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ORIGINAL ARTICLE

Lactic acid bacteria isolated from canine faeces

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Abstract

Aims: Lactic acid bacteria (LAB) were isolated and sequenced from the faeces of healthy dogs. Five of these strains were selected and further characterized to clarify the potential of these strains as probiotics for canine.
Methods and Results: LAB were found in 67% (14/21) of the canine faeces samples when plated on Lactobacilli Selective Media without acetic acid. Out of 13 species identified with partial 16S rRNA gene sequencing, *Lactobacillus fermentum* LAB8, *L. mucosae* LAB12, *L. rhamnosus* LAB11, *L. salivarius* LAB9 and *Weissella confusa* LAB10 were selected as candidate probiotic strains based on their frequency, quantity in faeces, growth density, acid tolerance and antimicrobial activity. The minimal inhibitory concentration values of these isolates were determined for 14 antibiotics. *L. salivarius* LAB9, *W. confusa* LAB10 and *L. mucosae* LAB12 were viable in pH 2 for 4 h (mLBS), indicating tolerance to acidity and thus the potential to survive in gastrointestinal tract of the canine. The LAB8-LAB12 strains showed antimicrobial activity against *Micrococcus luteus* A1 NCIMB86166.
Conclusions: Thirteen different LAB species were found from the faecal microbiota of the healthy canines. Five acid tolerant and antimicrobially active LAB strains with the capacity to grow to high densities both aerobically and anaerobically were chosen to serve as candidate probiotics.
Significance and Impact of the Study: The selected LAB strains are among the first host-specific LAB with antimicrobial activity isolated from canines that could serve as potential probiotics for canine use.

Introduction

Bacteria are frequently associated with subclinical abnormalities of canine gastrointestinal tract, such as small intestinal bacterial overgrowth (SIBO) (Delles *et al.* 1994; Rutgers *et al.* 1995). SIBO is detected in 30% of Beagles (Batt *et al.* 1991), causing luminal fluid loss and leading to diarrhoea (Delles *et al.* 1994; Rutgers *et al.* 1995). Selected probiotic bacteria have been reported to reduce diarrhoea in humans and animals (Dunne *et al.* 1999; Fooks *et al.* 1999; Jin *et al.* 1998). Lactic acid bacteria (LAB) constitute a major group of bacteria to be used as probiotics for animals (Biourge *et al.* 1998; Dunne *et al.* 1999). The probiotic characteristics of bacteria are linked to host specificity (Dunne *et al.* 1999; Ouwehand *et al.* 2002). To our knowledge, however, none of the commercial probiotic products for dogs are of canine origin. Also, many of these products contain *Enterococcus faecium* (Benyacoub *et al.* 2003; Chang and Liu 2002; Rinkinen *et al.* 2003), questioned for its safety (Franz *et al.* 1999; Rinkinen *et al.* 2003). In light of the need for probiotic bacteria for canine health, we isolated and characterized potential probiotic LAB strains from canine faeces of the seven most popular dog breeds in the USA (Anonymous 2002a) and in Scandinavia (Anonymous 2002b,c).

Materials and methods

Canine subjects

Seven dog breeds (German Shepherd, Golden Retriever, Jack Russell Terrier, Dachshund, German Shorthaired

Pointer, Border Collie and mixed breed) were selected on the basis of registration statistics in the USA (Anonymous 2002a) and in Scandinavia (Anonymous 2002b,c) in conjunction with the Fédération Cynologique Internationale breed nomenclature group (Anonymous 2002d). Three healthy individuals of each chosen breed from southern Finland were employed in the trial. Age (5·9 ± 3·5), gender (male = 8, female = 13) and nutrition factors varied in canine individuals. Owners were invited to relate the diet and health of their pet. Information from each examined dog was compiled on breed, birth year, health condition, medication and diet. Health facts allowed categorization as healthy or involving intestinal problems.

Specimen collection and isolation of bacteria

Fresh faeces samples were gathered in southern Finland within 4 h transportation distance to the laboratory and packed as full as possible in sterile 50 ml tubes (Cellstar; Greiner-Bio One GmbH, Frickenhausen, Germany). Serial 10-fold dilution of the faeces was prepared (up to 10^{-8}) and cultivated within 4 h of specimen collection on *Lactobacillus* Selective Agar (LBS, BBL; Becton Dickinson Microbiology System, Cockeysville, MD, USA) without the addition of acetic acid (mLBS) in order to decrease the selectivity and yield more strains. Plated samples were incubated in aerobic atmosphere for 48 h at 30°C. From each faecal sample, pure cultures of 13 ± 8 colonies were made on mLBS agar (48 h at 30°C).

Isolation of bacterial DNA

Total DNA was isolated from pure cultures grown overnight on mLBS plates according to the method described by Anderson and McKay (1983) without the phenol extraction. Lysozyme (Sigma-Aldrich, St Louis, MO, USA) was added at concentration of 100 mg ml^{-1} as well as 20 mg ml^{-1} of proteinase K (Finnzymes, Espoo, Finland), both for 1 h at 37°C.

The strains found in faeces ($n = 153$) were identified by partial 16S rRNA gene sequencing of a fragment amplified by PCR using chromosomal DNA as a template. PCR program consisted of 3 min in 94°C followed by 30 cycles of 45 s at 94°C, 60 s at 53°C and 60 s at 72°C, with a final extension step of 10 min at 72°C using universal primers pA 5′-AGA GTT TGA TCC TGG CTC AG-3′ and pE′ 5′-CCG TCA ATT CCT TTG AGT TT-3′ (Edwards *et al.* 1989). DNA Synthesis and Sequencing Laboratory, Institute of Biotechnology, Helsinki, Finland purified PCR products of 900 bp and sequenced them with the Autoread Sequencing Kit using an A.L.F. DNA Sequencer (Pharmacia, Piscataway, NJ, USA). The sequences obtained were compared against the National Center for Biotechnology Industry (NCBI) Blast Library (http://www.ncbi.nlm.nih.gov).

Characterization of the isolated bacteria

The identified strains were grown aerobically and anaerobically in mLBS broth for 48 h at 30°C. Growth density was measured by spectrophotometer (Bioscreen Labsystems, Helsinki, Finland) and the viable count by plating on mLBS agar with incubation for 48 h (time points 0, 12, 24 and 48 h) at 30°C. Acid tolerance of five selected strains in pH values 2, 4, and 7 were tested using survival at pH 5·7 (normal pH of modified LBS broth) as control. The acidity of mLBS was adjusted with 37% hydrochloric acid (Merck, Darmstadt, Germany). Strains were incubated (time points 0, 2, 4, 8, 24 h) in the presence of oxygen at 30°C, plated on mLBS and cultivated for 24 h.

MIC values of the LAB strains

The European Commission recommends for all bacterial products intended for use as food additives that the minimal inhibitory concentration (MIC) values of 14 specified antibiotics are determined (Anonymous 2003). Therefore, antibiotic sensitivities of LAB strains aimed for the feeding studies were tested (Table 2). Resistance to tested antibiotics was determined using breakpoint values set by the Scientific Committee on Animal Nutrition (SCAN). Colonies of overnight grown LAB strains on mLBS plates (BBL) were diluted in Müeller-Hinton broth (Difco Laboratories, Detroit, MI, USA) to obtain a suspension of 1 McFarland. The suspension was spread evenly with a sterile cotton swab over a Müeller-Hinton 5% blood agar plate (Tammertutka Ltd, Tampere, Finland) and E-test antibiotic strips (E-test; AB Biodisk, Solna, Sweden) were placed on top of cultured plate according to manufacturers instructions. The MIC values of the LAB strains were determined for ampicillin AM, ciprofloxacin CI, chloramphenicol CL, enrofloxacin EF, erythromycin EM, gentamicin GM, kanamycin KM, linezolid LZ, rifampicin RI, quinupristin/dalfopristin RP, streptomycin SM, tetracycline TC, trimethoprim TR and vancomycin VM. Duplicate plates were incubated at 37°C and read after 48 h.

Inhibition tests

LAB strains were tested for cross-inhibition by cross-streaking each strain over every other on an mLBS plate and then grown overnight at 30°C. Antimicrobial activities of the LAB strains were tested with well diffusion assay using *Micrococcus luteus* A1 NCIMB86166 (National Collection of Industrial and Marine Bacteria) as an indicator strain. For well diffusion assay, LAB strains were grown overnight in Difco™ Lactobacilli MRS broth (Becton Dickinson, Sparks, MD, USA) and in whey broth including 3% whey (Sigma-Aldrich), 1% caseinhydrolysate (Merck KGa A) and 1% yeast extract (Biokar Diagnostics, Beauvais, France) at 37°C. One millilitre of culture supernatant was boiled for 15 min and 100 μl was added to a well in the LB plate including 0·5% yeast extract (Biokar Diagnostics), 1% NaCl (J.T. Baker, Deventer, Holland), 1% tryptone (Biokar Diagnostics) and 1·5% agar (Oxoid Ltd, Hampshire, England). Part of the boiled supernatant was then treated with 0·5 mg ml^{-1} Proteinase K (Finnzymes) and 0·5 mg ml^{-1} Trypsin (Sigma-Aldrich) for 1 h at 37°C and after the protease inactivation by boiling for 15 min, 100 μl of the supernatant was added to an another well in LB plate. After diffusion of supernatants to wells, 7 ml of LB soft agar (0·7%) containing *M. luteus* (OD 0·1) was poured on top of agar surface and plates were grown overnight at 37°C.

Results

Bacterial isolation and identification

Of the 21 canine faecal samples examined, 14 (67%) contained LAB with an average bacteria concentration of $5{\cdot}8 \times 10^5 \pm 2{\cdot}1 \times 10^5$ CFU g^{-1} of wet weight. The faeces of all dog breeds investigated contained LAB except for that of the Dachshund. A section of the 16S rRNA gene of the 153 isolated strains was sequenced. A sequence similarity of ≥97% is regarded to be acceptable identification of the genus level (Drancourt *et al.* 2000). When the sequences of the 153 strains were compared with type strains in the NCBI genome Blast Library, sequence similarity values of 97% or more were obtained. Based on these similarity values, *Weissella confusa* (17·6%), *Pediococcus acidilactici* (17·0%), *Lactobacillus casei* (11·8%), *L. salivarius* (11·8%), *L. rhamnosus* (11·1%), *L. mucosae* (10·5%) and *L. fermentum* (9·2%) were the most frequently encountered bacteria in canine faeces (Table 1). No correlation between LAB and diet was observed.

Characterization of isolated bacteria

Bacterial strains were grown in mLBS broth in aerobic and anaerobic conditions at 30°C for 48 h. *L. fermentum* LAB8, *L. salivarius* LAB9, *W. confusa* LAB10, *L. rhamnosus* LAB11 and *L. mucosae* LAB12 of canine origin were classed as oxygen tolerant LAB and chosen for further characterization because of dominance in the canine LAB microbiota and capacity to grow to high densities. Average culture densities (OD_{600}) of LAB8-LAB12 were 1·4 ($1{\cdot}3 \times 10^9$ CFU ml^{-1}) ±0·2 ($\pm 1{\cdot}9 \times 10^8$ CFU ml^{-1}) after 12 h of aerobic cultivation in mLBS broth. *L. rhamnosus* LAB11 reached higher OD_{600} (1·6) and growth values ($5{\cdot}4 \times 10^9$ CFU ml^{-1}) in 12 h at 30°C, whereas

Table 1 Lactic acid bacteria species isolated from canine faeces. The partial 16S ribosomal gene sequence of only five strains has been deposited in the European Molecular Biology Laboratory gene bank

Bacteria species	Isolation % (isolates/total isolates)	Breed isolated from (number of isolates)	Accession number (strain number)
Weissella confusa	17·6 (27/153)	German Shepherd (19)	AJ508722 (LAB10)
		Jack Russell Terrier (8)	–
Pediococcus acidilactici	17·0 (26/153)	Golden Retriever (12)	–
		German Shorthaired Pointer (14)	–
Lactobacillus casei	11·8 (18/153)	German Shorthaired Pointer (15)	–
		German Shepherd (3)	–
Lactobacillus salivarius	11·8 (18/153)	Border Collie (16)	AJ508721 (LAB9)
		German Shorthaired Pointer (2)	–
Lactobacillus rhamnosus	11·1 (17/153)	German Shepherd (4)	–
		Jack Russell Terrier (13)	AJ508723 (LAB11)
Lactobacillus mucosae	10·5 (16/153)	Jack Russell Terrier (16)	AJ508724 (LAB12)
Lactobacillus fermentum	9·2 (14/153)	Golden Retriever (11)	AJ508720 (LAB8)
		Border Collie (2)	–
		German Shorthaired Pointer (1)	–
Lactobacillus reuteri	5·2 (8/153)	Jack Russell Terrier (5)	–
		German Shorthaired Pointer (3)	–
Weissella cibaria	3·3 (5/153)	Jack Russell Terrier (5)	–
Lactobacillus murinus	0·7 (1/153)	Golden Retriever (1)	–
Lactobacillus paraplantarum	0·7 (1/153)	German Shepherd (1)	–
Lactobacillus pentosus	0·7 (1/153)	German Shepherd (1)	–
Lactobacillus plantarum	0·7 (1/153)	Golden Retriever (1)	–

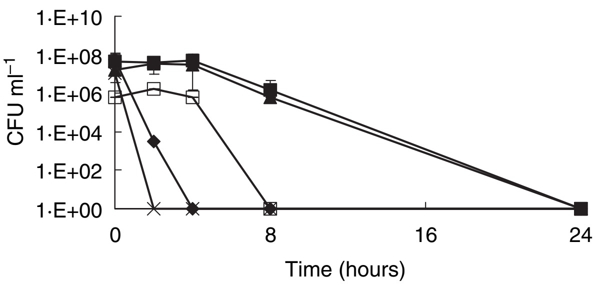

Figure 1 Survival of *Lactobacillus fermentum* LAB8 (◆), *L. salivarius* LAB9 (■), *Weissella confusa* LAB10 (▲), *L. rhamnosus* LAB11 (×) and *L. mucosae* LAB12 (□) incubated in LBS broth (BBL; Becton Dickinson Microbiology System) without addition of acetic acid for 24 h at 30°C. The pH was adjusted to 2 using 37% hydrochloric acid (Merck).

L. rhamnosus LAB11 incubated without oxygen rose to $4{\cdot}1 \times 10^9$ CFU ml^{-1} in identical incubation conditions.

Selected LAB8-LAB12 strains thrived at pH 4 and above for 48 h (data not shown). *L. salivarius* LAB9 and *W. confusa* LAB10 survived above 10^5 CFU ml^{-1} for 8 h and *L. mucosae* LAB12 lived for 4 h in pH 2, while the growth of *L. fermentum* LAB8 and *L. rhamnosus* LAB11 diminished in 2 h after the initiation of cultivation (Fig. 1).

Inhibition tests

Strains were tested on mLBS plates for mutual inhibition by cross streaking, revealing no interactive effect on growth (results not shown). The well diffusion assay revealed the antimicrobial activity of the LAB8-LAB12 against *M. luteus* A1 NCIMB86166 (Fig. 2). The strains produced more antimicrobial activity in MRS than in whey medium. Proteases did not decrease the antimicrobial activity of the LAB8-LAB12 strains grown on MRS or whey media. Interestingly, protease treatment of the growth supernatant of *L. mucosae* LAB12 resulted in increased antimicrobial activity.

MIC values of the LAB8-LAB12 strains

LAB8-LAB12 strains were resistant to vancomycin and amoxicillin whereas all tested strains showed sensitivity to erythromycin, chloramphenicol and ampicillin (Table 2). *L. fermentum* LAB8 was resistant to ciprofloxacin, enrofloxacin, gentamicin, kanamycin, quinupristin/dalfopristin, streptomycin, tetracycline and trimethoprim, *L. salivarius* LAB9 to ciprofloxacin, enrofloxacin, gentamicin, quinupristin/dalfopristin and tetracycline, *W. confusa* LAB10 to rifampin, quinupristin/dalfopristin and trimethoprim, *L. rhamnosus* LAB11 to gentamicin, kanamycin and trimethoprim and *L. mucosae* LAB12 to ciprofloxacin, enrofloxacin, linezolid and trimethoprim.

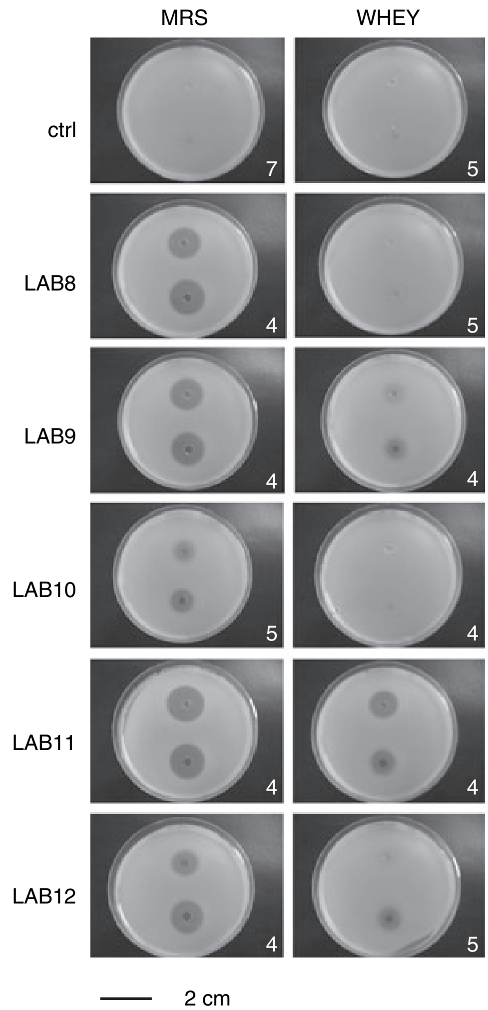

Figure 2 Antimicrobial activities of the *L. fermentum* LAB8, *L. salivarius* LAB9, *W. confusa* LAB10, *L. rhamnosus* LAB11 and *L. mucosae* LAB12 strains analysed with well diffusion assay. In each plate, *M. luteus* A1 NCIMB86166 was used as an indicator strain (bacterial lawn). Numbers at the right corner are pH-values measured from culture supernatants. Controls (ctrl) are cultivation broths without LAB. In each plate, lower well contains supernatant treated with proteases and upper well contains supernatant without treatment.

Discussion

Lactobacilli have been found in small quantities in all parts of the canine gastrointestine (Batt *et al.* 1991; Benno *et al.* 1992; Fujisawa and Mitsuoka 1996; Matsumoto and Baba 1972). Previous species identifications utilized mainly physiological characteristics resulting in a high risk of errors. Therefore, molecular-based methods for bacterial profiling is to be preferred (Drancourt *et al.* 2000; Greetham *et al.* 2002; Heilig *et al.* 2002), leading to the accurate identification of LAB isolated. Here, we employed partial 16S rRNA gene sequencing of fecal

Table 2 Minimal inhibitory concentration values of *L. fermentum* LAB8, *L. salivarius* LAB9, *W. confusa* LAB10, *L. rhamnosus* LAB11 and *L. mucosae* LAB12

Antibiotic	SCAN	MIC values (μg ml^{-1}) of LAB8-LAB12 strains				
		L. fermentum LAB8	*L. salivarius* LAB9	*W. confusa* LAB10	*L. rhamnosus* LAB11	*L. mucosae* LAB12
AM	2	0·5/0·75	1/1	0·38/0·5	0·125/0·38	0·016/0·25
CI	4	4/32 R	1·5/32 R	1·5/0·75	0·125/0·38	0·002/32 R
CL	16	0·75/2	6/3	0·5/4	0·75/5	0·016/2
EF	4	32/32 R	32/32 R	1/1·5	2/1·5	0·002/8 R
EM	4	0·016/0·047	0·047/0·047	0·016/0·064	0·016/0·023	0·016/0·016
GM	1	0·38/1 R	2·5/0·25 R	0·064/0·75	0·75/1 R	0·016/0·047
KM	32	8/48 R	4/8	1·5/24	32/32 R	0·016/2
LZ	4	0·75/1·5	2/3	1/3	0·38/2	0·016/4 R
RI	32	0·25/0·38	1/2	1/32 R	0·47/0·19	0·02/0·032
RP	4	0·75/6 R	4/4 R	0·25/2	1/4 R	0·02/0·25
SM	16	4/16 R	4/4	4/6	4/3	0·064/0·38
TC	16	2/24 R	8/48 R	1·1/4	0·125/1	0·016/3
TR	32	4/32 R	0·094/0·094	32/32 R	8/32 R	0·02/32 R
VM	4	256/256 R	256/256 R	256/256 R	256/256 R	0·016/256 R

Tested antibiotics were ampicillin (AM), ciprofloxacin (CI), chloramphenicol (CL), enrofloxacin (EF), erythromycin (EM), gentamicin (GM), kanamycin (KM), linezolid (LZ), rifampicin (RI), quinupristin/dalfopristin (RP), streptomycin (SM), tetracycline (TC), trimethoprim (TR), and vancomycin (VM). Resistance (R) was determined using breakpoint values set by Scientific Committee on Animal Nutrition.

isolates and showed LAB to be present in two-thirds of all faecal samples. Dachshund was the only breed in which LAB was not isolated. Maybe analysis of more samples from this breed would have led to isolation of LAB. Of the 13 species isolated in this study *L. fermentum* (Mitsuoka *et al.* 1976), *L. salivarius* (Fujisawa and Mitsuoka 1996), *L. reuteri* (Fujisawa and Mitsuoka 1996) and *L. murinus* (Greetham *et al.* 2002; Rinkinen *et al.* 2004) have previously been reported to be present in canine faeces. *L. reuteri* (Tzortzis *et al.* 2004), *L. mucosae* (Tzortzis *et al.* 2004) and *W. confusa* (Björkroth *et al.* 2002) have been isolated from canine clinical samples.

Stable and abundant growth of bacteria used for promoting health is required for reasons of cost and technological simplicity (Charteris *et al.* 1998). In this study, the isolated bacteria could grow in aerobic and anaerobic conditions (data not shown), suggesting that the tested bacteria are capable of surviving in the aerobic environment of industrial manufacturing and handling processes as well as the anaerobic conditions in the gut and intestine. For survival for passage into the small intestine, probiotic bacteria must tolerate the low pH in the stomach. With gastric acid stimulation, the pH of the gastric contents of canine gut can fall to a level of two or less, after which the pH gradually increases towards neutral throughout the small intestine and the colon (Wingfield and Twedt 1986). Microbial cultures used as probiotics have been proposed to be screened for their resistance to acidity and bile tolerance (Jin *et al.* 1998; Reid *et al.* 2003). Jin *et al.* (1998) reported that the *L. brevis* C10 strain isolated from chicken intestine could tolerate pH 2 for 3 h and scarcely survive in pH 1 for 2 h, while other tested *Lactobacillus* species perished at this acidity level. In this study, *L. salivarius* LAB9 and *W. confusa* LAB10 strains survived above 10^5 CFU ml^{-1} for 8 h at pH 2, emphasizing the probiotic potential of these strains.

The five tested LAB strains, LAB8-LAB12, demonstrated the capability to grow among each others presence showing that they may be included in one probiotic product. Simultaneous feeding of several probiotic strains may afford greater potential health benefit than does a single strain because of that the combined diversity of probiotic capacities of several strains is likely to be larger than that of one strain. Similarly, feeding of several probiotic strains occupying many different host binding sites could potentially lead to more effective competitive exclusion of pathogens, a mechanism proposed by Greetham *et al.* (2002) as a role of Lactobacilli to the benefit of the canine host.

Although LAB8-LAB12 strains were resistant to several antibiotics, they were also sensitive to many. Resistance was determined using the SCAN breakpoint values for Lactobacilli (Anonymous 2003), which have been questioned by Danielsen and Wind (2003). Using the breakpoint values set by Danielsen and Wind (2003), streptomycin, kanamycin and ciprofloxacin resistance would not have occurred in this study. Resistance to antibiotics is not of concern if the resistance is not mediated by transferable antibiotic markers. *L. casei* GG, a probiotic for human consumption, is on the market even though it is resistant to vancomycin (Tynkkynen *et al.* 1998). Some of the tested LAB were resistant to various antibiotics of clinical relevance, and might be considered to present a theoretical infection threat. However, no reports exist where LAB have been suggested to cause a septicemia in a dog, so the risk appears to be minimal. In addition, all the LAB strains tested were sensitive to ampicillin, which could be used as a drug if an unlikely systemic infection caused by fed probiotics should occur. One of the likely indications for probiotic therapy is prevention and treatment of antibiotic-associated diarrhoea. Therefore, the resistance against commonly used antibiotics (e.g. enrofloxacin/ciprofloracin and tetracyclin) can be regarded as a beneficial trait in the potential probiotics tested here.

Many LAB strains have the capacity to inhibit other bacteria either by producing antibacterial peptides or by secreting other inhibitory compounds, such as reuterin (Barefoot and Nettles 1993; Ross *et al.* 2002). Such a capacity has been suggested to contribute to the probiotic effects of LAB by inhibiting pathogens (Elliason and Tatini 1999). In MRS media, the LAB8-LAB12 strains inhibited *M. luteus*, which is sensitive to many antimicrobial peptides (Chatterjee *et al.* 2005; Thomas *et al.* 2000), showing that the strains have antimicrobial properties. Antimicrobial activities of the LAB8-LAB12 strains were not diminished by protease treatment indicating either protease resistance or a nonprotein nature of the antimicrobial substance. Part of the antibacterial activity observed may be because of lactic acid, but the differences of the antibacterial activity observed between the different strains are unlikely to be explained by differences in lactic acid amounts as acidification of the spent growth media did not correlate with antibacterial activity. For example, *W. confusa* LAB10 acidified MRS less than whey medium, but antimicrobial activity produced was higher in MRS compared with whey medium. In whey media, an interesting finding was that proteases enhanced the antimicrobial activity of the LAB12 strain similarly as found with *Propionibacterium jensenii* (Faye *et al.* 2002). This indicated that the inhibitory activity of strain LAB12 was an antibacterial peptide. The nature of the antimicrobial activity and the antimicrobial spectra of the LAB8-LAB12 strains will be analysed in future studies.

The probiotic addition to canine weaning food is thought to promote overall health over the dog's lifespan, and particularly the development of its immune system (Benyacoub *et al.* 2003; Biourge *et al.* 1998). Dog foods containing LAB are available at pet stores and from veterinarians in Finland (Anonymous 2004a,b). The origins of these LAB have not been named. *Ent. faecium*, the sole pharmaceutical probiotic on the commercial market for veterinary use in Finland, reportedly led to the increase of adhesion and colonization of *Campylobacter jejuni* on canine intestinal mucus (Rinkinen *et al.* 2003) emphasizing the need of a safer host-specific probiotic for canine. LAB isolated from canine intestine adhered to immobilized intestinal mucus (Rinkinen *et al.* 2000) and inhibited the adhesion of certain bacterial pathogens (Rinkinen *et al.* 2003). The LAB strains discussed here, present in healthy dogs, may possess the technical potential for serving as probiotics for dogs. To confirm this, feed with these canine-origin LAB will be fed to canine for an analysis of their *in vivo* impact.

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