PROBIOTIC PROPERTIES OF LACTIC ACID BACTERIA EVALUATED IN A GASTRO-INTESTINAL MODEL AND IN *IN VIVO* PIG TRIALS
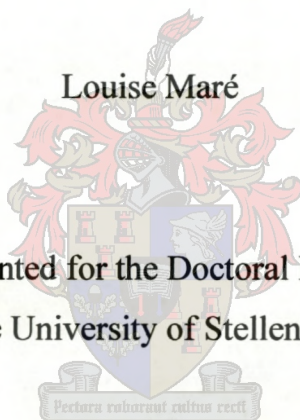
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Declaration

I, the undersigned hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Summary

This study describes the use of a gastro-intestinal model to screen lactic acid bacteria isolated from the gastro-intestinal tract of post-weaned piglets (raised on six different diets) for probiotic properties. Intestinal bacteria were isolated from the stomach, duodenum, jejunum, caecum, ileum and colon. The highest cell numbers (6 x 10^7 cfu/g) were isolated from the ileum. No significant differences in viable cell counts were recorded for piglets raised on the six diets.

Isolates with the best overall probiotic properties were identified as members of *Lactobacillus salivarius* and *Lactobacillus fermentum*. The two strains selected for further studies were *Lactobacillus plantarum* 423 (originally isolated from sorghum beer) and *Lactobacillus salivarius* 241 (isolated from pig intestine). *Enterococcus faecalis* FAIR E 92 was originally isolated from pig intestine and was included in this study as a non-pathogenic challenge strain. *L. plantarum* 423 produces a bacteriocin plantaricin 423, active against *E. faecalis* FAIR E 92.

L. plantarum 423 and *L. salivarius* 241 were included in the gastro-intestinal model and their adhesion to the mucus of porcine ileum studied with fluorescent-*in-situ*-hybridization (FISH). A decrease in viable cell numbers of *L. plantarum* 423 was recorded in the duodenum, jejunum and ileum in the presence of bile and pancreatic juice. However, higher cell numbers were recorded in the caecum and anterior colon, which suggested that strain 423 recovered from these stress factors. Plantaricin 423 was detected for up to 28 hours in the duodenum, jejunum, ileum and middle colon. Lower cell numbers (one log unit) of *L. salivarius* 241 were recorded in the gastro-intestinal model over seven days, compared to strain 423.

Piglets of one, 14 and 28-days-old were dosed with *L. plantarum* 423 and *L. salivarius* 241, separately and in combination (1:1). In a separate experiment, 14-day-old piglets were challenged twice with *E. faecalis* FAIR E 92, followed by dosage with strains 423 and 241. New-borne piglets dosed with *L. plantarum* 423 gained more weight (4 kg over 19 days) compared to piglets dosed with *L. salivarius* 241 (2.2 kg over 19 days), or a combination of the two strains (2 kg over 19 days). Piglets of 14 and 28-days-old, on the other hand, gained more weight when dosed with a combination of strains 423 and 241. The cell numbers of *E. faecalis* FAIR E 92 and other enterococci decreased drastically (two log units) when the

piglets were dosed with the latter two strains. Overall, piglets of various ages reacted differently when administered *L. plantarum* 423 and *L. salivarius* 241, separately or in combination.

Fluorescent-*in-situ*-hybridization (FISH) was used to study the *in vivo* adhesion of *L. plantarum* and *L. salivarius* to mucus in the stomach, duodenum, jejunum, ileum, caecum and colon. The highest number of *L. plantarum* cells was recorded in the ileum, whereas *L. salivarius* favoured adhesion to the duodenum. A decrease in cell numbers of *E. faecalis* in the ileum mucus was recorded when a combination of the probiotic strains 423 and 241 was administered. This study provided a reliable estimation of the presence and/or adhesion of *L. plantarum* and *L. salivarius* to various parts of the porcine gastro-intestinal tract, without the use of expensive cultivation techniques. Insight was gained into the co-evolution existing between probiotic bacteria and the porcine gastro-intestinal tract, emphasizing the use of gastro-intestinal models to study the dynamics of the gastro-intestinal tract.

Opsomming

Hierdie studie beskryf die gebruik van 'n gastro-intestinale model, om melksuurbakterieë wat geïsoleer is uit die spysverteringskanaal (SVK) van reeds gespeende varkies (gevoed op ses verskillende diëte) vir probiotiese eienskappe te toets. Ingewandsbakterieë is uit die maag, duodenum, jejunum, caecum, ileum en kolon geïsoleer. Die hoogste aantal selle (6 x 10^7 kve/g) is geïsoleer uit die ileum. Geen betekenisvolle verskille in lewensvatbare seltellings, vir varkies gevoed op ses verskillende voere is aangeteken nie.

Isolate met die beste algehele probiotiese eienskappe is as *Lactobacillus salivarius* en *Lactobacillus fermentum* geïdentifiseer. Vir verdere studie is twee isolate *Lactobacillus plantarum* (oorspronklik uit sorghum-bier geïsoleer) en *Lactobacillus salivarius* (uit die varkdermkanaal geïsoleer) geselekteer. *Enterococcus faecalis* FAIR E 92, oorspronklik uit die varkdermkanaal geïsoleer, is in hierdie studie as 'n nie-patogeniese indikator gebruik. *L. plantarum* 423 produseer 'n bakteriosien plantarisien 423 wat aktief is teen *E. faecalis* FAIR E 92.

L. *plantarum* 423 en *L. salivarius* 241 is ingesluit in die gastro-intestinale model, en vashegting van díe bakterieë aan die mukus van vark-ileum is met fluoresensie-*in-situ*-hibridisasie (FISH) bestudeer. 'n Afname in lewende selgetalle van *L. plantarum* 423 in die duodenum, jejunum en ileum is aangetoon in reaksie tot die byvoeging van gal en pankreatiese sappe. Hoër selgetalle is nietemin aangeteken in die caecum en voorste gedeelte van die kolon, wat 'n aanduiding gee dat isolaat 423, ten spyte van hierdie stres-faktore, oorleef. Plantaricin 423 is vir 'n tydperk (28 uur) in die duodenum, jejunum, ileum en sentrale kolon gevind. Laer selgetalle (een logaritmiese eenheid) van *L. salivarius* 241 is in die gastro-intestinale model oor 'n tydperk van sewe dae aangetoon, in vergelyking met isolaat 423.

Een, 14 en 28 dag oud varkies is met *L. plantarum* 423 en *L. salivarius* 241 (afsonderlik en in kombinasie 1:1) twee keer gedaag met *E. faecalis* FAIR E 92, opgevolg met dosering van 423 en 241. Pasgebore varkies het die hoogste gewigstoename getoon (4 kg oor 19 dae) na dosering met *L. plantarum* 423 in vergelyking met varkies gedoseer met *L. salivarius* 241 (2.2 kg oor 19 dae) of 'n kombinasie van die twee isolate (2 kg oor 19 dae). Daarenteen het veertien- en 28 dag oud varkies beter gewigstoename getoon na dosering met 'n kombinasie

van isolate 423 en 241. Die selgetalle van *E. faecalis* FAIR E 92 en ander enterococci het drasties afgeneem (twee logaritmiese eenhede) nadat die varkies met laasgenoemde twee isolate gedoseer is. Varkies van onderskeie ouderdom het verskillend gereageer na dosering met *L. plantarum* 423 en *L. salivarius* 241 afsonderlik of in kombinasie.

Fluoresensie-*in-situ*-hibridisasie (FISH) is gebruik om die *in vivo* vashegting van *L plantarum* en *L. salivarius* tot die vark mukus in die maag, duodenum, jejunum, ileum, caecum en kolon te bestudeer. Die hoogste telling van *L. plantarum* selle is aangeteken in die ileum, terwyl *L. salivarius* aanhegting tot die duodenum verkies het. 'n Afname in seltellings van *E. faecalis* in die ileum mukus was aangeteken na toediening met 'n kombinasie van probiotiese isolate 423 en 241. Hierdie studie het 'n betroubare bepaling van die voorkoms en/of vashegting van *L. plantarum* en *L. salivarius* isolate in verskeie gedeeltes van die vark-spysverteringskanaal voorsien, sonder die hulp van duur kwekings tegnieke. Probiotiese bakterieë is in 'n gastro-intestinale model, wat die natuurlike omgewing verteenwoordig, bestudeer. Insig oor die ko-evolusie tussen probiotiese bakterieë en die SVK van die vark is verkry. Die gebruik van 'n gastro-intestinale model om die dinamika van die SVK te bestudeer is met hierdie studie beklemtoon.

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Preface

This dissertation is presented as a compilation of manuscripts. Each chapter is introduced separately and is written according to the style of the respective journal. The addendum is added for additional information pertaining to this research.

Chapter 3   Marè, L., Wolfaardt, G.M. and Dicks, L.M.T. Screening of lactic acid bacteria isolated from the gastro-intestinal tract of post-weaned piglets for probiotic properties and the production of antimicrobial compounds. Prepared for publication in Journal of Animal Science.

Chapter 4   Marè, L. and Dicks, L.M.T. Survival of *Lactobacillus plantarum* and *Lactobacillus salivarius* in new-borne and post-weaned piglets, and in piglets challenged with *Enterococcus faecalis*. Prepared for publication in Letters in Applied Microbiology.

Chapter 5   Marè, L., Dicks, L.M.T. and Wolfaardt, G.M. Adhesion of *Lactobacillus salivarius* and *Lactobacillus plantarum* to the porcine gastro-intestinal tract, as detected by FISH. Prepared for publication in Applied and Environmental Microbiology.

Chapter 6   Marè, L. and Dicks, L.M.T. The study of probiotic lactic acid bacteria in a simple, but effective porcine gastro-intestinal model. Prepared for publication in Applied Microbiology and Biotechnology.

Addendum   Marè, L. and Du Toit, M. Why humans should swallow live bugs – Probiotics. 2002. The Southern African Journal of Epidemiology and Infection 17, 60-69.

               Marè, L. and Dicks, L.M.T. Evaluation of growth media for the isolation of lactic acid bacteria from pre- and post-weaned piglets. Prepared for publication in Letters in Applied Microbiology.

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CHAPTER 1

INTRODUCTION

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1. Introduction

The concept of probiotics probably evolved from a theory first proposed by Metchnikoff in 1908, which suggested that the long and healthy life span of Bulgarian peasants could be ascribed to the consumption of fermented milk products. Through the years many definitions for probiotics have been proposed (Lilly and Stilwell, 1965; Parker, 1974; Fuller, 1989; Salminen et al., 1998). Marteau et al. (2002) defined probiotics as "microbial cell preparations or components of microbial cells that have a beneficial effect on health and well-being". Recent definitions tend to focus more on specific effects, in particular stimulation of the immune system by well-defined strains (Isolauri et al., 2004).

Despite these definitions, the question arises whether or not any given microorganism can be considered a probiotic (Fioramonti et al., 2003). GRAS status, resistance to gastric acids and pancreatic secretions, adhesion to epithelial cells, antimicrobial activity, prevention of the adhesion of pathogenic bacteria, resistance to antibiotics, tolerance to food additives and stability in the food matrix are major criteria to consider (Havenaar and Huis In't Veld, 1992). The majority of commercially available probiotic preparations for animals include species of *Bacillus, Enterococcus, Lactobacillus, Bifidobacterium, Streptococcus* and yeasts such as *Saccharomyces* spp. (Strompfová et al., 2004).

Probiotics play an important role in animal health. The diets, microbiota and gastro-intestinal tract (GIT) interactions of mammals are complex, and are the result of millions of years of co-evolution between vertebrates and their microbiota (Konstantinov et al., 2004). Any major changes in lifestyle and diet place stress on the stability of these interactions and affect the entire GIT eco-physiology. A stable indigenous microbiota in the intestine prevents colonization by pathogens (Van der Waaij, 1989). This colonization resistance of pathogens is important for animals, especially at stressful times, such as weaning (Konstantinov et al., 2004).

During the past decade major efforts have been made to improve animal production. Antibiotics have been used to enhance performance and to control the spread of disease (Gustafson and Bowen, 1997). Increased concern about the potential of antibiotics in animal feed contributing to the spread of antibiotic resistant pathogens resulted in a ban placed on the use of low-dose antibiotics in animal feed. Several countries in Europe have already implemented strict legislation regarding the use of antibiotics in animal feed. Although the use of antibiotics as growth promoters in animal feed has not yet been banned in South Africa, legislation might be forthcoming in the near future (Ratcliff, 2000). The pig industry

is one of the major farming sectors that may be affected by this legislation, since antibiotics are widely used as a growth stimulant. Although this legislation can be viewed as a "precautionary principle", it must be carried through with the necessary risk assessments, especially in a country like South Africa with a diverse agricultural environment ranging from rural to high-technology commercial farming.

Natural alternatives to antibiotics in commercial swine operations, such as probiotic lactic acid bacteria, have been investigated (Turner et al., 2002). One of the major problems encountered in piggeries is the high mortality rate (*ca.* 20%) up to weaning age (Bäckström, 1973). The potential use of probiotics in pigs offer a means of reducing mortality rates (Turner et al., 2002).

Studies on the survival of administered probiotic bacteria have mostly been done on faecal samples (Lick et al., 2001). Cell counts in faeces do not accurately reflect survival of probiotic bacteria and more invasive sampling of the gastro-intestinal tract is needed (Lick et al., 2001). To ensure the selection of functional probiotic bacteria, the bacteria have to be studied within the porcine gastro-intestinal tract. A gastro-intestinal tract model was introduced to screen cultures for probiotic properties (Molly et al., 1993). One of the main criteria for the selection of probiotic strains is the ability to adhere to intestinal surfaces. The study of bacterial adhesion *in vivo* is difficult and research concentrated on the use of costly and labour intensive methods, including tissue culture techniques. The introduction of fluorescent-*in-situ*-hybridization (FISH) provided an alternative to detect individual microbial cells *in situ*, without cultivation (Amann et al., 1995).

2. Aims and objective of this study

The main aim of this study was to simulate the environment of the porcine gastro-intestinal tract in a model and to evaluate the adhesion and survival of probiotic bacteria to sections of the porcine gut.

In essence the study would have to include the following:

- Introduction of a porcine gastro-intestinal model to screen lactic acid bacteria isolated from the gastro-intestinal tract of post-weaned piglets (raised on six different diets) for probiotic properties.

- Oral administration of selected probiotic strains in piglets to determine the effect on weight gain, survival and adhesion *in vivo*. *Enterococcus faecalis* FAIR E 92 will be used as an indicator strain to determine the effect of bacteriocinogenic probiotic lactic acid bacteria.

- Fluorescent *in situ* hybridization (FISH) to study the colonization of probiotic strains *in vivo*. The adhesion studies will be done on mucus from all sections of the porcine gastro-intestinal tract.

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CHAPTER 2

PROBIOTICS IN PIGS: A REVIEW

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1. The probiotic concept

The concept of probiotics evolved at the beginning of the 20th century from a hypothesis first proposed by the Nobel Prize winning Russian scientist Elie Metchnikoff. He suggested that the long and healthy life span of Bulgarian peasants was due to the consumption of fermented milk products (Metchnikoff, 1908). During the last few decades, research on probiotics has expanded beyond bacteria isolated from fermented dairy products to normal microbiota of the intestinal tract (Sanders and Huis in't Veld, 1999).

Parker (1974) was the first to define the term "probiotic" as organisms or substances that contribute to the microbial equilibrium of the gut. More recently Vanbelle et al. (1990) defined probiotics as natural intestinal bacteria that, after oral administration in effective doses, are able to colonize the animal digestive tract, thus keeping or increasing the natural flora, preventing colonization of pathogenic organisms and securing optimal utility of the feed. Prebiotics are defined as non-digestible food ingredients that benefit the host by selectively stimulating the growth and/or activity of bacteria in the colon (Gibson and Roberfroid, 1995). Combinations of prebiotics and probiotics are referred to as synbiotics.

Ingestion of probiotic microorganisms alleviates or prevents various disorders such as lactose intolerance, rotavirus diarrhoea and atopy (Ouwehand et al., 2002). Despite this, the efficiency of probiotics remains questionable (Timmerman et al., 2004). Commercial probiotic products often do not meet expected standards in that the composition and viability of the strains may differ from information on the label (Hamilton-Miller et al., 1999; Hamilton-Miller and Shah et al., 2002; Weese, 2002; Fasoli et al., 2003). Another major issue in relation to the application of probiotics is the poor evidence for efficacy based on clinical trials (Klaenhammer and Kullen, 1999). Three issues interfere with the identification of specific health effects of probiotics (Klaenhammer and Kullen, 1999). Firstly, the complexity and variability of the gastro-intestinal environment in relation to gastro-intestinal diseases make it difficult to determine the effect probiotics have on health and disease. Secondly, confusion as to the identity, viability and properties of probiotics lead to strains being incorrectly identified. Lastly, single probiotic strains induce a multitude of effects among different hosts in a test population. A mono-strain probiotic is defined as containing one strain of a certain species whereas multi-strain probiotics contain more than one strain of the same species or genus. The term multi-species probiotics is used for preparations containing strains that belong to one or preferably more genera (Timmerman et al., 2004). Multi-species preparations have an advantage when compared to mono- and multi-strain probiotics

(Timmerman et al., 2004). Multi-species probiotics benefit from synergism due to the combination of characteristics from different species.

The concept of probiotics plays an important role in animal health. Pig rearing has become an intensive commercial industry. Economic losses, due to decreased health and performance brought about by intensive farming practices focusing on increased production and low costs, is very important. Major efforts have been made to find different ways to improve the rearing of pigs. Antibiotics have been used successfully for more than 50 years to enhance growth performance and control the spreading of disease (Gustafson and Bowen, 1997). Antibiotic resistance is as ancient as antibiotics, protecting antibiotic producing organisms from their own products (Phillips et al., 2004). Antibiotic resistant variants and species that are inherently resistant can dominate and populate host animals.

Increased concern exist about the potential of antibiotics in animal feed and their contribution to the growing list of antibiotic-resistant human pathogens (Corpet, 1996; Williams and Heymann, 1998). Although the use of antibiotics for growth promotion is still allowed in certain countries, including the United States, Australia and South Africa, several European countries have implemented strict legislation to prevent the incorporation of antibiotics in animal feed (Ratcliff, 2000). In 1986 Sweden was one of the first countries to ban the incorporation of low-dose antibiotics into animal feed.

The question remains, does the use of antibiotics in production animals pose a risk to human health? In a recent review, it was stated that the actual danger appears small and the low dosages used for growth promotion (generally below 0.2% per ton feed) could not be regarded as a hazard (Phillips et al., 2004). Antibiotics are used in animals and humans, and most of the resistance problem in humans arises from medicinal use. Resistance may develop in bacterial populations present in production animals, and resistant bacteria can contaminate animal-derived food, but adequate cooking destroys most bacteria. The rate at which these resistant bacteria colonize the human gut and transfer resistance genes remains unknown (Phillips et al., 2004). Growth-promoting antibiotics predominantly active against Gram-positive bacteria have very little or no effect on the antibiotic resistance of salmonellae and consequently on infections caused by salmonellae. Nevertheless, in some parts of the world, antibiotics used to treat animals and added to feed as growth promoters may have adverse effects when associated resistance is taken into account. The same antibiotics are often used to treat humans (Phillips et al., 2004). In contrast, Piddock (2002) could not find clear evidence that antibiotic-resistant bacteria isolated from animals, cause infections in humans, for example quinolone-resistant strains of *Salmonella* serovar Typhimurium DT104 are not

transmitted through production animals. The flouroquinolones used therapeutically in animals appear to pose little threat to human health. Flouroquinolone resistance was recorded in bacteria isolated from humans, in countries where the use of this growth promoter is banned such as Sweden, Finland and Canada (Rautelin et al., 1993; Sjögren et al., 1993; Gaudreau and Gilbert, 1998). Faecal flora isolated from a healthy person may contain antibiotic resistant enterococci, but most enterococci isolated from animals do not colonize the human intestine (Dupont and Steele, 1987; SCAN, 1996, 1998; Bezoen et al., 1999; Butaye et al., 1999; Acar et al., 2000). The situation with *Campylobacter* is even less clear. The banning of growth promoters, including macrolides, has little effect on antibiotic resistance in *Campylobacter* spp. colonizing humans (Travers and Barza, 2002). Risk analyses suggest that only some salmonellae and *Campylobacter* antibiotic resistance is acquired through animals (Phillips et al., 2004). *E. coli* resistance is more likely to be driven by antibiotic use in humans, although an animal origin for at least some clinical isolates cannot be excluded (Gulliver et al., 1999). The banning of antibiotic usage in animal feed remains a controversial issue especially in the way that it affects farming with production animals.

Many natural substances have been investigated as alternatives to conventional chemotherapeutic agents (Turner et al., 2002). Probiotics are one approach used to improve piglet health and deal with intestinal problems encountered during rearing (Vanbelle et al., 1990). Other approaches included acidification of feed or water (Chapman, 1988), altering dietary formulations for small piglets, the development of feeds with lower protein content (Lawrence, 1983), and vaccination with attenuated pathogens or with strains genetically modified (Greenwood and Tzipori, 1987; Trevallyn-Jones, 1987). The administration of growth hormones, somatostatin immunization and enzyme supplementation were also considered as alternatives to antibiotic treatment (Thacker, 1988). Treatment with psychopharmacological drugs (Björk et al., 1987), utilization of the lacto-peroxidase system (Reiter, 1985) and stimulation of hormone-like proteins (anti-secretory factors) capable of reversing intestinal hyper secretion to reduce symptoms of diarrhoea (Lönnroth et al., 1988) were proposed. Some esoteric substances such as zeolite reduced diarrhoea in piglets and increased feed uptake efficiency (Mumpton and Fishman, 1977).

Spray dried animal plasma (SDAP) was evaluated as an alternative to antimicrobial treatment (Torrallardona et al., 2003). SDAP may offer immune protection through its immunoglobulin fraction (Gatnau et al., 1995) or by preventing the adhesion of pathogenic bacteria to the gastro-intestinal mucosa due to the presence of glycoproteins (Nollet et al., 1999). Results suggested that SDAP might be an alternative to antibiotics since it provided

protection against *E. coli* K99 (Torrallardona et al., 2003). Further investigation into this product is required.

Natural substances that enhance growth performance and immune function in pigs include plant products such as seaweed, saponins extracted from certain desert plants, spices and herbs (Turner et al., 2002). Probiotic preparations may be incorporated in prophylactic agents and it is important to know the mode of action to anticipate the dosage levels (Jonsson and Conway, 1992). The use of probiotics should not exclude other alternatives and a combination of treatments may be complimentary and more effective.

2. Aspects relevant to the use of probiotics in pigs

To understand the effect probiotics have on piglets, a thorough understanding of aspects affecting the rearing of pigs and their digestive tract is needed.

2.1 Rearing of pigs

One of the major problems in the rearing of pigs is the high mortality rate (*ca.* 20%) up to weaning age (Bäckström, 1973). In piggeries pre-weaning mortality is caused by diarrhoea, overlay, splay leg, anaemia, bacterial septicaemia, necrotic enteritis, cold exposure and/or congenital defects (Fahy et al., 1987). Piglets in a piggery are often born immature, which renders them more vulnerable to infections.

Neonatal diarrhoea often manifests 48 h after birth and is largely attributed to the enterotoxic *E. coli* strains K88 (most frequent), K99, 987P or F41 (De Graaf and Mooi, 1986). *Salmonella* spp., *Campylobacter* spp., *Cryptosporidium*, transmissible gastroenteritis virus, rotavirus, porcine adenovirus and coronavirus may also cause diarrhoea (Tzipori, 1985; Fahy et al., 1987). The disease manifests by hypersecretion of fluids across the gut wall and into the lumen, triggering the host's immune system through the various toxins produced. Piglets are particularly susceptible to diarrhoea during the first three weeks after birth and at weaning age (21- to 28-days-old). During the first days, the piglet is protected by maternal immunoglobulins in the colostrum (Porter, 1969).

Post-weaning diarrhoea occurs approximately 4 to 10 days after weaning. Enteropathogenic *E. coli* is the major pathogen (Fahy et al., 1987). Many theories have been proposed as to why disease occurs at weaning. One hypothesis is the sudden deprivation of maternal antibodies and other protective factors in the sow's milk. Another possibility is

sudden changes in diet and/or a compromised metabolism (Fahy et al., 1987) that may lead to particles being metabolized by pathogens, which results in an increase of cell numbers. Changes in temperature, humidity and other environmental conditions may also affect the animal's immune system (Carghill, 1982), leading to diarrhoea (Björk et al., 1984). Traditionally, pigs have been weaned after 7 to 10 weeks, but piglets are now weaned after 3 to 4 weeks. At this young age the intestinal tract is not able to digest the diets developed for older pigs (Cranwell and Moughan, 1989). The correct feed formulation is thus of critical importance. Feed should contain easily digestible components. During the fattening stage (six months and older) swine dysentery is a problem and feed should be adapted to achieve desirable performances.

2.2 The porcine digestive tract

The length of the gastro-intestinal tract (GIT) in the newborn pig is only two meters compared to 20 meters in a mature animal (Slade, 2004). Probiotics need to resist low pH and proteolytic enzymes in the digestive tract. The retention time, mixing of the ingested material with gastric juices and previous digesta, influences the survival of the administered strains. In the anterior part of the small intestine, the most important defense is the fast flow rate that prevents microbial overgrowth, provided the microorganisms do not attach to the epithelium. Anaerobic bacteria, aerobic bacteria and coliform bacteria are present in the small intestine at concentrations ranging from 10^9 cfu/g, 10^8 cfu/g and 10^6 cfu/g, respectively (Wenk, 2001). Anaerobic bacterial numbers increase to 10^{11} cfu/g in the large intestine and aerobic bacteria to 10^9 cfu/g. Coliform bacteria remain at 10^6 cfu/g in the large intestine (Wenk, 2001). The presence of bile in this region also represses survival and activity of the microorganisms. In the caecum and large intestine probiotics have to compete with a stable indigenous microflora in the healthy host animal, but the passage rate is slower and the microorganisms establish easier (Jonsson and Conway, 1992).

2.2.1 Stomach

The entrance of the stomach has the same type of keratinized squamous non-secreting epithelium as the esophagus (Noakes, 1971). In this region epithelial cells are released continuously and are covered with intestinal bacterial cells including lactobacilli (Lipkin, 1987). Released squamous cells colonized by these bacteria may help to regulate the

composition of the digestive microflora by ensuring dominance of the lactic acid bacteria (Fuller et al., 1978; Barrow et al., 1980). In the stomach, gastric juices containing mucus, HCl, proteolytic enzymes and low pH are factors influenced by the age of the animal. The stomach pH may be as low as 2.0 in an adult pig, but as high as 5.0 in milk-fed piglets (Slade, 2004). The intestinal pH of pigs at different ages is listed in Table 1. The degree of mixing and the rate at which contents passes through the stomach influence the effectiveness of the digestion process. Mixing of the digesta depends on dry matter content and particle size. Liquid feed and finely ground feed are mixed more easily than drier or coarsely ground cereal diets (Maxwell et al., 1970).

Table 1

pH Values in the digestive tract of pigs

Age	Stomach	Small intestine		Caecum	Colon
		Anterior	Posterior		
Neonatal	4.0 - 5.9	6.4 – 6.8	6.3 – 6.7	6.7 – 7.7	6.6 – 7.2
Pre-weaned	3.0 – 4.4	6.0 – 6.9	6.0 – 6.8	6.8 – 7.5	6.5 – 7.4
Weaned	2.6 – 4.9	4.7 – 7.3	6.3 – 7.9	6.1 – 7.7	6.6 – 7.7
Adult	2.3 – 4.5	3.5 – 6.5	6.0 – 6.7	5.8 – 6.4	5.8 – 6.8

Compiled from Smith and Jones (1963), Smith (1965), Boucourt and Ly (1975), Clemens et al. (1975), Braude et al. (1976), Cranwell et al. (1976), Barrow et al. (1977), Schulze (1977), Schulze and Bathke (1977).

2.2.2 Small intestine

The acidified portions of digesta entering the duodenum are mixed with bile, pancreatic juice, enzymes and other substances. The pH increases in the small intestine, but variations are less than encountered in the stomach. The difference between piglets and adult pigs is less pronounced (Kidder and Manners, 1978). Variations are large in the duodenum (pH 2.0 to 6.0) and progressively smaller towards the ileum (pH 7.0 to 7.5). The activity of microflora in the distal part of the small intestine lowers the pH in this region (Friend et al., 1963). It normally takes 2.5 h for a food particle to pass through the small intestine (Kidder and Manners, 1978). At this flow rate, it is difficult for bacteria to multiply fast enough to prevent being diluted and probiotics should be administered in sufficient dosages. Attachment to

epithelial cells is a prerequisite for bacteria to colonize the small intestine. The mucus in this region may support rapid growth of *E. coli* with an *in vitro* generation time of 26 min (Jonsson and Conway, 1992). The intestinal content measured for the small intestine can be as much as 0.1, 0.6 and 20 L for very young, weaned and adult pigs, respectively (Vodovar et al., 1964).

2.2.3 Large intestine

The large intestine consists of the caecum, spiral colon and the distal colon. The rate of passage is slower compared to the small intestine, leading to the establishment of a dense and complex anaerobic microflora. The first part of a meal reaches the anus after 10 to 24 h, but the mean retention time is much more variable and can be two to four days (Kidder and Manners, 1978). The large intestine can hold volumes up to 0.04, 1.0 and 25.0 L for very young, weaned and adult pigs, respectively (Kidder and Manners, 1978). The pH of the large intestine remains at approximately 6.0 (Kidder and Manners, 1978).

2.3 Lactic acid bacteria indigenous to pigs

The pig is a monogastric animal in which the foregut (stomach and small intestine) is colonized by a relatively large variety of microflora. Bacteria in the small intestine survive low pH conditions better and bacterial numbers are generally high (10^7 to 10^9 cfu/ml) in this section of the GIT (Conway, 1989). Lactic acid bacteria, mostly *Lactobacillus* and *Streptococcus* spp. dominate the small intestine (Fuller et al., 1978). LAB in the foregut helps the young pig to decrease the stomach pH by the production of lactic acid and other organic acids, mainly from lactose (Cranwell et al., 1976; Barrow et al., 1977). LAB may regulate the microflora of the small intestine by migrating with the digesta passing down the GIT (Fuller et al., 1978). Gram-negative bacteria dominate the caecum (Robinson et al., 1981) and Gram-positive species the colon (Salinatro et al., 1977). Species often found in the porcine digestive tract are *Lactobacillus acidophilus*, *Lactobacillus delbreuckii*, *Lactobacillus fermentum*, *Lactobacillus reuteri*, *Lactobacillus salivarius*, *Enterococcus bovis*, *Enterococcus durans*, *Enterococcus faecalis*, *Enterococcus faecium*, *Streptococcus intestinalis*, *Streptococcus porcinus*, *Streptococcus salivarius*, *Bifidobacterium adolescentis* and *Bifidobacterium suis* (Raibaud et al., 1961; Zani et al., 1974; Barrow et al., 1977; Fuller et al., 1978; Collins et al., 1984; Robinson et al., 1984; Robinson et al., 1988).

The selection and establishment of the indigenous LAB in the neonatal pig develops progressively from birth (Sinkovics and Juhasz, 1974; Schulze, 1977). A succession of *Lactobacillus* spp. occurs in the small intestine (Tannock et al., 1990) *L. reuteri* colonize animals on the first day of birth, with the *L. acidophilus* group appearing one week after birth (Naito et al., 1995). Lysozyme in sow's milk has a significant effect on bacterial colonization of the pre-weaned piglet (Schulze and Müller, 1980). Colostrum from the sow's milk provides a protective effect against pathogen-induced diarrhoea (Ducluzeau, 1985). Adverse conditions may lead to changes in the intestinal flora. Markedly lower numbers of lactobacilli and bifidobacteria were detected in the foregut of piglets deprived of water and food for 72 h, while numbers of *E. coli* increased (Morishita and Ogata, 1970).

2.4 Detection and identification of lactic acid bacteria in the porcine gastro-intestinal tract

Understanding of the complex natural bacterial communities that colonize the GIT of monogastric mammals such as pigs and humans is far from complete. The identification of faecal flora by time-consuming methods where intestinal bacteria had to be isolated and cultured, revealed considerable species diversity (Moore and Holdeman, 1974; Salinatro et al., 1977; Moore et al., 1987). Reports on bacteria present in the caecum and colon are limited (Russel, 1979; Robinson et al., 1981; Croucher et al., 1983; Robinson et al., 1984). These methods only provide information on bacteria that are easily cultured and may present a biased view of microbial diversity (Pryde et al., 1999).

Over the past decade, molecular methods have been developed that may be used to study the diversity of the gut microflora (Wilson and Blitchington, 1996). Techniques such as genetic fingerprinting, gene sequencing, oligonucleotide probing and specific primer selection discriminate between closely related bacteria with varying degrees of success (McCartney, 2002). Molecular approaches based on PCR provided powerful tools to study the true phylogenetic diversity of microorganisms within environmental samples. These methods can be biased, are generally very labour intensive and expensive (MacGregor et al., 1997; Wise et al., 1997; Lloyd-Jones and Lau, 1998). Pryde and co-workers (1999), attempted to identify the main types of bacteria present in the porcine GIT by direct retrieval and analysis of small-ribosomal DNA (rDNA) sequences. The Pig Intestinal Microbiology Project included the analysis of a library of bacterial 16S rDNA sequences cloned from the GIT microorganisms of 24 pigs (Leser et al., 2002). This analysis identified phylotypes

(bacteria defined only by their 16S rDNA sequence) present in the GIT of Danish pigs. Specific probe sequences were designed for use in micro-arrays (biochips) to facilitate analysis of the intestinal microbiota. A combination of PCR and fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphism, led to new insights into the GIT ecology (Zoetendal et al., 2004). Additional taxonomic methods frequently used are temperature gradient gel electrophoresis (TGGE) and fluorescent-*in-situ*-hybridization (FISH).Whole cell or *in situ* hybridization involves detection of rRNA within intact cells (Amann et al., 1995). Analysis at single-cell level often provides a more detailed picture than dot blot hybridization. Cell morphology and occurrence of an uncultured organism can be determined and adhesion to intestinal mucus recorded. Quantification of the signal conferred by fluorescently marked rRNA-targeted oligonucleotides allows estimation of *in situ* growth rates of cells (Poulsen et al., 1993). The microscopic identification of single microbial cells with rRNA-targeted probes was first performed with radioactively labeled oligonucleotides (Giovannoni et al., 1988). After hybridization of whole fixed cells, the ^{35}S-labeled probe was visualized by micro-autoradiography. This proved to be a very time-consuming procedure and resulted in formation of silver grains several micrometers away from the target cell, preventing the exact *in situ* localization of small cells in clusters. As demonstrated, fluorescent probes yield good spatial resolution and can be instantly detected by epiflourescent microscopy (Bohlool and Schmidt, 1980). Fluorescently monolabeled rRNA targeted oligonucleotide probes allow for the identification of individual cells (DeLong et al., 1989). This made whole-cell hybridization with rRNA-targeted probes a suitable tool for determinative, phylogenetic and environmental studies (Amann et al., 1990).

FISH can be used to great effect in the identification of intestinal microorganisms. By applying fluorescently labeled oligonucleotides, individual whole fixed cells can be identified *in situ* (Delong et al., 1989; Amann et al., 1990). FISH can be implemented in the detection of probiotic bacteria since rDNA targeted specific oligonucleotide probes can be designed for the probiotic strains administered, that would enable detection of the cells in the mucus. Newly developed oligonucleotide probes targeting *L. reuteri* and *L. amylovorus* allowed for their rapid detection and quantification in the ileum and colon of piglets (Konstantinov et al., 2004). The addition of fermentable carbohydrates supports the growth of lactobacilli in the ileum and colon of weaning piglets. Future molecular biology studies on probiotics and gut flora will lead to a better understanding of the activity and function of microflora (McCartney, 2002). The quest will be to demonstrate the role of probiotic bacteria *in vivo*.

2.5 Immunology

In the healthy adult pig, immunoglobulins are released into the digestive tract and contribute to the host's defense against infection. This immune defense starts to function soon after birth and continues up to about 3 weeks of age (Jonsson and Conway, 1992). After 3 weeks, IgA is secreted and provides immune protection (Porter, 1969). Sow milk immunoglobulins inhibit the growth of *E. coli* (Wilson and Svendsen, 1971), adhesion to enterocytes (Nagy et al., 1979) and neutralizes toxins (Brandenburg and Wilson, 1973). At weaning, the piglet is suddenly deprived of milk antibodies and some non-immunological factors such as lactoferrin, transferrin, vitamin B_{12}-binding protein and the bifidus factor (Cranwell and Moughan, 1989). Although the immune system of piglets is fully functional at the time of weaning, it may need to be stimulated to prevent diarrhoea. Probiotics may stimulate the immune system (Perdigón et al., 1987; Shahani et al., 1989). Surface antigens such as CD4, CD8, DP, CD21, δγTCR, SWC3, SLA-DQ and activation markers such as IL-2r (CD25) are affected by weaning (Solano-Aguilar et al., 2001). Age and weaning time have a direct impact in the relative percentages of differentiated cells such as $C4^+$, $CD8^+$ or $CD4^+/CD8^+$ DP cells (Solano-Aguilar et al., 2001). A study conducted by Mason et al. (2003) showed that weaning is more stressful for younger piglets, with several differences relating to relative weight and suckling behaviours. It was difficult to determine whether smaller, weaker piglets experienced greater suffering compared to heavier, stronger piglets. This study concluded that all piglets experienced weaning stress, with large piglets finding it harder to adapt due to their nutritional demands, whilst smaller piglets, who received less milk during lactation, may suffer more from maternal separation (Mason et al., 2003). Hypersensitivity responses in the early-weaned piglet may be induced by dietary components. The intake of small amounts of certain proteins before weaning, particularly soy, sensitizes the immune system (Newby et al., 1984). Mild diarrhoea and some intestinal disturbances may result, leading to increased susceptibility to pathogenic infections.

3. Use of probiotics and prebiotics

The interest in probiotics increased during the 1940s, followed by a subsequent decline. However, interest is escalating again as can be seen from the number of recent publications on this topic. Emphasis has shifted from using milk fermented with microbes to selecting for

indigenous bacteria. The species used in probiotic products for pigs include *L. acidophilus*, *Lactococcus lactis*, *L. reuteri*, combinations of *Lactobacillus* spp., *E. faecalis*, *E. faecium*, *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus subtilis* var. toyoi, *Bifidobacterium bifidum*, *Bifidobacterium pseudolongum*, *Bifidobacterium thermophilus*, *Clostridium butyricum*, *Saccharomyces* spp. and other yeasts. Mixed combinations of organisms used, include *Pediococcus acidilactici*, *Lactobacillus plantarum*, *Lactobacillus casei*, *L. fermentum*, *Lactobacillus brevis*, *Lactobacillus delbreuckii* subsp. bulgaricus, *L. casei*, *Streptococcus salivarius* subsp. thermophilus, *L. plantarum*, *L. acidophilus* and *E. faecium* (Jonsson and Conway, 1992).

Lactobacilli are strong acid producers and seldom pathogenic (Sharpe et al., 1973; Sharpe, 1981). Although some strains of *E. faecalis* and *E. faecium* are pathogenic (Hardie, 1986; Mundt, 1986), non-pathogenic enterococci are incorporated in probiotic products (Strompfová et al., 2004). Many enterococci produce antimicrobial substances (enterocins) and have an effect on spoilage organisms (Cintas et al., 1997; Sabia et al., 2002). Enterococci can be used as probiotic organisms because of high growth rate, adhesion ability and production of enterocins (Maia et al., 2001). Non-pathogenic strains of certain *E. coli* can be administered to prevent subsequent colonization of other pathogenic bacteria in the GIT (Duval-Iflah et al., 1983). One of the best examples of probiotic *E. coli* is strain Nissle 1917 (EcN), serotype O6:K5:H1 (Blum et al., 1995). This strain lacks typical virulence genes and prevents the invasion of *Yersinia enterocolitica*, *Shigella flexneri*, *Legionella pneumophila* and *Listeria monocytogenes* (Altenhoefer et al., 2004).

Probiotic preparations should be administered soon after birth, when disease is anticipated (preventive or curative). Normal indigenous microflora of a healthy pig may not establish in the GIT when piglets are moved directly after birth into a scrupulously clean environment, or after antibiotic treatment. Preparations of LAB can be administered at these times to initiate the natural sequential colonization of the digestive tract (Cranwell et al., 1976). With normal pig rearing the piglets stay in close contact with the sow for the first weeks, and will be colonized by LAB. On farms with a high incidence of diarrhoea, it may be appropriate to introduce a probiotic strain as early as possible to colonize the digestive tract and prevent the proliferation of pathogens. The characteristics and mechanisms of action of the specific strains used will determine whether a single or continuous dosage is preferable.

Administration could be orally (although this could be very stressful to the animals), or dispensed in water or feed (pelleted or ground). Probiotic bacteria can be given as viable organisms in wet, frozen or freeze-dried preparations or pastes (Tournut, 1989), or as

fermented products (Pollman et al., 1984). Pelleting involves high temperatures and pressures that may be lethal to microorganisms (Jonsson and Conway, 1992). Some streptococci and *Bacillus* spp. are less affected by heat and may survive, but lactobacilli are more sensitive. Growth conditions of the bacteria, harvesting methods and exposure conditions prior to freeze-drying also influence survival of the cells.

Therapeutic doses are 10^9 to 10^{12} viable organisms per animal per day or 10^6 to 10^7 added to feed (Vanbelle et al., 1990). The number of organisms given should be sufficient to elicit a beneficial response in the host, but should not induce digestive disorders (Jonsson and Conway, 1992). The issue whether administered probiotic microorganisms are transient or adhere in the GIT, influences the dosage required. Transient strains need to be administered at higher levels than strains adhering to and multiplying in the GIT (Conway, 1989).

Prebiotics are often administered in conjunction with probiotics. The dominant prebiotics used are fructo-oligosaccharides (FOS), oligofructose and inulin trans-galacto-oligosaccharides, gluco-oligosaccharides, glyco-oligosaccharides, lactulose, lactitol, malto-oligosaccharides, xylo-oligosaccharides, stachyose and raffinose (Monsan and Paul, 1995; Orban et al., 1997; Patterson et al., 1997; Collins and Gibson, 1999; Patterson and Burkholder, 2003). Although mannan oligosaccharides (MOS) have been used as prebiotics, they do not enrich probiotic bacterial populations, but act by binding and removing pathogens from the intestinal tract and by stimulating the immune system (Spring et al., 2000). The oligomers, galacto-oligosaccharides, soybean oligosaccharides, lactosucrose, isomalto-oligosaccharides and palatinose revealed prebiotic potential (Manning et al., 2004).

4. Efficacy and mode of action of probiotics

Administration of probiotic products often gives inconclusive or conflicting results in host animals and determination of the mode of action becomes more difficult (Jonsson, 1985; Tuschy, 1986; Conway, 1989). One important factor to consider is that host susceptibility varies from one animal to the other. Evaluations of probiotic use should include the effect on microflora in the digestive tract. Performance and health can be evaluated by growth rates, feed utilization, number of deaths and occurrence of diarrhoea (Jonsson and Conway, 1992).

The clinical conditions in which efficacy of probiotics have been reported range from infectious, allergic and inflammatory to neoplastic, suggesting that a single mechanism of action is unlikely (Marteau and Shanahan, 2003). Various hypothesis exist explaining the mode of action of probiotics, but these remain speculatory (Vanbelle et al., 1990). Health

promoting advantages of probiotic preparations include production of antimicrobial substances, organic acids, and prevention of adhesion of pathogenic bacteria in the digestive tract. Other possible modes of action include production of metabolites able to neutralize bacterial toxins *in situ* or inhibition of their production. An increase in feed conversion by secretion of enzymes from the microflora, stimulation of the immune system, and proliferation in the GIT were also suggested as possible modes of action. Sakata et al. (2003) suggested that probiotics modify the metabolism in the microbial ecosystem of the large intestine by increasing the production of short chain fatty acids (SCFA). This leads to an increase in sodium and water absorption and a decrease in colonic activity. The SCFA act as modulators for required functions to ensure a healthy GIT. Multiple microbe-microbe and microbe-host interactions may account for the versatility of probiotic action (Marteau and Shanahan, 2003). The molecular details of host-flora interactions are poorly understood, and microbial signals that have been identified include bacterial formylated peptides such as f-met-leu-phe (Anton et al., 1998), lipopolysaccharides (LPS), peptidoglycan cell wall constituents and nucleotides (Marteau and Shanahan, 2003). The exact mechanism of action of probiotics remains largely unknown. Probiotics may contribute to host defense by reinforcing non-immunological defenses and stimulating both specific and non-specific host immune responses (Gill, 2003). Little is known about the relative importance of the probiotic-stimulated mechanisms in host protection.

5. Selection of potential probiotic strains

Probiotic strains are selected based on resistance to lytic enzymes in saliva (lysozyme) and digestive enzymes, growth at low pH and bile salts, and their ability to prevent colonization of pathogenic bacteria. Attachment or adhesion to brush border cells or colonization of the mucus is required, especially in the small intestine where the transit rate of intestinal contents is high. Stimulation of the immune system by the probiotic strains is required to increase cell-mediated immune response. Technological resistance and stability at high temperatures during pelleting, spraying etc. will ensure viability of the probiotic strains after dosage.

Cell adhesion is one of the selection criteria that remain controversial. This aspect was derived from the concept of virulence factors in pathogenic bacteria. Adherence promotes certain virulence activities like production of toxins (Edwards and Puente, 1998; Klemm and Schembri, 2000). Similar interactions could be beneficial for probiotic organisms such as

lactobacilli. Lactobacilli adhere to mucosal surfaces and thereby limits the adherence of pathogenic bacteria (Kotarski et al., 1997; Kirjavainen et al., 1998). Some lactobacilli lack the ability to bind to mucus *in vitro* (Jonsson et al., 2001). Since many of these non-binders were isolated from mucosal surfaces it may be assumed that the growth environment affect the adhesion properties of bacteria (Jonsson et al., 2001). The inclusion of mucin in the bacterial growth medium provided an evaluation method for studying adherence properties of probiotic bacteria. A strain with low mucus-binding activity could prove to adhere well to mucus within the intestinal tract (Jonsson et al., 2001).

The adhesion property of probiotic LAB is species-specific (Barrow et al., 1980). Host specificity is a desirable property for probiotic bacteria and is one of the selection criteria (Salminen et al., 1988; Saarela et al., 2000). Adhesion of LAB in relation to host specificity in human, canine, possum, bird and fish mucus were investigated *in vitro* (Rinkinen et al., 2003). Results indicated that the adhesion trait was not host specific but rather characteristic of the species. This suggests that animal models in probiotic adhesion assays may be more applicable to other host species than earlier thought and highlights the fact that the selection criteria for a probiotic may vary according to the application of the probiotic (Rinkinen et al., 2003).

Numerous papers have been published on the isolation and selection of potential probiotic strains (Nemcova et al., 1997; Chang et al., 2001; Gusils et al., 2002). Results obtained with *in vivo* feeding trials were variable because of the complexity of the intestine and variation between individual animals (Simon et al., 2003). Competitive exclusion products containing undefined cultures were effective in pigs (Fedorka-Cray et al., 1999; Genovese et al., 2000), but the possibility that these products may contain pathogens remains (Gillian et al., 2004). Individual probiotic strains need to be identified before inclusion in a probiotic product (Gillian et al., 2004).

Selection characteristics for prebiotics differ from those proposed for probiotics. Prebiotics should not be hydrolyzed by digestive enzymes or absorbed by mammalian tissues. Substances used as prebiotics must selectively enrich beneficial bacteria (Simmering and Blaut, 2001).

5.1 The use of gastro-intestinal models to screen cultures for probiotic properties

To select suitable probiotics, the strains have to be studied in the environment where they function. The intestinal tract of humans and animals is not readily available for research purposes. This lead to the development of various models simulating the gastro-intestinal tract (Miller and Wolin, 1981; Veilleux and Rowland, 1981; Edwards et al., 1985; Gibson et al., 1988; MacFarlane et al., 1989; Molly et al., 1993; Veenstra et al., 1993). The majority of semi-continuous fermentation systems used to study human colonic flora was adapted from the model described by Miller and Wolin in 1981 (Rumney and Rowland, 1992). This system was designed to maintain human colonic flora *in vitro* and mimic the periodic entry of fermentable substrates into the colon. Edwards et al. (1985) described an *in vitro* model of the anterior colon that was used to study the function of colonic bacteria. Five continuous cultures of faecal bacteria were maintained anaerobically in steady state for 21 days in a medium simulating the ileostomy effluent (Edwards et al., 1985). This system kept cells viable over long periods, with medium added hourly and formed the basis for development of other models to study the nutritional and ecological requirements of colonic bacteria. A three-stage continuous culture system for studying the effect of retention time on the ecology and metabolism of human colonic bacteria was described by MacFarlane and co-workers in 1989. This system was designed to reproduce spatial, temporal, nutritional- and physiochemical characteristics of the intestinal microbiota. It was validated on the basis of chemical and microbiological data obtained from intestinal contents of sudden death victims (MacFarlane et al., 1998). The development of a simulated human intestinal microbial ecosystem (SHIME) was described by Molly and co-workers in 1993. A five-stage reactor was developed to simulate the gastro-intestinal microbial ecosystem of humans. The small intestine was simulated by a two-step "fill and draw" system, the large intestine by a three-step reactor (Molly et al., 1993). This was the first model that included simulations of both the small and large intestine. This system was validated by monitoring fermentation fluxes and products, i.e. indicator bacterial groups, volatile fatty acids, enzyme activities and headspace gasses. Resulting patterns of microbial diversity and activity were analyzed and compared with data obtained from *in vivo* samples. *In vitro* data were representative of in vivo results obtained (Molly et al., 1993). A unique GIT model was developed at the TNO Nutrition and Food Research Organization, based in the Netherlands. It was the first *in vitro* model simulating both the small and large intestine, including features like peristaltic movements, physiological transit characteristics, nutrient absorption and water retention (Veenstra et al.,

1993). The model is a computer controlled system that simulates peristaltic mixing, water absorption and absorption of fermentation products in the large intestine (Minekus et al., 1999). High densities of microorganisms, comparable to those found in the colon, *in vivo*, were achieved by absorption of water and dialysis of metabolites trhough hollow-fibre membranes inside the reactor compartments. The dense content was mixed and transported by peristaltic movements (Minekus et al., 1999). These unique features made the TNO model very expensive to develop and operate. Potential applications included research on the digestibility of carbohydrates and other food ingredients, interactions of fats and proteins, stability of fat and sugar replacers, availability of minerals and survival of bacteria used in fermented foods and probiotics (Veenstra et al., 1993). The TNO model could be used in both animal nutrition and pharmaceutical research. This model can be applied to study the availability of heterocyclic aromatic amines in the gastro-intestinal tract. The TNO model is a powerful tool for research into the field of availability for absorption of mutagenic and anti-mutagenic components in food (Krul et al., 2000). The anti-mutagenic activity of green tea and black tea extracts was investigated in this model (Krul et al., 2001). Dairy products are a potential matrix for folate fortification to enhance folate consumption. Milk folate-binding proteins (FBP) are involved in folate bioavailability. With the use of the TNO model, it was shown that folic acid remains partly bound to FBP during passage through the small intestine, which reduces the bio-accessibility of folic acid from milk (Verwei et al., 2003). More recent research included studies of the absorption of mycotoxins in the GIT of pigs (Avantaggiato et al., 2003; Avantaggiato et al., 2004) and mechanistic studies on the intragastric formation of nitrosamines, resulting in obtaining valuable information regarding the human cancer risk for the combined intake of codfish and nitrate-containing vegetables (Krul et al., 2004). The behaviour of orally administered drug dosage under various physiological gastro-intestinal conditions can be monitored with the use of the TNO model and provides valuable information about the biopharmaceutical behaviour and efficacy of drug delivery systems (Blanquet et al., 2004). These *in vitro* models proved a popular tool for research concerning bacterial populations in the GIT and probiotic bacteria administered to animals and humans. Advantages in the use of *in vitro* models compared to *in vivo* animal trials and experiments include cost-effectiveness, rapid results, reproducibility and no ethical constraints (Veenstra et al., 1993). Probiotic or other bacteria used in various GIT models initially needed to be isolated to study adhesion The need for isolation was eliminated with the introduction of molecular detection methods, such as FISH. Intestinal samples can be introduced into a GIT model and probiotic strains studied in an environment simulating the GIT.

5.2 The safety of probiotic bacteria

Theoretically, probiotic bacteria may be responsible for side effects such as systemic infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals and gene transfer (Marteau, 2001). However, only a few cases of side effects in humans have been reported (Marteau, 2001). Limited information is available on the adverse effects of probiotics in animals, especially pigs. Future studies may focus on the degradation of the intestinal mucus layer by probiotics. No mucus degradation was observed in experiments with gnotobiotic rats (Ruseler-van Embden et al., 1995). Antibiotic resistance genes, especially those encoded by plasmids, can be transferred between organisms (Marteau, 2001). This raises the question whether resistance genes can be transferred by probiotics to endogenous flora or to pathogenic microorganisms. Risk of gene transfer depends on the genetic material transferred, nature of the donor and recipient strains and on selective pressure.

Probiotics currently used have been assessed as safe in fermented foods, but safety evaluation in microbial food supplements remains controversial since legislation differs between countries (Isolauri et al., 2004). The ability of specific probiotic strains to survive gastric conditions and adhere to intestinal mucosa following oral administration may entail the risk of bacterial translocation, bacteraemia and sepsis (Table 2).

Table 2

Potential clinical targets of probiotic intervention

Effect	Potential mechanism	Potential risks
Nutritional management of acute diarrhoea	Reduction in duration of rotavirus shedding, normalization of gut permeability and microbiota	Risk related to host and strain characteristics
Nutritional management of allergic disease and inflammatory bowel disease	Degradation/ structural modification of enternal antigens, normalization of properties of indigenous microbiota and gut barrier functions, local and systemic inflammatory response, increase in expression of mucins	Strains with pro-inflammatory effects, adverse effects on innate immunity, translocation, infection
Reducing the risk of infectious disease	Increase in IgA-secreting cells against rotavirus, the expression of mucins	Risk related to host and strain characteristics
Reducing the risk of allergic/inflammatory disease	Promotion of gut barrier functions, anti-inflammatory potential, regulation of the secretion of inflammatory mediators, promotion of the development of the immune system	Directing the microbiota towards other adverse outcomes, directing the immune responder type to other adverse outcomes

Modified from Isolauri et al. (2004)

6. General discussion

The large population of LAB present in the digestive tract of a healthy animal makes piglets ideal candidates for probiotic dosage. The genetic background, physiological health status and diet of the animal may influence the effectiveness of probiotic preparations (Jonsson and Conway, 1992). It appears difficult to establish a probiotic permanently in the digestive tract of the host animal. Most studies indicate that the indigenous microflora are very efficient in preventing new organisms from establishing permanently (Jonsson and Conway, 1992). More basic knowledge of the digestive ecosystem is needed to obtain consistent effects from probiotics. With the introduction of molecular based techniques such as FISH, this might be achieved in the future.

Some of the problems that remain to be solved include the mode of action of probiotics, dose-response relationship, better knowledge of the importance of adhesion, the chemical nature of the receptor sites of different probiotic strains and retaining viability. Specific conditions where probiotics can be incorporated, as alternatives to antibiotics need to be determined and production costs kept low for these products to become more attractive to the farmer.

7. References

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CHAPTER 3

Screening of lactic acid bacteria isolated from the gastro-intestinal tract of post-weaned piglets for probiotic properties and the production of antimicrobial compounds

Screening of lactic acid bacteria isolated from the gastro-intestinal tract of post-weaned piglets for probiotic properties and the production of antimicrobial compounds

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ABSTRACT: Lactic acid bacteria (LAB) isolated from the gastro-intestinal tract (GIT) of post-weaned (29- to 31-day-old) piglets were screened for probiotic properties and the production of broad-spectrum antimicrobial compounds. Piglets of the same age were raised on six different feedstuffs. Intestinal bacteria were isolated from the stomach, duodenum, jejunum, caecum, ileum and colon by using five different growth media. The highest cell numbers of intestinal bacteria were isolated from the ileum, followed by the caecum and colon. No significant differences in viable cell numbers were recorded for piglets raised on the different feedstuffs. Isolates with the best overall probiotic properties were identified as members of *Lactobacillus salivarius*. One strain (B3) was identified as a member of *Lactobacillus*, but the species remains unknown. These strains were resistant to porcine bile of 0.3 % to 0.6 % (v/v), but sensitive to bovine bile salts (0.3 % to 1.2 % w/v). *L. salivarius* and strain B3 were able to grow at pH 4.0. Both isolates produced antimicrobial compounds active against *Lactobacillus reuteri* and *Streptococcus thermophilus*.

Introduction

A new-born piglet first encounter microorganisms during birth when the upper part of the intestinal tract is infected with lactic acid bacteria from the sow's reproductive tract (Ratcliff, 1985). In a healthy animal, a well-balanced population of microflora in the gastro-intestinal tract (GIT) increases feed digestion and maximizes the absorption of nutrients, which in turn increases the immune response (Riise, 1981; Tannock, 1988). In stressed animals, the population of intestinal microflora is often altered, which results in a reduction of lactic acid bacteria and the proliferation of pathogens (Kyriakis et al., 1999). The symptoms are generally described as post-weaned diarrhoea (Kyriakis et al., 1999) and are often treated by adding pro-phylactic concentrations of antibiotics to the feed (Jonsson and Conway, 1992). The banning of antibiotics in animal feed in many European countries now prevents this practise (Jonsson and Conway, 1992), which in turn favours the inclusion of probiotic lactic acid bacteria in animal feed (Fuller, 1989). Probiotics are defined as natural intestinal bacteria, which colonize the digestive tract, thereby preventing the proliferation of intestinal pathogens (Vanbelle et al., 1990). Lactic acid bacteria most commonly included in probiotic preparations are strains from the *Lactobacillus acidophilus*-group, bifidobacteria and enterococci (Vanbelle et al., 1990). Concluded from previous studies on piglets (Pollmann et al., 1980; Tortuero et al., 1995), probiotics are most effective when administered at weaning age. The aim of this study was to screen various parts of the gastro-intestinal tract of post-weaned piglets for the presence of lactic acid bacteria with probiotic properties and to determine if the composition of the feedstuff has an influence on the intestinal lactic acid bacterial population.

Materials and Methods

Raising of Piglets

Piglets used in this study were of the South African Large White breed. Trials were done on a commercial farm, which placed some restrictions on the layout of the trial and the feed formulations. The metabolic energy (M.E.) for each feed was kept constant at 3185.57 Cal/kg.

Piglets with sows were kept in pens of 6.25 m². A farrowing crate (2.4 m by 0.9 m) was installed in the centre of each pen to prevent the sow from falling onto or trampling on the piglets. Each pen was furnished with a 260 Watt infrared element. Ammonia and methane

gasses emanating from the faeces were extracted through roof canopies. At the rear of the pen a flush channel, covered with a steel grid, was installed to remove the faeces. The steel grid was disinfected on a routine basis.

Twelve sows with their newborns were selected at random and kept in separate pens for the first ten days after birth. Sows received their normal feed and pre-farrowing treatments as part of a routine procedure. Piglets were fed in a creep feeder located at the back of the pens. Water was supplied with nipples. As from day 11, the piglets and sows were divided into six groups of six to 10 piglets per group. Each group was fed a specially formulated feedstuff (Table 1) for 18 to 20 days. All six feedstuffs contained the same raw materials, *viz*. grain sorghum, corn meal, wheat bran, fish meal and/or soy oil cake, whey powder, mono-calcium phosphate, limestone powder, salt, minerals and vitamins, methionine, lysine, zinc-oxide, and a growth stimulant, Olaquindox. In feeds B, D and F corn meal was replaced with precooked corn meal (Table 1). The piglets were weaned when 28 days old and moved to "follow-up" pens (6.25 m^2) A maximum of 14 piglets were housed in each pen.

Isolation of Lactic Acid Bacteria

Piglets with a body weight of between 10.0 and 10.5 kg were selected from each of the six groups (one piglet representative of each feedstuff). Sections of the stomach wall and contents of the stomach, duodenum, jejunum, caecum, posterior part of the colon, middle colon and anterior colon were sampled and kept in separate sterile vials at 8 °C during the two to four day transport to the laboratory. Samples of 5 g were resuspended in 50 ml sterile physiological salt solution and vortexed for 10 min in the presence of sterile glass beads (0.5 cm in diameter). The suspensions were kept at 25 °C for 1 h to allow a homogenous suspension of the microbial cells and were then serially diluted in sterile peptone water. The cell suspensions were plated onto MRS Agar (Biolab, Biolab Diagnostics, Wadeville, Gauteng, South Africa); MRS Agar (Biolab) adjusted to pH 4.6 with 1N HCl; Rogosa Agar (Biolab); LAMVAB Agar (Hartemink et al., 1997) and *Enterococcus* Selective Agar (Difco, Sparks, MD, USA), respectively. After 48 h of incubation at 37 °C, 660 colonies were selected at random from plates with between 30 and 100 colonies and streaked out on the same growth media that were used in the initial isolation of colonies. A total of 246 Gram-positive and catalase negative isolates were randomly selected for further studies.

Screening of Isolates for Probiotic Properties

All tests were done in duplicate.

Resistance to Bile Salts. Resistance to bile salts was tested according to the method described by Chateau et al. (1994), with some modifications. Porcine bile, collected from slaughtered piglets, was pasteurised for 30 min at 60 °C and then added to sterile MRS broth (Biolab) at 0.3 % to 2.6 % (v/v), with increments of 0.2 %. In a separate experiment, Oxgall (Oxoid, Basingstoke, Hampshire, England) was added to MRS broth (Biolab) at 0.3 %, 0.6 %, 0.8 %, 1.0 % and 1.2 % (w/v), respectively, and then autoclaved (15 min, 121 °C). The wells of a sterile ELISA plate were filled with 180 µl of the two bile-containing media and inoculated with 20 µl of an 18 h to 24 h-old culture. MRS broth (Biolab) without bile salts served as control. Incubation was at 37 °C. Optical density readings were taken at 580 nm every hour for 12 h in a microplate reader (Anthos 2001, Anthos Labtec Instruments, Salzburg, Germany).

Growth at Different pH. MRS broth (Biolab) was adjusted from pH 2.0 to 8.0 (increments of one pH unit) with 1 N HCl or 1 M NaOH, autoclaved and dispensed into wells of a sterile ELISA plate, as described elsewhere. MRS broth (Biolab) at pH 6.5 served as control. Optical density readings were recorded in a microplate reader at 580 nm every hour for 12 h.

Antibiotic Susceptibility. Strains resistant to low pH, were screened for susceptibility to 36 antibiotics (Table 2). A modified version of the agar disc diffusion method described by Kirby et al. (1966) was used. Strains were grown in MRS broth (Biolab) for 24 h at 37 °C and then plated onto the surface of MRS Agar (Biolab). Antibiotic discs were placed onto the surface and inhibition zones recorded after 24 h at 37 °C. To determine resistance of the strains to antibiotics currently incorporated into animal feed, the E-test (Viva Diagnostika, Cologne, Germany) was used. The antibiotics selected were ampicillin, benzylpenicillin, chloramphenicol, tetracycline, erythromycin, ciprofloxacin, streptomycin and gentamycin. MRS agar plates were seeded with viable cells suspended in sterile quarter-strength Ringers solution (Merck) to McFarland scale 0.5 (Viva Diagnostika, Cologne, Germany). Antibiotic strips were placed onto the surface of the plates according to the manufacturer's instructions. Plates were incubated at 37 °C and results were recorded after 48 h of incubation. The level of

resistance to each antibiotic was based on the breakpoint values as presented by Danielsen and Wind (2003), *viz.* ampicillin (4 μg/ml), benzylpenicillin (4μ g/ml), chloramphenicol (16 μg/ml), tetracycline (16 μg/ml), erythromycin (4 μg/ml), ciprofloxacin (> 32 μg/ml), streptomycin (> 256 μg/ml) and gentamycin (128 μg/ml).

Production of Antimicrobial Compounds. Isolates were cultured in MRS broth (Biolab) for 18 h at 37 °C, harvested (8000 x *g*, 10 min, 4 °C), the cell-free supernatants adjusted to pH 6.5 to 7.0 with sterile 1 M NaOH and then stored at –80 °C. The ability to produce antimicrobial compounds was tested by screening for activity against a panel of indicator strains (LMG culture collection, Laboratory of Microbiology, University of Gent, Belgium), including pathogenic strains of bacteria isolated from piglets. The agar spot-test method of Uhlman et al. (1992) was used. The plates were incubated for 12 to 18 h at 37 °C and examined for zones of growth inhibition. A zone of at least 1 mm in diameter was regarded as positive for the production of an antimicrobial compound. The presence of bacteriocins was confirmed by treating the cell-free supernatant with Proteinase K (Roche, Basel, Switzerland), as described by Green et al. (1997). Cell-free supernatants of the bacteriocin-producing *Lactobacillus plantarum* 423 (Van Reenen et al., 1998) and *Enterococcus faecalis* FAIR E 92 (Davidse et al., 2004) were used as controls.

Preliminary Identification of Strains with Probiotic Properties

Strains resistant to bile salts and with the ability to grow between pH 2.0 to 4.0 were identified to genus level according to their phenotypic characteristics (Sharpe, 1979). Production of CO_2 from the fermentation of glucose and gluconate was recorded by using the hot-tube method (Dicks and Van Vuuren, 1987). Further identification to species level was according to sugar fermentation reactions with the API 50CHL system (Bio-Mérieux, Lyon, France).

Results and Discussion

Formulation of six different feedstuffs

The metabolic energy recorded for the six feedstuffs were similar, but the percentage of fiber present in the feeds containing cooked corn meal was higher (7 % to 8 %), compared to the

feeds with raw corn meal (2 % to 3 %), (Table 1). Gelatinization is a transition process of starch that takes place during thermo-mechanical feed processing and leads to changes in the consistency of the feed, that leads to a higher percentage of fiber recorded (De Wet, 2000). The percentage available chloride may vary according to the salt content of the fish meal (determined by the quality and brand used). The aim of this study was not to evaluate the various feedstuffs through monitoring weight gain in the piglets, but to stimulate the presence of a variety of lactic acid bacteria within the gastro-intestinal tract of the piglets from which potential probiotic bacteria could be isolated.

Isolation of Lactic Acid Bacteria

The microbial population in faeces is not an accurate reflection of the diversity of microorganisms in the intestinal tract (Rall et al., 1970; Savage, 1983). Invasive sampling techniques proved to be more reliable. No significant differences in viable cell numbers were recorded for animals raised on the different feedstuffs. According to Rojas and Conway (1996), certain lactobacilli metabolize the mucus of the small intestine, and are not affected by the feed.

The average number of cells recorded in all parts of the GIT, with MRS (pH 6.5), ranged from 1×10^6 cfu/g to 6×10^7 cfu/g. Lower cell numbers, up to 1.1×10^7 cfu/g, was recorded with MRS adjusted to pH 4.6, suggesting that MRS at this lower pH is more selective. Cell numbers ranging from 1×10^6 cfu/g to 4×10^7 cfu/g were recorded with Rogosa medium. With LAMVAB medium lactobacilli cell numbers ranging from 1×10^6 cfu/g to 3.4×10^7 cfu/g were recorded. LAMVAB medium is highly selective for lactobacilli (Hartemink et al., 1997). Cell numbers of 1×10^7 cfu/g to 3×10^7 cfu/g were recorded with *Enterococcus* Selective Agar. The high cell numbers of enterococci and lactobacilli suggests that Enterococcus Selective Agar is not very selective.

The highest cell numbers of lactic acid bacteria (6×10^7 cfu/g) were isolated from the ileum (concluded from data obtained on various growth media), followed by the colon (5.8×10^7 cfu/g) and caecum (5×10^7 cfu/g). Cell numbers of 4×10^6 cfu/g and 2×10^6 cfu/g were isolated from the duodenum and jejunum, respectively. Higher cell numbers were recorded from the stomach wall (4×10^6 cfu/g) compared to the stomach content (2×10^6 cfu/g), suggesting that lactic acid bacteria favoured adhesion to the stomach wall. Little information

is available on the number of viable cells isolated from the contents of different sections of the porcine gastro-intestinal tract. According to Lick et al. (2001) most of the intestinal lactic acid bacteria are found in the ileum, which suggests that it is the preferred section for adhesion.

Recent findings suggested that enzyme supplementation to feed affects the numbers and species of lactic acid bacteria in the small intestine. Due to enzymatic activity and the release of fermentable substrates, the level of lactic acid increases and the concentration of short chain fatty acids decrease in the ileum (Högberg and Lindberg, 2004). No enzymes were included in the feedstuffs. However, the large number of lactic acid bacteria isolated from the ileum (6 x 10^7 cfu/g) suggests that adequate nutrients were available. Slightly higher cell numbers were obtained from piglets raised on fish meal and raw maize meal (data not shown). Optimal metabolism of lactobacilli is an important criteria, since these organisms are the most important of all probiotic bacteria in piglets (Pluske et al., 1997).

Resistance to Bile Salts

The requirements of an ideal probiotic for the inclusion in animal feed, includes resistance to bile salts (Conway et al., 1987; Lankaputhra et al., 1995; Jacobsen et al., 1999). Six of the 246 isolates (73, 76, 241, B1, B2, and B4) were sensitive to bovine bile at levels of 0.3 % to 1.2 % (w/v), while one isolate (B3) showed slight resistance. Most isolates were resistant to levels of 0.3 to 0.6 % (v/v) porcine bile. All isolates had a longer lag phase (between 2 h to 4 h) when grown in the presence of porcine-bile (0.6 % to 2.8 % v/v). The concentration and residence time of bile varies in the different sections of the GIT. Suckling piglets have less bile acid and thus also less active hydrolysis compared to weaned piglets (Harada et al., 1988; Kwekkeboom et al., 1990). The enzymes cholesterol 7 α- and 27-hydroxylases that determine the rate at which bile acid is formed is present at lower concentrations during the first 21 days of the suckling period, but increase in piglets between 21 and 49 days old (Lewis et al., 2000). Piglets consume high-fat milk, and have no problems with digestion of the fat. Neither the relatively lower bile acids nor the lower enzyme activity that regulates bile acid synthesis affects the ability of piglets to produce sufficient bile acids to digest and absorb milk fat (Lewis et al., 2000).

Growth at Different pH

Isolates 73, 76, 241, B1, B2, B3 and B4 were able to grow in MRS at pH 4.0, with OD_{580} - values ranging from 0.5 to 1.3. The highest OD_{580} - values at pH 4.0 which is within the pH range from 2.7 to 4.7 measured for weaned piglets, were recorded for isolates B3 and 241. One week after weaning the pH decreases to between 1.6 and 4.4 and reaches a value of between 1.7 and 3.9 two weeks later (Snoeck et al., 2004). The pH values recorded in the stomach one cm distal from the pylorus were between 5.1 and 6.5 in suckling piglets, between 4.2 and 6.1 at the moment of weaning, between 3.2 and 5.8 one week post weaning, and between 2.4 and 6.0 two weeks post weaning. From 1/16 of the length of the small intestine onwards, pH values lower than 4.6 were not recorded. In suckling pigs the change in pH along the small intestine was small and ranges from 6.5 to 6.8 (Snoeck et al., 2004). In the caecum and large intestine no significant changes in pH was observed during weaning, pH remained between 6.1 and 6.4 in the caecum and between 5.9 and 6.5 in the colon (Snoeck et al., 2004). The relatively high pH (remained above 2.5) in the stomach of a suckling and recently weaned piglet can be explained by several factors. The stomach of young piglets has not yet developed the capacity to secrete hydrochloric acid (Cranwell et al., 1976) The sow's milk does not stimulate this secretion and has considerable buffering capacity. Lastly, lactic acid production from lactobacilli in the upper alimentary tract of piglets partially suppresses hydrochloric acid production (Cranwell et al., 1976). This relatively high pH in the stomach, at weaning age may explain the isolation of only seven lactic acid bacteria from a total of 246.

Antibiotic Susceptibility

Results recorded when two isolates (241 and B3) that were most resistant to low pH, were tested against 36 different antibiotics are shown in Table 2. Results obtained with the E-test indicated that strain 241 was sensitive to ampicillin, benzylpenicillin and chloramphenicol, but resistant to the aminoglycosides, streptomycin and gentamycin. Results obtained from the antibiotic disc test and the E-test corresponds with sensitivity of the strain to ampicillin and chloramphenicol and resistance of the strain to streptomycin and gentamycin. Sensitivity or resistance to antibiotics needs to be taken into account when strains are included into animal feed (Sanders and Huis in't Veld, 1999).

Antimicrobial Compound Production

Gene-encoded, ribosomally synthesized antimicrobial peptides or bacteriocins are produced by LAB. Bacteriocins produced by LAB have a relatively narrow spectrum of inhibition, i.e. most are active against species only related to the antimicrobial peptide-producing bacteria (Klaenhammer, 1993). From the 246 isolates screened for antimicrobial properties, isolates 73, 76, 241, B1, B2, B3 and B4 inhibited the growth of *Lactobacillus reuteri* and *Streptococcus thermophilus*, but not *Lactobacillus acidophilus, Lactobacillus delbreuckii* subsp. bulgaricus, *Lactobacillus casei, Lactobacillus curvatus, Lactobacillus fermentum, Lactobacillus helveticus, Lactobacillus plantarum, Lactobacillus sakei, Leuconostoc mesenteroides* subsp. cremoris, *Bacillus cereus, Clostridium sporogenes, Clostridium tyrobutyricum, Enterococcus faecalis, Listeria innocua, Pediococcus pentosaceus, Propionibacterium acidipropionici, Propionibacterium* spp., *Staphylococcus carnosus, E. coli* K88 and *Salmonella choleraesuis. L. reuteri* is one of the dominating lactic acid bacteria in the gastro-intestinal tract of piglets up to 14 days old (Rojas and Conway, 1996). The use of *L. salivarius* strain 241 as a probiotic during the first two weeks should be considered very carefully. The strain will reduce the numbers of *L. reuteri* present in the gastro-intestinal tract, leading to an imbalance in the intestinal bacterial population. This imbalance could lead to the occurrence of diarrhoea and other diseases.

Preliminary Identification of Probiotic Strains

According to carbohydrate fermentation reactions determined with the API identification system, strains 73, 76 and 241 belong to the species *L. salivarius.* Strain B1 was identified as *L. fermentum,* B2 as *L. acidophilus.* Strains B3 and B4 could not be identified with the API identification system.

Concluded from this study, *L. salivarius* 241 and strain B3 revealed the best overall probiotic properties.

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Table 1. Composition of six formulated feed variations

Feed type[a]	Metabolic Energy (M.E)[b]	% Protein	% Fat	% Fiber	% Methionine	% Lysine	% Calcium	% Avail. P	% Avail. Na	% Avail. Cl
						Composition				
A	3185.75	15.40	4.09	3.12	0.36	0.89	0.73	0.75	0.16	0.25
B	3185.15	15.39	4.09	8.35	0.36	0.88	0.71	1.08	0.10	0
C	3185.60	15.39	6.11	2.73	0.26	0.86	0.72	0.78	0.15	0.54
D	3185.95	15.40	6.07	7.88	0.31	1.00	0.71	1.10	0.18	0
E	3185.60	15.39	4.93	2.95	0.31	0.83	0.71	0.78	0.16	0.38
F	3185.90	15.39	4.93	8.18	0.32	1.03	0.71	1.08	0.19	0.41

[a] A: Fish meal with raw corn meal, B: Fish meal with cooked corn meal, C: Soy meal with raw corn meal, D: Soy meal with cooked corn meal, E: Fish meal and soy meal with raw corn meal, F: Fish meal and soy meal with cooked corn meal

[b] Expressed as CAL/kg

Table 2. Antibiotic susceptibility of intestinal lactic acid bacteria (disc method)

Antibiotic (concentration) [a]	LAB Isolate	
	B3[b]	241[c]
Amikacin (30 µg)	+[d]	-[e]
Bacitracin (10 U)	-	+
Cefepime (30 µg)	+	-
Cefotaxime (30 µg)	+	-
Ceftazidime (30 µg)	+	-
Ceftriaxone (30 µg)	+	-
Cefuroxime (30 µg)	+	-
Erythromycin (15 µg)	-	+
Ofloxacin (5 µg)	+	-
Vancomycin (30 µg)	-	+

[a] Concentration of antibiotic present in disc (Oxoid)

[b] Lactic acid bacterium – not identified

[c] *L. salivarius* 241

[d] No inhibition zone visible – strain is resistant

[e] Inhibition zone visible – strain is susceptible

Other antibiotics tested (both strains showed similar results- no differences recorded in resistance or sensitivity of strains B3 and 241 when tested against these antibiotics): Colistin sulphate (25 µg), Nalidixic acid (30 µg), Cloxacillin (5 µg), Nystatin (100 U), Polymixin B (300 U), Methicillin (5 µg), Novobiocin (30 µg), Gentamycin (10 µg), Compound Sulphonamides (300 µg), Kanamycin (30 µg), Metronidazole (5 µg), Ampicillin (10 µg), Neomycin (30 µg), Nitrofurantoin (300 µg), Streptomycin (25 µg), Furazolidone (50 µg), Ciprofloxacin (5 µg), Rifampicin (5 µg), Sulphamethoxazole (100 µg), Clindamycin (2 µg), Tertracycline (30 µg), Chloramphenicol (30 µg), Fusidic acid (10 µg), Cephazolin (30 µg), Tobramycin (10 µg) and Oxacillin (1 µg)

CHAPTER 4

Survival of *Lactobacillus plantarum* and *Lactobacillus salivarius* in new-borne and post-weaned piglets, and in piglets challenged with *Enterococcus faecalis*

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Survival of *Lactobacillus plantarum* and *Lactobacillus salivarius* in new-borne and post-weaned piglets, and in piglets challenged with *Enterococcus faecalis*

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ABSTRACT

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Aims: Monitoring the survival (in terms of cell growth) and effect (in terms of bacteriocin activity) of *Lactobacillus plantarum* 423 and *Lactobacillus salivarius* 241 after oral administration of piglets. *L. plantarum* 423 produced plantaricin 423 active against *Enterococcus faecalis* FAIR E 92. This strain was administered as an indicator organism.

Methods and Results: One, 14, and 28-day-old piglets were dosed with *L. plantarum* 423 and *L. salivarius* 241, separately and in combination (mixed 1:1). Fourteen-day-old piglets were challenged twice with *E. faecalis* FAIR E 92, followed by dosage with probiotic strains. Average body weight, total faecal lactic acid bacteria and enterococci cell counts were recorded. Piglet age contributed to varied results recorded with *L. plantarum* 423 and *L. salivarius* 241, administered separately and in combination. A combination of these strains proved effective in lowering the faecal enterococci cell count.

Conclusions: New-borne piglets gained the most weight when dosed with *L. plantarum* 423 compared to *L. salivarius* 241 and a combination of the two strains. Piglets aged 14 and 28 days, recorded better body weight gain when dosed with a combination of strains 423 and 241.

Significance and Impact of the Study: Piglet age played a significant role in the effectiveness of *L. plantarum* 423 and *L. salivarius* 241 probiotic strains administered separately or in combination.

Keywords: *L. plantarum*, *L. salivarius*, probiotic activity, piglets, *E. faecalis* indicator

INTRODUCTION

In contrast to the gradual weaning of humans, piglets in a production environment are weaned at an early stage. Piglets are weaned when 26 to 28 days old and are put on a solid feed diet (Konstantinov *et al*. 2004). At weaning piglets experience a combination of stress factors such as separation from the sow, moving to new pens and a change in diet that can lead to diarrhoea, reduced growth and increased mortality (Spreeuwenberg *et al*. 2001). Symptoms of post-weaning diarrhoeal syndrome are alleviated by incorporating pro-phylactic concentrations of antibiotics to the feed (Kyriakis *et al*. 1999). The continuing use of antibiotics may result in the development of antibiotic-resistant bacteria that poses a health problem to other animals and humans.

Treatment of animals with probiotic lactic acid bacteria effects the composition of the microbial population in the gastro-intestinal tract (GIT) and limits the complications associated with weaning (Buddington *et al*. 2002; Teitelbaum and Walker 2002; Verstegen and Williams 2002; Hopkins and Macfarlane 2003). For these strains to survive in the gastro-intestinal tract, they need to be resistant to gastric juices and bile acids (Dunne 2001; Ohashi *et al*. 2004). Large numbers of viable probiotic bacteria detected in the faeces is usually an indication that the strains survived the harsh conditions (Fujiwara *et al*. 2001; Ohashi *et al*. 2001; Oozeer *et al*. 2002). Other techniques used to detect probiotic bacteria in faecal samples include the use of selective media, DNA probes and immunological techniques (Hartemink *et al*. 1997).

Orally administered probiotic lactic acid bacteria beneficially affects the growth of piglets by suppressing hemolytic coliforms, reducing scouring and stimulating the systematic immune response (Underdahl *et al*. 1982; Sarra *et al*. 1983; Tortuero *et al*. 1990; Tortuero *et al*. 1995). In one of the studies conducted on piglets fed with *Lactobacillus acidophilus* (Pollmann *et al*. 1980), an increase in body weight and feed conversion were recorded. However, this phenomenon seems to be strain or animal specific, since similar studies yielded no significant differences in body weight when piglets received probiotic lactic acid bacteria (Kornegay 1986; Nousiainen and Suoni

1991; Yuste *et al.* 1992). In a more recent study conducted on minipigs (Du Toit *et al.* 2003), probiotic lactic acid bacteria proved more effective when administered at weaning age.

This study describes the oral administration of probiotic lactic acid bacteria to one, 14 and 28-day-old piglets, monitoring of the bacteria in faecal samples and recording changes in body weight. Two probiotic strains, *Lactobacillus plantarum* 423 (Van Reenen *et al.* 1998) and *Lactobacillus salivarius* 241 were used. *L. plantarum* 423 produces plantaricin 423, a bacteriocin active against *Enterococcus faecalis* FAIR E 92 (Davidse *et al.* 2004). Survival of strain 423 in the gastro-intestinal tract could be monitored by screening for bacteriocin activity against strain FAIR E 92. Strain 241 was included to monitor the effect on body weight when administered alone or in combination with 423. The survival of strain 241 was not monitored directly. Strain 241 was included to determine the effect of a combination of probiotics.

MATERIALS AND METHODS

Bacterial strains and growth conditions

L. plantarum 423, isolated from sorghum beer (Van Reenen *et al.* 1998) and *L. salivarius* 241, isolated from the ileum of a 38-day-old piglet, were used in this study. *E. faecalis* FAIR E 92, isolated from pig faeces (Davidse *et al.* 2004) served as indicator organism sensitive to plantaricin 423 produced by *L. plantarum* 423. Strain 241 was not inhibited by plantaricin 423 and strain 423 was not sensitive towards any antimicrobial compound produced by strain 241. All strains were cultured in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37 °C.

Animal husbandry and feed formulations

Piglets of the South African Large White breed were used. Trails were done on a commercial farm, which limited the number of piglets available for inclusion in the

different experiments. All piglets had free access to water and feed throughout the trials. New-borne piglets were kept with sows in separate pens for the first 10 days. From day 11 piglets received a specially formulated crawl-feed diet that consisted of corn meal, wheat bran, fish meal, mono-calcium phosphate, feed chalk, salt, minerals, vitamins, zinc oxide, lysine, the growth stimulant Olaquindox (Feed Mix, Johannesburg, South Africa), pro-phylactic concentrations of Tylan (Eli Lilly, Johannesburg, South Africa), "Yellow pop" (starch in the form of maize meal, supplied by Feed Mix), whey powder and chemo-therapeutic concentrations of Mecadox (Ciba Speciality Chemicals, Isando, South Africa). Piglets were weaned at 28 days and moved to "follow-up" pens where they received the same diet for a further 10 days. The diet was then replaced with a growth-stimulating feed, which is identical to the crawl-feed, but without salt, zinc oxide, lysine, "yellow pop" and whey powder. The effect of Olaquindox, Tylan and Mecadox on *L. salivarius* 241, *L. plantarum* 423 and *E. faecalis* FAIR E 92 were tested *in vitro* at levels similar to those used in the feed (0.5 mg g^{-1} Olaquindox, 1 mg g^{-1}, Tylan and 0.5 mg g^{-1} Mecadox), separately and in combination.

Dosage of new-borne piglets with *L. plantarum* and *L. salivarius*

New-borne piglets were divided into three litters of 10, 9 and 7 animals, respectively. Five piglets of the first litter were dosed with 5 ml *L. plantarum* 423 (4 x 10^9 cfu ml^{-1}), five piglets of the second litter were dosed with *L. salivarius* 241 (2 x 10^9 cfu ml^{-1}) and four piglets of the third litter received a mixed population (1:1) of *L. plantarum* 423 and *L. salivarius* 241 (total cells in combination were 3 x 10^9 cfu ml^{-1}). Piglets were dosed on the day of birth and once a week for seven weeks. The rest of the piglets in each litter received no probiotics and served as controls. The average body weight of the piglets was recorded periodically from day one to day 47 (Fig. 1). Linear regression models (Y = a + b.X) were fitted for each treatment, where Y = average weight per pig and X = age in days. The test of parallelism was used to determine the differences between constants (a) and slopes (b). Both constants (intercepts, P = 0.012) and slopes (P = 0.007) differed significantly. Genstat for Windows (2000) Release 4.2 Fifth edition, Oxford: VSN International was used for statistical analyses. Faecal samples were not analyzed.

Dosage of 14-day-old piglets with *L. plantarum* and *L. salivarius* after infestation with *E. faecalis*

Seven litters consisting of between 7 to 10 piglets were used. All piglets were dosed with 5 ml *E. faecalis* FAIR E 92 (4×10^6 cfu ml^{-1}) when 14 days old and again at 22 days. On day 29, piglets from two litters each received 5 ml *L. plantarum* 423 (4×10^9 cfu ml^{-1}). Piglets from another two litters received *L. salivarius* 241 (2×10^9 cfu ml^{-1}). Piglets from the third set of two litters received 5 ml of a combination (1:1) of strains 423 and 241 (3×10^9 cfu ml^{-1}). Dosage with these probiotics was repeated after seven days and continued once a week for six weeks. The seventh litter served as the control and received only two *E. faecalis* FAIR E 92 dosages and no probiotics. Average body weight of the piglets was recorded on day 31 and on day 39 (Table 1). The experiment was designed as a completely randomised design (CRD). Analysis of variance (ANOVA) was used to test for differences between four treatments (Table 1). The data was acceptably normal with homogeneous treatment variances. Treatment means were separated using Fishers' protected t-test least significant difference (LSD) at the 5 % level of significance (Snedecor and Cochran 1980). Genstat for Windows (2000) Release 4.2 Fifth edition, Oxford: VSN International was used for statistical analyses. Faeces were collected at random. The first analysis of faecal samples was on 13-day-old piglets (one day before the start of the trial). The next analyses were done on day 15 (after the first dosage with *E. faecalis* FAIR E 92) and on day 23 (after the second dosage of *E. faecalis* FAIR E 92). Further isolations from faecal samples were done on the days the piglets were dosed with the probiotics.

Dosage of post-weaned piglets with *L. plantarum* and *L. salivarius*

L. plantarum 423 and *L. salivarius* 241 were administered separately and in combination once a week for 6 weeks to post-weaned (29-day-old) piglets. Seven litters of between 8 to 12 piglets were used. Piglets from two litters each received 5 ml *L. plantarum* 423 (4×10^9 cfu ml^{-1}). Piglets from another two litters received *L. salivarius* 241 (2×10^9 cfu ml^{-1}). Piglets from the third set of two litters received 5 ml of a combination (1:1) of strains 423

and 241 (3×10^9 cfu ml^{-1}). The seventh litter was the control and received no probiotics. The average body weight of all piglets was recorded. The experiment was designed as a completely randomised design (CRD). The statistical analyses were performed as described in the previous section. Faecal samples were collected from piglets after receiving probiotic dosages over three weeks.

Isolations from faecal samples

Litters that received no probiotic or *E. faecalis* FAIR E 92 dosages were included to serve as controls for lactic acid bacteria and enterococci isolations from faecal samples. Faeces were collected at random from day one to day 47 (Fig. 2). Faecal samples collected were diluted (10 % m/v) in 9 ml sterile Ringers solution, vortexed for 15 min, serially diluted in Ringers solution and plated out. MRS Agar (Biolab) was used for the isolation of lactobacilli. *Enterococcus* Selective Agar (Difco, Sparks, MD, USA) was used to isolate enterococci. Plates were incubated at 37 °C for 48 h.

RESULTS AND DISCUSSION

No significant responses in body weight gain of piglets after oral administration of probiotic lactic acid bacteria have been recorded in some previous studies (Cupere *et al*. 1992; Johnsson 1985; Kornegay 1986; Nousiainen and Suoni 1991; Yuste 1992). Results obtained in this study varied. Animal trials were done on a commercial farm in order to test the probiotics under conditions experienced in this environment. The exclusion of Mecadox, Olaquindox and Tylan from the feed was not allowed and considered as a high risk because of increased mortality rates. Olaquindox and Mecadox had no effect on *L. plantarum* 423, *L. salivarius* 241 and *E. faecalis* FAIR E 92 when tested *in vitro* at sub-therapeutic levels, separately and in combination. However, all three lactic acid bacteria strains were killed by Tylan when tested *in vitro*. Tylosin, the active component in Tylan reaches the stomach and anterior part of the colon but probiotic lactic acid bacteria colonize the ileum more frequently and in larger numbers compared to other parts of the GIT (Blake *et al*. 2003). Bacteria found in pigs that proved to be most sensitive to tylosin

were *Mycoplasma hyopneumonia*, *Bordetella bronchiseptica*, *Staphylococcus aureus* and *Erysipelothrix rhusiopathiae* (Sumano and Ocampo 1997). Reports from other studies indicated that lactobacilli isolated from pig faeces were resistant to tylosin (Whitehead and Cotta 2001). The effect of Tylan on orally administered strains would therefore be minimal taken the latter into account, especially since probiotic strains tend to favour colonization in the ileum.

Dosage of new-borne piglets with *L. plantarum* and *L. salivarius*

The average body weight of piglets recorded from day one to day 47, while receiving *L. plantarum* 423, *L. salivarius* 241, and a combination of both, is shown in Fig 1. The group dosed with *L. plantarum* 423 gave the best overall result and weighed 12 kg at the end of the trial, compared to between 9 and 10.5 kg recorded for piglets dosed with *L. salivarius* 241 and a combination of strains 423 and 241 (Fig. 1). Statistical analyses showed that the slopes of treatments 241 and 423 differed significantly (t $\leq$ 0.037) which indicates that the increase in weight of piglets dosed with 423 was significantly higher compared to piglets dosed with 241. No major differences in body weight were recorded between piglets dosed with *L. salivarius* 241, a combination of *L. plantarum* 423 and *L. salivarius* 241, and the control group (not dosed with probiotics). One exception was on day 33 when the control group recorded average body weights above 9 kg compared to the group that was dosed with a combination of strains 423 and 241 and recorded and average body weight of 6 kg. Water retention in pigs or feed intake changes on days preceding weighing influence weights recorded. Piglets dosed with *L. plantarum* 423 from birth, utilized this probiotic more effectively (gained more body weight) compared to *L. salivarius* 241 and a combination of strains 423 and 241. Concluded from these results, *L. salivarius* 241, which was isolated from a 38-day-old piglet did not lead to significant weight gain in new-borne piglets. The intestinal tract of new-borne piglets favoured *L. plantarum* 423 (isolated from sorghum beer). In suckling pigs the change in pH in the small intestine is little (Snoeck *et al.* 2004). *L. plantarum* 423 survived the stable pH in the small intestine of suckling pigs, while *L. salivarius* 241, isolated from an older piglet were not as effective in this environment. A previous study showed that *L.*

plantarum 299v survived passage through the gastro-intestinal tract irrespective of gastric acidity (Goossens *et al*. 2005). *L salivarius* 241 found the stable pH environment less favourable. The latter needs further investigation, especially since *L. salivarius* 241 was isolated from the ileum of a 38-day-old-piglet. The strain might be more adaptable to an environment with either a different pH as to the pH found in the small intestine of the suckling pig, or the strain might be more suited to an environment with an unstable pH.

Dosage of 14-day-old piglets with *L. plantarum* and *L. salivarius* after infestation with *E. faecalis*

E. faecalis FAIR E 92 was used as an indicator strain to determine the effectiveness of *L. plantarum* 423 in reducing the enterococci cell numbers. Plantaricin 423 was used as an indicator to determine the effect of *L. plantarum* 423 and not to reduce the total enterococci cell numbers present in the gastro-intestinal tract. Some enterococci present in the porcine gastro-intestinal tract are beneficial to the wellbeing of the piglet. After challenging piglets twice with *E. faecalis* FAIR E 92, followed by dosage with probiotic strains for 6 weeks, piglets dosed with a combination of strains 423 and 241 gained the most body weight (1.43 kg). Piglets that received only *L. plantarum* 423 or *L. salivarius* 241 gained 0.68 kg and 1.03 kg, respectively (Table 1). *L. salivarius* 241 proved more effective (recorded more body weight gain) when older (29 days) piglets were dosed with this strain compared to *L. plantarum* 423. This concurred with results indicating that *L. plantarum* 423 proved more effective when administered to new-borne piglets, as described elsewhere. *L. plantarum* 423 was isolated from sorghum beer and *L. salivarius* 241 from the porcine ileum. The possibility exists that strains 423 and 241 binds to different sections in the intestinal tract. This merits further investigation to determine whether some probiotic strains might prefer different parts of the gastro-intestinal tract as a niche. The ability of probiotic bacteria to adhere and colonize the intestinal mucosa, and therefore their efficacy to balance the endogenous microflora and to modulate the immune system, may be dependant on the age of the host under probiotic therapy (Kirjavainen *et al*. 1998). The intestinal environment of newborns may be immature and provide less sites for adherence (Kirjavainen *et al*. 1998). Strain 241 isolated from an

older piglet may not be able to find any suitable adhesion sites in the gastro-intestinal mucosa of new-borne piglets. The age effect seemed to be dependant on the bacterial strain and the age of the target host group should be taken into account when administering probiotics. Piglets dosed with *E. faecalis* FAIR E 92 only gained 0.42 kg (Table 1). Statistically, the results did not differ significantly, but it is clear that higher weight gain was recorded in piglets that received probiotic dosage(s) as compared to control litters. Access to the amount of litters used was restricted on the commercial farm. If more litters could have been included per treatment, results would have been more statistically correct. Nevertheless, the assumption can still be made that dosage with a combination of probiotics lead to higher weight gain.

Lactic acid bacteria cell numbers (ranging from 1×10^8 cfu g^{-1} to 1×10^9 cfu g^{-1}) were recorded from faeces of piglets (various ages) that received no probiotic dosages or *E. faecalis* FAIR E 92 (Fig.2). The lactic acid bacteria cell numbers decreased from 1×10^9 cfu g^{-1} on day one to 3×10^8 cfu g^{-1} on day 8, followed by an increase in lactic acid bacteria cell numbers to 4×10^9 cfu g^{-1} on day 13 (Fig.2). A decrease in lactic acid bacteria cell numbers (from 5×10^9 cfu g^{-1} on day 20 to 3×10^9 cfu g^{-1} during weaning) could be attributed to stress experienced during weaning, but more litters would have to be examined to reach a definite conclusion. However, an increase in lactic acid bacteria isolated from faeces of one to seven-day-old piglets and older, was described by Naito *et al.* (1995). The decrease in lactic acid bacteria cell numbers recorded from day one to day 8 could be attributed to the effect of the Tylan included in the feed on the natural lactic acid bacteria population. Results obtained from faecal samples collected from piglets that received no probiotics on day 1, indicated total enterococci cell numbers of 1×10^8 cfu g^{-1}.

After the first challenge with indicator strain 92 (on day 14), faecal enterococci cell numbers of 1×10^7 cfu g^{-1} were recorded and after the second challenge the cell numbers recorded were 2×10^7 cfu g^{-1}. These cell numbers were much lower compared to the enterococci cell numbers recorded on days 13 and 20 in faeces of control piglets that received no probiotics or *E. faecalis* FAIR E 92. The decrease in cell numbers could be attributed to the stress experienced when dosing piglets orally. Taken the latter into account, the presence of the indicator strain *E. faecalis* FAIR E 92 within GIT of these

piglets was shown with the increase in cell numbers after the first challenge. Dosage with probiotic cultures was resumed.

When the last isolation of faecal enterococci was done, some piglets in test litters were already weaned (Table 2). Piglets below a selected weight (4 kg) were not weaned as per standard procedure. In all instances higher enterococci cell numbers were recorded in faeces of piglets that were not yet weaned compared to those already weaned. The stress during weaning had a significant impact on the faecal enterococci cell numbers. This is in accordance with results reported by Robinson *et al.,* (1984), stating that weaning is accompanied by marked changes in the microbial populations in the GIT and therefore also in the faeces. The combination of probiotics (strains 423 and 241) proved to be more effective in lowering the faecal enterococci cell numbers of non-weaned piglets, compared to single strain probiotic treatments, but this is not reflected in weight gain data. Probiotic preparations may consist of single strains or may contain up to eight strains (Fuller 1989). Multiple strain probiotic preparations are active against a wider range of conditions and in more animal species (Fuller 1989). Well-designed multi-species probiotics may benefit from synergism when probiotic effects of the different species are combined (Timmerman *et al.* 2004). One of the synergistic effects is the adhesion and/or colonization of multi-species probiotics to different parts of the gastro-intestinal tract. This could explain why the combination probiotic 423 and 241 showed better results in lowering enterococci cell numbers. In post-weaned piglets, the faecal enterococci cell numbers were not lowered after treatment with the combination probiotic.

Dosage of post-weaned piglets with *L. plantarum* and *L. salivarius*

The average body weight recorded of pigs that received probiotic dosages from 23 days-old to 53 days-old is shown in Fig. 3. No major difference was recorded between body weights gained by the litter treated with probiotic strain 423 compared to the control litter (Fig. 3). No significant differences at 5 % level could be shown. The variance was small, therefore if more litters could have been included, statistical analyses could have indicated significant differences in weights recorded in piglets dosed with probiotics. Similar results

were recorded when *L. salivarius* 241 was dosed alone, or a combination of 241 and 423 was administered. Overall, piglets did not show definite body weight gain due to oral administering of probiotics. Other researchers have also recorded that daily body weight gain and feed conversions are not always increased with probiotic dosage but mortality rates are reduced (Vanbelle *et al.*, 1990). An increase in lactic acid bacteria cell numbers were recorded from faeces of piglets dosed with probiotics from 23 to 53 days old (2.0×10^9 cfu g^{-1}) compared to lactic acid bacteria cell numbers of 1×10^6 cfu g^{-1} faeces recorded for the control group that did not receive probiotics. The isolation of higher cell numbers of lactic acid bacteria from faeces of piglets dosed with probiotic bacteria indicated the presence of these bacteria in the GIT.

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Fig.1 Average body weight of piglets dosed with probiotic strains *L. plantarum* 423 and *L. salivarius* 241 and a combination of both (1:1)

* Average body weight of the piglets that received no probiotics

Table 1. Average body weight and average total weight gained by piglets dosed with *E. faecalis* FAIR E 92 on days 14 and 22 respectively, followed by probiotic dosage on days 29 and 36, respectively

Probiotic strain(s)	Weight recorded on:		Weight gained over 9 days
	Day 31	Day 39	
L. plantarum 423	5.58 kg [a]	6.53 kg	0.95 ± 0.71 [b] kg
L. salivarius 241	6.0 kg	7.03 kg	1.03 ± 0.50 [b] kg
Combination 423 and 241	6.58 kg	8.01 kg	1.43 ± 0.95 [b] kg
Control [c]	5.92 kg	6.34 kg	0.42 ± 0.06 [b] kg

[a] Average body weight of all piglets in litter (F probability = 0.543, CV % = 67.0)

[b] Means followed by the same letter did not differ significantly at the 5 % level

[c] Piglets received two dosages with strain 92 and no probiotics
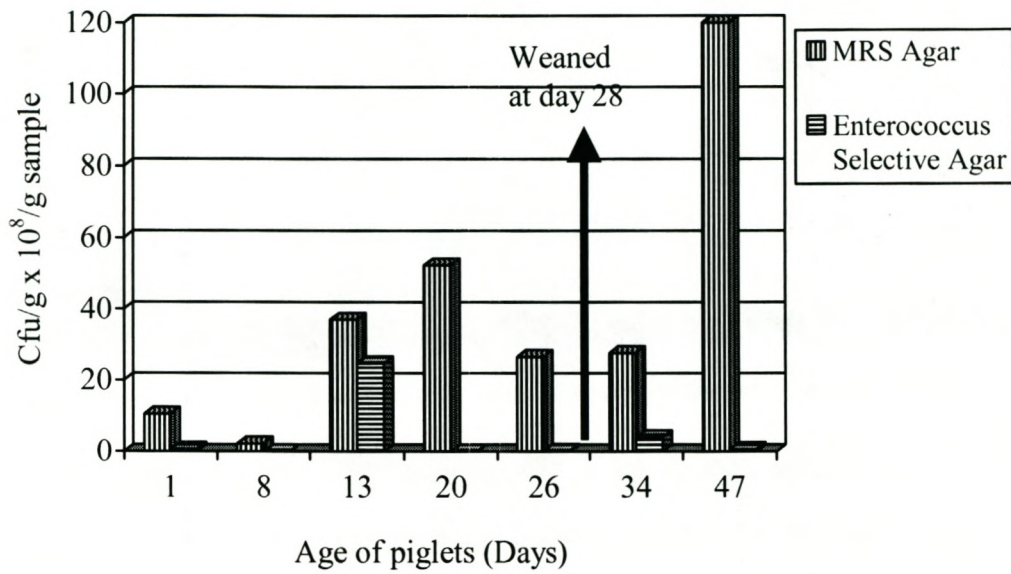
Fig. 2 Lactic acid bacteria and enterococci cell numbers isolated from piglet faeces samples collected from piglets that received no probiotics or *E. faecalis* FAIR E 92 (control)

Table 2. Faecal enterococci counts obtained on *Enterococcus* selective agar, after isolation from piglets dosed with *E. faecalis* FAIR E 92, followed by probiotic dosage with *L. plantarum* 423 and *L. salivarius* 241

Treatment	Weaned	Cfu x 10^6/g sample
Control [a]	Yes	0.41
Control	No	16.7
Probiotic 423	Yes	6.0
Probiotic 423	No	110.5
Probiotic 241	Yes	1.3
Probiotic 241	No	99
Combination 423 +241	Yes	2.22
Combination 423 + 241	No	2.38

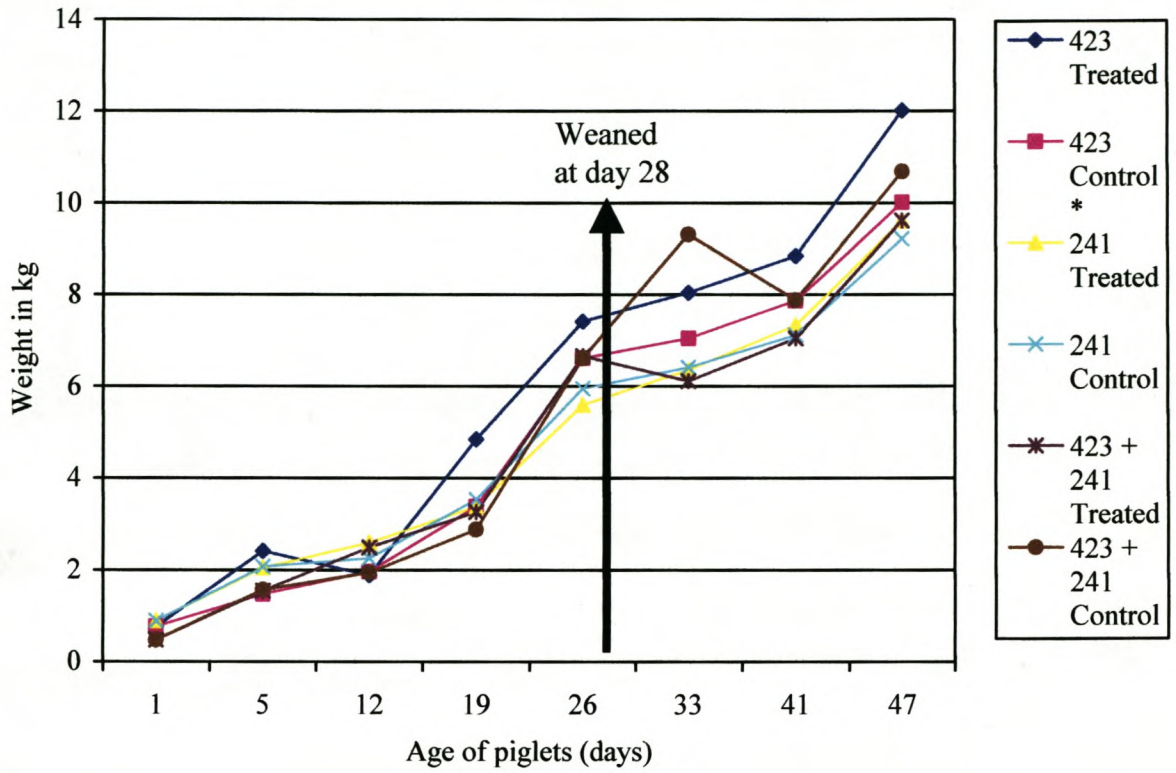[a] Piglets received no probiotic dosage
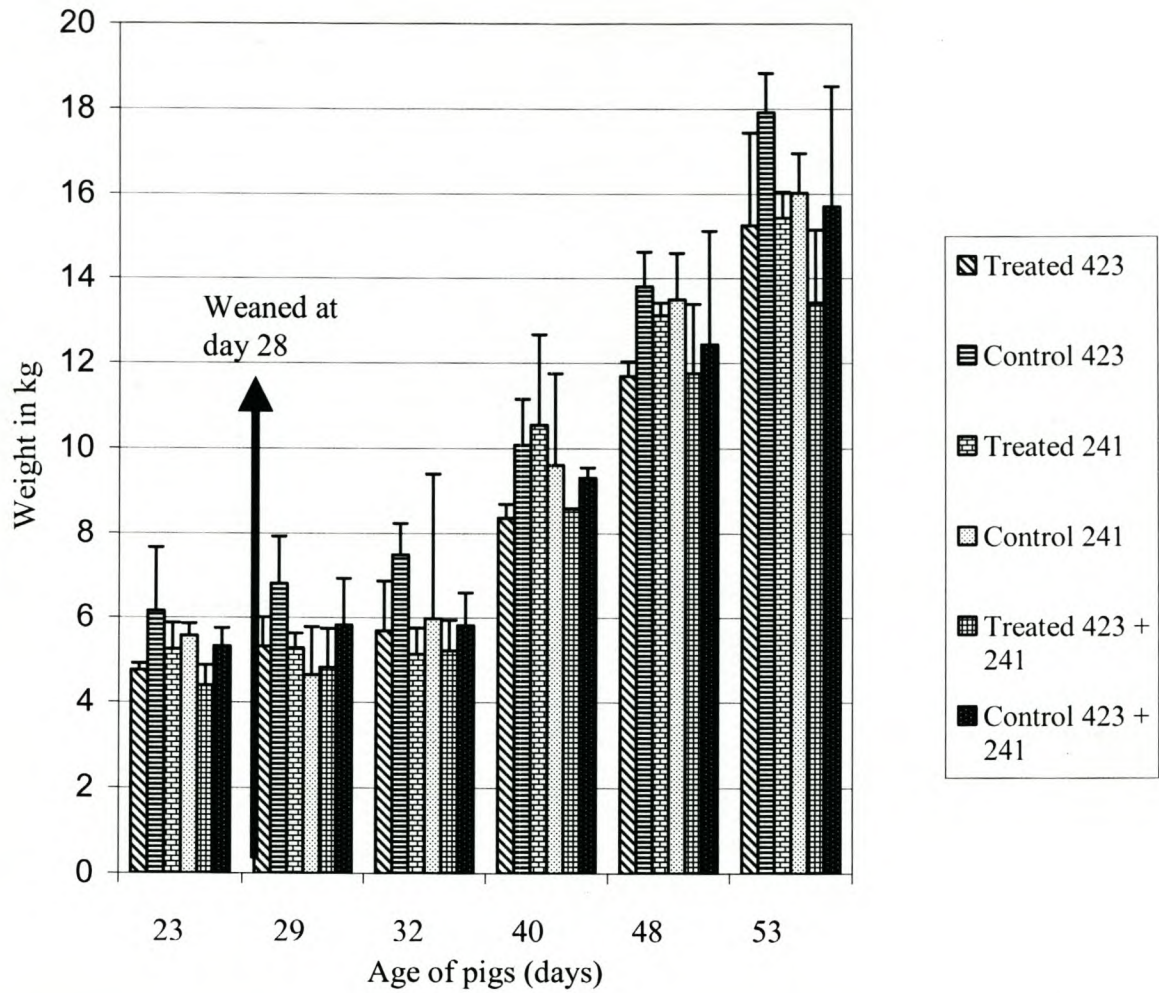
Fig. 3 Average body weight of pigs dosed with probiotic strains *L. plantarum* 423, *L. salivarius* 241 and a combination of 423 and 241 (1:1)

CHAPTER 5

Adhesion of *Lactobacillus salivarius* and *Lactobacillus plantarum* to the porcine gastro-intestinal tract, as detected by FISH

Adhesion of *Lactobacillus salivarius* and *Lactobacillus plantarum* to the porcine gastro-intestinal tract, as detected by FISH

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Abstract

The ability of probiotic bacteria to adhere to intestinal surfaces remains a controversial issue. Studying bacterial adhesion *in vivo* is difficult and is mostly done with costly and labour intensive methods, often involving tissue culture techniques. Fluorescent-*in-situ*-hybridization (FISH) provided a method to detect individual microbial cells *in situ*, without cultivation. This study describes the use of FISH for the detection of the probiotic bacteria *Lactobacillus plantarum* 423 and *Lactobacillus salivarius* 241 in the mucus of the porcine gastro-intestinal tract (GIT). The bacteria were administered orally to piglets. *L. plantarum* 423 produces a bacteriocin active against *Enterococcus faecalis* FAIR E 92, which was used as an indicator to determine the survival of strain 423 in the porcine GIT. Piglets were slaughtered and samples collected from the stomach, stomach wall, duodenum, jejunum, ileum, caecum and colon. The highest number of *L. plantarum* cells was detected in the ileum, whereas *L. salivarius* favoured adhesion to the duodenum. A decrease in cell numbers of *E. faecalis* in the ileum mucus was recorded when a combination of the probiotic strains 423 and 241 was administered. This study provided a reliable culture independent estimation of the presence and/or adhesion of probiotic cultures in various parts of the porcine GIT, without the use of time-consuming cultivation techniques.

INTRODUCTION

Antibiotics and antimicrobials are added to the diets of piglets to promote growth and to alleviate the effects of post-weaning diarrhoeal syndrome (PWDS) (26). PWDS is caused by a sudden imbalance of the intestinal flora coupled with an increase in pathogenic *Escherichia coli* strains and other pathogens (25). Concern exists worldwide that the continuing use of these substances will result in the proliferation of antibiotic-resistant bacteria that could affect humans and animals (33).

Probiotic lactic acid bacteria (LAB) can be used as alternatives to antibiotics in the treatment of PWDS and other diseases at weaning age (16). Probiotics are natural intestinal bacteria which, after oral dosage in effective doses, are able to establish and colonize the digestive tract, stabilize or increase the natural flora, prevent colonization of pathogenic organisms and secure optimal digestion of the feed (44, 50). Most probiotics developed for animals contain strains of *Lactobacillus* spp., *Streptococcus* spp. or *Bacillus* spp. and are included in the feed or administered orally in the form of viable cell suspensions (26). In a healthy animal, a well-balanced gastro-intestinal flora not only increases the digestion rate and absorption of nutrients, but also increases the animal's resistance to microbial infections (37, 52, 54). A successful probiotic strain prevents gastro-intestinal infections (1, 32), enhances immune response (23), and has anti-mutagenic and anti-carcinogenic properties (18, 35).

One of the main criteria for selecting probiotic strains is the ability to adhere to intestinal surfaces. Adherence to mucosal surfaces prolongs retention of probiotic cells in the gastro-intestinal tract, thereby preventing the colonization of pathogens (20, 22, 24, 27, 31, 45). Bacterial adhesion is based on non-specific physical interactions between two surfaces, which then enable specific interactions between adhesions (usually proteins) and complementary receptors (24). Mucus is continuously subjected to degradation, conversely new mucin glycoproteins (the major components of mucus) are constantly secreted (24). Recent papers suggested that most probiotic bacteria do not adhere to epithelial cells, in which case the continuous ingestion of probiotic cultures is a prerequisite (41).

Studies on the survival of administered probiotic bacteria are mostly done by analyzing faecal samples (28). Cell numbers in faeces are not a true indication of the survival of probiotic bacteria in the gastro-intestinal tract and do not reveal the number of cells that remain

colonized in the intestinal tract (28). Studies with intestinal cell lines (47) yielded valuable information on the colonization of these bacteria. The technique is laborious and special facilities are needed to maintain cell lines. Human ileostomy glycoproteins have been used as a model for small intestinal mucus to investigate the adhesion of probiotics but samples are not frequently available (48). Another method used to study the adhesion of probiotics, involved the use of *Lactobacillus fermentum* to inhibit the adhesion of radio-actively labeled *E. coli* 1107 to porcine ileal mucus (34). Molecular technology incorporating species-specific DNA probes designed from 16S and 23S rRNA sequences (14, 40, 53) provides another approach. Fluorescent-*in-situ*-hybridization (FISH) allows for specific detection of intact bacterial cells without cultivation (3, 12).

In this study two probiotic strains, *Lactobacillus plantarum* 423 and *Lactobacillus salivarius* 241, respectively and in combination, were administered orally to newborn piglets and piglets of 14 days old. *L plantarum* 423 produces a bacteriocin active against *Enterococcus faecalis* FAIR E 92 (51). Interactions between the probiotic cells and *E. faecalis* FAIR E 92 were studied in porcine mucus with FISH.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Lactobacillus plantarum* 423 was isolated from sorghum beer (51) and *Lactobacillus salivarius* 241 from the ileum of a 38-day-old piglet. *Enterococcus faecalis* FAIR E 92 was isolated from pig faeces (11) and served as an indicator organism sensitive to plantaricin 423, a bacteriocin produced by strain 423. Strain 241 is not inhibited by plantaricin 423 and strain 423 is not sensitive towards any antimicrobial compound produced by strain 241. FISH allowed detection of intact bacterial cells *in situ*, without cultivation (3, 12). All strains were cultured in MRS broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 37 °C.

Species-specific probes. Strains 423, 241 and FAIR E 92 were inoculated into 5 ml MRS broth (Biolab) and incubated overnight at 37 °C. One ml of each culture was centrifuged (8000 x g, 5 min, 20 °C) and the pellet suspended in 100 μl MilliQ water. Amplification of rDNA was done by using the TaKaRa Ex Taq^{Tm} kit (Takara Shozo Co. Ltd., Biomedical Group, Shiga, Japan). The reaction mixture was prepared as described by the manufacturers. The primers

selected for PCR reactions were 616V (5'–AGAGTTTGAT(CT)(AC)TGGCTCAG-3') and 985R (5'-CCGGTCCTCTCGTACT- 3') supplied by Roche, Mannheim, Germany. The PCR cycle was 4 min at 94 °C, 30 s at 94 °C, 30 s at 52 °C and 4 min at 72 °C, repeated 30 times, and once for 10 min at 72 °C. The PCR products were purified for sequencing by using the Quickstep^{Tm} PCR purification kit (MoBiTec, Edge Biosystems, Göttingen, Germany) and separated on a 1.0 % (w/v) agarose gel. Direct sequencing of the rDNA was done by using the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham, Pharmacia, Biotech, UK) and infrared (IR) labeled primers (MWG Biotech, Ebersberg, Germany). The primers used were described previously (29, 42). During sequencing, gel electrophoresis was performed on a LICOR DNA Sequencer, Model 4000L (MWG Biotech). The DNA probe for *L. plantarum* 423 (Table 1) was designed with the ARB software package (30). The complete 16S rRNA sequence of *L. plantarum* 423 was inserted into the database and a species-specific probe designed as described by Amann et al. (4). For the detection of *L. salivarius* 241 in porcine mucus, a previously published species-specific probe for *L. salivarius* (13) was used. The species-specific Efs probe for *E. faecalis* (5) was used for detection of the "indicator" strain *E. faecalis* FAIR E 92.

The probes were synthesized by Invitrogen Life Technologies (Inchinnan Business Park, Paisley, Scotland, UK). The EUB and LSAL probes were fluorescently marked with rhodamine (red) at the 5'-end, while LPLANG and Efs were marked with fluorescein (green) at the 5'-end. The sequences of the probes are listed in Table 1. The EUB 338 probe (2) was used as a positive control to determine the permeability of fixed cells in FISH.

Administering of lactic acid bacteria and sampling. Piglets of the South African Large White breed were used in this study. Trials were done on a commercial farm, which limited the number of pigs allowed to be included and therefore also the number of repeats. All piglets had free access to water and feed throughout the trials. New-borne piglets were kept with sows in separate pens for the first 10 days. From day 11 piglets received a specially formulated diet, "Formula 15", which consisted of corn meal, wheat bran, fish meal, mono-calcium phosphate, feed chalk, salt, minerals and vitamins, zinc oxide, lysine, the growth stimulant Olaquindox (Feed Mix, Johannesburg, South Africa), pro-phylactic concentrations of Tylan (Eli Lilly, Johannesburg, South Africa), "Yellow pop" (starch in the form of maize meal, supplied by Feed Mix), whey powder and chemo-therapeutic concentrations of Mecadox (Ciba Speciality Chemicals, Isando, South Africa). The effect of Olaquindox, Tylan and Mecadox on *L. salivarius*

241, *L. plantarum* 423 and *E. faecalis* FAIR E 92 were tested at levels similar to those used in feed (0.5 mg/g Olaquindox, 1 mg/g Tylan and 0.5 mg/g Mecadox).

New-borne piglets were divided into three litters of 10, 9 and 7 animals, respectively. Five piglets of the first litter were each dosed with 5 ml *L. plantarum* 423 (4 x 10^9 cfu/ml), five piglets of the second litter were dosed with *L. salivarius* 241 (2 x 10^9 cfu/ml) and four piglets of the third litter each received 5 ml of a mixed population (1:1) of *L. plantarum* 423 and *L. salivarius* 241 (3 x 10^9 cfu/ml). The piglets were dosed on the day they were born, followed by one dosage per week for 7 weeks. The rest of the piglets in each litter received no probiotics and served as controls.

In a separate experiment, seven litters of between 7 to 10, 14-day-old piglets each were used. The piglets of six litters each received an oral dosage of 5 ml *E. faecalis* FAIR E 92 (4 x 10^6 cfu/ml) on the first day of the trial and again seven days later. On day nine of the trial, piglets from two of the six litters each received 5 ml *L. plantarum* 423 (4 x 10^9 cfu/ml). Piglets from the other two litters received *L. salivarius* 241 (2 x10^9 cfu/ml). Piglets from the third set of two litters received 5 ml of a combination of strains 423 and 241 (1:1) at a concentration of 3 x 10^9 cfu/ml. The dosage was repeated after seven days and continued once a week for 6 weeks. The seventh litter received two dosages with *E. faecalis* FAIR E 92 (4 x 10^6 cfu/ml) as described elsewhere, but no probiotics, and served as control.

Piglets were weaned when 28 days old and then moved to "follow-up" pens where they received the same diet for a further 10 days. The diet was then replaced with "Formula 21", which is identical to "Formula 15", but without salt, zinc oxide, lysine, "yellow pop" and whey powder.

At the end of the experiment seven piglets were slaughtered; four piglets from the experiment with new-borne piglets (dosed with *L. plantarum* 423 and *L. salivarius* 214, separately and in combination) and three from the experiment where piglets were dosed with *E. faecalis* FAIR E 92, followed by probiotic dosage with strains 423 and 241 as described elsewhere. All piglets were healthy and showed no signs of diarrhoea. Samples were collected (10 to 15 cm sections) from the duodenum, jejunum, ileum, anterior-, middle- and posterior colon. All samples were stored in sterile sampling bottles at –20 °C until analyzed.

Detection of bacterial strains with FISH. The frozen samples were thawed and aseptically dissected to expose the mucus layer. The mucus layer was scraped with a microscope slide and collected into a sterile eppendorf tube (20). To this sample 500 µl PBS and 500 µl cold ethanol (99 % v/v) was added and vortexed for 1 min. The samples were kept overnight at –20 °C, thawed and again vortexed for 3 min. Five µl of each sample was spotted onto a FISH microscopic slide (Adcock Scientific, South Africa) and fixed with ethanol, as described by Beimfohr et al. (5). The slides were oven-dried at 60 °C for 35 min, cooled 18 to 20 °C and 20 µl lysozyme (10 mg/ml) spotted on each sample. After 20 min of incubation on ice, the slides were rinsed with distilled water and cells were dehydrated by immersion into 50 % ethanol, followed by 80 % and 100 % ethanol (3 min each). The slides were air-dried and then covered with 9 µl hybridization buffer (5M NaCl, 1M Tris/HCl pH 8.0, 0.01 % SDS and 25 % formamide) and 1 µl of the species-specific probe at a concentration of 500 ng/µl (Table 1). Hybridization was performed on the slides for 2.5 h at 46 °C in a moist chamber. The slides were then washed with 2 ml washing solution (5M NaCl, 1M Tris/HCl pH 8.0, 0.5M EDTA and 0.01 % SDS) and transferred to 50 ml washing solution at 48 °C. After 10 min, the slides were rinsed with sterile distilled water, air-dried and stored at –20 °C. Bacterial cells were examined with an epifluorescent microscope (Nikon, Eclipse E400), fitted with a Nikon high performance CCD digital camera (Cohu Inc., San Diego, CA). Twenty fields per sample were photographed and fluorescent cells in each field counted. More than 3000 digital images obtained with FISH were analyzed and fluorescent cells counted manually. The mean number of images per frame should be more than 10 and less than 100 to ensure accurate counting (7).

Certain limitations existed in this methodology. The sample size (part of the gastro-intestinal tract) varied up to 50% (pieces ranging from 10-15 cm were cut). This, and the fact that mucus could only be scraped from the section that was sampled, lead to the data obtained being semi-quantitative.

RESULTS

Development of FISH probes. Strains 423, 241 and FAIR E 92 were identified by 16S rRNA sequencing data as *L. plantarum*, *L. salivarius* and *E. faecalis*, respectively. These results concurred with previous identifications of strains 423 and FAIR E 92 (11, 51).

Detection of *L. plantarum, L. salivarius* and *E. faecalis* with FISH. Olaquindox and Mecadox had no effect on *L. plantarum* 423, *L. salivarius* 241 and *E. faecalis* FAIR E 92 when tested at sub-therapeutic levels. All three LAB strains were killed by Tylan when tested *in vitro*, against levels incorporated in feed.

Results obtained with FISH on bacterial cells in the mucus of four piglets slaughtered after receiving probiotic dosages from day one, are shown in Fig.1. Results are indicated as average cell numbers recorded in 20 fields with various probes for target organisms (LPLANG for *L. plantarum*, LSAL for *L. salivarius* and Efs for *E. faecalis*). *L. plantarum* cells in the mucus of each part of the GIT obtained from the control piglet (received no probiotics) were recorded with the use of the LPLANG probe. Low cell numbers (less than 5 per area) were recorded in the middle and posterior colon. The highest number of *L. plantarum* cells (140 per area), were recorded in the jejunum, followed by the ileum (30 cells per area) as shown in Fig. 1.

There was an increase in cells detected with the LPLANG probe in the ileum of the piglet dosed with *L. plantarum* 423 (115 cells per area compared to 70 cells per area in the jejunum of the control piglet), followed by the posterior colon (100 cells per area) (Fig. 1). After dosage with *L. plantarum* 423 cell numbers in the jejunum, decreased to 20 compared with 140 cells per area recorded in the jejunum of the control piglet. An increase in *L. plantarum* cells were recorded in the posterior colon with LPLANG, below 10 cells per area present in the control to 100 cells per area in the piglets dosed with *L. plantarum* 423. The number of *L. salivarius* cells detected per area, with the LSAL probe in the piglet dosed with *L. plantarum* 423 remained low (below 30) except for an increase in the posterior colon. After dosage with *L. plantarum* 423 and *L. salivarius* 241, the highest number of *L. plantarum* cells were detected with the LPLANG probe in the ileum (138 cells per area) (Fig. 1).

The number of *L. salivarius* cells detected with the LSAL probe in the duodenum of piglets dosed with strain 241, was the highest (80 cells per area), followed by the anterior colon (Fig. 1), compared to the control. High numbers of *L. plantarum* (84 cells per area) were recorded with the LPLANG probe in the duodenum of a piglet dosed with *L. salivarius* 241, but a drop in cells numbers per area were observed in the rest of the GIT (ranging from 12 in the jejunum to 20 in the ileum). The same was recorded for *E. faecalis* cells detected with the Efs probe (Fig. 1).

When *L. salivarius* 241 was administered with *L. plantarum* 423 (1:1), the cells recorded per area with the LSAL probe remained low, between 40 and 60 per area (Fig.1) in most parts of the GIT. Cells per area, of *L. plantarum* detected with the LPLANG probe were high in the ileum (140) as compared to 115 cells detected in the ileum of the control piglet. *E. faecalis* cells detected with the Efs probe remained low (under 40) throughout the GIT (Fig. 1).

Average cell numbers recorded with FISH done on mucus of three piglets slaughtered after dosage with *E. faecalis* FAIR E 92 followed by probiotic dosage are shown in Fig. 2. Cell numbers recorded in the control piglet receiving only dosage with *E. faecalis* FAIR E 92, but no probiotics, showed that the ileum contained the highest number of *E. faecalis* cells (80 cells per area detected with Efs), followed by the middle colon (30 cells per area). Based on results obtained with LPLANG probe, in piglet dosed with *L. plantarum* 423, after *E. faecalis* dosage, the highest number of *L. plantarum* cells were present in the ileum, while the highest number of *L. salivarius* cells, detected by LSAL probe were recorded in the duodenum. High numbers of *E. faecalis* cells were detected with the Efs probe in both the duodenum and ileum of the piglet dosed with *L. plantarum* 423 after dosing with *E. faecalis* FAIR E 92. *L. plantarum* 423 had no or little effect in reducing the cell numbers of *E. faecalis*. Piglets dosed with *L. salivarius* 241, recorded low cell numbers of *L. plantarum* in the ileum (20 cells per area) detected with the LPLANG probe. *L. salivarius* cells (detected with LSAL) were present in high numbers (50 cells per area) in the duodenum and the posterior colon. *E. faecalis* cell numbers per area remained low (below 50), throughout the GIT, as detected with the Efs probe (Fig. 2). The *E. faecalis* cells decreased in the ileum from 80 cells to 30 cells per area, when piglets were dosed with *L. salivarius* 241.

After piglets were initially dosed with *E. faecalis* FAIR E 92, followed by dosage with a combination of probiotic strains 423 and 241, cell numbers of *E. faecalis* in the ileum decreased to 30 per area (compared to 80 cells per area present in the control piglet sample). The numbers of *L. salivarius* detected with the LSAL probe remained high in the duodenum (70 cells per area) after dosage with a combination of strains 423 and 241.

Representative images obtained with FISH on various parts of the porcine GIT with the LPLANG probe are shown in Fig. 3 (A to D). These images were recorded in samples collected from a 47-day-old piglet dosed with *L. plantarum* 423 from new-borne age.

DISCUSSION

Evidence as to whether a probiotic strain adheres to the mucosa of the intestinal tract of the host can be gained from *in situ* analyses (8). Although it is believed that the maximum probiotic effect is achieved if the organisms adhere to the intestinal mucosal cells, there is little evidence that this occurs (41). The difficulties encountered with *in situ* adhesion studies (cell lines and radio-activity), were described elsewhere (34, 40, 47). Pigs at a commercial farm were used in this study and Mecadox, Olaquindox and Tylan could not be excluded from the feed. Tylosin, the active component in Tylan reaches the stomach and anterior part of the colon but probiotic LAB colonize the ileum more frequently and in larger numbers compared to other parts of the GIT (6). Bacteria found in pigs that proved to be most sensitive to tylosin were *Mycoplasma hyopneumonia*, *Bordetella bronchiseptica*, *Staphylococcus aureus* and *Erysipelothrix rhusiopathiae* (43). Reports from other studies showed that lactobacilli isolated from pig faeces were resistant to tylosin (54). The effect of Tylan on orally administered strains is therefore probably minimal, especially since probiotic strains tend to favour colonization in the ileum.

The presence of mucus in the samples interfered with the hybridization and detection of target cells, therefore a universal eubacterial probe (EUB 338) (2) was used as a positive control to establish the permeability of probes during hybridization, a critical step during FISH. It has been demonstrated that lactobacilli colonize the mucus layer of the small intestinal mucosa of piglets (39). Probes used in this study were species-specific for *E. faecalis* (5), and eubacteria (3) but species-specificity for the *L. plantarum* and *L. salivarius* probes need to be increased. Specificity was sufficient to distinguish these strains from other strains used in this study. Fish data obtained with these probes was therefore only applicable to this study.

Data obtained showed that high cell numbers of *L. plantarum* are naturally present in the jejunum. With the use of the LSAL probe it was determined that *L. salivarius* species were naturally present in the duodenum, jejunum, ileum (highest incidence shown here, 70 cells counted on average per area) and colon of the piglet that received no probiotic treatments (Fig. 1). The Efs probe used to detect *E. faecalis* FAIR E 92 showed that the highest number of cells were present in the ileum of the control piglet.

An increase in the *Lactobacillus* spp. after oral administration to piglets was recorded with FISH. This concurred with results obtained where LAB were administered to piglets and an increase in total lactobacilli cell numbers in the ileum was shown (15, 46). Data obtained in this study (Fig. 3) showed a larger number of fluorescent cells present in the ileum (Fig. 3 C) compared to other parts of the GIT (Fig. 3 A, B and D). The highest number of *L. plantarum* cells was recorded in the ileum, whereas *L. salivarius* favoured adhesion to the duodenum.

Results obtained concurred with other studies where it was proved that probiotic LAB colonize the ileum of the GIT more frequently and in larger numbers compared to other parts of the GIT (6, 19, 36). Several theories exist on the mechanism of action of LAB supplementation (46). These include production of antimicrobial substances, competition for adhesion and nutrients, immuno-stimulation and production of fewer toxic metabolites, such as ammonia and amines, by intestinal flora (9, 46). Despite these theories, it was documented that the beneficial effects of orally administrated LAB are related to changes in the gut flora (10, 17, 38, 49).

Results obtained from the experiment where *E. faecalis* FAIR E 92 was used as an indicator organism, showed a definite reduction in the number of *E. faecalis* cells detected with the Efs probe in the ileum of piglets treated with a combination of strains 423 and 241 (Fig. 2). This was the only part of the GIT where an overall reduction in the number of *E. faecalis* cells was recorded. These results concurred with studies where it was shown that probiotics function better in combination than alone (21).

During the last few years FISH was increasingly used to analyze bacterial communities in the GIT. The present study describes the expansion of this technique to study the adherence and/or presence of probiotic LAB to the mucosal layers of the porcine GIT. Although results obtained from this study were variable and certain limitations existed in the methodology as described previously, it provided an estimation of the presence and/or adhesion of probiotics in various parts of the porcine GIT. Although 20 fields were analised per sample, statistical analyses of cell numbers present in various parts of the gastro-intestinal tract, detected with FISH probes, could not be done. More than one intestinal sample needs to be analised from each part of the gastro-intestinal tract to do statistical analyses. A restricting factor was the amount of samples received from the commercial farm. However, these results demonstrated the presence of probiotics in various sections of the porcine gastro-intestinal tract. It indicates that *L. plantarum* 423 and *L. salivarius* 241 have the ability to attach to the porcine gastro-

intestinal tract. FISH proved a valuable method to study probiotic bacteria without cultivation. Results obtained in this study contributed towards our understanding of the adherence of probiotic lactic acid bacteria to mucosal layers. Strains 423 and 241 colonized different sections of the gastro-intestinal tract and may thus be used as multi-species probiotics.

Acknowledgements

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TABLE 1. Probe sequences for FISH detection of porcine intestinal mucosal bacteria

Probe	Target organism	Sequence	Reference
Efs	*E. faecalis*	5' –GGTGTTGTTAGCATTTCG- 3'	(5)
LPLANG	*L. plantarum* 423	5' –TATCATTGCCATGGTGA- 3'	This study
LSAL	*L. salivarius* 241	5' –GAATGCAAGCATTCGGTGTA- 3'	(13)
EUB 338	Bacteria	5' –GCTGCCTCCCGTAGGAGT- 3'	(3)
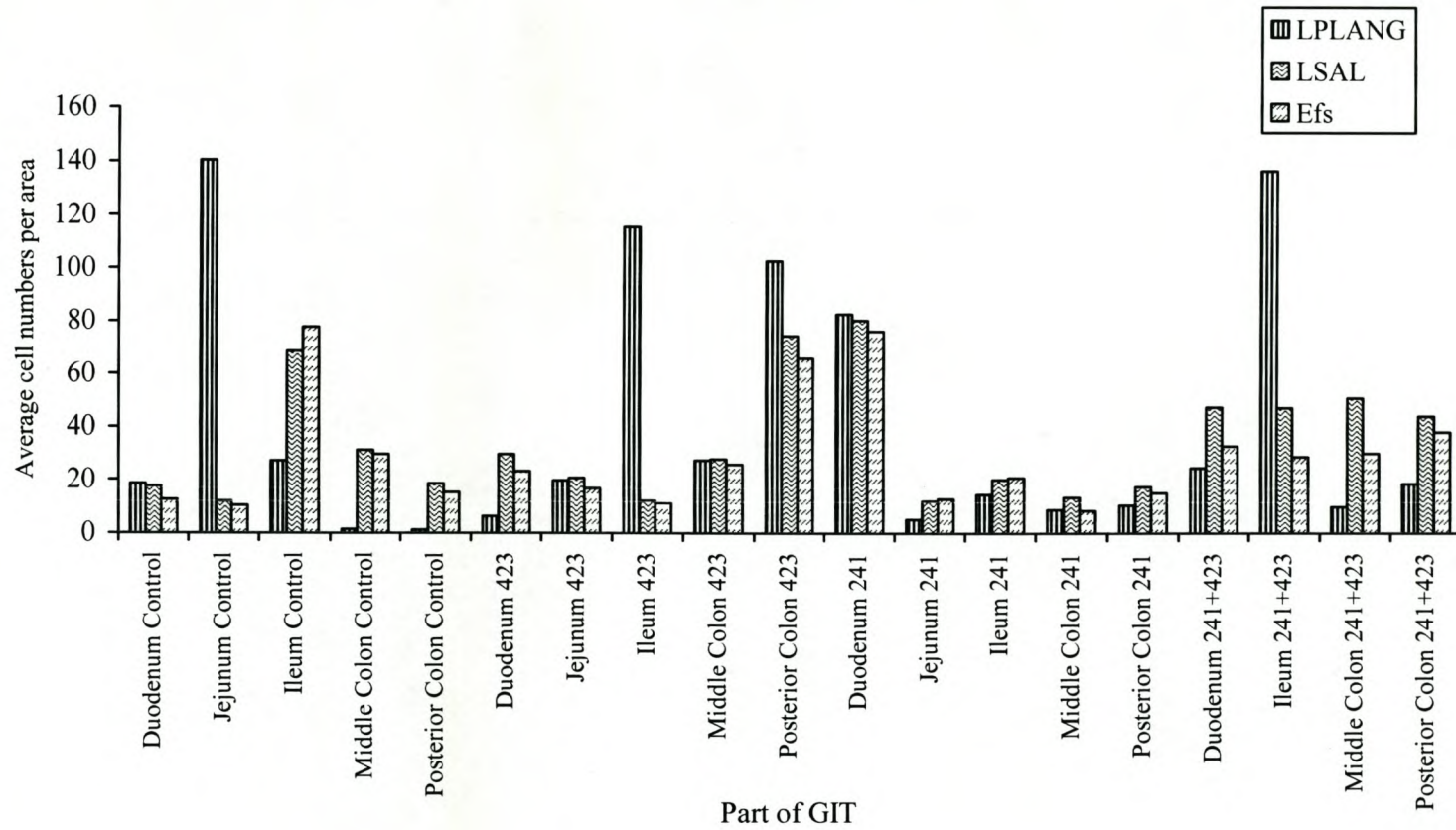
FIG. 1 Average cell numbers recorded with FISH on the mucus samples obtained from various parts of the GIT, after new-borne piglets up to 7 weeks old were dosed with probiotic strains *L. plantarum* 423 and *L. salivarius* 241, separately and in combination
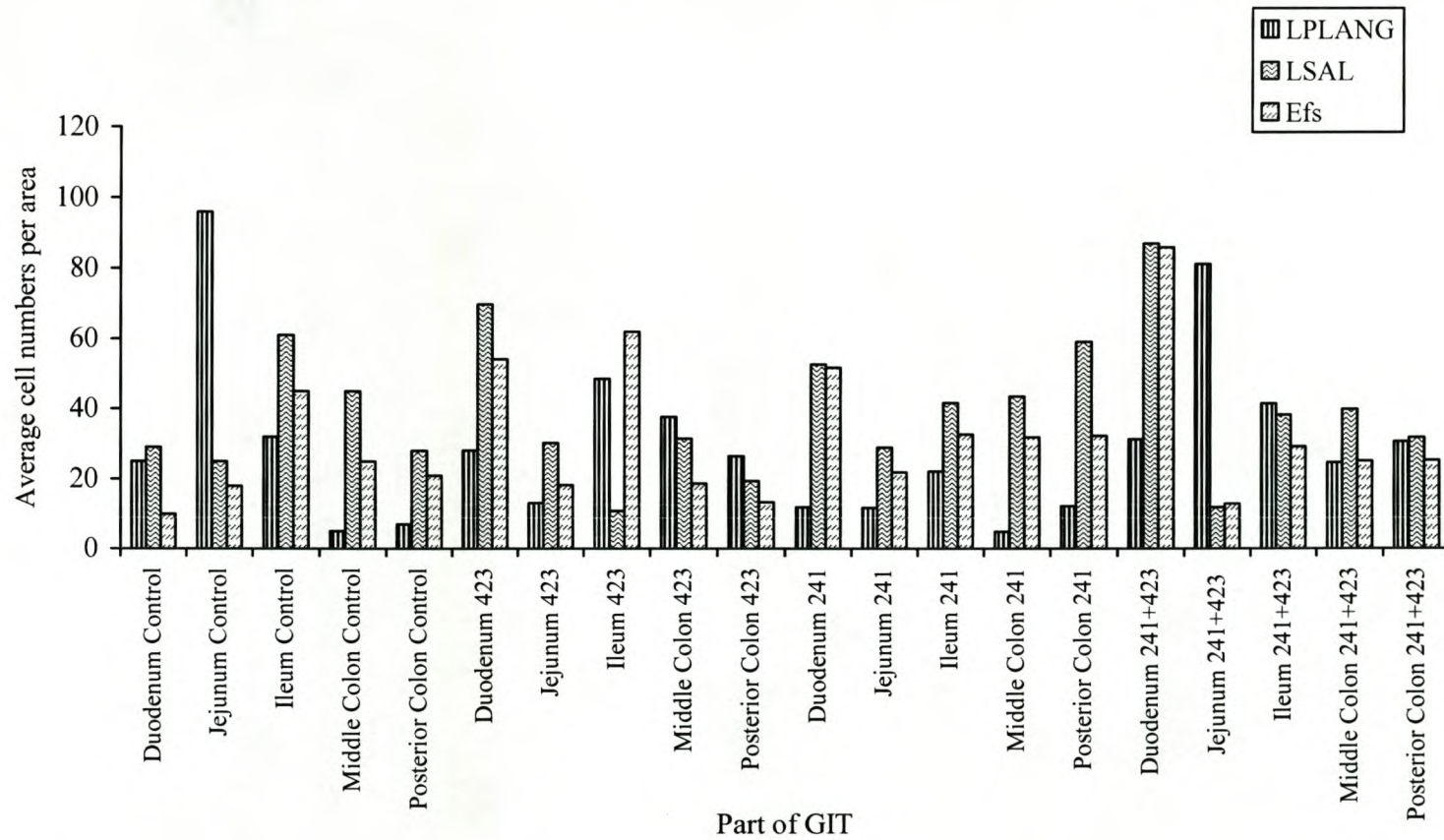
FIG. 2 Average cell counts recorded with FISH on mucus samples obtained from various parts of the GIT of 14-day-old piglets receiving two dosages with *E. faecalis* FAIR E 92, followed by probiotic dosages with *L. plantarum* 423 and *L. salivarius* 241, separately and in combination for six weeks
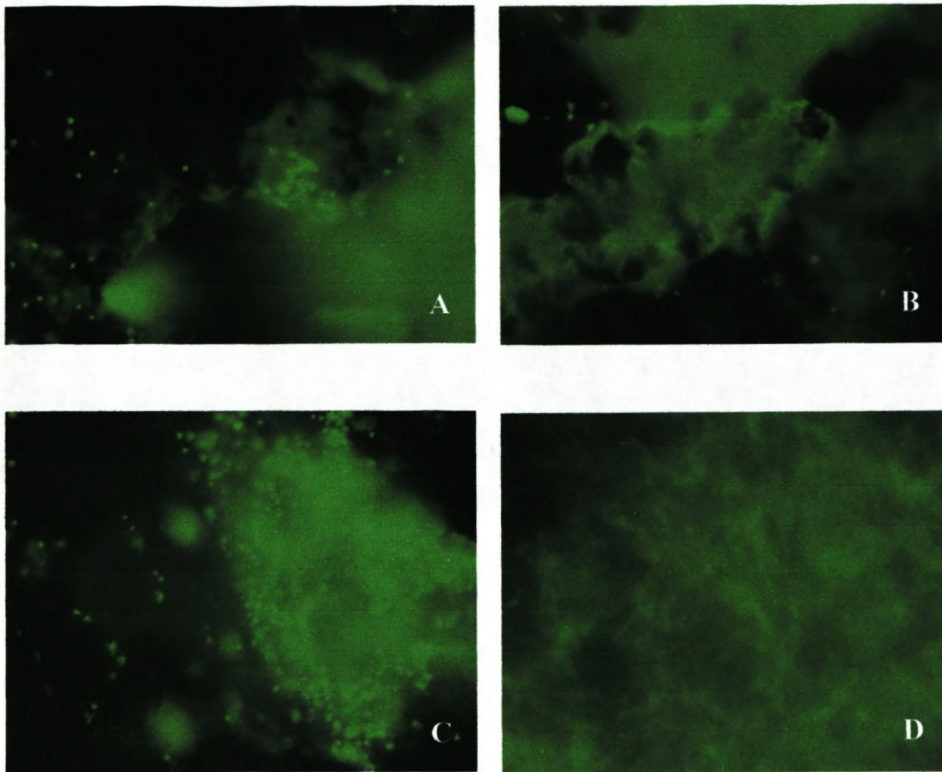
FIG. 3. FISH with LPLANG probe on Duodenum (A), Jejunum (B), Ileum (C) and Colon (D) of a 47day-old-piglet that was dosed with *L. plantarum* 423

CHAPTER 6

The study of probiotic lactic acid bacteria in a simple, but effective porcine gastro-intestinal model

The study of probiotic lactic acid bacteria in a simple, but effective porcine gastro-intestinal model

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Abstract

To evaluate probiotic bacteria they need to be studied in an environment simulating the gastro-intestinal tract (GIT). This paper describes the development of a porcine gastro-intestinal model to study the growth, survival and bacteriocin production of lactic acid bacteria. Two bacteriocin-producing strains, *Lactobacillus plantarum* 423 and *Lactobacillus salivarius* 241, were included in the gastro-intestinal model and adhesion of these strains to porcine ileum mucus studied with fluorescent-*in-situ*-hybridization (FISH). A decrease in *L. plantarum* 423 cell numbers in the duodenum, jejunum and ileum was recorded in reaction to the addition of bile and pancreatic juices. The cell numbers of strain 423 increased in the caecum and anterior colon, suggesting a recovery. Higher overall cell numbers of strain 423 were recorded in the gastro-intestinal model over seven days, compared to strain 241. Plantaricin 423, a bacteriocin produced by strain 423, was detected for up to 28 hours in the duodenum, jejunum, ileum and middle colon. Although the data obtained from these studies have to be validated with *in vivo* experiments, it provides a cost-effective alternative for the selection of probiotic strains.

Introduction

Intestinal microflora in healthy pigs compete with pathogenic bacteria for adhesion to the mucus or epithelial cells (Hill et al. 1986; Hillman et al. 1994). Lactic acid bacteria (LAB) maintain the balance in intestinal microbiota, enhance the digestion of feed, thereby increasing its nutritional value, reduce the activity of carcinogenic enzymes, lower serum cholesterol levels and stimulate the immune system (Fuller 1992). Probiotic lactic acid bacteria included in animal feed encourage weight gain, meat, egg or milk production, and prevent digestive disorders (Vanbelle et al. 1990). For probiotics to exert their beneficial effect, they should reach the large intestine in a viable state (Salminen et al. 1993). Orally administered, probiotic bacteria enter the large intestine after surviving the digestion processes in the small intestine. For persistence, probiotic cells need to adhere to the intestinal epithelium (Alander et al. 1999).

The main obstacle encountered by animal feed companies developing probiotic products is the selection of suitable bacterial strains. Selection criteria are based on properties such as bile and acid resistance, colonization, host specificity, production of antimicrobial substances, *in vitro* growth and stability during processing and storage, and resistance to additives and antibiotics (Alander et al. 1999).

Survival of probiotic bacteria in the gastro-intestinal tract (GIT) has not been thoroughly studied (Ohashi et al. 2004). Several gastro-intestinal models, most of which are based on the human GIT, have been developed (Miller and Wolin 1981; Edwards et al. 1985; Gibson et al. 1988; Molly et al. 1993; Veenstra et al. 1993). Advantages in the use of *in vitro* models compared to *in vivo* animal trials and experiments include cost-effectiveness, rapid results, reproducibility and no ethical constraints (Veenstra et al. 1993). Some models developed only focused on simulating the colon (Miller and Wolin 1981; Edwards et al. 1985; Gibson et al. 1988). The model developed in this study is based on the first model that simulated both the small and large intestines (Molly et al. 1993). Bacteria could be screened for probiotic properties while exposed to conditions encountered in the complete GIT. Although the model developed by Veenstra et al. (1993) simulated peristaltic movements, physiological transit characteristics, water retention and nutrient absorption, it proved costly to develop and operate.

With the introduction of molecular techniques, the adhesion properties of probiotic bacteria can be studied in a large population of mixed intestinal bacteria without isolation of single strains. Selected oligonucleotides identify specific bacterial species within a mixed community with the use of fluorescent-*in-situ*-hybridization (FISH) (Delong et al. 1989; Amann et al. 1990).

This study describes the development of a gastro-intestinal model simulating the porcine gastro-intestinal tract. The porcine digestive system has some anatomic differences to the human GIT, for instance a larger caecum. The physiology processes related to digestion is similar, both humans and pigs are monogastric and true omnivores (Swindle and Smith 2000). The gastro-intestinal model was used to study the growth, survival, and bacteriocin production of specific probiotic LAB under conditions encountered within the porcine GIT by using conventional culturing methods. Porcine ileum samples were incorporated into the ileum vessel. FISH was used to study the adhesion of probiotic strains to porcine ileum mucus.

Materials and Methods

Design of the gastro-intestinal model

The gastro-intestinal model developed is based on the human intestinal model described by Molly et al. (1993) and consisted of eight vessels representing, in order of sequence, the medium reservoir, stomach, duodenum, jejunum and ileum, caecum and anterior colon, middle colon, posterior colon and waste (Fig. 1). A ninth vessel attached to the duodenum served as a reservoir for pancreatic juices and bile salts. Seven peristaltic pumps (Aspen, East Sussex, U.K.), with a constant flow speed of 104 ml min^{-1}, were connected to digital time switches. Glass reactor vessels and peristaltic pumps were connected with autoclavable Nalgene tubing. Outlet ports were fitted with 0.22 μm filters. Samples were collected from vessels through thin Nalgene tubing, connected to a 5 ml sterile syringe and needle. The complete system was housed in a 37 °C incubator. Vessels could be detached from the system and autoclaved separately. The peristaltic pumps were cleaned with distilled water and sterilized by flushing with 30 % (v/v) ethanol, followed by larger volumes of sterile distilled water. The pH probes were rinsed thoroughly with sterile distilled water and wiped with a paper cloth immersed in 70 % (v/v) ethanol.

Bacterial strains and growth media

Lactobacillus plantarum 423 was isolated from sorghum beer (Van Reenen et al. 1998) and *Lactobacillus salivarius* 241 from the ileum of a 38-day-old piglet. *Enterococcus faecalis* FAIR E 92 was isolated from pig faeces (Davidse et al. 2004). All strains were cultured in MRS broth (Biolab, Biolab Diagnostics, Wadeville, South Africa) at 37 °C.

Inoculum

Probiotic cultures were inoculated into 10 ml MRS broth (Biolab) and incubated for 12 to 16 h at 37 °C. Twenty µl of the culture was inoculated into 200 ml MRS broth and incubated at 37 °C to $OD_{600} = 1 - 1.5$. The cultures were harvested (8000 x g, 15 min, 18 °C) and the pellets washed twice with 5 ml sterile distilled water, mixed (1:1) with 5 ml saliva buffer (Marteau et al. 1997). These cell suspensions were used to inoculate the stomach vessel (Fig. 1).

The reservoir vessel was filled with 1.5 L MRS broth (Biolab), supplemented with 4.65 g NaCl, 1.65 g KCl, 0.165 g $CaCl_2$ and 0.9 g $NaHCO_3$. The pH was adjusted to 4.0 with concentrated HCl and then autoclaved (121 °C, 20 min). The pancreatic-bile solution was prepared according to the method of Marteau et al. (1997) and contained 12 g $NaHCO_3$, 0.9 g pancreas acetone powder (Sigma) and 0.6 g Oxgall (Biolab) per liter. The pancreatic-bile solution was autoclaved (121 °C, 20 min) to lower the risk of contamination.

Operation of the gastro-intestinal model

The operational procedure was based on a "fill and draw" system (Kontula et al. 1998). A probiotic cell suspension (10 ml) was inoculated into the stomach vessel. At various stages throughout the run, samples were collected. Viable cell numbers were determined by plating onto MRS Agar (Biolab). Bacteriocin activity was determined according to the method described by Uhlman et al. (1992). *Enterococcus faecalis* FAIR E 92 (Davidse et al. 2004) is sensitive to plantaricin 423 and was used as an indicator strain.

Several preliminary experiments were performed to monitor the growth of *L. plantarum* 423 and *L. salivarius* 241 and production of plantaricin 423 in the model, before the addition of porcine ileum samples. An initial experiment involved inoculation with *L. plantarum* at 4×10^{10} cfu ml^{-1} and monitoring of cell growth over four days, at OD_{600}. In a separate experiment, *L. plantarum* was inoculated (4×10^{10} cfu ml^{-1}) and plantaricin 423 production monitored over four days.

Survival of *L. plantarum* 423 and production of plantaricin 423 were studied by inoculating 3×10^{10} cfu ml^{-1} and monitoring of cell growth over seven days. Survival of *L. salivarius* 241 in the gastro-intestinal model was studied by starting with an inoculation of 2×10^8 cfu ml^{-1}. Control vessels containing 200 ml MRS medium were inoculated with either *L. plantarum* 423 or *L. salivarius* 241 at cell numbers similar to that used in the model. These vessels received no daily supplementation of medium from the reservoir or bile and pancreatic juices.

Porcine ileum obtained from an abattoir was incorporated into the model. These samples were collected directly after slaughter. Samples were cut into 8 to 10 cm lengths and the intestinal content carefully rinsed with sterile distilled water. Pump operation and inoculation of probiotic cultures were done as for the preliminary experiments, until the contents reached the ileum and jejunum vessel (Fig. 1). Thereafter, an ileum sample was added, followed by incubation in the presence of probiotic strains. Samples were removed for analyses at various time intervals. The first mucus sample was taken after ileum samples had been incorporated for one hour and thereafter hourly, for six hours. No probiotic cells were added to ileum samples in the control vessel. Probiotic strains were inoculated at 4×10^{10} cfu ml^{-1} *L. plantarum* and 3×10^8 cfu ml^{-1} *L. salivarius*, respectively. On completion of the experimental run, the mucus was scraped off with a sterile microscopic slide and collected into a sterile Eppendorf tube (Jin et al. 2000). Adhesion of intestinal bacteria to the mucus was detected by FISH.

Analyses with FISH

To the mucus sample 500 µl PBS and 500 µl cold ethanol (99 % v/v) were added and the sample was vortexed for 1 min. These samples were kept overnight at –20 °C. Samples were thawed and then vortexed for 3 min and 5 µl of the sample spotted on a FISH microscopic slide

(Adcock Scientific, South Africa). The sample was fixed with ethanol, dried at 60 °C for 35 min, cooled to 21 °C and 20 µl lysozyme (10 mg ml⁻¹) added. Slides were incubated on ice for 20 min and the lysozyme carefully rinsed from the slides with sterile distilled water. Dehydration was performed by immersing the slides into 50 %, 80 % and 100 % ethanol for 3 min. Slides were air-dried and 9 µl hybridization buffer (5M NaCl, 1M Tris/HCl pH 8.0, 0.01 % SDS and 25 % formamide) added to each spot, with 1 µl of the strain-specific probe (500 ng µl⁻¹). The following rRNA-targeted oligonucleotide probes were used: Efs for *E. faecalis* FAIR E 92 (Beimfohr et al. 1993), LPLANG for *L. plantarum* 423 and LSAL for *L. salivarius* 241 (Ehrmann et al. 2002). The bacterial probe EUB 338 (Amann et al. 1990) was used as a positive control to determine probe permeability of fixed cells (Table 1). Probes were synthesized by Invitrogen life technologies, UK. The EUB and LSAL probes were fluorescently marked with Rhodamine (red) at the 5' terminal end while LPLANG and Efs, were marked with Fluorescein (green) at the 5' end. Hybridization was done at 46 °C for 2.5 h. Slides were incubated in a moist chamber containing excess hybridization buffer and unbound probe was washed from the slides with 2 ml washing solution (5M NaCl, 1M Tris/HCl pH 8.0, 0.5M EDTA and 0.01 % SDS). The washing solution was pre-heated to 48 °C in a water bath. Slides were immediately transferred into 50 ml washing solution and incubated at 48 °C for 10 min. A final wash was performed with distilled water. The slides were air-dried and kept at –20 °C. Fluorescence was detected with a microscope (Nikon, Eclipse E400), fitted for epifluorescence with a x 60/1.4 disc oil objective and red and green filters. Images were taken with a high performance CCD digital camera (Nikon, Cohu Inc., San Diego, CA).

Results

Growth of *L. plantarum* 423 in the gastro-intestinal model was determined over a period of four days. Optical density readings and viable cell numbers were recorded for up to 96 h (Fig. 2). In the duodenum, jejunum and ileum, a decrease in viable cell numbers was observed, while OD-values increased. In the caecum and anterior colon the OD-values and cell numbers decreased from 0.48 to 0.22 and 4 x 10⁸ cfu ml⁻¹ to 5 x 10⁷ cfu ml⁻¹, respectively (Fig. 2). In the middle colon, both OD-readings and cell numbers decreased, but from 56 h OD-values stabilized at 0.25

to 0.30 while viable cell numbers decreased to 0 or non-detectable levels at 73 h (Fig. 2). Plantaricin 423 production was detected from 4 h to 28 h.

The highest cell numbers of *L. plantarum* 423 was recorded in the posterior colon after 121.5 h (Table 2). In general, cell counts recorded for strain 423 were higher (in excess of 10^8 cfu ml^{-1}) compared to cell counts recorded with strain 241. Low cell numbers were recorded for *L. salivarius* 241 (2×10^4 cfu ml^{-1}) in the posterior colon after 149.5 h, (Table 2). After 7 days no *L. salivarius* 241 cells were detected in the jejunum, ileum, caecum and anterior colon. *L. salivarius* 241 cell numbers in the duodenum were variable, but from 29.0 to 101.5 h, cell numbers in the order of 10^8 cfu ml^{-1} were recorded. The highest cell numbers of *L. salivarius* (1.8×10^{10} cfu ml^{-1}) were recorded in the duodenum after 5.5 h.

Plantaricin 423 production was recorded in the control vessel for up to 73.5 h. Production of plantaricin 423 was recorded in all vessels up to 11.5 h. Before the daily addition of medium from the reservoir to the stomach, no production of plantaricin 423 was recorded from 23.5 h onwards in the caecum and anterior colon. After 29 h detectable levels of plantaricin 423 was only produced in the middle colon. With the daily addition of medium, plantaricin 423 was produced in the stomach up to 97.4 h. At 101.5 h, production of plantaricin 423 in all vessels decreased and no production could be detected after 225.5 h.

FISH images obtained with porcine ileum mucus samples collected during a run in the gastro-intestinal model shows adhesion of cells to porcine mucus in the form of clusters (Fig. 3 and Fig. 4).

Discussion

An important aspect of probiotics is their clinically proven efficiency (Alander et al. 1999). Any claim of a probiotic product should be supported by well-designed, randomized clinical trials. However, clinical trials are not always practical for screening purposes and other methods or tools are needed to evaluate probiotics. *In vitro* models provide a method of studying the effects of probiotics and their interactions on intestinal microbiota (Alander et al. 1999) The porcine gastro-intestinal model developed was based on the five-step multi-chamber reactor

simulating the human intestinal microbial ecosystem (Molly et al. 1993). A similar multi-chamber model was used in this study and each vessel simulating a specific section of the GIT could be operated as a separate unit. Similar residence times and volumes could be used as described for the simulated human intestinal microbial ecosystem (SHIME) developed by Molly et al. (1993).

Medium used in the reservoir simulated the saliva in the mouth and esophagus (Marteau et al. 1997). MRS was added to stimulate the growth of probiotic LAB. Some of the activity in the pancreatic-bile solutions might have been lost due to autoclaving. Ideally the inclusion of pancreatin and bile, sampled from a piglet would be the best simulation of this part of the gastro-intestinal tract, but contamination would have had an impact on the results obtained. The survival of strains under conditions similar to the GIT were monitored over a period of seven days and provided valuable information on the application frequency needed for probiotics, as strains not surviving for a required period (depending on probiotic preparation used) would have to be dosed repeatedly. Two probiotic strains were inoculated in the gastro-intestinal model. Mixed cultures are more likely to be successful probiotics, since individual strains of lactobacilli are predominant members of the gut microflora for only a short period of time (Tannock et al. 1990). Although contamination is always a factor to consider in any gastro-intestinal model, the system used proved to be effective because the vessels could be autoclaved separately. Probiotic LAB colonized the ileum more frequently and in larger numbers compared to other parts of the GIT (Pedersen and Tannock, 1989; Holzapfel et al. 1998; Blake et al. 2003). This study concentrated on the addition of ileum samples to study the adherence of probiotic strains. With the incorporation of ileum samples from slaughtered pigs, contamination levels rose but the probiotic bacteria present in the mucus could be identified and detected with FISH.

Results obtained over four days indicated that the OD_{600}-values were the highest after 12 h when contents entered the caecum and anterior vessel, after transportation through the duodenum, and the addition of pancreatic-bile (Fig. 2). *L. plantarum* 423 and *L. salivarius* 241 survived the bile/pancreatic environment and low pH conditions in the stomach. OD values decreased in the caecum and anterior colon in the 16 h to 28 h period from 0.4 to 0.2, but increased from 32 h onwards to a maximum of 0.38 (Fig. 2). Similar results were obtained with

plate counts. A decrease in cell numbers from 4×10^8 cfu ml^{-1}, to below 1×10^8 cfu ml^{-1} were recorded from 16 h to 20 h. This was in reaction to the pancreatic-bile solution that was added to the duodenum vessel. Cell numbers increased again at 24 h, suggesting that the cells survived the addition of pancreatic juices and bile salts. Similar results were reported by Marteau et al. (1997). Before this study, the effect of bile on microorganisms in the small intestine was thought to be low (Simon and Gorbach 1987). *In vitro* experiments indicated that conjugated bile salts, constituting the majority of bile salts in the small intestine, were less bactericidal than deconjugated bile salts (Floch et al. 1972; Northfield and McColl 1973; Heaton 1985; Simon and Gorbach 1987; Stewart et al. 1987). These findings support the importance of the sensitivity of microorganisms to bile as a selection step for potential probiotics (Gilliland et al. 1984; Fuller 1991; Havenaar et al. 1992). Bile stress for bacteria in the gastro-intestinal model is complex because bile concentrations and residence times vary in each vessel. Viable cell numbers decreased from 53 h, while OD$_{600}$ readings stabilized, indicating the presence of non-viable cells in the middle colon and posterior colon. It could be proposed that nutrients were depleted rapidly at this stage and cells were not able to retain their viability, but their presence was still detected by OD-readings.

Detectable levels of bacteriocin activity were recorded until contents reached the caecum and anterior colon vessel (up to 28 hours). Plantaricin 423 was produced even under low pH conditions and in the presence of pancreatic-bile.

Both probiotic cultures survived conditions in the gastro-intestinal model over seven days, with the daily addition of medium from the reservoir and pancreatic- and bile juices. Cell numbers recorded indicated that *L. plantarum* 423 survived better, compared to *L. salivarius* 241. Strain 241 was isolated from the intestinal tract of a piglet, while *L. plantarum* 423 was isolated from fermented sorghum beer. *L. salivarius* 241 favours growth in more anaerobic conditions and adhesion in the duodenum, while *L. plantarum* 423 adheres to the ileum. This explained the high survival rate of *L. plantarum* 423 over seven days in the jejunum and ileum, where cell numbers remained in the range 10^7 to 10^8 cfu ml^{-1} between 47.5 and 121.5 h (Table 2).

The addition of fresh reservoir medium played an important role in the production of plantaricin 423 over seven days. Production of plantaricin 423 was recorded again after addition of medium from the reservoir and production continued for up to 5 days. Feed ingested by the host animal provides nutrients for probiotic bacteria to survive passage through the GIT and remain effective under the conditions present in the GIT (Marteau et al. 1997). The addition of fresh medium simulated the daily feed intake supplying the nutrients necessary for bacterial growth.

Results obtained by FISH showed that cells of *L. plantarum* (detected with probe LPLANG), *L. salivarius* (detected with probe LSAL), *E. faecalis* (detected with probe Efs) and eubacteria (detected with EUB 338) is generally observed in control samples, i.e. (no probiotic cultures inoculated into the gastro-intestinal model (Figs. 3 and 4). With the addition of porcine ileum samples, no conclusions could be drawn regarding the adhesion of strains 423 and 241. These bacteria are normal inhabitants of the porcine ileum (Hillman et al. 1994). Cells were grouped closely together into clusters, making individual cell number determination difficult. Cells clustered together as the experimental run progressed, probably as a mechanism to survive the conditions within the gastro-intestinal model. Clustering of cells as a defense mechanism in extreme environmental conditions was observed by Miteva et al. (2004). Clustering is a strategy used by micro-organisms to enable them to be more competitive. It has not been observed regularly with lactic acid bacteria, but this study proved that these bacteria cluster together to survive passage through the porcine gastro-intestinal tract. This study contributed towards the understanding of the behavior of lactic acid bacteria and probiotic bacteria within the gastro-intestinal tract. The FISH method can be used as a cost-effective alternative (compared to cultivation of single bacteria *in vitro*) to depict the complex intestinal microbiota and select probiotic strains suitable to specific regions of the gastro-intestinal tract.

To validate the gastro-intestinal model developed, *in vivo* data must be collected and compared to *in vitro* results. A number of *in vivo* experiments will have to be performed, especially designed for this purpose. The porcine gastro-intestinal model assisted in the selection of possible probiotic strains that would survive conditions encountered in the GIT, without using experimental animals. Screening for probiotic micro-organisms is a lengthy task. Gastro-

intestinal models serve as an important tool to speed up product development. Other advantages of this model included cost effectiveness and simplicity. The monitoring of pH in the various vessels with the use of a computer program will assist in accurate reproducibility of results. This study provided insight into the survival, growth and adhesion of probiotic lactic acid bacteria in the small intestine of a pig, a niche that had not been extensively researched.

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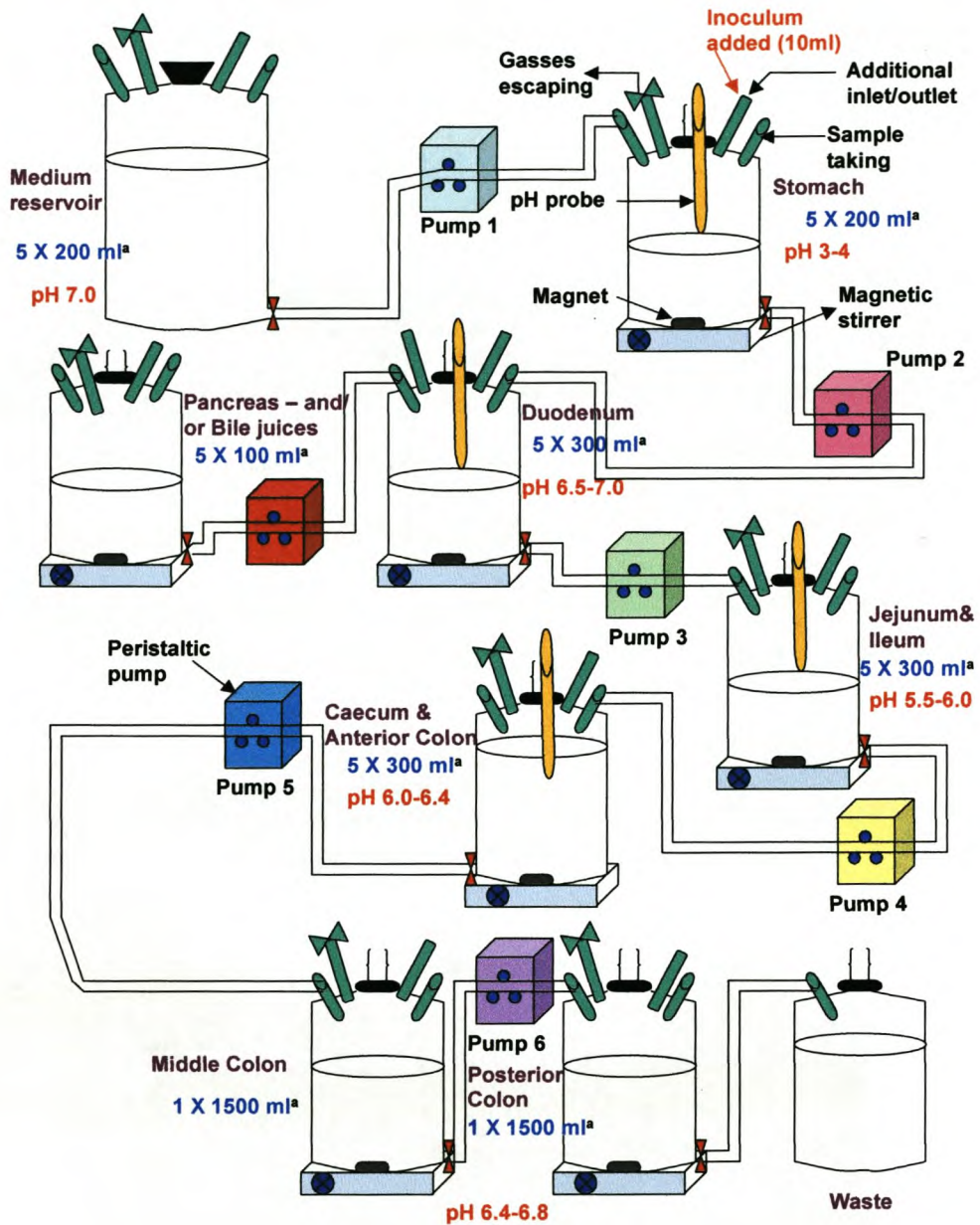
Fig. 1 Schematic presentation of a model simulating the porcine gastro-intestinal tract

[a] Flow volumes

Table 1. Probe sequences for target strains

Probe	Target organism	Sequence
Efs	*E. faecalis*	5' –GGTGTTGTTAGCATTTCG- 3'
LPLANG	*L. plantarum* 423	5' –TATCATTGCCATGGTGA- 3'
LSAL	*L. salivarius* 241	5' –GAATGCAAGCATTCGGTGTA- 3'
EUB 338	Bacteria	5' –GCTGCCTCCCGTAGGAGT- 3'

Fig. 2 Cell numbers and OD_{600} values recorded after inoculation of *L. plantarum* 423 in the porcine gastro-intestinal model

Table 2. Cell numbers in cfu ml^{-1} obtained after inoculation of *L. plantarum* 423 and *L. salivarius* 241, respectively, into MRS reservoir medium in the porcine gastro-intestinal tract

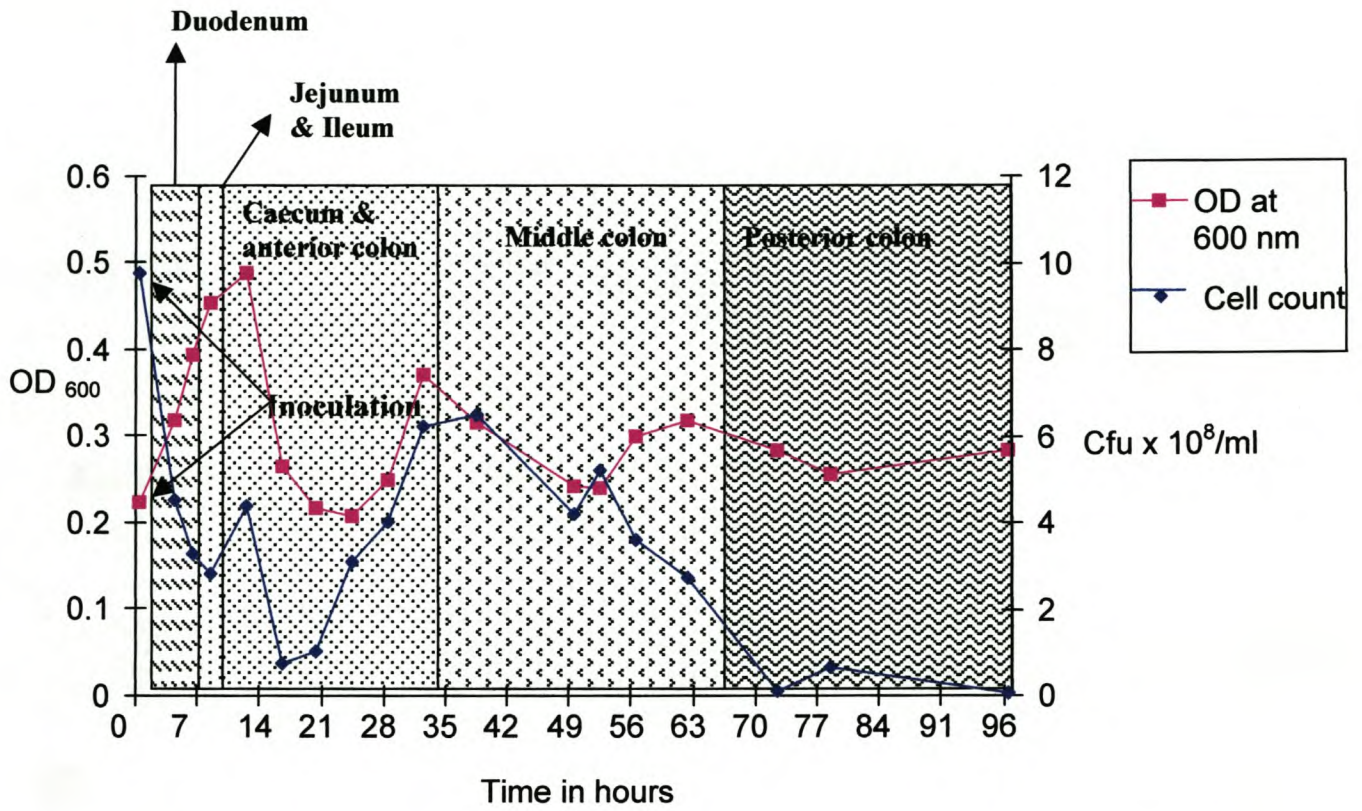| Time (h)	Stomach		Duodenum		Jejunum and Ileum		Caecum and Anterior Colon		Middle Colon		Posterior Colon		Control
	423	241	423	241	423	241	423	241	423	241	423	241	423/241[b]
1.25	8.3×10^8	3.9×10^8	$-$[a]	$-$	$-$	$-$	$-$	$-$	$-$	$-$	$-$	$-$	1.2×10^8
3.75	$-$	$-$	1.3×10^9	4.2×10^9	$-$	$-$	$-$	$-$	$-$	$-$	$-$	$-$	1.2×10^9
5.50	$-$	$-$	$-$	1.8×10^{10}	10.1×10^8	$-$	$-$	$-$	$-$	$-$	$-$	$-$	1.5×10^9
7.50	$-$	$-$	$-$	$-$	$-$	1.1×10^7	$-$	$-$	$-$	$-$	$-$	$-$	1.9×10^9
11.5	$-$	$-$	$-$	$-$	$-$	$-$	2.3×10^9	1.6×10^6	$-$	$-$	$-$	$-$	2.7×10^9
23.5	$-$	$-$	$-$	$-$	$-$	$-$	4.8×10^8	2.3×10^8	$-$	$-$	$-$	$-$	2.8×10^9
27.0	2.6×10^8	2.2×10^8	$-$	$-$	$-$	$-$	$-$	$-$	$-$	$-$	$-$	$-$	2.9×10^9
29.0	1.8×10^7	2.2×10^7	1.3×10^8	1.8×10^8	4.5×10^8	1.4×10^8	4.8×10^8	2.1×10^5	4.1×10^8	2.9×10^5	$-$	$-$	2.9×10^9
47.5	2.4×10^8	6.7×10^8	4.2×10^7	2.9×10^6	2.4×10^7	3.6×10^5	2.7×10^8	1.3×10^8	2.1×10^8	7.3×10^7	$-$	$-$	3.0×10^7
53.5	3.5×10^8	3.5×10^8	2.4×10^8	1.5×10^8	9.0×10^6	2.0×10^5	1.3×10^8	6.8×10^6	1.6×10^8	3.3×10^7	2.9×10^8	2.1×10^7	9.0×10^6
73.5	7.5×10^7	9.4×10^8	4.2×10^7	2.2×10^7	3.2×10^7	3.0×10^7	4.4×10^7	4.2×10^6	2.2×10^8	2.0×10^7	2.1×10^8	1.1×10^6	$-$
77.5	3.6×10^7	8.4×10^8	5.0×10^7	2.2×10^8	5.0×10^7	2.3×10^8	6.9×10^7	1.1×10^8	1.3×10^8	5.6×10^6	1.8×10^8	8.4×10^6	$-$
97.5	5.2×10^7	1.7×10^8	3.1×10^7	2.0×10^6	8.0×10^6	7.1×10^5	6.5×10^7	4.1×10^6	5.1×10^7	5.1×10^6	9.3×10^7	1.8×10^7	$-$
101.5	1.1×10^8	1.6×10^8	8.8×10^7	2.3×10^8	1.7×10^7	1.1×10^6	1.7×10^7	1.4×10^5	4.7×10^7	4.4×10^6	6.5×10^7	1.7×10^6	$-$
121.5	1.1×10^8	1.9×10^8	1.1×10^7	2.0×10^4	4.0×10^6	4.8×10^5	1.0×10^7	4.0×10^4	3.0×10^8	3.1×10^6	5.5×10^8	1.4×10^6	$-$
125.5	1.3×10^7	1.0×10^8	1.1×10^8	2.1×10^6	6.0×10^5	1.0×10^5	5.1×10^7	1.0×10^6	6.1×10^6	3.9×10^5	6.4×10^6	2.9×10^5	$-$
145.5	2.1×10^8	1.1×10^8	8.8×10^7	2.0×10^4	6.0×10^6	2.0×10^4	3.1×10^6	1.5×10^5	4.4×10^6	1.2×10^6	5.4×10^6	3.6×10^5	$-$
149.5	2.7×10^7	9.3×10^7	2.7×10^8	5.1×10^8	1.6×10^8	6.0×10^4	4.2×10^7	1.1×10^5	2.2×10^7	1.8×10^5	1.1×10^7	1.6×10^4	$-$
169.5	1.8×10^8	2.0×10^8	1.9×10^7	1.8×10^4	4.0×10^7	0	1.8×10^6	0	1.5×10^6	1.2×10^5	6.7×10^6	4.9×10^5	$-$

[a] Not Done

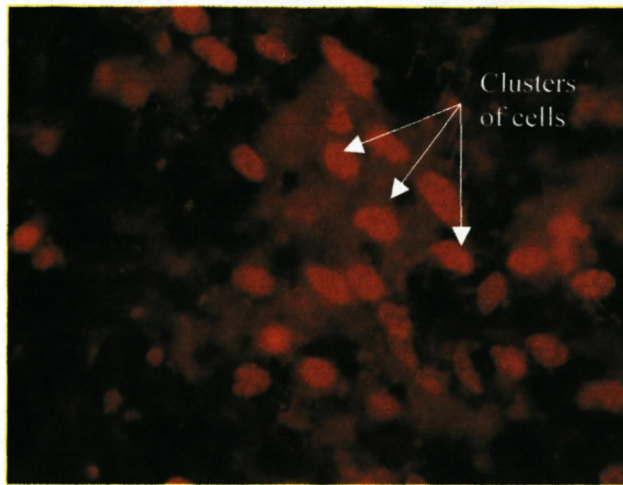[b] Average cell counts recorded from data of 241 and 423

127

Fig. 3 FISH with EUB 338 probe done on porcine ileum mucus samples collected from gastro-intestinal model
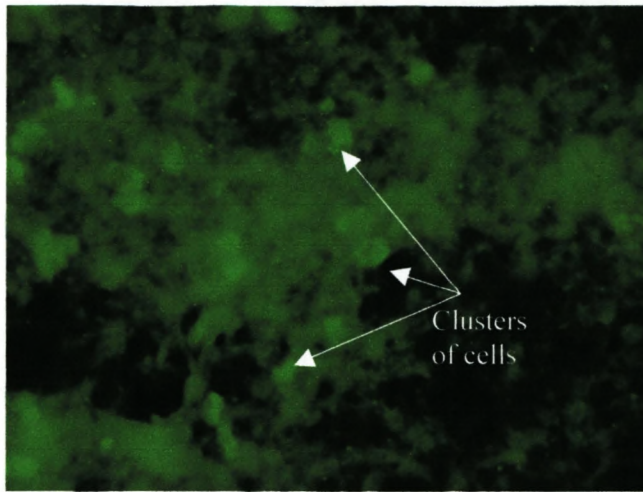
Fig. 4 FISH with LPLANG probe done on porcine ileum mucus samples collected from gastro-intestinal model

CHAPTER 7

General discussion and conclusions

The exclusion of antibiotic growth promoters from animal feeds resulted in the search for "natural" and "safe" alternatives (Ratcliff, 2000). This study concentrated on probiotics, an alternative to antibiotics. Bio-prospecting for potential probiotic strains mainly concentrated on the isolation of these strains from faeces. However, the microbial population in faeces does not accurately reflect the diversity of intestinal bacteria (Rall et al., 1970; Savage, 1983).

In this study, probiotic lactic acid bacteria (LAB) were isolated from the gastro-intestinal tract of piglets raised on six different feedstuffs. The six feedstuffs contained no antibiotics. No differences in viable cell numbers were recorded for animals, raised on the six different feeds. Research showed that certain LAB metabolize the mucus of the small intestine and are not affected by the feed ingested (Rojas and Conway, 1996). The highest cell numbers of LAB were recorded in the ileum (concluded from data obtained on various growth media), followed by the colon and caecum, suggesting that more growth requirements for LAB are available here compared to other parts of the gastro-intestinal tract (GIT). Little research data is available in the literature on the number of viable cells recorded from the porcine GIT. According to Lick et al. (2001) most intestinal LAB are found in the ileum, suggesting that it is the preferred section for adhesion, concurring with results obtained in this study

A total of 246 Gram-positive and catalase negative isolates were isolated and screened for probiotic properties that included resistance to bovine-, porcine bile and low pH, antibiotic susceptibility and antimicrobial compound production. The best probiotic properties were recorded with *Lactobacillus salivarius* 241, isolated from a piglet raised on a feed containing fish meal and cooked corn meal. Strain 241 was resistant to ampicillin and chloramphenicol. This strain could be administered as probiotic while piglets are treated with these antibiotics. *L. salivarius* 241 produced an antimicrobial compound active against *L. reuteri* and *Streptococcus thermophilus*. *L. reuteri* is one of the bacteria dominating in the GIT of piglets up to 14 days-old (Rojas and Conway, 1996).

The use of *L. salivarius* 241 as a probiotic during the first 14 days, must be considered with care, as this strain will reduce the numbers of *L. reuteri* naturally present in the GIT. This imbalance in the intestinal bacterial population may lead to the occurrence of diarrhoea and other diseases.

The latter strain, together with *L. plantarum* 423 (isolated from sorghum beer) was selected for further studies. *L. plantarum* 423 produces a bacteriocin, plantaricin 423 that proved active against *E. faecalis* FAIR E 92 (Van Reenen et al., 1998). *L. plantarum* 423 was included in this study to monitor the effectiveness of a probiotic strain through the decrease in enterococci cell numbers recorded in the porcine GIT. Plantaricin 423 was used as an indicator to determine the effect of *L. plantarum* 423 and not to reduce the total enterococci cell numbers present in the GIT. Some enterococci present in the porcine GIT are beneficial to the wellbeing of the piglet.

Piglets received feed containing sub-therapeutic doses of Tylan. The exclusion of this antibiotic from the feed was not permitted because trials were done on a commercial farm. The effect of Tylan on the probiotic strains administered were determined *in vitro*. Although Tylan proved active against strains 423, 241 and FAIR E 92, data published from previous research indicated that the effect of Tylan on these strains might be minimal *in vivo*. The inclusion of antibiotics in animal feed as a growth stimulant is still common practice in South Africa. The Tylan included in the feed during this study might have had an impact on the results, but the survival and adhesion of the probiotic strains *L. plantarum* 423 and *L. salivarius* 241 were tested under conditions found in most commercial farms in South Africa. Results are therefore very applicable to the local situation. Administration was done orally to ensure ingestion of probiotic strains. Piglets were dosed with probiotic strains *L. plantarum* 423 (isolated from sorghum beer) and *L. salivarius* 241, separately and in combination. Statistical analyses showed that new-borne piglets dosed with *L. plantarum* 423 gained the most weight compared to piglets dosed only with *L. salivarius* 241 or a combination of both strains. Piglets aged 14 and 28 days recorded better body weight gain when dosed with a combination of strains 423 and 241 compared to single dosages. A previous study showed that *L. plantarum* 299v survived

passage through the GIT irrespective of gastric acidity (Goossens et al., 2005). Oral administration is stressful to animals and this could explain variable weight gain results recorded with this study. The antibiotic (Tylan) included in the feed, could also account for variable results recorded. This concurred with statements by other researchers, who suggested that weight gain recorded in piglets after oral administration of probiotic LAB, are variable (Johnsson, 1985; Kornegay, 1986; Nousiainen and Suoni 1991; Cupere et al., 1992; Yuste, 1992). The use of weight gain as a measurement for the efficacy of a probiotic remains controversial as water retention and the amount of feed ingested before weighing, could affect results. Feed conversion and mortality rates are better parameters that can be used to measure the efficacy of probiotics. Results obtained with *in vivo* trials are often variable because of the complexity of the intestine and variation between animals (Simon et al., 2003). Feed conversion and mortality rates were not measured in this study because the aim was to investigate the survival and adhesion of probiotic strains when administered to piglets, and not to determine the efficacy of the probiotics *per se*.

After challenging piglets twice with *E. faecalis* FAIR E 92, followed by several dosages of probiotic strains 423 and 241 separately and in combination, it was recorded that piglets dosed with a combination gained more body weight compared to piglets dosed with 423 and 241 separately. A combination of probiotic strains 423 and 241 proved to be more effective in lowering the faecal enterococci count of non-weaned piglets, as compared to dosage with one species. Multi-species probiotics benefit from a synergism when different probiotic effects of the species are combined (Timmerman et al., 2004). One of these synergistic effects is the adhesion and/or colonization of probiotic bacteria to different parts of the gastro-intestinal tract. This could explain why the combination probiotic 423 and 241 showed better results in lowering enterococci counts. This study also indicated that *L. salivarius* adheres to the duodenum and *L. plantarum* to the ileum. The ability of probiotic bacteria to adhere to the intestinal mucosa may depend on the age of the host (Kirjavainen et al., 1998). The intestinal environment of new-bornes may provide less sites for adherence of probiotic strains (Kirjavainen et al., 1998). Concluded from results obtained in the present study, *L. plantarum* 423 proved more

effective in increasing weight gain when dosed to new-borne piglets. *L. salivarius* 241 proved more effective in 14-day-old piglets. One may hypothesise that there are more adhesion sites available for *L. salivarius* 241 in piglets of 14 days and older, compared to adhesion sites available in new-borne piglets. Although *L. salivarius* 241 was originally isolated from the ileum of a 38-day-old piglet, it favoured adhesion to the duodenum rather than to the ileum when it was administered orally. It could be speculated that the adherence preference of a probiotic strain might change when cultivated *in vitro* and this merits further investigation. With all of the latter taken into account, *L. plantarum* 423 should be administered to piglets during the first 14 days after birth, followed by *L. salivarius* 241. Higher enterococci cell numbers were recorded in faeces of non-weaned piglets compared to those who were weaned. Enterococci count is affected during weaning of piglets (Robinson et al., 1984).

Adhesion to mucosal surfaces, prolongs the time that probiotics can exert positive effects on the gastro-intestinal tract of the host and prevent the attachment of pathogens (Kirjavainen et al., 1998; Jin et al., 2000; Lee et al., 2000; Jonsson et al., 2001; Todoriki et al., 2001; Mukai et al., 2002). The ability to adhere was thought to correspond directly to the efficacy of the probiotic strain. There is very little evidence that exogenously administered probiotic adheres to mucosal surfaces (Servin and Coconnier, 2003). Some probiotics pass through the GIT without adhering, making the continuous ingestion of probiotic cultures a prerequisite (Servin and Coconnier, 2003). Adhesion studies remain difficult, since intestinal bacteria must be cultivated on host tissue cultures and/or various commercial cell lines.

With the introduction of fluorescent-*in-situ*-hybridization (FISH), the adhesion of probiotic bacteria could be studied *in situ*. In this study FISH was used to detect the adherence of probiotic LAB to the mucus layer of the porcine GIT. Probiotic bacteria were studied within a natural niche. Lactobacilli colonize the mucus layer of the small intestine of piglets (Rojas and Conway, 1996). After oral dosage of piglets with probiotic strains *L. plantarum* 423 and *L. salivarius* 241, an increase in the lactobacilli species present in the mucus were recorded. FISH results showed a larger number of fluorescent

cells present in the ileum compared to other parts of the GIT. Results obtained concurred with other studies where it was proved that overall, probiotic LAB colonize the ileum more frequently and in larger numbers as compared to other parts of the GIT (Pedersen and Tannock, 1989; Holzapfel et al., 1998; Blake et al., 2003). This study proved that association with the mucus takes place in the form of clusters of bacterial cells formed within the porcine mucus. The activity of microflora in the distal part of the small intestine lowers the pH in this region (Friend, 1963), making it favourable for LAB. The pig ileum is a more aerobic environment (Hartemink and Rombouts, 1999), resulting in a different bacterial composition as compared to the strictly anaerobic colon and this promotes the prevalence of aerobic and micro-aerophilic probiotic LAB in these sections of the GIT.

After piglets were dosed with *E. faecalis* FAIR E 92, a reduction in *E. faecalis* cells were recorded with the Efs probe in the ileum of piglets subsequently dosed with probiotic cultures 423, 241 and a combination of both. The ileum was the only part of the GIT where an overall reduction in the numbers of *E. faecalis* was recorded. The best reduction in *E. faecalis* cell numbers was shown in the ileum mucus of the slaughtered piglet dosed with a combination of probiotic cultures 423 and 241. This reduction alone is not adequate proof for the effectiveness of the probiotic strains tested; it only provides an indication that strain 423 continues to produce plantaricin 423 in the porcine GIT.

Claims made on labels of probiotic products should be supported by clinical trials. These trials are expensive and not always practical for screening purposes. Other methods or tools are needed to evaluate probiotics. *In vitro* models provide a tool for studying probiotics and their interactions on intestinal microbiota (Alander et al., 1999) without the use of animals or clinical trials. This study introduced a porcine gastro-intestinal model based on the five-step multi-chamber reactor simulating the human intestinal microbial ecosystem (Molly et al., 1993). The model developed by Molly et al. (1993), was the first that included both small and large intestine simulation vessels. A model simulating the human GIT could be used as a basis for developing a pig model,

because pigs are used as live animal models in surgical training, simulating humans (Taylor and Hammond, 2004).

The retention time of feed in the GIT of pigs varies between two to four days (Kidder and Manners, 1978). Results obtained with experimental runs in the gastro-intestinal model, over four days indicated that the OD_{600}-values determining cell growth, were the highest when contents entered the caecum and anterior vessel, after transportation through the duodenum, and the addition of pancreatic-bile juices. Probiotic cells survived the bile/pancreatic environment and low pH conditions in the stomach. Similar results were obtained with plate counts done on MRS, after serial dilution of samples obtained from the gastro-intestinal model over four days. A decrease in cell counts was recorded in reaction to the pancreatic-bile solution that was added to the duodenum vessel. Cell numbers increased again in the caecum and anterior colon, suggesting that probiotic cells survived the addition of pancreatic juices and bile. Bacteriocin activity was detected until contents reached the caecum and anterior colon vessel. Detectable levels (clear inhibition zones visible) of plantaricin 423 were recorded even under low pH conditions and in the presence of pancreatic juices and bile.

Experimental runs done over seven days, in the gastro-intestinal model provided data on the application frequency needed for probiotic strains, as the survival of strains under conditions similar to the GIT were monitored. Probiotic strains *L. salivarius* 241 and *L. plantarum* 423 survived conditions in the gastro-intestinal model over this period, with the daily addition of medium from the reservoir and pancreatic-bile vessel. *L. plantarum* 423 survived better (higher cell numbers recorded) compared to *L. salivarius* 241. Strain 241 was isolated from the gastro-intestinal tract of a piglet, while *L. plantarum* 423 was isolated from fermented sorghum beer. *L. salivarius* 241 favors growth in more anaerobic conditions. The adhesion of *L. plantarum* 423 and *L. salivarius* 241 in the small intestine was demonstrated with this study. To our knowledge, FISH had not previously been used to study the adherence of probiotic bacteria *in vivo* as well as *in vitro* in a gastro-intestinal model. The latter promotes these strains as good probiotic bacteria since adhesion to mucus in the small intestine, where transit rates are high, is required (Kidder and

Manners, 1978). Results obtained showed that probiotic cells cluster together in the mucus. This study provided new insight into the behaviour of LAB within the mucosal layer of the GIT. Lactic acid bacteria uses the phenomenon of clustering to survive this environment. Researchers showed that the adhesion trait of probiotic microorganisms are not host specific, but rather characteristic of the species (Rinkinen et al., 2003). This was confirmed in this study where *L. plantarum* 423, isolated from sorghum beer, adhered to the porcine intestinal mucus.

Vessels could be autoclaved separately, minimizing the contamination factor. A unique feature was the inclusion of ileum samples in the gastro-intestinal model to study the adherence of probiotic strains. Ileum samples from slaughtered pigs were added and probiotic bacteria present in the mucus were identified and detected with FISH. Results obtained by FISH showed that cells of *L. plantarum* (using probe LPLANG), *L. salivarius* (using probe LSAL), *E. faecalis* (using probe Efs) and other eubacteria (using EUB 338) could be detected. Cells were grouped into clusters and the presence of the latter strains in the porcine ileum mucus could be established. To our knowledge this is the first porcine gastro-intestinal model that incorporated ileum samples to study the adhesion of probiotic bacteria *in situ* with FISH. FISH can be used as a cost-effective alternative (compared to cultivation of single bacteria *in vitro*) to depict the complex intestinal microbiota and select probiotic strains suitable to specific regions of the GIT.

It is unlikely that a single product like probiotics will emerge as a direct replacement for antibiotic growth promoters. A combination of products may be considered together with a review of the various stress factors that may affect performance and disease, including nutrition, environment and management practices (Ratcliff, 2000).

It is difficult to achieve beneficial effects with probiotics because many factors could influence the effective results of these preparations. These include the genetic, physiological and health status of the animal, the diet and environment as well as the varying microbial load between herds (Jonsson and Conway, 1992). Probiotic preparations could vary with regard to the type of bacteria used, their viability and

physiological state and the form, time and levels of dosage administered. To obtain consistent results with probiotics, more knowledge of the microorganisms present in the digestive tract is needed.

There are many questions still to be answered regarding probiotics, the mode of action, the dose-response relationship, better knowledge regarding the importance of adhesion and the chemical nature of receptor sites for different probiotic strains, to mention a few. The determination of specific conditions in South Africa especially, where probiotics can be implemented, as alternatives to antibiotics are important. For the South African scenario the vital factor would be to keep the production costs for probiotic products as low as possible so that these products would become more attractive to the farmer including the small-scale rural farmers. Although certain limitations and shortcomings were identified in this study, the hypothesis stated could be proven. A gastro-intestinal model could be applied to evaluate the probiotic bacteria and *in vivo* results could be compared to *in vitro* results.

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ADDENDUM

ADDENDUM

ADDENDUM PART ONE

Current topics

Why Humans Should Swallow Live Bugs - Probiotics
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Running Headline: Probiotics for humans

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"A reader who has little knowledge of such matters may be surprised by my recommendation to absorb large quantities of microbes, as a general belief that microbes are harmful, but this belief is erroneous, there are many useful microbes, amongst which the lactic bacilli have the honourable place", a statement taken from the book "The prolongation of life. Optimistic studies", written by Metchnikoff in 1908, cited by Fuller.[1]

The consumption of live bacteria may be of benefit, providing they have GRAS (generally regarded as safe) status, e.g. the majority of lactic acid bacteria (LAB). Many probiotic bacteria have been described some of which have been extensively studied in clinical trials. The concept of administering probiotics had been thoroughly debated over the past decade. However, despite impressive lists of the potential benefits linked to the use of probiotics, they are not commonly part of the medical practitioner's prescription drugs. Many of the better known probiotics, especially from European origin, are available in health-food stores and grocery outlets. Although the general public consumes probiotics or probiotic-containing food (e.g. yoghurt), most people are generally ill informed concerning the possible benefits. This paper focuses on the recent probiotic information with emphasis on the bacterial species, characteristics of probiotic strains, potential benefits, safety aspects, applications of probiotic bacteria in human health and probiotic products available.

Definition and Development of the Probiotic Concept

The term "probiotic" is derived from Greek meaning "for life" and was first used to describe substances secreted by one organism which stimulates the growth of another.[2] The scientific study and value of probiotic bacteria was first realised in 1908 when Metchnikoff suggested that a fermented milk called "yahourth" provided health promoting effects ascribed to the bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*.[3] Parker termed probiotics as "organisms and substances which contribute to intestinal balance".[4] The definition was subsequently changed to "live microbial food supplements which beneficially affects host animals by improving its intestinal balance".[1] In 1992 the definition was extended to "a mono or mixed culture of live microorganisms, when applied to animal or human, beneficially affects the host by improving the properties of the indigenous microflora".[5] The latest definition reads "a microbial dietary adjuvant that beneficially affects the host physiology by modulating mucosal and systemic immunity as well as improving nutritional and microbial balance in the intestinal tract".[6] Two additional concepts, "prebiotics" and "synbiotics" have emerged through probiotic research. Prebiotics are defined as "non-

digestible food components that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, that have the potential to improve host health".[7] The term "prebiotics" was first used when it became evident that fructo-oligosaccharides, indigestible in the small intestine, could be metabolised by only a few bacterial species, notably bifidobacteria, and that the feeding of such oligosaccharides to experimental animals and humans increased the count of bifidobacteria in the intestinal tract.[8] Extensive research on oligosaccharides, especially fructo-oligosaccharides and inulin, has been performed.[8,9] Important to note is that the stimulation of microorganisms by these carbohydrates *in vivo* did not appear as selective as indicated by *in vitro* studies.[10] Synbiotics are described as "mixtures of probiotics and prebiotics that beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastro-intestinal tract".[7]

Microorganisms used as probiotics

A wide variety of microorganisms colonises the gastro-intestinal tract of humans, some beneficial and others detrimental.[6] Although microorganisms representing many different genera have been used as probiotics, most of the studies focused on lactobacilli, bifidobacteria, enterococci and the yeast *Saccharomyces boulardii*.[10]

Enterococci is often used as indicators of faecal contamination and have been implicated in outbreaks of foodborne illness.[11] However, enterococci is also used as probiotic treatment for gastroenteritis in humans, to improve the microbial balance of the intestine, but certain strains of enterococci are also known as nosocomial pathogens causing bacteraemia, endocarditis, urinary tract and other infections.[11] The application of enterococci as probiotics remains a somewhat controversial issue until the accumulation of more research data. The same holds for the inclusion of yeast in probiotic products. Microorganisms used as probiotics for humans are listed in Table 1.

A list of the properties of successful probiotic strains and their health effects are presented in Table 2. Probiotic bacteria can also be helpful in improving the nutritional quality of food and feed, act as metabolic stimuli of vitamin synthesis and enzyme production. Probiotics plays a role in preventing and alleviating some conditions and illnesses and in addition to the health effects mentioned in Table 2, probiotic bacteria enhances innate host defences by production of antimicrobial substances, assist in growth promotion and alleviate lactose intolerance.

Characteristics of probiotic bacteria and potential benefits

Some of the potential benefits of probiotic bacteria are closely related to special characteristics of these bacteria. The characteristics include antagonisms against pathogens by the production of bacteriocins and other antagonistic compounds, produced in smaller quantities like formic acid, CO_2, ammonia, fatty acids, ethanol, hydrogen peroxide, diacetyl, 2,3-butanediol, acetaldehyde, benzoate, bacteriolytic enzymes, antibiotics, and low-molecular weight metabolites.[26,27,28,29,30,31,32,33] Some LAB produce inhibitory substances that exert antagonism against a wide spectrum of microorganisms whereby the growth of undesirable pathogens may be inhibited. Certain probiotic strains stabilise the microflora in the intestinal tract by excluding the growth of enteropathogens. Lactic acid fermentation by LAB leads to the accumulation of organic acids (such as lactic acid and acetic acids) with a subsequent reduction in pH. The production of one or more of these antimicrobial substances may be of advantage to probiotic strains for their establishment and competitiveness with other organisms in the gastro-intestinal tract.

Bacteriocins

Bacteriocins are proteins or protein complexes with a bactericidal or bacteriostatic mode of action affecting closely related species.[34] Bacteriocin production by probiotic strains may advantageously contribute to their competitiveness, colonisation and growth in the gastro-intestinal tract. Bacteriocin production has been reported for most LAB genera, e.g. *Lactobacillus, Lactococcus, Enterococcus, Pediococcus, Leuconostoc* and *Carnobacterium*.[35] Certain strains of bifidobacteria, lactobacilli and enterococci associated with the intestinal tract of humans produce bacteriocins. *L. acidophilus, L. reuteri, L. casei, L. fermentum* and *L. plantarum* are typical representatives of the human gastro-intestinal tract with these features.[36,37,38,39,40,41,42,43,44,45]

Organic acids

Lactic acid is the main fermentation product of LAB, leading to a reduction in pH. Acetic acid, produced by heterofermentative species, is more effective against Gram-negative bacteria, moulds and yeast.[46,47] Both these acids are more effective in their undissociated form whereby they penetrate the microbial cell, reduce the intracellular pH and interfere with essential cell functions.[47] Bifidobacteria produce acetic and lactic acid at a ratio of 3:2 and may have some advantage over strains of lactobacilli by more effectively inhibiting intestinal Gram-negative pathogens or yeasts. For the treatment and control of *Candida* vaginitis,

L. reuteri, L. fermentum or *Bifidobacterium* spp. might be more effective as they produce more acetic acid than lactic acid.

Low-molecular weight metabolites

The best known low-molecular weight metabolites are diacetyl, CO_2 and acetaldehyde. Diacetyl and acetaldehyde are typical aroma compounds of fermented food products and beverages and are only antagonistic when present at high concentrations. The production of CO_2 is inhibitory mainly against aerobic microorganisms and may stimulate the growth of other microorganisms. Diacetyl, CO_2 and acetaldehyde will probably have little or no effect in the gastro-intestinal tract (GIT) of human and animals, although they may exert some antimicrobial activity when used in combination with other antimicrobials. The two most potent low-molecular weight substances that have been studied *in vitro* are reuterin produced by *L. reuteri* and a substance produced by *Lactobacillus* GG (*L. rhamnosus*).[47,48,49,50] Reuterin is a broad spectrum antimicrobial substance (produced from glycerol) effective against Gram-negative and Gram-positive bacteria, yeast, fungi and protozoa.[51] The *in vitro* conditions for production of reuterin are similar to the regions of the GIT where *L. reuteri* are natural inhabitants. *L. reuteri* has been isolated from humans and is able to colonise the intestinal mucosa. *Lactobacillus* GG is a human isolate that produces an unidentified antimicrobial substance reported to be active against Gram-positive anaerobic bacteria (*Clostridium* spp. and *Bifidobacterium* spp.), staphylococci, streptococci and Gram-negative bacteria including members of the family *Enterobacteriaceae* and the genus *Pseudomonas*.[47] Certain strains of bifidobacteria produce a broad-spectrum antimicrobial substance active against *Salmonella*, *Listeria*, *Campylobacter* and *Shigella* spp. which may act to protect the host against gastro-enteritis.[52] *L. acidophilus* LA1, isolated from humans, produces a non-bacteriocin antimicrobial substance active against Gram-positive and Gram-negative pathogens, *in vitro* and *in vivo*.[50] The screening of bacterial strains from human origin for the production of bacteriocins and low-molecular weight substances with broad spectrum antimicrobial activity is receiving much attention.

Latest applications of probiotic bacteria

At present, probiotic bacteria can be used for the treatment of a wide range of symptoms, diseases and illnesses. The most important applications will be described in more detail.

Stimulation of the immune system

The intake of probiotics stimulate cell-mediated immune effector functions.[53] Thus, enhanced production of interferon-γ by blood cells, enhanced phagocytosis by polymorphonuclear leukocytes (PMN) and to a lesser extent monocytes, and enhanced expression of complement receptors on PMNs are effects associated with individuals consuming probiotic bacteria.[53] It is likely that this is the result of probiotic bacteria being taken up across the small intestinal mucosa and being ingested by macrophages, that leads to the production of cytokines and other mediators stimulating cell-mediated immunity.[53] It has also been suggested that probiotics can function as adjuvants, i.e. agents that increase immune responses to other antigens administered concomitantly. However, responses to vaccination are only mildly increased during probiotic consumption and most likely depend on the occurrence of cross-reactive antibodies that are induced by the probiotic bacteria but also bind to structures unrelated to antigens.[53] A few studies have tested the clinical effect of probiotics on allergy conditions, but to date no effect has been convincingly proven.[53] *L. casei*, when injected intraperitoneally, activates the peritoneal macrophage and lymphocytes.[54, 55] *L. acidophilus*, *L. casei* and *L. delbrueckii* subsp. *bulgaricus* could play a role in the activation of cells involved in the non-specific immune response (the macrophages and phagocytes) but *L. casei* and *L. acidophilus* are more effective as they are able to survive and grow in the GIT.[55] Researchers reported an immunostimulatory effect of *E. faecium* M47 in humans by an increase in IgG levels.[56] *L. rhamnosus* GG may also be applied for the treatment of Crohn's disease .[56] When *L. rhamnosus* GG was orally administered to children with Crohn's disease, it resulted in an increased level of IgA specific for Crohn's disease.[57] *Cryptosporidium parvum* causes diarrhoea and may accelerate death in immunosuppressed individuals (e.g. AIDS). *L. reuteri* and *L. acidophilus* prevented infection of *C. parvum* in immunosuppressed mice.[58,59] As indicated by the above results, LAB have the potential to be used in combating diseases and probiotic treatment could be beneficial in conditions where stimulation of cell mediated effector functions is desired.

Anticarcinogenic properties

The anticarcinogenic properties of LAB or fermented milk products fall into three categories: 1) the inhibition of tumour cells, 2) the exclusion or inhibition of microorganisms that produce procarcinogenic enzymes (e.g. β-glucuronidase, β-glucosidase, azoreductase, nitroreductase), which convert procarcinogens into carcinogens, and 3) elimination of carcinogens. Various enzymes in the gut microflora modify ingested foreign compounds such as nitro aromatics,

azocompounds and nitrate, which can be metabolised to genotoxic and carcinogenic substances with enzymes produced by the anaerobic microflora in the colon.[59] Colon cancer is suggested to be directly linked to the type of diet with a higher incidence in diets high in meat, fat and protein and low dietary fibre.[60] A number of studies have shown that diet as well as antibiotics can change the microflora-associated characteristics. LAB and bifidobacteria have generally low activities of enzymes involved in carcinogen production.[10] Supplementation of galacto-oligosaccharides (GOS) and the synthetic disaccharide lactulose was shown to decrease faecal β-glucuronidase and increase lactobacilli counts in rats.[61,62] It was shown that *L. acidophilus* and *L. rhamnosus* GG decreased β-glucuronidase (an enzyme used to monitor mucosal carcinogenesis as this enzyme may convert procarcinogens into carcinogens) in carnivorous animals and humans.[63,64,65] LAB may also prevent or retard the initiation and promotion of tumours. Feeding fermented milk or live bacteria such as *L. acidophilus* and *L. delbrueckii* subsp.*bulgaricus* and/or *L. casei* suppresses Ehrlich ascites tumour or Sarcoma 180 in mice, but similar studies need to be done on humans.[66] Two independent studies on the treatment of human urinary bladder cancer by *L. casei* (Yakult) indicated that immunomodulatory effects of LAB and bifidobacteria cells may be used in the future to prevent and treat cancer of the human colon and bladder.[62,67] However, further studies in this field of research should be performed. Nitrates used in food processing are converted to carcinogenic nitrosamines in the GIT. Cellular uptake of nitrates by lactobacilli and bifidobacteria has been shown *in vitro*.[68] Two studies with probiotic milk products in patients undergoing radiotherapy for pelvic malignancies indicate that such products should be tested further to prevent therapy-related diarrhoea and clinical bowel discomfort symptoms.[69] Further identification and validation of biomarkers for risk of cancer is a prerequisite for future studies, to evaluate the potential of probiotics and prebiotics in man in relation to cancer.[10]

Cholesterol reduction

Heart disease and more specifically atherosclerosis are one of the major causes of death in humans. Hypercholesterolemia is considered the most common risk factor associated with atherosclerosis that leads to cardiovascular disease in modern industrialised countries.[70] Atherosclerosis is caused when cholesterol accumulates in the wall of the arteries to form plaques that inhibit blood flow, leading to clot formation and heart attack.[70] Cholesterol is synthesised in the liver at a rate the body requires. Alteration of the diet might have a significant effect on the serum cholesterol level.[70] A number of studies have examined the

potential of probiotic products to reduce serum cholesterol levels.[70] Unfortunately some of these studies had no suitable control or placebo groups, had a lack of run-in periods or very large doses of fermented dairy products (700-5000ml/day) were used. This made the actual results of these studies doubtful.[10] An important consideration in the evaluation of the studies was the fact that participation for a single week in a nutritional trial itself, may result in a reduction of cholesterol levels.[10] Furthermore, the analytical precision of serum cholesterol determination is another aspect that has to be considered.[10] A product containing *L. plantarum* 299v (ProViva®), that was tested in a parallel study with 30 subjects yielded a significant reduction in serum cholesterol levels, however the test group and the control group had similar cholesterol levels in the end.[71] A fermented milk product containing *E. faecium* and *S. thermophilus* (GAIO®) has been tested in three studies with parallel designs in altogether 214 subjects.[72,73,74] The randomisation of this trial was not always optimal. A small but significant reduction was found at some time-periods, but no difference was found after six months in any one of the studies.[74] GAIO® was also tested in a randomised crossover study and a reduction in serum cholesterol levels was found after six weeks.[74] The mechanisms whereby probiotics may reduce serum cholesterol levels are largely unknown.[10] Certain strains of bacteria have the capacity to assimilate cholesterol *in vitro*. However, it is clear that the effect of probiotics on cholesterol is still inconclusive. Long term studies are needed to document a sustained effect.[10] Another aspect that received attention was the role of bile salt deconjugation in the reduction of serum cholesterol levels.[75] Cholesterol and bile salt metabolism are closely linked, cholesterol is the precursor for synthesis and bile salts the water-soluble excretory end product.[75] The enterohepatic circulation (EHC) is a system by which bile acids are recycled and conserved from the liver, passed through the small intestine and reabsorbed in the distal ileum and returned to the liver via the portal veins.[76] One of the transformations of bile salts is their deconjugation during the EHC. Bile salt hydrolase (BSH) is the enzyme responsible for deconjugation of bile salts where glycine and taurine are split off, resulting in free or deconjugated bile salts. BSH activity was observed in *Lactobacillus, Enterococcus, Peptostreptococcus, Bifidobacterium, Clostridium* and *Bacteroides*.[76,77,78] It was hypothesised that deconjugation of bile salts will lower cholesterol, as free bile salts are not so easily reabsorbed and excreted with faeces.[79,80] The loss in bile salts increased the catabolism of cholesterol to bile acids and lead to lower cholesterol levels. The BSH hypothesis has definitely not been proved and further studies are necessary.

Alleviation of lactose intolerance

Beta-galactosidase (lactase) hydrolyses milk lactose to glucose and galactose. The presence of sufficient lactase activity in the small bowel mucosa is necessary for the newborn child to absorb lactose from breast milk.[10] Lactose, a milk carbohydrate, cannot be utilised by a large part of the Asian and African population, due to the lack of the intestinal mucosal enzyme β-galactosidase (lactase) or by a reduction in lactose activity caused by intestinal infection (e.g. rotavirus gastro-enteritis).[81] The symptoms of lactose maldigestion are flatulence, abdominal pain and diarrhoea. This enzyme, however is dramatically reduced in adult life in the majority of people.[10] It should be noted, however, that lactose malabsorption often occurs without symptoms of lactose intolerance.[10] Several studies have demonstrated that subjects with low intestinal lactase activity absorb lactose from yoghurt or milk containing *L. acidophilus* better than from milk.[82] High levels of β-galactosidase activity are found in yoghurt starter cultures *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*. Both species are sensitive to bile salts, leading to the release of high levels of β-galactosidase in the GIT. Bile increases the permeability of bacterial cells and bile tolerance may protect the bacteria against bile-induced permeability, preventing intracellular β-galactosidase to hydrolyse lactose.[83] *L. acidophilus* is bile resistant, has lower levels of β-galactosidase than the yoghurt starter cultures, is able to grow in the GIT, and may elevate β-galactosidase levels for longer time periods.[83] Fermented milk products with specific probiotic lactic acid bacteria improve lactose digestibility and absorption, and may reduce symptoms of lactose intolerance. This conclusion is in agreement with a recent review.[84]

Effect on blood pressure

Two independent studies have been published on the effect of fermented milk on blood pressure.[10] In the first study, *L. helveticus* and *Saccharomyces cerevisiae* were used and in the second one *L. casei* TMC 0409 and *S. thermophilus* TMC 1543 were investigated.[85,86] Both studies showed significant reductions in systolic blood pressure and in the first study, effects on diastolic blood pressure was also noted. It was suggested that the effect could be due to the formation of certain tripeptides that are inhibitors of ACE (Angio-tension-converting enzyme). These findings need to be confirmed.

Constipation alleviation

Constipation means complaints with bowel evacuations, low bowel emptying frequency and a slow transit through the large bowel. Based on early reports, various fermented milk products

can alleviate constipation but a lot of these reports were self-reported information by patients and is difficult to evaluate.[87] Whey fermented with *L. rhamnosus* GG did not change bowel movement frequency or hardness of stools in patients of a small placebo study.[88] Similar findings were recorded with yoghurt fermented by *L. rhamnosus* GG. Inulin and fructo-oligosaccharides also showed some dose-related laxation effects and could also be an aspect worth studying.[10] Further research is needed to substantiate these findings.

Irritable bowel syndrome

Irritable bowel syndrome (IBS) is a very common gastro-intestinal disorder, and is often the most frequent diagnosis given at a gastroenterologist's clinic.[10] Typical symptoms are flatulence, variations between constipation and diarrhoea and abdominal pains. There are different factors associated with the condition, e.g. food intake, malabsorption and psychosomatic influences.[10] Patients with IBS report pain with a lesser degree of abdominal distension than others.[10] In a double-blind RCT study on 61 subjects with IBS, no difference in tolerance was observed with unfermented milk containing *L. acidophilus* compared to ordinary milk.[89] A rose-hip drink with *L. plantarum* 299v (400ml/day) was tested in two RCTs with parallel designs. In one of the studies (40 patients), improvement of symptoms was significantly greater in the study group than in the control group.[90] In the other study (52 patients), flatulence was reduced in the test group compared with the placebo group.[91] Abdominal pain was reduced in both groups, even though the reduction was more rapid in the test group. No major change in gas bloating was shown. It could be concluded that an effect on some symptoms in IBS is reported with *L. plantarum* 299v but more controlled studies are needed for definite conclusions.

Diarrhoea

Many different diseases and treatments may induce diarrhoea. Diarrhoea may be related to viral and bacterial infections, radiotherapy or antibiotic use. The effect of probiotics on diarrhoea has been extensively studied and clinical trials have recently been reviewed.[92,93] Rotavirus causes acute diarrhoea in children and *L. rhamnosus* GG is the probiotic strain which has been most extensively studied to treat this condition. Milk products fermented with *L. rhamnosus* GG have been shown to shorten acute diarrhoea, especially when caused by rotavirus.[94,95] Since acute diarrhoea is self-limiting, seldom lasting for more than a week, the therapeutic effect is small, thus the duration of diarrhoea is usually shortened by approximately one day. However, the effect is reasonably well proven and has also been

replicated in other studies. The effect of *L. reuteri* SD2112 on acute diarrhoea in children were investigated in two separate studies and both demonstrated the same effect in the same order of magnitude as reported for *L. rhamnosus* GG.[96] It should be noted that only one of the above mentioned studies gives data on the extent of breast-feeding in different study groups.[94] Since breast-feeding effectively counteracts diarrhoea, it is very important to control this factor. Another well known disease associated with diarrhoea is Travellers' diarrhoea. A few placebo-controlled studies have failed to show effective prevention of infectious diarrhoea in adults.[93] These studies were performed with freeze-dried probiotic preparations, and no data have been published on the possible effects of probiotic food products in this respect. Treatment with antibiotics results in diarrhoea and abdominal discomfort in a variable fraction of patients, depending on age group and the antibiotic used.[10] In most cases, the cause of the diarrhoea is unknown, but a varying proportion of the cases are caused by *Clostridium difficile*. This toxin-producing species is not uncommon in the normal intestinal microflora. It is usually present in low numbers, after treatment with certain antibiotics, the lack of competition from other microbes in the normal intestinal flora permits *C. difficile* to reach high numbers.[10] The toxins of *C. difficile* may cause mild diarrhoea or a life-threatening disease called pseudo-membranous colitis.[10] A yoghurt containing bifidobacteria was shown to quite effectively reduce abdominal illness in volunteers consuming erythromycin for three days.[97] In other studies *L. acidophilus* and *L. rhamnosus* GG were used to treat antibiotic-associated diarrhoea.[98,99] These studies offered promising indications that probiotics could be useful against side effects of antibiotic treatment. Larger and better controlled studies are urgently needed. Severe *C. difficile* infection is treated with antibiotics active against anaerobic bacteria (vancomycin and metronidazol). In most cases the treatment is successful, but in 20% of patients, *C. difficile* is not eradicated and the patient is plagued by recurrent episodes of diarrhoea.[100] This condition, termed relapsing *C. difficile* infection, is difficult to treat. In a single placebo-controlled clinical study, *Saccharomyces boulardii* was utilised and by adding *S. boulardii* to the metronidazol or vancomycin treatment aiming to eradicate *C. difficile*, the risk of the patients relapsing was reduced by 50%.[100] The use of *L. rhamnosus* GG against relapsing *C. difficile* infection had only been evaluated in open trials. Although some successes were reported, more controlled studies are needed to investigate this potential application for probiotics.[100]

It is also important to look at the potential mechanisms involved in the control of diarrhoea. It was originally assumed that the ability of probiotic bacteria to shorten diarrhoea was dependant on their ability to colonise the intestine and "alter the balance" in such a way that

the pathogen would be eliminated. Specific probiotic strains such as *L. plantarum* 299 and 299v, *L. rhamnosus* 271 and GG have been proven to colonise human volunteers. This might relate to the fact that these species are prevalent on the normal human intestinal mucosa.[101] However, some probiotic bacteria do not colonise, but are eliminated at a rate that is similar to ingested inert particles, but probiotics that are not likely to colonise can still reduce diarrhoea. This can also be shown for the yeast *S. boulardii* active against *C. difficile* without colonising the intestine. Bifidobacteria also seem to reduce diarrhoea caused by antibiotic treatment without colonising the patient.[102,103] As mentioned before the colonisation or not of a probiotic remains a controversial issue. Another proposed effect has been that the probiotic induces an enhanced immune response against the microorganism causing the diarrhoea and that this leads to earlier resolution of the diarrhoeal disease. This has been proposed to be the mechanism of the effect of *L. rhamnosus* GG against rotavirus diarrhoea.[104] It seems that the exact mechanism by which probiotic intake reduces diarrhoea remains unknown.

Stomach ulcers

Helicobacter pylori cause peptic ulcers in humans. *L. acidophilus* LA1 was grown in milk and tested against *H. pylori* induced gastritis in a clinical study, with promising effect.[105] Suppression of the infection was determined by a standard breath test. However the effect was reversible, as seen also by a study with a probiotic *Clostridium* preparation. More recently *L. rhamnosus* GG together with antibiotic therapy was tested and fewer gastro-intestinal side effects could be seen in the probiotic group, suggesting that probiotics could be designed to improve *H. pylori* treatment results and prevent side effects.[105]

Inflammatory bowel disease

Another disease where the treatment with probiotic products showed encouraging results is inflammatory bowel disease (IBD). IBD may be caused or aggravated by alterations in the microbial flora. The distal ileum and colon are most frequently affected by the inflammatory process in patients with IBD, sites that harbour the largest populations of intestinal bacteria. Early studies with probiotic LAB *(L. reuteri* and *L. plantarum* 299v) showed some protective effect in chemically induced colitis in rodents. More recently studies in humans with ulcerative colitis given mesalazine or capsules containing a well defined *E. coli* strain, showed no difference in relapse rates, unfortunately no placebo group was included.[10] Only one placebo-controlled clinical study was performed on patients with pouchitis, a common long-term inflammation of the ileal reservoir created after surgical removal of the colon. Patients

on this therapy showed few relapses for 9 months (15%) as compared to 100% in the control group. This study further supports the potential role of probiotics in food products in IBD therapy and prophlylaxis.[106,107,108] A complex probiotic preparation containing 200 billion viable freeze-dried bacteria of four LAB strains per gram, three bifidobacteria and one strain of *S. thermophilus*, was tested in a clinical trial in patients showing allergy or intolerance of other origin to classical therapy with mesalamine or sulphasalazine.[106,107,108] The treated group showed reduced faecal pH and remained in remission. Well-designed large-scale randomised placebo-controlled clinical trials of different pro- and prebiotic preparations versus standard therapy in IBD and pouchitis should be undertaken in the future, since promising results were obtained in some studies.

Hepatic encephalopathy

Ammonia is produced in the GIT through bacterial urease and then absorbed and detoxified in the liver. Patients with liver failure have higher levels of ammonia, leading to encephalopathy. Patients who received *L. acidophilus* had lower levels of faecal urease and blood ammonia.[109] Very little research has however been done on this aspect.

Vaginitis

A very well known disease among woman is vaginitis. LAB, primarily *L. acidophilus,* grows in large numbers in the vagina and maintains the acidic pH. Administration of antibiotics or bacterial infections disturbs the ecological balance in the vagina, which could lead to candidal vaginitis caused by *Candida albicans* or urogenital infections caused by *Gardnerella vaginalis, Bacteroides bivius, Peptostreptococcus* spp. and *Chlamydia trachomatis.*[110,111] Hydrogen peroxide-producing lactobacilli may play a role in the prevention of bacterial vaginitis.[111] In a study where patients were given yoghurt containing *L. acidophilus*, candidal infections were reduced for the period that they received yoghurt.[112] Some success in the treatment of candidal infections with *L. rhamnosus* GG have also been reported.[113] The positive results obtained are worth exploring in future research.

Taking into account all the above mentioned applications and studies done, it is evident that although a lot of research has already been done on the use of probiotics in the treatment of various diseases and conditions, more well planned and well executed experiments are needed to be performed on human subjects to scientifically substantiate the use of probiotics.

The safety of probiotic bacteria

Probiotic bacteria may, theoretically, be responsible for four types of side effects: systemic infections, deleterious metabolic activities, excessive immune stimulation in susceptible individuals and gene transfer.[114] However, very few cases of adverse events have been reported in humans consuming probiotics.[114]

Rare cases of local or systemic infections, including septicemia and endocarditis due to lactobacilli, bifidobacteria or other LAB have been reported.[115] Most lactobacilli strains isolated from clinical cases belong to the species *L. rhamnosus*, *L. casei* or *L. paracasei* and *L. plantarum*. *E. faecium* and *E. faecalis* are more frequently involved in clinical infections, and there is concern over the emergence of vancomycin-resistant strains.[115] In most cases however, the organism appeared to have come from the patients' own microflora, but in a few cases the recent use of probiotics was mentioned as a potential cause. Case studies were done and it can be concluded that the risk of infection is not nil, but extremely low.[114] Nearly all patients with infections due to probiotic microorganisms or to microorganisms close to probiotics have had underlying conditions which predisposed them to infection, particularly abnormal heart valves in the case of endocarditis, and the presence of a catheter in cases of septicemia.[114] Other risk factors such as extremes of age, pregnancy, immunodeficiency or digestive lesions have not been identified as risk factors for probiotic infections.

If one admits that probiotics can promote metabolic activities in the gut that may have positive effects for health, one may also admit that they may induce other metabolic activities which may be detrimental to the host.[114] A study involving *L. acidophilus* and *Bifidobacterium* spp. administered to healthy humans with a terminal ileostomy drew attention to the potential risk of excessive deconjugation or dehydroxylation of bile salts in the small bowel by probiotics.[116] Excessive degradation of the intestinal mucus layer by probiotics may theoretically be detrimental but no mucus degradation was observed *in vitro* nor in gnotobiotic rats mono-associated with the test strains.[117]

No immunological side effect of a probiotic has been reported in man, except one case of auto-immune hepatitis which might have been enhanced by ingestion of large volumes of yoghurt.[118] Potential enhancement of other auto-immune diseases by probiotic consumption is a research field that should be studied more closely.

Some antibiotic resistance genes, especially those encoded by plasmids, can be transferred between microorganisms.[114] This property raises the question whether resistance genes can be transferred by probiotics to the endogenous flora or to pathogens. It was demonstrated that the plasmid pAMβ1, which codes for macrolide resistance, could be transferred from *L. reuteri* to *E. faecium* and *E. faecalis* in the mouse GIT.[119] The risk of gene transfer depends on the genetic material to be transferred (plasmids, transposons etc.), on the nature of the donor and recipient strains, on their concentrations and contacts, and on the selection pressure (especially the presence of antibiotics which can selectively promote growth of the transconjugants). It is difficult to assess *in vivo* and *in vitro*, and it is even more difficult to state what probability level of gene transfer is acceptable.[114]

Three approaches exist to assess the safety of a probiotic strain: studies on the intrinsic properties of the strain, studies on the pharmacokinetics of the strain (survival, activity in the intestine, dose-response relationships, faecal and mucosal recovery), and studies searching for interactions between the strain and host.[114] The selection criteria for probiotics are illustrated in Fig.1.

Philippe Marteau made three conclusions concerning the safety of probiotics:
1) the zero risk does not exist, and that acceptance of the concept that probiotics may not only have positive effects but potentially also side effects is important,
2) the safety of the current products is excellent, and
3) further epidemiological and clinical studies are useful for proper monitoring of the consumer's safety.[114]

Probiotic products available

As mentioned before probiotic products are available from retail outlets, usually supermarkets, grocery and health food stores. The probiotics are available to the consumer as powders or tablets, but most commonly milk-based products. LAB-supplemented foods available in different markets are listed in Table 3.

Many consumers purchase probiotic dairy products because they prefer the organoleptic and rheologic characteristics of these products in comparison to regular yoghurts. Whatever the reason, there are consumers who hold a firm believe that their health is improved by regular consumption of a probiotic. It becomes almost an article of faith. Our believe is that further

consumer education is needed for the correct and efficient use of probiotic products and that a fundamental knowledge of intestinal bacteria and their interactions with each other and with human hosts are a prerequisite for successful probiotic research and development.

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Table 1: **Microorganisms applied in human probiotic products[12]**

Lactobacillus spp.	*Bifidobacterium* spp.	Other lactic acid bacteria	Non-lactic acid bacteria
L. delbrueckii subsp. *bulgaricus*	*B. adolescentis*	*Streptococcus thermophilus*[¶]	*Sacchoromyces boulardii*[†]
	B. animalis	*Pediococcus acidilactici*[¶]	*Saccharomyces cerevisiae*[†]
L. acidophilus[¶]	*B. breve*	*Leuconostoc mesenteroides* subsp. *dextranicum*	*Bacillus cereus*[*,†]
L. casei	*B. bifidum*	*Enterococcus faecium*	*Propionibacterium freudenreichii*[*,†]
	B. longum		
L. johnsonii	*B. infantis*	*Lactococcus lactis*[¶]	*Escherichia coli* strain nissle
L. paracasei	*B. lactis*		
L. crispatus			
L. amylovorus			
L. plantarum			
L. rhamnosus			
L. gasseri			
L. reuteri			

[*]Main application for animals.

[†]Applied mainly as pharmaceutical preparations.

[¶]There is little known about the probiotic properties or the microorganisms are non-probiotic.

Table 2: **Properties of successful human probiotic strains and their health effects**

Strain	Health effect
Lactobacillus acidophilus (*johnsonii*) LA1 (LJ-1)	Immune enhancer, adjuvant in *Helicobacter pylori* treatment, adherence to human intestinal cells, balances intestinal microflora, elevation of gastritis, antagonistic against Gram (+) and (-) pathogens[13,14,15]
Lactobacillus acidophilus NCFB 1748	Reducing faecal enzyme levels, decreased faecal mutagenicity, prevention of radiotherapy diarrhoea, treatment of constipation[16,17,18,19]
Lactobacillus rhamnosus GG (ATCC 53013)	Prevention of acute and antibiotic-associated diarrhoea, treatment and prevention of rotavirus diarrhoea; treatment of relapsing *Clostridium difficile*, colonisation of human GIT, treatment of Crohn's disease, antagonistic against cariogenic bacteria and *Salmonella typhimurium*, vaccine adjuvant, lowering faecal enzyme activities, immune response modulation, alleviation of atopic dermatitis symptoms in children[18]
Lactobacillus casei Shirota	Treatment of rotavirus diarrhoea, balancing intestinal bacteria, prevention of intestinal disorders, lowering faecal enzyme levels, positively affecting superficial bladder cancer treatment, early colon cancer immune enhancer[18]
Bifidobacterium lactis Bb-12 (*B. animalis*)	Treatment of rotavirus and viral diarrhoea, balancing intestinal microflora, prevention of traveller's diarrhoea, modulation of intestinal flora, improvement of constipation, modulation of immune response, alleviation of atopic dermatitis symptoms in children[18]
Lactobacillus gasseri (ADH)	Survival of GIT, faecal enzyme reduction[13,14]
Lactobacillus reuteri (BioGaia Biologics)	Treatment of acute diarrhoea in children, shortening rotavirus diarrhoea in children, safe and well-tolerated in HIV-positive adults[18]
Lactobacillus acidophilus NCFM	Faecal enzyme reduction, survival[19]
Enterococcus faecium SF 68	Treatment of acute enteritis in adults, treatment of diarrhoea in paediatrics, lower blood ammonia[20,21,22,23]
Enterococcus faecium M74	Mainly animal studies, immune stimulation in humans, cholesterol reduction[24,25]
Lactobacillus plantarum DSM9843(299v)	Modulation of intestinal flora, increase in faecal short-chain fatty acid content[18]
Saccharomyces boulardii	Prevention of antibiotic-associated diarrhoea, treatment of *Clostridium difficile* colitis, prevention of diarrhoea in critically ill tube-fed patients[18]

Table 3: **LAB-supplemented foods currently available in different markets**[6]

Product or trade name	Origin	LAB culture
AB milk products	Denmark	*L. acidophilus, B. bifidum*
Acidophilus bifidus yogurt	Germany	*L. delbrueckii* subsp. *bulgaricus, S. thermophilus, L. acidophilus, B. bifidum* or *B. longum*
BA ®	France	*B. longum*
Bifidus milk	Germany	*B. bifidum* or *B. longum*
Bifidus milk with yogurt flavor	United Kingdom	*B. bifidum, B. longum*, or *B. infantis*
Bifidus yogurt	Many countries	*B. bifidum* or *B. longum*
Bifighurt®	Germany	*B. bifidum* or *B. longum*
Bifilakt® or Bifilact®	USSR	*Lactobacillus* spp., *Bifidobacterium* spp.
Biogarde®	Germany	*L. acidophilus, B. bifidum, S. thermophilus*
Bioghurt®	Germany	Similar to Biogarde®
Biokys®	Czechoslovakia	*B. bifidum, L. acidophilus, P. acidilactici*
Biomild®	Germany	*L. acidophilus, Bifidobacterium* spp.
Cultura®	Denmark	*L. acidophilus, B. bifidum*
Diphilus milk®	France	As above
Mil-Mil®	Japan	*B. bifidum, B. breve, L. acidophilus*
Progurt®	Chili	*Lactococcus lactis* biovar *diacetilactis, B. bifidum, Lactococcus lactis* subsp. *cremoris, L. acidophilus*
Sweet acidophilus milk	Japan	*L. acidophilus, B. longum*
Sweet bifidus milk	Japan/Germany	*Bifidobacterium* spp.
Ofilus®	France	*S. thermophilus, L. acidophilus, B. bifidum* or *Lactococcus lactis* subsp. *cremoris, L. acidophilus, B. bifidum*

Figure legends

Fig. 1 Selection criteria for probiotics[115]

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                  ┌──────────────────────────────────────┐
┌──────────────┐  │  Origin/Definition/Characterisation  │
│ General      ├──┤                                      │
│ aspects      │  ├──────────────────────────────────────┤
└──────────────┘  │  Strain & Genus safety properties    │
                  └──────────────────────────────────────┘

                  ┌──────────────────────────────────────┐
                  │ Activity and viability in products,  │
┌──────────────┐  │ adherence, invasive potential        │
│ Safety and   ├──┤                                      │
│ Stability    │  ├──────────────────────────────────────┤
└──────────────┘  │ Resistance to low pH, gastric juice, │
                  │ bile acid, pancreatic juice,         │
                  │ colonisation/ survival in vivo       │
                  └──────────────────────────────────────┘

                  ┌──────────────────────────────────────┐
                  │ Adherence to intestinal              │
                  │ epithelium/tissue/virulence          │
                  └──────────────────────────────────────┘
                               and/or
                  ┌──────────────────────────────────────┐
                  │ Antagonism to pathogens,             │
                  │ antimicrobial activity               │
                  └──────────────────────────────────────┘
                               and/or
┌──────────────┐  ┌──────────────────────────────────────┐
│ Functional   │  │ Stimulation/suppression of immune    │
│ and          ├──┤ response                             │
│ Physiological│  └──────────────────────────────────────┘
│ aspects      │               and/or
└──────────────┘  ┌──────────────────────────────────────┐
                  │ Selective stimulation of beneficial  │
                  │ bacteria and suppression of harmful  │
                  │ bacteria                             │
                  └──────────────────────────────────────┘
                               and/or
                  ┌──────────────────────────────────────┐
                  │ Clinical side effects,               │
                  │ volunteers/patients                  │
                  └──────────────────────────────────────┘
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ADDENDUM PART TWO

Evaluation of growth media for the isolation of lactic acid bacteria from pre- and post-weaned piglets

Evaluation of growth media for the isolation of lactic acid bacteria from pre- and post-weaned piglets

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ABSTRACT

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Aims: The development of low-cost growth media for the cultivation of lactic acid bacteria isolated from pig faeces.

Methods and results: Piglets of 11-, 30-, 70- and 106 days old were fed a commercial antibiotic-free diet for 10 days. Lactic acid bacteria were isolated from faeces after 14 days by using the following media MRS (De Man-Rogosa-Sharpe), pH 6.5; MRS, adjusted to pH 4.6 with 1 N HCl; LB (Luria Bertani); BHI (Brain-Heart-Infusion); Rogosa, (Oxoid); FM (faeces medium); CFM (crawl feed medium) and GFM (growth feed medium).

Conclusion: Lactic acid bacteria could be cultured on FM, CFM and GFM media. High cell counts were recorded with these media, but FM, CFM and GFM were not as specific for lactic acid bacteria, compared to MRS, and Rogosa.

Significance and impact of the study: The use of inexpensive alternative growth media such as FM, CFM and GFM reduces the costs involved in cultivation of lactic acid bacteria.

Keywords: lactic acid bacteria, pig faeces, alternative growth media.

INTRODUCTION

Lactic acid bacteria are found in various niches, including fermented food, beverages, soil, and the human and animal gastro-intestinal tract. Apart from their importance in fermentation processes and food preservation, certain intestinal lactic acid bacteria have probiotic properties (Havenaar and Huis in't Veld 1992; Muralidhara *et al.* 1977; Pollmann *et al.* 1980; Tannock 1990). Probiotics are preparations or products containing viable, defined microorganisms in sufficient numbers, which beneficially alters the microflora in the host and exerts health effects (Schrezenmeir and De Vrese 2001). Lactic acid bacteria are Gram-positive, anaerobic or facultative aerobic. Several strains of *Lactobacillus* spp. including *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus helveticus* have been included in animal feed (Salminen *et al.* 1993; Holzapfel *et al.* 2001; Reid 1999). Lactobacilli are one of the dominant microorganisms in the intestinal tract of pigs (Tannock 1990). Probiotic lactic acid bacteria are effective in piglets before and after weaning (Du Toit *et al.* 2003). Administering strains of lactic acid bacteria since birth establishes an ideal relationship between beneficial and pathogenic microorganisms.

Lactic acid bacteria are usually isolated from the faeces of piglets (Rall *et al.* 1970; Gilliland *et al.* 1975; Salanitro *et al.* 1977) and then screened for probiotic properties. Lactobacilli are generally isolated on rich, slightly acidic media such as MRS (De Man *et al.* 1960), LB and modified Rogosa medium (Mitsuoka *et al.* 1973; Kleesen *et al.* 1995; Minelli *et al.* 1993). Isolation media need to be selective to differentiate lactobacilli from bifidobacteria, streptococci and/or enterococci. Most lactobacilli are resistant to vancomycin while bifidobacteria, streptococci and enterococci are susceptible (Hartemink *et al.* 1997).

This study describes the isolation and cultivation of lactic acid bacteria from the faeces of piglets of various ages on growth media, including media prepared from faeces, crawl feed and growth feed. The latter growth media could provide a less expensive alternative for the cultivation of lactic acid bacteria.

MATERIALS AND METHODS

Animals

Piglets received a standard commercial diet with no antibiotics for 10 days before and during faecal sampling. Pig litters of 11, 30, 70 and 110 days old were kept in separate cages. The piglets were weaned at 28 days and faeces collected aseptically in sterile vials, from 21-, 40-, 80- and 120-day-old piglets.

Growth media

Eight different types of growth media were used for the isolation of lactic acid bacteria. These included MRS (Biolab, Biolab Diagnostics, Midrand, SA), MRS (Biolab) adjusted to pH 4.6 with 1N HCl, Rogosa (Oxoid, Basingstoke, Hampshire, England), LB (Biolab), BHI (Biolab), faeces medium (FM), crawl feed medium (CFM), and growth feed medium (GFM). No vancomycin was added as commercially available media were compared with media developed from faeces and feed. Faeces medium was prepared by suspending 100 g pig faeces in distilled water and blending for four minutes. The suspension was centrifuged at 8000 x g for 15 min at 15 – 20 °C. To 80 ml of the supernatant, 920 ml distilled water and 2.5 % w/v Agar (Biolab) was added. The medium was autoclaved (121 °C for 15 min). Crawl feed and growth feed media were prepared by suspending 100 g crawl feed or growth feed, respectively, in 200 ml distilled water, and left, without agitation, at 25 °C for 30 min. Suspensions were blended and centrifuged as described before. Eighty ml of the supernatant was added to distilled water (920 ml), supplemented with agar and autoclaved as described elsewhere.

Isolation and grouping of lactic acid bacteria

Faecal samples were suspended (10 % m/v) in 9 ml sterile Ringers solution, vortexed for 10 min, serially diluted and plated onto each of the eight growth media. After 18 h of incubation at 37 °C, plate counts were done, colonies selected at random and inoculated into MRS broth (Biolab). Cultivated cells were streaked out on MRS agar and isolates were grouped by gram stain reaction and catalase activity.

RESULTS AND DISCUSSION

Growth media

Bacterial strains isolated from piglet faeces indicated the presence of different lactic acid bacteria during the first 45 days (Pedersen and Tannock 1989). Bacterial counts obtained on eight different media, from piglets aged 21, 40, 80 and 120 days, are shown in Fig. 1. Eight different types of media were used, including MRS at pH 4.6 and 6.5 and Rogosa which is selective for lactic acid bacteria. MRS at pH 4.6 was included to favour the isolation of LAB surviving the low pH conditions in the stomach and posterior small intestine. The highest cell numbers recorded on MRS (pH 4.6) were at 80 days (8 x 10^8 cfu g^{-1} faeces) and 40 days (6 x 10^8 cfu g^{-1}), indicating that intestinal bacteria surviving at lower pH were present after weaning. One theory could be that intestinal bacteria, naturally restores the balance after the disturbance created during weaning. The highest cell numbers overall were recorded on BHI and LB, but this media favours the growth of most intestinal bacteria and is not very selective (Hartemink *et al*. 1997). The FM, CFM, BHI and LB, were not as selective for lactic acid bacteria, but LAB could be cultivated on these media.

Isolation and grouping of lactic acid bacteria

Not all colonies obtained from various media types were identified as lactic acid bacteria, but several Gram-positive and catalase negative strains were isolated on FM, GFM and CFM media.

Low cell numbers, 3 x 10^9 and 8 x 10^9 cfu g^{-1} were isolated at day 120 on Rogosa and MRS (pH 4.6) media, respectively, and at day 21 no bacteria could be isolated on these media. This could indicate the presence of low cell numbers of lactic acid bacteria at this age. The highest cell numbers at day 21 (1.5 x 10^9 cfu g^{-1} faeces) was recorded on BHI medium. This indicated the presence of a variety of intestinal bacteria at this age. Cell counts ranging from 5 x 10^8 cfu g^{-1} to 1 x 10^9 cfu g^{-1} were recorded with crawl feed medium, growth feed medium and faeces agar. Although bacteria isolated on these media were not all identified, some colonies selected from FM, GFM and CFM, were Gram-positive, catalase negative and could be cultured in MRS broth, suggesting that they might be lactic acid bacteria. FM, GFM and CFM could be used as inexpensive media to cultivate LAB

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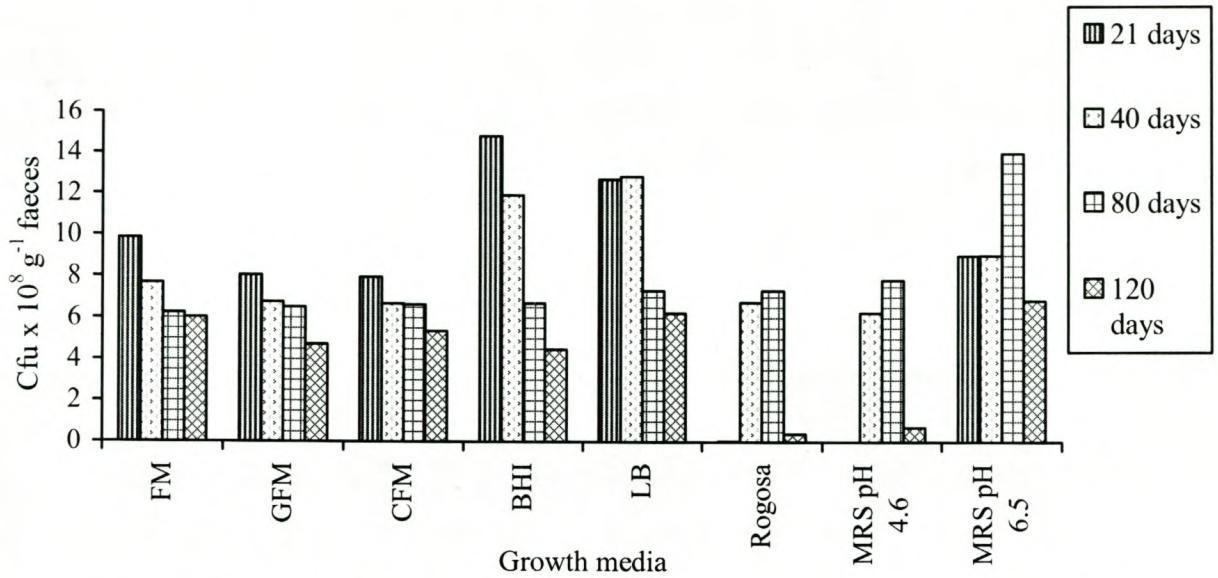
Fig.1 Bacterial counts obtained from piglets of four different ages cultured on various growth media types (FM = faeces medium, GFM = growth feed medium and CFM = crawl feed medium).

ADDENDUM PART THREE

3.1 Table 1. List of Intestinal samples collected from various parts of the GIT of piglets, after probiotic dosage with *L. plantarum* 423 and *L. salivarius* 241

Sample nr.	GIT	Treatment
1	Jejunum	Control
2	Duodenum	Control
3	Ileum	Control
4	Anterior colon	Control
5	Middle colon	Control
6	Posterior colon	Control
7	Duodenum	241[a]
8	Jejunum	241[a]
9	Ileum	241[a]
10	Anterior colon	241[a]
11	Middle colon	241[a]
12	Posterior colon	241[a]
13	Posterior colon	241[b]
14	Middle colon	241[b]
15	Anterior colon	241[b]
16	Ileum	241[b]
17	Jejunum	241[b]
18	Duodenum	241[b]
19	Duodenum	241 + 423
20	Ileum	241 + 423
21	Middle colon	241 + 423
22	Posterior colon	241 + 423
23	Duodenum	423
24	Ileum	423
25	Jejunum	423
26	Middle colon	423
27	Posterior colon	423
28	Duodenum	241 + 423
29	Jejunum	241 + 423
30	Ileum	241 + 423
31	Middle colon	241 + 423
32	Posterior colon	241 + 423
33	Duodenum	241
34	Jejunum	241
35	Ileum	241
36	Middle colon	241
37	Posterior colon	241
38	Duodenum	423
39	Jejunum	423
40	Ileum	423
41	Middle colon	423
42	Posterior colon	423

[a] Piglet was weak and ill when slaughtered
[b] Piglet was strong and healthy when slaughtered

3.2 List of digital images

Table 2. List of digital images obtained during the study described in chapter 5. Diskettes are available on request.

Sample nr.	Experiment	GIT	Treatment	Probes			
				EUB	LPLANG	LSAL	Efs
1	One	Jejunum	Control	8509-8568 [6][a]		9950-9991 [9]	
2	One	Duodenum	Control	8569-8628 [6]		8144-8189[12]	8190-8230[12]
3	One	Ileum	Control	8629-8672 [6]		0033-0072 [9]	
4	One	Anterior colon	Control	8673-8716 [6]		0073-0114 [9]	
5	One	Middle colon	Control	8730-8772 [6]		0115-0159 [9]	
6	One	Posterior colon	Control	8773-8815 [6]		0160-0200 [9]	
7	One	Duodenum	241[b]	8231-8271[12]	8272-8293[12]	0201-0241 [9]	
8	One	Jejunum	241[b]	8360-8380[12]	8339-8359[12]	0242-0282 [9]	
9	One	Ileum	241[b]	8950-8993 [6]		0338-0381 [10]	
10	One	Anterior colon	241[b]	8318-8338[12]	8994-9039[12]	0382-0423 [10]	
11	One	Middle colon	241[b]	9040-9085 [6]		0424-0463 [10]	
12	One	Posterior colon	241[b]	9086-9127 [6]		0464-0502 [10]	
13	One	Posterior colon	241[c]	9128-9169 [6]		0510-0550 [10]	
14	One	Middle colon	241[c]	9170-9211 [6]		0551-0591 [10]	
15	One	Anterior colon	241[c]	9212-9255 [6]		0592-0632 [10]	
16	One	Ileum	241[c]	8294-8317[12]	8477-8497[12]	0633-0673 [10]	
17	One	Jejunum	241[c]	9303-9344 [6]		1234-1273 [11]	
18	One	Duodenum	241[c]	8498-8518[12]	9345-9386[12]	1274-1315 [11]	
19	One	Duodenum	241 + 423	9387-9431 [6]		1316-1357 [11]	
20	One	Ileum	241 + 423	9432-9480 [6]		1358-1397 [11]	
21	One	Middle colon	241 + 423	9481-9523 [6]		1398-1439 [11]	
22	One	Posterior colon	241 + 423	9524-9564 [6]		1440-1479 [11]	
23	One	Duodenum	423	8519-8539[12]	8540-8560[12]	1492-1531 [11]	
24	One	Ileum	423	9600-9643 [6]		1532-1557 [11]	
25	One	Jejunum	423	9653-9693 [7]		1558-1599 [11]	
26	One	Middle colon	423	9694-9734 [7]		1600-1637 [11]	
27	One	Posterior colon	423	9735-9777 [7]		1638-1678 [11]	
28	Two	Duodenum	241 + 423	9778-9818 [7]		1679-1718 [11]	

29	Two	Jejunum	241 + 423	8624-8643[12]	8603-8623[12]	1719-1758 [11]	
30	Two	Ileum	241 + 423	8561-8581[12]	8582-8602[12]	1759-1798 [11]	
31	Two	Middle colon	241 + 423	9860-9990 [7]		1799-1839 [11]	
32	Two	Posterior colon	241 + 423	8644-8665[12]	8666-8686[12]	1840-1880 [11]	
33	Two	Duodenum	241	9950-9993 [8]		1881-1921 [11]	
34	Two	Jejunum	241	9994-0036 [8]		1922-1962 [11]	
35	Two	Ileum	241	0037-0081 [8]		1963-2004 [11]	
36	Two	Middle colon	241	0082-0124 [8]		2005-2046 [11]	
37	Two	Posterior colon	241	8687-8707[12]	8708-8728[12]	2047-2087 [11]	
38	Two	Duodenum	423	8771-8792[12]	8793-8812[12]	2088-2127 [11]	
39	Two	Jejunum	423	0167-0207 [8]		9012-9033[12]	8969-8990[12]
40	Two	Ileum	423	8750-8770[12]	8729-8749[12]	8991-9011[12]	8949-8968[12]
41	Two	Middle colon	423	8865-8885[12]	8886-8907[12]	9034-9055[12]	9056-9076[12]
42	Two	Posterior colon	423	8908-8927[12]	8928-8948[12]	9077-9098[12]	9099-9118[12]

[a] The number in parenthesis – diskette number
[b] Weak piglet
[c] Strong piglet