

Full Length Research Paper

Identification of oral strains of *Lactobacillus* species isolated from Mexican and French children

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The aim of the current study was to identify oral strains of *Lactobacillus rhamnosus* and *L. acidophilus* by PCR species-specific and other Lactobacilli by a biochemical test (API 50CH) from Mexican and French children with and without dental decay and to compare them using a RAPD-PCR analysis. Saliva samples were collected from Mexican and French children who were 6 to 12 years old. Children were either caries free or harboring carious lesions. Microorganisms were cultured in MRS media and Lactobacilli (*L. rhamnosus* and *L. acidophilus*) were identified by PCR species-specific. Other Lactobacilli strains were identified by a biochemical test (API 50CH). All Lactobacilli strains isolated in this study were compared using a molecular biology technique (RAPD-PCR). One hundred and sixty-three strains were isolated. The predominant species in French children with caries was *L. rhamnosus*, while *L. rhamnosus*, *L. acidophilus* and *L. brevis* were prevalent among the Mexican children. In children without caries, *L. acidophilus* was the predominant species identified among the Mexican population, whereas no Lactobacilli species were isolated from French children without caries. The RAPD-PCR results showed the same patterns of amplification between the type strain *L. rhamnosus* ATCC 9595 and wild strains isolated in this study, meanwhile *L. acidophilus* showed differences in the pattern of bands between the *L. acidophilus* ATCC 4656 strain and the wild strains isolated from the saliva of children with and without caries. Similar results were found with the API 50CH test. Even though this study does not investigate it, our results suggest that *L. rhamnosus* could be involved in both French and Mexican populations as a potential cariogenic agent.

Key words: *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, PCR.

INTRODUCTION

Lactobacilli belong to the normal flora of the oral cavity. They colonize sites in the mouth, that allow for mechanical retention (pits and fissures, margins of restorations, orthodontic devices) (Fosberg et al., 1991; Gonzalez-Cabezas et al., 2002; Kidd et al., 1995).

Although this genus represents only a low percentage of dental plaque bacteria, it is thought that there is an

overall increase in its presence in saliva prior to carious lesions. Because of this the *Lactobacillus* count has been widely used to determine carious risk factors (Bowden et al., 1997; Gabre et al., 1999; Gabris K et al., 1999; Nancy and Dorignac, 1992). This genus is involved in the progression of carious lesions (Tanzer et al., 2001) and carious dentin is the main ecological site of lactobacilli (Martin et al., 2002). Its collagen affinity (Mc Grady et al., 1995) and acidogenic and aciduric properties (Svensater et al., 1997) support this finding. Low pH of the ecological niche initiated by *Streptococcus mutans* is a selecting factor for these oral bacteria, which are characterized by

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a high acid tolerance (Ferjeskov, 1997). However, even if an association between lactobacilli and caries in humans has been shown, not much is known about the diversity of the species in relation to caries, or about their role in the disease (Bowden et al., 1997).

Members of the *Lactobacillus* genus are characterized as Gram-positive, facultative anaerobic, non-motile, non-spore-forming, rod-shaped and catalase-negative. Techniques for identifying the species include: carbohydrate fermentation, arginine hydrolysis and enzyme activity. Since such biochemical methods depend on environmental and culture conditions, they sometimes lead to ambiguous results or even misidentifications (Nigatu et al., 2000; Song et al., 1999). The increasing number of *Lactobacillus* strains with only slight variations makes the task more difficult (Quere et al., 1997). Genotypic methods are considered more reliable for identification purposes. The aim of the current study was to identify and to compare oral strains of *Lactobacillus* from Mexican and French children, with and without dental caries, by using both a combination of a biochemical test and genotypic analysis. For this purpose, we used PCR species-specific, the conventional biochemical API method and the RAPD-PCR method described by Richard et al. (2001)

METHODS

Bacterial strains and culture conditions

Two groups of children between 6 and 12 years old were included in the current study, one from Mexico and one from France. The oral health and nutritional habits of every child was evaluated. The Mexican group had 104 children (52 with dental caries and 52 caries free) and the French group had 84 children (42 with dental caries and 42 caries free). (In both cases, children that were caries free were considered the control group).

The parents of all the children included in this study gave signed consent to saliva collection after having the purpose of the study explained to them. Any child taking antibiotics during the previous 3 weeks was not included. Samples were processed under the same conditions in France and Mexico. Briefly, 2 ml of unstimulated saliva were collected by spitting and immediately taken to the laboratory. Samples were isolated by the streak plate procedure on MRS (Man, Rogosa and Sharpe) agar (Difco, France) for 6 days, at 37°C, in anaerobic conditions (BBL GasPak, Becton Dickinson and Company, USA).

A different colony was chosen after growth in MRS agar (1 to 2 different colonies). Typical colonies (white, smooth, with a diameter > 1 mm) were subcultured in MRS broth. Gram-positive, catalase-negative rods were stocked at -80°C in a glycerol-MRS mixture until use. The frozen Mexican strains were sent to the Laboratory of Microbiology at the Faculty of Dentistry in Bordeaux in order to be identified. The Lactobacilli strains most commonly found were compared with their corresponding ATCC strain. Microorganisms were cultured in MRS media and *Lactobacilli* (*L. rhamnosus* and *L. acidophilus*) were identified by PCR species-specific. Other Lactobacilli strains were identified by a biochemical test (API 50CH). All Lactobacilli strains isolated in this study were compared using a molecular biology technique (RAPD-PCR).

Genomic DNA preparation

The procedure was derived from the one described by Gasson and

Davis (1980) and was performed as follows. DNA was extracted from an overnight culture in 10 ml of MRS medium. Cells were collected by centrifugation (10000 g, 10 min, and 20°C). The pellet was washed twice in 10 ml of deionized water and resuspended in 900 µl TS buffer containing lysozyme (5 mg ml⁻¹ final concentration) and then incubated for 30 min at 37°C. The resulting protoplasts were collected by centrifugation (10000 g, 10 min, and 20°C). Nucleic acid and cell debris were separated by adding NaCl (1 M final concentration) at 4°C and centrifugation. DNA was extracted with phenol/chloroform (1:1), and sodium acetate (3 M pH 5.2) and ethanol was added to the upper phase. DNA pellet was dried with a vacuum pump and then resuspended in sterile water with RNase (10 mg ml⁻¹).

PCR species-specific of lactobacilli

The species-specific PCR for Lactobacilli was performed using the primers and conditions described by Walter et al. (2000). Briefly, the species-specific PCR for *L. acidophilus* was done using the primers which amplifies a 780 bp fragment from the 16S rRNA gene, AcI 16SI (sense) 5'-AGCTGAACCAACAGATTCAC-3' and AcI 16SII 5'-ACTACCAGGGTATCTAATCC-3' (antisense). The species-specific PCR for *L. rhamnosus* was done using also the primers which amplifies a 190 bp fragment from the 16S - 23S rRNA intergenic spacer region, PrI (sense) 5'-CAGACTGAAAGTCTGACGG-3' and RhaII 5'-GCGATGCGAATTTCTATTATT-3' (antisense).

Approximately 100 ng of genomic DNA were amplified with 2U of Taq polymerase (Qbiogene, France) in a 50 µl reaction mixture that contained reaction buffer (10 mM final concentration), 1.5 mM MgCl₂, 200 µM each deoxynucleoside triphosphate and 10 pmol each primer using a thermal cycler (Minicycler™ MJ Research). The conditions were 30 cycles of denaturation at 95°C (30 s), annealing at 62°C to *L. acidophilus* and 58°C to *L. rhamnosus* (30 s), and extension at 72°C (30 s). Ten µl of each amplification mixture were loaded on a 1.5% (w/v) agarose gel for 45 min at a constant voltage of 150 V in TEB (Tris 0.09 M, EDTA 2 mM, boric acid 0.09 M) buffer. The strains *L. rhamnosus* ATCC 9595 and *L. acidophilus* ATCC 4656 were used as positive control.

The PCR profiles were visualized after staining with ethidium bromide under ultraviolet light. A DNA molecular weight marker (Qbiogene, France) was used to evaluate the weight of the fragments. To assess the reproducibility of the species-specific PCR procedure, three separate trials starting from the same DNA preparation and using the same PCR reagents were performed.

Phenotypic characterization of isolates

Isolates from stock were subcultured in MRS broth and streaked on MRS plates before use. API 50CHL tests (Biomérieux, France) were carried out according to the manufacturer's instructions. Strains were incubated at 37°C according to Nigatu (2000) who found that the characteristic fermentation pattern of most of the oral species is not influenced by temperature. Arginine hydrolysis was tested using a specific medium (yeast extract 3 g l⁻¹, glucose 1 g l⁻¹, arginine 5 g l⁻¹ and 1 ml l⁻¹ of 1% bromocresol purple).

Genotypic characterization of isolates by RAPD-PCR amplification

As described previously (Richard et al., 2001), the RAPD-PCR used a primer targeting a conserved region of the *mle S* gene of *Lactococcus lactis* IL 1441; the region was NPVVYDP (amino acids 86 to 92) and the corresponding nucleotide sequence was an arbitrary primer, 9898 (sense) 5'-GCAGCCGG-3', the other primer was CIMA (antisense) 5'-GATCATAACAACCTGGAT-3' (nucleotides

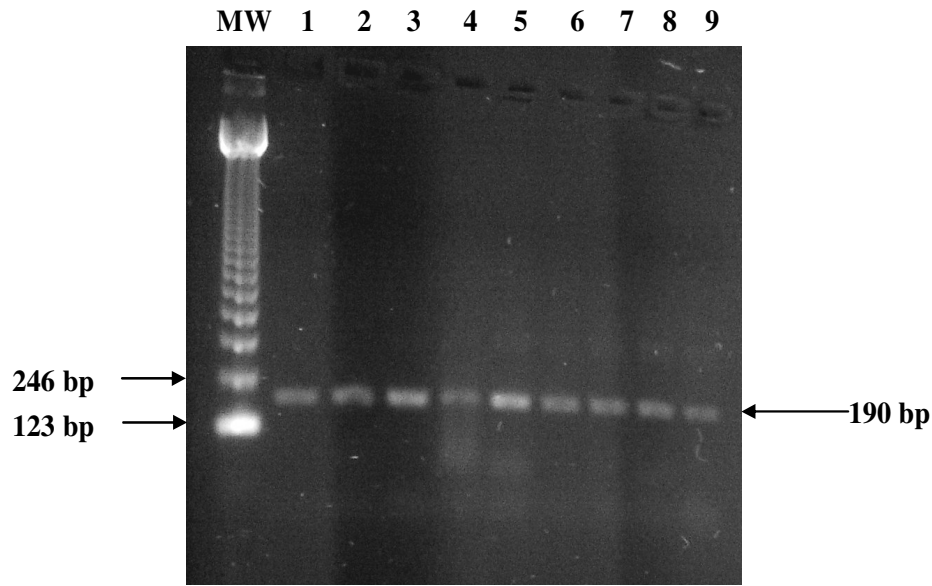


Figure 1. Species-specific PCR of *L. rhamnosus* (lines 2 to 9) strain isolated of children with caries, line 1 *L. rhamnosus* ATCC 9595. MW 123 bp DNA ladder.

276 to 255). Both were manufactured by Proligo (France). Approximately 100 ng of genomic DNA were amplified with 2.5 U of *Taq* polymerase (Qbiogene, France) in a 50 μ l reaction mixture using a thermal cycler (Minicycler™ MJ Research). The conditions were 30 cycles of denaturation at 96°C (30 s), annealing at 38°C (30 s), and extension at 72°C (2 min). Ten μ l of each amplification mixture were loaded on a 1% (w/v) agarose gel for 45 min at a constant voltage of 150 V in TEB (Tris 0.09 M, EDTA 2 mM, boric acid 0.09 M) buffer. The RAPD-PCR profiles were visualized after staining with ethidium bromide under ultraviolet light as previously described.

Statistical analysis

Chi square test was used to check the relationship between presence of Lactobacilli and caries activity. To compare the different species with the caries activity, we used a non-parametric analysis of variance (ANOVA) test.

RESULTS

All children with carious lesions were tested positive to Lactobacilli by culture in both the French and Mexican population. In the French group of children without caries, there were no positive cultures for lactobacilli, meanwhile in the Mexican group without caries, 76.9% had positive culture for lactobacilli.

In French children, only one lactobacilli colony morphology was observed, while in Mexican children 1 to 2 different colony morphologies were observed. Only one lactobacilli species from saliva per child (42 lactobacilli strains out of 42 children) were isolated in French population, while in the Mexican population there were isolated 1 to 2 lactobacilli species per child (76 lactobacilli strains out of 52 children) were isolated. Mexican children had only one lactobacilli strain in 54% and 46% of the

children had 2 lactobacilli strains. All Lactobacilli strains isolated in this study were tested by a PCR species-specific for both *L. rhamnosus* (Figure 1) and *L. acidophilus* (Figure 2) identification.

L. rhamnosus was only present in the saliva samples of children with carious lesions from both French and Mexican population in the following percentages: 85.72% in the French population and 39.5% in the Mexican population.

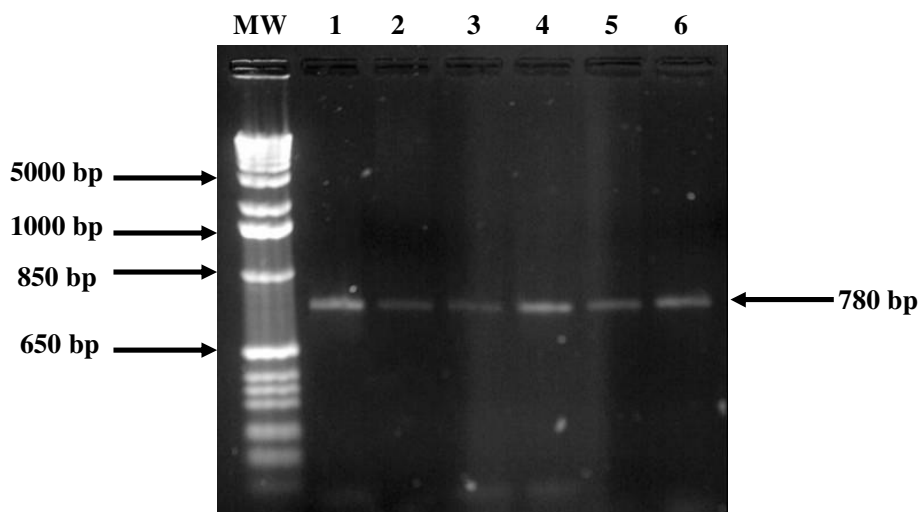
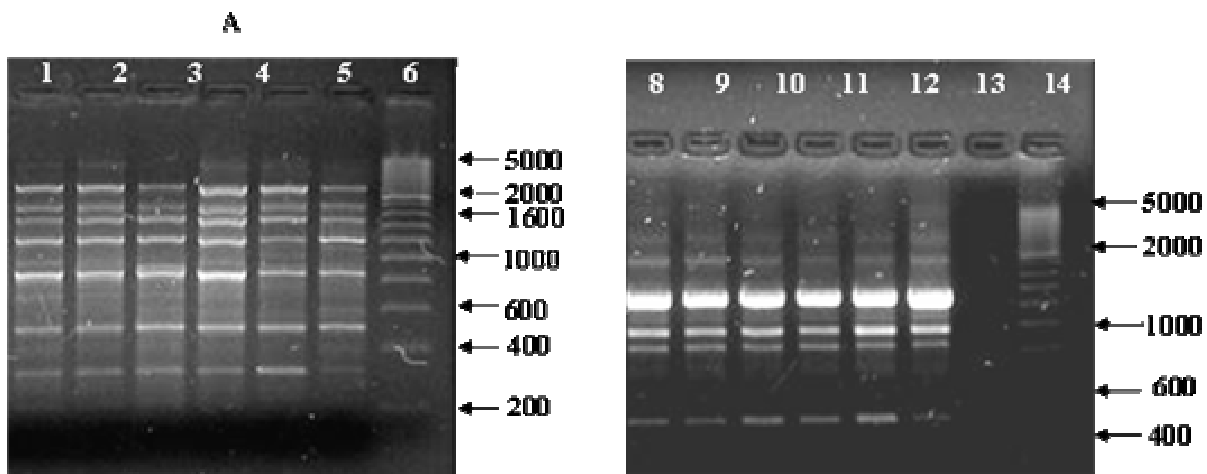
L. acidophilus was present in the saliva samples of both children with and without carious lesions from the Mexican population in the following percentages: 36.8% in the caries lesions group and 67% in the caries free group. No *L. acidophilus* strain was found in the French children population (Table 1).

All 163 Lactobacilli strains isolated in this study (42 from French children and 121 from Mexican children) were tested for PCR species specific for *L. rhamnosus* and *L. acidophilus*, and also were tested by both the API 50CH test and the RAPD-PCR technique. In every case there were a correlation between the biochemical and the molecular biology techniques.

The RAPD-PCR results showed the same patterns of amplification between the type strain *L. rhamnosus* ATCC 9595 and wild strains. The results for these strains showed the following bands: 2200, 1500, 1200, 850, 500 and 300 bp. Something similar was observed with *L. brevis* strains, they had a pattern characterized by the following bands: 1800, 1300, 950, 850 and 410 bp (Figure 3). Meanwhile *L. acidophilus* showed a two band pattern characteristic of 2400 and 550 bp between the *L. acidophilus* ATCC 4356 strain and the wild strains isolated from the saliva of children with and without caries. Bands with lower molecular weight were also present (Figure 4).

Table 1. Identification of *Lactobacillus* species isolated from Mexican and French children with and without caries

Species	Mexican strains (with caries) (76)	French strains (with caries) (42)	Mexican strains (without caries) (45)
<i>Lact. rhamnosus</i>	30 (39.5%)	36 (85.72%)	0
<i>Lact. brevis</i>	15 (19.8%)	1 (2.38%)	4 (9%)
<i>Lact. paracasei</i>	2 (2.6%)	2 (4.76%)	6 (13%)
<i>Lact. acidophilus</i>	28 (36.8%)	0	30 (67%)
<i>Lact. plantarum</i>	0	2 (4.76%)	5 (11%)
<i>Lact. salivarius</i>	0	1 (2.38%)	0
<i>Lact. Delbrueckii</i>	1 (1.3%)	0	0

**Figure 2.** Specie-specific PCR of *L. acidophilus* (lines 2 to 6) strain isolated of children with and without caries, line 1 *L. acidophilus* ATCC 4356. MW 1 Kb DNA plus ladder.**Figure 3.** RAPD-PCR of lactobacillus strain. (A) Fingerprints of *L. rhamnosus* ATCC 9595 (line 5), and representative lactobacillus isolated from saliva of children with caries (lines 1 to 4). (B) Representative fingerprint of *L. brevis* isolated from saliva of children with caries (lines 8 to 12), reactive control (line 13). Lines 6 and 14 MW 100 bp ladder.

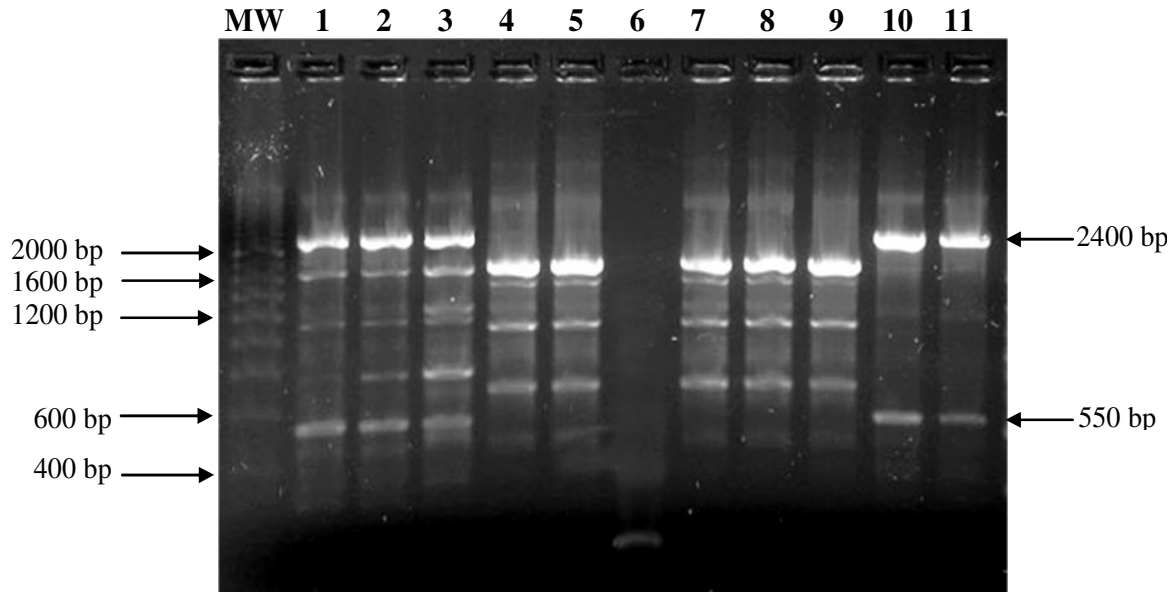


Figure 4. RAPD-PCR fingerprints of *L. acidophilus* (lines 1 to 3) and *L. paracasei* (lines 4, 5, 7 to 9) of isolated from children without caries, *L. acidophilus* ATCC 4356 lines 10 and 11. Line 6 negative control (no *Lactobacillus* DNA), MW 100 bp ladder.

In the French group with caries the predominant species was *L. rhamnosus* (85.72%) and no *L. acidophilus* was found. In the Mexican population with caries, the predominant species were *L. rhamnosus* (39.5%), *L. acidophilus* (36.8%) and *L. brevis* (19.8%).

In the French group of children without caries, there were no positive cultures for lactobacilli. In the Mexican group without caries, 76.9% had positive culture for lactobacilli and *L. acidophilus* (67%) was the predominant species isolated (Table 1).

In the French population, there was a statistically significant relationship between the presence of genus Lactobacilli and caries lesions, using the Chi square test with Fisher correction ($p < 0.0001$). In the Mexican population there was no statistically significant relationship between the presence of genus Lactobacilli and caries lesions. But, when we compared the species of Lactobacilli isolated in children with and without caries, *L. rhamnosus* ($p < 0.0001$) and *L. brevis* ($p = 0.0112$) were statistically related with caries. At the same time, there was no statistically significant relationship between *L. acidophilus* and caries lesions in children.

An ANOVA test allowed the comparison of different species and caries activity. It showed that, among French population *L. rhamnosus* was the only species related to caries. It also showed that, among the Mexican population *L. rhamnosus* was the most cariogenic species, followed by *L. brevis*. Meanwhile, using the same test, *L. acidophilus* was not related to caries activity.

DISCUSSION

The sampling method used in this study was chosen

according to Jenkins who advocates the use of unstimulated saliva for all studies of correlations between salivary factors and oral health (Jenkins and Edgar, 1989). We identified *L. rhamnosus* and *L. acidophilus* species by PCR species-specific test and all other Lactobacilli by the biochemical test API 50CH and then compared the molecular biology techniques (RAPD-PCR). Fitzsimons et al, (1999) has shown that sometimes it is difficult to identify a microorganism only by using the changes in pH as an indicator of growth in the presence of different sugars because of the various cut-off points used to determine a positive or a negative reaction. They obtained the most accurate results when both phenotypic and genotypic attributes were used to identify the species. Even all the *L. acidophilus* strains were identified by a PCR species-specific test at the beginning of the study due to, we found different bands in the analysis of the strains *L. acidophilus* by RAPD-PCR; we corroborated their presence by using again the species-specific PCR test.

L. rhamnosus was the most frequently identified species of microorganism among the two populations of children with caries. In the French population, mainly *L. rhamnosus* was isolated with no other lactobacilli, while in the Mexican population *L. rhamnosus* was isolated in some cases together with *L. acidophilus* and *L. brevis* as well. Our findings could be connected to those of Carlsson et al, (1975) who found that *L. casei* was more often identified in the oral cavities with carious lesions and fillings (). Other works have shown that *L. rhamnosus* could be associated with caries. Botha et al. (1998) found that *L. paracasei* and *L. rhamnosus* as the main etiological agents related with dentine caries. But they also identified a large number of *L. fermentum* as well as Marchant et al. (2001), which was not present in our samples (neither Mexican,

nor French). The reason for these differences in the results could be due to the fact that in both studies Lactobacilli came from decayed dentin samples instead of saliva samples.

On the other hand, our results are different from those of Muñoz-Jeldrez and Martinez (1980) who identified other Lactobacilli species in the saliva of 62 children, 7 to 10 years old. This study does not indicate the dental status of the children so; the species found in their study could not be associated with dental decay. Nevertheless, in our work we isolated *L. acidophilus* in a similar proportion among the Mexican population compared to the Chilean children, probably because both are Latin-American populations.

Colloca M et al, (2000) analyzed the lactobacillus of four areas of the mouth (teeth, tongue, gum and saliva) from healthy subjects. They found predominant species were *L. fermentum*, *L. plantarum*, *L. salivarius* and *L. rhamnosus*. *L. acidophilus* was only isolated from dental surfaces but *L. brevis* was not found. Another study by this group of researchers showed the presence of *L. plantarum* and *L. rhamnosus* in the teeth and saliva of patients with caries (Aumada et al., 1999). Our experimental data is different than those cited above, because we found *L. rhamnosus* only in the saliva samples of both French and Mexican children with caries, but not in both populations of caries free children. In the Mexican population we found *L. acidophilus* in saliva samples of children with and without caries. Some reports have documented the presence of probiotic strains of *L. acidophilus* that are able to compete and grow in the presence of similar bacteria, because they are able to produce some bacteriocins against other Lactobacilli that gave them a selective advantage against other bacteria (Percival, 1997).

Bjørndal and Larsen (2000) isolated more than 50% of *L. rhamnosus* in deep carious lesions. This species were followed by *L. brevis* (20%), *L. casei* (8.5%), *L. plantarum* (5%) and *L. acidophilus* (3 %).

Smith S. et al, (2001) isolated Lactobacilli in 62.5% of the saliva samples of patients with caries, finding the following species: *L. brevis* (24.6%), *L. fermentum* (18.5%), *L. casei* (16.9%), *L. delbrueckii* (15.4%), *L. plantarum* (9.23%), *L. acidophilus* (7.69%) and *L. salivarius* (1.54%). In both studies they found *L. brevis* in high percentages (between 20 and 25%) from caries patients. In the Mexican population with caries, we obtained similar results. *L. brevis* was isolated in 19.8% of the children participants. However, Smith S. et al. showed that *L. acidophilus* was found in 3 to 8% whereas we found it in 36.8% in the Mexican population.

In our study, *L. acidophilus* and *L. brevis* were only found in the Mexican samples. This finding was the main difference between French and Mexican populations. This difference may be due to the dietary habits that could include the intake of fermentative dairy products and probiotics in both populations. Montalto et al, (2004)

reported that an increase in the intake of probiotics is related to a rise in the Lactobacilli counts in saliva. *L. acidophilus* and *L. casei* are the most used Lactobacilli species in probiotics, so this could be a reason why they are present in the saliva samples in Mexican.

Conclusion

Even though this study does not investigate it, our results suggest that *L. rhamnosus* could be involved in both French and Mexican populations as a potential cariogenic agent.

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