Journal of Applied Microbiology 2003, **94**, 403–412

Characterization of intestinal lactobacilli as putative probiotic candidates

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2002/148: received 27 March 2002, revised 21 October 2002 and accepted 5 November 2002

ABSTRACT

H. ANNUK, J. SHCHEPETOVA, T. KULLISAAR, E. SONGISEPP, M. ZILMER AND M. MIKELSAAR. 2003.

Aims: To use antioxidative activity and antagonistic properties of lactobacilli against selected pathogens and members of the normal microflora as a basis for screening probiotic candidates.

Methods and Results: Antagonistic activity of lactobacilli against target bacteria in both microaerobic and anaerobic environments was tested. Production of antagonistic metabolites (ethanol, hydrogen peroxide (H_2O_2), acetic, lactic and succinic acid) by lactobacilli as well as their total antioxidative activity were assessed. In general, the lactobacilli tested were most effective against Gram-negative bacteria and their antagonistic activity was strain-specific. However, obligately heterofermentative lactobacilli had the strongest activity when tested in a microaerobic environment. Additionally, facultatively heterofermentative lactobacilli were equally effective in either milieu and produced significant levels of acetic and lactic acid. Moreover, obligately homofermentative lactobacilli had high H_2O_2 production and total antioxidative activity but weak antagonistic activity.

Conclusions: Antioxidative and antagonistic activity of intestinal lactobacilli is strain-specific but typically can be related to their fermentation type which may be used for rapidly screening large numbers of lactobacilli for probiotic candidates.

Significance and Impact of the Study: This study represents the first report on the utilization of group characteristics to screen lactobacilli intended for specific probiotic use. Such uses include the targeting of particular gut niches and pathogens as well as allowing for long-term benefits to the host.

Keywords: Lactobacilli, antagonistic and antioxidative activity, antimicrobial compounds.

INTRODUCTION

Probiotics, live microbial food supplements that beneficially affect the host by improving its microbial balance (Fuller 1989; Forestier *et al.* 2001), are quickly gaining interest as functional foods. An increasing number of food supplements as well as pharmaceutical preparations are being promoted with health claims based on several characteristics of certain strains of lactic acid bacteria (LAB), particularly from the genera *Lactobacillus*, *Enterococcus* and also the genus *Bifidobacterium* (McFarland and Elmer 1997; Kaur *et al.* 2002).

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Several selection criteria have been used for novel probiotic strains, which can be summarized into three groups: safety, functional and technological aspects (Saarela *et al.* 2000). For safety considerations it is important that new probiotic strains have a healthy human origin (Mattila-Sandholm *et al.* 1999). Functional aspects include such criteria as tolerance to gastric acidity and bile toxicity (Dunne *et al.* 2001), antioxidative activity (Kullisaar *et al.* 2002), production of antimicrobial compounds, ability to modulate immune responses and adhesion to gut tissues (Saarela *et al.* 2000). Furthermore, technological aspects include the ability of probiotic strains to withstand production under industrial conditions and survive in the final formulation of the appropriate food product (Saarela *et al.*

2000). Moreover, during this process, the ability of cultures to retain their functionality for the target area, usually the gastrointestinal tract, and co-existence with the indigenous microflora must be assessed.

The origin, habitat and species of *Lactobacillus* strains have a great impact on their value as probiotics. The classical division of the lactobacilli has been based on their fermentative characteristics: (1) obligately homofermentative lactobacilli (OHOL), (2) facultatively heterofermentative lactobacilli (FHEL) and (3) obligately heterofermentative lactobacilli (OHEL) (Kandler and Weiss 1986). However, it has been shown by the comparative 16S ribosomal RNA sequence analysis that phenotypically generated taxa frequently do not correspond phylogenetically (Holzapfel *et al.* 2001). All these taxonomic difficulties moreover complicate the possibility to predict the suitability of individual strains of the identified species for probiotics.

Lactobacilli have been shown to possess inhibitory activity towards the multiplication of enteropathogens (Drago *et al.* 1997). It is still not clear whether the antimicrobial mechanisms of lactobacilli differ according to the target bacteria involved or their associated virulence. The inhibitory activity of lactobacilli against commensals or pathogens either of Gram-negative or -positive origin has been found to be very variable and being more strain-specific concerning both pathogens and lactobacilli (Jacobsen *et al.* 1999).

Lactobacilli are highly competitive largely due to their production of several antimicrobial compounds such as certain organic acids, hydrogen peroxide (H_2O_2), carbon dioxide, diacetyl, acetaldehyde, reuterin and bacteriocins (Ouwehand 1998). Moreover, different antimicrobial compounds have shown to exert specific antagonistic properties against Gram-negative and -positive pathogens. For example, bacteriocins produced by LAB have specific inhibitory activity against Gram-positive bacteria (Abee *et al.* 1995). In contrast, Gram-negative pathogens are more sensitive against the organic acids produced by LAB (Alakomi *et al.* 2000). However, as the inhibition tests are predominantly carried out *in vitro*, their application to the gastrointestinal environment is unclear. For instance, differences in the production of putative antimicrobial compounds by lactobacilli cultivated in various media and atmospheric conditions have not been systematically investigated.

The aim of this study was to screen isolates of intestinal lactobacilli for their antioxidative activity and antagonistic properties against selected pathogens and members of indigenous microflora reflecting their potential use as probiotics. In addition, we aimed to discern any potential relationships between specific antimicrobial compounds produced by these candidate probiotics and their inhibitory activity under various environmental conditions against selected pathogens.

MATERIALS AND METHODS

Bacteria

The 35 intestinal lactobacilli strains used in this study (Table 1) were isolated from the faecal samples of 1–24-month-old healthy infants previously described in studies elsewhere (Sepp *et al.* 1997). Furthermore, five culture collection strains used in the study were purchased from both the American Type Culture Collection (ATCC) and the Deutsche Sammlung von Mikroorganismen (DSM), including *Lactobacillus acidophilus* ATCC 4356, *Lact. buchneri* ATCC 4005, *Lact. paracasei* subsp. *paracasei* DSM 5622, *Lact. rhamnosus* ATCC 53103 (GG) and *Lact. reuteri* DSM 20016). In addition a reference strain, of *Lact. reuteri*, isolated from a commercially available, fermented milk product (Filmjölk Bra; Arla för ICA Handlarna AB Solna, Sweden), was also used in the study. Moreover, for the identification of intestinal lactobacilli using molecular methods, the following culture collection strains were included: *L. delbrueckii* subsp. *delbrueckii* ATCC 9649, *L. brevis* ATCC 14869 and *L. fermentum* ATCC 14931. All lactobacilli strains were routinely grown in de Man–Rogosa–Sharpe (MRS) broth (Oxoid Ltd, Basingstoke, Hampshire, UK) for 24 h in a variable atmosphere incubator (IG 150; Jouan, France) with the following microaerobic atmosphere $CO_2/O_2/N_2$: 10/5/85 at 37°C.

The following target bacteria used in the present investigation were obtained from the culture collection of the Department of Microbiology, Tartu University, Estonia. This included three strains of intestinal *Escherichia coli* isolated from the faeces of healthy infants, one strain of *Enterococcus faecalis* and 11 *Lactobacillus* strains (three isolates from OHOL, five from FHEL and three from OHEL group). In addition, pathogenic bacteria were used, including a reference strain of *E. coli* K12 and clinical isolates of *Staphylococcus aureus*, *Shigella sonnei*, *Salmonella* Typhimurium (two strains) and 11 *E. coli* strains, which were freshly isolated from children suffering with recurrent urinary tract infection (UTI). All target bacteria were routinely grown in peptone broth (Difco, USA) or alternatively in the case of *Ent. faecalis* peptone broth supplemented with glucose for 18 h at 37°C and in the case of lactobacilli in MRS broth for 24 h in microaerobic environment at 37°C.

Identification of intestinal lactobacilli

Intestinal *Lactobacillus* strains were identified according to their morphological and cultural properties, catalase test (negative) and an API 50 CHL kit analysed by API LAB Plus software version 4.0 database (bioMérieux, Marcy l'Etoile, France). Internal-transcribed spacer polymerase chain reaction (ITS-PCR) followed by enzymatic restriction

Table 1 Intestinal *Lactobacillus* isolates and their identification based on API 50 CHL and ITS-PCR

Strains	Species identification by API	Quality of identification*	Identification by ITS-PCR
OHOL			
177-3	*Lactobacillus delbrueckii* subsp. *delbrueckii*	Good	Not confirmed†
A 11-4-1	*Lactobacillus delbrueckii* subsp. *delbrueckii*	Good	*Lactobacillus acidophilus*
A 3-6	*Lactobacillus acidophilus* 1	Good	*Lactobacillus acidophilus*
A 11-3-2	*Lactobacillus acidophilus* 1	Good	*Lactobacillus acidophilus*
821-3	*Lactobacillus acidophilus* 2	Good	*Lactobacillus acidophilus*
226-2	*Lactobacillus acidophilus* 3	Good	Not confirmed†
E29E-9-3	*Lactobacillus acidophilus* 3	Good	Not confirmed†
821-1	*Lactobacillus crispatus*	Very good	*Lactobacillus acidophilus*
180-2-1	*Lactobacillus salivarius* subsp. *salivarius*	Doubtful	*Lactobacillus salivarius* subsp. *salivarius*
FHEL			
11-2-8B	*Lactococcus lactis* subsp. *lactis*	Good	*Lactobacillus paracasei* subsp. *paracasei*
R23C-7-3	*Lactobacillus curvatus*	Very good	*Lactobacillus paracasei* subsp. *paracasei*
7-1	*Lactobacillus paracasei* subsp. *paracasei* 1	Very good	*Lactobacillus paracasei* subsp. *paracasei*
A 7-2-2	*Lactobacillus paracasei* subsp. *paracasei* 1	Good	*Lactobacillus paracasei* subsp. *paracasei*
180-2-2	*Lactobacillus paracasei* subsp. *paracasei* 3	Good	*Lactobacillus paracasei* subsp. *paracasei*
1-4-1A	*Lactobacillus paracasei* subsp. *paracasei* 3	Very good	*Lactobacillus paracasei* subsp. *paracasei*
253-2-1	*Lactobacillus plantarum*	Excellent	*Lactobacillus plantarum*
134-6-3	*Lactobacillus plantarum*	Excellent	*Lactobacillus plantarum*
277-7-1	*Lactobacillus plantarum*	Excellent	*Lactobacillus plantarum*
R28D-5-3	*Lactobacillus plantarum*	Excellent	*Lactobacillus plantarum*
E15C-8-1	*Lactobacillus rhamnosus*	Good	*Lactobacillus paracasei* subsp. *paracasei*
OHEL			
1-3	*Lactobacillus brevis*	Acceptable	*Lactobacillus buchneri*
13-2-1	*Lactobacillus brevis*	Acceptable	*Lactobacillus buchneri*
194-4	*Lactobacillus brevis*	Very good	*Lactobacillus brevis*
277-6-1	*Lactobacillus brevis*	Very good	*Lactobacillus brevis*
1-8	*Lactobacillus buchneri*	Very good	*Lactobacillus buchneri*
15-2-4A1	*Lactobacillus buchneri*	Very good	*Lactobacillus buchneri*
A7-2-1	*Lactobacillus buchneri*	Good	*Lactobacillus buchneri*
1-4-1B	*Lactobacillus buchneri*	Excellent	*Lactobacillus buchneri*
338-1-1	*Lactobacillus fermentum*	Very good	*Lactobacillus fermentum*
822-1-1	*Lactobacillus fermentum*	Very good	*Lactobacillus fermentum*
822-1-4	*Lactobacillus fermentum*	Very good	*Lactobacillus fermentum*
E56B-4-1	*Lactobacillus fermentum*	Very good	*Lactobacillus fermentum*
E23C-2-2	*Lactobacillus cellobiosus*	Good	*Lactobacillus fermentum*
11-0-1	*Lactobacillus coprophilus*	Very good	Not confirmed‡
51-3	*Lactobacillus coprophilus*	Good	Not confirmed‡

*Comments on the quality of identification derived from the ID% and the T index of the selected taxon.
†Not confirmed – did not correspond to the species identified by API, therefore an unknown species.
‡Not confirmed – reference strain was not available.
OHOL = Obligately homofermentative lactobacilli; FHEL = facultatively heterofermentative lactobacilli; OHEL = Obligately heterofermentative lactobacilli.

was used to confirm the species identification as demonstrated in previous studies (Drake *et al.* 1996; Jacobsen *et al.* 1999; Abdelgadir *et al.* 2001). DNA extraction from *Lactobacillus* isolates was performed as described by Alander *et al.* (1999) using lysozyme (Serva, Sweden; 20 mg ml^{-1}), mutanolysin (Sigma Chemical Co., St Louis, MOI, USA; 0·5 mg ml^{-1}) and proteinaseK solution (Fermentas, Lithuania; 14·6 mg ml^{-1}). Amplification of DNA was performed according to Jacobsen *et al.* (1999) in a reaction volume of 50 μl containing 1× *Taq* polymerase buffer (Fermentas), 1·5 U *Taq* polymerase (Fermentas), 0·5 μM of each primer (16S-1500F and 23S-32R; DNA Technology AS), 200 μM deoxynucleoside triphosphates, 2 mM $MgCl_2$ and 2 μl of extracted DNA. Furthermore, the PCR product was restricted as described by Zhong *et al.* (1998) using *Taq*I restriction enzyme (Fermentas). DNA fragments and a size marker (100 bp, DNA Ladder Plus, Fermentas) were separated by electrophoresis with a 2% agarose gel (field

strength 5 V cm^{-1}) in 1× TBE buffer. The banding pattern of the isolates was visually compared with that of the aforementioned *Lactobacillus* reference strains.

Antimicrobial testing

Antagonistic activity of lactobacilli against target bacteria on agar was assessed using a streak line procedure on plates containing MRS medium without tri-ammonium-citrate and sodium-acetate (pH 7·2). A single line of lactobacilli culture, grown in MRS broth for 48 h, was seeded in the middle of the agar plate. *Lactobacillus* strains were then cultivated for 48 h at 37°C in microaerobic environment and also in an anaerobic glove chamber (Sheldon Manufacturing, Inc., Shel LAB, Cornelius, OR, USA) with a gas mixture of $CO_2/H_2/N_2$: 5/5/90% and thereafter inactivated using chloroform gas for two hours. Target bacteria were cultured in peptone broth for 18 h at 37°C and seeded in duplicate perpendicular to the streak line of lactobacilli. Following incubation of the plates for 18 h at 37°C in aerobic environment, the width of the zone of inhibition (mm) of the target bacteria extending from the culture line of lactobacilli was measured (Mikelsaar *et al.* 1987).

Antagonistic activity in broth was determined in a modified MRS broth (without tri-ammonium-citrate and sodium-acetate, pH 7·2). Equal aliquots of lactobacilli and target bacteria suspensions in isotonic saline (10^9 CFU ml^{-1}) were co-incubated in modified MRS broth for 24 h at 37°C. Thereafter, broth pH was estimated, and the number of CFU ml^{-1} of viable target bacteria was semiquantitatively determined on peptone agar (Gould 1965).

Determination of organic acids and ethanol

The production of organic acids and ethanol (mmol l^{-1}) was estimated by gas chromatography as described by Holdeman *et al.* (1977). The gas chromatograph (Hewlett-Packard model 6890; Hewlett Packard Company, Wilmington, DE, USA) was equipped with a hydrogen flame ionization detector and an auto sampler (model 7683). The HP Chemical Station for GC System (A.06 revision) was used. Analyses were performed following cultivation of lactobacilli in modified MRS broth for 24 and 48 h in microaerobic environment and also in the anaerobic glove chamber.

Hydrogen peroxide production

Hydrogen peroxide (H_2O_2) production by lactobacilli was determined following culture of the strains in MRS broth for 24 h. Thereafter, manipulations were carried out under aerobic conditions at 4°C unless otherwise stated. The bacterial cells were washed and resuspended in isotonic saline. The density of bacterial suspension was adjusted to an absorbance of 1·1 at 600 nm by a spectrophotometer. H_2O_2 concentration was measured using the method of Ou and Wolff (1996). Briefly, 75 μl of test sample was mixed with 1·425 ml of FOX 1 (D-sorbitol and xylenol orange; Sigma) and ammonium ferrous sulphate hexahydrate (Sigma) and incubated at room temperature for 30 min. Flocculated protein and cells were removed by centrifugation and the absorbance was read at 560 nm. The concentration of H_2O_2 ($\mu g\ ml^{-1}$) was found by generating a standard curve and expressed as μg of H_2O_2 per ml of the sample.

Total antioxidative activity

The total antioxidative activity (TAA) of 25 randomly selected *Lactobacillus* strains was assessed using the linolenic acid test (Kullisaar *et al.* 2002). Lactobacilli were grown in MRS broth overnight and then pelleted by centrifugation (360 ×*g*, for 10 min), washed and resuspended in isotonic saline at 4°C. The density of the suspension was adjusted to an absorbance of 1·1 at 600 nm (*ca.* 10^9 CFU ml^{-1}) and the determination was carried out as described by Kullisaar *et al.* (2002). TAA was expressed as a percentage of inhibition by the sample of the peroxidation of the linolenic acid standard.

Statistical methods

The antagonistic activity for different fermentation groups of lactobacilli was compared using either Student's *t*-test or Mann–Whitney rank sum test. The choice of tests was made automatically using the computer software 'Statgraphics' (Statistical Graphics Corp., Rockville, MD, USA) according to the distribution of the data. The differences were considered significant when P value was $\leq 0{\cdot}05$. The Spearman rank order correlation test was used for comparisons between antimicrobial activity and the production of organic acids and H_2O_2. $P < 0{\cdot}05$ was used to indicate statistical significance.

RESULTS

Identification of intestinal lactobacilli

In total one-third of *Lactobacillus* strains differed from preliminary identification with API by molecular typing techniques (Table 1). In the case of the OHOL group, ITS-PCR reassigned five of nine strains despite a good identification level by analytical profile index (API). In contrast, the differentiation of the intestinal *Lact. rhamnosus* strain from *Lact. paracasei* subsp. *paracasei* was not successful by ITS-PCR. The same problem occurred for *Lact. reuteri* strains, intestinal *Lact. cellobiosus* and *Lact. fermentum*

isolates. The identity of two *Lact. coprophilus* strains could not be formally determined using ITS-PCR, as the reference strain was not available for comparison. However, the ITS-PCR profiles of both strains were different from the other OHEL group strains.

Antagonistic activity of lactobacilli on agar

Antagonistic activity against target bacteria was tested following growth of lactobacilli under both microaerobic and anaerobic environmental conditions (Table 2). The strongest inhibitory activity corresponded with the OHEL group grown in a microaerobic atmosphere, yet it was significantly decreased in an anaerobic environment ($P \leq 0{\cdot}001$). However, under anaerobic conditions the OHEL group retained the strongest antagonistic activity compared with other groups, although *Lact. brevis* and *Lact. coprophilus* had noticeably weaker antagonistic activity (data not shown). In contrast, the OHOL group had the weakest antimicrobial activity in both milieus, although the increase in an anaerobic environment was two and a half-fold. In the case of the FHEL group, similar and stable inhibitory values were noted using either atmospheric condition, although a significant difference ($P = 0{\cdot}008$) was observed for the inhibition of Gram-negative bacteria.

The inhibition of Gram-negative bacteria was stronger in both atmospheres than that of Gram-positive microbes ($P \leq 0{\cdot}001$). The antagonistic activity of lactobacilli against different test bacteria was found to be more dependent on the species of pathogen but not of particular strain as no discrepancy was found in case of *E. coli* K12, UTI *E. coli* strains and the intestinal *E. coli* isolates from healthy children. Furthermore, both *Staph. aureus* and *Ent. faecalis* had similar sensitivity against lactobacilli established in both environments.

The antagonistic activity of intestinal lactobacilli against target *Lactobacillus* strains was tested in a microaerobic environment (data not shown). There was no notable inhibition between the strains within the same fermentation groups. However, comparisons between groups demonstrated that FHEL and OHEL strains could inhibit the growth of OHOL strains in different ranges ($18{\cdot}3 \pm 6{\cdot}5$ and $21{\cdot}7 \pm 11{\cdot}2$ mm, respectively).

Antagonistic activity of lactobacilli in broth and its relationship with pH

The antagonistic activity of lactobacilli in broth was determined as the inhibition of growth of pathogens by $\log_{10}$ CFU ml^{-1} (Table 3). The strongest inhibition was demonstrated by FHEL group strains, in particular against Gram-negative pathogens. The OHOL group demonstrated the weakest inhibition against all target bacteria. In broth the inhibitory activity of lactobacilli from different fermentation groups against Gram-positive bacteria was equally weak among the groups.

It was found that the FHEL group strains lowered pH significantly more than the strains of the other two

Table 2 Antagonistic activity of lactobacilli on agar in microaerobic and anaerobic environments, expressed as inhibition zone values

	Inhibition zone values (mm) mean ± S.D.					
	OHOL (10 strains)		FHEL (13 strains)		OHEL (18 strains)	
Target bacteria	Microaerobic	Anaerobic	Microaerobic	Anaerobic	Microaerobic	Anaerobic
Escherichia coli K12	4·7 ± 4·8	11·9 ± 2·7	20·0 ± 5·9	18·0 ± 2·9	28·9 ± 6·6	21·4 ± 5·4
UTI *Escherichia coli**	3·8 ± 4·7	11·2 ± 3·0	18·3 ± 4·9	17·3 ± 2·9	26·5 ± 9·2	19·4 ± 4·9
Intestinal *Escherichia coli*†	4·1 ± 4·9	11·5 ± 2·8	18·6 ± 5·1	16·8 ± 3·9	28·3 ± 6·8	18·8 ± 7·0
Shigella sonnei	7·2 ± 4·7	14·6 ± 2·5	21·0 ± 4·7	20·7 ± 2·6	31·0 ± 6·6	22·8 ± 4·8
Salmonella Typhimurium‡	5·5 ± 4·6	12·7 ± 2·8	21·2 ± 6·2	18·5 ± 3·0	30·1 ± 5·3	22·1 ± 6·4
Average for Gram-negative bacteria	5·0 ± 4·7	12·4 ± 2·0	19·7 ± 5.3**	18·4 ± 3·0**	29·1 ± 6·4	21·0 ± 5·9
Staphylococcus aureus	2·6 ± 3·5	8·8 ± 2·6	15·9 ± 4·3	15·2 ± 2·9	23·4 ± 9·2	16·1 ± 4·5
Enterococcus faecalis	2·7 ± 3·6	9·7 ± 2·6	16·9 ± 3·9	16·5 ± 3·0	23·4 ± 9·6	17·6 ± 4·6
Average for Gram-positive bacteria	2·6 ± 3·4	9·3 ± 2·6	16·4 ± 4·1††	15·8 ± 3·0††	23·4 ± 9·3	16·8 ± 4·5
Average of the group	4·4 ± 4·5§	11·5 ± 3·1¶	18·9 ± 5·2§	17·7 ± 3·2¶	27·5 ± 7·8§	19·8 ± 5·8¶

*The mean of 11 urinary tract infection (UTI) *E. coli* strains.
†The mean of the intestinal *E. coli* strains.
‡The mean of two *Salm.* Typhimurium strains.
§A significant difference $P \leq 0{\cdot}001$.
¶A significant difference $P \leq 0{\cdot}001$.
**A significant difference $P = 0{\cdot}008$.
††No significant difference.

Table 3 The antagonistic activity of lactobacilli in broth (expressed as inhibition of growth of pathogens by $\log_{10}$ CFU ml^{-1}) and pH of broth after co-incubation for 24 h

	OHOL (10 strains)		FHEL (13 strains)		OHEL (18 strains)	
Target bacteria	log ↓ *	pH (mean ± S.D.)	log ↓ *	pH (mean ± S.D.)	log ↓ *	pH (mean ± S.D.)
Escherichia coli K12	0·4	3·8 ± 0·4	6·0	3·5 ± 0·1	1·5	3·9 ± 0·2
UTI *Escherichia coli*†	0·8	4·0 ± 0·4	2·9	3·8 ± 0·4	1·2	4·0 ± 0·4
Intestinal *Escherichia coli*‡	0·7	4·4 ± 0·4	2·9	4·0 ± 0·4	1·1	4·3 ± 0·5
Shigella sonnei	1·0	3·8 ± 0·4	6·6	3·5 ± 0·1	2·5	3·9 ± 0·2
Salmonella typhimurium§	1·2	4·5 ± 0·4	3·1	4·2 ± 0·5	1·2	4·4 ± 0·5
Average for Gram-negative bacteria	0·7¶	4·1 ± 0·5	2·9¶	3·8 ± 0·4	1·2¶	4·1 ± 0·4
Staphylococcus aureus	0·6	3·7 ± 0·3	1·1	3·4 ± 0·1	0·6	3·8 ± 0·2
Enterococcus faecalis	0	3·8 ± 0·2	0·5	3·6 ± 0·1	0·3	3·8 ± 0·1
Average for Gram-positive bacteria	0·2**	3·8 ± 0·3	0·6**	3·5 ± 0·1	0·4**	3·8 ± 0·2
Average of the group	0·5	4·0 ± 0·5††	1·1	3·8 ± 0·4††;‡‡	0·8	4·1 ± 0·4‡‡

*Inhibition of pathogen growth was calculated by subtracting the number of target bacteria reminaing in the co-incubation tube from the number in a control tube with only target bacteria. The result was expressed as $\log_{10}$ CFU ml^{-1}.
†The mean of 11 urinary tract infection (UTI) *E. coli* strains.
‡The mean of the intestinal *E. coli* strains.
§The mean of two *Salm. typhimurium* strains.
¶A significant difference $P \leq 0{\cdot}001$.
**No significant difference.
††A significant difference $P \leq 0{\cdot}001$.
‡‡A significant difference $P \leq 0{\cdot}001$.

lactobacilli groups, with a negative correlation ($P < 0{\cdot}05$) between the inhibitory activity of lactobacilli and the pH of the broth.

The production of organic acids and ethanol

Gas chromatographic analysis of metabolites produced by lactobacilli in a microaerobic atmosphere (Table 4) demonstrated that strains from the FHEL group produced abundant lactate following growth for 48 h. The production of lactic acid by OHOL and OHEL strains was lower, both differing significantly from FHEL strains ($P \leq 0{\cdot}001$). Both OHOL and FHEL strains produced small amounts of acetic acid, whereas OHEL strains yielded significantly more ($P \leq 0{\cdot}001$). Furthermore, OHEL strains produced ethanol in substantial amounts, whereas only trace amounts were detected for FHEL strains. All lactobacilli strains of different fermentation groups produced a small amount of succinic acid, yet larger differences were seen in the OHEL group, where some strains produced two to three times more succinic acid than the mean of the group.

Differences were found in the production of organic acids under a microaerobic and an anaerobic environment (Table 4). The higher amounts of acetic acid were yielded by OHOL and FHEL strains ($P \leq 0{\cdot}001$) and succinic acid by FHEL strains ($P = 0{\cdot}002$). In the case of lactic acid production, lower amounts were yielded by OHEL strains ($P = 0{\cdot}011$) with no significant differences between the other two groups. In addition, significantly higher levels of ethanol production were noted for OHEL strains grown in a microaerobic vs an anaerobic environment. However, within the OHEL group, particular strains were able to produce approximately three times more ethanol in anaerobic milieu compared with the mean of the group. Additionally, as a control, the metabolite profile of culture collection strains used in the study matched their fermentation types.

Spearman rank order correlation was calculated between the amount of organic acids and ethanol produced following growth for 24 h, and the inhibitory activity of target bacteria by lactobacilli in broth. A positive correlation ($P < 0{\cdot}05$) was found between lactic acid and acetic acid production and inhibition of bacterial viability for all pathogens. No correlation was found between both succinic acid or ethanol production and antimicrobial activity of lactobacilli in liquid media.

The production of H_2O_2 and TAA

The production of H_2O_2 by lactobacilli was found to be mainly strain-specific (data not shown) and differences in its production between the fermentation groups were less than was the case for organic acids. The amount of H_2O_2 produced (range/median) were as follows: OHOL > OHEL > FHEL (61–376/115·5, 28–287/106 and 14–230/47 μg ml^{-1}, respectively). A significant difference was observed between the OHOL and FHEL strains

Table 4 The production of organic acids and ethanol by lactobacilli after cultivation in broth for 24 and 48 h in microaerobic and anaerobic environments

		The amount of organic acids and ethanol (mmol l^{-1}) mean ± S.D.					
		OHOL (10 strains)		FHEL (13 strains)		OHEL (18 strains)	
Metabolites	Growth environment	24 h	48 h	24 h	48 h	24 h	48 h
Acetic acid	Microaerobic	0·8 ± 0·5	1·1 ± 0·4*	1·0 ± 0·5	1·5 ± 0·4†	2·4 ± 1·1	5·3 ± 5·0*,†
	Anaerobic	1·6 ± 0·7	2·1 ± 0·4	2·2 ± 0·4	2·8 ± 1·0	3·5 ± 1·4	4·5 ± 1·9
Lactic acid	Microaerobic	50·8 ± 30·6	81·3 ± 27·8‡	93·2 ± 20·6	129·6 ± 36·4‡,§	52·8 ± 35·7	76·8 ± 23·2§,¶
	Anaerobic	81·6 ± 21·5	91·1 ± 26·2	92·9 ± 15·9	113·8 ± 16·1	33·2 ± 34·8	46·9 ± 36·9¶
Succinic acid	Microaerobic	0·5 ± 0·2	0·6 ± 0·2	0·4 ± 0·1	0·5 ± 0·1	0·6 ± 0·3	0·8 ± 0·6
	Anaerobic	1·0 ± 0·6	0·7 ± 0·2	0·5 ± 0·1	0·7 ± 0·1	0·5 ± 0·1	0·6 ± 0·2
Ethanol	Microaerobic	–	–	traces	traces	98·5 ± 50·1	151·7 ± 26·1**
	Anaerobic	–	–	traces	traces	92·5 ± 125·9	115·8 ± 159·2**

*A significant difference $P \leq 0{\cdot}001$.
†A significant difference $P \leq 0{\cdot}001$.
‡A significant difference $P \leq 0{\cdot}001$.
§A significant difference $P \leq 0{\cdot}001$.
¶A significant difference $P \leq 0{\cdot}001$.
**A significant difference $P = 0{\cdot}003$.

($P = 0{\cdot}014$), in which the isolates from the first group yielded more H_2O_2.

Total antioxidative activity (Fig. 1) was measured for a randomly selected group of 25 strains of lactobacilli. The highest and most uniform TAA values were found for strains in the OHOL group. In the FHEL and OHEL groups the TAA values were highly strain-specific. Comparisons between fermentation groups noted a significant difference only between the OHOL and FHEL groups ($P = 0{\cdot}044$).

No correlation was found between H_2O_2 production, TAA and antimicrobial activity.

DISCUSSION

Despite the difficulties encountered in reliably characterizing probiotic strains using *in vitro* methods, the initial screening of strains in this manner remains a useful first step in the detection of probiotic candidates. We have found that the antagonistic properties of lactobacilli using well-standardized methods depend on their fermentation abilities and growth conditions. The latter predicts possible action in different parts of the gastrointestinal tract as both microaerobic and anaerobic conditions exist there, with the latter prevalent in the small and large intestine. Although OHOL group strains were found to have a relatively much stronger inhibitory activity in an anaerobic environment, they retained the weakest activity compared with the other groups. In addition, strains from the OHEL group had the strongest inhibitory activity when tested on a solid medium under microaerobic conditions, but high variability was noted when the test was carried out under anaerobic conditions and in broth. In particular, *Lact. brevis* and *Lact. coprophilus* had noticeably weaker antagonistic activity in anaerobic milieu. Axelsson (1998) showed that several strains of *Lact. brevis* ferment glucose poorly when grown in an anaerobic environment, which may associate with scarce production of antimicrobial metabolites. As the antagonistic activity of FHEL group strains was quite high and stable under different conditions and media they may be optimal for use as oral probiotics.

Some variability was noted in the inhibitory activity of different species within the three fermentation groups of lactobacilli. Therefore, precise identification of lactobacilli isolates is an important consideration in the selection of probiotic strains. Various methods have been used for the identification of lactobacilli isolates including carbohydrate fermentation as assessed by the API 50 CHL fermentation system (Johansson *et al.* 1995). In the present study we

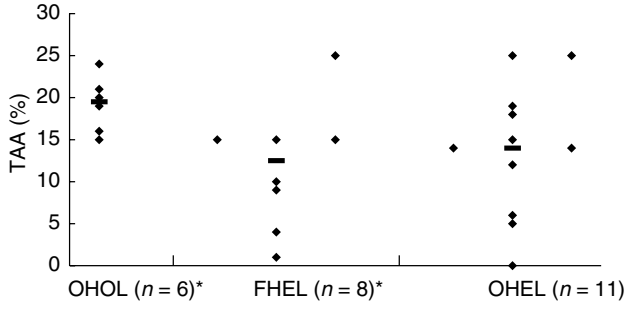

Fig. 1 Total antioxidative activity (%) of *Lactobacillus* strains. – Median for the particular fermentation group; * a significant difference $P = 0{\cdot}044$

utilized both phenotypic (API 50 CHL) and genotypic (ITS-PCR)-based systems for the identification of lactobacilli. Our results demonstrated agreement between these methods in only two-thirds of cases. API divides the *Lact. acidophilus* group into different biotypes, which do not allow the definition of a specific biotype for one species or vice versa (Klein *et al.* 1998). We showed that the differences between the biotypes 1 and 2 of *Lact. acidophilus* were not in agreement with ITS-PCR results which also found that biotype 3 was not identified as *Lact. acidophilus*. These latter strains may belong to the *Lact. acidophilus* group (*Lact. crispatus*, *Lact. amylovorus*, *Lact. gallinarum*, *Lact. gasseri* or *Lact. johnsonii*) and need further identification. However, in our study, no genotypic differences were noted between either *Lact. fermentum*, *Lact. cellobiosus* or *Lact. reuteri* using ITS-PCR. Furthermore, disagreement occurred between the methods for both *Lact. rhamnosus* and *Lact. paracasei* strains which are phylogenetically very similar. However, in this case successful phenotypic differentiation was provided by the API 50 CHL system. Thus, in the selection of probiotic lactobacilli both phenotypic and genotypic identification methods are important.

Several researchers have reported on highly antagonistic lactobacilli and associated this activity, among others, with production of organic acids caused by a resulting decrease in pH (Mishra and Lambert 1996; Ouwehand 1998). In our study a significant correlation was found between organic acid production and the inhibitory power of lactobacilli. According to the metabolite profiles of the fermentation groups, the main end product of glucose fermentation for both OHOL and FHEL is lactic acid. The OHEL group has additional production of acetic acid and/or ethanol and carbon dioxide. Correlation between antagonistic activity and lactic acid production was found in liquid media. It has been shown that acetic acid has stronger antagonistic activity than lactic acid (Ouwehand 1998). Similarly, we found correlation between the production of acetic acid and antagonistic activity in broth. Antimicrobial activity can also be related to the synergistic activity of different acids (Ouwehand 1998). In addition, the inhibitory activity of the culture collection strain of *Lact. reuteri* was found to be much higher in an anaerobic environment. This may be related to the production of reuterin by this species (Axelsson *et al.* 1989). Thus, the test milieu seems to be very important for expression of antimicrobial metabolites and subsequent selection of strains.

Inhibitory activity caused by the production of H_2O_2 by lactobacilli has been documented previously (Fontaine *et al.* 1996; Ocaña *et al.* 1999a). However, as lactobacilli produce several other antimicrobial compounds, the specific role of H_2O_2 is still unclear. In the present study no correlation was found between antagonistic activity of lactobacilli and the production of H_2O_2. In agreement with results from other investigators (Fontaine *et al.* 1996; Ocaña *et al.* 1999b), this property was found to be more strain-specific rather than species or fermentation type-specific.

However, in contrast to previous studies (Jacobsen *et al.* 1999), we found that the antimicrobial activity against Gram-positive and -negative pathogens was not the same. In both environments and in both methods used, Gram-negative bacteria were more sensitive against lactobacilli and this was not dependent on their fermentation group. Among Gram-negative bacteria *Sh. sonnei* was the most sensitive to the antagonistic activity of lactobacilli. This was readily apparent in liquid medium and as the pH of the broth following co-incubation was the lowest for that strain, it may be concluded that *Sh. sonnei* has less tolerance to an acidic environment.

In addition to microbial antagonistic activity, antioxidative activity also influences the survival of bacteria in a particular ecological niche. The microbial cell defences against the influence of severe oxidative stress may be useful for the host, helping to relieve the damaging effect of toxic-free radicals on human cells. The antioxidative effect of LAB has been reported only recently (Lin and Yen 1999). To date its beneficial effect for the human host has not been well described. In our study we found that according to preliminary screening of TAA, measured using the lipid peroxidation method, it varied broadly between the strains indicating strain-specificity for TAA. However, the OHOL group was found to have the highest activity compared with the other groups. Furthermore, OHOL group strains were not inhibitory against any species or isolate from the genus *Lactobacillus* although they themselves were influenced by FHEL and OHEL group strains. Thus, the OHOL group strains may be more suitable representatives for probiotic use in the gastrointestinal tract as a result of their lack of inhibitory activity against intestinal lactobacilli. As oxidative damage plays a crucial pathological role in the aetiology of illnesses such as cancer, cirrhosis, atherosclerosis, and arthritis (Lin and Yen 1999), the antioxidative capacity of the OHOL group would be a desirable characteristic of probiotic strains.

We conclude that the antimicrobial activity of lactobacilli *in vitro* is principally related to the production of antimicrobial metabolites such as acetic and lactic acid which is in turn dependent upon the fermentation type of lactobacilli and the growth milieu. Within the fermentation groups there exists a high degree of strain variability in antagonistic activity, indicating that other unidentified antimicrobial factors may also be active. However, initial probiotic selection could be based on the above-mentioned physiological groupings using the relevant biomarkers. Within the OHOL group, strains may be found with antioxidative activity useful for the defence of the gut against severe oxidative stress, while simultaneously being safe for indigenous intestinal lactobacilli in the course of prolonged use.

Representatives from both of the heterofermentative lactobacilli groups seem to be promising as highly antagonistic probiotic candidates against pathogens. As the expression of antagonistic activity depends on conditions within the ecological niche, OHEL group strains would be putatively more active in the microaerobic environment of the upper intestinal tract, whereas FHEL group isolates could potentially be used in all areas of the gut despite differences in growth milieu.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Estonian Science Foundation (Base Funding 0418 and DARMB0893). We thank Associate Professor Åsa Ljungh from Lund University for providing us with the reference strains of lactobacilli.

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