

Inhibitory effects of *Lactobacillus fermentum* on microbial growth and biofilm formation

Oxana V. Rybalchenko¹ · Viktor M. Bondarenko² · Olga G. Orlova¹ · Alexander G. Markov³ · S. Amasheh⁴

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Abstract Beneficial effects of Lactobacilli have been reported, and lactic bacteria are employed for conservation of foods. Therefore, the effects of a *Lactobacillus fermentum* strain were analyzed regarding inhibitory effects on staphylococci, *Candida albicans* and enterotoxigenic enterobacteria by transmission electron microscopy (TEM). TEM of bacterial biofilms was performed using cocultures of bacteriocin-producing *L. fermentum* 97 with different enterotoxigenic strains: *Staphylococcus epidermidis* expressing the *ica* gene responsible for biofilm formation, *Staphylococcus aureus* producing enterotoxin type A, *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Proteus mirabilis* producing thermolabile and thermostable enterotoxins determined by *elt* or *est* genes, and *Candida albicans*. *L. fermentum* 97 changed morphological features and suppressed biofilm formation of staphylococci, enterotoxigenic enterobacteria and *Candida albicans*; a marked transition to resting states, a degradation of the cell walls and cytoplasm, and a disruption of mature bacterial biofilms were observed, the latter indicating efficiency even in the phase of higher cell density.

Keywords Biofilm destruction · *Lactobacillus fermentum* 97 · Enterotoxigenic enterobacteria · Staphylococcus

Introduction

Bacterial biofilms are universal, occurring in aquatic and industrial water systems as well as a large number of environments and medical devices relevant to public health (Donlan and Costerton 2002). High-resolution microscopy techniques such as transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM) demonstrated that biofilms represent structures containing homogeneous deposits of cells and accumulated slime with complex communities of surface-associated cells enclosed in a polymer matrix containing water channels and were described for gram-positive and gram-negative microorganisms (O’Gara 2007; Rybalchenko et al. 2010; Sadowska et al. 2010).

Bacteria grow in matrix-enclosed biofilms adherent to surfaces in all nutrient sufficient aquatic ecosystems, and therefore these sessile bacterial cells deeply differ from single cells in suspension counterparts. Numerous studies demonstrated that biofilm-associated microorganisms are linked to several human diseases, such as inflammatory bowel disease, endocarditis and cystic fibrosis, and colonize a wide variety of medical devices (O’Gara 2007; Swidsinski et al. 2005). Though epidemiologic evidence points to biofilms as a source of several infectious diseases, the exact mechanisms by which biofilm-associated microorganisms elicit disease are only beginning to emerge (Holm and Vikström 2014).

The ultrastructure of single bacteria in suspension is well known, as the peculiarity of organization indicating cell specialization, cooperation in communities with

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✉ S. Amasheh
salah.amasheh@fu-berlin.de

¹ Faculty of Medicine, St. Petersburg State University, St. Petersburg, Russia

² Gamaleya Research Institute of Epidemiology and Microbiology, Moscow, Russia

³ Institute of General Physiology, St. Petersburg State University, St. Petersburg, Russia

⁴ Institute of Veterinary Physiology, Freie Universität Berlin, Oertzenweg 19b, 14163 Berlin, Germany

antagonistic microorganisms in the special organization of monoculture and mixed bacteria of different taxonomic groups has been demonstrated (Rybalchenko 2006). Whereas in contrast *Lactobacillus* were not sufficiently analyzed in this context, nevertheless some studies showed that lactobacilli are able to inhibit growth of gram-positive and gram-negative bacteria and yeasts (Kouakou et al. 2010; Rivas-Santiago et al. 2009). The main antagonistic qualities of lactobacilli are due to the antimicrobial peptides (AMP) or bacteriocin-like inhibitory substance (De Vos et al. 1995; Garver and Muriana 1993; Rybalchenko et al. 2014). Furthermore, AMP bactericidal nonspecific metabolites including lactic acid, hydrogen peroxide and lysozyme have been identified (Cotter et al. 2005; Diep and Nes 2002; Lebeer et al. 2008; Turovskiy et al. 2009). The synthesis and impacts of these inhibitory factors exemplify quorum-sensing (QS) processes (Miller and Bassler 2001; Holm and Vikström 2014).

Moreover, bacterial biofilm formation and expression of pathogenic factors are closely associated with the global regulatory system of prokaryotic cells. Recently it was found that QS-regulator factors of *Staphylococcus* biofilm formation include global regulation of the polysaccharide part of biofilms: δB —the activator of *icaR*-gene (Knobloch et al. 2004) and *SarA* (Tormo et al. 2005). As one regulation factor involved in the multicellular aggregation step of *S. aureus* biofilm formation, *rbf* was identified (Lim et al. 2004).

The purpose of this investigation was the transmission electron microscopic study of the structural organization and inhibition of bacterial biofilm formation of different enterotoxigenic bacteria under the influence of *Lactobacillus* antimicrobial compounds.

Materials and methods

Bacterial strains and growth conditions

In our work, following strains were used: *Lactobacillus fermentum* 97, *Salmonella typhimurium* 415, *Escherichia coli* M17, *Enterococcus faecium* SF68, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 13883, *Proteus mirabilis* ATCC 29906, *Citrobacter freundii* ATCC 8090, *Candida albicans* 624 and *Staphylococcus epidermidis* 193. Moreover, different species of clinical enterotoxigenic bacterial target strains were used: *Staphylococcus aureus*, producing enterotoxin type A; *Citrobacter freundii*, *Enterobacter cloacae*, *Klebsiella oxytoca*, *Proteus mirabilis*, producing thermolabile or thermostabile enterotoxins, determined by *elt* or *est* genes, and clinical strains of *Candida albicans*. The strains were received from the culture museum of

Gamaleya Federal Center of Epidemiology and Microbiology (Moscow). All cultures were plated on solid media: For cultivation of lactobacilli, *Lactobacillus* MRS Broth liquid media (HiMedia, Laboratories Pvt. Limited, India) was used, and Brain Heart Infusion Broth (HiMedia Laboratories Pvt. Limited, India) was used for all other indicator bacterial cultures. Unless stated otherwise, routine cultivation was performed overnight in a humid atmosphere at 37 °C and overnight (18–20 h) under standard incubation conditions.

Deferred antagonistic test on solid media

Deferred antagonistic test on solid media was carried out according to Tagg and Bannister (Tagg and Bannister 1979). *Lactobacillus fermentum* 97 was grown on Brain Heart Infusion Agar (HiMedia Laboratories Pvt. Limited, India) overnight as 1-cm-wide streak culture. Following the removal of visible growth, the plate was surface-sterilized by exposure to chloroform vapors and then air-dried for at least 30 min. Each studied bacterial culture indicator was applied at right angles to the streak of cultures by using a cotton-tipped swab. Plates were incubated and examined after 20 and 44 h of incubation for any zones of growth inhibition of the indicator bacteria.

Antagonistic activity assay

In order to determine effects of antagonistic activity including bacteriocin production, *Lactobacillus* was grown on the MRS agarized medium. Colonies have been grown during 2 days at 37 °C. Subsequently, a test strain ($1-2 \times 10^7$ cells at the exponential growth stage) was plated on top in 2.5 ml of soft meat-peptone agar prepared in the HiMedia (India).

Transmission electron microscopy (TEM)

Preparations for carrying out TEM analysis of ultrathin sections consisted in conservation of initial frame and the regional locating of microbial cells in an investigated biofilm. Areas of bacterial growth were cut out with agar and preliminary fixed in 2.5 % solution glutaraldehyde in Hanks buffer (pH 7.2) at 4 °C. It should be noted that the fixing solution was placed under agar plate. This method of fixation was used to preserve the integrity of the surface structures of microbial communities. After 24 h, 0.5 % aqueous agarose solution (30 °C) was placed on the surface to maintain biofilm structure. Secured agarose's biofilms were washed with distilled water and then fixed in 1.0 % OsO_4 aqueous solution for one day at 4 °C.

For dehydration, samples were entirely incubated in solutions of increasing alcohols concentration in a standard procedure and embedded in resin. For clear location and

orientation of sample analysis, a Pyramitome LKB-11800 was used (LKB, Sweden), which allowed correct focusing and an estimation of region location for further analysis. From regions of bacterial growth, ultrathin sections were obtained. Subsequently, preparations were analyzed employing a transmission electron microscope JEM-100C (JEOL, Japan).

Chemicals

All chemicals, unless otherwise noted, were purchased from Sigma-Aldrich.

Statistical analysis

Data are expressed as mean \pm standard error of the mean (SEM), and n indicates the number of experiments.

Results

Effects of *L. fermentum* 97 on biofilms generated by pathogens were studied morphophysiologicaly. Initially, the potentially inhibitory activity of *L. fermentum* 97 on growth of different pathogenic gram-positive and gram-negative bacteria was detected by the deferred antagonistic test, measuring the diameter of growth inhibition zones (Table 1). The test revealed growth inhibition of all microorganisms under investigation during 24 h, including *E. coli* M17, *E. faecium* SF68, *S. typhimurium* 415, *P. aeruginosa* ATCC 27853, *K. pneumoniae* ATCC 13883, *P. mirabilis* ATCC 29906, *C. freundii* ATCC 8090, *S. epidermidis* 193, *S. aureus* ATCC 25923 and *C. albicans* 624.

Subsequently, the ultrastructural organization of populations and individual cells of sensitive cultures was tested by

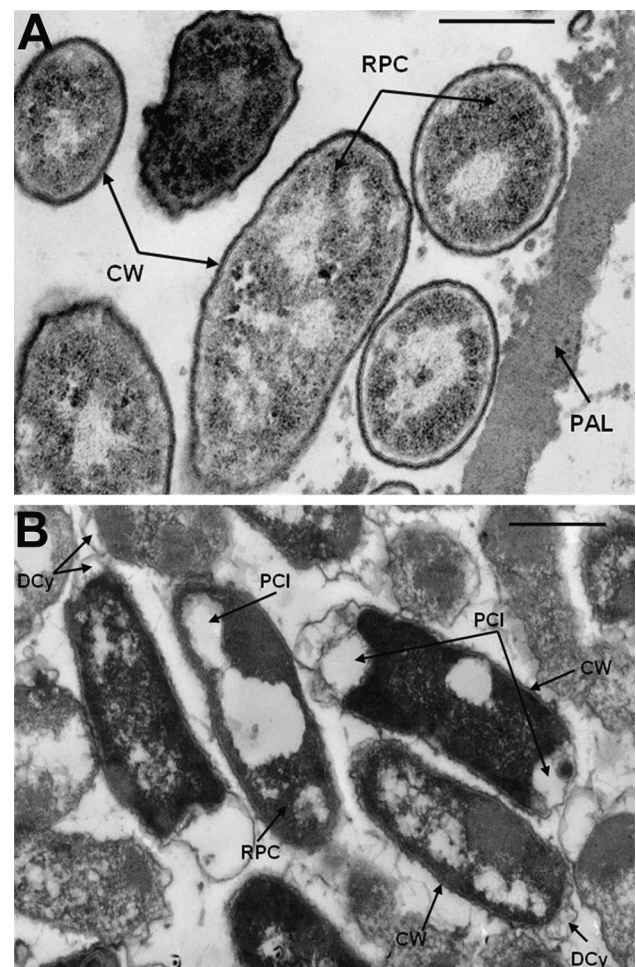


Fig. 1 Ultrathin sections of *E. coli* M17 biofilms; **a** control, surface structure complex of biofilm—polysaccharide amorphous layer (PAL); **b** after the antagonistic effect of *L. fermentum* 97: ribosomal protein complex (RPC), cell wall (CW), polar cytoplasmic inclusions (PCI), delamination of cytoplasm (DCy); scale bar is 1 μ m

Table 1 Assessment of antagonistic activity of *L. fermentum* 97 after 24-h growth (n = 5, respectively)

Microorganisms	Diameter of growth inhibition zones (mm)
<i>Escherichia coli</i> M17	17.5 \pm 0.45
<i>Enterococcus faecium</i> SF68	19.0 \pm 0.27
<i>Salmonella typhimurium</i> 415	26.5 \pm 0.63
<i>Pseudomonas aeruginosa</i> ATCC 27853	17.0 \pm 0.57
<i>Klebsiella pneumoniae</i> ATCC 13883	18.5 \pm 0.31
<i>Proteus mirabilis</i> ATCC 29906	17.5 \pm 0.45
<i>Citrobacter freundii</i> ATCC 8090	17.0 \pm 0.57
<i>Staphylococcus epidermidis</i> 193	21.0 \pm 0.58
<i>Staphylococcus aureus</i> ATCC 25923	20.0 \pm 1.10
<i>Candida albicans</i> 624	15.0 \pm 0.28

TEM. Employing this technique, changes of microbial cells caused by *Lactobacillus* antimicrobial compounds were visualized. Ultrathin changes in microbial cells, destruction of the cell wall and cytoplasm became apparent for all microorganisms under investigation, including *E. coli* M17 (Fig. 1a, b) and *S. aureus* ATCC 25923 (Fig. 2a, b, c), representing the gram-negative and gram-positive groups, respectively. At the population level, cell-to-cell interactions in the mixed populations changed the proportion of different morphotypes of cells and increased the relative number of lysed, involuted, and resting forms. The intensity of ultrastructural alterations in the cells correlated with the size of zones of bacterial inhibition (Table 1): If the diameter of inhibition zones of growth was wider, more cells have been destroyed, and cells from growth inhibition zones had multiple destructive changes in cell walls and cytoplasm.

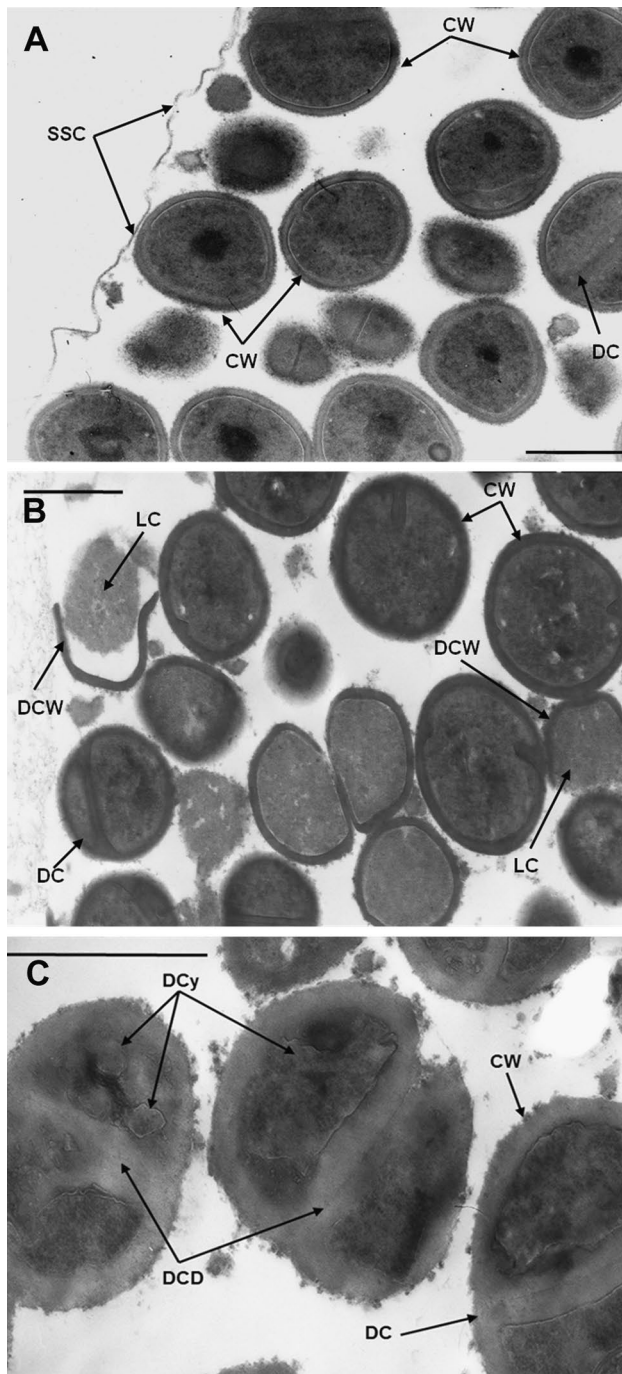


Fig. 2 Ultrathin sections of *S. aureus* ATCC 25923 biofilms. **a** Control, surface structure complex of biofilm (SSC); **b, c** after the antagonistic effect of *L. fermentum* 97; dividing cells (DC), cell wall (CW), delamination of cytoplasm (DCy) and delamination of cell wall (DCW), pore formation in cell membrane (PF), lysed cell (LC); damaged bacteria showing destroyed cell walls (**b**), and thickened cell walls and disturbed cell division (DCD)—(**c**); scale bar is 1 μ m

The ultrastructural changes in lactobacilli cells indicated possession of specific protecting mechanisms, associated with cell transformation into resting forms, and production

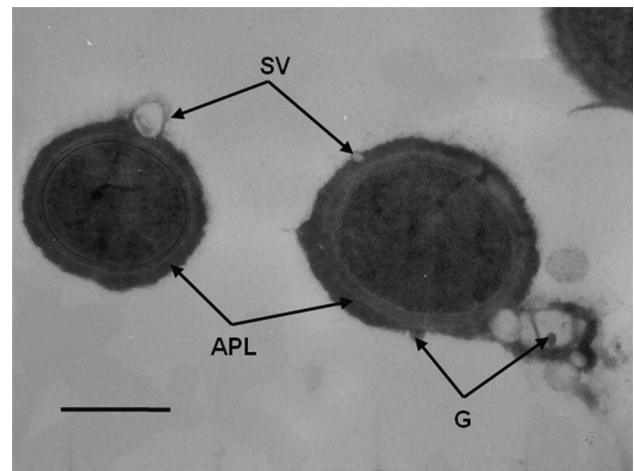


Fig. 3 Ultrathin section of *L. fermentum* 97 biofilm during antagonistic effect. Submembrane vesicles (SV), globules (G) and additional protecting layers (APL) on the cell surface; scale bar is 1 μ m

of submembrane vesicles, globules and additional protecting layers on the cell surface (Fig. 3).

The presented data show a specific character of cell-to-cell interactions between lactobacilli and the different opportunistic microorganisms at the cellular and population level. This fact suggests the existence of a special form of social behavior of microorganisms inside mixed microbial communities on the model of antagonistic interactions. TEM confirmed the various morphophysiological changes of target cells of *Enterobacteriaceae* and *Staphylococcus* during interaction with antimicrobial compounds of *L. fermentum* 97.

Discussion

Microbial colonization of the mucous membranes of the gastrointestinal and urogenital tracts depends on adhesion of prokaryotic cells and formation of colonies forming biofilms (Macfarlane 2008; Tenke et al. 2012).

Normal microflora prevents the spread of opportunistic pathogens bacteria from these biotopes and their translocation into the systemic circulation (Madden et al. 2005; Manzoni 2007). Previously, we have shown that lactobacilli can exhibit antagonistic properties and are able to destroy cells of opportunistic pathogenic bacteria (Rybalchenko 2006). Lactobacillus also can block the translocation of opportunistic pathogenic bacteria and penetration of their toxins from the mucosa into systemic circulation, perhaps by inhibition of bacterial transcytosis (Tuma and Hubbard 2003). However, currently there is no information regarding microbe-to-microbe interactions between Lactobacillus and other opportunistic pathogen bacteria during formation

of biofilms. Therefore, it is difficult to acquire a detailed comprehension of the mechanisms involved in the process of inhibition of biofilm formation by antimicrobial compounds.

Currently, nature, physicochemical properties and mechanism of action of bacteriocin-like inhibitory substance (BLIS) are the subject of intensive research (Belfiore et al. 2007; Pinto et al. 2013; Zaeim et al. 2014). Our study focusing on TEM indicates that antimicrobial compounds of *Lactobacillus*, possibly mediated by BLIS, are sufficient to inhibit bacteria of all different taxonomic groups under investigation.

The changes of microbial biofilms ultrastructure dependent on *Lactobacillus* antimicrobial compounds as demonstrated by TEM may allow us to understand these mechanisms of destruction of microbial communities (Rybalchenko et al. 2010, 2014). These investigations proved the fact that biofilms of bacteria as *E. coli* and *S. aureus* have complex surface structures. It can be supposed that the complex biofilm surface structure is one of the biological mechanisms for resistance to various stress factors. Further studies exhibited that microorganisms that are growing inside a biofilm are highly resistant to antimicrobial agents by one or more mechanisms.

Intestinal microbiota of rats, guinea pigs and mice has been shown to consist of biofilms of single bacterial species or mixed microbial communities of several bacterial species interacting cooperatively (Nielsen et al. 2003).

Bacterial cells within such biofilms can be physiologically heterogeneous because of the diversity of the micro-environment occurring within the biofilm structure. Cells on the surface of the structure are able to get access to nutrients, actively metabolize and begin to divide. Most part of the inside cells in biofilms possibly are in a state of rest (Rybalchenko et al. 2014). This concept of physiological heterogeneity within a biofilm, unlike the relative physiological synchrony of bacteria suspended as individual cells in broth culture, explains that vastly different susceptibilities to antibiotics are a result of heterogeneity of cells within biofilms.

In conclusion, this study revealed that probiotic strain *L. fermentum* 97 reduces growth and development of biofilms of different gram-negative and gram-positive opportunistic bacteria. The activity of *L. fermentum* 97 antimicrobial compounds against *Staphylococcus*, *Enterobacteriaceae*, including *E. coli*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Proteus* and clinical strains of *Candida albicans* are probably due to the destruction of the cell wall and cytoplasm by formation of transient pores in the cytoplasmic membrane. A clear understanding of this phenomenon is important for design and development of new antimicrobial medicine and probiotics.

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