Lactobacilli and Enterococci – Potential Probiotics for Dogs

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ABSTRACT. Forty strains of enterococci and forty strains of lactobacilli isolated from feces of 10 healthy dogs were tested for the antimicrobial activity, tolerance to bile and adhesion activity. The total count of fecal enterococci reached 5.5 log CFU/g and of lactobacilli 7.6 log CFU/g. Screening for production of bacteriocin-like substances showed an to partly inhibit the growth of *Enterobacter* sp. (hazy zones of inhibition). Ten strains of *Enterococcus* sp. and nine strains of *Lactobacillus* sp. were found without any inhibitory activity against all indicators used. Seven enterococcal strains and six lactobacilli with the broadest antimicrobial spectrum were selected for further probiotic assays. In the presence of 1 % bile, the survival rate of selected enterococci (71.7–97.5 %) was higher than that of lactobacilli (66.7–75.4 %). The adhesion of strains to human intestinal mucus (5.1–8.2 % by enterococci, 2.7–8.3 % by lactobacilli) was found to be similar as adhesion to canine intestinal mucus (3.7–10.6 % by enterococci, 2.1–6.0 % by lactobacilli). Strain AD1, one lactobacillus isolate, reduced the higher level of serum cholesterol and alanine aminotransferase after oral administration to dogs suffering from diseases of the gastrointestinal tract.

During the last years probiotics have been used with increasing frequency in nutrition and for prophylactic purposes. Probiotic has been defined as "preparation or product containing viable, defined microorganisms in sufficient numbers which alter the microflora in a compartment of the host and exert health effects in this host" (Schrezenmeir and de Vrese 2001). Microorganisms to be considered for probiotic use must be able to pass the stomach–duodenum barrier in a viable condition. They have to multiply at the site of destination in the intestine (Salminen *et al.* 1998). Additionally, they must be able to produce antagonistic metabolites against dominating saprophytic microflora resulting in a competitive growth. Antimicrobial activity of lactic acid bacteria, which are the most widely used as probiotics, is due to the production of organic acids (lactic and acetic acid in particular), carbon dioxide, ethanol, hydrogen peroxide, and biacetyl (De Vuyst and Vandamme 1994). However, an inhibition can also be caused by bacteriocins – ribosomally synthesized proteins or peptides with inhibitory activity against more or less related bacterial genera (Nes *et al.* 1996). Adhesion to the intestinal mucus belongs to the main properties of probiotic microorganisms (Ouwehand *et al.* 1999) and is considered to be important in transient colonization, antagonism against pathogens, modulation of the immune system and enhanced healing of damaged gastric mucus (Elliot *et al.* 1998; Alander *et al.* 1999). Potential probiotics need to have good technological properties, so that they can be cultured on a large scale. Finally, they must have an acceptable shelf life. These abilities are often found among lactic acid bacteria, *e.g.*, lactobacilli and enterococci, which are the most frequently used as animal feed supplements (including canine feed) or directly as probiotic preparations.

In our study, 40 strains of *Lactobacillus* spp. and 40 strains of *Enterococcus* spp. isolated from 10 healthy dogs of different age, breed and sex were studied for adhesion activity, tolerance against bile and production of bacteriocin-like inhibitory substances (BLIS) – major focal point of our study.

MATERIAL AND METHODS

Feces of 10 healthy dogs of 8 breeds, both sexes with median age 2 years (in the range from 3 months to 6 years) were serially diluted in phosphate buffered saline (PBS), plated on M-*Enterococcus* agar (*Becton Dickinson*, USA) and MRS agar (*Merck*, Germany) and incubated for 2 d at 37 °C. The total amount of enterococci and lactobacilli in canine feces was expressed as $\log_{10}$ of colony forming units (CFU) per g. Forty colonies from both genera were picked up and maintained on brain heart infusion (BHI) with agar

(*Becton Dickinson*) and MRS agar for further testing. Enterococci were genotyped by tDNA PCR followed by capillary electrophoresis according to Baelae *et al.* (2000) and Welsh and McClelland (1991).

Antimicrobial activity was detected using the agar diffusion test (Skalka *et al.* 1983). Overnight cultures were spotted onto the surface of plates containing 1.5 % (*W/V*) BHI for enterococci and Scheadler agar (*Becton Dickinson*) for lactobacilli. Medium pH was adjusted to 6.1 with phosphate buffer (g/L; KH_2PO_4 54, Na_2HPO_4 17.8). The plates were incubated in a CO_2 atmosphere for 1 d at 37 °C. After incubation, they were overlaid with 4 mL of appropriate soft agar (0.7 %, *W/V*) inoculated with 200 μL of the indicator strain and incubated under the same conditions for 1 d at 37 °C. Inhibition was detected as a clear zone around the tested organism. A complete list of indicator bacteria is given in Table I.

Table I. Antimicrobial activity[a] of lactobacilli (L) and enterococci (E)

Indicator strain	Source	No zone		<10 mm		≥10 mm	
		L	E	L	E	L	E
Gram-positive							
Enterococcus avium EA 5	feces of piglet	35.0	35.0	22.5	30.0	42.5	35.0
casseliflavus 20332 TS	collection[b]	92.5	80.0	7.5	20.0 (12.5)	0	0
durans 5A	feces of antelope	85.0	85.0	15.0	15.0 (15.0)	0	0
faecalis EE P4	feces of Japanese quail	65.0	30.0	22.5 (2.5)[e]	70.0 (2.5)	12.5 (10.0)	0
faecium EF 43	feces of piglet	52.5	27.5	12.5	47.5	35.0	15.0
Lactobacillus acidophilus LA 99	vegetable salad	52.5	45.0	15.0	52.5 (5.0)	32.5	2.5
johnsonii LJ 4982	vegetable salad	92.5	87.5	7.5	12.5	0	0
Micrococcus sp. 4898	fish salad	52.5	47.5	5.0	50.0 (5.0)	42.5	2.5
Staphylococcus aureus SA 2	fish salad	52.5	35.0	20.0	17.5	27.5	47.5
aureus SA 105	mastitis milk	100	100	0	0	0	0
lentus SL 163	feces of deer	37.5	50.0	60.0	50.0 (35.0)	2.5	0
xylosus SX 310	rumen content of calf	45.0	27.5	15.0	65.0 (5.0)	40.0	7.5
Streptococcus bovis AO 24/85	rumen content of calf[c]	37.5	35.0	30.0	65.0	32.5	0
bovis B 357	pigeon[d]	37.5	50.0	25.0	50.0 (50.0)	37.5	0
Lactobacillus lactis 96 RS	collection[b]	65.0	42.5	2.5	57.5	32.5	0
Gram-negative							
Escherichia coli W4	feces of dog	100	100	0	0	0	0
Enterobacter georgiviae EG3	pig slurry	25.0	100	32.5 (30.0)	0	42.5 (42.5)	0
Pseudomonas sp. E3	feces of dog	100	100	0	0	0	0
Salmonella enterica sv. enteritidis	feces pf pig	100	100	0	0	0	0
Yersinia sp. M8	feces of dog	100	100	0	0	0	0

[a]Per cent of isolates with inhibition zone of the indicated diameter (none, <10, ≥10 mm).
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[e]*In parentheses* – per cent of isolates showing hazy zones of inhibition.

Tolerance to bile was tested according to Gilliland and Walker (1990). Overnight cultures were inoculated (2 %) into MRS broth without and with ox bile (1 %; *Biomark*, India) and incubated at 37 °C for 1 d.

Viable cells of enterococci and lactobacilli were counted at time 0 and after a 1-d incubation on MRS agar plates.

Adhesion to human and canine mucus was estimated according to Ouwehand *et al*. (1999) in microtiterplate wells. The results are expressed as the average of at least three independent experiments in four parallels.

Human intestinal mucus was isolated from the healthy part of resected colonic tissue (Ouwehand *et al*. 2002). The *Joint Ethical Commitee of the University of Turku and Turku University Central Hospital* approved the use of human intestinal material. Resected tissue was gently washed in PBS with 1 ppm gelatin and mucus was collected by gently scraping the mucus with a rubber spatula. The mucus was centrifuged (13 000 g, 10 min) to remove cell debris and bacteria, and stored at –80 ºC until use.

Canine mucus was prepared from canine jejunal chyme according to Kirjavainen *et al*. (1998) and Ouwehand *et al*. (1999).

For in *vivo* experiment, blood samples of ill dogs with different diseases and disorders of the gastrointestinal tract (*see* Table III) were tested before application and after application (7 d) of *Lactobacillus* strain AD1 administered orally (daily dose 3 mL of 10^9 CFU/mL). The samples were used to determine biochemical parameters: cholesterol and alanine aminotransferase (EC 2.6.1.2; L-alanine:2-oxoglutarate aminotransferase).

RESULTS AND DISCUSSION

The total average counts of enterococci in feces of 10 healthy dogs reached 5.5 log CFU/g (range 3.3–7.0 log CFU/g) and of lactobacilli 7.6 log CFU/g (range 5.6–10.1 log CFU/g). Similar enterococcal counts in dog ileum and feces (5.0–6.0 log CFU/g) are mentioned in the study reported by Zentek *et al*. (1998). The average total counts of lactobacilli were lower than was reported by Strickling *et al*. (2000).

The isolates showed an inhibitory effect mainly against Gram-positive genera such as *Enterococcus*, *Lactobacillus*, *Staphylococcus*, *Streptococcus*, *Micrococcus* and *Lactococcus* (Table I). Moreover, 30 strains of lactobacilli partly inhibited the growth of *Enterobacter georgiviae* EG3 (hazy zone of inhibition). Ten strains of *Enterococcus* spp. and 9 strains of *Lactobacillus* sp. were found to be without any inhibition activity against all 20 indicator used. The broadest spectrum of inhibition was found in strains, which inhibited 14 indicator bacteria by enterococci and 12 indicators by lactobacilli. Bacteriocin-producing lactic acid bacteria were isolated from various diverse nondairy environments including intestine (Du Toit *et al*. 2000). The inhibitory spectrum of most bacteriocins includes Gram-positive food spoilage and/or food-borne pathogenic microorganisms; they are therefore widely used in food ecosystems as food preservatives (Caplice and Fitzgerald 1999). However, only limited information is available concerning their inhibitory activity against Gram-negative bacteria (Tomita *et al*. 1997). The production of substances by one type of microorganism that are antagonistic to other microbial types is often cited as one of the mechanism by which microbial communities are regulated (Tannock 1981). Although this inhibition may be related to the production of various metabolites, bacteriocin-like substances are one of these metabolites with broad antimicrobial activity (Klaenhammer 1993; Osuntoki *et al*. 2003). Seven strains of *Enterococcus* spp. (*E. hirae* EH2, *E. faecium* W1, EF4, EF01, *E. faecalis* EE4, *Enterococcus* sp. E01, E05) and six strains of *Lactobacillus* spp. (*Lactobacillus* sp. AX2, AD2, RO4, GO6, D1, AD1) with the broadest antimicrobial activity were selected for further probiotic characterization.

The ability of selected strains to tolerate the presence of 1 % bile is shown in Table II. The survival rate of enterococci ranged between 71.7 % (*E. hirae* EH2) and 97.5 % (*E. faecalis* EE4). Concerning lactobacilli, the survival rate was in the range from 67 (strain D1) to 75 % (strain AD1). Tolerance to bile salts is considered to be a prerequisite for colonization and metabolic activity of bacteria in the small intestine of the host (Havenaar *et al*. 1992). The resistance to bile salts varies among lactic acid bacteria and even between strains themselves (Xanthopoulos 1997). In our study, enterococci showed a higher resistance to bile than lactobacilli.

Adhesion to intestinal mucus of the tested enterococci ranged from 3.7 % (*E. hirae* EH2) to 10.6 % (*E. faecium* EF01) with canine mucus and from 5.1 % (strain E01) to 8.2 % (strain W1) with human mucus (Table II). The adhesion of selected lactobacilli was in the range from 2.1 (strain AD1) to 6.0 % (strain AD2) with canine mucus and from 2.7 (strain AD1) to 8.3 % (strain AD2) with human intestinal mucus. The isolates bind to human intestinal mucus in a similar manner to that observed for canine mucus. This suggests that the often mentioned species-specificity of probiotics (Casas *et al*. 1998) is not interfering with the *in vitro* adhesion of the tested strains to intestinal mucus. Similar results were obtained by Rinkinen *et al*. (2000).

Table II. Tolerance to 1 % bile (log CFU/g), and adhesion activity (%) of canine enterococci and lactobacilli[a]

Strain	Tolerance to bile		Adhesion activity	
	0 h	1 d	human mucus	canine mucus
***Enterococcus* sp.**				
E. hirae EH2	7.08 ± 0.40	5.08 ± 0.40	6.3 ± 2.8	3.7 ± 2.5
E. faecium W1	7.85 ± 0.12	7.05 ± 0.23	8.2 ± 3.9	4.9 ± 2.3
E. faecalis EE4	7.29 ± 0.43	7.11 ± 0.25	7.1 ± 4.3	7.6 ± 4.9
Enterococcus sp. E01	7.69 ± 0.18	6.67 ± 0.09	5.1 ± 3.3	6.2 ± 3.3
Enterococcus sp. E05	7.51 ± 0.33	7.12 ± 0.08	7.6 ± 3.5	7.3 ± 4.5
E. faecium EF4	7.85 ± 0.33	6.55 ± 0.05	5.3 ± 3.9	5.4 ± 1.8
E. faecium EF01	7.40 ± 0.25	6.36 ± 0.29	8.1 ± 4.8	10.6 ± 5.0
***Lactobacillus* sp.**				
AX2	9.86 ± 0.12	6.76 ± 0.28	4.6 ± 1.8	4.0 ± 1.3
AD2	9.60 ± 0.08	7.05 ± 0.25	8.3 ± 2.8	6.0 ± 2.3
RO4	9.75 ± 0.10	7.08 ± 0.30	3.9 ± 1.2	5.5 ± 5.5
GO6	9.50 ± 0.16	6.50 ± 0.23	5.0 ± 2.7	4.1 ± 4.1
D1	9.45 ± 0.24	6.30 ± 0.15	5.2 ± 2.0	3.4 ± 3.4
AD1	9.67 ± 0.15	7.29 ± 0.21	2.7 ± 1.9	2.1 ± 2.1

[a]For further details *see Materials and Methods*.

Lactobacillus sp. strain AD1 was used in *in vivo* experiments to investigate its influence on the level of cholesterol in serum and hepatic enzyme – alanine aminotransferase. It has been shown that *Lactobacillus* administration is associated with a decrease of both biochemical parameters (Table III). Several

Table III. Biochemical parameters before (*the first columns*) and after (*the second columns*) application of strain AD1 to ill dogs

Breed, sex, age	Diagnosis	CHO[a]		ALT[b]	
Boxer, bitch, 3 years	enteritis chronica	8.85	3.12	6.56	2.70
German Shepherd, bitch, 4 years	enteritis chronica	5.73	5.84	20.0	8.35
Miniature Pincher, dog, 2 years	gastroenteritis, hemorrhagis acuta	5.66	40.8	12.2	7.96
Maltése, bitch, 2 years	enteritis chronica	4.61	6.46	3.91	2.00
Golden Retriever, dog, 4 years	alergia alimentaria	4.70	5.53	1.04	6.00
Maltése, dog, 6 month	coprophagia	5.07	3.87	10.9	13.1

[a]Cholesterol, in mmol/L; normal range 3.25–6.50.
[b]Alanine aminotransferase, in μkat/L; normal values to 3.33.

studies in humans have suggested moderate cholesterol-lowering action of probiotic bacteria (Schaafsma *et al.* 1998; Larsen *et al.* 2000) but little is known about their effect on the blood hepatic enzymes.

Most lactobacilli and enterococci strains showed good probiotic properties. Strain AD1 of *Lactobacillus* sp. brought biochemical parameters of ill dogs to normal values.

REFERENCES

ALANDER M., SATOKARI R., KORPELA R., SAXELIN M., VILPPONEN-SALMELA T., MATTILA-SANDHOLM T., VON WRIGHT A.: Persistence of colonization of human colonic mucosa by a probiotic strain, *Lactobacillus rhamnosus* GG after oral consumption. *Appl.Environ.Microbiol.* **65**, 351–354 (1999).

BAELE M., BAELE P., VANEECHOUTTE M., STORMS V., BUTAYE P., DEVRIESE L., VERSCHRAEGEN G., GILLIS M., HAESEBROUCK F.: Application of tDNA-PCR for the identification of enterococci. *J.Clin.Microbiol.* **38**, 4201–4207 (2000).

CAPLICE E., FITZGERALD G.F.: Food fermentations: role of microorganisms in food production and preservation. *Internat.J.Food Microbiol.* **50**, 131–149 (1999).

CASAS I.A., EDENS F.W., DOBROGOSZ W.J.: *Lactobacillus reuteri*: an effective probiotic for poultry and other animals, pp. 475–518 in S. Salminen, A. von Wright (Eds): *Lactic Acid Bacteria: Microbiology and Functional Aspects*. Marcel Dekker, New York 1998.

DU TOIT M., FRANZ C.M.A.P., DICKS L.M.T., HOLZAPFEL W.H.: Preliminary characterization of bacteriocins produced by *Enterococcus faecium* and *Enterococcus faecalis* isolated from pig feces. *J.Appl.Microbiol.* **88**, 482–494 (2000).

ELLIOTT S.E., BURET A., MCKNIGHT W., MILLER M.J.S., WALLACE J.L.: Bacteria rapidly colonize and modulate healing of gastric ulcers in rats. *Amer.J.Physiol.* **275**, G425–G432 (1998).

GILLILAND S.E., WALKER D.K.: Factors to consider when selecting a culture of *Lactobacillus acidophilus* as a dietary adjunct to produce a hypocholesterolemic effect in humans. *J.Dairy Sci.* **73**, 905–911 (1990).

HAVENAAR R., TEN BRINK B., HUIS IN'T VELD J.H.J.: Selection of strains for probiotic use, pp. 209–221 in R. Fuller (Ed.): *Probiotics – the Scientific Basis*. Chapman and Hall, London 1992.

KLAENHAMMER T.R.: Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbial Rev.* **12**, 224–227 (1993).

LARSEN L.A., RABEN A., HAULRIK N., HANSEN A.S., MANDERS M., ASTROP A.: Effect of 8 week intake of probiotic milk products on risk factors for cardiovascular diseases. *Eur.J.Clin.Nutr.* **54**, 288–297 (2000).

NES I.F., DIEP D.B., HAVARSTEIN L.S., BRURNERG M.B., EIJDINK V., HOLO H.: Biosynthesis of bacteriocins in lactic acid bacteria. *Antonie van Leeuwenhoek* **70**, 113–128 (1996).

OSUNTOKI A.A., GBENLE G.O., OLUKOYA D.K.: Evidence for chromosomal determination of fungicidal activity in strains of *Lactobacillus brevis* and *Lactobacillus fermentum* isolated from fermented foods. *Folia Microbiol.* **48**, 56–58 (2003).

OUWEHAND A.C., KIRJAVAINEN P.V., SHORTT C., SAMINEN S.: Probiotics: mechanisms and established effects. *Internat.Dairy J.* **9**, 43–52 (1999).

RINKINEN M., MÄTTÖ J., SALMINEN S., WESTERMARCK E., OUWEHAND A.C.: *In vitro* adhesion of lactic acid bacteria to canine small intestinal mucus. *J.Anim.Physiol.Anim.Nutr.* **84**, 43–47 (2000).

SALMINEN S., VON WRIGHT A., MORELLI L., MARTEUAU P., BRASSART D., DE VOS W.M., FONDEN R., SAXELIN M., COLLINS K., MOGENSEN G., BIRKELAND S.E., MATTILA-SANDHOLM T.: Demonstration of safety of probiotics – a review. *Internat. J.Food Microbiol.* **44**, 93–106 (1998).

SCHAAFSMA G., MEULING W.J.A., VAN DOKKUM W., BOULEY C.: Effects of a milk product, fermented by *Lactobacillus acidophilus* and with fructo-oligosaccharides added, on blood lipids in male volunteers. *Eur.J.Clin.Nutr.* **52**, 436–440 (1998).

SCHREZENMEIR J., DE VRESE M.: Probiotics, prebiotics, and synbiotics – approaching a definition. *Amer.J.Clin.Nutr.* **73** (Suppl.), 361S–364S (2001).

STRICKLING J.A., HARMON D.L., DAWSON K.A., GROSS K.L.: Evaluation of oligosaccharide addition to dog diets: influences on nutrient digestion and microbial populations. *Anim.Feed Sci.Technol.* **86**, 205–219 (2000).

TANNOCK G.W.: Microbial interference in the gastrointestinal tract. *ASE Amer.J.Clin.Sci.* **2**, 2–34 (1981).

TOMITA H., FUJIMOTO S., TANIMOTO K., IKE Y.: Cloning and genetic organization of the bacteriocin 21 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pPD1. *J.Bacteriol.* **179**, 7843–7855 (1997).

WELSH J., MCCLELLAND M.: Genomic fingerprints produced by PCR with consensus tRNA gene primers. *Nucl.Acids Res.* **19**, 861–866 (1991).

XANTHOPOULOS V., LITOPOULOU-TZANETAKIS E., TZANETAKIS N.: *In vitro* study of lactobacillus species strains on bile tolerance and cholesterol removal, in *Lactic Acid Bacteria – Lactic 97*. Presses Universitaires de Caen, Caen 1997.

ZENTEK J., MOLITOR D., KAMPHUES J.: Prüfung intestinaler Effekte eines Probiotikums (*Enterococcus faecium*) bei Hunden. *Kleintierpraxis* **43**, 187–197 (1998).