# Fermentation of sugars and sugar-alcohols by plaque *Lactobacillus* strains

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ABSTRACT

***Objective*** The objective was to analyse the ability of *Lactobacillus* strains isolated from supragingival plaque of subjects with hyposalivation and from healthy controls to ferment sugars and sugar-alcohols.

***Material and methods***: 51 strains isolated from interproximal plaque from subjects with radiation-induced hyposalivation (25 strains), subjects with primary Sjögren's syndrome (16 strains) and from subjects with normal salivary secretion rate (10 strains) were tested. Growth and pH was determined after 24 and 48 h of anaerobic incubation in vials containing basal media with 1% of glucose, fructose, sucrose, mannitol, sorbitol or xylitol.

Results: No differences between strains isolated from hyposalivated subjects and controls were detected. All strains lowered the pH to < 5.0 from fructose and the majority of the strains from glucose and sucrose. A pH of < 5.5 was seen for 52 % of the strains using mannitol, 50 % using sorbitol and 36 % using xylitol. The ability to produce acids from sugars and sugar alcohols was highest among strains of *L. rhamnosus*, *L. casei* and *L. paracasei* and lowest among *L. fermentum* strains.

*Conclusion*: *Lactobacillus* strains are often able to ferment the sugar substitutes mannitol, sorbitol and xylitol to pH levels critical for enamel demineralisation.

***Clinical relevance:*** Our findings suggest that products containing mannitol, sorbitol and/or xylitol may contribute to the acidogenic potential of the dental plaque in hyposalivated subjects with high numbers of lactobacilli.

Keywords: fermentation, hyposalivation, lactobacillus, pH, sorbitol, xylitol

# INTRODUCTION

Subjects with hyposalivation often harbour high numbers of *Lactobacillus* spp. both in saliva and in supragingival plaque (Almståhl *et al.*, 2001, 2003, 2008, Eliasson *et al.*, 2006, Al-Nawas and Grötz 2006, Leung et al 2007**).** In a previous study, the prevalence of different *Lactobacillus* species in the supragingival plaque of subjects with hyposalivation due to primary Sjögren's syndrome or radiation therapy in the head and neck region and in controls with normal salivary secretion rates was analysed (Almståhl *et al.*, 2010). *L. fermentum* and *L. casei* were the most prevalent species in anterior plaque whereas *L. rhamnosus* and *L. fermentum* were the most prevalent in posterior plaque. Subjects with high *Lactobacillus* counts had a more acidogenic plaque than those with no or low numbers of lactobacilli in their plaque (Almståhl *et al*., 2010).

*Lactobacillus* spp. are able to ferment a wide range of carbohydrates resulting in acid-production (Kandler and Weiss, 1986). Strains of *L. plantarum* and *L. salivarius* have been found to ferment the sugar-substitutes sorbitol and xylitol leading to a pH < 5.5 (Badet *et al*., 2001), considered as the critical level for enamel demineralisation. It has also been shown that *Lactobacillus* strains can adapt to xylitol fermentation (Badet *et al*., 2004). Sorbitol and xylitol are common sugar substitutes in toothpaste and other fluoride-containing products, such as chewing gums and saliva-stimulating products, which are frequently used by subjects with hyposalivation (Almståhl *et al*., 2003). In subjects with hyposalivation, it is possible that lactobacilli are favoured by frequent access to sugar substitutes and can adapt to fermentation of them, giving them an advantage to other acidogenic microorganisms. Little is, however, known about the ability of different species and strains of lactobacilli to ferment carbohydrates and sugar alcohols.

Our hypothesis was that *Lactobacillus* strains able to ferment the sugar-substitutes mannitol, sorbitol and xylitol are frequently found in the supragingival plaque in subjects with hyposalivation due to primary Sjögren's syndrome (pSS) and subjects with radiation-induced hyposalivation (RT).

The aim of the present study was to analyse the fermentation patterns of *Lactobacillus* strains isolated from supragingival plaque from subjects with pSS, subjects with radiation-induced hyposalivation (RT) and controls with normal salivary secretion rate.

## MATERIAL AND METHODS

### *Supragingival plaque and* Lactobacillus *spp.*

The *Lactobacillus* strains included in the present study were isolated from supragingival plaque of anterior and posterior tooth surfaces from 6 RT subjects, 3 pSS subjects and from 5 healthy controls. Data from these subjects regarding stimulated salivary secretion rate and microflora (Eliasson *et al*., 2005, 2006, Almståhl et al., 2010) as well as the methods for plaque sampling and isolation and identification of *Lactobacillus* spp. have previously been presented (Almståhl *et al*., 2010).

Briefly, after refraining from interproximal tooth cleaning for three days, plaque was collected with sterile toothpicks from one upper anterior and one upper posterior interproximal area. After dilution and inoculation on Rogosa agar plates for 48 h (Almståhl et al., 2010), lactobacilli were randomly selected using a template with three circles representing 10% of

the agar surface area and all colonies within the circles were recultivated and saved (Almståhl et al 2010). Lactobacilli from 6 RT subjects (59 isolates), 3 pSS subjects (40 isolates) and 5 controls (11 isolates) were isolated and saved.

The 110 isolates were further identified using polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP).

*Lactobacillus isolates for fermentation tests*

Out of the 110 *Lactobacillus* isolates, 66 were selected for fermentation tests **(**22 from pSS subjects, 33 from RT subjects and 22 from controls). For the hyposalivated subjects, all isolates from plaque samples from which 1-2 isolates of *Lactobacillus* spp. had been collected, and about 50% of the isolates collected from plaque with ≥ 3 isolates, were included in the test. Isolates of the same species giving similar fermentation patterns (pH-values varying between 0 and 0.5 pH-units) were presumed to represent the same strain and were counted together. From respectively 3 pSS, 4 RT and 1 control plaque samples, 2 isolates were counted together and from 1 pSS and 1 RT plaque sample, 4 isolates were counted together. Fifty-one isolates (25 from RT subjects, 16 from pSS subjects and 10 from controls) of various species and fermentation patterns are therefore reported here (Table 1-**3 –** ~~4~~). Most of the isolates belonged to the species *L. fermentum*, *L. casei, L. rhamnosus* and *L. paracasei* or were unidentified.

*Fermentation tests*

*Lactobacillus* isolates were transferred from Cryobank tubes to Rogosa agar plates, which were incubated in 90 % $CO_2$ and 10 % $N_2$ at 36°C for 48 h. A representative colony was transferred to a vial with 5.0 ml of Basalmedia consisting of 5 g/L Thiotone-peptone (BBL

Microbiological Systems, Cockeyville, MD USA), 5 g/l trypticase-peptone (BBL), 5 g/l yeast extract (Becton, Dickinson and Company, Franklin lakes, NJ, USA), 20 ml salt solution (pH 7.4) and 1 % glucose (Difco, Detroit, MI, USA). The vial was incubated anaerobically until exponential growth phase (optical density 0.4-0.6) determined with a spectrophotometer (Novaspec II Spectrophotometer, Pharmacia Biotech) at a wavelength of 480 nm. Hundred microliters of the bacterial suspension was transferred to vials with 5.0 ml basalmedia with 1 % of glucose (Merck, Germany), fructose (Merck), sucrose (Difco), mannitol (Difco), sorbitol (Merck) or xylitol (kind gift from AB R. Lundberg, Sweden). The optical density (480 nm) and pH (pH-meter, Metrohm 632) of the solution was determined after 24 and 48 h of anaerobic incubation.

*Statistical methods*

The fermentation tests were performed twice for all isolates and a mean value was calculated. In cases where the pH differed more than 10 % between the two tests, the fermentation test was performed a third time and a mean was calculated from the three values. The mean value was used for the isolates (2-4) of the same species giving similar fermentation patterns and counted together. Differences in pH after sugar-fermentation between lactobacilli isolated from RT, pSS and controls, between lactobacilli isolated from anterior and posterior dental plaque and between different species of lactobacilli were analysed using ANOVA. When the ANOVA rejected the multi-sample hypothesis of equal means, multiple comparison testing was performed with Fisher's exact test. Testing was performed two-tailed and at the 1 % level.

# RESULTS

There were no significant differences in growth and acid formation between *Lactobacillus* isolates from anterior and posterior dental plaque. Neither were there any significant differences between species isolated from the different patients groups. Furthermore, the number of isolates from controls was low and therefore all isolates of different *Lactobacillus* species were grouped together irrespective of origin.

*Growth*

All *Lactobacillus* strains tested grew better with the sugars than with the sugar alcohols ($p < 0.01$ for all), and the growth was usually better with the monosaccharides than with sucrose **(**Figure**).** The mean growth from the three sugar-substitutes was of the same magnitude within each species, and the lowest mean values were usually seen with xylitol. After 48 h of incubation, the growth (optical density) was somewhat increased (0.04 - 0.3 units) from the sugars and to a lesser extent from the sugar alcohols where the mean values for *L. rhamnosus, L. casei* and the unidentified species were similar to the 24 h values. The overall patterns for the growth with sugars and sugar alcohols were, however not different.

*Fermentation of glucose, fructose and sucrose*

After 24 h of incubation, 46% of the *L. casei*, *L. fermentum*, *L. paracasei*, *L. rhamnosus*, *L. salivarius* and the unidentified strains counted together, could lower the pH to below 5.5 using glucose and 31% using sucrose. All strains tested lowered the pH to < 5.5 using fructose. As can be seen in Table 2, the highest pH from glucose, fructose and sucrose were seen for *L. fermentum* strains, while strains of *L. rhamnosus* displayed among the lowest pH-

values. After 48 h of incubation, the mean pH for most strains tested was about 0.2-0.8 units lower compared with the values obtained after 24 h (data not shown).

*Fermentation of mannitol, sorbitol and xylitol*

As for the sugars, *L. fermentum* displayed the highest pH-values and *L. rhamnosus* among the lowest at fermentation of sugar-alcohols (Table 3). Fifty-three percent of the *Lactobacillus* strains tested lowered the pH below 5.5 using mannitol. The ability to ferment mannitol was most common among the *L. rhamnosus* (88 %) and *L. paracasei* (86 %) strains and least common among the *L. fermentum* strains tested (15 %). Forty-nine percent of the *Lactobacillus* strains and especially strains of *L. paracasei* and *L. rhamnosus,* lowered the pH to ≤ 5.5 from sorbitol, while this ability was rarely seen for *L. fermentum.* A pH below 5.5 using xylitol was seen for 37 % of all strains. Among the *L. casei* and *L. paracasei* strains 61 % had this ability and 38 % of the *L. rhamnosus* strains. Only one of the *L. fermentum* strains could lower the pH to 5.5 using xylitol. For mannitol and sorbitol, the mean pH was 0.1-0.4 pH units lower after 48 h of incubation. The pH after xylitol fermentation had not decreased further after 48 h of incubation.

*Fermentation of sugar-substitutes in relation to individuals*

*Lactobacillus* with ability to lower the pH to < 5.5 using all 3 sugar-substitutes were found in one pSS subject, 4 RT subjects and 2 controls and *Lactobacillus* strains able to lower the pH using mannitol and sorbitol was found in one pSS subject and one RT subject.

# DISCUSSION

In the present study, the *in vitro* pH-lowering potential of *Lactobacillus* strains, isolated from supragingival plaque from subjects with hyposalivation due to primary Sjögren's syndrome (pSS) or radiation therapy in the head- and neck region (RT) and in healthy controls, was tested. Initially, it was our intention to compare the strains from hyposalivated subjects with those from controls. However, the strains from the controls were few and they showed similar patterns regarding growth and acid formation as those isolated from hyposalivated subjects. Therefore strains of the same species were grouped together regardless of origin.

## *Methodological considerations*

It is difficult to extrapolate the results from the *in vitro* experiments to the *in vivo* situation in the mouth. In our experiments, single strains of *Lactobacillus* were tested, while the dental plaque consists of many different microbial species (Aas *et al*., 2005), which compete for space and energy in the *in vivo* situation. In mature and complex biofilms, lactic acid produced by for example streptococci, *Actinomyces* and lactobacilli may be consumed by other bacteria like *Veillonella* species (Periasamy and Kolenbrander 2010). The pH decrease might therefore not be so large *in vivo*.

The *Lactobacillus* cells were in the exponential growth phase when they were transferred to vials with sugar or sugar alcohols, while the bacteria in the dental plaque are growing at a slow rate with less activity. Also, the time the *Lactobacillus* cells were exposed to the sugar or sugar alcohol, 48 h, differed from the *in vivo* situation. Studies on unstimulated saliva from healthy children after intake of a xylitol-containing product showed that the xylitol concentration was between 20 and 34 mg/ml one minute after the intake and remained at a

level of > 1 mg/ml for at least 16 min (Lif Holgerson *et al*., 2006). It is likely that the *Lactobacillus* strains in subjects with hyposalivation and a longer oral clearance time have access to a sugar or sugar-alcohol at concentrations of ≥ 1 mg/ml for even longer periods.

*Sugar alcohols*

It is well known that sucrose and other carbohydrates promote the growth of acidogenic and aciduric microorganisms, such as mutans streptococci and lactobacilli (Bradshaw and Marsh, 1998, Tenuta *et al*., 2006). Sugar-substitutes are therefore often used to replace sucrose, glucose and fructose. The most commonly used are mannitol, sorbitol and xylitol and the two latter are the most frequently used in Sweden. They can be found in oral health care products like toothpaste, fluoride-containing products, mouthwashes, chewing gums and tablets.

It has previously been found that frequent exposure to sorbitol lead to an increased number of sorbitol-fermenting bacteria and a significantly lower pH in dental plaque after frequent sorbitol exposure (Kalfas *et al*., 1990). The authors state that sorbitol has a cariogenic potential in subjects with impaired salivary secretion rates frequently using sorbitol-containing products (Kalfas *et al*., 1990). An *in vitro* study has shown that strains of *Lactobacillus plantarum* and *L. salivarius* are able to ferment sorbitol to a pH < 5.5 (Badet et al., 2001). In our study, 50 % of the tested strains were able to lower the pH ≤ 5.5 when using sorbitol. This ability was most common among *L. rhamnosus, L. casei* and *L. paracasei*. It is therefore possible that these lactobacilli reduce the plaque pH using sorbitol, increasing the risk of caries in hyposalivated subjects.

To our knowledge, the ability of dental plaque bacteria to adapt to xylitol has only been examined in healthy subjects with normal salivary secretion (Maguire *et al*., 2002). They

found that a two-week period of frequent xylitol use did not lead to adaptation of the dental plaque. However, it should be noticed that this was in healthy subjects who probably had very low numbers of lactobacilli. Badet *et al*., (2001) showed that strains of *Lactobacillus plantarum* and *L. salivarius* were able to ferment xylitol leading to a pH < 5.5. They also showed that *Lactobacillus* strains could adapt to xylitol fermentation (Badet *et al*., 2004). In our study, xylitol fermentation was seen for 36 % of the strains and this ability was most common among *L. casei* and *L. paracasei* strains. Eigthy-two % of our *Lactobacillus* strains were isolated from subjects who had suffered from hyposalivation for several years and probably had used fluoride-containing and saliva-stimulating products containing sugar alcohols for a long time. It is possible that these *Lactobacillus* strains had adapted to xylitol fermentation.

The ability of 13 probiotic strains of *Lactobacillus* to ferment sugars and sugar alcohols has been examined (Hedberg *et al*., 2008, Haukioja *et al*., 2008). Two strains of *L. plantarum* and *L rhamnosus*, respectively lowered the pH below 5.2 from sorbitol and one strain respectively of *L. reuteri*, *L. rhamnosus* and *L. johnsonii* from xylitol. Lactobacilli are frequently used as probiotics mostly to treat disturbances in the intestinal microflora (Servin 2004) but also for improving oral health (Näse et al 2001, Nikawa et al 2004, Krasse et al 2006, Caglar et al 2006). Milk containing *L. rhamnosus* GG reduced the number of mutans streptococci and caries lesions in children (Näse et al 2001) and *L. reuteri* reduced the levels of mutans streptococci in adults (Nikawa et al 2004, Caglar et al 2006) and could reduce gingivitis and plaque (Krasse et al 2006). Little is known about the effect of probiotic lactobacilli on the oral microflora of hyposalivated subjects.

In conclusion, the ability to produce acids from sugars and sugar alcohols was generally highest among *L. rhamnosus*, *L. casei* and *L. paracasei* and lowest among the *L. fermentum* strains tested. *Lactobacillus* strains often fermented the sugar substitutes mannitol, sorbitol and xylitol to pH levels critical for enamel demineralisation. It is therefore possible that frequent use of products containing these sugar alcohols may contribute to the pH-lowering potential of dental plaque in subjects with hyposalivation and high counts of lactobacilli.

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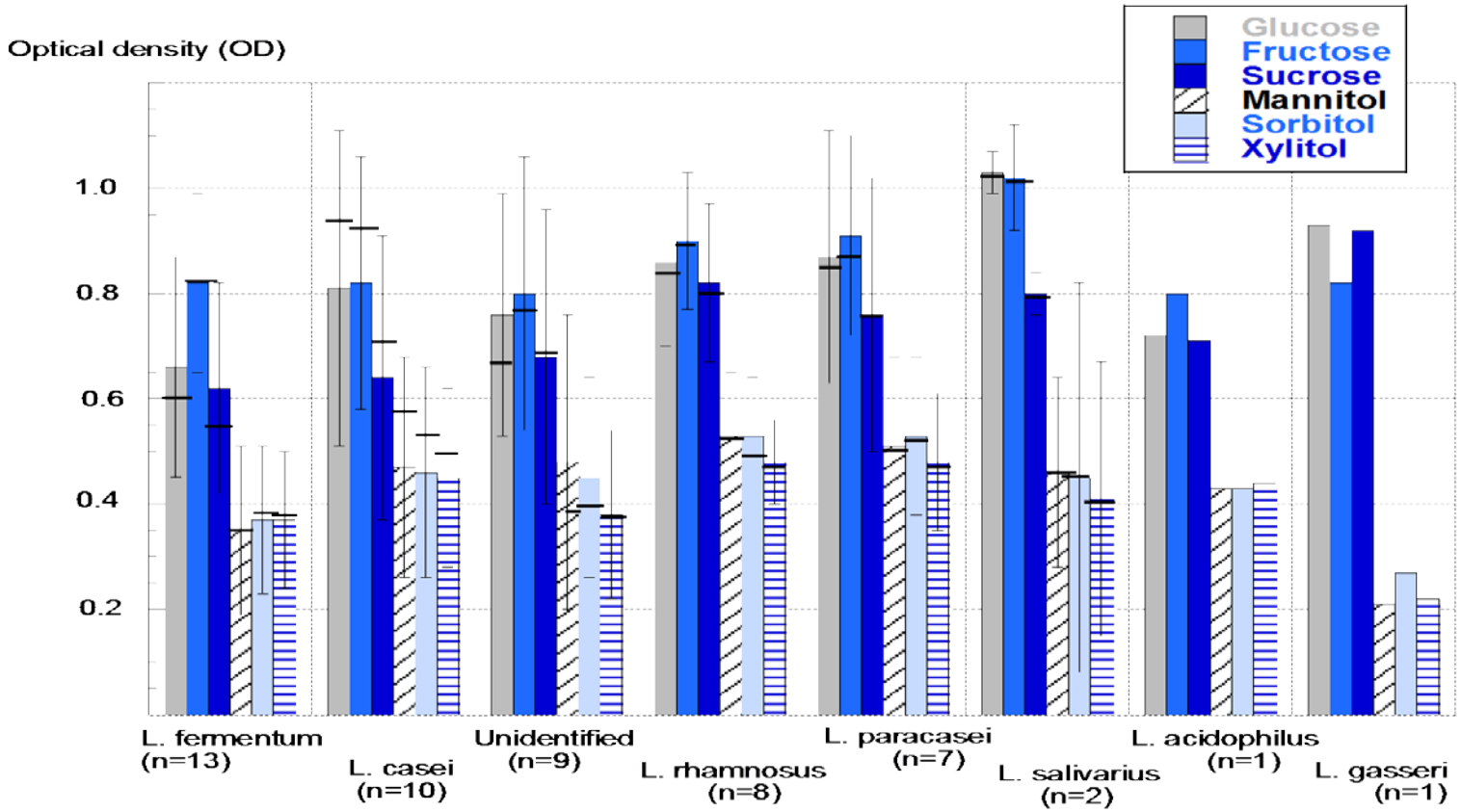
Optical density (OD)
Glucose
Fructose
Sucrose
Mannitol
Sorbitol
Xylitol
1.0
0.8
0.6
0.4
0.2
L. fermentum (n=13)
L. casei (n=10)
Unidentified (n=9)
L. rhamnosus (n=8)
L. paracasei (n=7)
L. salivarius (n=2)
L. acidophilus (n=1)
L. gasseri (n=1)

Table 1. Group and number of isolates subjected to fermentation tests.

| Group | Isolates selected for fermentation tests |
|---|---|
| pSS (n = 16) | *L. fermentum* (5), *L. casei* (3), *L. rhamnosus* (3), *L. paracasei* (2), *L. acidophilus* (1), *L. gasseri* (1), unidentified (1) |
| RT (n = 25) | *L. fermentum* (5), *L. rhamnosus* (5), *L. casei* (5), *L. paracasei* (3), *L. salivarius* (2), unidentified (6) |
| Controls (n = 10) | *L. fermentum* (3), *L. paracasei* (2), *L. casei* (2), unidentified (3) |

Table 2. pH after 24 h of fermentation of glucose, fructose, sucrose for different *Lactobacillus* species. Mean ± SD~~,~~ Mean ± SD, and median values are presented as well as proportions of strains giving a pH ≤ 5.5 (in parenthesis).

| Species | Glucose | Fructose | Sucrose |
|---|---|---|---|
| *L. fermentum* (n=12) | 5.5 ± 0.6[a] (4.6-6.4) (46) | 4.8 ± 0.3 [a](4.3-5.2) (100) | 5.8 ± 0.6 [a] (4.6-6.5) (31) |
| *L. casei* (n=10) | 4.7 ± 0.9 (4.1-6.5) (80) | 4.2 ± 0.3 [b] (3.9-4.7) (100) | 4.9 ± 0.6 [b] (4.0-5.9) (80) |
| *L. rhamnosus* (n=8) | 4.3 ± 0.2 (4.1-4.7) (100) | 4.2 ± 0.2 (4.0-4.5) (100) | 4.7 ± 0.5 (4.0-5.2) (100) |
| *L. paracasei* (n=7) | 4.5 ± 0.7 (4.1-6.1) (86) | 4.2 ± 0.2 [c](4.0-4.6) (100) | 4.8 ± 0.7 [c](4.0-6.1) (86) |
| *L. salivarius* (n=2) | 4.4 ± 0.6 (4.0-4.9) (100) | 4.2 ± 0.4 (3.9-4.5) (100) | 4.9 ± 0.0 (4.9-4.9) (100) |
| *L. acidophilus* (n=1) | 5.0 | 4.8 | 5.3 |
| *L. gasseri* (n=1) | 4.7 | 4.7 | 5.2 |
| Unidentified (n=9) | 5.1 ± 0.7 (4.1-6.2) (67) | 4.7 ± 0.5 [d,e] (4.1-5.3) (100) | 5.3 ± 0.6 (4.3-6.3) (67) |
| All 51 strains | 4.9 ± 0.8 (4.0-6.5) (75) | 4.4 ± 0.4 (3.9-5.3) (100) | 5.1 ± 0.7 (4.0-6.4) (71) |

a) higher compared with *L. rhamnosus* ($p < 0.001$), b) lower compared with *L. fermentum* ($p < 0.01$), c) lower compared with *L. fermentum* ($p < 0.001$ for both), d) lower compared with *L. casei* ($p < 0.01$), e) lower compared with *L. rhamnosus* ($p < 0.01$).

Table 3. pH after 24 of fermentation of mannitol, sorbitol and xylitol for different *Lactobacillus* species. Mean ± SD, and range are presented as well as proportions of strains giving a pH ≤ 5.5 (in parenthesis).

| Species | Mannitol | Sorbitol | Xylitol |
|---|---|---|---|
| *L. fermentum* (n=12) | 6.4 ± 0.6 [a] (5.1-6.9) (15) | 6.5 ± 0.5 [a](5.5-7.0) (8) | 6.5 ± 0.4 (5.7-7.0) (8) |
| *L. casei* (n=10) | 5.2 ± 0.7 (4.7-6.8) (80) | 5.6 ± 0.7 (5.0-7.1) (80) | 5.7 ± 0.6 (5.2-7.0) (70) |
| *L. rhamnosus* (n=8) | 5.1 ± 0.4 (4.8-6.0) (88) | 5.3 ± 0.3 (5.0-6.1) (88) | 5.6 ± 0.3 (5.2-6.1) (38) |
| *L. paracasei* (n=7) | 5.2 ± 0.8 (4.6-6.9) (86) | 5.3 ± 0.7 [b](4.7-6.8) (86) | 5.6 ± 0.6 (4.9-6.9) (57) |
| *L. salivarius* (n=2) | 5.5 ± 0.8 (4.9-6.0) (50) | 5.4 ± 0.6 (5.0-5.8) (50) | 5.7 ± 0.7 (5.3-6.1) (50) |
| *L. acidophilus* (n=1) | 6.2 | 5.9 | 6.3 |
| *L. gasseri* (n=1) | 6.1 | 6.1 | 6.2 |
| Unidentified (n=9) | 5.9 ± 0.8 (4.8-6.9) (33) | 6.1 ± 0.7 (5.2-7.0) (22) | 6.2 ± 0.6 (5.4-6.9) (33) |
| All 50 strains | 5.7 ± 0.8 (4.7-6.9) (52) | 5.8 ± 0.7 (4.8-7.1) (50) | 6.0 ± 0.6 (5.1-7.0) (36) |

a) higher compared with *L. rhamnosus*, *L. casei* ($p < 0.01$ for both), b) lower compared with *L. paracasei* ($p < 0.01$)