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ORIGINAL ARTICLE

Antagonistic activity of probiotic lactobacilli and bifidobacteria against entero- and uropathogens

P. Hütt[1], J. Shchepetova[1], K. Lõivukene[1,3], T. Kullisaar[2] and M. Mikelsaar[1]

1 Department of Microbiology, Medical Faculty, University of Tartu, Estonia
2 Department of Biochemistry, Medical Faculty, University of Tartu, Estonia
3 Laboratory of Clinical Microbiology, United Laboratories of Tartu University Clinics, Tartu, Estonia

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Correspondence
P. Hütt, Department of Microbiology, University of Tartu, Ravila 19, Tartu 50 411, Estonia. E-mail: pirje.hutt@ut.ee

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Abstract

Aim: To develop *in vitro* assays for comparing the antagonistic properties and anti-oxidative activity of probiotic *Lactobacillus* and *Bifidobacterium* strains against various entero- and urinary pathogens.

Methods and Results: The antagonistic activity of five probiotic lactobacilli (*Lactobacillus rhamnosus* GG, *Lactobacillus fermentum* ME-3, *Lactobacillus acidophilus* La5, *Lactobacillus plantarum* 299v and *Lactobacillus paracasei* 8700:2) and two bifidobacteria (*Bifidobacterium lactis* Bb12, *Bifidobacterium longum* 46) against six target pathogens was estimated using different assays (solid and liquid media, anaerobic and microaerobic cultivation) and ranked (low, intermediate and high). Bacterial fermentation products were determined by gas chromatography, and the total anti-oxidative activity of probiotics was measured using linolenic acid test. Pyelonephritic *Escherichia coli* was highly suppressed by GG and both bifidobacteria strains. Lactobacilli strains 8700:2, 299v and ME-3 were the most effective against *Salmonella enterica* ssp. *enterica* in microaerobic while ME-3 and both bifidobacteria expressed high activity against *Shigella sonnei* in anaerobic milieu. *Lact. paracasei*, *Lact. rhamnosus* and *Lact. plantarum* strains showed intermediate antagonistic activity against *Helicobacter pylori* under microaerobic conditions on solid media. The highest anti-oxidative activity was characteristic for *Lact. fermentum* ME-3 ($P < 0{\cdot}05$). No efficient antagonist against *Clostridium difficile* was found. The positive correlations between the pH, lactic acid production and anti-microbial activity for all tested probiotics were assessed.

Conclusions: Developed experimental assays enable to compare the anti-microbial and -oxidative activity of *Lactobacillus* and/or *Bifidobacterium* probiotics, which have been claimed to possess the ability of suppressing the growth of various enteric and urinary pathogens.

Significance and Impact of the Study: Screening *Lactobacillus* and *Bifidobacterium* sp. strains according to their activity in various environmental conditions could precede the clinical efficacy studies for adjunct treatment with probiotics in cure of different gastrointestinal and urinary tract infections.

Introduction

Several bacterial strains, belonging to the genera *Lactobacillus*, *Enterococcus*, *Streptococcus* and *Bifidobacterium* are currently available as probiotics. Probiotics have been defined as live microbial food supplements of human origin beneficially influencing human health by improving the intestinal microbial balance (Fuller 1989; Salminen *et al.* 1998; Salminen 2001). A number of issues of safety, functional and technological aspects need to be

considered in developing a probiotic product for the consumer (Saarela *et al.* 2000; FAO and WHO Guidelines 2002; Yeung *et al.* 2002). The evaluation of their functional validity, e.g. the beneficial effect of a particular probiotic strain, seems to be the most difficult aspect.

One of the most frequent health claims for probiotics concerns the putative reduction and prevention of infectious disease in the gastrointestinal tract (GIT). The effect of probiotic strains depends on their ability to survive during passage through the stomach, as well as their ability to persist and compete with pathogens in GIT. In the case of *Helicobacter pylori*, this Gram-negative spiral bacterium has the ability to infect gastric and duodenal mucosa and is mainly associated with chronic gastritis and peptic ulcer (Dunn *et al.* 1997).

Moreover, enteric pathogens infect the host in different atmospheric conditions of GIT, causing diarrhoeal disease. *Salmonella* spp. and *Clostridium difficile* cause inflammation in ileum and colon while *Shigella* sp. clearly prefers the colonic mucosa (Dupont 2005; Pegues *et al.* 2005). In addition, the colon has been considered the main reservoir of *Escherichia coli* strains causing urinary tract infections (Franz and Hörl 1999). In order to test the suppression of different pathogens by probiotic bacteria, it is necessary to consider their distinct environmental conditions in GIT.

In order to find strains of probiotic bacteria, which are antagonistically active against selected enteric pathogens, their individual and distinct metabolic properties should be considered. Previous research has clearly shown that the secreted compounds of particular species of lactic acid bacteria depend on the oxygen tension during growth, as well as their type of fermentation, e.g. obligately homofermentative (OHOL), obligately heterofermentative (OHEL) and facultatively heterofermentative (FHEL) (Annuk *et al.* 2003). However, at present there are few comparative studies in literature, which have elucidated a probiotic's anti-microbial effect under aerobic, microaerobic and anaerobic growth conditions (Jacobsen *et al.* 1999; Annuk *et al.* 2003).

Moreover, there is some evidence that lactic acid bacteria have an anti-oxidative potential (Kaizu *et al.* 1993; Peuhkuri *et al.* 1996; Amanatidou 2001; Kullisaar *et al.* 2002). This property could be helpful in allowing lactobacilli to colonize the intestines, as well as in the course of inflammation to protect the intestinal mucosa against excessive oxidative stress (Truusalu *et al.* 2004).

The EU-funded project 'EU and microfunction – functional assessment of interactions between the human gut microbiota and the host' (QLRT–2001-00135) aims to assess putative markers for determination of the efficacy of several well-characterized probiotics. In the context of the aforementioned project, the aim of the present study was to develop *in vitro* assays for comparing the antagonistic properties of five probiotic *Lactobacillus* and two *Bifidobacterium* strains against various entero- and uropathogens and measure the anti-oxidative activity of probiotics.

Materials and methods

Bacterial strains and culture conditions

The following well-known probiotic bacteria were selected: *Lactobacillus rhamnosus* GG (ATCC 53103; Valio, Helsinki, Finland), *Lactobacillus acidophilus* La5 (Chr. Hansen, Hørsholm, Denmark), *Lactobacillus plantarum* 299v (DSM 9843), *Lactobacillus paracasei* 8700:2 (DSM 13434; Probi, Lund, Sweden), *Bifidobacterium lactis* Bb12 (Chr. Hansen, Hørsholm, Denmark), and *Bifidobacterium longum* 46 (the University of Turku, Finland). These were obtained from the culture collection of the University of Turku. In addition to these, the strain of *Lactobacillus fermentum* ME-3 (DSM 14241) from the culture collection of Tartu University was also included. The antagonistic activity of probiotics was assessed against the following target bacteria: facultative anaerobic pyelonephritic strain of *E. coli* ATCC 700336, cystitic strain of *E. coli* ATCC 700414, *Salmonella enterica* ssp. *enterica* ATCC 13076, *Shigella sonnei* ATCC 25931, microaerobic *H. pylori* NCTC 11637 and strictly anaerobic *C. difficile* VPI 10463.

Lactobacilli were grown in de Man-Rogosa-Sharpe (MRS) broth (Oxoid) at 37°C under microaerobic conditions (10% CO_2; incubator IG-50 Jouan, France). Bifidobacteria were cultured on Wilkins-Chalgren agar (Oxoid) at 37°C for 48 h, in an anaerobic (gases $CO_2/H_2/N_2$: 5/5/90%) chamber (Seldon Manufacturing Inc., Shel Cornelius, OR, USA). *E. coli*, *Salm. enterica* ssp. *enterica* and *Sh. sonnei* were cultivated on peptone agar at 37°C for 18 h. The *H. pylori* strain NCTC 11637 was grown on Columbia Agar Base (Oxoid) supplemented with 7% horse blood and 1% Vitox (Oxoid) for 3–4 days at 37°C under microaerobic conditions. *C. difficile* was cultivated on the Wilkins-Chalgren agar (Oxoid) with 7% horse blood in an anaerobic chamber for 48 h.

Antagonistic activity assays

The antagonistic activity of probiotic lactobacilli and bifidobacteria was assessed using solid and liquid media under microaerobic and anaerobic conditions. Prior to final inoculation, the lactobacilli were precultivated three times in the respective environment. Bifidobacteria, as strict anaerobes, were grown only in an anaerobic environment.

Anti-microbial activity of lactobacilli *and* bifidobacteria *on agar plates*

The anti-microbial activity of probiotic lactobacilli and bifidobacteria against selected target bacteria was assessed using a streak line method. The following media were used: modified MRS medium (MRS medium without triammonium-citrate and sodium-acetate; pH 7·2) for lactobacilli, Wilkins-Chalgren agar with 7% horse blood for bifidobacteria and *C. difficile*, and Columbia Agar Base supplemented with 7% horse blood and 1% Vitox for *H. pylori*.

Lactobacilli and bifidobacteria were seeded in the middle of the agar plate using a 10 μl loop. Following the incubation in microaerobic/anaerobic environment at 37°C for 48 h for lactobacilli, or in anaerobic environment for bifidobacteria, the growth was inactivated with chloroform gas, which was used for 2 h. The following target bacteria were tested: *E. coli*, *Salm. enterica* ssp. *enterica* and *Sh. sonnei* grown in peptone water for 18 h (turbidity 10^9 CFU ml^{-1}), a *H. pylori* suspension in Brucella broth adjusted to McFarland density of 3–4, and a *C. difficile* suspension in saline adjusted to a McFarland density of 1. The target bacteria were seeded using a 1 μl loop, in duplicate, perpendicular to the streak line of lactobacilli and bifidobacteria on the respective media as described above, and were incubated at 37°C. *E. coli*, *Salm. enterica* ssp. *enterica* and *Sh. sonnei* were incubated in aerobic environment for 18 h, *H. pylori* was grown in microaerobic environment for 3 days, and *C. difficile* was incubated anaerobically for 2 days. The antagonistic activity of lactobacilli and bifidobacteria was estimated as the width of the inhibition zone (millimetre) of the target bacteria extending from the culture line of probiotic bacteria (Mikelsaar *et al.* 1987; Annuk *et al.* 2003). The inhibitory effect of lactobacilli and bifidobacteria was ranked as high (>25 mm), intermediate (13–25 mm) and low (0–12 mm).

Anti-microbial activity of probiotic lactobacilli *and* bifidobacteria *in broth*

Probiotic bacteria were preincubated as described above. The strains of *E. coli*, *Salm., enterica* ssp. *enterica* or *Sh. sonnei* in equal aliquots were co-incubated with the suspensions of lactobacilli or bifidobacteria in isotonic saline (10^9 CFU ml^{-1}) at 37°C for 24 h under microaerobic and/or anaerobic conditions. Thereafter, the number of colony forming units (CFU ml^{-1}) of the target Gram-negative bacteria was semi-quantitatively determined on peptone agar (Gould 1965). Inhibition of pathogen growth was calculated by subtracting the number of target bacteria remaining in the co-incubation tube from the number in a control tube with only target bacteria (Annuk *et al.* 2003). The result was expressed as $\log_{10}$ CFU ml^{-1}. The inhibition values of growth of pathogens by probiotics in liquid media were ranked as high, intermediate and low (decrease by 5·9–6·5, 3·4–5·8 and 0·6–3·2 $\log_{10}$ CFU ml^{-1}, respectively).

Antagonistic activity of the culture supernatants of lactobacilli

The inhibitory activity of nonconcentrated supernatants of lactobacilli cultures was assessed against facultative anaerobes; *E. coli*, *Salm. enterica* ssp. *enterica*, and *Sh. sonnei*. Lactobacilli were grown in the MRS broth with 0·15% agar supplement at 37°C for 48 h and then 10 μl of that culture was transferred into 3 ml of MRS broth. Following the incubation in microaerobic environment for 24 h and centrifugation (Jouan CR3, France) at 2000 ***g*** for 10 min, the supernatant was decanted. The pH of each supernatant was measured (Hanna Instruments HI 9024C, Singapore) and supernatants were sterilized using 0·20 μm millipore filters (Sarstedt, Nümbrecht, Germany). Prior to sterilization, half of the supernatant was neutralized to the pH of 7·0 ± 0·1 with NaOH (1 mol l^{-1}). The pathogens grown on peptone agar at 37°C for 18 h in aerobic environment were adjusted to 10^9 CFU ml^{-1} in isotonic saline. Both pH-adjusted and -unadjusted supernatants from the cultures of lactobacilli were placed into the well of a microtitre plate and were co-incubated under aerobic conditions with the suspensions of Gram-negative target bacteria at 37°C for 24 h. Thereafter, the number of CFU of the tested Gram-negative bacteria was semi-quantitatively determined on peptone agar (Gould 1965). All experiments were performed at least in triplicate.

Determination of organic acids

The production of organic acids was estimated by gas chromatography as described by Holdeman *et al.* (1977). The gas chromatograph (Hewlett-Packard model 6890) was equipped with a hydrogen flame ionization detector and an auto sampler (model 7683). The HP Chemical Station for the GC System (A.06 revision) was used. Analyses were performed following the cultivation of lactobacilli in microaerobic and anaerobic environment in modified MRS broth for 24 h and bifidobacteria under anaerobic conditions in MRS broth supplemented with cysteine for 24 h.

Total anti-oxidative activity

The total anti-oxidative activity (TAA) for the seven probiotic bacteria was assessed using the linolenic acid test (LA-test) as described previously by Kullisaar *et al.* (2002). Briefly, lactobacilli were grown in MRS broth

under microaerobic conditions and bifidobacteria in MRS broth supplemented with cysteine in anaerobic environment for 48 h. Probiotic bacteria were then pelleted by centrifugation (10 000 ***g*** for 10 min), washed twice and re-suspended in isotonic saline at 4°C. The density of suspension was adjusted to 10^9 CFU ml^{-1} using an absorbance of 1·1 at 600 nm. TAA was expressed as the inhibition percentage of the peroxidation of linolenic acid standard by the sample and it predominantly reflects the anti-oxidative status of the lipid fraction. Thereafter, we assessed a total anti-oxidative status (TAS) by using a commercially available kit (TAS, Randox Labaratories Ltd. Ardmore, UK) which is described elsewhere (Kullisaar *et al.* 2003). A water-soluble vitamin E (Trolox) served as a standard. This method is based on the inhibition of the absorbance of ferrylmyoglobin radicals of 2,2′-azinobis-ethylbenzothiazoline 6-sulfonate (ABTS+) generated by the activation of metmyoglobin peroxidase with H_2O_2, and indicates the anti-oxidative activity in water fractions. The total anti-oxidative values of probiotic bacteria were considered high if TAA was >20% and TAS > 0·1 mmol l^{-1}.

Statistical methods

The antagonistic activity of different probiotic bacteria was compared by using computer software to assess the Student's *t*-test or Mann–Whitney rank sum test, 'STATGRAPHICS' (Statistical Graphics Corp; USA). Differences were considered significant when *P*-value was <0·05. Pearson's correlation test was used to compare antagonistic activity and production of organic acids.

Results

Growth conditions and antagonistic activity of probiotic bacteria

Liquid media, microaerobic vs anaerobic environment
The antagonistic activity of lactobacilli and bifidobacteria against facultatively anaerobic Gram-negative target bacteria: uropathogenic *E. coli*, *Salm. enterica* ssp. *enterica* and *Sh. sonnei* was tested by co-cultivation experiments using microaerobic or anaerobic conditions. The antagonistic activity of *Lact. plantarum* 299v and *Lact. acidophilus* La5 were dependent on the environment of cultivation; *Lact. plantarum* 299v was more active in microaerobic and *Lact. acidophilus* La5 in anaerobic environment. The antagonistic activity of bifidobacteria as strict anaerobes was not tested in microaerobic conditions.

Compared with other probiotics *Lact. paracasei* 8700:2 expressed the highest antagonistic activity in microaerobic environment, whereas in anaerobic environment its inhibitory activity was nearly equal to that of *Lact. fermentum* ME-3, *Lact. rhamnosus* GG, and bifidobacteria strains, but exceeded significantly the inhibition value of *Lact. plantarum* 299v (Bb12 > 299v, $P = 0{\cdot}006$; B46 > 299v, $P = 0{\cdot}015$).

The pH-adjusted supernatants of probiotic lactobacilli did not suppress the growth of target bacteria. However, when pH-unadjusted supernatant of lactobacilli was tested, the inhibition of the growth of target bacteria differed and depended on the particular probiotic strain. Only two strains, namely *Lact. rhamnosus* GG and *Lact. plantarum* 299v, had significantly stronger suppression for their unadjusted supernatant compared with their whole cells in both microaerobic and anaerobic conditions (data not shown).

Solid media, microaerobic vs anaerobic environment
The assessment of difference in inhibitory activity of probiotic bacteria was based the results of cultivation on solid media or in liquid media and growth conditions: microaerobic *vs* anaerobic. As shown in Table 1 *Lact. plantarum* 299v demonstrated the highest antagonistic activity under microaerobic conditions. In contrast, *Lact. fermentum* ME-3 was the most active probiotic *Lactobacillus* when anaerobic precultivation was used. Anaerobically precultivated probiotic bifidobacteria did not suppress the growth of facultative anaerobic target bacteria cultivated in aerobic environment (data not shown).

Selected assays for ranking probiotics against target pathogens

The growth inhibition values of pathogens caused by probiotics in liquid media were estimated. Different probiotics were ranked as expressing high, intermediate and low activity at different atmospheric conditions mimicking the milieu in GIT (Table 2). Pyelonephritic strain of *E. coli* was highly suppressed by *Lact. rhamnosus* GG and both strains of bifidobacteria strains, but no significant activity was found against cystitic *E. coli*. The effective probiotics against *Salm. enterica* ssp. *enterica* were *Lact. paracasei* 8700:2, *Lact. plantarum* 299v and *Lact. fermentum* ME-3 showing high activity in microaerobic milieu. *Lact. fermentum* ME-3 and both bifidobacteria expressed high activity against *Sh. sonnei* in anaerobic milieu. The antagonistic activity against *H. pylori* was evaluated only on solid media. The highest antagonistic activity (Fig. 1) was expressed by *B. longum* 46 ($P < 0{\cdot}05$) anaerobically. However, under microaerobic conditions characteristic for the stomach, the high antagonistic activity was expressed by *Lact. rhamnosus* GG, *Lact. paracasei* 8700:2 and *Lact. plantarum* 299v. The inhibitory activity of probiotic bac-

Table 1 Antagonistic activity of probiotic lactobacilli grown in microaerobic and anaerobic environment against Gram-negative facultative anaerobes of enteral origin (two uropathogenic *E. coli*, *Salmonella enterica* ssp. *enterica*, *Sh. sonnei*) tested on modified MRS agar

	Inhibition zone values (mm) mean ± SD									
	Lact. acidophilus La5		*Lact. rhamnosus* GG		*Lact. paracasei* 8700:2		*Lact. plantarum* 299v		*Lact. fermentum* ME-3	
Gram-negative bacteria	M	A	M	A	M	A	M	A	M	A
E. coli ATCC 700336	2·4 ± 2·1	15·1 ± 1·1	22·9 ± 3·4	18·7 ± 1·5	18·7 ± 4·5	17·7 ± 2·1	27·5 ± 3·1	21·2 ± 1·1	23·3 ± 5·7	23·1 ± 2·4
E. coli ATCC 700414	1·4 ± 1·8	14·7 ± 1·2	21·9 ± 4·1	18·6 ± 1·6	17·9 ± 1·1	17·8 ± 2·0	25·8 ± 3·0	21·3 ± 1·1	20·8 ± 2·5	22·9 ± 1·7
Salmonella enterica ssp. *enterica* ATCC 13076	3·7 ± 2·8	16·1 ± 1·0	24·1 ± 4·3	20·2 ± 1·4	19·6 ± 2·5	18·9 ± 1·8	27·2 ± 3·5	22·2 ± 0·8	24·3 ± 5·3	24·1 ± 2·0
Sh. sonnei ATCC 25931	3·2 ± 2·6	16·1 ± 0·9	22·5 ± 1·8	19·9 ± 1·1	18·9 ± 1·7	19·4 ± 1·3	26·4 ± 2·9	22·4 ± 1·0	22·8 ± 4·2	24·2 ± 1·9
Mean	2·7 ± 2·4	15·5 ± 1·2*	22·9 ± 3·5	19·3 ± 1·5*	18·8 ± 2·8	18·4 ± 2·0	26·7 ± 3·1	21·8 ± 1·1*	22·8 ± 4·6	23·6 ± 2·0*

*A statistically significant difference between microaerobic and anaerobic growth conditions $P < 0{\cdot}001$; Microaerobic conditions: 229v > GG, $P < 0{\cdot}001$; GG > 8700:2, $P < 0{\cdot}001$; ME-3 > 8700:2, $P < 0{\cdot}001$; 8700:2 > La5, $P < 0{\cdot}001$; anaerobic conditions: ME-3 > 299v, $P < 0{\cdot}001$; 299v > GG, $P < 0{\cdot}001$; GG > 8700:2, $P = 0{\cdot}022$; 8700:2 > La5, $P < 0{\cdot}001$.
M, microaerobic conditions; A, anaerobic conditions; SD, standard deviation. All experiments were repeated at least three times.

Table 2 Ranking of probiotic strains compared with their antagonistic activity against target pathogens according to the atmospheric conditions of action

Target bacteria	Environment of action in liquid media	*Lact. acidophilus* La5	*Lact. rhamnosus* GG	*Lact. paracasei* 8700:2	*Lact. plantarm* 299v	*Lact. fermentum* ME-3	*B. lactis* Bb12	*B. longum* 46
E. coli ATCC 700336	Anaerobic	M	High	M	Low	M	High	High
E. coli ATCC 700414	Anaerobic	M	M	M	Low	M	M	M
Salm. enterica ssp.	Microaerobic	Low	M	High	High	High	ND	ND
enterica ATCC 13076	Anaerobic	M	M	M	Low	M	Low	Low
Sh. sonnei ATCC 25931	Anaerobic	M	M	M	Low	High	High	High

High activity – decrease by 5·9–6·5; intermediate 3·4–5·8 and low 0·6–3·2 $\log_{10}$ CFU ml^{-1}. The experiments were repeated at least three times. High *vs* low rank $P < 0{\cdot}05$; M, intermediate; ND, not determined.

teria against strictly anaerobic *C. difficile* reference strain was low (6–8 mm) expressed by both bifidobacteria strains and *Lact. paracasei* 8700:2.

Association between inhibitory activity of lactobacilli and production of short chain fatty acids and pH

There was no significant difference between the production of lactic, acetic or succinic acids of tested probiotic lactobacilli strains neither under anaerobic nor microaerobic conditions (Table 3). However, all tested lactobacilli produced to some extent more lactic acids under microaerobic than under anaerobic conditions. High amount of ethanol was produced by *Lact. fermentum* ME-3 in anaerobic environment than in microaerobic environment (96·3 mmol l^{-1} *vs.* 54·9 mmol l^{-1}). No ethanol was produced by *Lact. rhamnosus* GG, *Lact. plantarum* 299v and *Lact. acidophilus* La5.

The positive correlation between the production of lactic acid and the inhibitory activity of lactobacilli after cultivation under microaerobic conditions ($r = 0{\cdot}457$; $P = 0{\cdot}043$, $n = 20$) and bifidobacteria under anaerobic conditions ($r = 0{\cdot}862$, $P = 0{\cdot}005$, $n = 8$) were detected. The amount of acetic acid and the inhibitory activity of lactobacilli and bifidobacteria cultured under anaerobic conditions were negatively correlated ($r = -0{\cdot}428$, $P = 0{\cdot}006$; $r = -0{\cdot}862$, $P = 0{\cdot}006$, respectively).

Following the microaerobic cultivation, the pH values in liquid media correlated inversely with the antagonistic activity of all seven probiotic bacteria ($r = -0{,}530$, $P = 0{\cdot}016$ in broth; $r =$ -0,407, $P = 0{\cdot}043$ in supernatant).

Total anti-oxidative activity

Among all selected probiotic strains only *Lact. fermentum* ME-3 expressed high values of anti-oxidative activity; in comparison with other probiotics these values were significantly higher (Table 4). The TAS values of *B. longum* 46 did not differ significantly from those of *Lact. fermentum* ME-3 ($P = 0{\cdot}082$).

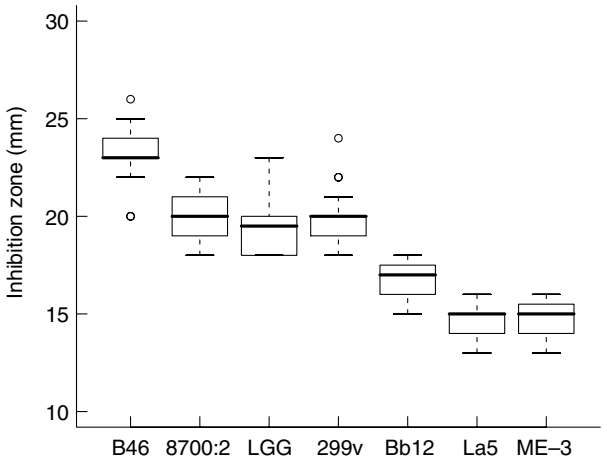

Figure 1 Antagonistic activity of probiotic bacteria against *H. pylori* reference strain on solid media under microaerobic condition. Data are median count (–) and distribution. Box displays 25th–75th quartile area, bars 10th–90th percentage area. La5 *Lact. acidophilus* La5, LGG *Lact. rhamnosus* GG, 8700:2 *Lact. paracasei* 8700 : 2; 299v *Lact. plantarum* 299v; ME-3 *Lact. fermentum* ME-3; B46 *B. longum* 46; Bb12 *B. lactis* Bb12 NS, nonsignificant; $P > 0{\cdot}05$.

Discussion

We have developed an experimental approach to compare the anti-microbial and anti-oxidative activity of probiotic strains of *Lactobacillus* and/or *Bifidobacterium* probiotics, which have been claimed to possess the ability to suppress the growth of various enteric and urinary pathogens. In the course of the EU-funded project 'EU and Microfunction' seven probiotic strains were selected. In the current study, cultivation was performed on solid and in liquid media under anaerobic and microaerobic conditions.

The antagonistic activity of probiotic *Lactobacillus* strains and *Bifidobacterium* sp. depends on the environmental growth conditions, e.g. aerobic/anaerobic conditions (Jacobsen *et al.* 1999; Annuk *et al.* 2003). However, the comparisons between solid and liquid media in particular atmospheric conditions did not help to rank different lactobacilli according to their antagonistic activity. On solid media in microaerobic conditions the highest anti-microbial potential was demonstrated by *Lact. plantarum* 299v, whereas in liquid media *Lact. paracasei* 8700:2 exhibited the highest activity. In contrast, in anaerobic conditions *Lact. fermentum* ME-3 had the strongest antagonistic activity on solid media, yet in liquid media three different probiotics were of equal potency. Therefore, we chose the method resembling the particular GIT niche of the target pathogen where the interaction of probiotic and pathogenic bacteria takes place.

Using this approach we found that high antagonists against *Salm. enterica* ssp. *enterica* were *Lact. paracasei* 8700:2, *Lact. plantarum* 299v and *Lact. fermentum* ME-3 in liquid media and in microaerobic environments.

There were differences found in probiotic potential concerning suppression of recurrent cystitis or pyelonephritis causing *E. coli* strains, which both usually reside in the anaerobic environment of the colon. For suppression of pyelonephritic *E. coli* strains, only three probiotics; *Lact. rhamnosus* GG and both bifidobacteria

Table 3 The production of organic acids by probiotic lactobacilli and bifidobacteria following cultivation in broth for 24 h under microaerobic and anaerobic conditions

		The amount of organic acids (mmol l^{-1}) mean ± SD						
		OHOL	FHEL			OHEL	Bifidobacteria	
Metabolites	Growth conditions	La5	LGG	299v	8700:2	ME-3	Bb12	B46
Acetic acid	M	2·7 ± 2·7	3·1 ± 3·2	6·6 ± 3·4	3·4 ± 3·6	6·8 ± 3·9	ND	ND
	A	2·4 ± 0·4	2·7 ± 1·3	8·2 ± 5·2	1·3 ± 1·8	5·1 ± 0·6	2·5 ± 0·3	37·2 ± 13·1
Lactic acid	M	60·1 ± 4·3	125·0 ± 9·6	118·9 ± 89·5	121·8 ± 10·0	103·3 ± 65·7	ND	ND
	A	35·8 ± 50·6	105·0 ± 43·0	72·3 ± 56·4	108·6 ± 39·5	59·0 ± 13·1	143·6 ± 95·4	23·0 ± 18·7
Succinic acid	M	1·0 ± 0·9	0·8 ± 1·2	0·8 ± 1·0	0·5 ± 0·7	1·8 ± 0·2	ND	ND
	A	0·7 ± 1·0	1·2 ± 0·01	1·9 ± 1·1	1·2 ± 0·1	1·6 ± 0·2	0·5 ± 0·5	0·5 ± 0·4
pH (range; median)	M	3·7–4·3 (3·9)	3·4–3·5 (3·5)	3·4–4·7 (3·5)	3·3–3·8 (3·5)	3·5–3·9 (3·8)	ND	ND
	A	3·8–3·9 (3·8)	3·4	3·4–4·1 (3·5)	3·3–3·4 (3·3)	3·8	3·3	3·8–4·2 (3·9)
Ratio (lactic/acetic acid)	M	22·3	40·3	18·0	35·8	15·2	ND	ND
	A	14·9	38·9	8·8	83·5	11·6	57·4	0·6

M, microaerobic condition; A, anaerobic condition, ND, not determined, OHOL, obligately homofermentative lactobacilli; OHEL, obligately heterofermentative lactobacilli; FHEL, facultatively heterofermentative lactobacilli; SD, standard deviation.
La5, *Lact. acidophilus* La5; LGG, *Lact. rhamnosus* GG; 8700:2, *Lact. paracasei* 8700:2; 299v, *Lact. plantarum* 299v; ME-3, *Lact. fermentum* ME-3; B46, *B. longum* 46; Bb12, *B. lactis* Bb12; All experiments were repeated at least three times.

Table 4 Anti-oxidative activity of probiotic lactic acid bacteria (mean ± SD)

Probiotic strains	TAA (%)	TAS (mmol l^{-1})
Lact. acidophilus La5	16 ± 4	0·08 ± 0·06
Lact. rhamnosus GG	16 ± 7	0·09 ± 0·03
Lact. plantarum 299v	12 ± 5	0·01 ± 0·02
Lact. paracasei 8700:2	15 ± 4	0·03 ± 0·03
Lact. fermentum ME-3	24 ± 4	0·18 ± 0·05
B. lactis Bb12	11 ± 6	0·03 ± 0·03
B. longum 46	11 ± 4	0·10 ± 0·08

TAA, the total anti-oxidative activity; TAS, total anti-oxidative status; SD, standard deviation.
All TAA values of tested probiotic bacteria *vs Lact. fermentum* ME-3 were significantly lower ($P < 0·05$), except TAS value of *B. longum* 46, $P = 0·082$. All experiments were repeated at least three times.

showed high potential. However, the tested probiotics seemed to possess intermediate potency for out-competing cystitis-causing *E. coli* from the large intestine. Reid *et al.* (2003) have claimed that some strains from the aforementioned species; *Lact. rhamnosus* GR-1 and *Lact. fermentum* RC-14 are useful for preventing and treating urogenital infections in women. This confirms once more the specificity of action of a particular probiotic strain.

Furthermore, the best antagonists against *H. pylori*, which colonizes the stomach mucosa, were the heterofermentative probiotics such as *Lact. rhamnosus* GG, *Lact. paracasei* 8700:2 and *Lact. plantarum* 299v. These probiotics were most active on solid media and in microaerobic environment resembling the stomach. In agreement with our *in vitro* results Tursi *et al.* (2004) recently demonstrated that a 10 days quadruple anti-*helicobacter* therapy with *Lact. casei* supplementation significantly increased the eradication rate of *H. pylori* infection. Cruchet *et al.* (2003) have shown that *Lact. johnsonii* La1 may interfere with *H. pylori* colonization in asymptomatic children and may be an effective alternative to modulate *H. pylori* infection.

Lactobacillus fermentum ME-3 and both *Bifidobacterium* strains expressed promising potential for the treatment of shigellosis, a disease typical for the large intestine. On the whole, the Gram-negative pathogen of *Sh. sonnei* and *Salm. enterica* ssp. *enterica* were the most susceptible target bacteria to indigenous lactobacilli similar to the results obtained in our previous study (Annuk *et al.* 2003). In clinical studies the anti-infectious potential of *Lact. rhamnosus* GG has been the most frequently assessed probiotic compared to the other probiotics tested in this study. It is important to mention that the results obtained by our *in vitro* experiments are in good agreement with clinical trials, which have substantiated the potential of *Lact. rhamnosus* GG for treatment of *Shigella* infections (Sepp *et al.* 1995). Alm (1983) demonstrated the enhancement for eradication of *Salmonella* in chronic carriers due to administration of *Lact. acidophilus*. Some placebo-controlled double-blind studies have shown the positive effect of *Lact. rhamnosus* GG by reducing the incidence of travellers' diarrhoea (Dupont 1997; Hilton *et al.* 1997).

In contrast to the positive therapeutic effect described in antibiotic-associated diarrhoea (Siitonen *et al.* 1990; Biller *et al.* 1995), the probiotic *Lact. rhamnosus* GG did not prevent *C. difficile* infection (Thomas *et al.* 2001). According to our results, the antagonistic activity of *Lact. rhamnosus* GG and other tested lactobacilli, as well as bifidobacteria, against *C. difficile*, was actually low. Some reports show the discrepancy between *in vitro* and *in vivo*, e.g. clinical testing of probiotics. Due to the enhancement of immune response and increase of indigenous lactobacilli counts by administration of probiotic strains the suppression of pathogens could be modulated in the host (Michalkiewicz *et al.* 2003; Monack *et al.* 2004; Songisepp *et al.* 2005).

Lactobacilli reveal different anti-microbial mechanisms shown *in vitro* assays. The antagonistic activity in liquid media is favoured by rapidly diffusing anti-microbial compounds, including organic acids and co-aggregation of different indigenous bacteria with pathogens (Türi *et al.* 1997; Annuk *et al.* 1999, 2001). Researchers have associated high antagonistic activity of lactobacilli with production of organic acids resulting in pH decrease (Ouwehand and Vesterlund 2004). In our previous study we have shown that indigenous lactobacilli produce organic acids and bacteriocins differently in microaerobic or anaerobic environments (Annuk *et al.* 2003). We revealed the positive correlations between the pH, amounts of produced lactic acid and rank of anti-microbial activity for all tested probiotics. Tested lactobacilli produced more lactic acid and had stronger inhibitory effect against selected target bacteria under microaerobic than under anaerobic conditions. Bacterial growth inhibition due to lactic acid can be explained by efficient leakage of hydrogen ions across the cell membrane causing acidification of cytoplasm and dissipation of pH gradient (Blom and Mørtvedt 1991).

Drago *et al.* (1997) described a lack of inhibitory effect for pH-adjusted cell-free supernatant of *Lact. paracasei* and *Lact. acidophilus*. In the present study no inhibitory effect was observed using pH-adjusted supernatant of lactobacilli against facultatively anaerobic Gram-negative pathogens.

High anti-oxidative activity values for *Lact. fermentum* ME-3 corresponding to high rank of anti-infectious potential by *in vitro* assays are important in treatment of infections. This was apparent in an experimental murine

typhoid model where probiotic *Lact. fermentum* ME-3 improved the anti-oxidativity of inflammatory gut mucosa and suppressed *Salmonella* Typhimurium infection (Mikelsaar *et al.* 2004; Truusalu *et al.* 2004).

The developed *in vitro* assays for testing of antagonistic and anti-oxidative activity of probiotic *Lactobacillus* strains and *Bifidobacterium* sp. towards selected entero- and urinary pathogens help to reveal their putative effect in various environmental conditions.

Screening *Lactobacillus* strains and *Bifidobacterium* sp. strains according to their activity in various environmental conditions could precede the clinical efficacy studies for adjunct treatment with probiotics in cure of different gastrointestinal and urinary tract infections.

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Authors' contributions

Pirje Hütt and Krista Lõivukene have been in charge of the microbiological experiments and analysing the results and preparing the manuscript. Jelena Shchepetova and Tiiu Kullisaar have been in charge of the biochemical analysis and writing the manuscript. Marika Mikelsaar is the main conductor of the probiotic research; for this paper she has been in charge of designing the study and reviewing the manuscript.

Competing interests

Marika Mikelsaar, Mihkel Zilmer, Tiiu Kullisaar, Heidi Annuk (Hynes) and Epp Songisepp are sharing the Estonian patent application: no. EE 2001 00356 29·06·01 and International Patent application: no. WO03002131.

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