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Sugar fermentation in probiotic bacteria – an *in vitro* study

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Introduction: Food supplemented with probiotic bacteria is a rapidly growing sector of the market. The aim of the present study was to evaluate and compare the acid production of selected probiotic strains available in commercial products.

Methods: Six *Lactobacillus* strains (*Lactobacillus plantarum* 299v and 931; *Lactobacillus rhamnosus* GG and LB21; *Lactobacillus paracasei* subsp. *paracasei* F19, and *Lactobacillus reuteri* PTA 5289) were cultivated at 37 °C in an anaerobic atmosphere on Man, Rogosa, Shape (MRS) agar for 48 h or MRS broth for 16 h. After centrifugation, the cells were washed and resuspended in sterile phosphate-buffered saline and immediately subjected to a fermentation assay with 12 different carbohydrates (nine sugars and three sugar alcohols) in microtiter plates with a pH indicator. The plates were examined for color changes after 24, 48, and 72 h of incubation under aerobic and anaerobic conditions. Three scores were used: negative (pH > 6.8); weak (pH 5.2–6.8), and positive (pH < 5.2). The strains were characterized with the API 50 CH system to confirm their identity.

Results: *L. plantarum* fermented all the sugars except for melibiose, raffinose, and xylitol. Both *L. rhamnosus* strains were generally less active although *L. rhamnosus* GG was slightly more active than strain LB21 in the 5% CO₂ setting. The latter strain exhibited negative reactions for sucrose, maltose, arabinose, and sorbitol under anaerobic conditions. The assays with *L. paracasei* and *L. reuteri* had negative or weak reactions for all tested sugars under both aerobic and anaerobic conditions.

Conclusion: The metabolic capacity to form acid from dietary sugars differed significantly between the various probiotic strains.

Key words: caries; fermentation; lactobacilli; probiotics; sugar

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The production of organic acids from dietary sugars is a key factor in the caries process. Low pH generated from acids challenges the homeostasis in the oral microbial community with a selection towards bacteria that induce caries (4, 15). *Lactobacilli*, however, may play a role in the maintenance of the microecological balance in the oral cavity (13, 22) and the use of probiotic strains has emerged as an alternative way of combating oral bacterial infections in analogy with other parts of the gastrointestinal tract (17). By definition, probiotics are a live microbial feed supplement that when consumed in adequate amounts beneficially

affects the host by improving its intestinal microbial balance as documented in clinical trials (20). Recent clinical studies with *Lactobacillus rhamnosus* and *Lactobacillus reuteri* strains have demonstrated that a regular intake can result in a reduction of mutans streptococci numbers in saliva and plaque (1, 6, 18, 19). Similarly, the prevalence of oral candida was reduced in elderly persons in connection with a daily intake of cheese containing *L. rhamnosus* (10).

To be effective against oral infections, probiotic bacteria need to adhere to the oral mucosa and dental tissues as a part of the biofilm and compete with the growth

of cariogenic bacteria or periodontal pathogens (7, 11). Bacteria differ in their metabolic capabilities and metabolic end products may serve as growth substrates or inhibit the growth of other species. The *Lactobacillus* group contains homofermentative and heterofermentative species and all are aciduric. Several strains produce low-molecular-weight antimicrobial substances with an inhibitory activity against a wide range of bacterial species, including oral streptococci (2, 12, 13, 16, 21).

The sector of the market involving live lactobacilli in foods and health products is growing rapidly. When ingested orally

it is feasible that these bacteria may attach to oral surfaces. It is therefore important to know the capacity of these bacteria to produce acid from dietary sugars to rule out deleterious effects on the teeth. The aim of the present study was to assess the acid production from various sugars and sugar alcohols by six probiotic lactobacillus strains that are available to consumers in over-the-counter products.

Material and methods

Bacterial strains

Six different strains of lactobacilli, *Lactobacillus plantarum* 299v (ProViva, Skåne Dairy, Malmö, Sweden), *L. plantarum* 931 (Essum, Umeå, Sweden), *L. rhamnosus* GG ATCC 53103 (Valio Ltd, Helsinki, Finland), *L. rhamnosus* LB21 (Essum), *Lactobacillus paracasei* subsp. *paracasei* F19 (Arla Ltd, Stockholm, Sweden), and *L. reuteri* PTA 5289 (BioGaia, Stockholm, Sweden), that are used in commercial products for oral consumption were selected. The strains were characterized by the API 50 CH system (BioMérieux SA, Marcy-l'Etoile, France) to confirm their identity.

Culture media

Man, Rogosa, Shape (MRS) agar and broth (Oxoid Ltd, Basingstoke, UK) were used for culturing the lactobacilli (14). A modified MRS broth without addition of carbohydrate was employed in the fermentation assay (pH 6.7). One litre of the medium contained 10 g tryptone (Difco, Detroit, MI), 5 g yeast extract (Difco), 10 g Lab-Lemco powder (Oxoid), 2 g di-potassium hydrogen phosphate (Merck, Darmstadt, Germany), 0.5 g sodium acetate $3H_2O$, 2 g di-ammonium hydrogen citrate, 0.2 g magnesium sulfate $7H_2O$ (Riedel-de Haën, Seelze, Germany), and 0.05 g manganese sulfate $4H_2O$ (BDH Chemicals Ltd, Poole, UK).

Culture technique

The lactobacilli were grown in an anaerobic atmosphere for 48 h at 37 °C. Pure colonies were picked and subcultured in 4.5 ml MRS broth for 16 h. To eliminate traces of glucose from the culture medium, the bacteria were centrifuged at 3500 rpm ($g = 1276$) for 5 min (Beckman Coulter Allegra™ X-22, rotor Conical C1015; Beckman Coulter AB, Bromma, Sweden), the medium was discarded, and the bacterial cells were suspended in sterile phos-

phate-buffered saline (PBS), sodium chloride 85 mM, potassium phosphate 25 mM (pH 7.4), centrifuged and resuspended in PBS. The optical densities were adjusted to 1.0 (650 nm) and these suspensions were immediately used in the fermentation assay.

Carbohydrates

Nine dietary sugars and three sugar alcohols were tested; glucose (Merck), fructose (Merck), lactose (May and Baker, Dagenham, UK), sucrose (BDH), maltose (Merck), melibiose (BDH), raffinose (Merck), trehalose (BDH), arabinose (Merck), mannitol (BDH), sorbitol (Merck), and xylitol (Finnsugar Trading Ltd, Helsinki, Finland) and prepared in 2% aqueous solutions and sterile filtered by 0.45 µm Millex -HA, a syringe-driven filter unit (Millipore, Bedford, MA).

Fermentation assay

The fermentation assay was performed in microtiter plates (96 MicroWell™ Plates Nunclon™Δ; Nunc, Roskilde, Denmark); 50 µl of modified MRS broth (without supplement of sugar) was mixed with 50 µl of each of the 12 carbohydrates in the wells. A sterile filtered 2% solution of bromocresol purple was used as pH indicator in the assay. The pH indicator was mixed with each of the bacterial strains (1 : 1) and 10 µl of the blend was added to each carbohydrate. The plates that were incubated in parallel at 37 °C both in an anaerobic condition with 10% H_2 , 5% CO_2 in N_2 , and in 5% CO_2 in air and were examined for color changes after 24, 48, and 72 h of incubation. Each combination of bacteria and carbohydrate was processed in triplicate and the experiment was repeated twice. For the negative control, bacteria were replaced by PBS.

pH indicator

The indicator turns purple at pH >6.8 and at pH <5.2 it turns yellow. Intermediate colors are exhibited at pH values inside the transition range. A pH >6.8 was considered negative, pH between 5.2 and 6.8 was considered weak, and pH < 5.2 was a positive reaction.

Results

Characterization of lactobacilli by the API 50 CH system

The *L. paracasei* subsp. *paracasei* strain and both strains of *L. plantarum* were

identified without difficulty by the API 50 CH system. *L. reuteri* gave a biochemical profile interpreted as *Lactobacillus fermentum* in the data base because *L. reuteri* is closely related to this species and cannot be distinguished from *L. fermentum* by biochemical analysis only. None of the *L. rhamnosus* strains were fully recognized by the API 50 CH.

Fermentation assay

The majority of fermentation reactions occurred at a higher velocity in anaerobic atmosphere than in the 5% CO_2 environment with some exceptions. The results from the fermentation assays are detailed in Table 1.

L. plantarum strains had the highest activity among the tested bacteria. After 24 h of incubation both strain 299v and 931 fermented glucose, fructose, lactose, sucrose, maltose, trehalose, and arabinose resulting in a final pH <5.2 in 5% CO_2 as well as under anaerobic conditions. In 5% CO_2 after 24 h, the sugar alcohol mannitol was equally fermented by the two strains while sorbitol was only digested by strain 931 and under anaerobic conditions only positive reactions with mannitol by strain 931 were found.

L. rhamnosus GG was slightly more active compared to strain LB 21 in the 5% CO_2 setting. Following 24 h of incubation the fermentation of glucose, fructose, mannitol, and trehalose resulted in pH values ranging between 5.2 and 6.8 for both strains. In contrast to *L. rhamnosus* LB21, strain GG reached a pH <5.2 after 48 h of incubation with glucose, fructose, sucrose, mannitol, trehalose, and maltose and in the presence of lactose and sorbitol the pH dropped below 5.2 after 72 h. Strain LB 21 exhibited only weak reactions and was not capable of lowering the pH below 5.2 with any of the carbohydrates, after incubation for up to 72 h. Anaerobic conditions increased the fermentation velocity of glucose, fructose, and trehalose as compared to 5% CO_2 and ended in pH values <5.2 after 24 h incubation for both strains.

L. paracasei F19 displayed only weak reactions and pH values ranging between 5.2 and 6.8 were reached after 72 h of incubation in 5% CO_2 with glucose, fructose, trehalose, and mannitol, while with the other carbohydrates the pH remained unaffected. Under anaerobic conditions a pH <5.2 was attained after 72 h with fructose and trehalose. Also sucrose, lactose, and sorbitol were fermented to some extent in the anaerobic

Table 1. Fermentation of sugars in 5% CO₂ and in an anaerobic atmosphere by selected probiotic lactobacillus strains

Strain	Glu	Fru	Lac	Suc	Malt	Ara	Mel	Raf	Tre	Man	Sorb	Xyl
5% CO ₂ atmosphere												
<i>L. plantarum</i> 299v	+++	+++	+++	+++	+++	+++	---	---	+++	+++	++	---
<i>L. plantarum</i> 931	+++	+++	+++	+++	+++	+++	---	---	+++	+++	+++	---
<i>L. rhamnosus</i> GG	+++	+++	---	---	---	---	---	---	+++	+++	++	---
<i>L. rhamnosus</i> LB21	+++	+++	---	---	---	---	---	---	+++	+++	---	---
<i>L. paracasei</i> F19	---	---	---	---	---	---	---	---	---	---	---	---
<i>L. reuteri</i> PTA5289	---	---	---	---	---	---	---	---	---	---	---	---
Anaerobic atmosphere												
<i>L. plantarum</i> 299v	+++	+++	+++	+++	+++	+++	---	---	+++	++	---	---
<i>L. plantarum</i> 931	+++	+++	+++	+++	+++	+++	---	---	+++	+++	++	---
<i>L. rhamnosus</i> GG	+++	+++	---	---	---	---	---	---	+++	+++	---	---
<i>L. rhamnosus</i> LB21	+++	+++	---	---	---	---	---	---	+++	+++	---	---
<i>L. paracasei</i> F19	---	---	---	---	---	---	---	---	---	---	---	---
<i>L. reuteri</i> PTA5289	---	---	---	---	---	---	---	---	---	---	---	---

Symbols in the table indicate: -, negative reaction (pH >6.8); ±, weak reaction (pH 5.2 to 6.8); +, positive reaction (pH <5.2) after 24, 48, and 72 h, respectively.

atmosphere with the pH ending between 5.2 and 6.8 after 72 h.

L. reuteri slowly generated weak reactions with glucose, lactose, sucrose, maltose, and melibiose when incubated in 5% CO₂. No pH <5.2 was detected with any of the sugars even after 72 h in this setting. In the anaerobic atmosphere pH values <5.2 were observed with sucrose and maltose after 72 h of incubation.

Discussion

Bacterial fermentation can be either health-promoting or harmful and the present study was performed to evaluate whether probiotic lactobacillus strains would exhibit a pH-lowering capacity that could increase the risk of dental caries. The strains were selected to represent a variety of products readily available for consumers. The *L. rhamnosus* and *L. paracasei* species belong to a closely related taxonomic group commonly used in dairy products (8). *L. plantarum* are found in fruit drinks while *L. reuteri* is the active bacterium in chewing gums, lozenges, and gruel for children. Promising but inconclusive results have so far been reported for these strains in a number of gastrointestinal conditions (23) and for *L. rhamnosus* and *L. reuteri* strains an inhibitory effect on oral pathogens has been reported (25). Neither of the two *L. rhamnosus* strains was fully identified with the API 50 CH system but as they were obtained from the producers we did not proceed with genotypic classification tools. A standard fermentation assay was used and applied under both aerobic and anaerobic conditions to mimic the newly established biofilm as well as the more complex *climax community* with a higher degree of *gram-negative* anaerobic bacteria (4).

It is possible that the present assay, however, did not fully disclose the true capacity of the tested strains because the *inducible enzyme systems* may not have been adequately reflected. Furthermore, an *in vitro* assay can never reflect the complexity of the live climax community with its mix of different cells and sugars. With these limitations the present findings displayed substantial differences in the metabolic activity between the tested lactobacilli under given conditions. Sucrose, fructose, and glucose are considered the most important carbohydrates involved in the caries process (15) and they were fermented dramatically differently by the various strains. Our results clearly demonstrated that the *L. plantarum* strains were most active, the *L. paracasei* and *L. reuteri* were almost inactive and the *L. rhamnosus* strains fell between these two groups. One of the tested strains, *L. rhamnosus* GG, has previously been described as not capable of fermenting either sucrose (16) or lactose (19) but we were able to demonstrate a slow but clear activity for both of these sugars after 48 and 72 h, respectively. Interestingly, the other *L. rhamnosus* strain showed no metabolic activity with sucrose while both *L. rhamnosus* strains were active with fructose. The findings that none of the *L. rhamnosus* strains, nor *L. paracasei* F19 or *L. reuteri* PTA 5289, displayed rapid reactions with sucrose were important because these strains are suggested as probiotic candidates for the prevention of oral infections. The negative reactions on xylitol were also encouraging because it seems unlikely that the probiotic lactobacilli would compromise the suggested antibacterial mechanisms of action of the sugar alcohol (24). The 48–72 h acid production of the *L. rhamnosus* species may be of limited clinical importance.

Although data obtained *in vitro* show that the bacteria can survive in saliva and adhere to saliva-coated surfaces (11), there are still very limited clinical data for or against a possible oral colonization with probiotic lactobacilli (5, 26). Furthermore, one should be cautious of drawing any clinical conclusions from an *in vitro* assay with pure *monocultures*, which may not be representative for the corresponding events in the oral biofilm. The next natural step would be to investigate the pH-lowering capacity of various probiotic bacteria in the dental plaque with the aid of an *in situ* micro-touch electrode.

One obstacle for the introduction of probiotics to the oral cavity could be that lactobacilli by tradition are associated with the development of dental caries. *Lactobacilli* constitute, however, only a small part of the oral microflora and no significant increase of the salivary counts has yet been demonstrated following daily administration of lactobacillus-derived probiotics (25). Secondly, modern molecular analyses have linked lactobacilli to carious dentine and the advancing front of caries lesions rather than to the early enamel demineralization (3, 9). Therefore, as young children with newly erupted teeth constitute the primary target groups for oral probiotic intervention, the present results with generally weak metabolic activity from dietary sugars, except for the two *L. plantarum* strains, would not present a safety problem.

In conclusion, the present findings clearly suggest that the metabolic activity differs between various probiotic strains.

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