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ORIGINAL ARTICLE

Polymerase chain reaction detection of *Lactobacillus acidophilus* in human oral cavity and fecal samples after 2-week consumption of yoghurt

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Abstract

Objective. To investigate whether short-term daily consumption of yoghurt leads to colonization by *Lactobacillus acidophilus* in a group of human subjects who were initially totally devoid of *L. acidophilus* in their oral cavities. ***Material and methods.*** Twenty-three volunteers consumed yogurt containing *L. acidophilus* during a 14-day trial stage. Oral and fecal samples were collected at the clearance stage and at the post-yoghurt intake stage until *L. acidophilus* was found. Standard polymerase chain reaction methods using specific primers were adopted for the detection and identification of *L. acidophilus.* ***Results.*** The isolation frequency decreased rapidly 72 h after stopping intake of yoghurt. After 1 week, *L. acidophilus* was absent in all oral samples. Non-significant differences were found between the survival rates of *L. acidophilus* in samples of saliva, plaque, tongue surface, and buccal mucosa. *L. acidophilus* was also found to remain in the gastrointestinal tract for longer than in the oral cavity. ***Conclusion.*** Allochthonous *L. acidophilus* is not likely to permanently colonize the oral cavity and intestine.

Key Words: *Colonization, human feces, Lactobacillus acidophilus, oral cavity, polymerase chain reaction detection, yoghurt*

Introduction

Probiotics are products containing living microorganisms. When consumed in adequate amounts, they benefit the health of the host [1]. The most abundantly used probiotic strains are those from the genera *Lactobacilli* and *Bifidobacteria*. *Lactobacilli* are commensal lactic acid-producing bacteria with a high aciduric potential. Some of them, such as *L. acidophilus* and *L. rhamnosus*, are particularly known to improve intestinal microbial health and have been used extensively in fermented milk products such as yogurt for many years [2]. They play an important role in the maintenance of health by stimulating natural immunity and contributing to the balance of microflora by interacting with other members of the flora [3,4].

For several decades now, interest in *L. acidophilus* has increased in the field of dental research. Modern molecular techniques have underscored the concept that bacteria are more associated with carious dentine and the advancing front of caries lesions than with initiation of the dental caries process [5]. Bacteria are often present in the dental plaque of adults [6]. *L. acidophilus* derived from consumer products was found to be most prone to co-aggregation with oral *Streptococci in vitro* [7]. Since the oral cavity represents the first part of the gastrointestinal tract, there is every reason to believe that at least some probiotic mechanisms may also play a role in this part of the system [8].

The colonization of the oral cavity by probiotics has been studied in several experiments utilizing different test strains. The report of Meurman et al. [9] on *L. rhamnosus GG* showed that the oral cavity could harbor *Lactobacilli* for up to 2 weeks after discontinuation of yoghurt consumption among nine adults. Yli-Knuttila et al. [10] found no colonization by *L. rhamnosus GG*, but the result indicated the possibility of colonization in some cases after 2 weeks of yoghurt consumption. After 14 days of intake, *L. reuteri* (LR-1, LR-2) was found in the saliva samples of 65–95% of 59 participants [11]. However, the study of Busscher [12] found that, after 1 week of consumption of bio-yoghurt containing the *L. casei*

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and *L. acidophilus* strains, salivary and interproximal plaque samples were free of both *Lactobacilli*.

Although several experiments have been conducted with *L. acidophilus* or other probiotic *Lactobacilli* strains, most of them only detected the bacteria in saliva in the oral cavity. *L. acidophilus* can also be found in many other parts of the mouth, such as plaque. However, there is limited information available on colonization by *L. acidophilus* in other sites of the oral cavity and in the human gastrointestinal tract.

This study aimed to investigate whether short-term daily consumption of yoghurt leads to colonization by *L. acidophilus* in the oral cavities of a group of human subjects who were initially totally devoid of *L. acidophilus*. Simultaneously, *L. acidophilus* detection in fecal samples was conducted to assess how long *L. acidophilus* can be harbored in the human gastrointestinal tract.

Material and methods

Subjects

A total of 23 healthy volunteers participated in this intervention study (12 women, 11 men; mean age 24.4 ± 2.3 years). All of them were students of Sichuan University. The inclusion criteria were good health and total lack of *L. acidophilus*. The exclusion criteria were poor oral hygiene, macroscopic caries, lost fillings, large marginal defects, treatment with a prescribed medicine, intolerance to milk products, severe constipation, diarrhea, and application of antibiotics within 2 weeks prior to the study. The Ethics Committee of the West China Stomatology College of Sichuan University approved the study protocol (No. 2009021). The subjects were informed of the true aim of the study, received both oral and written information about it, and gave their informed consent before the commencement of the research. Among the 23 participants, eight agreed to provide fecal samples.

One dentist performed the preliminary dental examinations. The clinical examination adopted World Health Organization criteria in a normally equipped dental surgery. The number of decayed/missing/filled teeth (DMFT) and the plaque index [13] for 10 teeth (11, 16, 17, 26, 27, 31, 36, 37, 46, and 47) were recorded. The volunteers were required to demonstrate plaque index < 1 after tooth brushing.

Study design

The study presumed that the potential effect of yoghurt on the oral environment was weak. Therefore, it controlled for the factors and characteristics causing microbial fluctuations that may have obscured the results. These included active caries, crowns, imperfect restorations [14,15], professional tooth cleaning [16], oral hygiene [17], use of antibacterial substances [18], intake of sugar-containing foods [19], and salivary sampling conditions [20].

The clinical research was divided into three phases (Figure 1). Each stage is indicated by a double-headed arrow. Arrowheads indicate the sampling days. The subjects were told not to use other probiotic bacteria-containing products or xylitol products before and during the study. They were required to use toothbrushes, fluoride toothpaste, and dental floss provided by the researchers. The use of other fluoride products was forbidden. The subjects were told to brush their teeth twice a day. All products containing probiotics were prohibited for 1 week (from Day 7 to Day 1). After the 1-week observation stage, the subjects started to consume 200 g of yoghurt every day for 2 weeks between breakfast and lunch (yoghurt intake stage; from Day 0 to Day 13). The yoghurt test food was manufactured to contain 4×10^9 cells each of *L. acidophilus*, *Streptococcus thermophilus*, *L. bulgaricus*, and *Bifidobacteria* per 100 g (Menniu Ltd., China). The subjects were then asked to stop taking yoghurt for 1 week (post-intake stage; Day 14 to Day 20). All samples were collected during the observation and post-intake stages.

Sampling of saliva, plaque of tooth surface, tongue, buccal mucosa and feces

Sampling for saliva, caries-free plaque (buccal surface of the left upper first molar), tongue, and buccal mucosa was performed five times (during the observation stage and on Days 13, 14, 16, and 20) between 08.00 and 11.00 (except on Day 13). On Day 13, the sampling was performed between 20.00 and 23.00. The participants were told to refrain from eating, drinking (except water), and smoking for 1 h prior to the sampling. The fecal samples were collected four times: on Days 1, 16, and 20, and 2 weeks after the yoghurt intake stage.

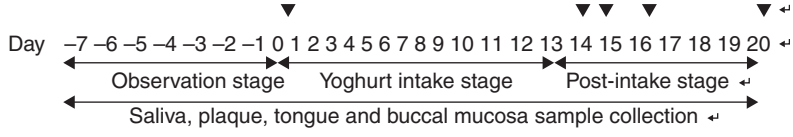

Figure 1. Experimental procedure for the clinical study. Each stage is indicated by a double-headed arrow. Arrowheads indicate the sampling days.

There was a protocol for each participant to follow before sampling fecal matter. Compliance was optimal in all participants. Each participant was given Eppendorf tubes containing 0.15 ml of Tris–EDTA (TE) buffer and cotton-tipped swabs parceled in a bio-clean cloth. Participants collected the fecal samples themselves using a cotton-tipped swab and then placed them in the Eppendorf tubes. The researchers transported the fecal samples to the laboratory, which were then frozen at −60°C for several minutes. All samples from the oral cavity were collected by the researchers following the same protocol. Subjects expectorated a sample of whole unstimulated saliva into sterile tubes. A 0.2-ml aliquot of the sample was vortexed with 0.15 ml of sterile, filtered TE buffer (10 mM Tris–HCl; 1 mM EDTA; pH 7.6). A 0.2-ml sample of this mixture was then taken, and 0.1 ml of 0.5 M NaOH was added [21]. The tongue sample was obtained by rotating 1 cm^2 of the center of the dorsum of the tongue for 5 s with cotton wool. The buccal mucosa and tooth surface were sampled using a swab brush. The swab brushes were swirled to remove adhering bacteria. The plaque was removed from the swab brush by washing and diluting in a tube containing 0.15 ml of TE buffer. From this, 0.2 ml of the solution was removed and placed in an individual Eppendorf tube, and 0.1 ml of 0.5 M NaOH was added. All final samples were transported to the refrigerator within a few minutes, where they were frozen at −60°C for microbiological assessment [21].

Polymerase chain reaction amplification

Isolation of bacterial DNA. DNA was isolated using the ColimnMate Bacteria gDNA Isolation Mini Kit (Watson Ltd., Shanghai, China). The samples, except for saliva, were homogenized in a 1.5-ml microcentrifuge and diluted with sterilized distilled water. DNA was extracted by following the manufacturer's instructions for the isolation kit and frozen at −20°C for analysis.

Amplification by polymerase chain reaction. The samples from the observation and post-intake stages were screened by means of *L. acidophilus*-specific polymerase chain reaction (PCR), using the following primers: LACFOR, 5′-TCTTGACATCTAGRGCAATC-3′; LACREV, 5′- GATTCGCTTGCCTTCGCAGG-3′ [22]. The strain of *L. acidophilus* isolated directly from the yoghurt used in this study served as the positive control, and the deionized water used in the same reaction was the negative control.

PCR was performed with thin-walled tubes and a DNA Engine Dyad Thermal cycler (Bio-Rad, Hercules, CA). One μl of the DNA template was added to the reaction mixture (50 μl final volume) containing 20 nmol of each primer, 40 nmol of deoxynucleoside triphosphates, and 1.25 U of TaKaRa Ex Taq polymerase (Takara Ltd., Dalian, China). In a hot-start protocol, the samples were preheated at 94°C for 5 min followed by amplification under the following conditions: denaturation at 94°C for 30 s; annealing at 59°C for 20 s; and elongation at 72°C for 20 s for each cycle. A total of 30 cycles were performed, followed by a final elongation step at 72°C for 7 min. The results of PCR amplification were examined by electrophoresis in 1% agarose gel with DL2000 DNA Marker (Takara Ltd.) as the molecular-weight marker. DNA was stained with ethidium bromide and visualized under short-wavelength UV light.

Statistical analysis

The positive rate of *L. acidophilus* in four sites of the oral cavity was analyzed by means of Fisher's exact probability test. $P < 0.05$ was considered statistically significant.

Results

Participant follow-up and health status at baseline

Compliance was optimal in all groups. All subjects (23/23) completed the study without any side-effects. Statistical analysis was performed using the data collected from the subjects. According to the clinical examination performed at the observation stage, the mean number of filled teeth for all participants was 2.1 [standard deviation (SD) 0.7], and the mean plaque index was 0.6 (SD 0.5). No *L. acidophilus* was found in the samples collected at the observation stage (Day 1).

PCR detection of oral and fecal samples

All participants were asked to stop taking yoghurt on Day 13. In the 12 h following the intervention, 23 participants (100%) were positive for *L. acidophilus* in their saliva, plaque, tongue, and buccal mucosa samples. After 24 h, nearly all participants were still carriers of *L. acidophilus*. However, the occurrence of *L. acidophilus* decreased gradually from Day 14. On Day 16, the positive rate of *L. acidophilus* had dropped significantly: seven participants (30.4%) showed positive results in their saliva samples, six (26.1%) in their plaque samples, six (26.1%) in their tongue samples, and four (17.4%) in their buccal mucosa samples. After 7 days, no participant was found to harbor the bacterium. The results are given in Table I. *L. acidophilus* was found in all eight fecal samples at Day 16. At Day 20, six of the eight fecal samples were

Table I. Number of *L. acidophilus* carriers found after different stages of yogurt intake.

	No. of *L. acidophilus* carriers				
Sampling site	Day 1	12 h (Day 13)	24 h (Day 14)	72 h (Day 16)	1 week (Day 20)
Saliva	0	23	21	7	0
Plaque	0	23	20	6	0
Buccal mucosa	0	23	20	6	0
Tongue	0	23	19	4	0

positive for *L. acidophilus*. Two weeks after yoghurt consumption, *L. acidophilus* was still detected in two fecal samples.

Different oral cavity sites for the establishment of L. acidophilus

Shortly after stopping taking yoghurt, *L. acidophilus* was found to remain in saliva, plaque, tongue, and buccal mucosa samples. However, the positive rate of *L. acidophilus* decreased at these four sites with time. The downward trend was not significant among the positive rates at these four sites, as shown by the Fisher's exact probability test.

Discussion

The oral cavity usually possesses native microbial flora to restore homeostasis. However, whether or not adventive *L. acidophilus* could colonize the human oral cavity through consumption of yoghurt is not very clear. In this study, the test participants introduced 10^8–10^9 acidophilus cells into their mouths daily for 2 weeks. This bacterium was only transiently present in the saliva, plaque, tongue, and buccal mucosa 72 h after stopping consumption of yoghurt. The findings of Busscher et al. [12] are in agreement with those of the current study, while those of Meurman et al. [9] are not. Zickert et al. [18] and Sharpe [23] believed that the colonization and growth of *Lactobacilli* in the mouth requires frequent sugar intake, and a low pH could help to reconcile this apparent contradiction. The subjects in the present study and those in the study of Busscher et al. were selected on the basis of the absence of *Lactobacilli* in their mouths and on their oral ecological conditions, such as the absence of active carious lesions. Sites with low pH are usually unfavorable for *Lactobacilli* growth. Conversely, the subjects in the study of Meurman et al. were not preselected, and their oral ecological conditions might have predisposed them to the growth of *Lactobacilli*. The results of earlier studies can be explained by the absence of adequate analysis methods in the early 1990s: the PCR primers for *Lactobacilli* verification used in the present study were not available then.

Another notable observation concerns the four niches in the oral cavity, namely saliva, plaque of tooth surface, tongue, and mucosa. Based on the results shown in Table I, the survival rate of *L. acidophilus* in the four niches decreased gradually starting on the day that yoghurt consumption was stopped. The survival rates of *L. acidophilus* in the four sites showed a non-significant difference.

Lactobacilli belong to the normal human mucosal flora of the mouth, although *L. acidophilus* is not known to be dominant in the oral cavity. Some studies indicated that *Lactobacillus* composition is subject-specific and that some identified species can be found in saliva and fecal matter of the same subject [24]. Ahrne et al. [25] reported that *L. acidophilus* could be found in the oral cavity of only 3/42 healthy volunteers. *L. acidophilus* is usually found in subjects with deep caries. *L. acidophilus* is detected transiently and unpredictably [24], and so it is considered allochthonous. In the current study, we avoided choosing subjects with poor oral hygiene, macroscopic caries, lost fillings, and large marginal defects. The healthy oral condition of the subjects may also have contributed to us not finding any *L. acidophilus* in the subjects.

Although there is overwhelming evidence that probiotics such as *L. acidophilus* can restore the physiological microbial equilibrium in several areas of the digestive tract [26], the duration of restoration varies. In general, probiotic *Lactobacilli* can persist in fecal samples for a few weeks to >1 month after their administration has ended [22,27,28]. The results of the present study demonstrated that *L. acidophilus* was found in all eight fecal samples in the first three days, and six of the eight fecal samples were positive for *L. acidophilus* in the first week. Two weeks after yoghurt consumption, *L. acidophilus* was still detected in two fecal samples. Whether this difference can be attributed to the *Lactobacilli* strains or to individual subjects should be explored in the future. Many studies have demonstrated that adventive *Lactobacilli* cannot be permanently retained in the human oral cavity or digestive tract.

By examining the fecal samples collected, *L. acidophilus* was found to stay in the gastrointestinal tract longer than in the oral cavity. Saliva and food clearance from the mouth is more rapid than that

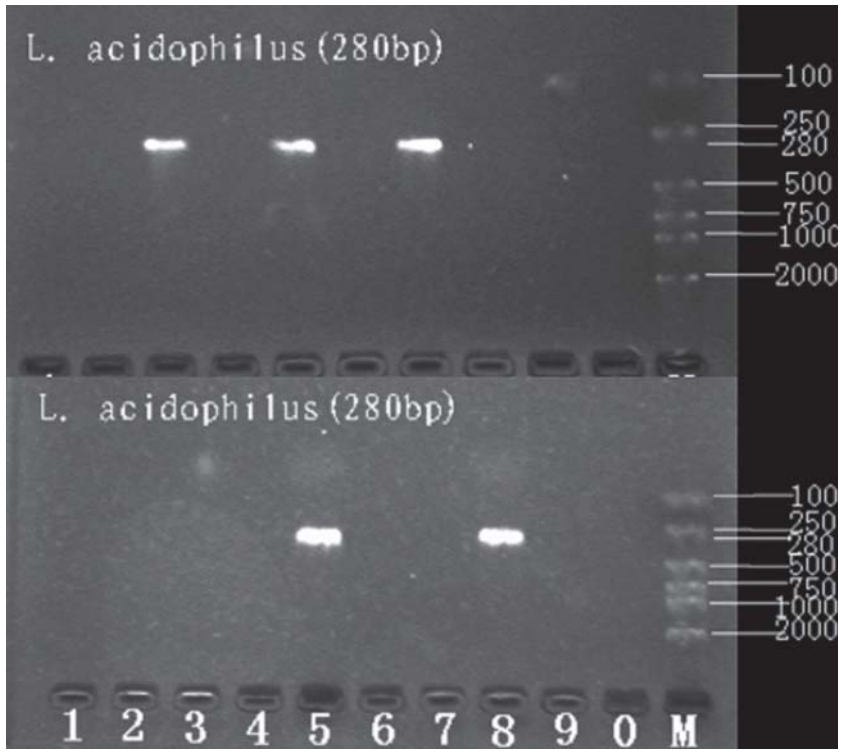

Figure 2. Upper: three positive saliva samples collected on Day 16. *Lanes 3, 5, and 7*: *L. acidophilus* (280 bp) was detected from saliva samples. Lower: two positive samples of buccal mucosa collected on Day 16. *Lanes 5, and 8*: *L. acidophilus* (280 bp) was detected from buccal mucosa samples. *Lane M* = molecular-weight marker (DL 2000); *Lane 0* = negative control.

from the intestine [29], and the contact time between yoghurt and the mouth is short. This suggests that some activities in the mouth may be weaker than those in the intestine. Species of the genus *Lactobacilli* can be cultivated from human feces with cell counts of up to 10^9 colony-forming units/g [30]. Sixteen *Lactobacilli* species are commonly isolated from fecal samples [31], but only the species *L. crispatus*, *L. gasseri*, *L. reuteri*, *L. ruminis*, and *L. salivarius* have been suggested to be truly autochthonous to the human gastrointestinal tract [32].

The current study only examined colonization by *L. acidophilus* in the oral cavity and in fecal samples. Other probiotics in yoghurt, such as *L. bulgaricus* and *Bifidobacteria*, should also be studied. Including other groups of participants, such as those having active caries lesions, is suggested in order to clarify the effect of *L. acidophilus* on persons with different oral conditions. The amount of *L. acidophilus* ingested by the participants may have had an effect on the amount and duration of stay of *L. acidophilus* in the oral cavity and fecal samples. Further research should be conducted in the future.

Conclusions

The following conclusions are offered based on the findings of the study. *L. acidophilus* can be detected in the oral cavity and in the human gastrointestinal tract shortly after consumption of probiotic ceases. It can also stay in the intestine longer than in the mouth. However, since it was not introduced into the oral cavities of our test participants, *L. acidophilus* in yoghurt is not likely to colonize the mouth and gastrointestinal tract when yoghurt is only consumed for a short time.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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