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ORIGINAL ARTICLE

Pathogen exclusion properties of canine probiotics are influenced by the growth media and physical treatments simulating industrial processes

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Abstract

Aims: Manufacturing process used in preparation of probiotic products may alter beneficial properties of probiotics. The effect of different growth media and inactivation methods on the protective properties of canine-originated probiotic bacteria against adhesion of canine enteropathogens was investigated.
Methods and Results: Three established dog probiotics, *Lactobacillus fermentum* VET9A, *Lactobacillus plantarum* VET14A and *Lactobacillus rhamnosus* VET16A, and their mixture were assessed using the dog mucus pathogen exclusion model. The pathogens used were *Enterococcus canis, Salmonella enterica* serovar Typhimurium and *Clostridium perfringens*. The effect of growth media, one reflecting laboratory and the other manufacture conditions, and viability (viable and heat inactivated, 80°C per 30 min) on the pathogen exclusion properties of probiotics were characterized. Greater pathogen exclusion percentages were noted for probiotics growing in conditions reflecting manufacture when compared to laboratory ($P < 0{\cdot}05$). Inactivation of probiotics by heat (80°C per 30 min) increased pathogen exclusion compared with their viable forms ($P < 0{\cdot}05$).
Conclusions: Manufacturing process conditions such as growth media, incubation temperature and pretreatment methods may significantly affect the protective properties of the tested strains.
Significance and Impact of the Study: Growing conditions and pretreatment methods should be carefully considered when designing new probiotics to reduce the risk of common infections in dogs. The studied probiotics are promising potential feed additives for dogs.

Introduction

Dogs face many diseases caused by enteropathogens, which may lead to gastrointestinal challenges and even death (Herstad *et al.* 2010; Marks *et al.* 2011). Specific canine bacterial pathogens are infective agents in well-documented zoonoses (Stafford *et al.* 2007) being a challenge for both pet owners and veterinary care. Antibiotic therapy can have a long-term effect on intestinal microbiota (Sullivan *et al.* 2001) and increase resistance to antibiotics (Damborg *et al.* 2008). The knowledge on commercial probiotic preparations for dogs is scarce, and few studies have been carried out to support their beneficial role in canine health (Kelley *et al.* 2009, 2010; Herstad *et al.* 2010; Bybee *et al.* 2011; Marsella *et al.* 2012).

Probiotic bacteria through adhesion and colonization of the mucosal surfaces may facilitate competition for binding sites and nutrients with pathogens and displacement of already adhered pathogens (Ouwehand and Salminen 2003; Collado *et al.* 2007; Ferreira *et al.* 2011). The use of probiotics as a single or as a combination of strains could improve the resilience of intestinal and mucosal microbiota against pathogens thus enhancing health and decreasing recovery time from pathogen-associated

diseases potentially improving survival of dogs in serious pathogen infections. Moreover, there is an increasing interest in the use of nonviable forms of probiotic bacteria or their cell extracts, to eliminate viability and shelf-life problems and to reduce the risks of microbial translocation and infection.

With these in mind, we assessed the *in vitro* effects of three established canine-originated dog probiotic strains, *Lact. fermentum* VET9A, *Lact. plantarum* VET14A and *Lact. rhamnosus* VET16A (Beasley *et al.* 2006; Manninen *et al.* 2006; Grześkowiak *et al.* 2013), alone and in mixture on the exclusion properties of pathogenic bacteria such as *Ent. canis, Salm. enterica* ser. Typhimurium and *Cl. perfringens* from dog jejunal mucus, selected because of their relevance in gastrointestinal infections and mortality in dogs (Marks *et al.* 2011). In addition, we investigated the effect of different growth media, one reflecting laboratory and the other manufacturing conditions, on the pathogen exclusion abilities of dog probiotic bacteria assessed in viable and heat-inactivated forms.

Materials and methods

Bacterial strains

The three established dog-originated probiotic strains used in the study were *Lact. fermentum* VET9A, *Lact. plantarum* VET14A and *Lact. rhamnosus* VET16A, which previously demonstrated probiotic characteristics (Beasley *et al.* 2006; Manninen *et al.* 2006; Grześkowiak *et al.* 2013) were provided by the Vetcare Ltd. (Mäntsälä, Finland).

The model dog pathogen used was *Ent. canis* (CCUG 46666^T), and human and animal pathogens such as *Cl. perfringens* (DSM 756) and *Salm. enterica* ser. Typhimurium (ATCC 14028).

For assays, dog probiotics were grown in de Man Rogosa Sharpe (MRS; Oxoid Ltd., Basingstoke, Hampshire, England) broth and incubated at 37°C under aerobic atmosphere. For a comparison of the effect of growth media on the exclusion properties, the parallel assays were carried out where the strains were grown in patented soy-based growth media (Patent application numbers FI 122247 B1 and PCT/FI2010/050538) provided by Galilaeus Ltd. (Kaarina, Finland) and incubated at 30°C under aerobic atmosphere. Soy-based growth medium contains mainly of soy meal as a source of nitrogen for the lactic acid bacteria strains. Strains have a carbon and mineral sources as well.

Model pathogen strain *Ent. canis* was grown in MRS broth and incubated at 37°C under aerobic atmosphere. Pathogens *Cl. perfringens* and *Salm. enterica* ser. Typhimurium were grown in Gifu anaerobic medium (GAM; Nissui Pharmaceutical, Tokyo, Japan) and incubated at 37°C under anaerobic atmosphere. For all bacteria, the culture was inoculated into broth in a 1·5-ml tube to a final concentration of one per cent and incubated for 18 h without agitation.

Dog mucus preparation

Mucus was prepared from jejunal chyme essentially as described earlier (Kirjavainen *et al.* 1998; Ouwehand *et al.* 2001). Dog mucus was dissolved (0·5 mg protein ml^{-1}) in HEPES – Hanks' buffer (HH; 10 mmol l^{-1}-HEPES, pH 7·4).

In vitro assay of pathogen adhesion to dog intestinal mucus

In brief, 100 μl (0·5 mg ml^{-1}) of dog jejunal mucus was immobilized onto 96 wells of polystyrene microtitre plates (Maxisorp, Nunc, Denmark) by incubation overnight at 4°C, as previously described (Grześkowiak *et al.* 2011b).

For adhesion assays, canine, *Ent. canis*, and human and canine, *Cl. perfringens* and *Salm. enterica* ser. Typhimurium, pathogen strains were metabolically labelled by the addition of 10 μl ml^{-1} tritiated thymidine (5-^{3}H-thymidine 1·0 mCi ml^{-1}; Amersham Biosciences, Little Chalfont, UK) to the culture media and were grown overnight. Radiolabelled bacterial absorbance (A_{600nm}) was adjusted to 0·25 $\pm$ 0·05 to standardize the bacterial concentration (10^8 cells ml^{-1}). The adhesion assessment of the bacterial pathogens was carried out as described previously (Grześkowiak *et al.* 2011a). Briefly, a suspension of 100 μl radioactively labelled bacteria single or in mixture was added to each well. The mixture was prepared by mixing bacterial solutions in equal proportions. After incubation at 20°C for 1 h, the wells were washed twice with 200 μl of HH to remove unbound bacteria. Bound bacteria were released and lysed by incubation at 60°C for 1 h with 250 μl of 1% sodium dodecyl sulphate (SDS) in 0·1 mol l^{-1} NaOH. Adhesion was assessed by quantifying the amount of radioactivity by liquid scintillation and was expressed as the percentage of radioactivity recovered after adhesion relative to the radioactivity of the bacterial suspension added to the immobilized mucus. Adhesion was determined in three independent experiments, and each assay was performed in triplicate to calculate intra-assay variation.

Treatments of the canine probiotic suspensions

The strain-specific abilities of the dog probiotics were tested in their viable and nonviable forms. To obtain nonviable forms, bacterial suspensions were divided into 1-ml aliquots and incubated at 80°C for 30 min to reflect

manufacturing conditions of the strain manufacturer, that is, Galilaeus Ltd.

Exclusion by inhibition assay

To test the ability of the studied probiotics to inhibit the adhesion of pathogens, the procedure described by Grześkowiak *et al.* (2011b) was used. In brief, unlabelled VET probiotics were added to the wells and incubated; they were then removed by washing with HH buffer. Radiolabelled pathogens were then added to the wells and incubated. Thereafter, the wells were washed and bound, bacteria were recovered after lysis, and radioactivity was measured.

Exclusion by displacement assay

The ability of the studied probiotics to displace pathogens already adhered was assessed as previously described (Grześkowiak *et al.* 2011b). In brief, radiolabelled pathogens were added to the wells containing mucus. After washing and removal of unbound pathogens, nonradiolabelled dog probiotics were added. Wells were incubated and washed, where after bound bacteria were recovered after lysis and radioactivity was measured.

Exclusion by competition assay

Competitive exclusion of the model pathogens by the studied probiotics was determined as previously described (Grześkowiak *et al.* 2011b). For the competition test, equal quantities of a given bacterial suspension of canine probiotics and radiolabelled pathogens were mixed and then added to intestinal mucus and incubated as previously indicated. The cells of the pathogen bound to the mucus were then removed, and adhesion was calculated.

Statistical analysis

Statistical analysis was carried out using the SAS for Windows 9.3 (SAS Institute, Inc., Cary, NC). Data were subjected to four-way ANOVA. All results are shown as the average of three independent experiments (each assay performed in triplicate); variation is expressed as standard deviation.

Results

In vitro pathogen adhesion to dog jejunal mucus

In our study, the most marked ability to adhere to dog jejunal mucus was detected for *Ent. canis* (16·22%, SD 1·45), followed by *Salm. enterica* ser. Typhimurium (11·62%, SD 1·50) and *Cl. perfringens* (8·93%, SD 1·47).

Pathogen exclusion by canine probiotics cultivated in MRS broth *vs.* soy-based growth media

The effect of the growth media on the pathogen exclusion abilities by probiotics is presented in Tables 1 and 2. Greater exclusion percentages of *Ent. canis* were noted for viable ($P < 0{\cdot}001$, $P = 0{\cdot}025$, respectively, for the two exclusion mechanisms: inhibition and competition) and nonviable ($P < 0{\cdot}001$, $P = 0{\cdot}002$, $P = 0{\cdot}001$, respectively, for the three exclusion mechanisms) probiotics cultivated in soy-based growth media compared with MRS broth. There was no difference in the exclusion of *Cl. perfringens* by viable ($P = 0{\cdot}293$ for the three mechanisms) and nonviable ($P = 0{\cdot}293$ for the three exclusion mechanisms) forms of probiotics cultivated in soy-based growth media *vs.* MRS broth. The exclusion of *Salm. enterica* ser. Typhimurium was more successful when viable probiotics were cultivated in soy-based growth media compared with MRS broth ($P < 0{\cdot}001$ for the three mechanisms). On the other hand, nonviable forms of probiotics showed greater exclusion percentages of *Salm. enterica* ser. Typhimurium when they were cultivated in MRS broth compared with soy-based growth media ($P = 0{\cdot}003$ for the three exclusion mechanisms).

Pathogen exclusion by canine probiotics cultivated in MRS broth

The ability to exclude the adhesion of pathogens from dog jejunal mucus differed between viable and nonviable forms of the tested probiotics single and in mixture grown in MRS broth (Table 1). The exclusion of *Ent. canis* by the tested viable probiotics and their mixture was different from their nonviable forms. Generally, nonviable forms of probiotics had greater inhibition ($P < 0{\cdot}001$) and displacement ($P = 0{\cdot}015$) percentages of *Ent. canis* than their viable forms. Only the exclusion by competition of *Ent. canis* did not differ ($P = 0{\cdot}295$) between viable and nonviable forms of probiotics. Nonviable single strains and mixture forms of probiotics had greater ability to inhibit, displace and compete with *Cl. perfringens* for adhesion than their viable forms ($P = 0{\cdot}042$). Also, greater exclusion percentages of *Salm. enterica* ser. Typhimurium were noted for probiotics when tested in nonviable compared with viable forms ($P < 0{\cdot}001$).

Pathogen exclusion by canine probiotics cultivated in soy-based growth media

The ability to exclude adhesion of pathogens from dog jejunal mucus differed between viable and nonviable forms of the tested probiotics single and in mixture grown in soy-based growth media (Table 2). Exclusion of

Table 1 Exclusion of the adhesion to jejunal mucus of pathogenic bacteria by the dog probiotics in viable and nonviable (heat inactivated for 30 min at 80°C) forms and cultivated in MRS broth

	MRS broth					
	Viable			Nonviable		
	*Ent. canis**	*Cl. perfringens*†	*Salm.* Typhimurium‡	*Ent. canis**	*Cl. perfringens*†	*Salm.* Typhimurium‡
Inhibition						
VET9	16·46 ± 6·08	13·32 ± 4·83	21·80 ± 1·63	33·58 ± 11·97	29·5 ± 10·46	25·14 ± 14·65
VET14	−26·09 ± 27·01	9·23 ± 3·42	10·05 ± 10·00	20·33 ± 13·16	9·47 ± 10·44	22·02 ± 7·41
VET16	−21·88 ± 27·22	−17·11 ± 24·31	−3·81 ± 2·98	27·20 ± 9·40	−5·91 ± 5·52	17·58 ± 9·21
MIX	−29·50 ± 6·97	1·57 ± 0·10	−8·98 ± 2·00	18·63 ± 11·38	−0·56 ± 13·83	14·90 ± 6·23
Displacement						
VET9	28·04 ± 16·32	3·23 ± 4·26	12·71 ± 6·28	47·15 ± 5·31	6·86 ± 14·72	20·36 ± 15·58
VET14	33·57 ± 9·28	11·54 ± 10·39	12·91 ± 8·15	41·95 ± 10·94	11·01 ± 8·56	22·58 ± 6·45
VET16	37·79 ± 6·75	17·22 ± 11·25	18·93 ± 2·62	46·05 ± 11·38	23·43 ± 12·62	27·83 ± 15·69
MIX	30·89 ± 10·33	15·89 ± 8·57	12·30 ± 4·95	32·93 ± 9·10	20·43 ± 17·36	35·75 ± 10·08
Competition						
VET9	22·11 ± 3·94	5·08 ± 4·41	12·06 ± 2·33	18·30 ± 0·79	6·73 ± 5·87	14·56 ± 2·63
VET14	17·81 ± 7·45	1·09 ± 4·70	4·02 ± 1·06	17·04 ± 3·38	6·83 ± 3·89	11·01 ± 5·46
VET16	18·20 ± 4·23	4·35 ± 3·84	7·37 ± 2·57	12·53 ± 2·66	5·97 ± 5·39	12·59 ± 5·92
MIX	20·15 ± 6·10	4·02 ± 3·65	3·29 ± 6·46	14·28 ± 2·92	9·59 ± 7·25	12·27 ± 7·24

Ent. canis, Enterococcus canis; *Cl. perfringens, Clostridium perfringens*; *Salm.* Typhimurium, *Salmonella enterica* ser. Typhimurium.
*Difference between viable *vs.* nonviable in inhibition ($P < 0·001$), displacement ($P = 0·015$) and competition ($P = 0·295$).
†Difference between viable *vs.* nonviable in inhibition, displacement and competition ($P = 0·042$).
‡Difference between viable *vs.* nonviable ($P < 0·001$).

Ent. canis by the tested viable probiotics and their mixture was different from their nonviable forms. In general, nonviable forms of probiotics had greater inhibition ($P < 0·001$) and displacement ($P < 0·001$) percentages of *Ent. canis* than their viable forms. Only the exclusion by competition of *Ent. canis* did not differ ($P = 0·855$) between viable and nonviable forms of probiotics. Nonviable single and mixture forms of probiotics had generally greater ability to inhibit, displace and compete with *Cl. perfringens* for adhesion then their viable forms ($P = 0·042$). Greater exclusion percentages of *Salm. enterica* ser. Typhimurium were noted for probiotics when tested in viable compared with nonviable forms ($P = 0·013$).

Discussion

Our results clearly demonstrate the importance of growth media and the value of *in vitro* studies on pathogen exclusion as means of providing quality control criteria for the later use of probiotics. We have recently demonstrated that these same probiotic bacteria of canine origin present different adhesive properties when cultivated in different media and inactivated by different treatments (Grześkowiak *et al.* 2013).

Colonization of the mucosal surfaces and competition with pathogens for the adhesion sites are possible protective mechanisms of probiotics action (Ouwehand and Vesterlund 2003). However, only a few studies exist describing *in vitro* probiotic adhesion and pathogen exclusion from canine mucus (Rinkinen *et al.* 2003; Vahjen and Manner 2003; Grześkowiak *et al.* 2013). Our results show that all the pathogens tested have the ability to bind to intestinal mucus, which thus benefits the pathogens in invading the host. We also demonstrate that the same canine-originated probiotics are able to successfully exclude pathogens from dog jejunal mucus and that these properties depend on the growth media and temperature, and viability of the probiotic strains, which may influence their *in vivo* effects, underlining the importance of control of the growth conditions, physiological treatments during manufacturing process affecting probiotic viability and also, the way of administration.

We found that the inhibition, displacement and competition percentages of *Ent. canis*, *Cl. perfringens* and *Salm.* enterica ser. Typhimurium differed when canine probiotics were cultivated in soy-based growth media compared with MRS broth. The reason for the laboratory growth media used was that the studied probiotics had previously been isolated and cultured from canine faeces using commercial laboratory growth media similar to MRS, thus providing a growth advantage on this particular medium (Beasley *et al.* 2006). However, the effect of soy-based growth manufacture medium and lower

Table 2 Exclusion of the adhesion to jejunal mucus of pathogenic bacteria by the dog probiotics in viable and nonviable (heat inactivated for 30 min at 80°C) forms and cultivated in soy-based growth media

	Soy-based growth media					
	Viable			Nonviable		
	*Ent. canis**	*Cl. perfringens*†	*Salm.* Typhimurium‡	*Ent. canis**	*Cl. perfringens*†	*Salm.* Typhimurium‡
Inhibition						
VET9	31·43 ± 13·46	14·24 ± 4·94	31·18 ± 15·37	63·59 ± 5·91	13·78 ± 6·58	23·88 ± 8·80
VET14	38·19 ± 1·82	2·11 ± 8·60	21·53 ± 7·45	56·08 ± 4·31	20·26 ± 8·12	19·43 ± 11·85
VET16	36·65 ± 8·96	−13·98 ± 10·99	8·28 ± 9·63	58·05 ± 5·31	−6·75 ± 16·35	7·69 ± 8·28
MIX	41·84 ± 13·40	−4·51 ± 8·91	17·75 ± 8·28	60·62 ± 14·51	1·29 ± 14·61	5·54 ± 12·33
Displacement						
VET9	37·15 ± 4·32	11·73 ± 9·33	18·45 ± 17·09	56·11 ± 3·77	13·68 ± 10·74	9·51 ± 10·98
VET14	30·97 ± 10·97	15·51 ± 5·45	18·39 ± 9·17	53·72 ± 7·19	13·56 ± 11·55	18·32 ± 14·02
VET16	38·92 ± 4·15	18·77 ± 7·30	25·86 ± 8·42	58·89 ± 1·41	21·06 ± 9·15	18·92 ± 3·24
MIX	23·35 ± 6·08	23·47 ± 2·94	31·30 ± 3·18	47·08 ± 6·06	15·93 ± 3·14	21·99 ± 2·31
Competition						
VET9	30·47 ± 1·01	9·88 ± 2·06	13·80 ± 3·22	34·50 ± 2·12	12·62 ± 1·22	11·31 ± 2·93
VET14	26·65 ± 1·85	6·56 ± 2·53	11·86 ± 3·97	26·71 ± 3·90	5·58 ± 1·98	9·81 ± 1·51
VET16	26·12 ± 2·63	3·84 ± 2·60	12·78 ± 3·03	25·99 ± 3·66	4·12 ± 1·38	8·11 ± 0·31
MIX	29·86 ± 3·30	9·19 ± 0·91	11·01 ± 3·36	28·72 ± 4·10	9·78 ± 4·32	8·84 ± 4·99

Ent. canis, Enterococcus canis; *Cl. perfringens, Clostridium perfringens*; *Salm.* Typhimurium, *Salmonella enterica* ser. Typhimurium.
*Difference between viable *vs.* nonviable in inhibition ($P < 0·001$), displacement ($P < 0·001$) and competition ($P = 0·855$).
†Difference between viable *vs.* nonviable in inhibition, displacement and competition ($P = 0·042$).
‡Difference between viable *vs.* nonviable in inhibition, displacement and competition ($P = 0·013$).

incubation temperature seemed to modulate the properties of studied probiotics in different manner, improving the percentage of pathogen exclusion from jejunal mucus, as demonstrated in our study.

Previous reports demonstrate that the viability of probiotics may affect the adhesion and pathogen exclusion (Ananta and Knorr 2009; Grześkowiak *et al.* 2013). Nonviable forms of probiotics may be less able to bind to intestinal mucosa but they are also less likely to improve their safety (Haller *et al.* 2000; Cross *et al.* 2004). In our work, the exclusion properties were dependent on the viability of probiotics. Heat-inactivated forms of the studied probiotics were more likely to exclude pathogens from dog jejunal mucus than viable forms. Moreover, nonviable forms of probiotics were found to be effective in the modulation of host immune system (Mastrangeli *et al.* 2009).

The present results suggest that the pathogen exclusion abilities by the mixture of dog probiotics were beneficial for mutual exclusion effect towards all tested pathogens, however, differed depending on the viability of probiotics and growth media used. The reason for using here a mixture of probiotic strains lies in the synergistic beneficial effect of the strains. Even though the pathogen exclusion percentages were not triplicated when a mixture of three probiotics was used compared with single strains, still however each strain exerts unique properties from which the host may benefit (Beasley *et al.* 2006; Manninen *et al.* 2006; Grześkowiak *et al.* 2013). Thus, it seems reasonable from veterinary practice and commercial point of view to use a mixture of probiotics than a single strains in animal health care. The phenomena of a mutual effect are common in probiotic nature, and multistrain probiotic products have been proposed and used especially in animals (Manninen *et al.* 2006; Collado *et al.* 2007; Garcia-Mazcorro *et al.* 2011). The canine probiotic effect appeared to depend also on the pathogen type used. Exclusion of pathogens by probiotics is based on bacteria-to-bacteria interactions, and these may highly depend on the growth media used (Kankaanpää *et al.* 2004; Muller *et al.* 2011). The differences in inhibition, displacement and competition of pathogens suggest different mechanisms of probiotic–pathogen interactions. Thus, further studies on these mechanisms should be conducted. In addition, different exclusion properties might also result from different cell surface protein expression due to different incubation temperature (37°C in MRS broth and 30°C in soy-based growth medium).

Dog probiotics used in our study fulfil the requirement of origin as they had been isolated from canine gut and tested using canine jejunal mucus (Beasley *et al.* 2006; Grześkowiak *et al.* 2013). Most studies demonstrate species specificity of probiotic properties (Christensen *et al.* 2002). Here, we also present that the property of one probiotic strain cannot be extrapolated to another.

The studied probiotics belong to lactobacilli, which in general have a good safety record. On the contrary,

numerous commercially available probiotic products for dog consumption contain enterococci, which have, however, notorious ability to rapidly develop, spread antibiotic resistance and favour the growth of potentially harmful microbes in humans (Bogø *et al.* 2003; Hammerum 2012). Therefore, new research on the identification of novel strains and the assessment of functional properties are being developed (Kelley *et al.* 2009; Herstad *et al.* 2011; Silva *et al.* 2013).

Taken together, our results support the importance of the impact of growth media and physical treatment methods on probiotic properties. The present findings demonstrate that the *in vitro* tested strains and their mixture have a potential as a successful probiotic feed additives in dogs' diet. Their positive effect against canine model enteropathogens was proven when probiotics were used in viable and nonviable forms. The most effective pathogen exclusion results were obtained when probiotics were grown in manufacturing media. However, we feel that there is still a need for more *in vitro* and *in vivo* studies with the special focus on manufacturing conditions to strengthen the potential role of probiotics in animal health and welfare.

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Conflict of interest

Shea Beasley is employed by Vetcare Ltd. None of the other authors had any conflict of interest to the studied probiotics.

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