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Lactobacilli Isolation from Dental Plaque and Saliva of a Group of Patients with Caries and Characterization of their Surface Properties

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The oral cavity is a complex ecosystem colonized by micro-organisms which play different roles. The aims of this study were to identify *Lactobacillus* strains from the teeth and saliva of children lacking in dental care and to study the surface characteristics related to the adhesion capability of these micro-organisms. The population considered is from Tucumán, Argentina. It is characterized by a dmfs index over 5 and an absence of dental care. Lactobacilli were isolated and identified by microscopic observations, biochemical tests and modified API CH 50. Bacterial surface studies included: hydrophobicity, acid and basic characteristics, blood cell agglutination and salt aggregation. Thirty strains were isolated. The major group was facultative heterofermentative. The surface characteristic studies did not indicate that lactobacilli are hydrophobic, neither do they show high basic nor acid charges. Lactobacilli isolated from saliva auto-agglutinated and also agglutinated ABO red blood cells. Salt aggregation was not a characteristic property. In this preliminary work, lactobacilli from teeth and saliva from this specific population were not demonstrated to have relevant adhesion properties.

Introduction

Lactic acid bacteria have been historically been described as cariogenic micro-organisms. They have been characterized thus mainly because of their acidurity and their capability of producing organic acids, such as lactic acid. These properties contribute to the cariogenic process. However, the beneficial properties of lactic acid bacteria have been thoroughly studied because they regulate the ecological balance of other areas. This regulation is not only carried out by organic acids, but may also be aided by the production of antagonistic substances, hydrogen peroxide [1], bacteriocins [2],

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competitive exclusion mechanism [3], nutrient competition [4] or stimulation of the immune system [5]. Some reports demonstrated the preventive role that lactobacilli exert over gastrointestinal [6,7], vaginal [8,9] and urinary tract infections [l0,11]. In these areas, lactobacilli have been described as 'beneficial' and 'probiotic' micro-organisms [12]. Hilton *et al.* [13] referred to the use of *Lactobacillus* GG isolated from the gastrointestinal tract (topically applied for the treatment of recurrent vaginitis).

The mechanism suggested for the adhesion of lactobacilli in the oral cavity is the aggregation capability of these micro-organisms [14]. Lactobacilli can co-agglutinate or agglutinate other micro-organisms making it possible for them to be eliminated by swallowing. There are some surface characteristics related to the adhesion capability that can be used to predict bacterial behaviour. Using this aforementioned manner of characterization, hydrophobicity of some oral strains of lactobacilli and streptococci has been described [15,16]. Some other properties, such as basic or acid characteristics of the cell surface [17] and haemagglutination [18], have not been applied to oral lactic acid bacteria.

In previous papers, we reported the isolation and characterization of lactobacilli from the tongue and gum of patients with caries [19], as well as the hard and soft tissues of the oral cavity of subjects with good oral health [20]. The aim of our present work was to isolate lactobacilli species from teeth and saliva of children with carious lesions, and to study and compare their properties. The group of patients involved belong to a small population of children who are characterized by a lack of oral care and hygiene, yet they are presently well nourished and consume a balanced diet.

Materials and methods

Population

Children in the study go to a government meal place. However, only 40.5% eat the four available daily meals. Despite this percentage, according to the program's nutritionist, the children have a midday diet balanced in terms of proteins, carbohydrates, vitamins and lipids. Although the children now have a balanced diet, this was not always been so. Samples were obtained from 20 patients between the ages of 3 and 11 years, who receive dental care from the same dentist. These patients, who belong to a low socio-economic level, had no antibiotic or corticoid consumption in the 20 days before sampling. Their nutritional, hygiene and oral health states were considered following the factors analysed by Newburn [21], and related by their parents. Dental caries and gingival indices were determined according to Löe and Sillness [22,23]. Salivary pH and secretion rate were also estimated. Consent of the patient's parents and the approval of the Institutional Ethics Committee were obtained before any samples were taken.

Most of the patients have never had dental care. Less than 15% of the children have a toothbrush and 10.2% say they brush their teeth more than once a day. Odontological index averages registered were: dmft 3.2 ± 2.3 (d: 3.2 ± 2.3, m: 0), dmfs 5.1 ± 2.9 (d: 5.1 ± 2.9, m: 0, f: 0). Plaque (PI) and Gingival (GI) indices were 1.43 ± 4.37 and 0.53 ± 0.23, respectively. The children generally consume sugar during meals rather than outside snacks. The children which participated in this study were the same as in a previous report, but the samples were taken some time later.

Collection and conservation of the samples

Sample collection was performed in the morning, at least 2 h after breakfast. Buccal, lingual, and occlusal sound surfaces of the left first and second mandible deciduous premolars (primary molars) were selected. Permanent teeth were present only in five children, and these permanent teeth were in healthy state. Teeth samples were taken with a dental spatula. An unstimulated saliva secretion of 5 min was aspired with a sterile syringe from the antero-vestibular area of the mouth. The volume obtained was registered in order to determine the CFU/ml isolated. The pH (6.99 ± 0.23) and secretion rate ($0.23\ \text{ml/min} \pm 0.14$) of saliva were considered in the normal range [21].

Teeth samples were collected in LBS (Lactobacilli Selective Media) [24] broth. Then, as saliva samples, they were cultured in LBS agar in microaerophylic conditions for up to 48 h at 37°C. Micro-organisms were stored at -70°C in milk-yeast extract (13% non-fat milk, 1% glucose, 1% yeast extract) for further conservation.

Lactobacilli isolation and identification

The micro-organisms were identified by their microscopic morphology, Gram-staining and biochemical properties. Gram-positive, catalase-negative, and indole- and nitrate-reduction-negative bacilli were selected. Lactobacilli identification was performed by standard tests and by the API CH 50 (Biomerieux, Marcy, L'Etoile, France) system, according to Bergey's Manual of Systematic Bacteriology [25].

Lactobacilli surface characteristics

The micro-organisms were sub-cultured in LAPTg (lactose, peptose, tryptose, glucose) [26] broth no more than three times before these studies. After 12 h incubation at 37°C, they were collected by centrifugation at 2000 × *g* and washed in saline solution.

Lactobacilli were analysed for their hydrophobicity. Hexadecane, xylene and toluene were used following the previously described [19] Microbial Adhesion to Hydrocarbons (MATH) method, first described by Rosemberg *et al.* [15] and modified by Geertsema *et al.* [16] and Sweet *et al.* [27]. Lactobacilli were classified in three groups depending on their hydrophobic property: low (0–35%), moderate (36–70%), and high (71–100%).

The MATH method was also applied to determine the basic and acid surface characteristics. Chloroform (Lewis acid) and ethyl acetate (Lewis base) were used as organic solvents [17]. Results are expressed as % of partition.

Aggregation in presence of ammonium sulphate was determined by the Salt Aggregation Test (SAT) [28]. Lactobacilli were classified as cited previously [19] into three categories: those that aggregate with 0.2–2 M ammonium sulphate, those that auto-aggregate in presence or absence of salt, and those with no aggregation property.

Haemagglutination test [18] was performed in microplates [19]. Titres were expressed as the inverse of the last dilution of bacteria producing agglutination.

Statistical analysis

Statistica software, version 5.0 was used to calculate the correlation coefficients between the results of the partition index with the three hydrophobic solvents. This software was also used to perform the matrix plot.

Results

Although 20 subjects were considered, lactobacilli were isolated only from 14 of the subjects. Only in four patients lactobacilli were obtained from both, the teeth and saliva, but the microflora did not coincide between them. Thirty microaerophilic lactobacillus strains were isolate, belonging to ten different species (Table 1). When percentages of isolates from different areas were calculated from the total number of isolates of non-healthy mouths [19], 22% was from the teeth and 28% was from saliva, compared to 38% from the tongue and only 12% from gums. If metabolic groups were identified, facultative heterofermentative lactobacilli prevailed. Only one obligate heterofermentative strain was isolated from teeth, but this species was not present in saliva.

L. plantarum and *L. casei* sp *rhamnosus* were the predominant micro-organisms in teeth and saliva, reported at present, and also in gums, as previously reported [19]. Saliva not only contained the strains isolated from the other areas, but also contained *L. delbrueckii* sp *delbrueckii*, *L. salivarius*, *L. alimentarius*, and *L. coryniformis* sp *torquens*.

Hydrophobic partition was analysed in the study of lactobacilli surface characteristics. Table 2 shows that results with the three hydrophobic solvents employed (hexadecane, toluene, xylene) were similar ($P < 0.05$). Almost 90% of the isolated strains revealed low hydrophobicity (values between 0 and 35% of partition). The matrix plot showing this result, and the correlation coefficient between the results obtained with the three hydrophobic solvents are between 0.85 and 0.89, as shown in Figure 1.

Strains with basic and acidic surface characteristics determined with chloroform and ethyl acetate, respectively, are also shown in Table 2. Most of the isolates (ten of 13) from teeth showed low charged surfaces (values between 0 and 35% partition). In the saliva isolates, nine of 17 strains showed low acid surface characteristics (between 0 and 35% partition with chloroform). Ten of 17 strains showed low basic surface characteristics (between 0 and 35% partition values with ethyl acetate). Only five strains (numbered 109, 123, 531, 532, 44) showed either differences between acid or basic surface characteristics. The correlation coefficient between the results of partition with hexadecane and chloroform was 0.57, and between chloroform and ethyl acetate 0.37.

Very few strains aggregated in the presence of ammonium sulphate: only one strain from teeth and two strains from saliva. However, autoaggregation was an important property for the isolates from saliva, since 88% of the lactobacilli analysed aggregated. Autoaggregating strains were also obtained from the teeth (69%). All isolates from saliva and the teeth that salt-aggregate, also autoaggregate.

Table 2 also presents the results of human red blood cell agglutination. Lactobacilli showed low titres for the aggregation with A, B and O erythrocyte groups. The *L. alimentarius* strain (strain 54) lacking on surface charges was the only strain that agglutinated cells of A and B groups with high titres. Saliva strains with this characteristic were present in higher numbers than in teeth.

Table 1. Percentage *of Lactobacillus* species isolated form teeth and saliva of subjects with caries

Species	Metabolic group	Isolated strains N°(%)[1]	
		Teeth	Saliva
L. delbrueckii sp *delbrueckii*	Obligate	—	1 (5.9) (1.7)
L. delbrueckii sp *lactis*	Homofermenters	1 (7.7) (1.7)	1 (5.9) (1.7)
L. salivarius		—	2 (11.8) (3.3)
L. alimentarius	Facultative	—	2 (11.8) (3.3)
L. casei sp *casei*	Heterofermenters	1 (7.7) (1.7)	—
L. casei sp *rhamnosus*		3 (23.1) (5.0)	5 (29.4) (8.3)
L. coryniformis sp *torquens*		—	1 (5.9) (1.7)
L. paracasei sp *paracasei*		3 (23.1) (5.0)	1 (5.9) (1.2)
L. plantarum		4 (30.8) (6.7)	4 (23.5) (6.7)
L. fermentum	Obligate Heterofermenters	1 (7.7) (1.7)	—
Total[2]		13 (22)	17 (28)

[1]First number in brackets indicates the partial percentage referring to the area of the mouth, and second number in brackets indicates percentage referring to the total number of lactobacilli isolated (60).
[2]Total number of strains per locale; % of total number of strains (in brackets).

Table 2. Adhesion-related properties of *Lactobacillus* strains isolated from teeth and saliva of 20 subjects with caries from Tucumán, Argentina

Isolated strains[1]	Hydrophobicity (%)			Surface charge (%)		SAT (M)[2]	Autaggregation[3]	Haemagglutination[4]
	Hexadecane	Xylene	Toluene	Chloroform	Ethyl acetate			
Strains from teeth								
L. delbrueckii sp *lactis* (63)	27	30	24	55	0	2	+	0
L. casei sp *casei* (78)	7	10	4	21	0	0	+	A,B,C (1,1,1)
L. casei sp *rhamnosus* (201)	26	30	32	14	14	0	0	0
L. casei sp *rhamnosus* (202)	38	33	33	52	32	0	+	0
L. casei sp *rhamnosus* (65)	0	0	9	0	3	0	W	A,B (1.1)
L. fementum (42)	25	21	20	75	54	0	0	0
L. paracas. sp *paracasei* (2)	4	7	10	0	0	0	W	0
L. paracas. sp *paracasei* (30)	7	7	2	0	14	0	W	0
L. paracas. sp *paracasei* (31)	0	0	0	0	0	0	W	0
L. plantarum (20)	0	0	0	0	27	0	W	0
L. plantarum (21)	0	0	0	0	10	0	W	0
L. plantarum (13)	20	8	12	4	42	0	0	0
L. plantarum (79)	52	39	44	0	38	0	0	0
Strains from saliva								
L. alimentarius (54)	22	15	21	0	0	0	0	A,B (4,4)
L. alimentarius (109)	33	30	26	77	15	2	+	A,B (2,1)
L. casei sp *rhamnosus* (27)	28	15	24	42	61	0	+	0
L. casei sp *rhamnosus* (30)	42	25	24	67	62	0	+	O(1)
L. casei sp *rhamnosus* (62)	0	0	6	0	0	0	+	0
L. casei sp *rhamnosus* (123)	7	8	12	48	0	2	+	A,B,O (2,2,1)
L. casei sp *rhamnosus* (76)	12	10	11	0	0	0	0	0
L. coryn. sp *coryniformis* (46)	33	42	33	65	50	0	+	0
L. delbr sp *delbrueckii* (531)	14	10	18	33	0	0	+	A,B,O (1,1,1)
L. delbruecki sp *lactis* (532)	21	14	25	61	0	0	+	A,B,O (1,1,1)
L. paracas. sp *paracasei* (12)	12	15	11	12	39	0	+	0
L. plantarum (10)	17	14	16	4	18	0	+	0
L. piantarum (11)	18	15	13	15	17	0	+	0
L. plantarum (44)	24	17	14	33	0	0	+	0
L. plantarum (45)	22	29	29	20	45	0	+	0
L. salvarius (29)	32	28	23	50	44	0	+	0
L. salivarius (661)	35	43	50	73	62	0	+	A,B (1, 1)

[1]Numbers in brackets indicate the laboratory internal nomenclature.
[2]SAT: Salt Aggregation Test. Numbers indicate the minimal molar ammonium sulphate concentration that produced aggregation.
[3]+: autoaggregation, 0: no autoaggregation.
[4] A, B, O: Blood groups that lactobacilli aggregated with. Numbers in brackets indicate the correspondence titres. Titres are the inverse of the last bacterial dilution that produced haemagglutination. 0: without haemagglutination.

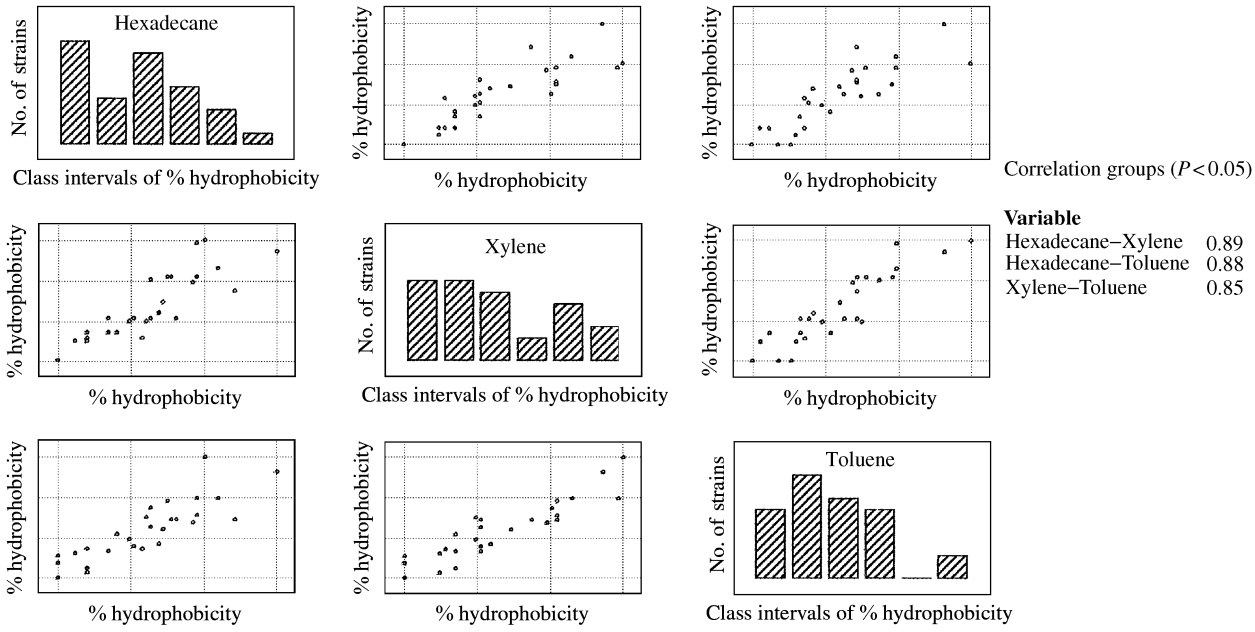

Figure 1. Matrix plot showing the relationship between the results obtained with the three organic solvents to test the hydrophobicity of the cellular surface by the MATH method. The correlation coefficients obtained with the three solvents are showed in the side. First row of graphs: Hexadecane–Xylene, Hexadecane–Toluene relations. Second row: Xylene–Hexadecane, Xylene–Toluene relations. Third row: Toluene–Hexadecane, Toluene–Xylene. Matrix plot combines the histogrames of each one of the represented variables, with dispersion graphs of pairs of those variables. The histograms (diagonal) represent the distribution of the values of each variable, grouped in six class intervals, from 0 to 100%. The height of the columns in each class is the number of strains in each interval.

Discussion

The oral cavity is a complex ecosystem constituted by different surfaces and a great number of micro-organisms that can be eliminated by swallowing or can adhere themselves to hard or soft tissues of the mouth in the oral cavity. This ability of bacteria to adhere to these tissues of the mouth is considered to be the first step in colonising the environment of the mouth. Bacterial adherence has been suggested to be the result of specific and non-specific mechanisms [29]. Non-specific binding involves electrostatic or hydrophobic interactions of lower affinity than that involved in specific binding.

In this paper, the isolation of different lactobacilli strains from teeth and saliva from children that have almost the same nutritional state of health is reported with the objective of screening and predicting bacterial adhesive properties. The samples obtained from teeth were taken from the dental surfaces, which means from the dental plaque, but not from the caries or cavities. The dental plaque is a highly complex biofilm of hundreds of species. The surface properties of lactobacilli evaluated in the present paper can be important in order to understand the interactions of the micro-organisms which are part of the community of the dental plaque biofilm. The micro-organisms present in this biofilm can be beneficial or detrimental, depending on the ecological or environmental conditions of the oral cavity. The adhesion capability of lactobacilli to a biofilm is of particular importance when the dental plaque is covering the dentine. However, the adhesion to dentine by itself or to collagen, one of the major components of dentine, could be important in the development of a carious lesion, as discussed by McGrady *et al*. [30].

The phenotypic identification of lactobacilli from definite oral areas has not been previously reported only in a more general sense. [3l] From the results obtained, is not possible to conclude that some specific species is isolated from a particular area: there was a variety of species isolated from these environments, showing a wide diversity and heterogeneity of species in all the patients studied, thus indicating the degree of biodiversity that exist in the micro-organisms isolation, even though the patients showed the same nutritional pattern. The teeth, saliva and the gums are slightly dominated by the most frequent isolates: *L. casei* ssp *rhamnosus* and *L. plantarum* [19]. The results referring to the metabolic group in each one of the areas shows that the three groups were present in teeth, but the obligated heterofermentative was not in saliva. The species of lactobacilli reported in this paper agree with those published by Carlsson and Gothefors [3l], who find *L. casei*, *L. rhamnosus* and occasionally *L. acidophilus* and *L. fermentum* in children with carious lesions.

Strains showed low hydrophobicity with hexadecane, xylene and toluene and there was also a high degree of similarity of the behaviour of the strains in response to the three solvents employed, as demonstrated by the coefficient correlation obtained, (between 0.88 and 0.92). Since these techniques had previously been used by other researchers to predict adhesive properties [15], these results could indicate that the isolated strains have low adhesion and colonisation capacity with respect to the oral tissues. These observations did not coincide with those previously obtained for lactobacilli isolated from healthy patients [20].

Basic and acidic surface characteristics were studied by the use of the chloroform and ethyl acetate partition methods. The results showed some differences with each of the two solvents for some of the strains (only four or five strains), however almost half of the lactobacilli did not register surface charges. The correlation coefficient obtained between the results of chloroform and ethyl acetate partition was around 0.57. These results differ from those reported by Pelletier *et al.* [71], who determined that highly hydrophobic lactobacilli had a high affinity for chloroform and a low affinity for ethyl acetate.

Haemagglutination capability was tested based on the hypothesis that the receptors of epithelial mammalian cells (where micro-organisms would adhere) have a similar ontogenetic origin as erythrocytes [29]. Some pathogenic micro-organisms showed the capability of agglutinating red blood cells by the presence of special structures on their cell walls such as fimbria, pilli, or specific hydrocarbon receptors. Piette and Idziak [32] attributed this ability to the presence of lectins on the surface of the non-pathogenic bacteria. Among the strains studied, few of them agglutinate with human cells. Previously, Andrew *et al*. [18] reported the haemagglutinating capability of vaginal lactobacillus strains, classifying them in different groups according to the pattern obtained. Mukai *et al.* [33] studied the haemagglutination of *L. reuteri* isolated from the gut, and related this property to the adhesion capability of the strain.

Autoaggregation is important for salivary lactobacilli. This characteristic could promote the adhesion to surfaces [15] or the elimination of micro-organisms by swallowing. This paper reports that almost all of the lactobacilli isolated from patients with caries do no show relevant surface characteristics related to adhesion. This finding is in contrast to previous findings for lactobacilli isolated from individuals with healthy mouths, which showed higher adhesion-related properties [20]. More studies are being performed to understand the adhesion ability and the ecological role of the lactobacilli in the oral cavity.

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