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Original Article

Probiotic Candidates among Dairy *Lactobacilli* and *Streptococcus Thermophiles* Strains for Control of the Oral Pathogen *Porphyromonas Gingivalis*

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Abstract

Introduction: The gram-negative bacterium *Porphyromonas gingivalis* is a major causative agent of periodontitis in adults. It is also associated with disorders of the cardiovascular and endocrine systems, rheumatoid arthritis, pancreatic cancer, and Alzheimer's disease. Lactic acid bacteria (LAB) present in the oral cavity or introduced as probiotic preparations can support successful treatment of periodontitis due to their antagonism with the pathogen.

Aim: The aim of this study was in vitro assessment of the antimicrobial activity of *Lactobacillus spp.* and *Streptococcus thermophilus* against *P. gingivalis.*

Materials and methods: The antimicrobial effect of lactobacilli or *S. thermophilus* from the LBB Culture collection against *P. gingivalis* DSM 20709 was evaluated with the well diffusion assay on Wilkins Chalgren blood agar. Inhibition of the pathogen was evaluated by measuring the diameter of clear zones around the wells.

Results: Application of milk fermented with selected LAB resulted in **a** bacteriostatic effect. The most active culture was *S. thermophilus* 187/4, followed by *L. delbr.* ssp. *bulgaricus* (LBB.B1054, C3/2 and LBB.B120), *L. helveticus* LBB.H48/1 and *L. rhamnosus* I-1/13. The respective reconstituted freeze-dried preparations had a stronger inhibitory effect on the pathogen with the formation of clear bactericidal zones. The effect of milk acidified with lactic acid was apparent with minimal bactericidal zone observed at concentration of 0.1%. The effectiveness of the assay was confirmed with Elgydium and Eludril.

Conclusions: *P. gingivalis* DSM 20709 was sensitive to the metabolites produced in fermented milk by selected strains of *L. delbr.* ssp. *bulgaricus*, *L. helveticus*, *L. rhamnosus*, and *S. thermophilus*. Reconstituted freeze dried fermented milk had a stronger inhibitory effect compared to fresh samples. Lactic acid produced by lactic acid bacteria was the key component for inhibition of the pathogen.

Keywords

inhibition, *Lactobacillus*, periodontitis, *Streptococcus thermophilus*

INTRODUCTION

Following caries, periodontitis is the second, most frequent chronic inflammatory disease of the oral cavity. It is initiated as inflammation of the gingiva followed by tooth loss and bone destruction. Periodontal disease has not only stomatological consequences, but it can also affect the cardio-vascular system, the endocrine system, the joints, etc. One of the causative agents of chronic periodontitis in adults is *Porphyromonas gingivalis*. Its major virulent factors are lipopolysaccharides (LPS), capsular polysaccharide (CPS), fimbriae responsible for adherence to salivary proteins attachment and a group of cell surface cysteine proteinases – gingipains.[1] Also, this pathogen has the capability of biofilm formation, which makes it one of the main members of the core "red complex" consortium in periodontitis[2], along with *Treponema denticola* and *Tannerella forsythia*. Presently, extensive data of case-controlled studies have been accumulated proving a statistically significant relationship between the presence of *P. gingivalis* and periodontal diseases.[3] Furthermore, Sato et al.[4] showed that *P. gingivalis* is responsible for the aggravation of collagen-induced arthritis mainly due to the synthesis of peptidyl arginine deiminase that produces rheumatoid arthritis-related auto-antigens. Their results from experiments with mice demonstrated that *P. gingivalis* was a mediator between periodontitis and rheumatoid arthritis through its activity on the intestinal immune system and microflora. In a review by Öğrendik[5], pancreatic cancer is determined as the fourth leading cause of cancer-related mortality worldwide and that oral pathogens are linked to the etiology of the disease. Singhrao et al.[6] discuss the potential link between *P. gingivalis* periodontal infection and Alzheimer's disease.

Periodontitis therapy involves local cleaning and smoothing of dental root surfaces, application of oral hygiene preparations and frequently – systemic antibiotic therapy. Recently, great interest has been focused on the application of probiotic bacteria and their effect on oral pathogens. Numerous studies have shown that the consumption of fermented milk products, lactic acid bacteria probiotic preparations and lactobacilli in particular can serve as auxiliary tools in the successful treatment of periodontitis.[7-11] Most importantly, *in vitro* studies are followed by randomized confirmatory control trials. Matsubara et al.[12] have reviewed 12 such trials up to March, 2016 and have reached the conclusion that oral administration of probiotics is safe and effective treatment to accompany antibiotic therapy.

AIM

The aim of this study was in vitro assessment of the antimicrobial activity of *Lactobacillus spp.* and *S. thermophilus* against *P. gingivalis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The antimicrobial effect of lactic acid bacteria was evaluated against *P. gingivalis* DSM 20709 as a test culture. Lactobacilli and *S. thermophilus* were derived from the LBB Culture collection (LB Bulgaricum Plc., Sofia, Bulgaria) with the exception of *L. rhamnosus* NBIMCC 507 (National Bank for Industrial Microorganisms and Cell Cultures – Bulgaria) and *L. reuteri* which was isolated from a commercial probiotic oral food supplement **(Table 1)**. *P. gingivalis* was propagated in Wilkins Chalgren broth (CONDA) under anaerobic conditions at 37°C for up to 72 hours. Enumeration of the viable cells of the pathogen and the well-diffusion assay were performed on Wilkins Chalgren blood agar (Wilkins Chalgren agar supplemented with 5.0% defibrinated sheep blood). Dairy lactobacilli and *S. thermophilus* were grown in sterile 10.0% reconstituted skim milk powder for 16 hours at 37°C and used either fresh or followed by freeze-drying. Thermal inactivation of *L. delbr.* ssp. *bulgaricus* LBB.B1054 prior to lyophilisation was carried out for 1 hour at 63°C.

Agar well diffusion assay

The agar well diffusion assay was performed as described by Soleimani et al.[13] with minor modifications. Mid log-phase culture of *P. gingivalis* (10^5-10^6 cfu/ml) was spread on the surface of Wilkins Chalgren blood agar. Wells (6.0 mm) were punched in the agar and filled with 50 µl of 1. fermented milk sample; 2. reconstituted (with distilled water to 10.0% dry matter) freeze-dried fermented milk; 3. non-inoculated milk (negative control); 4. milk, acidified with lactic acid (0.1-7.0% final concentration) or 5. one of the commercial preparations Eludril Classic antiplaque mouth rinse or ELGYDIUM ANTI-PLAQUE toothpaste (both products of Pierre Fabre ORAL CARE, France) as positive controls. The inoculated plates were incubated anaerobically at 37°C for 4-5 days. The inhibition of the pathogen was evaluated by measuring the diameter of clear zones (mm) around the wells in which no growth (bactericidal effect) or supressed growth (bacteriostatic effect) of *P. gingivalis* DSM 20709 was observed. Average values for four parallel wells were recorded.

RESULTS

In the agar well diffusion assay, fermented milk or reconstituted freeze-dried fermented milk containing viable LAB was introduced into the wells. Therefore, a limited zone of LAB growth around the wells was observed for some strains, followed either by a clear zone of bactericidal effect against the pathogen or a zone of bacteriostatic effect with partially inhibited growth of *P. gingivalis*. The application of milk

fermented with the selected LAB resulted in strain-specific bacteriostatic effect observed around the wells **(Table 1)**. The most active culture was *S. thermophilus* 187/4, followed by three strains of *L. delbr.* ssp. *bulgaricus* (B1054, C3/2 and B120), *L. helveticus* LBB.H48/1, and *L. rhamnosus* I-1/13. The inhibitory effect of these strains was comparable to the *L. reuteri* strain isolated from a commercial probiotic preparation. Another eight strains had no visible effect on *P. gingivalis*. For some of the strains, like *L. rhamnosus* I-4/1 and *L. rhamnosus* NBIMCC 507, the correct assessment of the inhibitory effect was difficult due to the growth of the LAB around the well.

Although freeze-dried fermented milk preparations were reconstituted with water to obtain the same dry weight as before lyophilisation, when introduced into the wells, a stronger inhibitory effect on the pathogen was observed compared to initial fermented milks with the formation of clear bactericidal zones **(Table 1)**. In the case of *L. delbr.* ssp. *bulgaricus* LBB.B1054 a valid assessment of the inhibitory effect of the freeze-dried preparation was only possible after heat inactivation of the culture prior to lyophilisation to eliminate the growth of LAB around the well.

As with fermented milks, the reconstituted freeze-dried preparation of *S. thermophilus* 187/4 showed the strongest inhibition of the pathogen among all tested preparations **(Fig. 1)**. An inhibitory effect was also confirmed for freeze-dried preparations of *L. delbr.* ssp. *bulgaricus* LBB.B1054 and C3/2, *L. helveticus* LBB.H48/1 and *L. rhamnosus* I-1/13, again to a much bigger extent than observed for initial fermented milks. For strains *L. delbr.* ssp. *lactis* LBB.L1266, *L. rhamnosus* I-4/1, and *L. rhamnosus* NBIMCC 507

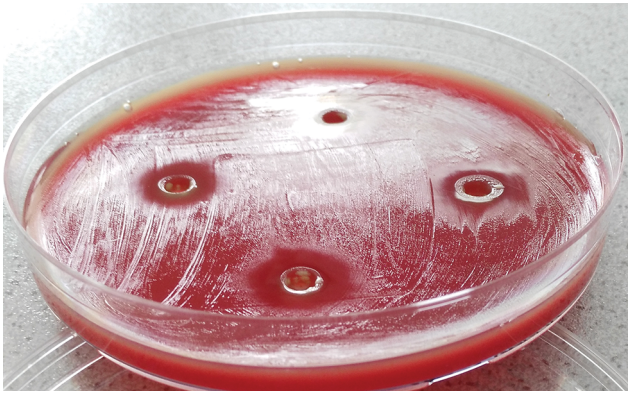

Figure 1. Inhibition of *P. gingivalis* DSM 20709 growth within clear zones around wells loaded with reconstituted freeze-dried milk fermented with *S. thermophilus* 187/4 (agar well diffusion assay, four parallel wells).

inhibition of the pathogen was only found with the application of freeze-dried preparations, confirming the overall higher activity of reconstituted freeze dried fermented milks compared to "fresh" fermented milk.

In the agar well diffusion assay, milk as control had a negligible effect on *P. gingivalis* **(Table 2)**. However, the effect of milk acidified with lactic acid was apparent with minimal bactericidal zone observed at concentration of lactic acid as low as 0.1%. As positive controls, the two preparations used for mouth hygiene, Elgydium (tooth paste) and Eludril (mouth rinse) gave the maximal antibacterial effect observed in this study – inhibition zone of 7.0 mm **(Table 2)**.

Table 1. Inhibition of *P. gingivalis* DSM 20709 by selected lactobacilli and *S. thermophilus* strains

Strain	Inhibition zone (mm)*	
	Fermented milk	Freeze-dried fermented milk**
L. delbr. ssp. *bulgaricus* LBB.B1054	(2.0)****	2.5 + (0.5)***
L. delbr. ssp. *bulgaricus* C3/2	(3.0)	1.0 + (1.5)
L. delbr. ssp. *bulgaricus* 53/8	0****	ND
L. delbr. ssp. *bulgaricus* LBB.B130	0	ND
L. delbr. ssp. *bulgaricus* LBB.B120	(1.0)****	ND
L. delbr. ssp. *bulgaricus* 69/6P	0	ND
L. delbr. ssp. *lactis* LBB.L1266	(0.5)	2.0
L. helveticus LBB.H48/1	(2.0)	2.0 + (1.0)
L. helveticus 2096/6	(0.5)	ND
L. rhamnosus I-1/13	(1.0)****	3.0
L. rhamnosus I-4/1	0****	4.0
L. rhamnosus NBIMCC 507	0****	2.0 + (1.0)
S. thermophilus 187/4	(10.0)	4.0 + (0.5)
S. thermophilus C2	0	(1.0)
L. reuteri (commercial preparation)	(3.0)	ND

* bactericidal effect (bacteriostatic effect presented in parenthesis), average from two independent trials; ** freeze-dried preparations reconstituted in water to 10% dry matter; *** *L. delbr.* ssp. *bulgaricus* LBB.B1054 was thermally inactivated before freeze-drying; **** growth of LAB around the well; ND: not determined

Table 2. Effect of lactic acid and commercial mouth hygiene products on the growth of *P. gingivalis* DSM 20709

Controls	Inhibition zone (mm)*
Milk	(0.5)
Milk, 0.1% lactic acid	1.0
Milk, 1.0% lactic acid	1.5
Milk, 3.0% lactic acid	4.0
Milk, 5.0% lactic acid	3.0
Milk, 7.0% lactic acid	6.0
Elgydium (tooth paste)	7.0
Eludril (mouth rinse)	7.0

* bactericidal effect (bacteriostatic effect presented in parenthesis), average from two independent trials

DISCUSSION

Several studies emphasize the relation between altered oral microflora and the presence of periodontal disease. Van Essche et al.[14] performed a large scale study to demonstrate the prevalence of bacterial isolates antagonistic to *P. gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Aggregatibacter actynomycetemcomitans* in samples from healthy individuals compared to patients with periodontitis. Comparing these isolates with seven commercially available probiotic bacteria, the authors found that the effect of the latter was much stronger and that all of the probiotic strains inhibited *P. gingivalis*. Kõll-Klais et al.[15] found that *P. gingivalis* was inhibited by oral lactobacilli and that in healthy individuals a prevalence of obligatory homofermentative species was observed compared to chronic periodontitis patients. Both authors[14,15] attribute the effect of lactic acid bacteria on the pathogens mainly to the production of large amounts of organic acids, such as lactic and acetic acid, and especially to the inability of *P. gingivalis* to grow at pH bellow 6.5.[16] In terms of dietary nutrition, a comprehensive study of 942 subjects confirmed that consumption of lactic acid foods may have a beneficial effect on periodontal diseases.[8]

In our study, milk acidified with lactic acid clearly and in concentration-dependent manner inhibited the growth of *P. gingivalis* forming clear bactericidal zones around the wells **(Table 2)**. However, although all tested lactobacilli and *S. thermophilus* strains produced lactic acid to coagulate milk, the presence and extent of pathogen inhibition was strain-specific. This indicated the presence of other metabolites that play an essential role in the inhibition of *P. gingivalis*. One example is the synthesis of hydrogen peroxide that is a function of the activity of specific oxidases in *L. delbrueckii* strains.[17] Moreover, in our experiments, reconstituted freeze-dried preparations of milks fermented with lactic acid bacteria showed stronger inhibitory effect than the initial fermented milks. Although with the same lactic acid content, fresh fermented milk cultures generated only zones of bacteriostatic effect on the pathogen, while the corresponding reconstituted freeze-dried preparations formed clear zones of bactericidal effect. This may be attributed to the destruction of a portion of the bacterial cells during lyophilisation and the liberation of intracellular content, including inhibitory substances **(Table 1)**. Alternatively, stress factors may accumulate in lactic acid bacteria in the freezing process that increase the activity of the freeze-dried preparations. Many species of lactic acid bacteria are capable of producing antibacterial peptides such as bacteriocins.[18] Bacteriocin production has been also described for *L. delbr.* ssp. *Bulgaricus.*[19] Interestingly, bacilli were found to produce a cold-shock protein-like bacteriocin.[20] It may be possible that cold shock and freezing may increase the production and availability of such antibacterial substances in the lactic acid bacteria cultures included in our experiment.

In our study, growth of lactobacilli around the well on Wilkins Chalgren blood agar was observed, especially with *L. delbr.* ssp. *bulgaricus* and *L. rhamnosus* strains, but this did not result in significantly larger inhibition zones which may be explained by the sensitivity of *P. gingivalis* only to metabolites already accumulated in fermented milk or reconstituted freeze-dried fermented milk preparations. Also, in the case of *L. delbr.* ssp. *bulgaricus* LBB.B1054, where the culture was heat-inactivated prior to lyophilisation, clearly *P. gingivalis* was inhibited by heat-stable metabolites rather than by viable cells. The complexity of the mechanism of inhibition of *P. gingivalis* by lactobacilli is well demonstrated in the study of Samot and Badet[10] where 52 of a total of 66 autochthonous oral lactobacilli slightly inhibited the growth of this pathogen, but only 7 produced hydrogen peroxide, the inhibiting activity was independent of Proteinase K treatment, and higher activity was observed for facultatively heterofermentative cultures.

Strong inhibition of *P. gingivalis* was also observed for *S. thermophilus* 187/4. Although in yoghurt, cocci are weaker acid producers, Zhu et al.[9] found that both *S. thermophilus* and *L. delbr.* ssp. *bulgaricus* inhibited the growth of *P. gingivalis*, but only if yogurt bacteria were inoculated first as early colonizers. This suggests that the mechanism of antagonism between *S. thermophilus* and *P. gingivalis* may be competition for substrate. Another study showed that *S. thermophilus* may reduce oral malodour by inhibition of *P. gingivalis* growth and neutralizing volatile sulfur compounds produced by the pathogen.[11] Again, in our study, the same effect of activation of the inhibitory effect by freeze-drying was observed with *S. thermophilus* 187/4 with bacteriostatic effect of fermented milk enhanced to bactericidal effect of the lyophilized preparation **(Table 1)**.

Stamatova et al.[7] tested the inhibitory activity of lactobacilli against five clinical isolates of *P. gingivalis* and determined that *L. casei* ATCC 344 was the most potent culture inhibiting four of the pathogen isolates, followed by *L. rhamnosus* strain that showed the highest activity against three of the *P. gingivalis* isolates. Nevertheless, the authors

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observed only "slight inhibition of growth" of *P. gingivalis* by 40% of the tested lactobacilli and no inhibition of the pathogen by *L. delbr.* ssp. *bulgaricus* strains. This discrepancy with our results for *L. delbr.* ssp. *bulgaricus* LBB.B1054 and C3/2 can be explained by the difference in the antimicrobial activity test method which in the experiment of Stamatova et al.[7] was streak line method on Brucella agar, while in our study we used the well-diffusion assay testing directly fermented milk preparations on Wilkins Chalgren blood agar and *P. gingivalis* DSM 20709 as a test culture. Notably, with the agar-overlay method, Stamatova et al.[7] have observed inhibitory activity of *L. delbr.* ssp. *bulgaricus* strains against other oral pathogens such as some species of streptococci and *A. actynomycetemcomitans*.

In vitro selection of potential probiotic lactic acid bacteria should be followed by clinical trials. The results of this study supported the performance of initial trials with freeze-dried preparations of *L. delbr.* ssp. *bulgaricus* LBB. B1054 and *S. thermophilus* 187/4 with encouraging results showing favourable change in the species distribution of bacterial species in patients with periodontitis, decrease of *P. gingivalis* counts and improvement of periodontal status after additional intake of the probiotic preparations.[21,22]

CONCLUSIONS

The agar well diffusion assay showed that *P. gingivalis* DSM 20709 was sensitive to the metabolites produced in fermented milk by several species of lactobacilli (*L. delbr.* ssp. *bulgaricus, L. helveticus,* and *L. rhamnosus*) and *S. thermophilus* in a strain-specific manner, with *L. delbr.* ssp. *bulgaricus* and *S. thermophilus* being the typical microflora of yoghurt. Reconstituted freeze dried fermented milk samples had a stronger inhibitory effect compared to fresh fermented milks which suggests that a better effect may be achieved by application of probiotic preparations in a lyophilized form. Clearly lactic acid, produced by lactic acid bacteria, was among the active metabolites inhibiting the pathogen. The heat-treated preparation of *L. delbr.* ssp. *bulgaricus* LBB. B1054 preserved its inhibitory activity emphasizing that it is the metabolites rather than viable cells that were the mediator of the effect of *P. gingivalis* inhibition for this strain.

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Кандидаты в пробиотики из молочных штаммов Lactobacilli и *Streptococcus Thermophiles* для борьбы с оральным патогеном *Porphyromonas Gingivalis*

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Резюме

Введение: Грамотрицательная бактерия *Porphyromonas gingivalis* является основной причиной пародонтита у взрослых. Это также связано с нарушениями сердечно-сосудистой и эндокринной систем, ревматоидным артритом, раной поджелудочной железы и болезнью Альцгеймера.

Молочнокислые бактерии (МКБ), присутствующие в полости рта и принимаемые в качестве пробиотиков, могут сопровождать успешное лечение пародонтита из-за их антагонизма с возбудителем.

Цель: Целью данного исследования было выполнить *in vitro* оценку антимикробной активности *Lactobacillus spp*. и *Streptococcus thermophilus* против *P. gingivalis*

Материалы и методы: Антимикробный эффект лактобацилл или *S. thermophilus* из коллекции культур МКБ против *P. gingivalis* DSM 20709 оценивали с использованием метода диффузии в лунках кровяного агара Уилкинса-Чалгрена. Ингибирование патогенов оценивали путём измерения диаметра чистых участков вокруг лунок с агаром.

Результаты: Применение молока, ферментированного выбранным МКБ, вызывало бактериостатический эффект. Наиболее активной культурой была *S. thermophilus* 187/4, за ней следовала *L. delbr. ssp. bulgaricus* (LBB.B1054, C3/2 и LBB.B120), *L. helveticus* LBB.H48/1 и *L. rhamnosus* I-1/13. Соответствующие лиофилизированные препараты, восстановленные из сухого молока, оказывали более сильное ингибирующее действие на возбудителя с образованием чистых бактерицидных зон. Эффект молока, подкисленного молочной кислотой, был очевиден с минимальными бактерицидными зонами, наблюдаемыми при концентрациях 0.1%. Эффективность анализа подтверждена препаратами Elgydium и Eludril.

Заключение: *P. gingivalis* DSM 20709 был чувствителен к метаболитам, продуцируемым в ферментированном молоке отобранными штаммами *L. delbr. ssp. bulgaricus, L. helveticus, L. rhamnosus* и *S. thermophilus*. Лиофилизированное ферментированное молоко, восстановленное из сухого молока, имело более сильный ингибирующий эффект, чем свежие образцы. Молочная кислота, продуцируемая молочнокислыми бактериями, была ключевым компонентом в подавлении патогена.

Ключевые слова

ингибирование, *Lactobacillus*, пародонтит, *Streptococcus thermophilus*