.

Ducatelle, R., Eeckhaut, V., Haesebrouck, F., Van Immerseel, F. (2015). A review on prebiotics and probiotics for the control of dysbiosis: present status and future perspectives. *Animal* 9, p 43-48, DOI: 10.1017/S1751731114002584.

Evans, T.C., Jehle, D. (1991). The red blood cell distribution width. *J*. *Emerg*. *Med*. 9, p 71–74, DOI: 10.1016/0736-4679(91)90592-4.

Evtushenko, L.I., Takeuchi, M. (2006). The family *Microbacteriaceae*. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K., Stackebrant, E. (Eds.). The prokaryotes volume 3 Archaea Bacteria:

Firmicutes, Actinomycetes. Springer, Berlin, Germany, p 1020-1098, DOI: 10.1007/0-387-30743-5_43.

Fairbrother, J.M., Nadeau, E., Gyles, C.L. (2005). *Escherichia coli* in post weaning diarrhea in pigs: an update on bacterial types, pathogenesis, and prevention strategies. *Anim*. *Health Res*. *Rev*. 6, p 17–39, DOI: 10.1079/AHR2005105.

Falagas, M.E., Kasiakou, S.K, Saravolatz, L.D. (2005). Colistin: The revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin*. *Infect*. *Dis*. 40, p 1333-1341, DOI: 10.1086/429323.

Fasoli, S., Marzotto, M., Rizzotti, L., Rossi, F., Dellaglio, F., Torriani, S. (2003). Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis. *Int*. *J*. *Food Microbiol*. 82(1), p 59–70, DOI: 10.1016/S0168-1605(02)00259-3.

Feberwee, A., Hampson, D.J., Phillips, N.D., La, T., van der Heijden, H.M.J.F., Wellenberg, G.J., Dwars, R.M., Landman, W.J.M. (2008). Identification of *Brachyspira hyodysenteriae* and other pathogenic *Brachyspira* species in chickens from laying flocks with diarrhea or reduced production or both. *J*. *Clin*. *Microbiol*. 46(2), p 593-600, DOI: 10.1128/JCM.01829-07 (2008).

Garrity, G.M., Bell, J.A., Lilburn, T. (2015). *Pseudomonadaceae*. In: Whitman, W.B. (Ed.). Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley & Sons in association with Bergey's Manual Trust, New Jersey, USA, DOI: 10.1002/9781118960608.fbm00232.

Gasparich, G.E. (2014). The family Entomoplasmataceae. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (2014). The prokaryotes: Firmicutes and Tenericutes. Springer, Berlin, Germany, p 505-514, DOI: 10.1007/978-3-642-30120-9_390.

Gheisar, M.M., Hosseindoust, A., Kim, I.H. (2016). Effects of dietary *Enterococcus faecium* on growth performance, carcass characteristics, faecal microbiota, and blood profile in broilers. *Veterinarni Medicina* 61, p 28-34, DOI: 10.17221/8680-VETMED.

Gleckman, R., Blagg, N., Joubert, D.W. (1981). Trimethoprim: Mechanisms of action, antimicrobial activity, bacterial resistance, pharmacokinetics, adverse reactions, and therapeutic indications. *Pharmacotherapy* 1, p 14-19, DOI: 10.1002/j.1875-9114.1981.tb03548.x.

Glöckner, F.O., Zaichikov, E., Belkova, N., Denissoya, L., Pernthaler, J., Pernthaler, A., Amann, R. (2000). Comparative 16S rDNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of Actinobactereria. *Appl*. *Environ*. *Microbiol*. 66(11), p 5053–5065, DOI: 10.1128/AEM.66.11.5053-5065.2000.

Gong, J., Si, W., Forster, R.J., Huang, R., Yu, H., Yin, Y., Yang, C., Han, Y. (2007). 16S rDNA gene-based analysis of mucosa-associated bacterial community and phylogeny in the chicken gastrointestinal tracts: from crops to ceca. *FEMS Microbiol*. *Ecol*. 59(1), p 147–157, DOI: 10.1111/j.1574-6941.2006.00193.x.

Gordon, R.J., Lowy, F.D. (2008). Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection. *Clin*. *Infect*. *Dis*. 46, S350–S359, DOI: 10.1086/533591.

Graczyk, S., Pliszczak-Krol, A., Kotonski, B., Wilczek, J., Chmielak, Z. (2003). Examinations of haematological and metabolic changes mechanisms of acute stress in turkeys. *Electronic J*. *Polish Agri*. *Uni*. 6, p 1-10.

Greene, A. (2014). The family *Desulfuromonadaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.). The prokaryotes: Deltaproteobacteria and Epsilonproteobacteria. Springer, Berlin, Germany, p 143-155, DOI: 10.1007/978-3-642 39044-9_380.

Gupta, R.S., Mahmood, S., Adeolu, M. (2013). Erratum: A phylogenomic and molecular signature based approach for characterization of the phylum *Spirochaetes* and its major clades: proposal for a taxonomic revision of the phylum. *Front*. *Microbiol*. 4, Article ID: 322, DOI: 10.3389/fmicb.2013.00322.

Hedemann, M.S., Højsgaard, S., Jensen, B.B. (2003). Small intestinal morphology and activity of intestinal peptidases in piglets around weaning. *J*. *Anim*. *Physiol*. *Anim*. *Nutr*. 87, p 32-41, DOI: 10.1046/j.1439-0396.2003.00405.x.

Higgins, S.E., Higgins, J.P., Wolfenden, A.D., Henderson, S.N., Torres-Rodriguez, A., Tellez, G, Hargis, B. (2008). Evaluation of a *Lactobacillus*-based probiotic culture for the reduction of *Salmonella* enteritidis in neonatal broiler chicks. *Poult*. *Sci*. 87(1), p 27–31, DOI: 10.3382/ps.2007-00210.

Höltzel, A., Gänzle, M.G., Nicholson, G.J., Hammes, W.P., Jung, G. (2000). The first low molecular weight antibiotic from lactic acid bacteria: reutericyclin, a new tetramic acid. *Angew*. *Chem*. *Int*. *Ed*. 39, p 2766–2768, DOI: 10.1002/1521-3773(20000804)39:15<2766::AID-ANIE2766>3.0.CO;2-G.

Horwitz, W. (2001). International Official Methods of Analysis, Association of Official Analytical Chemists (AOAC) 17th ed (AOAC Int., 2001).

Janda, J.M. (2006). New members of the family *Enterobacteriaceae*. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K., Stackebrandt, E. (Eds.). The prokaryotes: Volume 6 Proteobacteria: Gamma Subclass. Springer, Berlin, Germany, p 5-40, DOI: 10.1007/0-387-30746-X_1.

Kaakoush, N.O. (2015). Insights into the role of *Erysipelotrichaceae* in the human host. *Front*. *Cell*. *Infect*. *Microbiol*. 5, Article ID: 84, DOI: 10.3389/fcimb.2015.00084.

Kabir, S.M.L., Rahman, M.M., Rahman, M.B., Hosain, M.Z., Akand, M.S.I., Das, S.K. (2005). Viability of probiotics in balancing intestinal flora and effecting histological changes of crop and caecal tissues of broilers. *Biotechnol*. 4(4), p 325-330, DOI: 10.3923/biotech.2005.325.330.

Kabir, S.M.L. (2009). The role of probiotics in the poultry industry. *Int*. *J*. *Mol*. *Sci*. 10, p 3531-3546, DOI: 10.3390/ijms10083531.

Kabir, S.M.L., Rahman, M.M., Rahman, M.B., Ahmed, S.U. (2004). The dynamics of probiotics on growth performance and immune response in broilers. *Int*. *J*. *Poult*. *Sci*. 3, p 361–364, DOI: 10.3923/ijps.2004.361.364.

Kämpfer, P., Wohlgemuth, S., Scholz, H. (2014). The family *Brucellaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.). The prokaryotes: Alphaproteobacteria and Betaproteobacteria. Springer, Berlin, Germany, p 155-178, DOI: 10.1007/978-3-642-30197-1_299.

Klaenhammer, T.R., Kullen, M.J. (1999). Selection and design of probiotics. *Int*. *J*. *Food Microbiol*. 50, p 45–57, DOI: 10.1016/S0168-1605(99)00076-8.

Kurtoğlu, F., Kurtoğlu, V., Çelik, T., Keçeri, T., Nizamlioğlu, M. (2005). Effects of dietary boron supplementation on some biochemical parameters, peripheral blood lymphocytes, splenic plasma cells and bone characteristics of broiler chicks given diets with adequate or inadequate cholecalciferol (vitamin D3) content. *Br*. *Poult*. *Sci*. 46, p 87-96, DOI: 10.1080/00071660400024001.

Lamošová, D.M., Máčajová, M., Zeman, M. (2004). Effects of short-term fasting on selected physiological functions in adult male and female Japanese quail. *Acta Vet*. *Brno* 73, p 9-16, DOI: 10.2754/avb200473010009.

Lee, K., Lillehoj, H.S., Siragusa, G.R. (2010). Direct-fed microbials and their impacts on the intestinal microflora and immune system of chickens. *J*. *Poul*. *Sci*. 47, p 106-114, DOI: 10.2141/jpsa.009096.

Leeson, S., Summers, J.D. (2009). Commercial poultry nutrition (3rd edition). Nottingham University Press, Nottingham, England.

Li, L-L., Hou, Z-P., Li, T-J., Wu, G-Y., Huang, R-L., Tang, Z-R., Yang, C-B., Gong, J., Yu, H., Kong, X-F., Pan, E., Ruan, Z., Xhu, W-Y., Deng, Z-Y., Xie, M., Deng, J., Yin, F-G., Yin, Y-L. (2008). Effects of dietary probiotic supplementation on ileal digestibility of nutrients and growth performance in 1- to 42-day-old broilers. *J*. *Sci*. *Food Agric*. 88(1), p 35–42, DOI: 10.1002/jsfa.2910.

Lory, S. (2014). The family *Staphylococcaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.). The Prokaryotes: Firmicutes and Tenericutes, Springer, Berlin, Germany, p 363-366, DOI: 10.1007/978-3-642-30120-9_350.

Lund, M., Bjerrum, L., Pedersen, K. (2010). Quantification of *Faecalibacterium prausnitzii*- and *Subdoligranulum* variabile-like bacteria in the cecum of chickens by real-time PCR. *Poult*. *Sci*. 89, p 1217–1224, DOI: 10.3382/ps.2010-00653.

Magnusson, J., Schnürer, J. (2001). *Lactobacillus coryniformis* subsp. *coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. *Appl*. *Environ*. *Microbiol*. 67, p 1–5, DOI: 10.1128/AEM.67.1.1-5.2001.

Martínez, I., Wallace, G., Zhang, C., Legge, R., Benson, A.K., Carr, T.P., Moriyama, E.N., Walter, J. (2009). Diet-induced metabolic improvements in a hamster model of hypercholesterolemia are strongly linked to alterations of the gut microbiota. *Appl*. *Environ*. *Microbiol*. 75(12), p 4175–4184, DOI: 10.1128/AEM.00380-09.

Martínez, I., Lattimer, J.M., Hubach, K.L., Case, J.A., Yang, J., Weber, C.G., Louk, J.A., Rose, D.J., Kyureghian, G., Peterson, D.A., Haub, M.D., Walter, J. (2013). Gut microbiome composition is linked to whole grain-induced immunological improvements. *ISME J*. 7(2), p 269-280, DOI: 10.1038/ismej.2012.104.

Matur, E., Eraslan, E. (2012). The impact of probiotics on the gastrointestinal physiology. In: Brzozowski, R. (Ed.). New Advances in the Basic and Clinical Gastroenterology, Intech, London, UK, p 51-74, DOI: 10.5772/34067.

McBride, M.J. (2014). The Family *Flavobacteriaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds). The Prokaryotes: Other Major Lineages of Bacteria and the Archaea, Springer, Berlin, Germany, p 643-676, DOI: 10.1007/978-3-642-38954-2_130.

Medhanie, G.A., Mcewen, S.A., Slavic, D., Guerin, M.T. (2013). *Brachyspira* spp. and avian intestinal spirochaetosis: an epidemiological review. *World's Poult*. *Sci*. *J*. 69, p 541-552, DOI: 10.1017/S0043933913000561.

Miles, R.D., Butcher, G.D., Henry, P.R., Littell, R.C. (2006). Effect of antibiotic growth promoters on broiler performance, intestinal growth parameters, and quantitative morphology. *Poult*. *Sci*. 85, p 476-485, DOI: 10.1093/ps/85.3.476.

Montagne, L., Pluske, J.R., Hampson, D.J. (2003). A review of interactions between dietary fibre and the intestinal mucosa and their consequences on digestive health in young nonruminant animals. *Anim*. *Feed Sci*. *Technol*. 103, p 95-117, DOI: 10.1016/S0377-8401(03)00163-9.

Mountzouris, K.C., Tsirtsikos, P., Kalamara, E., Nitsch, S., Schatzmayr, G., Fegeros, K. (2007). Evaluation of the efficacy of a probiotic containing *Lactobacillus*, *Bifidobacterium*, *Enterococcus*, and *Pediococcus* strains in promoting broiler performance and modulating cecal microflora composition and metabolic activities. *Poult*. *Sci*. 86, p 309–317, DOI: 10.1093/ps/86.2.309.

Mountzouris, K.C., Tsitrsikos, P., Palamidi, I., Arvaniti, A., Mohnl, M., Schatzmayr, G., Fegeros, K. (2010). Effects of probiotic inclusion levels in broiler nutrition on growth performance, nutrient digestibility, plasma immunoglobulins, and cecal microflora composition. *Poult*. *Sci*. 89(1), p 58-67, DOI: 10.3382/ps.2009-00308.

Mountzouris, K.C., Balaskas, C., Xanthakos, I., Tzivinikou, A., Fegeros, K. (2009). Effects of a multi-species probiotic on biomarkers of competitive exclusion efficacy in broilers challenged with *Salmonella* Enteritidis. *Br*. *Poult*. *Sci*. 50, p 467–478, DOI: 10.1080/00071660903110935.

Nagy, B., Fekete, P.Z. (2015). Enterotoxigenic *Escherichia coli* in veterinary medicine. *Int*. *J*. *Med*. *Microbiol*. 295, p 443–454, DOI: 10.1016/j.ijmm.2005.07.003.

Nourmohammadi, R., Hosseini, S.M., Saraee, H., Arab, A., Arefinia, H. (2011). Plasma thyroid hormone concentrations and pH values of some GI-tract segments of broilers fed on different dietary citric acid and microbial phytase levels. *J*. *Anim*. *Vet*. *Adv*. 10, p 1450-1454, DOI: 10.3923/javaa.2011.1450.1454.

Ohimain, E.I., Ofongo, R.T.S. (2012). The effect of probiotic feed supplementation on chicken health and gut microflora: a review. *Int*. *J*. *Anim*. *Veter*. *Adv*. 4, p 135-143.

Olnood, C.G., Beski, S.S.M., Choct, M., Iji, P.A. (2015). Novel probiotics: Their effects on growth performance, gut development, microbial community and activity of broiler chickens. *Anim*. *Nutr*. 1, p 184-191, DOI: 10.1016/j.aninu.2015.07.003.

Onyango, E.M., Hester, P.Y., Stroshine, R., Adeola, O. (2003). Bone densitometry as an indicator of percentage tibia ash in broiler chicks fed varying dietary calcium and phosphorus levels. *Poult*. *Sci*. 82, p 1787-1791, DOI: 10.1093/ps/82.11.1787.

Pallmann, P., Schaarschmidt, F., Hothorn, L.A., Fischer, C., Nacke, H., Priesnitz, K.U., Schork, N.J. (2012). Assessing group differences in biodiversity by simultaneously testing a user-defined selection of diversity indices. *Mol*. *Ecol*. *Resour*. 12(6), p 1068-1078, DOI: 10.1111/1755-0998.12004.

Palm, N.W., de Zoete, M.R., Cullen, T.W., Barry, N.A., Stefanowski, J., Hao, L., Degnan, P.H., Hu, J., Peter, I., Zhang, W., Ruggiero, E., Cho, J.H., Goodman, A.L., Flavell, R.A. (2014).

Immunoglobulin A coating identifies colitogenic bacteria in inflammatory bowel disease. *Cell* 158(5), p 1000–1010, DOI: 10.1016/j.cell.2014.08.006.

Patterson, J.A., Burkholder, K.M. (2003). Application of prebiotics and probiotics in poultry production. *Poult*. *Sci*. 82, p 627–631, DOI: 10.1093/ps/82.4.627.

Pluske, J.R., Williams, I.H., Aherne, F.X. (1996). Villous height and crypt depth in piglets in response to increases in the intake of cows' milk after weaning. *Anim*. *Sci*. 62, p 145-158, DOI: 10.1017/S1357729800014429.

Pourakbari, M., Seidavi, A., Asadpour, L., Martínez, A. (2016). Probiotic level effects on growth performance, carcass traits, blood parameters, cecal microbiota, and immune response of broilers. *An*. *Acad*. *Bras*. *Ciênc*. 88, p 1011-1021, DOI: 10.1590/0001-3765201620150071.

Presnell, J.K., Schreibman, M.P. (1997). Humason's animal tissue techniques (5th edition). Johns Hopkins University Press, Maryland, USA.

Rabson, A., Roitt, I., Delves, P. (2005). Really Essential Medical Immunology (2nd edition). Blackwell Publishing, New Jersey, USA.

Rama Rao, S.V., Reddy, V. (2001). Utilisation of different phosphorous sources in relation to their fluorine content for broilers and layers. *Br*. *Poult*. *Sci*. 42, 376-383, DOI: 10.1080/00071660120055368.

Ramos, C.L., Thorsen, L., Schwan, R.F., Jespersen, L. (2013). Strain-specific probiotics properties of *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus brevis* isolated from Brazilian food products. *Food Microbiol*. 36, p 22-29, DOI: 10.1016/j.fm.2013.03.010.

Reece, F.N., Deaton, J.W., May, J.D., May, K.N. (1971). Cage *versus* floor rearing of broiler chickens. *Poul*. *Sci*. 50, p 1786–1790, DOI: 10.3382/ps.0501786.

Rehman, H.U., Vahjen, W., Awad, W.A., Zentek. J. (2007). Indigenous bacteria and bacterial metabolic products in the gastrointestinal tract of broiler chickens. *Arch*. *Anim*. *Nutr*. 61, p 319–335, DOI: 10.1080/17450390701556817.

Rinttilä, T., Apajalahti, J. (2013). Intestinal microbiota and metabolites- Implications for broiler chicken health and performance. *J*. *Appl*. *Poult*. *Res*. 22, p 647-658, DOI: 10.3382/japr.2013-00742.

Robert, C., Brasseur, P.Y., Dubois, M., Delahaut, P., Gillard, N. (2016). Development and validation of rapid multiresidue and multi-class analysis for antibiotic and anthelmintics in feed by ultra-high-performance liquid chromatography coupled to tandem mass spectrometry. *Food Addit*. *Contam*. *Part A* 33(8), p 1312-1323, DOI: 10.1080/19440049.2016.1207808.

Rosenberg, E. (2014a). The family *Brachyspiraceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.). The Prokaryotes: Other Major Lineages of Bacteria and the Archaea. Springer, Berlin, Germany, p 485-486, DOI: 10.1007/978-3-642-38954-2_157.

Rosenberg, E. (2014b). The Family *Chitinophagaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.). The Prokaryotes: Other Major Lineages of Bacteria and the Archaea. Springer, Berlin, Germany, p 493-495, doi: 10.1007/978-3-642-38954-2_137.

Saddler, G.S., Bradbury, J.F. (2015). *Xanthomonadaceae* fam. nov. In: Whitman, W.B. (Ed.). Bergey's Manual of Systematics of Archaea and Bacteria, John Wiley & Sons in association with Bergey's Manual Trust, New Jersey, USA, DOI: 10.1002/9781118960608.fbm00237.

Saiyed, M.A., Joshi, R.S., Savaliya, F.P., Patel, A.B., Mishra, R.K., Bhagora, N.J. (2015). Study on inclusion of probiotic, prebiotic and its combination in broiler diet and their effect on carcass characteristics and economics of commercial broilers. *Vet*. *World* 8(2), p 225-231, DOI: 10.14202/vetworld.2015.225-231.

Santos, F.B.O., Sheldon, B.W., Santos, A.A., Ferket, P.R. (2008). Influence of housing system, grain type, and particle size on *Salmonella* colonisation and shedding of broilers fed triticale or corn-soybean meal diets. *Poult*. *Sci*. 87, p 405-420, DOI: 10.3382/ps.2006-00417.

Satomi, M., Fujii, T. (2014). The family *Oceanospirillaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds). The Prokaryotes: Gammaproteobacteria Springer, Berlin, Germany, p 491-527, DOI: 10.1007/978-3-642-38922-1_286.

Scholz-Ahrens, K.E., Ade, P., Marten, B., Weber, P., Timm, W., Açil, Y., Glüer, C.C., Schrezenmeir, J. (2007). Prebiotics, probiotics, and synbiotics affect mineral absorption, bone mineral content, and bone structure. *J*. *Nutr*. 137(3), 838S-846S, DOI: 10.1093/jn/137.3.838S.

Seiser, P.E., Duffy, L.K., McGuire, A.D., Roby, D.D., Golet, G.H., Litzow, M.A. (2000). Comparison of pigeon guillemot, *Cepphus columba*, blood parameters from oiled and un-oiled areas of Alaska eight years after the Exxon Vadez oil spill. *Mar*. *Pollut*. *Bull*. 40(2), p 152-164, DOI: 10.1016/S0025-326X(99)00194-0.

Sen, S., Ingale, S.L., Kim, Y.W., Kim, J.S., Kim, K.H., Lohakare, J.D., Kim, E.K., Kim, H.S., Ryu, M.H., Kwon, M.H., Kwon, I.K., Chae, B.J. (2012). Effect of supplementation of *Bacillus subtilis* LS 1-2 to broiler diets on growth performance, nutrient retention, caecal microbiology and small intestinal morphology. *Res*. *Vet*. *Sci*. 93(1), p 264–268, DOI: 10.1016/j.rvsc.2011.05.021.

Sepp, T., Sild, E., Hõrak, P. (2010). Hematological condition indexes in greenfinches: effects of captivity and diurnal variation. *Physiol*. *Biochem*. *Zool*. 83, p 276–282, DOI: 10.1086/648580.

Shim, Y.H., Shinde, P.L., Choi, J.Y., Kim, J.S., Seo, D.K., Pak, J.I., Chae, B.J., Kwon, I.K. (2010). Evaluation of multi-microbial probiotics produced by submerged liquid and solid substrate fermentation methods in broilers. *Asian-Australasian J*. *Anim*. *Sci*. 23(4), p 521–529, DOI: 10.5713/ajas.2010.90446.

Shukla, S., Mishra, P. (2015). *Pseudomonas aeruginosa* infections in broiler chicks in Jabalpur. *Int*. *J*. *Ext*. *Res*. 6, p 37-39.

Singh, K.M., Shah, T., Deshpande, S., Jakhesara, S.J., Koringa, P.G., Rank, D.N., Joshi, C.G. (2012). High through put 16S rDNA gene-based pyrosequencing analysis of the fecal microbiota of high FCR and low FCR broiler growers. *Mol*. *Biol*. *Rep*. 39(12), p 10595-10602, DOI: 10.1007/s11033-012-1947-7.

Sobieraj, M., Boone, D.R. (2006). *Syntrophomonadaceae*. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K., Stackebrant, E. (Eds). The Prokaryotes: volume 4 Bacteria: Firmicutes, Cyanobacteria, Springer, Berlin, Germany, p 1041-1049, DOI: 10.1007/0-387-30744-3_37.

Stackebrandt, E. (2014). The family *Aerococcaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds). The prokaryotes: Firmicutes and Tenericutes, Springer, Berlin, Germany, p 3-6, doi: 10.1007/978-3-642-30120-9_349.

Sun, X., McElroy, A., Webb, K.E., Sefton, A.E., Novak, C. (2005). Broiler performance and intestinal alterations when fed drug-free diets. *Poult*. *Sci*. 84, p 1294–1302.

Thota, V.R., Dacha, S., Natarajan, A., Nerad, J. (2011). *Eggerthella lenta* bacteremia in a Crohn's disease patient after ileocecal resection. *Future Microbiol*. 6, p 595–597, DOI: 10.2217/fmb.11.31.

Ue, H., Matsuo, Y., Kasai, H., Yokota, A. (2011). *Demenquina globuloformis* sp. nov., *Demequina oxidasica* sp. nov. and *Demequina aurantiaca* sp. nov., actinobacteria isolated from marine environments, and proposal of *Demequinaceae* fam. nov. *Int*. *J*. *Syst*. *Evol*. *Microbiol*. 61, p 1322-1329, DOI: 10.1099/ijs.0.024299-0.

Vartoukian, S.R., Palmer, R.M., Wade, W.G. (2007). The division "*Synergistes*". *Anaerobe* 13, p 99-106, DOI: 10.1016/j.anaerobe.2007.05.004.

Verbarg, S., Göker, M., Scheuner, C., Schumann, P., Stackebrandt, E. (2014). The families *Erysipelotrichaceae* emend., *Corprobacillaceae* fam. nov., *Turicibacteraeae* fam. nov. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrandt, E., Thompson, F. (Eds.). The prokaryotes: Firmicutes and Ternericutes, Springer, Berlin, Germany, p 79-105, DOI: 10.1007/978-3-642-30120-9_205.

Wei, S., Lilburn, M., Yu, Z. (2016). The bacteriomes of ileal mucosa and cecal content of broiler chickens and turkeys as revealed by metagenomic analysis. *Int*. *J*. *Microbiol*. 2016, Article ID: 4320412, DOI:10.1155/2016/4320412.

Willis, W.L., Isikhuemhen, O.S., Ibrahim, S.A. (2007). Performance assessment of broiler chickens given mushroom extract alone or in combination with probiotic. *Poult*. *Sci*. 86, p 1856–1860, DOI: 10.1093/ps/86.9.1856.

Xu, Z.R., Hu, C.H., Xia, M.S., Zhan, X.A., Wang, M.Q. (2003). Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of male broilers. *Poult*. *Sci*. 82(6), p 1030-1036, DOI: 10.1093/ps/82.6.1030.

Yegani, M., Korver, D.R. (2008). Factors affecting intestinal health in poultry. *Poult*. *Sci*. 87, p 2052-2063, DOI: 10.3382/ps.2008-00091.

Yu, B., Liu, J.R., Hsiao, F.S., Chiou, P.W.S. (2008). Evaluation of *Lactobacillus reuteri* Pg4 strain expressing heterologous β-glucanase as a probiotic in poultry diets based on barley. *Anim*. *Feed Sci*. *Technol*. 141, p 82–91, DOI:10.1016/j.anifeedsci.2007.04.010.

Zakrzewski, M., Proietti, C., Ellis, J.J., Hasan, S., Brion, M-J., Berger, B., Krause, L. (2017). Calypso: a user-friendly web-server for mining and visualizing microbiome–environment interactions. *Bioinformatics* 33(5), p 782-783, DOI: 10.1093/bioinformatics/btw725.

Zaman, Q., Khan, M.Z., Islam, N., Muhammad, G. (1995). Experimental furazolidone toxicosis in broiler chicks: effect of dosage, duration and age upon clinical signs and some blood parameters. *Acta Vet*. *Hung*. 43, p 359-367.

Zhang, Y.J., Li, S., Gan, R.Y., Zhou, T., Xu, D.P., Li, H.B. (2015). Impacts of gut bacteria on human health and diseases. *Int*. *J*. *Mol*. *Sci*. 16(4), p 7493-7519, DOI: 10.3390/ijms16047493.

Zulkifli, I., Che Norma, M.T., Chong, C.H., Loh, T.C. (2000). Heterophil to lymphocyte ratio and tonic immobility reactions to preslaughter handling in broiler chickens treated with ascorbic acid. *Poult. Sci*. 79, p 402-406, DOI: 10.1093/ps/79.3.402.

Chapter 5

Interaction of pathogenic and probiotic bacteria with epithelial cells

Abstract

Changes in the proteome of epithelial cells from the ileum of broilers were recorded when exposed to pathogenic and probiotic bacteria, respectively. The pathogenic strain of *Salmonella enterica* Enteritidis (strain 147), was cytotoxic and invaded Caco-2 cells. In addition, strain 147 destroyed the claudin-3 tight junctions between Caco-2 cells, resulting in the disruption of the monolayer. Viable cells of *Lactobacillus salivarius* DPN181, *Lactobacillus crispatus* DPN167, *Lactobacillus gallinarum* DPN164, *Lactobacillus johnsonii* DPN184, *Enterococcus faecalis* DPN94 and *Bacillus amyloliquefaciens* DPN123, on the other hand, were not cytotoxic to Caco-2 cells. The probiotic cells adhered to the intestinal epithelial cells but did not invade the cells. The probiotic cells decreased the number of claudin-3 junctions, but the monolayer was not disrupted. Metabolites of *L. johnsonii* DPN184, *L. salivarius* DPN181, *L. gallinarum* DPN164, *E. faecalis* DPN94 and *B. amyloliquefaciens* DPN123 significantly decreased the efficiency at which *S.* Enteritidis 147 invaded Caco-2 cells. *Salmonella* Enteritidis 147 exposed to broiler epithelial cells resulted in the up-regulation of eight proteins (lysozyme C and G, cathelicidin 2 and 3, myeloid protein 1, trypsin inhibitor CITI-1, gallinacin-2 and ubiquitin-fold modifier 1) and the down-regulation of three proteins (glutaredoxin-1, gallicin-7 and vigilin). Upregulated proteins have broad spectrum antimicrobial activity, act as chemotactic compounds, inhibitors of microbial enzymes, and play critical roles during stress responses. Down-regulated proteins play vital roles in activation of natural killer cells, regulation of apoptosis and antimicrobial defence systems. Broiler epithelial cells exposed to the multi-species probiotic resulted in the up-regulation of three proteins (transgelin 2/3, elongation factor-1 beta and anterior gradient 2) and the down-regulation of four proteins (carnitine O-acetyltransferase, adenylate kinase 2, superoxide dismutase [Cu-Zn] and protein SET). Upregulated proteins were involved in cell proliferation, cell migration and healing, and cytoskeleton regulation, whereas down-regulated proteins play important roles in fatty acid transport, energy homeostasis, nucleotide metabolisms, free-radical elimination and signal transduction.

Introduction

The microbiota of the chicken gastrointestinal tract (GIT) plays an important role in regulating digestion and adsorption of nutrients, stimulation of the immune system and preventing the colonisation of pathogens (Lan *et al*., 2005; Schneitz, 2005; Brisbin *et al*., 2008; Yegani and Korver, 2008; Choct, 2009; Lee *et al*., 2010; Chambers and Gong, 2011). Each section of the GIT has its own unique physiochemical characteristics and is inhabited by a specific microbial community (Dethlefsen *et al*., 2007). Development of the intestinal microbiome starts at hatching when chicks are exposed to intestinal microbes deposited onto the surface of eggshells (Rinttilä and Apajalahti, 2013). Exposure to these microorganisms is critical to establish a healthy gut microbiome. The first strains that colonise the GIT dictate bird development over the entire life span, as these bacteria modulate the development of the immune system and the GIT microbiome (Apajalahti *et al*., 2004). How changes in the composition of microbiome relate to changes in metabolic functions and host development remains to be elucidated (Oakley *et al*., 2014).

Microorganisms have co-evolved with the host in a symbiotic or pathogenic manner. Evolution of either symbionts or pathogens are similar, however, the establishment of a symbiosis requires more time and evolutionary processing (Steinert *et al*., 2000). Unravelling the interactions which occur between the symbiotic microbiome, the host and pathogenic bacteria will provide insight in disease development and may provide measures to prevent infections (Bäumler and Sperandio, 2016; Pickard *et al*., 2017). *Salmonella enterica* causes salmonellosis in humans. *Salmonella* Enteritidis is associated with poultry, whereas *S*. Typhimurium has a wider species range, including poultry, pigs and cattle (Hugas and Beloeil, 2014). Circulation of multidrug-resistant *Salmonella* clones and spreading of mobile genetic elements encoding antibiotic resistance from poultry to humans is a great concern. Health problems in the GIT of broilers typically occur between 20 to 30 days of age and causes wet litter, nonspecific enteritis, poor weight gain, and a decrease in the digestion and absorption of nutrients (Wilson *et al*., 2005; Teirlynck *et al*., 2011). Systemic infections normally start from the intestinal phase were bacteria enter the blood stream and spread to various organs such as the liver and spleen (Kaur and Jain, 2012). In the past, the spreading of foodborne pathogens in broilers were controlled using antibiotic feed additives. However, the use of antibiotics has been banned in Europe, due to the selection of antimicrobial resistant bacteria (Chang *et al*., 2015). In addition, antibiotics have a profound impact on the microbiome composition and can lead to the expansion of pathogenic populations (Bäumler and Sperandio, 2016). Alternative feed additives, including short- and medium-chain fatty acids (Van Immerseel *et al*., 2006), manno-oligosaccharides (Berge and Wierup, 2012), probiotics (Prado-Rebolledo *et al*., 2016; Neveling *et al*., 2017), and prebiotic dietary fibres (Vermeulen *et al*., 2017) have been proposed to control *Salmonella* and other food-borne related pathogens.

Numerous researchers have characterised the proteomic changes which occur in *Salmonella* during interaction with eukaryotic cells, however, the proteomic changes which occur in host cells have rarely been studied (Yang *et al*., 2015; Qi *et al*., 2017). Epithelial cells infected with *S*. Typhimurium revealed that during early infection *Salmonella* stimulates Rho-family GTPases leading to the activation of mitogen-activated protein kinase (MAPK) and NF-B signalling (Bruno *et al*., 2009). In late stage of infection, *Salmonella* induces the activation of the transcription factor STAT3, required for bacterial

replication (Hannemann *et al*., 2013). *Salmonella* infection in HeLa cells led to the induction of host integrin signalling and glycolytic pathways and up-regulation of PARP1 (Qi *et al*., 2017). Additionally, proteomic analysis of macrophages in response to *Salmonella* infection showed increased levels of superoxide dismutase, indicating oxidative stress in host mitochondria (Shi *et al*., 2009). Researchers studying the effect of probiotic *Enterococcus faecium* CGMCC 2516 on the liver proteome of broiler concluded that probiotics improved metabolism of nutrients and decreased inflammatory responses (Zheng *et al*., 2016). *Lactobacillus mucosae* LM1 interaction with intestinal porcine epithelial cells (IPEC-J2) led to the up-regulation of proteins involved in tight junction assembly, actin organisation, and genetic information processing were upregulated in epithelial cells (Pajarillo *et al*., 2017). Elucidating the proteomic changes which occur in host cells in response to probiotic and pathogenic bacteria will provided researchers with greater insight into the crosstalk which occurs between the host and bacterium.

Cytotoxicity, cell adhesion and invasion, as well as changes in claudin-3 tight junctions of Caco-2 cells in response to *S*. Enteritidis 147 and a multi-species probiotic, consisting of *Lactobacillus salivarius* DPN181, *Lactobacillus crispatus* DPN167, *Lactobacillus gallinarum* DPN164, *Lactobacillus johnsonii* DPN184, *Enterococcus faecalis* DPN94 and *Bacillus amyloliquefaciens* DPN123 was determined. The ability of probiotic strains to decrease *Salmonella* invasion of Caco-2 cells was also studied. Proteomic changes induced by pathogenic and probiotic bacteria on epithelial cells collected from the ileum of broilers are reported.

Materials and Methods

Cell culture and bacterial growth conditions

Colonic epithelial cells (Caco-2 ACC 169) were grown to passage 42 in minimal essential medium (MEM), supplemented with 10 % (v/v) foetal calf serum (FCS), 1 % (v/v) non-essential amino acids (NEAA) and 1 % (v/v) sodium-pyruvate (Thermo Fisher Scientific, Massachusetts, USA). Currently, no avian epithelial cell line is commercially available, thus Caco-2 cells were used as a eukaryotic model to study the cytotoxicity, adhesion and invasion, and effect on tight junctions by probiotic and pathogenic bacteria. A previous study on the invasion of Caco-2 cells by *Campylobacter jejuni* (Hänel *et al*., 2004) has shown that these cells may be used as a model to study the colonisation of broilers. Incubation was at 37 °C under humidified (95 % relative humidity, RH, 5 % CO_2) atmosphere. Strains with probiotic properties were isolated from healthy free-range broilers (Neveling *et al*., 2017). *Lactobacillus johnsonii* DPN184, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164 and *E*. *faecalis* DPN94 were grown in MRS (de Man, Rogosa and Sharpe) broth (Merck, Darmstadt, Germany) at 37 °C in an anaerobic cabinet (Jacomex GP Concept, Dagneux, France), in the presence of N_2 (84 %), H_2 (8 %) and CO_2 (8 %). *Bacillus amyloliquefaciens* DPN123 was grown in BHI (Brain Heart Infusion) broth (Merck), at 37 °C under aerobic conditions using a rotating wheel. *Salmonella enterica* serovar Enteritidis 147, isolated from egg white (Methner *et al*., 1995), was grown in LB (Luria-Bertani) broth (Merck) at 37 °C under aerobic conditions. The *Salmonella* strain was considered pathogenic as the strain evaded the avian immune system to infect the egg during development in the oviduct (Methner *et al*., 1995).

Determination of short chain fatty acids

Production of short chain fatty acids (SCFA) butyric, propionic, acetic, isovaleric and lactic acid was determined as described by Vermeulen *et al*. (2017). Lactic acid bacteria (*L*. *johnsonii* DPN184, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *L*. *crispatus* DPN167 and *E*. *faecalis* DPN94) were inoculated in MRS broth (1%, v/v) and incubated at 37 °C under anaerobic condition for 24 h. *Bacillus amyloliquefaciens* DPN123 was inoculated in BHI broth (1 %, v/v) and incubated at 37 °C under aerobic conditions using a rotating wheel for 24 h. *Salmonella* Enteritidis 147 was inoculated in LB broth (1 %, v/v) and incubated at 37 °C under aerobic conditions using a rotating wheel for 24 h. Overnight cultures were centrifuged (10 000 × *g*, 10 min, 4 °C), the supernatant collected, and filter-sterilised using a 0.22 µm Millex-HV Millipore filter (Merck Millipore, Billerica, Massachusetts). The concentration of butyric, propionic, acetic and isovaleric acid was determined using GC-MS (Vermeulen *et al*., 2017). In short, SCFA were extracted from bacterial supernatants using diethyl ether. Methyl hexanoic acid was added as internal standard to the extract. The extracts were analysed using a GC-2014 gas chromatograph (Shimadzu, Kyoto, Japan), equipped with a capillary fatty acid-free EC-1000 Econo-Cap column (dimensions: 25 mm × 0.53 mm, film thickness 1.2 mM; Alltech, Laarne, Belgium), a flame ionisation detector and a split injector. The injection volume was 1 µl and the temperature set from 110 to 160 °C, with a step-wise increase of 6 °C/min. The carrier gas was N_2, the injector was set at 100 °C and the detector at 220 °C. The lactic acid concentration was quantified using the D/L-lactic acid assay kit (Megazyme Inc., Illinois, USA), according to the manufacturer's instructions.

Cytotoxicity tests

The cytotoxicity of *L*. *salivarius*, *L*. *johnsonii*, *L*. *crispatus*, *L*. *gallinarum*, *E*. *faecalis*, *B*. *amyloliquefaciens* and *S*. Enteritidis 147 towards Caco-2 cells were determined using the neutral red (NR) assay, as described by Repetto *et al*. (2008). Absorbance readings (540 nm) were used to determine the release of NR from lysed cells, as described by Balls *et al*. (1987). Caco-2 cells (suspended in MEM, supplemented with 10 %, v/v, FCS; 1 %, v/v, NEAA and 1 % v/v sodium-pyruvate) were seeded in a 96 well microtiter plate (1 × 10^5 cells/well) and incubated at 37 °C in humidified atmosphere for 24 h. Lactic acid bacteria (*L*. *johnsonii* DPN184, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *L*. *crispatus* DPN167 and *E*. *faecalis* DPN94) were inoculated in MRS broth (1%, v/v) and incubated at 37 °C under anaerobic condition for 24 h. *Bacillus amyloliquefaciens* DPN123 was inoculated in BHI broth (1 %, v/v) and incubated at 37 °C under aerobic conditions using a rotating wheel for 24 h. *Salmonella* Enteritidis 147 was inoculated in LB broth (1 %, v/v) and incubated at 37 °C under aerobic conditions using a rotating wheel for 24 h. Overnight bacterial cultures were centrifuged (8000 × *g*, 37 °C, 10 min), and cells suspended in equal volumes of MEM, serially diluted, plated onto respective media and incubated at 37 °C for 24 h. Cell numbers were expressed as CFU/ml. Bacterial cell-free supernatants were lyophilised, suspended in equal volumes of MEM and the pH adjusted to pH 7.5 with 1 M NaOH. The supernatants were then treated with catalase (3 mg/mL) and pronase (3 mg/mL), respectively, and incubated at 37 °C for 2 h. Pronase and catalase were inactivated by exposing the suspensions to 80 °C for 15 min. All treated supernatants were filter-sterilised through

a 0.22 µm filter membrane (Merck) before adding to Caco-2 cells. Bacterial cells and treated supernatants were added to Caco-2 cells and incubated under humidified atmosphere, as described elsewhere, for 3 h. The cells were washed twice with 200 µl Hank's balanced salt solution (HBSS; Ca^{2+}, Mg^{2+}, without phenol red; Thermo Fisher Scientific). Viable Caco- 2 cells were stained with 150 µl neutral red [1 ml neutral red (0.5 %, m/v) added to 80 ml MEM] and incubated at 37 °C in humidified atmosphere, as before, for 3 h. Cells were then washed twice with pre-warmed 200 µl HBSS. Neutral red bound to Caco-2 were removed by adding 100 µl NR desorb (1 % v/v glacial acetic acid, 50 % v/v ethanol and 49 % v/v H_2O), plates were protected from light by covering in foil, and shaken with an orbital shaker (50 rpm) for 20 sec. The NR released from the cells was recorded spectrophotometrically at 540 nm ± 10 nm.

Adhesion and invasion of bacterial cells

Bacterial adhesion and invasion of Caco-2 cells by probiotic bacteria and *S*. Enteritidis were determined as described by Darfeuille-Michaud *et al*. (1999), with minor modifications. Caco-2 cells (suspended in MEM supplemented with 10 % FCS, 1 % NEAA, 1 % sodium-pyruvate) were seeded in a 96-well microtiter plate (1×10^4 cells/well) and incubated at 37 °C in humidified atmosphere for 24 h. Lactic acid bacteria (*L*. *johnsonii* DPN184, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *L*. *crispatus* DPN167 and *E*. *faecalis* DPN94) were inoculated in MRS broth (1%, v/v) and incubated at 37 °C under anaerobic condition for 24 h. *Bacillus amyloliquefaciens* DPN123 was inoculated in BHI broth (1 %, v/v) and incubated at 37 °C under aerobic conditions using a rotating wheel for 24 h. *Salmonella* Enteritidis 147 was inoculated in LB broth (1 %, v/v) and incubated at 37 °C under aerobic conditions using a rotating wheel for 24 h. Bacterial cells were collected from an overnight culture by centrifugation (8000 × *g*, 10 min, 37 °C), washed twice with sterile phosphate-buffered saline (PBS, pH 7.5), and resuspended in MEM. Bacterial suspensions of *L*. *johnsonii* DPN184, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *E*. *faecalis*, *B*. *amyloliquefaciens* DPN123, *S*. Enteritidis 147 (1×10^6 CFU per well) were separately added to washed Caco- 2 cells (100 bacterial cells :1 Caco-2 cell), centrifuged (1500 × *g*, 37 °C, 10 min), and incubated at 37 °C in humidified atmosphere for 2 h. After incubation, non-adherent bacteria were removed by washing the cells twice with HBSS. Cells were lysed in the presence of 100 µl 1 % (v/v) Triton-X-100, and shaken at 100 rpm for 10 min. Lysed cells were serially diluted in HBSS and plated onto respective growth media, followed by incubation at 37 °C for 48 h to calculate the number of adhered and invaded cells. The number of cells that invaded were determined using the gentamicin-protective assay (Friis *et al*., 2005), with some modifications. After non-adherent bacteria were removed, 200 µl MEM (supplemented with 50 µg/ml gentamycin and 50 µg/ml amoxicillin) was added to the Caco-2 cells and incubated at 37 °C for 2 h. Cells were washed twice with HBSS and disrupted by adding 100 µL 1 % (v/v) Triton X-100 to each well. Plates were shaken at 100 rpm at 37 °C for 10 min, serially diluted with HBSS, and plated on respective bacterial media. After 24 to 48 h of incubation, enumerated bacterial colonies were counted to determine the number of invaded bacteria. The number of cells adhered to Caco-2 cells were determined by subtracting the total invaded cells from the total adhered and invaded cell counts.

Inhibition of *S. enterica* invasion

Caco-2 cells were seeded in a 96-well plate (1 x 10^5 cells/well) and grown for 24 h at 37 °C under humidified atmosphere. Lactic acid bacteria (*L. johnsonii* DPN184, *L. salivarius* DPN181, *L. gallinarum* DPN164, *L. crispatus* DPN167 and *E. faecalis* DPN94) were inoculated in MRS broth (1%, v/v) and incubated at 37 °C under anaerobic condition for 24 h. *Bacillus amyloliquefaciens* DPN123 was inoculated in BHI broth (1 %, v/v) and incubated at 37 °C under aerobic conditions using a rotating wheel for 24 h. *Salmonella* Enteritidis 147 was inoculated in LB broth (1 %, v/v) and incubated at 37 °C under aerobic conditions using a rotating wheel for 24 h. Supernatants of overnight-grown probiotic cultures (*L. johnsonii* DPN184, *L. salivarius* DPN181, *L. gallinarum* DPN164, *E. faecalis* DPN94 and *B. amyloliquefaciens* DPN123) were collected by centrifugation (10 000 × *g*, 10 min, 4 °C), the pH adjusted to pH 7.5 with 1 M NaOH and filter sterilised using a 0.2 µm filter membrane (Merck). Sterilised probiotic supernatants were inoculated with *S.* Enteritidis 147 (1 × 10^7 CFU/ml) and incubated at 37 °C under aerobic conditions using a rotating wheel for 4 h. After incubation, bacterial cells were collected (8000 × *g*, 30 °C, 10 min) and suspended in MEM. Caco-2 cells were inoculated with *S.* Enteritidis (1 × 10^6 CFU/well) exposed to probiotic supernatants for 4 h. Prior to incubation, the microtiter plate containing the Caco-2 and bacterial cells were centrifuged (1500 × *g* for 10 min at 37 °C) to force cells closer to each other and incubated 37 °C for 3 h under humidified atmosphere. After incubation, Caco- 2 cells (1 x 10^5 cells) were washed three times with HBSS and 200 µl MEM (supplemented with 50 µg/ml gentamycin) added and incubated at 37 °C for 1 h under humidified atmosphere. Cells were washed three times with 200 µl HBSS, and cells lysed by adding 100 µl 1% (v/v) Triton-X-100 shaken at 50 rpm for 10 min with an orbital shaker. After shaking, cells were serially diluted, plated onto LB agar and incubated for 24 h at 37 °C. The efficiency at which *S.* Enteritidis invaded the Caco-2 cells was determined by comparing to untreated cells.

Changes in Caco-2 tight junctions

Caco-2 cells were suspended in MEM (10 % v/v FCS, 1 % v/v NEAA, 1 % v/v Na-pyruvate) and seeded at 1 x10^5 cells/well, containing a sterile glass disk and incubated at 37 °C at 5 % CO_2 atmosphere for 12 days. An overnight bacterial culture (10^9 CFU/ml) was centrifuged (8000 × *g* for 5 min at 37 °C) and suspended in MEM (10 % FCS, 1 % NEAA, 1 % Na-pyruvate). Bacteria were added to Caco-2 cells (1x10^6 CFU/well), centrifuged (1500 × *g* for 10 min at 37 °C), and incubated at 37 °C in 5 % CO_2 atmosphere for 4 h. After incubation, cells were washed twice with 200 µl HBSS.

Caco-2 cells were fixed to glass surfaces by submersing glass disks in 4 % (m/v) paraformaldehyde, dissolved in 0.1 M phosphate buffer (pH 7.2) for 10 min at 25 °C. After fixation, cells were washed three times with PBS and permeabilized by submersion into 0.2 % (v/v) Triton X-100, suspended in 0.1 M phosphate buffer (pH 7.2). After 5 min of incubation at 25 °C, cells were rinsed twice with PBS and incubated in the presence of 1 % (m/v) bovine serum albumin (BSA), suspended in 0.1 M phosphate buffer (pH 7.2) for 30 min at 25 °C to block non-specific binding sites. Cells were rinsed twice with PBS and incubated in the presence of 200 µg/ml anti-claudin 3 antibodies (Abcam, Cambridge, UK), suspended in 0.1 M phosphate buffer (pH 7.2), for 16 h at 4 °C. After incubation, cells were rinsed twice with PBS. Binding of the primary antibody was detected using the Dako REAL™

EnVision™ peroxidase/DAB detection kit (Agilent, California, USA), as per manufacturer's instructions. Cells were counter-stained with haematoxylin and visualized using a Nikon SMZ800 (Nikon Corporation, Tokyo, Japan) stereomicroscope, equipped with a Nikon DS-Fi1 digital camera (Nikon Corporation).

Changes in the proteome of ileal cells

The use of animals during this study was approved by the Ethical Commission of the Faculty of Veterinary Science and Bioscience Engineering of Ghent University, Belgium (EC2016/32). Overnight bacterial cultures were centrifuged (8000 × *g* for 5 min at 25 °C) and cells suspended in HBSS. The feed of 30-day old broilers (Ross 308) was withdrawn 12 h before the intestinal loop operational procedure, and water removed two hours prior. Broilers were anaesthetised using a mask with continues supply of oxygen (1.5-2 L/min) and isoflurane (1.5-3 %). The abdominal cavity was surgically opened with an incision of 2 inches. The ileum was exposed, and three loops were made, with small spacer loops in-between, using Vicryl 4/0 surgical suture (Figure 1). The first loop was injected with 1 ml *S*. *enterica* serovar Enteritidis 147 (6×10^8 CFU), the second loop with 1 ml of the multi-species probiotic (1×10^8 CFU *L*. *johnsonii* DPN184, 1×10^8 CFU *L*. *salivarius* DPN181, 1×10^8 CFU *L*. *crispatus* DPN167, 1×10^8 CFU *L*. *gallinarum* DPN164, 1×10^8 CFU *E*. *faecalis* DPN94 and 1×10^8 CFU *B*. *amyloliquefaciens* DPN123) and the third loop (negative control) with 1 ml HBSS. The ileum was repositioned in the abdominal cavity and the peritoneum, muscles and skin were sutured. The site of incision was covered with aluminium spray, and broilers were administered intramuscularly a dose of ketamine (10 mg/kg) and diazepam (2 mg/kg), 10 ml glucose solution, and awoken. After 6 h, broilers were euthanised by intravenous injection of an overdose of sodium pentobarbital. Respective loops were collected and rinsed in HBSS (4 °C) supplemented with cOmplete™ EDTA-free protease inhibitor cocktail (Roche, Holding AG, Basel, Switzerland).

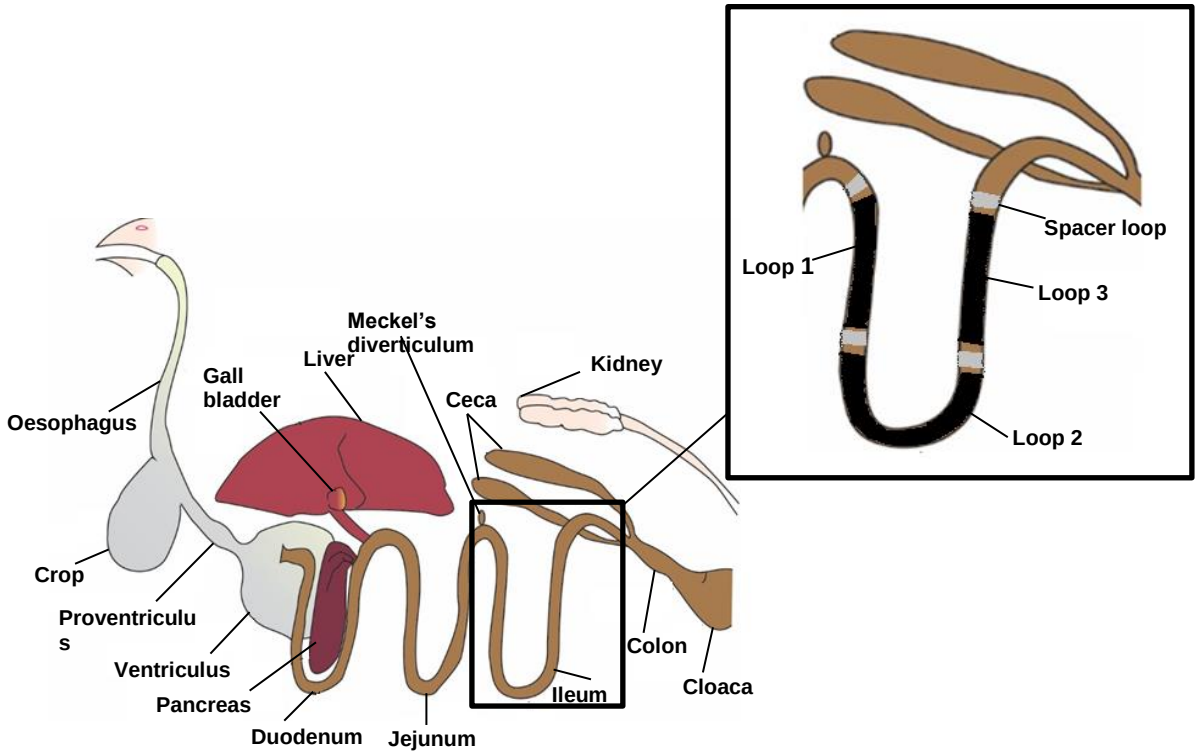

Figure 1: Graphical illustration of the intestinal loop experiment. Three loops were made in the ileum using surgical suture, with spacer loops in-between treatment loops.

The epithelial layer of the ileum was collected by scraping with sterile surgical blades and suspended in 8 M urea in 50 mM Tris-HCl, supplemented with protease inhibitor cocktail (pH 8). Cells were sonicated four times at 30 secs intervals (4 °C), at 40 % output power using the Omni-Ruptor 400 (OMNI international, Kennesaw, California). Tissue debris was removed by centrifugation (5000 × *g*, 10 min, 4 °C), and insoluble proteins removed at 16 000 × *g* for 30 min at 4 °C. The fraction containing soluble proteins were dialysed using Vivaspin 2 dialysis cassettes (Sartorius AG, Göttingen, Germany), with a molecular weight cut-off (MWCO) of 3 kDa, as per manufacturer's instructions. Protein extracts were loaded on the cassette and centrifuged (4000 × *g*, 1 h, 4 °C). The dialysed extracts were washed twice using 8 M urea (suspended in 50 mM ammonium bicarbonate, pH 8). Washed proteins were collected by centrifuging the dialysis cassettes upside-down (4000 × *g*, 2 min, 4 °C), and the protein concentration determined using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific). The disulphide bonds of proteins (50 µg) was reduced in the presence of 10 mM DTT (dithiothreitol), at 60 °C for 30 min. Proteins were acylated in the presence of 20 mM iodoacetamide, for 30 min at 25 °C in the dark and digested with Pierce™ trypsin protease (1:50 w/w ratio to protein) for 18 h at 37 °C. Digestion was terminated using formic acid (0.1 % v/v). Peptide samples were dried using a Savant™ SpeedVac™ high capacity concentrator (Thermo Fisher Scientific, Massachusetts, USA). Peptides were dissolved (0.5 µg/µl) in 15 µL of 2 % (v/v) acetonitrile with 0.1 % TFA. Samples were analysed via LC−MS/MS on an Ultimate 3000 RSLC nano LC (Thermo Fisher Scientific) in-line connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific). The sample mixture was first loaded on a trapping column (100 µm i.d. × 20 mm, 5 µm beads C18 Reprosil-HD, Dr. Maisch, Ammerbuch-

Entringen, Germany). After flushing from the trapping column, the sample was loaded on an analytical column (75 µm i.d. × 150 mm, 3 µm beads C18 Reprosil-HD, Dr. Maisch). Peptides were loaded with loading solvent (0.1 % v/v TFA in dH_2O) and separated with a linear gradient from 98% solvent A (0.1 % v/v formic acid in dH_2O) to 55 % solvent B (0.1 % v/v formic acid in dH_2O/acetonitrile, 20:80 v/v) in 30 min at a flow rate of 300 nL/min. This was followed by a 5 min wash step reaching 99 % solvent B.

The mass spectrometer was operated in data-dependent, positive ionisation mode, automatically switching between MS and MS/MS acquisition for the 10 most abundant peaks in each MS spectrum. The source voltage was 3.4 kV, and the capillary temperature was 275 °C. One MS1 scan (m/z 400−2000, AGC target 3×10^6 ions, maximum ion injection time 80 ms) acquired at a resolution of 70 000 (at 200 m/z) was followed by up to 10 tandem MS scans (resolution 17 500 at 200 m/z) of the most intense ions fulfilling predefined selection criteria (AGC target, 5×10^4 ions; maximum ion injection time, 60 ms; isolation window, 2 Da; fixed first mass, 140 m/z; spectrum data type, centroid; underfill ratio, 2 %; intensity threshold, 1.7×10^4; exclusion of unassigned, 1, 5−8, >8 charged precursors; peptide match preferred; exclude isotopes, on; dynamic exclusion time, 20 s). The HCD collision energy was set to 25 % normalised collision energy, and the polydimethylcyclosiloxane background ion at 445.120025 Da was used for internal calibration (lock mass).

Statistical and bioinformatic analysis

GraphPad Prism 6 (GraphPad Software Inc., California, USA) was used to perform statistical analyses. Data of adhesion and invasion abilities, cytotoxicity and inhibition of *Salmonella* invasion were analysed by one-way ANOVA to determine the significance of the main effects and interactions. The mean variances were compared using the Turkey test. Differences were considered significant if p values were less than 0.05. Bioinformatics analyses were performed using MaxQuant (Tyanova *et al*., 2015) and Perseus (Tyanova *et al*., 2016) software. The LC-MS/MS raw data were processed with MaxQuant (version 1.6.0.1) and the peptides were identified from the MS/MS spectra searched against the UniProt database (*Gallus gallus*, UP000000539) using Andromeda (version 1.5.6.0) search engine (Cox *et al*., 2011). The following peptide bond cleavages: arginine or lysine followed by any amino acid, and up to two missed cleavages were allowed. Cysteine carbamidomethylation was set as a fixed modification. Unique and razor peptides and acetylated and/or oxidised peptides were quantified. The false discovery rate for peptide identification was calculated with a reversed-sequence decoy library and set to 0.01.

In Perseus (version 1.6.0.7) (Tyanova *et al*., 2016), data was filtered to remove potential contaminants, reverse decoy sequences, and proteins only identified by site. Peptides were removed that were only quantified in two of the five biological replicates. Missing values were imputed based on a normal distribution (width 0.3, down-shift 1.8), and LFQ values log2 transformed. ANOVA analysis was performed on LFQ intensity log2 ratios to determine differentially accumulated proteins ($p > 0.05$). In Perseus a heatmap of differentially accumulated proteins were generated with hierarchical clustered using Euclidean distance. Heatmap colours are based on the combined Z-scored (log2) LFQ values. Multivariate analyses were carried out by an untargeted principal component analysis (PCA), the obtained data was visualized using XLSTAT (XLSTAT, New York, USA). Differentially accumulated

proteins were further investigated using STRING 10.5 (Szklarczyk *et al*., 2017) to analyse protein-protein associations using the Markov Cluster Algorithm (MCL) and gene ontology information. Gene pairs that were either coexpressed or involved in experimentally validated protein–protein interactions with a medium to high score (score ≥ 0.4) are reported.

Results and Discussion

The GIT is densely populated with microorganisms that closely interact with the host. Understanding how different genera interact with the host will provide insight into methods to control detrimental microbiome shifts during diseases development. Cytotoxicity of pathogenic and probiotic cells towards Caco-2 cells were determined at varying MOI levels (Figure 2). At very high MOI levels (1:1000, Caco-2: bacteria) *S*. Enteritidis 147 and *E*. *faecalis* DPN94 were more cytotoxic than *L*. *crispatus* DPN167, *L*. *gallinarum* DPN164, *B*. *amyloliquefaciens* DPN123, *L*. *johnsonii* DPN184 and *L*. *salivarius* DPN181 (Figure 2). *Lactobacillus johnsonii* DPN184 and *L*. *salivarius* DPN181 were more cytotoxic towards Caco-2 cells than *L*. *crispatus* DPN167. At high MOI levels (1:100) *S*. Enteritidis was more cytotoxic towards Caco-2 cells than any of the probiotic strains (Figure 2). *Enterococcus faecalis* DPN94 was more cytotoxic than *L*. *crispatus* DPN167. At medium MOI levels (1:10), *S*. Enteritidis 147 was more cytotoxic than any of the probiotic strains, except for *E*. *faecalis* DPN94. *Lactobacillus gallinarum* DPN164, *L*. *johnsonii* DPN184 and *E*. *faecalis* DPN94 were more cytotoxic than *L*. *crispatus* DPN167 (Figure 2). At low MOI levels (1:1) *S*. Enteritidis 147 was more cytotoxic towards Caco-2 cells than *L*. *crispatus* DPN167 and *L*. *johnsonii* DPN184. *Lactobacillus gallinarum* DPN164, *B*. *amyloliquefaciens* DPN123, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94 and *L*. *salivarius* DPN181 were more cytotoxic than *L*. *crispatus* DPN167. *Enterococcus faecalis* DPN94 were more cytotoxic than *L*. *johnsonii* DPN184 and *L*. *crispatus* DPN167 at low MOI levels (Figure 2). Increase in MOI levels did not result in increased cytotoxicity for probiotic strains *L*. *gallinarum* DPN164, *B*. *amyloliquefaciens* DPN123 and *L*. *salivarius* DPN181. Increase in MOI levels for *S*. Enteritidis 147 resulted in increased cytotoxicity ($p < 0.05$). Probiotic bacterium *L*. *crispatus* DPN167 was less cytotoxic at MOI 1:1 than at MOI 1:1000 and 1:100 ($p < 0.05$). *Lactobacillus johnsonii* DPN184 was less cytotoxic at MOI 1:1 than at MOI 1:10, 1:100 and 1:1000 ($p < 0.05$). *Enterococcus faecalis* DPN94 cells were more cytotoxic at MOI 1:10 than MOI 1:1 and were more cytotoxic at MOI 1000:1 than MOI 1:10 ($p < 0.05$).

Probiotic strains *L*. *gallinarum* DPN164, *B*. *amyloliquefaciens* DPN123, *L*. *johnsonii* DPN184, *L*. *crispatus* DPN167 and *L*. *salivarius* DPN181 showed low cytotoxicity towards Caco-2 cells at different MOI levels. At very high MOI levels (1:1000), *E*. *faecalis* DPN94 was highly cytotoxic towards Caco- 2 cells, however, cytotoxicity decreased with the lowering of MOI levels. *Enterococcus faecalis* DPN94 was more cytotoxic than other lactic acid bacteria (*L*. *johnsonii* DPN184, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164 and *L*. *crispatus* DPN167). *Enterococcus faecalis* DPN94 is facultatively anaerobic whereas the other LAB strains are anaerobic in nature. This enables *Enterococcus* cells to be metabolically active in the presence of Caco-2 cells, leading to the production of metabolites such as lactic acid and acetic acid which lowers the extracellular pH, leading to cell death. Cytotoxicity of pathogenic *S*. Enteritidis 147 towards Caco-2 cells increased with increasing MOI levels. The

cytotoxicity is undoubtedly due to cell membrane destruction during adhesion and invasion (Shah *et al*., 2011).

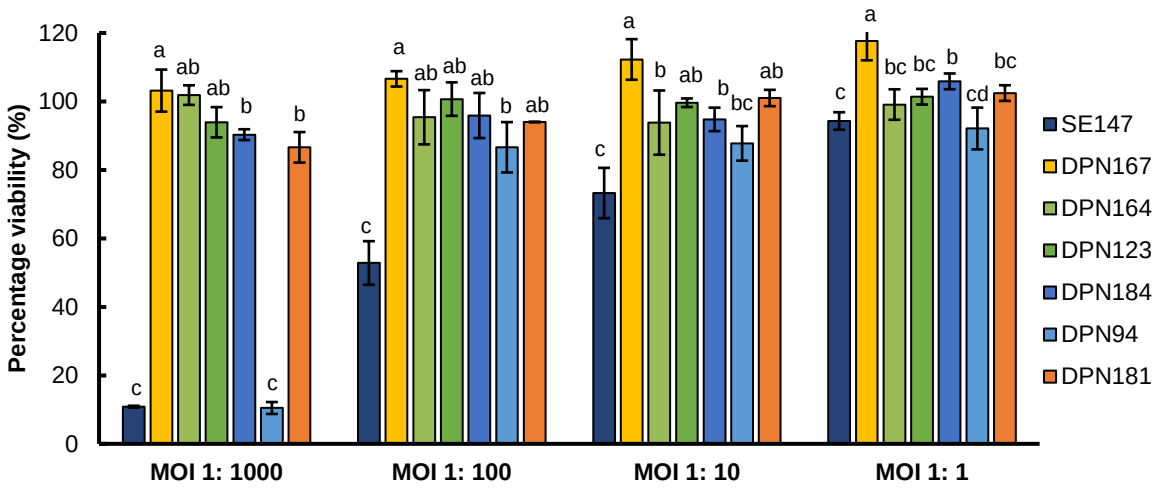

Figure 2: Cytotoxicity of bacterial cells towards Caco-2 cells at varying multiplicity of infection (MOI) levels. SE147- *S*. Enteritidis 147, DPN167- *L*. *crispatus*, DPN164- *L*. *gallinarum*, DPN123- *B*. *amyloliquefaciens*, DPN184- *L*. *johnsonii*, DPN94- *E*. *faecalis* and DPN181- *L*. *salivarius*. Error bars indicate standard deviations, and bars with different superscripts (a, b, c) differ significantly ($p < 0.05$).

Cytotoxicity of metabolites produced by pathogenic and probiotic bacteria towards Caco-2 cells is shown in Figure 3. The cytotoxicity of untreated supernatant from *S*. Enteritidis and *B*. *amyloliquefaciens* DPN123 were significantly lower than that from *L*. *crispatus* DPN167, *L*. *gallinarum* DPN164, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94 and *L*. *salivarius* DPN181 (Figure 3). The concentration of SCFA produced by bacterial strains were determined by GC-MS (Table 1). All LAB produced high levels of acetic acid (1946-2187 mg/L) and lactic acid (3427-3909 mg/L) (Table 1). Pathogenic *S*. Enteritidis 147 produced low levels of lactic acid, propionic acid and butyric acid (Table 1). Isovaleric acid was only produced by *B*. *amyloliquefaciens* DPN123 and *S*. Enteritidis 147. After pH adjustment, the cell-free supernatant of *L*. *salivarius* DPN181 was more cytotoxic than that of the other bacterial strains (Figure 3). In addition, pH-adjusted supernatant from *L*. *crispatus* DPN167 and *L*. *gallinarum* DPN164 were more cytotoxic than that of *B*. *amyloliquefaciens* DPN123, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94 and *S*. Enteritidis 147. After catalase treatment, the cell-free supernatant of *L*. *salivarius* DPN181 was more cytotoxic than that of *L*. *gallinarum* DPN164, *B*. *amyloliquefaciens* DPN123, *L*. *johnsonii* DPN184 and *S*. Enteritidis 147. In addition, cytotoxicity of *L*. *crispatus* DPN167 was higher than that of *L*. *johnsonii* DPN184. After pronase treatment, the cytotoxicity of supernatant from *L*. *salivarius* DPN181 was significantly higher than that of all bacterial strains (Figure 3). In addition, catalase treated supernatant from *L*. *crispatus* DPN167 was more cytotoxic than that of *B*. *amyloliquefaciens* DPN123, *L*. *johnsonii* DPN184, *L*. *salivarius* DPN181 and *S*. Enteritidis 147.

Cytotoxicity of untreated supernatant from LAB were due to high levels of lactic and acetic acid, which decreases the extracellular pH leading to Caco-2 cell death. Cytotoxicity of SCFA *in vitro* is not

a true reflection of its effect and role *in vivo*. The membrane of eukaryotic cells contains SCFA receptors, and these molecules acts as signalling molecules between the microbiome and the host (Morrison and Preston, 2016). In addition, SCFA plays important roles in local, intermediary and peripheral metabolism, and modulate immune development (Corrêa *et al*., 2016; Morrison and Preston, 2016). The untreated supernatants of all bacterial strains were more cytotoxic towards Caco-2 cells than the respective pH-adjusted supernatants ($p < 0.05$). Pronase and catalase treatment did not alter the cytotoxicity of *L*. *crispatus* DPN167, *L*. *gallinarum* DPN164 and *L*. *johnsonii* DPN184 compared to pH-adjusted supernatants. Catalase treatment increased cytotoxicity of *S*. Enteritidis 147, *B*. *amyloliquefaciens* DPN123 and *E*. *faecalis* DPN94 as compared to their respective pH adjusted supernatants ($p < 0.05$). Pronase treatment decreased cytotoxicity of *L*. *salivarius* DPN181, and increased cytotoxicity of *S*. Enteritidis 147 as compared to their respective pH adjusted supernatants ($p < 0.05$). Cytotoxicity recorded after pH adjustment, catalase and pronase treatment is probably due to membrane components such as peptidoglycan, polysaccharide and glycoproteins which known to be cytotoxic towards Caco-2 cells (Oda *et al*., 1983; Fichera and Giese, 1994).

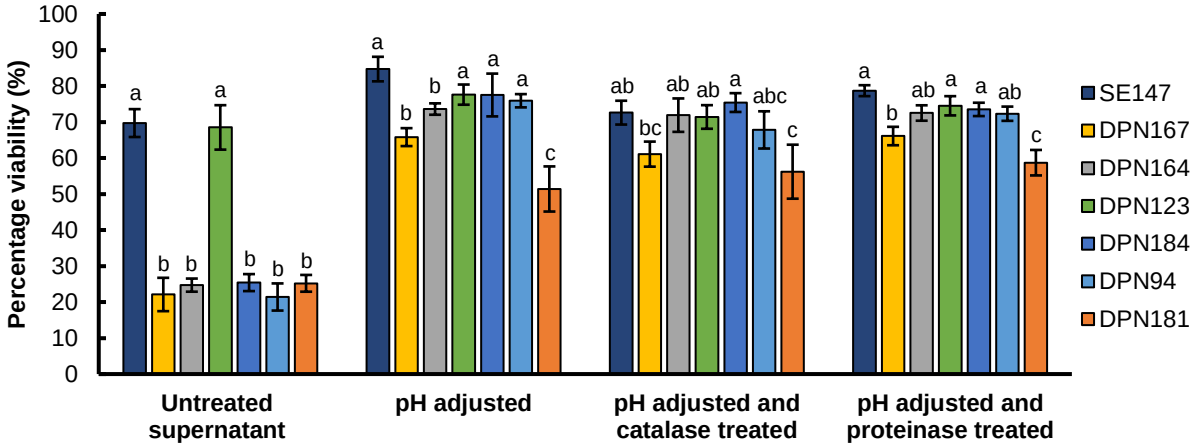

Figure 3: Cytotoxicity of bacterial supernatant (i.e. untreated supernatant; pH 7.5 adjusted; pH adjusted, and catalase treated; and pH adjusted, and proteinase treated) towards Caco-2 cells. SE147- *S*. Enteritidis 147, DPN167- *L*. *crispatus*, DPN164- *L*. *gallinarum*, DPN123- *B*. *amyloliquefaciens*, DPN184- *L*. *johnsonii*, DPN94- *E*. *faecalis* and DPN181- *L*. *salivarius*. Error bars indicate standard deviations, and bars with different superscripts (a, b, c) differ significantly ($p < 0.05$).

Table 1: Concentration (standard deviation) of short chain fatty acids (SCFA) i.e. acetic, propionic, butyric, isovaleric and DL-lactic acid produced by bacterial strains.

	Short chain fatty acids (mg/l)				
	Acetic acid	Propionic acid	Butyric acid	Isovaleric acid	DL-Lactic acid
S. Enteritidis 147	438.7 (5.7)	163.6 (4.3)	6.9 (0.2)	8.1 (0.5)	11.3 (12.1)
L. *crispatus* DPN167	2030.1 (71.9)	0	0	0	3449.7 (77.3)
L. *gallinarum* DPN164	2186.7 (20.2)	0	0	0	3850.3 (170.8)
B. *amyloliquefaciens* DPN123	1236.5 (14.9)	0	0	32.5 (1.2)	695.4 (21.9)
L. *johnsonii* DPN184	2034.8 (46.4)	0	0	0	3741.6 (11.1)
E. *faecalis* DPN94	2053.6 (29.5)	0	0	0	3426.8 (133.5)
L. *salivarius* DPN181	1945.6 (17.2)	0	0	0	3908.8 (30.3)

The ability of pathogenic and probiotic bacteria in adhering and invading Caco-2 cells is shown in Figure 4. Pathogenic *S*. Enteritidis 147 had higher ability to adhere to Caco-2 than any of the probiotic strains. Probiotic bacterium *L*. *crispatus* DPN167 had higher ability to adhere to Caco- 2 cells than *L*. *gallinarum* DPN164, *B*. *amyloliquefaciens* DPN123, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94, and *L*. *salivarius* DPN181 (Figure 4). In addition, *E*. *faecalis* DPN94 had a higher ability to adhere to Caco-2 cells than *L*. *gallinarum* DPN164 and *B*. *amyloliquefaciens* DPN123, *L*. *johnsonii* DPN184 and *L*. *salivarius* DPN181. Invasion of Caco-2 cells by *S*. Enteritidis 147 was significantly higher than that of the probiotic strains (Figure 4). *Salmonella* Enteritidis 147 adhered (75.37 % ± 6.95) and invaded (14.61 % ± 5.54) Caco- 2 cells at high efficiency, whereas probiotic bacteria adhered to Caco-2 cells with varying efficiency (16.30-58.29 %) but did not invade Caco-2 cells (0.002-0.2 %). Probiotic cells that remain after antibiotic treatment, are probably due to the ineffectiveness in killing all bacteria after 2 h treatment. Pathogenic *Salmonella* adheres and invade epithelial cells with high efficiency due to encoding for several virulence factors such as lipopolysaccharide, flagella, fimbriae, and type III secretion systems (T3SS) (Schmidt and Hensel, 2004; Haiko and Westerlund-Wikström, 2013). The T3SS have been shown to be important during infection as these proteins manipulate host cell cytoskeleton to facilitate invasion (Coombes *et al*. 2005; Patel and Galán, 2005; Boyen *et al*. 2006; Coburn *et al*., 2007). Recently an alternative T3SS-1-independent invasion pathway has been elucidated, in which outer membrane proteins Rck and PagN invasins mediates a Zipper-like entry into the host cell (Velge *et al*., 2012).

The exact mechanisms by which probiotic bacteria adhere to epithelial cells remains to be elucidated. It is thought that bacteria initially adhere to the epithelial surface by nonspecific physical interactions (i.e. steric and hydrophobic interactions), which result in reversible attachment to the cell surface (Wang *et al*., 2017). Researchers are indifferent on the role cell surface hydrophobicity plays in adhesion to the mucosal surface, however, it is believed that that high cell surface hydrophobicity results in high adhesion ability (Kos *et al*., 2003; Muñoz-Provencio *et al*., 2009). Numerous adhesion mechanisms and molecules have been identified in the probiotic bacteria which assists in adhesion such as surface-layer (S-layer) proteins (Ljungh and Wadstrom, 2006), cell wall-anchored mucus-

binding proteins (Roos and Jonsson, 2002; Buck *et al*., 2005; Boekhorst *et al*., 2006), cell-surface collagen-binding proteins (Aleljung *et al*., 1994), mannose-specific adhesins (Pretzer *et al*., 2005), carbohydrate-cell surface proteins (Spillmann and Burger, 1996) and mucus-binding pilin (Kankainen *et al*., 2009).

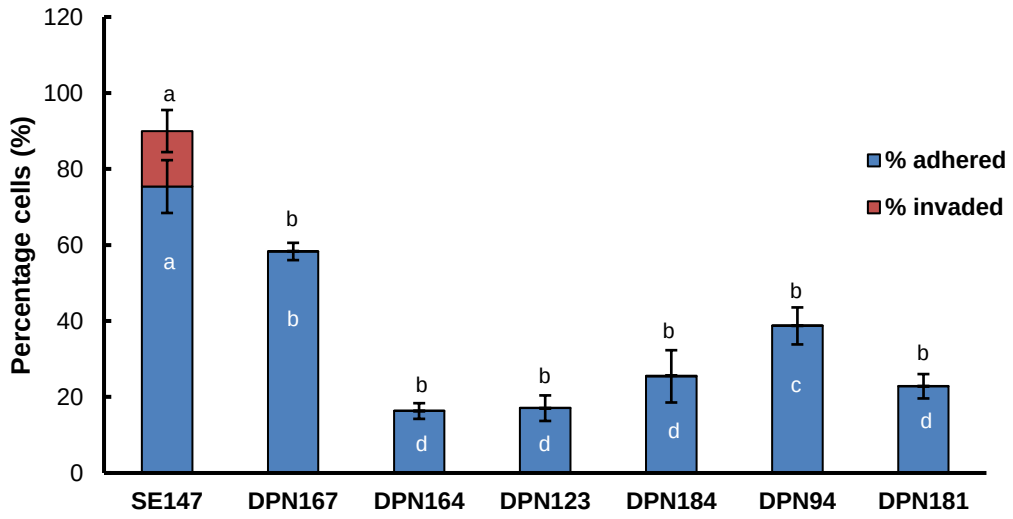

Figure 4: Percentage bacterial cells that invaded and adhered to Caco-2 cells. SE147- *S*. Enteritidis 147, DPN167- *L*. *crispatus*, DPN164- *L*. *gallinarum*, DPN123- *B*. *amyloliquefaciens*, DPN184- *L*. *johnsonii*, DPN94- *E*. *faecalis* and DPN181- *L*. *salivarius*. Error bars indicate standard deviations. Bars with different superscripts (a, b, c) differ significantly ($p < 0.05$), white subscripts indicate differences in adhesion and black differences in invasion data.

The ability of probiotic metabolites in inhibiting *S*. Enteritidis invasion is shown in Figure 5. Invasion efficiency of *S*. Enteritidis 147 significantly decreased after exposure to metabolites originating from *L*. *johnsonii* DPN184, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *E*. *faecalis* DPN94 and *B*. *amyloliquefaciens* DPN123. Probiotic strains probably decreased *Salmonella* invasion due to the production of antimicrobial compounds such as SCFA, hydrogen peroxide and lipopeptides (Neveling *et al*., 2017). Short-chain fatty acids have strong inhibitory effects against Gram-negative bacteria (Alakomi *et al*., 2000; Makras *et al*., 2006). Undissociated acids enters the cell and dissociates inside the cytoplasm, leading to lowering of the intracellular pH or accumulation of the ionized form, leading to cell death (Ouwehand, 1998; Russell and Diez-Gonzalez, 1998). In addition, metabolites from the LAB have been shown to decrease *Salmonella* infections chickens by modulating virulence gene expression (Yang *et al*., 2014a; Muyyarikkandy and Amalara, 2017).

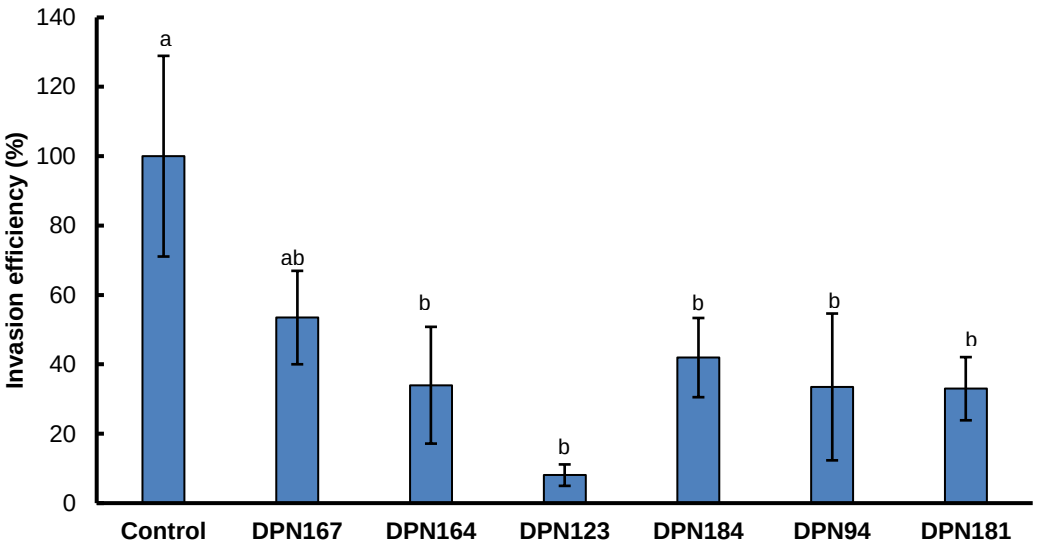

Figure 5: *Salmonella* Enteritidis 147 invasion efficacy after exposure to bacterial metabolites originating from control (untreated *S*. Enteritidis 147 cells), DPN167- *L*. *crispatus*, DPN164- *L*. *gallinarum*, DPN123- *B*. *amyloliquefaciens*, DPN184- *L*. *johnsonii*, DPN94- *E*. *faecalis* and DPN181- *L*. *salivarius*. Error bars indicate standard deviations, and bars with different superscripts (a, b, c) differ significantly ($p < 0.05$).

Claudins are proteins that form either paracellular barrier or pores which control cell permeability (Furuse *et al*., 1998; Chiba *et al*., 2008). The effect of pathogenic and probiotic bacteria on claudin-3 TJ in Caco-2 cells were assessed to determine bacterial strains ability to disrupt monolayer integrity (Figure 6). Untreated Caco-2 cells were used as control. Bacterial strains had varying degrees of TJ loss. Probiotic bacteria showed lower levels of claudin-3 TJ loss as compared to *S*. Enteritidis. Probiotic bacteria decreased claudin-3 TJ, however, did not disrupt perijunctional actin, and cell contraction and monolayer disruption were absent. Probiotic bacteria decreased TJ by producing SCFA, hydrogen peroxide and antimicrobial lipopeptides. Pathogenic *S*. Enteritidis 147 disrupted claudin-3 TJ and extracellular matrix destruction was evident. *Salmonella* causes actin cytoskeleton reorganisation during invasion leading to monolayer destruction (Jepson *et al*., 1995; Haraga *et al*. 2008).

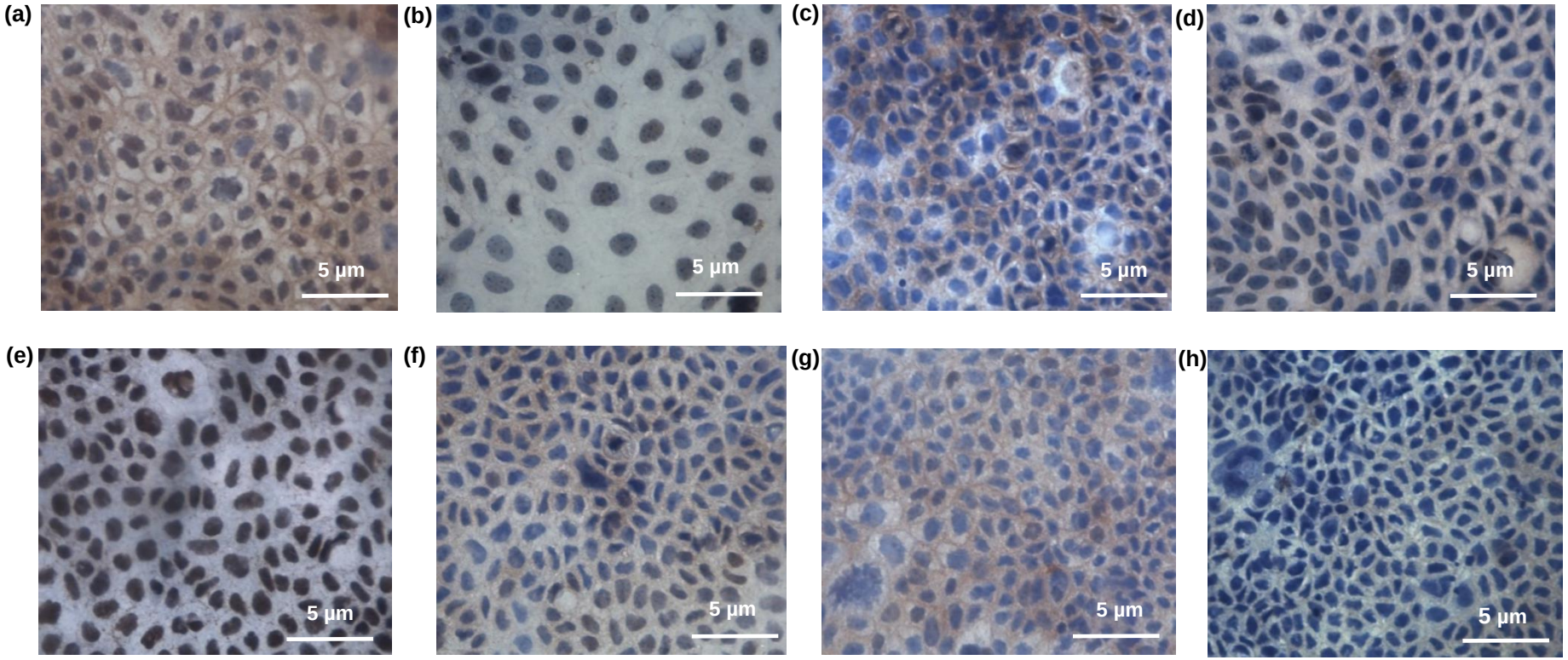

Figure 6: Claudin-3 tight junctions of Caco-2 cells after exposure to (a) untreated, (b) *S*. Enteritidis 147, (c) *L*. *crispatus* DPN167, (d) *L*. *gallinarum* DPN164, (e) *B*. *amyloliquefaciens* DPN123, (f) *L*. *johnsonii* DPN184, (g) *E*. *faecalis* DPN94 and (h) *L*. *salivarius* DPN181.

Following proteomic analysis, 432 proteins were identified in *Salmonella* treated epithelial cells and among them 8 proteins were found to be up-regulated and 3 proteins down-regulated (Table 2) as compared to control epithelial cells (untreated). In epithelial cells exposed to the multi-species probiotic, 456 proteins were identified of which 3 proteins were upregulated and 4 proteins were down regulated (Table 3) as compared to the control group.

Table 2: Differentially expressed proteins in broilers epithelial cells after exposure to *S*. *enterica* serovar Enteritidis 147.

Accession	Peptides	Score	Anova (p)*	Fold (Log2)	Average Normalised Abundances		Protein names
					Control	Salmonella	
LYSC_CHICK	11	171.67	0.006	1.14	2,56E+07	2,91E+07	Lysozyme C
CTHL2_CHICK	7	126.49	0.011	1.15	2,58E+07	2,96E+07	Cathelicidin-2
MIM1_CHICK	17	323.31	0.017	1.13	2,96E+07	3,33E+07	Myeloid protein 1
ISK1L_CHICK	5	72.591	0.033	1.11	2,54E+07	2,81E+07	Trypsin inhibitor ClTI-1
CTHL3_CHICK	6	200.35	0.030	1.11	2,78E+07	3,08E+07	Cathelicidin-3
GLL2_CHICK	3	16.389	0.023	1.10	2,65E+07	2,93E+07	Gallinacin-2
LYG_CHICK	5	41.498	0.024	1.07	2,55E+07	2,74E+07	Lysozyme G
UFM1_CHICK	3	17.222	0.043	1.06	2,47E+07	2,61E+07	Ubiquitin-fold modifier 1
GLRX1_CHICK	3	47.144	0.048	0.99	2,78E+07	2,75E+07	Glutaredoxin-1
GLL7_CHICK	2	19.97	0.020	0.11	2,51E+07	2,76E+06	Gallinacin-7
VIGLN_CHICK	5	30.395	0.027	0.10	2,65E+07	2,54E+06	Vigilin

Table 3: Differentially expressed proteins in broilers epithelial cells after exposure to the multi-species probiotic (*L*. *crispatus* DPN167, *L*. *gallinarum* DPN164, *L*. *salivarius* DPN181, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94 and *B*. *amyloliquefaciens* DPN123).

Accession	Peptides	Score	Anova (p)*	Fold	Average Normalised Abundances		Protein names
					Control	Probiotic	
A0A1D5PDK5_CHICK	4	32.98	0.049	1.14	2,24E+07	2,54E+07	Transgelin-2/Transgelin-3
EF1B_CHICK	1	6.14	0.049	1.06	2,32E+07	2,46E+07	Elongation factor 1-beta
E1C937_CHICK	1	6.14	0.041	1.05	2,36E+07	2,48E+07	Anterior gradient 2
A0A1D5Q004_CHICK	1	6.07	0.037	0.96	2,42E+07	2,32E+07	Carnitine O-acetyltransferase
F1NJ73_CHICK	2	11.78	0.049	0.94	2,41E+07	2,26E+07	Adenylate kinase 2
SODC_CHICK	2	13.80	0.049	0.93	2,49E+07	2,32E+07	Superoxide dismutase [Cu-Zn]
F2Z4L4_CHICK	1	6.58	0.018	0.11	2,31E+07	2,44E+06	Protein SET

Principle components analysis (PCA) biplots of differentially expressed proteins in epithelial cells of the different treatment groups were used to determine similarity between biological repeats and associations of proteins with treatment groups (Figure 7). Biplot of *Salmonella* treated cells formed two separate clusters (Figure 7a), i.e. *Salmonella* and control treated, and biological repeats were highly similar within treatments. Epithelial cells exposed to *Salmonella* were associated with the presence of

myeloid protein 1, cathelicidin-2 and 3, lysozyme C and G, gallinacin-2 and 7, ubiquitin-fold modifier 1 and trypsin inhibitor ClTI-1. The control treated birds were associated with glutaredoxin-1 and vigilin. The PCA biplot of probiotic treated cells compared to control cells formed three clusters (Figure 7b). The first cluster consisted of control treated cells (con 1-5) and were associated with adenylate kinase 2, superoxide dismutase [Cu-Zn] and carnitine O-acetyltransferase. The second cluster consisted of probiotic treated cells (pro 2 and 3) and were associated with transgelin-2/3. The third cluster consisted of probiotic treated cells (pro 1, 4 and 5) were associated with the presence of protein SET, elongation factor 1-beta and anterior gradient 2. A heat-map plot of hierarchical clustering of differentially expressed protein intensities were generated to discern global proteomic changes (Figure 8). As indicated in the diagrams of *Salmonella* vs control treated, three clusters were present (Figure 8a). The cluster 1 (con 2, con 3, con 4 and con 5), cluster 2 (sal 2), cluster 3 (sal 1, sal 3, sal 4, sal 5 and con 1) (Figure 8a). Results indicate that *Salmonella* exposure had a profound effect on ileum epithelial proteome and the treatment could be differentiated from the control group. Moreover, the protein expression patterns within each cluster was similar. Heatmap comparison of the multi-species probiotic and control groups formed three cluster; cluster 1 (pro 2 and pro 3), cluster 2 (con 1, con 3, pro 1, con 2 and con 4) and cluster 3 (pro 4, con 5 and pro 5) (Figure 8b). Differentially expressed proteins in epithelial cells exposed to the multi-species probiotic could be used to distinguish treatment from the control group. Based on the heat map and PCA plots, treatments formed separate clusters from the control group, and differentially expressed proteins could be used to differentiate treatments from each other.

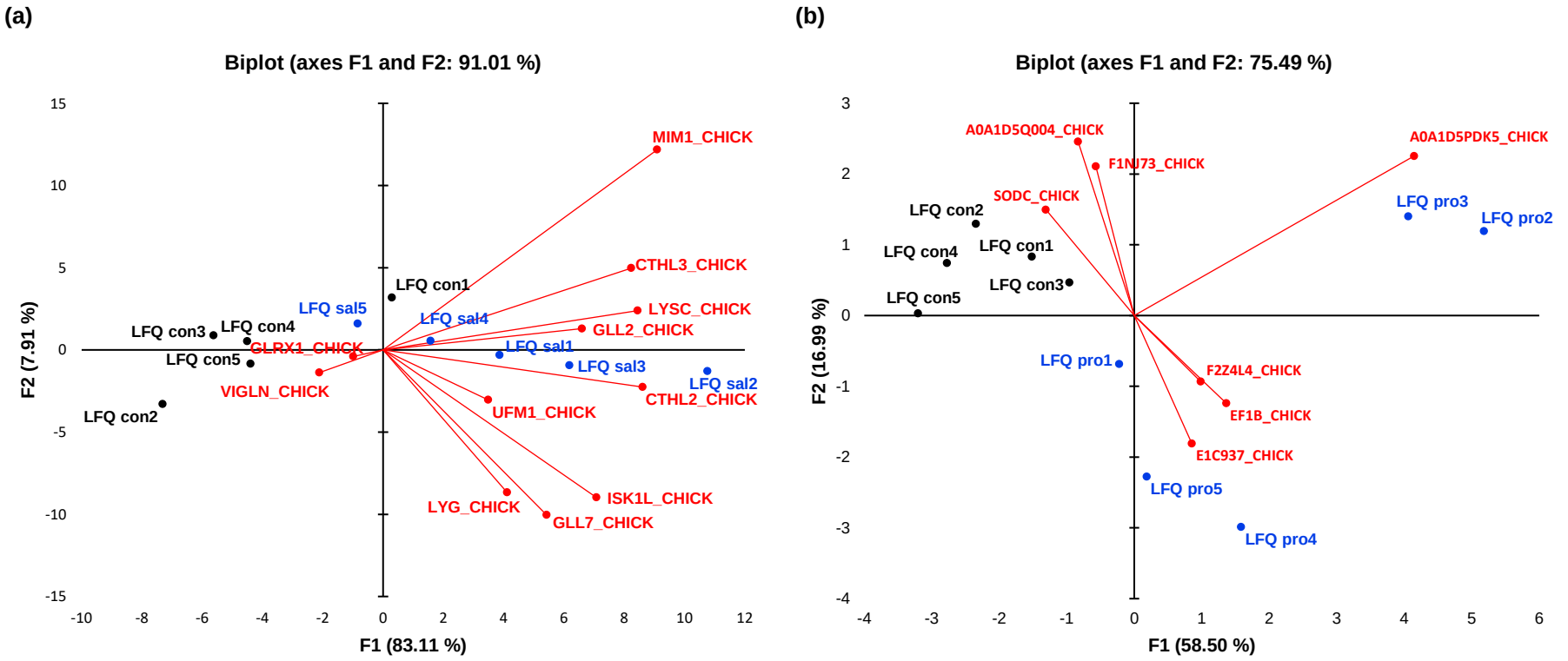

Figure 7: Principle components analysis biplots of differentially expressed proteins in broiler epithelial cells exposed (a) *Salmonella* Enteritidis 147 and the (b) multi-species probiotic (*L*. *crispatus* DPN167, *L*. *gallinarum* DPN164, *L*. *salivarius* DPN181, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94 and *B*. *amyloliquefaciens* DPN123), as compared to the control (untreated) expression levels.

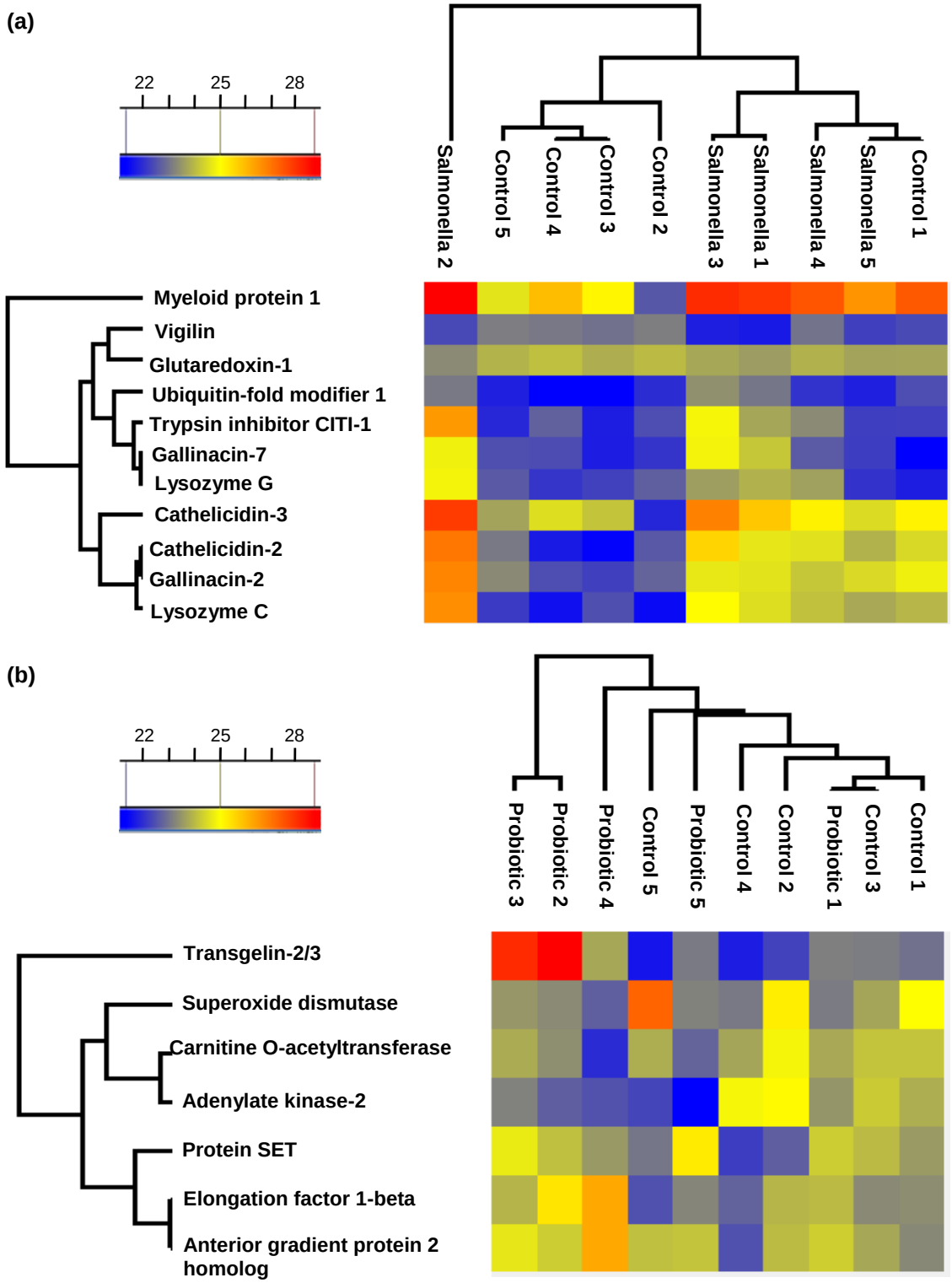

Figure 8: Heat-map of proteins differentially accumulated in the epithelial cells of broilers in response to (a) *S*. Enteritidis 147 and (b) the multi-species probiotic. Proteins differentially accumulated between treatment and control groups were identified using the label-free quantification (LFQ) method (ANOVA, $p < 0.05$). Euclidean distance clustering was used to visualize variations in the protein abundance between the samples and is shown as normalised LOG2 LFQ intensities in a heat map.

Proteins usually do not work alone, but rather interact with other proteins. Evaluation of protein-protein interactions provides additional information about the proteins systematic function. Protein interaction networks of differentially expressed proteins were analysed using STRING software (Huang *et al*., 2009). In *Salmonella* treated epithelial cells, only 3 differentially expressed proteins did not form part of the interactive network (*SPINK2*, *LYG1* and *HDLBP*). Interactive network consisted of 20 nodes and 36 edges (Figure 9a), which formed three clusters consisting of cluster 1 (*GAL2*, *5* and *7*; *DEFB1*, *CATHL2*, *LYZ*, *LECT2*, *MYBL1* etc.), cluster 2 (*GLRX* and *TXNDC3*) and cluster 3 (*UFSP2*, *UFM1* and *UBXN7*). These proteins were involved in biological processes such as defence responses to bacteria, multi-organism processes, response to stress, biological processes, response to stimulus, cytolysis, biological regulation, metabolic processes, defence to gram-negative bacteria, cellular processes and macromolecule metabolic processes (Figure 9b). Functions of these proteins included lysozyme activity, chromatin binding activity and molecular functions. In broiler epithelial cells exposed to the multi-species probiotic, only 1 differentially expressed protein did not form part of the interactive network (*AGR2*). The interactive network consisted of 15 nodes and 30 edges and formed a single cluster (Figure 10). These proteins had unknown biological and molecular functions, however, were involved in biological pathways such as ribosome, purine metabolism, pyrimidine metabolism and metabolic pathways (Figure 10b).

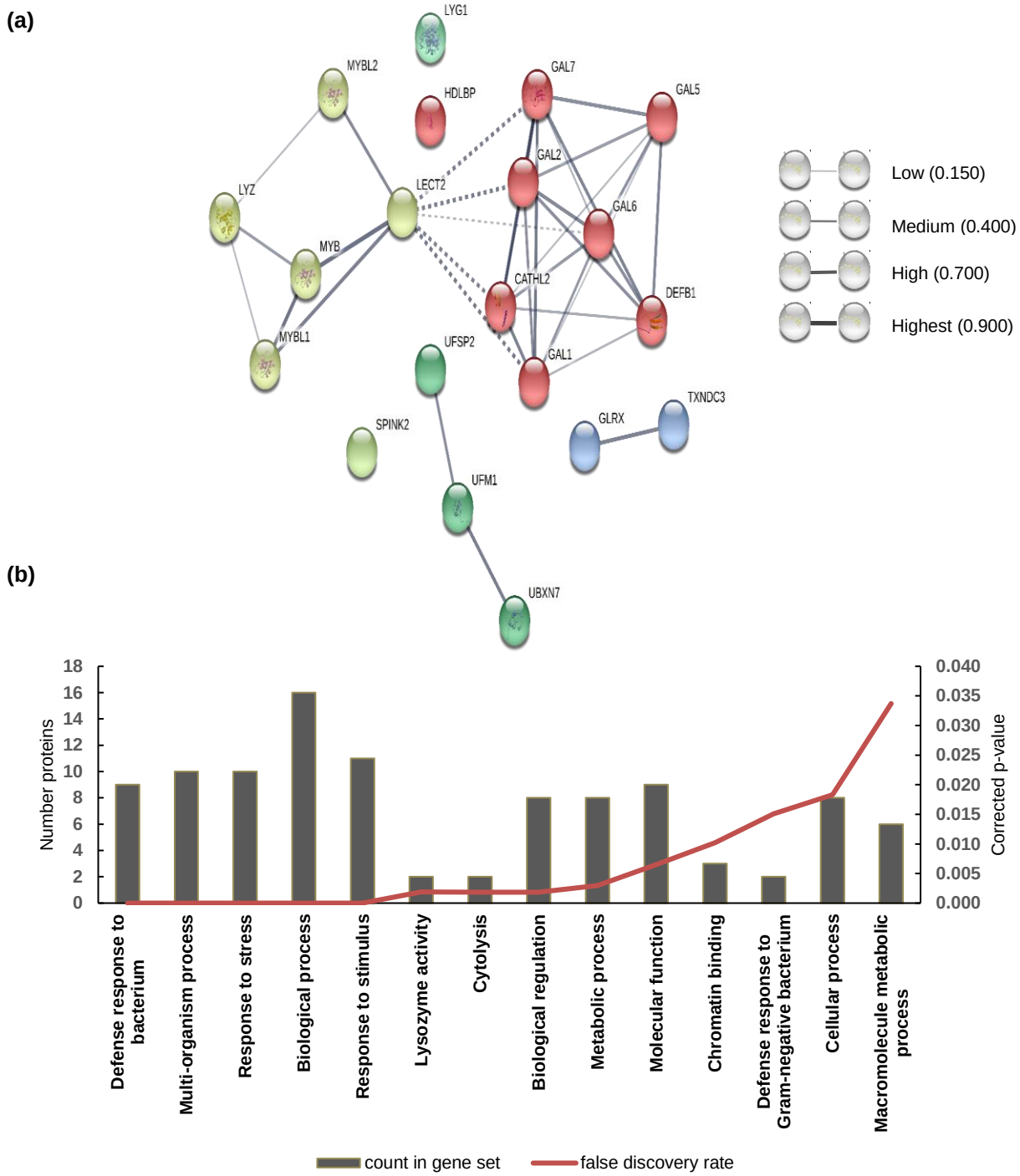

Figure 9: STRING network analysis (a) of differentially expressed proteins s in broiler ileum epithelial cells after exposure to *S*. Enteritidis 147 with two added nodes of predicted interacting proteins. In the network, nodes represent proteins, and lines with different shading represent different levels of predicted association and dash-lines represent inter-cluster edges. Ontology analysis (b) of identified proteins at a depth of biological process and molecular function.

Table 4: STRING analysis protein node classification, UniProt ID and protein name for differentially expressed and predicted interacting proteins in epithelial cells exposed to *S*. Enteritidis 147.

Input		
Gene name	**UniProt ID**	**Protein name**
HBLBP	VIGLN_CHICK	Vigilin (1267 aa)
LECT2	MIM1_CHICK	Myeloid protein 1 (327 aa)
LYZ	LYSC_CHICK	Lysozyme C (147 aa)
SPINK2	ISK1L_CHICK	Serine peptidase inhibitor (Kazal type 2)
GLRX	GLRX1_CHICK	Glutaredoxin-1 (60 aa)
LYG1	LYG_CHICK	Lysozyme g precursor (211 aa)
CATHL2	CTHL2_CHICK	Cathelicidin-2 (154 aa)
GAL2	GLL2_CHICK	Gallinacin-2
GAL7	GLL7_CHICK	Gallinacin-7 (67 aa)
UFM1	UFM1_CHICK	Ubiquitin-fold modifier 1 (85 aa)
Predicted functional partners		
Gene name	**UniProt ID**	**Protein name**
MYB	MYB_CHICK	Transcriptional activator Myb
GAL1	GLL1_CHICK	Antimicrobial peptide CHP1 (74 aa)
UBXN7	N.A.	Uncharacterised protein (492 aa)
MYBL1	MYBA_CHICK	Myb-related protein A
DEFB1	GLL3_CHICK	Gallinacin-3 (80 aa)
TXNDC3	N.A.	Uncharacterised protein (592 aa)
GAL6	GLL6_CHICK	Gallinacin-6 (67 aa)
MYBL2	MYBB_CHICK	Myb-related protein B
UFSP2	UFSP2_CHICK	Ufm1-specific protease 2
GAL5	GLL_CHICK	Gallinacin-5 (66 aa)

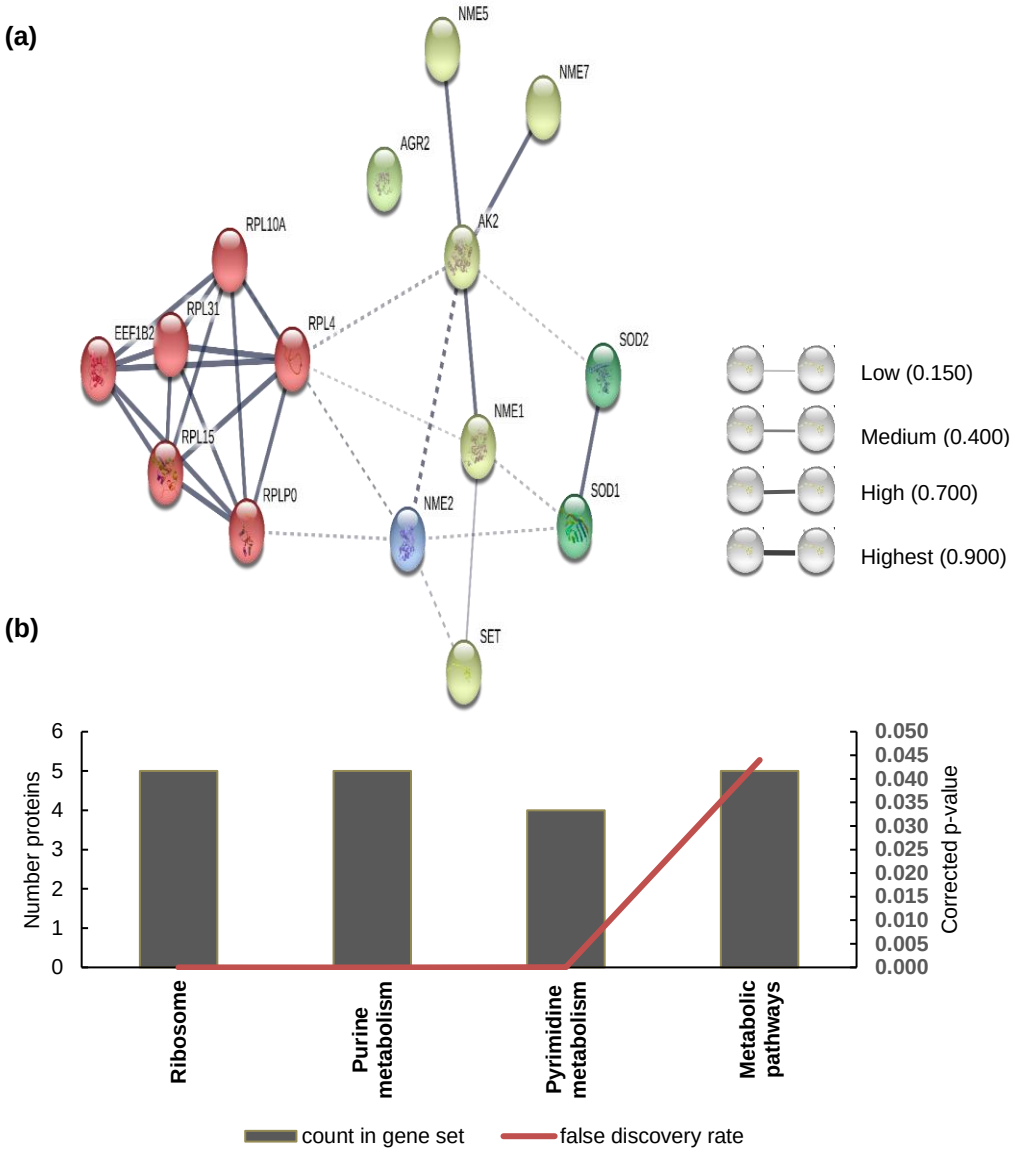

Figure 10: STRING network analysis (a) of differentially expressed proteins in broiler ileum epithelial cells in response to the multi-species probiotic with two added nodes of predicted interacting proteins. In the network, nodes represent proteins, and lines with different shading represent different levels of predicted association and dash-lines represent inter-cluster edges. Ontology analysis (b) of identified proteins were at a depth of KEGG pathways.

Table 5: STRING analysis protein node classification, UniProt ID and protein name of differentially expressed and predicted interacting proteins in broiler epithelial cells after exposure to the multi-species probiotic.

Input		
Gene name	**Uniprot ID**	**Protein name**
AK2	F1NJ73_CHICK	Uncharacterised protein (189 aa)
SET	F2Z4L4_CHICK	Protein SET (277 aa)
EEF1B2	EF1B_CHICK	Elongation factor 1-beta (224 aa)
ARG2	E1C937_CHICK	Uncharacterised protein (172 aa)
SOD1	SODC_CHICK	Superoxide dismutase (155 aa)
Predicted functional partners		
Gene name	**Uniprot ID**	**Protein name**
SOD2	F1NT19_CHICK	Superoxide dismutase (224 aa)
RPL15	RL15_CHICK	Ribosomal protein L15 (206 aa)
RPLP0	RLA0_CHICK	60S acidic ribosomal protein P0
RPL10A	F6SU35_CHICK	Ribosomal protein (184 aa)
NME2	A0A1I7Q438_CHICK	Nucleoside diphosphate kinase
NME1	F1N910_CHICK	Nucleoside diphosphate kinase A (158 aa)
RPL4	Q5ZII1_CHICK	60S ribosomal protein L4 (421 aa)
RPL31	F1P4V6_CHICK	Uncharacterised protein (126 aa)
NME7	E1C3P8_CHICK	Uncharacterised protein (376 aa)
NME5	A0A1D5P3Y3_CHICK	Uncharacterised protein (210 aa)

Salmonella Enteritidis 147 induced the up-regulation of lysozyme C (1.14 fold) and G (1.07 fold), cathelicidin 2 (1.15 fold) and 3 (1.11 fold), myeloid protein 1 (1.13 fold), trypsin inhibitor CITI-1 (1.11 fold), gallinacin-2 (1.10 fold) and ubiquitin-fold modifier 1 (1.06 fold) as compared to untreated broiler epithelial cells (Table 2). In addition, *Salmonella* induced down-regulation of glutaredoxin-1 (0.99 fold), gallicin-7 (0.11 fold) and vigilin (0.10 fold).

Lysozyme is an antimicrobial enzyme, part of the innate immune system, which catalyses the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, a major component of gram-positive bacterial cell membrane. Hydrolysis of peptidoglycan compromises the cell wall resulting in lysis (Callewaert and Michiels, 2010; Lelouard *et al*., 2010). Degraded peptidoglycan fragments in-turn activates nucleotide-binding oligomerization (NOD) receptors in mucosal epithelial cells, which leads to secretion of chemotactic and activating factors for neutrophils and macrophages (Ragland and Criss, 2017). Cathelicidins are antimicrobial peptides part of the host innate defence system, which possess broad spectrum of activity against bacteria (Travis *et al*., 2000), fungi (Shin *et al*., 2000), parasites (Giacometti *et al*., 2000), and enveloped viruses (Falla *et al*., 1996). Cathelicidins are stored in the secretory granules of neutrophils, macrophages and non-myeloid cells and released upon leukocyte activation (Zanetti *et al*., 1995; Zanetti, 2005). Cathelicidin-2 (CATH-2) is an arginine-lysine rich peptide with both immunomodulatory and broad-spectrum antibacterial activity, however its mode of action is still unclear (Xiao *et al*., 2006; van Dijk *et al*., 2009; Schneider *et al*., 2016). Cathelicidins mainly localizes at the membrane surface and upon binding permeabilize the bacterial cell (Schneider *et al*., 2016). In addition, besides the membrane target, cathelicidins can cross the membrane without damage and reach intracellular targets such as ribosomes, DNA or other intracellular molecules resulting in inhibition of various processes such as DNA synthesis, RNA synthesis, protein synthesis and protein folding (Brogden, 2005; Nguyen *et al*., 2011).

Myeloid protein 1 (Mim-1) has 60 % identity with the 16 kDa human leukocyte chemotactic protein LECT 2 which exhibits chemotactic activity, plays multifunctional roles in cell growth and differentiation, damage/repair processes, carcinogenesis and autoimmune diseases (Suzuki *et al*., 1993; Segawa *et al*., 2001; Allen and Hebbes, 2003). The Mim-1 gene is expressed in myeloid cells of the chicken haematopoietic system and in myeloblasts and promyelocytes (Ness *et al*., 1989). In chickens Mim-1 functions as a chemotactic factor for heterophils and plays an active role in lymphokine mediated protection against *Salmonella* infections (Bischoff *et al*., 2001). Expression is reduced in differentiated granulocytes, but the protein is stored as granules, and secreted upon induced exocytosis from polymorphonuclear neutrophils (Terashima *et al*., 1996; Grigoryev *et al*., 1998). The trypsin inhibitor CITI-1 is a *Gallus gallus* specific protein capable of inhibiting enzyme such as trypsin, acrosin and plasmin. The precise function is yet to be elucidated, however, their function is similar to that of Kazal-type inhibitors (Laskowski and Kato, 1980; Jonakova *et al*., 1992). Kazal-type inhibitors regulate the host-defence system and inhibit microbial proteinases (Bourin *et al*., 2011). Proteinase inhibitors play a role in defence against extrinsic peptidases produced by pathogens to prevent damage and evasion of host defence systems (Proaño Bolaños *et al*., 2017). In addition, proteinase inhibitors might indirectly act as regulators of processing reactions of peptides (cationic α-helical antimicrobial peptides), allowing

them to be released, so they can display their activity and protect from invading microorganisms (Lai *et al*., 2002; Chen and Shaw, 2003).

Ubiquitin-fold modifier 1 (Ufm1) is a member of the ubiquitin-like protein family, with unknown biological function (Li *et al*., 2017). Protein modifications by ubiquitin-like proteins, are critical for cell cycling, stress responses, signal transduction, immune responses, transcription, DNA repair, autophagy, cell proliferation and differentiation, apoptosis and endoplasmic reticulum (ER) regulation (Weissman, 2001; Kerscher *et al*., 2006; Hochstrasser, 2009; Yoo *et al*., 2015). Previously, increased expression of Ufm1 in response to lipopolysaccharide, alleviated the inflammatory responses and inhibited the nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB) (Li *et al*., 2017). Vigilin is a multi-KH domain protein which binds RNA-and is localized in the nuclear envelope, nucleus and rough endoplasmic reticulum (ER) (Wintersberger *et al*., 1995; Frey *et al*., 2001; Vollbrandt *et al*., 2004). The exact biological functions remain to be elucidated, however, is involved in translational control (Hilgendorf *et al*., 2001), nuclear export of tRNA (Kruse *et al*., 1998), cytoplasmic transport of RNA (Vollbrandt *et al*., 2004), metabolism of specific mRNAs (Cunningham *et al*., 2000), RNAi-mediated vigilin silencing (Zhou *et al*., 2008), sterol metabolism (Brykailo *et al*., 2007), carcinogenesis (Yang *et al*., 2014b) and formation of heterochromatin (Wang *et al*., 2005).

Glutaredoxin-1 (GRX-1) is an oxidoreductase enzyme part of the endogenous antioxidant defence system (Sun *et al*., 2017). The precise function remains to be elucidated, however, is involved in many cellular processes such as antioxidation (Liu *et al*., 2015; Sun *et al*., 2017), anti-apoptosis (Yu *et al*., 2012; Sun *et al*., 2017), regulation of cell differentiation (Bräutigam *et al*., 2011 and 2013), inflammation (Reynaert *et al*., 2007; Maki *et al*., 2015), protein folding (Wiita *et al*., 2007; Berndt *et al*., 2008) and regulation of transcription factor activity (Bandyopadhyay *et al*., 1998). Overexpression of GRX-1 inhibits oxidative stress and apoptosis (Liu *et al*., 2015; Sun *et al*., 2017). Glutaredoxin-1 inhibited apoptosis by activating Ref-1 an intracellular redox-active molecule, which in turn activated the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) which stimulated the immune system (Daily *et al*., 2001a; Daily *et al*., 2001b). Recently, GRX1 have been shown to regulate apoptosis through the apoptosis signal-regulating kinase-1 (ASK1) cascade, a mitogen-activated protein kinase kinase kinase (MAPKKK) that is bound to the reduced state of GRX1 (Akterin *et al*., 2006), when oxidised, ASK1 is liberated and can initiate the MAPK cascade leading to activation of c-Jun N-terminal kinase (JNK) or p38 (Ichijo *et al*., 1997; Song and Lee, 2003a), as well translocation of death-associated protein (Daxx) (Song and Lee, 2003b). Gallinacins are small cysteine-rich antimicrobial peptides part of the beta-defensin family which play an important role in the innate immune system against bacterial infections (Lynn *et al*., 2004; Sugiarto and Yu, 2004). Gallinacins are abundant in cells that are involved in the innate immune system (Ganz, 2003; Xiao *et al*., 2004), and exhibit broad spectrum of antimicrobial activity (Higgs *et al*., 2005). Gallinacins are responsible for recognizing bacteria in the initial stages of infection and induce immune cells such as dendritic cells to mature and respond to the infection at later stage (Ganz, 2003), and recruit macrophages, granulocytes, and lymphocytes to the site of infection (Welling *et al*., 1998; Kagan *et al*., 1990; Satchell *et al*., 2003).

Broiler epithelial cells exposed to the multi-species probiotic resulted in the up-regulation of transgelin 2/3 (1.14 fold), elongation factor-1 beta (1.06 fold) and anterior gradient 2 (1.05 fold) proteins.

In addition, probiotics led to the down-regulation of carnitine O-acetyltransferase (0.96 fold), adenylate kinase 2 (0.94 fold), superoxide dismutase [Cu-Zn] (0.93 fold) and protein SET (0.11 fold) (Table 3). Transgelin-2 is expressed in both smooth and non-smooth muscle cells (Meng *et al*., 2017). Transgelin-2regulates actin cytoskeleton dynamics by stabilizing actin fibres and participate in processes involving actin cytoskeleton remodelling such as cell proliferation, differentiation, migration, and apoptosis (Dvorakova *et al*., 2014). Transgelin-2 is highly expressed in B-cells, however, the precise role remains to be elucidated (Na *et al*., 2016). The immunological synapse is a dynamic and organized junction between T-cells and antigen presenting cells, which is critical for initiating the adaptive immune system (Dustin, 2008). The actin cytoskeleton plays a major role in T-cell reorganisation during immunological synapse formation, and transgelin-2 expressed in T-cells, stabilises cortical F-actin, promoting T-cell activation in response to antigen stimulation (Na *et al*., 2016).

Elongation factor 1B-α (EF1B-α) is an important protein which plays regulatory roles in cell growth, apoptosis and tumorigenesis (Thornton *et al*., 2003). Overexpression of EF1B leads to increased rates of translation and protein synthesis, resulting in enhanced cell proliferation (Al-Maghrebi *et al*., 2005). EF1B complex in eukaryotes consist of three subunits i.e. EF1B-α, EF1B-γ and EF1B-β (Sasikumar *et al*., 2012; Choi *et al*., 2016). EF1Bα has been found essential for cell growth (Hiraga *et al*., 1993), and mutation within the subunit enhanced lowered translation efficiency and increased translation errors (Carr-Schmid *et al*., 1999). It has been suggested that EF1Bα promotes nucleotide exchange in EF1A by disrupting interactions between GDP with the P-loop and switch regions of EF1A (Guerrucci *et al*., 1999).

Anterior gradient 2 (AGR2) is a disulphide isomerase which is widely expressed in mucus-producing and endocrine-responsive cells (Persson *et al*., 2005; Brychtova *et al*., 2011). Expression of AGR2 has been shown to increase cell migration and metastatic behaviour, regulate mucin production and accelerate wound healing (Ramachandran *et al*., 2008; Wang *et al*., 2008; Gupta *et al*., 2013; Zhu *et al*., 2017). Carnitine O-acetyltransferase (CRAT) is an enzyme which plays an important role in energy homeostasis and fat metabolism (Jogl and Tong, 2003). The enzyme catalyses the reversible transfer of acyl groups from an acyl-CoA thioester to carnitine and regulates the ratio of acyl-CoA/CoA (Ramsay *et al*., 2001). The activity of CRAT is essential for the cell cycle to proceed from the G1 phase to the S phase (Brunner *et al*., 1997). The most important biological function of CRAT is transport of fatty acids for β-oxidation (McGarry and Brown, 1997; Ramsay *et al*., 2001). Fatty acids are oxidised for energy production in the mitochondrial matrix, however, fatty acids are accumulated in the cytoplasm, and require transport from the cytoplasm to the mitochondrial matrix (Jogl *et al*., 2004). The carnitine shuttle system of which CRAT is a member, helps fatty acids cross the mitochondrial membrane to accumulate in the mitochondrial matrix (Jogl *et al*., 2004). Adenylate kinase 2 (AK2) is a nucleoside monophosphate kinase that catalyse the phosphorylation of AMP by using ATP or GTP as phosphate donors. By constantly monitoring phosphate nucleotide levels inside the cell, ADK plays a fundamental role in regulating cellular energy homeostasis (Dzeja and Terzic, 2009). Although the specific role of AK2 in the regulation of adenine nucleotide metabolism and energy homeostasis is yet to be elucidated, AK2 may mediate energetic coupling between the mitochondria and endoplasmic reticulum (Burkart *et al*., 2010). Adipocyte differentiation and B cell differentiation has been associated

with increase in AK2 levels (Wilson-Fritch *et al*., 2003). Adenylate kinase is linked to the induction of the unfolded protein response (UPR) and is required for the full induction of the UPR, in response to acute accumulation of misfolded proteins in mature adipocytes (Burkart *et al*., 2010).

Superoxide dismutase (SOD) destroys radicals which are toxic, they are ubiquitous metalloproteins which catalyses the dismutation of superoxide anions to oxygen and hydrogen peroxide (Fridovich, 1989). Superoxide dismutase [Cu-Zn] (SOD1) is a dimeric protein with Cu and Zn metal ions essential for its catalytic function in protecting against oxidative stress associated with mitochondrial respiration (Perry *et al*., 2010; Reddi and Culotta, 2013). Over-expression of SOD1 inhibited apoptosis and reduced oxidative damage (Dimmeler *et al*., 1999; Landis and Tower, 2005).

The phosphoprotein SET is localized in the nucleus and cytoplasm and plays a critical role in regulation of signal transduction (Irie *et al*., 2012). The SET protein together with nuclear phosphoprotein pp32 forms an inhibitor subunit for histone acetyltransferase complex, and upon binding to histones prevents their acetylation (Seo *et al*., 2001). The SET protein is a potent inhibitor of protein phosphatase 2A (PP2A), a cellular serine/threonine phosphatase involved in the regulation of a variety of cellular processes and signal transductions (Li *et al*., 1996; Millward *et al*., 1999; Zolnierowicz, 2000), Protein kinase D2 (PKD2) in T cells activates interleukin-2 promoter and T cell death and is a substrate for SET protein. Protein kinase D2 regulates PP2A activity in activated T cells through phosphorylation of Ser171 of SET protein (Irie *et al*., 2012).

Proteins upregulated in response to *S*. Enteritidis 147 were involved in the innate immune system to fight off invasion and infection which included lysozyme C and G, cathelicidins 2 and 3, gallinacins 2, trypsin inhibitor CITI-1, ubiquitin-fold modifier and myeloid protein 1. These proteins possessed broad spectrum antimicrobial activity, acted as chemotactic compounds, inhibitors of microbial enzymes, and played critical roles in stress responses. In addition, *Salmonella* exposure down-regulated expression of vigilin, gluteradoxin-1 and gallinacins 7. These proteins play vital roles in activation of natural killer cells, regulation of apoptosis and antimicrobial defence systems. *Salmonella* interacted with the host in a pathogenic manner, as differentially expressed proteins were involved in activation of the innate immune system and other host defence responses. Broiler epithelial cells exposed to the multi-species probiotic (*B*. *amyloliquefaciens* DPN123, *E*. *faecalis* DPN94, *L*. *salivarius* DPN181, *L*. *crispatus* DPN167, *L*. *johnsonii* DPN184 and *L*. *gallinarum* DPN164) resulted in the up-regulation of elongation factor 1Bα, anterior gradient 2 and transgelin 2/3 in which were involved in cell proliferation, cell migration and healing, and cytoskeleton regulation. In addition, the multi-species probiotic resulted in the down-regulation of carnitine O-acetyltransferase, adenylate kinase-2, superoxide dismutase and phosphoprotein SET which play important roles in fatty acid transport, energy homeostasis, nucleotide metabolisms, free-radical elimination and signal transduction. The multi-species probiotic interacted with broiler epithelial cells in a symbiotic manner, as differentially expressed proteins were not involved in host defence responses but rather normal cellular and metabolic processes.

Conclusions

Interactions which occur between epithelial cells and pathogenic and probiotic bacteria were elucidated. *Salmonella* Enteritidis 147 was cytotoxic, adhere and invade with high efficiency and disrupted claudin- 3 tight junctions of Caco-2 cells. Probiotic bacteria were not cytotoxic; however, their fermentative end-products were. Probiotic bacteria adhered to Caco-2 cells but were unable to invade them, and reduced claudin-3 tight junctions but did not disrupt monolayer integrity. In addition, metabolites of *L*. *johnsonii* DPN184, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *E*. *faecalis* DPN94 and *B*. *amyloliquefaciens* DPN123 significantly decreased *S*. Enteritidis 147 invasion efficiency of Caco-2 cells. Broiler ileum epithelial cells exposed to *S*. Enteritidis 147 resulted in the up-regulation (lysozyme C and G, cathelicidin 2 and 3, myeloid protein 1, trypsin inhibitor CITI-1, gallinacin-2 and ubiquitin-fold modifier 1) and down-regulation (glutaredoxin-1, gallicin-7 and vigilin) of various proteins. Upregulated proteins have broad spectrum antimicrobial activity, act as chemotactic compounds, inhibitors of microbial enzymes, and play critical roles during stress responses. Down-regulated proteins play vital roles in activation of natural killer cells, regulation of apoptosis and antimicrobial defence systems. *S*almonella Enteritidis 147 interacted with epithelial cells in a pathogenic manner and induced differential expression of proteins related to the immune system. Broilers epithelial cells exposed to the multi-species probiotic resulted in the up-regulation (transgelin 2/3, elongation factor-1 beta and anterior gradient 2) and down-regulation (carnitine O-acetyltransferase, adenylate kinase 2, superoxide dismutase [Cu-Zn] and protein SET) of various proteins. Upregulated proteins were involved in cell proliferation, cell migration and healing, and cytoskeleton regulation, whereas down-regulated proteins play important roles in fatty acid transport, energy homeostasis, nucleotide metabolisms, free-radical elimination and signal transduction. The multi-species probiotic interacted with epithelial cells in a symbiotic manner, as no detrimental proteomic changes were induced in the epithelial cells. The study provided insight into the initial interaction which occurs between epithelial cells and pathogenic and probiotic bacteria.

References

Akterin, S., Cowburn, R.F., Miranda-Vizuete, A., Jiménez, A., Bogdanovic, N., Winblad, B., Cedazo-Minguez, A. (2006). Involvement of glutaredoxin-1 and thioredoxin-1 in β-amyloid toxicity and Alzheimer's disease. *Cell Death Differ*. 13, p 1454-1465, DOI: 10.1038/sj.cdd.4401818.

Alakomi, H-L., Skyttä, E., Saarela, M., Mattila-Sandholm, T., Latva-Kala, K., Helander, I.M. (2000). Lactic acid permeabilizes gram-negative bacteria by disrupting the outer membrane. *Appl*. *Environ*. *Microbiol*. 66(5), p 2001–2005.

Aleljung, P., Shen, W., Rozalska, B., Hellma, U., Ljungh, A., Wadström, T. (1994). Purification of collagen-binding proteins of *Lactobacillus reuteri* NCIB 11951. *Curr*. *Microbiol*. 28(4), p 231–236.

Allen, S.C.H., Hebbes, T.R. (2003). Myb induced myeloid protein 1 (Mim-1) is an acetyltransferase. *FEBS Letters* 534, p 119-124, DOI: 10.1016/S0014-5793(02)03803-6.

Al-Maghrebi, M., Anim, J.T., Olalu, A.A. (2005). Up-regulation of eukaryotic elongation factor-1 subunits in breast carcinoma. *Anticancer Res*. 25(3C), p 2573-2577.

Apajalahti, J., Kettunen, A., Graham, H. (2004). Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. *World's Poultry Sci*. *J*. 60(2), p 223–232, DOI: 10.1079/WPS200415.

Balls, M., Riddell, R.J., Horner, S.A., Clothier, R.H. (1987). The frame approach to the development validation and evaluation of *in vitro* alternative methods. In: Goldber, A.M. (Ed), *In vitro* methods in toxicology—approaches to validation alternative methods in toxicology. Mary Ann Liebert Inc., New York, p. 45–58.

Bandyopadhyay, S., Starke, D.W., Mieyal, J.J., Gronostajski, R.M. (1998). Thioltransferase (glutaredoxin) reactivates the DNA-binding activity of oxidation-inactivated nuclear factor I. *J*. *Biol*. *Chem*. 273(1), p 392-397.

Bäumler, A.J., Sperandio, V. (2016). Interactions between the microbiota and pathogenic bacteria in the gut. *Nature* 535(7610), p 85-93, DOI: 10.1038/nature18849.

Berge, A.C., Wierup, M. (2012). Nutritional strategies to combat Salmonella in monogastric food animal production. *Animal* 6(4), p 557-564, DOI: 10.1017/S1751731111002217.

Berndt, C., Lillig, C.H., Holmgren, A. (2008). Thioredoxins and glutaredoxins as facilitators of protein folding. *Biochim*. *Biophys*. *Acta*. 1783(4), p 641-650, DOI: 10.1016/j.bbamcr.2008.02.003.

Bischoff, K.M., Pishko, E.J., Genovese, K.J., Crippen, T.L., Holtzapple, C.K., Stanker, L.H., Nisbet, D.J., Kogut, M.H. (2001). Chicken mim-1 protein, p33, is a heterophil chemotactic factor present in *Salmonella* Enteritidis immune lymphokine. *J*. *Food Prot*. 64(10), p 1503-1509.

Boekhorst, J., Helmer, Q., Kleerebezem, M., Siezen, R. J. (2006). Comparative analysis of proteins with a mucus-binding domain found exclusively in lactic acid bacteria. *Microbiol*. 152, p 273–280, DOI: 10.1099/mic.0.28415-0.

Bourin, M., Gautron, J., Berges, M., Attucci, S., Le Blay, G., Labas, V., Nys, Y., Rehault-Godbert, S. (2011). Antimicrobial potential of egg yolk ovoinhibitor, a multidomain Kazal-like inhibitor of chicken egg. *J*. *Agric*. *Food Chem*. 59(23), p 12368-12374, DOI: 10.1021/jf203339t.

Boyen, F., Pasmans, F., Van Immerseel, F., Morgan, E., Adriaensen, C., Hernalsteens, J-P., Decostere, A., Ducatelle, R., Haesebrouck, F. (2006). Salmonella Typhimurium SPI-1 genes promote intestinal but not tonsillar colonisation in pigs. *Microbes Infect*. 8(14–15), p 2899–2907, DOI: 10.1016/j.micinf.2006.09.008.

Bräutigam, L., Jensen, L.D., Poschmann, G., Nyström, S., Bannenberg, S., Dreij, K., Lepka, K., Prozorovski, T., Montano, S.J., Aktas, O., Uhlén, P., Stühler, K., Cao, Y., Holmgren, A., Berndt, C. (2013). Glutaredoxin regulates vascular development by reversible glutathionylation of sirtuin 1. *Proc*. *Natl*. *Acad*. *Sci*. *USA*. 110(50), p 20057-20062, DOI: 10.1073/pnas.1313753110.

Bräutigam, L., Schütte, L.D., Godoy, J.R., Prozorovski, T., Gellert, M., Hauptmann, G., Holmgren, A., Lillig, C.H., Berndt, C. (2011). Vertebrate-specific glutaredoxin is essential for brain development. *Proc*. *Natl*. *Acad*. *Sci*. *USA*. 108(51), p 20532-20537, DOI: 10.1073/pnas.1110085108.

Brisbin, J.T., Gong, J., Sharif, S. (2008). Interactions between commensal bacteria and the gut-associated immune system of the chicken. *Anim*. *Health Res*. *Rev*. 9(1), p 101–110. DOI: 10.1017/S146625230800145X.

Brogden, K.A. (2005). Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat*. *Rev*. *Microbiol*. 3, p 238–250, DOI: 10.1038/nrmicro1098.

Brunner, S., Kramar, K., Denhardt, D.T., Hofbauer, R. (1997). Cloning and characterization of murine acetyltransferase: evidence for a requirement during cell cycle progression. *Biochem*. *J*. 322(2), p 403-410.

Bruno, V.M., Hannemann, S., Lara-Tejero, M., Flavell, R.A., Kleinstein, S.H., Galán, J.E. (2009). *Salmonella* Typhimurium type III secretion effectors stimulate innate immune responses in cultured epithelial cells. *PLoS Pathog*. 5, e1000538, DOI: 10.1371/journal.ppat.1000538.

Brychtova, V., Vojtesek, B., Hrstka, R. (2011). Anterior gradient 2: a novel player in tumor cell biology. *Cancer Lett*. 304(1), p 1–7, DOI: 10.1016/j.canlet.2010.12.023.

Brykailo, M.A., Corbett, A.H., Fridovich-Keil, J.L. (2007). Functional overlap between conserved and diverged KH domains in *Saccharomyces cerevisiae* SCP160. *Nucleic Acids Res*. 35(4), p 1108–1118, DOI: 10.1093/nar/gkl1160.

Buck, BL., Altermann, E., Svingerud, T., Klaenhammer, TR. (2005), Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. *Appl*. *Environ*. *Microbiol*. 71(12), p 8344-8351, DOI: 10.1128/AEM.71.12.8344-8351.2005.

Burkart, A., Shi, X., Chouinard, M., Corvera, S. (2010). Adenylate kinase 2 links mitochondrial energy metabolism to the induction of the unfolded protein response. *J*. *Biol*. *Chem*. 286(6), p 4081-4089, DOI: 10.1074/jbc.M110.134106.

Callewaert, L., Michiels, C.W. (2010). Lysozymes in the animal kingdom. *J Biosci*. 35(1), p 127–60.

Carr-Schmid, A., Valente, L., Loik, V.I., Williams, T., Starita, L.M., Kinzy, T.G. (1999). Mutations in elongation factor 1beta, a guanine nucleotide exchange factor, enhance translational fidelity. *Mol*. *Cell Biol*. 19(8), p 5257–5266, DOI: 10.1128/MCB.19.8.5257.

Chambers, J.R., Gong, J. (2011). The intestinal microbiota and its modulation for *Salmonella* control in chickens. *Food Res*. *Int*. 44(10), p 3149 –3159, DOI: 10.1016/j.foodres.2011.08.017.

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M., Hanage, W.P. (2015). Antibiotics in agriculture and the risk to human health: how worried should we be? *Evol*. *Appl*. 8(3), p 240-247, DOI: 10.1111/eva.12185.

Chen, T., Shaw, C. (2003). Identification and molecular cloning of novel trypsin inhibitor analogs from the dermal venom of the Oriental fire-bellied toad (*Bombina orientalis*) and the Europeanyellow-bellied toad (*Bombina variegata*). *Peptides* 24(6), p 873–880, DOI: 10.1016/S0196-9781(03)00165-7.

Chiba, H., Osanai, M., Murata, M., Kojima, T., Sawada, N. (2008) Transmembrane proteins of tight junctions. *Biochim*. *Biophys*. *Acta* 1778(3), p 588–600, DOI: 10.1016/j.bbamem.2007.08.017.

Choct, M. (2009). Managing gut health through nutrition. *Br*. *Poult*. *Sci*. 50(1), p 9–15, DOI: 10.1080/00071660802538632.

Choi, Y.S., Lee, H.J., Kang, B.S. (2016). Assembly of human translational elongation factor 1B is based on the interaction of the glutathione transferase-like domains from alpha and gamma subunits. FASEB J. 30(1), 1067.1-1067.1.

Coburn, B., Sekirov, I., Finlay, B.B. (2007). Type III secretion systems and disease. *Clin*. *Microbiol*. *Rev*. 20(4), p 535-539, DOI: 10.1128/CMR.00013-07.

Coombes, B.K., Coburn, B.A., Potter, A.A., Gomis, S., Mirakhur, K., Li, Y., and Finlay, B.B. (2005). Analysis of the contribution of *Salmonella* pathogenicity islands 1 and 2 to enteric disease progression using a novel bovine ileum loop model and a murine model of infectious enterocolitis. *Infect*. *Immun*. 73(11), p 7161– 7169, DOI: 10.1128/IAI.73.11.7161-7169.2005.

Corrêa-Oliveira, R., Fachi, J.L., Vieira, A., Sato F.T., Vinolo, M.A.R. (2016). Regulation of immune cell function by short-chain fatty acids. *Clin*. *Transl*. *Immunology* 5(4), e73, DOI: 10.1038/cti.2016.17.

Cox, J., Neuhauser, N., Michalski, A., Scheltema, R.A., Olsen, J.V., Mann, M. (2011). Andromeda: a peptide search engine integrated into the MaxQuant environment. *J*. *Proteome Res*. 10(4), p 1794-1805, DOI: 10.1021/pr101065j.

Cunningham, K.S., Dodson, R.E., Nagel, M.A., Shapiro, D.J., Schoenberg, D.R. (2000). Vigilin binding selectively inhibits cleavage of the vitellogenin mRNA 3′-untranslated region by the mRNA endonuclease polysomal ribonuclease 1. *Proc*. *Natl*. *Acad*. *Sci*. *USA*. 97(23), p 12498–12502, DOI: 10.1073/pnas.220425497.

Daily, D., Vlamis-Gardikas, A., Offen, D., Mittelman, L., Melamed, E., Holmgren, A., Barzilai, A. (2001a). Glutaredoxin protects cerebellar granule neurons from dopamine-induced apoptosis by dual activation of the ras-phosphoinositide 3-kinase and jun n-terminal kinase pathways. *J*. *Biol*. *Chem*. 276, p 21618–21626, DOI: 10.1074/jbc.M101400200.

Daily, D., Vlamis-Gardikas, A., Offen, D., Mittelman, L., Melamed, E., Holmgrens, A., Barzilai, A. (2001b). Glutaredoxin protects cerebellar granule neurons from dopamine-induced apoptosis by activating NF- kB via Ref-1. *J*. *Biol*. *Chem*. 276(2), p 1335-1344, DOI: 10.1074/jbc.M008121200.

Darfeuille-Michaud, A., Aubel, D., Chauviere, G., Rich, C., Bourges, M., Servin, A., Joly, B. (1990). Adhesion of enterotoxigenic Escherichia coli to the human colon carcinoma cell line Caco-2 in culture. *Infect*. *Immun*. 58(4), p 893-902.

Dethlefsen, L., McFall-Ngai, M., Relman, D.A. (2007). An ecological and evolutionary perspective on human–microbe mutualism and disease. *Nature* 449, p 811–818, DOI: 10.1038/nature06245.

Dimmeler, S., Hermann, C., Galle, J., Zeiher, A.M. (1999). Up-regulation of superoxide dismutase and nitric oxide synthase mediates the apoptosis-suppressive effects of shear stress on endothelial cells. *Arterioscler*. *Thromb*. *Vasc*. *Biol*. 19(3), p 656-664, DOI: 10.1161/01.ATV.19.3.656.

Dustin, M.L. (2008). Hunter to gatherer and back: immunological synapses and kinapses as variations on the theme of amoeboid locomotion. *Annu*. *Rev*. *Cell Dev*. *Biol*. 24, p 577–596, DOI: 10.1146/annurev.cellbio.24.110707.175226.

Dvorakova, M., Nenutil, R., Bouchal, P. (2014). Transgelins, cytoskeletal proteins implicated in different aspects of cancer development. *Expert Rev*. *Proteomics* 11(2), p 149–165, DOI: 10.1586/14789450.2014.860358.

Dzeja, P., Terzic, A. (2009). Adenylate kinase and AMP signalling networks: metabolic monitoring, signal communication and body energy. *Int*. *J*. *Mol*. *Sci*. 10(4), p 1729–1772, DOI: 10.3390/ijms10041729.

Falla, T.J., Karunaratne, D.N., Hancock, E.W. (1996). Mode of action of the antimicrobial peptide indolicidin. *J. Biol. Chem*. 271(32), p 19298-19303, DOI: 10.1074/jbc.271.32.19298.

Fichera, G.A., Giese, G. (1994). Non-immunologically-mediated cytotoxicity of *Lactobacillus casei* and its derivative peptidoglycan against tumor cell lines. *Cancer Lett*. 85(1), p 93–103, DOI: 10.1016/0304-3835(94)90244-5.

Frey, S., Pool, M., Seedorf, M. (2001). Scp160p, an RNA-binding, polysome-associated protein, localizes to the endoplasmic reticulum of *Saccharomyces cerevisiae* in a microtubule-dependent manner. *J. Biol. Chem*. 276, p 15905–15912, DOI: 10.1074/jbc.M009430200.

Fridovich, I. (1989). Superoxide dismutase: An adaptation to a paramagnetic gas. *J. Biol. Chem*. 264(14), p 7761–7764.

Friis, L.M., Pin, C., Pearson, B.M., Wells, J.M. (2005). *In vitro* cell culture methods for investigating *Campylobacter* invasion mechanisms. *J. Microbiol. Methods* 61(2), p 145-160, DOI: 10.1016/j.mimet.2004.12.003.

Furuse, M., Sasaki, H., Fujimoto, K., Tsukita, S. (1998). A single gene product, claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *J. Cell Biol*. 143(2), p 391–401, DOI: 10.1083/jcb.143.2.391.

Ganz, T. (2003). Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol*. 3(9), p 710-720, DOI: 10.1038/nri1180.

Giacometti, A., Cirioni, O., Barchiesi, F., Ancarani, F., Scalise, G. (2000). *In vitro* anti-cryptosporidial activity of cationic peptides alone and in combination with inhibitors of ion transport systems. *J. Antimicrob. Chemother*. 45(5), p 651-654, DOI: 10.1093/jac/45.5.651.

Grigoryev, S.A., Woodcock, C.L. (1998). Chromatin structure in granulocytes a link between tight compaction and accumulation of a heterochromatin associated protein (MENT). *J. Biol. Chem*., 273, (1998), p 3082–3089, DOI: 10.1074/jbc.273.5.3082.

Guerrucci, M.A., Monnier, A., Delalande, C., Bellé, R. (1999). The elongation factor-1 delta (EF-1 delta) originates from gene duplication of an EF-1 beta ancestor and fusion with a protein-binding domain. *Gene* 233(1-2), p 83-87, DOI: 10.1016/S0378-1119(99)00153-5.

Gupta, A., Wodziak, D., Tun, M., Bouley, D.M., Lowe, A.W. (2013). Loss of anterior gradient 2 (Agr2) expression results in hyperplasia and defective lineage maturation in the murine stomach. *J. Biol. Chem*. 288, p 4321-4333, DOI: 10.1074/jbc.M112.433086.

Haiko, J., Westerlund-Wikström, B. (2013). The role of the bacterial flagellum in adhesion and virulence. *Biology* 2(4), p 1242-1267, DOI:10.3390/biology2041242.

Hänel, I., Müller, J., Müller, W., Schulze, F. (2004). Correlation between invasion of Caco-2 eukaryotic cells and colonisation ability in the chick gut in *Campylobacter jejuni*. *Vet. Microbiol*. 101(2), p 75-82, DOI: 10.1016/j.vetmic.2004.04.004.

Hannemann, S., Gao, B., Galán, J.E. (2013). *Salmonella* modulation of host cell gene expression promotes its intracellular growth. *PLoS Pathog*. 9, e1003668, DOI: 10.1371/journal.ppat.1003668.

Haraga, A., Ohlson, M.B., Miller, S.I. (2008). Salmonellae interplay with host cells. *Nat. Rev. Microbiol*. 6(1), p 53–66, DOI:10. 1038/nrmicro1788.

Higgs, R., Lynn, D.J., Gaines, S., McMahon, J., Tierney, J., James, T., Lloyd, A.T., Mulcahy, G., O'Farrelly, C. (2005). The synthetic form of a novel chicken beta-defensin identified *in silico* is predominantly active against intestinal pathogens. *Immunogenetics* 57(1-2), p 90-98, DOI: 10.1007/s00251-005-0777-3.

Hilgendorf, I., Gellersen, O., Emmrich, J., Mikkat, U., Rohwedel, J., Krammer, H.J., Müller, P.K., Kruse, C. (2001). Estradiol has a direct impact on the exocrine pancreas as demonstrated by enzyme and vigilin expression. *Pancreatology* 1(1), p 24–29, DOI: 10.1159/000055788.

Hiraga, K., Suzuki, K., Tsuchiya, E., Miyakawa, T. (1993). Cloning and characterization of the elongation factor EF-1 beta homologue of Saccharomyces cerevisiae, EF-1 beta is essential for growth. *FEBS Lett*. 316(2), p 165–169, DOI: 10.1016/0014-5793(93)81208-H.

Hochstrasser, M. (2009). Origin and function of ubiquitin-like proteins. *Nature* 458, p 422–429, DOI: 10.1038/nature07958.

Huang da, W., Sherman, B.T., Lempicki, R.A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat*. *Protoc*. 4(1), p 44-57, DOI: 10.1038/nprot.2008.211.

Hugas, M., Beloeil, P. (2014). Controlling *Salmonella* along the food chain in the European Union – progress over the last ten years. *Euro Surveill*. 19(19), pii=20804. https://doi.org/10.2807/1560-7917.ES2014.19.19.20804.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signalling pathways. *Science* 275(5296), p 90–94, DOI: 10.1126/science.275.5296.90.

Irie, A., Harada, K., Araki, N., Nishimura, Y. (2012). Phosphorylation of SET protein at Ser171 by protein kinase D2 diminishes its inhibitory effect on protein phosphatase 2A. *PLoS ONE* 7(12), e51242, DOI: 10.1371/journal.pone.0051242.

Jepson, M.A., Collares-Busato, C.B., Clark, M.A., Hirst, B.H., Simmons, N.L. (1995). Rapid Disruption of epithelial barrier function by *Salmonella* typhimurium is associated with structural modification of intercellular junctions. *Infect*. *Immun*. 63(1), p 356–359.

Jogl, G., Hsiao, Y.S., Tong, L. (2004). Structure and function of carnitine acyltransferases. *Ann*. *N.Y*. *Acad*. *Sci*. 1033(1), p 17-29, DOI: 10.1196/annals.1320.002.

Jogl, G., Tong, L. (2003). Crystal structure of carnitine acetyltransferase and implications for the catalytic mechanism and fatty acid transport. *Cell* 112(1), p 113-122, DOI: 10.1016/S0092-8674(02)01228-X.

Jonáková, V., Calvete, J.J., Mann, K., Schäfer, W., Schmid, E.R., Töpfer-Petersen, E. (1992). The complete primary structure of three isoforms of a boar sperm-associated acrosin inhibitor. *FEBS Lett*. 297(1-2), p 147–150, DOI: 10.1016/0014-5793(92)80347-J.

Kagan, B.L., Selsted, M.E., Ganz, T., Lehrer, R.I. (1990). Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proc*. *Natl*. *Acad*. *Sci*. *USA*. 87(1), p 210-214.

Kankainen, M., Paulin, L., Tynkkynen, S., von Ossowski, I., Reunanen, J., Partanen, P., Satokari, R., Vesterlund, S., Hendrickx, A.P., Lebeer, S., De Keersmaecker, S.C., Vanderleyden, J., Hämäläinen, T., Laukkanen, S., Salovuori, N., Ritari, J., Alatalo, E., Korpela, R., Mattila-Sandholm, T., Lassig, A., Hatakka, K., Kinnunen, K.T., Karjalainen, H., Saxelin, M., Laakso, K., Surakka, A., Palva, A., Salusjärvi, T., Auvinen, P., de Vos, W.M. (2009). Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human-mucus binding protein. *Proc*. *Natl*. *Acad*. *Sci*. *USA*. 106(40), p 17193–17198, DOI: 10.1073/pnas.0908876106.

Kaur, J., Jain, S.K. (2012). Role of antigens and virulence factors of *Salmonella enterica* serovar Tyhphi in its pathogenesis. *Microbiol*. *Res*. 167(4), p 199-210, DOI: 10.1016/j.micres.2011.08.001.

Kerscher, O., Felberbaum, R., Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annu*. *Rev*. *Cell Dev*. *Biol*. 22, p 159–180, DOI: 10.1146/annurev.cellbio.22.010605.093503.

Kos, B., Susković, J., Vuković, S., Simpraga, M., Frece, J., Matosić, S. (2003). Adhesion and Aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *J*. *Appl*. *Microbiol*. 94(6), p 981–987, DOI: 10.1046/j.1365-2672.2003.01915.x.

Kruse, C., Grünweller, A., Willkomm, D.K., Pfeiffer, T., Hartmann, R.K., Müller, P.K. (1998). tRNA is entrapped in similar, but distinct, nuclear and cytoplasmic ribonucleoprotein complexes, both of which contain vigilin and elongation factor 1 α. *Biochem*. *J*. 329, p 615–621, DOI: 10.1042/bj3290615.

Lai, R., Liu, H., Lee, W.H., Zhang, Y. (2002). Identification and cloning of a trypsin inhibitor from skin secretions of Chinese red-belly toad *Bombina maxima*. *Comp*. *Biochem*. *Physiol*. *B*: *Biochem*. *Mol*. *Biol*. 131(1), p 47–53, DOI: 10.1016/S1096-4959(01)00479-1.

Lan, Y., Verstegen, M.W.A., Tamminga, S., Williams, BA. (2005). The role of the commensal gut microbial community in broiler chickens. *World's Poult*. *Sci*. *J*. 61, p 95–104, DOI: 10.1079/wps200445.

Landis, G.N., Tower, J. (2005). Superoxide dismutase evolution and life span regulation. *Mech*. *Ageing Dev*. 126(3), p 365-379, DOI: 10.1016/j.mad.2004.08.012.

Laskowski, M., Kato, I. (1980). Protein inhibitors of proteinases. *Annu*. *Rev*. *Biochem*. 49, p 593–626, DOI: 10.1146/annurev.bi.49.070180.003113.

Lee, K., Lillehoj, H.S., Siragusa, G.R. (2010). Direct-fed microbials and their impact on the intestinal microflora and immune system of chickens. *J*. *Poult*. *Sci*. 47(2), p 106–114, DOI: 10.2141/jpsa.009096.

Lelouard, H., Henri, S., De Bovis, B., Mugnier, B., Chollat-Namy, A., Malissen, B., Méresse, S., Gorvel, J.P. (2010). Pathogenic bacteria and dead cells are internalized by a unique subset of Peyer's patch dendritic cells that express lysozyme. *Gastroenterology*. 138(1), p 173–84, DOI: 10.1053/j.gastro.2009.09.051.

Li, M., Makkinje, A., Damuni, Z. (1996). The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A. *J*. *Biol*. *Chem*. 271, p 11059–11062, DOI: 10.1074/jbc.271.19.11059.

Li, Y-Y., Zhang, G-Y., He, J-P., Zhang, D-D., Kong, X-X., Yuan, H-M., Chen, F-L. (2017). Ufm1 inhibits LPS-induced endothelial cell inflammatory responses through the NF-κB signalling pathway. *Int*. *J*. *Mol*. *Med*. 39(5), p 1119-1126, DOI: 10.3892/ijmm.2017.2947.

Liu, X., Jann, J., Xavier, C., Wu, H. (2015). Glutaredoxin 1 (Grx1) protects human retinal pigment epithelial cells from oxidative damage by preventing AKT glutathionylation. *Invest*. *Ophthalmol*. *Vis*. *Sci*. 56(5), p 2821-2832, DOI: 10.1167/iovs.14-15876.

Ljungh, A., Wadstrom, T. (2006). Lactic acid bacteria as probiotics. *Curr*. *Issues Intest*. *Microbiol*. 7(2), p 73–90.

Lynn, D.J., Higgs, R., Gaines, S., Tierney, J., James, T., Lloyd, A.T., Fares, M.A., Mulcahy, G., O'Farrelly, C. (2004). Bioinformatic discovery and initial characterisation of nine novel antimicrobial peptide genes in the chicken. *Immunogenetics* 56(3), p 170-177, DOI: 10.1007/s00251-004-0675-0.

Maki, K., Nagai, K., Suzuki, M., Inomata, T., Yoshida, T., Nishimura, M. (2015). Temporal changes in glutaredoxin 1 and protein S-glutathionylation in allergic airway inflammation. *PLoS ONE* 10(4), e0122986, DOI: 10.1371/journal.pone.0122986

Makras, L., Triantafyllou, V., Fayol-Messaoudi, D., Adriany, T., Zoumpopoulou, G., Tsakalidou, E., Servin, A., De Vuyst, L. (2006). Kinetic analysis of the antibacterial activity of probiotic lactobacilli towards *Salmonella enterica* serovar Typhimurium reveals a role for lactic acid and other inhibitory compounds. *Res*. *Microbiol*. 157(3), p 241–247, DOI: 10.1016/j.resmic.2005.09.002.

McGarry, J.D., Brown, N.F. (1997). The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. *Eur*. *J*. *Biochem*. 244(1), p 1-14, DOI: 10.1111/j.1432-1033.1997.00001.x.

Meng, T., Liu, L., Hao, R., Chen, S., Dong, Y. (2017). Transgelin-2: A potential oncogenic factor. *Tumour Biol*. 39(6), p 1-7, DOI: 10.1177/1010428317702650.

Methner, U., Al-Shabibi, S., Meyer, H. (1995). Experimental oral infection of specific pathogen-free laying hens and cocks with *Salmonella* Enteritidis strains. *Zentralbl Veterinarmed B* 42(1-10), p 459-469, DOI: 10.1111/j.1439-0450.1995.tb00737.x.

Millward, T.A., Zolnierowicz, S., Hemmings, B.A. (1999). Regulation of protein kinase cascades by protein phosphatase 2A. *Trends Biochem*. *Sci*. 24(5), p 186–191, DOI: 10.1016/S0968-0004(99)01375-4.

Morrison, D.J., Preston, T. (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* 7(3), p 189-200, DOI: 10.1080/19490976.2015.1134082.

Muñoz-Provencio, D., Llopis, M., Antolín, M., de Torres, I., Guarner, F., Pérez-Martínez, G., Monedero, V. (2009). Adhesion properties of *Lactobacillus casei* strains to resected intestinal fragments and components of the extracellular matrix. *Arch*. *Microbiol*. 191(2), p 153–161, DOI: 10.1007/s00203-008-0436-9.

Muyyarikkandy, M.S., Amalaradjou, M.A. (2017). *Lactobacillus bulgaricus*, *Lactobacillus rhamnosus* and *Lactobacillus paracasei* attenuate *Salmonella* Enteritidis, *Salmonella* Heidelberg and

Salmonella Typhimurium colonisation and virulence gene expression *in vitro*. *Int*. *J*. *Mol*. *Sci*. 18(11), pii: E2381. DOI: 10.3390/ijms18112381.

Nan, B-R., Kwon, M-S., Chae, M-W., Kim, H-R., Kim, C-H., Jun, C-D., Park, Z-Y. (2016). Transgellin-2 in B-cells controls T-cell activation by stabilizing T cell-B cell conjugates. *PLoS ONE* 11(5), e0156429, DOI: 10.1371/journal.pone.0156429.

Ness, S.A., Marknell, A., Graf. T. (1989). The *v-myb* oncogene product binds to and activates the promyelocyte-specific *mim-1* gene. *Cell* 59(6), p 1115-1125.

Neveling, D.P., Van Emmenes, L., Ahire, J.J., Pieterse, E., Smith, C., Dicks, L.M.T. (2017). Safety assessment of antibiotic and probiotic feed additives for *Gallus* gallus *domesticus*. *Sci*. *Rep*. 7(1), Article ID 12767, DOI: 10.1038/s41598-017-12866-7.

Nguyen, L.T., Haney, E.F., Vogel, H.J. (2011). The expanding scope of antimicrobial peptide structures and their modes of action. *Trends Biotechnol*. 29(9), p 464–472, DOI: 10.1016/j.tibtech.2011.05.001.

Oakley, B.B., Lillehoj, H.S., Kogut, M.H., Kim, W.K., Maurer, J.J., Pedroso, A., Lee, M.D., Collett, S.R., Johnson, T.J., Cox, N.A. (2014). The chicken gastrointestinal microbiome. *FEMS Microbiol*. *Lett*. 360(2), p 100-112, DOI: 10.1111/1574-6968.12608.

Oda, M., Hasegawa, H., Komatsu, S., Kambe, M., Tsuchiya, F. (1983). Anti-tumor polysaccharide from *Lactobacillus* sp. *Agric*. *Biol*. *Chem*. 47(7), p 1623–1625, DOI: 10.1080/00021369.1983.10865826.

Ouwehand, A.C. (1998). Antimicrobial components from lactic acid bacteria, in Salminen S, von Wright A (eds): Lactic Acid Bacteria: Microbiology and Functional Aspects. New York, Dekker, p 139–159.

Pajarillo, E.A.B., Kim, S.H., Valeriano, V.D., Lee, J.Y., Kang, D-K. (2017). Proteomic view of the crosstalk between *Lactobacillus mucosae* and intestinal epithelial cell in co-culture revealed by Q Exactive-based quantitative proteomics. *Front*. *Microbiol*. 8, 2459, DOI: 10.3389/fmicb.2017.02459.

Patel, J.C., Galán, J.E. (2005). Manipulation of the host actin cytoskeleton by *Salmonella* — all in the name of entry. *Curr*. *Opin*. *Microbiol*. 8(1), p 10–15, DOI: 10.1016/j.mib.2004.09.001.

Perry, J.J., Shin, D.S., Getzoff, E.D., Tainer, J.A. (2010) The structural biochemistry of the superoxide dismutases. *Biochim*. *Biophys*. *Acta*. 1804(2), p 245–262, DOI: 10.1016/j.bbapap.2009.11.004.

Persson, S., Rosenquist, M., Knoblach, B., Khosravi-Far, R., Sommarin, M., Michalak, M. (2005). Diversity of the protein disulphide isomerase family: Identification of breast tumor induced Hag2and Hag3 as novel members of the protein family. *Mol*. *Phylogenet*. *Evol*. 36(3), p 734–740, DOI: 10.1016/j.ympev.2005.04.002.

Pickard, J.M., Zeng, M.Y., Caruso, R., Núñez, G. (2017). Gut microbiota: Role in pathogen colonisation, immune responses, and inflammatory disease. *Immunol*. *Rev*. 279(1), p 70-89, DOI: 10.1111/imr.12567.

Prado-Rebolledo, O.F., Delgado-Machuca, J.J., Macedo-Barragan, R.J., Garcia-Márquez, L.J., Morales-Barrera, J.E., Latorre, J.D., Hernandez-Velasco, X., Tellez, G. (2016). Evaluation of a selected lactic acid bacteria-based probiotic on *Salmonella enterica* serovar Enteritidis

colonisation and intestinal permeability in broiler chickens. *Avian Pathol*. 46(1), p 90-94, DOI: 10.1080/03079457.2016.1222808.

Pretzer, G., Snel, J., Molenaar, D., Wiersma, A., Bron, R.A., Lambert, J., de Vos, W.M., van der Meer, R., Smits, M.A., Kleerebezem, M. (2005). Biodiversity-based identification and functional characterization of the mannose-specific adhesin of *Lactobacillus plantarum*. *J. Bacteriol*. 187(17), p 6128–6136, DOI: 10.1128/JB.187.17.6128-6136.2005.

Proaño Bolaños, C., Li, R., Zhou, M., Wang, L., Xi, X., Tapia, E.E., Coloma, L.A., Chen, T., Shaw, C. (2017). Novel Kazal-type Protease inhibitors from the skin secretion of the Splendid leaf frog, *Cruziohyla calcarifer*. *EuPA Open Proteom*. 15, p 1-13. DOI: 10.1016/j.euprot.2017.02.001

Qi, L., Hu, M., Fu, J., Liu, Y., Wu, M., Yu, K., Liu, X. (2017). Quantitative proteomics analysis of host epithelial cells infected by *Salmonella enterica* serovar Typhimurium. *Proteomics* 17, p 13-14, DOI: 10.1002/pmic.201700092.

Ragland, S.A., Criss, A.K. (2017). From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. *PLoS Pathog*. 13(9), e1006512, DOI: 10.1371/journal.ppat.1006512.

Ramachandran, V., Arumugam, T., Wang, H., Logsdon, C.D. (2008). Anterior gradient 2 is expressed and secreted during the development of pancreatic cancer and promotes cancer cell survival. *Cancer Res*. 68(19), p 7811–7818, DOI: 10.1158/0008-5472.CAN-08-1320.

Ramsay, R.R., Gandour, R.D., van der Leij, F.R. (2001). Molecular enzymology of carnitine transfer and transport. *Biochim*. *Biophys*. *Acta*. 1546(1), p 21-43, DOI: 10.1016/S0167-4838(01)00147-9.

Reddi, A.R., Culotta, V.C. (2013). SOD1 integrates signals from oxygen and glucose to repress respiration. *Cell* 152(1-2), p 224–235, DOI: 10.1016/j.cell.2012.11.046.

Repetto, G., del Peso, A., Zurita, J.L. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat*. *Protoc*. 3(7), p 1125-1131, DOI: 10.1038/nprot.2008.75.

Reynaert, N.L., Wouters, E.F., Janssen-Heininger, Y.M. (2007). Modulation of glutaredoxin-1 expression in a mouse model of allergic airway disease. *Am*. *J*. *Respir*. *Cell Mol*. *Biol*. 36, p 147–151, DOI: 10.1165/rcmb.2006-0259RC.

Rinttilä, T., Apajalahti, J. (2013). Intestinal microbiota and metabolites – Implications for broiler chicken health and performance. *J*. *Appl*. *Poult*. *Res*. 22(3), p 647-658, DOI: 10.3382/japr.2013-00742.

Roos, S., Jonsson, H. (2002). A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiol*. 148(2), p 433–442, DOI: 10.1099/00221287-148-2-433.

Russell, J.B., Diez-Gonzalez, F. (1998). The effects of fermentation acids on bacterial growth. *Adv*. *Microb*. *Physiol*. 39, p 205–234, DOI: 10.1016/S0065-2911(08)60017-X.

Sasikumar, A.N., Perez, W.B., Kinzy, T.G. (2012). The many roles of the eukaryotic elongation factor 1 complex. Wiley Interdiscip. Rev. RNA 3(4), p 543-555, DOI: 10.1002/wrna.1118.

Satchell, D.P., Sheynis, T., Shirafuji, Y., Kolusheva, S., Ouellette, A.J., Jelinek, R. (2003). Interactions of mouse Paneth cell alpha-defensins and alpha-defensin precursors with membranes. Prosegment inhibition of peptide association with biomimetic membranes. *J*. *Biol*. *Chem*. 278(16), p 13838-13846, DOI: 10.1074/jbc.M212115200.

Schmidt, H., Hensel, M. (2004). Pathogenicity islands in bacterial pathogenesis. *Clin*. *Microbiol*. *Rev*. 17(1), p 14-56, DOI: 10.1128/CMR.17.1.14-56.2004.

Schneider, V.A.F., Coorens, M., Ordonez, S.R., Tjeerdsma-van Bokhoven, J.L.M., Posthuma, G., van Dijk, A., Haagsman, H.P., Veldhuizen, E.J.A. (2016). Imaging the antimicrobial mechanism(s) of cathelocidin-2. *Sci*. *Rep*. 6, 32948, DOI: 10.1038/srep32948.

Schneitz, C. (2005). Competitive exclusion in poultry—30 years of research. *Food Control* 16(8), p 657–667, DOI: 10.1016/j.foodcont.2004.06 .002.

Segawa, Y., Itokazu, Y., Inoue, N., Saito, T., Suzuki, K. (2001). Possible changes in expression of chemotaxin LECT2 mRNA in mouse liver after concanavalin A induced hepatic injury. *Biol*. *Pharm*. *Bull*. 24(4), p 425–428, DOI: 10.1248/bpb.24.425.

Seo, S.B., McNamara, P., Heo, S., Turner, A., Lane, W.S., Chakravarti, D. (2001). Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. *Cell* 104(1), p 119–130, DOI: 10.1016/S0092-8674(01)00196-9.

Shah, D., Zhou, X., Addwebi, T., Davis, M.A., Orfe, L., Call, D.R., Guard, J., Besser, T.E. (2011). Cell invasion of poultry-associated *Salmonella enterica* serovar Enteritidis isolates is associated with pathogenicity, motility and proteins secreted by the type II secretion system. *Microbiol*. 157(Pt. 5), p 1428-1445, DOI: 10.1099/mic.0.044461-0.

Shi, L., Chowdhury, S.M., Smallwood, H.S., Yoon, H., Mottaz-Brewer, H.M., Norbeck, A.D., McDermott, J.E., Clauss, T.R., Heffron, F., Smith, R.D., Adkins, J.N. (2009). Proteomic investigation of the time course responses of RAW 264.7 macrophages to infection with *Salmonella enterica*. *Infect*. *Immun*. 77(8), p 3227–3233, DOI: 10.1128/IAI.00063-09.

Shin, S.Y., Kang, S.W., Lee, D.G., Eom, S.H., Song, W.K., Kim, J.I. (2000). CRAMP analogues having potent antibiotic activity against bacterial, fungal, and tumor cells without hemolytic activity. *Biochem*. *Biophys*. *Res*. *Commun*. 275(3), p 904-909, DOI: 10.1006/bbrc.2000.3269.

Song, J.J., Lee, Y.J. (2003a). Differential role of glutaredoxin and thioredoxin in metabolic oxidative stress-induced activation of apoptosis signal-regulating kinase 1. *Biochem*. *J*. 373(3), p 845–853, DOI: 10.1042/BJ20030275.

Song, J.J., Lee, Y.J. (2003b). Role of the ASK1-SEK1-JNK1-HIPK1 signal in Daxx trafficking and ASK1 oligomerization. *J*. *Biol*. *Chem*. 278(47), p 47245–47252, DOI: 10.1074/jbc.M213201200.

Spillmann, D., Burger, M.M. (1996). Carbohydrate-carbohydrate interactions in adhesion. *J*. *Cell*. *Biochem*. 61(4), p 562-568, DOI: 10.1002/(SICI)1097-4644(19960616)61:4<562::AID-JCB9>3.0.CO;2-M.

Steinert, M., Hentschel, U., Hacker, J. (2000). Symbiosis and pathogenesis: evolution of the microbe-host interaction. *Naturwissenschaften* 87(1), p 1-11, DOI: 10.1007/s001140050.

Sugiarto, H., Yu, P.L. (2004). Avian antimicrobial peptides: the defense role of beta-defensins. *Biochem*. *Biophys*. *Res*. *Commun*. 323(3), p 721-727, DOI: 10.1016/j.bbrc.2004.08.162.

Sun, J., Wei, X., Lu, Y., Cui, M., Li, F., Lu, J., Liu, Y., Zhang, X. (2017). Glutaredoxin 1 (GRX1) inhibits oxidative stress and apoptosis of chondrocytes by regulating CREB/HO-1 in osteoarthritis. *Mol*. *Immunol*. 90, p 211-218, DOI: 10.1016/j.molimm.2017.08.006.

Suzuki, K., Yamakawa, Y., Matsuo, Y., Kamiya, T., Minowada, J., Mizuno, S. (19993). Isolation and amino acid sequence of a chemotactic protein, LECT/interleukin 8, from a human myeloid leukemia cell line, ML-1. *Immunol*. *Lett*. 36(1), p 71–81.

Szklarczyk, D., Morris, J.H., Cook, H., Kuhn, M., Wyder, S., Simonovic, M., Santos, A., Doncheva, N.T., Roth, A., Bork, P., Jensen, L.J., von Mering, C. (2017). The STRING database in 2017: quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res*. 45, D362-D368, DOI: 10.1093/nar/gkw937.

Teirlynck, E., Gussem, M.D.E., Dewulf, J., Haesebrouck, F., Ducatelle, R., Van Immerseel, F. (2011). Morphometric evaluation of “dysbacteriosis” in broilers. *Avian Pathol*. 40(2), p 139–144, DOI: 10.1080/03079457.2010 .543414.

Terashima, M., Badruzzaman, M., Tsuchiya, M., Shimoyama, M. (1996). Exocytosis of argine-specific ADP-ribosyltransferase and p33 induced by A23187 and calcium or serum-opsonized zymosan in chicken polymorphonuclear leukocytes. *J*. *Biochem*. 120(6), p 1209–1215.

Thornton, S., Anand, N., Purcell, D., Lee, J. (2003). Not just for housekeeping: protein initiation and elongation factors in cell growth and carcinogenesis. *J*. *Mol*. *Med*. 81(9), p 536-548, DOI: 10.1007/s00109-003-0461-8.

Travis, S.M., Anderson, N.N., Forsyth, W.R., Espiritu, C., Conway, B.D., Greenberg, E.P., McCray, P.B., Lehrer, R.I., Welsh, M.J., Tack, B.F. (2000). Bactericidal activity of mammalian cathelicidin-derived peptides. *Infect*. *Immun*. 68(5), p 2748-2755.

Tyanova, S., Temu, T., Carlson, A., Sinitcyn, P., Mann, M., Cox, J. (2015). Visualization of LC-MS/MS proteomics data in MaxQuant. *Proteomics* 15(8), p 1453–1456, DOI: 10.1002/pmic.201400449.

Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M.Y., Geiger, T., Mann, M., Cox, J. (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nat*. *Methods* 13(9), p 731-740, DOI: 10.1038/nmeth.3901.

van Dijk, A., Molhoek, E.M., Veldhuizen, E.J., Bokhoven, J.L., Wagendorp, E., Bikker, F., Haagsman, H.P. (2009). Identification of chicken cathelicidin-2 core elements involved in antibacterial and immunomodulatory activities. *Mol*. *Immunol*. 46(13), p 2465–2473, DOI: 10.1016/j.molimm.2009.05.019.

Van Immerseel, F., Russell, J.B., Flythe, M.D., Gantois, I., Timbermont, L., Pasmans, F., Haesebrouck, F., Ducatelle, R. (2006). The use of organic acids to combat *Salmonella* in poultry: a mechanistic explanation of the efficacy. *Avian Pathol*. 35(3), p 182-188, DOI: 10.1080/03079450600711045.

Velge, P., Wiedemann, A., Rosselin, M., Abed, N., Boumart, Z., Chaussé, A.M., Grépinet, O., Namdari, F., Roche, S.M., Rossignol, A., Virlogeux-Payant, I. (2012). Multiplicity of *Salmonella* entry mechanisms, a new paradigm for *Salmonella* pathogenesis. *Microbiologyopen*. 1(3), p 243-258, DOI: 10.1002/mbo3.28.

Vermeulen, K., Verspreet, J., Courtin, C.M., Haesebrouck, F., Ducatelle, R., Van Immerseel, F. (2017). Reduced particle size wheat bran is butyrogenic and lowers *Salmonella* colonisation, when added to poultry feed. *Vet*. *Microbiol*. 198, p 64-71, DOI: 10.1016/j.vetmic.2016.12.009.

Vollbrandt, T., Willkomm, D., Stossberg, H., Kruse, C. (2004). Vigilin is co-localized with 80S ribosomes and binds to the ribosomal complex through its C-terminal domain. *Int*. *J*. *Biochem*. *Cell Biol*. 36(7), p 1306–1318, DOI: 10.1016/j.biocel.2003.11.006.

Wang, Q., Zhang, Z., Blackwell, K., Carmichael, G.G. (2005). Vigilins bind to promiscuously A-to-I-edited RNAs and are involved in the formation of heterochromatin. *Curr*. *Biol*. 15(4), p 384–391, DOI: 10.1016/j.cub.2005.01.046.

Wang, R., Jiang, L., Zhang, M., Zhao, L., Hao, Y., Guo, H., Sang, Y., Zhang, H., Ren, F. (2017). The adhesion of *Lactobacillus salivarius* REN to a human intestinal epithelial cell line requires S-layer proteins. *Sci*. *Rep*. 7, Article ID 44029, DOI: 10.1038/srep44029.

Wang, Z., Hao, Y., Lowe, A.W. (2008). The adenocarcinoma-associated antigen, AGR2, promotes tumor growth, cell migration, and cellular transformation. *Cancer Res*. 68(2), p 492–497, DOI: 10.1158/0008-5472.CAN-07-2930.

Weissman, A.M. (2001). Themes and variations on ubiquitylation. *Nat*. *Rev*. *Mol*. *Cell Biol*. 2(3), p 169–178., DOI: 10.1038/35056563.

Welling, M.M., Hiemstra, P.S., van den Barselaar, M.T., Paulusma-Annema, A., Nibbering, P.H., Pauwels, E.K., Calame, W. (1998). Antibacterial activity of human neutrophil defensins in experimental infections in mice is accompanied by increased leukocyte accumulation. *J*. *Clin*. *Invest*. 102(8), p 1583-1590, DOI: 10.1172/JCI3664.

Wiita, A.P., Perez-Jimenez, R., Walther, K.A., Gräter, F., Berne, B.J., Holmgren, A., Sanchez-Ruiz, J.M., Fernandez, J.M. (2007). Probing the chemistry of thioredoxin catalysis with force. *Nature* 450, p. 124-127, DOI: 10.1038/nature06231.

Wilson, J., Tice, G., Brash M.L., Hilaire, S.St. (2005). Manifestations of Clostridium perfringens and related bacterial enteritides in broiler chickens. *World's Poult*. *Sci*. *J*. 61(3), p 435–449, DOI: 10.1079/wps200566.

Wilson-Fritch, L., Burkart, A., Bell, G., Mendelson, K., Leszyk, J., Nicoloro, S., Czech, M., Corvera, S. (2003). Mitochondrial biogenesis and remodelling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Mol*. *Cell*. *Biol*. 23(3), p 1085–1094, DOI: 10.1128/MCB.23.3.1085-1094.2003.

Wintersberger, U., Kühne, C., Karwan, A. (1995). Scp160p, a new yeast protein associated with the nuclear membrane and the endoplasmic reticulum, is necessary for maintenance of exact ploidy. *Yeast* 11(10), p 929–944, DOI: 10.1002/yea.320111004.

Xiao, Y., Cai, Y., Bommineni, Y.R., Fernando, S.C., Prakash, O., Gilliland, S.E., Zhang, G. (2006). Identification and functional fharacterization of three fhicken fathelicidins with potent antimicrobial activity. *J*. *Biol*. *Chem*. 281(5), p 2858–2867, DOI: 10.1074/jbc.M507180200.

Xiao, Y., Hughes, A.L., Ando, J., Matsuda, Y., Cheng, J.F., Skinner-Noble, D., Zhang, G. (2004). A genome-wide screen identifies a single beta-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. *BMC Genomics* 5(1), 56, DOI: 10.1186/1471-2164-5-56.

Yang, W.L., Wei, L., Huang, W.Q., Li, R., Shen, W.Y., Liu, J.Y., Xu, J.M., Li, B., Qin, Y. (2014b). Vigilin is overexpressed in hepatocellular carcinoma and is required for HCC cell proliferation and tumor growth. *Oncol*. *Rep*. 31(5), p 2328–2334, DOI: 10.3892/or.2014.3111.

Yang, X., Brisbin, J., Yu, H., Yin, F., Zhang, Y., Sabour, P., Sharif, S., Gong, J. (2014a). Selected lactic-acid-producing bacterial isolates with the capacity to reduce *Salmonella* translocation and virulence gene expression in chickens. *PLoS One* 9(4), e93022, DOI: 10.1371/journal.pone.0093022.

Yang, Y., Hu, M., Yu, K., Zheng, X., Liu, X. (2015). Mass spectrometry-based proteomic approaches to study pathogenic bacteria-host interactions. *Prot*. *Cell* 6(4), p 265-274, DOI: 10.1007/s13238-015-0136-6.

Yegani, M., Korver, D.R. (2008). Factors affecting intestinal health in poultry. *Poult*. *Sci*. 87(10), p 2052–2063, DOI: 10.3382/ps.2008-00091.

Yoo, H.M., Park, J.H., Jeon, Y.J., Chung, C.H. (2015). Ubiquitin-fold modifier 1 acts as a positive regulator of breast cancer. *Front*. *Endocrinol*. 6, Article 36, DOI: 10.3389/fendo.2015.00036.

Yu, H.T., Yue, L.L., Zhang, C.J. (2012). Grx1 antagonized high glucose-Induced apoptosis in endothelial cells through inhibition of jnk pathway. *Adv*. *Mater*. *Res*. *Trans*. *Tech*. *Publ*. (345), p 257-262, DOI: 10.4028/www.scientific.net/AMR.345.257.

Zanetti, M. (2005). The role of cathelicidins in the innate host defences of mammals. *Curr*. *Issues Mol*. *Biol*. 7(2), p 179-196.

Zanetti, M., Gennaro, R., Romeo, D. (1995). Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS Lett* 374(1), p 1–5, DOI: 10.1016/0014-5793(95)01050-O.

Zheng, A., Luo, J., Meng, K., Jianke, L., Bryden, W.L., Chang, W., Zhang, S., Wang, L.X.N., Liu, G., Yao, B. (2016). Probiotic (*Enterococcus faecium*) induced responses of hepatic proteome improves metabolic efficiency of broiler chickens (*Gallus gallus*). *BMC Genomics* 17, 89, DOI: 10.1186/s12864-016-2371-5.

Zhou, J., Wang, Q., Chen, L.L., Carmichael, G.G. (2008). On the mechanism of induction of heterochromatin by the RNA-binding protein vigilin. *RNA* 14(9), p 1773–1781, DOI: 10.1261/rna.1036308.

Zhu, Q., Mangukiya, H.B., Mashausi, D.S., Guo, H., Negi, H., Merugu, S.B., Wu, Z., Li, D. (2017). Anterior gradient 2 is induced in cutaneous wound and promotes wound healing through its adhesion domain. *FEBS J*. 284(17), p 2856-2969, DOI: 10.1111/febs.14155.

Zolnierowicz, S. (2000). Type 2A protein phosphatase, the complex regulator of numerous signalling pathways. *Biochem*. *Pharmacol*. 60(8), p 1225–1235, DOI: 10.1016/S0006-2952(00)00424-X.

Chapter 6

Controlling *Salmonella* colonisation in broilers with antibiotic and probiotic feed additives

Abstract

Salmonella is a considered a high risk in poultry and high intestinal cell numbers may lead to contaminated meat and the outbreak of salmonellosis. *Salmonella* is a natural inhabitant of the broiler GIT and rarely cause mortalities, but rather poses a threat as food-borne pathogen for humans. Various feed additives, including probiotics, are used to control cell numbers of *Salmonella* in broilers. Here we report on the effect a multi-species probiotic (*L*. *crispatus* DPN167, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94 and *B*. *amyloliquefaciens* DPN123) has on the colonisation of *Salmonella enterica* serovar Enteridis A9 in the intestinal tract of broilers, in comparison to infected broilers treated with oxytetracycline. The first group of broilers (119 birds) was administered orally via feed with 2 to 8.9 × 10^7 CFU of the multi-species probiotic per day, from day 1, for 29 days. The second group (119 broilers) received orally 4 to 31 mg oxytetracycline per day, over the same period. Broilers in groups 3 (119 birds) acted as a positive control (*Salmonella* infected) and 4 (119 birds) acted as a negative control (uninfected). Birds from groups 3 and 4 were not treated with the probiotic or oxytetracycline. On days 9 and 10, broilers in group 1, 2 and 3 were infected with 9 × 10^7 CFU of *S*. Enteritidis A9. Broilers in group 4 were not infected with *Salmonella*. The growth performance, immune organ weight, erythrocyte and leukocyte cell counts, and serum interferon gamma levels of broilers infected with *S*. Enteritidis A9 (groups 1, 2 and 3) correlated with values recorded for uninfected broilers (group 4). Within the first few days after infection (days 11-14), the faecal cell numbers of *S*. Enteritidis A9 of broilers treated with oxytetracycline (group 2) decreased from 7.954 to 0.857 (Log CFU) and remained at this level for 4 days. However, cell numbers increased from day 19 and onwards to levels equivalent to those recorded for broilers from the probiotic and positive control treatment groups (groups 1 and 3, respectively). Broilers that received the multi-species probiotic (group 1) had similar levels of *Salmonella* in their cecum as untreated infected broilers (group 3). However, the viable cell numbers of *Salmonella* decreased on day 29 to levels recorded in uninfected broilers (group 4). Treatment with the multi-species probiotic and oxytetracycline increased serum *Salmonella* bactericidal activity and T lymphocyte responses in broilers infected with *Salmonella*. In addition, probiotic use increased serum lysozyme levels. In conclusion, antibiotic and probiotic feed additives increased broiler immune responses in response to *Salmonella* infection.

Introduction

Salmonella infection is responsible for huge financial losses in the poultry industry (Wigley *et al*., 2001; Vo *et al*., 2006; Kabir, 2010; Obukhovska, 2013). Newly hatched chicks are the most susceptible to infections, especially during the first 24 h after hatching (Milner and Shaffer, 1952). At this age the gut microbiome is not fully developed and offers no protection in the form of competitive exclusion, thus no resistance, to the colonisation by *Salmonella.* The mucosa, cecal tonsils and Peyer's patches are usually heavily infected, followed by infection of the liver, spleen, lungs, gizzard, ovaries and yolk sac (Chappell *et al*., 2009; Dunkley *et al*., 2009; Kabir, 2010). One of the first symptoms of *Salmonella* infection is diarrhoea (Shivaprasad, 2000; Awad *et al*., 2017), accompanied by a decrease in feed intake (Marcq *et al*., 2011). Not all birds infected with *Salmonella* show symptoms (Cosby *et al*., 2015; Dar *et al*., 2017) and enter the meat market, with the potential of causing widespread salmonellosis (CDC, 2015).

In the past, *Salmonella* infections were controlled by adding antimicrobial additives to the feed. Since many of these additives contained antibiotics that may contribute to the developing of antibiotic resistance amongst bacteria, this practice has been banned, at least in Europe (Phillips *et al*., 2004; Singer *et al*., 2016). Since the banning, many alternatives to antibiotics have been proposed, e.g. probiotics (Adhikari and Kim, 2017; Wang *et al*., 2017), prebiotics (Adhikari and Kim, 2017), synbiotics (Pandey *et al*., 2015; Dunislawska *et al*., 2017), enzymes (Yang *et al*., 2009; Kiarie *et al*., 2013), essential oils (Alcicek *et al*., 2004; Brenes and Roura, 2010) and fatty acids (Huff *et al*., 2010). Commercially available probiotics include PoultryStar® (*E*. *faecium*, *P*. *acidilactici*, *B*. *animalis*, *L*. *salivarius*, and *L*. *reuteri* and prebiotic fructooligosaccharides), CLOSTAT™ (*B*. *subtilis*) and Floramax® (*L*. *salivarius* and *P*. *parvulus*). PoultryStar® administration decrease colonisation of *C*. *jejuni* and *S*. Enteritidis (Sterzo *et al*., 2007; Ghareeb *et al*., 2012). CLOSTAT™ improved feed conversion, intestinal morphology, enhanced immune responses, and inhibited GIT colonisation by *C*. *jejuni*, *E*. *coli* and *S*. Minnesota (Teo and Tan, 2007; Melegy *et al*., 2011; Lourenco *et al*., 2012; Abudabos *et al*., 2013). Floramax® B11 improved weight gain, bone characteristics, intestinal morphology, immune responses and decreased *S*. Enteritidis colonisation (Gutierrez-Fuentes *et al*., 2013; Prado-Rebolledo *et al*., 2017).

Salmonella enterica is an important group of gastrointestinal pathogens that are leading causes of foodborne diseases such as gastroenteritis and diarrhoea (Gal-Mor *et al*., 2014). Several serovars of *S*. *enterica* subsp. *enterica*, specifically *S*. Enteritidis, *S*. Typhimurium and *S*. Gallinarum, are major avian pathogens that cause severe morbidity and mortality in broilers and food-borne illnesses in humans (Ribeiro *et al*., 2007). It is important to control gastrointestinal microbiome homeostasis to prevent colonisation by pathogens. Microbiome homeostasis is influenced by various factors i.e. diet and feed additives, farm conditions and practices, and the composition of the gut microbiome (Stanley *et al*., 2014). The microbiome comprises trillions of microorganisms localised primarily at the distal end of the GIT (Oakley *et al*., 2014), and together the community mediates digestion, controls gut homeostasis, and prevents colonisation of pathogenic bacteria (Pan and Yu, 2014; Ohland and Jobin, 2015).

This study aimed to determine the ability of a multi-species probiotic (*L*. *crispatus* DPN167, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94 and *B*. *amyloliquefaciens* DPN123) and antibiotic (oxytetracycline) in inhibiting colonisation of *Salmonella enterica* subsp. *enterica* serovar Enteritidis A9 *in vivo* in broilers.

Materials and Methods

Animals and housing

Ethical approval to conduct the study was granted by the Research Ethics Committee: Animal Care and Use of Stellenbosch University, Stellenbosch (registration number SU-ACUD15-00016). All experiments were performed in accordance with relevant guidelines and regulations. Four hundred and seventy-six day old as-hatched Cobb 500 broiler chicks were separated into 68 cages of 2 m^2 each (7 birds per cage) and housed in a temperature-controlled poultry rearing facility at Mariendahl experimental farm, Stellenbosch University. Each cage had equal number of sexes (4 females and 3 males) and weighed the same. Each treatment group consisted of 17 cages (119 broilers). Each cage was equipped with feeders and automatic water dispensers. Humidity, temperature and light were controlled according to the Cobb Broiler Management Standards (Cobb Vantress, Colchester, UK) and the South African Animal Welfare Act. Broilers from the control uninfected group was housed in the same rearing facility, however, was isolated from the infected treatment groups and handled separately to prevent cross contamination of *Salmonella*.

Preparation of probiotics

Probiotic feed additive (consisting of *L*. *crispatus* DPN167, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94 and *B*. *amyloliquefaciens* DPN123) was produced as previously described (Neveling *et al*., 2017). Probiotic strains were cultured in molasses medium, which consisted of 5.0 % (w/v) molasses, 0.3 % (w/v) yeast extract, 0.2 % (w/v) peptone, 0.004 % (w/v) $MnSO_4$, 0.001 % (w/v) Na-citrate, 0.4 % (w/v) K_2HPO_4 and 0.02 % (v/v) Tween80. The medium was sterilised at 121 °C for 15 min, cooled to 25 °C, the upper phase removed from the sediment and again autoclaved. Bacterial strains were grown separately, freeze dried and combined as a dry power. Thioglycolate (0.15 %, w/v) was added to the growth medium of *L*. *crispatus* DPN167 and *L*. *johnsonii* DPN184 to create an anaerobic environment. Incubation was for 3 to 4 days at 37 °C. Cells were harvested (8000 × g, 10 min, 4 °C), washed with sterile PBS (0.8 %, w/v, NaCl; 0.02 %, w/v, KCl; 0.142 %, w/v, Na_2HPO_4; 0.024 %, w/v, KH_2PO_4; pH 7.5) and resuspended in sterile cryoprotectant (10 %, w/v, lactose and 10.0 %, w/v, sucrose, autoclaved at 121 °C for 10 min and cooled to 4 °C). The number of viable cells per gram freeze-dried culture was determined by plating onto MRS Agar (Biolab) or BHI Agar (Biolab) and incubated at 37 °C for 24 h under aerobic and anaerobic conditions. The strains were combined to yield a total cell count of 2.8 × 10^8 CFU/g freeze-dried powder, consisting of 2.6 × 10^7 CFU/g *L*. *crispatus* DPN167, 3.6 × 10^7 CFU/g *L*. *salivarius* DPN181, 1.3 × 10^8 CFU/g *L*. *gallinarum* DPN164, 1.9 × 10^7 CFU/g *L*. *johnsonii* DPN184, 5.1 × 10^7 CFU/g *E*. *faecalis* DPN94 and 1.9 × 10^7 CFU/g *B*. *amyloliquefaciens* DPN123.

Preparation of feed

Diets were formulated according to Cobb guidelines (Cobb Vantress). Broilers were fed a three-ration feed (Table 1). The starter diet was provided at 244 g per bird (over 10 days), grower diet at 1183 g per bird (over 14 days) and a finisher diet at 583 g per bird (over 4 days).

Table 1: Broiler starter, grower and finisher feed composition.

Ingredients	**Starter** (g/100 g)	**Grower** (g/100 g)	**Finisher** (g/100 g)
Maize	50.638	52.360	55.170
Soybean full fat	26.000	30.000	30.000
Soybean 46	17.838	11.837	8.397
L-lysine HCl	0.266	0.119	0.096
DL methionine	0.382	0.297	0.254
L-threonine	0.120	0.165	0.033
Vitamin premix	0.250	0.250	0.250
Limestone	1.399	1.267	1.159
Salt	0.266	0.268	0.275
Monocalcium phosphate	1.981	1.757	1.563
Sodium bicarbonate	0.133	0.133	0.127
Sunflower oil	0.727	1.546	2.676

Feeding trials

Feed of broilers from group 1 were supplemented with the multi-species probiotic. Average daily intake of the multi-species probiotic from day 1 to 29 during the different feeding stages is listed in Table 2. Broilers in group 1 received via feed between 2 and 8.9×10^7 CFU daily of the multi-species probiotic for 29 days; consisting of *L*. *crispatus* DPN167 (1.9×10^6 to 8.2×10^6 CFU), *L*. *salivarius* DPN181 (2.6×10^6 to 1.1×10^7 CFU), *L*. *gallinarum* DPN164 (9.3×10^6 to 4.1×10^7 CFU), *L*. *johnsonii* DPN184 (1.2×10^6 to 6.0×10^6 CFU), *E*. *faecalis* DPN94 (3.7×10^6 to 1.6×10^7 CFU) and *B*. *amyloliquefaciens* DPN123 (2.6×10^6 to 6.0×10^6 CFU). Feed of broilers from group 2 was supplemented with 200 mg oxytetracycline per kg feed (Virbac, Centurion, South Africa). Average daily intake of oxytetracycline from day 1 to 29 during the different feeding stages is listed in Table 2. Broilers consumed on average 4 (day 1) to 31 mg (day 29) oxytetracycline daily for 29 days. Broilers from group 3 (infected) and 4 (uninfected) received feed without antibiotics and probiotics.

Table 2: Average daily feed, probiotic and antibiotic consumption rates.

Feed	Probiotic concentration (CFU/g feed)	Days	Daily average feed consumption (g)	Daily average probiotic consumption (CFU)	Daily average oxytetracycline consumption (mg)
Starter	1.01×10^6	0	140	1.41×10^8	28
		1			
		2			
		3			
		4			
		5			
		6			
		7			
		8	30	3.03×10^7	6
		9	35	3.53×10^7	7
		10	39	3.93×10^7	7.8
Grower	6.82×10^5	11	44	3.00×10^7	8.8
		12	50	3.41×10^7	10
		13	55	3.75×10^7	11
		14	61	4.16×10^7	12.2
		15	67	4.57×10^7	13.4
		16	73	4.98×10^7	14.6
		17	80	5.46×10^7	16
		18	86	5.87×10^7	17.2
		19	93	6.34×10^7	18.6
		20	100	6.82×10^7	20
		21	107	7.30×10^7	21.4
		22	115	7.85×10^7	23
		23	122	8.32×10^7	24.4
		24	130	8.87×10^7	26
Finisher	2.42×10^5	25	137	3.32×10^7	27.4
		26	144	3.49×10^7	28.8
		27	151	3.66×10^7	30.2
		28	157	3.80×10^7	31.4

Infection of broilers with *Salmonella*

On day 5, the absence of *Salmonella* in broilers were confirmed, 10 chicks were randomly selected and euthanised by cervical dislocation. Their ceca were sterilely removed, placed in a Sterilin™ homogenising bag (Thermo Fisher Scientific, Massachusetts, USA) containing 100 ml peptone buffered water [1 % (m/v) peptone, 0.5 % (m/v) NaCl, 0.35 % (m/v) Na_2HPO_4, 0.15 % (m/v) KH_2PO_4, pH 7.2), and homogenised using a stomacher (Interscience, St Nom la Bretêche, France) for 5 min. The homogenised ceca were serially diluted in peptone buffered water and plated on SS agar supplemented with novobiocin (0.004 % m/v) and incubated at 37 °C for 24 h. In addition to direct plating, homogenised samples were enriched in peptone buffered water at 37 °C for 18 h, and thereafter in tetrathionate brilliant green bile (TGB) broth (Merck) supplemented with novobiocin (0.004 % m/v) at 37 °C for 24 h. After enrichment samples were serially diluted and plated on SS agar and incubated at 37 °C for 24 h. No *Salmonella* growth on SS agar after direct plating and enrichment indicated the absence of *Salmonella*.

On day 9 and 10, broilers from groups 1, 2 and 3 were infected with 200 µl (9×10^7 CFU) *Salmonella enterica* subsp. *enterica* serovar Enteritidis A9 by oral gavage. Broilers from group 4 (uninfected untreated) received sterile peptone buffered water instead of *Salmonella*. *Salmonella* Enteritidis A9 was grown aerobically in LB broth at 37 °C for 8 h, cells collected by centrifugation (8000 × g for 10 min at 37 °C), washed three times with sterile PBS, and reconstituted in peptone buffer water to a concentration of 4.5×10^8 CFU/ml. Viable cell numbers were determined by serial diluting and plating on *Salmonella-Shigella* (SS) agar (Merck) and incubating at 37 °C for 24 h.

Salmonella colonisation of the cecum

On days 11 (days post infection, dpi 1), 14 (dpi 4), 19 (dpi 9), 21 (dpi 11), 25 (dpi 15) and 28 (dpi 18) colonisation of *Salmonella* in the cecum was followed. At each time point one broiler per cage was euthanised by cervical dislocation, and the ceca processed as described above. Direct plating was used to determine the CFU per cecum, and enrichment steps used to confirm the absence of *Salmonella* in samples negative after direct plating. Ceca samples absent of *Salmonella* after direct plating but present after enrichment were assumed to contain 10^1 CFU/cecum. Samples absent of *Salmonella* after enrichment were considered *Salmonella* free. *Salmonella* isolates were identified by morphological (growth on SS agar) and biochemical characteristics (urease test), and identification confirmed by DNA homology tests. *Salmonella* isolates were analysed for urease by methods previously described (Christensen, 1946). Isolates were inoculated in urease broth [2 % (m/v) urea, 0.95 % (m/v) Na_2HPO_4, 0.91 % (m/v) KH_2PO_4, 0.01 % (m/v) yeast extract and 0.001 % (m/v) phenol red, pH 6.8] and incubated at 37 °C for 12 h, and thereafter inspected for colour change. *Salmonella* produce urease which results in media colour change from red to yellow. DNA was isolated from strains using the Zymo DNA extraction kit (Zymo Research, Irvine, California), according to the manufacturer's instructions. The 16S rDNA gene was amplified by PCR using DNA primers 8F: (5′-CACGGATCCAGACTTTGATYMTGGCTCAG-3′) and 1512R: (5′-GTGAAGCTTACGGYTAGCTTGTTACGACTT-3′), according to the method of Neveling *et al*. (2012). The 16S rDNA gene was purified using the QIAquik PCR purification kit (Qiagen, Hilden, Germany),

according to the manufacturer's instructions. The 16S rDNA gene was sequenced using the BigDye Terminator V3.1 sequencing kit (Applied Biosystems, Foster City, California), as per manufacturer instructions. Primers 8F, 1512R, 1100R (5′-AGGGTTGCGCTCGTTG-3′) and 520R (5′-ACCGCGGCTGCTGGC-3′) were used to sequence the 16S rDNA fragment (Neveling *et al*., 2012). MEGA7 software (Kumar *et al*., 2016) was used to align and construct the 16S rDNA gene, and BLAST analysis (Altschul *et al*., 1990) was performed to determine sequence similarity.

Growth performance

Visual health and growth performance of the birds were evaluated based on daily feed consumption and changes in body mass. Weight of broilers and feed intake were recorded weekly and reported as an average of the pen. All the birds were weighed and the change in body mass of each cage calculated relevant to the mass recorded on day 1. Average feed conversion ratio (FCR) was calculated from the feed intake (FI) and body weight gain (BWG).

Haematology and immune organ weights

Blood was collected from broilers on days 18 (8 dpi) and 29 (19 dpi). Blood samples were collected from one broiler per cage (68 cages), from the brachial vein into a sterile syringe coated with 100 U of heparin, and aliquoted into 1ml K_2-EDTA BD Vacutainer® blood collection tube (Becton, Dickinson and Company, New Jersey, USA). Automated full blood counts were performed using the Celldyne 3700CS haematology analyser (Abbott Diagnostics, Illinois, USA). The erythrocyte counts and their related parameters, i.e. haemoglobin content, haematocrit value, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and erythrocyte distribution width were determined. In addition, the total leukocyte counts were recorded. On days 14 (dpi 4), 21 (dpi 11) and 28 (dpi 18), the spleen and bursa of Fabricius of one birds per cage was excised and weighed.

Serum interferon gamma levels

On day 15 (dpi 5), blood was collected from one broiler per cage (68 cages) from the brachial vein using a sterile syringe, and aliquoted into a 1ml BD Vacutainer® SST™ serum separation tube (Becton, Dickinson and Company), allowed to clot for 10 minutes at room temperature and then centrifuged at 1300 x g for 15 min at 4 °C. Serum was collected, and interferon gamma levels determined using the chicken IFN- γ ELISA Kit (Elabscience, Texas, USA), as per manufacturer's instructions.

Serum bactericidal and lysozyme activity

On days 11 (dpi 1), 14 (dpi 4), 20 (dpi 10), and 29 (dpi 19), SST blood was collected, and serum stored at -80 °C. Bactericidal activity of serum was determined against *Salmonella* Enteritidis A9 and *Escherichia coli* DH5α by methods previously described (Boyd *et al*., 2014). Briefly, an overnight culture of *S*. Enteritidis A9 and *E*. *coli* DH5α were inoculated (1 % v/v) separately in LB broth (Merck) and grown for 6 h at 37 °C. Bacteria in exponential growth (OD 0.4 to 0.6) were serially diluted in saline to 1×10^6 CFU/ml. The bactericidal test was conducted in a microtiter plate and each well contained 50 µl serum, 50 µl of Hanks's balanced salt solution (HBSS), 100 µl of LB broth and 10 µl of either *S*. Enteritidis A9

(10^4 CFU) or *E. coli* DH5α (10^4 CFU). Serum bactericidal activity was measured in triplicate. In control wells serum was replaced with HBSS, and supplemented with or without bacteria, which acted as controls for normal and no growth, respectively. Plates were incubated in a humidified (60 % RH) incubator at 37 °C for 18 h. Absorbance readings were measured at 690 nm before and after incubation, using a spectrophotometer (DU730 UV-Vis spectrophotometer, Beckman, California, USA).

Serum lysozyme levels were determined using an agar assay previously described (Lie *et al*., 1986). Briefly, the bacterial suspension was obtained by diluting 0.5 g of lyophilised *Micrococcus luteus* ATCC 4698 (Merck) in 20 ml sterile sodium-phosphate buffer (pH 6.3). Lysozyme assay plates were prepared by dissolving 1 g of medium EEO Agarose (Merck) in 100 ml sodium phosphate buffer (pH 6.3); after boiling in a water bath for 15 min, the solution is cooled to 60 ° C and constantly stirred, to which 200 µL of the *M. luteus* suspension is added. For each Petri dish (L × W × H; 120 mm × 120 mm × 17 mm) 20 ml of the agar was poured and allowed to solidify. Finally, 25 holes were made equidistant from each other on the agar plate. Lysozyme (Merck) was serially diluted in sodium phosphate buffer (pH 6.3) at concentrations ranging from 8 mg/ml to 64 µg/ml. Lysozyme standards and serum test samples were pipetted (10 µl) into the wells and incubated in a humidified chamber at 37 °C for 16 h. After incubation, zones of lysis were recorded by measuring the diameter of the halos, and the lysozyme concentrations of serum samples determined from the lysozyme standard curve.

T lymphocyte responsiveness

The proliferative response of T lymphocytes was assessed using the phytohaemagglutinin (PHA) test as described by Kean and Lamont, (1994) and Smits *et al*., (1999). Response induced by the mitogen was evaluated on day 17 (dpi 7), 24 h after administration. Briefly, in birds with feather cover, a 1-cm patch of skin on the mid-patagium of the left wing was plucked clean of feathers (Smits and Williams, 1999). Bare skin of the left-wing web was swabbed with alcohol and injected with 100 µl PHA (100 µg) subcutaneously using a 27-G needle. Patagium thickness was measured accurate to 0·01 mm, using a gauge micrometer (Dyer OD gauge 0·01 mm, The Dyer Company, Lancaster, PA). Four measurements of patagium thickness were taken before injection, and again 24 h after. The T lymphocyte responsiveness was calculated as the difference between the thickness of the patagium before and after injection.

Statistical analyses

GraphPad Prism 6 (GraphPad Software Inc., California, USA) was used to perform statistical analyses. Data of growth performance, lymphoid organ weight, haematological parameters, serum interferon gamma levels, and cell-mediated immune responses were analysed by one-way ANOVA to determine the significance of the main effects and interactions. The mean variances were compared using the Tukey post-hoc test. Differences were considered significant if p-values were less than 0.05. The serum bactericidal activity, serum lysozyme concentration, average log CFU *Salmonella* counts in the ceca of broilers from the treatment groups were analysed by two-way ANOVA to determine the significance of the main effects and multiple comparison done using either LSD or Games-Howell post-hoc test. The occurrence of *Salmonella* in the ceca (positive vs negative birds) of broilers from the

different treatment groups were compared using the Chi-square test and expressed as percentage infected (positive/total chickens x 100).

Results and Discussion

Growth performance

The weekly feed consumption rate of birds from group 4 (untreated uninfected) was significantly higher than birds from group 3 (infected untreated) within the first week (Table 3). No differences were observed between the remaining treatment groups. These initial differences might be due to the experimental setup, as birds from group 4 was separated from infected birds (groups 1, 2 and 3). In addition, birds were only infected with *Salmonella* on days 9 and 10, and thus differences are not due to *Salmonella*. The weekly weight gain, weekly feed consumption and the feed conversion ratio (FCR) of broilers in the different treatment groups were similar for the remaining of the 4-week protocol (Table 3). These results suggest that *Salmonella* administration did not negatively affect broiler growth performance, in accordance with literature (Ribeiro *et al*., 2007; Park and Kim, 2014; Olnood *et al*., 2015). Other researchers have shown that the benefits of probiotics (*B. cereus* or *S. boulardii*) on growth performance in broilers infected with *Salmonella* is not evident at a young age (14 days), but rather observed at a much older age (47 day) (Gil de los Santos *et al*., 2005). Current results are in accordance with literature that *Salmonella* infection does not negatively influence broiler growth performance.

Immune organ weight

The bursa of Fabricius is an organ that is unique to birds and is the major site for B cell differentiation and maturation (Masteller *et al*., 1997). The spleen plays an important role in red blood cell turnover (Mebius and Kraal, 2005) and contains B cells and macrophages (Jeurissen, 1993; Jia and Pamer, 2009, Swirski *et al*., 2009). The relative immune organ weights and the bursa: spleen ratio did not differ significantly between broilers from the different treatment groups on days 14 (dpi 4), 21 (dpi 11) and 28 (dpi 18) (Table 4). Results agree with other studies, showing that *Salmonella* infection and treatment with different antibiotics (zinc-bacitracin; virginiamycin) and probiotics (*B. subtilis*; multi-species consisting of *L. johnsonii*; *L. acidophilus*, *L. fermentum*, *L. plantarum* and *E. faecium*; and multi-species consisting of *L. jensenii*, *L. plantarum*, *L. fermentum* and *L. casei*) do not alter the immune organ weights of broilers (Chen *et al*., 2012; Olnood *et al*., 2015; Park and Kim, 2015; Sadeghi *et al*., 2015).

Table 3: Mean (± standard deviation) weekly feed intake (grams), weekly broiler weight (grams) and feed conversion ratio (FCR) for day 0-28 of broilers from the different treatment groups i.e. group 1 (broilers infected with *Salmonella* receiving probiotics as treatment), group 2 (broilers infected with *Salmonella* receiving oxytetracycline as treatment), group 3 (broilers infected with *Salmonella* receiving no treatment) and group 4 (uninfected broilers receiving no treatment).

	Weekly feed intake				**Average chick weight**				**FCR**
Treatment	Day 0-7	Day 7-14	Day 14-21	Day 21-28	Day 7	Day 14	Day 21	Day 28	Day 0-28
Group 1 (Probiotics)	121.6 ± 6.9	283.6 ± 41.3	555.7 ± 76.1	1029.2 ± 103.4	147.0 ± 7.9	366.9 ± 42.5	794.8 ± 89.4	1472.7 ± 96.4	1.408 ± 0.075
Group 2 (Antibiotics)	119.6 ± 7.3	319.1 ± 30.3	578.5 ± 72.0	984.2 ± 80.1	150.6 ± 6.7	387.8 ± 32.0	816.9 ± 88.1	1516.8 ± 121.9	1.379 ± 0.038
Group 3 (infected untreated)	118.7 ± 5.0	304.5 ± 41.2	570.7 ± 81.8	989.4 ± 130.8	144.9 ± 5.5	367.7 ± 41.6	791.1 ± 116.2	1411.1 ± 162.6	1.433 ± 0.069
Group 4 (uninfected)	125.1 ± 8.3	303.6 ± 51.8	527.3 ± 75.0	974.3 ± 86.5	148.4 ± 7.3	371.8 ± 47.8	782.1 ± 87.6	1456.3 ± 149.2	1.398 ± 0.055
Group difference (p value)	**0.050**	**0.143**	**0.259**	**0.461**	**0.122**	**0.467**	**0.777**	**0.451**	**0.118**

Table 4: Relative (± standard deviation) immune organ weights (spleen %, bursa % and spleen: bursa ratio) of broilers on days 14 (dpi 4), 21 (dpi 11) and 28 (dpi 18) from the different treatment groups i.e. group 1 (broilers infected with *Salmonella* receiving probiotics as treatment), group 2 (broilers infected with *Salmonella* receiving oxytetracycline as treatment), group 3 (broilers infected with *Salmonella* receiving no treatment) and group 4 (uninfected broilers receiving no treatment).

	Spleen %			**Bursa %**			**Spleen: Bursa ratio**		
Treatment	**Day 14**	**Day 21**	**Day 28**	**Day 14**	**Day 21**	**Day 28**	**Day 14**	**Day 21**	**Day 28**
Group 1 (Probiotics)	0.085 ± 0.017	0.090 ± 0.044	0.082 ± 0.021	0.223 ± 0.050	0.234 ± 0.049	0.217 ± 0.046	0.398 ± 0.110	0.403 ± 0.233	0.400 ± 0.148
Group 2 (Antibiotics)	0.083 ± 0.017	0.079 ± 0.016	0.083 ± 0.015	0.243 ± 0.044	0.212 ± 0.060	0.220 ± 0.071	0.351 ± 0.086	0.402 ± 0.140	0.414 ± 0.125
Group 3 (infected untreated)	0.082 ± 0.019	0.081 ± 0.017	0.092 ± 0.020	0.214 ± 0.070	0.234 ± 0.064	0.229 ± 0.048	0.096 ± 0.034	0.363 ± 0.103	0.419 ± 0.137
Group 4 (uninfected)	0.075 ± 0.014	0.084 ± 0.024	0.087 ± 0.019	0.218 ± 0.066	0.244 ± 0.049	0.228 ± 0.039	0.366 ± 0.098	0.355 ± 0.127	0.384 ± 0.082
Group difference (p value)	**0.3527**	**0.3879**	**0.804**	**0.5185**	**0.5509**	**0.9039**	**0.3727**	**0.6658**	**0.9195**

Salmonella colonisation of the cecum

On days 11 (dpi 1), 14 (dpi 4), 19 (dpi 9), 21 (dpi 11) and 25 (dpi 15) *Salmonella* cell counts in the cecum of broilers from group 4 (uninfected untreated) was significantly lower than levels in birds from the other treatment groups (Figure 1). Broilers receiving oxytetracycline had significantly lower *Salmonella* cell counts in their cecum on days 11 (dpi 1) and 14 (dpi 4), however, from day 19 (dpi 9) onwards the *Salmonella* counts were similar to that of birds from groups 1 (probiotics) and 3 (infected untreated). These results show that antibiotics initially decreased *Salmonella* levels, but that prolonged use may have indirectly favoured *Salmonella* colonisation by disrupting homeostasis of the GIT microbiome. Antibiotic use causes imbalances in the GIT microflora, indirectly favouring *Salmonella* colonisation (Ribeiro *et al*., 2007). On day 28, *Salmonella* cell counts in broilers from the group 4 was significantly lower than birds from groups 2 and 3, however, did not differ from birds receiving probiotic (Figure 1).

Occurrence of *Salmonella* (positive vs negative birds) in broilers from groups 1, 2 and 3 did not significantly differ on days 11 (dpi 1, $p= 0.473$), 14 (dpi 4, $p= 0.109$), 19 (dpi 9, $p= 0.258$), 21 (dpi 11, $p= 0.578$), 25 (dpi 15, $p= 0.624$) and 28 (dpi 18, $p= 0.104$). However, the occurrence of *Salmonella* in birds from group 4 (*Salmonella* uninfected and untreated) significantly differed from birds from group 1, 2 and 3 on days 11, 14, 19, 25 and 28 ($p < 0.00002$). The overall effect of treatment showed that broilers receiving antibiotics had significantly lower *Salmonella* cell counts in their cecum than birds from groups 1 and 3 ($p > 0.05$). In addition, broilers group 4 had no *Salmonella* cell present as compared to birds from groups 1, 2 and 3 ($p > 0.05$). Broilers receiving probiotics had similar levels of *Salmonella* in their cecum as birds from group 3 (infected untreated). The main effect of time showed that *Salmonella* counts in broilers decreased significantly over time ($p > 0.05$), however, from days 25 and onwards *Salmonella* counts did not significantly decrease.

Varying results have been reported with regards to the ability of probiotics in reducing *Salmonella* colonisation, probably due to experimental differences and differences in *Salmonella* and probiotic strains used. Numerous researchers have shown that different probiotics (*E*. *faecium*; dual-strain *L*. *salivarius* and *P*. *parvulus*; multi-species *L*. *acidophilus*, *L*. *fermentum*, *L*. *plantarum* and *E*. *faecium*; multi-species *L*. *jensenii*, *L*. *plantarum*, *L*. *fermentum* and *L*. *casei*; *L*. *johnsonii*; and *L*. *plantarum*) are able to decrease *Salmonella* colonisation (Audisio *et al*., 2000; Higgins *et al*., 2008; Chen *et al*., 2012; Olnood *et al*., 2015; Foltz *et al*., 2017), while others have shown that their respective probiotics were are unable (Ribeiro *et al*., 2007; Mountzouris *et al*., 2009; Murate *et al*., 2015). Contradicting results suggest that product- and time-specific testing is required as no general interpretation is possible or applicable.

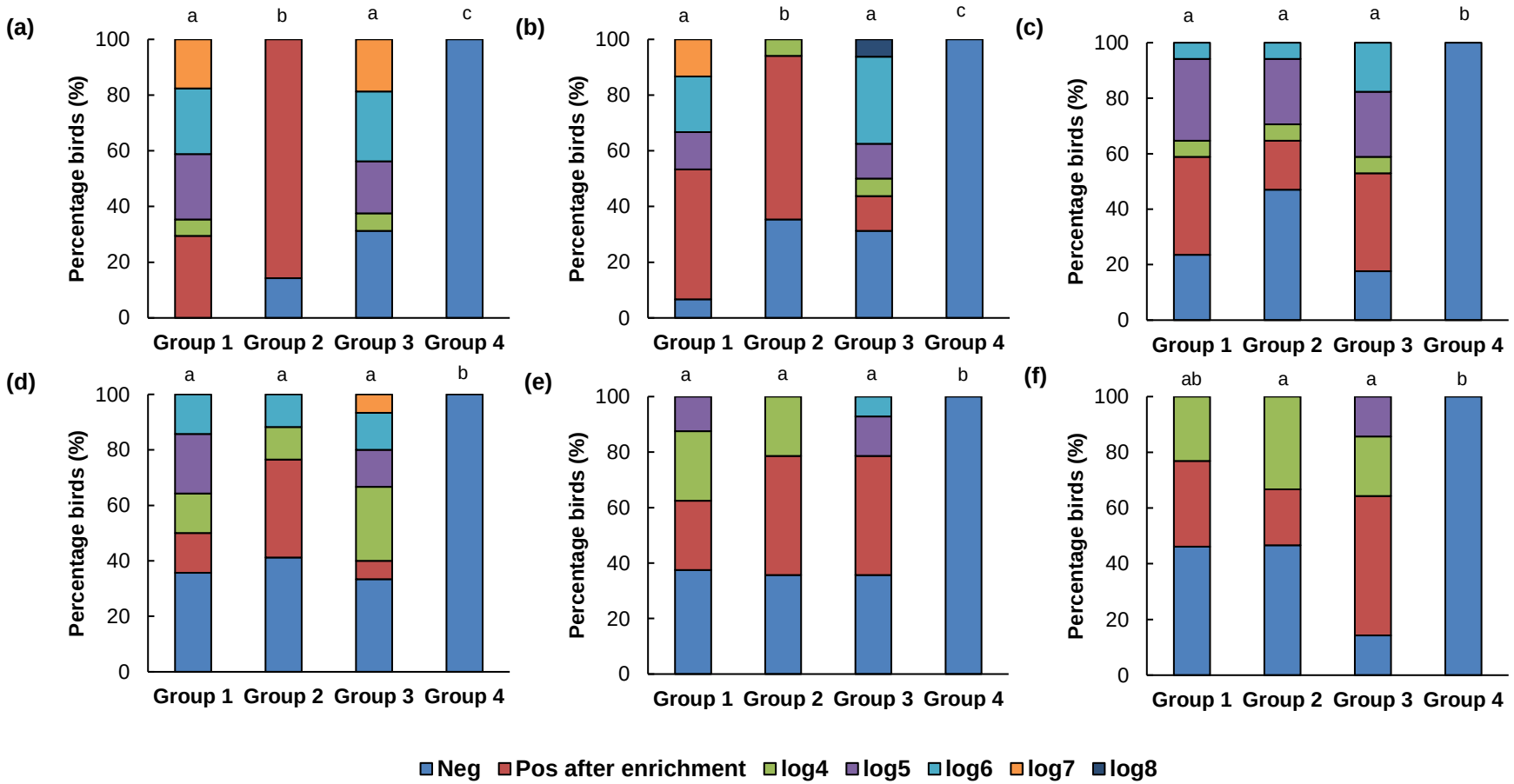

Figure 1: Percentage broilers from group 1 (broilers infected with *Salmonella* receiving probiotics as treatment), group 2 (broilers infected with *Salmonella* receiving oxytetracycline as treatment), group 3 (broilers infected with *Salmonella* receiving no treatment) and group 4 (uninfected broilers receiving no treatment) with cecum *Salmonella* counts ranging from log4-log8 CFU after direct plating, and percentage birds positive (log1 CFU) and negative after enrichment on days 11 (a), 14 (b), 19 (c), 21 (d), 25 (e) and 28 (f). Bars with different superscripts (a, b, c) differ significantly ($p < 0.05$).

Haematology

Red blood parameters of broilers from the different treatment groups on days 18 (dpi 8) and 29 (dpi 19) did not differ significantly (Table 5). The mean (± standard deviation) total white blood cell counts (10^6/µl) of broilers from the different treatment groups on days 18 and 29 did not significantly differ ($p > 0.05$), in line with research on probiotic (*B. subtilis*) and antibiotic (oxytetracycline and neomycin, or virginiamycin) treatment in similar models (Park and Kim, 2015; Abudabos *et al*., 2016). On days 18 and 29, the total white blood cell counts for broilers from the different treatment groups were as follows; group 1 (179.58 ± 33.82; 215.69 ± 13.41), group 2 (182.30 ± 38.96; 207.94 ± 12.62), group 3 (182.76 ± 30.83; 212.27 ± 13.98) and group 4 (183.38 ± 38.96; 212.93 ± 18.56), respectively.

Serum bactericidal and lysozyme activity

Serum bactericidal activity against *E. coli* of broilers from the different treatment groups did not significantly differ at any time point assessed (Figure 2a). In addition, the serum bactericidal activity against *S.* Enteritidis did not significantly differ on days 14 (dpi 4), 20 (dpi 10) and 24 (dpi 14) (Figure 2b). Broilers receiving oxytetracycline had significantly ($p= 0.017$) higher serum bactericidal activity against *Salmonella* (27.96 % ± 10.92), compared to birds from group 4 (16.15 % ± 10.38) on day 11 (dpi 1). On day 29 (dpi 19), broilers receiving probiotics (15.06 % ± 15.83) had significantly higher serum bactericidal activity against *Salmonella*, compared to birds from group 3 (3.47 % ± 3.15). The overall effect of treatment showed that broilers receiving probiotics had significantly higher serum bactericidal activity against *Salmonella* as compared to birds from group 4 ($p= 0.031$). The effect of time showed that broiler serum bactericidal activity against *Salmonella* increased from days 11 to 20, and thereafter significantly decreased to levels below day 11. Current results agree with a previous study which showed that probiotics increased broiler serum bactericidal activity (Alaqaby *et al*., 2014).

Lysozyme is abundant in secretions and the cytoplasmic granules of macrophages and polymorphonuclear neutrophils (PMNs) and forms part of the innate immune system (Ragland and Criss, 2017). The enzyme catalyses the hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan, a major component in the cell walls of Gram- positive bacteria (Ragland and Criss, 2017). Hydrolysis of the cell wall, in turn, compromises the integrity of bacterial cell leading to cell lysis (Ellison and Giehl, 1991). *In vitro*, lysozyme is generally considered effective against some Gram-positive bacteria, but ineffective against Gram-negative bacteria (Cunningham *et al*., 1991). However, *in vivo* lysozyme may indirectly affect Gram-negative bacteria (May *et al*., 2012; Wells *et al*., 2015). In addition, lysozyme hydrolysis products enhance immunoglobulin A secretion, macrophage activation, and rapid clearance of bacterial pathogens (Kawano *et al*., 1981, Clarke *et al*., 2010). Broilers from the different treatment groups had similar serum lysozyme levels on days 14 (dpi 4), 20 (dpi 10) and 29 (dpi 19) (Figure 3). On day 11, broilers receiving probiotics (12.321 µg/ml ± 5.694) had significantly ($p= 0.022$) higher serum lysozyme concentrations, compared to birds from group 4 (7.781 µg/ml ± 1.545). The overall effect of treatment showed that probiotic treated broilers had significantly higher serum lysozyme levels as compared to broilers from the other treatment groups ($p= 0.018$). The main effect of time showed that days 11 and 14, and days 21 and 28 did not differ from each other. Serum lysozyme levels were significantly higher

in broilers at days 14 and 20, as compared to days 21 and 28 ($p > 0.05$). Findings agree with others studies that probiotics increase broiler serum lysozyme levels (Jung *et al*., 2010; Abd El Tawab *et al*., 2015). Broilers infected with pathogens (*C*. *perfringens*; *S*. Gallinarum) and treated with probiotics (*S*. *cerevisiae*; *S*. *cerevisiae*, *B*. *subtilis* and *L*. *acidophilus)* had significantly increased serum lysozyme concentrations as compared to control birds (Jung *et al*., 2010; Abd El Tawab *et al*., 2015).

Table 5: Erythrocyte parameters of broilers from the different treatment groups at day 18 (dpi 8) and 29 (dpi 19), i.e. mean (± standard deviation) erythrocyte count (RBC) and haemoglobin content (HGB), haematocrit value (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and erythrocytes cell distribution width (RDW).

	RBC (10^6 /µl)		**HGB (g/dl)**		**HCT (%)**		**MCV (f)**		**MCH (pg)**		**MCHC (g/dl)**		**RDW (%)**	
Treatment	Day 18 (dpi 8)	Day 29 (dpi 19)	Day 18 (dpi 8)	Day 29 (dpi 19)	Day 18 (dpi 8)	Day 29 (dpi 19)	Day 18 (dpi 8)	Day 29 (dpi 19)	Day 18 (dpi 8)	Day 29 (dpi 19)	Day 18 (dpi 8)	Day 29 (dpi 19)	Day 18 (dpi 8)	Day 29 (dpi 19)
Group 1 (Probiotics)	2.32 ± 0.13	2.45 ± 0.20	13.52 ± 0.69	14.32 ± 0.86	21.11 ± 1.33	21.48 ± 1.33	90.86 ± 3.34	87.56 ± 2.84	58.32 ± 1.98	58.35 ± 1.99	64.15 ± 1.78	66.67 ± 1.68	12.424 ± 0.78	12.39 ± 0.57
Group 2 (Antibiotics)	2.28 ± 0.13	2.40 ± 0.17	13.44 ± 0.78	14.08 ± 0.99	20.82 ± 1.39	20.82 ± 1.55	90.97 ± 3.74	86.61 ± 2.87	58.84 ± 1.81	58.66 ± 2.18	64.61 ± 1.47	67.70 ± 1.63	12.60 ± 1.09	12.34 ± 0.03
Group 3 (infected untreated)	2.32 ± 0.13	2.40 ± 0.13	13.64 ± 0.78	14.25 ± 0.72	21.11 ± 1.33	21.05 ± 1.01	90.91 ± 2.67	87.49 ± 2.38	58.80 ± 1.55	59.28 ± 2.04	64.69 ± 1.84	67.72 ± 2.06	12.43 ± 0.74	12.06 ± 0.90
Group 4 (uninfected)	2.26 ± 0.17	2.33 ± 0.14	13.41 ± 0.93	13.83 ± 0.69	20.74 ± 1.31	20.69 ± 1.33	91.71 ± 2.64	88.17 ± 2.26	59.30 ± 1.24	59.27 ± 1.60	64.65 ± 1.33	66.94 ± 2.04	12.52 ± 0.04	12.24 ± 0.72
Group difference (p value)	**0.639**	**0.292**	**0.868**	**0.427**	**0.814**	**0.410**	**0.854**	**0.473**	**0.439**	**0.509**	**0.763**	**0.332**	**0.926**	**0.626**

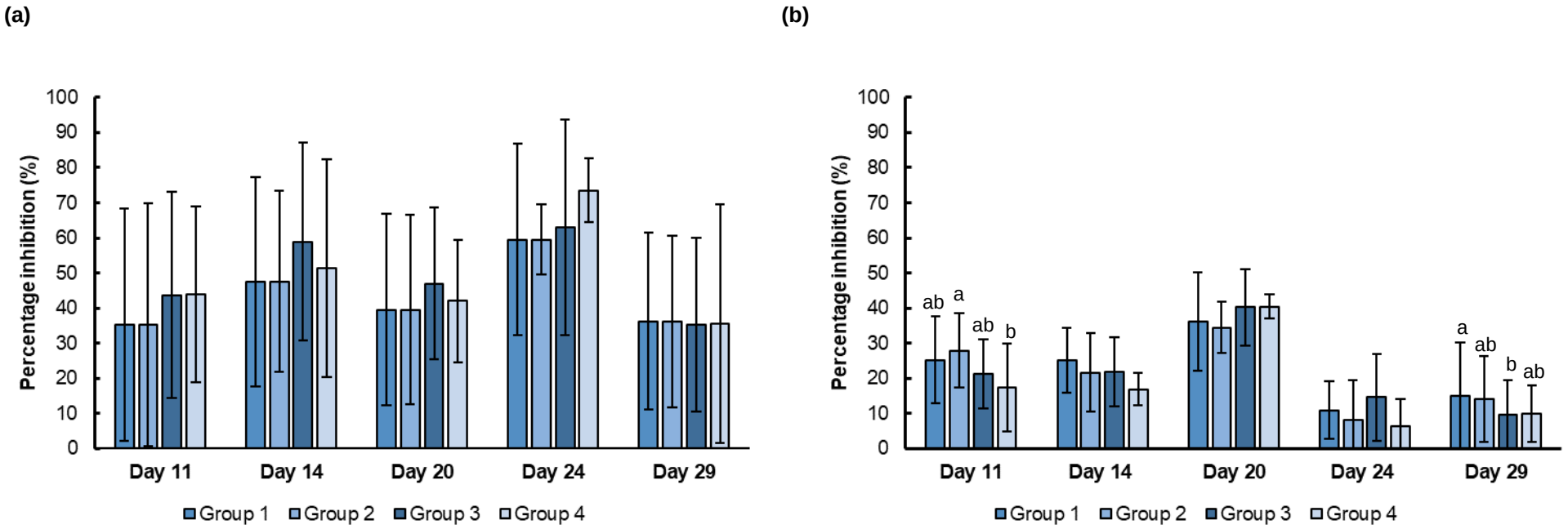

Figure 2: Serum bactericidal activity of broilers from the different treatment groups i.e. group 1 (broilers infected with *Salmonella* receiving probiotics as treatment), group 2 (broilers infected with *Salmonella* receiving oxytetracycline as treatment), group 3 (broilers infected with *Salmonella* receiving no treatment) and group 4 (uninfected broilers receiving no treatment) against (a) *E. coli* DH5α and (b) *S. enterica* Enteritidis A9 on days 11 (dpi 1), 14 (dpi 4), 20 (dpi 10), 24 (dpi 14) and 29 (dpi 19). Error bars indicate standard deviations, and bars with different superscripts (a, b, c) differ significantly ($p < 0.05$).

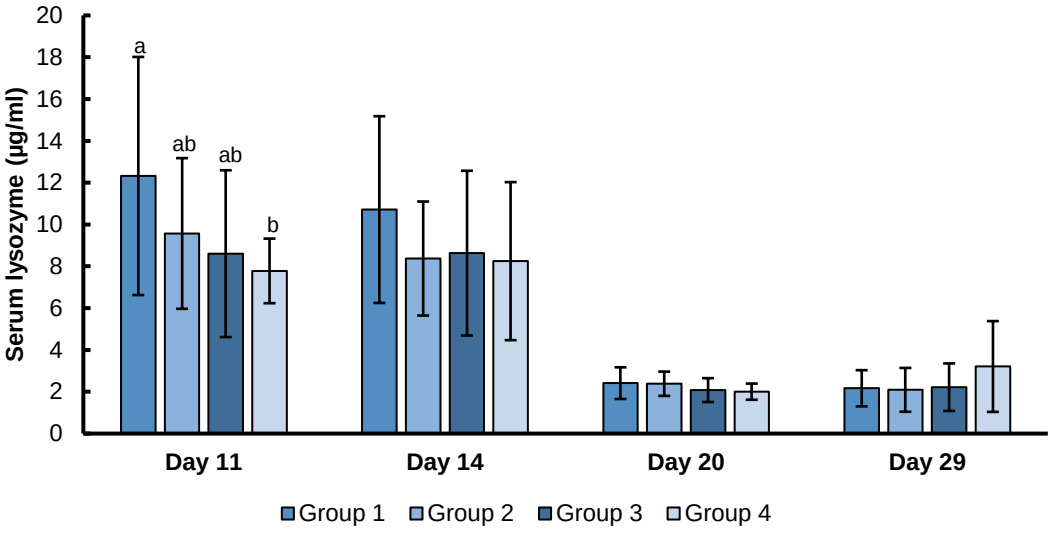

Figure 3: Serum lysozyme concentrations of broilers from the different treatment groups i.e. group 1 (broilers infected with *Salmonella* receiving probiotics as treatment), group 2 (broilers infected with *Salmonella* receiving oxytetracycline as treatment), group 3 (broilers infected with *Salmonella* receiving no treatment) and group 4 (uninfected broilers receiving no treatment) on days 11 (dpi 1), 14 (dpi 4), 20 (dpi 10) and 29 (dpi 19). Error bars indicate standard deviations, and bars with different superscripts (a, b, c) differ significantly ($p < 0.05$).

Serum interferon gamma

Interferon gamma (IFN-γ) stimulates macrophages to secrete oxidants with antimicrobial activity and is produced by natural killer cells and Th1-lymphocytes (Romagnani, 1999; Fasina *et al*., 2008). On day 18 (dpi 8), serum interferon gamma concentrations (pg/ml) of broilers from the different treatment groups i.e. group 1 (22.86 ± 14.69) group 2 (32.35 ± 34.73), group 3 (19.85 ± 10.77) and group 4 (29.95 ± 27.65) did not significantly differ ($p= 0.512$). Previous studies have shown that *Salmonella* Typhimurium induces intestinal mucosal inflammatory responses and upregulates IFN- γ levels in the ileum 10 days post-infection (Fasina *et al*., 2008). *Salmonella enterica* induced expression of IFN-γ in cecal tonsils of broilers were decreased by treatment with a multi-species probiotic (*L*. *acidophilus*, *L*. *fermentum*, *L*. *plantarum* and *E*. *faecium*) (Chen *et al*., 2012). *Salmonella* infection in this study did not lead to increased IFN-γ levels in the serum as no significant differences were observed between infected and infected birds, suggesting that infection rather induces the releases of proinflammatory cytokines at the site of infection such as the mucosal surface as previously reported (Chen *et al*., 2012).

Cell-mediated immune responsiveness

The phytohaemagglutinin (PHA) assay is a method used to measure T-cell-mediated immune responsiveness in birds (Goto *et al*., 1978; Kidd *et al*., 1993; Smits and Williams, 1999; Tella *et al*., 2008). Swelling in response to PHA mitogen correlates with the potentiation of phagocytic activity of macrophages or enhanced delayed type hypersensitivity due to elevated number of lymphocytes with high functional competency in the lymphoid organs (Awais *et al*., 2018). The PHA test reflects the ability

of an individual to cope with a T-cell-mediated immune response and is a measure of immunocompetence but also a wider index of health status (Alonso-Alvarez and Tella, 2001). In the site of PHA injection, $CD5^+$ and $CD8^+$ lymphocytes accumulate (Goto *et al*., 1978; Tella *et al*., 2008).

Broilers receiving probiotics and antibiotics had significantly ($p > 0.05$) higher T-cell-mediated immune responses as compared to broilers from the control Sal+ and control Sal- treatment groups at day 17 (dpi 7) (Figure 4). These findings agree with previous studies showing that probiotics (*B*. *subtilis* and *B*. *licheniformis*; *L*. *fermentum* and *S*. *cerevisiae; B*. subtilis) use in broilers lead to increased T-cell immune responses (Bai *et al*., 2013; Hosseini *et al*., 2017; Sikandar *et al*., 2017). Enhanced T-cell responses have been reported to correlate with increased rates of *Salmonella* clearance (Beal *et al*., 2004; Kogut *et al*., 2005). In addition, results also showed that immune improvements facilitated by probiotics compared well with antibiotic, suggesting that probiotics could replace antibiotics as growth promoters.

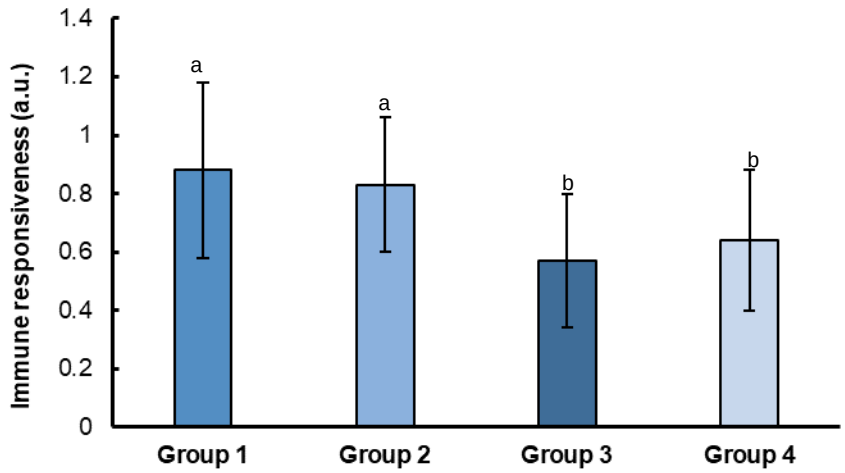

Figure 4: Immune T-cell responsiveness of broilers from the different treatment groups i.e. group 1 (broilers infected with *Salmonella* receiving probiotics as treatment), group 2 (broilers infected with *Salmonella* receiving oxytetracycline as treatment), group 3 (broilers infected with *Salmonella* receiving no treatment) and group 4 (uninfected broilers receiving no treatment) on day 17 (dpi 7). Error bars indicate standard deviations, and bars with different superscripts (a, b, c) differ significantly ($p < 0.05$).

Conclusions

Administration of *Salmonella* Enteritidis A9 did not alter broiler growth performance, immune organ weights, haematological parameters, serum interferon gamma levels and serum bactericidal activity against *E. coli*. *Salmonella* colonisation in the cecum of broilers receiving oxytetracycline was lower on day 11 (dpi 1) and 14 (dpi 4), however, from day 19 (dpi 9) onwards levels increased to that of the other treatment groups. Continuous use of antibiotics probably led to disruption of homeostasis in the GIT microbiome, indirectly favouring *Salmonella* colonisation. In addition, antibiotics use increased serum bactericidal activity against *Salmonella* on day 11 (dpi 1) and increased T-lymphocyte responses on day 17 (dpi 7). Broilers receiving probiotics had similar levels of *Salmonella* in their cecum on day 29 (dpi 19) as compared to birds from groups 3 (infected untreated) and 4 (infected). Despite *Salmonella* levels being similar to that of the control infected group, a reduction in *Salmonella* occurred for levels to be similar to that of the uninfected birds. In addition, probiotics increased broiler serum bactericidal activity against *Salmonella* on day 29 (dpi 19), increased serum lysozyme levels on day 11 (dpi 1), and increased T-lymphocyte responses on day 17 (dpi 7). In conclusion, antibiotic and probiotic feed additives increased broiler immune responses in response to *Salmonella* infection.

References

Abd El Tawab, A.A., Amar, A.M., Elshorbagy, A., Mostafa, E.W. (2015). Impact of probiotic and prebiotic on the lysozyme response of broilers against clostridial infection. *Benha Veterinary Medical Journal* 28(2), p 208-213.

Abudabos, A.M., Alyemni, A.H., Al Marshad, M.B.A. (2013). *Bacillus subtilis* PB6 based probiotic (CloSTAT™) improves intestinal morphological and microbiological status of broiler chickens under *Clostridium perfringes* challenge. *Int. J. Agri. Biol*. 15(5), p 978-982.

Abudabos, A.M., Murshed, M.A., Qaid, M.M., Abdelrahman, A.G. (2016). Effect of probiotics on serum biochemical and blood constituents in chicken challenged with *Salmonella enterica* subsp. Typhimurium. *Trop. J. Pharm. Res*. 15(3), p 461-467, DOI: 10.4314/tjpr.v15i3.5.

Adhikari, P.A., Kim, W.K. (2017). Overview of prebiotics and probiotics: Focus on performance, gut health and immunity- A review. *Ann. Anim. Sci*. 17(4), p 949-966, DOI: 10.1515/aoas-2016-0092.

Alaqaby, A.R.A., Glaskovich, A.A., Kapitonova, E.A., Losev, E. (2014). Study the effect of using probiotic (Vetlactoflorum) on some of biochemical and immunological parameters of broiler chickens. *Bas. J. Vet. Res*. 1(1), p 166-179.

Alcicek, A., Bozkurt, M., Cabuk, M. (2004). The effect of a mixture of herbal essential oils, an organic acid or a probiotic on broiler performance. *S.A. J. Anim. Sci*. 34(4), p 217-222.

Alonso-Alvarez, C., Tella, J.L. (2001). Effects of experimental food restriction and body-mass changes on the avian T-cell-mediated immune response. *Can. J. Zool*. 79, p 101-105, DOI: 10.1139/cjz-79-1-101.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990). Basic local alignment search tool. *J. Mol. Biol*. 215(3), p 403-410, DOI: 10.1016/S0022-2836(05)80360-2.

Audisio, M.C., Oliver, G., Apella, M.C. (2000). Protective effect of *Enterococcus faecium* J96, a potential probiotic strain, on chicks infected with *Salmonella* Pullorum. *J. Food Protect*. 63(10), p 1333-1337, DOI: 10.4315/0362-028X-63.10.1333.

Awad, W.A., Hess, C., Hess, M. (2017). Enteric pathogens and their toxin-induced disruption of the intestinal barrier through alteration of tight junctions in chickens. *Toxins* 9(2), e60, DOI: 10.3390/toxins9020060.

Awais, M.M., Akhtar, M., Anwar, M.I., Khaliq, K. (2018). Evaluation of *Saccharum offinarum* L. bagasse-derived polysaccharides as native immunomodulatory and anticoccidial agents in broilers. *Vet. Parasitol*. 249, p 74-81, DOI: 10.1016/j.vetpar.2017.11.012.

Bai, S.P., Wu, A.M., Ding, X.M., Lei, Y., Bai, J., Zhang, K.Y., Chio, J.S. (2013). Effects of probiotic-supplemented diets on growth performance and intestinal immune characteristics of broiler chickens. *Poult. Sci*. 92(3), p 663-670, DOI: 10.3382/ps.2012-02813.

Barrow, P.A., Jones, M.A., Smith, A.L., Wigley, P. (2012). The long view: *Salmonella*-the last forty years. *Avian Pathol*. 41(5), p 413-420, DOI: 10.1080/03079457.2012.718071.

Beal, R.K., Powers, C., Wigley, P., Barrow, P.A., Smith, A.L. (2004). Temporal dynamics of cellular, humoral and cytokine responses in chickens during primary and secondary infection with *Salmonella enterica* serovar Typhimurium. *Avian Pathol*. 33(1), p 25–33, DOI: 10.1080/03079450310001636282.

Boyd, M.A., Tennant, S.M., Saague, V.A., Simon, R., Muhsen, K., Ramachandran, G., Cross, A.S., Galen, J.E., Pasetti, M.F., Levine, M.M. (2014). Serum bactericidal assays to evaluate typhoidal and nontyphoidal *Salmonella* vaccines. *Clin. Vaccine Immunol*. 21(5), p 712-721, DOI: 10.1128/CVI.00115-14.

Brenes, A., Roura, E. (2010). Essential oils in poultry nutrition: Main effects and modes of action. *Anim. Feed Sci. Technol*. 158(1-2), p 1-14, DOI: 10.1016/j.anifeedsci.2010.03.007.

CDC (2015). Reports of *Salmonella* Outbreak Investigations from 2015. http://www.cdc.gov/salmonella/outbreaks-2015.html.

Chappell, L., Kaiser, P., Barrow, P., Jones, M.A., Johnston, C., Wigley, P. (2009). The immunobiology of avian systemic salmonellosis. *Vet. Immunol. Immunopathol*. 128(1-3), p 53–59, DOI: 10.1016/j.vetimm.2008.10.295.

Chen, C-Y., Tsen, H-Y., Lin, C-L., Yu, B., Chen, C-S. (2012). Oral administration of a combination of select lactic acid bacteria strains to reduce the *Salmonella* invasion and inflammation of broiler chicks. *Poult. Sci*. 91(9), p 2139-2147, DOI: 10.3382/ps.2012-02237.

Christensen, W.B. (1946). Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. *J. Bacteriol*. 52(4), p 461-466.

Clarke, T.B., Davis, K.M., Lysenko, E.S., Zhou, A.Y., Yu, Y., Weiser, J.N. (2010). Recognition of peptidoglycan from the microbiota by Nod1 enhances systemic innate immunity. *Nat. Med*. 16(2), p 228–231, DOI: 10.1038/nm.2087.

Coburn, B., Grassi, G.A., Finlay, B.B. (2006). *Salmonella*, the host and disease: a brief review. *Immunol. Cell Biol*. 85(2), p 112-118, DOI: 10.1038/sj.icb.7100007.

Cosby, D.E., Cox, N.A., Harrison, M.A., Wilson, J.L., Buhr, R.J., Fedorka-Cray, P.J. (2015). *Salmonella* and antimicrobial resistance in broilers: A review. *J. Appl. Poult. Res.* 24(3), p 408-426, DOI: 10.3382/japr/pfv038.

Cox, N.A., Frye, J.G., McMahon, W., Jackson, C.R., Richardson, J., Cosby, D.E., Mead, G., Doyle, M.P. (2015). *Salmonella*. In: Salfinger, Y., Tortorello, M.L. (Eds.). Compendium of Methods for the Microbiological Examination of Foods. American Public Health Association, Washington, USA.

Cunningham, F.E., Proctor, V.A., Goetsch, S.J. (1991). Egg-white lysozyme as a food preservative: An overview. *World's Poult. Sci. J.* 47(2), p 141–163, DOI: 10.1079/WPS19910015.

Dar, M.A., Ahmad, S.M., Bhat, S.A., Ahmed, R., Urwat, U., Mumtaz, P.T., Bhat, S.A., Dar, T.A., Shah, R.A., Ganai, N.A. (2017). *Salmonella* typhimurium in poultry: a review. *World's Poult. Sci. J.* 73(2), p 345-354, DOI: 10.1017/S0043933917000204.

Donado-Godoy, P., Gardner, I., Byrne, B.A., Leon, M., Perez-Gutierrez, E., Ovalle, M.V., Tafur, M.A., Miller, W. (2012). Prevalence, risk factors, and antimicrobial resistance profiles of *Salmonella* from commercial broiler farms in two important poultry-producing regions of Colombia. *J. Food. Protect*. 75(5), p 874-883, DOI: 10.4315/0362-028X.JFP-11-458.

Dunislawska, A., Slawinska, A., Stadnicka, K., Bednarczyk, M., Gulewicz, P., Jozefiak, D., Siwek, M. (2017). Synbiotics for broiler chickens- *In vitro* design and evaluation of the influence on host and selected microbiota populations following *in ovo* delivery. *PLoS ONE* 12(1), Article ID: e0168587, DOI: 10.1371/journal.pone.0168587.

Dunkley, K.D., Callaway, T.R., Chalova, V.I., McReynolds, J.L., Hume, M.E., Dunkley, C.S., Kubena, L.F., Nisbet, D.J., Ricke, S.C. (2009). Foodborne *Salmonella* ecology in the avian gastrointestinal tract. *Anaerobe* 15(1-2), p 26–35, DOI: 10.1016/j.anaerobe.2008.05.007.

Ellison, R.T., Giehl, T.J. (1991). Killing of gram-negative bacteria by lactoferrin and lysozyme. *J. Clin. Invest*. 88(4), p 1080–1091, DOI: 10.1172/JCI115407.

Fasina, Y.O., Holt, P.S., Moran, E.T., Moore, R.W., Conner, D.E., McKee, S.R. (2008). Intestinal cytokine response of commercial source broiler chicks to *Salmonella* Typhimurium infection. *Poult. Sci*. 87(7), p 1335-1346, DOI: 10.3382/ps.2007-00526.

Foltz, K.L., Ritzi, M.M., Barrett, N.W., Evans, N.P., Collins, D., Sriranganathan, N., Mahsoub, H., Dalloul, R.A., Sewell, J., Persia, M.E. (2017). Efficacy of *Lactobacillus plantarum* supplementation in broilers challenged with avian pathogenic *Escherichia coli* and *Salmonella* Typhimurium. *J. Appl. Res*. 26(3), p 316-324, DOI: 10.3382/japr/pfw074.

Gal-Mor, O., Boyle, E.C., Grassi, G.A. (2014). Same species, different diseases: how and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. *Front. Microbiol*. 5, Article ID: 391, DOI: 10.3389/fmicb.2014.00391.

Ghareeb, K., Awad, W.A., Mohnl, M., Porta, R., Biarnés, M., Böhm, J., Schatzmayr, G. (2012). Evaluating the efficacy of an avian-specific probiotic to reduce the colonisation of *Campylobacter jejuni* in broiler chickens. *Poult. Sci*. 91(8), p 1825-1832, DOI: 10.3382/ps.2012-02168.

Gil de los Santos, J.R., Storch, O.B., Gil-turnes, C. (2005). *Bacillus cereus* var. toyoii and *Saccharomyces boulardii* increased feed efficiency in broilers infected with *Salmonella* Enteritidis. *Brit*. *Poult*. *Sci*. 46(4), p 494-497, DOI: 10.1080/00071660500181461.

Goto, N., Kodama, H., Okada, K., Fujimoto, Y. (1978). Suppression of phytohemagglutinin skin response in thymectomized chickens. *Poult*. *Sci*. 57(1), p 246–250, DOI: 10.3382/ps.0570246.

Gutierrez-Fuentes, C.G., Zuñiga-Orozco, L.A., Vicente, J.L., Hernandez-Velasco, X., Menconi, A., Kuttappan, V.A., Kallapura, G., Latorre, J., Layton, S., Hargis, B.M., Téllez, G. (2013). Effect of a lactic acid bacteria based probiotic, Floramax-B11®, on performance, bone qualities, and morphometric analysis of broiler chickens: An economic analysis. *Biol*. *Syst*. 2(3), 113. doi:10.4172/2329-6577.1000113.

Hallstrom, K., McCormick, B.A. (2011). *Salmonella* interaction with and passage through the intestinal mucosa: through the lens of the organism. *Front*. *Microbiol*. 2, e88, DOI: 10.3389/fmicb.2011.00088.

Higgins, S.E., Higgins, J.P., Wolfenden, A.D., Henderson, S.N., Torres-Rodriguez, A., Tellez, G., Hargis, B. (2008). Evaluation of a *Lactobacillus*-based probiotic culture for the reduction of *Salmonella* Enteritidis in neonatal broiler chicks. *Poult*. *Sci*. 87, p 27-31, DOI: 10.3382/ps.2007-00210.

Hosseini, S.M., Chamani, M., Mousavi, S.N., Hosseini, S.A., Sadeghi, A. (2017). Effects of dietary physical form and dietary inclusion of probiotic and enzyme on growth performance, cellular and humoral immunity, and relative weights of lymphoid organs at early period of broiler chickens fed triticale-based diets. *S*. *Afr*. *J*. *Anim*. *Sci*. 47(6), p 776-784, DOI: 10.4314/sajas.v47i6.5.

Huff, G.R., Farnell, M.B., Huff, W.E., Rath, N.C., Solis De Los Santos, F., Donoghue, A.M. (2010). Bacterial clearance, heterophil function, and hematological parameters of transport-stressed turkey poults supplemented with dietary yeast extract. *Poult*. *Sci*. 89(3), p 447-456, DOI: 10.3382/ps.2009-00328.

Hugas, M., Beloeil, P. (2014). Controlling *Salmonella* along the food chain in the European Union – progress over the last ten years. *Euro Surveill*. 19(19), pii=20804, DOI: 10.2807/1560-7917.ES2014.19.19.20804.

Jeurissen, S.H.M. (1993). The role of various compartments in the chicken spleen during an antigen-specific humoral response. *Immunol*. 80(1), p 29-33.

Jia, T., Pamer, E.G. (2009). Immunology: Dispensable but not irrelevant. *Science* 325(5940), p 549–550, DOI: 10.1126/science.1178329.

Jones, F., Axtell, R.C., Tarver, F.R., Rives, D.V., Scheideler, S.E., Wineland, M.J. (1991). Environmental factors contributing to Salmonella colonisation of chickens. In: Blankenship, L. (Ed.). Colonisation control of human bacterial enteropathologens in poultry. Academic Press, Massachusetts, USA, p 3-21, DOI: 10.1016/B978-0-12-104280-6.50011-3.

Jung, B-G., Ko, J-H., Lee, B-J. (2010). Dietary supplementation with a probiotic fermented four-herb combination enhances immune activity in broiler chicks and increases survivability against *Salmonella* Gallinarum in experimentally infected broiler chicks. *J*. *Vet*. *Med*. *Sci*. 72(12), p 1565-1573.

Kabir, S.M.L. (2010). Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *Int*. *J*. *Environ*. *Res*. *Public Health* 7(1), p 89–114, DOI: 10.3390/ijerph7010089.

Kawano, M., Namba, Y., Hanaoka, M. (1981). Regulatory factors of lymphocyte-lymphocyte interaction. I. Con A-induced mitogenic factor acts on the late G1 stage of T-cell proliferation. *Microbiol*. *Immunol*. 25(5), p 505–515, DOI: 10.1111/j.1348-0421.1981.tb00052.x.

Kean, R.P., Lamont, S.J. (1994). Effect of injection site on cutaneous basophil hypersensitivity response to phytohemagglutinin. *Poult*. *Sci*. 73(11), p 1763-1765, DOI: 10.3382/ps.0731763.

Kiarie, E., Romero, L.F., Nyachoti, C.M. (2013). The role of added feed enzymes in promoting gut health in swine and poultry. *Nut*. *Res*. *Rev*. 26(1), p 71-88, DOI: 10.1017/S0954422413000048.

Kidd, M.T., Anthony, N.B., Newberry, L.A., Lee, S.R. (1993). Effect of supplemental zinc in either a corn-soybean or a milo corn-soybean meal diet on the performance of young broiler breeders and their progeny. *Poult*. *Sci*. 72(8), p 1492-1499, DOI: 10.3382/ps.0721492.

Kogut, M.H., Rothwell, L., Kaiser, P. (2005). IFN-γ priming of chicken heterophils upregulates the expression of proinflammatory and Th1 cytokine mRNA following receptor-mediated phagocytosis of *Salmonella enterica* serovar Enteritidis. *J*. *Interferon Cytokine Res*. 25(2), p 73–81, DOI: 10.1089/jir.2005.25.73.

Kumar, S., Stecher, G., Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol*. *Biol*. *Evol*. 33(7), p 1870-1874, DOI: 10.1093/molbev/msw054.

Lie, O., Syed, M., Solbu, H. (1986). Improved agar plate assays of bovine lysozyme and haemolytic complement activity. *Acta*. *Vet*. *Scand*. 27(1), p 23-32.

Lourenco, M.C., Kuritza, L.N., Westphal, P., Muniz, E., Pickler, L., Santin, E. (2012). Effects of *Bacillus subtilis* in the dynamics of infiltration of immunological cells in the intestinal mucosa of chickens challenged with *Salmonella* Minnesota. *Int*. *J*. *Poult*. *Sci*. 11(10), p 630-634, DOI: 10.3923/ijps.2012.630.634.

Marcq, C., Cox, E., Szalo, I.M., Théwis, A., Beckers, Y. (2011). *Salmonella* Typhimurium oral challenge in mature broilers: bacteriological, immunological, and growth performance aspects. *Poult*. *Sci*. 90, p 59-67, DOI: 10.3382/ps.2010-01017.

Masteller, E.L., Pharr, G.T., Funk, P.E., Thompson, C.B. (1997). Avian B cell development. *Int*. *Rev*. *Immunol*. 15(3-4), p 185-206, DOI: 10.3109/08830189709068176.

May, K.D., Wells, J.E., Maxwell, C.V., Oliver, W.T. (2012). Granulated lysozyme as an alternative to antibiotics improves growth performance and small intestinal morphology of 10-day-old pigs. *J*. *Anim*. *Sci*. 90(4), p 1118–1125, DOI: 10.2527/jas.2011-4297.

Mebius, R.E., Kraal, G. (2005). Structure and function of the spleen. *Nat*. *Rev*. *Immunol*. 5, p 606–616, DOI:10.1038/nri1669.

Melegy, T., Khaled, N.F., El-Bana, R., Abdellatif, H. (2011). Effect of dietary supplementation of *Bacillus subtilis* PB6 (CLOSTAT™) on performance, immunity, gut health and carcass traits in broilers. *J*. *Am*. *Sci*. 7(12), p 891-898.

Milner, K.C., Shaffer, M.F. (1952). Bacteriologic studies of *Salmonella* infection in chicks. *J*. *Infect*. *Dis*. 90(1), p 81-96.

Mountzouris, K.C., Balaskas, C., Xanthakos, I., Tzivinikou, A., Fegeros, K. (2009). Effects of a multi-species probiotic on biomarkers of competitive exclusion efficacy in broilers challenged with *Salmonella* Enteritidis. *Brit*. *Poult*. *Sci*. 50(4), p 467-478, DOI: 10.1080/00071660903110935.

Murate, L.S., Paião, F.G., de Almeida, A.M., Berchieri, A., Shimokomaki, M. (2015). Efficacy of prebiotics, probiotics, and synbiotics on laying hens and broilers challenged with *Salmonella* Enteritidis. *J*. *Poult*. *Sci*. 52(1), p 52-56, DOI: 10.2141/jpsa.0130211.

Namata, H., Welby, S., Aerts, M., Faes, C., Abranhantes, J.C., Imberechts, H., Vermeersch, K., Hooyberghs, J., Méroc, E., Mintiens, K. (2009). Identification of risk factors for the prevalence and persistence of *Salmonella* in Belgian broiler chicken flocks. *Prev*. *Vet*. *Med*. 90(3-4), p 211-222, DOI: 10.1016/j.prevetmed.2009.03.006.

Neveling, D.P., Endo, A., Dicks, L.M.T. (2012). Fructophilic *Lactobacillus kunkeei* and *Lactobacillus brevis* isolated from fresh flowers, bees and bee-hive. *Curr*. *Microbiol*. 65(5), p 507-515, DOI 10.1007/s00284-012-0186-4.

Oakley, B.B., Lillehoj, H.S., Kogut, M.H., Kim, W.K., Maurer, J.J., Pedroso, A., Lee, M.D., Collett, S.R., Johnson, T.J., Cox, N.A. (2014). The chicken gastrointestinal microbiome. *FEMS Microbiol*. *Lett*. 360(2), p 100-112, DOI: 10.1111/1574-6968.12608.

Obukhovska, O. (2013). The natural reservoirs of *Salmonella* Enteritidis in populations of wild birds. *Online J*. *Public Health Inform*. 5(1), e171.

Ohland, C.L., Jobin, C. (2015). Microbial activities and intestinal homeostasis: A delicate balance between health and disease. *Cell*. *Mol*. *Gastroenterol*. *Hepatol*. 1(1), p 28-40, DOI: 10.1016/j.jcmgh.2014.11.004.

Olnood, C.G., Beski, S.S.M., Choct, M., Iji, P.A. (2015). Use of *Lactobacillus johnsonii* in broilers challenged with *Salmonella sofia*. *Animal Nutrition* 1(3), p 203-212, DOI: 10.1016/j.aninu.2015.07.001.

Pan, D., Yu, Z. (2014). Intestinal microbiome of poultry and its interaction with host and diet. *Gut Microbes* 5(1), p 108-119, DOI: 10.4161/gmic.26945.

Pandey, K.R., Naik, S.R., Vakil, B.V. (2015). Probiotics, prebiotics and synbiotics- a review. *J*. *Food Sci*. *Technol*. 52(12), p 7577-7587, DOI: 10.1007/s13197-015-1921-1.

Park, J.H., Kim, I.H. (2014). The effects of the supplementation of *Bacillus subtilis* RX7 and B2A strains on the performance, blood profiles, intestinal *Salmonella* concentration, noxious gas emission, organ weight and breast meat quality of broiler challenged with *Salmonella* typhimurium. *J*. *Anim*. *Physiol*. *Anim*. *Nutr*. 99(2), p 326-334, DOI: 10.1111/jpn.12248.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R., Waddell, J. (2004). Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J*. *Antimicrob*. *Chemother*. 53(1), p 28-52, DOI: 10.1093/jac/dkg483.

Prado-Rebolledo, O.F., de Jesus Delgado-Machuca, J., Macedo-Barragan, R.J., Garcia-Márquez, L.J., Morales-Barrera, J.E., Latorre, J.D., Hernandez-Velasco, X., Tellez, G. (2017). Evaluation of a selected lactic acid bacteria-based probiotic on *Salmonella enterica* serovar Enteritidis

colonisation and intestinal permeability in broiler chickens. *Avian Pathol*. 46(1), p 90-94, DOI: 10.1080/03079457.2016.1222808

Ragland, S.A., Criss, A.K. (2017). From bacterial killing to immune modulation: recent insights into the functions of lysozyme. *PLoS Pathogen* 13(9), e1006512, DOI: 10.1371/journal.ppat.1006512.

Ribeiro, A.M.L., Vogt, L.K., Canal, C.W., Cardoso, M.R.I., Labres, R.V., Streck, A.F., Bessa, M.C. (2007). Effects of prebiotics and probiotics on the colonisation and immune response of broiler chickens challenged with *Salmonella* Enteritidis. *Rev*. *Bras*. *Cienc*. *Avic*. 9(3), p 193-200, DOI: 10.1590/S1516-635X2007000300009.

Romagnani, S. (1999). Th1/Th2 cells. *Inflamm*. *Bowel Dis*. 5(4), p 285-294, DOI: 10.1097/00054725-199911000-00009.

Sadeghi, A.A., Shawrang, P., Shakorzadeh, S. (2015). Immune response of *Salmonella* challenged broiler chickens fed diets containing Gallipro®, a *Bacillus subtilis* probiotic. *Probiotics Antimicrob*. Proteins 7(1), p 24-30, DOI: 10.1007/s12602-014-9175-1.

Shaw, K.S., Cruz-Cano, R., Jiang, C., Malayil, L., Blythe, D., Ryan, P., Sapkota, A.R. (2016). Presence of animal feeding operations and community socioeconomic factors impact salmonellosis incidence rates: An ecological analysis using data from the Foodborne Diseases Active Surveillance Network (FoodNet), 2004-2010. *Environ*. *Res*. 150, p 166-172, DOI: 10.1016/j.envres.2016.05.049.

Shivaprasad, H.L. (2000). Fowl typhoid and pullorum disease. Rev. Sci. Tech. 19(2), p 405-424.

Sikandar, A., Zaneb, H., Younus, M., Masood, S., Aslam, A., Shah, M., Rehman, H. (2017). Growth performance, immune status and organ morphometry in broilers fed *Bacillus subtilis*-supplemented diet. *S*. *Afr*. *J*. *Anim*. *Sci*. 47(3), p 378-388, DOI: 10.4314/sajas.v47i3.14.

Singer, A.C., Shaw, H., Rhodes, V., Hart, A. (2016). Review of antimicrobial resistance in the environment and its relevance to environmental regulators. *Front*. *Microbiol*. 7, e1728, DOI: 10.3389/fmicb.2016.01728.

Smits, J.E., Bortolotti, G.R., Tella, J.L. (1999). Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. *Funct*. *Ecol*. 13(4), p 567-572, DOI: 10.1046/j.1365-2435.1999.00338.x.

Smits, J.E., Williams, T.D. (1999). Validation of immunotoxicology techniques in passerine chicks exposed to oil sands tailings water. *Ecotoxicol*. *Environ*. *Saf*. 44(1), p 105–112, DOI: 10.1006/eesa.1999.1806.

Snoeyenbos, G.H., Weinack, O.M., Smyser, C.F. (1978). Protecting chicks and poultry from salmonellae by oral administration of “normal” gut microflora. *Avian Dis*. 22(2), p 273-287.

Stanley, D., Hughes, R.J., Moore, R.J. (2014). Microbiota of the chicken gastrointestinal tract: influence on health, productivity and disease. *Appl*. *Microbiol*. *Biotechnol*. 98(10), p 4301–4310, DOI: 10.1007/s00253-014-5646-2.

Sterzo, E.V., Paiva, J.B., Mesquita, A.L., Freitas Neto, O.C., Berchieri, S. (2007). Organic acids and/or compound with defined microorganisms to control *Salmonella enterica* serovar Enteritidis experimental infection in chickens. *Rev*. *Bras*. *Cienc*. *Avic*. 9(1), p 69-73, DOI: 10.1590/S1516-635X2007000100010.

Swirski, F.K., Nahrendorf, M., Etzrodt, M., Wildgruber, M., Cortez-Retamozo, V., Panizzi, P., Figueiredo, J.L., Kohler, R.H., Chudnovskiy, A., Waterman, P., Aikawa, E., Mempel, T.R., Libby, P., Weissleder, R., Pittet, M.J. (2009). Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science* 325(5940), p 612–616, DOI: 10.1126/science.1175202.

Tella, J.L., Lemus, J.A., Carrete, M., Blanco, G. (2008). The PHA test reflects acquired T-cell mediated immunocompetence in birds. *PLoS ONE* 3(9), Artcile ID: e3295, DOI: 10.1371/journal.pone.0003295.

Teo, A.Y., Tan, H-M. (2007). Evaluation of the performance and intestinal gut microflora of broiler fed on corn-soy diets supplemented with *Bacillus subtilis* PB6 (CloSTAT™). *J*. *Appl*. *Poult*. *Res*. 16(3), p 296-303, DOI: 10.1093/japr/16.3.296.

Traub-Dargatz, J.L., Ladely, S.R., Dargatz, D.A., Fedorka-Cray, P.J. (2006). Impact of heat stress on the fecal shedding patterns of *Salmonella enterica* Typhimurium DT104 and *Salmonella enterica* Infantis by 5-week-old male broilers. *Foodborne Pathog*. Dis. 3(2), p 178–183, DOI: 10.1089/fpd.2006.3.178.

Vandeplas, S., Dauphin, R.D., Beckers, Y., Thonart, P., Théwis, A. (2010). *Salmonella* in chicken: current and developing strategies to reduce contamination at farm level. *J*. *Food*. *Protect*. 73(4), p 774-785, DOI: 10.4315/0362-028X-73.4.774.

Vo, A.T., van Duijkeren, E., Fluit, A.C., Heck, M.E., Verbruggen, A., Maas, H.M., Gaastra, W. (2006). Distribution of *Salmonella* enterica serovars from humans, livestock and meat in Vietnam and the dominance of *Salmonella* Typhimurium phage type 90. *Vet*. *Microbiol*. 113(1-2), p 153-158, DOI: 10.1016/j.vetmic.2005.10.034.

Wang, H., Ni, X., Qing, X., Liu, L., Lai, J., Khalique, A., Li, G., Pan, K., Jing, B., Zeng, D. (2017). Probiotic enhanced intestinal immunity in broilers against subclinical necrotic enteritis. *Front*. *Immunol*. 8, Article ID: 1592, DOI: 10.3389/fimmu.2017.01592.

Wells, J.E., Berry, E.D., Kalchayanand, N., Rempel, L.A., Kim, M., Oliver, W.T. (2015). Effect of lysozyme or antibiotics on faecal zoonotic pathogens in nursery pigs. *J*. *Appl*. *Microbiol*. 118(2), p 1489–1497, DOI: 10.1111/jam.12803.

Wigley, P., Berchieri, A., Page, K.L., Smith, A.L., Barrow, P.A. (2001). *Salmonella enterica* serovar Pullorum persists in splenic macrophages and in the reproductive tract during persistent, disease free carriage in chickens. *Infect*. *Immun*. 69, p 7873–7879, DOI: 10.1128/IAI.69.12.7873-7879.2001.

Wischmeyer, P.E., McDonald, D., Knight, R. (2016). Role of the microbiome, probiotics, and 'dysbiosis therapy' in critical illness. *Curr*. *Opin*. *Crit*. *Care* 22(4), p 347-353, DOI: 10.1097/MCC.0000000000000321.

Yang, Y., Iji, P.A., Choct, M. (2009). Dietary modulation of gut microflora in broiler chickens: A review of the role of six kinds of alternatives to in-feed antibiotics. *World's Poult*. *Sci*. *J*. 65(1), p 97-114, DOI: 10.1017/S0043933909000087.

Chapter 7

General Discussion and Conclusions

Antibiotics used as feed additives select for antibiotic resistance which poses a great threat to humans (Phillips *et al*., 2004; Aarestrup, 2015). This is worrisome as development of novel antibiotics for clinical treatment is time-consuming, creating the need for novel feed additives which do not cause detrimental after-effects (Conly and Johnston, 2005). Probiotics have been proposed as an alternative feed additive for broilers to improve health and growth performance, and to prevent the colonisation of pathogens (Gadde *et al*., 2017; Alagawany *et al*., 2018; Baldwin *et al*., 2018). The aim of this study was to develop a novel multi-species probiotic for *Gallus gallus domesticus* to replace antibiotic feed additives. The objectives were to assess the safety of probiotics and antibiotics in broiler diets, to elucidate the proteomic changes induced in broiler ileum epithelial cells in response to probiotics or pathogenic bacteria, and to determine the ability of probiotics and antibiotics in reducing *Salmonella* colonisation in the ceca of broilers.

The multi-species probiotic was designed specifically for each section of the gastrointestinal tract (GIT), to ultimately strive for complete colonisation. The multi-species probiotic consisted of *L*. *crispatus* DPN167 (isolated from the crop, proventriculus and ventriculus), *B*. *amyloliquefaciens* DPN123 (duodenum), *L*. *gallinarum* DPN164 (jejunum and ileum), *E*. *faecalis* DPN94 (jejunum and ileum), *L*. *johnsonii* DPN184 (cecum) and *L*. *salivarius* DPN181 (colon). All strains showed high tolerance towards acidic conditions and bile salts and produced exopolysaccharides. Bacteria employ numerous mechanisms to tolerate harsh environmental conditions. This includes surrounding their outer membranes with exopolysaccharides (EPS) (Kumar *et al*., 2007). In addition, EPS also modulates the microbiome composition by selectively promoting the growth of beneficial bacterial (Patten and Laws, 2015; Caggianiello *et al*., 2016). Gram-positive bacteria tolerate acidic conditions by restoring the internal pH by extruding H+ using the F0F1-ATPase efflux system (Cotter *et al*., 2001; Fortier *et al*., 2003; Corcoran *et al*., 2005). Bile salt hydrolase (BSH) was produced by *E*. *faecalis* DPN94 and *L*. *crispatus* DPN167. The prevalence of BSH genes is high in intestinal microorganisms, suggesting that BSH plays an important role in adaptation to the GIT environment (Jones *et al*., 2008). Bile salt hydrolase activity has also been suggested to contribute to bile salts tolerance (Begley *et al*., 2006), and indirectly decrease serum cholesterol levels (Tsai *et al*., 2014; Geng and Lin, 2016).

Bacillus amyloliquefaciens DPN123 produced extracellular amylase. Amylase producing bacteria can increase starch degradation in the intestine, ultimately improving broiler growth performance (Onderci *et al*., 2006; Li *et al*., 2015). Supplementation of broiler feed with amylase is known to improve digestibility of nutrients and improve growth performance (Onderci *et al*., 2006; Tang *et al*., 2013). Phytase enzymes were produced by *B*. *amyloliquefaciens* DPN123 and *E*. *faecalis* DPN94. Phytic acid exerts antinutritive effects by sequestering essential cations which includes calcium, magnesium, iron, and zinc, reducing their bioavailability (Graf and Eaton, 1990; Adeola and Cowieson, 2011). Production of phytases is beneficial as they release energy from anti-nutritive phytic acid, leading to improved broiler growth (Askelson *et al*., 2014). In addition, phytases reduce the

antinutritional effect of phytic acid, improving the bioavailability of phosphorous, calcium, magnesium, iron, and zinc (Dersjant-Li *et al*., 2015).

Hydrogen peroxide (H_2O_2) was produced by *L*. *johnsonii* DPN184, *L*. *salivarius* DPN181 and *L*. *crispatus* DPN167. Hydrogen peroxide production has been previously identified in these species (Ocaña *et al*., 1999; Tomás *et al*., 2003; Pridmore *et al*., 2008; Mitchell *et al*., 2015). Hydrogen peroxide inhibits the growth of pathogens such as *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Listeria monocytogenes* (Dahiya and Speck, 1968; Watson and Schubert, 1969; Siragusa and Johnson, 1989; Stern *et al*., 2006; Neal-McKinney *et al*., 2012). *Bacillus amyloliquefaciens* DPN123 produced antimicrobial lipopeptides (surfactin and iturinA1) which had antimicrobial activity against *M*. *luteus*, *L*. *monocytogenes*, Enteroinvasive *E*. *coli* and *S*. Enteritidis. Bacteria which produce antimicrobial compounds can modulate the GIT microbiome composition, by preventing colonisation of pathogenic bacteria which induces microbiome dysbiosis (Dobson *et al*., 2012; Xu *et al*., 2018).

Enterococcus faecalis DPN94 genome encoded for *cad*, *ace*, *slyA*, *asa1*, *EF3314*, *EF0109*, *cob*, *asp1*, *efaA*, *gelE* and *cpd*, however, *cylA*, *cylB* and *cylM* were not present. Virulence factors should not be considered true virulence determinants, but rather auxiliary factors that facilitate colonisation in the GIT (Toledo-Arana *et al*., 2001; Pillar and Gilmore, 2004). Cytolysin has a dual function as haemolytic toxin and bacteriocin (Ike *et al*., 1987 and 1990; Huycke and Gilmore, 1995; Shankar *et al*., 2002). Clinical *Enterococcus* isolates have a high prevalence of *cylA* genes, whereas prevalence is low or absent in environmental isolates (Creti *et al*., 2004). *In vitro* cytotoxicity test showed that probiotic bacteria (*L*. *crispatus* DPN167, *L*. *salivarius* DPN181, *L*. *gallinarum* DPN164, *L*. *johnsonii* DPN184, *E*. *faecalis* DPN94 and *B*. *amyloliquefaciens* DPN123) were not cytotoxic towards Caco-2 cells; however, their fermentative end-products were. Metabolites such as lactic acid and acetic acid lowered the external pH causing cell death. Cytotoxicity of short-chain fatty acids (SCFA) *in vitro* is not a true reflection of its effect and role *in vivo*, as SCFA receptors exist on eukaryotic membranes and molecules acts as signalling molecules between the microbiome and the host, plays important roles in local, intermediary and peripheral metabolism, and modulate host immune development (Corrêa *et al*., 2016; Morrison and Preston, 2016). Probiotic bacteria adhered to the Caco-2 cells with varying efficacy but were unable to invade them and reduced claudin-3 tight junctions but did not disrupt monolayer integrity. Probiotic strains were thus considered safe due to inability to invade epithelial cells.

Interaction of probiotic and pathogenic with the mucosal surface is well known, however, the proteomic crosstalk which occurs due to their interaction remains largely unknown. The multi-species probiotic upregulated transgelin 2/3, elongation factor-1 beta and anterior gradient 2, and down-regulated carnitine O-acetyl transferase, adenylate kinase 2, superoxide dismutase Cu-Zn and protein SET in broiler epithelial cells *in vivo*. Upregulated proteins are involved in cell proliferation, cell migration, healing, and cytoskeleton structure regulation and down-regulated proteins are involved in fatty acid transport, energy homeostasis, nucleotide metabolisms, free-radical elimination and signal transduction. Results indicated that the multi-species probiotic interacted with the ileum epithelial cells in a symbiotic manner, as differentially expressed proteins were not involved in host defence responses but rather involved in normal cellular and metabolic processes. Previously, *L*. *fermentum* I5007 induced

proteomic changes in Caco-2 cells beneficial for gut integrity which included voltage-dependent anion channel 1, glutathione transferase, and heat shock protein gp96 (Yang *et al*., 2007).

Broiler epithelial cells exposed to *S*. Enteritidis upregulated lysozyme C and G, cathelicidin 2 and 3, myeloid protein 1, trypsin inhibitor CITI-1, gallinacin-2 and ubiquitin-fold modifier 1, and down-regulated glutaredoxin-1, gallicin-7 and vigilin. Differentially expressed proteins are involved in various biological processes such as defence responses, responses to stress and cytolysis. *In vitro Salmonella* was cytotoxic towards Caco-2 cells, adhere and invade them with high efficacy and decreased claudin-3 tight junctions between Caco-2 cells which led to monolayer destruction. The genome of *Salmonella* encodes for virulence factors such as lipopolysaccharide, flagella, fimbriae, and type III secretion systems to adhere and invade epithelial cells (Schmidt and Hensel, 2004; Haiko and Westerlund-Wikström, 2013). Results indicated that *S*. Enteritidis negatively interacted with epithelial cells which resulted in the expression of proteins related to the innate immune system and other host defence responses. Previous studies indicated that *Salmonella* liposaccharides induce proteomic changes in broiler serum, upregulating α1-acid glycoprotein, a chemokine CCLI10, and cathelicidin-2, and down-regulating interferon-stimulated gene-12-2 protein. Differentially expressed proteins are associated with immunomodulation, cytokine changes, and defence responses (Packialakshmi *et al*., 2016). Results from this study agree with previous studies that *Salmonella* interaction with host induces host immune responses (Baptista *et al*., 2013; Packialakshmi *et al*., 2016).

Supplementation of broiler feed with the multi-species probiotic or antibiotic combination (sulphadiazine, colistin and trimethoprim) had no effect on the weight gain, feed intake, feed conversion ratios, relative lymphoid organ weights, relative gizzard weights, tibia bone parameters and haematological parameters. Broilers receiving antibiotics had higher levels of lymphocyte and basophil counts, larger villi area, but these effects were transient and only statistically significant on day 19. A higher basophil count is characteristic of a pro-inflammatory response and may be the result of sensitivity to antibiotics or the presence of bacteria that elicits an immune response. Transiently higher lymphocyte counts are indicative of a response to the presence of specific immune provoking bacteria. Antibiotic use in broilers are well known to induce a shift in the microbiome composition (Torok *et al*., 2011; Costa *et al*., 2017; Li *et al*., 2017). It is possible that antibiotics indirectly selected for pathogen colonisation which elicited the immune system. Broilers receiving probiotics had reduced *L*. *monocytogenes* bioluminescence in the ileum at 3.5 h after administration of the pathogen. These results indicated that the multi-species probiotic inhibited the metabolic activity of *Listeria* in the GIT, most likely by means of competitive exclusion or by the production of antimicrobial compounds (Höltzel *et al*., 2000; Magnusson and Schnürer, 2001). *Bacillus amyloliquefaciens* DPN123 produce lipopeptides which have antimicrobial activity against *Listeria*, and the lactic acid bacterial strains produce hydrogen peroxide and high levels of lactic acid which inhibit the growth of *L*. *monocytogenes*.

The microbiome of broilers receiving antibiotics had significantly lower levels of *Enterobacteriaceae*, and higher levels of unclassified Clostridiales, *Brucellaceae*, *Synergistaceae*, *Erysipelotrichaceae* and *Coriobacteriaceae* in their cecum on day 29. Results concur with previous studies that antibiotics alter the microbiome structure and functions in broilers (Torok *et al*., 2011; Costa *et al*., 2017; Li *et al*., 2017). The multi-species probiotic did not alter the microbiome of healthy broilers.

Probiotics accelerate microbiome maturation whereas antibiotic decrease maturation (Gao *et al*., 2017). Understanding how microbiota changes relate to metabolic changes in the host needs to be elucidated. The mechanism by which specific bacterial species modulate the GIT environment is required to improve our understanding (Wei *et al*., 2016; Borda-Molina *et al*., 2018). In future, the use of multi-omics approaches may enhance our understanding.

Controlling the presence of *Salmonella* in the GIT of broilers is important for food safety as these microorganisms possess a threat for humans as food-borne pathogens. Administration of *Salmonella enterica* serovar Enteritidis in broilers did not negatively affect broiler growth performance, as no significant differences were observed between infected and uninfected birds. These results are in accordance with results reported by other researchers (Ribeiro *et al*., 2007; Park and Kim, 2014; Olnood *et al*., 2015). Administration of an antibiotic (oxytetracycline) and multi-species probiotic in broilers infected with *Salmonella* did not significantly alter the immune organ weights, haematological parameters or serum interferon gamma levels. *Salmonella* colonisation in the cecum of broilers receiving oxytetracycline was lower at day 11 (days post infection, dpi 1) and 14 (dpi 4) as compared to untreated and probiotic groups, however, from day 19 and onwards *Salmonella* counts increased and were similar to the other treatment groups. Continuous use indirectly selected for *Salmonella* colonisation by altering the GIT microbiome. At day 29, *Salmonella* levels in the cecum of broilers receiving probiotics were similar to that of the infected and uninfected control birds.

Broilers receiving oxytetracycline had significantly higher serum bactericidal activity against *Salmonella* as compared to control birds infected and untreated on day 11 (dpi 1). The serum bactericidal activity against *Salmonella* from broilers from the probiotic treatment group was higher as compared to the *Salmonella*-infected control birds on day 29 (dpi 19). Broilers receiving the multi-species probiotic had higher serum lysozyme concentrations as compared to uninfected broilers on day 11 (dpi 1). Broilers receiving the antibiotic and probiotic feed additives had higher T lymphocyte responses as compared to broilers from the control treatment groups on day 17 (dpi 7). These results are in accordance with results reported by other researchers that probiotics increase broiler immune responses during pathogenic infection (Jung *et al*., 2010; Alaqaby *et al*., 2014; Hosseini *et al*., 2017).

The multi-species probiotic showed *in vitro* tolerance towards simulated GIT conditions and was able to adhere to epithelial cells. In addition, the probiotic possessed numerous beneficial characteristics and its daily use as a feed additive was deemed safe, as probiotic use did not negatively affect the performance of healthy birds. The multi-species probiotic crosstalk with broiler epithelial cells did not induce negative proteomic changes. The probiotic also increased immune responses of broilers infected with *Salmonella* suggesting that the multi-species probiotic can be used as an alternative feed additive to improve broiler health and performance. With the increase in occurrence of poultry-related food-borne pathogenic infections it is imperative to control the presence of these pathogens in broilers. The multi-species probiotic can be used as a feed additive for broilers to increase immune responses and inhibit colonisation of *Listeria* and *Salmonella*. The probiotic compares well with benefits reported of commercial probiotics PoultryStar® (*E. faecium*, *P. acidilactici*, *B. animalis*, *L. salivarius*, and *L. reuteri* and prebiotic fructooligosaccharides) (Sterzo *et al*., 2007; Ghareeb *et al*., 2012), CLOSTAT™ (*B. subtilis*) (Teo and Tan, 2007; Melegy *et al*., 2011; Lourenco *et al*., 2012; Abudabos *et al*., 2013) and

Floramax® (*L. salivarius* and *P. parvulus*) (Gutierrez-Fuentes *et al*., 2013; Prado-Rebolledo *et al*., 2017).

Further research is required to fully understand the role probiotics play in improving broiler health and growth performance. The microbiome composition of broilers with dysbiosis needs be elucidated so that an *in vivo* model can be designed to determine whether probiotics can restore homeostasis. In addition, the role members of the microbiome play in broiler physiology needs to be elucidated to better understand the crosstalk which occur between the host and the microbiome. Elucidating these unknowns will provide greater insight into the role probiotics play in improving broiler health and growth.

References

Aarestrup, F.M. (2015). The livestock reservoir for antimicrobial resistance: a personal view on changing patterns of risks, effects of interventions and the way forward. *Phil. Trans. R. Soc. B* 370, e20140085, DOI: 10.1098/rstb.2014.0085.

Abudabos, A.M., Alyemni, A.H., Al Marshad, M.B.A. (2013). *Bacillus subtilis* PB6 based probiotic (CloSTAT™) improves intestinal morphological and microbiological status of broiler chickens under *Clostridium perfringes* challenge. *Int. J. Agri. Biol*. 15(5), p 978-982.

Adeola, O., Cowieson, A.J. (2011). Board-invited review: Opportunities and challenges in using exogenous enzymes to improve nonruminant animal production. *J. Anim. Sci*. 89(10), p 3189–3218, DOI: 10.2527/jas.2010-3715.

Alagawany, M., Abd El-Hack, M.E., Farag, M.R., Sachan, S., Karthik, K., Dhama, K. (2018). The use of probiotics as eco-friendly alternatives for antibiotics in poultry nutrition. *Environ. Sci. Pollut. Res*. In Press, DOI: 10.1007/s11356-018-1687-x.

Alaqaby, A.R.A., Glaskovich, A.A., Kapitonova, E.A., Losev, E. (2014). Study the effect of using probiotic (Vetlactoflorum) on some of biochemical and immunological parameters of broiler chickens. *Bas. J. Vet. Res*. 1(1), p 166-179.

Askelson, T.E., Campasino, A., Lee, J.T., Duong, T. (2014). Evaluation of phytate-degrading *Lactobacillus* culture administration to broiler chickens. *Appl. Environ. Microbiol*. 80(3), p 943-950, DOI: 10.1128/AEM.03155-13.

Baldwin, S., Hughes, R.J., Thu Hao Van, T., Moore, R.J., Stanley, D. (2018). At-hatch administration of probiotic to chickens can introduce beneficial changes in gut microbiota. *PLoSONE* 13(3), e0194825, DOI: 10.1371/journal.pone.0194825.

Baptista, A.A.S., Donato, T.C., Souza, E.E., Gonçalves, G.A.M., Garcia, K.C.O.D., Rodrigues, J.C.Z., Sequeira, J.L., Filho, R.L.A. (2013). Induction of immune response in broiler chickens immunized with recombinant FliC and challenged by *Salmonella* Typhimurium. *Pesq. Vet. Bras*. 33(10), p 1215-1221, DOI: 10.1590/S0100-736X2013001000007.

Begley, M., Hill, C., Gahan, C.G.M. (2006). Bile salt hydrolase activity in probiotics. *Appl. Environ. Microbiol*. 72(3), p 1729–1738, DOI: 10.1128/AEM.72.3.1729-1738.2006.

Borda-Molina, D., Seifert, J., Camarinha-Silva, A. (2018). Current perspectives of the chicken gastrointestinal tract and its microbiome. *Comput*. *Struct*. *Biotechnol*. *J*. 16, p 131-139, DOI: 10.1016/j.csbj.2018.03.002.

Caggianiello, G., Kleerebezem, M., Spano, G. (2016). Exopolysaccharides produced by lactic acid bacteria: from health-promoting benefits to stress tolerance mechanisms. *Appl*. *Microbiol*. *Biotechnol*. 100(9), p 3877-3886, DOI: 10.1007/s00253-016-7471-2.

Conly, J.M., Johnston, B.L. (2005). Where are all the new antibiotics? The new antibiotic paradox. *Can*. *J*. *Infect*. *Dis*. *Med*. *Microbiol*. 16(3), p 159-160.

Corcoran, B.M., Staton, C., Fitzgerald, G.F., Ross, R.P. (2005). Survival of probiotic lactobacilli in acidic environments is enhanced in the presence of metabolizable sugars. *Appl*. *Environ*. *Microbiol*. 71(6), p 3060-3067, DOI: 10.1128/AEM.71.6.3060-3067.2005.

Corrêa-Oliveira, R., Fachi, J.L., Vieira, A., Sato F.T., Vinolo, M.A.R. (2016). Regulation of immune cell function by short-chain fatty acids. *Clin*. *Transl*. *Immunology* 5(4), e73, DOI: 10.1038/cti.2016.17.

Costa, M.C., Bessegatto, J.A., Alfieri, A.A., Weese, J.S., Filho, J.A.B., Oba, A. (2017). Different antibiotic growth promoters induce specific changes in the cecal microbiota membership of broiler chicken. *PLoS ONE* 12(2), e0171642, DOI: 10.1371/journal.pone.0171642.

Cotter, P.D., Hill, C. (2003). Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiol*. *Mol*. *Biol*. *Rev*. 67(3), p 429-453, DOI: 10.1128/MMBR.67.3.429-453.2003.

Creti, R., Imperi, M., Bertuccini, L., Fabretti, F., Orefici, G., Di Rosa, R., Baldassarri, L. (2004). Survey for virulence determinants among *Enterococcusfaecalis* isolated from different sources. *J*. *Med*. *Microbiol*. 53(1), p 13-20, DOI: 10.1099/jmm.0.05353-0.

Dahiya, R.S., Speck, M.L. (1968). Hydrogen peroxide formation by lactobacilli and its effect on *Staphylococcus aureus*. *J*. *Dairy Sci*. 51(10), p 1568-1572, DOI: 10.3168/jds.S0022-0302(68)87232-7.

Dersjant-Li, Y., Awati, A., Schulze, H., Partridge, G. (2015). Phytase in non-ruminant animal nutrition: a critical review on phytase activities in the gastrointestinal tract and influencing factors. *J*. *Sci*. *Food Agric*. 95(5), p 878-896, DOI: 10.1002/jsfa.6998.

Dobson, A., Cotter, P.D., Ross, R.P., Hill, C. (2012). Bacteriocin production: a probiotic trait? *Appl*. *Environ*. *Microbiol*. 78(1), p 1-6, DOI: 10.1128/AEM.05576-11.

Fortier, L.C., Tourdot-Maréchal, R., Diviès, C., Lee, B.H., Guzzo, J. (2003). Induction of *Oenococcus oeni* H+-ATPase activity and mRNA transcription under acidic conditions. *FEMS Microbiol*. *Lett*. 222(2), p 165-169, DOI: 10.1016/S0378-1097(03)00299-4.

Gadde, U., Oh, S.T., Lee, Y.S., Davis, E., Zimmerman, N., Rehberger, T., Lillehoj, H.S. (2017). The effects of direct-fed microbial supplementation, as an alternative to antibiotics, on growth performance, intestinal immune status, and epithelial barrier gene expression in broiler chickens. *ProbioticsAntimicro*. *Prot*. 9, p 397-405, DOI: 10.1007/s12602-017-9275-9.

Gao, P., Ma, C., Sun, Z., Wang, L., Huang, S., Su, X., Xu, J., Zhang, H. (2017). Feed-additive probiotic accelerate yet antibiotics delay intestinal microbiota maturation in broiler chicken. *Microbiome* 5, e91, DOI: 10.1186/s40168-017-0315-1.

Geng, W., Lin, J. (2016). Bacterial bile salt hydrolase: an intestinal microbiome target for enhanced animal health. *Anim*. *HealthRes*. *Rev*. 17(2), p 148-158, DOI: 10.1017/S1466252316000153.

Ghareeb, K., Awad, W.A., Mohnl, M., Porta, R., Biarnés, M., Böhm, J., Schatzmayr, G. (2012). Evaluating the efficacy of an avian-specific probiotic to reduce the colonisation of *Campylobacter* jejuni in broiler chickens. *Poult*. *Sci*. 91(8), p 1825-1832, DOI: 10.3382/ps.2012-02168.

Graf, E., Eaton, J.W. (1990). Antioxidant functions of phytic acid. *Free Radic*. *Biol*. *Med*. 8(1), p 61–69, DOI: 10.1016/0891-5849(90)90146-A.

Gutierrez-Fuentes, C.G., Zuñiga-Orozco, L.A., Vicente, J.L., Hernandez-Velasco, X., Menconi, A., Kuttappan, V.A., Kallapura, G., Latorre, J., Layton, S., Hargis, B.M., Téllez, G. (2013). Effect of a lactic acid bacteria based probiotic, Floramax-B11®, on performance, bone qualities, and morphometric analysis of broiler chickens: An economic analysis. *Biol*. *Syst*. 2(3), 113, DOI: 10.4172/2329-6577.1000113.

Haiko, J., Westerlund-Wikström, B. (2013). The role of the bacterial flagellum in adhesion and virulence. *Biology* 2(4), p 1242-1267, DOI:10.3390/biology2041242.

Höltzel, A., Gänzle, M.G., Nicholson, G.J., Hammes, W.P., Jung, G. (2000). The First low molecular weight antibiotic from lactic acid bacteria: reutericyclin, a new tetramic acid. *Angew*. *Chem*. *Int*. *Ed*. 39, 2766–2768, DOI: 10.1002/1521-3773(20000804)39:15<2766::AID-ANIE2766>3.0.CO;2-G.

Hosseini, S.M., Chamani, M., Mousavi, S.N., Hosseini, S.A., Sadeghi, A. (2017). Effects of dietary physical form and dietary inclusion of probiotic and enzyme on growth performance, cellular and humoral immunity, and relative weights of lymphoid organs at early period of broiler chickens fed triticale-based diets. *S*. *Afr*. *J*. *Anim*. *Sci*. 47(6), p 776-784, DOI: 10.4314/sajas.v47i6.5.

Huycke, M.M., Joyce, W.A., Gilmore, M.S. (1995). *Enterococcus faecalis* cytolysin without effect on the intestinal growth of susceptible enterococci in mice. *J*. *Infect*. *Dis*. 172(1), p 273–276.

Ike, Y., Clewell, D.B., Segarra, R.A., Gilmore, M.S. (1990). Genetic analysis of the pAD1 hemolysin/bacteriocin determinant in *Enterococcus faecalis*: Tn917 insertional mutagenesis and cloning. *J*. *Bacteriol*. 172(1), p 155–163.

Ike, Y., Hashimoto, H., and Clewell, D.B. (1987) High inci-dence of hemolysin production by Enterococcus (Strepto-coccus) faecalis strains associated with human parenteralinfections. J Clin Microbiol 25: 1524–1528.

Jones, B.V., Begley, M., Hill, C., Gahan, C.G., Marchesi, J.R. (2008). Functional and comparative metagenomic analysis of bile salt hydrolase activity in the human gut microbiome. Proceedings of the National Academy of Sciences of the United States of America 105: 13580–13585.

Jung, B-G., Ko, J-H., Lee, B-J. (2010). Dietary supplementation with a probiotic fermented four-herb combination enhances immune activity in broiler chicks and increases survivability against *Salmonella* Gallinarum in experimentally infected broiler chicks. *J*. *Vet*. *Med*. *Sci*. 72(12), p 1565-1573.

Kumar, A.S., Mody, K., Jha, B. (2007). Bacterial exopolysaccharides- a perception. *J*. *Basic Microbiol*. 47(2), p 103-117, DOI: 10.1002/jobm.200610203.

Li, J., Hao, H., Cheng, G., Liu, C., Ahmed, S., Shabbir, M.A.B., Hussain, H.I., Dai, M., Yuan, Z. (2017). Microbial shifts in the intestinal microbiota of *Salmonella* infected chickens in response to enrofloxacin. *Front*. *Microbiol*. 8, e1711, DOI: 10.3389/fmicb.2017.01711.

Li, Y., Xu, Q., Huang, Z., Lv, L., Liu, X., Yin, C., Yan, H., Yuan, J. (2015). Effect of *Bacillussubtilis* CGMCC1.1086 on the growth performance and intestinal microbiota of broilers. *J*. *Appl*. *Microbiol*. 120(1), p 195-204, DOI: 10.1111/jam.12972.

Lourenco, M.C., Kuritza, L.N., Westphal, P., Muniz, E., Pickler, L., Santin, E. (2012). Effects of *Bacillus* subtilis in the dynamics of infiltration of immunological cells in the intestinal mucosa of chickens challenged with *Salmonella* Minnesota. *Int*. *J*. *Poult*. *Sci*. 11(10), p 630-634, DOI: 10.3923/ijps.2012.630.634.

Magnusson, J., Schnürer, J. (2001). *Lactobacilluscoryniformis* subsp. *coryniformis* strain Si3 produces a broad-spectrum proteinaceous antifungal compound. *Appl*. *Environ*. *Microbiol*. 67, 1–5, DOI: 10.1128/AEM.67.1.1-5.2001 (2001).

Melegy, T., Khaled, N.F., El-Bana, R., Abdellatif, H. (2011). Effect of dietary supplementation of *Bacillus subtilis* PB6 (CLOSTAT™) on performance, immunity, gut health and carcass traits in broilers. *J*. *Am*. *Sci*. 7(12), p 891-898.

Mitchell, C., Fredricks, D., Agnew, K., Hitti, J. (2015). Hydrogen-peroxide producing lactobacilli are associated with lower levels of vaginal IL 1β, independent of bacterial vaginosis. *Sex*. *Transm*. *Dis*. 42(7), p 358-363, DOI: 10.1097/OLQ.0000000000000298.

Morrison, D.J., Preston, T. (2016). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. *Gut Microbes* 7(3), p 189-200, DOI: 10.1080/19490976.2015.1134082.

Neal-McKinney, J.M., Lu, X., Duong, T., Larson, C.L., Call, D.R., Shah, D.H., Konkel, M.E. (2012). Production of organic acids by probiotic *Lactobacilli* can be used to reduce pathogen load in poultry. *PLoSONE* 7(9), e43928, DOI: 10.1371/journal.pone.0043928.

Ocaña, V., Pesce de Ruiz Holgado, A. & Nader-Macías, M. (1999). Selection of vaginal H2O2-generating *Lactobacillus* species for probiotic use. *Curr*. *Microbiol*. 38(5), p 279-284, DOI: 10.1007/PL00006802.

Olnood, C.G., Beski, S.S.M., Choct, M., Iji, P.A. (2015). Use of *Lactobacillusjohnsonii* in broilers challenged with *Salmonellasofia*. *AnimalNutrition* 1(3), p 203-212, DOI: 10.1016/j.aninu.2015.07.001.

Onderci, M., Sahin, N., Sahin, K., Cikim, G., Aydín, A., Ozercan, I., Aydín, S. (2006). Efficacy of supplementation of α-amylase-producing bacterial culture on the performance, nutrient use, and gut morphology of broiler chickens fed a corn-based diet. *Poult*. *Sci*. 85(3), p 505-510, DOI: 10.1093/ps/85.3.505.

Packialakshmi, B., Liyanage, R., Lay, O.J., Makkar, S.K., Rath, N.C. (2016). Proteomic changes in chicken plasma induced by *Salmonella typhimurium* lipopolysaccharides. *Proteomics Insights* 7, p 1–9, DOI: 10.4137/PRI.S31609.

Park, J.H., Kim, I.H. (2014). The effects of the supplementation of *Bacillussubtilis* RX7 and B2A strains on the performance, blood profiles, intestinal *Salmonella* concentration, noxious gas emission,

organ weight and breast meat quality of broiler challenged with *Salmonella* typhimurium. *J*. *Anim*. *Physiol*. *Anim*. *Nutr*. 99(2), p 326-334, DOI: 10.1111/jpn.12248.

Patten, D.A., Laws, A.P. (2015). *Lactobacillus*-produced exopolysaccharides and their potential health benefits: a review. *Benef*. *Microbes* 6(4), p 457-471, DOI: 10.3920/BM2014.0117.

Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R., Waddell, J. (2004). Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J*. *Antimicrob*. *Chemother*. 53(1), p 28-52, DOI: 10.1093/jac/dkg483.

Pillar, C.M., Gilmore, M.S. (2004). Enterococcal virulence-pathogenicity islands of *E*. *faecalis*. *Front*. *Biosci*. 9, p 2335-2346, DOI: 10.2741/1400.

Prado-Rebolledo, O.F., de Jesus Delgado-Machuca, J., Macedo-Barragan, R.J., Garcia-Márquez, L.J., Morales-Barrera, J.E., Latorre, J.D., Hernandez-Velasco, X., Tellez, G. (2017). Evaluation of a selected lactic acid bacteria-based probiotic on *Salmonella enterica* serovar Enteritidis colonisation and intestinal permeability in broiler chickens. *Avian Pathol*. 46(1), p 90-94, DOI: 10.1080/03079457.2016.1222808.

Pridmore, R.D., Pittet, A-C., Praplan, F., Cavadini, C. (2008). Hydrogen peroxide production by *Lactobacillus johnsonii* NCC 533 and its role in anti-*Salmonella* activity. *FEMS Microbiol*. *Lett*. 283(2), p 210-215, DOI: 10.1111/j.1574-6968.2008.01176.x.

Ribeiro, A.M.L., Vogt, L.K., Canal, C.W., Cardoso, M.R.I., Labres, R.V., Streck, A.F., Bessa, M.C. (2007). Effects of prebiotics and probiotics on the colonisation and immune response of broiler chickens challenged with *Salmonella* Enteritidis. *Rev*. *Bras*. *Cienc*. *Avic*. 9(3), p 193-200, DOI: 10.1590/S1516-635X2007000300009.

Schmidt, H., Hensel, M. (2004). Pathogenicity islands in bacterial pathogenesis. *Clin*. *Microbiol*. *Rev*. 17(1), p 14-56, DOI: 10.1128/CMR.17.1.14-56.2004.

Shankar, N., Baghdayan, A.S., Gilmore, M.S. (2002). Modulation of virulence within a pathogenicity island in vancomycin-resistant *Enterococcus faecalis*. *Nature* 417(6890), p 746–750, DOI: 10.1038/nature00802.

Siragusa, G.R., Johnson, M.G. (1989). Inhibition of Listeria monocytogenes growth by the lactoperoxidase-thiocyanate-H_2O_2 antimicrobial system. *Appl*. *Environ*. *Microbiol*. 55(11), p 2802-2805.

Stern, N.J., Svetoch, E.A., Eruslanov, B.V., Perelygin, V.V., Mitsevich, E.V., Mitsevich, I.P., Pokhilenko, V.D., Levchuk, V.P., Svetoch, O.E., Seal, B.S. (2006). Isolation of a *Lactobacillussalivarius* strain and purification of its bacteriocin, which is inhibitory to *Campylobacterjejuni* in the chicken gastrointestinal system. *Antimicrob*. *AgentsChemother*. 50(9), p 3111–3116, DOI: 10.1128/AAC.00259-06.

Sterzo, E.V., Paiva, J.B., Mesquita, A.L., Freitas Neto, O.C., Berchieri, S. (2007). Organic acids and/or compound with defined microorganisms to control *Salmonella enterica* serovar Enteritidis experimental infection in chickens. *Rev*. *Bras*. *Cienc*. *Avic*. 9(1), p 69-73, DOI: 10.1590/S1516-635X2007000100010.

Tang, D.F., Iji, P., Choct, M., Ru, Y.J. (2013). Effect of α-amylase on performance and nutrient utilization of broilers fed diets based on cassava and soybean meal. 24th Annual Australian Poultry Science Symposium, 17-20 February 2013, p 88-91, Sydney, New South Wales, Australia.

Teo, A.Y., Tan, H.M. (2007). Evaluation of the performance and intestinal gut microflora of broilers fed on corn-soy diets supplemented with *Bacillus subtilis* PB6 (CloSTAT). *J*. *Appl*. *Poult*. *Res*. 16(3), 296–303, DOI: 10.1093/japr/16.3.296.

Toledo-Arana, A., Valle, J., Solano, C., Arrizubieta, M.J., Cucarella, C., Lamata, M., Amorena, B., Leiva, J., Penadés, J.R., Lasa, I. (2001). The enterococcal surface protein, Esp, is involved in *Enterococcusfaecalis* biofilm formation. *Appl*. *Environ*. *Microbiol*. 67(10), p 4538-4545, DOI: 10.1128/AEM.67.10.4538-4545.2001.

Tomás, M.S.J., Bru, E., Nader-Macías, M.E. (2003). Comparison of the growth and hydrogen peroxide production by vaginal probiotic lactobacilli under different culture conditions. *Am*. *J*. *Obstet*. *Gynecol*. 188(1), p 35-44, DOI: 10.1067/mob.2003.123.

Torok, V.A., Allison, G.E., Percy, N.J., Ophel-Keller, K., Hughes, R.J. (2011). Influence of antimicrobial feed additives on broiler commensal posthatch gut microbiota development and performance. *Appl*. *Environ*. *Microbiol*. 77(10), p 3390-3390, DOI: 10.1128/AEM.02300-10.

Tsai, C-C., Lin, P-P., Hsieh, Y-M., Zhang, Z-Y., Wu, H-C., Huang, C-C. (2014). Cholesterol-lowering potentials of lactic acid bacteria based on bile-salt hydrolase activity and effect of potent strains on cholesterol metabolism *invitro* and *invivo*. *Sci*. *WorldJ*. 2014, e690752, DOI: 10.1155/2014/690752.

Watson, J.A., Schubert, J. (1969). Action of hydrogen peroxide on growth inhibition of *Salmonella typhimurium*. *J*. *Gen*. *Microbiol*. 57(1), p 25-34, DOI: 10.1099/00221287-57-1-25.

Wei, S., Lilburn, M., Yu, Z. (2016). The bacteriomes of ileal mucosa and cecal content of broiler chickens and turkeys as revealed by metagenomic analysis. Int. *J*. *Microbiol*. 2016, Article ID: 4320412, DOI:10.1155/2016/4320412.

Xu, S., Lin, Y., Zeng, D., Zhou, M., Zeng, Y., Wang, H., Zhou, Y., Zhu, H., Pan, K., Jing, B., Ni, X. (2018). *Bacillus licheniformis* normalise the ileum microbiota of chickens infected with necrotic enteritis. *Sci*. *Rep*. 8, 1744, DOI: 10.1038/s41598-018-20059-z.

Yang, F., Wang, J., Li, X., Ying, T., Qiao, S., Li, D., Wu, G. (2007). 2-DE and MS analysis of interactions between *Lactobacillus fermentum* I5007 and intestinal epithelial cells. *Electrophoresis* 28, p 4330-4339, DOI: 10.1002/elps.200700166.