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Colonization of the Mouse Upper Gastrointestinal Tract by *Lactobacillus murinus*: A Histological, Immunocytochemical, and Ultrastructural Study

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Abstract *Lactobacillus* is normally present in animals and humans colonizing several epithelia, mainly those belonging to the upper gastrointestinal tract. Most of the information about the distribution of *Lactobacillus* in mice has been obtained by bacterial culture and characterization, and only few reports have described the direct presence of these bacteria in tissues, especially in the gastric mucosa. In this study, we have characterized and evaluated the location and detailed relationship between *Lactobacillus* and epithelia using a combination of histological, molecular, immunocytochemical and ultrastructural methods. Normal Balb/c mice were sacrificed to study esophagus and stomach. Partial 16S rRNA gene sequencing, Gram, and P.A. Schiff staining allowed us to demonstrate that *Lactobacillus murinus* isolated from each animal colonize not only the epithelium of the forestomach but also that belonging to the distal esophagus. The pattern of colonization was linear over the keratinized epithelium, and also in a vertical way of focal bacterial aggregates. This was confirmed by transmission electron microscopy, and the nature of bacteria was further assessed by immunocytochemistry. Our results indicate that *L. murinus* can colonize the stomach and the esophagus epithelia in a biofilm-like manner, possibly acting as a defense barrier against colonization by other bacteria.

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Introduction

Normal microbiota is present in humans and animals, and it is an important defensive barrier against exogenous pathogens. In normal conditions, it regulates the delicate ecological relationship among endogenous bacteria, yeasts and phages, and also contributes to nutrition and to elicit protective immune responses [11]. This led to use some of the members of normal microbiota, mainly belonging to the genus *Lactobacillus*, as probiotics for medical purposes [6]. A detailed knowledge about the physical location of *Lactobacillus* in the gastrointestinal tract is needed to better understand both biology and ecology. However, most of the studies have been based on the isolation and characterization of these bacteria [1, 16, 18], and few data on the relationship between *Lactobacillus* and its interaction with epithelia comprising organs of the whole upper gastrointestinal tract, not only the stomach, have been reported [15, 17]. Thus, in this study, a straightforward study was performed to characterize the *Lactobacillus* strain normally present in the esophagus and in the stomach of Balb/c mice, and to examine its topographic distribution, using combined genetic, histological, immunocytochemical, and ultrastructural methods.

Materials and Methods

Ten 12-week-old, specific-pathogen-free Balb/c mice (5 males and 5 females) were obtained from the bioterium of the University of La Plata, School of Veterinary Medicine,

Argentina, maintained in individual boxes, and fed on pellets ad libitum. One day before the experiment, solid diet was suppressed to reduce the presence of food in the mouse stomach during dissection, and animals were fed ad libitum with a solution of 1.25 % sucrose in distilled water to avoid starvation. This sucrose concentration was similar to that of carbohydrates contained in pellets. All the procedures applied on these animals met the criteria set by the University of Buenos Aires Laboratory Animal Welfare Guidelines. Mice were euthanized by CO_2 asphyxiation, and the upper gastrointestinal tract was carefully dissected. The stomach was cut open in the middle and rinsed free of ingesta. After having removed the contents of the stomach, one half was applied over the surface of the LAPTG medium in plastic Petri dishes and then removed. Plates were incubated 48 h at 37 °C in a 5 % CO_2—95 % air atmosphere. The LAPTG medium contained 1.5 % peptone, 1 % triptone, 1 % yeast extract, 1 % glucose, and 1.5 % agar (Difco®). Suitable small pieces of the gastric mucosa were minced in cold, freshly prepared 4 % formaldehyde from paraformaldehyde and 1 % glutaraldehyde in 1× phosphate-buffered saline (PBS) composed of 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4.2H_2O$, 2.0 mM KH_2PO_4, until 0.5 mm pieces were obtained. The fixation was performed for 1 h at 4 °C and then, tissues were post-fixed in 1 % osmium tetroxide and routinely embedded in Vestopal. Slides were obtained with glass knives and grids were stained with uranyl acetate and lead citrate. Specimens were examined with a Zeiss EM-109-T transmission electron microscope at 80 kV. The other half, including the whole esophagus, was fixed in Bouin's fluid for 24 h. Tissues were carefully embedded in paraffin so as to completely observe their histology, and consecutive slides were obtained. Some of them were stained with Hematoxylin-Eosin; and Gram, Periodic Acid Schiff (P.A. Schiff) or peroxidase-antiperoxidase (PAP) anti-*Lactobacillus* were performed in adjacent slides. For the labeling of *Lactobacillus*, a rabbit anti-*Lactobacillus casei* serum was prepared by rabbit inoculation with four boosters of a suspension of formaldehyde-killed *L. casei* provided by Guillermo Oliver (CERELA–CONICET, Tucumán, Argentina). The PAP method was performed as described previously [8]. Briefly, endogenous peroxidase was blocked with 2 % hydrogen peroxide in 0.05 N Tris–HCl buffers. In order to reduce non-specific background, slides were incubated with 5 % normal goat serum in 0.05 N Tris–HCl. The primary serum against *L. casei* was used at a dilution of 1:500 in 0.05 N Tris–HCl. The second and the third sera were, respectively, goat anti-rabbit immunoglobulins (1:50) and peroxidase-antiperoxidase produced in rabbit (1:250) (Dako®). In between incubations, slides were washed thoroughly with 0.05 N Tris–HCl and developed under microscopic control using 0.03 % 3-3′ diaminobenzidine, 2 % hydrogen peroxide, and 0.05 N Tris–HCl. Slides were slightly counterstained with methylene blue, and routinely mounted. To carry out microbiological and molecular analysis, approximately ten colonies were cultured in LAPTG broth for 96 h at 37 °C. Gram staining was performed on slides to confirm the presence of Gram-positive rods and 2 ml of cultured broth were spun down, washed three times with 1× PBS, and processed for 16S rRNA gene partial sequencing as follows: total genomic DNA was extracted from bacteria using Master Pure DNA Purification kit, Epicentre®, following the manufacturer's instructions. Polimerase-Chain Reaction (PCR) (Promega®) was employed to amplify the 16S rRNA gene using universal primers 16SF (5′-ACGGCTACCTTGTTACGACTT-3′) and 16SR (5′-AGAGTTTGATCATGGCTCAG-3′) as described by Weisburg et al. [20]. Sequencing of the 1.6 kb PCR product was performed on both DNA strands using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems®). Sequences were analyzed using the Blast V2.0 software (http://www.ncbi.nlm.nih.gov/BLAST/).

Results and Discussion

The analysis of the genomic sequence obtained showed 99.5 % of identity with that described in Gene Bank nucleotide sequence as belonging to *L. murinus* strain SWP05 (Accession number HQ668465.1). *L. murinus* was first isolated by Raibaud et al. [14] and described as a new species by Hemme et al. [3, 7]. It has been identified in 38 % of cultures obtained from saliva, tongue, teeth, and mucosa of normal Balb/c mice as part of the indigenous oral microbiota [19], it has also been isolated from the upper gastrointestinal tract [16] and together with *L. reuteri*, it constituted 72 % of all *Lactobacillus* species cultured from the intestinal content of normal C57/Bl6 mice [12]. However, those findings do not indicate how the colonization occurs. In this study, the Gram staining of esophagus and stomach tissues, allowed us to observe that the distribution of bacteria followed different patterns. Some bacteria were attached linearly to the keratinized layer of both organs' epithelia. However, this pattern was not uniform, since periodically, *L. murinus* were entrapped in groups. Some of these groups of rods did not show a particular orientation in the space while others mainly adopted a vertical-growing pattern, from the keratinized epithelial layer towards the lumen of the organs (Fig. 1a, b). This was observed not only in the keratinized forestomach epithelium but also in different parts of the esophagus and, especially, in its distal limit, near the epithelium of the forestomach. The P.A. Schiff technique detected that bacteria were surrounded by a mucopolysaccharide matrix (data not shown). The characterization of the rods as *Lactobacillus* was confirmed by the PAP method (Fig. 2a). The specificity of this technique was

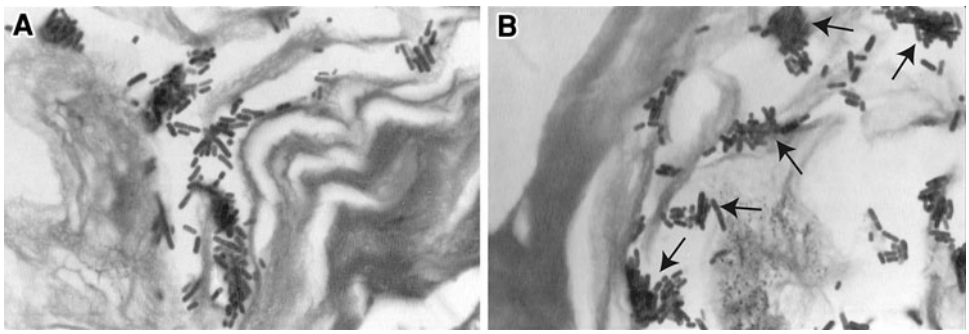

Fig. 1 Gram staining performed on slides obtained from paraffin-embedded esophagus and stomach of normal Balb/c mice. **a** Gram-positive rods covering the keratinized distal esophagus epithelium. Bacteria are forming clumps although some of them show a linear adherence (×650). **b** Gram-positive rods over the keratinized forestomach epithelium. Note the predominant vertical growth pattern of aggregation (*arrows*) separated by empty areas (×650)

confirmed by a previous study, where we demonstrated that the polyclonal serum used as a primary, only reacted with *L. casei, L. salivarius* and *L. murinus*, but not with *E. coli*, *Klebsiella sp*, *Pseudomonas aeruginosa* or *Staphylococcus aureus* (unpublished results). Moreover, adjacent slides treated with normal rabbit serum as a primary did not show any immunolabeling at all. Ultrastructural studies, using transmission electron microscopy (TEM), showed rods with a thick cell wall-a typical feature in Gram positive bacteria-, from which small "spikes" aroused, probably being adhesines. The amorphous matrix was also observed by this method, surrounding the bacteria and maintaining them in a sticky fashion (Fig. 2b). It is interesting to observe that, TEM also allowed us to further confirm that bacteria were not tightly adhered to the eukaryotic cells surface, but rather separate from the epithelium border and immersed in an amorphous material. TEM—instead of Scanning Electron Microscopy (SEM)—was chosen, since TEM thin sections allow a detailed study of the epithelium ultrastructure, as well as the bacteria growing on it in a perpendicular, 2-dimension image; while SEM would have only permitted the observation of bacterial distribution on the epithelial surface at a low magnification. Taken together, the results reported here indicate that, *L. murinus* is normally present in the esophagus and the forestomach of Balb/c mice, colonizing the epithelium not always as a linear attachment but, more often, forming periodic clumps of vertical growth with empty spaces between and into them. This suggests that, in this model, *L. murinus* colonized the upper gastrointestinal tract as biofilm-like structures. Biofilms are composed of bacterial populations that colonize inert materials and tissues, developing into an exopolysaccharide matrix [10]. They are very useful for the maintenance of bacterial population life-cycle and their resistance, for example, to antibiotics, and most, if not all, bacterial species produce them [4, 5, 9]. *L. murinus* is known to produce biofilms in vitro [13], and the biochemical basis of *Lactobacillus* biofilms has been partially characterized [18]. Furthermore, some molecular approaches have reported on the interaction between *Lactobacillus* and the differentiation of normal epidermal keratinocytes in in vitro assays [2]. Other authors have also described the existence of *Lactobacillus* in the

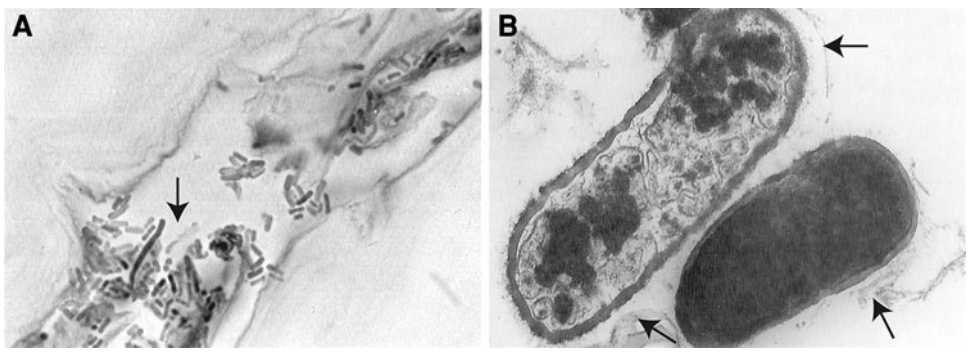

Fig. 2 **a** PAP immunolabeling of rods colonizing the epithelium of the forestomach using a primary serum against *Lactobacillus*. Positive staining is evident in rods (*arrow*). The slide was slightly counterstained with methylene blue (×650). **b** Transmission electron microscopy image of two rods in the mouse gastric mucosa. A thick wall (characteristic of Gram-positive bacteria) and tiny spikes (probably adhesins) arising from the cell wall are observed. Note the amorphous extracellular matrix surrounding both rods (*arrows*) (×40,000)

stomach of mice by low-power histological studies [15, 17], but without further elaboration about the detailed disposition of bacteria.

In summary, here we show that *L. murinus* (identified by genetics and immunocytochemistry methods) colonizes not only the forestomach keratinized epithelium but also the lower part of the esophagus, an organ usually considered as a mere transition duct between the pharynx and the stomach. Bacteria, embedded in a mucopolysaccharide matrix, are not tightly associated to epithelia, but appear as proliferations growing to the lumen in a vertical manner. The combined methods used in this study, confirm and deepen previous hypothesis and, hopefully, will allow a better comprehension of the colonization of the esophagus and the stomach epithelium of Balb/c mice by *L. murinus*.

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