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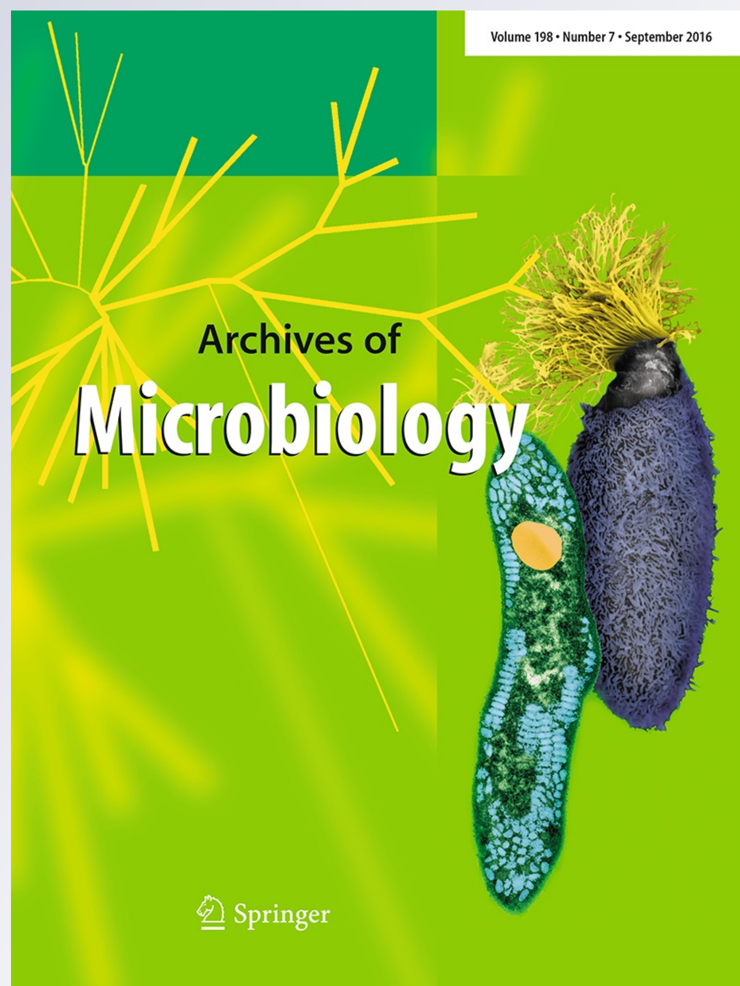

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ORIGINAL PAPER

Biofilms of vaginal *Lactobacillus reuteri* CRL 1324 and *Lactobacillus rhamnosus* CRL 1332: kinetics of formation and matrix characterization

María Cecilia Leccese Terraf[1] · María Silvina Juárez Tomás[1] · Lucie Rault[2,3] · Yves Le Loir[2,3] · Sergine Even[2,3] · María Elena Fátima Nader-Macías[1]

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Abstract Adhesion and biofilm formation are strain properties that reportedly contribute to the permanence of lactobacilli in the human vagina. The kinetics of biofilm formation and the chemical nature of the biofilm matrix formed by *Lactobacillus reuteri* CRL (Centro de Referencia para Lactobacilos Culture Collection) 1324 and *Lactobacillus rhamnosus* CRL 1332, vaginal beneficial strains, were evaluated in this work. Crystal violet-stained microplate assay and techniques of epifluorescence, electron and confocal microscopy were applied. The highest density and complexity of biofilms of both vaginal lactobacilli were observed at 72 h of incubation. Protease, proteinase K, α-chymotrypsin and trypsin treatments efficiently detached *L. reuteri* CRL 1324 biofilm that was also partially affected by α-amylase. However, *L. rhamnosus* CRL 1332 biofilm was slightly affected by protease, proteinase K and α-amylase. Confocal microscopy revealed greater amount of polysaccharides in *L. rhamnosus* CRL 1332 biofilm matrix than in *L. reuteri* CRL 1324 biofilm matrix. The results indicate that proteins are one of the main components of the *L. reuteri* CRL 1324 biofilm, while the biofilm matrix of *L. rhamnosus* CRL 1332 is composed of carbohydrates and proteins. The results obtained support the knowledge, understanding and characterization of two biofilm-forming vaginal *Lactobacillus* strains.

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Introduction

Biofilms are defined as microbial communities enchased in a self-synthesized extracellular polymeric matrix, growing attached to a biotic or abiotic surface (Costerton et al. 1995). Biofilms are a strategy of some organisms to persist in harsh environments (Stoodley et al. 2002). The associated matrix and the microbial behavior indeed promote the microbial resistance to antimicrobial agents, immune system and stress conditions (Boles et al. 2004).

The composition of the extracellular biofilm matrix, which was evaluated mainly in pathogenic strains, varies among different microbial species and depends on environmental conditions (Lasa and Penadés 2006). Poly-*N*-acetylglucosamine (PNAG), a surface polysaccharide, is the main component of the *Staphylococcus epidermidis* biofilm matrix (Izano et al. 2008). Extracellular DNA is a major structural component of the biofilm matrix of *Staphylococcus aureus* (Izano et al. 2008; Kaplan et al. 2012) and of genital pathogen *Neisseria gonorrhoeae* (Falsetta et al. 2011). Some studies have highlighted the role of proteins as constituents of the biofilm matrix, as for example in the opportunistic pathogens *Shewanella* sp. HRCR-1 (Cao et al. 2011) and *Acinetobacter baumannii* (Rahbar et al. 2010).

In contrast, biofilm formation was not studied extensively in nonpathogenic bacteria such as different *Lactobacillus* species (Lebeer et al. 2007; Vélez et al. 2010). In some *Lactobacillus* strains, structures or components that potentially favor their approach to mucosal surfaces,

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adhesion and subsequent colonization was evidenced, including external structures such as S-layer proteins (Wang et al. 2008), aggregation-promoting factors-like proteins of *Lactobacillus acidophilus* NCFM (Goh and Klaenhammer 2010) and mucus-binding proteins of some *Lactobacillus reuteri* strains (Mackenzie et al. 2010). Different studies have demonstrated the presence of pili in *Lactobacillus rhamnosus* GG and their involvement in adhesion (Lebeer et al. 2012), which could also be related to the biofilm formation.

The studies of the human microbiome confirmed that the vaginal tract of healthy women is dominated by different *Lactobacillus* species (Human Microbiome Project Consortium 2012; Romero et al. 2014). Lactobacilli can interfere with pathogens by different mechanisms such as the formation of a barrier that prevents pathogen colonization, the production of antimicrobial compounds or the stimulation of the immune system (Boris and Barbés 2000; Nader-Macías et al. 2008). Specific Lactobacilli (e.g., *L. rhamnosus* GR-1 and *L. reuteri* RC-14) strains are being applied in several probiotic products to restore the human vaginal microbiome for the prevention or treatment of urogenital infections (Martínez et al. 2009; Vicariotto et al. 2012).

Biofilm formation in beneficial *Lactobacillus* strains is a valuable characteristic that can favor bacterial colonization when a probiotic product is administered to the host (Walter et al. 2007, Vélez et al. 2010). However, few works have dealt with this property in urogenital tract isolates (Martín et al. 2008; Malik et al. 2013). Recently, the in vivo biofilm formation by vaginal lactobacilli was evidenced (Ventolini 2015; Ventolini et al. 2015). The studies were mostly based on microscopic observation of wet mounts obtained from vaginal samples.

To characterize and go further on the knowledge of the biofilm formation, in vitro conditions should be also assayed. The in vitro biofilm formation by vaginal *L. reuteri* CRL (Centro de Referencia para Lactobacilos Culture Collection) 1324 and *L. rhamnosus* CRL 1332 in media without Tween 80 was previously evidenced (Leccese Terraf et al. 2012). These microorganisms exhibit different characteristics to be considered as probiotic candidates. *L. reuteri* CRL 1324 and *L. rhamnosus* CRL 1332 produce H_2O_2 and show high ability to auto-aggregate, adhere and form biofilm on mucin (Juárez Tomás et al. 2011; Leccese Terraf et al. 2014). Moreover, *L. reuteri* CRL 1324 inhibits some urogenital pathogens in vitro by lactic acid production and exerts a preventive effect against vaginal colonization by *Streptococcus agalactiae* in a murine experimental model (De Gregorio et al. 2014, 2015). The objective of this work was to further study the biofilms produced by these strains through the evaluation of their morphological characteristics, the kinetics of formation and the chemical characterization of the biofilm matrix.

Materials and methods

Bacterial strains, media and growth conditions

Lactobacillus reuteri CRL 1324 (from the Centro de Referencia para Lactobacilos Culture Collection, Tucumán, Argentina) and *L. rhamnosus* CRL 1332, originally isolated from human vagina in Tucumán, Argentina (Ocaña et al. 1999), were used in this study. The microorganisms were stored in milk yeast extract (13 % nonfat milk, 0.5 % yeast extract and 1 % glucose) at −70 °C. Before the experiments, each strain was transferred from the frozen stock culture to De Man-Rogosa-Sharpe (MRS) broth (De Man et al. 1960), incubated for 24 h at 37 °C and subcultured in the same medium at 37 °C for 12 h. A third subculture in MRS without Tween 80 (MRS_{-TWEEN}) was performed at 37 °C for 14 h. MRS_{-TWEEN} was used to favor biofilm formation of the microorganisms, according to previous results (Leccese et al. 2012). All cultures were performed under static conditions.

Kinetics of biofilm formation by vaginal lactobacilli (VL)

Microplate assay

Biofilm formation by each individual strain was assayed by the crystal violet-stained microplate assay taking samples at different times (Leccese Terraf et al. 2012). To prepare the inoculum of each strain, cell pellets from the third subculture were washed and resuspended in saline up to an optical density at 540 nm ($OD_{540\ nm}$) of 1.5 (around 2.4×10^8 colony forming units per milliliter (CFU/mL)). These initial levels of CFU/mL were selected in a previous work, when evaluating the effect of inoculum concentration on biofilm formation (Leccese Terraf et al. 2012). The number of viable bacterial cells used for the biofilm assay is physiologically related to the concentration in the human urogenital tract, where *Lactobacillus* are present in 10^7–10^8 CFU/mL of vaginal fluid (Boris and Barbés 2000; Borges et al. 2013). Two hundred μL of bacterial suspensions was inoculated into 5 mL of MRS_{-TWEEN} broth, and aliquots of 200 μL were added to 96-well polystyrene microplates (a microplate for each time point). The microplates were incubated under static conditions at 37 °C. The kinetics of biofilm formation was assessed by taking samples at 6, 24, 48 and 72 h.

The quantification of the biofilm formed was carried out according to the technique previously described, using 30 % acetic acid as crystal violet eluent (Leccese Terraf et al. 2012, 2014). Additionally, sterile culture medium was always included as negative control.

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Epifluorescence microscopy

The *L. reuteri* CRL 1324 and *L. rhamnosus* CRL 1332 bacterial suspensions used as inoculums were prepared as described above, and 200 µL of each suspension was inoculated into 5 mL of MRS_{-TWEEN} broth. Biofilms were grown on glass coverslips placed in Petri dishes (6 cm diameter) containing 5 mL of each culture, under static conditions at 37 °C. Samples were taken at 6, 24, 48 and 72 h of incubation and washed with 5 mL PBS. Later, 5 mL of 0.001 % (w/v) acridine orange (Sigma-Aldrich, St. Louis, Missouri, USA) was added, incubated for 15 min and washed with PBS. The biofilms formed on the glass coverslips were analyzed by epifluorescence microscopy at different magnifications (50×, 100× and 400×), with a Carl Zeiss Axio Scope A1 microscope (Gottingen, Germany) fitted with an appropriate filter for acridine orange. Images were processed using AxioVision Release 4.8 software.

Experimental design and statistical analysis

A 2x4 complete factorial design was applied to evaluate the effects of the following factors on the kinetics of biofilm formation: microorganism (*L. reuteri* CRL 1324 and *L. rhamnosus* CRL 1332) and incubation time (6, 24, 48 and 72 h). Each strain was assayed in four independent experiments, each condition in triplicate.

An analysis of variance (ANOVA) using the general linear model was applied to determine the main and interaction effects of the factors on biofilm formation. A significant difference between mean values of the response of interest (OD_{570nm}) was determined by Tukey's test. A p value < 0.05 was considered as statistically significant. The statistical analysis was performed using Minitab 16 Statistical Software.

Characterization of the biofilm matrix

Microplate assay

The chemical nature of the biofilm matrix was evaluated by treating the biofilms formed with different chemical agents, as described by Izano et al. (2008), with some modifications. The biofilms were grown in 96-well microtiter plates for 72 h as described above, rinsed once with PBS (pH 7.4) and then treated with 200 µL of different enzymes and inorganic compounds (Table 1). Control biofilms were incubated with 200 µL of the appropriate solvent for each chemical agent (Table 1). Microplates were incubated for 1 h at 37 °C, and later, the biofilms were rinsed with PBS (pH 7.4) and stained with crystal violet. The dye was extracted with acetic acid (30 %), and the OD_{570nm} was determined (Leccese Terraf et al. 2012, 2014).

Scanning electron microscopy (SEM)

Biofilms were grown on glass coverslips submerged in Petri dishes (6 cm diameter) containing MRS_{-TWEEN} (5 mL) under static aerobic conditions for 72 h at 37 °C. The broth was then removed and the biofilms were carefully rinsed with PBS. The biofilms formed were treated with 5 mL of the different chemical agents listed in Table 1. Control biofilms were incubated with 5 mL of the appropriate solvent for each agent. After 1 h at 37 °C, the treated biofilms were rinsed with PBS buffer (pH 7.4). The ultrastructure of treated biofilm was assessed by SEM, according to the technique described by Leccese Terraf et al. (2012). Images were taken with high vacuum in a Zeiss Supra 55VP scanning electron microscope (Carl Zeiss, Oberkochen, Germany). All the visual fields on the samples were recorded, and some representative areas were photographed at different magnifications: 2000× (2.00 K×), 7000× (7.00 K×), 20,000× (20.00 K×), 50,000× (50.00 K×) and 80,000× (80.00 K×).

Experimental design and statistical analysis

A 2x9 complete factorial design was applied to evaluate the effects of two *Lactobacillus* strains (*L. reuteri* CRL 1324 and *L. rhamnosus* CRL 1332) and nine different treatments (Table 1) on the biofilms formed. The complete experimental design was repeated four times, each condition in triplicate.

A mixed linear model of ANOVA was applied where strain and chemical treatment were considered as fixed effects, and the assay repetitions and triplicates were included in the model as random effects. To establish the differences between the means of the different combinations of the factors studied, the LSD Fisher test of p values was applied. A p value <0.05 was considered as statistically significant. Statistical analysis of this experimental design was performed by using InfoStat version 2012 Software.

Morphological evaluation of biofilm by confocal microscopy

Biofilms were grown in 8-well Labtek™ chamber slides (NalgeNunc International, Naperville, IL, USA) in MRS_{-TWEEN}, under static aerobic conditions at 37 °C. After 72 h of incubation, the culture supernatant was discarded and biofilm was allowed to dry for 30 min at room temperature. Staining was performed in darkness at room temperature. Biofilms were stained using the fluorescent nucleic acid stain Syto 9 from the LIVE/DEAD BacLight stain kit (Molecular Probes Inc., Leiden, The Netherlands) for 30 min which allowed staining of bacteria. In addition,

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Table 1 Different chemical agents used for the characterization of the biofilm matrix of vaginal lactobacilli

Chemical agents	Concentration	Solvent	Effect
Type 14 protease[a]	1 mg/mL	PBS pH 7.4	Protease mixture (at least 10 proteases) cleaving several peptide bonds
Proteinase K[a]	1 mg/mL	PBS pH 7.4	Endolytically cleaves the peptide bonds of aliphatic, aromatic or hydrophobic amino acids[c]
Trypsin[a]	1 mg/mL	PBS pH 7.4	Specifically cleaves the peptide bonds of lysine and arginine[c]
α-Chymotrypsin[a]	1 mg/mL	Buffer Tris–HCl $CaCl_2$ 0.01 M pH 8	Serine protease that hydrolyzes peptide bonds with aromatic or large hydrophobic side chains (Tyr, Trp, Phe, Met, Leu) on the carboxyl end of the bond
Cellulase (from *Aspergillus* sp.)[a]	Enzymatic activity ≥1000	Aqueous solution	[1,4-(1,3:1,4)-β-D-glucan-4-glucanohydrolase][d]
α-Amylase[a]	1 mg/mL	PBS pH 5.9	(1,4-α-D-glucan-glucanohydrolase)[d]
Sodium metaperiodate[a]	10 mM	Acetate buffer pH 4.5	It modifies the polymeric chain of the PNAG by oxidizing the carbons bearing vicinal hydroxyl groups, cleaving the C3 C4 bonds of the GlcNAc residue[c]
Bovine DNase I[a]	0.1 mg/mL	0.15 mg $CaCl_2 \cdot 2H_2O$ and 8.77 mg NaCl in 1 mL water; pH 8	Endonuclease that acts on phosphodiester bonds adjacent to pyrimidines
Recombinant human DNase I[b]	1 mg/mL	0.15 mg $CaCl_2 \cdot 2H_2O$ and 8.77 mg NaCl in 1 mL water; pH 8	Endonuclease that acts on phosphodiester bonds adjacent to pyrimidines

[a] From Sigma-Aldrich, St. Louis, Missouri, USA

[b] From Roche, Buenos Aires, Argentina

[c] Chaignon et al. (2007)

[d] Rowe et al. (2010)

concanavalin A and rhodamine-B-isothiocyanate (RITC) were used to stain polysaccharides and proteins, respectively. Images were acquired using the confocal Nikon C1Si microscope (Nikon, Tokyo, Japan) with an excitation wavelength of 488 for Syto 9, of 543 nm for RITC and 632 nm for concanavalin A, with 100× magnification lens. Emission of fluorescence was monitored at 515 ± 15 nm for Syto 9, 590 nm ± 25 for rhodamine and 650 nm for concanavalin A. Image analysis was performed with Image J (National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/).

Results and discussion

Biofilm formation of beneficial lactobacilli reportedly promotes their mucosal colonization and persistence; moreover, this phenomenon can mask epithelial cell receptors, thus preventing the mucosal adherence of pathogens (Martín et al. 2008). In this work, the kinetics and matrix characterization of biofilm formed by vaginal lactobacilli were evaluated through in vitro experiments. Quantitative (crystal violet-stained microplate assay, using polystyrene as hydrophobic substratum) and qualitative (fluorescence, scanning electronic and confocal microscopy, using glass as hydrophilic substratum) techniques were used. The characteristics of the support or matrix used can influence the outcome of biofilm formation (Di Bonaventura et al. 2008). However, the results obtained with quantitative and qualitative techniques can be evaluated in a complementary way.

Kinetics of biofilm formation by VL

Biofilm formation was dependent on VL strain and incubation time, i.e., the biofilm formation was different for the two strain assayed and through the incubation time. In the specific case of *L. reuteri* CRL 1324, OD_{570nm} values (i.e., biofilm formed) were significantly higher at 72 h compared with 6 h of incubation (Fig. 1a). For *L. rhamnosus* CRL 1332, biofilm density was significantly higher at 72 h compared with 6, 24 and 48 h of incubation. Through epifluorescent microscopy, a low cell density of the two strains was observed on the glass surface at 6 h, corresponding to the initial stages of microbial adhesion to the support (Fig. 1b). *L. reuteri* CRL 1324 formed the biofilm faster than *L. rhamnosus* CRL 1332, reaching a higher density at

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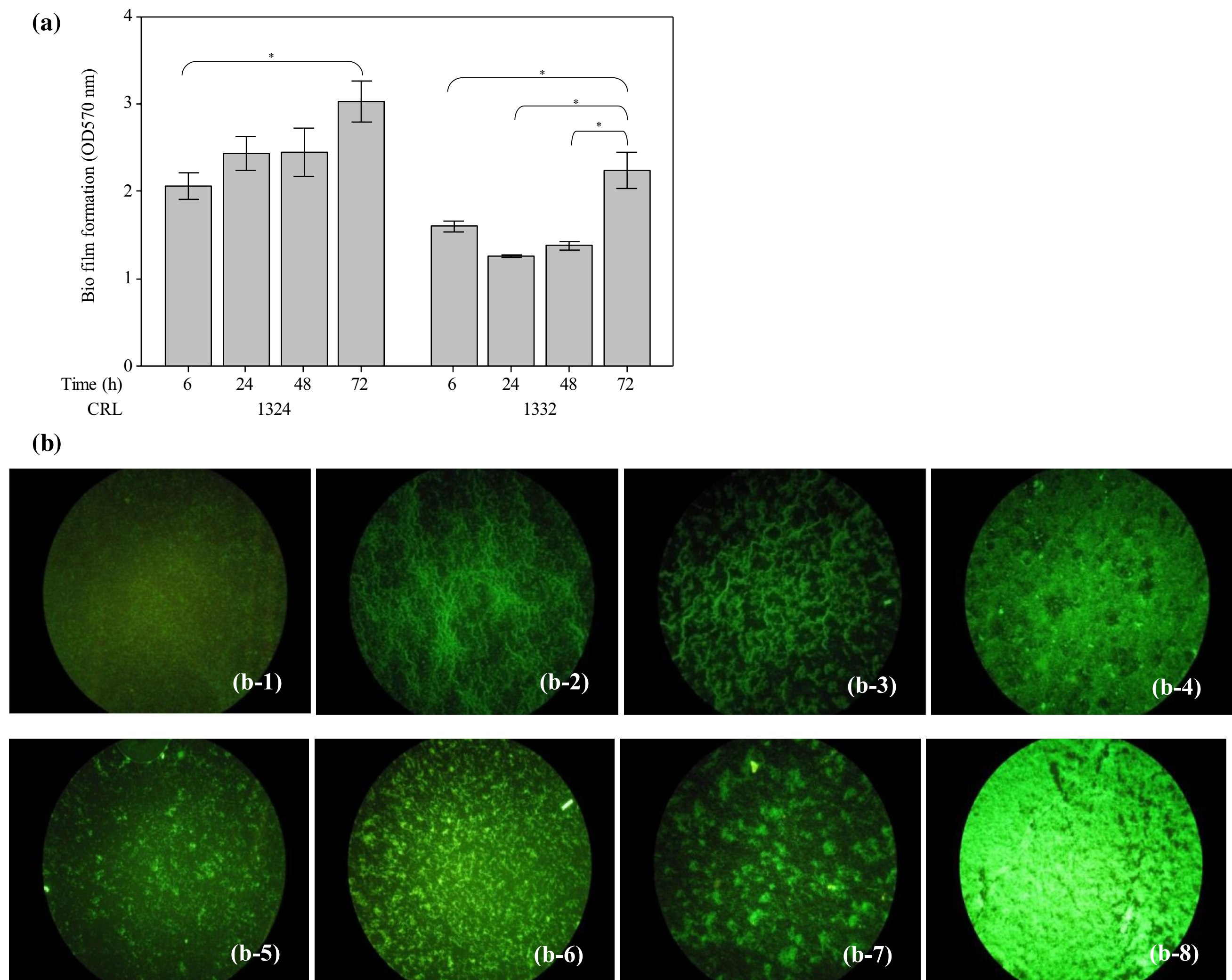

Fig. 1 Kinetics of biofilm formation by vaginal *Lactobacillus* strains in MRS broth without Tween 80. The data show the mean OD_{570nm} values ± SE obtained from 30 % acetic acid solubilized crystal violet-stained biofilms from the microplates at 6, 24, 48 and 72 h of incubation (**a**). *Significant differences ($p < 0.05$) in biofilm formation between the different incubation times for a same *Lactobacillus* strain, according to Tukey's test. Fluorescent microscopy of the kinetic of biofilm formation (**b**) by *L. reuteri* CRL 1324 (**b1**–**4**) and *L. rhamnosus* CRL 1332 (**b5**–**8**), at different incubation times: 6 h (**b1**, **b5**), 24 h (**b2**, **b6**), 48 h (**b3**, **b7**) and 72 h (**b4**, **b8**). Microphotographs are representative of the full biofilms formed and of at least three independent experiments. Magnification: ×50

24 and 48 h. The mature VL biofilm was formed at 72 h of incubation (Fig. 1a, b).

Only a few studies have evaluated the biofilm formation by lactobacilli along the incubation time (Furukawa et al. 2011). To the best of our knowledge, the kinetics of formation of VL biofilm has not yet been reported. Furukawa et al. (2011) studied mixed-species biofilm formation between *Lactobacillus plantarum* ML11-11 (an isolate from Fukuyama pot vinegar) and *Saccharomyces cerevisiae* BY474, using yeast extract–peptone–dextrose (YPD) broth as culture media, polystyrene microplates as support and 30 °C incubation temperature. Biofilm production increased over time, and a thick mixed-species biofilm was formed after 12 h. In this work, VL biofilm formation was slower than *L. plantarum–S. cerevisiae* biofilm; however, some of the culture conditions employed were different.

Characterization of the biofilm matrix

The extracellular matrix of the biofilm provides spatial organization and structural stability during the biofilm development (Jiao et al. 2011). The biofilm matrix is a macromolecular complex that can be formed by exopolysaccharides, proteins and DNA (Muscariello et al. 2013).

Protease, proteinase K, trypsin and α-chymotrypsin efficiently detached *L. reuteri* CRL 1324 biofilm, indicating that proteins are the main components of the biofilm matrix of this microorganism (Fig. 2a). The SEM observations

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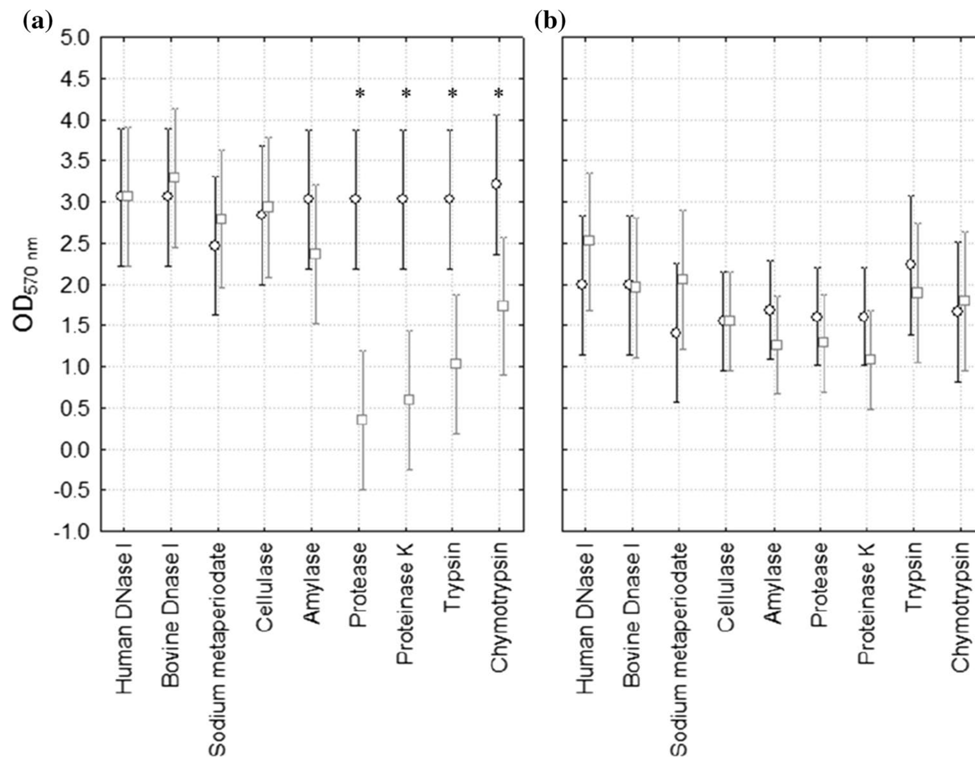

Fig. 2 Effects of chemical agents on biofilm formed by vaginal lactobacilli. Detachment of *L. reuteri* CRL 1324 (**a**) and *L. rhamnosus* CRL 1332 (**b**) 72-h-old biofilms formed on microplates and treated with protease, proteinase K, α-chymotrypsin, trypsin, cellulase, α-amylase, sodium metaperiodate, bovine DNase I and recombinant human DNase I (in Table 1, the concentrations of chemical agents are included). The data show the mean OD_{570nm} values ± 95 % confidence intervals obtained from 30 % acetic acid solubilized crystal violet-stained biofilms treated with several agents (*square*) or with the appropriate buffer (control biofilms, *circle*). *Significant differences ($p < 0.05$) between the biofilm formed in the presence of each chemical agent and the corresponding control biofilm, according to the LSD Fisher test

of *L. reuteri* CRL 1324 PBS control biofilm evidenced a complex three-dimensional structure formed by dense cell aggregates joined to an extracellular matrix, channels and towers (Fig. 3a–d). However, in the proteinase K-treated biofilm, which was included as an example of protease-treated biofilm, a flat structure was obtained because the biofilm was efficiently dispersed into single cells or into small cell groups, with a complete loss of the matrix (Fig. 3e–h). The amylase treatment slightly, but not significantly, affected the attachment of *L. reuteri* CRL 1324 biofilm to microplates (Fig. 2a). In agreement, observation of biofilms formed at 72 h by confocal microscopy revealed a low and rather homogeneous presence of polysaccharides in the biofilm, as indicated by a low intensity of concanavalin A (Fig. 5c). These results support the idea that polysaccharides are not major components of *L. reuteri* CRL 1324 biofilm matrix.

The OD_{570nm} mean values of biofilm formed by *L. rhamnosus* CRL 1332 were slightly, but not significantly lower in the presence of protease, proteinase K and amylase; however, *L. rhamnosus* biofilm was not affected by cellulose or sodium metaperiodate (Fig. 2b). Indeed, none of the treatments resulted in a significant loss of biofilm of this strain, when performing the microplate assay. These results suggest that *L. rhamnosus* CRL 1332 biofilm matrix probably relies on several components and, even though one component of the matrix is degraded by a given treatment, the other components maintain the biofilm cohesion. SEM images of the *L. rhamnosus* CRL 1332 biofilm treated with α-amylase are included as example (Fig. 4), but similar patterns were obtained after proteinase K and protease treatments (data not shown). The PBS control biofilm was evidenced as a soft biofilm that did not completely cover the surface of the coverslip with dense cell aggregates held together by a minimal extracellular matrix and surrounded by void areas (Fig. 4a–d). In contrast, simple bacterial cell chains were observed after the treatment with α-amylase (Fig. 4e–h). In agreement, confocal microscopy revealed

Fig. 3 Scanning electron microscopy of the *L. reuteri* CRL 1324 biofilm treated with proteinase K (1 mg/mL). Microphotographs show the 72-h-old biofilm formed by *L. reuteri* CRL 1324 incubated 1 h with control PBS buffer (pH 7.4) (**a**–**d**) and proteinase K (**e**–**h**) at different magnifications: 2.00 K× (**a**, **e**), 7.00 K× (**b**, **f**), 20.00 K× (**c**, **g**) and 50.00 K× (**d**, **h**) ▶

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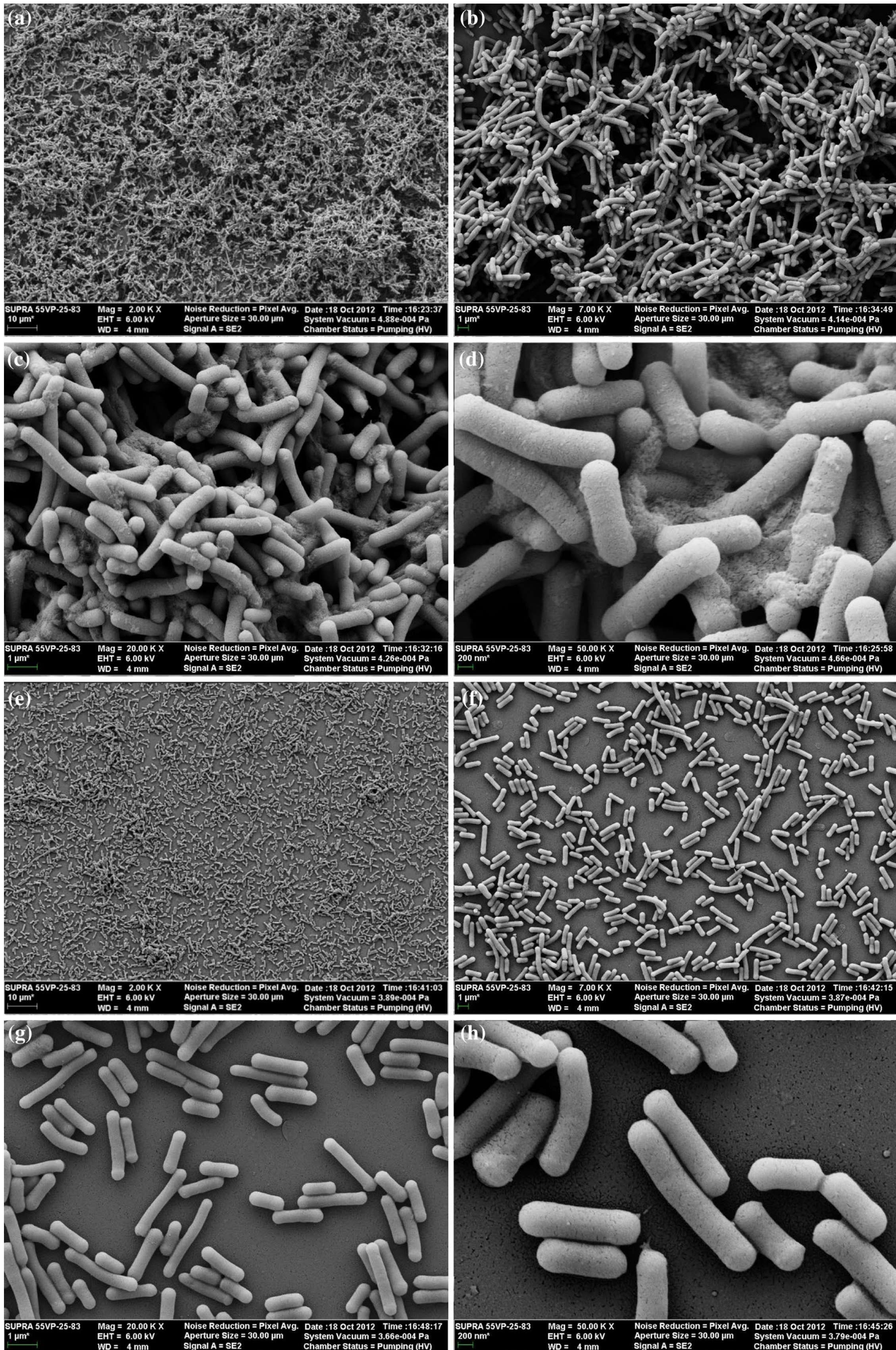

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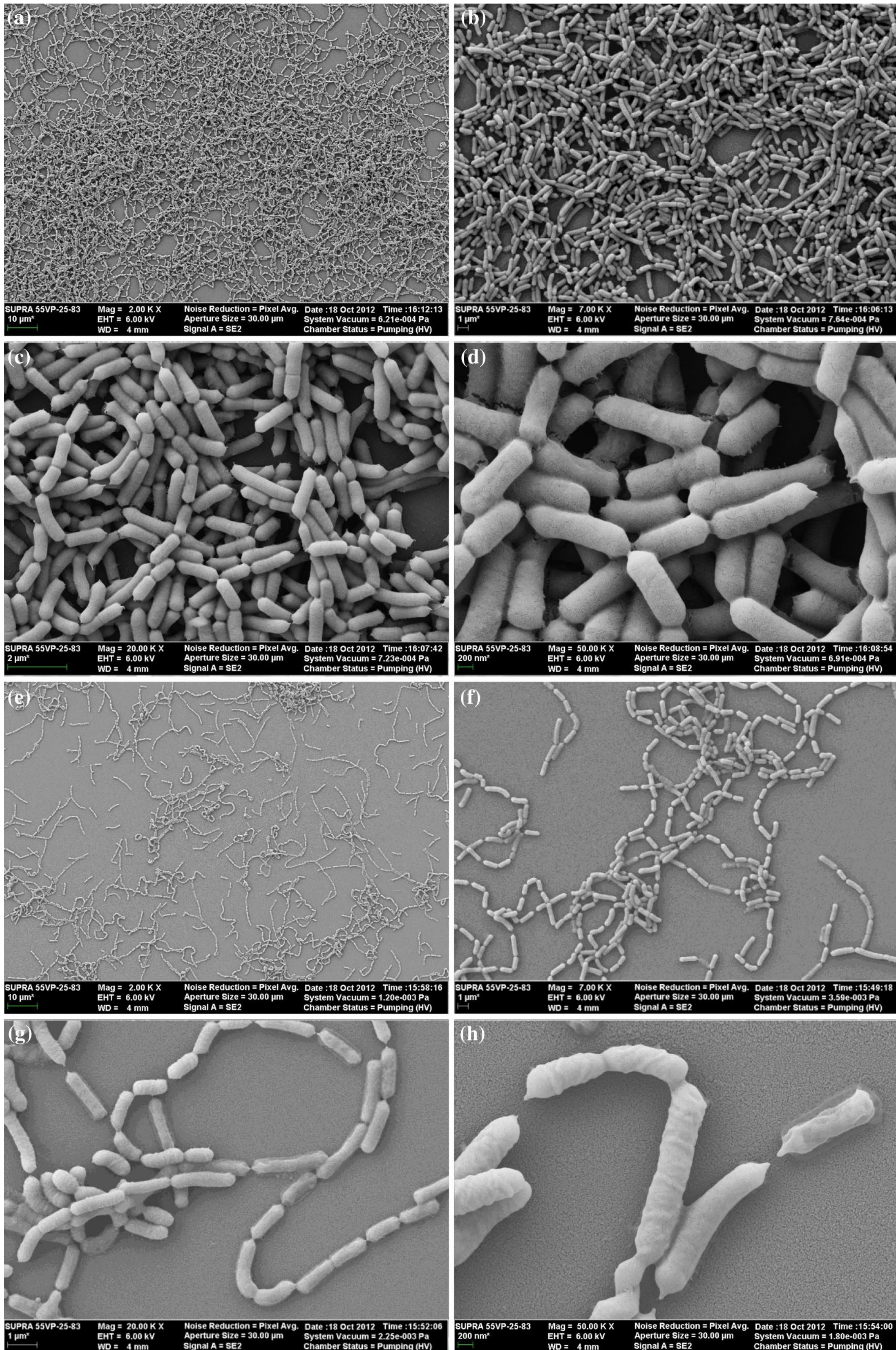

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◀**Fig. 4** Scanning electron microscopy of the *L. rhamnosus* CRL 1332 biofilm treated with α-amylase (1 mg/mL). Microphotographs show the 72-h-old biofilm formed by *L. rhamnosus* CRL 1332 incubated for 1 h with control PBS buffer (pH 5.9) (**a**–**d**) and α-amylase (**e**–**h**) at different magnifications: 2.00 K× (**a**, **e**), 7.00 K× (**b**, **f**), 20.00 KX (**c**, **g**) and 50.00 K× (**d**, **h**)

the presence of polysaccharides in *L. rhamnosus* CRL 1332 biofilm matrix, dispersed within the biofilm or forming aggregates (Fig. 5f).

From the results obtained through microplate assay and microscopy techniques, it appears that the treatment effects on biofilm attachment on different supports can vary with the support used. In the case of *L. rhamnosus* CRL 1332, biofilm matrix composition (carbohydrates and proteins) could be evidenced mainly through SEM and confocal microscopy, using glass as substratum.

Several reports have characterized the biofilm matrix of pathogenic microorganisms, but few studies have described the components of biofilm matrixes of beneficial or probiotic bacteria (Habimana et al. 2009; Muscariello et al. 2013). The results reported in this work are in partial agreement with those published by Muscariello et al. (2013), showing that the biofilm produced by *L. plantarum* (a vegetal strain) was sensitive to proteinase K and DNaseI, thus confirming the presence of protein factors and extracellular DNA as functional components participating in the maintenance of biofilm integrity.

The chemical nature (proteins and carbohydrates) of biofilm matrixes of vaginal *Lactobacillus* strains can be relevant in the vaginal tract. Proteomic studies of cervical–vaginal fluids demonstrated the presence of several serine proteases mainly with immunological functions, as well as inhibitors of serine and cysteine proteases and other proteases (Zegels et al. 2009). α-Amylase is also present in vaginal fluid, where its activity is associated mainly with glycogen degradation, which can favor the proliferation of lactobacilli and prevent urogenital pathogens growth (Nasioudis et al. 2015). Thus, the formation and stability of the biofilms of potentially probiotic VL will depend on the complex balance between these different components.

It is important to point out that *L. reuteri* CRL 1324 and *L. rhamnosus* CRL 1332 biofilms are stable at pH 4.5 (pH of the acetate buffer used to solubilize sodium metaperiodate). As an example, Fig. 6 shows the *L. reuteri* CRL 1324 biofilm incubated in the presence of acetate buffer and treated with sodium metaperiodate. These results could suggest the resistance of these biofilms to the low pH (4.0–4.5) of the healthy urogenital tract (Larsen 1993).

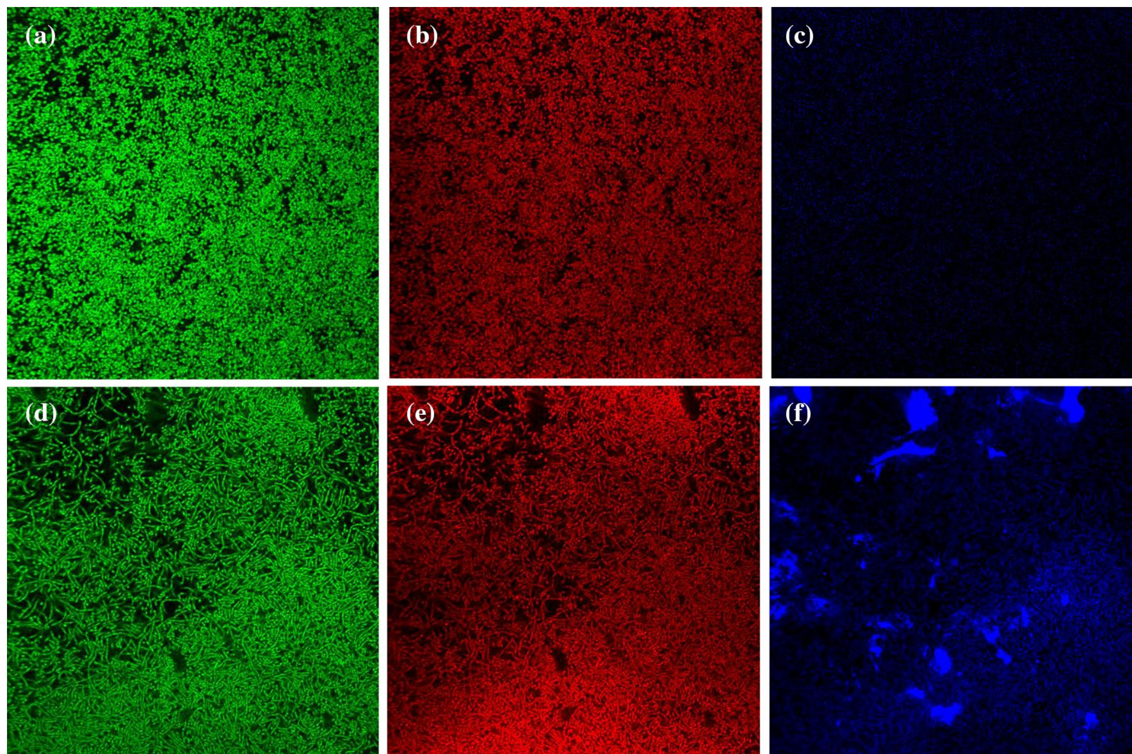

Fig. 5 Fluorescent confocal microscopy of *L. reuteri* CRL 1324 and *L. rhamnosus* CRL 1332 biofilms. Analysis was performed on 72-h-old biofilm formed by *L. reuteri* CRL 1324 (**a**–**c**) and *L. rhamnosus* CRL 1332 (**d**–**f**) after staining with Syto 9 (**a**, **d**), RITC (**b**, **e**) and concanavalin A (**c**, **f**) (see "Materials and methods" for details)

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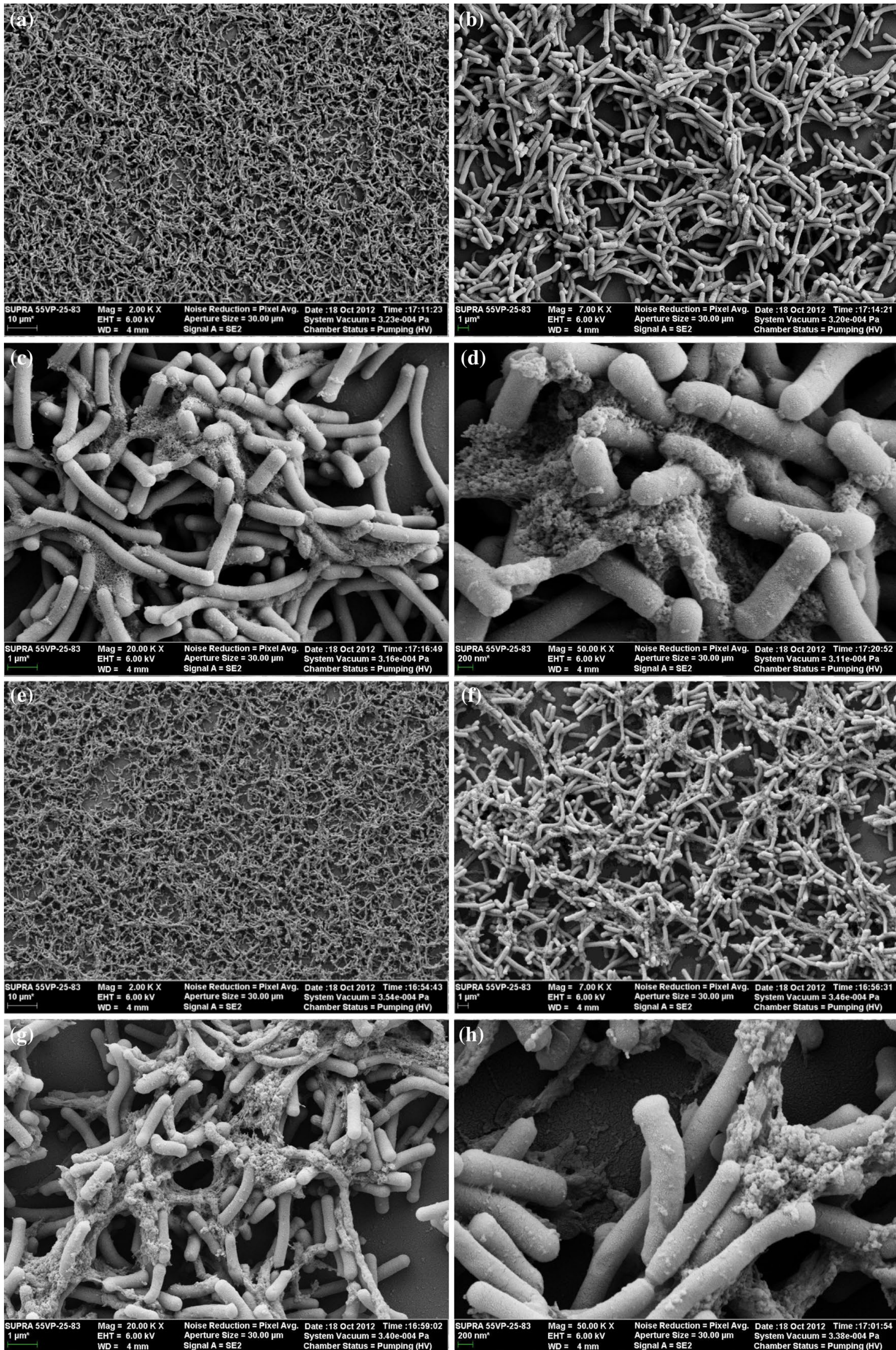

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Fig. 6 Scanning electron microscopy of the *L. reuteri* CRL 1324 biofilm treated with sodium metaperiodate (10 mM). Microphotographs show the 72-h-old biofilm formed by *L. reuteri* CRL 1324 incubated 1 h with control acetate buffer (pH 4.5) (**a**–**d**) and sodium metaperiodate (**e**–**h**) at different magnifications: 2.00 K× (**a**, **e**), 7.00 K× (**b**, **f**), 20.00 K× (**c**, **g**) and 50.00 K× (**d**, **h**)

Bovine DNaseI and recombinant human DNaseI showed no significant effects on the attachment of the biofilm formed by *L. reuteri* CRL 1324 and *L. rhamnosus* CRL 1332. In contrast, in *Lactococcus lactis* MG1363, a higher extracellular DNA concentration at the base of the biofilm was suggested, which was released from cell lysis (Habimana et al. 2009).

In this work, two biofilm-forming strains of two *Lactobacillus* species have been investigated. However, because of the low number of strains assayed, the behavior cannot be generalized at the species level. Further studies should be performed to evaluate a higher number of strains belonging to *L. reuteri* or *L. rhamnosus* species, in order to determine whether the biofilm formation is specific for each strain or specie.

Conclusions

This is the first work directed to evaluate the formation of biofilm during the incubation time and the chemical nature of the biofilm matrixes of beneficial *Lactobacillus* strains, autochthonous from human vagina. The treatments applied differentially affected the biofilm formed by the two VL under study. *L. reuteri* CRL 1324 was able to produce a mainly proteinaceous-type biofilm, which was evidenced through both quantitative and qualitative assays. From qualitative assays using glass as substratum, carbohydrates and proteins showed to be the components of *L. rhamnosus* CRL 1332 biofilm matrix. These findings provide new insights into biofilm formed by vaginal bacteria considered as probiotic candidates. Further studies are being performed to evaluate the formation of VL biofilm in conditions that mimic the physiological conditions of the urogenital tract.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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