

**Characterization of the adhesion genes of probiotic
lactic acid bacteria**

by

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Declaration

I the undersigned hereby declare that the work contained in this thesis is my own original work and has not been submitted for any degree or examination in any university and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references.

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Summary

One of the key selection criteria for potential probiotics is the ability to adhere and colonise the host gastrointestinal tract (GIT). Probiotics compete for receptor sites at the host intestinal surface, preventing the colonisation of pathogens, thereby protecting the host from infection. In addition, several important intestinal functions are mediated by the binding of probiotics to host tissue. However, the molecular mechanisms and genotypic characterization of adhesive elements have not received as much attention as other aspects of probiotic research. The present study aims to contribute to this area of research.

The first part of the study focused on monitoring the expression of mucus adhesion genes *mub*, *mapA*, adhesion-like factor *EF-Tu* and bacteriocin gene *plaA* of *Lactobacillus plantarum* 423, as well as *mub*, surface layer protein (*slp*) and *EF-Tu* of *Lactobacillus acidophilus* ATCC 4356 when grown in the presence of mucin, bile, pancreatin and at low pH. Real time PCR was used. *mub*, *mapA* and *EF-Tu* of strain 423 were up-regulated in the presence of mucus and expression increased under increasing concentrations of mucus. Expression of *mapA* was up-regulated under normal gut conditions (0.3%, w/v, bile; 0.3%, w/v, pancreatin; pH 6.5) and at higher levels of bile (1.0%, w/v) and pancreatin (1.0%, w/v). Expression of *mub* was down-regulated in the presence of bile and pancreatin at pH 6.5, whilst the expression of *EF-Tu* and *plaA* remained unchanged. At pH 4.0, the expression of *mub* and *mapA* remained unchanged, whilst *EF-Tu* and *plaA* were up-regulated. Expression of *mapA* was down-regulated in the presence of 0.1% (w/v) cysteine, suggesting that the gene is regulated by a mechanism of transcription attenuation that involves cysteine. In the case of *L. acidophilus* ATCC 4356, none of the genes were up-regulated under increasing concentrations of mucin, whilst only *slp* and *EF-Tu* were up-regulated under normal and stressful gut conditions *in vitro*.

In the second part of the study, male Wistar rats were used to evaluate which section of the gastrointestinal tract are colonised by *L. plantarum* 423 and *Enterococcus mundtii* ST4SA and determine the effect of adhesion. Fluorescent *in situ* hybridization (FISH) incorporating strain specific oligonucleotide probes indicated strong fluorescent signals for *L. plantarum* 423 along the intestinal lining of the ileum and the cecum. *L. plantarum* 423 did not colonise the colon as indicated by real time PCR. Fluorescent signals were recorded for *E. mundtii* ST4SA across the epithelial

barrier of cecum and colonic tissue, suggesting that translocation took place. Real time PCR revealed highest cell numbers of strain ST4SA in the cecum and the colon. Haemotoxylin eosin staining of rat tissue revealed no change in morphology or any toxic effects induced upon adhesion of the strains. 16S rDNA PCR and denaturing gradient gel electrophoresis (DGGE) revealed a decrease in enterobacterial species whilst the lactic acid bacterial content remained unchanged. Strains 423 and ST4SA agglutinated yeast cells *in vitro*, indicating the possible presence of mannose receptors. It is well known that these receptors play a crucial role in the elimination of type 1 fimbriated strains of *E. coli*. It is thus safe to speculate that mannose receptors may have played a role in diminishing the enterobacterial content in the gut.

The third part of the study encompassed characterization of cell surface proteins of *L. plantarum* 423 and their role in adhesion to Caco-2 cell lines. The strain lacks the typical surface layer protein whilst a multifunctional “intracellular” protein, elongation factor Tu (EF-Tu) and glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and triosephosphate isomerase (TPI) were detected. Removal of surface proteins reduced adherence of strain 423 to Caco-2 cell lines by 40%, suggesting that these proteins play a role in adhesion. The ability of strain 423 to competitively adhere, exclude and displace *Clostridium sporogenes* LMG 13570 and *Enterococcus faecalis* LMG 13566 from Caco-2 cell lines, was studied. Adhesion of *C. sporogenes* LMG 13570 and *E. faecalis* LMG 13566 was inhibited by 70% and 90%, respectively. Strain 423 excluded *C. sporogenes* LMG 13570 from Caco-2 cells by 73% and displaced the pathogen by 80%. *E. faecalis* LMG 13566 was excluded by 60% and displaced from Caco-2 cells by 90%. Despite removal of the surface proteins, *L. plantarum* 423 was still capable of competitively adhering to Caco-2 cells and reduced adherence of *C. sporogenes* LMG 13570 by 50% and *E. faecalis* LMG 13566 by 70%.

Opsomming

Een van die sleutelkriteria vir die seleksie van 'n potensiële probiotikum is die vermoë van die selle om aan die gasheer se gastro-intestinale weg vas te heg en te koloniseer. Probiotika kompteer vir reseptorsetels op die oppervlak van die gasheer se ingewande en verhoed sodoende die koloniserings van patogene en daaropvolgende infeksies. Aanvullend tot bogenoemde word verskeie belangrike intestinale funksies tydens vashegting van die probiotiese selle aan die gasheer gestimuleer. Ten spyte hiervan geniet die molekulêre meganismes en genotipiese karakterisering van adhesie-elemente relatief min aandag in vergelyking met baie ander aspekte van navorsing op probiotika.

Die eerste gedeelte van die studie fokus op die uitdrukking van die gene *mub*, *mapA* en die adhesie-tipe faktor *EF-Tu* wat vir hegting aan mukus kodeer, asook die bakteriosien-geen *plaA* van *Lactobacillus plantarum* 423. Die uitdrukking van *mub*, die *slp*-geen wat kodeer vir produksie van seloppervlak proteïene, en *EF-Tu* van *Lactobacillus acidophilus* ATCC 4356 is ook bestudeer. Laasgenoemde studies is gedoen tydens groei in die teenwoordigheid van mukus, galsoute, pankreatien en verlaagde pH. Intydse ("Real time") polimerase kettingreaksie (PKR) is gebruik. *mub*, *mapA* en *EF-Tu* van stam 423 se regulering is opwaarts aangepas in die teenwoordigheid van mukus, met die uitdrukking proporsioneel eweredig tot die verhoging in mukus vlakke. Die uitdrukking van *mapA* het onder normale toestande (0.3%, m/v, gal; 0.3%, m/v, pankreatien; pH 6.5) en in die teenwoordigheid van hoër vlakke galsoute (1.0%, m/v) en pankreatien (1.0%, m/v) toegeneem. Die uitdrukking van *mub* het afgeneem in die teenwoordigheid van galsoute en pankreatien by pH 6.5, terwyl die uitdrukking van *EF-Tu* and *plaA* onveranderd gebly het. In 'n omgewing van pH 4.0 het die uitdrukking van *mub* en *mapA* onveranderd gebly, terwyl die uitdrukking van *EF-Tu* en *plaA* gestimuleer is. Uitdrukking van *mapA* is onderdruk in die teenwoordigheid van 0.1% (w/v) sisteïen, wat daarop dui dat die geen deur 'n meganisme van transkripsie-attenuasie in die teenwoordigheid van sisteïen gereguleer word. In die geval *L. acidophilus* ATCC 4356 is geen uitdrukking nie in die teenwoordigheid van hoë mukusvlakke gestimuleer nie. Die uitdrukking van *slp* en *EF-Tu* is onder normale en stresvolle toestande in die ingewande gestimuleer.

In die tweede afdeling van die studie is manlike Wistar rotte gebruik om te bepaal presies watter deel van die gastro-intestinale weg deur *L. plantarum* 423 en

Enterococcus mundtii ST4SA gekoloniseer word. *In situ* fluorisensie hibridisasie (FISH) met stam-spesifieke oligonukleotiedpeilers het aangetoon dat *L. plantarum* 423 op die mukosa van die ileum en die sekum vestig. *L. plantarum* 423 het nie die dikderm gekoloniseer soos deur intydse PKR voorspel is nie. Fluoriserende selle van *E. mundtii* ST4SA is op epiteelselle van die sekum en kolon waargeneem en dui dus op translokasie van die selle. Intydse PKR het hoë selgetalle van stam ST4SA in die sekum en die kolon aangetoon. Haemotoksilien-eosin kleuring van die weefsel het geen verandering in morfologie of enige toksiese defekte getoon as gevolg van adhesie nie. 16S rDNA PKR en denaturende gradient-gel elektroforese (DGGE) het op 'n verlaging in aantal ingewandsbakterieë gedui, terwyl die selgetalle vir melksuurbakterieë onveranderd gebly het. Binding van stamme 423 en ST4SA aan gisselle dui op die moontlike voorkoms van mannose reseptore. Dit is algemeen bekend dat mannose reseptore 'n essensiele rol speel in die eliminasië van *E. coli* stamme met tipe-1 fimbrae. Dit is dus veilig om te spekuleer dat reseptore vir mannose 'n belangrike rol speel in die verwydering van ingewandsbakterieë.

Die derde gedeelte van die studie het die karakterisering van sel-oppervlak proteïene van *L. plantarum* 423, en hul rol in die vashegting aan Caco-2 selle, behels. Stam 423 ontbreek die tipiese oppervlak-proteïene, terwyl 'n multi-funksionele intrasellulêre proteïen, verlengingsfaktor TU (EF-Tu) en die glikolitiese ensieme gliserinaldehyd 3-fosfaat dehidrogenase (GAPDH) en trifosfaat isomerase (TPI) opgespoor is. Verwydering van oppervlak-proteïene het die vashegting van stam 423 aan Caco-2 sellyne met 40% verminder en dui daarop dat hierdie proteïene 'n rol speel in adhesie. Die vermoë van stam 423 om kompetend vas te heg en *Clostridium sporogenes* LMG 13570 en *Enterococcus faecalis* LMG 13566 van Caco-2 selle te verplaas, is bestudeer. Vashegting van *C. sporogenes* LMG 13570 en *E. faecalis* LMG 13566 is onderskeidelik met 70% en 90% ge-inhibeer. Stam 423 het suksesvol met *C. sporogenes* LMG 13570 vir vashegting aan Caco-2 selle gekompeteer en het 73% van die selle verplaas en 80% van die patogeen verplaas. *E. faecalis* LMG 13566 is met 60% uitgeskakel en is teen 90% verplaas. Ten spyte van die verwydering van oppervlak-proteïene was *L. plantarum* 423 nogsteeds daartoe in staat om kompetend aan Caco-2 selle vas te heg en die kolonisering van *C. sporogenes* LMG 13570 met 50% en *E. faecalis* LMG 13566 met 70% te verminder.

To Vernon Ramiah

*My personal source of
strength and inspiration*

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Chapter 1

Introduction

Motivation for the study

Probiotic properties of lactic acid bacteria have initiated various discussions, resulting in the increased growth of research in this field, one of which has attempted to understand the role of adhesion of these organisms.

The strong adhesive ability of some *Lactobacillus* spp. to host intestinal tissue has been demonstrated (9, 11). However, controversy on the importance of this property for probiotic potential arose, since the ability of a pathogen to adhere to host tissue is considered a virulence factor (2). It was later shown that unlike pathogens, the adhesive ability of lactobacilli is not a universal property and that adhesion does not elicit infection (1, 7). It was thus concluded that the high adhesive properties of lactobacilli are not a virulence trait and do not pose as a potential risk factor (7). Adhesion of probiotics to intestinal mucosa is now considered one of the main selection criteria (10, 12).

The ability of probiotics to inhibit or eliminate diarrhoea is possibly the most validated health benefit reported (3) and is discussed in Chapter 2. This effect depends on the adhesion of probiotic bacteria to host intestinal tissue and subsequent colonization of the gastrointestinal tract. Lactic acid bacteria and pathogens compete for receptor sites at the intestinal surface. Competition for these receptors will diminish the opportunity for pathogenic colonization and thus protect the host from infection (5). The molecular mechanism surrounding host-microbial interactions was demonstrated by the colonization of *Bacteroides thetaiotaomicron* (major commensal of human and mouse intestinal microflora) to germ-free mice (6). Several important intestinal functions, including mucosal barrier reinforcement, xenobiotic metabolism, nutrient absorption, detoxication and postnatal intestinal maturation facilitated by mere adhesion of the strain, was reported (6). Adhesion of probiotics also play a substantial role in stimulating the immune system by enhancing the natural immune

response (15). In addition to the inhibition of pathogenic colonization and immune stimulation, the adhesion of probiotics is also imperative to prolong their persistence and beneficial role in the gastrointestinal tract.

Protein and carbohydrate molecules play a substantial role in mediating the adhesion of lactobacilli to host surfaces (13). However, molecular characterization of adhesive elements have not received much attention as only a few of these molecules have been characterized. More than 300 peer-reviewed articles on probiotics are published annually, indicating the significant interest in this field (8). However, very limited number of these reviews focus on the mechanisms of probiotic action, particularly with respect to the mechanisms of adhesion (8). Most of the research conducted on adhesion focuses primarily on *in vitro* adhesion of the potential probiotic to adhesion models such as cell lines or mucus preparations. These studies provide significant insight on the adhesive ability of the probiotic. However, it may not provide adequate information on the mechanisms of adhesion, particularly at the molecular level. Most probiotics are selected based on their superior phenotypic ability (tolerance to bile, acid, antimicrobial activity, etc.) and not on their unique ability to confer a defined health benefit. Similar probiotics may have different mechanisms of action against different pathogens. Understanding the molecular mechanisms of probiotic action, particularly the mechanisms of adhesion, will provide the scientific rationale for selection of the best species or strains that target a specific health problem.

In light of the abovementioned, this research focused on characterization of the adhesive properties of *Lactobacillus plantarum*, *Enterococcus mundtii* and *Lactobacillus acidophilus* at the molecular level. Most of the research in the present investigation was conducted on *L. plantarum*, as the organism displays strong adhesive ability and excellent probiotic potential. Examination of *in vivo* gene expression of certain lactobacilli indicated that expression of 3 *Lactobacillus reuteri* and 72 *L. plantarum* genes were induced in the murine gastrointestinal tract (4, 14). Most of the gut-inducible *L. plantarum* genes were involved in nutrient assimilation, stress response and modification of cell surface protein composition. Such responses indicate that the organism is metabolically active and adaptive to the gastrointestinal tract (4, 14).

Efficient colonization of a probiotic can only be facilitated by overcoming digestive stress conditions such as tolerance to bile, pancreatic juice and low pH. *In*

vitro expression of adhesion genes under digestive stress conditions have not been previously studied. The first part of study focused on the adhesion gene expression of *Lactobacillus plantarum* 423 and *Lactobacillus acidophilus* 4356 when cultured in the presence of varying concentrations of mucus, bile, pancreatin and pH. The role of the surface layer protein (Slp) in adhesion was studied by investigating the regulation of the gene in the presence of mucus.

In vitro analysis provides valuable preliminary information on the properties of a potential probiotic. However, *in vitro* analysis is not a complete representation of the actual results, making *in vivo* analysis essential. The second part of the study included the use of rat models to determine which section of the gastrointestinal tract was colonised by *L. plantarum* 423 and *Enterococcus mundtii* ST4SA. *L. acidophilus* was not considered in this part of the study as it was only incorporated in the first section to determine the role of Slp in adhesion. Molecular techniques such as fluorescent *in situ* hybridization (FISH), 16S rDNA PCR and denaturing gradient gel electrophoresis (DGGE) were used to evaluate adhesion of strains 423 and ST4SA to enterocytes as well as the effect on the natural microflora of rats.

In the last part of the study, the surface proteins of *L. plantarum* 423 were characterized, with interest in distinguishing the presence of a surface layer protein. The role of the surface proteins in adhesion of the strain to Caco-2 cell lines was also examined. The ability of the strain to competitively exclude *Clostridium sporogenes* LMG 13570 and *Enterococcus faecalis* 13566 from Caco-2 cell lines, was also examined.

References

1. **Apostolou, E., P. V. Kirjavainen, M. Saxelin, H. Rautelin, V. Valtonen, S. J. Salminen, A. C. Ouwehand.** 2001. Good adhesion properties of probiotics: a potential risk for bacteremia? FEMS Immunology and Medical Microbiology. **31**:35-39.
2. **Baddour, L. M., G. D. Christensen, W. A. Simpson, E. H. Beachey.** 1990. Microbial adherence, p. 9-25. In G. L. Mandell, R. G. Jr. Douglas, J. E. Bennett (ed.), Principles and practice of infectious diseases. Churchill Livingstone, New York.

3. **Boyle, R. J., R. M. Robins-Browne, M. L. K. Tang.** 2006. Probiotic use in clinical practice: what are the risks? *American Journal of Clinical Nutrition.* **83**:1256-1264.
4. **Bron, P. A., C. Grangette, A. Mercenier, W. M. de Vos, M. Kleerebezem.** 2004. Identification of *Lactobacillus plantarum* genes that are induced in the gastrointestinal tract of mice. *Journal of Bacteriology.* **186**:5721-5729.
5. **De Vries, M. C., E. E. Vaughan, M. Kleerebezem, W. M. De Vos.** 2005. *Lactobacillus plantarum* - survival, functional and potential probiotic properties in the human intestinal tract. *International Dairy Journal.* **16**:1018-1029.
6. **Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, J. I. Gordon.** 2001. Molecular analysis of commensal host-microbial relationships in the intestine. *Science.* **291**:881-884.
7. **Kirjavainen, P. V., E. M. Tuomola, R. G. Crittenden, A. C. Ouwehand, D. W. S. Harty, L. F. Morris, H. Rautelin, M. J. Playne, D. C. Donohue, S. J. Salminen.** 1999. In vitro adhesion and platelet aggregation properties of bacteremia-associated lactobacilli. *Infection and Immunity.* **67**:2653-2655.
8. **Marco, M. L., S. Pavan, M. Kleerebezem.** 2006. Towards understanding molecular modes of probiotic action. *Current Opinion in Biotechnology.* **17**:204-210.
9. **Ouwehand, A. C.** 2005. Measurement of bacterial adhesion - in vitro evaluation of different methods. *Journal of Microbiological Methods.* **60**:225-233.
10. **Ouwehand, A. C., P. V. Kirjavainen, C. Shortt, S. Salminen.** 1999. Probiotics: mechanisms and established effects. *International Dairy Journal.* **9**:43-52.
11. **Ouwehand, A. C., P. V. Kirjavainen, M. M. Gronlund, E. Isolauri, S. J. Salminen.** 1999. Adhesion of probiotic microorganisms to intestinal mucus. *International Dairy Journal.* **9**:623-630.
12. **Salminen, S., M. Laine, A. von Wright, J. Vuopio-Varkila, T. Korhonen, T. Mattila-Sandholm.** 1996. Development of selection criteria for probiotic strains to assess their potential in functional foods: a Nordic and European approach. *Bioscience Microflora.* **15**:61-67.

13. **Tuomola, E. M., A. C. Ouwehand, S. J. Salminen.** 2000. Chemical, physical and enzymatic pre-treatments of probiotic lactobacilli alter their adhesion to human intestinal mucus glycoproteins. *International Journal of Food Microbiology*. **60**:75-81.
14. **Walter, J., N. C. K. Heng, W. P. Hammes, D. M. Loach, G. W. Tannock, C. Hertel.** 2003. Identification of *Lactobacillus reuteri* genes specifically induced in the mouse gastrointestinal tract. *Applied and Environmental Microbiology*. **69**:2044-2051.
15. **Yokokura, T., K. Nomoto, T. Shimizu, K. Nomoto.** 1986. Enhancement of hematopoietic response of mice by subcutaneous administration of *Lactobacillus casei*. *Infection and Immunity*. **52**:156-160.

Chapter 2

Literature review

2.1 A brief history on probiotics

The therapeutic use of lactic acid bacteria was applied in the early centuries to improve the storage quality, nutritive value and flavour of perishable foods, without the existence of these microorganisms being recognised. Lactic acid fermentations served as a valuable food source for people of different cultures and played a pivotal role in prolonging the life of early descendents (206). Russian scientist Elie Metchnikoff first proposed the curative benefits of lactic acid bacteria at the beginning of the 20th century (159). This facilitated a profusion of experiments leading to their prevalent use as dairy starter cultures, incorporation in a number of functional foods to pharmaceutical applications with promoted health benefits (151, 206).

Lactic acid bacteria are Gram-positive, catalase negative and asporogenous. Fermentable sugars are converted to lactic acid via one of two metabolic pathways, i.e. the glycolytic pathway and pentose-phosphate pathway (8). The first pure culture of a lactic acid-producing bacterium, classified as “*Bacterium lactis*” was isolated from rancid milk in 1873 by J. Lister (8). This led to a progression in the classification of lactic acid bacteria isolated from various sources, including the gastrointestinal tract (GIT) of humans and animals. Four genera that predominantly compose this group include *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Enterococcus*, with lactobacilli and enterococci being the major commensals of the human GIT (8, 250).

The “Bulgarian bacillus” isolated from Bulgarian yoghurt by a Swiss scientist, was the first strain to be incorporated in human trials. However, the strain was incapable of surviving gastric transit (159, 191). This necessitated the need for isolates to be of human origin as it was perceived that these bacteria would overcome digestive stress conditions and maintain a competitive advantage in the gut (159). Subsequently, the selection of human derived isolates as compared to those of fermented origin, gained attention. Bifidobacteria were first isolated from the faeces of breast fed infants (156).

Bifidobacteria are obligate anaerobes and constitute a major part of the intestinal microflora (250). They are phylogenetically distinct from lactic acid bacteria as they exclusively degrade hexoses via the fructose-6-phosphate pathway (8). They play a fundamental role in colonisation of the human gut and implement favourable health effects (181). As a result, bifidobacteria as probiotics have gained considerable interest and remain one of the major starter cultures in the dairy industry (96).

The first report of a *Lactobacillus* sp. included *Lactobacillus acidophilus*, isolated from infant faeces by Moro in 1900 (75). Various “*acidophilus*” strains have subsequently been isolated from the intestinal tract of humans and animals. However, molecular taxonomic methods indicated that these “*acidophilus*” strains were composed of at least six different species, namely *L. acidophilus*, *L. crispatus*, *L. gallinarum*, *L. amylovorus*, *L. johnsonii* and *L. gasseri* (29, 59, 106, 120). Molecular analytical methods indicated that *L. casei*, *L. paracasei* and *L. rhamnosus* are the prevalent *Lactobacillus* spp. in the human gut (160, 226). *Lactobacillus casei* was used as a probiotic for the first time in 1935 when strain Shirota was included in a yoghurt preparation called Yakult. The strain is resistant to gastric and bile acid and is documented to generate health benefits when ingested (145).

One of the most extensively researched probiotic microorganisms is *L. rhamnosus* GG (LGG) (76, 216). The strain is of human origin and was identified from a selection of lactobacilli as possessing the best probiotic characteristics (70). Strain GG is capable of tolerating bile salts, pancreatic enzymes and gastric acid. It produces antimicrobial compounds and adheres to cells of the small intestine. The strain also survives passage through the gastrointestinal tract where it colonises and persists for days. Consequently, LGG was the first probiotic strain reported to colonise the human gastrointestinal tract (73).

2.2 Mechanisms of probiotic action

The mode of action by which a probiotic eradicates a pathogen can be summarised as suppression of viable cell numbers, altering the metabolism of pathogens and stimulation of the host immune system (61, 139). Suppression of viable cells may be facilitated by the production of antimicrobial compounds such as bacteriocins, organic acids and hydrogen peroxide (113, 195). The antagonistic activity of these compounds against Gram-positive and Gram-negative bacteria have been demonstrated *in vitro*. Bacteriocins play a significant role in the food industry where they prevent spoilage

by harmful microorganisms thereby improving the quality of food. However, not much is known about their activity *in vivo* (141, 239).

Competition for nutrients also reduces pathogenic cell numbers as probiotics may utilise nutrients that may otherwise have been consumed by pathogens (61). Evidence for this occurring *in vivo* is also lacking. The effect of probiotics on microbial metabolism in the gut was demonstrated by the consumption of *L. acidophilus*. Suppression in the activity of selected enzymes such as β -glucuronidase, nitroreductase and azoreductase was observed (71). Further mechanisms for the alteration of microbial metabolism remain to be elucidated.

The immune system is basically composed of acquired immunity (B lymphocytes and sensitised T lymphocytes) as part of the specific immune response and innate immunity that consists mainly of macrophages and natural killer (NK) cells as part of the non-specific immune response (172). *L. casei* Shirota enhanced proliferation of phagocytes such as macrophages and neutrophils in the bone marrow and spleen of mice (256). Direct activation of macrophages by strain Shirota augmented the bactericidal effect of macrophages on pathogenic bacteria such as *Pseudomonas aeruginosa* (152). Secretory IgA is a defence molecule produced as part of the acquired immune system in the intestine. The molecule plays a key role in maintaining a barrier against infection caused by pathogenic bacteria and viruses (51). Lactating mice treated with *Bifidobacterium lactis* Bb-12 showed significantly higher levels of total fecal IgA as compared to the controls (60). In addition, anti β -lactoglobulin IgA was higher in the faeces and in the milk (60). The acquired immunity (B lymphocytes) promoting effects of probiotics were also demonstrated by strain Shirota in a rabbit model. The probiotic not only prevented proliferation of enterohemorrhagic *E. coli* O157:H7, but also increased the intestinal antibody titre against O157:H7 and Shiga toxin produced by the pathogen (173).

Competitive adherence to bacterial adhesion sites on epithelial surfaces is another important mechanism of action by probiotics (2, 34). Competition for these sites will not only diminish the opportunity for pathogens to proliferate, but more importantly protect the host from infection (190). The mucus layer plays a pivotal role in maintaining a protective lining over intestinal tissue, thereby preventing intestinal permeability (51). Pathogens produce mucinase which degrades the mucosal layer and compromise intestinal epithelial integrity, leading to infection (83). The ability to degrade mucus is thus considered a valuable indicator of pathogenicity and local

toxicity of lumen bacteria (51). Probiotic bacteria have exhibited the ability to bind to intestinal mucus. For example, 45% of *Lactobacillus rhamnosus* strains and 30% of *Bifidobacterium lactis* strains adhered to stool mucus after oral administration to humans (112). Lactobacilli adhere and propagate on the mucosal layer, as shown for the small intestine of pigs (194). The lack of mucinase enzymes in probiotic bacteria and the inability of certain strains to degrade mucus have promoted their safe use (99). This property has been demonstrated for probiotic strains *L. casei* GG, *L. acidophilus*, *B. bifidum* (200), *L. rhamnosus* and *B. lactis* (257). In addition, probiotics have demonstrated the remarkable ability to stimulate mucin production as another effective mechanism to prevent pathogenic colonisation (134). Probiotic strains *L. plantarum* 299v and *L. rhamnosus* GG demonstrated up-regulation of *muc2* and *muc3* genes in human colonic HT-29 cells (134). Alternate mechanisms were proposed for the probiotic yeast, *Saccharomyces boulardii*. The yeast produces a protease capable of degrading the toxin A receptor for *Clostridium difficile* in animals, thus preventing adherence of the pathogen (187). A combination of all the processes by which probiotics hinder the colonisation of pathogens is referred to as colonisation resistance (195). Consequently, some probiotic strains have been selected purely based on their strong adhesive ability (195).

2.3 Selection criteria for potential probiotics

Health promoting effects of probiotics may either be acquired through incorporation in food components (functional foods) or as non-food preparations. In either situation the safety of the probiotic is of ultimate concern to the consumer. For this reason, *Lactobacillus* spp. and *Bifidobacterium* spp. have become popular choices due to their long history of safe use in the fermentation industry and being natural inhabitants of the gastrointestinal tract. These properties have conferred them with generally regarded as safe (GRAS) status (5, 94). This forms the basis for one of the first selection criteria for a potential probiotic. The safety assessment of each potential probiotic strain is imperative. Extrapolation of data from closely related strains is unacceptable (47). The strain should be well characterized and safety should be thoroughly evaluated *in vitro*, followed by *in vivo* studies. The efficacy of the probiotic should be evaluated by at least one human trial which should be double blind and placebo controlled (47, 84).

The efficiency of a probiotic depends on its viability and subsequent survival in the gastrointestinal tract. Strains of human origin are preferred over fermented origin as human isolates may possess a selective advantage in overcoming gastrointestinal conditions (47, 159). A potential probiotic will have to overcome harsh conditions in the GIT, such as survival at low pH, tolerance to bile acids and pancreatic juice, and efficiently colonise enterocytes. Secretion of more than two litres of gastric acid by the stomach daily constitutes a defense mechanism against most ingested microorganisms (41). The pH of gastric juice can reach values as low as 1.5 (41). Hence, survival of gastric transit by a probiotic is dependent on tolerance to low pH during a transit time of 1 to 4h (6).

Organisms that survive gastric transit proceed to the small intestine where they encounter further stress associated with bile acids and pancreatic juice. Bile acids are synthesized from cholesterol in the liver and are secreted into the duodenum in a conjugated form (91). Conjugated bile acids then undergo various chemical modifications in the colon almost solely by microorganisms (89). Conjugated and deconjugated forms of bile acids are inhibitory to both Gram-positive and Gram-negative bacteria. However, the deconjugated form is more inhibitory to Gram-positive bacteria (52, 127). It is thus imperative for potential probiotic strains to be tested for their ability to tolerate bile acids. A comparative study on the ability of *Lactobacillus* and *Bifidobacterium* strains to endure various concentrations of bovine, porcine and human bile was conducted (240). Although the tested strains exhibited resistance to bovine bile, porcine bile proved more inhibitory to both groups. However, despite the resistance patterns to bovine and porcine bile, all strains grew under relevant physiological concentrations of human bile (240). The bile salt hydrolase gene (*bsh*) present in intestinal lactobacilli such as *L. acidophilus*, *L. casei* and *L. plantarum* codes for a bile salt hydrolase that hydrolyses bile acids to more soluble deconjugated bile salts (67). This may be one of the mechanisms by which intestinal lactobacilli counteract toxic concentrations of bile acids.

Adhesion of a probiotic to enterocytes is important for colonisation and prolonging the beneficial role in the host. Of all available probiotics, *L. rhamnosus* GG has the longest retention time in the GIT (47, 72). The importance of this trait was demonstrated by the ability of *L. rhamnosus* GG to shorten the duration of rotavirus associated diarrhoea in infants (100). In a separate experiment, *L. bulgaricus* which does not adhere and colonise the GIT had no effect on alleviating rotavirus diarrhoea

(203). Intestinal mucus and human cell lines HT-29 and Caco-2 serve as valuable tools for assessing the adhesive properties of potential probiotic strains. The use of mucus and cell lines have been used to elucidate mechanisms of enteropathogen adhesion (15, 33) and assess lactic acid bacteria (LAB) on their adhesive properties (81, 177, 246).

In addition to the medico-scientific (*in vitro* and *in vivo* assessment of the efficacy in terms of human health) selection for probiotic bacteria, a few technological criteria need to be met if the probiotic is to be commercialised (32). Probiotics are available in either frozen concentrated or freeze-dried forms, depending on the application. Freeze-dried preparations should be conducted with cryoprotection to ensure that the probiotic retains its viability during storage (maintain high shelf-life). If the organism is incorporated into functional foods, it should be capable of proliferating to high cell densities in inexpensive media under robust conditions. In either situation the probiotic should maintain its viability during storage and use (32).

2.4 Beneficial role of probiotics in health

The availability of antibiotics in the 1950's facilitated their prevalent use as growth stimulants for animals and therapeutic agents (61). Growing concern over the years on the emergence of multi-drug resistant pathogens and associated side effects of antibiotic use has led to consumers and manufacturers seeking alternate measures. Probiotics pose an attractive alternative to overcome these problems. The mechanisms by which probiotics function, particularly in eliminating pathogens are diverse. Chances for the emergence of pathogens resistant to probiotics are thus minimal (51). In addition, the need for re-establishing the normal gut microflora after antibiotic therapy is eliminated (61, 195). The use of probiotic therapy may also prove a cheaper option compared to antibiotics (61). Probiotics have been documented to benefit the host in a number of ways and new health benefits are reported incessantly.

Intestinal infections caused by pathogenic microorganisms such as *Escherichia coli*, *Vibrio cholerae*, *Shigella* spp., *Campylobacter* spp., *Clostridium difficile* and rotavirus are the main causes of death in developing countries (172). Even in developed countries such as the United States, 21-37 million cases of diarrhoea occur annually in a population of 16.5 million children (69). The overuse of antibiotics has resulted in an increase of nosocomial infections caused by multi-drug resistant pathogens. The adverse side effects of antibiotic therapy enhances the need for

probiotics. Consequently, the most prevalent use of probiotics has involved the treatment of intestinal infections in humans and animals (61). Rotaviral diarrhoea, characterized by vomiting and watery stool, is predominant in children aged between 6 to 24 months. Treatment includes fluid replacement to counteract dehydration and nutritional deficiency. The effect of different probiotics for treatment of rotaviral diarrhoea was investigated in various double-blind placebo-controlled trials (100, 222). *L. rhamnosus* GG significantly reduced the duration of diarrhoea in infants aged 1-3 months compared to the placebo group. Similar results were obtained when *L. reuteri* SD 2222 was consumed by patients aged 6-36 months (222).

Clostridium difficile is a Gram-positive bacterium that causes colitis by the production of toxins. Treatment with metronidazole and vancomycin is effective, but there were incidences of recurrence of the disease in some patients (231). The pathophysiology of the disease, particularly the recurrence, is not clearly defined. Production of spores are considered a contributing factor to the recurrence but no conclusions have been derived (149). Re-treatment with antibiotics is usually prescribed. The probiotic yeast *Saccharomyces boulardii* exhibited positive results in effectively managing *C. difficile* infection in mice and humans (231). The probiotic prevented the adherence of *C. difficile* to cells *in vitro* (238) and also stimulated the IgA immune response to toxin A in mice (189). The efficacy of the probiotic in inhibiting recurrence of the disease was demonstrated on patients with renal failure, whereby 5 out of 7 patients tested showed an improvement (186). In 3 separate studies, 8 of 11 adults, 2 of 4 children and 5 of 9 adults were cured from *C. difficile* associated diarrhoea by treatment with *L. rhamnosus* GG (13, 16, 77).

Shigellosis, caused by *Shigella dysenteriae* 1, is a highly contagious infection characterized by fever, diarrhoea and bloody mucoid stools (130). Frequency of epidemic outbreaks and the fatality rate of young children pose a major concern in developing countries. Furthermore, the emergence of strains that are resistant to multiple antibiotics, is increasing. Rat studies have proven successful in understanding the pathogenesis of the disease and to evaluate the effect of probiotics. A combination of *L. rhamnosus* and *L. acidophilus* exhibited a protective role in reducing inflammation of rat tissue and aid in eliminating infection (158).

Campylobacter jejuni is a Gram-negative bacterium frequently isolated from animal faeces. Infection caused by the organism usually results in enteritis and is characterized by abdominal pain, diarrhoea and fever. Food sources contaminated

with *C. jejuni* is one of the most common causes of diarrhoea amongst humans (201). *Bifidobacterium breve* was evaluated for the treatment of *C. jejuni* induced enteritis in 133 patients aged between 6 months to 15 years. The strain did not alleviate symptoms, but reduced the number of pathogens detected in the faeces and shortened the duration of diarrhoea (242). A combination of *L. acidophilus*, *L. fermentum*, *L. crispatus* and *L. brevis* was successful in completely eradicating the pathogen in different sections of a simulated chicken digestive system (30).

The antagonistic effect of lactobacilli on *E. coli* is frequently used to select potential probiotic strains (39). A strain of *L. salivarius* suppressed the growth of *E. coli* in the gut of new born rats (39). Similar results were obtained with cultures of *L. acidophilus* and *L. lactis* in new born pigs (118, 165). In addition to lactobacilli, *B. thermophilum*, *B. pseudolongum* and *E. faecium* C63 were successful in protecting pigs against *E. coli* induced diarrhoea (110, 247).

Prolonged treatment with antibiotics such as clindamycin, cephalosporin and penicillin disturb the endogenous bacterial flora which facilitates abnormal proliferation of opportunistic enteropathogens (172). This imbalance in normal gut microflora causes diarrhoea commonly referred to as antibiotic associated diarrhoea and occurs in about 20% of treated patients (172). Probiotic cultures of *L. rhamnosus* GG (180), *B. longum* (40) and *E. faecium* SF68 (25) were administered to patients on antibiotic treatment. A significant decrease in the incidence of antibiotic associated diarrhoea was reported in the double-blind placebo controlled trials. *C. difficile* is usually present in the intestine at very low levels and antibiotic treated patients may become susceptible to the associated infection (172). Diarrhoea may progress to pseudomembranous enteritis and recurrence of the disease may occur. *S. boulardii* was concomitantly consumed with vancomycin and recurrence of the disease was considerably reduced compared to the placebo group (148). The probiotic produces proteolytic enzymes that digests toxin A or B of the pathogen. This important mechanism of action prevents adsorption of the toxin to receptors on the intestinal mucopithelium (148).

Probiotics have also demonstrated their efficacy in the treatment of traveller's diarrhoea which may be acquired by the ingestion of contaminated food or water. Occurrence of the disease is more prevalent in residents from developed countries travelling to developing countries. Most of the reported cases (80-85%) have been initiated by bacterial pathogens such as enterotoxigenic *E. coli* (ETEC) and

Campylobacter jejuni (88, 255). Due to the diversity in the cause and etiology of the disease, antibiotic therapy proves complex and controversial. A probiotic supplement containing *S. boulardii*, *L. acidophilus* and *B. bifidum* provided a safe and effective alternative in the prevention of traveller's diarrhoea in 12 controlled clinical trials (147).

Helicobacter pylori causes gastritis and peptic ulcers and is thus considered to be a risk factor for gastric cancer (92). Presence of the organism in most individuals is usually asymptomatic. However, the organism has been associated with a high mortality rate (137). Numerous *in vitro* studies have verified the ability of various lactobacilli or their metabolic products to eliminate or inhibit the pathogen. Some of these strains include *L. acidophilus* CRL 639, the metabolic product lactisyn (133), *L. johnsonii* LA1 (153) and *L. salivarius* WB1004 (3). Some researchers have claimed that the high lactate production of lactobacilli is the major factor for eradication of *H. pylori* (3, 154). Other researches have shown inhibitory effects by the production of antibacterial compounds such as microcin. *L. johnsonii* LA1 not only destroyed free-floating *H. pylori* cells but also those attached to epithelial cells *in vitro* (153). Mechanisms of competitive adhesion have been demonstrated by some strains of *L. reuteri*. These strains contain a surface glycolipid binding protein that is homologous to that of *H. pylori* and competes for receptor sites on the host (163). *L. salivarius* WB1004 inhibited the adhesion of *H. pylori* to human and mouse gastric epithelial cells (109). In human trials, treatment with a probiotic strain of *L. acidophilus* resulted in a higher percentage of eradication of the pathogen compared to groups treated with antibiotics (27). Supplementation of fermented milk with the probiotic *L. casei* DN-114 001 inhibited proliferation of *H. pylori* in children with gastritis (234).

The gastrointestinal barrier is composed of physical (epithelial cells with the mucosal layer) and functional (immune cells) components (51). This barrier plays a fundamental role in restricting the colonisation of pathogens, prevents the translocation of foreign antigens and regulates the antigen specific immune response (208). Any disturbance in this impressive array of intestinal defences will result in gut barrier dysfunction and inflammation (101). Probiotics have displayed various mechanisms in counteracting inflammation and these include degradation of enteral antigens, stabilisation of normal microflora during infection and reduction in the production of inflammatory mediators (101). During infection, the healthy host-microbe interaction is compromised and an imbalance in the intestinal microflora is

accompanied by inflammation (51, 101). Counteracting infection, thereby reducing the generation of antigens and restoring microbial balance, constitutes the rationale for probiotic therapy (101). Probiotics are thus useful for the treatment of inflammatory bowel diseases (IBD) such as irritable bowel syndrome (IBS), Crohn's disease and ulcerative colitis (101). Interleukin-10 (IL-10) is a pleiotropic molecule involved in anti-inflammatory reactions. IL-10 gene deficient mice develop chronic colitis similar to that observed for patients with Crohn's disease. Increased permeability as assessed by mannitol flux was observed in IL-10 gene deficient mice in an Ussing chamber. Permeability was eliminated after 4 weeks of treatment with the probiotic combination VSL#3 which contains *Bifidobacterium* spp., *Lactobacillus* spp. and *Streptococcus* spp. (136). The probiotic effect was mediated by a soluble proteinaceous component as similar results were observed by treatment with the VSL#3 cell-free culture medium. The effect was abolished after treatment of the culture medium with proteinase, indicating the involvement of a protein molecule in mediating the response (136).

L. bulgaricus was the first probiotic reported to possess anti-tumour properties (19). β -glucuronidase, β -glucosidase, azoreductase and nitroreductase, enzymes produced by enteric bacteria, are involved in the production of carcinogens from innocuous complexes. Probiotic bacteria mediate anticarcinogenic properties by suppressing bacteria that produce these enzymes and degrade carcinogens in the gastrointestinal tract. A reduction in the release of these enzymes were observed in individuals that consumed milk fermented with *Lactobacillus* spp. and *Bifidobacterium* spp. (22, 73). Nitroreductase is responsible for the production of N-nitrosamine, which is a carcinogen. Degradation of N-nitrosamine was observed by various *Lactobacillus* spp. (198) and a decrease in the production of the enzyme was observed in human subjects after consumption of fermented milk containing *L. acidophilus* (71). Oral administration of carcinogens N-methyl-N'-nitro-N-nitrosoguanidin (MNNG) and 1,2 dimethylhydrazine (DMH) resulted in DNA damage of gastrointestinal cells in rats within 24h. Consumption of *L. casei* 8h before exposure to the carcinogens prevented DNA damage (185). It is hypothesized that LAB metabolites are involved in anticarcinogenic effects as heat treatment of *L. acidophilus* resulted in loss of protection against the carcinogens (MNNG) and (DMH) (185).

LAB are also implicated in having anticholesterolaemic effects. Probiotics can assimilate cholesterol for their own metabolism, thereby reducing cholesterol levels and improve cardiovascular health (232). The serum cholesterol levels in rats fed with skim milk fermented with *L. acidophilus* were significantly lower than that of rats fed with untreated skim milk (82). It was suggested that bacterial metabolites present in fermented milk prevented cholesterol metabolism by the body (82). Direct assimilation of cholesterol in the growth medium was demonstrated for *L. acidophilus* (66). It was later suggested by some researchers that assimilation occurred as a result of cholesterol precipitation in laboratory media, at different pH. This was revealed by *in vitro* assays performed at pH 6.0 and lower (115). However, feeding trials with *L. acidophilus* considerably reduced the serum cholesterol levels of pigs fed with cholesterol (66).

There is also evidence for the therapeutic role of probiotics in alleviating high blood pressure. It was proposed that the proteolytic action of probiotic bacteria on casein during milk fermentation facilitates the production of bioactive peptides that may suppress the blood pressure of hypertensive individuals (235). This theory was proven on studies conducted on hypertensive rats (167) and a clinical trial on humans (86). Tripeptides, isoleucine-proline-proline and valine-proline-proline, identified as by-products of milk fermentation by *Saccharomyces cerevisiae* and *L. helveticus*, have been recognized as the active compounds for alleviating high blood pressure. The compounds function as angiotensin-1- converting enzyme inhibitors (86, 167). Calpis, a Japanese company has produced a functional food product called Ameal-S, based on this technology (207).

The beneficial role of probiotics has also been exemplified in improving the digestion of humans and animals. In the former, the most widely explored aspect has been the compensation for lactase in lactose intolerant individuals (51). These individuals possess a congenital deficiency for the enzyme β -galactosidase in the brush borders of the small intestine (197). As a result, undigested lactose proceeds to the colon where it is utilized by colonic bacteria resulting in abdominal discomfort and diarrhoea (140). The presence of LAB in yoghurt generates the lactase enzyme either in culture or during growth in the gastrointestinal tract. This enzyme facilitates lactose digestion and thus eases the symptoms in lactose intolerant individuals (140). These individuals digested lactose more efficiently in yoghurt preparations as compared to the same amount of lactose in milk (62). This was determined by the

hydrogen breath analysis method, as a measure of hydrogen secretion in the breath is correlated with colonic fermentation and lactose maldigestion (125). Among the first clinical trials conducted with lactobacilli, the associated relief in constipation was observed. Supplements of *L. acidophilus* favourably enhanced the bowel function of constipated patients (78). LAB are also valuable generators of vitamins, especially the B complex vitamins as well as enzymes that aid in digestion (140).

2.5 *Lactobacillus plantarum*

2.5.1 Ecological diversity

The genus *Lactobacillus* encompasses over one hundred different species that inhabit a range of environmental niches (229). However, only a limited number of species are encountered in fermented foods and in the human gastrointestinal tract and one of them includes *L. plantarum* (28). One of the most significant lactic acid bacterial strains to be incorporated in meat (37, 65), plant, vegetable (111, 179, 199, 227, 249) and dairy fermentations is *L. plantarum* (49), indicating the versatile nature of this bacterium.

Whole genome sequence data of *L. plantarum* WCFS1 revealed a large number of genes encoding surface-anchored proteins and regulatory functions (116). The genome contains two lifestyle adaptation islands which encode proteins responsible for the adaptation of the organism to its environment. The first is 3080-3260 kb and encodes proteins solely involved in sugar transport, metabolism and regulation. The second is 2600-3000 kb and encodes a large number of extracellular peptides. This valuable information indicates the incredible ability of this microorganism to exist and adapt to a vast range of habitats (17, 116)The ecological diversity of this microorganism is further justified by the genome size (approximately 3 308 274bp), one of the largest known genomes of lactic acid bacteria (116)

2.5.2 Probiotic properties

The numerous reports documenting the use of *L. plantarum* in various food fermentations, functional foods as well as its natural occurrence in the human gastrointestinal tract, provides an indication on the general safety of the microorganism. However, this cannot be accepted as a general consensus and the possible role in infection needs to be thoroughly evaluated for each new strain prior to its use as a probiotic. One of the concerns of *L. plantarum* has been the association

with infective endocarditis (221). However, the number of cases implicating the role of *L. plantarum* in the disease is very limited. Furthermore, problems were associated with obtaining a positive isolate (98). The possible role in infective endocarditis is a trait shared by other lactic acid bacteria and is not solely associated with *L. plantarum* (98).

The correlation between bacterial translocation and septic morbidity is increasing (202). This necessitates the need to examine the passage of bacteria across the intestinal barrier and its isolation from sterile locations such as blood (204). Research conducted on *L. plantarum* NCIMB, the human saliva isolate, indicated no adverse translocation across the intestinal barrier of mice. Conversely, administration of the strain reduced the translocation of endogenous microbiota in mice suffering from colitis (184). In another study, *L. plantarum* 299V did not influence bacterial translocation and postoperative septic morbidity in surgical patients (150). Intravenous injection of *L. plantarum* 299V to rats did not result in bacteremia as no detection of the probiotic was observed in the heart and the blood, 96 h after injection. This indicates that even if bacterial translocation took place, no infection occurred, illustrating the safety of the probiotic (1). Another recent report showed that in 66 of 100 000 bacteremia cases, 48 isolates were confirmed to be *Lactobacillus* strains of which 26 were *L. rhamnosus*. *L. plantarum* was not involved in any of the bacteremia cases (205).

Various inhibitory compounds that exhibit hostile activity against a range of Gram-positive, Gram-negative and fungal species, are produced by *L. plantarum* strains. Some plantaricins reported to date include plantaricin A (45), plantaricin B (253), plantaricin C (74), plantaricin F (58) and plantaricins S and T (104). Some plantaricins display a broad antimicrobial spectrum against Gram-positive and Gram-negative bacteria. Two examples include bacteriocins ST26MS and ST28MS, produced by *L. plantarum* strains isolated from molasses (241). Novel antifungal compounds were isolated from the culture filtrate of the sourdough *L. plantarum* strain 21B. Thin layer chromatography and spectroscopic analysis indicated antifungal compounds such as phenyllactic and 4-hydroxy-phenyllactic acids which almost completely inhibited various *Eurotium*, *Penicillium*, *Endomyces*, *Aspergillus*, *Monilia* and *Fusarium* spp. (121). Novel and diverse inhibitory compounds produced by *L. plantarum* strains are continually being reported, depicting the importance of this trait for probiotic potential.

Stimulation of the immune system facilitated by adhesion of the probiotic, is another valuable consequence of probiotic administration. *L. plantarum* 299V increased the production of interleukin-10 (IL-10) by macrophages and T cells (183). Rats administered with a combination of *L. plantarum* and *E. coli* showed higher levels of serum IgA, and IgM and IgA antibody levels against *E. coli*, in comparison to rats that were administered with *E. coli* alone. These results signify that *L. plantarum* competes with *E. coli* for intestinal colonisation and plays a pivotal role in stimulating the immune system (87).

The ability of *L. plantarum* to reduce cholesterol levels was also depicted. Rats treated with metabolites of *L. plantarum* I-UL4, isolated from the culture medium, showed significantly lower levels of total plasma cholesterol in comparison to the control group (53). Humans with slightly high cholesterol levels showed a decrease in both the LDL-cholesterol and fibrinogen levels in the blood after consumption of probiotic 299V (24). Expression of the bile salt hydrolase enzyme (*bsh*) by *L. plantarum* is proposed to contribute to the lowering of cholesterol levels (169). This enzyme hydrolyses bile acids in cholesterol to deconjugated bile salts, which are more readily excreted in the faeces as compared to conjugated bile salts (237). The presence of this gene in *L. plantarum* enables the organism to tolerate toxic levels of bile acids (36). *L. plantarum* displays probiotic properties by its ability to survive acid and bile, adhere and colonise the gastrointestinal tract, produce antagonistic compounds, and have GRAS status, as indicated by the number of investigations conducted on this bacterium.

2.5.3 Adhesins of *L. plantarum* and their role in probiotic potential

One of the most essential roles proposed for probiotics is the ability to eliminate intestinal infections caused by enterotoxigenic *E. coli* (ETEC), the causative agent of travellers' diarrhoea (190). These pathogenic ETEC strains express type 1 fimbriae that interact with mannose receptors on the host cell surface. Mannose specific adhesins have been described for *L. plantarum* and this mechanism of adhesion plays an important role in eliminating type 1 fimbriated *E. coli* by competing for the same receptor sites at the epithelial surface (2, 188). Cell surfaces of yeast are covered by α -mannoside oligosaccharides. Deletion of the mannose specific adhesin (Msa) of *L. plantarum* prevented the organism from agglutinating yeast cells (188). In addition, Msa (LP1229) contains three mucus binding domains, indicating the importance of

this molecule for mediating adhesion of the microorganism to host surfaces (188). Factors involved in the binding of *L. plantarum* Lp6 to rat intestinal mucus indicated mannose specific adhesins to be the most significant factor (230). Mannose adhesion is possibly one of the most significant probiotic traits of *L. plantarum*.

Chemical and enzymatic pre-treatments of 31 strains of *L. plantarum* indicated that lectin-like adhesins and proteinaceous cell surface structures mediate adhesion of the strains to Caco-2 cell lines and mucin (236). Adhesion of *L. plantarum* strains 299V, CBE, BMCM12, Col4S and T25 were strongly inhibited when cells were treated with trypsin, lithium chloride and methyl- α -D-mannoside (236). A large number of extracellular proteins (~223) are encoded by the *L. plantarum* genome. Of these proteins, a large proportion contains domains involved in the attachment of the cell to its surface (17). Three domains are involved in the adherence to collagen, 1 with a chitin binding domain, 1 with fibronectin and 7 involved in the adhesion to mucus (17). These data reveal the high adhesive ability of this microorganism, an important characteristic for potential probiotics. This characteristic is verified by the ability of the probiotic *L. plantarum* strain 299V to adhere to rectal mucosa of healthy as well as critically ill patients on antibiotic treatment (114). Strain 299V further demonstrated that pre-treatment with the probiotic prevented intestinal permeability in rats, induced by *E. coli*, supporting the concept that adhesion of probiotics exert beneficial effects in the gut (138).

2.5.4 Implications in health

Due to their excellent probiotic potential, *L. plantarum* strains 299 and 299V have been incorporated in a number of human trials. Table 1 outlines the effect of *L. plantarum* on the health status of healthy volunteers and patients as assessed by *in vivo* studies. A considerable decrease in Gram-negative anaerobic fecal bacteria was observed in healthy volunteers who consumed daily intakes of an oatmeal soup containing different strains of lactobacilli. The main *Lactobacillus* strains that were recovered after days 1 and 11 were *L. plantarum* strains 299 and 299V. This indicates survival of gastric transit, prolonged retention and the exceptional ability of this microorganism to colonise the gastrointestinal tract, particularly when there is competition amongst other strains (105).

L. plantarum is available as health adjuncts either as capsules or incorporated in a drink. A few popular products available as capsules include Plantadophilus,

manufactured by New Health Education, Bio-Kult, manufactured by the Finchley Clinic and ProBion, manufactured by the Healing Arc. The positive effects on human health associated with the consumption of *L. plantarum* is developing into a promising field for marketing future strains as potential probiotics and health adjuncts.

Table 1. Effect of *L. plantarum* on healthy volunteers and patients in various trials

Dosage	Duration	Number of Subjects	Effect	Ref.
Unknown	13 days	151	Six times reduction in fecal enterobacteria	(111)
1×10^{10}	6 weeks	30	9.6% reduction in LDL-cholesterol and 13.5% reduction in fibrinogen	(24)
Unknown	4 weeks	18	Increase in weight and improved natural immune response in children with HIV	(42)
5.0×10^{10}	38 days	20	1/3 reduction in recurrence of <i>Clostridium difficile</i> associated diarrhoea	(254)
2.0×10^{10}	4 weeks	40	Reduction in symptoms of IBS (abdominal pain, bloating and flatulence)	(170)

Source: Adapted from De Vries *et al.*, 2005 (43)

2.6 Enterococci as potential probiotics

2.6.1 Safety of enterococci as potential probiotics

The use of enterococci as potential probiotics is a very controversial issue due to their implication in nosocomial infections and superinfections such as endocarditis, bacteremia, urinary tract, intra-abdominal and pelvic infections (57). Further causes of concern is the presence of virulence factors such as invasins, haemolysin and the resistance to a range of antibiotics (166). *Enterococcus faecalis* and *Enterococcus*

faecium are the two major species associated with enterococcal infections, with the former being accountable for more than 80% of human infections (103). The remaining *Enterococcus* spp., including *E. mundtii*, are rarely associated with infections (164). In addition enterococci are opportunistic pathogens that usually cause infection in patients with an existing underlying disease or in immunocompromised individuals (161). The potential of these opportunistic pathogens to cause disease in healthy humans is highly unlikely. Furthermore, the mortality of healthy humans associated with enterococcal infections is extremely low (55). Enterococci are ubiquitous lactic acid bacteria that have a long history of safe use in various food fermentations such as meat (57), cheese (131) and olives (12). They also constitute part of the autochthonous microflora of the GIT of humans and animals (57), indicating their general safety.

The use of enterococci as potential probiotics will probably always remain controversial. It is thus imperative that when an enterococcal strain is to be considered as a probiotic, rigorous *in vitro* evaluation will have to be conducted which should be confirmed by *in vivo* analysis. The detection of any virulence genes or antibiotic resistance genes is of cardinal importance and it should be noted that these traits are strain specific. The presence of these genes ought to be carefully evaluated, particularly since the transfer of these genes to other strains forms part of the main controversy for using enterococci as probiotics (55).

2.6.2 Enterococcal adhesins

Enterococci are gastrointestinal commensals, which reflects the need for adhesive components to promote binding to eukaryotic tissue and facilitate colonisation. This ensures survival, as non-binding enterococci would be eliminated with the high flow rates of the lumen (103). It is therefore, not surprising that evolutionary pressure for colonisation and survival has resulted in organisms capable of expressing more than one type of adhesin (85). The concept of multiple adhesins in Gram-negative bacteria, especially in the *Enterobacteriaceae* family has been recognised for over 20 years. However, this concept of multiple adhesins has been extended to Gram-positive bacteria and is suggested to be implicated in specific purposes, not necessarily pathogenic (46, 85). Adhesion of a pathogen to host tissue is considered the first step to elicit infection (9). However, in the case of probiotics, adhesion is considered as a positive response in assisting the host in eliminating possible infectious agents. Hence,

the mechanisms of adhesion and molecules that mediate this function play an important role in establishing if the outcome of adhesion is possibly pathogenic or probiotic. The aggregation substance, enterococcal surface protein and surface carbohydrates are adherence factors of enterococci which are considered to be virulence factors. In contrast to the detailed characterization of these adhesins, limited research has been conducted on other adhesion mechanisms in enterococci (103).

A few factors need to be taken into consideration when investigating the role of adhesion of enterococci as a pathogenic trait. During an infectious disease, pathogens colonise host tissue by engaging their surface bound adhesins with host receptors. In certain instances, recognition of host receptors is specific in targeting a particular tissue or organ (90). For example, *Bordetella pertusis* is capable of discriminating between cilia and macrophages and between humans and other mammalian cells (212). Hence, if adhesins have a specific target site it can be considered significant in playing a role in pathogenesis (57). Certain adhesins are reported to be toxins by interacting with a repertoire of host cells. On the one hand adhesins mediate attachment of the bacterium whilst at the same time induce cellular intoxication by introducing the enzymatically active toxic subunit (90, 209). Molecular and physiological characterization of adhesins are imperative in determining its effect on the host. Plasmids usually encode genes conferring additional characteristics to an organism. They are known to harbour virulence genes which are transferred between strains by elements such as transposons. The presence of a gene encoding an adhesin on a plasmid may be considered a virulence factor, as it could be transferred to other strains (103). Hence, the location of the adhesin gene is critical when investigating adhesins in enterococci.

2.6.2.1 Aggregation substance

Aggregation substances (AS) in *E. faecalis* are surface bound adhesins encoded by pheromone induced-plasmids. Aggregation substances are produced in response to sex pheromones which are small peptides excreted by plasmidless recipient strains of *E. faecalis* (48). Aggregation substances cause clumping, altering the surface of the donor bacterium which facilitates plasmid transfer (48). In eukaryotes, AS mediates the adhesion of *E. faecalis* to cultured renal tubular cells and an Arg-Gly-Asp-Ser amino acid motif is thought to be involved in the binding (119). This amino acid motif is also present in fibronectin and facilitates binding to eukaryotic receptors known as

integrins (119). *E. faecalis* cells expressing AS were internalised by enterocytes to a higher degree as compared to non-expressing mutant strains (174). Electron micrographs indicated that AS interacted with enterocyte microvilli. Localisation of enterococci occurred within membrane bound vacuoles in the cytoplasm, indicating that AS is an important virulence factor (174).

2.6.2.2 Enterococcal surface protein

The enterococcal surface protein (Esp) is a chromosomally encoded adhesin (55). Esp is a 202kDa bacterial surface protein that is composed of repeating units at the central core, a slightly divergent C-terminal cell wall anchor domain and a globular N-terminal domain (219). The number of central repeats between *E. faecalis* isolates were found to vary between 3 and 11 and is hypothesised to serve as a retractable arm extending the N-terminal globular domain through the cell wall to the surface (219). Esp contributed to the colonisation of *E. faecalis* in the urinary tract of mice. However, no histopathological changes were induced to mice tissue upon adhesion of the strain (220). Esp also plays a role in adherence to abiotic surfaces and biofilm formation by increasing cell surface hydrophobicity. The protein exhibits characteristics of surface protein receptors designated “microbial surface components recognising adhesive matrix molecules”(MSCRAMMs) which mediates binding to extracellular matrix proteins and promotes colonisation (243). Incidence of Esp was shown to be higher in clinical isolates of *E. faecalis* compared to isolates from healthy individuals (219).

2.6.3 Implications of enterococci in health

Enterococci are known to produce enterocins which generally belong to the class II bacteriocins (57). They exhibit antimicrobial activity against other enterococci, strains of *Listeria monocytogenes* (68) and *Clostridium* spp. (56). Enterocin AS-48, produced by *E. faecalis* S-48, was the first enterocin purified to homogeneity (63, 142). Enterocin ST4SA, produced by *E. mundtii* ST4SA, is resistant to treatment at 80°C for 90min and incubation in MRS broth at pH 1.0 to 10.0. The bacteriocin is also active against a number of food-borne pathogens including *Bacillus cereus*, *Clostridium sporogenes*, *Enterococcus faecalis* and *Listeria monocytogenes* (117). One particular study showed the inhibitory activity of mundticins against *Listeria innocua* and two *Salmonella enteritidis* strains isolated from cheese. This was one of

the very few studies testing the antimicrobial activity of enterocins against *Salmonella* spp (50). The use of these microorganisms for treatment of food-borne infections denotes an important implication for health.

E. faecium SF68 is a probiotic strain that has *in vitro* inhibitory activity against *E. coli*, *Salmonella* spp., *Shigella* spp. and *Enterobacter* spp. Strain SF68 was selected as a probiotic as it is a commensal of the human intestine, has a short lag phase and generation time and is moderately resistant to antibiotics. Additional characteristics include tolerance to low pH and resistance to bile salts (11, 126). *E. faecium* SF68 is used to treat diarrhoea and was successful for the treatment of enteritis in adults and children (11, 126). Further health benefits associated with probiotic SF-68 includes the reduction of blood ammonia and improving the mental state and psychometric performance of patients with hepatic encephalopathy. It was suggested that SF-68 modified the intestinal microflora, thereby reducing blood ammonia levels and the associated disorders in these patients (132).

2.7 Adhesion studies

Initial experiments conducted to gain insight on components that mediate adhesion of lactobacilli indicated that protein and carbohydrate molecules play a substantial role in this aspect (2, 245). Boiling, autoclaving and enzymatic treatment with trypsin and pepsin reduced the adhesion of *L. acidophilus* LA1 to intestinal mucus and *L. rhamnosus* GG to intestinal mucosal cells, respectively (245). The importance of protein molecules in mediating adhesion was demonstrated after treatment of different *Lactobacillus* spp. with proteases, as adhesion was significantly reduced in these strains (38, 81). The interaction of carbohydrate moieties in cell attachment was depicted after treatment of different lactobacilli with metaperiodate, as this causes oxidation of carbohydrates. Treatment with metaperiodate decreased adhesion of the lactobacilli to cell lines (81).

Various mechanisms have been described for the anchoring of proteins to the surfaces of Gram-positive bacteria (Fig. 2.1). The cell envelope of Gram-positive bacteria is composed of a cell membrane surrounded by a peptidoglycan (PG) layer and cell wall polymers such as teichoic acids, teichuronic acid and other neutral or acidic polysaccharides (168, 217, 244). A common anchoring mechanism is the sortase-dependent mechanism via the LPXTG-motif (leucine, proline, X represents any amino acid, threonine and glycine). Proteins with this mechanism contain a

carboxyl terminal LPXTG motif, a hydrophobic region and a tail of charged amino acids (a, Fig. 2.1). The LPXTG sequence is covalently linked to the peptide cross bridges of PG via a membrane associated sortase enzyme (182, 244). The mucus binding protein (MUB) (196), other putative mucus binding proteins (18), cell envelope proteases (215) and exoenzymes such as fructosyltransferase (248) are lactobacilli surface proteins characterized with this motif. The GW-motif (repeats of glycine and tryptophan) was first identified in *Listeria monocytogenes* but has also been described for *Lactobacillus* spp. (23). Proteins with the GW-motif contain glycine and tryptophan repeats in the carboxyl terminus which anchors the protein to cell wall teichoic acids (107) (b, Fig. 2.1). Proteins with the LysM domain have been described for various bacterial species and mediates binding to PG via the C-terminus (10) (c, Fig. 2.1). It was thus suggested that this domain binds to a common component in PG such as the glycan strands (228). Other mechanisms include the direct binding of proteins to the cell membrane via a common cysteine containing lipobox (d, Fig. 2.1) (233) or an α -helical transmembrane anchor (e, Fig. 2.1) (44).

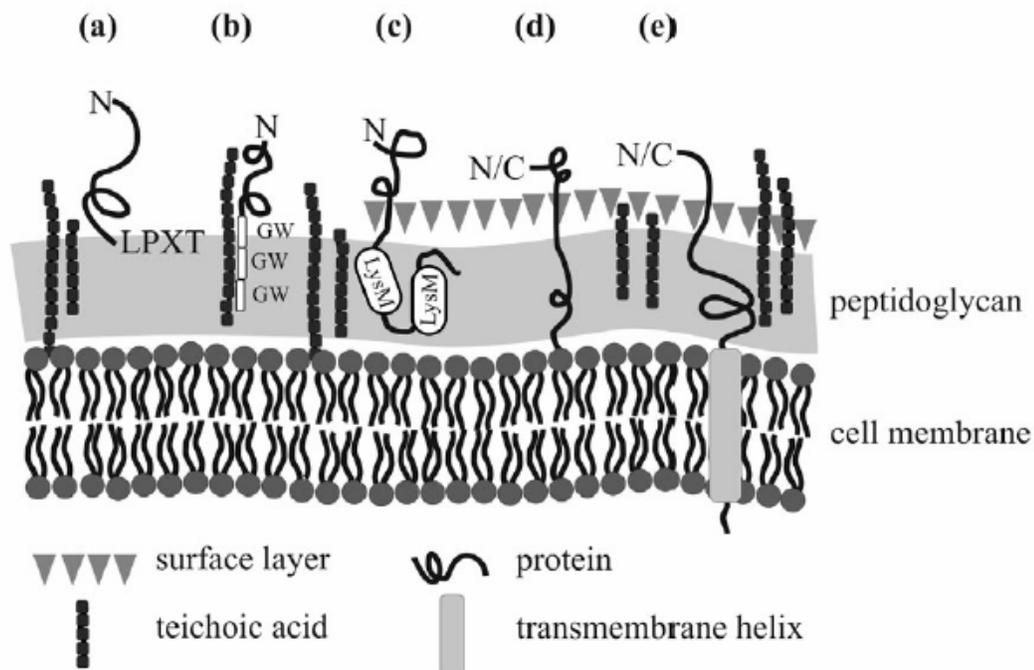


Fig. 2.1. Different mechanisms of protein anchoring to the Gram-positive cell surface. a: anchoring via the LPXTG-motif; b: anchoring via the GW-motif; c: anchoring via the C-terminus of the LysM domain; d: direct binding of proteins to the cell membrane via a common cysteine containing lipobox; e: or an α -helical

transmembrane anchor. N: amino terminal domain and C refers to the carboxyl terminal domain of proteins.

Molecular characterization of adhesive proteins in lactobacilli have not received much attention. Most of the research conducted on adhesion focuses primarily on phenotypic aspects such as *in vitro* adhesion of potential probiotic cells to adhesion models such as cell lines (Caco-2 and HT-29) or mucus preparations. Adhesion proteins characterized to date includes the mucus binding protein (Mub) (196), mucus adhesion promoting protein (MapA) (211), anchorless multifunctional proteins elongation factor Tu (EF-Tu) (80) and GroEl (14), surface layer proteins (Slp) (21, 252) and the aggregation promoting factors (apf1 and apf2) (102, 251).

2.7.1 Mub

The mucus binding protein is a high molecular mass (358 kDa), cell surface protein isolated and characterized from *L. reuteri* 1063 (196). Strain 1063, isolated from the small intestine of a pig, exhibited strong adherence to small intestinal mucus preparations of pigs and hens. Treatment of the strain with proteinase K eradicated the adhesion to mucus, indicating that binding was mediated by a proteinaceous substance. Mub was isolated from the culture supernatant and the surface of strain 1063, after multiple wash steps. Mub was the first *Lactobacillus* adhesion protein shown to contain the same domain organization as most of the characterized adhesins from other Gram-positive bacteria. These include a signal peptide, C-terminal anchoring sequences containing the LPXTG domain, repeated regions displaying adhesion properties and an N-terminal region, often with an uncharacterized function (196). Mub contains unique features, such as being one of the largest bacterial cell surface proteins identified, the corresponding *Mub* gene has two possible translation start sites, and the membrane anchor sequence at the C terminus contains a cysteine residue. The presence of two types of amino acid residue repeats (MBP-Mub1 and MBP-Mub2) and an N-terminal region of >500 amino acids indicates that Mub is a multifunctional protein. Inhibition of binding by the glycoproteins fetuin and asialofetuin suggests interaction with carbohydrate components in mucus. However, this inhibition of binding affected MBP-Mub2, indicating interaction of MBP-Mub1 with other carbohydrate structures of mucus. Furthermore, binding was shown to be pH dependent at just below neutral pH with both repeats having different pH optima. These data indicate the adaptation to different niches in the gastrointestinal tract (196).

Mub homologues have also been described for *L. acidophilus*, *L. gasseri*, *L. johnsonii* and *L. plantarum*, indicating the importance of this protein for the adherence and colonisation of lactobacilli (4).

2.7.2 MapA

The mucus adhesion promoting protein, MapA, is a cell surface protein isolated from *L. reuteri* 104R, originally thought to be *L. fermentum* 104R. The mechanism by which this protein anchors to the cell surface has not been determined (211). MapA has a molecular mass of 26kDa and a theoretical pI of 9.7. MapA shares a high similarity with the collagen binding protein, CnBP, of *L. reuteri* NCIB 11951 and the bacterial surface protein, BspA, of *L. fermentum*. BspA and CnBP are recognized for their binding potential to type 1 collagen and fibronectin which are mammalian receptors for many pathogens. Hence, these adhesion proteins participate as potential antagonistic competitors. *In vitro* experiments exhibited the ability of purified MapA to bind to Caco-2 cell lines and was also responsible for the binding of *L. reuteri* to these cell lines (157). MapA was suggested to aid *L. reuteri* DSMZ 20016 in colonising mice tissue. The best possible probiotic potential of this protein was demonstrated by the antagonistic activity against *Campylobacter jejuni*, by competing for the Peb1 receptor on HeLa cells (193).

2.7.3 Elongation factor-Tu and GroEl

The description of EF-Tu as an adhesion molecule occurred as an attempt to distinguish the surface proteins of *L. johnsonii* (NCC533) La1 that mediated attachment to epithelial cell lines or mucin (80). The EF-Tu protein is a GTP binding protein that functions during protein synthesis but also plays a role in the adhesion of *L. johnsonii* La1 to human intestinal cell lines and mucus. The importance of the protein in mediating attachment was reflected by the ability of the purified protein to inhibit binding of strain La1 to mucin by up to 40% (80). EF-Tu of *L. johnsonii* does not possess an identifiable signal sequence and also lacks the first methionine at the amino terminal. The protein also showed an apparent lack of the LPXTG peptidoglycan anchor motif or a lipoprotein motif, to demonstrate its localization on the surface of the bacterium. The presence of EF-Tu on different regions of bacteria is well documented. An EF-Tu recombinant molecule bound to HT-29 cells at neutral

pH using sCD14 as a ligand. A CD14 dependent pro-inflammatory response was also elicited upon adhesion to HT-29 cells (80).

GroEl is an 'intracellular' protein functioning in protein folding. However, the protein was identified on the cell surface and the culture medium of *L. johnsonii* La1 and binds to mucin and human epithelial cells at acidic pH (14). Probiotic potential of this protein in *L. johnsonii* was demonstrated by the ability to stimulate interleukin-8 secretion in macrophages and aggregate cells of the gastric pathogen *Helicobacter pylori* (14). EF-Tu and GroEl are referred to as anchorless multifunctional proteins as no established signal sequence or anchoring motif is present in their predicted sequences (35).

2.7.4 Surface layer proteins

Surface layers (S-layers) are crystalline structures composed of protein or glycoprotein subunits with molecular masses ranging from 40 to 200kDa (225). Lactobacilli surface layers are among the smallest known with molecular masses ranging from 25 to 71kDa (210). The unique property of lactobacilli S-layer proteins is its highly basic nature with pI ranging from 9.35-10.4 (210). This may occur as a result of a higher percentage of positively charged amino acid residues (12.5%). Glycan structures have been identified from the S-layer proteins of different Gram-positive bacteria (218). However, lactobacilli S-layers appear to be non-glycosylated with only the glycan structure of *L. buchneri* been reported (143, 162). The amino acid composition of lactobacilli S-layer proteins share some degree of homology with other S-layers, particularly in the high content of hydrophobic amino acids residues. This value ranges from 31.9% to 38.7%, a typical feature of all S-layer proteins (224). S-layer protein subunits are non-covalently attached to each other and the cell wall. These proteins can thus be extracted from the cell surface by chaotropic agents such as guanidine hydrochloride or a high concentration of salts, such as lithium chloride (224). A general functional property for all S-layer proteins has not been determined. However, proposed functions include protective coats, cell shape determinants, cell adhesion and surface recognition and molecule and ion traps (210, 225). S-layer protein genes of *Lactobacillus* spp. that have been cloned and characterized includes *L. acidophilus* (21), *L. brevis* (252), *L. helveticus* (26) and *L. crispatus* (223). S-layer proteins have also been described for *L. amylovorus* (20), *L. buchneri* (64), *L. gallinarum* (143), *L. kefir* and *L. parakefir* (144). However, their genes have not yet

been sequenced. The functional role of lactobacilli S-layers are poorly characterized with the only proposed function being attachment to various surfaces. Location of the adhesive domain of the S-layer protein has been described for *L. brevis* ATCC 8287 (SlpA) and *L. crispatus* JCM 5810. The receptor binding domain of *L. brevis* was established in the N-terminal region of SlpA comprising of amino acid residues 96 to 176 (97). Binding to fibronectin was also proven to be mediated by the N-terminal domain consisting of amino acid residues 96 to 245 (97). In *L. crispatus* the N-terminal section of the Slp (CbsA) adhered to collagen and laminin, components of the subintestinal extracellular matrix (7).

2.7.5 Aggregation promoting factors

Aggregation promoting factors (APF) are surface proteins described for *L. johnsonii* and *L. gasseri*. These proteins contribute to the aggregation phenotype of these strains and are thus designated accordingly. The amino acid compositions, physical properties and genetic organization of these proteins are similar to the S-layer proteins of other lactobacilli, suggesting a similar role in these species. APF demonstrate a highly alkaline iso-electric point and display important features associated with the more typical S-layer proteins of other lactobacilli. These proteins contain all the necessary characteristics exhibited by surface proteins, such as limited sulfur containing amino acid residues, high content of serine and threonine and a high content of hydrophobic amino acids. These proteins can be removed by treatment with LiCl, indicating anchorage to the cell wall by electrostatic or specific non-covalent interactions. The APF proteins of both species are encoded by two tandem genes, separated by a short intergenic region. A possible explanation for the presence of two genes may be attributed to the fact that these organisms encounter various habitats and competition for substrates, which may facilitate selective pressure for APF variation. The 3' segment of the corresponding genes are almost identical indicating possible functionality of the C-terminal region in anchorage of the APF protein to the underlying cell wall. APF was shown to be essential for maintaining the cell shape of *L. gasseri* (102, 251).

2.8 Mechanisms of adhesion

Protein molecules, (38, 80, 81, 196, 211) lipotechoic acids and mannose residues (2, 79) act as major adhesins. This illustrates the importance of receptor mediated

binding interactions that occur between probiotics and the host cell. The ability of certain strains to exhibit strong adherence is an indication that these strains contain the necessary receptors to recognize host tissue receptors. Adhesion of probiotic microorganisms to intestinal mucus revealed no changes to mucus derived from subjects of different ages. The data indicated no change in the availability of receptors for these strains, clarifying the mechanism of receptor mediated adhesion (177). The different mechanisms of binding and recognition of certain receptors is further justified by the binding of *L. acidophilus* LA1 to mucus secreted by the HT-29 cells, whereas its adherence to Caco-2 cells occurs via a proteinaceous adhesion promoting factor (15).

Other mechanisms such as electrical charges and hydrophobic interactions may also contribute to the binding of lactobacilli to host tissue. The Slp confers hydrophobicity to a cell. However, adhesion to hydrophobic surfaces does not always proceed according to expectations as hydrophobic strains do not always adhere to hydrophobic surfaces as predicted by surface thermodynamics. One study investigating the adhesion of *Lactobacillus* spp. to silicone rubber and glass showed that certain strains exhibiting hydrophobic properties, such as *L. acidophilus* 4356 and *L. fermentum* B54 adhered in higher proportions to hydrophilic glass than to hydrophobic silicone rubber. It was suggested that other factors play a more significant role in adhesion to various surfaces as compared to the interfacial free energies (155). Other studies also indicated no correlation between cell surface hydrophobicity and adherence to intestinal mucus. Highly adherent bacteria demonstrated fairly low surface hydrophobicities. These researchers stated that cell surface hydrophobicity was not an accurate measure of adhesive potential and should not be used as an appropriate marker for potential adhering microorganisms (177, 214).

The adhesion to intestinal cell lines provides a good indication of the adhesive properties of a strain. However, it may not take into account the adhesion to the mucus layer covering the intestinal cells (176). Intestinal mucus plays a significant role in maintaining a barrier between the beneficial and pathogenic bacteria. Mucus serves as a medium for the adhesion of commensals whilst preventing the binding of harmful bacteria (171). Mucus is a viscous, high molecular weight material secreted by goblet cells (213). It is composed of mucin which is a glycoprotein characterized by a high molecular weight, high carbohydrate content and O-glycosidic bonds between N-

acetylgalactosamine and either serine or threonine in the peptide backbone (54). *MUC2* and *MUC3* are the predominant genes encoding mucin in humans. *MUC2* is expressed in goblet cells of the small and large intestine and is the main mucin component present in the colon (31). *MUC3* is more prevalent in the small intestine and is secreted by goblet cells and enterocytes (31). Mucus serves a dual function, it may prevent the adhesion of pathogenic bacteria by forming a gel on the intestinal surface, thereby protecting the intestinal cells, but may also serve as a nutrient source and matrix for colonisation of probiotic bacteria (171).

The adhesion of *Lactobacillus* strains to intestinal epithelial cells *in vitro* up-regulated the *MUC3* gene, stimulating the production of mucus (134). The up-regulation of mucin encoding genes, particularly during enteric infections is another important mechanism of action by probiotics (93). Probiotic bacteria stimulated the production of mucus upon adhesion to adenocarcinoma cell lines. *L. plantarum* 299V adhered to HT-29 cells, which increased the transcription and secretion of MUC2 mucin. Similarly, an increase in MUC3 mucin production was observed upon adhesion of *L. rhamnosus* GG to Caco-2 cell lines. These probiotic strains proved efficient in inhibiting the adherence of an enteropathogenic *E. coli* strain. It was proposed that the ability of these probiotics to hinder pathogenic colonisation was mediated through the ability to increase expression of MUC2 and MUC3 (135, 146). However, the molecular mechanisms underlying the adhesion or stimulation of mucin expression is still unclear. It is still uncertain if mucin stimulation by probiotics are facilitated by the activation of intestinal cells by specific molecules or by stimulation of the immune system (213). The mucus layer, colonisation by probiotics and subsequent stimulation of the mucus genes are all mechanisms that maintain epithelial integrity and prevent the translocation of antigens and bacteria from the lumen content. Damage of the epithelial lining and intestinal permeability ultimately leads to pathogenesis in many circumstances (138).

Lipotechoic acid (LTA), a molecule associated with the surface of *L. johnsonii* La1 participated in the attachment to Caco-2 intestinal cells and further demonstrated an immunomodulatory effect on gut homeostasis. Competitive experiments also revealed that LTA was not the only molecule involved in the binding to intestinal epithelial cells. This is a further demonstration on the importance of receptor mediated adherence for the specificity of adhesion (79).

2.9 *In vitro* adhesion models

In vitro model systems have proved efficient for providing a good measure on the adhesive ability of a potential probiotic. Tissue culture cell lines and mucus preparations are the most frequent *in vitro* models utilized. HT-29 and Caco-2 cell lines are the most commonly used for this purpose as these cells are able to differentiate and express morphological and physiological characteristics that closely resemble human enterocytes (246). The competence of HT-29 cells was evaluated when cells were grown in the presence of methotrexate. The cells differentiated into goblet cells and secreted mucus. Antibodies raised against the mucus reacted with normal human gastric and colonic mucus designating the close proximity of these cells (124). HT-29 cell lines provided valuable information on the adherence mechanisms of *L. plantarum*. Binding of *L. plantarum* strains to HT-29 cells were inhibited by methyl- α -D-mannoside providing evidence for a mannose specific adherence mechanism for this binding (2). HT-29 cell models were also taken a step further into understanding gastrointestinal immune stimulation by the adhesion of probiotics. Binding of *L. casei* 3260 and *L. rhamnosus* GG to the colonic cell line increased Th1 type cytokine levels and decreased Th2 type cytokine levels. Balancing of the Th1/Th2 cytokine level may prove valuable for cancer treatment by probiotics. However, this effect is critically dependent on the binding capacity of the probiotic to the cancer cell (122).

The specific adhesion of some strains to Caco-2 cell lines illustrates the importance of employing this cell line as an *in vitro* adhesion model. *L. rhamnosus* LC-705 was shown to efficiently bind to Caco-2 cells, whilst the adhesion to intestinal mucus was very poor. These observations indicate that certain strains possess specific receptors for binding to this cell line. It was also suggested that the availability in the amount of certain receptors may also play a crucial role (123). In addition to the utilization of this cell line in establishing the adhesive properties of potential probiotics, it was also successfully employed in recognising the elongation factor Tu as a novel adhesin like factor in *L. johnsonii* LA1 (80).

The mucus layer covering epithelial cells serves as an important site for colonisation and may also contain specific receptors required for this purpose. The use of mucus as an *in vitro* adhesion model also proves important. An accurate source of mucus can be obtained from intestinal scrapings of desired sources. However, it may be difficult to obtain mucus from human tissue which led to the isolation of human

mucus from fecal samples. This serves as a very practical source, particularly for studying adhesion at an individual level. However, this form of mucus is easily degradable and important measures should be undertaken to ensure proper analysis (175). Alternatives include mucus derived from animal sources which also serves as a useful and convenient method of analysis. The addition of pig intestinal mucus to MRS growth medium reflected the binding activity of various *L. reuteri* strains. The binding activity of poorly adherent strains were significantly increased after growth in the mucin supplemented medium (108). A comparative study observing the adhesion of LAB to diseased and healthy human intestinal mucosa revealed that all the tested strains adhered more efficiently to intestinal mucus as compared to whole tissue, emphasizing the importance of this *in vitro* model (178).

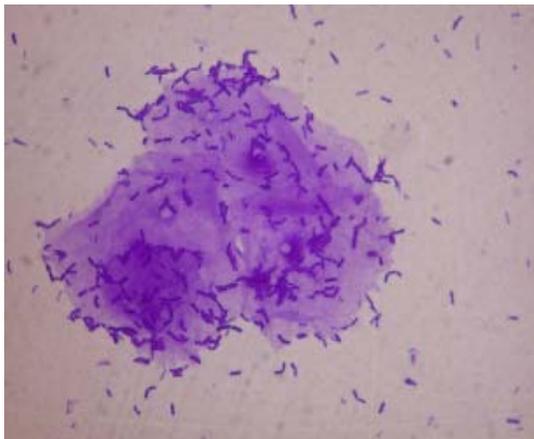


Fig. 2.2. *In vitro* adhesion model illustrating the adhesion of *L. fermentum* to chicken epithelial cells. Adhesion was observed using phase contrast microscopy after cells were stained with 0.5% crystal violet (129).

2.10 *In vivo* adhesion models

Data obtained by *in vitro* testing has proven its functional and practical use, particularly in obtaining valuable preliminary information on the properties of a potential probiotic. However, *in vitro* testing does not provide an accurate representation of conditions *in vivo*, which is imperative for the safety evaluation of a potential probiotic. Strains that exhibit strong adherence *in vitro* do not necessarily colonise the gut *in vivo*. In some situations, non-adhesive strains may still influence pathogenic colonisation and microbial changes, by the production of antimicrobial compounds (176). This necessitates the need for *in vivo* analysis. Exposure to bile,

pancreatic juice and varying pH are some of the digestive stress conditions that probiotics need to overcome to efficiently colonise the gut. Pretreatment of potential probiotic strains to canine jejunal chyme dramatically reduced adhesion of the strains to intestinal mucus. It was thus suggested that the selection criteria for adhesion of probiotics should be altered to include “adhesion after exposure to digestive factors”. It was also emphasized that adhesion properties should be further assessed after transit through an intestinal or animal model (192). The use of animal models for assessing the adhesive capability of an organism has most often analysed cell counts from fecal samples. This provides an effective indication on the ability of the organism to colonise as well as the duration of colonisation (128).

Rat and mouse models are more prevalent choices for the adoption of *in vivo* testing and have been incorporated in numerous trials (138, 184, 258). These models are often used for evaluation of the safety and efficacy of a potential probiotic in the gut. Prior to the incorporation of a probiotic in food and pharmaceutical applications, every concern surrounding its safety for human consumption needs to be tested and resolved. One of these main concerns is bacterial translocation and the possibility of septicemia, particularly since there have been some reports on the association of lactobacilli with bacteremia (204). Although the causative agents of these diseases were unlikely to be LAB, each new potential strain introduced to the market needs to be thoroughly assessed. Rat and mouse models provide an accurate and comprehensive means for this purpose. They have been successfully utilized in a number of trials to evaluate the safety potential and gain vital information on probiotic properties. Criteria used to examine any deleterious effect of a probiotic includes any adverse effect on the general health status of the animal, alterations in the feed intake and body weight, changes in differential blood cell count and changes in intestinal mucosal morphology (villus height, crypt depth, epithelial cell height and mucosal thickness). Bacterial translocation can be assessed by the detection of any viable bacteria recovered from blood and tissue samples such as the mesenteric lymph nodes, liver and spleen (95, 184, 258). β -glucuronidase, β -glycosidase, azoreductase, nitroreductase and nitrate reductase are bacterial enzymes that have been implicated in generating mutagens and carcinogens (197). Measurement of the fecal enzymatic content of these enzymes is another safety evaluation trait examined in these models.

In conclusion, *in vitro* assays are extremely useful for the pre-selection of potential probiotic strains. However, the proof of efficacy of the probiotic can only be evaluated

through *in vivo* analysis and should be granted at least one well designed human trial (84).

References

1. **Adawi, D., G. Molin, S. Ahrne, B. Jeppsson.** 2002. Safety of the probiotic strain *Lactobacillus plantarum* DSM 9843 (=strain 299V) in an endocarditis animal model. *Microbial Ecology in Health and Disease*. **14**:50-53.
2. **Adlerberth, I., S. Ahrne, M. L. Johansson, G. Molin, L. A. Hanson, A. E. Wold.** 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. *Applied and Environmental Microbiology*. **62**:2244-2251.
3. **Aiba, Y., N. Suzuki, A. M. A. Kabir, A. Takagi, Y. Koga.** 1998. Lactic acid mediated suppression of *Helicobacter pylori* by the oral administration of *Lactobacillus salivarius* as a probiotic in a gnotobiotic murine model. *American Journal of Gastroenterology*. **93**:2097-2101.
4. **Alterman, E., W. M. Russell, M. A. Azcarate-Peril, R. Barrangou, B. L. Buck, O. McAuliffe, N. Souther, A. Dobson, T. Duong, M. Callanan, S. Lick, A. Hamrick, R. Cano, T. D. Klaenhammer.** 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proceedings of the National Academy of Science of the United States of America*. **102**:3906-3912.
5. **Ammor, M. S., A. B. Florez, B. Mayo.** 2007. Antibiotic resistance in non-enterococcal lactic acid bacteria and bifidobacteria. *Food Microbiology*. **24**:559-570.
6. **Ammor, M. S., B. Mayo.** 2007. Selection criteria for lactic acid bacteria to be used as functional starter cultures in dry sausage production: An update. *Meat Science*. **76**:138-146.
7. **Antikainen, J., L. Anton, J. Sillanpaa, T. K. Korhonen.** 2002. Domains in the S-layer protein CbsA of *Lactobacillus crispatus* involved in adherence to collagens, laminin and lipoteichoic acids and in self-assembly. *Molecular Microbiology*. **46**:381-394.
8. **Axelsson, L.** 1998. Lactic acid bacteria: Classification and physiology, p. 1-72. *In* S. Salminen, A. Von Wright (ed.), *Lactic acid bacteria: microbiology and functional aspects*. Marcel Dekker, Inc., New York.

9. **Baddour, L. M., G. D. Christensen, W. A. Simpson, E. H. Beachey.** 1990. Microbial adherence, p. 9-25. *In* G. L. Mandell, R. G. Jr. Douglas, J. E. Bennett (ed.), Principles and practice of infectious diseases. Churchill Livingstone, New York.
10. **Bateman, A., M. Bycroft.** 2000. The structure of a LysM domain from *E. coli* membrane-bound lytic murein transglycosylase D (MltD). *Journal of Molecular Biology.* **299**:1113-1119.
11. **Bellomo, G., A. Mangiagle, L. Nicastro, G. Frigerio.** 1980. A controlled double-blind study of SF68 strain as a new biological preparation for the treatment of diarrhoea in pediatrics. *Current Therapeutic Research.* **28**:927-934.
12. **Ben Omar, N., A. Castro, R. Lucas, H. Abriouel, N. M. K. Yousif, C. M. A. P. Franz, W. H. Holzapfel, R. Perez-Pulido, M. Martinez-Canamero, A. Galvez.** 2004. Functional and safety aspects of Enterococci isolated from different Spanish foods. *Systematic and Applied Microbiology.* **27**:118-130.
13. **Bennet, R. G., B. Laughon, J. Lindsay.** 1990. *Lactobacillus* GG treatment of *Clostridium difficile* infection in nursing home patients. Abstract of the third International Conference on Nosocomial Infection, Atlanta, Georgia.
14. **Bergonzelli, G. E., D. Granato, R. D. Pridmore, L. F. Marvin-Guy, D. Donnicola, I. E. Corthesy-Theulaz.** 2006. GroEl of *Lactobacillus johnsonii* La1 (NCC533) is cell surface associated: potential role in interactions with the host and the gastric pathogen *Helicobacter pylori*. *Infection and Immunity.* **74**:425-434.
15. **Bernet, M. F., D. Brassart, J. R. Neeser, A. L. Servin.** 1994. *Lactobacillus acidophilus* LA1 binds to culture human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria. *Gut.* **35**:483-489.
16. **Biller, J. A., A. J. Katz, A. F. Flores, T. M. Buie, S. L. Gorbach.** 1995. Treatment of recurrent *Clostridium difficile* colitis with *Lactobacillus* GG. *Journal of Pediatric Gastroenterology.* **21**:224-226.
17. **Boekhorst, J., M. Wels, M. Kleerebezem, R. J. Siezen.** 2006. The predicted secretome of *Lactobacillus plantarum* WCFS1 sheds light on interactions with its environment. *Microbiology.* **152**:3175-3183.

18. **Boekhorst, J., Q. Helmer, M. Kleerebezem, R. J. Siezen.** 2006. Comparative analysis of proteins with a mucus binding domain found exclusively in lactic acid bacteria. *Microbiology*. **152**:273-280.
19. **Bogdonov, I. G., P. Popkhirstov, L. Marinov.** 1962. Anticancer effect of *antibioticum bulgaricum* on sarcoma 180 and on the select form of Ehrlich carcinoma. Abstract VIII. International Cancer Congress.364.
20. **Boot, H. J., C. P. A. M. Kolen, B. Pot, K. Kersters, P. H. Pouwels.** 1996. The presence of two S-layer protein-encoding genes is conserved among species related to *Lactobacillus acidophilus*. *Microbiology*. **142**:2375-2384.
21. **Boot, H. J., C. P. A. M. Kolen, J. M. van Noort, P. H. Pouwels.** 1993. S-layer protein of *Lactobacillus acidophilus* ATCC 4356: purification, expression in *Escherichia coli* and nucleotide sequence of the corresponding gene. *Journal of Bacteriology*. **175**:6089-6096.
22. **Bouhnik, Y., B. Flourie, C. Andrieux, N. Bisetti, F. Briet, J. C. Rambaud.** 1996. Effects of *Bifidobacterium* sp. fermented milk ingested with or without inulin on colonic bifidobacteria and enzymatic activities in healthy humans. *European Journal of Clinical Nutrition*. **50**:269-273.
23. **Braun, L., S. Dramsi, P. Dehoux, H. Bierne, G. Lindahl, P. Cossart.** 1997. InlB: an invasion protein of *Listeria monocytogenes* with a novel type of surface association. *Molecular Microbiology*. **25**:285-294.
24. **Bukowska, H., J. Pieczul-Mroz, M. Jastrzebska, K. Chelstowski, M. Naruszewicz.** 1998. Decrease in fibrinogen and LDL-cholesterol levels upon supplementation of diet with *Lactobacillus plantarum* in subjects with moderately elevated cholesterol. *Atherosclerosis*. **137**:437-438.
25. **Buydens, P., S. Debeucklaere.** 1996. Efficacy of SF68 in the treatment of acute diarrhoea: a placebo controlled trial. *Scandinavian Journal of Gastroenterology*. **31**:887-891.
26. **Callegari, M. L., B. Riboli, J. W. Sanders, P. S. Cocconcelli, J. Kok, G. Venema, L. Morelli.** 1998. The S-layer gene of *Lactobacillus helveticus* CNRZ 892: cloning, sequence and heterologous expression. *Microbiology*. **144**:719-726.
27. **Canducci, F., A. Armuzzi, F. Cremonini, G. Cammarota, F. Bartolozzi, P. Pola, G. Gasbarrini, A. Gasbarrini.** 2000. A lyophilised and inactivated

- culture of *Lactobacillus acidophilus* increases *Helicobacter pylori* eradication rates. *Alimentary Pharmacology and Therapeutics*. **14**:1625-1629.
28. **Cataloluk, O., B. Gogebakan.** 2004. Presence of drug resistance in intestinal lactobacilli of dairy and human origin. *FEMS Microbiology Letters*. **236**:7-12.
 29. **Cato, E. P., W. E. C. Moore, J. L. Johnson.** 1983. Synonymy of strains of "*Lactobacillus acidophilus*" group A2 (Johnson *et al.*, 1980) with the type strain of *Lactobacillus crispatus* (Brygoo and Aladame 1953) Moore and Holdeman 1970. *International Journal of Systematic Bacteriology*. **33**:426-428.
 30. **Chang, M. H., T. C. Chen.** 2000. Reduction of *Campylobacter jejuni* in a simulated chicken digestive tract by lactobacilli cultures. *Journal of Food Protection*. **63**:1594-1597.
 31. **Chang, S. K., A. F. Dohrman, C. B. Basbaum, S. B. Ho, T. Tsuda, N. W. Toribara, J. R. Gum, Y. S. Kim.** 1994. Localisation of mucin (MUC2 and MUC3) messenger RNA and peptide expression in human normal intestine and colon cancer. *Gastroenterology*. **107**:28-36.
 32. **Charteris, W. P., P. M. Kelly, L. Morelli, J. K. Collins.** 1998. Ingredient selection criteria for probiotic microorganisms in functional dairy foods. *International Journal of Dairy Technology*. **51**:123-136.
 33. **Chavier, G., M. H. Coconnier, S. Kerneis, A. Derfeuille-Michaud, B. Joly, A. L. Servin.** 1992. Competitive exclusion of diarrheagenic *Escherichia coli* (ETEC) from human enterocyte-like Caco-2 cells by heat-killed *Lactobacillus*. *FEMS Microbiology Letters*. **91**:213-218.
 34. **Chen, X., J. Xu, J. Shuai, J. Chen, Z. Zhang, W. Fang.** 2007. The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* 0157:H7 and *Salmonella typhimurium*. *International Journal of Food Microbiology*. **115**:307-312.
 35. **Chhatwal, G. S.** 2002. Anchorless adhesins and invasins of Gram-positive bacteria: a new class of virulence factors. *Trends in Microbiology*. **10**:205-208.
 36. **Christiaens, H., R. J. Leer, P. H. Pouwels, W. Verstraete.** 1992. Cloning and expression of a conjugated bile acid hydrolase gene from *Lactobacillus plantarum* by using a direct plate assay. *Applied and Environmental Microbiology*. **58**:3792-3798.

37. **Cocolin, L., M. Manzano, C. Cantoni, G. Comi.** 2000. Development of a rapid method for the identification of *Lactobacillus* spp. isolated from naturally fermented Italian sausages using a polymerase chain reaction-temperature gradient gel electrophoresis. *Letters in Applied Microbiology*. **30**:126-129.
38. **Coconnier, M. H., T. R. Klaenhammer, S. Kernies, M. F. Bernet, A. L. Servin.** 1992. Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell lines in culture. *Applied and Environmental Microbiology*. **58**:2034-2039.
39. **Cole, C. B., R. Fuller.** 1984. A note of the effect of host specific fermented milk on the coliform population of the neonatal rat gut. *Journal of Applied Bacteriology*. **56**:495-498.
40. **Colombel, J. F., A. Cortot, C. Neut, C. Romond.** 1987. Yoghurt with *Bifidobacterium longum* reduces erythromycin induced gastrointestinal effects. *Lancet*. **2**:43.
41. **Conway, P. L., S. L. Gorbach, B. R. Goldin.** 1987. Survival of lactic acid bacteria in the human stomach and adhesion to intestinal cells. *Journal of Dairy Science*. **70**:1-12.
42. **Cunningham-Rundles, S., S. Ahrne, S. Bengmark, R. Johann-Liang, F. Marshall, L. Metakis, C. Califano, A. Dunn, C. Grasse, G. Hinds, J. Cervia.** 2000. Probiotics and immune response. *The American Journal of Gastroenterology*. **95**:22-25.
43. **De Vries, M. C., E. E. Vaughan, M. Kleerebezem, W. M. De Vos.** 2005. *Lactobacillus plantarum* - survival, functional and potential probiotic properties in the human intestinal tract. *International Dairy Journal*. **16**:1018-1029.
44. **Desvaux, M., E. Dumas, I. Chafsey, M. Hebraud.** 2006. Protein cell surface display in Gram-positive bacteria: from single protein to macromolecular protein structure. *FEMS Microbiology Letters*. **256**:1-15.
45. **Diep, D. B., L. S. Havarstein, J. Nissen-Meyer, I. F. Nes.** 1994. The gene encoding plantaricin A, a bacteriocin from *Lactobacillus plantarum* C11, is located on the same transcription unit as an agr-like regulatory system. *Applied and Environmental Microbiology*. **60**:160-166.

46. **Duguid, J. P., D. C. Old.** 1980. Adhesive properties of *Enterobacteriaceae*, p. 187-217. In E. H. Beachey (ed.), Bacterial adherence (receptors and recognition, series B). vol. 6. Chapman & Hall, Ltd., London.
47. **Dunne, C., L. O. Mahony, L. Murphy, G. Thornton, D. Morrissey, S. O'Halloran, M. Feeny, S. Flynn, G. Fitzgerald, C. Daly, B. Kiely, G. C. O'Sullivan, F. Shanahan, J. K. Collins.** 2001. In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. American Journal of Clinical Nutrition. **73**:386-392.
48. **Dunny, G. M.** 1990. Genetic functions and cell-cell interactions in the pheromone-inducible plasmid transfer system of *Enterococcus faecalis*. Plasmid. **4**:689-696.
49. **Ercolini, D., P. J. Hill, C. E. R. Dodd.** 2003. Bacterial community structure and location in Stilton cheese. Applied and Environmental Microbiology. **69**:3540-3548.
50. **Ferreira, A. E., N. Canal, D. Morales, D. B. Fuentefria, G. Corcao.** 2007. Characterisation of enterocins produced by *Enterococcus mundtii* from human faeces. Brazilian Archives of Biology and Technology. **50**:249-258.
51. **Fioramonti, J., V. Theodorou, L. Beuno.** 2003. Probiotics: what are they? What are their effects on gut physiology. Best Practice and Research Clinical Gastroenterology. **17**:711-724.
52. **Floch, M. H., H. J. Binder, B. Filburn, W. Gershengoren.** 1972. The effect of bile acids on intestinal microflora. American Journal of Clinical Nutrition. **25**:1418-1426.
53. **Foo, H. L., T. C. Loh, P. W. Lai, Y. Z. Lim, C. N. Kufli, G. Rusul.** 2003. Effects of adding *Lactobacillus plantarum* I-UL4 metabolites in drinking water of rats. Pakistan Journal of Nutrition. **2**:283-288.
54. **Forstner, J. F., G. G. Forstner.** 1994. Gastrointestinal mucus, p. 1255-1283. In R. L. Johnson (ed.), Physiology of the gastrointestinal tract. Raven, New York.
55. **Franz, C. M. A. P., M. E. Stiles, K. H. Schleifer, W. H. Holzapfel.** 2003. Enterococci in foods - a conundrum for food safety. International Journal of Food Microbiology. **88**:105-122.
56. **Franz, C. M. A. P., U. Schillinger, W. H. Holzapfel.** 1996. Production and characterisation of enterocin 900, a bacteriocin produced by *Enterococcus*

- faecium* BFE 900 from black olives. International Journal of Food Microbiology. **29**:255-270.
57. **Franz, C. M. A. P., W. H. Holzapfel, M. E. Stiles.** 1999. Enterococci at the crossroads of food safety? International Journal of Food Microbiology. **47**:1-24.
58. **Fricourt, B. V., S. F. Barefoot, R. F. Testin, S. S. Hayasaka.** 1994. Detection and activity of plantaricin F, an antibacterial substance from *Lactobacillus plantarum* BF001 isolated from processed channel catfish. Journal of Food Protection. **57**:698-702.
59. **Fujisawa, T., Y. Benno, T. Yaeshima, T. Mitsuoka.** 1992. Taxonomic study of the *Lactobacillus acidophilus* Group, with recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonymy of *Lactobacillus acidophilus* group A3 (Johnson *et al.*, 1980) with the type strain of *Lactobacillus amylovorus* (Nakamura 1981). International Journal of Systematic Bacteriology. **42**:487-491.
60. **Fukushima, Y., Y. Kawata, K. Mizumachi, J. Kurisaki, T. Mitsuoka.** 1999. Effect of Bifidobacteria feeding on faecal flora and production of immunoglobulins in lactating mouse. International Journal of Food Microbiology. **46**:193-197.
61. **Fuller, R.** 1989. Probiotics in man and animals. Journal of Applied Bacteriology. **66**:365-378.
62. **Gallagher, C. R., A. L. Moleson, J. H. Caldwell.** 1974. Lactose intolerance and fermented dairy products. Journal of American Diet Association. **65**:418-419.
63. **Galvez, A., G. Gimenez-Gallego, M. Maqueda, E. Valdivia.** 1989. Purification and amino acid composition of peptide antibiotic AS-48 produced by *Streptococcus (Enterococcus) faecalis* subsp. *liquefaciens* S-48. Antimicrobial Agents and Chemotherapy. **33**:437-441.
64. **Garrote, G. L., L. Delfederico, R. Bibiloni, A. G. Abraham, P. F. Perez, L. Semorile, G. L. De Antoni.** 2004. Lactobacilli isolated from kefir: evidence of the presence of S-layer proteins. Journal of Dairy Research. **71**:222-230.
65. **Gevers, D., M. Danielsen, G. Huys, J. Swings.** 2003. Molecular characterisation of *tet(M)* genes in *Lactobacillus* isolates from different types

- of fermented dry sausage. *Applied and Environmental Microbiology*. **69**:1270-1275.
66. **Gilliland, S. E., C. R. Nelson, C. Maxwell.** 1985. Assimilation of cholesterol by *Lactobacillus acidophilus*. *Applied and Environmental Microbiology*. **49**:377-381.
67. **Gilliland, S. E., M. L. Speck.** 1977. Deconjugation of bile acids by intestinal lactobacilli. *Applied and Environmental Microbiology*. **33**:15-18.
68. **Giraffa, G.** 1995. Enterococcal bacteriocins: Their potential as anti-Listeria factors in dairy technology. *Food Microbiology*. **12**:291-299.
69. **Glass, R. I., J. F. Lew, R. E. Gangarosa, C. W. LeBaron, M. S. Ho.** 1991. Estimates of morbidity and mortality rates for diarrhoeal diseases in American children. *Journal of Pediatrics*. **118**:27-33.
70. **Goldin, B. R., S. L. Gorbach.** 1987. "*Lactobacillus* GG: a new strain with properties favourable for survival, adhesion and antimicrobial activity in the gastrointestinal tract". *FEMS Microbiology Review*. **46**:72.
71. **Goldin, B. R., S. L. Gorbach.** 1984. The effect of milk and lactobacillus feeding on human intestinal bacterial enzyme activity. *American Journal of Clinical Nutrition*. **39**:756-761.
72. **Goldin, B. R., S. L. Gorbach.** 1992. Probiotics for humans, p. 355-376. *In* R. Fuller (ed.), *Probiotics, the scientific basis*. Chapman and Hall, London.
73. **Goldin, B. R., S. L. Gorbach, M. Saxelin, S. Bakarar, L. Gualtieri, S. Salminen.** 1992. "Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract". *Digestive Diseases and Sciences*. **37**:121-128.
74. **Gonzalez, B., P. Arca, B. Mayo, J. E. Suarez.** 1994. Detection, purification and partial characterisation of plantaricin C, a bacteriocin produced by a *Lactobacillus plantarum* strain of dairy origin. *Applied and Environmental Microbiology*. **60**:2158-2163.
75. **Gonzalez, S. N., M. C. Appela, N. Romero, A. A. Pesce de Ruiz Holgado, G. Oliver.** 1989. Superoxide dismutase activity in some strains of lactobacilli: Induction by manganese. *Chemical Pharmaceutical Bulletin*. **37**:3026-3028.
76. **Gorbach, S. L.** 2000. Probiotics and gastrointestinal health. *American Journal of Gastroenterology*. **95**:S2-S4.
77. **Gorbach, S. L., T. Chang, B. Goldin.** 1978. Successful treatment of relapsing *Clostridium difficile* colitis with *Lactobacillus* GG. *Lancet*. **ii**:1519.

78. **Graf, W.** 1983. Studies on the therapeutic properties of acidophilus milk. Symposia of Swedish Nutrition Foundation. **XV**.
79. **Granato, D., F. Perotti, I. Masserey, M. Rouvet, M. Golliard, A. Servin, D. Brassart.** 1999. Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. Applied and Environmental Microbiology. **65**:1071-1077.
80. **Granato, D., G. E. Bergonzelli, R. D. Pridmore, L. Marvin, M. Rouvet, I. E. Corthesy-Theulaz.** 2004. Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533(La1) to human intestinal cells and mucins. Infection and Immunity. **72**:2160-2169.
81. **Greene, J. D., T. R. Klaenhammer.** 1994. Factors involved in adherence of lactobacilli to human Caco-2 cells. Applied and Environmental Microbiology. **60**:4487-4494.
82. **Grunewald, K. K.** 1982. Serum cholesterol levels in rats fed skim milk fermented by *Lactobacillus acidophilus*. Journal of Food Science. **47**:2078-2079.
83. **Guandalini, S., L. Pensabene, M. A. Zikri, J. A. Dias, L. G. Casali, H. Hoekstra, S. Kolacek, K. Massar, D. Micelic-Turk, A. Papadopoulou, J. S. de Sousa, B. Sandhu, H. Szajewska, Z. Wiezman.** 2000. *Lactobacillus* GG administered in oral rehydration solution to children with acute diarrhoea: a multicentre European trial. Journal of Pediatric Gastroenterology and Nutrition. **30**:54-60.
84. **Guarner, F., G. J. Schaafsma.** 1998. Probiotics. International Journal Food Microbiology. **39**:237-238.
85. **Hasty, D. L., I. Ofek, H. S. Courtney, R. J. Doyle.** 1992. Multiple Adhesins of Streptococci. Infection and Immunity. **60**:2147-2152.
86. **Hata, Y., M. Yamamoto, M. Ohni, K. Nakajima, Y. Nakamura, T. Takano.** 1996. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. American Journal of Clinical Nutrition. **64**:767-771.
87. **Herias, M. V., C. Hessle, E. Telemo, T. Midtvedt, L. A. Hanson, A. E. Wold.** 1999. Immunomodulatory effects of *Lactobacillus plantarum* colonising the intestine of gnotobiotic rats. Clinical Experimental Immunology. **116**:283-290.

88. **Hill, D. R.** 2000. Occurrence and self treatment of diarrhoea in a large cohort of Americans travelling to developing countries. *American Journal of Tropical Medicinal Hygiene*. **62**:585-589.
89. **Hill, M. J., B. S. Draser.** 1968. Degradation of bile salts by human intestinal bacteria. *Gut*. **9**:22-27.
90. **Hoepelman, A. I. M., E. I. Tuomanen.** 1992. Consequences of microbial attachment: directing host cell functions with adhesins. *Infection and Immunity*. **60**:1729-1733.
91. **Hoffman, A. F., G. Molino, M. Milanese, G. Belforte.** 1983. Description and stimulation of a physiological pharmacokinetic model for the metabolism and enterohepatic circulation of bile acids in man. *Journal of Clinical Investigation*. **71**:1003-1022.
92. **Hohenberger, P., S. Gretschel.** 2003. Gastric cancer. *Lancet*. **362**:305-315.
93. **Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, J. I. Gordon.** 2001. Molecular analysis of commensal host-microbial relationships in the intestine. *Science*. **291**:881-884.
94. **Hsu, C. A., R. C. Yu, C. C. Chou.** 2005. Production of galactosidase by bifidobacteria as influenced by various cultures conditions. *International Journal Food Microbiology*. **104**:197-206.
95. **Huang, Y., L. Kotula, M. C. Adams.** 2003. The in vivo assessment of safety and gastrointestinal survival of an orally administered novel probiotic, *Propionibacterium jensenii* 702, in a male Wistar rat model. *Food and Chemical Toxicology*. **41**:1781-1787.
96. **Hughes, D. B., D. G. Hoover.** 1991. Bifidobacteria: their potential for use in American dairy products. *Food Technology*. **4**:74-83.
97. **Hynonen, U., B. Westerlund-Wikstrom, A. Palva, T. K. Korhonen.** 2002. Fibronectin-binding function in the SfpA surface protein of *Lactobacillus brevis*. *Journal of Bacteriology*. **184**:3360-3367.
98. **Isenberg, D.** 1977. *Lactobacillus* infective endocarditis. *Proceedings of the Royal Society of Medicine*. **70**:278-281.
99. **Isolauri, E.** 2003. Probiotics for infectious diarrhoea. *Gut*. **52**:436-437.
100. **Isolauri, E., M. Juntunen, T. Rautanen, P. Sillanaukee, T. Koivula.** 1991. A human *Lactobacillus* strain (*Lactobacillus casei* sp. strain GG) promotes recovery from acute diarrhoea in children. *Pediatrics*. **88**:90-97.

101. **Isolauri, E., P. V. Kirjavainen, S. Salminen.** 2002. Probiotics: a role in the treatment of intestinal infection and inflammation. *Gut*. **50**:54-59.
102. **Jankovic, I., M. Ventura, V. Meylan, M. Rouvet, M. Elli, R. Zink.** 2003. Contribution of aggregating-promoting factor to maintenance of cell shape in *Lactobacillus gasserii* 4B2. *Journal of Bacteriology*. **185**:3288-3296.
103. **Jett, B. D., M. M. Huycke, M. S. Gilmore.** 1994. Virulence of Enterococci. *Clinical Microbiology Reviews*. **7**:462-478.
104. **Jimenez-Diaz, R., R. M. Rios-Sanchez, M. Desmazeaud, J. L. Ruiz-Barba, J. C. Piard.** 1993. Plantaricins S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. *Applied and Environmental Microbiology*. **59**:1416-1424.
105. **Johansson, M. L., G. Molin, B. Jeppsson, S. Nobaek, S. Ahrne, S. Bengmark.** 1993. Administration of different *Lactobacillus* strains in fermented oatmeal soup: In vivo colonisation of human intestinal mucosa and effect on the indigenous flora. *Applied and Environmental Microbiology*. **59**:15-20.
106. **Johnson, J. L., C. F. Phelps, C. S. Cummins, J. London, F. Gasser.** 1980. Taxonomy of the *Lactobacillus acidophilus* group. *International Journal of Systematic Bacteriology*. **30**:53-68.
107. **Jonquieres, R., H. Bierne, F. Fiedler, P. Gounon, P. Cossart.** 1999. Interaction between the protein InlB of *Listeria monocytogenes* and lipoteichoic acid: a novel mechanism of protein association at the surface of Gram-positive bacteria. *Molecular Microbiology*. **34**:902-914.
108. **Jonsson, H., E. Strom, S. Roos.** 2001. Addition of mucin to the growth medium triggers mucus-binding activity in different strains of *Lactobacillus reuteri* in vitro. *FEMS Microbiology Letters*. **204**:19-22.
109. **Kabir, A. M. A., Y. Aiba, A. Takagi, S. Kamiya, T. Miwa, Y. Koga.** 1997. Prevention of *Helicobacter pylori* infection by lactobacilli in a gnotobiotic murine model. *Gut*. **41**:49-55.
110. **Kimura, N., M. Yashikane, A. Kobayashi, T. Mitsuoka.** 1983. An application of dried bifidobacteria preparation to scouring animals. *Bifidobacteria Microflora*. **2**:41-55.

111. **Kingamkono, R., E. Sjogren, U. Svanberg.** 1999. Enteropathogenic bacteria in faecal swabs of young children fed on lactic acid-fermented cereal gruels. *Epidemiology and Infection.* **122**:23-32.
112. **Kirjavainen, P. V., A. C. Ouwehand, E. Isolauri, S. J. Salminen.** 1998. The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiology Letters.* **167**:185-189.
113. **Klaenhammer, T. R.** 2000. Probiotic bacteria: Today and Tomorrow. *Journal of Nutrition.* **130**:415S-416S.
114. **Klarin, B., M. L. Johansson, G. Molin, A. Larsson, B. Jeppsson.** 2005. Adhesion of the probiotic bacterium *Lactobacillus plantarum* 299V onto the gut mucosa in critically ill patients: a randomised open trial. *Critical Care.* **9**:285-293.
115. **Klaver, F. A. M., R. V. Meer.** 1993. The assumed assimilation of cholesterol by lactobacilli and *Bifidobacterium* is due to their bile salt-deconjugating activity. *Applied and Environmental Microbiology.* **59**:1120-1124.
116. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. K. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proceedings of the National Academy of Science of the United States of America.* **100**:1990-1995.
117. **Knoetze, H., S. D. Todorov, L. M. T. Dicks.** 2007. Characterisation of bacteriocin ST4SA, produced by *Enterococcus mundtii* ST4SA isolated from soybeans. Unpublished.
118. **Kohler, E. M., E. M. Bohl.** 1964. Prophylaxis of diarrhoea in newborn pigs. *Journal of the American Veterinary Medical Association.* **144**:1794-1797.
119. **Kreft, B., R. Marre, U. Schramm, R. Wirth.** 1992. Aggregation substance of *Enterococcus faecalis* mediates adhesion to cultured renal tubular cells. *Infection and Immunity.* **60**:25-30.
120. **Lauer, E., O. Kandler.** 1980. *Lactobacillus gasseri* sp. nov., a new species of the subgenus *Thermobacterium*. *Zentralblatt für Bakteriologie Mikrobiologie und Hygiene Series 1 Abstract Original.* 75-78.

121. **Lavermicocca, P., F. Valerio, A. Evidente, S. Lazzaroni, A. Corsetti, M. Gobbetti.** 2000. Purification and characterisation of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. *Applied and Environmental Microbiology*. **66**:4084-4090.
122. **Lee, J., K. T. Hwang, K. Y. Park.** 2004. Adhesion of lactic acid bacteria can modulate the secretion of cytokines on HT-29 colon adenocarcinoma cells. *Journal of Korean Association of Cancer Prevention*. **9**:36-41.
123. **Lehto, E. M., S. Salminen.** 1997. Adhesion of two *Lactobacillus* strains, one *Lactococcus* and one *Propionibacterium* to cultured human intestinal Caco-2 cell line. *Bioscience and Microflora*. **16**:13-17.
124. **Lesuffleur, T., A. Barbat, E. Dussaulx, A. Zweibaum.** 1990. Growth adaptation to methotrexate of HT-29 human colonic carcinoma cells associated with their ability to differentiate into columnar adsorptive and mucus-secreting cells. *Cancer Research*. **50**:6334-6343.
125. **Levitt, M. D., R. M. Donaldson.** 1970. Use of respiratory hydrogen (H₂) excretion to detect carbohydrate malabsorption. *Journal of Clinical Laboratory Medicine*. **75**:937-945.
126. **Lewenstein, A., G. Frigerio, M. Moroni.** 1979. Biological properties of SF68, a new approach for the treatment of diarrhoeal diseases. *Current Therapeutic Research*. **26**:967-981.
127. **Lewis, R., S. Gorbach.** 1972. Modification of bile acids by intestinal bacteria. *Archives of International Medicine*. **130**:545-549.
128. **Lick, S., K. Drescher, K. J. Heller.** 2001. Survival of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* in the terminal ileum of fistulated Gottingen minipigs. *Applied and Environmental Microbiology*. **9**:4137-4143.
129. **Lin, W. H., B. Yu, S. H. Jang, H. Y. Tsen.** 2007. Different probiotic properties of *Lactobacillus fermentum* strains isolated from swine and poultry. *Anaerobe*. **13**:107-113.
130. **Lindberg, A. A., T. Pal.** 1993. Strategies for development of potential candidate Shigella vaccines. *Vaccine*. **11**:168-179.
131. **Litopolou-Tzanetaki, E., N. Tzanetakis, A. Vafopoulou-Mastrojiannaki.** 1993. Effect of type of lactic starter on microbiological, chemical and sensory characteristics of Feta cheese. *Food Microbiology*. **10**:31-41.

132. **Loguercio, C., C. Del Vecchio Blanco, M. Coltorti.** 1987. *Enterococcus* lactic acid bacteria strain SF-68 and lactulose in hepatic encephalopathy: A controlled study. *Journal of International Medical Research.* **15**:335-343.
133. **Lorca, G. L., T. Wadstrom, G. F. Valdez, A. Ljungh.** 2001. *Lactobacillus acidophilus* autolysins inhibit *Helicobacter pylori* in vitro. *Current Microbiology.* **42**:39-44.
134. **Mack, D. R., S. Ahrne, L. Hyde, S. Wei, M. A. Hollingsworth.** 2003. Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells *in vitro*. *Gut.* **52**:827-833.
135. **Mack, D. R., S. Michail, S. Wei, L. McDougall, M. A. Hollingsworth.** 1999. Probiotics inhibit enteropathogenic *E. coli* adherence in vitro by inducing mucin gene expression. *American Journal of Physiology and Gastrointestinal Liver Physiology.* **276**:941-950.
136. **Madsen, K., A. Cornish, P. Soper.** 2001. Probiotic bacteria enhance murine and human intestinal epithelial barrier function. *Gastroenterology.* **121**:580-591.
137. **Malfertheiner, P., F. Megraud, C. O'Morain, A. P. S. Hungin, R. Jones, A. T. R. Axon, D. Y. Graham, G. N. J. Tytgat.** 2000. Current concepts in the management of *Helicobacter pylori* infection-the Maastricht 2-2000 consensus report. *Alimentary Pharmacology and Therapeutics.* **16**:167-180.
138. **Mangell, P., P. Nejdfor, M. Wang, S. Ahrne, B. Westrom, H. Thorlacius, B. Jeppsson.** 2002. *Lactobacillus plantarum* 299V inhibits *Escherichia coli* - induced intestinal permeability. *Digestive Diseases and Sciences.* **47**:511-516.
139. **Marco, M. L., S. Pavan, M. Kleerebezem.** 2006. Towards understanding molecular modes of probiotic action. *Current Opinion in Biotechnology.* **17**:204-210.
140. **Marcon, M. J.** 1997. Probiotics in health and medicine: moving from fashion to scientific validity. *Clinical Microbiology Newsletter.* **19**:89-96.
141. **Mare, L., G. M. Wolfaardt, L. M. T. Dicks.** 2006. Adhesion of *Lactobacillus plantarum* 423 and *Lactobacillus salivarius* 241 to the intestinal tract of piglets, as recorded with fluorescent in situ hybridisation (FISH) and production of plantaricin 423 by cells colonised to the ileum. *Journal of Applied Microbiology.* **100**:838-845.

142. **Martinez-Bueno, M., M. Maqueda, A. Galvez, B. Samyn, J. van Beeumen, J. Coyette.** 1994. Determination of the gene sequence and the molecular structure of the enterococcal peptide antibiotic AS-48. *Journal of Bacteriology*. **176**:6334-6339.
143. **Masuda, K., T. Kawata.** 1983. Distribution and chemical characterisation of regular arrays in the cell walls of strains of the genus *Lactobacillus*. *FEMS Microbiology Letters*. **20**:145-150.
144. **Masuda, K., T. Kawata.** 1981. Characterisation of a regular array in the wall of *Lactobacillus buchneri* and its reattachment to the other wall components. *Journal of General Microbiology*. **124**:81-90.
145. **Matsuzaki, T.** 1998. Immunomodulation by treatment with *Lactobacillus casei* strain shirota. *International Journal of Food Microbiology*. **41**:133-140.
146. **Mattar, A. F., D. H. Teitelbaum, R. A. Drongowski, F. Yongyi, C. M. Harmon, A. G. Coran.** 2002. Probiotics up-regulate MUC2 mucin gene expression in a Caco-2 cell-culture model. *Pediatric Surgery International*. **18**:586-590.
147. **McFarland, L. V.** 2007. Meta-analysis of probiotics for the prevention of traveller's diarrhoea. *Travel Medicine and Infectious Disease*. **5**:97-105.
148. **McFarland, L. V., C. M. Surawicz, R. N. Greenberg, G. W. Elmer, K. A. Moyer, S. A. Melcher, K. E. Bowen, J. L. Cox.** 1995. Prevention of beta-lactam-associated diarrhoea by *Saccharomyces boulardii* compared with placebo. *American Journal of Gastroenterology*. **90**:439-448.
149. **McFarland, L. V., G. W. Elmer, C. M. Surawicz.** 2002. Breaking the cycle: treatment strategies for 163 cases of recurrent *Clostridium difficile* disease. *American Journal of Gastroenterology*. **97**:1769-1775.
150. **McNaught, C. E., N. P. Woodcock, J. MacFie, C. J. Mitchell.** 2002. A prospective randomised study of the probiotic *Lactobacillus plantarum* 299V on indices of gut barrier function in elective surgical patients. *Gut*. **51**:827-831.
151. **Mercenier, A., S. Pavan, B. Pot.** 2003. Probiotics as biotherapeutic agents: present knowledge and future prospects. *Current Pharmaceutical Design*. **9**:175-191.

152. **Miake, S., K. Nomoto, T. Yokokura, M. Mutai, K. Nomoto.** 1985. Protective effect of *Lactobacillus casei* on *Pseudomonas aeruginosa* infection in mice. *Infection and Immunity*. **48**:480-485.
153. **Michetti, P., G. Dorta, P. H. Wiesel, D. Brassart, E. Verdu, M. Herranz, C. Felley, N. Porta, M. Rouvet, A. L. Blum, I. Corthesy-Theulaz.** 1999. Effect of whey-based culture supernatant of *Lactobacillus acidophilus (johnsonii)* LA1 on *Helicobacter pylori* infection in humans. *Digestion*. **60**:203-209.
154. **Midolo, P. D., J. R. Lambert, R. Hull, F. Luo, M. L. Grayson.** 1995. In vitro inhibition of *Helicobacter pylori* NCTC 11637 by organic acids and lactic acid bacteria. *Journal of Applied Bacteriology*. **79**:475-479.
155. **Millsap, K. W., G. Reid, H. C. van der Mei, H. J. Busscher.** 1996. Adhesion of *Lactobacillus* species in urine and phosphate buffer to silicone rubber and glass under flow. *Biomaterials*. **18**:87-91.
156. **Mitsuoka, T.** 1982. Recent trends in research on intestinal flora. *Bifidobacteria Microflora*. **1**:3-24.
157. **Miyoshi, Y., S. Okada, T. Uchimura, E. Satoh.** 2006. A mucus adhesion promoting protein, MapA, mediates the adhesion of *Lactobacillus reuteri* to Caco-2 human intestinal epithelial cells. *Bioscience Biotechnology Biochemistry*. **70**:1622-1628.
158. **Moorthy, G., M. Phil, M. R. Murali, S. N. Devaraj.** 2007. Protective role of lactobacilli in *Shigella dysenteriae* 1-induced diarrhoea in rats. *Nutrition*. **23**:424-433.
159. **Morelli, L.** 2000. *In vitro* selection of probiotic lactobacilli: A critical appraisal. *Current Issues in Intestinal Microbiology*. **1**:59-67.
160. **Morelli, L., C. Cesena, C. de Haen, L. Gozzini.** 1998. Taxonomic *Lactobacillus* composition of faeces from human newborns during the first few days. *Microbial Ecology*. **35**:205-212.
161. **Morrison, D., N. Woodford, B. Cookson.** 1997. Enterococci as emerging pathogens of humans. *Journal of Applied Microbiology Symposium Supply*. **83**:89-99.
162. **Moschl, A., C. Schaffer, U. B. Sleytr, P. Messner, R. Christian, G. Schulz.** 1993. Characterisation of the S-layer glycoproteins of two lactobacilli, p. 281-

284. In T. J. Beveridge, S. F. Koval (ed.), *Advances in bacterial paracrystalline surface layers*. Plenum, New York, USA.
163. **Mukai, T., T. Asasaka, E. Sato, K. Mori, M. Matsumoto, H. Ohoi.** 2002. Inhibition of binding of *Helicobacter pylori* to the glycolipid receptors by probiotic *Lactobacillus reuteri*. *FEMS Immunology and Medical Microbiology*. **32**:105-110.
164. **Mundy, L. M., D. F. Sahn, M. Gilmore.** 2000. Relationships between Enterococcal virulence and antimicrobial resistance. *Clinical Microbiology Reviews*. **13**:513-522.
165. **Muralidhara, K., G. G. Sheggeby, P. R. Elliker, D. C. England, W. E. Sandine.** 1977. Effect of feeding lactobacilli on the coliform and lactobacillus flora of intestinal tissue and faeces from pigs. *Journal of Food Protection*. **40**:288-295.
166. **Murray, B. E.** 1990. The life and times of the Enterococcus. *Microbiology Reviews*. **3**:46-65.
167. **Nakamura, Y., N. Yamamoto, K. Sakai, T. Takano.** 1995. Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin-I- converting enzyme. *Journal of Dairy Science*. **78**:1253-1257.
168. **Navarre, W. W., O. Schneewind.** 1999. Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiology Molecular Biology Reviews*. **63**:174-229.
169. **Nguyen, T. D. T., J. H. Kang, M. S. Lee.** 2007. Characterisation of *Lactobacillus plantarum* PH04, a potential probiotic bacterium with cholesterol-lowering effects. *International Journal of Food Microbiology*. **113**:358-361.
170. **Niedzielin, K., H. Kordecki, B. Birkenfeld.** 2001. A controlled, double-blind, randomized study on the efficacy of *Lactobacillus plantarum* 299V in patients with irritable bowel syndrome. *European Journal of Gastroenterology and Hepatology*. **13**:1143-1147.
171. **Nielsen, E. M., J. Schlundt, A. Gunvig, B. L. Jacobsen.** 1994. Epithelial, mucus and lumen subpopulations of *Escherichia coli* in the large intestine of conventional and gnotobiotic rats. *Microbial Ecology in Health Disease*. **7**:263-273.

172. **Nomoto, K.** 2005. Prevention of infection by probiotics. *Journal of Bioscience and Bioengineering*. **100**:583-592.
173. **Ogawa, M., K. Shimizu, K. Nomoto, M. Takahashi, M. Watanuki, R. Tanaka, T. Tanaka, T. Hamabata, S. Yamasaki, Y. Takeda.** 2001. Protective effect of *Lactobacillus casei* strain Shirota on Shiga toxin-producing *Escherichia coli* 0157:H7 infection in infant rabbits. *Infection and Immunity*. **69**:1101-1108.
174. **Olmsted, S. B., G. M. Dunny, S. L. Erlandsen, C. L. Wells.** 1994. A plasmid-encoded surface protein of *Enterococcus faecalis* augments its internalisation by cultured intestinal epithelial cells. *Journal of Infectious Diseases*. **170**:1549-1556.
175. **Ouwehand, A. C., E. Isolauri, P. V. Kirjavainen, S. J. Salminen.** 1999. Adhesion of four *Bifidobacterium* strains to human intestinal mucus from subjects in different age groups. *FEMS Microbiology Letters*. **172**:61-64.
176. **Ouwehand, A. C., P. V. Kirjavainen, C. Shortt, S. Salminen.** 1999. Probiotics: mechanisms and established effects. *International Dairy Journal*. **9**:43-52.
177. **Ouwehand, A. C., P. V. Kirjavainen, M. M. Gronlund, E. Isolauri, S. J. Salminen.** 1999. Adhesion of probiotic microorganisms to intestinal mucus. *International Dairy Journal*. **9**:623-630.
178. **Ouwehand, A. C., S. J. Salminen, P. J. Roberts, J. Ovaska, E. Salminen.** 2003. Disease dependent adhesion of lactic acid bacteria to the human intestinal mucosa. *Clinical and Diagnostic Laboratory Immunology*. **10**:643-646.
179. **Oyetayo, V. O., B. Osho.** 2004. Assessment of probiotic properties of a strain of *Lactobacillus plantarum* isolated from fermenting corn slurry. *Food, Agriculture and Environment*. **2**:132-134.
180. **Pant, A. R., S. M. Graham, S. J. Allen, S. Harikul, A. Sabchareon, L. Cuevas, C. A. Hart.** 1996. *Lactobacillus* GG and acute diarrhoea in young children in the tropics. *Journal of Tropical Pediatrics*. **42**:16-25.
181. **Park, M. S., K. H. Lee, G. E. Ji.** 1997. Isolation and characterisation of two plasmids from *Bifidobacterium longum*. *Letters in Applied Microbiology*. **25**:5-7.

182. **Paterson, G. K., T. J. Mitchell.** 2004. The biology of Gram-positive sortase enzymes. *Trends in Microbiology*. **12**:89-95.
183. **Pathmakanthan, S., C. K. Li, J. Cowie, C. J. Hawkey.** 2004. *Lactobacillus plantarum* 299V: beneficial in vitro immunomodulation in cells extracted from inflamed human colon. *Journal of Gastroenterology and Hepatology*. **19**:166-173.
184. **Pavan, S., P. Desreumaux, A. Mercenier.** 2003. Use of mouse models to evaluate the persistence, safety and immune modulation capacities of lactic acid bacteria. *Clinical and Diagnostic Laboratory Immunology*. **10**:696-701.
185. **Pool-Zobel, B. L., B. Bertram, M. Knoll, R. Lambertz, C. Neudecker, U. Schillinger, P. Schmezer, W. H. Holzappel.** 1993. Antigenotoxic properties of lactic acid bacteria *in vivo* in the gastrointestinal tract of rats. *Nutrition Cancer*. **20**:271-282.
186. **Popoola, J., A. Swann, G. Warwick.** 2000. *Clostridium difficile* in patients with renal failure - management of an outbreak using biotherapy. *Nephrology Dialysis and Transplantation*. **15**:571-574.
187. **Pothoulakis, C., C. P. Kelly, M. A. Joshi, N. Gao, C. J. O'Keane, I. Castagliuolo, J. T. Lamont.** 1993. *Saccharomyces boulardii* inhibits *Clostridium difficile* toxin A binding and enterotoxicity in rat ileum. *Gastroenterology*. **104**:1108-1115.
188. **Pretzer, G., J. Snel, D. Molenaar, A. Wiersma, P. A. Bron, J. Lambert, W. M. de Vos, R. van der Meer, M. A. Smits, M. Kleerebezem.** 2005. Biodiversity-Based identification and functional characterisation of the mannose specific adhesin of *L. plantarum*. *Journal of Bacteriology*. **187**:6128-6136.
189. **Qamar, A., A. Aboudola, M. Warny, P. Michetti, C. Pothoulakis, J. T. LaMont, C. P. Kelly.** 2001. *Saccharomyces boulardii* stimulates intestinal immunoglobulin A immune response to *Clostridium difficile* toxin A in mice. *Infection and Immunity*. **69**:2762-2765.
190. **Reid, G., J. Burton.** 2002. Use of *Lactobacillus* to prevent infection by pathogenic bacteria. *Microbes Infection*. **4**:319-324.
191. **Rettger, L. F., M. N. Levy, L. Weinstein, J. E. Weiss.** 1935. *Lactobacillus acidophilus* and its therapeutic action., Yale University Press, New Haven, CT.

192. **Rinkinen, M., J. Matto, S. Salminen, E. Westermarck, A. C. Ouwehand.** 2000. *In vitro* adhesion of lactic acid bacteria to canine small intestinal mucus. *Journal of Animal Physiology and Animal Nutrition.* **84**:43-47.
193. **Rojas, M.** 1996. Studies on an adhesion promoting protein from *Lactobacillus* and its role in the colonisation of the gastrointestinal tract. PhD thesis, Goteborg University, Goteborg, Sweden.
194. **Rojas, M., P. L. Conway.** 1996. Colonisation by lactobacilli of piglet small intestinal mucus. *Journal of Applied Bacteriology.* **81**:474-480.
195. **Rolfe, R. D.** 2000. The role of probiotic cultures in the control of gastrointestinal health. *Journal of Nutrition.* **130**:396S-402S.
196. **Roos, S., H. Jonsson.** 2002. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiology.* **148**:433-442.
197. **Rowland, I. R.** 1992. Metabolic interactions in the gut, p. 29-53. *In* R. Fuller (ed.), *Probiotics: the scientific basis.* Chapman and Hall, London.
198. **Rowland, I. R., P. Grasso.** 1975. Degradation of N-nitrosamine by intestinal bacteria. *Applied Microbiology.* **29**:7-12.
199. **Ruiz-Barba, J. L., J. C. Piard, R. Jimenez-Diaz.** 1991. Plasmid profiles and curing of plasmids in *Lactobacillus plantarum* strains isolated from green olive fermentations. *Journal of Applied Bacteriology.* **71**:417-421.
200. **Ruseler-van Embden, J. G., L. M. van Lieshou, M. J. Gosselink, P. Marteau.** 1995. Inability of *Lactobacillus casei* strain GG, *L. acidophilus* and *Bifidobacterium bifidum* to degrade intestinal mucus glycoproteins. *Scandinavian Journal of Gastroenterology.* **30**:675-680.
201. **Ryan, K. J., C. G. Ray.** 2004. *Sherris Medical Microbiology.* McGraw Hill. p. 8385-8529.
202. **Saadia, R., M. Schein, C. MacFarlane, K. D. Boffard.** 1990. gut barrier function and the surgeon. *British Journal of Surgery.* **77**:487-492.
203. **Saavedra, J., N. Bauman, I. Onung, J. Perman, R. Yolken.** 1994. Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. *Lancet.* **47**:709-710.
204. **Salminen, M. K., H. Rautelin, S. Tynkkynen, T. Poussa, M. Saxelin, V. Valtonen, A. Jarvinen.** 2004. *Lactobacillus* bacteremia, clinical significance

- and patient outcome with special focus on probiotic *L. rhamnosus* GG. *Clinical Infectious Disease*. **38**:62-69.
205. **Salminen, M. K., S. Tynkkynen, H. Rautelin, M. Saxelin, M. Vaara, P. Ruutu, S. Sarna, V. Valtonen, A. Jarvinen.** 2002. *Lactobacillus* Bacteremia during a rapid increase in probiotic use of *Lactobacillus rhamnosus* GG in Finland. *Clinical Infectious Diseases*. **35**:1155-1160.
206. **Salminen, S., A. Von Wright.** 1998. Lactic acid bacteria: microbiology and functional aspects, p. 589-601. *In* S. Salminen, A. Von Wright (ed.), *Food science and technology*. Marcel Dekker, Inc., New York.
207. **Sanders, M. E.** 2000. Considerations for use of probiotic bacteria to modulate human health. *Journal of Nutrition*. **130**:384-390.
208. **Sanderson, I. R., W. A. Walker.** 1993. Uptake and transport of macromolecules by the intestine: possible role in clinical disorders (an update). *Gastroenterology*. **104**:622-639.
209. **Sansonetti, P.** 1991. Genetic and molecular basis of epithelial cell invasion by *Shigella* species. *Review of Infectious Diseases*. **13**:285-292.
210. **Sara, M., U. B. Sleytr.** 2000. S-layer proteins. *Journal of Bacteriology*. **182**:859-868.
211. **Satoh, E., R. J. Leer, M. Rojas, P. L. Conway, P. H. Pouwels.** 2000. The gene encoding the adhesion promoting protein MapA from *Lactobacillus reuteri* 104R is part of an operon whose expression is controlled by a mechanism of transcription attenuation, involving cysteine. Unpublished. **Genebank Accession Number AJ 293860**.
212. **Saukkonen, K., W. N. Burnette, V. Mar, H. R. Masure, E. Tuomanen.** 1992. Pertussis toxin has eukaryotic-like carbohydrate recognition domains. *Proceedings of the National Academy of Science of the United States of America*. **89**:118-122.
213. **Saulnier, N., M. A. Zocco, S. D. Caro, G. Gasbarrini, A. Gasbarrini.** 2006. Probiotics and small bowel mucosa: molecular aspects of their interactions. *Genes and Nutrition*. **1**:107-116.
214. **Savage, D. C.** 1992. Growth phase, cellular hydrophobicity and adhesion in vitro of lactobacilli colonising the keratinising gastric epithelium in the mouse. *Applied and Environmental Microbiology*. **58**:1992-1995.

215. **Savijoki, K., H. Ingmer, P. Varmanen.** 2006. Proteolytic systems of lactic acid bacteria. *Applied Microbiology Biotechnology*. **71**:394-406.
216. **Saxelin, M.** 1997. *Lactobacillus* GG - a human probiotic strain with thorough clinical documentation. *Food Reviews International*. **13**:293-313.
217. **Schaffer, C., P. Messner.** 2005. The structure of secondary cell wall polymers: how Gram-positive bacteria stick their cell walls together. *Microbiology*. **151**:643-651.
218. **Schaffer, C., T. Wugeditsch, C. Neuninger, P. Messner.** 1996. Are S-layers glycoproteins and lipopolysaccharides related? *Microbiology Drug Resistance*. **2**:17-23.
219. **Shankar, V., A. Baghdayan, M. Huycke, G. Lindahl, M. Gilmore.** 1999. Infection-derived *Enterococcus faecalis* strains are enriched in *esp*, a gene encoding a novel surface protein. *Infection and Immunity*. **67**:193-200.
220. **Shankar, V., C. V. Lockett, A. S. Baghdayan, C. Drachenberg, M. S. Gilmore, D. E. Johnson.** 2001. Role of *Enterococcus faecalis* surface protein Esp in the pathogenesis of ascending urinary tract infection. *Infection and Immunity*. **69**:4366-4372.
221. **Shinar, E., E. Leitersdorf, R. Yevin.** 1984. *Lactobacillus plantarum* endocarditis. *Journal of Molecular Medicine*. **62**:1173-1174.
222. **Shornikova, A. V., I. A. Casas, E. Isolauri, H. Mykkanen, T. Vesikari.** 1997. *Lactobacillus reuteri* as a therapeutic agent in acute diarrhoea in young children. *Journal of Pediatric Gastroenterology and Nutrition*. **24**:399-404.
223. **Sillanpaa, J., B. Martinez, J. Antikainen, T. Toba, N. Kalkkinen, S. Tankka, K. Lounatmaa, J. Keranen, M. Hook, B. Westerlund-Wikstrom, P. H. Pouwels, T. K. Korhonen.** 2000. Characterisation of the collagen-binding S-layer protein CbsA of *Lactobacillus crispatus*. *Journal of Bacteriology*. **182**:6440-6450.
224. **Sleytr, U. B.** 1997. Basic and applied S-layer research: an overview. *FEMS Microbiology Reviews*. **20**:5-12.
225. **Sleytr, U. B., T. J. Beveridge.** 1999. Bacterial S-layers. *Trends in Microbiology*. **7**:253-260.
226. **Song, Y. L., N. Kato, C. X. Liu, Y. Matsumiya, H. Kato, K. Watanabe.** 2000. Rapid identification of 11 human intestinal *Lactobacillus* species by multiplex PCR assays using group- and species-specific primers derived from

- the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA. *FEMS Microbiology Letters*. **187**:167-173.
227. **Spano, G., G. Chieppa, L. Beneduce, S. Massa.** 2004. Expression analysis of putative *arcA*, *arcB*, and *arcC* genes partially cloned from *Lactobacillus plantarum* isolated from wine. *Journal of Applied Microbiology*. **96**:185-193.
228. **Steen, A., G. Buist, K. J. Leenhouts, M. El Khattabi, F. Grijpstra, A. L. Zomer, G. Venema, O. P. Kuipers, J. Kok.** 2003. Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents. *Journal of Biological Chemistry*. **278**:23874-23881.
229. **Stiles, M. E., W. H. Holzapfel.** 1997. Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology*. **36**:1-29.
230. **Sun, J., G. W. Le, Y. H. Shi, G. W. Su.** 2007. Factors involved in the binding of *Lactobacillus plantarum* Lp6 to rat intestinal mucus. *Letters in Applied Microbiology*. **44**:79-85.
231. **Surawicz, C. M.** 2003. Probiotics, antibiotic-associated diarrhoea and *Clostridium difficile* diarrhoea in humans. *Best Practice and Research Clinical Gastroenterology*. **17**:775-783.
232. **Survarna, V. C., V. U. Bobby.** 2005. Probiotics in human health: A current assessment. *Current Science*. **88**:1744-1748.
233. **Sutcliffe, I. C., D. J. Harrington.** 2002. Pattern searches for the identification of putative lipoprotein genes in Gram-positive bacterial genomes. *Microbiology*. **148**:2065-2077.
234. **Sykora, J., K. Valeckova, J. Amlerova, K. Siala, P. Dedek, S. Watkins, J. Varvarovska, F. Stozicky, P. Pazdiora, J. Schwarz.** 2005. Effects of a specially designed fermented milk product containing probiotic *Lactobacillus casei* DN-114 001 and the eradication of *H. pylori* in children: a prospective randomised double-blind study. *Journal of Clinical Gastroenterology*. **39**:692-698.
235. **Takano, T.** 1998. Milk derived peptides and hypertension reduction. *International Dairy Journal*. **8**:375-381.
236. **Tallon, R., S. Arias, P. Bressollier, M. C. Urdaci.** 2007. Strain and matrix dependent adhesion of *L. plantarum* is mediated by proteinaceous bacterial compounds. *Journal of Applied Microbiology*. **102**:442-451.

237. **Taranto, M. P., F. Sesma, A. P. R. Holdago, G. F. Valdez.** 1997. Bile salts hydrolase plays a key role on cholesterol removal by *Lactobacillus casei*. *Biotechnology Letters*. **19**:845-847.
238. **Tasteyre, A., M. C. Barc, T. Karjalainen, P. Bourlioux, A. Collignon.** 2002. Inhibition of in vitro cell adherence of *Clostridium difficile* by *Saccharomyces boulardii*. *Microbial Pathogens*. **32**:219-225.
239. **Ten Brink, B., M. Minekus, J. Bol, J. H. J. Huis in t'Veld.** 1987. Production of antimicrobial compounds by lactobacilli. *FEMS Microbiology Reviews*. **46**:64.
240. **Thornton, G. M.** 1996. Probiotic bacteria. Selection of *Lactobacillus* and *Bifidobacterium* strains from the healthy human gastrointestinal tract; characterisation of a novel *Lactobacillus*-derived antibacterial protein. PhD thesis. National University of Ireland, Cork, Ireland.
241. **Todorov, S. T., L. M. T. Dicks.** 2005. *Lactobacillus plantarum* isolated from molasses produces bacteriocins active against Gram-negative bacteria. *Enzyme and Microbial Technology*. **36**:318-326.
242. **Tojo, M., T. Oikawa, Y. Morikawa, N. Yamashita, S. Iwata, Y. Satoh, J. Hanada, R. Tanaka.** 1987. The effects of *Bifidobacterium breve* administration on *Campylobacter* enteritis. *Acta Pediatric Japan*. **29**:160-167.
243. **Toledo-Arana, A., J. Valle, C. Solano, M. L. Arrizubieta, C. Cacarella, M. Lamata, B. Amorena, J. Leiva, J. R. Penades, I. Lasa.** 2001. The enterococcal surface protein, Esp, is involved in *Enterococcus faecalis* biofilm formation. *Applied and Environmental Microbiology*. **67**:4538-4545.
244. **Ton-That, H., L. A. Marraffini, O. Schneewind.** 2004. Protein sorting to the cell wall envelope of Gram-positive bacteria. *Biochimica et Biophysica Acta*. **1694**:269-278.
245. **Tuomola, E. M., A. C. Ouwehand, S. J. Salminen.** 2000. Chemical, physical and enzymatic pre-treatments of probiotic lactobacilli alter their adhesion to human intestinal mucus glycoproteins. *International Journal of Food Microbiology*. **60**:75-81.
246. **Tuomola, E. M., S. J. Salminen.** 1998. Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. *International Journal of Food Microbiology*. **41**:45-51.

247. **Underdahl, N. R., A. Torres-Medina, A. R. Doster.** 1982. Effect of *Streptococcus faecium* C63 in control of *Escherichia coli* induced diarrhoea in gnotobiotic pigs. *American Journal of Veterinary Research.* **43**:2227-2232.
248. **Van Hijum, S. A., G. H. van Geel-Schutten, H. Rahaoui, M. J. van der Maarel, L. Dijkhuizen.** 2002. Characterisation of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high-molecular-weight inulin and inulin oligosaccharides. *Applied and Environmental Microbiology.* **68**:4390-4398.
249. **Van Reenen, C. A., L. M. T. Dicks, M. L. Chikindas.** 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology.* **84**:1131-1137.
250. **Vaughan, E. E., M. C. De Vries, E. G. Zoetendal, K. Ben-Amor, A. D. L. Akkermans, W. M. De Vos.** 2002. The intestinal LABs. *Antonie van Leeuwenhoek.* **82**:341-352.
251. **Ventura, M., I. Jankovic, D. C. Walker, R. D. Pridmore, R. Zink.** 2002. Identification and characterisation of novel surface proteins in *Lactobacillus johnsonii* and *Lactobacillus gasseri*. *Applied and Environmental Microbiology.* **68**:6172-6181.
252. **Vidgren, G., I. Palva, R. Pakkanen, K. Lounatmaa, A. Palva.** 1992. S-layer protein gene of *Lactobacillus brevis*: cloning by polymerase chain reaction and determination of the nucleotide sequence. *Journal of Bacteriology.* **174**:7419-7427.
253. **West, C. A., P. J. Warner.** 1988. Plantaricin B, a bacteriocin produced by *Lactobacillus plantarum* NCDO 1193. *FEMS Microbiology Letters.* **49**:163-165.
254. **Wullt, M., M. J. Hagslatt, I. Odenholt.** 2003. *Lactobacillus plantarum* 299V for the treatment of recurrent *Clostridium difficile*-associated diarrhoea: A double blind, placebo-controlled trial. *Scandinavian Journal of Infectious Diseases.* **35**:365-367.
255. **Yates, J.** 2005. Traveller's diarrhoea. *American Family Physician.* **71**:2095-2100.

256. **Yokokura, T., K. Nomoto, T. Shimizu, K. Nomoto.** 1986. Enhancement of hematopoietic response of mice by subcutaneous administration of *Lactobacillus casei*. *Infection and Immunity*. **52**:156-160.
257. **Zhou, J. S., P. K. Gopal, H. S. Gill.** 2001. Potential probiotic lactic acid bacteria *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017) and *Bifidobacterium lactis* (HN019) do not degrade gastric mucin in vitro. *International Journal of Food Microbiology*. **63**:81-90.
258. **Zhou, J. S., Q. Shu, K. J. Rutherford, J. Prasad, P. K. Gopal, H. S. Gill.** 2000. Acute oral toxicity and bacterial translocation studies on potentially probiotic strains of lactic acid bacteria. *Food and Chemical Toxicology*. **38**:153-161.

Part of the research in Chapter 3 has been published in the International Journal of Food Microbiology (Addendum). Chapters 3, 4 and 5 are written according to the style of Applied and Environmental Microbiology.

Chapter 3

Expression of mucus adhesion genes of *Lactobacillus plantarum* 423 and *Lactobacillus acidophilus* ATCC 4356 under digestive stress conditions *in vitro*, as monitored with real time PCR

Abstract

Expression of the mucus adhesion genes *mub* and *mapA*, adhesion-like factor *EF-Tu* and bacteriocin gene *plaA* by *Lactobacillus plantarum* 423, and expression of *mub*, *slp* and *EF-Tu* by *Lactobacillus acidophilus* ATCC 4356, grown in the presence of mucin, bile, pancreatin and at low pH, was studied by real time PCR. *mub*, *mapA* and *EF-Tu* of strain 423 were up-regulated in the presence of mucus, proportional to increasing concentrations. Expression of *mapA* by strain 423 was up-regulated in the presence of 0.3% (w/v) bile and 0.3% (w/v) pancreatin at pH 6.5. Similar results were recorded in the presence of 1.0% (w/v) bile and 1.0% (w/v) pancreatin at pH 6.5. Expression of *mub*, on the other hand, was down-regulated in the presence of bile and pancreatin at pH 6.5, whilst the expression of *EF-Tu* and *plaA* remained unchanged under these conditions at pH 6.5. Expression of *mapA* and *mub* by strain 423 remained unchanged at pH 4.0, whilst expression of *EF-Tu* and *plaA* were up-regulated. Expression of *mapA* was down-regulated in the presence of 0.1% (w/v) cysteine, suggesting that the gene is regulated by a mechanism of transcription attenuation that involves cysteine. In the case of strain ATCC 4356, none of the genes were up-regulated under increasing concentrations of mucin, whilst *slp* and *EF-Tu* were up-regulated under normal and stress conditions. Expression of *mub* was not affected under normal conditions but was down-regulated under stress conditions.

Introduction

Lactic acid bacteria are regarded probiotic if they successfully compete with intestinal pathogens and restore the normal microbiota. One of the first biological barriers encountered by probiotic bacteria is low pH in the stomach. Those cells that survive such extreme conditions are exposed to further stress in the small intestine, inflicted by bile salts and pancreatic juice (7).

Probiotic bacteria compete with intestinal microorganisms for adhesion to mucus and epithelial cells by competition for receptor sites. The production of antimicrobial agents such as organic acids, diacetyl, hydrogen peroxide and bacteriocins aid in the survivability of these microorganism in the digestive tract (19, 20, 29). A few strains produce specific adhesion proteins. The best studied examples are the mucus binding protein (Mub) and the mucus adhesion promoting protein (MapA) produced by *Lactobacillus reuteri* (28, 31). Five proteins homologous to the Mub protein of *L. reuteri* 104R have been described for *Lactobacillus acidophilus* NCFM (3). Mub homologues have also been reported for *Lactobacillus gasseri*, *Lactobacillus johnsonii* and *Lactobacillus plantarum* (3).

An adhesion-like protein, classified as elongation factor Tu (EF-Tu), was isolated from *Lactobacillus johnsonii* NCC 533 (La1) (15). EF-Tu facilitates the transfer of aminoacyl-tRNA to the A-site of ribosomes during protein synthesis (12). However, the EF-Tu protein also binds to mucin and mediates the colonisation of strain NCC 533 to human intestinal cells and mucus (15). The mechanism by which EF-Tu interacts with mucin is not well understood. An EF-Tu protein isolated from *L. plantarum* shared 84% homology with the EF-Tu protein of *L. johnsonii* NCC 533 (15).

Surface layers (S-layers) are crystalline structures composed of protein or glycoprotein subunits with molecular masses ranging from 25 to 71 kDa for *Lactobacillus* spp. (30). These S-layer proteins (Slp) consist of a high content of hydrophobic amino acid residues with values ranging from 31.9% to 38.7% (32). A general functional property for all S-layer proteins has not been determined. Several functions have been postulated, such as protection against harsh environmental conditions (21), phage receptor sites (16) and adhesion to intestinal cells (8). The Slp of *L. acidophilus* ATCC 4356 has been characterized and expressed in *Escherichia coli* (6).

Lactobacillus plantarum and *Lactobacillus acidophilus* are common inhabitants of the human gastrointestinal tract and are also incorporated as starter cultures in the production of several types of fermented foods (2, 13). Strain 423, isolated from sorghum beer produces a bacteriocin, plantaricin 423, resistant to treatment at 80°C and incubation in MRS broth at pH 1.0 to 10.0 (34). The genes encoding plantaricin, a typical class IIa peptide, have been sequenced (35). The peptide is active against food-borne pathogens, including *Bacillus cereus*, *Clostridium sporogenes*, *Enterococcus faecalis*, *Listeria* spp. and *Staphylococcus* spp. (34).

Previous studies on adhesion of probiotic bacteria have focussed mainly on phenotypic aspects, such as *in vitro* models with mucus or epithelial cells (4, 24, 33). No previous investigation has attempted to explore the expression of adhesion genes when cells are exposed to stress conditions simulating the gastrointestinal tract. The present study investigated the expression of *mub*, *mapA*, *EF-Tu* and the structural gene of plantaricin 423 (*plaA*) of *L. plantarum* 423, as well as the expression of *mub*, *slp* and *EF-Tu* of *L. acidophilus* ATCC 4356 when cultured in the presence of bile salts, pancreatic juice and low pH. Up-regulation of the genes when strains were cultured under varying conditions of mucin, was also examined. Gene expression was monitored with real time PCR.

Materials and Methods

Bacterial strains and culture conditions

Lactobacillus plantarum 423 and *Lactobacillus acidophilus* ATCC 4356 were cultured in De Man Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37°C, without aeration. Gene expression studies were conducted in MRS broth (Biolab) supplemented with porcine mucin (Sigma, Mannheim, Germany), bile and pancreatin, to simulate normal and stressful gut conditions (Table 1). The effect of cysteine on the expression of *mapA* was examined in MRS broth (Biolab), supplemented with 1.0 g/l L-cysteine HCl.

Acidocin production

L. acidophilus ATCC 4356 was cultured in MRS broth (Biolab) for 18 h (OD_{600} = approximately 4.0). The culture was centrifuged at $10\,000 \times g$ for 10 min and the supernatant collected. The pH of the cell-free culture supernatant was adjusted to 5.5 with 0.5 M HCl and then boiled for 10 min. Production of the bacteriocin, acidocin was tested against *Lactobacillus sakei* LMG 13558 for zones of inhibition on MRS

agar plates, as described by Ivanova *et al.* (17). The bacteriocin was treated with Proteinase K, trypsin, α -amylase and lipase and again tested for activity.

Primer design and PCR

Primers used in this study are listed in Table 2. Primers for the amplification of *plaA* were designed from the structural gene encoding plantaricin 423 (35). All other primer sequences for *L. plantarum* 423 were designed from the genome sequence published for *L. plantarum* WCFS1 (accession number NC 004567). The *mub*, *EF-Tu* and *GDPH* (glyceraldehyde 3-phosphate dehydrogenase gene) primer sequences for *L. acidophilus* ATCC 4356 were designed from the genome sequence published for *L. acidophilus* NCFM (accession number NC 006814). Primers for the Slp of strain ATCC 4356 have been designed from the published sequence (6). Primers were designed with the Primer Designer Program, version 1.01 (Scientific and Educational Software). The GC content of the primers ranged between 30 and 40% and the T_m from 55 to 65°C. Primer sets were designed to produce an amplicon of approximately 150bp. Primer dimer formation was checked by agarose gel electrophoresis (2% agarose) and melting curve analysis. A standard curve consisting of the Log template concentration plotted against the Ct value (crossing points of different standard dilutions), was constructed for each primer set. Efficiencies were calculated by $E = 10^{(-1/S)} - 1$, where S = slope of the standard curve.

RNA isolation and cDNA synthesis

Total RNA was isolated from 500 μ l of 18 h cultures (OD_{600} = approximately 4.0), using the RNeasy Mini Kit (Qiagen, Valencia, California, USA). RNA was stabilised in two volumes of RNAprotect bacteria reagent (Qiagen), prior to isolation. Cells were harvested ($5000 \times g$, 10 min, 4°C) and lysed by incubation with lysozyme (6 μ l of 50 mg/ml lysozyme per 100 μ l TE buffer) for 20 min at 37°C. RNA was resuspended in 40 μ l DEPC-treated distilled water. Residual DNA was removed with turbo DNase treatment, according to instructions from the supplier (Ambion, USA). RNA (0.2 μ g) was transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) and gene specific primers. The reaction occurred for 30 min at 55°C.

Real time PCR

Standard curves were used to determine expression efficiencies between the target and reference genes. The standard curves and their efficiencies were calculated similar to the primer efficiencies. Target concentrations were adjusted by normalising against

the glyceraldehyde 3-phosphate dehydrogenase gene, which served as the endogenous reference, by dividing the average concentration of target DNA by the average concentration of reference DNA. The relative gene expression levels were compared by dividing the normalised target concentration by the control sample (cells grown in MRS, medium 1), and *vice versa*.

Real time PCR reactions were performed in a LightCycler instrument (Roche), with Relative Quantification Software (version 5.32; Roche) and SYBR Green technology (Sigma). Each 20 μ l reaction mixture consisted of 10 μ l SYBR Green Supermix, 1 μ l of each primer at 5 μ M, 7 μ l nuclease-free water and 1 μ l diluted cDNA. Five dilutions of cDNA were prepared. A negative control (no template) was included in each run. Synthesis of cDNA and real time PCR was performed in triplicate for each gene. The PCR conditions were as follows: Initial denaturation at 95°C for 15 min, followed by 40 cycles of amplification at 50°C for 10 sec and 72°C for 15 sec. At the end of each run a melting curve was performed from 95°C to 45°C (0.2°C.s⁻¹).

Results

All primers and standard curves displayed an efficiency of above 80% (Figs. 1, 2, 3 and 4). A single product-specific melting curve was obtained for each primer set, indicating that primers were designed with optimal efficiency and were effective in targeting and amplifying only the genes of interest. Both strains survived exposure to 0.5 M HCl at pH 2.0 which reveals that the strains are capable of surviving gastric transit. *L. acidophilus* 4356 produces the bacteriocin acidocin that is active against *L. sakei*. The bacteriocin was completely inactivated by treatment with Proteinase K, trypsin and α -amylase, indicating its glyco-proteinaceous nature.

Expression of *mub*

Genes are considered to be up- or down-regulated if their relative expression levels are at least two-fold higher or lower respectively, when compared to the control sample or the calibrator (9). The *mub* gene of *L. plantarum* 423 was expressed 80-fold more when grown in the presence of 0.01% (w/v) mucin and 144-fold more when grown in the presence of 0.05% (w/v) mucin. However, the gene was suppressed when cells were grown under normal gut conditions, i.e. 0.3% (w/v) bile, 0.3% (w/v) pancreatin, pH 6.5, and conditions simulating stress (1%, w/v, bile; 1%, w/v,

pancreatin; pH 6.5). Expression was down-regulated 7-fold under normal intestinal conditions and 34-fold under stressful conditions (Table 3). Expression of *mub* remained unchanged at pH 4.0 (results not shown).

In the case of *L. acidophilus* ATCC 4356, the *mub* gene was not up-regulated in the presence of 0.01% or 0.05% (w/v) mucin. Expression of the gene did not seem to be altered under normal gut conditions (0.3%, w/v, bile; 0.3%, w/v, pancreatin; pH 6.5). However, the gene was down-regulated 18 fold when cells were cultured under stressful gut conditions (Table 4).

Expression of *EF-Tu*

The *EF-Tu* gene of *L. plantarum* 423 was significantly induced when cells were grown in medium supplemented with mucin. The gene was expressed 33-fold higher in the presence of 0.01% (w/v) mucin and 105-fold higher in the presence of 0.05% (w/v) mucin (Table 3). Expression of the gene was not affected by varying concentrations of bile and pancreatin. Cells grown at pH 4.0 expressed *EF-Tu* 6-fold higher compared to the control sample (cells grown in MRS) (results not shown).

The *EF-Tu* gene of *L. acidophilus* ATCC 4356 was only up-regulated under normal (44-fold) and stressful (14-fold) gut conditions (Table 4). No change in expression was monitored in the presence of mucus.

Expression of *mapA*

The *mapA* gene of strain 423 was up-regulated when cells were incubated in the presence of mucin. Expression was 6 and 8-fold higher in the presence of 0.01% (w/v) mucin and 0.05% (w/v) mucin, respectively. The *mapA* gene was significantly up-regulated under normal and stressful conditions. Under normal conditions, *MapA* was expressed 25-fold higher when compared to the control sample (Table 3). At pH 4.0, the gene was up-regulated twice as much as the control (results not shown). In the presence of 0.1% (w/v) cysteine, *mapA* was considerably down-regulated (17-fold) (results not shown).

Expression of *plaA*

Expression of *plaA* was not influenced by changes in bile, pancreatin and pH (Table 3).

Expression of *slp*

The Slp of *L. acidophilus* ATCC 4356 does not seem to be stimulated by mucin as no up-regulation of the gene was monitored when the strain was cultured under

varying concentrations of mucin (Table 4). However, the gene was induced 33-fold under normal *in vitro* conditions and 4-fold under stressful conditions.

Discussion

Colonisation of the intestinal tract is considered a prerequisite for probiotic bacteria (26). Most studies report on the adhesion of probiotic bacteria to intestinal cell lines (1, 10, 14). Although this may be a useful indication of adhesion, it is not always a reflection of the ability of the bacteria to adhere to mucus covering the intestinal cells (25). The mucus layer serves a dual function. It may prevent the adhesion of pathogenic bacteria, thereby protecting the intestinal cells, but also serves as a nutrient source and matrix for colonisation of probiotic bacteria (23). Adhesion to mucosa is important for proliferation of probiotic cells, especially in the small intestine with high flow rates (36). Furthermore, adhesion stimulates the immune system (25). In at least one study (11), it has been claimed that damaged gastric mucosal cells are repaired by the adherence of probiotic lactobacilli.

From first studies, it was concluded that the EF-Tu molecule is restricted to the cytoplasm of prokaryotes. However, later studies have shown that this molecule is also associated with the membrane of *E. coli* (18) and the periplasm of *Neisseria gonorrhoeae* (27). In lactobacilli, EF-Tu may function as an “envelope associated protein,” which may be released from the cell when the organism experiences osmotic shock (22). Granato *et al.* demonstrated the presence of EF-Tu on the outer surface of 5 *Lactobacillus* and 2 *Bifidobacterium* strains. The concentration of EF-Tu on the surface of the strains differed, suggesting possible differences in expression (15). In this study, up-regulation of *EF-Tu* of *L. plantarum* 423 in the presence of mucus suggests that the EF-Tu molecule may also play a role in adhesion. This correlates well with work done by Granato *et al.* (15). Furthermore, expression of the gene was not affected by digestive stress conditions *in vitro* and may thus contribute to the colonisation of the strain in the digestive tract. Conversely, in *L. acidophilus* ATCC 4356, *EF-Tu* was not up-regulated in the presence of porcine mucus. However, *EF-Tu* was up-regulated under normal and stressful conditions. These results suggest that the EF-Tu molecule may only mediate adhesion and facilitate colonisation in certain lactobacilli strains.

None of the genes tested for *L. acidophilus* 4356 were up-regulated in the presence of porcine mucin. Induction of *slp* only occurred under normal digestive conditions.

This suggests that one of the main functional roles of Slp in *L. acidophilus* may not be associated with the adherence of the strain to mucus. With regards to strain 423, all the mucus adhesion genes were significantly up-regulated in the presence of mucus and expression levels increased with increasing concentrations of mucus. Under normal physiological concentrations of bile and pancreatin, expression of the *mub* gene was affected, the *mapA* gene was over-expressed and the *EF-Tu* gene remained stable. Similar results were obtained when *L. plantarum* 423 was exposed to digestive stress. This suggests that, whilst the expression of certain genes, whose products interact with mucus, may be affected by bile and pancreatin, others genes are switched on, enabling the strain to adapt to physiological conditions and adhere to the gastrointestinal tract.

The pH of the gut lumen is 6.5, but it has been postulated that it becomes slightly more acidic closer to the mucus-covered surface, owing to the sulfated content and sialic residues of mucin (5, 15). Hence, expression of the adhesion genes of strain 423 were also investigated at pH 4.0. Variations in pH did not influence the expression of the genes negatively, as none were down-regulated at lower pH (results not shown).

Regulation of *mapA* was also investigated. Expression of *mapA* from *L. reuteri* 104R is regulated by a mechanism of transcription attenuation, involving cysteine (31). Our studies indicate that *mapA* of *L. plantarum* 423 is down-regulated in the presence of 0.1% (w/v) cysteine. Hence, cysteine participates as an effector molecule and represses transcription of *mapA* in the presence of excess cysteine.

Conclusions

Lactobacillus plantarum 423 complies with most of the major criteria required to be classified as a probiotic. Expression of *plaA* was not affected by digestive stress conditions *in vitro* and strain 423 survived exposure to HCl at pH 2.0. Expression of adhesion genes by strain 423 was positively regulated in the presence of mucus. Variations in pH did not affect expression of the adhesion genes, whilst bile and pancreatin only affected the *mub* gene. *L. plantarum* 423 is capable of adapting to conditions in a normal healthy intestine and has the necessary genetic characteristics required for an effective probiotic.

Lactobacillus acidophilus ATCC 4356 survived exposure to HCl at pH 2.0. The organism may not display strong adhesive properties as none of the adhesion genes were up-regulated in the presence of mucus. However, up-regulation of *slp* and *EF-Tu*

in the presence of bile and pancreatin indicated that the strain has the ability to adapt to physiological conditions. Other adhesion genes may be involved in effective colonisation of strain ATCC 4356 to the gastrointestinal tract. Results of the present investigation support the theory of receptor mediated adhesion as similar genes in different organisms react differently to surface components such as mucus.

References

1. **Adlerberth, I., S. Ahrne, M. L. Johansson, G. Molin, L. A. Hanson, A. E. Wold.** 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. *Applied and Environmental Microbiology*. **62**:2244-2251.
2. **Ahrne, S., S. Noback, B. Jeppsson, I. Adlerberth, A. E. Wold, G. Molin.** 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. *Journal of Applied Microbiology*. **85**:88-94.
3. **Alterman, E., W. M. Russell, M. A. Azcarate-Peril, R. Barrangou, B. L. Buck, O. McAuliffe, N. Souther, A. Dobson, T. Duong, M. Callanan, S. Lick, A. Hamrick, R. Cano, T. D. Klaenhammer.** 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proceedings of the National Academy of Science of the United States of America*. **102**:3906-3912.
4. **Apostolou, E., P. V. Kirjavainen, M. Saxelin, H. Rautelin, V. Valtonen, S. J. Salminen, A. C. Ouwehand.** 2001. Good adhesion properties of probiotics: a potential risk for bacteremia? *FEMS Immunology and Medical Microbiology*. **31**:35-39.
5. **Blum, S., R. Reniero, E. J. Schiffrin, R. Crittenden, T. Mattila-Sandholm, A. C. Ouwehand, S. Salminen, A. Von Wright, M. Saarela, M. Saxelin, K. Collins, L. Morelli.** 2000. Adhesion studies for probiotics: need for validation and refinement. *Trends in Food Science and Technology*. **10**:405-410.
6. **Boot, H. J., C. P. A. M. Kolen, J. M. van Noort, P. H. Pouwels.** 1993. S-layer protein of *Lactobacillus acidophilus* ATCC 4356: purification, expression in *Escherichia coli* and nucleotide sequence of the corresponding gene. *Journal of Bacteriology*. **175**:6089-6096.
7. **Bron, P. A., M. Marco, S. M. Hoffer, E. van Mullekom, W. M. de Vos, M. Kleerebezem.** 2004. Genetic characterization of the bile salt response in

- Lactobacillus plantarum* and analysis of responsive promoters *in vitro* and *in situ* in the gastrointestinal tract. *Journal of Bacteriology*. **186**:7829-7835.
8. **Chen, X., J. Xu, J. Shuai, J. Chen, Z. Zhang, W. Fang.** 2007. The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* 0157:H7 and *Salmonella typhimurium*. *International Journal of Food Microbiology*. **115**:307-312.
 9. **Desroche, N., C. Beltramo, J. Guzzo.** 2005. Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni*. *Journal of Microbiology Methods*. **60**:325-333.
 10. **Dunne, C., L. O. Mahony, L. Murphy, G. Thornton, D. Morrissey, S. O'Halloran, M. Feeny, S. Flynn, G. Fitzgerald, C. Daly, B. Kiely, G. C. O'Sullivan, F. Shanahan, J. K. Collins.** 2001. *In vitro* selection criteria for probiotic bacteria of human origin: correlation with *in vivo* findings. *American Journal of Clinical Nutrition*. **73**:386-392.
 11. **Elliot, S. N., A. Buret, W. McKnight, M. J. S. Miller, J. L. Wallace.** 1998. Bacteria rapidly colonise and modulate healing of gastric ulcers in rats. *American Journal of Physiology*. **275**:425-432.
 12. **Gaucher, E. A., M. M. Miyamoto, S. A. Benner.** 2001. Function-structure analysis of proteins using covarion-based evolutionary approaches: Elongation factors. *Proceedings of the National Academy of Science of the United States of America*. **98**:548-542.
 13. **Gilliland, S. E., T. E. Staley, L. J. Bush.** 1984. Importance of bile tolerance of *Lactobacillus acidophilus* used as a dietary adjunct. *Journal of Dairy Science*. **67**:3045-3051.
 14. **Granato, D., F. Perotti, I. Masserey, M. Rouvet, M. Golliard, A. Servin, D. Brassart.** 1999. Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. *Applied and Environmental Microbiology*. **65**:1071-1077.
 15. **Granato, D., G. E. Bergonzelli, R. D. Pridmore, L. Marvin, M. Rouvet, I. E. Cortesy-Theulaz.** 2004. Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533(La1) to human intestinal cells and mucins. *Infection and Immunity*. **72**:2160-2169.

16. **Ishiguro, E. E., W. W. Kay, T. Ainsworth, J. B. Chamberlain, R. A. Austen, J. T. Buckley, T. J. Trust.** 1981. Loss of virulence during culture of *Aeromonas salmonicida* at high temperature. *Journal of Bacteriology*. **148**:333-340.
17. **Ivanova, I., V. Miteva, T. S. Stefanova, A. Pantev, I. Budakov, S. Danova, P. Montcheva, I. Nikolova, X. Dousset, P. Boyaval.** 1998. Characterisation of a bacteriocin produced by *Streptococcus thermophilus* 81. *International Journal of Food Microbiology*. **42**:147-158.
18. **Jacobson, G. R., J. P. Rosenbusch.** 1976. Abundance and membrane association of elongation factor Tu in *E. coli*. *Nature*. **261**:23-26.
19. **Klaenhammer, T. R.** 2000. Probiotic bacteria: Today and Tomorrow. *Journal of Nutrition*. **130**:415S-416S.
20. **Morelli, L.** 2000. *In vitro* selection of probiotic lactobacilli: A critical appraisal. *Current Issues in Intestinal Microbiology*. **1**:59-67.
21. **Munn, C. B., E. E. Ishiguro, W. W. Kay, T. J. Trust.** 1982. Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. *Infection and Immunity*. **36**:1069-1075.
22. **Nakamura, J., D. Ito, K. Nagai, Y. Umehara, M. Hamachi, C. Kumagai.** 1997. Rapid and sensitive detection of hiochi bacteria by amplification of hiochi bacterial common antigen gene by PCR method and characterisation of the antigen. *Journal of Fermentation and Bioengineering*. **185**:7019-7023.
23. **Nielsen, E. M., J. Schlundt, A. Gunvig, B. L. Jacobsen.** 1994. Epithelial, mucus and lumen subpopulations of *Escherichia coli* in the large intestine of conventional and gnotobiotic rats. *Microbial Ecology in Health Disease*. **7**:263-273.
24. **Ouwehand, A. C.** 2005. Measurement of bacterial adhesion - in vitro evaluation of different methods. *Journal of Microbiological Methods*. **60**:225-233.
25. **Ouwehand, A. C., P. V. Kirjavainen, C. Shortt, S. Salminen.** 1999. Probiotics: mechanisms and established effects. *International Dairy Journal*. **9**:43-52.
26. **Ouwehand, A. C., P. V. Kirjavainen, M. M. Gronlund, E. Isolauri, S. J. Salminen.** 1999. Adhesion of probiotic microorganisms to intestinal mucus. *International Dairy Journal*. **9**:623-630.

27. **Porcella, S. F., R. J. Belland, R. C. Judd.** 1996. Identification of an EF-Tu protein that is periplasm-associated and processed in *Neisseria gonorrhoeae*. *Microbiology*. **142**:2481-2489.
28. **Roos, S., H. Jonsson.** 2002. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiology*. **148**:433-442.
29. **Saarela, M., G. Mogensen, R. Fonden, J. Matto, T. Mattila-Sandholm.** 2000. Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology*. **84**:197-215.
30. **Sara, M., U. B. Sleytr.** 2000. S-layer proteins. *Journal of Bacteriology*. **182**:859-868.
31. **Satoh, E., R. J. Leer, M. Rojas, P. L. Conway, P. H. Pouwels.** 2000. The gene encoding the adhesion promoting protein MapA from *Lactobacillus reuteri* 104R is part of an operon whose expression is controlled by a mechanism of transcription attenuation, involving cysteine. Unpublished. **Genebank Accession Number AJ 293860**.
32. **Sleytr, U. B.** 1997. Basic and applied S-layer research: an overview. *FEMS Microbiology Reviews*. **20**:5-12.
33. **Tuomola, E. M., A. C. Ouwehand, S. J. Salminen.** 2000. Chemical, physical and enzymatic pre-treatments of probiotic lactobacilli alter their adhesion to human intestinal mucus glycoproteins. *International Journal of Food Microbiology*. **60**:75-81.
34. **Van Reenen, C. A., L. M. T. Dicks, M. L. Chikindas.** 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology*. **84**:1131-1137.
35. **Van Reenen, C. A., M. L. Chikindas, W. H. Van Zyl, L. M. T. Dicks.** 2003. Characterization and heterologous expression of a class IIa bacteriocin, plantaricin 423 from *Lactobacillus plantarum* 423, in *Saccharomyces cerevisiae*. *International Journal Food Microbiology*. **81**:29-40.
36. **Wadstrom, T.** 1998. Adherence traits and mechanisms of microbial adhesion in the gut. *Baillier's Clinical Tropical Medicine and Communicable Disease*. **3**:417-433.

Table 1: Modification of MRS broth (Biolab) to simulate gastric conditions.

Medium ^a	Mucin ^b %	Bile % (w/v)	Pancreatin %	pH ^c
1	0	0	0	7
2	0.01	0	0	7
3	0.05	0	0	7
4	0.01	0.3	0.3	6.5
5	0.01	1	1	6.5
6	0.01	0.3	0.3	4

^a Medium 4 simulates normal gut conditions and media 5 and 6 stress conditions.

^b Porcine mucus (Sigma, Manheim, Germany)

^c Adjusted with 0.5 M HCl

Table 2: Primers used in the study

Primer set	Sequence 5' to 3'	Size of amplicon (bp)
Bac 423F RT Bac 423R RT	GACATTTACATTGAGTAGGAACTAG GTAACCCCATACCATAGTATTTAC	147
Mub 423F RT Mub 423R RT	GTAGTTACTCAGTGACGATCAATG TAATTGTAAAGGTATAATCGGAGG	150
Map 423F RT Map 423R RT	TGGATTCTGCTTGAGGTAAG GACTAGTAATAACGCGACCG	156
EFTu 423F RT EFTu 423R RT	TTCTGGTCGTATCGATCGTG CCACGTAATAACGCACCAAC	161
GDPH 423F RT GDPH 423R RT	ACTGAATTAGTTGCTATCTTAGAC GAAAGTAGTACCGATAACATCAGA	140
Mub 4356F RT Mub 4356R RT	GTAATCGTGTTCTACATATACATAG GGTATAAAGTTAACAGCATTGTTC	152
Slp 4356F RT Slp 4356R RT	GCACAACGCATACTACTACG CTTGCAACAGCCTTACCGT	150
EFTu 4356F RT EFTu 423R RT	GGTGCTATCTTAGTTGTTGC CAACCAAGTCGATCAATTCT	150
GDPH 4356F RT GDPH 4356R RT	CTATCGTTTACTCAGTAAACCAAGA CGTGGATAGTAGTCATAGTACCAAC	149

Table 3: Expression of *mub*, *mapA*, *EF-Tu* and *plaA* of *L. plantarum* 423

Genes	Medium	Av. Ct	Quant.*	<i>GDPH</i> Av. Ct	Quant.	Normalised	Set to Control
<i>mub</i>	1	24.98	38.9	21.64	57.5	0.68	1
	2	24.5	53.7	27.93	1	53.7	80
	3	23.61	97.7	27.91	1	97.7	144
	4	25.28	31.6	19.19	316	0.1	7
	5	27.86	5.4	18.88	316	0.02	34
<i>mapA</i>	1	26.4	41.7	21.62	398.1	0.1	1
	2	25.78	60.3	23.64	104.7	0.6	6
	3	25.73	61.7	24.16	74.1	0.8	8
	4	26.95	30.2	26.94	12.02	2.5	25
	5	27.1	27.5	25.91	23.4	1.2	12
<i>EF-Tu</i>	1	20.3	66.1	22.76	21.9	3	1
	2	19.36	117.5	28.06	1.2	97.9	33
	3	17.63	346.7	27.94	1.1	315.2	105
	4	17.85	302	18.64	269	1.1	2.7
	5	17.44	398	18.48	295	1.3	2.3
<i>plaA</i>	1	20.62	91.2	23.97	15.5	5.8	1
	2	20.21	114.8	23.89	16.2	7.1	1.2
	3	20.19	117.5	24.42	11.5	10.2	1.8
	4	24.3	10	27.45	1.4	7.1	1.2
	5	22.36	32.4	26.58	2.6	12.5	2.2

*Calculation of the quantity of each gene was determined from the standard curves constructed for the specific genes.

Table 4: Expression of *mub*, *slp* and *EF-Tu* of *L. acidophilus* ATCC 4356

Genes	Medium	Av. Ct	Quant.*	<i>GDPH</i> Av. Ct	Quant.	Normalised	Set to Control
<i>mub</i>	1	30.74	6.17	30.88	2.5	2.5	1
	4	32.26	2.4	31.99	1.3	1.9	1.3
	5	31.5	3.8	27.14	27.5	0.14	18
<i>slp</i>	1	19.76	2.1	30.88	2.63	0.8	1
	4	15.25	34.67	31.99	1.3	26.7	33
	5	15.9	23	29.3	7.2	3.2	4
<i>EF-Tu</i>	1	24.63	1.7	26.98	12.9	0.13	1
	4	21.13	14.13	29.59	2.5	5.7	44
	5	18.42	72.4	25.16	39.8	1.82	14

*Calculation of the quantity of each gene was determined from the standard curves constructed for the specific genes.

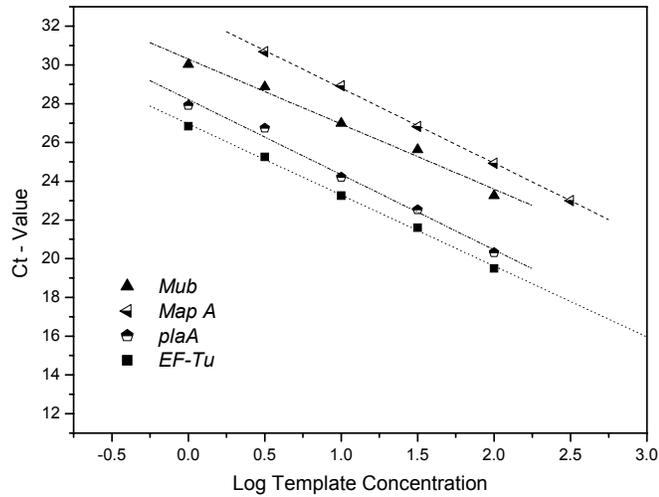


FIG. 1. Standard curves of the different genes for *L. plantarum* 423. *mub* ($y = -3.356x + 30.302$), *EF-Tu* ($y = -3.664x + 26.952$), *plaA* ($y = -3.88x + 28.218$), *mapA* ($y = -3.876x + 32.68$).

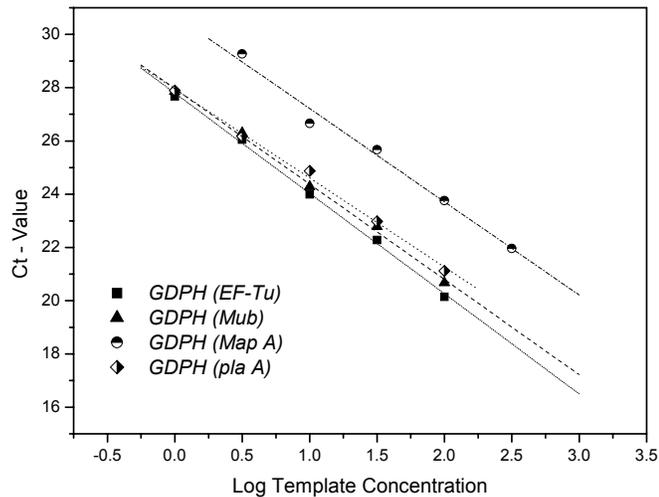


FIG. 2. Standard curves of glyceraldehyde 3-phosphate dehydrogenase for the different genes of *L. plantarum* 423. *EF-Tu* ($y = -3.766x + 27.796$), *mub* ($y = -3.58x + 27.958$), *mapA* ($y = -3.504x + 30.72$), *plaA* ($y = -3.346x + 27.952$).

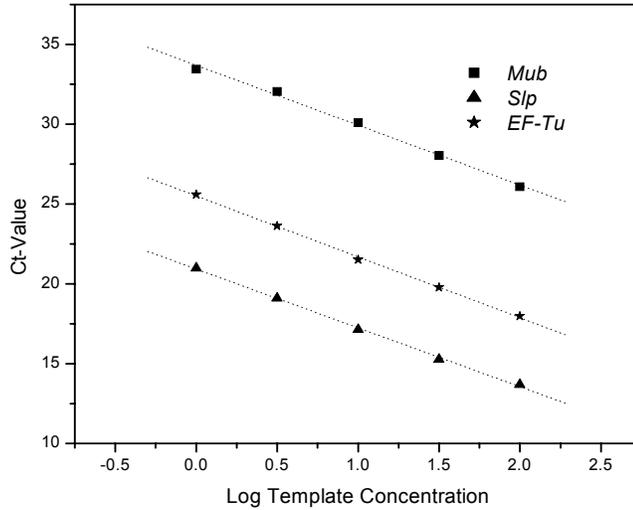


FIG. 3. Standard curves of the different genes for *L. acidophilus* 4356. *mub* ($y = -3.75x + 33.688$), *slp* ($y = -3.684x + 20.926$), *EF-Tu* ($y = -3.81x + 25.5$).

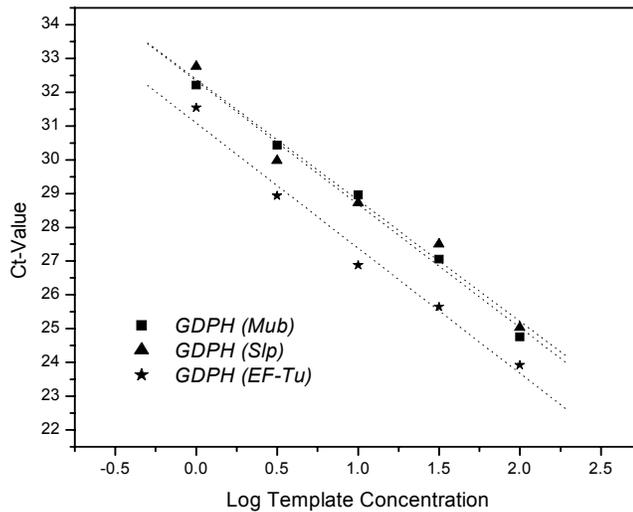


FIG. 4. Standard curves of glyceraldehyde 3-phosphate dehydrogenase for the different genes of *L. acidophilus* 4356. *mub* ($y = -3.654x + 32.338$), *slp* ($y = -3.584x + 32.384$), *EF-Tu* ($y = -3.706x + 31.088$).

Chapter 4

Evaluation of the adhesive properties of the probiotics *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA to rat intestinal tissue

Abstract

The adhesive properties of probiotic strains *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA were evaluated in male Wistar rat models using fluorescent *in situ* hybridization (FISH) incorporating fluorescently labeled strain-specific oligonucleotide probes. Tissue samples collected from the intestinal tract of rats were subjected to real time PCR (RT-PCR) to quantify strains in different sections of the gastrointestinal tract. Results obtained with FISH indicated that *L. plantarum* 423 adhered strongly to the surfaces of the ileum and the cecum, but did not colonise the colon. These results were confirmed with RT-PCR. *E. mundtii* ST4SA adsorbed to the epithelial cells of the cecum and colon, but penetrated the cells. No changes in tissue morphology or other toxic defects were observed when tissue samples were stained with haematoxylin eosin, suggesting that strains 423 and ST4SA are non-inflammatory and non-toxic. 16S rDNA PCR and denaturing gradient gel electrophoresis (DGGE) was conducted to evaluate the effect of strains 423 and ST4SA to the natural microflora of rats. Data indicated that the lactic acid bacteria remained unchanged, whereas the *Enterobacteriaceae* population showed a clear decline. Strains 423 and ST4SA agglutinated yeast cells when tested *in vitro*, indicating the possible presence of mannose receptors on their cell surfaces. Type 1 fimbriated strains of *E. coli* use the same receptors for binding to epithelial cells. It is thus possible that strains 423 and ST4SA compete against certain *E. coli* and other enterobacteriaceae for colonisation.

Introduction

The adhesion of probiotic bacteria to intestinal mucosa facilitates a number of health benefits to the host. These include exclusion of pathogens, restoration of natural microflora after antibiotic treatment, reduction in carcinogens, lowering of blood pressure (15), stimulation of the immune system (26) and a decrease in cholesterol levels (7). An effective probiotic is often evaluated by its ability to colonise the intestinal tract or its ability to compete against pathogens for adhesion sites (14). Exclusion of pathogens by probiotic bacteria provides a healthy gut with balanced microflora (30).

The mucosal layer in the intestinal tract plays an essential role in maintaining a barrier against the interaction of toxic substances and pathogenic bacteria from the lumen content. Previous reports have indicated that pathogenic bacteria like *Salmonella typhi* (18) and *Escherichia coli* (33) may damage the intestinal barrier and increase permeability at the mucosal surface. Other studies have shown that the adhesion of lactobacilli to the mucosal surface prevents the translocation of pathogens (1) and thereby inhibits intestinal inflammation (21). Lactic acid bacteria and pathogenic bacteria compete for receptor sites at the mucosal surface. One of these receptors includes the mannose receptor and mannose specific adherence has been demonstrated in many *L. plantarum* strains (28). Type 1 fimbriated *E.coli* also adhere to mannose residues on the intestinal epithelial cells of humans and rats (2, 13). The adhesion of probiotic lactic acid bacteria, such as *L. plantarum*, to host intestinal tissue may thus directly hinder the colonisation of pathogenic *E. coli* strains.

Cell counts from fecal samples illustrate the survival rate of administered probiotics (19). However, this does not provide an indication of the adherence of cells to the intestinal tissue or the number of cells that remain colonised. Fluorescent *in situ* hybridization (FISH) has been used to detect lactobacilli in pig mucus and epithelial tissue (22). The technique has also been successfully used to detect and quantify predominant groups of anaerobic bacteria in human fecal samples (10).

Lactic acid bacteria are used as starter cultures in a variety of fermented products and are generally regarded as safe. These organisms are common inhabitants of a number of environmental niches, including the human and animal gastro-intestinal tract. *L. plantarum* 423 and *Enterococcus mundtii* ST4SA were isolated from sorghum beer and soybean flour, respectively (17, 35). Both strains produce bacteriocins that are resistant to treatment at 80°C and incubation in MRS broth at pH 1.0 to 10.0. The

bacteriocins are also active against a number of food-borne pathogens, including *Bacillus cereus*, *Clostridium sporogenes*, *Enterococcus faecalis* and *Listeria monocytogenes* (17, 35).

In this study, we report on the adhesion of two probiotic strains, *L. plantarum* 423 and *E. mundtii* ST4SA, to rat intestinal tissue. FISH with strain-specific probes were used to detect strains 423 and ST4SA. Real time PCR with strain-specific primers was used to quantify strains in different sections of the gastrointestinal tract. 16S rDNA PCR and DGGE was used to determine the effect of the probiotics on enterobacteriaceae and lactic acid bacteria, naturally present in the intestinal tract of the rats.

Materials and Methods

Bacterial strains and dosage preparation

Lactobacillus plantarum 423, *Enterococcus mundtii* ST4SA and *L. plantarum* WCFS1 were cultured in De Man Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37°C to OD_{600nm} = 4.0. Ten ml cultures of *L. plantarum* 423 and *E. mundtii* ST4SA were harvested (10000 × g, 10 min at 4°C), washed twice in sterile physiological water, resuspended in 10% sterile skim milk and lyophilised in separate vials. One vial lyophilised cells of each strain was resuspended in 1 ml sterile physiological water, serially diluted and plated onto MRS (Biolab) agar to determine the number of viable cells. Lyophilised cells in the other vials were suspended in a predetermined volume of sterile physiological solution to yield 1 × 10⁸ cfu per 200 µl.

Treatment of animals and tissue collection

Approval to work with Wistar rats was obtained from the Ethics Committee of the University of Stellenbosch (ethics reference number: 2005B02003). Four-week-old male Wistar rats were divided into four groups (A, B, C, D) with six rats per group. The rats were contained in a controlled environment (23 ± 2°C) and had free access to feed and drinking water. Probiotics were administered via intragastric gavage. Group A received 200 µl *L. plantarum* 423, group B 200 µl *E. mundtii* ST4SA and group C a combination of the two strains (100 µl each). Group D served as the control and received 200 µl sterile physiological water. The rats were dosed once a day for 14 days. Changes in body weight were monitored every alternate day. At the end of the 14 day trial, the rats were anaesthetised with an overdose (1 ml) of sodium

pentobarbitone (200 mg/ml). Blood was drawn via cardiac puncture of the right ventricle. The rats were dissected and samples of the duodenum, jejunum, ileum, cecum and colon were removed aseptically. Approximately 2 cm sections were placed in cassettes and immersed in 4% paraformaldehyde in 0.1 M PBS (0.2 M NaH₂PO₄, 0.2 M Na₂HPO₄, pH 7.2). The remaining tissue samples were homogenised in 0.1 M PBS pH7.2 (0.3 g/ml) and stored at -20°C.

Construction of strain-specific probes and FISH

Primers Bac423 and MundST4SA were used to amplify the structural genes encoding bacteriocin 423 and peptide ST4SA, respectively (Table 1). Primers were designed with the Primer Designer Program, version 1.01 (Scientific and Educational Software) to yield a GC-content between 35 and 45%. PCR amplicons were labelled via the universal labelling system of the ULYSIS nucleic acid labelling kits (Molecular ProbesTm, Invitrogen Corporation, Carlsbad, California, USA). The Alexa Fluor 568 dye (Invitrogen) with a maximum absorbance at 576nm and emission at 600 nm was used.

Excised tissue fixed in 4% (v/v) paraformaldehyde (pH 7.2) for a minimum of 24 h were dehydrated in a series of 70%, 90%, 95 and 98% (v/v) ethanol for 12.5 h, followed by 3.5 h of clearing in xylol and 4 h of impregnation in paraffin wax (Histosec). The tissues were sectioned with a R Jung microtome (Heidelberg, Germany) in 5 µm increments and placed on Poly-L-Lysine coated microscope slides (Lasec, Johannesburg, South Africa). Tissue sections were then deparaffinized and dehydrated by incubation in xylene for 5 min, followed by submersion in 98% (v/v) ethanol for 3 min and then in 95% (v/v) ethanol for 3 min. This was followed by a 3 min submersion in 80% and 50% ethanol, respectively. The slides with sections were rinsed in sterile distilled water and submerged for 20 min in 0.1% (w/v) trypsin in PBS. Incubation was at 37°C. Slides were rinsed with sterile distilled water and dried for 20 min at 46°C (31). Slides were treated with 20 µl lysozyme (20 mg/ml) for 20 min at room temperature, rinsed with sterile distilled water and air dried. Slides were then covered with 50 µl hybridization buffer (5 mol/L NaCl; 1 mol/L Tris.Cl pH 8.0; 0.01%, w/v, SDS and 25%, v/v, formamide) and 5 µl of the strain-specific probe (50 ng/µl). Hybridisation was for 2.5 h at 46°C in a moist chamber. The slides were submerged in 50 ml wash buffer (5 mol/L NaCl; 1 mol/L Tris.Cl pH 8.0; 0.01%, w/v, SDS and 0.5 mol/L EDTA, pH8.0) for 10 min at 48°C (5). Slides were given a final rinse with sterile distilled water, air dried in the dark and stored at -20°C, covered with

fluorescent mounting medium (Dako Cytomation) to prevent photobleaching. Tissue sections from group D rats served as the controls.

The tissue sections were observed through an Olympus cell^R system attached to an IX-81 inverted fluorescence microscope, equipped with a 10× lens (UPlan FLN, Olympus), a 20× lens (LucPlan FLN, Olympus), a 40× lens (LucPlan FLN, Olympus) and a 60× lens (PlanApoN 1.42 N.A. oil immersion) (Olympus Biosystems GMBH, Germany). Samples were visualized with 400x and 600x magnification. The sample was excited through an excitation filter for Texas Red (572nm). Cell^R imaging software was used for the image acquisition and analysis.

Haematoxylin and eosin staining (H&E)

Tissue sections were prepared for haematoxylin-eosin (H&E) staining similar to the method used to prepare slides for FISH. All samples were analysed by PathCare veterinary pathologists (Cape Town, South Africa).

Quantification of strains with real time PCR

Homogenised tissue (300 µl) were inoculated into 100 ml MRS (Biolab) and incubated for 18 h at 37°C, without aeration. Plasmid isolation was performed using the QIAprep Spin Miniprep Kit (Qiagen[®], Valencia, California, USA), according to the manufacturer's instructions and eluted in 100 µl sterile milliQ water.

Primers used for the amplification of the genes encoding bacteriocin 423 and antimicrobial peptide ST4SA, are listed in Table 1. Primer sets were designed to produce amplicons smaller than 150bp. Primer dimer formation was checked by agarose gel electrophoresis (2% w/v, agarose) and melting curve analysis.

Standard curves were used for quantification and consisted of Log template concentration of DNA plotted against the Ct-value (crossing points of different standard dilutions). Efficiencies were calculated by $E = 10^{(-1/S)} - 1$, where S = slope of the standard curve. Real time PCR reactions were performed in a LightCycler instrument (Roche, Mannheim, Germany), with Relative Quantification Software (version 5.32; Roche) and SYBR Green technology (Sigma, Missouri, USA). Each 20 µl reaction mixture consisted of 10 µl SYBR Green Supermix (Sigma), 1 µl of each primer at 5 µM, 7 µl nuclease-free water and 1 µl diluted plasmid DNA. Five dilutions of plasmid DNA were prepared. A negative control with sterile Milli Q water, was included in each run. Isolation of plasmid plasmid DNA and RT-PCR was performed in duplicate for each of the two strains in each tissue sample. PCR conditions were as follows: initial denaturation at 95°C for 15 min, followed by 40 cycles of

amplification at 50°C for 10 sec and 72°C for 15 sec. At the end of each run a melting curve was performed from 95°C to 45°C (0.2°C.s⁻¹).

16SrDNA PCR and denaturing gradient gel electrophoresis (DGGE)

Homogenized tissue samples from different sections of the intestinal tract (300 µl) were inoculated into 10 ml MRS broth (Biolab) and 10 ml MacConkey broth (Biolab) at 37°C to OD_{600nm} = 1.8, for the isolation of lactic acid bacteria (9) and *Enterobacteriaceae* (23), respectively. DNA isolated from homogenised tissue samples of group D rats inoculated into 10 ml MRS broth (Biolab) and 10ml MacConkey broth (Biolab), served as the controls. 16S rDNA amplification was carried out in a Gene Amp[®] PCR 9700 system (Applied Biosystems, California, USA), with primers 341FGC (5'-CGCCCCGCCGCGCGGGCGGGCGGGGC GGGGGCACGGGGGGCCTACGGGAGGCAGCAG -3') and 534R (5'-ATTACCGCGGCTGCTGG -3'), designed to target conserved 16S rDNA sequences (24). Primers were selected to produce 193bp PCR amplicons encompassing the V3 region of the 16SrDNA gene. The forward primer was modified with a GC-clamp to impart melting stability to the DNA fragments for efficient DGGE analysis. The PCR mix included the following: 5 µl of 10 × buffer, 3 µl of 25 mM MgCl₂, 5 µl of 5 µM 341FGC, 5 µl of 5 µM 534R, 4 µl of 2.5 mM DNTP's, 0.5 µl of *Taq* polymerase enzyme (Takara, Shiga, Japan) and 1 µl of genomic DNA (100 ng/µl). Each reaction was adjusted to a final volume of 50 µl with sterile milli Q water.

DGGE was performed with a 10% (w/v) polyacrylamide gel (37.5:1, acrylamide:bisacrylamide) with a gradient ranging from 30% – 60%. Usually, 100% denaturant corresponds to 7 M urea and 40% (w/v) acrylamide. The 30% gradient contained 5 ml of 40% (w/v) polyacrylamide, 1 ml of 10 × TAE, 2.4 ml formamide, 2.5 g urea and was adjusted to a final volume of 20 ml with distilled water. The 60% gradient contained 5 ml of 40% (w/v) polyacrylamide, 1 ml of 10 × TAE, 4.8 ml formamide, 5.0 g urea and was adjusted to a final volume of 20 ml with distilled water. Polymerisation was catalysed by 140 µl of 10% ammonium persulfate and 14 µl of TEMED. Electrophoresis was at a constant 100V for 16 h at 60°C in the DCode[™] Universal Mutation Detection System (Bio-Rad, Laboratories, USA). Electrophoresis buffer consisted of 0.5 × TAE (20 mM Tris-acetate pH 7.4, 10 mM sodium acetate and 0.5 mM EDTA pH 7.4) (24, 25).

Agglutination of yeast cells

L. plantarum 423, *E. mundtii* ST4SA and *Lactobacillus plantarum* WCFS1 were cultured in MRS broth (Biolab) at 37°C to $OD_{600nm} = 1.8$ (1×10^6 CFU/ml). Cells were harvested, ($10000 \times g$, 10 min at 4°C), washed twice in sterile physiological water and resuspended in PBS (pH 7.2). Cells were then serially diluted with PBS (pH 7.2) in a 96-well microtitre plate. An equal volume of PBS was added to the cells, followed by the addition of double the volume of 1% (w/v) commercially available bakers' yeast (*Saccharomyces cerevisiae*). The plates were shaken on an orbital platform shaker for 5 min and incubated for 16 h at 4°C. Agglutination was observed by light microscopy under $400 \times$ magnification. The agglutination titre was calculated according to the method described by Adlerberth *et al.* (3). *L. plantarum* WCFS1, treated the same way, was used as the positive control.

Results

FISH

Fig. 1 illustrates FISH images composed of the fluorescent image, phase image (no fluorescence) and the overlaid image (fluorescent signal overlaid on the phase image), respectively. This was conducted to obtain a clear view of the fluorescent signals on each of the tissue samples.

FISH depicted intense fluorescence of *L. plantarum* 423 along the epithelial lining of the cecum and villi of the small intestinal tissue of group A rats (Fig. 1A). Samples from all six rats investigated, displayed similar results (data not shown). The specificity of probe 423 was exemplified by FISH conducted on the intestinal tissue of the control rats. The fluorescent intensity in the control samples was not as intense or evident as the test samples (Fig. 1C). In the case of rats treated with *E. mundtii* ST4SA, clear intracellular fluorescence within the intestinal tissue was visible and much less along the epithelial lining (Fig. 1D). Tissue from all six rats were also investigated for strain ST4SA and similar results were observed in all samples (data not shown).

Haematoxylin and Eosin Staining (H&E)

H&E stains revealed no morphological changes or toxic defects induced to the rat tissue upon adhesion of *L. plantarum* 423 and *E. mundtii* ST4SA (Fig. 2).

Real time PCR

The standard curves displayed an efficiency of above 90% (Fig. 3) and a single product specific melting curve was obtained for each primer set. This indicates that primers were designed with optimal efficiency and were effective in targeting the genes of interest.

L. plantarum 423 colonised the ileum and cecum in highest numbers and did not adhere to the colon (Table 2). Results were recorded by the highest detection of the bacteriocin gene of strain 423 in the ileum and cecum tissue samples. Highest numbers of *E. mundtii* ST4SA was recorded in the cecum, followed by the colon. Little to no colonisation occurred in the small intestine, as expected with enterococci.

16SrDNA PCR and denaturing gradient gel electrophoresis (DGGE)

Colonisation of the strains to the intestinal tract significantly altered the enterobacterial composition of microbiota in the gut. This was evident by the adhesion of *L. plantarum* 423 to the ileum and cecum. The probiotic potential of strain 423 is further emanated by the displacement of *Enterobacteriaceae* in the colon, despite the non-adherence of strain 423 to this section of the digestive tract (Fig. 4A, blue arrow). The *Enterobacteriaceae* that were dislodged from the cecum by strains 423 and ST4SA is indicated in Fig. 4A and B, respectively (red brackets). Species that were detached from the colon by the probiotic action of strain ST4SA is indicated by the blue arrow (Fig. 4B). Results appear similar for both strains. The lactic acid bacteria population remained unaffected by the adhesion and colonisation of strains 423 and ST4SA (Figs. 4C and D).

Agglutination of yeast cells

Mannose-rich polysaccharides are a major component in the cell wall of *Saccharomyces cerevisiae*. Bacteria with mannose-specific adhesins agglutinate yeast in a mannose specific manner (3). *L. plantarum* 423 and *E. mundtii* ST4SA was evaluated for its ability to agglutinate yeast cells and display mannose adherence. Both strains exhibited strong agglutination of yeast cells. *E. mundtii* ST4SA displayed agglutination by at least four titre steps and *L. plantarum* 423 by three titre steps (results not shown).

Discussion

The ability of probiotic strains to adhere to gastrointestinal tissue is of extreme importance to prolong beneficial health effects in the host. Various techniques are

used to evaluate the adhesive properties of potential probiotic cells, including a number of *in vitro* studies with intestinal cell lines or mucus (12, 20, 27). The percentage survival of ingested cells has also been used as an indication of adhesion (11). However, not much information is available on the adhesion of probiotics to the intestinal tract *in vivo*. The present study successfully reports the use of FISH to evaluate the adhesion of two potential probiotic strains to rat intestinal tissue.

L. plantarum 423 displays strong adhesive properties, as indicated by the intense fluorescent signal along the epithelial lining of the rat intestinal tissue (Fig. 1A). Real time PCR indicated higher numbers of strain 423 in the ileum and cecum, compared to the colon. These results correlated well with FISH conducted on colonic tissue of group A rats, as no discernible fluorescent signal was observed on the epithelial lining (Fig. 1B), indicating no binding to the colon. These results suggest that gastrointestinal conditions and receptors in the small intestine and anterior colon favour the colonisation of strain 423. *L. plantarum* is a common inhabitant of a variety of food fermentation processes (8). Hence, its abundance in the cecum could be attributed to the high level of fermentation and digestion that occurs within this organ. The complete genome sequence of *L. plantarum* WCFS1 reveals the presence of a number of genes involved in the metabolism of various sugars, utilization of peptides and the formation of different amino acids (16). The small intestine is thus the ideal location for this organism, due to the abundance of micronutrients to stimulate the growth of *L. plantarum*. Adhesion of the organisms to this section of the intestinal tract will also assist the host in digestion and nutrient absorption. The mucus binding protein (Mub) isolated from *Lactobacillus reuteri* 1063 adhered to pig mucus within a pH range of 3-7.4 when studied *in vitro* (29). Mub homologues have also been described for *L. plantarum* 423 (4). Hence, the small intestine may provide optimal pH conditions for the binding of organisms that contain adhesion proteins such as Mub. This reflects the remarkable potential of *L. plantarum* as an efficient coloniser of the gastrointestinal tract.

Accurate FISH analysis is dependent on a highly-specific probe as well as sample preparation. Significant research has been conducted on probe specificity (6, 10). However, not much has been done in evaluating and comparing various sample preparations. Previous reports have used mucus epithelial scrapings and homogenized samples to determine the presence of specific species (10, 22). However, this method does not provide an accurate representation of adhesion and proves ineffective for

determining the exact localization of the target organism. The present report makes use of homogenized tissue, mucus scrapings and formaldehyde fixed tissue as a comparative means of accurate detection. FISH conducted on homogenized samples and tissue scrapings proved vastly inaccurate as no significant differentiation in fluorescent intensity could be documented between the test and control samples. This was due to non-specific binding of the probe to cellular debris, particularly in the control samples. In addition, this method of analysis would have merely provided an indication of the presence of these strains in the various samples and is thus not an accurate indication of adhesion. This means of analysis proved crucial for evaluating the adhesion mechanism of *E. mundtii* ST4SA. FISH on formaldehyde fixed tissue of group B rats indicated translocation of strain ST4SA and intracellular activity. This data would not have been observed in the other sample preparations. Hence, sample preparation is imperative for high-quality FISH analysis, particularly when more than mere detection is of question.

Translocation of enterococci across the intestinal barrier has been previously reported and was also shown to occur across vaginal epithelium (37). It has been suggested that the preferred site for translocation of *E. faecalis* in mice is the cecum and the colon (38). According to the translocation model, adherent luminal bacteria may be phagocytosed by intestinal epithelial cells or intraepithelial leukocytes (36). Spontaneous translocation of indigenous bacteria is thought to occur continuously in a healthy host (32). These bacteria may either exit through the apical side of the epithelial cells or migrate in phagocytes to mesenteric lymph nodes where they proliferate, spread to distant sites and cause infection (36). However, chances of this occurring is minimal, as these bacteria are generally killed en route (32). It has been suggested that this process may be beneficial to the host for stimulating protective immunity (32). Hence, the translocation of enterococci is not an uncommon phenomenon. However, it may only pose a serious threat if the enterococci are detected in sterile sites such as blood and if the organism elicits infection. In a separate study, the *in vivo* assessment on the safety of *E. mundtii* ST4SA in male Wistar rat models was evaluated. No deleterious effects such as weight loss or gastrointestinal disorders on the general health status of the rats were observed (Kim Ten Doeschate, personal communication). More importantly, no bacteremia has been detected in any of the rats and no adverse effects have been noted in haematological analysis. Macroscopic examination of the visceral organs and histological analysis of

tissues indicated no abnormalities (this study). The strain also exhibited no β -glucuronidase activity *in vitro* and *in vivo* and was thus concluded to be non-toxic and safe for human consumption (34).

Real time PCR denoted the presence of strain ST4SA in the cecum in highest numbers, followed by the colon. *E. mundtii* is a lactic acid enterobacterium. Hence, its presence in the cecum is expected due to the fermentation processes that occur within this organ and being an enterobacterium would elucidate its presence in the colon.

Strains 423 and ST4SA were successful in modifying the gut microbiota in a positive manner. DGGE exhibited the absence of certain *Enterobacteriaceae* in the gut of rats fed with strains 423 and ST4SA, whilst the lactic acid bacterial content remain unchanged. Both strains also exhibited agglutination of yeast cells indicating the possible presence of mannose receptors. This is of paramount importance in the gut for excluding type 1 fimbriated pathogenic *E. coli* strains and other enterobacteria that display mannose adherence and might have contributed to the dislodging of possible *E. coli* strains in the gut.

Conclusions

L. plantarum 423 exhibits strong adhesive potential for a successful probiotic. The organism colonises the ileum and cecum of rats and positively influences the natural microflora by inhibiting enterobacteria whilst not affecting the lactic acid bacterial content. Strain 423 also displays the possible presence of mannose receptors. Histological analysis indicated no morphological changes induced to rat tissue upon adhesion of the strain indicating the safety of adhesion.

E. mundtii ST4SA displays translocation in the cecum and colon of rat tissue. This is a common phenomenon of enterococci. However, only poses a problem if the organism elicits infection. Safety evaluation of strain ST4SA indicated no infection or detrimental health effects induced to Wistar rats. Histological analysis of the intestinal tissue indicated no morphological changes or deleterious effects. This denotes that translocation of the organism does not affect the tissue and organism as a whole. The strain also positively influences the natural microflora by inhibiting enterobacteria whilst not affecting the lactic acid bacterial content. The strong possibility of mannose receptors was observed for *E. mundtii* ST4SA, an important trait for potential probiotics.

References

1. **Adawi, D., G. Molin, S. Ahrne, B. Jeppsson.** 1999. Modulation of the colonic bacterial flora affects differently bacterial translocation and liver injury in an acute liver injury model. *Microbial Ecology Health and Disease*. **11**:47-54.
2. **Adlerberth, I., L. A. Hanson, C. Svanborg.** 1995. Adhesins of *Escherichia coli* associated with extra-intestinal pathogenicity confer binding to colonic epithelial cells. *Microbes Pathogen*. **18**:373-385.
3. **Adlerberth, I., S. Ahrne, M. L. Johansson, G. Molin, L. A. Hanson, A. E. Wold.** 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. *Applied and Environmental Microbiology*. **62**:2244-2251.
4. **Alterman, E., W. M. Russell, M. A. Azcarate-Peril, R. Barrangou, B. L. Buck, O. McAuliffe, N. Souther, A. Dobson, T. Duong, M. Callanan, S. Lick, A. Hamrick, R. Cano, T. D. Klaenhammer.** 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proceedings of the National Academy of Science of the United States of America*. **102**:3906-3912.
5. **Beimfor, C., A. Krause, R. Amann, W. Ludwig, K. H. Schleifer.** 1993. *In situ* identification of lactococci, enterococci and streptococci. *Systematic and Applied Microbiology*. **16**:450-456.
6. **Bouvier, T., P. A. del Giorgio.** 2003. Factors influencing the detection of bacterial cells using fluorescence in situ hybridisation (FISH): A quantitative review of published reports. *FEMS Microbiology Ecology*. **44**:3-15.
7. **Bukowska, H., J. Pieczul-Mroz, M. Jastrzebska, K. Chelstowski, M. Naruszewicz.** 1998. Decrease in fibrinogen and LDL-cholesterol levels upon supplementation of diet with *Lactobacillus plantarum* in subjects with moderately elevated cholesterol. *Atherosclerosis*. **137**:437-438.
8. **De Vries, M. C., E. E. Vaughan, M. Kleerebezem, W. M. De Vos.** 2005. *Lactobacillus plantarum* - survival, functional and potential probiotic properties in the human intestinal tract. *International Dairy Journal*. **16**:1018-1029.

9. **Dellaglio, F., V. Bottazzi, L. D. Troatelli.** 1973. Deoxyribonucleic acid homology and vase composition in some thermophilic lactobacilli. *Journal of General Microbiology*. **74**:289-297.
10. **Franks, A. H., H. J. M. Harmsen, G. C. Raangs, G. J. Jansen, F. Schut, G. W. Welling.** 1998. Variations of bacterial populations in human feces measured by fluorescent in situ hybridisation with group-specific 16S rRNA-targeted oligonucleotide probes. *Applied and Environmental Microbiology*. **64**:3336-3345.
11. **Fujiwara, S., Y. Seto, A. Kimura, H. Hashiba.** 2001. Establishment of orally administered *Lactobacillus casei* SBT2055SR in the gastrointestinal tract of humans and its influence on intestinal microflora and metabolism. *Journal of Applied Microbiology*. **90**:343-352.
12. **Granato, D., G. E. Bergonzelli, R. D. Pridmore, L. Marvin, M. Rouvet, I. E. Corthesy-Theulaz.** 2004. Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533(La1) to human intestinal cells and mucins. *Infection and Immunity*. **72**:2160-2169.
13. **Herias, M. V., T. Midtvedt, L. A. Hanson.** 1995. Role of *Escherichia coli* P fimbriae in intestinal colonisation in gnotobiotic rats. *Infection and Immunity*. **63**:4781-4789.
14. **Kirjavainen, P. V., A. C. Ouwehand, E. Isolauri, S. J. Salminen.** 1998. The ability of probiotic bacteria to bind to human intestinal mucus. *FEMS Microbiology Letters*. **167**:185-189.
15. **Klaenhammer, T. R.** 2000. Probiotic bacteria: Today and Tomorrow. *Journal of Nutrition*. **130**:415S-416S.
16. **Kleerebezem, M., J. Boekhorst, R. van Kranenburg, D. Molenaar, O. P. Kuipers, R. Leer, R. Tarchini, S. A. Peters, H. M. Sandbrink, M. W. E. J. Fiers, W. Stiekema, R. M. K. Lankhorst, P. A. Bron, S. M. Hoffer, M. N. N. Groot, R. Kerkhoven, M. de Vries, B. Ursing, W. M. de Vos, R. J. Siezen.** 2003. Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proceedings of the National Academy of Science of the United States of America*. **100**:1990-1995.
17. **Knoetze, H., S. D. Todorov, L. M. T. Dicks.** 2007. Characterisation of bacteriocin ST4SA, produced by *Enterococcus mundtii* ST4SA isolated from soybeans. Unpublished.

18. **Kops, S. K., D. K. Lowe, W. M. Bement, A. B. West.** 1996. Migration of *Salmonella typhi* through intestinal epithelial monolayers: An *in vitro* study. *Microbiology Immunology*. **40**:799-811.
19. **Lick, S., K. Drescher, K. J. Heller.** 2001. Survival of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* in the terminal ileum of fistulated Gottingen minipigs. *Applied and Environmental Microbiology*. **9**:4137-4143.
20. **Mack, D. R., S. Ahrne, L. Hyde, S. Wei, M. A. Hollingsworth.** 2003. Extracellular MUC3 mucin secretion follows adherence of *Lactobacillus* strains to intestinal epithelial cells *in vitro*. *Gut*. **52**:827-833.
21. **Mao, Y., S. Nobaek, B. Kasravi, D. Adawi, U. Stenram, G. Molin, B. Jeppsson.** 1996. The effects of *Lactobacillus* strains and oat fiber on methotrexate-induced enterocolitis in rats. *Gastroenterology*. **111**:334-344.
22. **Mare, L., G. M. Wolfaardt, L. M. T. Dicks.** 2006. Adhesion of *Lactobacillus plantarum* 423 and *Lactobacillus salivarius* 241 to the intestinal tract of piglets, as recorded with fluorescent *in situ* hybridisation (FISH) and production of plantaricin 423 by cells colonised to the ileum. *Journal of Applied Microbiology*. **100**:838-845.
23. **Meade, H. M., S. R. Long, C. B. Ruvkun, S. E. Brown, F. M. Ausubel.** 1982. Physical and genetic characterisation of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn5 mutagenesis. *Journal of Bacteriology*. **149**:114-122.
24. **Muyzer, G., E. C. de Waal, A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology*. **59**:695-700.
25. **Myers, R. M., S. G. Fischer, L. S. Lermann, T. Maniatis.** 1985. Nearly all single base substitutions in DNA fragments joint to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Research*. **13**:3131-3145.
26. **Ouwehand, A. C., P. V. Kirjavainen, C. Shortt, S. Salminen.** 1999. Probiotics: mechanisms and established effects. *International Dairy Journal*. **9**:43-52.

27. **Ouwehand, A. C., P. V. Kirjavainen, M. M. Gronlund, E. Isolauri, S. J. Salminen.** 1999. Adhesion of probiotic microorganisms to intestinal mucus. *International Dairy Journal*. **9**:623-630.
28. **Pretzer, G., J. Snel, D. Molenaar, A. Wiersma, P. A. Bron, J. Lambert, W. M. de Vos, R. van der Meer, M. A. Smits, M. Kleerebezem.** 2005. Biodiversity-Based identification and functional characterisation of the mannose specific adhesin of *L. plantarum*. *Journal of Bacteriology*. **187**:6128-6136.
29. **Roos, S., H. Jonsson.** 2002. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiology*. **148**:433-442.
30. **Salinatro, J. P., I. G. Blake, P. A. Muirhead.** 1977. Isolation and identification of faecal bacteria from adult swine. *Applied and Environmental Microbiology*. **33**:79-84.
31. **Schurter, M. J., D. P. LeBrun, K. J. Harrison.** 2002. Improved technique for fluorescence in situ hybridisation analysis of isolated nuclei from archival, B5 or formalin fixed, paraffin wax embedded tissue. *Journal of Clinical Pathology: Molecular Pathology*. **55**:121-124.
32. **Shanahan, F.** 2002. The host-microbe interface within the gut. *Best Practice and Research Clinical Gastroenterology*. **16**:915-931.
33. **Spitz, J., R. Yuhan, A. Koutsouris, C. Blatt, J. Alverdy, G. Hecht.** 1995. Enteropathogenic *Escherichia coli* adherence to intestinal epithelial monolayers diminishes barrier function. *American Journal of Physiology*. **268**:G374-G379.
34. **Tendoeschate, K., R. Smith, L. M. T. Dicks.** 2007. *In vivo* assessment of the safety of orally administered novel probiotics, *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 in a male Wistar rat model. Unpublished.
35. **Van Reenen, C. A., L. M. T. Dicks, M. L. Chikindas.** 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology*. **84**:1131-1137.
36. **Wells, C. L., M. A. Maddaus, R. L. Simmons.** 1988. Proposed mechanisms for the translocation of intestinal bacteria. *Review of Infectious Diseases*. **10**:958-978.

37. **Wells, C. L., R. P. Jechorek, L. B. Twiggs, D. C. Brooker.** 1990. Recovery of viable bacteria from pelvic lymph nodes of patients with gynecologic tumors. *Journal of Infectious Diseases*. **162**:1216-1218.
38. **Wells, C. L., S. L. Erlandsen.** 1991. Localisation of translocating *Escherichia coli*, *Proteus mirabilis* and *Enterococcus faecalis* within cecal and colonic tissues of monoassociated mice. *Infection and Immunity*. **59**:4693-4697.

Table 1. Strain-specific primers used in the design of probes to detect *L. plantarum* 423 and *E. mundtii* ST4SA. Primers were also used for real time PCR

Primer set	Sequence 5' to 3'	Size of amplicon (bp)
Bac 423F RT Bac 423R RT	GACATTTACATTGAGTAGGAACTAG GTAACCCATTACCATAGTATTTAC	147
Mund ST4SA F Mund ST4SA R	ATGTCACAAGTAGTAGGTGG AGCTAAATTTCGCAGCAGA	123

Table 2. Quantification of strains by using real time PCR

Strain	Section	Av. Ct	Quant.*	Set to calibrator [#]
423	Duodenum	30.76	2.8	1
	Ileum	25.99	60	22
	Cecum	25.76	70	26
	Colon	30.84	2.7	1
ST4SA	Jejunum	27.01	1.3	1
	Ileum	26.29	2.1	1.6
	Cecum	19.21	251	193
	Colon	22.26	32	24

* Calculation of the quantity of each bacteriocin gene in specific tissue sections were determined by equations obtained in the standard curve.

[#] Samples that contained the lowest quantity of the bacteriocin gene served as the calibrator sample.

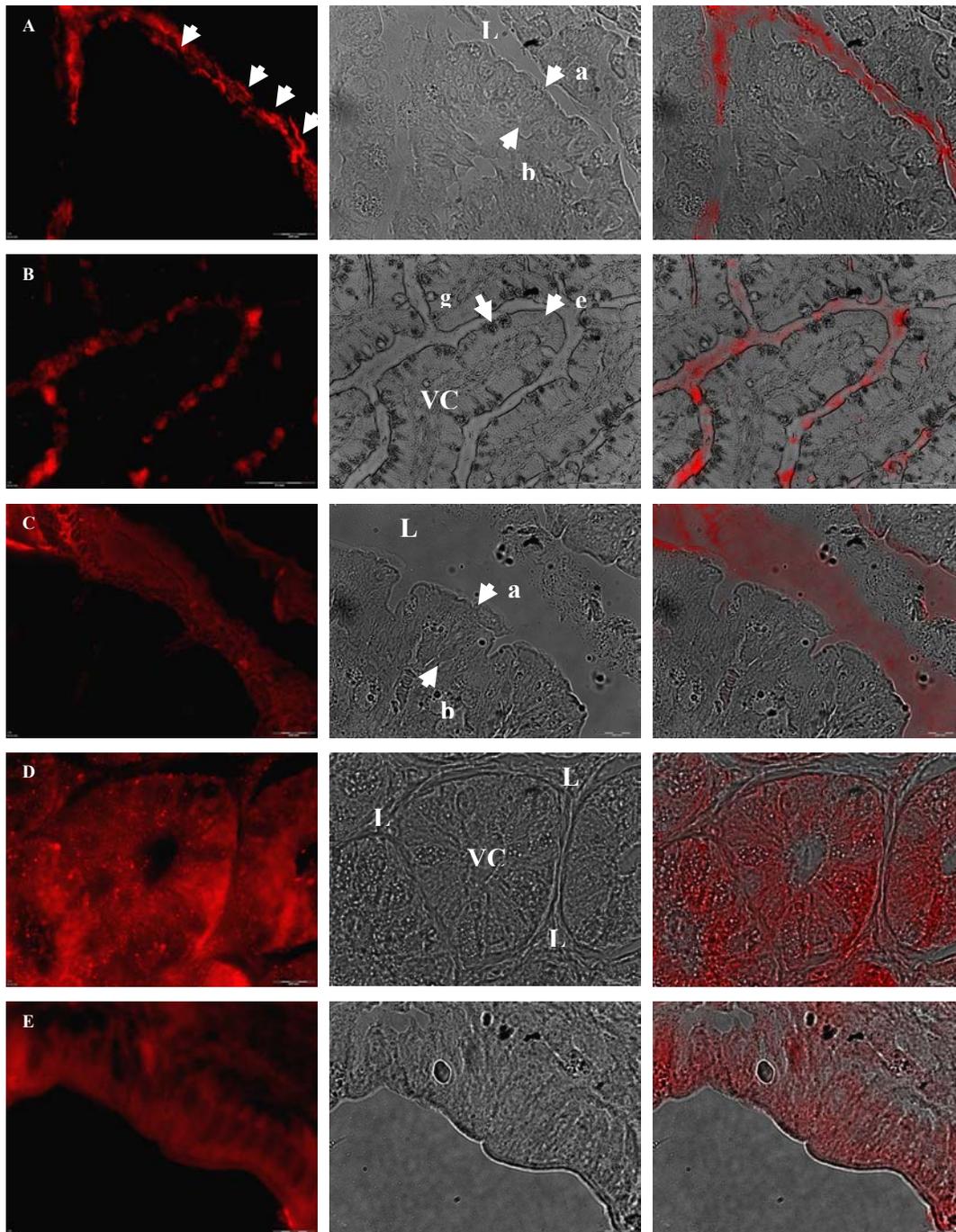


FIG. 1. FISH images of *L. plantarum* 423 and *E. mundtii* ST4SA adhered to rat intestinal tissue under 600x magnification. Fluorescent images are shown on the left, phase images in the middle and overlaid images on the right. A: *L. plantarum* 423 adhering to the cecum. Arrows indicate the strong fluorescent signal recorded along the epithelial lining. B: Strain 423 detected in the lumen content of the colon, with no binding to colonic tissue. C: FISH on cecum tissue of group D rats with probe 423. D: FISH indicating intracellular activity of strain ST4SA (indicated with arrows). E:

FISH on cecum tissue of group D rats with probe ST4SA. L = lumen, a = apical side of the enterocyte, b = basal side of enterocyte, VC = villus core, g = goblet cell, e = enterocyte.

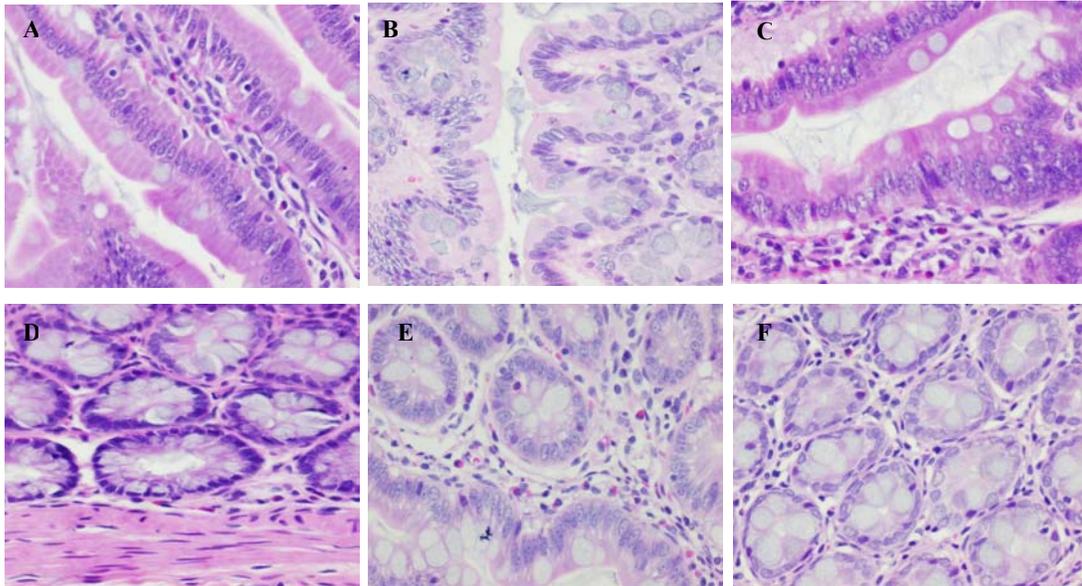


FIG. 2. H&E stains of rat tissue. A: colonic tissue of rats administered with *L. plantarum* 423; B: colonic tissue of rats administered with *E. mundtii* ST4SA; C: colonic tissue of control rats; D: tissue from the ileum of rats administered with strain 423; E: tissue from the ileum of rats administered with *E. mundtii* ST4SA; F: tissue from the ileum of control rats. No morphological changes can be visualized upon adhesion of the strains.

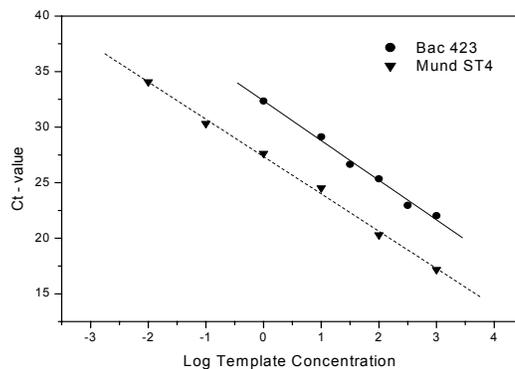


FIG. 3. Standard curves for Bac 423 and Mund ST4SA. Bac 423 ($y = -3.5694x + 32.361$). Mund ST4SA ($y = -3.361x + 27.353$).

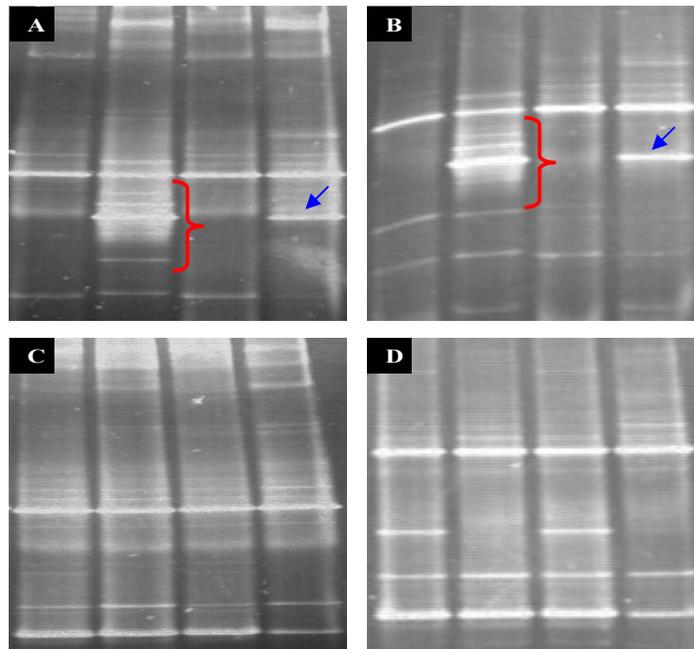


FIG. 4. DGGE profiles of lactic acid bacteria and *Enterobacteriaceae* in intestinal tissue samples, after treatment with *L. plantarum* 423 and *E. mundtii* ST4SA. DGGE profiles of intestinal tissue sample from group D rats served as the controls. A: Effect of strain 423 on the enterobacteriaceae (left to right, cecum, cecum control, colon, colon control); B: effect of strain ST4SA on the enterobacteriaceae (left to right, cecum, cecum control, colon, colon control); C: effect of strain 423 on the lactic acid bacteria population (left to right, cecum, cecum control, colon, colon control); D: effect of strain ST4SA on the lactic acid bacteria population (left to right, cecum, cecum control, colon, colon control).

Chapter 5

Surface proteins of *Lactobacillus plantarum* 423 may confer binding to Caco-2 cell lines and lead to competitive exclusion and displacement of *Clostridium sporogenes* LMG 13570 and *Enterococcus faecalis* LMG 13566

Abstract

Surface proteins of *Lactobacillus plantarum* 423 were characterized to determine their possible role in adhesion to Caco-2 cells. A multifunctional “intracellular” protein, elongation factor Tu (EF-Tu), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and triosephosphate isomerase (TPI) were detected. Removal of surface proteins reduced adherence of strain 423 to Caco-2 cells by 40%, suggesting that these proteins play a role in adhesion. Adherence of *Clostridium sporogenes* LMG 13570 and *Enterococcus faecalis* LMG 13566 to Caco-2 cells were inhibited by 70% and 90% respectively, when incubated with strain 423. Strain 423 excluded *C. sporogenes* LMG 13570 from Caco-2 cells by 73% and displaced the opportunistic pathogen by 80%. *E. faecalis* LMG 13566 was excluded by 60% and displaced from Caco-2 cells by 90%. Despite removal of the surface proteins, *L. plantarum* 423 was still capable of competitively adhering to Caco-2 cells and reduced adherence of *C. sporogenes* LMG 13570 by 50% and *E. faecalis* LMG 13566 by 70%.

Introduction

Probiotic therapy has become an attractive alternative to overcome problems associated with antibiotics, such as emergence of multi-drug resistant pathogens. The complex mechanisms by which they function in eliminating pathogens, minimises the chances for the development of resistance against probiotics (16). In addition, the use of probiotics may possibly eliminate the need to re-establish the normal gut microflora as often required after antibiotic therapy (17, 38). Probiotics also benefit the host in a number of ways, for example stimulate the immune system (50), alleviate problems associated with inflammatory bowel diseases (27), reduce cholesterol levels (23) and lower high blood pressure (25). However, the most prevalent health benefit has been their role in the treatment of diarrhoea (5, 11, 42). This effect is dependent on the ability of probiotics to adhere to intestinal enterocytes and prevent colonisation of pathogens. Adhesion to the gastrointestinal tract is also a pre-requisite for potential probiotics to prevent elimination by peristalsis (34).

Several functions have been proposed for lactobacilli surface proteins, one of which includes the role in cell adhesion and surface recognition (39, 45). The absence of an outer membrane and the presence of multiple peptidoglycan layers has resulted in different strategies for anchoring proteins to the cell surface (46). Three main strategies have been identified and they include covalent attachment (e.g., the LPXTG containing proteins) (36), charge interactions (e.g., Slp) (32) and hydrophobic interactions (30). Surface layer proteins (Slp) are regular structures covering the cell surface and are composed of monomeric protein units known as the S-protein (45). Common features of all lactobacilli Slp is their ability to spontaneously crystallize into a two dimensional structure outside the bacterial cell surface (44) and their extraction with charged reagents such as guanidine hydrochloride and lithium chloride (32). Genes encoding Slp of *Lactobacillus acidophilus* (9), *Lactobacillus brevis* (49), *Lactobacillus helveticus* (12) and *Lactobacillus crispatus* (43) have been sequenced. Slp have also been described for *Lactobacillus amylovorus* (8), *Lactobacillus buchneri* (19), *Lactobacillus gallinarum* (32), *Lactobacillus kefir* and *Lactobacillus parakefir* (33), but none of the genes encoding these Slp have been sequenced. *L. acidophilus* group B strains, *Lactobacillus johnsonii* and *Lactobacillus gasseri* do not appear to have characteristic Slp (8) but rather aggregation promoting factors (APF) (48). APF was removed by treatment with 5 M LiCl and displayed a high alkaline

isoelectric point. They also appear to be anchored by specific non-covalent interactions and share many characteristics with typical Slp of other lactobacilli (48).

In addition to Slp and APF, anchorless multifunctional proteins have also been described for lactobacilli (4, 22). EF-Tu and GroEl are intracellular proteins that function in protein synthesis and protein folding. However, these proteins have been identified on the cell surface and culture medium of *L. johnsonii* La1 where they mediate adhesion to mucin and human epithelial cells (4, 22). EF-Tu and GroEl are referred to as anchorless multifunctional proteins as no established signal sequences or anchoring motifs are present in their predicted sequences (14). Surface enzymes such as PrtB of *Lactobacillus delbrueckii* subsp. *bulgaricus*, has been described (21). A novel bacterial surface protein, BspA, has also been identified in *Lactobacillus fermentum* strain BR11. This protein shares significant homology with family III of bacterial solute binding proteins (47).

The role of surface proteins in conferring adherence of lactobacilli to human enterocytes, have been demonstrated for different species (10, 13). The present study characterized the surface proteins of *L. plantarum* 423 and determined the role in adhesion to Caco-2 cell lines. The ability of *L. plantarum* 423 to competitively exclude and displace *Clostridium sporogenes* LMG 13570 and *Enterococcus faecalis* LMG 13566 from Caco-2 cells was also investigated.

Materials and methods

Bacterial strains and culture conditions

L. plantarum 423, *Lactobacillus plantarum* WCFS1 and *Lactobacillus acidophilus* ATCC 4356 were cultured in De Man Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37°C, without aeration. *C. sporogenes* LMG 13570 was cultured in tryptone soy broth (TSB, Biolab) and *E. faecalis* LMG 13566 in brain heart infusion broth (BHI, Biolab) at 37°C without aeration. *L. plantarum* 423, *C. sporogenes* LMG 13570 and *E. faecalis* LMG 13566 were cultured for 18 h until OD₆₀₀ = 1.8, which is equivalent to approximately 1 × 10⁶ CFU/ml. Cells were harvested (10000 × g, 10 min, 4°C), washed twice with PBS and resuspended in 100 µl PBS for adhesion assays.

SDS-PAGE of surface proteins, purification and characterization

L. plantarum 423, *L. plantarum* WCFS1 and *L. acidophilus* ATCC 4356 were inoculated into 10 ml of MRS broth (Biolab) and cultured for 18 h (OD₆₀₀ = 1.8).

Cells were harvested ($10000 \times g$, 10 min, 4°C) and washed twice with ice-cold sterile distilled water. Cells were treated with 2 ml 4 M guanidine-HCl for 1 h and 2 ml 5 M LiCl for 30 min. Both reactions were incubated at 37°C (9). Supernatants were separated by SDS-PAGE (10%, w/v) as described by Laemmli (29). Protein bands were visualised by silver staining using the ProteoSilverTM Plus Silver Stain Kit (Sigma, Steinheim, Germany). Bands with molecular weights between 20 and 45 kDa were excised, destained with the ProteoSilverTM Plus Silver Stain Kit (Sigma) and prepared for identification by mass spectrometry, according to the method of Shevchenko *et al.* (41). Intact protein bands were digested with sequencing grade modified trypsin (Promega, Madison, USA). The procedure included reduction and acetamidation steps prior to tryptic digestion (41). Digested peptides were analysed with Waters API Q-TOF Ultima fitted with a Waters CapLC Gradient and an Atlantis dC18 Column. 5 μl samples were injected at a flow rate of 200 nl/min. Purification and characterization of surface proteins were performed in duplicate.

Culturing of Caco-2 cells

Caco-2 cells (Highveld Biological Pty. Ltd., Kelvin, Johannesburg) were cultured in Minimal Essential Medium (MEM) Earle's Base (Highveld Biological), supplemented with 10% (v/v) fetal bovine serum (Sigma), 100 U ml⁻¹ penicillin and 100 U ml⁻¹ streptomycin (Sigma), in the presence of 5% CO₂. Cells were cultured in 12-well plates (Multiwell_{TM}, Becton Dickinson Labware, NJ, USA) at 1×10^5 cells per well at 37°C , until confluency.

Competitive, exclusion and displacement adhesion assays

Surface proteins of *L. plantarum* 423 were removed by treatment with 4 M guanidine-HCl for 30 min prior to adhesion assay to determine their role in adherence to Caco-2 cells. 100 μl (approximately 1×10^6 cfu) of *L. plantarum* 423 (with and without surface proteins) was added to Caco-2 cells (1×10^5 Caco-2 cells per well) in separate wells and incubated for 2 h at 37°C . After incubation, bacterial cells were withdrawn from the wells and Caco-2 cells were washed twice with 1 ml sterile PBS. Adhering cells were treated with 1 ml 0.5% (v/v) Triton X-100 for 3 min on ice, followed by serial dilutions and plating onto MRS agar (Biolab).

Competition for adherence of *L. plantarum* 423 (with and without surface proteins), *C. sporogenes* LMG 13570 and *E. faecalis* LMG 13566 to Caco-2 cells were studied by inoculating each well (1×10^5 Caco-2 cells) with 100 μl (approximately 1×10^6 cfu) of strain 423 and 100 μl (approximately 1×10^6 cfu) of

either *C. sporogenes* LMG 13570 or *E. faecalis* LMG 13566. After 2 h incubation at 37°C, the bacterial cells were withdrawn from the wells and the Caco-2 cells washed with two volumes of 1 ml sterile PBS, followed by treatment with 1 ml 0.5% (v/v) Triton X-100. Ten-fold dilutions of adhering bacterial cells were prepared and plated onto MRS agar (Biolab) for enumeration of *L. plantarum* 423, on TSB (Biolab) plates for *C. sporogenes* LMG 13570 and on BHI (Biolab) plates for *E. faecalis* LMG 13566.

The ability of *L. plantarum* 423 to competitively exclude each opportunistic pathogen from adhering to Caco-2 cells was determined by inoculating each well (1×10^5 Caco2 cells) with 100 μ l of strain 423 (approximately 1×10^6 cfu). After 1 h of incubation at 37°C, 100 μ l of either *C. sporogenes* LMG 13570 or *E. faecalis* LMG 13570 was added to *L. plantarum* 423 in separate wells and incubated for a further 60 min. Non-adhering cells were removed by washing twice with sterile PBS, followed by treatment with 0.5% (v/v) Triton X-100, serially diluted and plated out onto the respective agar media.

In a separate experiment, the ability of *L. plantarum* 423 to displace *C. sporogenes* LMG 13570 and *E. faecalis* LMG 13570 from Caco-2 cells was determined by first incubating Caco-2 cells with 100 μ l of either *C. sporogenes* LMG 13570 or *E. faecalis* LMG 13570 for 1 h at 37°C, followed by incubation in the presence of *L. plantarum* 423 for a further 60 min. Cells were serially diluted and plated onto respective agar media. All adhesion assays were performed in triplicate.

Results

SDS-PAGE of surface proteins, purification and characterization

L. plantarum 423 does not possess an apparent S-layer protein as present in most *Lactobacillus* spp. Treatment of bacteria with LiCl is a routine procedure to extract S-layer proteins. However, treatment of *L. plantarum* 423 with LiCl yielded only a few proteins, with the highest molecular weight approximately 20kDa. This indicated no evidence for the presence of a distinct S-layer protein (Fig. 1). Treatment of cells with guanidine-HCl proved more efficient, as observed by a greater variety of proteins being extracted (Fig. 1).

Dominant protein bands 1, 2 and 3 of *L. plantarum* 423 (lane 6, Fig. 1) were excised, purified and characterized by mass spectrometry and Mascot MS/MS ion searches. In the latter, confidence that a protein has been identified correctly may be

derived from multiple matches to peptides from a subject protein in the database. The score is based on an absolute probability (P) that the match between the protein in question and the subject protein in the database is a random event. Therefore, the higher the score the greater the chance the match is not a random event. Results indicated that band 1 reflected significant homology to the elongation factor Tu (EF-Tu) of other *L. plantarum* strains, as depicted by a score of 443. Band 2 revealed extensive homology to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein of other *L. plantarum* strains with a score of 471. Band 3 indicated homology to triosephosphate isomerase (TPI) of other *L. plantarum* strains with a score of 110. In addition, molecular sizes of the proteins correlated well with the respective matches in the database, confirming the identity of the proteins.

Competitive, exclusion and displacement adhesion assays

Treatment of *L. plantarum* 423 with guanidine-HCl reduced adhesion of the strain to Caco-2 cells by 40%. *L. plantarum* 423 demonstrated its probiotic potential by inhibiting the adherence of *C. sporogenes* LMG 13570 and *E. faecalis* LMG 13566 to Caco-2 cells by 70% and 90%, respectively. Strain 423 excluded *C. sporogenes* LMG 13570 by 73% and displaced the pathogen by 80%. *E. faecalis* LMG 13566 was excluded by 60% and displaced by 90%. Despite the removal of the surface proteins, *L. plantarum* 423 was still capable of competitively adhering to Caco-2 cells and reduced adherence of *C. sporogenes* LMG 13570 by 50% and *E. faecalis* LMG 13566 by 70%.

Discussion

L. johnsonii and *L. gasseri* are two *Lactobacillus* spp. previously reported to not possess a distinct Slp (8). Both species contain novel surface proteins (APF) which share similar characteristics to typical Slp of most lactobacilli (48). The present study emphasizes the absence of a Slp in another *Lactobacillus* species, *L. plantarum*. Characterization of surface proteins of *L. plantarum* 423 revealed the presence of an “intracellular” multifunctional protein (EF-Tu) and surface glycolytic enzymes (GAPDH and TPI). EF-Tu, GAPDH and TPI do not contain a signal sequence or anchoring motif in their predicted sequences to explain their presence on the surface of the bacterium. The mechanism of adherence of these proteins to the surface of the producer cell is not well understood. However, their detection on the surfaces of other

Gram-positive bacteria have been reported (3, 22). These proteins were removed by treatment with guanidine-HCl, suggesting non-covalent attachment to the cell wall.

The EF-Tu protein binds to GTP and functions during protein synthesis (20). Initially it was thought that the molecule is restricted to within the cytoplasm of prokaryotes. However, later studies have shown that the EF-Tu molecule is also associated with the membrane of *E. coli* (28) and the periplasm of *Neisseria gonorrhoeae* (37). In lactobacilli, EF-Tu may function as an “envelope associated protein”, which may be released from cells during osmotic shock (35). The role of EF-Tu as an adhesion molecule was described for *L. johnsonii* La1 (NCC 533) when the molecule mediated attachment of the strain to Caco-2 cells and mucus. The importance of the protein in mediating attachment was reflected by the ability of the purified protein to inhibit binding of strain La1 to mucin by up to 40% (22). In addition, the EF-Tu protein of *L. johnsonii* NCC 533 shares 84% homology with an EF-Tu protein isolated from *L. plantarum*, suggesting functional similarity between these molecules (22). Up-regulation of EF-Tu in the presence of mucus suggests that this molecule participates as a major adhesin in *L. plantarum* 423.

GAPDH functions in glycolysis, catalyzing the breakdown of glucose to release carbon as an energy source. However, other functions for the enzyme, such as activation of transcription (51) and initiation of apoptosis (24) have been described. The position of the enzyme on the surface of Gram-positive bacteria has also been depicted, with involvement in pathogen-host interactions (3, 7, 40). GAPDH has also been detected in the cytoplasmic fraction and cell surface of *Streptococcus pneumoniae* (3). Recent studies have shown that some strains of *L. acidophilus* and *L. crispatus* have GAPDH as a major constituent of their extracellular proteome (26). The GAPDH of *L. crispatus* ST1 binds to lipoteichoic acid (LTA) at very low pH, suggesting that negatively charged LTA may be involved in the anchoring of the protein to surfaces (1). TPI also functions in the glycolytic pathway, catalyzing the reversible interconversion of triose phosphate isomers facilitating efficient energy production (2). Genome analysis of *L. plantarum* WCFS1 revealed over 200 putative extracellular proteins with 57 predicted to be secreted into their surrounding medium or associated to the cell wall by unknown mechanisms (6). This indicates the importance of extracellular proteins in the cross-talk between lactobacilli and their host. Surface enzymes such as GAPDH and TPI can be classified as multifunctional proteins that may be released from the cell and assist the organism to adapt to changes

in the environment. Hence, GAPDH and TPI of *L. plantarum* 423 may function as adhesins which play a crucial role in colonisation of the organism to its environment.

Caco-2 cells express several markers that are distinctive of normal small intestinal villi and have served as excellent *in vitro* models for understanding the mechanisms involved in adherence of probiotic bacteria and invasion of pathogens, as depicted by the number of reviews (15, 18, 22, 31). Caco-2 cells provide an excellent system for understanding not only the mechanisms by which commensals adhere to the intestine but also how these bacteria interact with pathogens that compete in the same environment. *L. plantarum* 423 portrayed exceptional probiotic potential by competitively adhering to Caco-2 cells and reduced the adherence of *C. sporogenes* LMG 13570 and *E. faecalis* LMG 13566 significantly. The strain was more successful in displacing both opportunistic pathogens from Caco-2 cells. This is important attribute for a potential probiotic for counteracting an adhering pathogen in the case of infection.

Conclusions

Lactobacillus plantarum 423 does not possess a Slp, but a multifunctional protein, EF-Tu and glycolytic enzymes GAPDH and TPI were detected. Recent evidence has shown the presence of these proteins on surfaces of Gram-positive bacteria and additional functions have been described. EF-Tu, GAPDH and TPI can be classified as multifunctional proteins that are released from the cell to assist *L. plantarum* 423 with adapting to changes in the environment. Removal of surface proteins reduced adherence of strain 423 to Caco-2 cells by 40% suggesting that EF-Tu, GAPDH and TPI may function as adhesins in *L. plantarum* 423. Strain 423 exhibited excellent probiotic potential by competitively adhering to Caco-2 cells in the presence of *C. sporogenes* LMG 13570 and *E. faecalis* LMG 13570. More importantly, strain 423 displaced *C. sporogenes* LMG 13570 by 80% and *E. faecalis* LMG 13570 by 90%.

References

1. **Antikainen, J., V. Kupannen, K. Lahteenmaki, T. K. Korhonen.** 2007. pH-dependent association of enolase and glyceraldehyde 3-phosphate dehydrogenase of *Lactobacillus crispatus* with the cell wall and lipoteichoic acids. *Journal of Bacteriology*. **189**:4539-4543.
2. **Becker, P., W. Hufnagle, G. Peters, M. Herrman.** 2001. Detection of differential gene expression in biofilm-forming versus planktonic populations

- of *Staphylococcus aureus* using micro-representational difference analysis. Applied and Environmental Microbiology. **67**:2958-2965.
3. **Bergmann, S., M. Rohde, S. Hammerschmidt.** 2004. Glyceraldehyde 3-phosphate dehydrogenase of *Streptococcus pneumoniae* is a surface-displayed plasminogen-binding protein. Infection and Immunity. **72**:416-2419.
 4. **Bergonzelli, G. E., D. Granato, R. D. Pridmore, L. F. Marvin-Guy, D. Donnicola, I. E. Corthesy-Theulaz.** 2006. GroEl of *Lactobacillus johnsonii* La1 (NCC533) is cell surface associated: potential role in interactions with the host and the gastric pathogen *Helicobacter pylori*. Infection and Immunity. **74**:425-434.
 5. **Biller, J. A., A. J. Katz, A. F. Flores, T. M. Buie, S. L. Gorbach.** 1995. Treatment of recurrent *Clostridium difficile* colitis with *Lactobacillus* GG. Journal of Pediatric Gastroenterology. **21**:224-226.
 6. **Boekhorst, J., M. Wels, M. Kleerebezem, R. J. Siezen.** 2006. The predicted secretome of *Lactobacillus plantarum* WCFS1 sheds light on interactions with its environment. Microbiology. **152**:3175-3183.
 7. **Boel, G., H. Jin, V. Pancholi.** 2005. Inhibition of cell surface export of group A streptococcal anchorless surface dehydrogenase affects bacterial adherence and antiphagocytic properties. Infection and Immunity. **73**:6237-6248.
 8. **Boot, H. J., C. P. A. M. Kolen, B. Pot, K. Kersters, P. H. Pouwels.** 1996. The presence of two S-layer protein-encoding genes is conserved among species related to *Lactobacillus acidophilus*. Microbiology. **142**:2375-2384.
 9. **Boot, H. J., C. P. A. M. Kolen, J. M. van Noort, P. H. Pouwels.** 1993. S-layer protein of *Lactobacillus acidophilus* ATCC 4356: purification, expression in *Escherichia coli* and nucleotide sequence of the corresponding gene. Journal of Bacteriology. **175**:6089-6096.
 10. **Boris, S., J. E. Suarez, C. Barbes.** 1997. Characterisation of the aggregation promoting factor from *Lactobacillus gasseri*, a vaginal isolate. Journal of Applied Microbiology. **83**:413-420.
 11. **Boyle, R. J., R. M. Robins-Browne, M. L. K. Tang.** 2006. Probiotic use in clinical practice: what are the risks? American Journal of Clinical Nutrition. **83**:1256-1264.
 12. **Callegari, M. L., B. Riboli, J. W. Sanders, P. S. Cocconcelli, J. Kok, G. Venema, L. Morelli.** 1998. The S-layer gene of *Lactobacillus helveticus*

- CNRZ 892: cloning, sequence and heterologous expression. *Microbiology*. **144**:719-726.
13. **Chen, X., J. Xu, J. Shuai, J. Chen, Z. Zhang, W. Fang.** 2007. The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* 0157:H7 and *Salmonella typhimurium*. *International Journal of Food Microbiology*. **115**:307-312.
 14. **Chhatwal, G. S.** 2002. Anchorless adhesins and invasins of Gram-positive bacteria: a new class of virulence factors. *Trends in Microbiology*. **10**:205-208.
 15. **Finlay, B. B., S. Falkow.** 1990. *Salmonella* interactions with polarized intestinal Caco-2 epithelial cells. *Journal of Infectious Diseases*. **162**:1096-1106.
 16. **Fioramonti, J., V. Theodorou, L. Beuno.** 2003. Probiotics: what are they? What are their effects on gut physiology. *Best Practice and Research Clinical Gastroenterology*. **17**:711-724.
 17. **Fuller, R.** 1989. Probiotics in man and animals. *Journal of Applied Bacteriology*. **66**:365-378.
 18. **Gaillard, J. L., P. Berche, J. Mounier, S. Richard, P. Sansonetti.** 1987. In vitro model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. *Infection and Immunity*. **55**:2822-2829.
 19. **Garrote, G. L., L. Delfederico, R. Bibiloni, A. G. Abraham, P. F. Perez, L. Semorile, G. L. De Antoni.** 2004. Lactobacilli isolated from kefir: evidence of the presence of S-layer proteins. *Journal of Dairy Research*. **71**:222-230.
 20. **Gaucher, E. A., M. M. Miyamoto, S. A. Benner.** 2001. Function-structure analysis of proteins using covarion-based evolutionary approaches: Elongation factors. *Proceedings of the National Academy of Science of the United States of America*. **98**:548-542.
 21. **Gilbert, C., D. Atlan, B. Blanc, R. Portalier, L. Lapierre, B. Mollet.** 1996. A new cell surface proteinase: sequencing and analysis of the *prtB* gene from *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Journal of Bacteriology*. **178**:3059-3065.
 22. **Granato, D., G. E. Bergonzelli, R. D. Pridmore, L. Marvin, M. Rouvet, I. E. Corthesy-Theulaz.** 2004. Cell surface-associated elongation factor Tu

- mediates the attachment of *Lactobacillus johnsonii* NCC533(La1) to human intestinal cells and mucins. *Infection and Immunity*. **72**:2160-2169.
23. **Grunewald, K. K.** 1982. Serum cholesterol levels in rats fed skim milk fermented by *Lactobacillus acidophilus*. *Journal of Food Science*. **47**:2078-2079.
 24. **Hara, M. R., N. Agrawal, S. F. Kim.** 2005. S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 binding. *Natural Cell biology*. **7**:665-674.
 25. **Hata, Y., M. Yamamoto, M. Ohni, K. Nakajima, Y. Nakamura, T. Takano.** 1996. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *American Journal of Clinical Nutrition*. **64**:767-771.
 26. **Hurmala, V., S. Edelman, J. Antikainen, M. Baumann, K. Lahteenmaki, T. K. Korhonen.** 2007. Extracellular proteins of *Lactobacillus crispatus* enhance activation of human plasminogen. *Microbiology*. **153**:1112-1122.
 27. **Isolauri, E., P. V. Kirjavainen, S. Salminen.** 2002. Probiotics: a role in the treatment of intestinal infection and inflammation. *Gut*. **50**:54-59.
 28. **Jacobson, G. R., J. P. Rosenbusch.** 1976. Abundance and membrane association of elongation factor Tu in *E. coli*. *Nature*. **261**:23-26.
 29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**:680-685.
 30. **Leenhouts, K., G. Buist, J. Kok.** 1999. Anchoring of proteins to lactic acid bacteria. *Antonie van Leeuwenhoek*. **76**:367-376.
 31. **Lehto, E. M., S. Salminen.** 1997. Adhesion of two *Lactobacillus* strains, one *Lactococcus* and one *Propionibacterium* to cultured human intestinal Caco-2 cell line. *Bioscience and Microflora*. **16**:13-17.
 32. **Masuda, K., T. Kawata.** 1983. Distribution and chemical characterisation of regular arrays in the cell walls of strains of the genus *Lactobacillus*. *FEMS Microbiology Letters*. **20**:145-150.
 33. **Masuda, K., T. Kawata.** 1981. Characterisation of a regular array in the wall of *Lactobacillus buchneri* and its reattachment to the other wall components. *Journal of General Microbiology*. **124**:81-90.

34. **Morelli, L.** 2000. *In vitro* selection of probiotic lactobacilli: A critical appraisal. *Current Issues in Intestinal Microbiology*. **1**:59-67.
35. **Nakamura, J., D. Ito, K. Nagai, Y. Umehara, M. Hamachi, C. Kumagai.** 1997. Rapid and sensitive detection of hiochi bacteria by amplification of hiochi bacterial common antigen gene by PCR method and characterisation of the antigen. *Journal of Fermentation and Bioengineering*. **185**:7019-7023.
36. **Navarre, W. W., O. Schneewind.** 1994. Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in Gram positive bacteria. *Molecular Microbiology*. **14**:115-121.
37. **Porcella, S. F., R. J. Belland, R. C. Judd.** 1996. Identification of an EF-Tu protein that is periplasm-associated and processed in *Neisseria gonorrhoeae*. *Microbiology*. **142**:2481-2489.
38. **Rolfe, R. D.** 2000. The role of probiotic cultures in the control of gastrointestinal health. *Journal of Nutrition*. **130**:396S-402S.
39. **Sara, M., U. B. Sleytr.** 2000. S-layer proteins. *Journal of Bacteriology*. **182**:859-868.
40. **Seifert, K. N., W. P. McArthur, A. S. Bleiweis, L. J. Brady.** 2003. Characterisation of group B streptococcal glyceraldehyde 3-phosphate dehydrogenase: surface localisation, enzymatic activity and protein-protein interactions. *Canadian Journal of Microbiology*. **49**:350-356.
41. **Shevchenko, A., M. Wilm, O. Vorm, M. Mann.** 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Analytical Chemistry*. **68**:850-858.
42. **Shornikova, A. V., I. A. Casas, E. Isolauri, H. Mykkanen, T. Vesikari.** 1997. *Lactobacillus reuteri* as a therapeutic agent in acute diarrhoea in young children. *Journal of Pediatric Gastroenterology and Nutrition*. **24**:399-404.
43. **Sillanpaa, J., B. Martinez, J. Antikainen, T. Toba, N. Kalkkinen, S. Tankka, K. Lounatmaa, J. Keranen, M. Hook, B. Westerlund-Wikstrom, P. H. Pouwels, T. K. Korhonen.** 2000. Characterisation of the collagen-binding S-layer protein CbsA of *Lactobacillus crispatus*. *Journal of Bacteriology*. **182**:6440-6450.
44. **Sleytr, U. B., P. Messner.** 1988. Crystalline surface layers in prokaryotes. *Journal of Bacteriology*. **170**:2891-2897.

45. **Sleytr, U. B., T. J. Beveridge.** 1999. Bacterial S-layers. *Trends in Microbiology*. **7**:253-260.
46. **Stahl, S., M. Uhlen.** 1997. Bacterial surface display: trends and progress. *Trends in Biotechnology*. **15**:185-192.
47. **Turner, M. S., P. Timms, L. M. Hafner, P. M. Giffard.** 1997. Identification and characterisation of a basic cell surface-located protein from *Lactobacillus fermentum* BR11. *Journal of Bacteriology*. **179**:3310-3316.
48. **Ventura, M., I. Jankovic, D. C. Walker, R. D. Pridmore, R. Zink.** 2002. Identification and characterisation of novel surface proteins in *Lactobacillus johnsonii* and *Lactobacillus gasseri*. *Applied and Environmental Microbiology*. **68**:6172-6181.
49. **Vidgren, G., I. Palva, R. Pakkanen, K. Lounatmaa, A. Palva.** 1992. S-layer protein gene of *Lactobacillus brevis*: cloning by polymerase chain reaction and determination of the nucleotide sequence. *Journal of Bacteriology*. **174**:7419-7427.
50. **Yokokura, T., K. Nomoto, T. Shimizu, K. Nomoto.** 1986. Enhancement of hematopoietic response of mice by subcutaneous administration of *Lactobacillus casei*. *Infection and Immunity*. **52**:156-160.
51. **Zheng, L., R. G. Roeder, Y. Luo.** 2003. S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component. *Cell*. **114**:255-266.

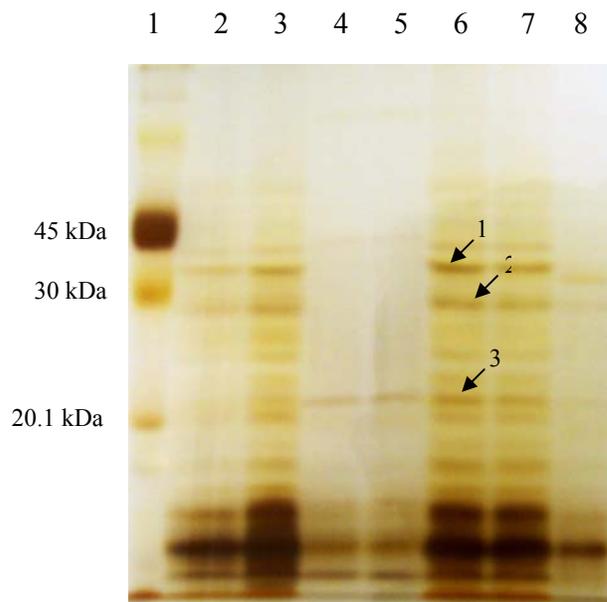


FIG. 1. SDS-PAGE of surface proteins of *L. plantarum* 423. Lane 1: High range protein molecular weight marker; lanes 2 and 3: Guanidine-HCl extraction of *L. plantarum* WCFS1; lane 4: LiCl extraction of *L. plantarum* WCFS1; lane 5: LiCl extraction of *L. plantarum* 423; lanes 6 and 7: Guanidine-HCl extraction of *L. plantarum* 423; lane 8: Guanidine-HCl extraction of *L. acidophilus* ATCC 4356. Band 1 is EF-Tu, band 2 is GAPDH and band 3 is TPI.

Chapter 6

Discussion and concluding remarks

General discussion and conclusions

The human gastrointestinal system is inhabited by a dynamic and complex microbial ecosystem. The advent of molecular techniques has facilitated a greater understanding of the complex mechanisms that occur in bacterial-host interactions and as a result we have gained a greater appreciation for the indigenous microflora (probiotics). Much attention has focused on the molecular basis of crosstalk between enteric pathogens and the intestinal epithelium (4-7). Excluding stimulation of the immune system, the molecular mechanisms of probiotic adhesion, still remain largely unknown. Advances in molecular technology will undoubtedly contribute to this area of research and provide superior quantitative and qualitative analysis on the adhesive properties of potential probiotics. The present study aimed to contribute to this area of research and reports on the use of current molecular techniques to provide insight on the molecular mechanisms of adhesion of potential probiotic strains *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA.

Probiotic adhesion to host tissue is mediated by non-specific mechanisms (hydrophobic interactions and electrostatic forces) and specific interactions involving protein or carbohydrate molecules. The latter has proven critical for probiotic action as these molecules recognise receptors that may also be specific for the colonisation of pathogens. In addition to phenotypic evaluation of the adhesive potential of a probiotic, detection of genes encoding these adhesive molecules should also become a focus to determine the ability of a particular probiotic to target specific receptors. Research on the expression of these genes under digestive stress conditions should also be a priority as, although it may be useful to ascertain the presence of these genes, it may serve no purpose if these genes are not up-regulated under appropriate conditions.

This study encompasses the first report on expression of mucus adhesion genes under digestive stress conditions *in vitro*. Results concluded that *L. plantarum* 423 possess all the necessary requirements to be an efficient probiotic. Mucus adhesion genes *mub*, *mapA* and *EF-Tu* were detected and the genes were expressed under different conditions, indicating the ability of the strain to adapt to physiological conditions and adhere to the gastrointestinal tract.

A general functional role for Slp have not been described. Several functions have been proposed, including surface recognition and cell adhesion. The Slp of *L. crispatus* ZJ001 contributed to adhesion of the strain to HeLa cells. Functional role of the Slp in adhesion was confirmed by an antibody-mediated inhibition assay with polyclonal antibodies (2). In the present study, the role of the Slp in adhesion to mucus was investigated for *L. acidophilus* ATCC 4356. *slp* was not up-regulated in the presence of mucus, but was expressed under normal and stressful gut conditions *in vitro*. Characterization of the surface proteins of *L. plantarum* 423 did not reveal the presence of Slp. Despite the lack of Slp strain 423 efficiently adhered to Caco-2 cell lines and enterocytes of rats. These data reveal that the Slp may contribute to the adhesion of certain strains to cell surfaces. The present study does not support Slp as a major contributor to adhesion of probiotics to host surfaces. Investigation of the surface proteins of *L. plantarum* 423 revealed EFTu, GAPDH and TPI to be major contributors to adhesion of the strain to Caco-2 cells. *EFTu* was up-regulated in the presence of mucus and is present on the surface of the strain. This suggests that EFTu may function as a novel adhesin in *L. plantarum* 423. It can be concluded that *L. plantarum* 423 possesses all the genetic requirements necessary to be an efficient coloniser of the GIT and exhibits exceptional characteristics as a future probiotic.

Numerous studies have incorporated radioactive labeling as a sensitive means of analysis for monitoring adhesion of probiotics to mucus or cell lines (3, 8, 10). Real time PCR is a safe, rapid and sensitive technique that negates the dangers associated with radioactive labeling. A previous study utilized the technique to quantify the adhesion of *Bifidobacterium* spp. to Caco-2 cells and also outlined advantages of the technique in comparison to radioactive labeling (1). In the present study quantitative real time (qRT) PCR was successfully applied to evaluate adhesion gene expression of *L. plantarum* 423 and quantify strain 423 and *E. mundtii* ST4SA in the gastrointestinal tract of male Wistar rats. In addition to *in vitro* applications, the technique proved successful *in vivo* for delivering accurate data within a short space of time and avoided

the tediousness associated with conventional culture based methods. The present study supports the use of RT PCR as a robust technique that delivers precise, reproducible quantitative and qualitative data. The technique should be applied more regularly in future applications.

Examination of the adhesive potential of a probiotic should extend beyond phenotypic evaluation to gain a greater perspective on how the microorganism functions. FISH conducted on formaldehyde fixed tissue of rats administered with *E. mundtii* ST4SA revealed translocation of the strain. Translocation of enterococci is a common phenomenon and has been described previously (11, 12). However, this may only pose a problem if the organism elicits infection. Safety evaluation of *E. mundtii* ST4SA concluded the strain to be safe for human consumption (9). However, to reduce any possible risk that may be associated with the translocation of strain ST4SA, suggestions into the use of the purified antimicrobial peptide for future probiotic applications could be considered. This would have not been evident by only taking phenotypic studies into consideration. Molecular genome-based approaches provide greater insight on the functional properties of a probiotic and allows for a more selective view for their probiotic action.

Future recommendations

Understanding the full potential of probiotics for therapeutic applications requires thorough investigations on molecular mechanisms of probiotic action, particularly interactions with the host. Enhanced understanding of these molecular interactions will also enable researchers to foresee any possible adverse effects that may be generated by a particular probiotic. This will permit for the selection of the best strains that target specific problems, whilst simultaneously eliminating possible associated risks. Molecular analytical tools such as real time PCR and FISH serve as versatile, highly-sensitive techniques for delivering rapid data in a variety of *in vitro* and *in vivo* applications. The techniques were successfully applied in understanding the molecular mechanisms of adhesion of *L. plantarum* 423 and *E. mundtii* ST4SA.

Research goals of the present study addressed key issues with regards to evaluating the adhesion of probiotics to host tissue. Although the objectives have been achieved, greater emphasis is required in this area of research. Future recommendations will encompass characterising novel surface proteins of *L. plantarum* 423 and evaluate specific interactions of these proteins with host surface

receptors. Inactivation of the adhesion genes and competitive experiments with specific pathogens should provide a greater perspective on the specificity of these genes in combating a particular pathogen. Stimulation of the immune system by strain ST4SA should be considered for future research to determine if translocation of the strain exhibits a positive influence to the host.

Unraveling the intricacies of probiotic host interactions will provide greater insight on the functional attributes of these remarkable microorganisms. This will allow for industry and consumers to choose scientifically validated probiotics as a natural alternative for overcoming various health problems.

References

1. **Candela, M., G. Seibold, B. Vitali, S. Lachenmaier, B. J. Eikmanns, P. Brigidi.** 2005. Real-time PCR quantification of bacterial adhesion to Caco-2 cells: competition between bifidobacteria and enteropathogens. *Research in Microbiology*. **156**:887-895.
2. **Chen, X., J. Xu, J. Shuai, J. Chen, Z. Zhang, W. Fang.** 2007. The S-layer proteins of *Lactobacillus crispatus* strain ZJ001 is responsible for competitive exclusion against *Escherichia coli* 0157:H7 and *Salmonella typhimurium*. *International Journal of Food Microbiology*. **115**:307-312.
3. **Collado, M. C., J. Meriluoto, S. Salminen.** 2007. *In vitro* analysis of probiotic strain combinations to inhibit pathogen adhesion to human intestinal mucus. *Food Research International*. **40**:629-636.
4. **DeVinney, R., D. Knoechel, B. Finlay.** 1999. Enteropathogenic *Escherichia coli*: cellular harassment. *Current Opinion in Microbiology*. **2**:83-88.
5. **Finlay, B., P. Cossart.** 1997. Exploitation of mammalian host cell functions by bacterial pathogens. *Science*. **276**:718-725.
6. **Galan, J., J. Pace, M. Hayman.** 1992. Involvement of the epidermal growth factor receptor in the invasion of cultured mammalian cells by *Salmonella typhimurium*. *Nature*. **357**:588-589.
7. **Goosney, D., D. Knoechel, B. Finlay.** 1999. Enteropathogenic *E. coli*, *Salmonella* and *Shigella*: masters of host cell cytoskeletal exploitation. *Emerging Infectious Diseases*. **5**:216-223.

8. **Rinkinen, M., E. Westermarck, S. Salminen, A. C. Ouwehand.** 2003. Absence of host specificity for *in vitro* adhesion of probiotic lactic acid bacteria to intestinal mucus. *Veterinary Microbiology*. **97**:55-61.
9. **Tendoeschate, K., R. Smith, L. M. T. Dicks.** 2007. *In vivo* assessment of the safety of orally administered novel probiotics, *Enterococcus mundtii* ST4SA and *Lactobacillus plantarum* 423 in a male Wistar rat model. Unpublished.
10. **Tuomola, E. M., A. C. Ouwehand, S. J. Salminen.** 2000. Chemical, physical and enzymatic pre-treatments of probiotic lactobacilli alter their adhesion to human intestinal mucus glycoproteins. *International Journal of Food Microbiology*. **60**:75-81.
11. **Wells, C. L., R. P. Jechorek, L. B. Twiggs, D. C. Brooker.** 1990. Recovery of viable bacteria from pelvic lymph nodes of patients with gynecologic tumors. *Journal of Infectious Diseases*. **162**:1216-1218.
12. **Wells, C. L., S. L. Erlandsen.** 1991. Localisation of translocating *Escherichia coli*, *Proteus mirabilis* and *Enterococcus faecalis* within cecal and colonic tissues of monoassociated mice. *Infection and Immunity*. **59**:4693-4697.

Addendum

Short communication

Expression of the mucus adhesion genes *Mub* and *MapA*, adhesion-like factor *EF-Tu* and bacteriocin gene *plaA* of *Lactobacillus plantarum* 423, monitored with real-time PCR

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Abstract

Expression of the mucus adhesion genes *Mub* and *MapA*, adhesion-like factor *EF-Tu* and bacteriocin gene *plaA* by *Lactobacillus plantarum* 423, grown in the presence of bile, pancreatin and at low pH, was studied by real-time PCR. *Mub*, *MapA* and *EF-Tu* were up-regulated in the presence of mucus, proportional to increasing concentrations. Expression of *MapA* was up-regulated in the presence of 3.0 g/l bile and 3.0 g/l pancreatin at pH 6.5. Similar results were recorded in the presence of 10.0 g/l bile and 10.0 g/l pancreatin at pH 6.5. Expression of *Mub* was down-regulated in the presence of bile and pancreatin, whilst the expression of *EF-Tu* and *plaA* remained unchanged. Expression of *Mub* and *MapA* remained unchanged at pH 4.0, whilst expression of *EF-Tu* and *plaA* were up-regulated. Expression of *MapA* was down-regulated in the presence of 1.0 g/l l-cysteine HCl, suggesting that the gene is regulated by transcription attenuation that involves cysteine.

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Keywords: *Lactobacillus plantarum*; Gene expression; Real-time PCR

1. Introduction

Lactic acid bacteria are regarded probiotic if they successfully compete with intestinal pathogens and restore the normal microbiota. One of the first biological barriers encountered by probiotic bacteria is low pH in the stomach. Those cells that survive such extreme conditions are exposed to further stress in the small intestine, inflicted by bile salts and pancreatic juice (Bron et al., 2004).

Probiotic bacteria compete with intestinal micro-organisms for adhesion to mucus and epithelial cells by production of antimicrobial agents such as organic acids, diacetyl, hydrogen peroxide and bacteriocins (Klaenhammer, 2000; Morelli, 2000; Saarela et al., 2000). A few strains produce specific adhesion proteins. The best studied examples are the mucus adhesion-promoting protein (MapA; Genebank accession number AJ 293860) and the mucus-binding protein (Mub) produced by *Lactobacillus reuteri* (Roos and Jonsson, 2002). Five proteins

homologous to the Mub protein of *L. reuteri* 104R have been described for *Lactobacillus acidophilus* NCFM (Alterman et al., 2005). Mub homologues have also been reported for *Lactobacillus gasseri*, *Lactobacillus johnsonii* and *Lactobacillus plantarum* (Alterman et al., 2005).

The collagen binding protein (CnBP) produced by *L. reuteri* interacts with mucin but does not play a role in adhesion (Aleljung et al., 1994; Roos et al., 1996). An adhesion-like protein, classified as elongation factor Tu (EF-Tu), was isolated from *L. johnsonii* NCC 533 (La1) (Granato et al., 2004). EF-Tu facilitates the transfer of aminoacyl-tRNA to the A-site of ribosomes (Gaucher et al., 2001). The EF-Tu protein also binds to mucin and mediates colonisation of strain NCC 533 to human intestinal cells and mucus (Granato et al., 2004). The mechanism by which EF-Tu interacts with mucin is not well understood. An EF-Tu protein isolated from *L. plantarum* shared 84% homology with the EF-Tu protein of *L. johnsonii* NCC 533 (Granato et al., 2004).

L. plantarum is used as a starter culture in the production of several types of fermented foods and is considered a natural inhabitant of the intestinal tract (Ahrne et al., 1998). Strain 423,

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isolated from sorghum beer (Van Reenen et al., 1998) produces a bacteriocin, plantaricin 423, resistant to treatment at 80 °C and incubation in MRS broth at pH 1.0 to 10.0 (Van Reenen et al., 1998). The genes encoding plantaricin, a typical class IIa peptide, have been sequenced (Van Reenen et al., 2003). The peptide is active against food-borne pathogens, including *Bacillus cereus*, *Clostridium sporogenes*, *Enterococcus faecalis*, *Listeria* spp. and *Staphylococcus* spp. (Van Reenen et al., 1998). Strain 423 survived gastro-intestinal conditions simulated in an in vitro model and adhered to intestinal mucus (unpublished data).

Previous studies focussed mainly on physiological aspects, such as in vitro adhesion of probiotic cells to CaCo-2 cell lines or mucus (Tuomola et al., 2000; Apostolou et al., 2001; Ouwehand, 2005). Little is known about the expression of genes involved in adhesion, especially when cells are exposed to stress conditions simulating the gastro-intestinal tract. The present study looks at the expression of *Mub*, *MapA*, *EF-Tu* and the structural gene of plantaricin 423 (*plaA*) when the strain is cultured in the presence of bile salts, pancreatic juice and low pH. Expressions of these genes were monitored by using real-time PCR.

2. Materials and methods

2.1. Bacterial strains and culture conditions

L. plantarum 423, isolated from sorghum beer (Van Reenen et al., 1998), was cultured in De Man Rogosa Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37 °C. Gene expression studies were done in MRS broth (Biolab), supplemented with mucin, bile and pancreatin (Table 1). Regulation of the *MapA* gene was studied in MRS broth (Biolab), supplemented with 0.1% (w/v) l-cysteine HCl.

2.2. Primer design and PCR

Primers used in this study are listed in Table 2. Primers for the amplification of *plaA* were designed from the structural gene encoding plantaricin 423 (Van Reenen et al., 2003). All other primer sequences were designed from the genome sequence published for *L. plantarum* WCFS1 (accession number NC 004567). Primers were designed with the Primer Designer Program, version 1.01 (Scientific and Educational

Table 2
Primers used in the study

Primer set	Sequence 5' to 3'	Size of amplicon (bp)
Bac 423F RT	GACATTTACATTGAGTAGGAAGCTAG	147
Bac 423R RT	GTAACCCCATTACCATAGTATTAC	
Mub 423F RT	GTAGTTACTCAGTGACGATCAATG	150
Mub 423R RT	TAATTGTAAAGGTATAATCGGAGG	
Map 423F RT	TGGATTCTGCTTGAGGTAAG	156
Map 423R RT	GACTAGTAATAACGCGACCG	
EFTu 423F RT	TTCTGGTCGTATCGATCGTG	161
EFTu 423R RT	CCACGTAATAACGCACCAAC	
GDPH 423F RT	ACTGAATTAGTTGCTATCTTAGAC	140
GDPH 423R RT	GAAAGTAGTACCGATAACATCAGA	

Software). The GC-content of the primers ranged between 30 and 40% and the T_m from 55 to 65 °C. Primer sets were designed to produce an amplicon of approximately 150 bp. Primer dimer formation was checked by agarose gel electrophoresis (2% agarose) and melting curve analysis. A standard curve consisting of the Log template concentration of DNA plotted against the Ct value (crossing points of different standard dilutions) was constructed for each primer set. Efficiencies were calculated by $E = 10^{(-1/S)} - 1$, where S=slope of the standard curve.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from 500 µl of an 18-h-old culture (OD₆₀₀=approximately 4.0), by using the RNeasy Mini Kit (Qiagen, Valencia, California, USA). RNA was stabilised in two volumes of RNA protect bacteria reagent (Qiagen), prior to isolation. Cells were harvested (5000×g, 10 min, 4 °C) and lysed by incubation with lysosyme (6 µl of 50 mg/ml lysosyme per 100 µl of TE buffer) for 20 min at 37 °C. RNA was resuspended in 40 µl DEPC-treated H₂O. Residual DNA was removed with turbo DNase treatment, according to the instructions from the supplier (Ambion, USA). RNA concentrations were measured in triplicate using a NanoDrop spectrophotometer (Inqaba Biotec, USA). Exactly 0.2 µg RNA was transcribed into cDNA, using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany) and gene specific primers. The reaction took 30 min at 55 °C.

2.4. Real-time PCR

The standard curve method was used for quantification as the efficiencies between the targets and the reference gene were not comparable (Giulietti et al., 2001). Standard curves were constructed by plotting the Ct-value against the log template value of cDNA of each gene. Target concentrations were adjusted by normalising against the glyceraldehyde-3-phosphate gene, which served as endogenous reference, by dividing the average concentration of target DNA by the average concentration of reference DNA. The relative gene expression levels were compared by dividing the normalised target concentration by the control sample (cells grown in MRS, medium 1), and vice versa.

Table 1
Modification of MRS broth (Biolab) to simulate gastric conditions

Medium ^a	Mucin % (w/v) ^b	Bile % (w/v)	Pancreatin % (w/v)	pH ^c
1	0	0	0	7
2	0.01	0	0	7
3	0.05	0	0	7
4	0.01	0.3	0.3	6.5
5	0.01	1.0	1.0	6.5
6	0.01	0.3	0.3	4

^aMedium 4 simulates normal gut conditions and media 5 and 6 stress conditions.

^bPorcine mucus (Sigma, Mannheim, Germany).

^cAdjusted with 0.5 M HCl.

Real-time PCR reactions were performed in a LightCycler instrument (Roche), with Relative Quantification Software (version 5.32; Roche) and SYBR Green technology (Sigma). Each 20 μ l reaction mixture consisted of 10 μ l SYBR Green Supermix, 1 μ l of each primer at 5 μ mol, 7 μ l nuclease-free water and 1 μ l diluted cDNA. Five dilutions of cDNA were prepared for the standard curves. A negative control was included in each run. Synthesis of cDNA and real-time PCR was performed in triplicate for each gene. The PCR conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 40 cycles of amplification at 50 °C for 10 s and 72 °C for 15 s. At the end of each run a melting curve was performed from 95 °C to 45 °C (0.2 °C s⁻¹).

3. Results

All primers and standard curves displayed an efficiency of between 80 and 100% (Figs. 1 and 2). A single product-specific melting curve was obtained for each primer set, which indicates that primers were designed with optimal efficiency and were effective in targeting and amplifying only the genes of interest. *L. plantarum* 423 survived exposure to 0.5 M HCl at pH 2.0.

3.1. Expression of *Mub*

Genes are considered to be up- or down-regulated if their relative expression levels are at least two-fold higher or lower respectively, compared to the control sample or the calibrator (Desroche et al., 2005). The *Mub* gene was expressed approximately 80-fold more when grown in the presence of 0.01% (w/v) mucin and 144-fold more when grown in the presence of 0.05% (w/v) mucin (Table 3). However, the gene was suppressed when cells were grown under normal gut conditions (0.3%, w/v, bile and 0.3%, w/v, pancreatin, pH 6.5) and conditions simulating stress (1%, w/v, bile and 1%, w/v, pancreatin, pH 6.5). Expression was down-regulated approximately seven-fold under normal intestinal conditions and about

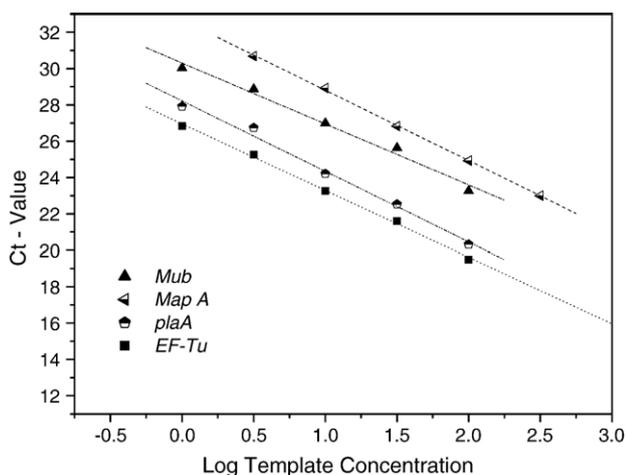


Fig. 1. Standard curves of the different genes. *Mub* ($y = -3.356x + 30.302$), *EF-Tu* ($y = -3.664x + 26.952$), *plaA* ($y = -3.88x + 28.218$), *MapA* ($y = -3.876x + 32.68$).

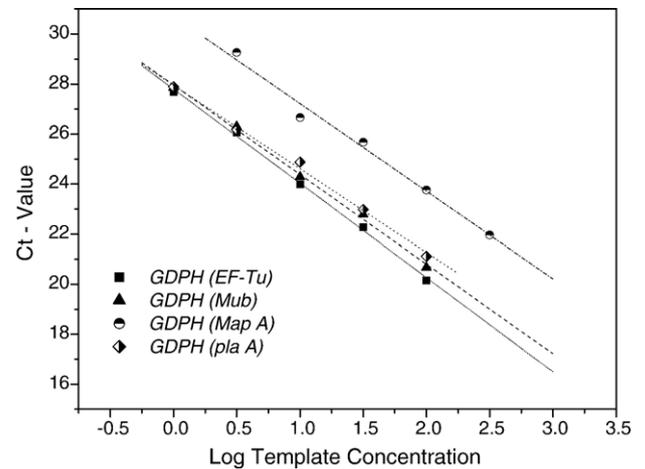


Fig. 2. Standard curves of glyceraldehyde-3-phosphate for the different genes. *EF-Tu* ($y = -3.766x + 27.796$), *Mub* ($y = -3.58x + 27.958$), *MapA* ($y = -3.504x + 30.72$), *plaA* ($y = -3.346x + 27.952$).

30-fold under stressful conditions. Expression of *Mub* remained unchanged at pH 4.0.

3.2. Expression of *MapA*

The *MapA* gene was up-regulated when cells were incubated in the presence of mucin. Expression was six- and eight-fold higher in the presence of 0.01% (w/v) mucin and 0.05% (w/v) mucin, respectively (Table 3). The *MapA* gene was significantly up-regulated under normal and stressful conditions. Under normal conditions, *MapA* was expressed 25-fold higher compared to the control (cells grown in MRS). However, under stressful

Table 3

Expression of *Mub*, *MapA*, *EF-Tu* and *plaA*, as recorded by real-time PCR

Genes	Medium ^a	Av. Ct ^b	Amt.	GDPH Av. Ct	Amt.	Normalised	Set to control
<i>Mub</i>	1	24.98	38.9	21.64	57.5	0.68	1
	2	24.5	53.7	27.93	1	53.7	80
	3	23.61	97.7	27.91	1	97.7	144
	4	25.28	31.6	19.19	316	0.1	7
	5	27.86	5.4	18.88	316	0.02	34
<i>MapA</i>	1	26.4	41.7	21.62	398.1	0.1	1
	2	25.78	60.3	23.64	104.7	0.6	6
	3	25.73	61.7	24.16	74.1	0.8	8
	4	26.95	30.2	26.94	12.02	2.5	25
	5	27.1	27.5	25.91	23.4	1.2	12
<i>EF-Tu</i>	1	20.3	66.1	22.76	21.9	3	1
	2	19.36	117.5	28.06	1.2	97.9	33
	3	17.63	346.7	27.94	1.1	315.2	105
	4	17.85	302	18.64	269	1.1	2.7
	5	17.44	398	18.48	295	1.3	2.3
<i>plaA</i>	1	20.62	91.2	23.97	15.5	5.8	1
	2	20.21	114.8	23.89	16.2	7.1	1.2
	3	20.19	117.5	24.42	11.5	10.2	1.8
	4	24.3	10	27.45	1.4	7.1	1.2
	5	22.36	32.4	26.58	2.6	12.5	2.2

^aSee composition in Table 1.

^bAv. Ct = average crossing point at which a significant increase in fluorescence is detected.

conditions, *MapA* was expressed 12-fold higher (Table 3). At pH 4.0, the gene was up-regulated twice as much compared to the control sample. In the presence of 0.1% (w/v) l-cysteine HCl, *MapA* was considerably down-regulated (approximately 17-fold).

3.3. Expression of *EF-Tu*

EF-Tu was induced when cells were grown in medium supplemented with mucus. The gene was expressed 33-fold higher in the presence of 0.01% (w/v) mucin and approximately 100-fold higher in the presence of 0.05% (w/v) mucin (Table 3). Expression of the gene was not affected by varying concentrations of bile and pancreatin. Cells grown at pH 4.0 expressed *EF-Tu* six-fold higher compared to cells grown under normal conditions.

3.4. Expression of *plaA*

Expression of *plaA* was not influenced by changes in bile, pancreatin and pH (Table 3).

4. Discussion

Colonisation of the intestinal tract is considered a prerequisite for probiotic bacteria (Ouwehand et al., 1999a). Most studies report on the adhesion of probiotic bacteria to intestinal cell lines (Adlerberth et al., 1996; Granato et al., 1999; Dunne et al., 2001). Although this may be a useful indication of adhesion, it is not always a reflection of the ability of the bacteria to adhere to mucus covering the intestinal cells (Ouwehand et al., 1999b). The mucus layer serves a dual function. It may prevent the adhesion of pathogenic bacteria, thereby protecting the intestinal cells, but also serves as a nutrient source and matrix for colonisation of probiotic bacteria (Nielsen et al., 1994). Adhesion to mucosa is important for proliferation of probiotic cells, especially in the small intestine with high flow rates (Wadstrom, 1998). Furthermore, adhesion stimulates the immune system (Ouwehand et al., 1999b). In at least one study (Elliot et al., 1998), it has been claimed that damaged gastric mucosal cells are repaired by the adherence of probiotic lactobacilli.

From first studies, it was concluded that the EF-Tu molecule is restricted to the cytoplasm of prokaryotes. However, later studies have shown that this molecule is also associated with the membrane of *E. coli* (Jacobson and Rosenbusch, 1976) and the periplasm of *Neisseria gonorrhoeae* (Porcella et al., 1996). In lactobacilli, EF-Tu may function as an “envelope associated protein,” which may be released from the cell when the organism experiences osmotic shock (Nakamura et al., 1997). Granato et al. (2004) demonstrated the presence of EF-Tu on the outer surface of five *Lactobacillus* and two *Bifidobacterium* strains. The concentration of EF-Tu on the surface of the strains differed, suggesting possible differences in expression (Granato et al., 2004). In this study, up-regulation of *EF-Tu* in the presence of mucus suggests that the EF-Tu molecule may also play a role in adhesion. This correlates well with work done by Granato et al. (2004).

Under normal physiological concentrations of bile and pancreatin, expression of the *Mub* gene was affected, the *MapA* gene was over-expressed and the *EF-Tu* gene remained stable. Similar results were obtained when *L. plantarum* 423 was exposed to digestive stress. This suggests that, whilst the expression of certain mucus genes may be affected by bile and pancreatin, other mucus genes are switched on, enabling the strain to adapt to physiological conditions and adhere to the gastro-intestinal tract.

The pH of the gut lumen is 6.5, but it has been postulated that it becomes slightly more acidic closer to the mucus-covered surface, owing to the sulfated content and sialic residues of mucin (Blum et al., 2000; Granato et al., 2004). Hence, expressions of the adhesion genes were also investigated at pH 4.0. Variations in pH did not influence the expression of the genes negatively, as none were down-regulated at lower pH.

Regulation of *MapA* was also investigated. Expression of *MapA* from *L. reuteri* 104R is regulated by a mechanism of transcription attenuation, involving cysteine (information published in Genbank, accession number AJ 293860). Our studies indicate that the *MapA* gene of *L. plantarum* 423 is down-regulated in the presence of 0.1% (w/v) l-cysteine HCl. Cysteine participates as an effector molecule and represses transcription of *MapA* in the presence of cysteine.

5. Conclusion

L. plantarum 423 complies with all the major criteria required to be classified as a probiotic. Expression of *plaA* was not affected by digestive stress conditions in vitro and strain 423 survived exposure to HCl at pH 2.0. Expression of adhesion genes by strain 423 was positively regulated in the presence of mucus. Variations in pH did not affect expression of the adhesion genes, whilst bile and pancreatin only affected the *Mub* gene. *L. plantarum* 423 is capable of adapting to conditions in a normal healthy intestine and has the genetic characteristics required for an effective probiotic.

References

- Adlerberth, I., Ahrne, S., Johansson, M.L., Molin, G., Hanson, L.A., Wold, A.E., 1996. A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. *Applied and Environmental Microbiology* 62, 2244–2251.
- Ahrne, S., Nobaek, S., Jeppsson, B., Adlerberth, I., Wold, A.E., Molin, G., 1998. The normal *Lactobacillus* flora of healthy human rectal and oral mucosa. *Journal of Applied Microbiology* 85, 88–94.
- Aleljung, P., Shen, W., Rozalska, B., Hellman, U., Ljung, A., Wadstrom, T., 1994. Purification of collagen binding proteins of *Lactobacillus reuteri* NCIB 11951. *Current Microbiology* 28, 231–236.
- Alterman, E., Russell, W.M., Azcarate-Peril, M.A., Barrangou, R., Buck, B.L., McAuliffe, O., Souther, N., Dobson, A., Duong, T., Callanan, M., Lick, S., Hamrick, A., Cano, R., Klaenhammer, T.D., 2005. Complete genome sequence of the probiotic lactic acid bacterium *Lactobacillus acidophilus* NCFM. *Proceedings of the National Academy of Science* 102, 3906–3912.
- Apostolou, E., Kirjavainen, P.V., Saxelin, M., Rautelin, H., Valtonen, V., Salminen, S.J., Ouwehand, A.C., 2001. Good adhesion properties of probiotics: a potential risk for bacteremia. *FEMS Immunology and Medical Microbiology* 31, 35–39.

- Blum, S., Reniero, R., Schiffrin, E.J., Crittenden, R., Mattila-Sandholm, T., Ouwehand, A.C., Salminen, S., Von Wright, A., Saarela, M., Saxelin, M., Collins, K., Morelli, L., 2000. Adhesion studies for probiotics: need for validation and refinement. *Trends in Food Science and Technology* 10, 405–410.
- Bron, P.A., Marco, M., Hoffer, S.M., Van Mullekom, E., De Vos, W.M., Kleerebezem, M., 2004. Genetic characterization of the bile salt response in *Lactobacillus plantarum* and analysis of responsive promoters in vitro and in situ in the gastrointestinal tract. *Journal of Bacteriology* 186, 7829–7835.
- Desroche, N., Beltramo, C., Guzzo, J., 2005. Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni*. *Journal of Microbiological Methods* 60, 325–333.
- Dunne, C., Mahony, L.O., Murphy, L., Thornton, G., Morrissey, D., Halloran, S.O., Feeney, S., Flynn, Fitzgerald, G., Daly, C., Kiely, B., Sullivan, G.C.O., Shanahan, F., Collins, J.K., 2001. In vitro selection criteria for probiotic bacteria of human origin: correlation with in vivo findings. *American Journal of Clinical Nutrition* 73, 386–392.
- Elliot, S.N., Buret, A., McKnight, W., Miller, M.J.S., Wallace, J.L., 1998. Bacteria rapidly colonise and modulate healing of gastric ulcers in rats. *American Journal of Physiology* 275, 425–432.
- Gaucher, E.A., Miyamoto, M.M., Benner, S.A., 2001. Function-structure analysis of proteins using covarion-based evolutionary approaches: elongation factors. *Proceedings of the National Academy of Science of the United States of America* 98, 548–552.
- Granato, D., Perotti, F., Masserey, I., Rouvet, M., Golliard, M., Servin, A., Brassart, D., 1999. Cell surface-associated lipoteichoic acid acts as an adhesion factor for attachment of *Lactobacillus johnsonii* La1 to human enterocyte-like Caco-2 cells. *Applied and Environmental Microbiology* 65, 1071–1077.
- Granato, D., Bergonzelli, G.E., Pridmore, R.D., Marvin, L., Rouvet, M., Corthesy-Theulaz, I.E., 2004. Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. *Infection and Immunity* 72, 2160–2169.
- Giulietti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R., Mathieu, C., 2001. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* 25, 386–401.
- Jacobson, G.R., Rosenbusch, J.P., 1976. Abundance and membrane association of elongation factor Tu in *E. coli*. *Nature* 261, 23–26.
- Klaenhammer, T.R., 2000. Probiotic bacteria: today and tomorrow. *Journal of Nutrition* 130, 415S–416S.
- Morelli, L., 2000. In vitro selection of probiotic lactobacilli: a critical appraisal. *Current Issues in Intestinal Microbiology* 1, 59–67.
- Nakamura, J., Ito, D., Nagai, K., Umehara, Y., Hamachi, M., Kumagai, C., 1997. Rapid and sensitive detection of hiochi bacteria by amplification of hiochi bacterial common antigen gene by PCR method and characterisation of the antigen. *Journal of Fermentation and Bioengineering* 185, 7019–7023.
- Nielsen, E.M., Schlundt, J., Gunvig, A., Jacobsen, B.L., 1994. Epithelial, mucus and lumen subpopulations of *Escherichia coli* in the large intestine of conventional and gnotobiotic rats. *Microbial Ecology in Health and Disease* 7, 263–273.
- Ouwehand, A.C., 2005. Measurement of bacterial adhesion — in vitro evaluation of different methods. *Journal of Microbiological Methods* 60, 225–233.
- Ouwehand, A.C., Kirjavainen, P.V., Gronlund, M.M., Isolauri, E., Salminen, S.J., 1999a. Adhesion of probiotic micro-organisms to intestinal mucus. *International Dairy Journal* 9, 623–630.
- Ouwehand, A.C., Kirjavainen, P.V., Shortt, C., Salminen, S., 1999b. Probiotics: mechanisms and established effects. *International Dairy Journal* 9, 43–52.
- Porcella, S.F., Belland, R.J., Judd, R.C., 1996. Identification of an EF-Tu protein that is periplasm-associated and processed in *Neisseria gonorrhoeae*. *Microbiology* 142, 2481–2489.
- Roos, S., Jonsson, H., 2002. A high-molecular-mass cell-surface protein from *Lactobacillus reuteri* 1063 adheres to mucus components. *Microbiology* 148, 433–442.
- Roos, S., Aleljung, P., Robert, N., Lee, B., Wadstrom, T., Lindberg, M., Jonsson, H., 1996. A collagen binding protein from *Lactobacillus reuteri* is part of an ABC transporter system? *FEMS Microbiology Letters* 144, 33–38.
- Saarela, M., Mogensen, G., Fonden, R., Mattu, J., Mattila-Sandholm, T., 2000. Probiotic bacteria: safety, functional and technological properties. *Journal of Biotechnology* 84, 197–215.
- Tuomola, E.M., Ouwehand, A.C., Salminen, S.J., 2000. Chemical, physical and enzymatic pre-treatments of probiotic lactobacilli alter their adhesion to human intestinal mucus glycoproteins. *International Journal of Food Microbiology* 60, 75–81.
- Van Reenen, C.A., Chikindas, M.L., Dicks, L.M.T., 1998. Isolation, purification and partial characterization of plantaricin 423, a bacteriocin produced by *Lactobacillus plantarum*. *Journal of Applied Microbiology* 84, 1131–1137.
- Van Reenen, C.A., Chikindas, M.L., Van Zyl, W.H., Dicks, L.M.T., 2003. Characterization and heterologous expression of a class IIa bacteriocin, plantaricin 423 from *Lactobacillus plantarum* 423, in *Saccharomyces cerevisiae*. *International Journal of Food Microbiology* 81, 29–40.
- Wadstrom, T., 1998. Adherence traits and mechanisms of microbial adhesion in the gut. *Baillier's Clinical Tropical Medicine and Communicable Disease* 3, 417–433.