Isolation, characterization and strain-specific detection of canine-derived *Lactobacillus acidophilus*

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ACADEMIC DISSERTATION IN MICROBIOLOGY

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Front cover: Transmission electron microscopy image of *Lactobacillus acidophilus* LAB20 (photo by Yurui Tang)

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and manuscripts. They are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.

I **Yurui Tang**, Titta J. K. Manninen and Per E. J. Saris. (2012). Dominance of *Lactobacillus acidophilus* in the facultative jejunal lactobacilli microbiota of fistulated beagles. Applied and Environmental Microbiology. 78(19): 7156–7159.

II **Yurui Tang** and Per E. J. Saris. (2013). Strain-specific detection of orally administered canine jejunum-dominated *Lactobacillus acidophilus* LAB20 in dog feces by real-time PCR targeted to the novel surface layer protein. Letters in Applied Microbiology. 57(4):330-335.

III **Yurui Tang** and Per E. J. Saris. (2014). Viable intestinal passage of a canine jejunal commensal strain *Lactobacillus acidophilus* LAB20 in dogs. Submitted to Current Microbiology.

IV **Yurui Tang**, Veera Kainulainen, Thomas Spillmann, Susanne Kilpinen, Justus Reunanen, Marita Hämäläinen, Reetta Satokari, Per E. J. Saris. (2014). The canine intestinal isolate *Lactobacillus acidophilus* LAB20 adheres to intestinal mucus and epithelial cells, and suppresses LPS-induced interleukin-8 release of enterocytes. Submitted to Applied and Environmental Microbiology.

The author's contribution in articles:

I Modified the abstract, introduction, materials and methods, results and discussion based on Manninen's original manuscript, and wrote the final article in collaboration with the co-author and corresponding author.

II Performed all experimental works. Wrote the manuscript and interpretation of the results with corresponding author.

III Performed all experimental works. Wrote the manuscript and interpretation of the results with corresponding author.

IV Constructed the EPS mutant (SAA658), performed observation of the cell structure using TEM for Figure 1, wrote most of the manuscript and interpreted the results in collaboration with the co-authors and corresponding author.

Abbreviations

aa	Amino acid
AD	Atopic dermatitis
AMP	Ampicillin
ATP	Adenosine triphosphate
Bp	Base pair
DC	Dendritic cell
EFSA	European Food Safety Authority
EM	Electron microscope
EPS	Extracellular polysaccharide
Erm	Erythromycin
etc.	et cetera
Fbp	Fibronecting-binding protein
GIT	Gastrointestinal tract
GRAS	Generally recognized as safe
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
Ig	Immunoglobulin
kb	Kilobase
LAB	Lactic acid bacteria
LB	Luria Bertani medium
LBS	*Lactobacillus* selective medium
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAMP	Microorganism-associated molecular patterns
mLBS	Modified LBS (acetic acid omitted)
MRS	de Man, Rogosa and Sharpe medium
Mub	Mucin-binding protein
NA	Nutrient agar
NCBI	National Center for Biotechnology Information
NFκB	Nuclear factor-kappa B
N-terminal	Aminoterminal
OD	Optical density
PCR	Polymerase chain reaction
PG	Peptidoglycan
PM	Phenotype Microarrays
rep-PCR	Repetitive sequence-based PCR
rRNA	Ribosomal ribonucleic acid
TEM	Transmission electron microscopy
TNF	Tumor necrosis factor
SIBO	Small intestinal bacterial overgrowth
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SCFA	Short-chain fatty acid
SIBO	Small intestinal bacterial overgrowth
S-layer protein	Surface layer protein
PG	Peptidoglycan
vs.	Versus
WTA	Wall teichoic acid

Abstract

Lactobacilli are commensal gastrointestinal microbes commonly utilized in probiotic products, as they are believed to bestow multiple beneficial health effects to the host. Most well-studied lactobacilli have been isolated from feces. However, fecal isolates do not reflect the microbiota present in the upper gut, since different niches provide different microbial habitats. The fistulated dog model facilitates investigation of the microbiota in fresh intestinal samples without disturbing the physiology of the canine gut.

In this study, jejunal lactobacilli from five permanently fistulated beagles were studied. We found that facultative *Lactobacillus* strains were abundant in the jejunal microbiota, and *L. acidophilus* was the dominant species. Repetitive sequence-based polymerase chain reaction (rep-PCR) fingerprint profiles of *L. acidophilus* isolates revealed one predominant strain, named LAB20.

Adhesion is an important factor in bacterial colonization of the host gut. In order to adhere, compete, and dominate within the host, numerous bacterial cell-surface factors are required to interact with the host mucosa. In this study, the protein profile of LAB20 was studied using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and cell structure was observed via transmission electron microscope (TEM). A surface (S) layer protein was revealed from LAB20. S-layer proteins form crystalline arrays of proteinaceous subunits in the outer layer of the cell wall and are involved in mediating bacterial adhesion to host surfaces. Inverse PCR revealed the DNA sequence of the LAB20 S-layer protein, alignment with other lactobacilli S-layer protein genes showed it was a novel one.

The discovery of this novel S-layer protein in LAB20 enabled us to develop a strain-specific detection method. Real-time PCR primers targeting the variable region of the S-layer protein gene were used to detect and quantify LAB20 in dog intervention studies. We found that LAB20 persisted in one dog for over 6 weeks after the feeding period (6×10^8 CFU daily for 5 days), whereas the five dogs in the other study maintained high LAB20 numbers only during the feeding period (10^8 CFU daily for 3 days). Cultivation of fecal samples demonstrated that LAB20 transits through the dog gut and can be identified based on colony morphotype.

TEM revealed a putative extracellular polysaccharide (EPS) layer that comprised LAB20's outermost structure. Using antisense RNA strategy, EPS production was manipulated to investigate its potential impact on the ability of LAB20 to adhere to mucus and epithelial cells. LAB20 displayed significantly higher adhesion in canine cecal mucus relative to the EPS mutant SAA658 and could adhere to Caco-2 and HT-29 epithelial cells. This suggests that wild-type EPS plays an integral role in the adhesion of LAB20 in the host gut. Moreover, LAB20 attenuated lipopolysaccharide (LPS)-induced interleukin (IL)-8 production in HT-29 cells, which indicates that LAB20 could be a probiotic candidate with anti-inflammatory properties.

In conclusion, this study investigated the surface structure, persistence, adhesion ability, and probiotic potential of LAB20, the dominant *L. acidophilus* strain in the canine small intestine. Our results suggest that LAB20 has potential as a canine probiotic.

Introduction

1. Lactic acid bacteria and probiotics

1.1 Lactic acid bacteria and their beneficial health effects

Lactic acid bacteria (LAB) have been used in traditional foods and to carry out fermentation since ancient times, when people were not aware of their existence. Naturally occurring fermented milk was desired for its pleasant flavor and longer shelf life. By 1857, Louis Pasteur had discovered LAB, identifying their role in fermentation. Since then, LAB have been isolated using bacterial cultivation techniques and added to food to facilitate fermentation. By 1919, Orla-Jensen had classified LAB based on cellular morphology, mode of glucose fermentation, growth temperature ranges of growth, and sugar utilization patterns; even in the modern taxonomic era, these are considered very important classification criteria (Atte Von Wright 2011). The LAB are recognized as Gram-positive, low-GC, aerotolerant, generally non-sporulating, non-respiring rods or cocci, which are devoid of cytochromes and genuine catalase and produce lactic acid as major carbohydrate fermentation product (Atte Von Wright 2011). With the help of molecular biological tools, mounting numbers of LAB are being discovered, including non-culturable species. According to the current taxonomic classification, LAB belong to the phylum *Firmicutes*, class Bacilli, and order *Lactobacillales*, and are divided into different families, including *Aerococcaceae*, *Carnobacteriacea*, *Enterococcaceae*, *Lactobacillaceae*, *Leuconostocaceae*, and *Streptococcaceae* (http://www.uniprot.org/taxonomy/186826).

LAB are widespread in the environment and predominant in the human and animal gastrointestinal tract (GIT). Profound investigations are revealing the beneficial functions of non-pathogenic LAB. By producing an array of antibacterial agents, such as acidic compounds and bacteriocins, LAB can inhibit spoilage and the growth of pathogenic microorganisms (Mills et al. 2011, Dalié et al. 2010). Non-pathogenic LAB can improve enzymatic digestion of lactose, and provide vitamins and other essential nutrients (Masood et al. 2011). In addition, when interacting with mammalian epithelial cells, non-pathogenic LAB can enhance the immune system and relieve allergy symptoms (van Baarlen et al. 2013).

1.2 Criteria for a probiotic

Probiotics are “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Food and Agriculture Organization-WHO 2002). The earliest scientific report on probiotic bacteria dates to 1907, when Elie Metchnikoff described a correlation between ingestion of the lactic acid-producing bacteria in yogurt and enhanced longevity in Bulgarians and other populations (Metchnikoff 1907). An increasing number of studies seeks to unveil the mechanisms underlying the beneficial effects of probiotics confer to the host and further to investigate the clinical effectiveness

of probiotics for various diseases. It has been reported that probiotics may help suppress diarrhea, alleviate lactose intolerance and post-operative complications, reduce the symptoms of irritable bowel syndrome (IBS), prevent inflammatory bowel disease (IBD), and exhibit antimicrobial and anti-colorectal cancer activities (Fontana et al. 2013). Probiotics are primarily utilized as food supplement. Despite their putative benefits, the European Food Safety Authority (EFSA) has refused hundreds of applications for probiotic health claims. This highlights the need to carefully follow regulatory guidelines. To provide strong evidence for probiotic efficacy, carefully designed clinical trials with sufficient numbers of subjects are needed. Moreover, without a clear understanding of probiotics' mechanisms, the quest to develop these bacteria as clinical drugs will prove even more arduous (Sanders et al. 2013).

To be successfully utilized, probiotics and probiotic candidates must generally possess certain characteristics. For example, probiotic candidates should be capable of tolerating gastrointestinal conditions (gastric acid and bile) and maintaining themselves in the GIT by adhering to mucus or gastrointestinal epithelial cells; they should also confer beneficial effects upon the host via microbe-host interactions or the exclusion of pathogens. Given these characteristics, some probiotics manage to survive the harsh conditions of the stomach and small intestine. After reaching the lower gut, they must conquer the potential challenges of a continuously renewed mucus layer, occupied adhesion sites, competition from indigenous microbes, and host immune defenses. It is possible that administering a sufficient does of probiotics within the proper period could compensate for insufficient tolerance or adhesion ability. Thus, the viability and amount of probiotic microorganisms are emphasized in the definition of probiotics. The essential function of probiotics is to benefit host health. This could be accomplished by preventing pathogen invasion, producing antimicrobial substances like bacteriocins, aiding digestion to provide better nutrition, and/or reinforcing immune defenses. In addition, probiotics should be non-pathogenic, non-toxic and free of significant adverse side effects. From a technical point of view, an adequate number of viable cells of the probiotic candidate should be present in the delivery product. Therefore, the candidate must be compatible with the product matrix and its processing and storage conditions (Fontana et al. 2013).

When considering the potential health benefits of probiotics, it is notable that probiotic effects tend to be strain-specific (Williams 2010). Strain-specificity may depend on the structure of the bacterial outer membrane, which determines adhesion capability in the host gut, and contains various microorganism-associated molecular patterns (MAMPs) that trigger the host immune responses (Konstantinov et al. 2008, Yasuda et al. 2008, Grangette et al. 2005). On the other hand, probiotic strains can develop sophisticated responses and adaptations in response to the stresses and signals of the host environment. The coordinated expression or suppression of genes can alter cellular processes, such as cell division, membrane composition and transport systems (Sengupta et al. 2013). Modifications to the macromolecular composition of the bacterial cell envelope contribute to variation in adhesion capability in different hosts. In addition, since various host species

provide different anatomical and physiological environment, and have different dietary preferences, a probiotic strain isolated from one host is not necessarily benificial to another (Ley et al. 2008, Eckburg et al. 2005, Dogi and Perdigón 2006). Therefore, host specificity is considered a desirable property for probiotic bacteria (Salminen et al. 1998, Saarela et al. 2000).

1.3 Mechanisms of probiotic action

The mechanisms underlying the beneficial effects of probiotics have been studied extensively during recent decades, although the history of probiotics dates back to the early 1900s (Morelli and Capurso 2012). The putative mechanisms are likely to be multi-factorial and to differ according to strain. The major mechanisms can be assigned to three modes of action. First, probiotics can facilitate a balanced and healthy microbial ecology in the GIT via the promoting competitive exclusion of pathogenic bacteria. This may occur either through direct inhibitory or competitive activity or through the probiotic strain's influence upon the indigenous commensal microbiota (Lebeer et al. 2008, Corr et al. 2009). Second, probiotics can strengthen epithelial barrier function by modulating signaling pathways that lead to enhanced mucus or defensin production, preventing apoptosis, or increasing tight junction function (Oh et al. 2010). Third, probiotics can modulate the immune system of the host, particularly in the small intestine, which harbors fewer microorganisms and so provides more adhesion sites for transient probiotics (Gareau et al. 2010). By activating dendritic cells (DCs) and interacting with epithelial cells and macrophages, probiotics can mediate the release of cytokine, and consequently induce polarization of the T cell response in the GIT (Bron et al. 2011, Coombes and Powrie 2008). Different *Lactobacillus* strains, for example, can elicit a wide range of cytokine responses in immune cells (van Baarlen et al. 2013, Maassen et al. 2000) and, therefore, regulate the innate and adaptive immune responses. Differences in profiles and amounts of host immunostimulatory molecules induced by lactobacilli are suggested to be contributed by bacterial strain-specific metabolism and structures (Lee et al. 2013). To maintain the delicate balance between necessary and excessive immune defense, probiotics should be carefully chosen to improve the host's ability to fight infections by up-regulating immune function or alleviate the onset of intestinal inflammation and autoimmunity by down-regulating the immune response. Recent reports have suggested that probiotics also have effects on the host's enteric nervous system and brain signaling (Collins et al. 2012) and that by inactivating carcinogens, they decrease cancer risk (Sanders et al. 2013). A summary of the potential mechanisms of probiotic action is presented in **Fig. 1**.

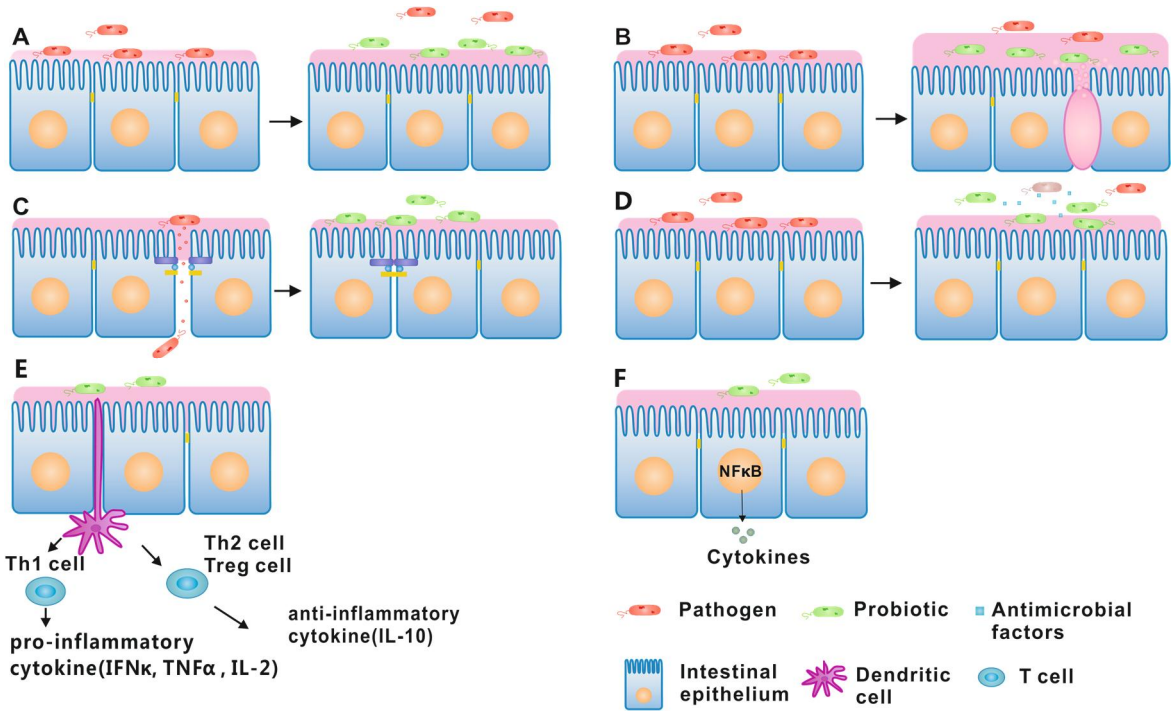

Figure 1. Potential probiotic mechanisms of action. A) Probiotics provide resistance to pathogen colonization by blocking entry into epithelial cells. B) They stimulate goblet cells to release mucus, thus strengthening mucus barrier. C) They maintain the intercellular integrity of tight junctions, thereby preventing the passage of molecules and pathogen invasion. D) They produce antimicrobial factors to kill pathogens. E) They stimulate the immune system by signaling dendritic cells to activate pro- or anti-inflammatory responses. F) They initiate TNF production in epithelial cells and inhibit or activate nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) to influence cytokine production. Adapted from Gareau et al., 2010.

2. Microbiota of the canine gut

2.1 Symbiosis of gut microbiota and the host

Microbes can be found everywhere, from the skin surface to the oral cavity and the urinary and genital tracts. The GIT harbors the largest microbial population; the human gut contains 40,000 bacterial species (Frank and Pace 2008). This abundance is due to the unique physiological characteristics of the GIT, such as being connected to the outer environment, containing various nutritional substrates, and having a large surface area.

The symbiotic relationship between GI microbes and the host is crucial for host health, as it is necessary for the proper function of nutritional, immunological, developmental, and physiologic processes in animals. Germ-free animals exhibit increased requirements for energy and vitamins B and K, decreased immune defenses, impaired intestinal structure and morphology, and delayed gastric motility relative to conventional animals (Claus et al. 2011, Tlaskalová-Hogenová et al. 2011). The resident microbiota can facilitate the digestion of complex carbohydrates, thus providing additional nutrients. The

primary end products of the fermentation process, such as acetate, propionate, and butyrate (short-chain fatty acid, or SCFA), provide energy for host epithelial cell growth and metabolism and also have immuneomodulatory properties. A balanced microbiota can prevent pathogen invasion by creating a physiologically restrictive environment, in which competition for nutrients and mucosal adhesion sites is stiff. Host genetic background, the immune response and dietary preferences can shape individual core microbiomes. Taken together, these findings indicate that microbiota is essential and the symbiosis between a host and its resident microbiome helps maintain health.

2.2 Microbiota composition of the canine gut

Bacterial numbers and composition vary among the compartments of the GIT. In the canine stomach (~pH 2 when empty), acidic conditions restrict the bacterial community to very low numbers, only 10^1 to 10^6 colony forming units (cfu)/g of content survive in this harsh environment (Benno et al. 1992, Hooda et al. 2012). Culture-based studies have reported that a mixture of aerobes and anaerobes, dominated by Gram-positive bacteria, inhabits the stomach. The bile salts and enzymes secreted into the small intestine, which facilitate digestion, limit the bacteria in the duodenum and jejunum to around 10^5 cfu/ml of content. *Eubacterium*, *Bacteroides*, *Clostridium*, *Fusobacterium*, *Bifidobacterium*, and *Lactobacillus* spp. are predominant in the canine duodenum and jejunum (Hermanns et al. 1995, Johnston 1999). In the distal small intestine and the large intestine, a more diverse microbiota encompassing greater numbers of bacteria (10^9 to 10^{10} cfu/g of content) is present (Hooda et al. 2012). In 1977, 84 bacterial species within 27 genera were cultivated from the ileal, cecal, and colonic content of dogs. The predominant genera included *Bacteroides*, *Bifidobacterium*, *Fusobacterium*, *Peptostreptococcus*, *Eubacterium*, *Clostridium*, *Peptococcus*, and *Lactobacillus* (Davis et al. 1977).

However, cultivation assays provide limited information about the gut microbiota because the majority of microbes cannot be cultured without detailed knowledge of their growth requirements. With the aid of molecular-based techniques, GI microbial ecology can be studied in more detail (Table 1). Although there have been some previous studies on mucosa and digesta samples from various segments of the canine GIT, most studies have focused only on bacteria from canine fecal samples (Hooda et al. 2012).

Table 1. Predominant bacterial groups in the canine gastrointestinal tract (presented as percentage of sequences)

Reference	Sample type	Method	Dog number (n) and age	Actinobacteria	Bacteroidetes	Firmicutes	Fusobacteria	Proteobacteria
(Hand et al. 2013)	Fecal	V1-V3* region 16S rRNA gene pyrosequencing	n = 11 1-11 years	0.33	33.36	15.81	39.17	11.31
(Garcia-Mazcorro et al. 2011)	Fecal	V1-V3 region 16S rRNA gene pyrosequencing	n = 12 0.7-10.2 years	0.9-2.0	0.1-1.1	97.5	0.1-0.8	0.1
(Handl et al. 2011)	Fecal	V1-V3 region 16S rRNA gene pyrosequencing	n = 12 0.7-10.2 years	1.8	2.2	95	0.3	-
(Swanson 2010)	Fecal	Whole genome pyrosequencing	n = 12 1.7 years	1	37-38	31-35	7-9	13-15
(Middelbos et al. 2010)	Fecal	V3 region 16S rRNA gene pyrosequencing	n = 6 1.7 years	0.8-1.4	32-34	15-28	24-40	5-6
(Suchodolski et al. 2009)	Jejunal mucosa samples	16S rRNA gene pyrosequencing	n = 5 2 years	11.2	6.2	15	5.4	46.7
(Xenoulis et al. 2008)	Duodenal biopsies	16S rRNA gene pyrosequencing	n = 9 2.7-6years	1.0	11.2	46.4	3.6	26.6
(Suchodolski 2008)	Duodenum, jejunum, ileum and colon contents	V1-V3 region 16S rRNA gene pyrosequencing	n = 6 3.6-7 years	-	12.4	47.7	16.6	23.3

* *V1-V3 are hypervariable regions on 16S rRNA, which enable distinguish of bacterial species.*

2.3 Probiotic intervention studies on dogs

2.3.1 Effects on diarrhea and microbiota shifts

Animal models and human studies have been used to investigate the impact of probiotics upon many gastrointestinal diseases, including IBS, IBD, infectious diarrhea, small intestinal bacterial overgrowth (SIBO), and antimicrobial-associated and nosocomial diarrhea (Sanders et al. 2013, Gareau et al. 2010). Findings on the efficacy of probiotics for bowel diseases have been inconsistent, due to variation in the strains and doses studied and small or heterogeneous trial populations. Additionally, in the absence of generally agreed upon biomarkers for certain diseases, such as IBS and allergy, it is difficult to obtain comparable data from various intervention studies. However, promising results continue to encourage researchers to study probiotic functions.

Many gastrointestinal diseases are associated with diarrhea. Diarrhea results from the stimulation of mucosal fluid secretion when mucosal absorptive capacity is diminished. It can be stimulated by dysfunctional immune responses or enterotoxins released from microbes. Canine intervention studies of probiotics effects on clinical diarrhea are limited relative to human clinical trials. In one randomized, double-blind parallel study, ingestion of a probiotic cocktail reduced convalescence time for acute, self-limiting diarrhea in dogs (the period of abnormal stools was reduced from 2.2 to 1.3 days) (Herstad et al. 2010). In a dog model of non-specific dietary sensitivity (NSS), *L. acidophilus* strain DSM 13241 improved fecal consistency, fecal dry matter, and defecation frequency and increased fecal lactobacilli and bifidobacteria while decreasing the number of *C. perfringens* and *Escherichia* spp. (Pascher et al. 2008). Another study reported that, compared to a placebo, the canine-derived probiotic *B. animalis* strain AHC7 significantly shortened the resolution rate of acute idiopathic diarrhea in dogs (Kelley et al. 2009). In addition to clinical signs evaluation, intestinal cytokine patterns have been studied in dogs with food-responsive diarrhea (FRD). However, intestinal cytokine patterns were not associated with the improved clinical features observed after treatment with a probiotic cocktail (Sauter et al. 2006). In another study with large sample size, the ability of the probiotic *E. faecium* SF68 to reduce the duration of chronic diarrhea was investigated in 217 cats and 182 dogs in an animal shelter. While cats fed SF68 had fewer episodes of diarrhea, no significant reduction was found in dogs (Bybee et al. 2011). Due to the inadequacy of the research base, our knowledge of the effects of probiotics in dogs with clinical symptoms is too restricted to draw reliable conclusions.

Probiotic intervention studies have also been conducted on healthy dogs, to investigate probiotic-induced shifts in the microbiota. Most studies have found a decrease in potentially pathogenic bacteria and an increase in LAB. In one study, dietary supplementation with *B. amyloliquefaciens* CECT 5940 and *E. faecium* CECT 4515 had no effect on fecal scores or digestibility coefficients compared with the control group, but it is possible that it stabilized the fecal microbiota by decreasing pathogenic Clostridia (González-Ortiz et al. 2013). In another study, probiotic *L. acidophilus* strain DSM13241 increased the number of fecal lactobacilli and decreased the number of Clostridia. In addition, it improved immune function in dogs by increasing hematocrit levels, hemoglobin concentrations, serum IgG levels, and the number of red blood cells, neutrophils, and monocytes (Baillon et al. 2004). In addition to culture-based studies, pyrosequencing has been used to study the fecal microbiota of healthy cats and dogs (Garcia-

Mazcorro et al. 2011). After probiotic feeding, no changes in the predominant bacterial phyla in dog feces or no significant changes in immune markers were found. However, an increased abundance of probiotic bacteria was found in the feces, consistent with culture-based analyses. Some canine-derived strains that have potential as probiotics have also been investigated in intervention studies. The canine feces-derived strain *L. animalis* LA4 led to an increase in fecal lactobacilli while reducing enterococci (Biagi et al. 2007). Canine-derived strain *L. fermentum* AD1 increased the number of fecal lactobacilli and enterococci as well as total proteins and lipids, and reduced serum glucose levels (Strompfová et al. 2006). Another study reported that the canine colon-derived strain *B. animalis* AHC7 significantly reduced carriage of Clostridia in dogs (O'Mahony et al. 2009). One study of canine fecal LAB administration resulted in jejunal bacterial population changes, and an indigenous LAB strain became dominant after probiotic feeding had ended (Manninen et al. 2006).

Prebiotics are substrates that can facilitate the growth and function of probiotics when used with probiotics, this combination is termed symbiotic. Symbiotic combinations have rarely been studied in dogs. In one study, *L. fermentum* CCM 7421 was administrated with inulin. The fecal microbiota of dogs fed with this combination contained less Clostridia and higher numbers of LAB than that of a control group. However, the inulin supplement did not intensify probiotic efficacy (Strompfová et al. 2012). One obstacle to using probiotics is that probiotic candidates generally cannot persist in the GIT after administration stops. In one study, however, the canine-derived strain *E. faecium* EE3 persisted in dog feces for 3 months after a 1 week administration, accompanied by decreased *Staphylococci* and *Pseudomonas*-like bacteria and increased LAB (Marcináková et al. 2006).

2.3.2 Effects on general immune function

Probiotics can benefit the host by interacting with the intestinal mucosa, thus modulating the host immune system. Several probiotic effector molecules are involved in immune interactions, including bacterial cell wall component, such as peptidoglycan, polysaccharides, and specific proteins (Klaenhammer et al. 2012). Furthermore, probiotics can indirectly influence the gut immune response by affecting the endogenous commensal microbiota. The mechanisms underlying the probiotic-regulated immune response have been studied primarily using *in vitro* cell-culture models that may not accurately reflect *in vivo* conditions.

Compared to human trials, many fewer studies have explored the effects of proibiotics on immune function in dogs. One study demonstrated that supplementation with *E. faecium* SF68 increased fecal IgA and canine distemper virus (CDV) vaccine-specific circulating IgG and IgA; this was the first time that dietary probiotic LAB were shown to enhance specific immune functions in young dogs (Benyacoub 2003). A recombinant strain of *L. casei* engineered to produce biologically active canine granulocyte macrophage colony stimulating factor (cGM-CSF) increased serum canine corona virus (CCV)-specific IgG (Chung et al. 2009).

2.3.3 Effects on skin disease

Probiotic studies on canine skin problems are rare. Marsella et al. have studied the effects of *L. rhamnosus* strain GG upon atopic dermatitis (AD) in atopic beagles. The results indicate that *L. rhamnosus* strain GG decreased allergen-specific IgE (Marsella 2009). A follow-up study, three years after *L. rhamnosus* strain GG exposure had been discontinued, found that exposure to

probiotics in early life had long-term clinical and immunological effects in this canine model of AD (Marsella et al. 2012). Later, expression of filaggrin, a key protein for the skin barrier function by preventing percutaneous transfer of allergens, was used as a biomarker for AD. Probiotic exposure did not alter filaggrin expression in canine skin biopsy samples (Marsella et al. 2013).

2.3.4 Effects on parasites

The effects of probiotics on eukaryotic pathogens have been little studied. Recent studies have shown that gut commensal microflora can interfere with the life cycle of the intestinal parasitic nematode *Trichuris muris* and provide indirect protective immunostimulation against non-gut parasites, such as *Toxoplasma gondii* (Benson et al. 2009). Probiotic intervention studies to reduce the viability or infectivity of various eukaryotic pathogens have been conducted using cell culture and animal models, primarily mice (Travers et al. 2011). The results have been inconsistent, with protection against parasites varying according to the probiotic strain tested. In the only dog model, Simpson et al. studied to date, Simpson et al. (Simpson et al. 2009) found that *E. faecium* SF68 failed to affect giardia cyst shedding or the innate and adaptive immune responses in dogs with chronic, naturally acquired, subclinical giardiasis.

3. Adherence of *Lactobacillus* in the gastrointestinal tract

Lactobacilli are present in variable amounts throughout the human GIT; they represent about 1% of microorganisms in the nutrient-rich luminal content but only 0.01% of total culturable counts from feces (Dal Bello et al. 2003, Tannock et al. 2005). The proportion of lactobacilli also varies significantly among individuals (Maukonen et al. 2008). Lactobacilli are the most often used probiotics in foods, fermentation, and pharmaceutical preparations (Sanders 1999). It has been reported that they adhere to and interact with host gastrointestinal surfaces via various bacterial cellular structures, some of which are also involved in mediating the host immune response (Strompfová et al. 2006). Various *in vitro* model systems are utilized in routine adhesion experiments, such as Caco-2 or HT-29 human-derived colorectal adenocarcinoma cells (von Kleist et al. 1975), immobilized intestinal mucus (Roos and Jonsson 2002, Vesterlund et al. 2005), and immobilized extracellular matrices (Lindgren et al. 1992). Detection methods such as quantitative culturing (Mack et al. 1999), microscopic enumeration (Tuomola and Salminen 1998), radiolabelling (Bernet et al. 1993) immunological detection, and fluorescent *in situ* hybridization (FISH) provide good resolution in adhesion assays (Maré et al. 2006).

3.1 The intestinal mucosa

The intestinal mucosa consists of a one-cell thick epithelial layer and the underlying lamina propria. The lamina propria is a sterile connective tissue that contains various immune cells. The epithelial layer separates the highly colonized intestinal lumen from the lamina propria, preventing the passage of “non-self” entities, such as bacteria and food components, from the former to the latter. The epithelial layer induces pro-inflammatory host responses while maximizing nutrient absorption via its large surface area (O'Hara and Shanahan 2006). More than 80% of intestinal epithelial cells are columnar cells involved in nutrient absorption and metabolic functions. Tight junctions maintain a selective impermeable barrier between neighboring epithelial cells (Balda and Matter 2008). In addition, Paneth cells and goblet cells in the epithelium support the integrity of the epithelial barrier

via innate immune defenses (McCracken and Lorenz 2001). For example, Paneth cells at the bottom of intestinal crypts produce various antimicrobials, such as defensins and lysozyme, to prevent close contact between microorganisms and the crypt's proliferative cells (Ouellette and Bevins 2001, Bevins and Salzman 2011). Goblet cells produce a complex mixture of glycosylated proteins (mucins), thus forming a protective mucus layer on the epithelium, that prevents direct contact with luminal microorganisms (McCracken and Lorenz 2001, Bevins and Salzman 2011, Wells et al. 2011). The composition of the mucus layer is dynamic, reflecting a balance between production, degradation, and physical erosion (Sengupta et al. 2013). The mucus layer can shorten the bacterial residence time in the GIT, thereby preventing the colonization of epithelial cells by undesired bacteria. On the other hand, mucus can serve as a habitat for commensal bacteria, such as lactobacilli (Kirjavainen et al. 1998, Ouwehand et al. 2001, Servin 2004). The antimicrobial-saturated mucus layer, along with epithelial cells and immune defenses, establish the epithelial barrier that is critical to intestinal health.

3.2 Cell surface structures of *Lactobacillus* associated with adhesion

The fundamental structure of the lactobacillar cell envelope consists of a bilipid plasma membrane embedded with proteins and surrounded by a cell wall. The bacterial cell wall consists of multiple layers of peptidoglycan (PG) decorated with teichoic acids (wall teichoic acids, WTAs, and lipoteichoic acids, LTAs) and proteins anchored to the cell wall through various mechanisms. Sometimes polysaccharides, proteinaceous filaments called pili, and an additional paracrystalline layer of surface (S)-layer proteins that encompasses the PG layer are present as well (Sengupta et al. 2013). These components vary in terms of appearance and structure among different bacterial strains. In lactobacilli, they display species and strain-specific characteristics, playing crucial roles in host-microbe interactions and adaptation to the changing host environment. Moreover, the surface properties of lactobacilli can be modified in response to environmental challenges (Taranto et al. 2003, Fozo et al. 2004).

3.2.1 Mucus binding proteins

Lactobacillus adhesion to mucus involves mucus-binding proteins (Mubs). Thus far, functionally characterized lactobacilli mucus adhesins include the Mub of *L. reuteri* 1063 (Roos and Jonsson 2002), the Mub of *L. acidophilus* NCFM (Buck et al. 2005), and the lectin-like mannose-specific adhesion (Msa) of *L. plantarum* WCFS1 (Pretzer et al. 2005). These three Mubs share a similar mucus-binding domain that has also been identified in several species of LAB, implying the domain is a LAB-specific functional unit (Sengupta et al. 2013). However, high levels of genetic heterogeneity exist among Mubs of different strains, resulting in strain-specific diversity in the ability of bacteria to adhere to mucus (Mackenzie et al. 2010). Proteins containing Mub repeats are abundant in lactobacilli that inhabit the GIT, suggesting that the Mub repeat is a functional unit that may be an evolutionary adaptation for survival in the GIT. Mub and Mub-like proteins have also been shown to contribute to autoaggregation in *L. reuteri* strains (Mackenzie et al. 2010).

3.2.2 Sortase-dependent proteins

In Gram-positive bacteria, a subgroup of surface proteins that contain the C-terminal motif LPxTG is recognized by sortase (SrtA). Cleavage between the T and G residues results in the formation of a

covalent link between the threonine carboxyl group and an amino group provided by cell wall cross-bridges of peptidoglycan precursors. The resulting surface protein is incorporated into the cell envelope and displayed on the bacterium's surface (Marraffini et al. 2006). These surface proteins are commonly called sortase-dependent proteins. Functionally characterized sortase-dependent proteins include the Mub of *L. reuteri* 1063 (Roos and Jonsson 2002), the Msa of *L. plantarum* WCFS1 (Pretzer et al. 2005), and the Mub of *L. acidophilus* NCFM (Buck et al. 2005), all of which are mucus adhesins. The lipoprotein signal peptidase (LspA) of *L. salivarius* UCC118 and the lactobacillus epithelium adhesion (LEA) of *L. crispatus* ST1 are reported to mediate adhesion to epithelial cells (Claesson et al. 2006, van Pijkeren et al. 2006) (Edelman et al. 2012). Although most sortase-dependent proteins of lactobacilli are reported to have capacity to bind to mucus, they do not necessarily have affinity to mucus components (Vélez et al. 2007). More studies will be needed to reveal the function of putative lactobacilli sortase-dependent proteins.

3.2.3 Surface layer proteins

The S-layer proteins of lactobacilli generally self-assemble into monomolecular crystalline arrays exhibiting a morphologically similar lattice structure; they represent 10-15% of total proteins in the bacterial cell wall (Antikainen et al. 2002, Jakava-Viljanen et al. 2002, Åvall-Jääskelainen and Palva 2005). S-layers can be found in several species of *Lactobacillus*, as well as in other bacterial species and Archaea. The biological functions of S-layers are diverse, ranging from serving as a protective coat to providing molecule and ion traps, surface recognition of hydrolase, and adhesion sites (Hynönen and Palva 2013a). Although the biological functions of most S-layers remain unknown, some *Lactobacillus* S-layer proteins, including the CbsA of *L. crispatus* JCM 5810 (Toba et al. 1995, Sillanpää et al. 2000, Antikainen et al. 2002), the Slp of *L. helveticus* R0052 (Johnson-Henry 2007), the SlpA of *L. brevis* ATCC 8287 (Vidgren 1992, Åvall-Jääskelainen 2002, Hynönen 2002), and the SlpA of *L. acidophilus* NCFM (Buck et al. 2005) have been shown to mediate adhesion to epithelial cells, and extracellular matrices (Vidgrén et al. 1992, Hynönen et al. 2002, de Leeuw et al. 2006). In addition, it has been suggested that S-layer proteins have a lectin-like ability to interact with glycoproteins and polysaccharides, thus influencing interactions between lactobacilli and other microorganisms (Golowczyc et al. 2009).

3.2.4 Proteins mediating adhesion to the extracellular matrix

The extracellular matrix (ECM) composed of various proteins, including laminin, collagen, and fibronectin, surrounds intestinal epithelial cells and is referred to as connective tissue. When the mucosa is damaged, the ECM can be exposed to and colonized by undesirable microbes (Styriak et al. 2003). Some lactobacilli have the ability to adhere to this matrix and can occupy binding sites in the gut, competing with pathogens for receptors (Styriak et al. 1999, Neeser et al. 2000, Lorca et al. 2002). The fibronectin-binding protein (FbpA) of *L. acidophilus* NCFM and the collagen-binding protein (CnBP) of *L. reuteri* NCIB11951 may facilitate binding of these strains to ECM (Aleljung et al. 1994) (Buck et al. 2005). Other examples of lactobacilli binding to collagen include the previously discussed S-layer proteins of *L. crispatus* (CbsA) (Antikainen et al. 2002), and *L. brevis* ATCC 8287 (SlpA) (Hynönen et al. 2002).

3.2.5 Nonprotein adhesins (LTA and EPS)

Teichoic acids are the second major component of the lactobacillus cell wall, accounting for up to half of the cell wall's dry weight (Kleerebezem et al. 2010). They are anionic polymers made up of repeating units of glycerol- or ribitol-phosphate they can be covalently linked to PG, in the case of WTA or attached to the cytoplasmic membrane via lipid anchors, in the case of LTA. LTAs contribute to the cell wall with their hydrophobic character, influencing its adhesiveness. TAs vary in terms of sugars and number of phosphate residues. The variation reflects multiple-factors, such as the strain species, stage or rate of growth, and nutrient availability in the medium (Delcour et al. 1999). In *L. johnsonii* NCC 533, LTA has been reported to mediate adhesion to Caco-2 cells (Granato et al. 1999).

Cell wall polysaccharides are neutral polysaccharides that can form an outer capsule by covalently binding to PG (in the case of capsular polysaccharide, CPS), loosely associating with the cell wall (in the case of wall polysaccharide, WPS) or being released into the extracellular medium (in the case of extracellular polysaccharides, EPS). However, it is difficult to provide distinct definitions for the various classes of cell wall polysaccharides. In lactobacilli, EPS generally refers to extracellular polysaccharides attached to the cell wall or released into the surrounding medium (Sengupta et al. 2013). The composition of EPSs varies with regard to the nature of the sugar monomers as well as their linkages, distribution, and substitution. This variability contributes to the structural variety observed in the *Lactobacillus* cell wall (Reeves et al. 1996, Wicken et al. 1983). EPS usually consists of heteropolysaccharides, although some strains of lactobacilli are capable of synthesizing homopolysaccharides (Tieking et al. 2005). Some polysaccharide chains are components of glycoproteins, providing anchorage for S-layer proteins and contributing extra complexity to bacterial cell wall architecture (Francius et al. 2008). The specific functions of EPS in the cell wall remain unclear, although it has been reported to mediate interactions between lactobacilli and the environment and to promote bacterial adhesion and biofilm formation (Lebeer et al. 2011). In *L. acidophilus* CRL639, adhesion to components of the ECM has been associated with production of different types of EPS (Lorca et al. 2002). The carbohydrates on the *L. acidophilus* BG2FO4 cell wall have been reported to be partly responsible for adhesion of this strain to Caco-2 cells and to mucus secreted by HT29-MTX cells (Coconnier et al. 1992).

Aim of the study

The main objectives of this study were to isolate potential probiotic strain for canine use. *Lactobacillus* strains from canine jejunal chyme were investigated (I). By developing a strain-specific detection method (II), one particular strain *L. acidophilus* LAB20 was further studied for its properties that facilitate it to be predominant in canine lactobacilli (III and IV). The detailed objectives of the research were to:

1. Exploit the *Lactobacillus* community in the jejunal chyme of fistulated dogs. The dominant strain (LAB20) was selected for further study of its cellular surface structure, which may facilitate its dominance in the canine gut.

2. Detect LAB20 from the feces of dogs to which LAB20 has been orally administered, using strain-specific detection primers in real-time PCR.

3. Evaluate whether LAB20 has the capacity to adhere to mucus or intestinal cells and potential immunomodulatory effects.

4. Modulate the extracellular polysaccharide (EPS) production of LAB20 using antisense RNA, to investigate its effect on bacterial binding ability.

Materials and Methods

1. Strains, primers, and plasmids

The strains used in this study are presented in Table 2, PCR primer sequences are listed in Table 3, and bacterial plasmids are listed in Table 4.

Table 2. Bacterial strains in this study.

Strain	Reference/Source	Used in
Lactobacillus acidophilus LAB20	This work	I, II, III, IV
Lactobacillus acidophilus LAB48	Abbas Hilmi et al., 2007	I
Lactobacillus acidophilus HAMBI80	HAMBI	I
Lactobacillus acidophilus 74-2	Danisco Ltd.	I, II
Lactobacillus acidophilus ATCC4356	ATCC	I
Lactobacillus rhamnosus Lc-705	Valio Ltd.	II
Lactobacillus rhamnosus GG	Valio Ltd.	II, IV
Lactobacillus crispatus ATCC33820	ATCC	II
Lactobacillus crispatus 119MI	Cultor Ltd.	II
Lactobacillus helveticus 53/7	Valio Ltd.	II
Lactobacillus reuteri CHCC1956	CHCC	II
Lactobacillus salivarius ATCC11742T	ATCC	II
Lactobacillus acidophilus HAMBI1448	HAMBI	II
Lactococcus lactis ATCC7962	ATCC	II
Escherichia coli TG-1	Genesit Ltd.	I, II
Lactobacillus acidophilus SAA658	This work	IV

ATCC: American Type Culture Collection. HAMBI: Culture collection from the University of Helsinki, Faculty of Agriculture and Forestry, Division of Microbiology

Table 3. Sequences of PCR primers used in this study.

Primer name	Sequence 5'-3'	Used in
pA	AGAGTTTGATCCTGGCTCAG	I
pE	CCGTCAATTCCTTTGAGTTT	I
$(GTG)_5$-primer	GTGGTGGTGGTGGTG	I
Usl-1 forward	GAATYGTKAGCGCTSCTGCTGC	I

Usl-2 reverse	GTAAACGTAWGCGTTGTGCTTC	I
UpInverse 1	TTTAGACCAATACGGTAACG	I
UpInverse 2	AGCACCTGCACCAGTTAAGTC	I
Inverse 1	TACATCAACGCTGCTAACATC	I
Inverse 2	TTTAACGCTGTCAGTACCAA	I
RT1	TCAGGCTACACTACTATT	II, III
RT2	CTACACCAGTAAGTTCAA	II, III
EPF	AAAGCGCGCTGCTTGTGGGGGT	IV
EPR	ATCATTTTTCCTCTTACCCTGATTCATATTGTACTAAC	IV
EAF	AGTACAATATGAATCAGGGTAAGAGGAAAAATGAT	IV
EAR	TTTGATATCTGATAAACATACCGCCCATGC	IV

Table 4. Plasmids used in this study.

Plasmid	Relevant properties	Reference	Used in
pLEB767	3.1 kb, pBluescript + partial S-layer gene of LAB20, Amp^r	This study	I
pLEB579	2.9 kb, Cloning vector, Erm^r	Beasley et al. 2004	III

2. Methods

The methods used in this study are presented in Table 5. Detailed descriptions of the methods are presented in the Materials and Methods sections of publications I-II, and manuscripts III and IV. Unpublished methods are presented in chapters 2.1-2.4.

Table 5. Methods used in this study.

Method	Used and described in	Reference
Strain isolation	I, II, III	Shea Beasley et al. 2004
Basic DNA techniques, including PCR, enzyme modifications, electrophoresis, plasmid isolations	I, II, III, IV	Ausubel et al. 1987; Sambrook et al. 1989; Anderson and McKay 1983.
Partial 16S rRNA gene sequencing	I	Edwards et al. 1989

SDS PAGE	II	Sambrook et al. 1989
TEM	IV	
N-terminal sequencing	II	
Rep-PCR	I	
Genomic DNA isolation	I, II, III, IV	Anderson and McKay 1983
Fermentation of milk	II, III	
Real-time PCR	II, III	
Overlap PCR	IV	
Attenuation assay and ELISA	IV	
Cell and mucus adhesion assay	IV	

2.1 Phenotypic microarray test.

The effect of pH on the growth profile of LAB20 was determined using the phenotypic microarray (PM) system from Biolog (Hayward, CA). Reagents, media, and PM10 MicroPlates were purchased from Biolog, and PM experiments were conducted according to the manufacturer's instructions. PM plates were incubated at 37°C and recorded for 60 h. Data from a single experiment were analyzed with Omnilog-PM software from Biolog.

2.2 Growth rates.

The pH of mLBS broth was adjusted to 5.0, 6.5, 7.0, 8.0, or 9.0 with HCl or NaOH, and then media was filtered with a 0.45 µm filter. In each well of a Honeycomb plate (Growth Curves Ltd, Helsinki Finland), 300 µl broth was inoculated with 6 µl LAB20 overnight culture, except for controls wells, which contained only broth. Three replicates were performed for each treatment group. The Honycomb plate was incubated in Microbiology Reader Bioscreen C (Growth Curves Ltd, Helsinki Finland) at 37°C for 30 h. Growth was measured every 40 min by optical density (OD) at 600 nm. Maximum cell density (OD_{max}) was determined when growth curves reached stationary phase.

2.3 Transmission electron microscopy.

Bacterial cells from overnight LAB20 culture were fixed using 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at room temperature for one hour. Samples were then stained via incubation in 2% glutaraldehyde, 0.1% ruthenium red in 0.1 M sodium cacodylate at 4°C for one hour. After washing with 0.1 M sodium cacodylate buffer, the samples were post-fixed by incubation in 2% osmium tetroxide containing 0.1% ruthenium red in sodium cacodylate buffer for 3 hours at room temperature. Samples were washed again with 0.1 M sodium cacodylate prior to dehydration with a series of ethanol solutions increasing from 50% to 100%, and finally with 100% acetone. Then they were plastic embedded by successive incubation in 30% Epon in acetone for 3 hours, 70% Epon in acetone overnight, 100% Epon for 3 hours repeated twice, and finally fresh 100% Epon resin. After polymerization of the resin (60°C for 18 hours) ultrathin sections (60 nm) were

cut. For negative staining, an overnight culture of LAB20 was loaded to a copper grid, and negative staining was performed with phosphotungstic acid (2% [wt/vol] in H2O). Sections post-stained with uranyl acetate and lead citrate were imaged using a TecnaiF20 transmission electron microscope (TEM, FEI Corp.) operating at 200 kV.

2.4 Expression analysis of LAB20 genes associated with binding when grown in the presence of mucin

Two hundred µl LAB20 overnight culture was inoculated to 10 ml mLBS7 broth supplemented with 0, 0.05, or 0.25% procine mucin (Sigma) and grown at 37°C overnight. Total RNA was extracted from 10 ml 0%, 0.05%, and 0.25% mucin-cultured LAB20 (GeneJET RNA Purification Kit, Thermo Scientific, Finland). Then the first strand of cDNA was synthesized using reverse transcription PCR (RT-PCR; RevertAid RT kit, Thermo Scientific, Finland). cDNA was diluted 1:10 and used as a real-time PCR template, with a Tm of 58°C. The independent-samples *t* test was used to determine statistically significant differences ($P < 0.05$). The results of technical replicates are shown as means ± standard deviations.

Results and Discussion

1. Prevalence of *L. acidophilus* in canine jejunal chyme (I)

1.1. Lactobacilli in the jejunal chyme of five fistulated beagles

Due to difficulties in sampling the intestine, most host-derived probiotic strains are isolated from fecal samples (Baillon et al. 2004). However, it has been reported that the fecal microbiota are different from the upper intestinal microbiota, both in terms of species composition and cell numbers (Mentula et al. 2005). To study the prevalence of lactobacilli in the canine small intestine and identify potential probiotic candidates that could dominate in the canine gut, we used the jejunal fistulated dog model. The fistulated dog model enables investigation of the intestinal microbiota without disturbing intestinal motility or microflora (Harmoinen et al. 2001).

Jejunal chyme specimens from five dogs (A, B, C, D, and E) were plated on nutrition agar (NA) and mLBS plates, and then incubated aerobically. With a view toward the convenient manufacture of probiotics, aerobic/facultative anaerobic bacteria were chosen for study. The total jejunal bacteria was around 3×10^7 CFU/ml in each dog, whereas the number of lactobacilli selected with mLBS plates varied (from 7×10^4 to 8×10^7 CFU/ml) (Fig.1 of Study I). Previously, the microbial composition in canine jejunal chyme was had been studied by Mentula et al. (2005). In their study, only 30 CFU/g lactobacilli were found, and in only one canine jejunal sample of 22 dogs. The small number of lactobacilli detected may be a result of sample treatment, as they plated samples that had been frozen without adding cryoprotectants. In our study, fresh jejunal chyme was plated within 3 hours, thus avoiding freeze damage to bacterial cells. In addition, we utilized less selective mLBS plates, which resulted with abundant *Lactobacillus* isolates.

Approximately 20 colonies from mLBS plate of each dog were identified using partial 16S rRNA gene sequencing, yielding a total of 74 isolates that clustered into four species. *L. acidophilus* was dominant in four dogs, and isolates from dogs D and E were confined to *L. acidophilus* strains. *L. murinus* was dominant in dog C and also found in dogs A and B. However, *L. johnsonii* was detected only in dog A, and only minor *L. reuteri* counts were identified in dogs B and C. The results indicate that facultative jejunal lactobacilli consist of a limited number of species (Table 1 of Study I). In another study that used a fistulated dog model (Rinkinen et al. 2004), *L. murinus* and *L. reuteri* were also detected, whereas *S. alactolyticus* was the dominant culturable LAB. The small number of bacterial species in the small intestine could result from the challenges posed by bile salts and enzymes and the rapid transit time of the intestinal contents.

1.2. Rep-PCR typing of isolated *L. acidophilus* strains

To analyze variation among the *L. acidophilus* strains isolated, rep-PCR with the $(GTG)_5$-primer pair were performed. Fifty-one fragment profiles were generated from 54 *L. acidophilus* jejunal isolates. None of the isolates had profiles identical to *L. acidophilus* strains from other host (Fig2. of Study I). Further, in eight distinct profiles identified, the majority of isolates presented the same fragment profile, suggesting there could be a single dominant *L. acidophilus* strain in the jejunum. This representative strain (shown in lane A13 of Fig2. in Study I) was named as LAB20 and selected for further study.

1.3. LAB20 growth optimization (Unpublished)

To optimize the growth condition for LAB20, the Biolog PM test was used. Although some substrates, such as sucrose and L-Lyxose, enhanced LAB20 growth, the most dramatic increase in growth occurred in response to pH (**Fig. 1**). Bacterial density and the growth rate of LAB20 gradually increased with pH (from 3.5 to 10.0), peaking at pH 9.5. This suggests that LAB20 cultivation could be optimized by adjusting the pH in the mLBS medium. However, in the PM test, LAB20 was grown in a defined culture formula, not in mLBS. Therefore, the optimal pH (9.5) identified using the microarray may not be the optimal pH for LAB20 growth in mLBS medium.

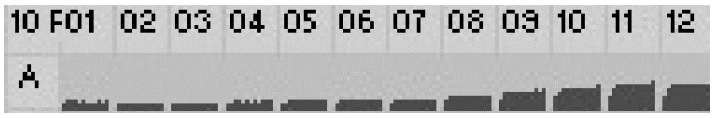

Figure. 1 Growth of LAB20 in PM10 microplates (Biolog). Data are presented in a PM kinetics graph for different pHs. A01 = pH 3.5, A02 = pH 4.0, A03 = pH 4.5, A4 = pH 5.0, A05 = pH 5.5, A06 = pH 6.0, A07 = pH 7.0, A08 = pH 8.0, A09 = pH 8.5, A10 = pH 9.0, A11 = pH 9.5, and A12 = pH 10.0.

To test the effect of pH upon LAB20 growth in mLBS medium, broth of varying pH was inoculated with LAB20 and incubated using the Bioscreen system (**Fig. 2**). Growth curves for LAB20 grown in mLBS medium with different initial pHs were obtained. Broth with a pH of 7.0 yielded the fastest growth and highest cell density (i.e. the shortest lag phase and exponential phase time) at OD 600 nm. In contrast, broth at pH 5.0, 6.2, 8.0 and 9.0 did not represent optimal growth conditions. Therefore, mLBS at pH 7.0 was used as the optimized LAB20 growth medium.

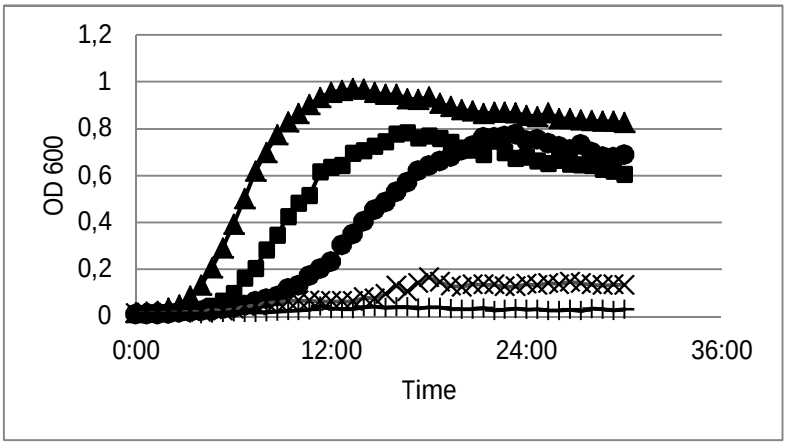

Figure. 2 Growth curves for LAB20 in mLBS broth with different initial pHs, 5.0 (●), 6.2 (■), 7.0 (▲), 8.0 (×), 9.0 (+).

2. Surface structures of LAB20 (II)

2.1. Identification of S-layer protein as a surface component of LAB20

Surface structures could play an essential role in LAB20's dominance in the canine small intestine, since bacterial adhesion in the gut is most likely associated with bacterial surface structures

(Jakava-Viljanen and Palva 2007, von Ossowski et al. 2011, Lebeer et al. 2010). Therefore, LAB20 protein profiles were studied, both for whole cells and LiCl-extracted proteins. The protein profiles revealed one major band with a molecular mass of approximately 50 kDa (Fig. 1 of Study II). The proportion of the protein in the profile and its putative extracellular location indicated that it could be an S-layer protein, which typically is extracted using LiCl and represent 10-15% of proteins in a profile (Frece et al. 2005).

To verify the presence of the putative S-layer protein on LAB20 cells, the N-terminus of the protein was sequenced. Its NH_2-terminal sequence (Ala-Asp-Ala-Thr-Thr-Thr-Thr-Thr-Ala) was 78% identical to that of the *L. crispatus* S-layer protein N-terminus. In addition, a degenerate primer pair (Usl-1 and Usl-2) was used to amplify the partial S-layer protein gene from LAB20 (Jakava-Viljanen and Palva 2007). Then, the sequence was completed using inverse PCR. The predicted open reading frame (ORF) and other gene elements are described in Study II. In general, amino acid sequences of S-layer proteins in related species are remarkably similar (Hynönen and Palva 2013b, Hagen et al. 2005). Comparison to other *Lactobacillus* S-layer protein sequences (Fig. S1 of Study II) demonstrated that LAB20's S-layer protein is novel. The ClustalW multiple alignment program revealed higher levels of similarity in the signal peptide and C-terminal regions, which are predicted to anchor the protein to the bacterial cell wall. Great variability was observed in the N-terminal region, which is responsible for interactions with the environment (Smit et al. 2001, Hynönen and Palva 2013b). The S-layer protein of LAB20 clustered with the S-layer proteins of *L. crispatus* MH315 and *L. acidophilus* 30SC.

2.2. Electron microscopy images of LAB20 (Unpublished)

To visualize the surface structure of LAB20, cells from an overnight culture were studied using electron microscopy (EM). Negative staining revealed an interesting tube-like structure in the LAB20 cell wall (**Fig. 3**). However, this structure was rarely present, indicating that it may form only under certain circumstances. It is possible that LAB20 cells use these structures to communicate, as they were present in cells in contact with one another. Alternatively, the cells may have responded to some stimulus by secreting substances, suggested by the higher density at the tip of the tube. Further investigation is needed to learn more about the formation and function of this tube-like structure.

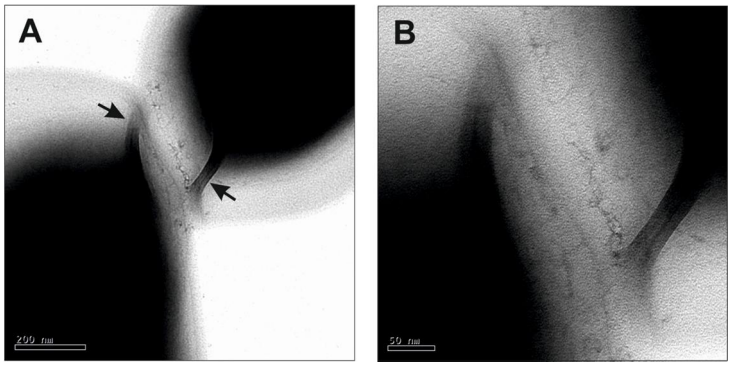

Figure. 3 EM images of negative-stained LAB20 cells. Tube-like structure (arrows) was found in two cells in close contact (A). An enlarged view of the tube-like structure is shown in panel B. The scale bar in panel A represents 200 nm, and that in panel B represents 50 nm.

TEM was used to visualize cellular structures in the LAB20 cell wall. The cell wall was coated in an S-layer protein envelope (Fig. 4), revealed upon further study to consist of novel S-layer proteins (chapter 2.1). In addition, the outermost structure of the LAB20 cell was found to be a putative extracellular polysaccharide (EPS) layer. This EPS layer was thicker when cell were grown at low pH (pH 5.0 vs. optimized pH 7.0 culture conditions). It has been reported that environmental stress leads to various changes in gene expression facilitating a cell's adaptation to its environment (Lebeer et al. 2008). Many resistance mechanisms arise from changes in lactobacillar cell surface structures, which contribute to maintaining cell integrity under stressful conditions (Sengupta et al. 2013). With regard to the role of EPS in stress resistance, microarray expression analyses indicate that *L. acidophilus* and *L. reuteri* genes involved in EPS biosynthesis are suppressed after exposure to bile, however, the underlying mechanism remains unclear (Whitehead et al. 2008, Pfeiler et al. 2007). In the dairy industry, EPS from lactic acid bacteria could improve the viscosity and texture of fermentation products. Therefore, many studies aimed at optimizing the production of lactobacillar EPS have been performed. Optimum lactobacillar EPS production is typically observed under acidic conditions, in the range of pH 4.0 to 5.8 (Mozzi et al. 2003, van den Berg et al. 1995). Consistent with these studies, LAB20's EPS layer was thicker in an acidic culture. Generally, EPS mediates interactions between lactobacilli and the environment and promotes bacterial adhesion and biofilm formation (Sengupta et al. 2013), its role in responding to acid pressure is less clear.

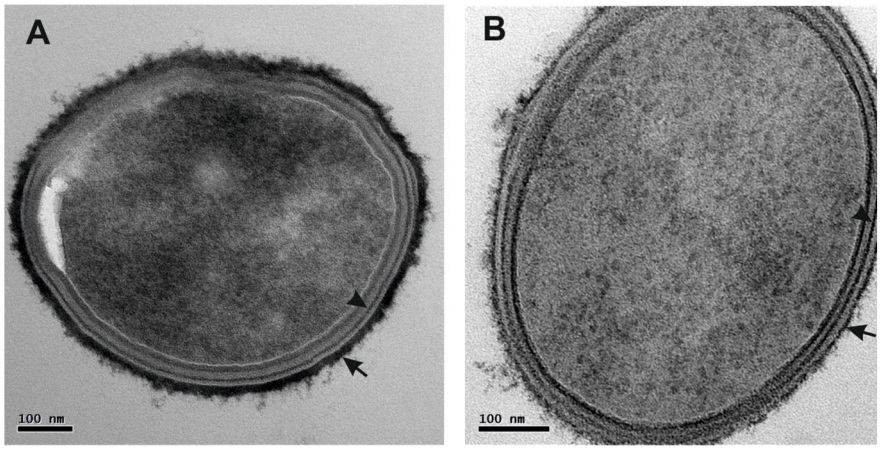

Figure 4. TEM images of LAB20 cells grown in mLBS medium at (A) pH 5 and (B) pH 7. The S-layer protein envelope was present in LAB20 cells grown under both conditions (arrow heads). The extracelluar polysaccharide layer (arrows) was thicker at pH 5. The scale bar in the panels represents 100 nm.

3. Strain-specific detection of LAB20 in dog feces (II, III)

3.1. Real-time PCR assay development

A real-time PCR assay to detect LAB20 on strain level was developed, and validated in a preliminary dog feeding study. A strain-specific primer set was constructed and targeted to the variable region of the novel LAB20 S-layer protein gene. This variable region is located 85 amino acid (aa) after the LAB20 signal sequence, and primer pair RT1 and RT2 generates a 163 base pair (bp) amplicon. The specificity of these primers was verified by comparing the target sequence in GenBank, and using 11 *Lactobacillus* strains phylogenetical closely and distantly related to LAB20 as PCR templates. Null results for both indicate that the primer pair targets to LAB20 specifically.

To further validate the detection assay, an intervention study was conducted to detect LAB20 in dog feces. Fermented milk containing LAB20 was fed to a single domestic dog as a food supplement for 5 days. Its feces were collected over the feeding period and the 6 weeks that followed. Prior to LAB20 exposure, the primer pair detected $10^{0.59}$ DNA copies g^{-1} in the feces, and LAB20 counts peaked after the third feeding, at $10^{7.20}$ copies g^{-1}. Preliminary 454 sequencing data indicates there is a single copy of the S-layer protein gene in the LAB20 genome. Therefore, targeting it could generate more reliable results than targeting a multicopy gene, such as the one for 16s rRNA (Masco et al. 2007). Interestingly, LAB20 was detected from fecal sample even 6 weeks post-administration, which indicates that this strain can persist in the dog gut for a reasonably long period. By contrast, many probiotic intervention studies have reported shorter persistence periods, from 3 days to 5 weeks post-administration (Manninen et al. 2006). Because most probiotic strains fail to persist in the gut, probiotic products should contain very high numbers of bacterial to compensate. Therefore, a probiotic with a reasonable persistence period could be meaningful, not only to reduce the frequency of ingestion required, but also because it implies that the probiotic strain can adapt and thrive in the GIT.

3.2. Dog intervention study

To determine if LAB20 could transit through and persist in the dog gut, five pet dogs raised in different families were included in an intervention study. A previously developed, strain-specific real-time PCR assay was used to detect LAB20 in fecal samples. The baseline for real-time PCR detection varied from 0 to $10^{2.98}$ copies g^{-1} in dog feces. By feeding days 2 and 3, significantly higher numbers of LAB20 was detected in fecal samples. However, these numbers dropped to baseline levels after feeding ceased. This result is not consistent with the findings of the previous dog assay, in which LAB20 was able to persist in the dog gut 6 weeks post-administration (II). However, in this intervention study, fewer LAB20 cells (10^8 CFU vs. 5×10^8 CFU) were fed to dogs over a shorter feeding period (3 days vs. 5 days). Without access to biopsy samples from pet dogs, it was difficult to determine whether LAB20 was unable to colonize in the canine gut, or because it colonized the canine gut but was not shed in the feces in high numbers.

Thus far, most putative probiotic isolates have not been able to persist in recipients' guts after administration ends (Weese and Anderson 2002, Manichanh et al. 2010). Apparently, the microbial ecosystem is rather stable, reflecting host adaptation over time. Additionally, a unique individual gut microbiome develops as a result of the host's genetic background, immune responses, and dietary preferences (Roessler et al. 2008, Turnbaugh et al. 2009, Bron et al. 2011). Thus, it is difficult for a bacterial strain to gain a foothold in the pre-existing microbial ecosystem. However, in a previous study (I), we found that LAB20 was dominant in the guts of five dogs. This may indicate that LAB20 is a canine commensal bacterium, able to adapt in different dogs. Therefore, the mechanisms underlying the ability of LAB20 to adhere to the canine gut mucosa are of particular interest.

Most putative canine probiotics have been isolated from dog feces (Biagi et al. 2007, Manninen et al. 2006, Strompfová et al. 2004). In one study, isolates from dog colon commensal bacteria were studied, but only *B. animalis* AHC7 was selected for dog intervention study, because it could transit the murine GIT in high numbers. The results indicated that *B. animalis* AHC7 consumption could

improve canine GI health by reducing Clostridia carriage (O'Mahony et al. 2009). Endogenous strains are interesting to investigate, not only because they may adapt and thrive better than isolates from dog feces, but also because they influence other commensal microbes and the host immune response. It has been reported that commensal bacteria can influence their neighbors via direct inhibition, competition activity, or their metabolism products, thus shaping microbial ecology in the host gut (Vacharaksa and Finlay 2010). The small intestine accounts for a large proportion of the immunomodulatory capacity of the body, and the population size of endogenous microbiota is relatively small at this site (Bron et al. 2011). Therefore, LAB20, which is dominant in the canine jejunum, could potentially benefit the host by modulating the host immune response.

4. LAB20 cells adhere to mucus, Caco-2 and HT-29 cell lines and regulate lipopolysaccharide-induced interleukin-8 production (IV)

4.1. Adhesion to mucus of different origins

Mucus is the protective layer for underlying epithelium and contains mucin glycoproteins and diverse antimicrobial molecules. The viscous mucus layer is continually renewed to resist microbial passage; meanwhile, it provides epitopes for bacterial adhesion (McGuckin et al. 2011). The adhesion ability of LAB20 was test using mucus samples collected from different sources (canine duodenum, jejunum, ileum, cecum, and colon; and porcine and human colon). LAB20's adhesion to canine colonic mucus was significant greater ($P < 0.05$) than its adhesion to other types of mucus (Fig.2 in Study IV). By contrast, the human-derived strain *L. rhamnosus* GG adhered best to human mucus ($P < 0.05$). This indicates that mucus adhesion efficiency of bacteria may correlate to a specific host.

To study the impact of EPS on LAB20's mucus adhesion, a LAB20 EPS mutant (SAA658) was constructed by cloning an overlapping partial *epsE* gene and S-layer promoter region into the pLEB579 vector and transforming it into competent LAB20 cells. An antisense RNA strategy was used, since creating a knockout mutant was not possible due to the low transformation efficiency of LAB20. SAA658 showed reduced adhesion to all mucus samples relative to the LAB20 wild-type strain. This suggests that EPS could potentially modulate the adhesion of LAB20 to mucus.

Various mucins constitute the main components of mucus. The complex oligosaccharides arrays on the central glycosylated domains of mucins represent multiple potential ligands for microbial adhesion. The expression of glycosyltransferases differs between various GIT and can be modulated by the innate and adaptive immune responses (McGuckin et al. 2011). In addition to mucins, proteomic studies have shown that a large number of proteins add complexity to mucus (Johansson et al. 2009). The complexity and variable composition of mucus may explain the varying adhesion efficiency of LAB20 to canine mucus from various intestinal compartments.

4.2. Adhesion to epithelial cells

Caco-2 and HT-29 cells are derived from human colon adenocarcinomas. They are widely used as *in vitro* models for studying the adhesion of bacteria to GIT epithelial cells. The HT-29 cell line differs from the Caco-2 cell line in that it contains a small proportion (<5%) of mucus-secreting and columnar absorptive cells (Gagnon et al. 2013, Huet et al. 1995). The adherence of LAB20 and SAA658 to Caco-2 and HT-29 cells at different growth stages was included. For both strains,

adhesion was more efficient with 21-day-old than 3-day and 8-day old HT-29 and Caco-2 (Fig.3 in Study IV). Wild-type LAB20 displayed significantly higher adhesion than the EPS mutant SAA658 ($P < 0.05$). The putative EPS modifications in SAA658 could be responsible for its reduced adhesion to epithelial cells. However, manipulation of the outermost bacterial polysaccharide layer may also impact the presentation of surface proteins, which have the potential to bind to epithelial cells (Schneitz et al. 1993).

4.3. Attenuation assay

To investigate the potential probiotic effects of LAB20, lipopolysaccharide (LPS) induced interleukin-8 (IL-8) production was evaluated by incubating LAB20 with HT-29 cells. IL-8 is a pro-inflammatory chemokine that recruits neutrophils into the mucosa (Mitsuyama et al. 1994). The result showed that IL-8 expression in HT-29 cells was attenuated in the presence of LAB20 (Fig. 2 of Study IV) and displayed significant ($P < 0.05$) reduction in higher LPS stimulation. Culture medium, freeze-dried LAB20, and the EPS mutant strain SAA658 were employed as controls, either no or reduced attenuation of IL-8 expression was observed for these controls. The fact that IL-8 was not reduced by exposure to freeze-dried cells suggests that IL-8 attenuation is associated with LAB20 bacterial structures or metabolic products. To study the role of LAB20's putative EPS layer in IL-8 attenuation (Fig. 1 of Study IV, panel A), strain SAA658 was constructed using antisense RNA to alter EPS production in LAB20 cells. Although EPS was not eliminated in SAA658, the appearance of the putative EPS layer adjunct to the cell wall was modified (Fig. 1 of Study IV, panel B). The reduced IL-8 attenuation associated with SAA658 implies that the EPS structure of LAB20 may be responsible for the reduction in IL-8. SAA658 also displayed a significantly reduced ability to adhere; therefore, the reduction in IL-8 attenuation associated with SAA658 may attributable to reduced adhesion and interaction with HT-29 cells. Several studies have showed that bifidobacteria and lactobacilli can reduce the severity of inflammation in rodent models and patients with IBD (Madsen et al. 2001, McCarthy et al. 2003, Claes et al. 2011), but anti-inflammatory properties vary among strains.

Probiotic candidates are exploited for their anti-inflammatory and immunoregulatory effects, which influence enteric infections and mucosal inflammation. *L. paracasei* CNCM I-4034 and its supernatant have been found to reduce the production of *Salmonella typhi*-induced IL-6, IL-8, IL-12p70, and tumor necrosis factor α (TNF-α) in human intestinal DCs (Bermudez-Brito et al. 2012). In an IBD animal model, the anti-inflammatory effects of *L. delbrueckii* subsp. *lactis* CNRZ327 result from modulation of the production of transforming growth factor β (TGF-β), IL-6, and IL-12 in colonic tissue (Santos Rocha et al. 2014). These results provide insight into treating IBD with probiotic bacteria that modulate cytokine production. Based on our findings, the probiotic candidate LAB20 has the potential to decrease IL-8 production in inflammation.

5. Transcription level changes of *Mub*, *Fbp*, and S-layer protein genes during co-incubation with porcine mucin (Unpublished)

It has been reported that multiple cell surface proteins contribute to bacterial adhesion to intestinal cells *in vitro*. For instance, *L. acidophilus* NCFM's fibronectin-binding protein (Fbp), mucus-binding protein (Mub), and S-layer protein are involved in adhesion to Caco-2 cells (Buck et al.

2005). However, exposure to bile and acid stress *in vivo* could alter the bacterial surface structures. To investigate whether mucin influences the transcription of genes associated with mucus binding, LAB20 was co-cultured with 0, 0.05 and 0.25% porcine mucin. The expression of *Mub* was significantly down-regulated ($P < 0.05$) in both the 0.05% and 0.25% mucin cultures; S-layer protein gene transcription also decreased in the presence of mucin, but only significantly in the 0.25% culture. Expression of *Fbp* was up-regulated in 0.05% mucin and significantly down-regulated in 0.25% mucin (**Fig. 5**). This suggests that mucin present in the GIT may mediate transcription of genes associated with mucus binding. Therefore, the presence of mucin may affect LAB20 adhesion *in vivo*.

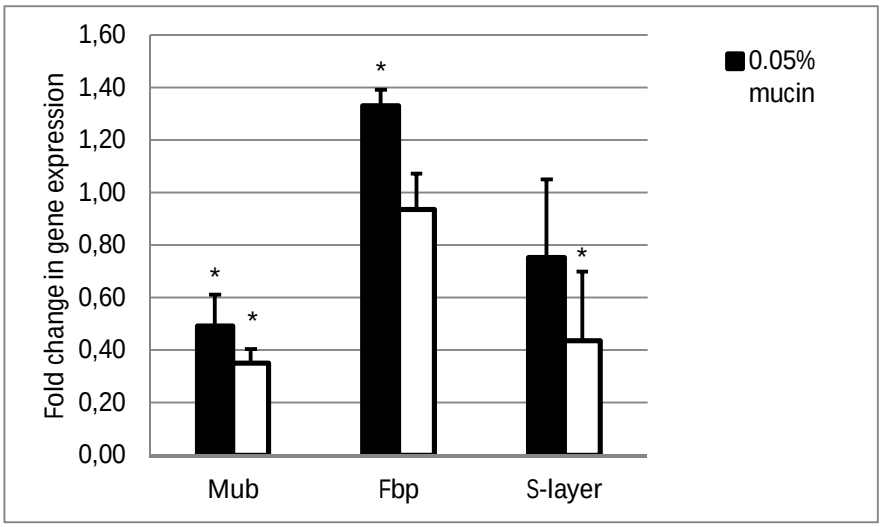

Figure 5. Expressioin of the LAB20 *Mub*, *Fbp* and S-layer protein genes when co-cultured with mucin (0, 0.05, or 0.25%). The values represent the fold change in expression relative to LAB20 cells grown without mucin (set at 1.00). Expression of target genes was normalized to expression of the 16S rRNA gene. The results of three technical replicates with three parallels are expressed as means ± standard deviations. Statistically significant differences are indicated with an asterisk ($P < 0.05$).

Conclusion

In this study, facultative *Lactobacillus* strains were found abundant in the jejunal microbiota of five fistulated beagles, and *L. acidophilus* was dominant. LAB20, the strain with the most common *L. acidophilus* rep-PCR fingerprint, was selected as a representative strain, and we investigated the chariacteristics contributing to its abundance in the canine small intestine. This strain was found to grow poorly *in vitro*; therefore, its growth was optimized by raising the pH of the culture medium to 7.0. Using TEM, SDS-PAGE, and N-terminal sequencing, a novel S-layer protein was identified in LAB20. In addition, a real-time PCR assay specifically targeted to LAB20 was developed, enabling the detection and quantification of this strain. In one dog intervention study that included a single animal, the strain-specific detection was verified, and LAB20 was found to persist in the dog intestine over 6 weeks after the probiotic feeding period had ended. In another intervention study that included five dogs, high LAB20 counts were found only during the feeding period. Fecal sample plating revealed that LAB20 is able to survive passage through the canine intestine. We also found its colony morphotype was distinct from that of other lactobacilli. LAB20 adhered to mucus and epithelial cells and elicited an anti-inflammatory response in HT-29 cells. Both adhesion and IL-8 attenuation in intestinal epithelial cells appeared to be associated with bacterial EPS. Our results indicate that LAB20 has potential as a canine probiotic candidate with anti-inflammatory property, the capacity to adhere to mucus and intestinal cell lines, and the ability to survive passage through the GIT.

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