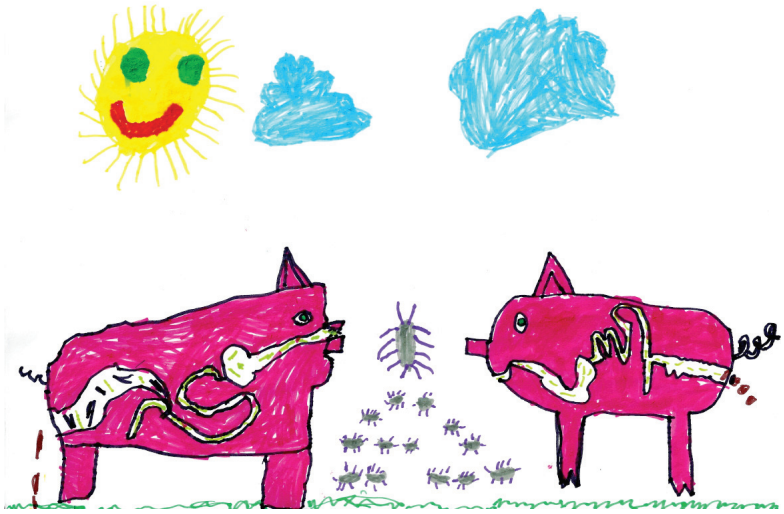
DISSERTATIONES SCHOLA DOCTORALIS SCIENTIAE CIRCUMIECTALIS,
ALIMENTARIAE, BIOLOGICAE. UNIVERSITATIS HELSINKIENSIS

7/2014

TANJA LÄHTEINEN

In Search of Health-Promoting Microbes: *In Vitro* and *In Vivo* Studies in Swine



VETERINARY MICROBIOLOGY AND EPIDEMIOLOGY
DEPARTMENT OF VETERINARY BIOSCIENCES
FACULTY OF VETERINARY MEDICINE
DOCTORAL PROGRAMME IN FOOD CHAIN AND HEALTH
UNIVERSITY OF HELSINKI

Department of Veterinary Biosciences
Faculty of Veterinary Medicine
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Finland

IN SEARCH OF HEALTH-PROMOTING MICROBES
IN VITRO AND *IN VIVO* STUDIES IN SWINE

Tanja Lähteinen

ACADEMIC DISSERTATION

To be presented, with the permission of the Faculty of Veterinary Medicine of the University of Helsinki, for public examination in Walter auditorium, EE-building, Agnes Sjöberginkatu 2, Helsinki, on November 14th 2014, at 12 noon.

Helsinki 2014

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Published in *Dissertationes Schola Doctoralis Scientiae Circumiectalis, Alimentarie, Biologicae*

ISBN 978-951-51-0336-9 (paperback)
ISSN 2342-5423 (print)

ISBN 978-951-51-0337-6 (PDF)
ISSN 2342-5431 (Online)
http://ethesis.helsinki.fi

Cover illustration: Veera & Teemu Lähteinen

Layout: Tinde Päivärinta/PSWFolders Oy

Hansaprint
Helsinki 2014

To all the bacteria
that got stressed-out,
damaged or even consumed
during the course of this work.

Contents

Abstract

Lactobacilli are important members of the commensal microbiota of both man and animals, contributing to the health and well-being of the host. Several *Lactobacillus* strains are known to possess health-promoting characteristics, warranting their recognition as probiotics, defined by WHO as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host". Dietary supplementation with lactobacilli to enhance the health and productivity of the host has generated much interest in the production animal sector, especially after the prohibition of in-feed antimicrobials. In the swine rearing industry, disturbances in gastro-intestinal (GI) health, such as diarrhea, are very common, causing significant financial losses and compromising animal welfare. The use of lactobacilli as dietary probiotics to maintain and restore a balanced intestinal microbiota during various stressful situations, like the weaning of piglets, might help to prevent the development of GI infections. In addition to their use as probiotics, lactobacilli are also considered as good candidates for antigen carriers in vaccine applications. In particular, those strains carrying a surface (S) layer are attractive vaccine vectors, as the production of the antigenic epitope in each protein subunit as part of the S-layer lattice would enable the expression of a large number of antigens on the surface of the bacterial cell. This, in turn, would be expected to enhance the immune response generated by the vaccine. While the use of lactobacilli in a variety of ways to maintain and improve the health and productivity of swine has been widely examined, there are several aspects such as the selection process of the strains used as well as the functional mechanisms behind the observed effects that still remain to be clarified. Thus, comprehensive studies regarding the use of lactobacilli as a probiotic and / or vaccine vector in various animal species are warranted.

The main aim of this work was to characterize the probiotic potential of lactobacilli for use in swine production, by using both *in vitro* and *vivo* methods. A total of 94 lactic acid bacterial (LAB) isolates, originating from porcine small intestine and feces, were first screened for selected properties considered as important for putative probiotic microbes. In general, the isolates tolerated well low pH and bile, and showed highly variable adhesion capacities towards porcine enterocytes collected from five different intestinal sections. While the LAB isolates adhered more efficiently to large intestinal enterocytes, compared to those collected from the small intestine, the isolation site of the strain had no influence on the adhesion preferences of the strains towards enterocytes of different origins. The spent culture filtrates collected from the isolates inhibited the growth of several intestinal pathogens. While this inhibition was mainly due to organic acids, some of the isolates appeared to produce also other inhibitory substances. The predominating phylotypes identified among the isolates were *Lactobacillus reuteri* and *Lactobacillus salivarius*, of which the former generally had the best adhesion capacity, whereas the latter was one of the best inhibitors of pathogen growth. However, the properties assessed showed extensive variability, even between strains of the same species.

With respect to the porcine lactobacilli evaluated in the first part of this work, six strains were selected for use in a multispecies bacterial supplementation, which was assessed in a feeding trial performed in recently weaned piglets. Additionally, a *Lactobacillus* strain possessing a surface (S) layer, namely *Lactobacillus brevis* ATCC 8287, was used in the feeding trial as a monostrain supplementation. While both supplementations did induce some alterations on the mRNA levels of selected cytokines in the intestinal mucosa, more pronounced effects were evident with the multispecies supplementation. The *L. brevis* supplementation induced a non-significant increase

in piglet body weight, but no such effect was observed for the multispecies supplementation. None of the supplemented strains could be isolated alive from feces, although the *L. brevis* strain was detected in the large intestinal digesta as well as in the mucosa of small and large intestines using techniques unable to differentiate between dead and live cells. Based on these results, it seems that the ability of these strains to survive and colonize within the porcine gut appears to be limited, although both types of supplementations exerted some immunomodulatory effects in the intestinal mucosa. While these supplementations may be suitable for use as probiotics in swine, additional studies will be needed to explore the effects of the strains on piglet health and immune status in more detail. Additionally, the suitability of the *L. brevis* strain for use as a vaccine vector will need to be further assessed.

In the final part of this work, S-layer protein-carrying *Lactobacillus amylovorus* strains of swine origin, as well as the type strain (DSM 20531^T) were characterized for certain probiotic properties. Additionally, the role of the S-layer proteins of each strain in adherence to IPEC-1 cells was addressed. While none of the *L. amylovorus* strains adhered to porcine intestinal mucus, more variability was observed in their adherence to IPEC-1 cells, with some strains demonstrating a good adhesion capacity to these cells. Interestingly, the adhesion efficiency of the strains to IPEC-1 cells did not strictly correlate with their ability to inhibit adhesion of an *Escherichia coli* strain to the same cells. Thus, apart from competition for binding sites, other mechanisms are also involved in the ability of lactobacilli to inhibit pathogen adhesion. The extent of cytokine induction by the strains in human MoDC was of varying intensity, and did not clearly deviate towards Th_1 or Th_2 phenotypes. Instead, the induced cytokine response included Th1 favoring (IL-12), Th2 favoring (IL-10) as well as proinflammatory (TNF-α, IL-6, IL-1β, IP-10/CXCL10) cytokines. In all of the strains, one major S-layer protein (named SlpA) was recognized, and these proteins were found to share a high sequence similarity with the *L. acidophilus* NCFM SlpA protein. In addition, two of the strains carried additional S-layer like proteins on their surfaces (named SlpB and SlpC). Unexpectedly, none of the major S-layer proteins was found to solely mediate adhesion of the strains to IPEC-1 cells.

List of original publications

This thesis is based on the following original publications referred to in the text by their Roman numerals (I-IV):

I Lähteinen T, Malinen E, Koort JMK, Mertaniemi-Hannus U, Hankimo T, Karikoski N, Pakkanen S, Laine H, Sillanpää H, Söderholm H and Palva A. (2010) Probiotic properties of *Lactobacillus* isolates originating from porcine intestine and feces. Anaerobe 16(3):293-300.

II Lähteinen T, Lindholm A, Rinttilä T, Junnikkala S, Kant R, Pietilä TE, Levonen K, von Ossowski I, Solano-Aguilar G, Jakava-Viljanen M and Palva A. (2014) Effect of *Lactobacillus brevis* ATCC 8287 as a feeding supplement on the performance and immune function of piglets. Vet. Immunol. Immunopathol. 158(1-2):14-25

III Lähteinen T, Rinttilä T, Koort JMK, Kant R, Levonen K, Jakava-Viljanen M, Björkroth J and Palva A. Effect of a multispecies *Lactobacillus* formulation as a feeding supplement on the performance and immune function of piglets. (Submitted)

IV Hynönen U, Kant R, Lähteinen T, Pietilä TE, Beganović J, Smidt H, Uroić K, Åvall-Jääskeläinen S, Palva A. (2014) Functional characterization of probiotic surface layer protein-carrying *Lactobacillus amylovorus* strains. BMC Microbiol. 14(1):199 doi: 10.1186/1471-2180-14-199

The original publications are reprinted with the kind permission of the publishers.
In addition, some unpublished results are presented.

Abbreviations

ADWG	Average daily weight gain
ATCC	American Type Culture Collection
AUC	Area under the growth curve
ARP	Area reduction percentage
BMDC	Bone marrow derived dendritic cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
CXCL	(C-X-C motif) ligand
CTLA	Cytotoxic T-lymphocyte antigen
CWF	Cell wall fragment
DAPI	4', 6-diaminido-2-phenylindole dilactate
DC	Dendritic cell
DC-SIGN	Dendritic cell specific C-type lectin intercellular adhesion molecule 3-grabbing non-integrin
DFI	Daily feed intake
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen
ECM	Extracellular matrix
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic *Escherichia coli*
ELISA	Enzyme-linked-immunosorbent-assay
ETEC	Enterotoxigenic *Escherichia coli*
FAO	Food and Agriculture Organization of the United Nations
FCR	Feed conversion ratio
FCS	Fetal calf serum
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
FISH	Fluorescence *in situ* hybridisation
GI	Gastrointestinal
GM-CSF	Granulocyte macrophage-colony stimulating factor
HBSS	Hanks balanced salt solution
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HRP	Horse radish peroxidase
IEC	Intestinal epithelial cell
IFA	Indirect immunofluorescence assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-12-RB2	Interleukin 12 receptor beta 2 subunit

IP	Interferon gamma-induced protein
ISR	16S-23S intergenic spacer region
kDa	Kilodalton
LAB	Lactic acid bacteria
LB	Luria Bertani medium
MIC	Minimum inhibitory concentration
MoDC	Monocyte derived dendritic cell
MOI	Multiplicity of infection
MPN	Most probable number
mRNA	Messenger ribonucleic acid
MRS	de Man Rogosa Sharpe medium
MyD-88	Myeloid differentiation primary response gene 88
NaOH	Sodium hydroxide
NF-κB	Nuclear factor κB
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
pBD	Porcine beta-defencin
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
*p*I	Isoelectric point
PPIB	Peptidyl-prolyl cis-trans isomerase B
qPCR	Quantitative polymerase chain reaction
QPS	Qualified presumption of safety
RNA	Ribonucleic acid
RPL32	Ribosomal protein L32
rpm	Revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
S-layer	Surface layer
SCF	Spent culture filtrate
Slp	Surface layer protein
Th	T helper cell
T_{reg}	Regulaory T cell
TEER	Transepithelial electric resistance
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UPGMA	Unweighted pair group method with arithmetic averages
WGS	Whole genome sequencing
WHO	World Health Organization

1 Introduction

The main aim of livestock farming is to produce safe foodstuffs for human consumption. While the importance of animal health and welfare is being increasingly appreciated in animal production, the intensive management practices of modern animal farming often subject the animals to severe stress, leading to health problems and reduced productivity. For example, diarrhea and other disturbances in gastro-intestinal (GI) health are very common in the swine rearing industry, where they are responsible for significant financial losses and compromised animal welfare (Reid and Friendship, 2002). In addition to direct health problems to the host animal, pathogenic bacteria can also be transferred along the food chain, thus endangering also human health. In an attempt to manage the problems caused by GI and other infections, sub-therapeutic levels of antibiotics have long been incorporated into animal feed (Dibner and Richards, 2005); this both effectively promotes the growth of the animals and reduces animal mortality and morbidity (Cromwell, 2002). However, the worldwide concern about the growing problem of antibiotic resistance and about the transfer of resistance genes from animal to human microbiota (Aarestrup, 2002) has led to a gradual discontinuation of growth-promoting antibiotics throughout Europe. For example, Sweden prohibited the use of antibiotic feed additives in 1986 (Wierup, 2001), Denmark in 2000 (Aarestrup et al., 2010), and from the beginning of 2006, this prohibition has been implemented throughout the European Union. After the withdrawal of these antibiotics, increases in mortality and morbidity, especially due to enteric infections, have been reported in pig farms (Callesen, 2002; Casewell et al., 2003). Additionally, the use of antibiotics as therapeutic agents has increased (Callesen, 2002; Casewell et al., 2003). Although these negative consequences on the pig production may be only transient (Aarestrup et al., 2010), it is clear that alternative ways are urgently needed to promote the health of production animals and to reduce the increasing use of therapeutic antibiotics.

Various dietary strategies have been claimed to improve gut health and disease resistance of production animals, including swine (Roselli et al., 2005; Thacker, 2013). One such approach involves the supplementation of animal feeds with beneficial microbes. The concept that harmful gut microbes could be suppressed and displaced by beneficial ones to improve the health of the host first appeared over a century ago. Since then, the concept of beneficial microbes, which are often called "probiotics", has evolved considerably, as have the definition for these microbes (Isolauri et al., 2002; Dobrogosz et al., 2010). According to the World Health Organization, probiotics are defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (WHO/FAO, 2001).

One of the health benefits associated with probiotic consumption is modification of the gastro-intestinal (GI) microbiota in such a way that there is increased resistance to colonization by pathogenic bacteria (McCracken and Gaskins, 1999; Lalles et al., 2007; Ohashi and Ushida, 2009). The commensal GI tract microbiota is a highly complex community, consisting of an immense number of different species of microbes. In humans, the gut microbiota has generally been estimated to consist of around 500-1000 different microbial species (Yatsunenko et al., 2012), and estimations of the species diversity of swine intestinal microbiota have been in the same range (Leser et al., 2002; Lamendella et al., 2011). The gut microbiota is of crucial importance to the host, for example providing protection against extrinsic microbes. This is emphasized by the fact that germfree animals, devoid of commensal microbiota, are much more susceptible to infections, especially to gastro-intestinal infections. For example, the lethal dose of *Salmonella enteritidis* in a germfree mouse is as low as ten bacteria delivered *per os*, whereas

in conventional mice 10^9 bacteria are required to evoke a lethal infection (Collins and Carter, 1978). The colonization resistance provided by the commensal intestinal microbiota is illustrated also by studies conducted with newly hatched chickens. In commercial poultry settings, the development of the commensal gut microbiota during the first weeks of life is delayed, e.g. rendering the hatchlings highly vulnerable to *Salmonella* infections (Schneitz, 2005). However, providing the gut contents of an adult bird to the chicks has been shown to be a very effective way to protect the young birds against *Salmonella*, and other intestinal infections (Nurmi and Rantala, 1973; Schneitz, 2005; Dobrogosz et al., 2010), and this phenomenon is referred to as "competitive exclusion" (CE). In other production animals, the development of the commensal microbiota after birth is not as prone to disturbances as it is in poultry, and thus the practice of CE is not as widespread elsewhere. However, probiotic products containing defined bacterial strains and aimed at increasing the disease resistance of the animals are being used also in several other species, including swine (Bernardeau et al., 2006; Gaggia et al., 2010).

Several different types of microbes, i.e. bacteria, yeasts and molds, have been evaluated as animal probiotics, but the genus *Lactobacillus* has been one of the most widely applied genera (Nousiainen et al., 2004; Gaggia et al., 2010). Lactobacilli are prominent members of the commensal intestinal microbiota of both humans and animals, and are considered to be beneficial for the host. In piglets, a reduction in the abundance of intestinal lactobacilli has been observed to occur around weaning (Konstantinov et al., 2006a; Su et al., 2008b) and this decline has been considered to predispose the piglets to GI disturbances, e.g. diarrhea. The restoration of a balanced gut microbiota after the application of probiotic microbes could help to prevent the development of GI infections around weaning. Indeed, positive health effects including reductions in diarrhea prevalence have been described in swine after consumption of lactobacilli and other probiotics, although positive effects have not been detected in all studies that have been performed (Nousiainen et al., 2004; Lalles et al., 2007; Bosi and Trevisi, 2010; Kenny et al., 2011). The functional mechanisms behind the putative health-promoting effects of probiotic lactobacilli are for the most part unclear. Consequently, research aimed at revealing the putative positive health effects of probiotic consumption as well as the molecular mechanisms leading to these outcomes is very important and can be used to guide the selection of bacterial strains used in probiotic feed additives intended not only for swine but also for other animals.

2 Literature review

2.1 The gastrointestinal tract of swine

The digestive tract of swine is classified as monogastric and shares many anatomical and physiological similarities with the human GI-tract (Miller and Ullrey, 1987; Heinritz et al., 2013). Anatomically the porcine GI-tract can be divided into four parts: the esophagus, the stomach, the small intestine (which is further divided into the duodenum, the jejunum and the ileum) as well as the large intestine (which is further divided into the cecum, the colon and the rectum; Figure 1). At the time of birth, the lengths of the porcine small and large intestine are around four and one meters, respectively, while at maturity the corresponding measures lie in the range from 18 to 23 meters and four to seven meters (McCance, 1974; Miller and Ullrey, 1987).

Similarly to humans, pigs are omnivorous colon fermenters. However, while humans lack a distinct cecum, pigs exhibit also significant cecal fermentation. This difference is illustrated by the fact that the short chain fatty acids (SCFA) produced by the large intestinal microbiota of swine may provide up to 30% of the host energy requirements for maintenance (Rerat et al., 1987), whereas in humans only about 10% of this requirement is provided by SCFA produced in the colon (Bergman, 1990). The diet of feral pigs is very diverse, consisting mainly of vegetation like fruits, grasses, forbs, corn, roots and tubers, but also of animal matter including both invertebrates and small vertebrates (Baber and Coblentz, 1987; Taylor, 1999). In commercial pig production, a highly palatable and concentrated feed is provided to maximize growth efficiency; the main ingredients in swine fodder are different grains like maize and barley and various sources of proteins such as soybean and fish meal as well as whey powder.

Like in all mammals, the GI microbiota of swine is a highly complex community consisting mainly of bacteria, but also of archae and eukaryotic microbes as well as viruses. The total number of microbial cells in the GI tract is estimated to be around 10^{14} cells, which is ten times more than the number of cells of the host organism (Luckey, 1972; Savage, 1977). The GI microbiota is crucial for the health and normal development of the host; it provides resistance to colonization by harmful microbes, influences intestinal structure and physiology and promotes the development of the immune system (reviewed by Shanahan (2002)). In general, the GI-tract microbiota of swine resembles that of humans; e.g. the predominant phyla in both of these host species are Firmicutes and Bacteroidetes (Mahowald et al., 2009; Isaacson and Kim, 2012; Heinritz et al., 2013). However, there are some interesting differences between the intestinal microbiota of swine and of humans. For example, LAB and in particular lactobacilli, are among the most abundant phylotypes in the gut microbiota of swine, while in the human intestine, lactobacilli are present at considerably lower levels (Heinritz et al., 2013).

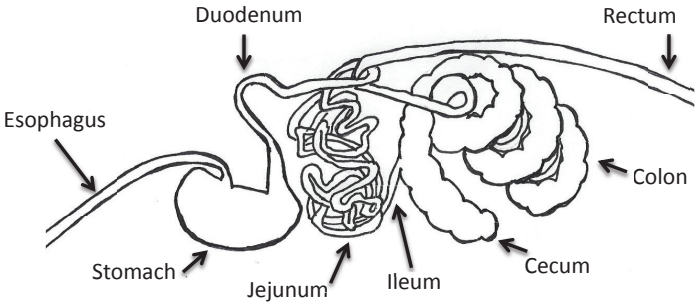


Figure 1. A schematic representation of the GI-tract of swine

2.2 The genus *Lactobacillus*

2.2.1 Overview

The genus *Lactobacillus* is an important group of LAB belonging to the phylum Firmicutes. Members of the genus *Lactobacillus* are Gram-positive rod shaped bacteria that obtain the energy they need for the maintenance and macromolecule synthesis from substrate-level phosphorylation (i.e. fermentation), forming lactic acid as the primary metabolic end product. This highly heterogeneous genus consists of over 150 species inhabiting diverse ecological niches. Lactobacilli are ubiquitous in natural environments and are present in all places where substrates rich in carbohydrates are available, i.e. in soil, sewage and plant material. Lactobacilli are also essential members of the commensal microbiota of humans and animals, e.g. they can be found in the GI tract and are present in feces of several different host species. For centuries, lactobacilli have been used as starter cultures in the production of fermented foods, particularly in dairy products, but also in vegetable and meat products. In addition to foods intended for human consumption, *Lactobacillus* fermentation can also be used to produce animal feeds, such as silage (Holzer et al., 2003; Meieregger et al., 2011). Furthermore, as lactobacilli have been associated with various health-promoting properties, they are widely used as probiotics for both humans and animals (Barrangou et al., 2012).

Two basic fermentative pathways are utilized by lactobacilli. The homofermentative pathway (Embden-Meyerhof-Parnas pathway) produces almost exclusively lactic acid as the end product, whereas in the heterofermentative pathway (phosphoketolase pathway) also CO_2 and ethanol are produced. Based on these pathways, lactobacilli can be divided into three distinct groups; (1) obligatory homofermentative species, (2) facultative heterofermentative species, which can use both of the pathways, and (3) obligatory heterofermentative species (Hammes and Hertel, 2009).

The various environmental habitats of lactobacilli are reflected in the high genomic diversity of the genus. The size of the *Lactobacillus* genome ranges usually from 1.8 to 3.3 Mbp (Canchaya et al., 2006; Kant et al., 2011a; Lukjancenko et al., 2012), the smallest reported so far being that of *Lactobacillus iners* which is only 1.3 Mbp (Macklaim et al., 2011). The G+C content of the *Lactobacillus* genomes is typically low (Barrangou et al., 2012) but also diverse, ranging from 32% to 55% (Axelsson, 2004). The taxonomy of lactobacilli is known to be complex and constantly changing; the information obtained from new studies and the recent availability of whole genome sequences (WGS) have enabled more conclusive analyses of the phylogenetic relationships of lactobacilli species (Makarova et al., 2006; Claesson et al., 2008; Kant et al., 2011a). It is evident that the phylogeny of lactobacilli species does not correlate well with their phenotypes (Felis and Dellaglio, 2007), and further revisions of the taxonomy have been proposed (Axelsson, 2004; Makarova et al., 2006).

2.2.2 Lactobacilli in the gut microbiota of swine

At birth, the sterile environment of the womb changes to a situation in which there is a constant microbial exposure, leading to colonization of the newborn animal. The establishment of the host microbiota proceeds sequentially, as a succession of microbial populations, until a relatively stable climax community is formed (Savage, 1977; Isaacson and Kim, 2012). In most suckling animals, lactobacilli are one of the first colonizers of the gut (Savage, 1977; Berg, 1996), and this has been confirmed in swine as well (Smith, 1965; Fuller et al., 1978; Ducluzeau, 1983; Konstantinov et al., 2006a). The colonizing bacteria, including lactobacilli, originate from the rearing environment, like maternal feces (Tannock et al., 1990; Nousiainen et al., 2004). The

microbial succession of different intestinal compartments and feces of piglets has been examined in several studies (Kenworthy and Crabb, 1963; Tannock et al., 1990; Swords et al., 1993; Naito et al., 1995; Melin et al., 1997; Konstantinov et al., 2004b; Konstantinov et al., 2006a), although the experiments have been generally conducted using culture-based methods, which are unable to detect the majority of gut microbes (Vaughan et al., 2000). Substantial individuality in the colonization process of the piglet intestine has been described during the first two weeks of life, this being reflected by differences in the gut microbiota of littermates and penmates (Thompson et al., 2008). In addition, the developing microbiota of piglets is influenced also by management practices (Davis, 2012).

During the suckling period, the numbers of lactobacilli in the ileum of piglets seem to remain rather constant (Konstantinov et al., 2006a), but marked changes in both the abundance and community structure of lactobacilli occur at weaning. Weaning, which occurs at approximately three to four weeks of age in commercial swine production units, exposes the piglets to severe social, environmental and nutritional stresses. The weaning transition causes a significant decrease in the overall GI tract *Lactobacillus* population (Franklin et al., 2002; Konstantinov et al., 2006a; Pieper et al., 2006; Su et al., 2008a), and changes in the diversity and structure of lactobacilli community have also been described (Bateup et al., 1998; Janczyk et al., 2007; Su et al., 2008a). However, at least some of these changes are transient (Janczyk et al., 2007).

Investigations of the GI tract microbial composition of post-weaning swine conducted with high-throughput molecular techniques have revealed lactobacilli as being among of the most abundant phylotypes recognized in the intestine (Pryde et al., 1999; Leser et al., 2002; Hill et al., 2005) and feces (Kim et al., 2011). The cell count of lactobacilli in the porcine intestine, based on bacterial culture and fluorescent *in situ* hybridization (FISH), is at the level of 10^8 CFU per gram of digesta (Konstantinov et al., 2004a; Castillo et al., 2006). The predominant *Lactobacillus* species in the swine intestine include e.g. *L. amylovorus*, *L. reuteri*, *L. johnsonii*, *L. mucosae* and *L. salivarius* (Leser et al., 2002; Hill et al., 2005; Konstantinov et al., 2006a; Mann et al., 2014). Although the GI tract microbiota of adult swine is relatively stable over time, dietary and environmental factors can induce changes in the composition of the microbiota, including the lactobacilli community (Fuller, 1989; Bauer et al., 2006; Rist et al., 2013).

2.2.3 Lactobacilli as animal probiotics

The lactobacilli in the GI tract have long been considered as being beneficial for the host (Tannock, 1990), and several species of lactobacilli are known to possess properties considered important for probiotic microbes (Reid, 1999). Consequently, the oral supplementation of these bacteria is hypothesized to improve the gut health of the host (Fuller, 1989; Meieregger et al., 2011), and the use of lactobacilli as probiotics for both man and animals has been extensively studied in the last decades (Simon et al., 2001; Nousiainen et al., 2004; Bernardeau et al., 2006; de Vrese and Schrezenmeir, 2008; Turpin et al., 2010). Although the use of microbial feed supplements for farm animals was explored as early as 1925, this practice was not exploited commercially until the 1960s and 1970s, coinciding with increased concerns over the widespread and uncontrolled use of antibiotic growth promoters (Fuller, 1999; Reid and Friendship, 2002; Meieregger et al., 2011). In swine, *L. acidophilus* was one of the first microbes reported to stimulate the growth when supplemented into the feed (King, 1968). Currently, around 20 different species of microbes are authorized in the EU as feed additives for animal nutrition, including several *Lactobacillus* species, such as *L. brevis*, *L. plantarum*, *L. rhamnosus* and *L. salivarius* (EU, 2014).

Several health effects have been proposed for beneficial microbes, but as the goals of probiotic consumption depend on the target host species, some of these are not relevant in the veterinary field, or at least not in production animals (Table 1). On the other hand, some effects desirable in production animals may be irrelevant or even unwanted in humans. These include e.g. increased body weight gain and improvements in feed efficiency (Simon et al., 2001; Bernardeau and Vernoux, 2013). In addition to beneficial effects for the host animal itself, one further aim of using probiotic microbes in production animals would be to reduce the carriage of microbes which are not harmful to the hosts themselves, but can lead to human infection if they pass in to the food chain (Reid and Friendship, 2002; Doyle and Erickson, 2012).

The mechanisms behind the health enhancing effects of probiotics are largely unknown, but several modes of action have been proposed (reviewed by Fooks and Gibson (2002); Vanderpool et al. (2008); Ohashi and Ushida (2009) and Kenny et al. (2011)). These include e.g. 1) effects on intestinal microbiota, such as stimulation of indigenous lactobacilli or other beneficial bacteria, 2) inhibition of harmful microbes via competition for nutrients or receptors for adhesion, or via production of antagonistic substances, 3) stimulation and / or modulation of the host immune function, 4) regulation of enterocyte functions, like the maintenance of the epithelial barrier 5) stimulation and / or modulation of intestinal nutrition physiology, like absorption and secretion activity, or enhancement of short-chained fatty acid (SCFA) production.

Table 1. Proposed health effects of probiotics and their relevance in the veterinary field, as estimated by the author.

Proposed effect (adapted from de Vrese and Schrezenmeir (2008) and Chassard et al. (2011)	**Relevance in veterinary medicine**
Very well-established effects with valid scientific proof	
Prevention and alleviation of certain types of diarrhea (e.g. rotavirus, antibiotic-associated, traveler's)	High
Alleviation of lactose intolerance	Low
Well-established effects / effects observed in certain target groups	
Modulation of the microbiota (usually intestinal)	High
Immunomodulation / - regulation	High
Prevention of respiratory tract infections	High
Beneficial effects in inflammatory diseases of the GI tract (e.g. inflammatory bowel disease, bacterial overgrowth)	Intermediate (mainly comp. animals[1])
Prevention and alleviation of allergies / atopic diseases in infants	Intermediate (mainly comp. animals)
Treatment of urogenital infections	Intermediate (mainly comp. animals)
Effects not well-established with insufficient scientific proof	
Normalization of passing stool and stool consistency (e.g. constipation)	Intermediate (mainly comp. animals)
Alleviation of autoimmune diseases	Intermediate (mainly comp. animals)
Prevention of cancer	Intermediate (mainly comp. animals)
Prevention of ischemic heart disease	Low
Reducing of blood cholesterol	Low
Caries prevention	Intermediate (mainly comp. animals)

[1] companion animals

2.2.4 Lactobacilli as vaccine vectors

In addition to the use of lactobacilli as probiotics, interest in their use as delivery vectors for vaccine antigens has increased during the last decades (Bermudez-Humaran et al., 2011; Wells, 2011a). Many of the properties considered important for probiotics (see section 2.2.1.) also apply to the use of bacteria as potential vaccine delivery vectors, thus several *Lactobacillus* species are good candidates for both applications (Mercenier, 1999).

One important advantage of using live bacterial vaccine vectors would be the ability to administer these vaccines mucosally, e.g. *per os*. Mucosal administration is expected to generate improved and more appropriate local immune response than the traditional parenteral route, giving better protection against infectious agents that mostly enter the body mucosally (Mercenier, 1999; Fujkuyama et al., 2012). In addition, mucosal administration is more convenient and cheaper than the parenteral route (Mercenier, 1999; Holmgren et al., 2003). Traditionally, certain pathogenic bacteria, e.g. *Salmonella* and *Listeria*, have been extensively investigated for use as vaccine vectors (Detmer and Glenting, 2006; Mohamadzadeh et al., 2008), but the comparatively lower intrinsic immunogenicity of lactobacilli compared to attenuated pathogens is considered favorable, since it may result in fewer side-effects in the vaccinated host (Pouwels et al., 1998; Mercenier, 1999; Wells and Mercenier, 2008). At the same time, however, lactobacilli are able to stimulate and / or modulate host immune responses, as demonstrated by several studies performed in both humans and animals (reviewed by Corthesy et al. (2007) and Wells (2011b)). This adjuvant-like property has been demonstrated during infections, e.g. as increased pathogen-specific Ig-titers (Link-Amster et al., 1994; Kaila et al., 1995; Vlasova et al., 2013), and also as enhancement or modulation of the immune response stimulated by vaccination (Licciardi and Tang, 2011; Maidens et al., 2013). However, studies performed on pigs are extremely scarce; one study examining the effect of *L. rhamnosus* supplementation on porcine reproductive and respiratory syndrome (PRRS) vaccination could not detect any impact on the resulting immune response (Kritas and Morrison, 2007).

The *Lactobacillus* species mostly explored as vaccine vectors include *L. plantarum*, *L. casei*, *L. helveticus* and *L. acidophilus* (Wells, 2011a). Various antigens have been successfully expressed in lactobacilli (Mohamadzadeh et al., 2008; Wells, 2011a), including also those originating from pathogens infecting swine, e.g. transmissible gastroenteritis virus (Ho et al., 2005), classical swine fever virus and porcine parvovirus (Xu et al., 2011). In addition, lactobacilli have been investigated as carriers for DNA vaccines (Li et al., 2007). Most of the immunization studies performed with recombinant lactobacilli have been conducted in mouse models (Mohamadzadeh et al., 2008; Wells, 2011a), but promising results have been obtained also in swine (Xu et al., 2011).

2.2.5 Surface layer proteins of lactobacilli

Surface (S) layers are cell envelope structures found on the outermost surface of many bacteria and most archaea, completely covering the cells (Sleytr et al., 2014). In addition, several *Lactobacillus* species including e.g. *L. amylovorus*, *L. crispatus, L. acidophilus*, *L. brevis* and *L. helveticus* are known to possess an S-layer (Hynönen and Palva, 2013). These crystalline bidimensional arrays are composed of identical protein or glycoprotein subunits assembled to form a regular porous structure, which can be aligned in square, oblique or hexagonal symmetry, although only the last two of these possible arrangements have so far been observed in S-layers of lactobacilli (Åvall-Jääskelainen and Palva, 2005). The S-layer proteins of lactobacilli are among the smallest known, with molecular weights ranging from 25 to 71 kDa (Hynönen and Palva, 2013), while in other

bacterial species the size of these proteins is much larger, up to 200 kDa (Sara and Sleytr, 2000). The thickness of the S-layer is generally 5-20 nm, and the size of the pores, which occupy 30% to 70% of the S-layer surface, is in the range of approximately 2-8 nm (Sleytr et al., 2014). The sequence similarities between S-layer proteins of different bacterial species are generally low (Sleytr et al., 2014), and this is the case also in lactobacilli; homology is found only between genes of related species (Hynönen and Palva, 2013). Despite this, some common features are present in the amino acid composition of S-layer proteins, such as their high content of hydrophobic amino acids as well as the low amount of sulfur containing amino acids (Sleytr et al., 2014). A characteristic which is specific to lactobacillar S-layers is the higher abundance of positively charged amino acid residues compared to that of negatively charged residues, leading to high theoretical isoelectric point (p*I*) values (i.e. 9.35-10.4) (Åvall-Jääskelainen and Palva, 2005).

The S-layer subunit proteins attach to each other and to the underlying cell wall structures by non-covalent interactions. It is possible to achieve complete detachment of the S-layer and disintegration into the monomer subunits e.g. with high concentrations of chaotropic agents disrupting the interactions of non-covalent forces (Sleytr et al., 2014). Isolated S-layer protein subunits have a high intrinsic propensity to recrystallize into regular lattices e.g. on solid supports or even in suspension after the removal of the disrupting agent, thus they are very poorly water-soluble. Two distinct structural regions have been identified in S-layer proteins; the first being involved in the binding of the S-layer to the cell wall, and the other being responsible for the S-layer assembly (Sleytr et al., 2014). The locations of these regions in the S-layer protein vary between different species of lactobacilli (Hynönen and Palva, 2013).

Although no common biological role for S-layers has been identified, various functions have been postulated, including protection from environmental factors, cell shape maintenance, binding or sieving of large molecules and modulation of the host immune responses (Hynönen and Palva, 2013; Sleytr et al., 2014). In addition, mediation of adhesion to host structures has commonly been proposed as a function of lactobacillar S-layers. Attempts to produce completely S-layer negative *Lactobacillus* mutants have been unsuccessful (Boot et al., 1996a; Martinez et al., 2000; Palva A. unpublished results), emphasizing the necessity of this structure for its host cell. For this reason, protein level methods (e.g. labelled subunits or recombinantly expressed proteins) have to be used when examining the role of S-layers in bacterial adhesion. In some *Lactobacillus* species, S-layers have been shown to mediate the adhesion to different targets, like extracellular matrix (ECM) proteins and epithelial cells (Mobili et al., 2010; Hynönen and Palva, 2013).

The structural properties of the S-layer, as well as the self-assembly tendency of the subunits mean that these structures are attractive candidates for a wide range of applications, including vaccine carriers. The ability to produce the antigenic epitope in each S-layer subunit as part of the overall lattice structure would enable bacteria with such chimeric S-layers to display a large number (e.g., ~5×10^5/bacterium) of antigenic molecules on their cell surface (Sleytr et al., 2007). Small model peptides have already been successfully expressed in each monomeric subunit of the S-layer of *L. brevis* ATCC 8287 (Åvall-Jääskeläinen et al., 2002) and *L. acidophilus* ATCC 4356 (Smit et al., 2002). In the future, increasing knowledge about the structure and biology of *Lactobacillus* S-layers will help in the utilization of these structures in the development of efficient vaccines for veterinary use.

2.3 Discovering probiotic microbes

2.3.1 How to choose the best strains?

The vast species and strain diversity of lactobacilli and other potentially beneficial microbes ensures that there is no shortage of candidate probiotics. However, as it is impossible to test large numbers of different strains in *in vivo* feeding trials, some kind of preliminary selection is necessary. The commonly stated selection criteria for probiotic microbes involve several features related to safety aspects as well as functional and technological properties (Table 2).

The first step in the development of a probiotic product is the isolation of the candidate strain(s). The origin of the strain is an important factor to be considered, e.g. for human probiotics, strains of human origin are preferred (Collins and Gibson, 1999; Saarela et al., 2000; Ouwehand et al., 2011). It has been claimed that at least some of the functional properties of probiotics, such as adhesion to the intestinal epithelium, are host species specific, indicating that strains isolated from the intended target species would show better performance as compared to those isolated from other species (Bengmark, 1998; Saarela et al., 2000). However, the results of *in vitro* studies assessing the host specificity of lactobacilli adherence have been conflicting, with both supporting findings (Fuller, 1973; Barrow et al., 1980; Mäyrä-Mäkinen et al., 1983; Nemcova et al., 1997), as well as negative reports (Rinkinen et al., 2000; Nikoskelainen et al., 2001; Rinkinen et al., 2003). Nevertheless, since host species specific diversification of lactobacilli strains has been observed (Oh et al., 2010; Frese et al., 2011; Guinane et al., 2011), it appears prudent to prefer strains originally isolated from the target species.

Safety is obviously one of the most important requirements for a possible probiotic (Saarela et al., 2000; von Wright, 2005; Chassard et al., 2011). While lactobacilli have a long history of safe use in food products, they are living micro-organisms, and theoretically could be capable of evoking unwanted side-effects, at least in susceptible individuals. As reviewed by Bernardeau et al. (2006) and Sanders et al. (2010), cases of lactobacillar infections, e.g. bacteremia, peritonitis and pneumonia, have been reported in humans, but considering the huge quantities of probiotic products consumed, these infections seem to be extremely rare occurring mainly in immunocompromised patients. On the other hand, as far as the author is aware, there are no reports of animal infections caused by lactobacilli, even though *Weissella confusa* (basonym *Lactobacillus confusus*) has been isolated from an otitis sample obtained from a dog (Björkroth et al., 2002) as well as from a case of systemic infection in a mona monkey (*Cercopithecus mona*) (Vela et al., 2003). With respect to the assessment of safety, an unequivocal taxonomic identification of the strain is an important prerequisite (WHO/FAO, 2002; Vankerckhoven et al., 2008), as a number of bacterial species, including several lactobacilli, have received a qualified presumption of safety (QPS) status in the European Union (EFSA, 2007). Micro-organisms to be used as feed additives are authorized according to the European Parliament and Council Regulation (EC) No 1831/2003, and this protocol requires thorough safety assessment, including toxicity studies performed using the target species (von Wright, 2005; Anadón et al., 2006; Meieregger et al., 2011). However, a full safety assessment is required only for strains without a QPS status (Meieregger et al., 2011; Salminen and von Wright, 2012).

Although non-viable microbes have demonstrated some health enhancing effects (Ouwehand and Salminen, 1998), it is generally assumed that viability is important for the functional properties of probiotic microbes (Chassard et al., 2011; Ouwehand et al., 2011). Consequently, the candidate strain has to be able to survive industrial manufacturing conditions,

as well as to maintain high viable cell numbers in the final product during storage (Saarela et al., 2000; Meieregger et al., 2011). This requirement makes the technological properties of the strain (Table 2) extremely important in the selection process, and it has been claimed that the current probiotics have been chosen mostly based on these characteristics (Lacroix and Yildirim, 2007). For the most part, probiotics of intestinal origin are highly sensitive to many environmental stresses, such as the extremes of temperature, oxygen stress and the mechanical shearing forces encountered during industrial processing (Lacroix and Yildirim, 2007; Meieregger et al., 2011), These are factors which pose significant challenges to the process optimization. Moreover, in addition to affecting the cell yield and viability of the strain, manufacturing procedures and also the food matrix into which the bacteria are to be incorporated may have an influence on the functional properties of the strain (Pessi et al., 1998; Deepika et al., 2009; Grzeskowiak et al., 2011; Deepika et al., 2012), further emphasizing the importance of the optimization of production process.

It is important to recognize that the characteristics of a bacterial strain are specific to that particular strain, meaning that even strains belonging to the same species can have divergent properties (Pineiro and Stanton, 2007; Marteau, 2011). Thus, properties known to exist in one strain cannot be extrapolated to all strains of the same species. Consequently, appropriate *in vitro* and *in vivo* experiments need to be performed for each candidate strain to guide the selection process, and the final validation of the health benefits can only be obtained in carefully controlled clinical trials (Chassard et al., 2011).

Table 2. Commonly used criteria for selection of probiotic microbes. Adapted from Saarela et al., (2000); Chassard et al., (2011); Meieregger et al., (2011)

Safety aspects	Functional properties	Technological properties
Origin (preferably target species)	GI tract survival (acid, bile)	Easily propagated
Identification	Adhesion	Maintains high viability
QPS status	Pathogen inhibition	Sustains production processes
Antibiotic resistance	Immuno-stimulation / -modulation	Stable during storage
Virulence factors	Metabolic activities	Favorable / no adverse effects on product quality
Toxicity (e.g. acute and chronic)	Anti-carcinogenesis / -mutagenesis	

2.3.2 *In vitro studies*

2.3.2.1 *Tolerance of low pH and bile*

Probiotics are usually consumed orally, and their expected biological functions occur in the intestine. After ingestion, the probiotic encounters the acidic environment of the stomach; the gastric pH of a suckling piglet can reach values below three, and even lower values, less than two, have been recorded after weaning (Moughan et al., 1991; Snoeck et al., 2004). After transit through the stomach, the acid stress is followed by exposure to bile in the duodenum. Apart for their digestive functions, both of these factors are also antimicrobial defense mechanisms of the GI-tract, causing stress to transiting microbes (Dunne et al., 2001; Upadrasta et al., 2011). Exposure to an acidic environment, leading to a reduction in bacterial cytoplasmic pH, can decrease the activity of pH sensitive enzymes and damage the cell membrane and

macromolecules, such as DNA and proteins (van de Guchte et al., 2002; Cotter and Hill, 2003). The antimicrobial effect of bile on the other hand is mainly due to the disruption on cell membrane integrity, but it may also influence macromolecular stability (Begley et al., 2005).

The acid and bile tolerance of lactobacilli isolated from various sources, including swine, have been extensively assessed (e.g. De Angelis et al. (2006); Yun et al. (2009); Guo et al. (2010); Zhang et al. (2013)), and while lactobacilli of intestinal origin usually show high tolerance of low pH and bile (Morelli, 2000), extensive strain-to-strain variability is evident. The *in vitro* experiments used in these tests have been based on exposing the strains to low pH or to bile for a limited time period, after which the bacterial population surviving the stress has been estimated, e.g. by plate count or turbidity measurements. Additionally, methods based on growth curve parameters have also been used (Morelli, 2000). Typically, the lowest pH values used in the experiments have been pH 2, and the bile concentration has ranged from 0.1% to 5%, but most often below 0.5%. Interestingly, differences according to the origin of the bile have been reported, as lactobacilli and bifidobacteria were inhibited more by porcine bile than by bovine bile (Dunne et al., 2001). Nonetheless, the ability of these kinds of static experiments, using constant pH and / or bile concentration, to accurately predict the *in vivo* GI-tract survivability of the strains has been criticized (Morelli, 2000), as several factors related to the food matrix, ingestion and digestion have been observed to affect strain survival (Conway et al., 1987; Charteris et al., 1998; Upadrasta et al., 2011). Thus, dynamic models possibly capable of simulating the GI-tract conditions more precisely have been developed (Marteau et al., 1997; Mainville et al., 2005; Ceuppens et al., 2012; Van den Abbeele et al., 2012).

2.3.2.2 Adhesion

The intestinal motility causes a constant flow of the gut luminal contents, flushing the microbiota towards the distal intestine, and ultimately out of the host. In order to resist this flow and to maintain their population density at constant levels, bacteria need to either multiply rapidly, and / or to adhere to intestinal surfaces (Fuller, 1999; Morelli, 2000). Thus, adhesiveness is often considered to be an important characteristic for a candidate probiotic, as adhesive strains are generally assumed to have better abilities to at least temporarily colonize the intestine of the host, and to exist in an intimate contact with host cells, leading to more efficient functionality (Blum et al., 1999; Gueimonde and Salminen, 2006; Chassard et al., 2011).

Several *in vitro* methods have been used for the assessment of the adherence of lactobacilli to intestinal structures (Blum et al., 1999; Morelli, 2000; Gueimonde and Salminen, 2006; Velez et al., 2007; Van Tassell and Miller, 2011). The main difference between these methods is the component of the intestinal mucosa being used as the substratum of adhesion. A schematic representation of the intestinal epithelium is shown in Figure 2. The intestinal epithelial cells, i.e. enterocytes, are covered by a mucus layer, a continuous viscous gel matrix consisting mainly of complex glycoproteins called mucins (Van Tassell and Miller, 2011). This layer protects the underlying cells, creating a physical barrier to bacteria-host interactions. Thus, mucus adhesion might be the first step in bacterial colonization and interaction with the host. The ability of lactobacilli to adhere to porcine mucus has been assessed in several studies, and both commercially available mucins as well as mucus isolated from freshly collected intestines have been used (Li et al., 2008; Macias-Rodriguez et al., 2009; Iniguez-Palomares et al., 2011; Carasi et al., 2014).

The most widely applied method for the assessment of bacterial adhesion to intestinal epithelial cells is to use tissue culture cells as the target of adhesion (Lahtinen and Ouwehand, 2009). The human derived carcinoma cell lines Caco-2 and HT29 (Rousset, 1986) have commonly been used in adhesion assays (Ouwehand and Salminen, 2003), including also those performed with lactobacilli originating from swine (Kim et al., 2007; Li et al., 2008; Zhang et al., 2013). However, the porcine derived cell lines IPEC-1 (Gonzalez-Vallina et al., 1996) and IPEC-J2 (Rhoads et al., 1994) have been reported to support the adhesion of swine pathogenic *Escherichia coli* strains better than the human derived cell line INT-407 (Koh et al., 2008). Therefore, these swine specific cell lines might also be more appropriate for the adhesion assays performed with porcine lactobacilli. While it is known that these tissue culture cell models do show morphological and functional differentiation and possess the characteristics of mature enterocytes (Chantret et al., 1988; Gonzalez-Vallina et al., 1996; Diesing et al., 2011), it is still somewhat unclear how well they represent intact intestinal epithelial cells. Freshly harvested intestinal tissue pieces and cells have consequently been postulated to represent a closer model of the intestinal epithelium (Morelli, 2000; Ouwehand et al., 2002). Both intestinal pieces as well as isolated enterocytes have been used to examine the adhesion of lactobacilli isolated from swine (Barrow et al., 1980; Mäyrä-Mäkinen et al., 1983; Lin et al., 2007; Guo et al., 2010). However, even this approach has its disadvantages, including practical difficulties such as availability and preservation of specimens. In addition, variability between animals from which the tissue samples are collected is likely to exist, and this would likely cause variations in bacterial adhesion as well. Furthermore, when using enterocytes collected from the intestine, the separation of cells from each other exposes the basolatelar sides of the cells to bacteria, which is not a relevant target of adhesion in the intact intestine (Ouwehand and Salminen, 2003). The use of whole intestinal tissue pieces preserves the intestinal wall architecture and may include also the covering mucus in addition to the enterocytes, providing perhaps a more natural model of the intestinal wall.

In addition to the intestinal cells and mucus, also ECM components, including type IV collagen, laminin and fibronectin, have been used as the target of adhesion studies performed with lactobacilli (Styriak et al., 2003; Jakava-Viljanen and Palva, 2007).

The results of a large number of studies assessing the adhesion of lactobacilli to intestinal structures highlight their highly variable and strain specific adhesion pattern, swine lactobacilli being no exception (Barrow et al., 1980; Mäyrä-Mäkinen et al., 1983; Kim et al., 2007; Lin et al., 2007; Guo et al., 2010; Zhang et al., 2013). However, the lack of standardization in the assay protocols complicates comparison of the results obtained in the different studies. Several factors have been observed to affect the results of adhesion studies; these include e.g. the buffer used, the pH, the presence of spent culture supernatant, the incubation time as well as the growth phase and concentration of the bacteria (Blum et al., 1999; Ouwehand and Salminen, 2003). Thus there is an obvious need for standardized methods to evaluate lactobacillar adhesion to intestinal structures. However, as at present it is impossible to say which method most accurately models the *in vivo* intestine, it is probably sensible to use more than one method when assessing the adhesion ability of lactobacilli and other bacteria.

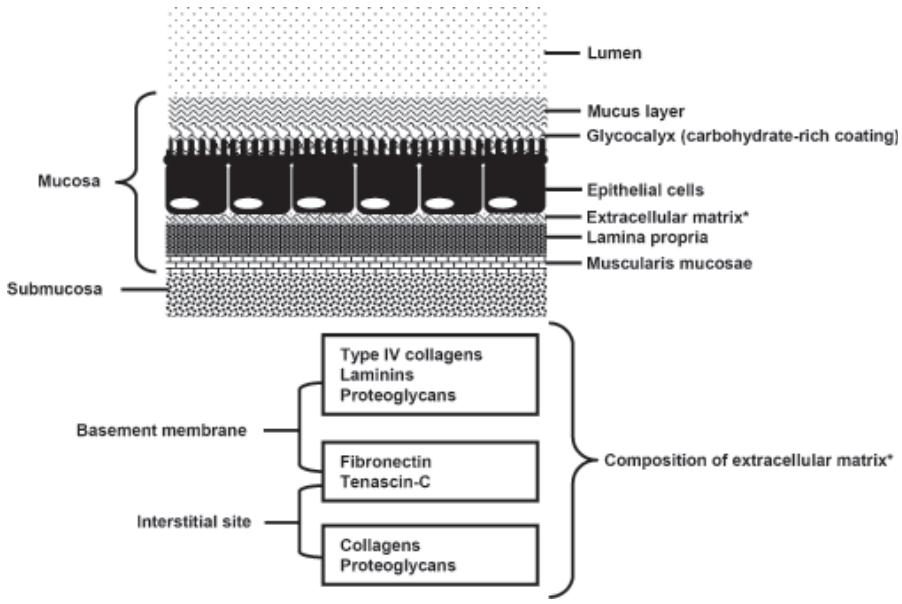


Figure 2. Schematic representation of the components of the intestinal mucosa and submucosa. Components of the extracellular matrix are indicated with an asterisk. The Figure is reproduced from Velez et al. (2007) with permission granted by John Wiley and Sons.

2.3.2.3 Pathogen inhibition

The ability to inhibit pathogenic microbes is considered to be highly desirable property for probiotics, as this characteristic is anticipated to confer better gut health for the host (Vandenbergh, 1993; Servin and Coconnier, 2003; De Vuyst and Leroy, 2007). Generally, the *in vitro* studies have assessed the ability of probiotic microbes to inhibit two different aspects of pathogen function: their adhesion to intestinal structures, and their growth (Fernandez et al., 2003; Servin and Coconnier, 2003).

As adhesion to the intestinal epithelium is the first step in the pathogenesis of many GI-tract infections (Gyles and Prescott, 2010), inhibition of pathogen adhesion by probiotic microbes could be assumed to reduce the occurrence of these diseases (Holzapfel et al., 1998). While the mechanisms by which probiotics, including lactobacilli, inhibit the adhesion of pathogens have been poorly characterized, several possible modes of action have been proposed, e.g. competition for adhesion sites and steric hindrance, as well as stimulation of mucin production by intestinal cells (Servin and Coconnier, 2003; Servin, 2004). The *in vitro* methods applied to assess inhibition of pathogen adhesion by probiotics have used the same substrata for adhesion as the regular adhesion assays, i.e. swine intestinal mucus (Jin et al., 2000; Collado et al., 2007), tissue culture cells (Bogovic Matijasic et al., 2006; Zhang et al., 2013) including IPEC-1 (Roselli et al., 2007), isolated enterocytes (Spencer and Chesson, 1994), as well as resected *ex vivo* jejunal tissue (Bogovic Matijasic et al., 2006). Three experimental set-ups have commonly been used: i) competition (simultaneous addition of the probiotic and the pathogen), ii) exclusion (addition of the probiotic prior to the pathogen) and iii) displacement (addition of the pathogen prior to the probiotic). Similarly to adhesiveness, inhibition of pathogen adhesion by lactobacilli displays extensive variability; this is a species and / or strain dependent property, being furthermore

affected by the assay methodology (Spencer and Chesson, 1994; Collado et al., 2007; Roselli et al., 2007; Zhang et al., 2013).

The ability of lactobacilli to inhibit the growth of other microbes is well-established, and this property has for a long time been utilized in food preservation. Inhibition of pathogen growth would be highly desirable also for lactobacilli when they are used as probiotics (Saarela et al., 2000). The microbial growth inhibition caused by lactobacilli is primarily due to the production of organic acids, mainly lactic acid, and concomitant lowering of the pH (Nes et al., 2012). The inhibitory action of lactic acid is likely caused by the undissociated form of this acid, which is favored at low pH, and which can cross the cell membrane entering the cell cytoplasm. Subsequently, as the pH inside the cell is higher than outside the cell, the molecule will dissociate, disrupting intracellular pH homeostasis and affecting metabolic processes of the cell (Brul and Coote, 1999; Nes et al., 2012). Lactic acid has also been shown to permeabilize the outer membrane of Gram-negative bacteria, and sensitizing them to other antimicrobial compounds, like lysozyme (Alakomi et al., 2000). In addition to weak organic acids (such as lactic acid), also other metabolic products produced by lactobacilli, such as free fatty acids, ammonia, ethanol, diacetyl and hydrogen peroxide, may contribute to the growth inhibition, although to a lesser extent (Vandenbergh, 1993; Nes and Johnsborg, 2004). Furthermore, certain strains of lactobacilli produce specific antimicrobial compounds, including bacteriocins and reuterin (Nes et al., 2012). While reuterin has a broad antimicrobial spectrum, acting against Gram-positive and Gram-negative bacteria, fungi, protozoa and even viruses (Nes et al., 2012), bacteriocins are primarily targeted against closely related bacteria, although a broader target specificity including also pathogenic bacteria has been recognized for some members of this group of antimicrobials (Jack et al., 1995; Servin, 2004; Nes et al., 2012).

The *in vitro* screening of antimicrobial properties has usually been based on assessing the growth inhibition of selected bacteria, or so called "indicator organisms", in solid or in liquid medium (Cabo et al., 1999; Papagianni et al., 2006). The agar diffusion assay, resembling the disc diffusion assay for antibiotic susceptibility, has been a very commonly applied method for investigating the antimicrobial properties of lactobacilli (De Mitchell and Kenworthy, 1976; du Toit et al., 2000; Klose et al., 2010a; Klose et al., 2010b). In this method, the growth inhibition of the indicator organism is quantified by measuring the growth-free zone around the lactobacilli spotted on the agar, or in wells containing the spent culture filtrate (SCF) (Davidson and Parish, 1989). Several factors, including the diffusion rate of the antimicrobial compounds and the composition of the agar are known to affect the results of agar based methods (Davidson and Parish, 1989; Piddock, 1990). In addition, measurement of the inhibition zones can be difficult and subjective. Unexpectedly, when the same *Lactobacillus* strains were tested with both the agar spot and the well diffusion methods, the results obtained did not correlate well with each other (Hernández et al., 2005). Consequently, liquid-medium methods, which eliminate the diffusion related problems, have been proposed to be more accurate and reliable (Cabo et al., 1999). In these assays, the indicator organism is grown in liquid media supplemented with lactobacillar SCF (Kim et al., 2007; Bernardeau et al., 2009; Guo et al., 2010), or alternatively co-cultured in the same test tube with the *Lactobacillus* strain (Drago et al., 1997; Annuk et al., 2003; Fernandez et al., 2003). Growth inhibition of the indicator organism can be quantified in several ways, including plate count (Drago et al., 1997; Moslehi-Jenabian et al., 2011) and bioluminescence (Vesterlund et al., 2004), but measurement of optical density is a simple and widely applied method (Davidson and Parish, 1989; Turcotte et al., 2004). Turbidometry has often been used as an end-point method, when the quantification of the growth inhibition is performed after a

lengthy incubation period (e.g. overnight), at the stationary growth phase of the indicator (Daba et al., 1991; Parente et al., 1995; Cabo et al., 1999; Lee et al., 2003). However, kinetic recording of the indicator growth over the whole incubation period gathers more information on the effects caused by the inhibitory compounds (Davidson and Parish, 1989; Skyttä and Mattila-Sandholm, 1991), and several growth curve parameters can be used to quantify the growth inhibition of the indicator organism (Adams and Hall, 1988; Mattila and Sandholm, 1989; Skyttä and Mattila-Sandholm, 1991). While the methods involving liquid media have some advantages over agar based methods, their results may also be affected by several factors, such as the inoculum size of the indicator and incubation parameters (Davidson and Parish, 1989; Piddock, 1990).

The ability of porcine lactobacilli to inhibit the growth of various swine pathogens, including different strains of *E. coli*, *Salmonella*, *Listeria*, *Staphylococcus*, *Brachyspira* and *Clostridium* have been observed in several studies (du Toit et al., 2000; Chang et al., 2001; De Angelis et al., 2006; Kim et al., 2007; Lin et al., 2007; Guo et al., 2010; Klose et al., 2010a; Klose et al., 2010b). However, as in the case of adhesion assays, the results from different studies are difficult to compare due to methodological differences. Furthermore, as pathogen inhibition in the complex gut environment is likely to be affected by the whole intestinal microbiota, fermentation models simulating the GI-tract conditions, including possible changes in the microbiota composition after the addition of lactobacilli, have been developed (Chassard et al., 2011).

2.3.2.4 Immunological effects

The co-evolution of the commensal microbiota with its host has led to a finely tuned crosstalk between the partners in this symbiotic relationship (Artis, 2008; Neish, 2009). This dynamic interaction occurs for the most part at the epithelial surfaces, and it is orchestrated by the immune system. Commensal microbiota, including lactobacilli, is known to regulate the immune system functions of the host, with both immunostimulatory as well as anti-inflammatory effects being observed (Corthesy et al., 2007; Wells, 2011b; van Baarlen et al., 2013). Additionally, lactobacilli and other commensals have an important role in the establishment of immune tolerance in the intestine (Round et al., 2010; Finamore et al., 2012). Beneficial immunomodulatory properties would be a highly desirable characteristic for lactobacilli aimed to be used as probiotics, as these could lead to e.g. increased disease resistance via enhanced immune response against pathogens and / or alleviation of allergic or inflammatory symptoms (Saarela et al., 2000; Meijerink and Wells, 2010; Chassard et al., 2011).

The immunomodulatory actions of lactobacilli can be triggered by several mechanisms, including influencing the maturation and functions of dendritic cells (DCs) (Borchers et al., 2009; Wells, 2011b). These sentinel cells have a central role in the activation of immune responses, as they are the most potent antigen presenting cells, and have the ability to activate naïve T-cells (Kelsall et al., 2002; Mildner and Jung, 2014). Additionally, DCs direct the activation of T cells in such a way to result in the triggering of an appropriate type of immune response (e.g. Th_1 / Th_2 / Th_{17} / T_{reg}) and this guidance occurs mainly through the secretion of cytokines, as well as via costimulatory signals provided by cell surface molecules (Mildner and Jung, 2014). *In vitro* co-culture assays have been extensively used in assessing the effects of lactobacilli on DC maturation and cytokine production. Since intestinal DCs are difficult to obtain, these experiments have usually been conducted with DCs derived from peripheral blood monocytes (MoDCs), or from bone marrow (BMDCs) (Borchers et al., 2009). It appears that there are at least some differences in the responses of DCs originating from the different compartments of the body (Hart et al., 2004; O'Mahony et al., 2006; Fink and Frokiaer, 2008), so it remains to be

determined how well the results obtained with peripheral DCs can be extrapolated to those of intestinal origin. Considering the species origin of the cells, human and murine DCs have by far been the most extensively applied, with swine-derived DCs seldom being used. The priming effect of lactobacilli on DCs can be estimated by measuring the production of selected cytokines (e.g. interleukin (IL) 10, IL-12 or tumor necrosis factor (TNF) α) or the expression of certain surface markers (e.g. cluster of differentiation (CD) molecules like CD40, CD80 and CD86) (Mohamadzadeh et al., 2005; Smits et al., 2005; Konstantinov et al., 2008b; Verbeek et al., 2010; Gad et al., 2011). In addition, co-incubation of the lactobacilli-primed DCs with Th cells has been used to reveal the resulting modulations of T cell differentiation (Mohamadzadeh et al., 2005; Smits et al., 2005; Konstantinov et al., 2008b).

In addition to modifying the functions of immune cells like DCs, lactobacilli can interact with intestinal epithelial cells (IECs) (Borchers et al., 2009; van Baarlen et al., 2013). While the intestinal epithelium is a crucial barrier between the gut lumen and underlying host tissues, it is also an active player in the regulation of intestinal homeostasis and immunity (Artis, 2008). Lactobacilli and other commensals have been shown to influence the functions of the IEC, such as their production of signaling molecules e.g. cytokines (Artis, 2008; Borchers et al., 2009). The *in vitro* experiments assessing the possible alterations in IEC cytokine expression induced by lactobacilli have mainly used the same tissue culture cells as the adhesion studies, namely Caco-2 cells (Morita et al., 2002; Roselli et al., 2006) and HT- 29 cells (McCracken et al., 2002), as well as two cell lines originating from swine, IPEC-J2 (Liu et al., 2010), and PIE (Hosoya et al., 2011). Furthermore, as it is evident that in the intestinal environment a close interaction occurs also between IECs and lamina propria DCs, *in vitro* experiments combining these two cell types in a transwell culture system have also been conducted (Zeuthen et al., 2008; Villena et al., 2014).

Similarly to the other potentially probiotic characteristics of lactobacilli, considerable species- and strain-specific differences have been observed in the *in vitro* immunomodulative properties of lactobacilli (reviewed e.g. by Borchers et al. (2009); Meijerink and Wells (2010); Wells (2011b)). Priming of DCs towards the production of regulatory T cells (Smits et al., 2005), Th_2 cells (Konstantinov et al., 2008b) as well as Th_1 cells (Mohamadzadeh et al., 2005) has been described, and the bacterial dose used affects these responses (Smits et al., 2005; Konstantinov et al., 2008b; Gad et al., 2011). While epithelial cell lines have been unresponsive to some strains of lactobacilli (O'Hara et al., 2006; Candela et al., 2008), stimulation of these cells by lactobacilli can result to production of pro- and / or anti-inflammatory cytokines (Roselli et al., 2006; Zeuthen et al., 2008). Moreover, lactobacilli have been observed to modulate the inflammatory responses of DCs and IECs induced by pathogenic bacteria (Vizoso Pinto et al., 2009; Villena et al., 2014), indicating possible advantageous outcomes for the host.

2.3.2.5 Antibiotic resistance

Resistance to antibiotics is an ancient protection mechanism of bacteria (D'Costa et al., 2011), which nowadays is widely disseminated in bacterial communities due to the modern over-use of antibiotics. In addition to pathogenic bacteria, also commensals and environmental bacteria as well as those used in the food industry are known to harbor antibiotic resistance determinants (Riesenfeld et al., 2004; Sommer et al., 2010; Devirgiliis et al., 2013), and the horizontal transfer of resistance genes from these reservoirs to pathogens poses a major threat to both human and animal health. Consequently, evaluating the antibiotic resistance patterns and mechanisms exhibited by potentially probiotic bacteria is an integral part of the safety assessment (Saarela et al., 2000; von Wright, 2005; Clementi and Aquilanti, 2011). According to the FEEDAP Panel

(Panel on Additives and Products or Substances used in Animal Feed), strains carrying an acquired antibiotic resistance resulting from genes located in transmissible elements are not acceptable for use as feed additives (EFSA, 2012).

A broad variety of *in vitro* techniques, including both agar and broth-based methods, have been used to assess the antibiotic resistance of lactobacilli (Salminen et al., 2006; Klare et al., 2007; Mayrhofer et al., 2008; Rabia and Shah, 2011; Mayrhofer et al., 2014), limiting the comparability and interpretation of the results. However, the technical guidance of the FEEDAP panel includes instructions for methods suitable for determination of minimum inhibitory concentration (MIC), as well as the microbiological breakpoints for selected antibiotics categorizing potential probiotics, including lactobacilli, as resistant (EFSA, 2012).

Although lactobacilli are naturally resistant to a wide range of antibiotics, this resistance is generally not due to transmissible elements but instead it is of the intrinsic type, not considered to be a safety concern (Saarela et al., 2000; Rabia and Shah, 2011). For instance, intrinsic resistance towards vancomycin is common among species of lactobacilli (Saarela et al., 2000; Gueimonde et al., 2013). However, also resistance genes located in mobile genetic elements potentially able to be transferred to other microbes have been found in lactobacilli (Rabia and Shah, 2011; Devirgiliis et al., 2013; Gueimonde et al., 2013), and transmission of some of the elements to other bacteria has been shown to occur (Feld et al., 2008; Devirgiliis et al., 2009).

In addition to the safety concerns regarding lactobacillar antibiotic resistance, this property can also be utilized when isolating lactobacilli from complex communities, e.g. the GI-tract. For example, in an *in vivo* feeding trial, the re-isolation of the supplemented strain among the commensal gut microbiota can be highly challenging. The usage of strains harboring resistance to particular antibiotics would enable antibiotic selection in the bacterial culture, increasing the probability of detecting the right strain(s) among the indigenous lactobacilli (Casey et al., 2007; De Angelis et al., 2007; Walsh et al., 2008).

2.3.3 *In vivo studies*

After the preliminary selection of the candidate strain(s) by *in vitro* methods, the functionality of the putative probiotic strain has to be validated in *in vivo* trials. These should be preferably performed in the intended target host species, although mechanistic studies focusing on molecular level functions of the strain are often performed in rodent models (Mileti et al., 2009; Pagnini et al., 2010; Castillo et al., 2013). The authorization process of microbial feed additives, based on Regulation (EC) No. 1831/2003, requires that the efficacy of the supplement is established, and usually three target animal studies showing statistically significant benefits on relevant parameters are required. The FEEDAP panel has issued technical guidance on the performance of efficacy trials on target animals (EFSA, 2011).

To be able to deduce whether the strain tested actually "works", i.e. confers desirable outcomes to the host, specific measurable endpoints have to be defined. In production animals, and to some extent also in companion animals as well as in humans, the use of probiotic microbes differ from the use of most pharmaceuticals, as the main aim is to maintain and improve health rather than to cure a clinical disease. However, animal health and well-being is a complex concept which is not always easily measured (Clark et al., 1997; Meieregger et al., 2011). In addition, in the case of production animals, productivity is of major importance, and while this aspect does not necessarily strictly correlate with animal well-being (Frazer, 1993; Clark et al., 1997), it needs to be taken into account in probiotic feeding trials. Indeed, the measures most commonly used in feeding trials performed in production animals relate to the productivity of the animal, but

also other endpoints, like alterations in the composition of the intestinal microbiota and in the immune function of the host are commonly used (Bosi and Trevisi, 2010; Kenny et al., 2011; Meieregger et al., 2011).

Numerous feeding trials using potentially probiotic *Lactobacillus* strains have been performed in swine, and as can be seen from Table 3, the results have been highly variable. Interpretation of these results is hampered by the fact that several aspects in the study design, including those related to the supplemented microbe or to the host animal, can affect the outcomes of *in vivo* feeding trials (Bernardeau et al., 2006; Lim and Tan, 2009; Rijkers et al., 2010).

While the bacterial species and strain used are obviously important variables in probiotic feeding trials, the bacterial dose administered to the animals can also influence the results obtained (Li et al., 2012; Suo et al., 2012; Wen et al., 2012; Zhu et al., 2014). However, probiotic functionality in terms of dose-dependence has not been extensively investigated in feeding trials performed in swine, and the dosages of bacteria used have typically been in the range from 10^8 to 10^{10} CFU, with these being administered daily or at different time intervals, e.g. once a week (Table 3). In addition, although using only single strain supplementation has been a common approach, multi-strain or multi-species preparations have also been investigated in several studies (Table 3). The combination of probiotic strains possessing characteristics that complement each other might help to overcome the problems related to species– and strain–specificity of the beneficial properties (Collado and Salminen, 2009). Moreover, as host related factors also contribute to the host–microbe interaction, the performance of a probiotic microbe is likely to vary between different host individuals. Consequently, multistrain /–species products might function more effectively and more consistently than a monostrain product. Since most of the studies that have been performed using combinations of different strains were not specifically designed to compare the effects of a multistrain supplementation to those of the same strains applied singly, it is difficult to evaluate the possible differences in beneficial outcomes between these approaches. However, there are some indications that multistrain products may perform better that their monostrain counterparts (Nousiainen et al., 2004; Timmerman et al., 2004; Collado and Salminen, 2009; Chapman et al., 2011).

In addition to factors related to the strain(s) used in the supplementation, there are also several practical issues that can influence the results of an *in vivo* feeding trial. The production environment and management practices are known to impact on the gut microbiota composition of swine (Davis, 2012), thus these factors probably also affect the interplay between the supplemented strain and the commensal microbiota. In addition, the hygiene level of the production facility can play an important role, since exposure to pathogenic bacteria and the subsequent infection incidence can be very variable in different environments. Consequently, the most pronounced effects of probiotic supplementation might be observed in facilities with lower level of hygiene and / or in circumstances inflicting stress on the animals (Fuller, 1989; Thomke and Elwinger, 1998; Kritas and Morrison, 2007), and the same has been proposed also for in-feed antimicrobials (Fuller, 1989; Dritz et al., 2002; Kenny et al., 2011). In addition, the time frame of probiotic application, including the age of the animals used and the length of the trial, has differed in the various experiments performed, and is likely to have an impact on the observed outcomes of probiotic supplementation (Simon et al., 2001; Taras et al., 2007; Gaggia et al., 2010).

Table 3. Examples of bacterial feeding trials performed in swine, estimating the effects of either a monostrain Lactobacillus preparation or a multistrain preparation, containing at least one *Lactobacillus* strain.

Bacterial strains used[1]	Daily dosage / animal	Age[2] or category	Main effects observed	Reference
L. acidophilus	$2x10^{12}$ CFU	2 days	Increased numbers of lactobacilli and coliforms in the stomach, tended to reduce growth	Pollmann et al., 1980
L. acidophilus LAC-300	$3x10^{8}$ CFU / $3x10^{9}$ CFU	Neonatal and weaned	Increased ADWG, no effect on FCR	Abe et al., 1995
L. brevis 1E1	$5x10^{9}$ CFU	Neonatal	Decreased numbers of *E. coli* and coliforms in the small intestine Increased villous height : crypt depth ratio Certain changes in the intestinal leucocyte populations	Gebert et al., 2011
L. casei subsp. *casei* CECT 4043	10^{9} CFU/g feed[3]	21 days	Increased ADWG and DFI Decreased numbers of fecal coliforms	Guerra et al., 2007
L. fermentum I5007	$2x10^{9}$ CFU	3 weeks	Increased ADWG and FCR Increased proportion of DC4+ cells in blood Increased expression of TNF-α and IFN-γ in the ileum No effect on serum cytokine levels	Wang et al., 2009
L. gasseri LF221 *L. gasseri* K7 (applied separately)	$5x10^{10}$ CFU	21 days	Both strains isolated from feces and adhered to the small intestinal mucosa; high variability between different host individuals Transient increase in the numbers of fecal lactobacilli	Bogovic Matijasic et al., 2006
L. paracasei	$2x10^{9}$ CFU	Neonatal and 36 days	No effect on growth, blood immunoglobulin concentrations or immune cell numbers No change in total counts of selected bacterial groups	Herich et al., 2002
L. plantarum ZJ316	$1x10^{9}$ / $5x10^{9}$ / $1x10^{10}$ CFU	35 days	Increased ADWG and FCR Increased villous height in the small intestine Reduced incidence of diarrhea Better pork quality Dose-dependence of the observed effects	Suo et al., 2012

Table 3 cont.

Bacterial strains used[1]	Daily dosage / animal	Age[2] or category	Main effects observed	Reference
L. salivarius B1	$5x10^9$ – $1,5x10^{10}$ CFU (increasing dose administered on days 0, 7 and 11)	Neonatal	Increased numbers of intraepithelial lymphocytes and IgA-producing cells Increased expression of IL-6 in the ileum and TLR 2 in the duodenum and ileum	Zhang et al., 2011
L. sobrius S1	$5x10^9$ – $1,5x10^{10}$ CFU (increasing dose administered on days 0, 7 and 11)	7 days	Minor changes in the microbial community of the large intestine	Su et al., 2008a
L. salivarius B1 *Bacillus subtilis* RJGP16 (applied separately or in combination)	$5x10^9$ – $1,5x10^{10}$ CFU (increasing dose administered on days 0, 7 and 11)	Neonatal	Increased intestinal expression of TLR-2, pBD-2 and IL-6 Co-administration of the strains modified responses Increased numbers of IgA-producing cells	Deng et al., 2013
L. rhamnosus GG *L. sobrius* 001^T (applied separately)	10^{10} CFU	21 days	Cross-reactivity of serum IgA; supplementation strain-specific IgA found in the control group as well	Casini et al., 2007
L. plantarum 423 *L. salivarius* 241 (applied separately or in combination)	$2x10^{10}$ CFU $1x10^{10}$ CFU $1.5x10^{10}$ CFU (in total; combination) dosing once a week	Neonatal and 28 days	Both strains adhered to the small intestinal mucosa, with differing localization which was affected by the age of the piglets	Maré et al., 2006
L. fermentum applied separately or in combination with: *L. gasseri*, *L. reuteri*, and *L. acidophilus*	10^9 / 10^{10} / 10^{11} CFU (separately) 10^8 CFU (in total; combination)	4 weeks	Increased ADWG and apparent digestibility of crude protein, no effect on FCR Transient increased in serum ovalbumin-specific IgG levels Dose-dependence of the observed effects	Yu et al., 2008
L. plantarum 4.1 *L. reuteri* 3S7 (applied in combination)	10^{10} CFU (in total)	Sows and piglets	Both strains isolated from sow and piglet feces Decreased numbers of fecal *Enterobacteriaceae* Increased numbers of fecal lactobacilli in piglets	De Angelis et al., 2007

Table 3 cont.

Bacterial strains used[1]	Daily dosage / animal	Age[2] or category	Main effects observed	Reference
L. plantarum ATCC 14917 *L. fermentum* DSM 20016 *Enterococcus faecium* ATCC 19434 (applied in combination)	10^8 CFU/kg feed[3] (each)	5 weeks	Increased ADWG (1st trial) No effect on performance parameters (2nd trial)	Veizaj-Delia et al., 2010
L. acidophilus *L.delbrueckii* subsp. *bulgaricus* *B. subtilis* *Saccharomyces cervisiae* (applied in combination)	$9x10^8$ CFU $2x10^6$ CFU $9x10^8$ CFU $8x10^6$ CFU (0.3% in feed)	7 days	No effect on ADWG Decreased numbers of coliforms in the ileum and bifidobacteria in the colon No effect on blood lymphocyte and neutrophil numbers	Shim et al., 2005
L. murinus DPC6002 *L. murinus* DPC6003 *L. pentosus* DPC6004 *L. salivarius* DPC6005 *Pediococcus pentosaceus* DPC6006 (applied separately or all in combination)	$3x10^{10}$ CFU (each or in total; combination)	5-6 weeks	Differential survival and persistence of the strains in the GI-tract Variability in the excretion of the strains between host individuals Reduced numbers of fecal *Enterobacteriaceae*	Gardiner et al., 2004
L. salivarius *L. reuteri* *Bifidobacterium thermophilum* *E. faecium* (applied in combination)	10^{11} CFU (in total)	4 weeks	No change in performance parameters Decreased pH in the stomach and small intestine Alterations in the gut microbiota (e.g. increased numbers of colonic lactobacilli)	Mair et al., 2010a
			Increased expression of certain genes, e.g. cell turnover markers (in colon and blood) and TGF-β (in mesenteric lymph nodes)	Mair et al., 2010b

Table 3 cont.

Bacterial strains used[1]	Daily dosage / animal	Age[2] or category	Main effects observed	Reference
L. murinus DPC6002 *L. pentosus* DPC6004 *L. salivarius* DPC6005 *P. pentosaceus* DPC6006 (applied in combination)	5x10^9 CFU (in total)	6 weeks	Differential survival of the strains through the GI-tract, as well as adherence to the intestine wall Increased numbers of ileal *Enterobacteriaceae* Increased IL-8 expression in the ileum Decreased CD25 and CTLA-4 induction on immune cells	Walsh et al., 2008
L. acidophilus KNU 31 *B. subtilis* KNU 42 *S. cerviciae* KNU 55 *Aspergillus oryzar* KNU 48 (applied in combination)	10^4/10^{10} CFU 10^6/10^{11} CFU 10^4/10^6 CFU 10^5/10^9 (0.3 / 0.6% in feed)	Weaned piglets	Increased ADWG, DFI, FCR and apparent digestibility Decreased numbers of fecal *Clostridium* Dose-dependent effect on intestinal morphology (villus height and villus height : crypt ratio)	Choi et al., 2011a
L. acidophilus KNU 31 *B. subtilis* KNU 42 *S. cerviciae* KNU 55 *Aspergillus oryzar* KNU 48 (applied in combination)	10^{10} CFU 10^{11} CFU 10^9 CFU 10^9 CFU (0.3% in feed)	Weaned piglets	Increased ADWG, DFI and FCR Decreased numbers of fecal coliforms and *Clostridium*, increased numbers of fecal lactobacilli The production method of the probiotic preparation influenced the outcomes	Choi et al., 2011b
L. acidophilus C3 *L. plantarum* 1K8 *L. plantarum* 3K2 *E. faecium* 6H2 *P. pentosaceus* D7 (applied in three different mixtures)	10^8 CFU 10^8 CFU 10^8 CFU 10^{10} CFU 10^8 CFU (600 ppm in feed)	21-23 days	Increased ADWG, DFI and FCR in the first two weeks after weaning (no difference between the various mixtures) Increased numbers of lactic acid bacteria in the digesta, but no effect on *E. coli* counts Decrease in diarrhea incidence	Giang et al., 2010

[1] *L.* = *Lactobacillus* [2]Age at the start of trial [3]Feed consumption not reported

2.3.3.1 Effects on productivity parameters

A high level of biological functioning can be considered as one aspect of animal well-being (Frazer, 1993), thus the assessment of certain performance parameters to detect changes in productivity is frequently used to estimate the possible effects of bacterial supplementation. Productivity measures are also extremely important for the producer, as higher productivity yields better financial returns. The average daily weight gain (ADWG) is the most commonly utilized measurement of productivity, used mainly in fattening farm animals (Meieregger et al., 2011). Other parameters that are often used are the daily feed intake (DFI), as well as the feed conversion ratio (FCR), which quantifies the relation between DFI and ADWG, measuring the efficiency in converting food mass into body mass. Depending on the production category of the animal, the usual range for FCR is 1 : 1.5-4 for swine and poultry, and 1 : 6-7 for cattle (Meieregger et al., 2011), with high-performing animals showing lower ratios indicating that less feed is needed to produce the same amount of meat. In production animals not in the fattening category, other parameters, such as those related to reproduction (e.g. the litter size and weight, sow weight loss after birth) can be used to assess the effects of probiotic supplementation (Böhmer et al., 2006).

The mechanisms behind the improved performance caused by bacterial supplementation are not fully understood, but some of the putative actions of probiotics possibly contributing to enhanced host growth are summarized in Figure 3. Lactobacilli and other beneficial microbes have been proposed to contribute to the nutrition of the host e.g. by synthesizing vitamins and breaking down nutritional components that the host is unable to metabolize, possibly improving nutrient bioavailability (Kopp-Hoolihan, 2001; Turpin et al., 2010; Kenny et al., 2011; Ezema, 2013). However, as the cell numbers of supplemented bacteria are low compared to that of the commensal microbiota, the relevance of their possible contribution to nutrition may be minor. Modification of the host gut physiology, such as increasing the production of growth factors, might also be a mechanism through which lactobacilli can enhance host growth (Turpin et al., 2010). In addition, stabilization of the gut microbiota resulting in a reduced pathogen load has been postulated to induce increased growth (Kenny et al., 2011).

In swine, highly variable results have been obtained on studies assessing the ability of various *Lactobacillus* supplementations to beneficially affect the productivity, with some studies showing improved performance while others have not detected any benefits (Table 3). With regard to growth promotion, the percentage improvements in the weight gain of starter piglets has often ranged from around 2 to 10% (Fuller, 1989; Meieregger et al., 2011), and while several studies have failed to achieve statistical significance, a general tendency towards an elevated growth rate in piglets supplemented with probiotics has been reported in numerous studies, as reviewed by Simon et al. (2001). The FEEDAP panel has issued scientific opinions regarding the ability of certain *Lactobacillus* preparations to enhance swine growth performance and some products have been concluded to increase the growth of piglets (EFSA, 2008, 2013). Studies performed in growing-finishing pigs are rarer, and the responses in growth enhancement have usually been of lower magnitude than those observed in young piglets (Nousiainen et al., 2004).

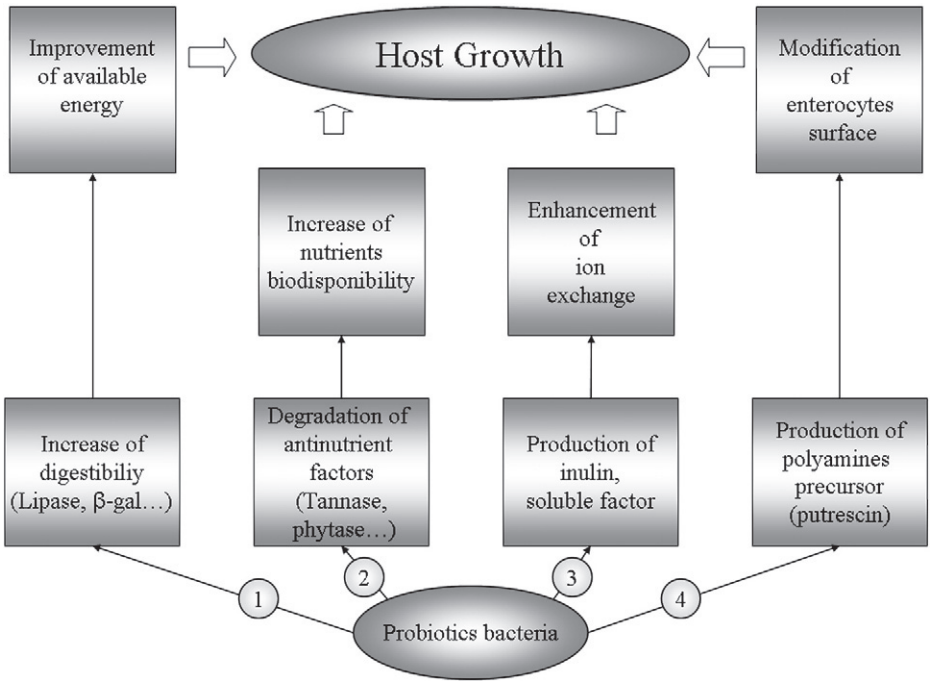


Figure 3. Probiotics can improve host growth by increasing biodisponibility of macronutrients (1), degradation of antinutrient factors (2), enhancement of mineral absorption (3), and production of growth factors (4). The other squares indicate the mechanism involved in host growth. The Figure is reprinted from Turpin et al. (2010) with permission granted by Elsevier.

2.3.3.2 Effects on gut microbiota

The GI-tract microbiota is known to be a significant factor affecting the health and well-being of the host, and positive effects on this complex community are believed to be one of the mechanisms behind probiotic action (Simon et al., 2001; Gueimonde and Salminen, 2006; Bosi and Trevisi, 2010). Consequently, the possible alterations of the gut microbiota attributable to lactobacilli consumption have been commonly monitored in porcine feeding trials. While it is not so straightforward to determine what kind of changes in the GI-tract microbiota can be considered as favorable for the host (Simon et al., 2001), an increase in the numbers of "beneficial" microbes, such as lactic acid bacteria and bifidobacteria is often interpreted as a positive outcome after lactobacilli supplementation (Simon et al., 2001; Nousiainen et al., 2004; Meieregger et al., 2011). Additionally, a reduction in the numbers of potentially pathogenic microbes, like enterobacteria including *E. coli*, has been proposed to be an advantageous change (Simon et al., 2001; Nousiainen et al., 2004; Meieregger et al., 2011). Both of these responses have been described in swine after supplementation with lactobacilli (Table 3). However, since a large part of these studies have been based on bacterial culture methods, the results are biased towards cultivable species, and provide only a partial picture of the changes occurring in the intestinal microbiota. The use of culture-independent molecular techniques allows a more complete description of the possible effects bacterial supplementation might have on the intestinal microbiota composition.

In addition to general changes in the composition of the intestinal microbiota, also the fate of the administered strain is of interest. Survivability of the feeding strain through the GI-tract, and possibly even colonization of the host intestine have been stated as necessary characteristics for a probiotic microbe (Vaughan et al., 1999; Marco et al., 2006). While the GI-transit survivability has been demonstrated for certain strains of lactobacilli in feeding trials performed in swine (Table 3), this factor has not been investigated in the majority of the trials. Moreover, colonization of the intestine by the supplemented strain has been evaluated even more rarely in swine and only for short periods of time (Pedersen and Tannock, 1989; Gardiner et al., 2004; De Angelis et al., 2007). While permanent colonization of the host intestine by an allochthonous (i.e. exogenous) strain is easily achieved in gnotobiotic animals (Servin and Coconnier, 2003; Smith et al., 2007), the colonization resistance exerted by the commensal microbiota seems to be strong as the exogenous strain administered usually disappears from the intestine after discontinuation of consumption (Bezkorovainy, 2001; Ouwehand and Salminen, 2003; Isolauri et al., 2004). Even the adhesive *L. rhamnosus* strain GG does not seem to permanently colonize the human GI-tract (Bezkorovainy, 2001), nonetheless its persistence has been reported in colonic mucosa even although it was not recovered from fecal samples (Alander et al., 1999). In addition, the age of the host at the time of probiotic administration may be an important factor, as *L. rhamnosus* strain GG was found to persist for as long as 24 months in babies when administered to their mothers during late pregnancy (Schultz et al., 2004). This implies that application of probiotics in the neonatal period, when the commensal microbiota is not yet fully developed, may be optimal for intestinal colonization with the desired bacterial strain. Interestingly, a strain of *L. acidophilus* (LAB20) isolated from a dog was found to persist in the intestine of a dog at least for six weeks (Tang and Saris, 2013), thus there probably exist differences in colonization ability attributable to the bacterial strain as well as the host species.

2.3.3.3 Effects on host immune function

The *in vivo* immune modulating actions of lactobacilli have been demonstrated in several trials, performed for the most part in mouse models as well as in humans, and have included shifts in the balance of different T cell types, stimulation of regulatory immune mechanisms (e.g. generation of T_{reg} cells), alterations in cytokine expression patterns, increases in the production of antibodies and in the activity of certain immunological cells (e.g. natural killer cells, macrophages and other phagocytes) as well as modifications in the functions of the intestinal barrier (Borchers et al., 2009; Meijerink and Wells, 2010; Wells, 2011b; van Baarlen et al., 2013). In lactobacilli feeding trials performed in swine, the most commonly measured immunological parameter has been the expression levels of cytokine genes in the intestine or other tissues, but also immune cell populations in the gut mucosa or peripheral blood as well as immunoglobulin concentrations have been investigated in some of the trials (Table 3).

While lactobacilli and other probiotic microbes obviously do have the capability to modify host immune responses, the question still remain open; are the observed effects actually beneficial for the host? When taking into account the complexity of the immune system, interpretation of the data obtained from the *in vivo* studies is highly problematic and mere immunostimulation or some other alteration does not necessarily represent a positive health effect. A more detailed understanding of the immune system mechanisms are needed to unravel the consequences of the changes that probiotic microbes induce in the host immune function.

2.3.3.4 Effects on disease occurrence

As a reduced occurrence of diseases, especially of diarrheal diseases, is one of the health claims for beneficial microbes, this outcome has been frequently explored in probiotic feeding trials, both in humans and animals (Fuller, 1989; Simon et al., 2001). Reductions in disease incidence or prevalence would lead to improved animal welfare and often also to increased productivity. GI infections are very common in the pig production, causing significant financial losses. Diarrheal diseases especially in the neonatal and post-weaning period are a major problem, which traditionally has been combated with the use of therapeutic and in-feed antimicrobials (Bomba et al., 2012). While there are several different bacteria and viruses that can cause neonatal and post-weaning diarrhea in piglets, different enterotoxigenic *E. coli* (ETEC) strains possessing F4, F5, F6, F7 and F18 fimbriae are those most commonly detected (Melkebeek et al., 2013).

Two different study designs can be applied to assess the ability of a bacterial supplement to reduce disease occurrence *in vivo*. In field trials, the efficacy of the potential probiotic is estimated in regular production farms, i.e. under "real life conditions". However, for practical reasons, these kinds of studies are very challenging to perform, and a large number of animals is needed to achieve significant and biologically relevant results. Therefore, studies in which the animals are deliberately challenged with infectious organisms to cause a disease are often used to estimate the disease preventive ability of lactobacilli and other probiotic microbes. In the challenge studies that have been performed in swine, different *E. coli* strains have been the most often used pathogens, but strains of *Salmonella* have also been used (Table 4). In several studies, the supplemented probiotic strain has been able to alleviate the symptoms and reduce the fecal excretion of the pathogen, as well as to modify the pathogen induced immune responses of the host animal (Table 4). The mechanisms behind the protective effect of probiotic bacteria against pathogens remain unresolved, although several have been postulated, including competition for adhesion sites or nutrients, production of antibacterial substances that inhibit pathogen growth and survival, prevention of pathogen actions by agglutinating with the pathogen and inhibition of the production of pathogen toxins (Fuller, 1989; Nousiainen et al., 2004; Turpin et al., 2010). In the future, more detailed studies will help to reveal the interactions between the supplemented probiotic and the pathogen that occur in the complex environment of the host gut microbiota.

Table 4. Examples of pathogen challenge trials evaluating the efficacy of different *Lactobacillus* preparations against the pathogen.

Bacterial strains used[1]	Daily dosage	Age[2] or category	Main effects observed	Reference
L. amylovorus DSM 16698 **Ch**[3]: *E. coli* ETEC K88 O149 Fac	10^{10} CFU 1.5x10^{10} CFU	3 weeks	Improved ADWG Reduced ETEC levels in the ileum Longer duration of diarrhea	Konstantinov et al., 2008a
L. fermentum I5007 **Ch**: *E. coli* K88ac	2x10^{9} CFU 2x10^{9} CFU	3 weeks	Only minor and transient alleviation of symptoms, no effect on *E. coli* induced growth depression Modulation of the *E. coli* induced alterations in the expression of TNF-α and IFN-γ in the small intestine	Wang et al., 2009
L. plantarum CJLP243 **Ch**: *E. coli* ETEC K88ac	10^{8} / 10^{9} / 10^{10} CFU 10^{9} CFU	19-21 days	Dose-dependent increase in ADWG and DFI Alleviation of elevated rectal temperature and diarrhea Modulation of the serum cytokine response	Lee et al., 2012
L. rhamnosus GG **Ch**: *E. coli* ETEC K88ac	10^{11} CFU 10^{10} CFU	19 days	Reduced incidence of diarrhea Increased numbers of fecal lactobacilli and bifidobacteria Decreased numbers of fecal coliforms Increased sIgA levels in the intestinal mucosa Modulation of the serum cytokine response	Zhang et al., 2010
L. rhamnosus GG **Ch**: *E. coli* ETEC F4	10^{10} / 10^{12} CFU 10^{10} CFU	22 days	No effect on ADWG or DFI Alleviation on diarrhea; low dose more efficient Changes in intestinal and fecal microbiota Attenuation of immunological responses in the intestine	Li et al., 2012
			Modulation of cytokine expression and immune cell populations in the intestinal mucosa and blood; dose-dependence of these changes	Zhu et al., 2014
L. rhamnosus GG **Ch**: *E. coli* O149:Fac	6x10^{9} CFU 1.5x10^{10} CFU	21 days	Reduced ADWG and DFI Tended to worsen diarrhea, increase fecal excretion of ETEC and decrease villus height Reduced total IgA in the serum No effect on the levels of colonic LAB or enterobacteria	Trevisi et al., 2011
L. murinus DPC6002 *L. murinus* DPC6003 *L. pentosus* DPC6004 *L. salivarius* DPC6005 *P. pentosaceus* DPC6006 (applied all in combination) **Ch**: *S.* Typhimurium PT12	4x10^{9} CFU / 4x10^{10} CFU (in total) 1x10^{8} CFU	Weaned piglets	Reduced incidence, severity and duration of diarrhea Improved ADWG Reduced excretion of *S.* Typhimurium	Casey et al., 2007

[1] *L.* = *Lactobacillus* [2]Age at the start of trial [3]Ch: pathogen used in the challenge

2.3.4 Correlation of in vitro and in vivo studies

As the purpose of the *in vitro* tests is to predict which of the candidate probiotic strains would perform best in the target animal species, the correlation of the results obtained in these screening tests to the *in vivo* findings is very important. However, it is a very difficult task to systematically and extensively evaluate this matter. Additionally, studies which have estimated the correlation of *in vitro* and *in vivo* results are, for the most part, based on experiments performed in humans or mouse, and only few studies executed in swine have evaluated this topic.

The tests for tolerance of low pH and bile are intended to demonstrate which strains will be able to survive the transit through the host GI-tract. Indeed, several studies performed both in humans (Pochart et al., 1992; Jacobsen et al., 1999; Dunne et al., 2001) and swine (Gardiner et al., 2004; De Angelis et al., 2007) have shown that strains exhibiting *in vitro* tolerance to low pH and bile are able to survive intestinal passage. Additionally, the *in vitro* survival results obtained in a dynamic model of the human GI-tract correlated well with *in vivo* data (Marteau et al., 1997). However, appropriate *in vivo* comparison of strains with differing performances in the *in vitro* stress tolerance tests has not usually been performed in these studies. Furthermore, those strains that are known to survive human GI-tract transit *in vivo*, e.g. *L. rhamnosus* GG (Goldin et al., 1992; Alander et al., 1999), have sometimes performed poorly in *in vitro* tests (Mattila-Sandholm et al., 1999; Morelli, 2000; Mainville et al., 2005). Thus, at least some of the *in vitro* test conditions used in the stress tolerance assays may well be too harsh as compared to the *in vivo* situation. Additionally, as the food matrix protects the supplemented bacteria during gastric passage (Conway et al., 1987; Charteris et al., 1998), strains with poor *in vitro* acid tolerance may well be able to tolerate the *in vivo* GI-tract environment.

Good adhesiveness to the intestinal wall structures has been considered to be an important property for probiotic microbes mainly for three reasons: adherent strains might 1) more easily colonize the host intestine, 2) exclude pathogens by competing for adhesion receptors, 3) induce immune modulatory actions via their close contact with host cells (Ouwehand and Salminen, 2003; Lahtinen and Ouwehand, 2009). With regard to colonization, as already discussed in section 2.2.3.2, it seems however that even adhesive strains cannot permanently colonize the host intestine. Based on studies performed in humans (Jacobsen et al., 1999; Morelli, 2000) and in various animals (i.e. chickens (Fuller, 1978), mouse (Zarate et al., 2002) and fish (Sugimura et al., 2011)) it seems that *in vitro* adhesion ability of a bacterial strain may predict *in vivo* adhesion and / or persistence in the host intestine. However, the adhesion ability by itself is apparently not sufficient to ensure a prolonged persistence of the strain in the gut environment. This is illustrated by results obtained in mouse showing that also non-adherent strains can temporarily colonize the host intestine (Hautefort et al., 2000). On the other hand, studies performed in humans (Morelli, 2000) and swine (Nousiainen et al., 2004) have revealed that adhesive strains do not necessarily succeed in colonization. Consequently, the correlation between *in vitro* adhesiveness and transient gut colonization is not clear. Interestingly, when comparing isogenic variants of *L. crispatus* differing in their *in vitro* aggregation and adhesion abilities, the low-adhesive mutant was not detected in the feces or biopsies of human volunteers, while the more adhesive parent strain was found (Cesena et al., 2001), and the same was observed also in experiments conducted in mice (Voltan et al., 2007). Future studies with a similar approach of using isogenic strains differing only in their adhesion ability may help clarify the correlation of *in vitro* adhesiveness to *in vivo* colonization capacity.

While *in vivo* challenge studies performed in swine have shown that lactobacilli and other probiotics are able to exclude pathogens, and there is some evidence that lactobacilli could use the same attachment sites as pathogens (Lebeer et al., 2008), the importance of probiotic adhesion in pathogen exclusion is still rather unclear. A non-adhering mutant of the strain *L. plantarum* 299v was, in contrast to the wild type, unable to inhibit the adhesion of an *E. coli* strain to HT-29 cells, which indicates that adhesion ability may be important for pathogen exclusion (Mack et al., 2003). However, it appears that even if a strain has a high adherence ability, it may not necessarily inhibit pathogen adhesion to cultured intestinal epithelial cells (Bibiloni et al., 1999), and also other mechanisms have been proposed to be involved in pathogen exclusion, including coaggregation with the pathogen (Lebeer et al., 2008).

In addition to the colonization ability and / or pathogen exclusion properties, adhesiveness is also considered important for the immunomodulatory actions of a probiotic strain. Obviously, stimulation of the immune system requires an intimate contact between the bacteria and host cells, and this typically occurs via specific receptors, e.g. pattern recognition receptors (PRRs) binding to certain structures on the microbial surface, inducing signaling pathways that trigger immunological responses (Murphy, 2012). As reviewed by Lebeer et al. (2010), these kinds of interactions also take place between probiotic bacteria and the host. However, this does not reveal whether adhesion to enterocytes is necessary to obtain the immunological actions of probiotics. While studies performed in humans have given some indications that highly adhesive strains have a greater effect on the immune system than their less adhesive counterparts (Ouwehand and Salminen, 2003), it cannot be concluded for certain that these immunological differences are specifically due to the differing adherence properties of the strains. Thus far, few studies have been performed with isogenic strains; in one such study the aggregating and adhesive strain *L. crispatus* M247 induced changes at the mRNA levels of Toll-like receptor (TLR) 2 and TLR 4 in the colonic mucosa of mice, while the less adhesive mutant strain had no effect on these parameters (Voltan et al., 2007). This observation indicates that an aggregative and / or adhesive phenotype is important in modulating murine enterocyte responsiveness, but for the most part, the role of adhesiveness in probiotic immunomodulation still remains to be elucidated.

The comparison of *in vitro* and *in vivo* immunomodulatory properties of probiotic bacteria is complicated by the extreme complexity of the host immune system, as only a few parts of this vast network can be examined in laboratory experiments. However, at least some correlations seem to exist between certain *in vitro* measures and *in vivo* immune functionality. In chickens, provision of lactobacilli strains which enhanced the proliferation of suboptimally stimulated lymphocytes *in vitro* also increased the specific humoral immune responses *in vivo* (Koenen et al., 2004). In mouse models of experimentally induced inflammatory colitis, the strains inducing in PBMCs a higher release of IL-10, considered as anti-inflammatory, and a lower release of IL-12, viewed as Th_1 stimulating, showed better *in vivo* protective ability against colitis (Foligne et al., 2007). Additionally, *in vitro* induction of inflammatory cytokine production on DCs predicted the ability of different *Lactobacillus* species to confer protection from induced colitis in mice (Mileti et al., 2009). In the latter study, strains displaying immunostimulatory properties exacerbated the development of induced colitis, emphasizing the need to characterize properly the *in vivo* immunomodulative properties of different strains in various disease conditions. While these results indicate that the *in vitro* immunomodulatory responses observed for lactobacilli may indeed be predictive of *in vivo* functions, more comparative studies will be needed before any firm conclusions can be made.

3 Aims of the study

The objective of this study was to explore the possibility of using lactobacilli as health-promoting microbes and/or a recombinant vaccine vector in swine; to obtain host-specific *Lactobacillus* strains originating from the swine intestine as well as to characterize certain properties associated with probiosis of the strains and to evaluate the suitability of selected *Lactobacillus* strains for use as a viable probiotic / vaccine vector in swine. Additionally, the potential role of the surface layer proteins of *Lactobacillus amylovorus* strains in adhesion was examined. This study was part of a project aiming to develop a *Lactobacillus* vectored vaccine against porcine post-weaning diarrhea and edema disease caused by *E. coli* F18+ strains.

The specific aims of the subprojects were:

1. to examine the *in vitro* probiotic properties of lactic acid bacterial strains isolated from the feces and intestine of swine,
2. to evaluate the potential effects of *Lactobacillus brevis* ATCC 8287 as a feeding supplement on the performance and immune function in piglets,
3. to evaluate the potential effects of a multispecies *Lactobacillus* supplementation (containing *L. amylovorus*, *L. johnsonii*, *L. mucosae*, *L. salivarius* and two strains of *L. reuteri*) on the performance and intestinal cytokine expression in piglets and
4. to characterize the putative probiotic properties of eight *L. amylovorus* strains and to assess the role of the surface layer proteins of the strains in adherence to porcine intestinal epithelium cells *in vitro*

4 Materials and methods

4.1 Bacterial strains and culture conditions (I-IV)

A total of 94 LAB were isolated (I) from the digesta and feces of swine, with some of these *Lactobacillus* strains being used in further studies (III, IV). Digesta samples were from the duodenum, jejunum and ileum of six swine, and fecal samples from the rectum of two sows. For the isolation of swine *Lactobacillus* strains the samples were cultured on de Man Rogosa Sharpe (MRS) or Rogosa liquid and solid media (Difco, Becton-Dickinson (BD), USA) incubated anaerobically (Anaerocult A, Merck, Germany) at 37°C for 48 h.

Six LAB isolates identified as surface layer-carrying *L. amylovorus* strains were selected for Study IV (Table 5). The seven *Lactobacillus* strains used in the porcine feeding trial (II, III; Table 5) were selected on the basis of the *in vitro* characteristics associated with probiotic properties.

Additionally, several strains from culture collections were used (Table 5). The pathogens used in Study I and IV were selected based on food hygienic relevance and / or significance to swine health. Furthermore, two *Escherichia coli* strains were used in the gene expression analysis in Study IV (Table 5).

All bacterial strains were stored at −80°C; the LAB strains in MRS broth (Difco) and the pathogens and *E. coli* strains in tryptic soy broth (TSB; Difco) or in Luria-Bertani (LB) broth (Difco), containing 15% glycerol.

The *Lactobacillus* strains (I-IV) were grown from stocks anaerobically in MRS broth at 37°C for 24h, and, when needed, subcultured in MRS solid media at 37°C for 48h.

The pathogenic bacteria used in the growth inhibition assay (I, IV) were grown from stocks in blood agar (Tammer-Tutkan maljat OY, Finland) or tryptic soy agar (TSA; Difco) at 37°C for 24 h and subcultured in TSB. The enterotoxigenic *E. coli* (ETEC) strain used in the adhesion assays (IV) was cultured in LB broth (Difco). The broth cultures of the pathogens were incubated at 37°C with agitation (200 rpm). The *E. coli* strains used in the genome expression analyses (IV) were cultivated in LB broth or in M9ZB medium (Studier, 1991) with added kanamycin (30 μg/ml) when appropriate.

4.2 Identification of lactic acid bacteria (I, III)

The putative identification of the porcine LAB isolates as members of the genus *Lactobacillus* (I) was performed with Gram-staining and a polymerase chain reaction-enzyme-linked-immunosorbent-assay (PCR-ELISA) as described by Jakava-Viljanen and Palva (2007).

LAB isolates confirmed as S-layer positive (n=9) were identified by 16S ribosomal RNA (rRNA) gene sequencing as described by Jakava-Viljanen and Palva (2007). For the remaining isolates, preliminary identification down to the species level was performed by sequencing the first 500 base pairs of the 16S rRNA gene. For selected isolates (n=11), sequencing of the whole 16S rRNA gene was performed in order to confirm the preliminary identification. The chromosomal DNA was extracted with standard methods, and the amplification PCR product purified with the Qiaquick PCR purification kit (Qiagen Co., Germany). The primers used in the 16S rRNA sequencing analyses are listed in Table 6. Sequencing was performed with an ABI Prism 310 or 3700 Genetic Analyzer (Applied Biosystems Inc., USA), and the Basic Local Alignment Search Tool (BLAST) program was used for comparing the 16S sequences to the gene bank database.

In addition to sequencing of the 16S rRNA gene, whole genome sequencing (WGS) was performed to those *Lactobacillus* isolates used in Study III. The WGS was performed in the Institute of Biotechnology with the method described by Kant et al. (2011b).

Table 5. Strains used in this work.

Strain	Origin, reference or source	Study
***Lactobacillus* strains**		
L. brevis ATCC 8287	Green fermented olives, ATCC[a]	II
L. mucosae GLR 1167 (LAB 4)	Swine feces, DVB[c]	III
L. salivarius GRL 1169 (LAB 33)	Swine ileum, DVB[c]	III
L. johnsonii GRL 1171 (LAB 81)	Swine ileum, DVB[c]	III
L. reuteri GRL 1168 (LAB 26)	Swine duodenum, DVB[c]	III
L. reuteri GRL 1170 (LAB 49)	Swine jejunum, DVB[c]	III
L. amylovorus GRL 1112 (LAB 2)	Swine feces (Jakava-Viljanen and Palva, 2007), DVB[c]	III, IV
L. amylovorus GRL 1114 (LAB 8)	Swine feces (Jakava-Viljanen and Palva, 2007), DVB[c]	IV
L. amylovorus GRL 1115 (LAB 13)	Swine ileum (Jakava-Viljanen and Palva, 2007), DVB[c]	IV
L. amylovorus GRL 1116 (LAB 16)	Swine jejunum (Jakava-Viljanen and Palva, 2007), DVB[c]	IV
L. amylovorus GRL 1117 (LAB 31)	Swine jejunum (Jakava-Viljanen and Palva, 2007), DVB[c]	IV
L. amylovorus GRL 1118 (LAB 52)	Swine jejunum (Jakava-Viljanen and Palva, 2007), DVB[c]	IV
L. amylovorus DSM 16698	Swine feces (Konstantinov et al., 2006b), DVB[c]	IV
L. amylovorus DSM 20531^T	Fermented corn silage, DSM[b]	IV
Intestinal pathogens		
Escherichia coli ERF 2014; O141, F18+	DVB[c]	I, IV
Escherichia coli F4+ (ETEC)	(Roselli et al., 2007)	IV
Escherichia coli ATCC 43894; O157 (EHEC)	ATCC[a]	I, IV
Salmonella Typhimurium ATCC14028	ATCC[a]	I, IV
Listeria monocytogenes R14-2-2	DVB[c]	I, IV
Yersinia enterocolitica R5-9-1	DVB[c]	I, IV
Strains used in gene expression studies		
Escherichia coli DH5αF'	(Woodcock et al., 1989)	IV
Escherichia coli BL21 (DE3)	EMD Millipore	IV

[a] American Type Culture Collection
[b] Deutsche Sammlung von Mikroorganismen
[c] Culture collection of the Department of Veterinary Biosciences / Veterinary Microbiology and Epidemiology, University of Helsinki, Finland

Table 6. Primers used in this work.

Oligonucleotide sequence (5´- 3´)	T_m °C	Target gene / species	Reference	Study
Primers used in 16S rRNA amplification (I)				
f AGAGTTTGATCCTGGCTCAG r ACGGCTACCTTGTTACGACTT	55	16S rRNA / universal	Doré et al., 1998	I
Primers used in 16S rRNA sequencing: first 500 base pairs (I)				
f AGAGTTTGATCCTGGCTCAG r CACCGCTACACATGGAG	50	16S rRNA / universal 16S rRNA / *Lactobacillus*	Doré et al., 1998 Heilig et al., 2002	I
Additional primers used in whole 16S rRNA sequencing				
f CAGC(AC)GCCGCGGTAAT(AT)C r CCCCGTCAATTCCTTTGAGTTT f AAACTCAAAGGAATTGACGGGG r ACGGCTACCTTGTTACGACTT	50	16S / 18S rRNA / universal 16S / 18S rRNA / universal 16S / 18S rRNA / universal 16S rRNA / universal	Apajalahti et al., 2001 This study Doré et al., 1998	UB[1]
Primers used in PCR screening of fecal isolates (II, III)				
f CTGATGGTACAAAGGCAGGTT r CCAGCAGCATAGACTGTTGA	60	S-layer / *L. brevis*	This study	II
f TATGTCTGGCTTAAAAAGCACTTG r TCAATGTACTAACTCCTGACTTC	59	ISR / *L. amylovorus*	This study	III
f ACGGACTTGACGTTGGTTTA r CCGAAGCCATCTTTTAAATTTGA	59	16S rRNA / *L. mucosae*	This study	III
f ACGAAACTTTCTTACACCGAAT r GATCATGCGATCCTTAGAGATA	62	16S rRNA / *L. salivarius*	This study	III
f CTTGAATAACAAGCCAAGCATA r CCCATCATTGCCTTTTATCA	57	ISR / *L. johnsonii*	This study	III
f AACGGAACCTACACATCGAA r CCTTCATAACTTAACCTAAACAATC	62	ISR / *L. reuteri*	This study	III

Table 6 cont.

Oligonucleotide sequence (5´- 3´)	T_m °C	Target gene / species	Reference	Study
Primers used in real-time qPCR analysis (II, III)				
f TCCTACGGGAGGCAGCAGT r GGACTACCAGGGTATCTAATCCTGTT	58	16S rRNA / total bacteria	Nadkarni et al., 2002	II, III
f AGCAGTAGGGAATCTTCCA r CACCGCTACACATGGAG	60	16S rRNA / *Lactobacilli*	Rinttilä et al., 2004	III
Primers used in S-layer gene amplification (IV)				
f GCGGCCATGGATGCCGTTCAATCAGCTACT r CGGCTCGAGAAAGTTTGAAGCCTTAACGTAAG	62	*slpA* / *L. amylovorus* DSM 16698	This study	IV
f GCGGCCATGGCTGACAGCAATGAAACTGG r GCGGCTCGAGTTGAGCTGCGCTCAAGTTAG	63	*slpB* / *L. amylovorus* DSM 16698	This study	IV
f CGTAGCCATGGCAGATACTAATGCTGTAAACA AAATG r TCGCACTCGAGCTTCTTTGCGGCAGCTT	62	*slpC* / *L. amylovorus* DSM 16698	This study	IV
f AGTACCCATGGCTGCTACTACTATTAACGCTGGTT r TCGCACTCGAGAAAGTTTGAAGCCTTTACGTAA GT	63	*slpA* / *L. amylovorus* DSM 20351^T	This study	IV
f GCGGCCATGGACGTTAACACTAACATTGTTT r GCGGCTCGAGGAAGTTTGCCTTCTTTACGT	58	*slpA* / *L. amylovorus* GRL 1112	This study	IV
f GTCATCCATGGCTACTACAGTTAATATCAACGG TAAC r TCGCACTCGAGAAAGTTTGAAGCCTTAACGT	60	*slpA* / *L. amylovorus* GRL 1114	This study	IV
f GCGGCCATGGCTGACGCTACTACAACTACTAC r GCGGCTCGAGAAAGTTTGCAACCTTAACGTA	60	*slpA* / *L. amylovorus* GRL 1115	This study	IV
f CTACGCCATGGCTGACGCTACTACAACTACTAC TG r GTCTGCTCGAGAAAGTTTGAAGCCTTTACGTAA GT	60	*slpA* / *L. amylovorus* GRL 1116	This study	IV
r CTACGCCATGGCTGTAGCAATTAAGGGTAACA CT r GTAGTCTCGAGGAAGTTTGCCTTCTTAACGTAT TG	64	*slpA* / *L. amylovorus* GRL 1117	This study	IV
f CTACGCCATGGACACACCAACAGTTGATACTG r GTAGTCTCGAGTTGAGCTGCGCTCAAGTT	60	*slpB* / *L. amylovorus* GRL 1117	This study	IV
f GCGGCCATGGCTGATGCTACTAACAGTAATGC r CGGCTCGAGAAAGTTTGAAGCCTTAACGTAAG	62	slpA / *L. amylovorus* GRL 1118	This study	IV

[1]unbublished

4.3 *In vitro* assays for examining probiotic properties

4.3.1 Acid and bile tolerance (I)

The ability of the LAB isolates to tolerate low pH and bile was determined by exposing each isolate (OD_{600}: 1) to either pH-adjusted MRS broth (pH 2.0 or pH 4.0) or MRS broth containing 0.3% ox gall. The isolates were incubated in these stress conditions for three hours at 37°C in a microwell plate, after which a 7-step dilution series (dilution factor of 25) was generated in buffered MRS (acid tolerance test) or regular MRS (bile tolerance test) on the same microwell plate. Five parallel series were used for each isolate. Following anaerobic incubation, the wells were examined for growth and the OD600 values were measured (iEMS Reader MF; Thermo Labsystems, Finland). The size of the bacterial population surviving the exposure to each stress condition was estimated by the most probable number (MPN) method in MS Excel as described by Briones and Reichardt (1999).

4.3.2 Bacterial adhesion (I, IV)

For the adhesion assay performed in Study I, intestinal epithelial cells were collected from healthy pigs aged two to three months. The entire intestine was divided to the anatomical sections (duodenum, jejunum, ileum, proximal colon and distal colon), and the sections were washed with PBS until clean. The enterocytes were removed by mechanical disruption, washed two to three times by centrifugation, and resuspended in Hanks Balanced Salt Solution (HBSS; Gibco BRL, Life Technologies Inc., USA) supplemented with lactalbumin (Difco), fetal bovine serum (Gibco BRL), dimethyl sulphoxide (DMSO; Sigma-Aldrich, USA) and glycerol, and stored at -80°C until used in the adhesion assay. For the assay, the enterocyte suspension and the OD-adjusted LAB isolates (OD_{600}: 1) were incubated in a microwell plate, after which the unattached bacteria were removed by washing. Bacterial adhesion was quantified by examining Giemsa-stained microscopic slides under a light microscope (Leica; Microsystems GmbH, Germany) and calculating the average number of attaching bacteria per enterocyte, using twenty visually undamaged enterocytes. In the case when the number of adhering bacteria in one enterocyte exceeded twenty, it was scored as twenty in the calculations, because in such cases it was too difficult to calculate the exact number of bacteria. For each intestinal section, a positive and a negative control strain were selected from the isolates and the consistency of the adherence level of these was monitored in individual test runs.

For the mucus adhesion assay conducted in Study IV, porcine intestinal mucus was isolated from the small intestine of a two-months-old piglet with a protocol modified from MacAdam et al. (2000). Briefly, the intestinal sections washed with protease inhibiting saline solution were scraped gently to collect the mucus, after which the insoluble material and cellular debris were separated by centrifugation. The crude mucus was homogenized, concentrated by filtration, and clarified by centrifugation and several filtration steps. Purification of the mucus was performed by gel filtration chromatography monitoring the A280 nm values of 5 ml fractions. Protein-containing fractions were dialyzed against water and assayed for total protein by the method of Bradford (Bio-Rad Protein Assay, Bio-Rad, USA) using bovine serum albumin (BSA) as a standard, and for glycoproteins using the Crypton™ Glycoprotein Staining Kit (Thermo Scientific, USA), with porcine gastric mucins (Sigma), horse radish peroxidase (HRP) and soybean trypsin inhibitor (Thermo Scientific) as standards. The void volume fractions with high glycoprotein content were pooled, lyophilized and stored at -20°C until used in the adhesion assay. In addition to the porcine intestinal mucus, porcine gastric mucins (type II, Sigma) were used in the adhesion

assay. For the assay, the mucus was immobilized into polystyrene microtiter plate wells (Thermo Scientific). The *L. amylovorus* cells used in the mucus adhesion assay were labelled with a nucleic acid binding fluorescent stain by incubating the bacteria with SYTO®9 (Molecular Probes, USA), after which the OD-adjusted (OD_{600}: 0.1, 0.25, 0.5 and 1.0) bacterial suspension was added to the mucus coated wells. After incubation and removing the unbound bacteria by washing, the bound bacteria were lysed with sodium dodecyl sulfate (SDS) in NaOH, and the input (added) and output (remaining) fluorescence values were measured in a microplate reader (Victor Multilabel Plate Reader; Perkin Elmer, USA). The background fluorescence from mucus-coated wells without bacteria (for outputs) and from wells filled with PBS (for inputs) were subtracted from the values. The adherence was expressed as the proportion (%) of the original fluorescence added.

The cell adhesion assays conducted in Study IV were performed using IPEC-1 cells, a non-transformed continuous cell line (Gonzalez-Vallina et al., 1996). The cells were cultured at 39 ºC and 5% CO2 using Dulbecco's modified eagle medium/Ham's F12 Nutrient Mixture (DMEM/Ham's F-12 [1:1]) supplemented with fetal calf serum (FCS; Integro, Netherlands), insulin-transferrin-selenium, HEPES (all PAN-Biotech, Germany) and epidermal growth factor (BD). In the assay, the cells were seeded (2 x 10^5 /ml) into a Transwell system (Thincerts™, 1 µm pore size, diameter 10 mm; Greiner bio-one, Germany) and allowed to differentiate for 4-5 days, until the transepithelial electric resistance (TEER) value was ≥1 kΩcm2. The *L. amylovorus* strains used in the cell adhesion assay were metabolically labelled with 3H-thymidine, and added to the wells after OD-adjustment ($OD6_{00}$: 0.25, 0.5 or 1). After incubation, the unbound bacteria were removed by washing, the bound bacteria lysed with SDS in NaOH, and the radioactivity of the lysed samples (output) was measured by liquid scintillation counting. The input radioactivity values were determined by liquid scintillation counting of the OD-adjusted cell suspensions in DMEM/Ham's F-12 [1:1] treated with SDS in NaOH. The adherence was expressed as the proportion (%) of the original radioactivity added after subtracting the background radioactivity from IPEC-1 cells incubated without bacteria (for outputs) and from DMEM/Ham's F-12 [1:1] medium (for inputs).

4.3.3 Inhibition of pathogen adhesion (IV)

The ability of the *L. amylovorus* strains to inhibit the adhesion of the *E. coli* ETEC F4+ strain to IPEC-1 cells was investigated in the adhesion inhibition assay (IV). The ETEC strain was metabolically labelled with 3H-thymidine, and the expression of F4 fimbriae was confirmed with an agglutination test. Three different setups were used in the adhesion inhibition assay, with differing orders of addition of the bacterial cells to the IPEC-1 cells: 1) exclusion, where the lactobacilli (OD_{600}: 6) were allowed to adhere for 1 hour before adding the ETEC strain (OD_{600}: 0.6); 2) displacement, where the ETEC strain was allowed to adhere for 1 hour, and the unbound bacteria were removed with washing before the addition of lactobacilli; 3) competition, where both bacteria species were added simultaneously. In all of the assays, the cells were lysed after incubation and washing by SDS in NaOH, after which the radioactivity was measured by liquid scintillation counting. The proportion of adherent ETEC cells (%) in the presence or absence of the *L. amylovorus* strains was calculated as in the IPEC-1 cell adhesion experiments, and the inhibition (%) was calculated according to the formula: [adherence (no La) – adherence (with La)] / adherence (no La) x 100 %, where La indicates *L. amylovorus*.

4.3.4 Antimicrobial activity (I, III, IV)

The ability of the LAB isolates (I) and the *L. amylovorus* strains (IV) to inhibit the growth of selected intestinal pathogens was investigated with a turbidometric assay essentially as described by Skyttä and Mattila-Sandholm (1991), using filtration-sterilized (0.22 μm) spent culture filtrates (SCF) of the strains. The inhibitory effects of the SCF were examined by following the growth of the pathogens in TSB media supplemented (10% vol/vol) with the filtrates using an automatic reader Bioscreen C (Growth Curves Oy/Ab, Finland). Growth rate of the pathogens was quantified using the area under the growth curve (AUC) value of the logarithmic growth phase, which was automatically processed by the software (BioLink (Growth Curves Oy/Ab) or Research Express (Transgalactic Ltd., Finland)). Inhibition caused by the SCF was expressed as an area reduction percentage (ARP) calculated with the following formula: $(AUC_{Co} - AUC_{SCF}) / AUC_{Co} \times 100$, in which AUC_{Co} represents pathogen growth in TSB media supplemented with 10% MRS medium and AUC_{SCF} represents pathogen growth in TSB media supplemented with the culture filtrate. For each LAB strain assayed, one to three independent experiments with fresh SCF were performed, and in each assay two to three parallel wells were used.

In order to estimate the relationship between the ARP value and CFU counts, ten-fold dilution series prepared from each of the pathogens were incubated in the Bioscreen microwell plate, the ARP values of the dilutions were plotted against the corresponding logCFU reductions and linear regression was used to generate regression formulas for the pathogens, as described by Skyttä and Mattila-Sandholm (1991).

Two different approaches were adopted to monitor the effect of the filtrate pH on the pathogen growth inhibition. In the first approach, the culture filtrates collected from the LAB strains were categorized into six pH groups, and for each group two different pH controls were used in the Bioscreen assay: MRS medium with pH adjusted to the group mean either with lactic acid or with HCl. The second approach involved using both pH-adjusted (pH 6.2) and non-adjusted culture filtrates.

To verify the lack of antagonistic effects of the *Lactobacillus* strains used in Study III towards each other, the ability of each strain to grow in the presence of the SCF collected from the other strains was monitored with the turbidometric Bioscreen assay with a similar experiment setup as described for the intestinal pathogens.

4.3.5 Stimulation of cytokine production by dendritic cells (IV)

Monocyte-derived dendritic cells (MoDC) were generated from purified and *in vitro* cultured human monocytes using a method described by (Veckman et al. (2004) with minor modifications. Briefly, peripheral blood mononuclear cells were first isolated from leukocyte-rich buffy coats by two density gradient centrifugations and magnetic beading using anti-CD3 and anti-CD19 beads (Dynal Invitrogen, Life Technologies, USA). Thereafter, the monocytes were allowed to adhere to 24-well plates (Falcon, BD, Franklin Lakes, New Jersey) in the presence of RPMI 1640 (Sigma) supplemented with HEPES, penicillin, streptomycin and L-glutamine, and differentiation was induced by maintaining the cells in RPMI 1640 (with supplements as described above) containing FCS, human recombinant granulocyte macrophage-colony stimulating factor (GM-CSF; Gibco Life Technologies, USA), and human recombinant IL-4 (Gibco Life Technologies). MoDCs were used on day 7 in the experiments, with cells of four donors being were used in each experiment. The OD-normalized *L. amylovorus* strains were added to human moDCs (multiplicity of infection (MOI) 1, 10, and 100), using medium without bacteria as a control.

After incubation, the cell culture supernatants were collected and stored at -20°C until analyzed by Bio-Rad's Bio-Plex Pro Cytokine assay using the Bio-Plex -200 platform (Bio-Rad, Hercules, CA). Quantification of human TNF-α, IL-1β, IL-6, IL-10, and IL-12 was performed according to the manufacturer's instructions. Human IP-10/CXCL10 was measured separately with the OptEIA ELISA kit (BD, Franklin Lakes, New Jersey) using samples diluted with sample matrix RPMI 1640 medium.

4.3.6 Antimicrobial susceptibility testing (III)

The antibiotic resistance of the *Lactobacillus* strains used in Study III was investigated against five antibiotics: G-penicillin, kanamycin, vancomycin, tobramycin and enrofloxacin. Eight-step dilution series (dilution factor of two) were prepared from the antibiotics in MRS to the honeycomb microwell plates (Growth Curves Oy/Ab), with the highest concentration of the antibiotic being as follows: 16 µg/ml for G-penicillin, 1024 µg/ml for kanamycin and vancomycin, 512 µg/ml for tobramycin and 128 µg/ml for enrofloxacin. Turbidity-adjusted lactobacilli (Mac Farland standard: 0.5) were inoculated (10% vol/vol) into the wells, and the growth of the strains was followed for 48h with a Bioscreen C (Growth Curves Oy/Ab).

4.4 *In vivo* feeding trial (II, III)

4.4.1 Trial design and sample collection (II, III)

The bacterial strains used in the porcine feeding trial are listed in Table 5. For the trial, the cell density of *L. brevis* ATCC 8287 was adjusted to 10^{10} CFU/ml, and the cell density of each strain (n=6) included in the multispecies supplement was adjusted to 1.67 x 10^9 CFU/ml (total bacterial cell count 10^{10} CFU/ml). The density adjusted cell suspensions were aliquoted as 1 ml glycerol stocks, and stored at -80°C until used in the trial.

Thirty commercially-bred piglets of both genders (12 Finnish Landrace, 6 Finnish Yorkshire, and 12 backcrossing of Finnish Landrace X Finnish Yorksire sow and Finnish Landrace or Finnish Yorkshire boar) with mean body weights of 9.6 ± 1.7 kg were used in the trial. After weaning at 4-5 weeks of age, the piglets were transported to the animal facilities of the National Veterinary and Food Research Institute EELA (later Finnish Food Safety Authority Evira). Based on litter origin, the piglets were divided into three groups (n=10), each of which was housed in similar but separate holding facilities and allowed to acclimatize for 12 days. During the three week feeding trial, the two treatment groups received daily a piece of wheat bun containing the thawed aliquot of the supplemented bacteria (*L. brevis* or the multispecies preparation), while the control group was provided with a wheat bun to which a 1-ml aliquot of PBS and glycerol had been added. The feeding trial was approved by the ethics committee of National Veterinary and Food Research Institute EELA (No 5/03).

The health status of the piglets as well as any signs of possible GI disturbances were monitored daily. Individual weighing as well as sample collection was performed according to the timeline shown in Figure 4. Serum was separated from the blood samples (II), and stored at -80°C. Fecal samples (II, III) were processed immediately for bacterial culturing or stored at -80°C for real-time qPCR. After slaughtering, the intestinal luminal pH was measured (II, III) after which digesta samples were collected (II, III) from four compartments of the intestine; the jejunum (2-4 meters cranially from the ileocecal junction), the ileum (2 meters cranially from the ileocecal junction), the cecum, and the spiral colon, and stored at -80°C. For the pathological investigation (II, III), pieces of intestinal walls were cut from the same compartments and

immersed in 10% buffered formalin. Additionally, biopsies for immunofluorescence studies (II) were also cut from the same locations, as well as from the jejunal and ileal Peyer's patches, and stored at -80°C. For the cytokine assay (II, III), mucosal samples from the four intestinal compartments were scraped from the intestine wall with a scalpel, resuspended in the RNA stabilization reagent RNAlater (Ambion, USA), and stored at -20°C.

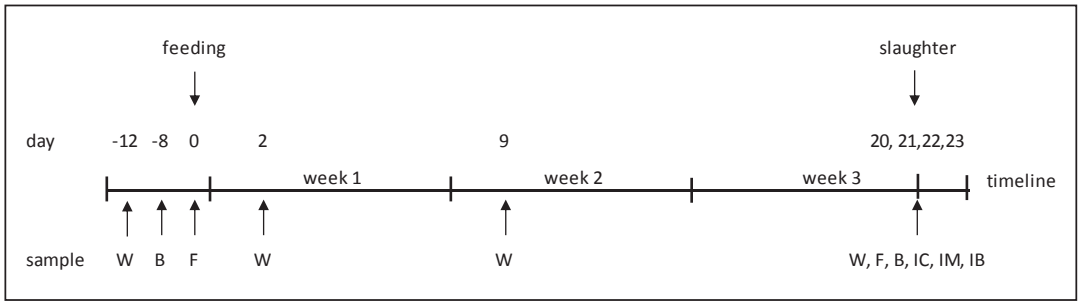


Figure 4. Weighing and sample collection schedule during the porcine feeding trial. W = weighing, F = fecal sample, B = blood sample, IC = intestinal contents (digesta) sample, IM = intestinal mucosal sample, IB = intestinal biopsy sample

4.4.2 *Detection of the supplemented strains in intestinal and fecal samples (II, III)*

Culture techniques were used to investigate if the lactobacilli strains fed to the piglets would be able to survive through the porcine intestine (II, III). Prior to culturing, pooled fecal samples were generated by combining either all ten individual pre-feeding samples within the study groups or two to four individual samples per group from pigs that had been slaughtered on the same day. Pooled samples were cultured in MRS liquid and solid media, and colonies with a morphology resembling the supplemented strains were selected; 14 colonies from the pre-feeding samples from each group, as well as 30 (control and multispecies groups) and 42 (*L. brevis* group) colonies from the slaughter samples. The isolates were identified with species-specific PCR analysis using Dynazyme II DNA Polymerase and primers listed in Table 6.

To confirm the identity of the fecal isolates, some of the PCR products were further selected for sequencing (III). The purified PCR products were sequenced with the original amplification primers and Big Dye Terminator v3.1 Cycle Sequencing Reaction Kit (Applied Biosystems, USA). After precipitating and washing, the PCR products were resuspended in Template suppression reagent (Applied Biosystems, USA) and evaluated by ABIPrism 310 Genetic Analyzer (Applied Biosystems). The sequences obtained were analyzed by comparison with the GenBank sequence file.

To determine whether the fecal isolates represented the same strains as those fed to the piglets, selected isolates were further analyzed with pulsed-field gel electrophoresis (PFGE), essentially as previously described by Vihavainen and Björkroth (2009) (III). Agarose embedded bacterial cells were lysed with lysozyme (Sigma, USA), mutanolysin (Sigma) and proteinase K (Finnzymes), after which the DNA was digested with *Sma*I endonuclease (New England Biolabs, USA). The separation of restriction fragments was performed on SeaKem Gold agarose (Lonza Rockland) at 14°C and 6 V/cm with the PFGE switching and running times as follows: 0.5-6 s and 16 h (*L. reuteri*); 0.5-10 s and 16 h (*L. johnsonii*); 0.5-15 s and 18 h (*L. mucosae*); 0.5-20 s and

20 h (*L. amylovorus* and *L. salivarius*). Images of ethidium bromide-stained gels were captured, and the pattern similarity was estimated visually. The image of *L. reuteri* run was imported into BioNumerics (version 5.1; Applied Maths, Sint-Martens-Latem, Belgium), the gel was normalized against the low-range PFG marker (New England Biolabs), and a dendrogram of PFGE patterns was constructed using the unweighted pair group method with arithmetic averages (UPGMA) and the band based Dice similarity coefficient with a position tolerance of 1.8%.

The levels of the fed *L. brevis* strain in the digesta samples as well as in the fecal samples taken at slaughter were analyzed with real-time qPCR (II) using the same S-layer gene-flanking primers as employed in the analysis of the isolates cultured from the fecal samples. The bacterial cell lysis, DNA isolation and real-time qPCR amplifications were performed as described in section 4.4.3. Ten-fold dilution series of 0.1 pg to 10 ng of *L. brevis* genomic DNA were used as standards in conjunction with the intestinal DNA samples in the same real-time qPCR run.

Indirect immunofluorescence assay (IFA) was used to localize the *L. brevis* cells in different intestinal compartments (II). Thin-sections cut from the Tissue-Tek O. C. T. (Sakura Finetek, USA) embedded frozen intestinal wall pieces were fixed with paraformaldehyde and treated with a blocking solution containing BSA (Sigma Life Sciences, USA) and incubated with rabbit anti-SlpA serum. After removing unbound antibodies by washing, further incubation with goat anti-rabbit Alexa Fluor 488 F(ab')2 Fragment secondary antibody (Life Technologies, USA) was performed, after which the sections were embedded in ProLong Gold Antifade Reagent (Life Technologies, USA) including DAPI (4', 6-diaminido-2-phenylindole dilactate) nucleic acid stain for recognizing epithelial cell DNA. A Leica DM 4000 B microscope (Leica Microsystems, USA) equipped with epifluorescence excitation modules was used to examine the stained sections. The presence of *L. brevis* was determined by two independent investigators counting those fluorescent bacteria that resembled the *L. brevis* cell staining morphology in 20 randomly selected fields (in three to five sections) at 400x magnification. Controls were obtained by omitting the primary antibody. For histological clarity parallel sections were stained with hematoxylin and eosin. Confocal images were taken with a Leica TCS SP5 microscope (Leica Microsystems, Germany) using the Leica Application Suite Advanced Fluorescence Lite 2.6.0 (LAS AF Lite; Leica, Germany) software. The images were studied with the public domain software Image J.

4.4.3 Quantification of total bacteria and the genus Lactobacillus from digesta and fecal samples (II, III)

The fecal and intestinal digesta samples collected from the piglets at slaughter were analyzed for the abundance of total bacteria (II, III) and the genus *Lactobacillus* (III) with real-time qPCR. Bacterial cells were collected from the digesta or feces by centrifugation, lysed by incubation with proteinase K, and disrupted by bead beating twice in a FastPrep®-24 cell disruption instrument (MP Biomedicals, Inc., USA) with sterile glass beads (Ø=0.1 mm; Scientific industries inc., USA). Genomic DNA was isolated from the samples by phenol-chloroform-isoamyl alcohol extraction and chloroform-isoamyl alcohol purification with standard methods. The template DNA was diluted 1:1000 or 1:100 for the real-time qPCR amplifications performed with the primers listed in Table 6, and SYBR Green Master Mix (Roche Diagnostics, Germany). A melting curve analysis was carried out in conjunction with each amplification run by slowly cooling from 95°C to 60°C, with fluorescence collection at 0.3°C intervals. Ten-fold dilution series of 0.1 pg to 10 ng of *L. crispatus* genomic DNA were used as a standard in conjunction with the intestinal DNA samples in the same real-time qPCR run.

4.4.4 Detection of serum immunoglobulins by ELISA (II)

Serum samples taken before the start of the feeding trial and at slaughter were analyzed for total IgA and IgG concentrations using a commercial pig IgA or IgG ELISA Quantitation Kit (Bethyl Laboratories, USA), according to the manufacturer's instructions. Two-fold serial dilutions were prepared from the serum samples (from 1:400 to 1:6400 for IgA and from 1:20 000 to 1:160 000 for IgG). The dilutions of HRP conjugate used in the assays were 1:120 000 for IgA and 1:20 000 for IgG. The optical density at 450 nm was measured with a Multiskan EX device (Thermo Scientific, Finland) and Sigmoid logistics was utilized to calculate the Ig concentrations of the samples from the linear range of the calibration curve (Ascent Software for iEMS and Multiskan EX; Thermo Scientific, Finland).

L. brevis-specific IgG antibodies were determined from the serum samples taken at slaughter with a whole-cell ELISA method essentially as described by Lindholm et al. (2004). Briefly, frozen pellets of *L. brevis* cells were resuspended in serum samples diluted in PBS (1:10) and then incubated. The unbound antibodies were removed by washing, and 1:1000 diluted HRP-conjugated goat anti-pig IgG (Bethyl Laboratories) was added. After incubation and washing, the cells were resuspended in substrate buffer and loaded on microwell titer plates. 3,3',5,5'-Tetramethylbenzidine (TMB; Sigma, Germany) was used as the substrate solution as described by Laitinen et al. (2002). The concentration values of specific IgG were expressed as arbitrary units (AU) per milligram total IgG.

4.4.5 Cytokine gene expression in the intestinal mucosa (II, III)

Preliminary analysis of the expression of selected immunological genes in the intestinal mucosal samples of five piglets from both the control group and the *L. brevis* supplementation group (II) was performed essentially as described by Solano-Aguilar et al. (2008). The genes chosen for the analysis included those of cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p35, IL12p40, IL-18, TNF-α, IFN-α, IFN-γ), immunological receptors (TLR-9, IL-12-RB2), and the adapter protein MyD-88 as well as the transcription factor NF-κB. Total RNA was extracted from the homogenized mucosal samples with a phenol/chloroform-based method (Trizol reagent; Invitrogen, USA), after which the samples were treated with DNase in the presence of RNase inhibitor (Ambion, USA). The absence of genomic DNA was confirmed with a real-time qPCR amplification of the ribosomal protein L32 (RPL32) housekeeping gene. Quantification of the RNA was performed using Bioanalyzer 2100 and the RNA 6000 Kit (Agilent Technologies, USA), and 7.5-10 μg of RNA was used for cDNA synthesizing with SuperScript II RT (Invitrogen) and oligo(dT) (Invitrogen), with 50 ng of this cDNA being used for the real-time qPCR amplifications performed with an iCycler iQ Real-Time Detection System (Bio-Rad, USA). The primers and probes used in the real-time qPCR runs can be found in the DGIL Porcine Translational Research Database available at http://www.ars.usda.gov/services/docs.htm?docid=6065. Data for each gene assayed were adjusted for the housekeeping gene RPL32, and the adjusted values were analyzed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001), with control group piglets as the comparison group.

The gene expression levels of eight selected cytokines, i.e., IL-1β, IL-4, IL-6, IL-8, TGF-β1, IFN-α, IFN-γ and TNF, were further analyzed (II, III) with the QuantiGene 2.0 Plex Assay (Affymetrix/Panomics, USA), according to the manufacturer's instructions. This assay combines branched DNA signal amplification and multianalyte bead technologies, and enables quantification of mRNA signals without an RNA isolation step. Homogenization of the samples

was done in a homogenization solution (QuantiGene 2.0 Sample Processing Kit; Affymetrix/ Panomics) using a FastPrep®-24 instrument (MP Biomedicals, Inc.) with a CryoPrep adapter™ (MP Biomedicals, Inc.) and zirconium–silica beads (Ø=0.1 mm; Biospec Products, USA). After incubation at 65°C, the supernatants were collected with centrifugation and used in the assay. Samples were run with a Bio-Plex 200 array system (Bio-Rad Laboratories, USA) using the low RP1 target values. After subtracting the background fluorescence from the values obtained for the test samples, each gene marker was normalized to the geometric means of hypoxanthine phosphoribosyltransferase 1 (HPRT1) and peptidyl-prolyl cis-trans isomerase B (PPIB) housekeeping genes. Normalized values were averaged for treatment groups, and the fold change calculated using the control group as the comparison.

4.5 The surface layer proteins of *Lactobacillus amylovorus* strains (IV)

4.5.1 Genes encoding the S-layer proteins: Detection and expression analysis (IV)

The putative *slp* genes were identified *in silico* in the draft genomes of the *L. amylovorus* strains based on homology with *L. acidophilus slp* gene sequences. Standard SDS-PAGE analysis of the *L. amylovorus* strains was used to estimate the molecular weights of the S-layer proteins. The expressed *slp* genes were identified on the bases of these molecular weights, and additionally, using aminoterminal sequencing and/or peptide mapping analysis performed at the Institute of Biotechnology (University of Helsinki, Finland).

4.5.2 Adherence of the S-layer protein-coated cell wall fragments (IV)

The role of the *L. amylovorus* Slp:s in adherence to IPEC-1 cells was examined using a protein presentation system based on purified *L. amylovorus* cell wall fragments (CWF) as S-layer protein carriers. The S-layer proteins used in the adhesion assays were produced by cloning the expressed *slp* genes as *Nco*I-*Xho*I –fragments in *E. coli* DH5αF' and expressing the proteins in *E. coli* BL21 (DE3) as C-terminal hexahistidine tag-fusions, as described in the pET system manual (Merck KGaA, Germany) and as previously reported by Åvall-Jääskeläinen et al. (2008). The oligonucleotide sequences used in the PCR reactions, carried out with Phusion High-Fidelity DNA polymerase (Thermo Scientific), are listed in Table 6. After purification with His Trap HP columns (GE Healthcare, UK), the pooled protein fractions were dialyzed against deionized water, centrifuged and stored in aliquots at -80°C until used to coat the cell wall fragments (CWF) purified from the *L. amylovorus* cells as described by Åvall-Jääskeläinen et al. (2008). Before coating, the recombinant S-layer proteins were solubilized in guanidine hydrochloride (GuHCl), dialyzed, and centrifuged to remove large protein aggregates. After incubating the S-layer proteins with the CWF (ratio 1:4 (W/W)), the coated CWF were collected by centrifugation and analyzed by SDS-PAGE in order to estimate the success of the coating. The absence of large protein aggregates was verified by examining the uranyl acetate stained preparations by JEOL 1200-EX II transmission electron microscope. The uncoated CWF used as controls were labeled by EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. The Slp-coated or uncoated CWF were added to the IPEC-1 cells grown on Thincert™ wells and incubated, after which the unbound material was removed by washing and the IPEC-1 cells were fixed with paraformaldehyde. The Slp-coated CWF were detected by indirect immunofluorescence staining with Slp-specific immunoglobulins (GE Healthcare, UK) and AlexaFluor488-conjugated secondary antibodies

(Life Technologies), and the uncoated cell walls were detected by staining with AlexaFluor488-conjugated streptavidin (Life Technologies). The bottoms of the Thincert™ wells were observed in a Leica DM 4000B epifluorescence microscope (Leica Microsystems, Wetzlar, Germany). The mean number of adherent CWF was quantified from 20 randomly selected fields of 3.5×10^4 μm^2, and representative photographs were taken with the Olympus DP70 digital camera system with the cellP imaging software (Olympus Corp., Tokyo, Japan).

4.6 Statistical analyses (I-III)

SPSS versions 12.0.1 (I), 18.0 (II) or 21.0 (III) for Windows (SPSS Inc., Chicago, IL, USA) were used for statistical analysis of the data. As the tests of normality showed that the data did not follow a normal distribution (I), or the datasets were too small to assess the distribution (II , III), nonparametric tests were utilized in the analyses. The p-values ≤ 0.05 were considered significant.

In order to estimate differences between several test groups, the Kruskall-Wallis test (for independent samples) or Friedman's test (for dependent samples) was applied (I), with suitable post hoc analysis. When appropriate, the Bonferoni correction was used when estimating statistical significance. Differences between two test groups were estimated with the Mann-Whitney U test (I, II, III), or, for related samples within the groups, the Wilcoxon Signed-Rank test (II). For categorical data, cross-tabulation with Pearson Chi-Square test or Fisher's exact test was used (I). Principal component analysis (PCA) was used to reduce the dimensions of the data when the antimicrobial assay results were interpreted (I), and regression formulas for the relationships between ARPs and CFU counts were calculated for each indicator organism used in the antimicrobial activity tests (I, IV).

5 Results and discussion

5.1 Isolation and identification of lactic acid bacteria (I, III)

In total, 29, 26, 31 and 8 LAB strains were isolated from the swine duodenum, jejunum, ileum and feces, respectively. The isolates were putatively identified as *Lactobacillus* strains with a PCR-ELISA assay, and partial sequencing of the 16S rRNA gene confirmed this identity for 89 isolates, while five of the isolates were identified as *Streptococcus alactolyticus* (GenBank accession numbers for the best BLAST hits are shown in Table 1 in I). The identification of 11 isolates was further confirmed with sequencing of the whole 16S rRNA gene. The largest identification groups were *L. reuteri* (n=46), *L. salivarius* (n=14), *L. mucosae* (n=10), *L. johnsonii* (n=8) and *L. amylovorus* (n=6), all of which have previously been identified as abundant phylotypes in the swine intestinal microbiota (Leser et al., 2002; Mann et al., 2014).

For the swine originating strains used in the *in vivo* feeding trial (Study III), the WGS confirmed the preliminary identification based on 16S rRNA sequencing (Kant et al., 2011b, personal communication Kant R.).

5.2 *In vitro* probiotic properties

5.2.1 Acid and bile tolerance (I)

The results of each stress tolerance test were categorized initially into four classes: good, moderate, poor and no survival (Table 7). For the Pearson Chi-Square test analysis of the acid tolerance test results, the categories of moderate and good survival were combined, as were the categories of poor or no survival to minimize the number of cells with an expected count less than five in the cross tabulation table.

The majority of the isolates tolerated exposure to bile and pH 4.0 well with no significant reductions in MPN values, whereas more variation in tolerance to pH 2.0 was observed (Table 7). This is in line with previous studies performed with lactobacilli of swine origin, reporting typically good tolerance to pH 3.0 to 4.0, whereas pH 2.0 has been more poorly tolerated by the isolates (De Angelis et al., 2006; Yun et al., 2009; Guo et al., 2010; Zhang et al., 2013). Considering the influence of the isolation site of the strain on the acid and bile tolerance, strains sourced from the human GI tract have been reported to tolerate low pH and bile better than those originating from other environments (Morelli, 2000; Upadrasta et al., 2011). However, to the knowledge of the author, the putative differences between strains isolated from different intestinal compartments have not been investigated previously. In this work, the intestinal isolates tended to tolerate pH 2.0 better than the fecal isolates (Pearson Chi-Square $p=0.062$), but the tolerance to pH 4.0 (Pearson Chi-Square $p=0.247$) or to bile (Pearson Chi-Square $p=0.943$) did not differ according to the isolation sites. At the species level, the largest identification group, namely *L. reuteri*, tolerated pH 2.0 (Fisher's exact test $p<0.0005$) and bile (Fisher's exact test $p=0.004$) better than the others, but no such difference was observed with respect to pH 4.0 tolerance (Fisher's exact test $p=0.361$). However, there was extensive variation between strains of the same species, a phenomenon observed also in other studies comparing multiple strains of one single species isolated from various environments, including swine intestine and different foodstuffs (Parente et al., 2010; Zhang et al., 2013). Although specific strains of *L. reuteri* isolated from swine have been previously shown to be resistant to exposure to low pH and bile (Lee et al., 2009; Guo et al., 2010; Seo et al., 2010), no systematic comparison to other species of lactobacilli has been performed.

Table 7. Proportion (%) of the lactobacilli isolates in each stress survival category according to the origins of the isolates. The values in brackets represent the number of isolates.

	Stress survival category											
	Good[a]			Moderate[b]			Poor[c]			No[d]		
Isolation site	pH 2.0	pH 4.0	Bile	pH 2.0	pH 4.0	Bile	pH 2.0	pH 4.0	Bile	pH 2.0	pH 4.0	Bile
Duodenum	41.4 (12)	62.1 (18)	86.2 (25)	20,7 (6)	37.9 (11)	13.8 (4)	6.9 (2)	0	0	31.0 (9)	0	0
Jejunum	15.4 (4)	57.7 (15)	80.8 (21)	26.9 (7)	30.8 (8)	19.2 (5)	11.5 (3)	7.7 (2)	0	46.2 (12)	3.8 (1)	0
Ileum	26.7 (8)	56.7 (17)	83.3 (25)	30.0 (9)	36.7 (11)	16.7 (5)	3.3 (1)	6.6 (2)	0	40.0 (12)	0	0
Feces	0	75.0 (6)	87.5 (7)	12.5 (1)	25.0 (2)	12.5 (1)	0	0	0	87.5 (7)	0	0

[a] no reduction in the MPN value compared with the control
[b] a maximum of 3 logs reduction in the MPN value
[c] a maximum of 6 logs reduction in the MPN value
[d] more than 6 logs reduction in the MPN value

5.2.2 Adhesion (I, IV)

The adhesion capacity of the LAB isolates to swine enterocytes collected from the small intestine (i.e. duodenum, jejunum and ileum) as well as from the large intestine (i.e. proximal and distal colon) was very variable (Table 2 in I), with better adherence to the colonic enterocytes compared to those from small intestinal origin (Friedman test $p<0.001$; Fig. 1 in I). Examples of adherent and non-adherent strains are shown in Figure 5. The high strain-to-strain variability with respect to lactobacillar adherence has also been observed in previous studies (Lin et al., 2007; Guo et al., 2010), but the possible variation in adhesion to enterocytes collected from different intestinal sections has rarely been addressed before this study. Barrow et al. (1980) observed that two swine originating LAB isolates (identified as *L. fermentum* and *Streptococcus salivarius*) adhered to the squamous epithelial cells of the mouth, esophagus and stomach, but not to the columnar epithelium of the stomach, duodenum, ileum or cecum. In addition, Li et al. (2008) compared intestinal mucus isolated from the three compartments of the small intestine (i.e. duodenum, jejunum and ileum) in terms of lactobacillar adhesion, and adhesion to ileal mucus was found to be more efficient than that to duodenal or jejunal mucus. In addition to the origin of the enterocytes, also the isolation site of the strain could affect its adherence pattern, and better adhesion to that part of the intestine from which the strain was originally isolated might be anticipated. While some previous studies have described these kinds of adhesion preferences (Barrow et al., 1980; Jin et al., 1996), in this study the origin of the isolate did not seem to influence its adhesion properties to enterocytes collected from the different intestinal sections.

At the species level, those isolates identified as *L. reuteri* showed a generally good adherence capacity, with a statistically significantly higher level of adhesion to duodenal enterocytes compared to *L. amylovorus* and *L. mucosae* (Kruskall-Wallis $p=0.05$; Fig. 2A in I). In addition, the adhesion of *L. reuteri* isolates to the proximal colon cells was better than that of *L. johnsonii* or *L. amylovorus* isolates (Kruskall-Wallis $p=0.024$; Fig. 2D in I) and to the distal colon cells better than that of *L. johnsonii* isolates (Kruskall-Wallis $p=0.032$; Fig. 2E in I). Although certain strains of *L. reuteri* have shown very good *in vitro* adherence to different intestinal cells (Guo et

al., 2010; Zhao et al., 2012; Zhang et al., 2013), no proper comparison to other species has been performed.

The adhesion capacity of the *L. amylovorus* isolates (n=8) to porcine mucus as well as to IPEC-1 cells were further characterized. All of these strains showed relatively poor adherence to porcine gastric mucins as well as to isolated intestinal mucus of swine origin, as typically around 1% to 2% of the original amount of cells remained mucin-bound (Fig. 1 in IV). In previous studies strains with such a low-level adherence have been considered as non-adhering (Kirjavainen et al., 1998; Ouwehand et al., 1999; Collado et al., 2005). In addition, the lack of consistent dose-response of binding, and the high variability in different replications of the adhesion experiments point to non-specific binding due to hydrophobic interactions, which are known to interfere with interpretation of mucus binding studies (Laparra and Sanz, 2009; Van Tassell and Miller, 2011). Despite the lack of mucus binding, the *L. amylovorus* GRL-strains have previously been shown to adhere to ECM proteins (Jakava-Viljanen and Palva, 2007). The adherence of the *L. amylovorus* strains to IPEC-1 cells showed more variability as well as dose-responsivity in comparison to the mucus adhesion results. The previously reported adhesion of *L. amylovorus* DSM 16698 (Roselli et al., 2007) was confirmed, and the adhesion of strains GRL 1112 and GRL 1115 was at a similar level (Fig. 2 in IV). When comparing the adhesion results obtained for the GRL-strains determined with either IPEC-1 cells or isolated porcine enterocytes, it seems that that these methods do not correlate well with each other. Those strains displaying the highest adhesion ability to IPEC-1 cells (namely GRL 1112 and GRL 1115) were poorly adhesive to isolated small intestinal enterocytes, although strain GRL 1115 adhered strongly to colonic enterocytes (Table S1 in I). On the other hand, the strain exhibiting the best adherence to the enterocytes isolated from the small intestine (namely GRL 1114; Table S1 in I), adhered only weakly to the IPEC-1 cells (Fig. 2 in IV).

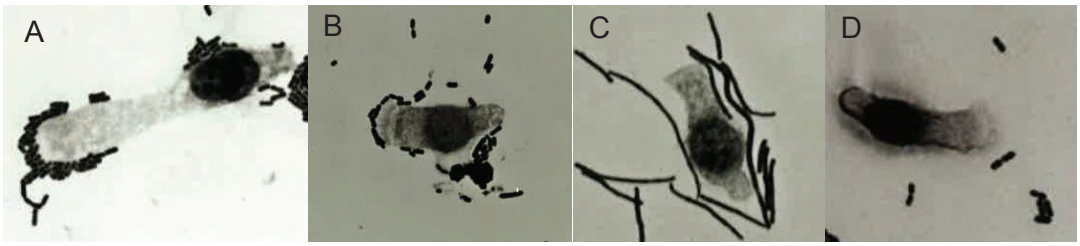


Figure 5. Examples of adhering (A, B) and non-adhering (C, D) lactobacilli. Enterocytes collected from porcine duodenum (A) and ileum (B-D).

5.2.3 *Inhibition of pathogen adhesion (IV)*

The ability of the *L. amylovorus* strains to inhibit the adhesion of *E. coli* ETEC F4+ to IPEC-1 cells was investigated with three different experimental set-ups (i.e. competition, exclusion and displacement). In both the competition and in the exclusion assays, four of the *L. amylovorus* strains (DSM 16698, GRL 1112, GRL 1115 and GRL 1118) were able to clearly inhibit the adhesion of the *E. coli* ETEC F4+ (Fig. 3A and 3B in IV), but no inhibition of ETEC adhesion was detected in the displacement assay (Fig. 3C in IV). Several previous studies have evaluated the ability of lactobacilli to inhibit pathogen adhesion (Jin et al., 2000; Fernandez et al., 2003; Bogovic Matijasic et al., 2006; Collado et al., 2007; Dhanani and Bagchi, 2013; Zhang et al., 2013), and these three different set-ups have commonly been used. In the competition assay,

simultaneous addition of the *Lactobacillus* and the pathogen will result in competition for the previously empty binding sites, while the other two set-ups explore the possible displacement of previously bound bacteria. In the exclusion assay, *Lactobacillus* cells are allowed to bind to the target before the addition of the pathogen, while the reversed order of adding the different bacteria is used in the displacement assay. When considering that lactobacilli are normal residents of the swine intestinal microbiota, i.e. they are continuously present, whereas the presence of pathogens is usually transient in the swine gut, it may be that the exclusion assay which assesses the ability of lactobacilli to protect intestinal cells from becoming colonized by pathogens is the most appropriate simulation of the *in vivo* situation. Moreover, it might be anticipated that the greatest inhibition of pathogen adhesion by lactobacilli would be obtained in the exclusion and / or competition assays, while displacement of pathogens already attached to the intestinal structures would be less likely to occur. Indeed, this was observed with the *L. amylovorus* strains used here, as inhibition of ETEC adhesion was observed in the competition and exclusion assays, but none of the strains were able to remove previously bound ETEC from IPEC-1 cells. A higher inhibition of pathogen adhesion in the exclusion and / or competition assays compared to the displacement assay has been noted also in some previous studies (Jin et al., 2000; Fernandez et al., 2003; Bogovic Matijasic et al., 2006; Zhang et al., 2011), but not in others (Collado et al., 2007; Dhanani and Bagchi, 2013).

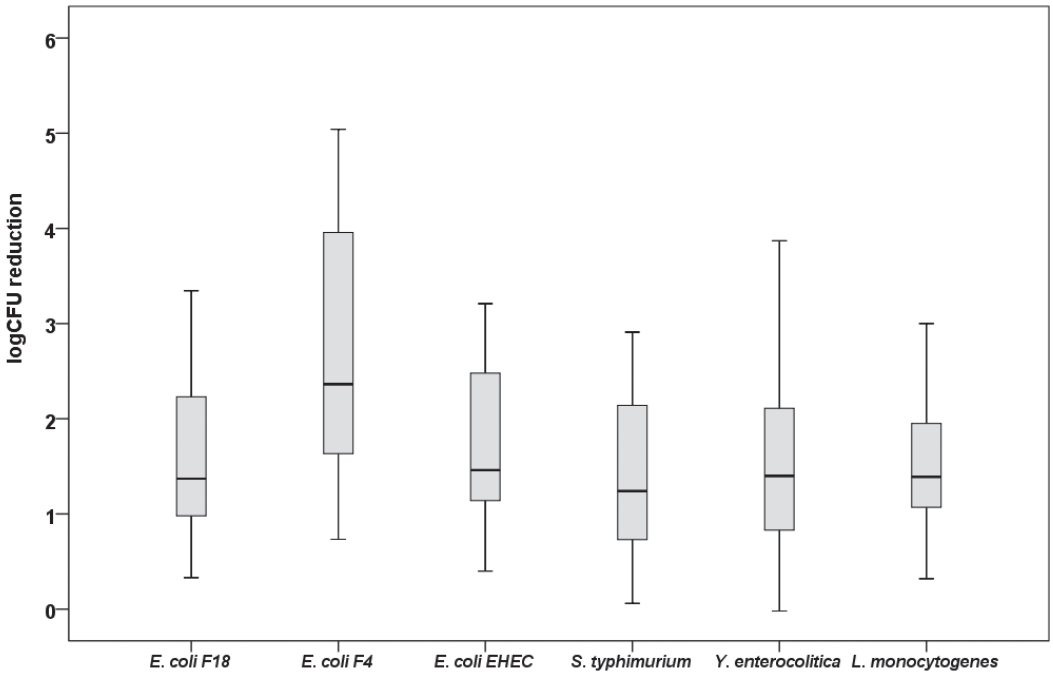
As competition for the same adhesion sites is believed to be one mechanism behind pathogen exclusion, the ability to inhibit the adhesion of pathogens could be expected to correlate with adhesiveness of the strain. To some extent, this was the case with the *L. amylovorus* strains used in this study, as three (namely DSM 16698, GRL 1112 and GRL 1115) of the four strains which inhibited ETEC adhesion also exhibited adhesion ability to IPEC-1 cells. The inhibition of pathogen adhesion demonstrated by the poorly adherent strain GRL1118 suggests that in addition to competition for adhesion receptors other mechanisms must also be involved in the inhibition. These could include coaggregation with the pathogen or secreted inhibitory factors (Lebeer et al., 2008). The culture filtrate of the strain GRL 1118 did not inhibit the growth of the ETEC strain more than those of the other GRL strains (see 5.2.4), but the possible production of substances specifically capable of inhibiting adherence was not investigated.

5.2.4 Antimicrobial activity against intestinal pathogens (I, IV)

The spent culture filtrates collected from the LAB isolates were assayed against six indicator pathogens (I, unpublished results). The growth inhibitions caused by the SCF were quantified using the area under the growth curve (AUC) value, which combines several points of time in the indicator growth curve, and has been shown to be a reliable parameter for determining changes occurring during bacterial growth (Skyttä and Mattila-Sandholm, 1991). The log CFU reductions in pathogen numbers caused by the culture filtrates were estimated using the regression formulas generated for each pathogen.

In accordance with previous studies estimating the antimicrobial properties of porcine lactobacilli (du Toit et al., 2000; Chang et al., 2001; De Angelis et al., 2006; Kim et al., 2007; Lin et al., 2007; Guo et al., 2010; Klose et al., 2010a; Klose et al., 2010b), a variable degree of inhibition in the growth of the indicator pathogens was caused by the SCF collected from the porcine LAB (I, unpublished results). When the different indicator pathogens were compared against each other, *E. coli* ETEC F4+ and *E. coli* EHEC were inhibited statistically significantly more than the other pathogens (Friedman test $p<0.001$; Fig. 6). The culture filtrates of the eight *L.*

amylovorus strains were also assayed against the same pathogens (IV), and similarly, the growth of *E. coli* ETEC F4+ was the most efficiently inhibited by these SCF (Fig. 4 in IV). As far as is known, the magnitude of growth inhibition exhibited by different indicator pathogens has not been systematically compared previously, but strains of *E. coli* have been extensively inhibited by lactobacilli also in other studies (Guo et al., 2010; Klose et al., 2010a).

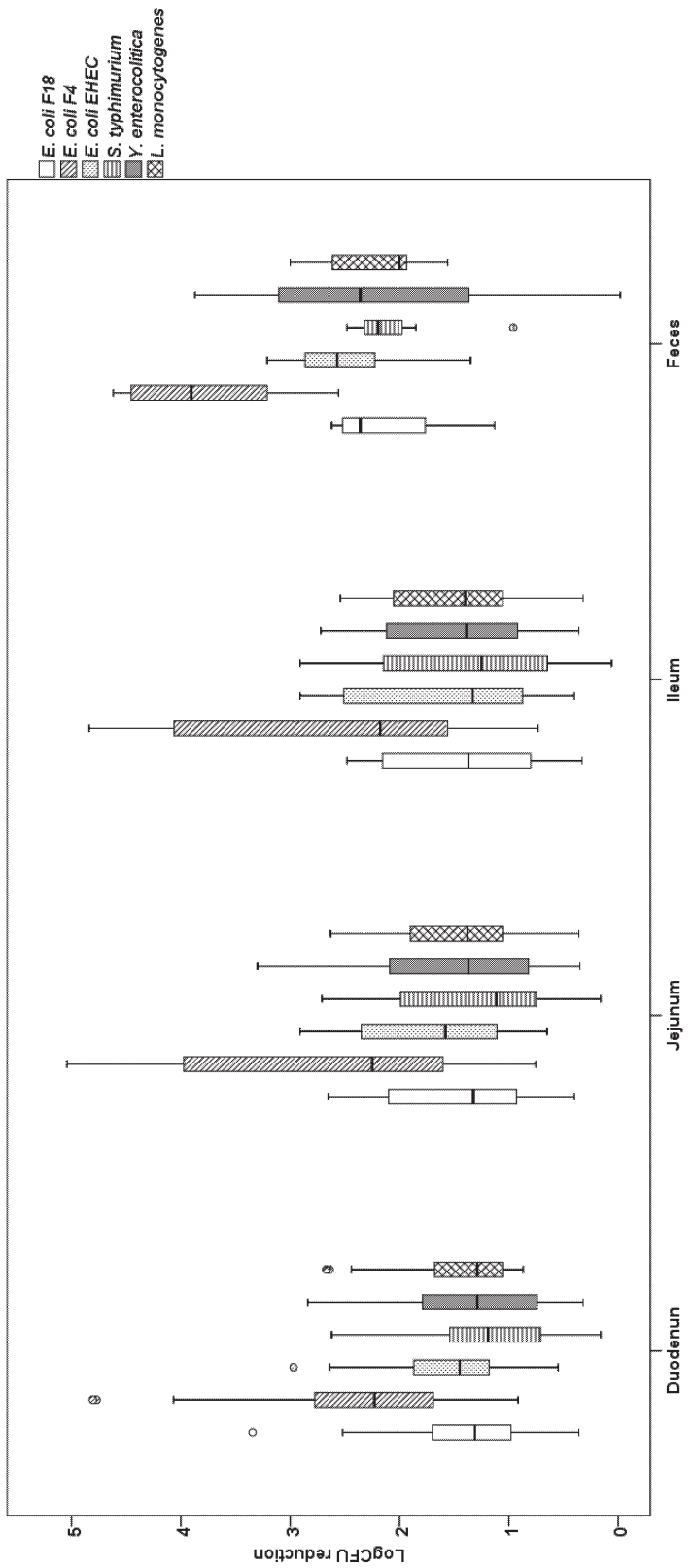


	E. coli F18	*E. coli* F4	*E. coli* EHEC	*S.* Typhimurium	*Y. enterocolitica*
***E. coli* F4**	**0.000**				
***E. coli* EHEC**	**0.002**	**0.000**			
***S.*Typhimurium**	0.504	**0.000**	**0.000**		
Y. enterocolitica	1.000	**0.000**	**0.001**	0.880	
L. monocytogenes	1.000	**0.000**	**0.016**	0.107	1.000

Figure 6. A box-and-wishkers plot illustrating the log CFU reductions achieved by the six indicator pathogens cultured in TSB media supplemented with the SCF collected from the porcine LAB isolates. Each box shows the median, quartiles and extreme values within a category. Friedman's test post hoc values for pairwise comparisons (SPSS, 2012) with a Bonferroni correction for multiple comparisons are shown; statistically significant values in bold.

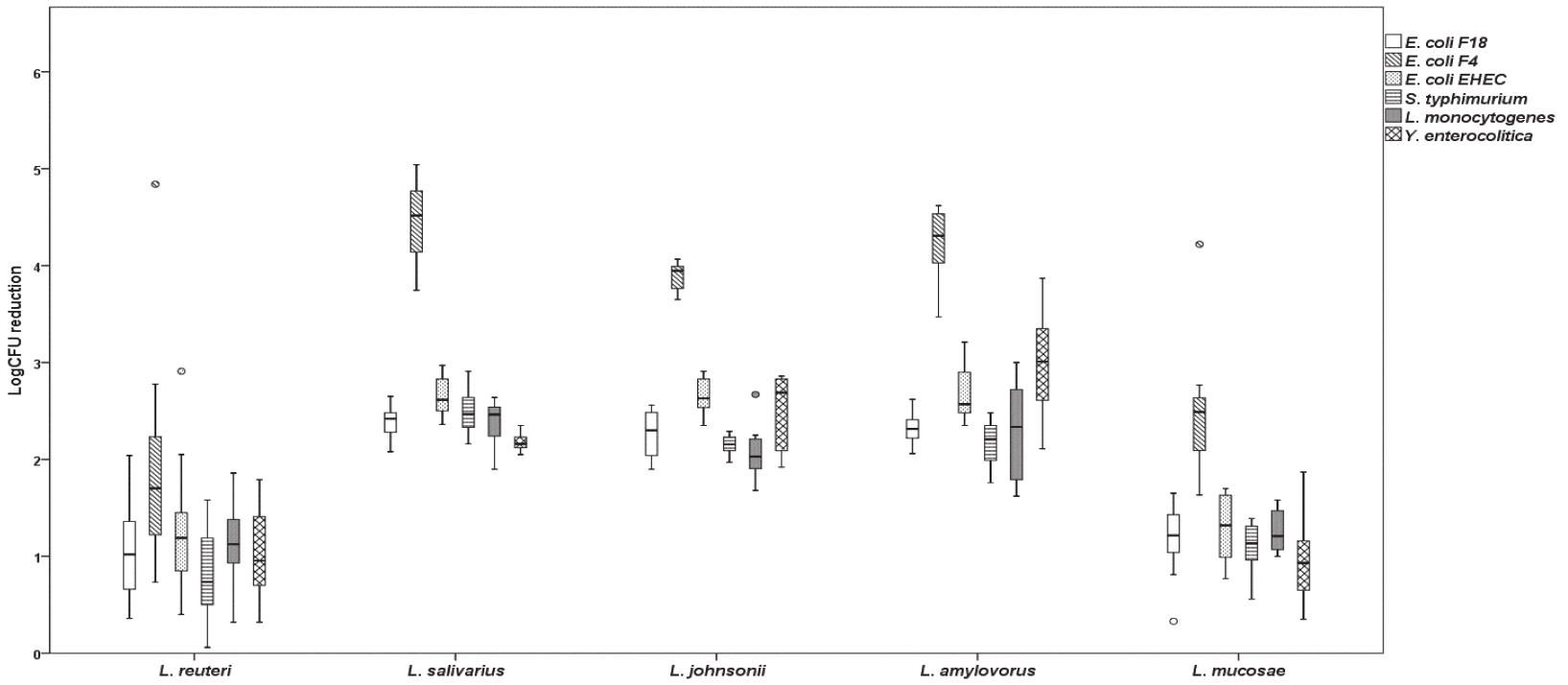
For most of the indicator pathogens, the isolation site of the LAB strain did not influence the growth inhibition caused by the SCF, except for *E. coli* EHEC and *L. monocytogenes*, both of which were inhibited more by the fecal isolates (Kruskal-Wallis p=0.037 (*E. coli* EHEC) and p=0.016 (*L. monocytogenes*); Fig. 7). However, the small number of fecal isolates as well as the fact that these isolates represented different *Lactobacillus* species compared to the intestinal isolates may have influenced this finding. At the species level, when comparing the five largest *Lactobacillus* identification groups, a statistically significant difference was found in the inhibition of all the indicator pathogens (Kruskall-Wallis p<0.001). Generally, the isolates identified as *L. salivarius*, *L. johnsonii* or *L. amylovorus* inhibited the indicator pathogens more than the isolates identified as *L. reuteri* or *L. mucosae* (Fig. 8). Apparently, no systematic comparisons of different species of lactobacilli in terms of pathogen growth inhibition have been performed previously, but three *L. salivarius* strains have been found to inhibit several swine pathogens, including five *E. coli* strains and a *Brachyspira pilosicoli* strain, generally more than the other *Lactobacillus* species investigated (Klose et al., 2010a)

The pH of the culture filtrates collected from the LAB isolates (I), and from the *L. amylovorus* strains (IV) varied from 3.62 to 5.36 and from 3.8 to 4.5, respectively. In order to assess the effect of the pH value of the SCF on the pathogen growth inhibition, two different experimental set-ups were used. In Study I, the pathogen growth inhibition evoked by the SCF was compared to those caused by the pH controls (MRS medium with pH adjusted with lactic acid or with HCl). It was evident that most of the growth inhibition caused by the SCF was due to lactic acid and low pH, but certain filtrates inhibited the growth of the indicators more than the pH controls (Table S1 in I). Thus, some of the LAB isolates appeared to produce other inhibitory substances in addition to organic acids. On the other hand, the pathogen growth inhibition caused by the pH-adjusted (pH 6.2) SCF of the *L. amylovorus* strains (IV) was low, indicating that most of the pathogen growth inhibition was due to acidity of the SCF (data not shown). Alternatively, inhibitory compounds with activity at acidic but not at neutral pH might have been produced, as has been observed for Lactocin S produced by *Lactobacillus sakei* (Mortvedt-Abildgaard et al., 1995). These results are in line with previous studies showing that neutralization of the SCF often, although not invariably, abolishes the growth inhibition of pathogens caused by the filtrate (Lin et al., 2007; Guo et al., 2010; Klose et al., 2010a).



	Duodenum	Jejunum	Ileum
Jejunum	1.000 / 1.000		
Ileum	1.000 / 1.000	1.000 / 1.000	
Feces	0.057 / **0.012**	**0.050** / **0.031**	**0.031** / 0.021

Figure 7. A box-and-wishkers plot illustrating the log CFU reductions achieved by the six indicator pathogens cultured in TSB media supplemented with the SCF collected from the porcine LAB isolates originating from the duodenum, jejunum, ileum and feces of swine. Each box shows the median, quartiles and extreme values within a category. Kruskall-Wallis test *post hoc* values for pairwise comparisons (Dunn's 1964) with a Bonferroni correction for multiple comparisons are shown for *E. coli* EHEC and *L. monocytogenes*; statistically significant values in bold.



	L.reuteri	*L. salivarius*	*L. johnsonii*	*L. amylovorus*
L. salivarius	**0.000** / **0.000** / **0.000** / **0.000** / **0.000** / **0.000**	-		
L. johnsonii	**0.000** / **0.035** / **0.000** / **0.001** / **0.000** / **0.001**	1.000 / 0.728 / 1.000 / 1.000 / 1.000 / 1.000	-	
L. amylovorus	**0.001** / **0.001** / **0.001** / **0.002** / **0.000** / **0.001**	1.000 / 1.000 / 1.000 / 1.000 / 1.000 / 1.000	1.000 / 1.000 / 1.000 / 1.000 / 1.000 / 1.000	-
L. mucosae	1.000 / 0.384 / 1.000 / 1.000 / 1.000 / 1.000	**0.002** / **0.040** / **0.003** / **0.001** / **0.001** / **0.001**	**0.028** / 1.000 / **0.018** / 0.233 / **0.001** / 0.076	**0.061** / 0.466 / **0.047** / 0.226 / **0.001** / **0.046**

Figure 8. A box-and-wishkers plot illustrating the log CFU reductions achieved by the six indicator pathogens cultured in TSB media supplemented with the SCF collected from the porcine LAB isolates identified as *L. reuteri*, *L. salivarius*, *L. johnsonii*, *L. amylovorus* or *L. mucosae*. Each box shows the median, quartiles and extreme values within a category. Kruskall-Wallis test *post hoc* values for pairwise comparisons (Dunn's 1964) with a Bonferroni correction for multiple comparisons are shown for each indicator pathogen (the order as follows: *E. coli* F18 / *E. coli* F4 / *E. coli* EHEC / *S.* Typhimurium / *Y. enterocolitica* / *L. monocytogenes*); statistically significant values in bold.

5.2.5 Correlations between the properties tested (I)

The possible association between the antimicrobial activity, adherence to the enterocytes and stress tolerance of the LAB strains was investigated with the Spearman's rank-correlation test. For this analysis, a relative survival was calculated from the stress tolerance data of each isolate with the following formula: MPN (stress) / MPN (control), and the logarithmic of this value was used in the correlation analysis. Additionally, an overall average adhesion was calculated for each isolate from the adhesion values to enterocytes of different intestinal origins. In an attempt to reduce the dimensions of the antimicrobial activity data, a principal components analysis (PCA) was performed. In the correlation matrix, all variables had correlation coefficients greater than 0.68, indicating that the correlations between the different variables were sufficient to include all of the variables in the analysis. The overall Kaiser-Meyer-Olkin (KMO) measure (0.89) and the individual KMO measures (all greater than 0.7) showed the adequacy of sampling, and the Bartlett's test of sphericity was statistically significant ($p<0.005$), thus the inhibitory data was suitable for PCA. In PCA, only one component with an eigenvalue greater than one was revealed, and this component explained 85.1% of the total variance.

In the correlation tests, only negative correlations were found between the different properties, and all of them were relatively weak. The PCA values of the antimicrobial activity tests correlated negatively with the averaged adhesion (Spearman's rho -0.346, $p=0.001$), indicating that the strains showing the highest adhesion ability generally exhibited lower inhibitory capacity of the indicator pathogens. To some extent, this was also illustrated when comparing the largest identification groups (see 5.2.2 and 5.2.4). Additionally, the PCA values correlated negatively with the tolerance towards pH 2 (Spearman's rho -0.519, $p<0.0005$) as well as with the tolerance toeards pH 4 (Spearman's rho -0.220, $p=0.034$). Thus, those strains with the greatest ability to inhibit the growth of the indicator pathogens generally seemed to show lower tolerance towards low pH.

5.2.6 Stimulation of cytokine production by dendritic cells (IV)

The possible stimulation of immune signaling in human MoDCs by the *L. amylovorus* strains was assessed at three different bacterial concentrations. Induction of cytokine production was observed at the bacteria/DC ratio of 100:1, with substantial strain-dependent differences between the levels of cytokines induced (Fig. 5 in IV). The cytokine profiles induced by the strains were not clearly skewed towards Th_1 or Th_2 phenotypes. Instead, a mixed type of response, including Th1 favoring (IL-12), Th2 favoring (IL-10) as well as proinflammatory (TNF-α, IL-6, IL-1β, IP-10/CXCL10) cytokines, was stimulated by most of the strains. Similar cytokine profiles have been demonstrated also for *L. gasseri* strains (Luongo et al., 2013), while induction of proinflammatory cytokine profiles without high IL-12 expression has also been described for some strains of this *Lactobacillus* species (Stoeker et al., 2011). Interestingly, the strain NCFM of *L. acidophilus*, which is a close relative of *L. amylovorus,* has been shown to stimulate the differentiation of human DCs towards Th_2 generation via interaction between the SlpA protein and the DC-SIGN molecule (dendritic cell specific C-type lectin intercellular adhesion molecule 3-grabbing non-integrin) (Konstantinov et al., 2008b). Although the SlpA proteins of the *L. amylovorus* strains used in this study show a high similarity with the NCFM SlpA (Additional file 2 in IV), the Th_2 favoring action of NCFM was not observed with the present *L. amylovorus* strains. On the other hand, a skewing towards Th1 type has also been observed to be caused by *L. acidophilus* NCFM

in both murine BMDCs (Weiss et al., 2010) and human MoDCs (Gad et al., 2011), emphasizing the divergent effects observed for the same strains with different experimental designs.

By using different bacterial doses in DC stimulation experiments, a clear dose-dependent effect has been observed (Smits et al., 2005; Konstantinov et al., 2008b; Gad et al., 2011). For example, the ratio of IL-10 to IL-12 can even be reversed with increasing bacterial loads (Konstantinov et al., 2008b). Smits et al. (2005) observed optimal *in vitro* priming of regulatory DCs cells with MOI values of 1, whereas higher concentrations of bacteria evoked lower effect. This contrasts with the results of the present study, in which no notable induction of any of the cytokines measured was observed at the lower MOI values of 10 and 1, as compared to the negative control (data not shown).

5.2.7 Antibiotic susceptibility (III)

To evaluate if antibiotics could be used as selective agents in the bacterial culture of porcine fecal samples in Study III, the susceptibilities of the strains used in the feeding trial to five antibiotics was tested with the broth microdilution method (Table 8). All of the strains were sensitive to G-penicillin, which seems to be a common feature among lactobacilli (Klare et al., 2007; Nawaz et al., 2011; Gueimonde et al., 2013). Four strains expressed resistance towards vancomycin, concurring with previous results obtained for human and food isolates (Klare et al., 2007; Nawaz et al., 2011) and also with the fact that many species of lactobacilli are intrinsically resistant to this antibiotic (Saarela et al., 2000; Gueimonde et al., 2013). Similarly, lactobacilli often exhibit resistance towards inhibitors of nucleic acid synthesis, such as fluoroquinolones (Karapetkov et al., 2011; Gueimonde et al., 2013), and this was also the case for the porcine strains examined in this study, although the MIC values were somewhat variable. The most extensive variation was observed in the susceptibility of the strains to kanamycin and tobramycin; previously the resistance of lactobacilli isolated from different food and animal sources towards aminoglycosides (i.e. gentamycin, tobramycin and kanamycin) has also been described to be variable (Nawaz et al., 2011; Bujnakova et al., 2014; Klose et al., 2014). No single antibiotic, which could have been used as a selective agent for isolation of the six strains from the porcine fecal samples, could be identified amongst the tested antibiotic agents.

Table 8. Minimal inhibitory concentration (MIC) values (μg/ml) of the *Lactobacillus* strains for selected antibiotics assayed using microdilution method, with two parallel wells for each dilution series of the antibiotics.

Strain	Penicillin G	Enrofloxacin	Kanamycin	Tobramycin	Vancomycin
L. mucosae GLR 1167	<0.125	16	32	8	512
L. salivarius GRL 1169	<0.125	2	256	64	>1024
L. johnsonii GRL 1171	<0.125	32	256	128	<8
L. reuteri GRL 1168	<0.125	16	32	128	512
L. reuteri GRL 1170	<0.125	8	64	8	512
L. amylovorus GRL 1112	<0.125	8	64	64	<8

5.3 *In vivo* feeding trial (II, III)

5.3.1 Animal performance and growth (II, III)

During the adaptation period, signs of mild diarrhea were recorded in some of the piglets, and a total of seven piglets (three in both the control and multispecies supplementation groups and one in the *L. brevis* group) had to be treated with the combination of trimethoprim and sulfadiazine (Tribrissen, Schering-Plough A/S, Denmark). On the other hand, all piglets remained healthy during the actual feeding trial, exhibiting no clinical signs of diarrhea or any other adverse effects.

When evaluating the possible beneficial health effects of bacterial feed supplements in meat-producing animals, weight gain during the feeding trial is a commonly used measure, as improved growth would be highly desirable outcome of probiotic supplementation. In swine, enhanced growth rates have been observed both with monostrain (Abe et al., 1995; Guerra et al., 2007) and multistrain/-species (Choi et al., 2011a; Choi et al., 2011b) supplementations. On the other hand, a failure to promote growth has also been reported with both types of supplementation (Shim et al., 2005; Mair et al., 2010a). In the present study, no statistically significant differences in the mean body weight (BW) or ADWG could be detected between the control and the two supplementation groups at any of the measurement points, although piglets receiving *L. brevi*s supplementation had a numerically greater final body weight than the piglets in the other two groups (Table 9). This concurs with an earlier study, in which a different *L. brevis* strain (1E1) also induced a statistically non-significant growth increase in neonatal piglets (Gebert et al., 2011). The high variability in growth promotion observed after various bacterial supplementations can be attributed to several factors, e.g. inherent characteristics of the species or strain being used. Additionally, host-specific properties like age and health status can affect the responses of the host to bacterial supplementation (Taras et al., 2007; Gaggia et al., 2010). Furthermore, it has been speculated that the hygienic conditions in experimental institutes may be too favorable to allow the appearance of any clear health benefits or growth promotion after bacterial supplementation, as the environmental pathogen load in these sites is presumably lower compared to that present in commercial intensive rearing units (Simon et al., 2001).

Table 9. Effects of a monostrain (*L. brevis*) and a multistrain *Lactobacillus* supplementations on the growth of piglets and digesta pH. All values represent mean ± standard deviation.

	Growth			pH			
Group	Initial BW	Final BW	ADWG	Jejunum	Ileum	Cecum	Colon
Control	9.48 ± 1.88	20.26 ± 3.58	0.44 ± 0.09	6.4 ± 0.4	6.5 ± 0.3	6.0 ± 0.2	6.0 ± 0.2
L. brevis	9.39 ± 1.66	21.21 ± 4.10	0.48 ± 0.11	6.4 ± 0.3	6.7 ± 0.4	6.0 ± 0.2	6.0 ± 0.3
Multispecies	10.04 ± 1.55	20.66 ± 4.49	0.42 ± 0.12	6.5 ± 0.2	6.6 ± 0.2	5.9 ± 0.2	6.1 ± 0.2
p-value[1]	0.627	0.619	0.415	0.967	0.237	0.642	0.779

[1] P-values for Kruskall-Wallis test

5.3.2 Detection of the supplemented strains in the intestine and feces (II, III)

To investigate the survivability of the strains fed to the piglets, bacterial colonies isolated from the fecal samples were analyzed with species-specific PCR (II, III). A total of 144 colonies (56 from the *L. brevis* group and 44 from the control and multispecies groups, respectively) were analyzed by *L. brevis* S-layer specific PCR; however, no positive results were found. On the other hand, each of the bacterial species included in the multispecies formulation was found among the fecal isolates analyzed (44 colonies from both the control and multispecies supplementation groups) (Table 3 in III). However, in PFGE, none of the strains isolated from the supplementation group fecal samples had an identical *Sma*I pattern (genotype) to the strains which had been fed to the piglets, even though one *L. reuteri* isolate from the slaughter sample did resemble *L. reuteri* GRL 1170 (Fig. 9).

This inability to isolate any of the strains fed to the piglets from the fecal samples was unexpected and surprising. Previously, using a similar technique, the same *L. brevis* strain that was used in the present trial was isolated from human stool samples in a small-scale intervention study (Rönkä et al., 2003), and as well from feces of mice that had received the strain via an oral gavage (unpublished data from the laboratory of A. Palva). In addition, the *Lactobacillus* strains included in the multispecies supplementation had all been originally isolated from the intestine and feces of swine, and based on the *in vitro* screening tests for tolerance of low pH and bile it was expected that the strains would be able to survive in the porcine GI-tract. However, as the bacterial culturing method used with the pooled fecal samples may have lacked sufficient sensitivity to detect the strains, it cannot be concluded with certainty that the supplemented strains were unable to survive passage through the intestine of swine. Previously, certain *Lactobacillus* strains have been observed to survive transit through the GI tract of pigs (Table 3), but high variability have been observed in the excretion levels of the strain fed between different host individuals (Gardiner et al., 2004).

To investigate the distribution of *L. brevis* cells in the porcine intestine in more detail, the presence of this strain in the intestinal lumen and mucosa of the piglets from the control and *L. brevis* supplementation groups was examined with real-time qPCR analysis of the digesta and fecal samples as well as with histological examination of cryosections using IFA (II). With both of these methods, *L. brevis* was found as the most abundant in the large intestine of piglets from the *L. brevis* supplementation group. In this group, genomic DNA of *L. brevis* was detected in the cecal digesta of four piglets as well as in the colonic digesta and feces of seven piglets while only one piglet from the control group had any detectable amounts of *L. brevis* DNA in its cecum (Fig. 1 in II). The inability to detect *L. brevis* in every piglet of the supplementation group may be due to the detection limit of the real-time qPCR assay (10^4 genomes g^{-1}) or host-related genetic factors influencing the ability of the strain to colonize the intestinal microbiota. On the other hand, when examining the cecal and colonic cryosections, *L. brevis*-like cells were detected in samples from every piglet in the supplementation group and with high cell numbers, but accurate and reliable enumeration of individual cells was problematic owing to the large cell clusters that had formed (Fig. 2 in II). In addition, nonspecific staining of smaller-sized bacterial cells further complicated *L. brevis* cell counting; thus, numerical data for the large intestine cryosections is not presented. In the small intestine, genomic DNA of *L. brevis* was not found in the digesta of either the control or the supplementation group ($<10^4$ genomes g^{-1}). However, *L. brevis*-like cells were detected in the jejunal and ileal cryosections from both groups, with the jejunum sections of the supplementation group displaying statistically significantly higher cell numbers compared

to the control group (Mann-Whitney U-test $p<0.001$), while no difference was observed in the ileum (Fig. 3 in II). Taken together, the results of the real-time qPCR analysis of the digesta and detection *L. brevis*-like cells with intact cell morphology in cryosections suggest that this strain may be capable of colonizing both the large, and to a lesser extent also the small, intestines of weaned piglets. However, the viability of the strain in the piglet intestine should be further confirmed.

Interestingly, potentially indigenous *L. brevis* cells were also detected in a few control group piglets both in cryosections (with *L. brevis* SlpA-specific antiserum), as well as in the large intestinal digesta samples (with real-time qPCR). While this *Lactobacillus* species does not seem to be a prominent member of the porcine gut microbiota (Hill et al., 2002; Leser et al., 2002; Hill et al., 2005), it has been isolated previously from different porcine samples (Krause et al., 1995; Martin et al., 2009; Gebert et al., 2011; Qi et al., 2011), thus the *L. brevis*-like cells detected in samples from the control group were in all likelihood indigenous strains of this *Lactobacillus*.

In an attempt to examine whether the *L. brevis* strain used would adhere more efficiently to Peyer's patches as compared to the regular intestinal wall areas, the small intestinal cryosections containing and not containing Peyer's patches were examined for the presence of *L. brevis*-like cells with IFA (II). While it has been reported that several *Lactobacillus* species seem to be able to

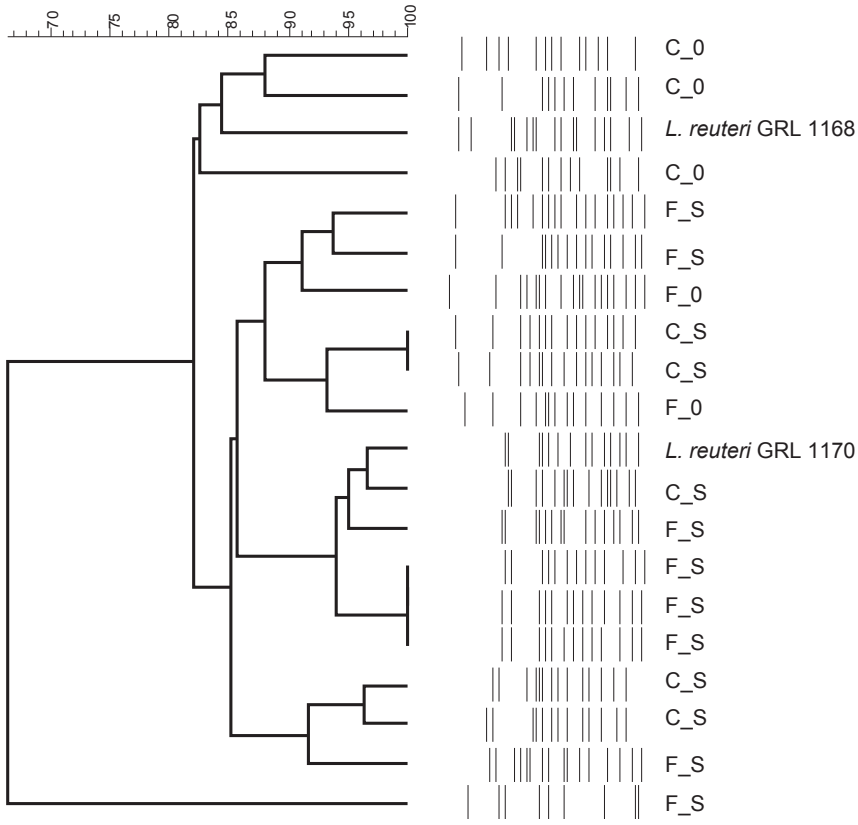


Figure 9. Dendrogram representing the Dice coefficient-derived percentages of similarity among the distinct SmaI PFGE patterns obtained for fecal L. reuteri isolates. The source of isolates is listed on the right. C=control group; F=multispecies supplementation group; 0=sample taken before the start of the trial; S=sample taken at slaughter.

adhere specifically to Peyer's patches (Perdigon et al., 2000; Plant and Conway, 2001; Edelman et al., 2002), the mucosal numbers of *L. brevis*-like cells were similar in both types of cryosections (Fig. 3 in II), suggesting that this strain lacks the capacity to adhere specifically to these tissue structures.

5.3.3 Effects of the supplementations on the bacterial and lactobacillar counts (II, III)

In order to reveal any major effects of the bacterial supplements on the GI-tract microbiota, the total numbers of bacteria (II, III) and lactobacilli (III) were quantified with real-time qPCR. While increased numbers of digestal or fecal lactobacilli and / or other bacterial species considered beneficial for the host have been detected in some previous porcine feeding trials (Pollmann et al., 1980; De Angelis et al., 2007; Choi et al., 2011b), the multispecies supplementation used in the present study did not influence the abundance of lactobacilli in either the intestinal contents or feces of the piglets (Fig. 2 in III). However, increased numbers of total bacteria were detected in the jejunum of the multispecies supplementation group, as compared to the control group (Mann-Whitney U-test p=0.023; Fig. 2 in III), suggesting that some changes had occurred in the indigenous microbiota of this intestinal compartment due to the multispecies supplementation. In the *L. brevis* supplementation group, the quantities of bacteria remained similar to the control group in all intestinal compartments investigated (Table 2 in II), which is in agreement with a previous porcine feeding trial performed with *L. amylovorus* DSM 16698^T (Konstantinov et al., 2008a).

5.3.4 Immunological effects of the supplementations (II, III)

To determine whether the *L. brevis* supplement could induce systemic humoral immune responses, total IgA and IgG as well as *L. brevis*-specific IgG concentrations were measured from serum samples of the *L. brevis* and the control group (II). While in both groups the concentrations of serum IgA and IgG increased during the feeding trial, no statistically significant differences could be observed between the groups (Fig. 5 in II). Additionally, the levels of *L. brevis*-specific IgG remained similar in both groups (data not shown). These results indicate that the *L. brevis* strain used does not elicit a measurable humoral immune response, or alternatively, that the length of the bacterial supplementation was too short to induce any detectable antibody response. The detection of *L. brevis*-specific IgG antibodies in piglets from the control group is likely due to either cross-reactivity or the stimulation of antibody production by an autochthonous *L. brevis* strain. Correspondingly, the presence of serum IgA antibodies that recognize *L. rhamnosus* GG, considered an atypical bacterium of the porcine microbiota, has been demonstrated also in piglets that did not receive this strain as a feed supplement (Casini et al., 2007). Furthermore, IgA antibodies able to recognize *L. amylovorus* DSM 16698^T have been detected in both the supplementation and control groups (Konstantinov et al., 2008a), thus in swine it seems that it may be rather common to detect cross-reactive antibodies binding to various *Lactobacillus* species.

The potential effects of the *L. brevis* supplement on the expression of selected immunological genes were first screened by investigating the intestinal mucosal samples of five piglets from the *L. brevis* and the control groups by real-time qPCR (data not shown). However, as the RNA extracted from the samples was of low quality and quantity, an alternative method, QuantiGene 2.0 Plex Assay (Affymetrix/Panomics, USA), permitting mRNA quantitation without RNA extraction, was used for the analysis of the mucosal samples.

Examination of the gene expression of eight cytokines (i.e. IL-1β, IL-4, IL-6, IL-8, TGF-β1, IFN-α, IFN-γ and TNF) in the intestinal mucosa revealed that although both types of supplementations did induce some alterations in the expression of these cytokines, more pronounced effects were triggered by the multispecies preparation (Fig. 4 in II; Fig. 3 in III). The mRNA levels of the cytokine TGF-β1 were downregulated in the ileum by both of the supplementations (Mann-Whitney *U* test p=0.015 (*L. brevis* group) and <0.001 (multispecies group)), and also in the jejunum (p<0.001) and the colon (p=0.017) by the multispecies formulation. This contrasts to previous results obtained for another type of multispecies supplementation containing four bacterial strains which exerted no effect on the expression of this cytokine in the intestine of weaned piglets (Mair et al., 2010b). Moreover, increased production of TGF- β1 in the Caco-2 cell line has been reported after contact with gut commensals, including *Lactobacillus* strains (Zeuthen et al., 2008). Despite the downregulation of the anti-inflammatory TGF-β1 cytokine, no visual changes were observed in the pathological investigation of the porcine intestinal walls, suggesting that the supplementations had not induced any inflammation in the intestinal wall.

Interestingly, the mRNA levels of the cytokines IL-6 and IL-4 were upregulated in the cecum by both types of supplementation (Fig. 4 in II; Fig. 3 III), although statistical significance was reached only by the IL-4 change in the multispecies group (p=0.035) and the IL-6 change in the *L. brevis* group (p=0.023). While changes in the expression of IL-6 in the small intestinal mucosa of piglets have been reported to occur e.g. during weaning (Pie et al., 2004) as well as after bacterial supplementation (Zhang et al., 2011; Deng et al., 2013), there does not seem to be any data available on the expression patterns of this cytokine in the colon of piglets. With regard to the increased cecal mRNA expression of IL-4 stimulated by the multispecies supplementation, this change was mainly attributable to two individual piglets in that group, and when the Mann-Whitney *U* test analysis was performed without these piglets, no difference between the groups was any longer apparent (data not shown). However, no special features were observed in the pathological investigation of the cecum wall samples from these two individuals. It does seem that the intestinal expression of this major Th_2 cytokine has not been investigated in other bacterial feeding studies performed in swine, and little is known about the function of IL-4 in the porcine intestine. In human intestinal cell lines, IL-4 has been found to reduce the barrier function of the epithelial monolayer, resulting in its increased permeability to macromolecules (Colgan et al., 1994; Berin et al., 1999). However, there are indications that species differences exist in the expression and functions of IL-4 (Reddy et al., 2000; Raymond and Wilkie, 2004; Bautista et al., 2007), and differences between swine breeds have been observed (Verfaillie et al., 2001; de Groot et al., 2005), reflecting possible genetic factors influencing immune function. Thus, the functions of IL-4 in the porcine intestine remain to be explored in further studies. In addition to the increased mRNA expression of IL-4, also the mRNA expression of IFN-α was upregulated in the cecum of the multispecies supplementation group (p=0.001). While the production of interferons has traditionally been considered to be primarily induced by viruses, also bacteria, including lactobacilli, are known to stimulate their production (Kitazawa et al., 1994; Bogdan et al., 2004).

In the colon, the multispecies formulation induced a downregulated mRNA expressions of IL-8 (p=0.008) and TNF (p=0.001). Previously, a five-strain bacterial supplementation has been reported to increase the expression of IL-8 in the ileum of weaned piglets, but the large intestinal compartments were not investigated (Walsh et al., 2008). In addition, the expression of TNF in the colon and the small intestine of weaned piglets was not altered by a four-strain supplement

(Mair et al., 2010b). These dissimilarities in the results of various studies are probably due to differences in the bacterial strains used, as well as in the experimental designs, such as the genetic background of the animals, as these factors can affect the results of immunological studies (Joling et al., 1993; de Groot et al., 2005; Flori et al., 2011). One limitation of the present study was the heterogeneity of the study population, as the piglets represented a mixture of different breeds as well as genders, and this may well have exerted an influence on the outcomes. Moreover, when considering the complexity of the mucosal immune system, it is very difficult to draw any firm conclusions about the results from cytokine expression studies, as is predicting what would be the concrete health consequences of increased or decreased gene expression of different cytokines triggered by bacterial supplementation. Consequently, reasonable speculation on these phenomena will need to be based on more detailed information about the immune system networks.

5.4 The surface layer proteins of *Lactobacillus amylovorus* strains (IV)

5.4.1 Genomic characterization and expression analysis of L. amylovorus S-layer proteins (IV)

In the *in silico* analysis of the *L. amylovorus* genomes, several *slp* genes were revealed in each of the strains. The presence of multiple S-layer protein genes is common in lactobacilli, and has previously been reported e.g. in strains of *L. acidophilus* (Boot et al., 1995) as well as those closely related to *L. acidophilus*, including *L. amylovorus* (Boot et al., 1996b), and also in strains of *L. brevis* (Jakava-Viljanen et al., 2002) and *L. crispatus* (Sillanpää et al., 2000). The *L. amylovorus slp* genes with high homology to *L. acidophilus* NCFM *slpA* and *slpB* (Konstantinov et al., 2008b) were also named *slpA* and *slpB*, respectively, and the third type of *slp*-like gene detected was designated *slpC*. For both *slpA* and *slpB*, most of the strains carried only one gene homologue, but two distinct homologues of *slpA* and *slpB* were detected in GRL 1117 and DSM20531, respectively. There was more variation in the number of *slpC*-type genes, as one to three homologues were recognized in the *L. amylovorus* strains (Fig. 6 in IV). A phylogenetic tree constructed based on the deduced amino acid sequences of the *slpA*, *slpB* and *slpC* gene products indicated that the SlpA-like sequences have diversified most during the evolution, while the SlpB-type proteins have remained more similar to each other and the predicted SlpC-type proteins have formed three distinct groups (Fig. 6 in IV, the deduced amino acid sequences of the *L. amylovorus* Slp proteins are presented in Additional file 1 in IV).

Comparison of the sequence data of the *slp* genes with the results of the aminoterminal sequencing or the peptide mapping analysis performed for the *L. amylovorus* S-layer proteins revealed that the major S-layer protein bands seen in the surface protein profiles of the strains (Fig. 7 in IV) were all encoded by *slpA*-like genes. The amino acid sequences of the SlpA proteins were found to be very similar to that of the *L. acidophilus* NCFM SlpA protein, especially in the carboxy terminal region. This may reflect the role of the C-terminal part in cell wall binding, previously described in *L. acidophilus* (Smit et al., 2001) and *L. crispatus* (Antikainen et al., 2002). Bands representing the products of *slpB*- and *slpC*-like genes were also identified in the surface protein profiles of the strains: the former in strains GRL 1117 and DSM 16698 but the latter only in strain DSM 16698 (Fig. 7 in IV). While multiple S-layer genes are commonly carried by lactobacilli, simultaneous expression of more than one of these gene seems rare, but this phenomenon has been previously described for *L. brevis* ATCC 14869 (Jakava-Viljanen et al., 2002) and *L. acidophilus* NCMF (Goh et al., 2009).

5.4.2 The role of L. amylovorus S-layer proteins in adherence (IV)

To explore if the *L. amylovorus* S-layer proteins have a role in the adherence capacity of the strains to IPEC-1 cells, a protein presentation system based on the propensity of S-layer proteins to recrystallize in a native manner on purified CWF was developed (an electron micrograph of purified CWF: Fig. 8B in IV). In an attempt to minimize the presence of Slp protein aggregates in the adhesion assay, the dilute Slp protein fraction was separated from the precipitated protein by centrifugation, as has been described previously (Åvall-Jääskeläinen et al., 2008). The method applied to the purification of the CWF preserves the covalently attached polymeric components like teichoic acids and polysaccharides, which ensures the proper self-assembly of the recombinant Slp:s and leads to the presentation of the S-layer proteins in the native and symmetric organization observed on the surface of bacterial cells. In the adhesion assays, the detection of the Slp-coated CWF was performed with an indirect immunofluorescence staining using Slp-specific antibodies. Uncoated CWF were used as negative controls, but as attempts to produce functional antibodies against purified cell wall fragments were unsuccessful, these were biotinylated prior to the adhesion assay, and detected with labeled streptavidin after the assay.

While none of the uncoated CWF adhered to the IPEC-1 cells, at least a low level of adherence was demonstrated for all of the Slp coated CWF. However, the adherence level of the coated CWF did not correlate to that of the bacterial strain from which the proteins had originated. For example, the adhesion of CWF coated with SlpA from the strains DSM 16698 (Fig. 8D in IV), GRL 1112 or GRL 1115 was poor, even though the intact cells of these strains were adhesive to IPEC-1 cells (Fig. 2 in IV). On the other hand, the CWF coated with Slp:s from the weakly adhering strains GRL 1117 (Fig. 8H in IV), DSM 20531^T (Fig. 8G in IV) and GRL 1118 did display affinity for IPEC-1 cells. The SlpB of the strain DSM 16698 as well showed some adhesiveness (Fig. 8E in IV), when compared to SlpA (Fig. 8D in IV) or SlpC (Fig. 8F in IV) of the same strain.

These results indicate that none of the *L. amylovorus* S-layer proteins examined in this study solely mediate adhesion of the strains to IPEC-1 cells. However, also other non-covalently attached cell wall components may have been removed during the preparation of the CWF, and that these structures may well have a role in the mediation of adherence. This methodological limitation also applies to experiments based on chemical extraction of the S-layer from the bacterial surface; while this procedure has been shown to decrease the adhesion of lactobacilli to different targets (Hynönen and Palva, 2013), providing indirect evidence that the S-layer structure is involved in the adhesion process, a role for other non-covalently attached molecules removed in the extraction process cannot be excluded.

The highly efficient binding of the S-layer protein originating from the weakly adhesive strain GRL 1117 indicates that some component(s) on the surface of this strain shield(s) the S-layer and thus prevents the adhesion of the strain to IPEC-1 cells. This kind of phenomenon has been observed in *L. rhamnosus* GG: the exopolysaccharide component shields the mucus-binding fimbriae, reducing the adhesive capacity of the strain for mucus (Kankainen et al., 2009; Lebeer et al., 2009). While genes putatively participating in exopolysaccharide synthesis have been identified in all of the *L. amylovorus* strains studied (unpublished data from the laboratory of A. Palva), no biochemical evidence of their presence has been described so far. Thus, the reason for reduced adhesiveness of the intact cells of GRL 1117 compared to that of the SlpA-protein originating from this strain remains to be explained.

6 Conclusions and future aspects

The importance of the gut microbiota to the health and well-being of the host has been increasingly recognized during the last decade, and the use of microbial supplements to modulate the composition and function of this complex community has generated much interest in the fields of human and veterinary medicine. While various types of microbial products have been used in production animals, including swine, to maintain and improve the health and productivity of the host animal, several factors related to the selection process of the microbes as well as the functional mechanisms behind the observed effects still remain unrevealed. Thus, comprehensive studies regarding the use of probiotic microbes on various animal species are warranted. The main aim of this study was to characterize the probiotic potential of porcine lactobacilli for use in swine production. In addition, as lactobacillar S-layers are attractive candidates for antigen carriers in vaccine vectors, strains of lactobacilli possessing an S-layer were also investigated in this work.

I. The *in vitro* screening assays for selected traits commonly considered important for a putative probiotic led to the following conclusions:
    1) the probiotic properties of porcine LAB strains, such as acid and bile tolerance, adherence to host structures, ability to inhibit pathogens as well as immunostimulatory patterns vary considerably, even between strains of the same species. This finding emphasizes the need for careful selection of the putative probiotic strain,
    2) the porcine LAB isolates examined in this study generally adhered more efficiently to large intestinal enterocytes, in comparison to those collected from the small intestine. While the isolation site of the strain did not affect its adhesion preferences towards enterocytes of different origins, this may be influenced by the fact that the strains were isolated from intestinal digesta, and it would be interesting to explore this aspect also with strains originating from the mucosa of different intestinal sections,
    3) the ability to inhibit pathogen adhesion was not strictly correlated with the adhesive capacity of the porcine *Lactobacillus* strain. Thus, in addition to competition for the same adhesion sites, also other mechanisms are involved in the inhibition of pathogen adhesion,
    4) while the SCF of the LAB isolates inhibited the growth of several pathogens, this inhibition was mainly due to low pH value of the filtrate. However, some of the isolates seemed to produce also other inhibitory compounds, but the nature of these substances remains to be investigated in future studies and
    5) some of the properties correlated negatively with each other; for example, LAB strains showing high adhesion ability to enterocytes generally exhibited lower ability to inhibit pathogen growth. Although these correlations were only weak, this finding may indicate that it may be difficult to discover strain(s) that would possess all the characteristics considered desirable for probiotic bacteria. Indeed, no single strain showing excellent performance in all of the different properties tested in this work was detected.

II. The *in vivo* feeding trial led to the following conclusions:
    1) both of the supplementations used (i.e. a monostrain preparation containing *L. brevis* ATCC 8287, and a multistrain preparation containing six lactobacilli of porcine origins) were well tolerated by piglets, and produced no adverse effects. The supplementations did not seem to exert any growth enhancing effects; however, this aspect needs to be explored in more detail in clinically relevant settings,
    2) both supplementations induced some alterations of cytokine mRNA expression in the intestinal mucosa, with more pronounced changes being produced by the multistrain preparation, especially in the large intestinal area. Despite these immunological alterations in the intestinal mucosa, neither of the supplementations caused mucosal inflammation. The health consequences of these changes in mucosal cytokine expression will need to be clarified in future studies,
    3) the supplemented strains may not be able to survive transit through the swine GI-tract; however, as the method used may have lacked sufficient sensitivity, the gut transit tolerance of these strains will need to be investigated further in more detail. Nevertheless, it seems probable that these strains are not able to efficiently compete with the indigenous gut microbiota of weaned piglets. In future studies, it would be interesting to investigate the ability of these strains to colonize and / or to survive passage through the GI-tract of suckling piglets, and
    4) both of the supplementations examined in this work may be suitable for use as probiotics in swine, but as the efficacy of these putative probiotics under intensive production systems cannot be evaluated based on these preliminary results, more comprehensive studies will still be needed to assess their possible effects on porcine health and immunity. Additionally, the potential use of the *L. brevis* strain as a vaccine carrier for swine pathogens is worthy of further investigations e.g. with immunization studies performed using a recombinant strain expressing suitable antigens, such as the receptor binding domain of the FedF adhesin of F18+ *E. coli* strains causing oedema disease and / or weaning diarrhea.

III. The genomic characterization of the S-layer protein-carrying *L. amylovorus* strains led to the following conclusions:
    1) several *slp* –like genes are carried by each of the strains; these genes were named *slpA*, *slpB* and *slpC*. In all of the strains, the major Slp protein was encoded by the *slpA* gene, and this protein group shared high sequence similarity with the *L. acidophilus* NCFM SlpA protein. In addition to the major S-layer protein SlpA, two strains carried also other S-layer-like proteins on their surface, namely SlpB and / or SlpC and
    2) as the adherence level of the CWF coated with *L. amylovorus* Slp proteins did not correlate to that of the bacterial strains from which the proteins had originated, it seems apparent that none of the major Slp proteins alone mediate the adhesion of the strain to IPEC-1 cells. However, the putative role of other non-covalently attached cell wall components possibly removed during the preparation of the CWF should be examined in further studies.

7 Acknowledgements

This work was carried out in the Department of Veterinary Biosciences, Faculty of Veterinary Medicine, University of Helsinki. The financial support received for this research from the University of Helsinki, the Ministry of Agriculture and Forestry, the Academy of Finland, the Finnish Veterinary Foundation, the Finnish Foundation of Veterinary Research, the Finnish Graduate School on Applied Biosciences and the Walter Ehrström Foundation is gratefully acknowledged. I thank the heads of the Department, Lars-Axel Lindberg, Antti Sukura and Airi Palva, for providing such excellent working and educational facilities.

I am deeply grateful to my main supervisor Professor Airi Palva for her advice, guidance and support during the ups and downs of the research process and for her much-needed encouragement during the completion of this dissertation. I want to gratefully acknowledge my supervisor Professor Johanna Björkroth for her very valuable expertise and advice during the course of this work. I want to express my deep gratitude to my supervisor University lecturer Joanna Koort for her patient help and counselling; your door was always open to me, whether I needed help in research related issues or in knitting. I am also thankful to Docent Ilkka Palva for stimulating scientific discussions, and I'm deeply sorry that he is no longer here with us. I sincerely thank the official reviewers of this thesis, Professor Tapani Alatossava and Docent Maria Saarela, for their constructive and valuable comments that helped to improve the quality of this thesis. Ewen MacDonald is gratefully acknowledged for the revision of the English language.

I am extremely grateful to all my co-authors for their contributions; without your help this work would never have been completed. I especially want to thank Erja Malinen for her generous help with the statistics and the SPSS programme, Agneta Lindholm for her serene but enthusiastic attitude which helped me to calm down even in tight situations, Ulla Hynönen for her kind encouragement and willingness to answer my endless questions, Miia Jakava-Viljanen for her invaluable help with the practical aspects of the animal trial and Gloria Solano-Aguilar for her generous help during my visit to BHNRC. Over these years, I have had the opportunity to work with exceptional people, and I want to warmly thank all of the former and present members of Airi Palva's research group for their companionship and for creating such a pleasant and enjoyable atmosphere in the laboratory as well as around the lunch table. Special thanks to my bald-headed roommate for immunological discussions. I also want to express my gratitude to Sinikka Ahonen, Esa Pohjolainen, Anja Osola and Ulla Viitanen, the personnel in EELA (later Evira) for their help in conducting the animal trial, the staff in BHNRC (USDA) for helping me during my visit, and all the others who have offered their skilful technical assistance throughout this work. The secretaries in our Department are also gratefully acknowledged for handling of numerous practical things over the years.

Finally, I want to thank all my friends and family for support and encouragement. I especially want to thank my husband Ville, without whom I would still be working on the Figures of Study I, and my children Teemu and Veera, without whom there would be much less joy in my life.

Vantaa, August 2014

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