Adherence Capability to Different Cultured Cell Lines of *Streptococcus sp.* Strains Isolated from Pozol a Prehispanic Mexican Fermented Beverage

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**Rec date:** Oct 29, 2014; **Acc date:** Dec 16, 2014; **Pub date:** Dec 23, 2014

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**Abstract**

Pozol is an acid fermented pre-hispanic beverage, consumed as part of the diet of ethnic groups in Southern and Southeastern Mexico. *Streptococcus sp.* a major component of pozol microbiota, was analyzed to assess their in vitro adherence ability to HEp-2, HeLa, HT-29 and Caco-2 cell lines. Adhesion tests were performed in 35 strains, and four of them with adherence to the different cell lines were analyzed using a Scanning Electron Microscope (SEM). Thirty-one (89%) strains could adhere to at least one of the cell lines, adherence on Caco-2 cells was the most frequently observed (63%). Diffuse and aggregative adherence phenotypes similar patterns to those described for *Escherichia coli*, were observed in the trial. The SEM analysis showed in one of the strains, an amorphous structure in which a large number of bacteria were included. The SEM images of other three strains, showed the presence of bacterial projections that connect them with each other and with the cells. The results showed that *Streptococcus sp.* strains isolated from pozol, adhere to different epithelial cell lines likely through structures which may correspond to exopolysaccharides and/or surface adhesins in bacteria. The adherence ability of these bacteria to different cultured cells could be associated with different epithelial cell colonization and the possible use of these lactic acid bacteria as probiotics if the safe use is confirmed.

**Keywords:** *Streptococcus sp.*, Cell lines; SEM

**Introduction**

“Pozol” is a pre-Hispanic, fermented drink prepared by cooking corn grain with 1% lime solution (nixtamalization). The corn grains are washed and ground to obtain a dough, which is made into balls, wrapped in banana leaves and left to ferment at room temperature during two to seven days. The fermented dough is dissolved in water and consumed as food or a refreshing drink, as much by the indigenous population as by the mixed-race population in the Southeast of Mexico (Yucatán, Quintana Roo, Campeche, Tabasco, Chiapas and Oaxaca) and Guatemala [1]. Different microorganisms including fungi, yeasts and Lactic Acid Bacteria (LAB), have been isolated from different pozol samples [2-4]. LAB are comprised of Gram-positive bacteria whose main characteristic is lactic acid production during sugar fermentation [5]. Studies relating to the characterization of microorganism components of the “pozol” biota, have shown that species of the genus *Streptococcus* constitute between 25 and 75% of the above-mentioned biota [2,6,7].

Analysis of the 16S subunit from the ribosomal DNA (rDNA) of *Streptococcus* strains isolated from “pozol” identified the existence of four different species of which *S. infantarius* was the predominant group [8]. LAB adapt very well to conditions in the gastrointestinal tract, in particular those microorganisms defined as probiotics that are ingested in adequate quantities and promote a positive effect on health [9,10]. One important characteristic that allows colonization of host epithelial cells is the adherence ability of microorganisms. However, the characteristics, origin of the structures and events that occur during interaction between bacteria and cells when LAB adherence is taking place are not fully defined [11-13].

Related to the factors that contribute to Gram-positive bacterial adherence is limited. Studies of some oral bacteria, such as *Actinomyces naeslundii* [14], *S. parasanguinis* [15] and *S. salivarius* [16-18] suggest that fimbriae may participate in the adherence process of such microorganisms.

In this study the in vitro adherence to different cultured cells lines of *Streptococcus sp.* strains isolated from “pozol”, was evaluated, and the cell adherence patterns of the bacteria, were also analyzed by Scanning Electron Microscope (SEM).

**Methods**

Thirty-five strains of *Streptococcus sp.* (not completely characterized) isolated from different “pozol” samples [8], were they evaluated in vitro to determine their cell adherence ability. In the assay *Escherichia coli* O42 (aggregative adherence), *E. coli* E2348/69 (localized adherence), *E. coli* 55784 (diffuse adherence), and *E. coli* HB101 (non-adherent) were used as control strains [19,20].

**Adherence tests**

The adherence assay described by Cravioto et al. [21] with some modifications was used. Briefly, HEp-2 (pharyngeal carcinoma), HeLa
(cervical carcinoma), HT-29 (human colon adenocarcinoma) and Caco-2 (human colon carcinoma) cultured cells, were utilized in the study. Briefly, cells were grown on 72 cm² plastic bottles in MEM (Minimum Essential Medium) (GIBCO, New York, USA) media for HEp-2 and HeLa cells; likewise, DMEM (Dulbecco’s Modified Eagle Medium) (Sigma, St. Louis, USA) to HT-29 and Caco-2 cells. In 24-well plates (Corning, New York, USA) containing 10 mm plastic lentils (Thermo Scientific, New York, USA) was added 1 ml of a cellular suspension (2.5 X 105 cells ml⁻¹) by triplicate.

Results

Adherence to cultured cells

Of the 35 Streptococcus sp. strains analyzed, 89% (31/39) showed adherence and of these 13% (4/31) adhered to all four cell lines, 19% (6/31) to three, 13% (4/31) to two, and 55% (17/31) only to one cell line (Table 1). Analysis of each of the cell lines showed that 13 (37%) strains adhered to HEp-2 cells, 10 (29%) to HeLa cells, 14 (40%) to HT-29 cells, and 22 (63%) to Caco-2 cells (Table 2). Qualitative analysis by light Microscopy at the different preparations showed phenotypes like to diffuse (LDA), and aggregative (LAA) adherences (Table 1); which, were similar to those described to Escherichia coli [19]. In HEp-2 cells, in nine of the isolates the identified patterns were similar to diffuse adherence (LDA) and aggregative adherence (LAA) in four strains. For HeLa cells, seven strains showed LDA and three LAA patterns. In Caco-2 cells, 12 isolates showed LDA patterns (Figure 1a) and 10 LAA patterns (Figure 1b). In the HT-29 cells (Figure 1c), the 15414 strain displayed an adherence pattern that is similar to localized adherence (LIA) reported previously in E. coli diarrheogenic strains [19].

<table>
<thead>
<tr>
<th>Cell Cultured Lines</th>
<th>Strains</th>
<th>Hep-2</th>
<th>HeLa-3</th>
<th>HT-29</th>
<th>Caco-2</th>
</tr>
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<tbody>
<tr>
<td>15133</td>
<td>-</td>
<td>LDA</td>
<td>LDA</td>
<td>LAA</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1: Adherence patterns on Caco-2 cells identified in Streptococcus sp. strains isolated from pozol; a) Like aggregative adherence pattern with chain formation of 25245 strain; b) Like diffuse adherence of A12203 strain; c) Like localized adherence to HT29 cells of 15414 strain. Preparations were Giemsa stained and observed under light microscopy 100X.
Table 1: Adherence on different cultured cells of Streptococcus sp. strains isolated from pozol samples, LDA=Like Diffuse Adherence, LAA=Like Aggregative Adherence, LLA=Like Localized Adherence, - =Non Adherent.

<table>
<thead>
<tr>
<th>Adherence Phenotypes (%)</th>
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<tbody>
<tr>
<td>Cell Culture</td>
</tr>
<tr>
<td>Hep-2</td>
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<tr>
<td>HeLa</td>
</tr>
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</table>

Sung-Mee and Dung-Soon [31] and Lee et al. [32], when they bacteria; likewise, some cells of strain 25137 (LAA) forming Discussion

89% of the Streptococcus sp. strains studied were adherent to different microcolonies appeared deeply embedded in the brush border to mucosal cells (Figure 2b). In addition, elongated fiber that connect bacteria to the cell (Figure 2a and 2c, respectively), were observed in strains A12203 (LDA) and 25245 (LAA). With regard to strain 15124 (LAA), extended structures were observed that connect bacteria to each other and other smaller ones that link these bacteria to the cell. In the non-adherent strain 25109, no structures or prolongations it were identified (Figure 2d).

Table 2: Adherence phenotypes of Streptococcus sp. on different cultured cell lines. LDA=Like Diffuse Adherence, LAA=Like Aggregative Adherence, LLA=Like Localized Adherence.

<table>
<thead>
<tr>
<th></th>
<th>HT-29</th>
<th>Caco-2</th>
<th>Non-adherent</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>5</td>
<td>1</td>
<td>14 (40)</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>0</td>
<td>22 (63)</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4 (11)</td>
</tr>
</tbody>
</table>

Scanning Electron Microscopy (SEM) strains 25137, 25245, A12203, and 15124 adherent to the four cell lines (Table 1), showed elongated fiber plus-like interconnecting to bacteria; likewise, some cells of strain 25137 (LAA) forming microcolonies appeared deeply embedded in the brush border to mucosal cells (Figure 2b). In addition, elongated fiber that connect bacteria to the cell (Figure 2a and 2c, respectively), were observed in strains A12203 (LDA) and 25245 (LAA). With regard to strain 15124 (LAA), extended structures were observed that connect bacteria to each other and other smaller ones that link these bacteria to the cell. In the non-adherent strain 25109, no structures or prolongations it were identified (Figure 2d).

Adherence to intestinal mucosa is a prerequisite of probiotic microorganisms to colonize the epithelium and compete with enteropathogenic bacteria found in such spaces [23,24]. Adherence of lactic bacteria in animal models is complicated and on occasion, difficult to evaluate. Bearing this in mind, some different in vitro systems have been adopted [25-27] and of these cultured intestinal epithelial cells are the more common [24,28-30]. In the current study, 89% of the Streptococcus sp. strains studied were adherent to different cell lines but with a higher frequency on intestinal HT-29 (40%) and Caco-2 (63%) cell lines. These results are similar to those reported by Sung-Mee and Dung-Soon [31] and Lee et al. [32], when they examined the adherence properties of LAB to Caco-2 and HT-29 cells and Lactobacillus strains to HT-29 cells. They observed that 28 (49%) of their samples adhered with a superior adhesion ability to that observed in L. rhamnosus GG [33]. These results and the observed in this current study, show that in general, LAB showed structures that contribute to the adherence of bacteria to intestinal cells.

Results of adherence test to HEp-2 (37%) and HeLa (29%) cells, demonstrate that Streptococcus sp. strains isolated from pozol, are able to colonize both the intestinal epithelium and others as the pharyngeal and cervical epithelia. The fact that the analyzed bacteria showed adherence in the presence of D-Manosse indicates that the elements related to adherence are resistant to mannos, probably in a similar way to that described for Gram-negative bacteria [19,21,34]. The adherence tests also revealed the presence of diffuse, aggregative and localized phenotypes, similar to those described for E. coli [19,34,35]. These same adherence phenotypes, have been identified in lactic acid bacteria strains isolated from cooked meat products [36], and in strains of Lactobacillus paracasei isolated from a fermented drink (honey water) obtained from an agave plant (Hernández-Ramírez – personal communication).

The strains described as 15124, 25139, 25421 and 25142 displayed adherence of the LDA (Like Difuse) and LAA (Like Aggregative) phenotypes in the four epithelial cell lines; however, some other (15133, 25245, 25148, 25233, 25137, 15414, A57103, A57206, A12203, and A54201) strains, showed different adherence patterns. Regarding adhesion of bacteria to intestinal, epithelial cells, passive electrostatic, and hydrophobic type forces, as well as covalent types of interactions induced by specific adhesins and their respective receptors may be playing a role [37]. In relation to adherence of Lactobacillus as probiotic, has not yet been well defined the adherence factors and therefore has been suggested that proteins, glycoproteins, teichoic or lipoteichoic acids mediate the interaction bacteria-cells [38-40]. With this in mind, it has been proposed that some components of the cell wall induce different types of bacteria-cell interactions, explaining the presence of different adherence phenotypes [41-43]. Lévesque, et al. [17] reported in a strain of S. salivarius, the presence of genes that code for a protein related to bacterial adherence. On the other hand, Sara and Uwe [44], Logan, et al. [45], Ossowski, et al. [33] and Sanchez, et al. [46] in different BAL strains, also have described the participation of bacteria surface proteins in promoting adhesion to intestinal tissues.

SEM conducted as part of this current study, revealed that strain 25137 forms a matrix that consists probably of exopolysaccharides with bacteria aggregates (Figure 2b). Maldonado, et al. [47] reported that LAB strains secrete polysaccharides, involved with the adhesion to intestinal mucosa, the formation of bacterial agglomerates, and prolongations that contribute to the cells adhesion. However, is possible the participation of different structures in the bacteria cell adherence, this because the same procedure with strains 25245 (LAA), A12203 (LDA) and 15124 (LAA), showed the presence of filament-
type structures that connect bacteria to each other and to epithelial cells (Figure 2a and 2c). The results of this study indicate that even between bacteria of the same genus and species, adherence could be related to different structures and events making the characterization of these adhesins important to determine.

The biochemical characterization of Streptococcus sp. of the strains of our study show that they are members of Streptococcus boris/S. equinus complex (SBSEC) species, however, molecular studies are necessary to define the species. Overall SBSEC group members are considered as putative pathogens, however, PCR and Southern blotting analyses of S. macedonicus ACA-DC 198, indicated the absence of several Streptococcus pyogenes pathogenicity genes [48]. PCR test conducted in our strains to identify emm (M protein), SpeA/SpeC (erythrogenic toxin), sic (complement inhibitory protein) and otf (opacity factor), showed negative results in all strains.

Although it requires more studies, one could propose that our strains can be considered as potential probiotic bacteria.

Acknowledgement

The authors would like to thank the General Office of Academic Personnel Affairs – UNAM (DGAPA) and the project “Uso Potencial como Probióticos de Bacterias Lácticas Aisladas del Pozol Bebida Fermentada de Uso Tradicional en México” (PAPIIT-IN219611-3) for their support.

In addition, N.L. Ramírez-Chavarrin and C.A. Eslaiva-Campos are grateful for the support of the Program for Post-Doctoral Grants at the Faculty of Medicine, UNAM-DGAPA and UNAM-CONACYT.

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