ISSN 0026-2617, Microbiology, 2013, Vol. 82, No. 3, pp. 364–372. © Pleiades Publishing, Ltd., 2013.

EXPERIMENTAL ARTICLES

# Dairy Biofilm: Bacterial Community Diversity Assessment and Impact of the *Lactococcus lactis* Bio Adhesion on Biofilm Growth[1]

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Received July 27, 2012

**Abstract**—Biofilms are the most common mode of bacterial growth in nature. Their formation occurs on organic or inorganic solid surfaces in contact with a liquid, on gas-liquid and liquid-liquid boundaries as well. The aims of this study were, by combining cell enumeration, scanning electron microscopy and denaturing gel gradient electrophoresis (DGGE), to characterize the structural dynamics of dairy biofilm growth in the environments with a nutrient flow, and to evaluate the impact of adhesion of *Lactococcus lactis* on the biofilm community depending on the incubation time. Significantly higher values of biofilm volume and thickness were observed under dynamic conditions after 55 h. The populations of gram-positive bacteria and fungi exhibited a significantly higher biofilm organization after 2 days of cultivation than that of gram-negative bacteria. Also, results showed that *Lc. lactis* was able to adhere to silicone surface and the produced biofilm in which the number of adhered gram-positive and gram-negative bacteria decreased by nine orders of magnitude after 48 h of contact. This study constitutes a step ahead in developing the strategies to prevent microbial colonization by lactococcal protective biofilm.

*Keywords:* dairy biofilm, ESEM, *Lactococcus lactis*, DGGE, protective biofilm

**DOI:** 10.1134/S002626171303017X

Life in biofilm is a part of the ecological cycle for the vast majority of bacteria and yeasts found in the environment. The term biofilm is commonly used to describe bacteria in the attached state surrounded by an extracellular matrix of polysaccharides [1]. Clearly, biofilms can become a health hazard by harboring pathogenic bacteria [2] capable of attaching to various surfaces, such as stainless steel [3, 4] and silicon rubber [5]. Several approaches have been used to identify and characterize the bacterial elements and genetic determinants involved in biofilm development. For instance, plate counting has been used to quantify sessile cells on abiotic surfaces [6]. Recently, a Flow cell system was used to study biofilm formation [7] and their structures have been investigated by scanning electron microscopy (SEM) [8]. Fast and reliable molecular techniques based essentially on the analysis of rDNA amplified sequences, such as denaturing gradient gel electrophoresis (DGGE) provided the tools to determine microbial presence and diversity in biofilms [9, 10]. Characterization of the chemistry of surface by goniometry [11, 12] was recently developed and used to characterize biofilm interaction with the substrate. The main challenge in the food industry is to avoid contamination of raw materials, plants and products by pathogens and spoilage organisms. Nowadays, protective biofilm formation on food industry surfaces or medical devices can also be beneficial because their presence may effectively modify the physico-chemical properties of the substrates and reduce adhesion of the undesirable planktonic microorganisms [13]. Indeed, the biofilms of lactic acid bacteria received considerable attention for their potential use in the settlement of a competitive flora [5] by synthesis of antagonistic compounds such as acids, bacteriocins, or biosurfactants whose molecules consist of proteins, polysaccharides and phosphates, with regard to an anti-adhesive effect [5, 14]. *Lactococcus lactis* is most frequently used for fermentation and preservation purposes because has no present any detrimental effect on the organoleptic properties of processed foods, making it a suitable candidate for the creation of protective biofilms [15, 16].

The purpose of the present work was to investigate the kinetics of development of a dairy biofilm by environmental scanning electron microscopy (ESEM) and DGGE under flow conditions. The impact of *Lc. lactis* bio adhesion on biofilm growth on silicone was also determined.

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## MATERIALS AND METHODS

**Flow-silicone system biofilm experiments.** The biofilm was cultivated in continuous-flow silicone system with the channel (total length of 1 m and diameter of 1 cm). The flow system was inoculated with fresh raw milk. After inoculation, the medium flow was stopped for 1 h to allow bacterial adhesion, and thereafter the medium was pumped at 1.3 mL $min^{-1}$ by using a peristaltic pump (ISMATEC ID 871). Biofilm development was assayed at 2, 7, 11, 15, 27, 42 and 55 h by analysis of 1 $cm^2$ of silicone sections. Two independent experiments with two replicates were made for the assessment of dairy biofilm development at each sampling time.

**Application of *Lc. lactis* on silicone.** The cells of *Lc. lactis* were prepared by overnight shaking on M17 [17] and were harvested by centrifugation at 6000 *g* for 10 min. The cell pellets were then quickly washed twice and were resuspended in sterile saline water (0.9%). The harvested cells suspensions were adjusted to a concentration of $10^6$ cell $mL^{-1}$ and the system with 55-h biofilm was inoculated. Analysis of silicone section (1 $cm^2$) was carried out after 3, 6, 24 and 48 h of contact. Two independent experiments with two replicates were made at each sampling time.

**Isolation of microorganisms and enumeration of viable cells.** The number of bacterial cells of adhered to the silicone sections was determined after 2, 7, 11, 15, 27, 42 and 55 hours of cultivation. Initially, 1 $cm^2$ of silicone section were immersed three times in 5 mL of sterile saline water (0.9%), to remove the planktonic cells, followed by the removal of the adhered cells with sterile swabs. The swabs were transferred to the test tubes containing 10 mL of saline solution and stirred vigorously for one min [18]. Serial dilutions were plated on selective agar media. After that, the isolates were picked from the agar plates and characterized by PCR 16s rRNA gene sequencing.

**16S rDNA amplification and identification of biofilm community.** Genomic DNA from biofilm cultures were isolated by the phenol extraction method as reported by Cherif et al. [9]. The 16S rDNA regions were amplified using the primers F: S-D-Bact-0008-a-S-20 (5'-AGAGTTTGATCCTGGCTCAG-3') and R: S-D-Bact-1495-a-S-20 (5'-CTACGGCTACCTTGTTACGA-3') (Invitrogen). Fungal were plated on agar, incubated for 3 to 7 days at 30°C and the rDNA ITS regions 1 and 2 were amplified using the primers ITS1 and ITS4 [11]. The PCR conditions were: (94°C for 4 min) 1×, (94°C for 45 s, 55°C for 1 min, 72°C for 2 min) 30×, (72°C for 7 min) 1×. DNA sequencing was performed using PRIMM (PRIMM Biotech), according to the manufacturer's instructions. The GenBank BLASTN tools were used for sequence analysis.

**Environmental scanning electron microscopy (ESEM) analysis.** The specimens of silicone were gently washed three time with sterile distilled water and the images were obtained on an Environmental Scanning Electron Microscope (ESEM Quanta 200) equipped with a tungsten filament (FEI). The signal was collected using a Gaseous Secondary Electron Detector (GSED).

**DGGE analysis and sequencing**. Total DNA was extracted from dairy biofilm samples, PCR amplification targeting 16S rRNA genes was performed using the universal primers specific to the bacteria domain: 907r (5'-CCGTCAATTCCTTTGATGTTT-3') and 357f (5'-TACGGGAGGCAGCAG-3'). PCR experiments were performed as previously described [9]. DGGE analysis was performed using a Gradient Delivery System Model ''INGENY phorU''. After electrophoresis, the dominant bands were manually excised and eluted in 80 μL of sterilized distilled water overnight at 37°C under light shaking. Eluted DNA (15 μL) was used as a template to reamplify the band of interest using the same universal primer without the GC-clamp. The amplified products were purified with the QIAquick PCR Purification Kit (Qiagen) and sequenced by PRIMM; Applied Biosystems. The obtained sequences were compared with 16S rRNA gene sequences in the NCBI database using the BLASTn search program to identify the nearest relatives of the excised dominant bands. The phylogenetic tree was constructed by means of ''Clustal X'' program.

## RESULTS AND DISCUSSION

**Biofilm development.** Growth and microbial diversity of a dairy biofilm in a Flow Displacement System was observed 2, 7, 11, 15, 27, 42 and 55 h after inoculation; the steady-state was achieved at 55 h. In fact, after the initial adhesion of cells at 2 h, low rate of adhered cells was assessed on silicone surface. After 15 h, the rate of adhered cell increased to $10^5$ cell $cm^{-2}$. After 27 and 42 h of contact, the rate of adhered cells increased to $10^7$ and $10^9$ cell $cm^{-2}$ respectively. Indeed, the steady-state was observed at 55 h with a high rate of adhered cells ($10^{11}$ cell $cm^{-2}$). Many studies have previously reported that microbial attachment and biofilm development occurs in three or five-step processes [19–21]. ESEM observations under a stronger magnification (Fig. 2a) and Matlab analysis evidenced that silicone surface was covered by a uniform and compact biofilm constituting by a large numbers of various rod-shaped bacteria with rounded ends and some cocci, and the steady-state was observed at 55 h. Also, observations revealed that the biofilm formed from 2 h to 55 h had different morphological characteristics and the biofilm's volume increased during incubation irrespective of the hydrodynamic flow. Usually, the hydrodynamic conditions can be evidenced by the increased fluid flow towards or parallel to a substratum surface resulting in faster adhesion of microorganisms due to higher mass transport despite the presence of higher fluid shear stimulating their

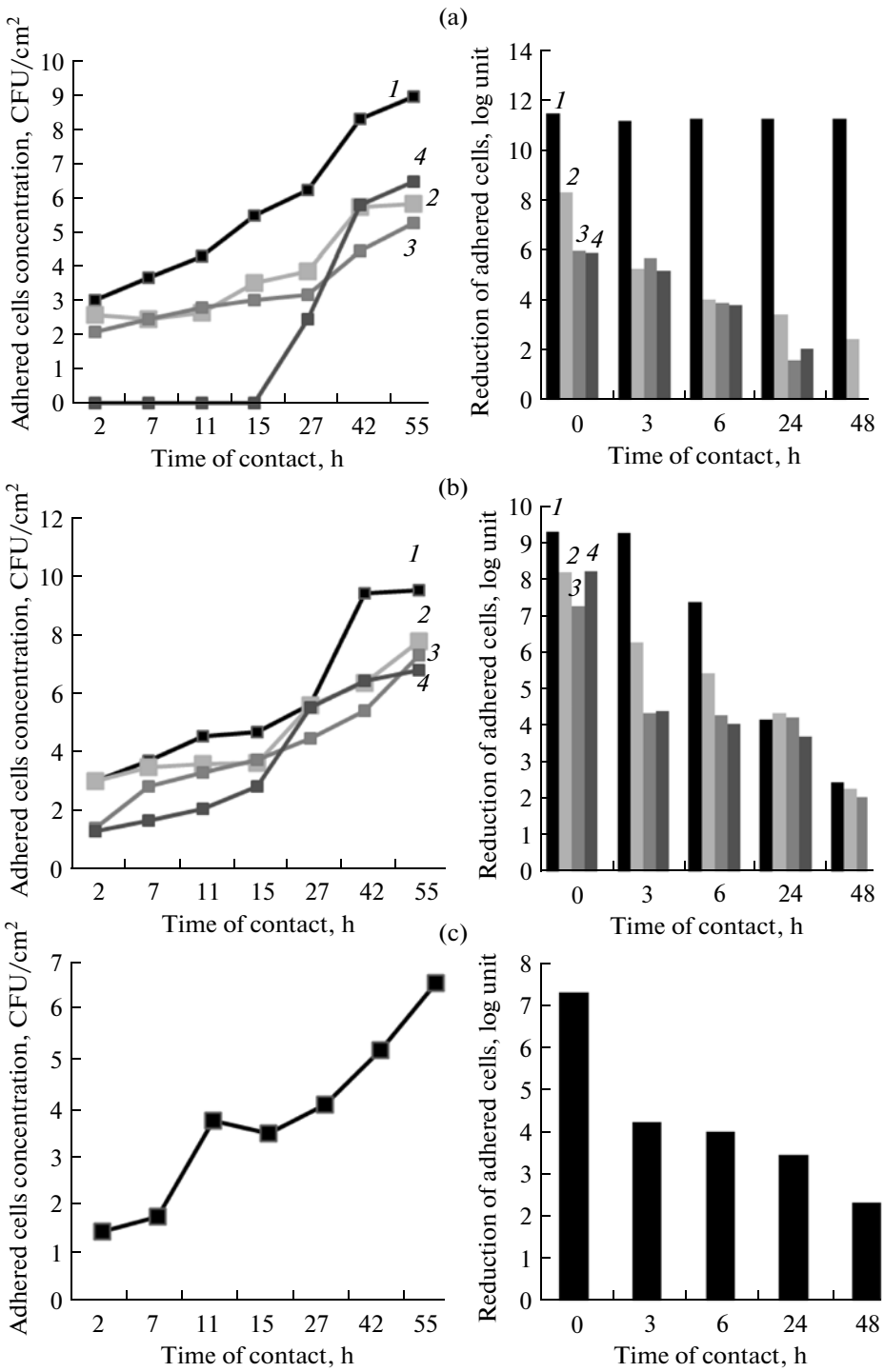


**Fig. 1.** Assessment of biofilm development and impact of *Lc. lactis* adhesion on adhered cells (a) gram-positive population (Lactic acid bacteria (*1*) *staphylococci* (*2*) mesophilic *Bacillus* (*3*), thermophilic *Bacillus* (*4*)); (b) gram-negative population *Enterobacteria* (*1*), total colifom (*2*), fecal coliform (*3*), *Pseudomonas* (*4*)); (c) fungal population.

detachment [22]. Hence, even if few works have been carried out on the investigation of dairy biofilm [3], our study evidenced the dynamic of biofilm formation and characterization of attached population composed with multiple species.

**Ecology and identification of the isolates.** The ecological profiles of the biofilms, in terms of broad groups are shown in Table 1. According to plate counts, gram-negative bacteria (Fig. 1b) exhibited a significantly higher biofilm population after 2 days of

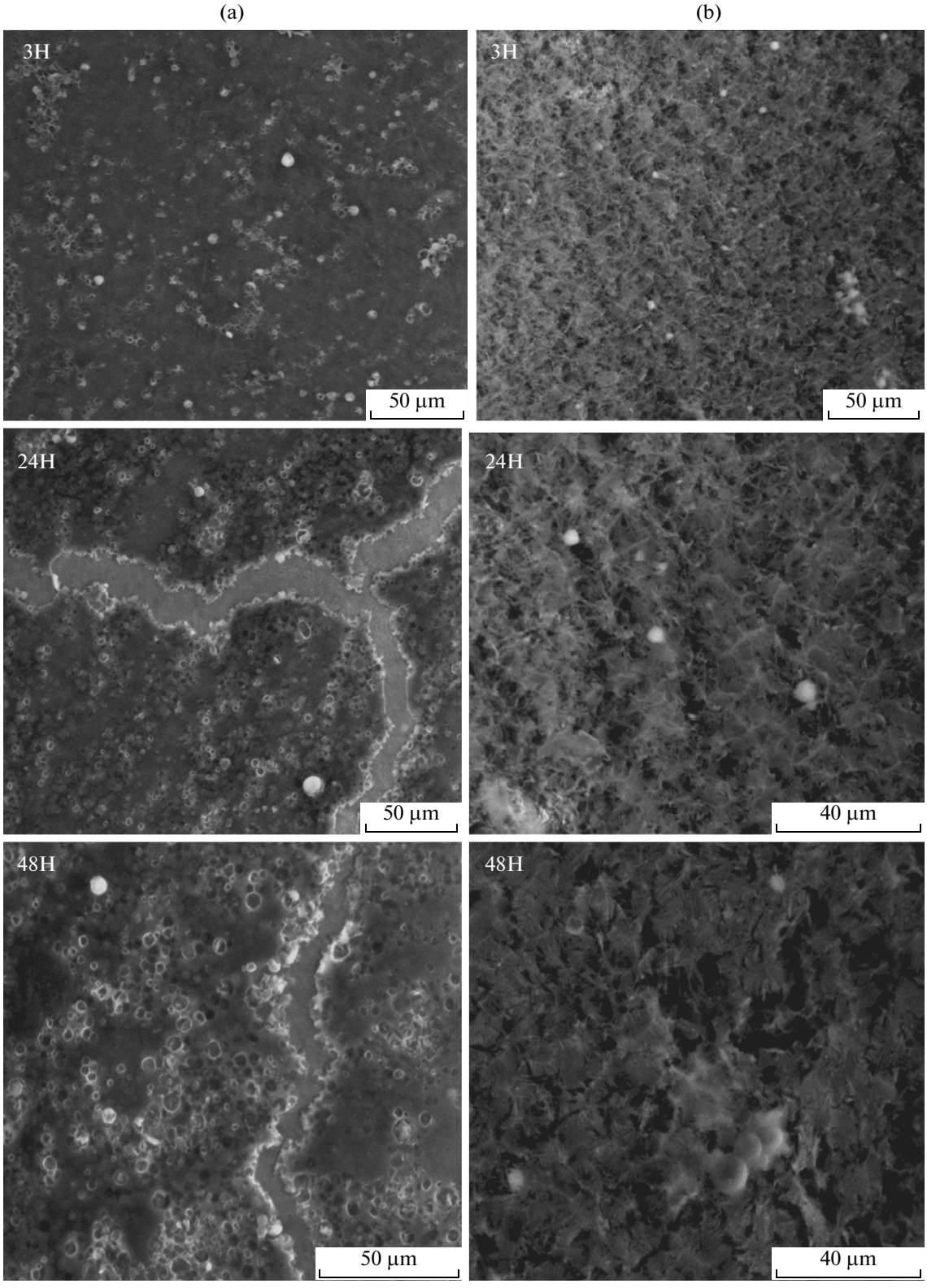


**Fig. 2.** Electron micrographs of dairy biofilm adhered onto substrata (a) and impact of *Lc. lactis* adhesion on adhered cells (b) visualized by ESEM.

**Table 1.** Ecology and 16s sequencing of dairy biofilm isolates

| 16S rDNA sequence analysis Similarities | | | % total subpopulation Biofilm formation $T_{55}$ |
|---|---|---|---|
| GRAM +ve | | | |
| Lactic acid bacteria | | | 46–60 |
| | *Laciococcus* spp. *lactis lactis* | (98%) | |
| | *Lactobacillus laciis* | (97%) | |
| | *Lactobacillus paracasei* | (98%) | |
| *Bacilli* | | | 14 |
| | *Bacillus cereus* I | (98%) | |
| | *Bacillus weithanpansis* | (96%) | |
| | *Bacillus licheniformis* | (97%) | |
| *Staphylococci* | | | 12 |
| | *Staphylococcus xylosus* | (99%) | |
| GRAM-ve | | | |
| Colifoms | | | 17 |
| | *Enterobacter sakazaki* | (98%) | |
| | *Serratia liquefaciens* | (98%) | |
| | *Enterobacter cloacae* | (98%) | |
| *Pseudomonas* | | | 12 |
| | *Chyseomonas luteola* | (96%) | |
| | *Pseudomonas fluorescens* | (98%) | |
| | *Pseudomonas putida* | (98%) | |
| Yeasts | | | 20 |
| | *Candida zylanoides* | (96%) | |
| | *Candida albicans* | (98%) | |
| | *Saccharomyces cerevisiae* | (98%) | |
| Fungi | | | 8 |
| | *Geotrichum capilatum* | (96%) | |
| | *Aliernaria* | (96%) | |

cultivation than gram-positive bacteria (Fig. 1a). Our results at 2–27 h demonstrated that gram-positive anaerobes and aerobes were the most predominant comprising 43–64% the total flora followed by gram-negative aerobes (1–19%). Recent analysis of bacterial communities in raw milk carried out by Raats et al. [23], demonstrated that gram-positive bacteria prevailed in the farm tanks during the incubation and *Lactococcus lactis* was the most abundant species. Indeed, the predominance of gram-positive bacteria may be due to exopolysaccharides (EPS) production and the cellular structure enhancing adherence and resistance to flow displacement and sanitization [22]. Moreover, the dominance of gram-positive bacteria in the microbial community might be due to the composition of indigenous raw milk communities [24]. Interestingly, our results showed that *Micrococcacea* adhered later than the gram-positive bacteria because they were first detected after 15 h of contact. Indeed, Soussa et al. [25] reported that late adhesion of *Staphylococcus* resulted probably from its unique surface features, manifesting themselves in its capacity for flocculation. Gram-negative anaerobes were detected in all samples (45%). Yeast and Fungi constituted 14% of the total population. This data were consistent with the previous finding of Fadda et al. [26], who revealed the low occurrence of yeast in raw milk resulting in the low level of adhered cells on the surface. Gram-negative bacteria were, although insignificantly, more adherent to silicone than gram-positive ones. Apparently, the biofilm formed at different time revealed species diversity, and the difference gradually increased with incubation time indicating that the microbial community varied during the biofilm formation and growth. Results of 16S rDNA sequencing of microbial diversity of a dairy biofilm showed that gram-positive bacteria was constituted essentially by Lactic Acid bacteria and the strains were identified as

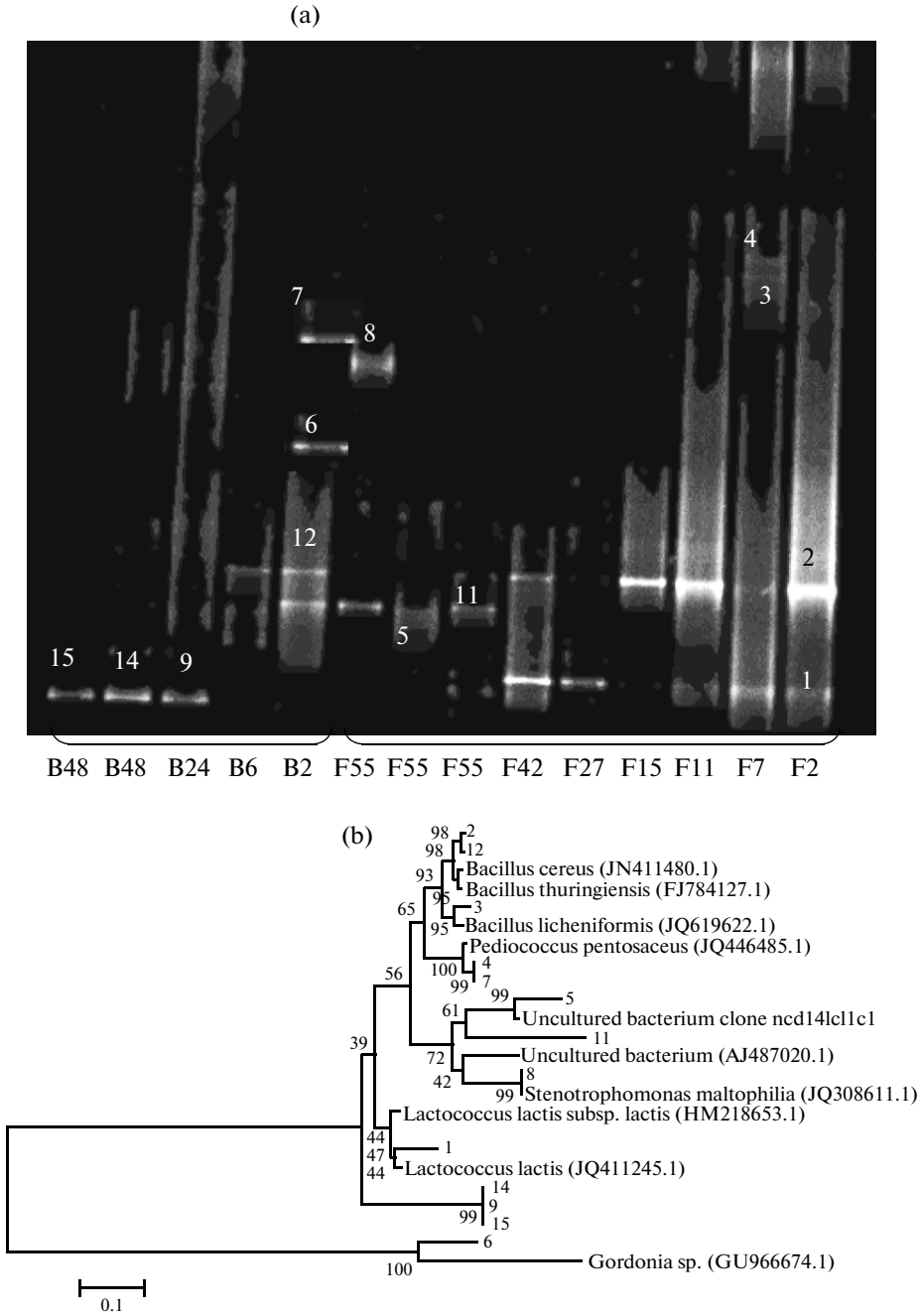


**Fig. 3.** DGGE patterns of 16S rDNA fragments amplified from dairy biofilm formation (lanes F2 to F55) (a), and impact of *Lc. lactis* bio adhesion on adhered cells (B2 to B48) (b). Dendrogram of the microbial communities from biofilm samples. The numbers in parentheses are GenBank accession numbers.

*Lactococcus lactis*, *Lb. pentosus* and *Lb. lactis* (100% similarity) (Table 1). The *Bacillus* group was identified as *B. cereus* and *B. licheniformis* (98% similarity), and the *Micrococcacea* group was essentially represented by *Staphylococcus xylosus* strain (100% similarity). Moreover, gram-negative bacteria were essentially represented by coliformic bacteria especially *Enterobacter sakazaki* and *E. cloacea* (98% similarity) and *Pseudomonas* identified as *P. putida* and *P. fluorescence* (98% similarity). *Candida albicans* and *C. zylanoides* were predominant strains on the yeast group (99% similarity). The ecological profile of biofilm development were shown to be very different at the 5 sampling times suggesting that the biofilm was remarkably unstable over time. This finding is in agreement with the reports of Van Loosdrecht et al. [21] observed that the microbial composition of a biofilm varied depending on the time of contact with dairy surface. Moreover, Bore et al. [3] reported that the dominance of gram-positive bacteria such as *Micrococcacea*, Lactic

**Table 2.** Sequences analysis of (16S rRNA genes) majors bands recovered from DGGE profiles of biofilm communities

| DGGE band number | Most related organisms Relative in NCBI database | GenBank accession no. | Similarity, % |
|---|---|---|---|
| 1 | *Lactococcus lactis* | JQ411245.1 | 97 |
| 2 | *Bacillus cereus* | JN411480.1 | 98 |
| 3 | *Bacillus licheniformis* | JQ619622.1 | 95 |
| 4 | *Pediococcus pentosaceus* strain 16 | JQ446485.1 | 100 |
| 5 | Uncultured bacterium clone ncd14lcl1c1 | HM259537.1 | 98 |
| 6 | *Gordonia* sp. | GU966674.1 | 100 |
| 7 | *Pediococcus pentosaceus*, strain 16 | JQ446485.1 | 100 |
| 8 | *Stenotrophomonas maltophilia* | JQ308611.1 | 99 |
| 9 | *Lactococcus lactis* subsp. *lactis s* | HM218653.1 | 99 |
| 10 | Uncultured bacterium clone 3-41049 | JN 319832.1 | 96 |
| 11 | Uncultured bacterium (*Burkholderia* sp.) | AJ487020.1 | 95 |
| 12 | *Bacillus thuringiensis* | FJ784127.1 | 98 |
| 14 | *Lactococcus lactis* subsp. *lactis* | HM218653.1 | 99 |
| 15 | *Lactococcus lactis* subsp. *lactis* | HM218653.1 | 99 |

Acid Bacteria, *Bacilli*, rather than gram-negative bacteria was probably due to their higher resistance and adherence to surface.

**Impact of *Lc. lactis* adhesion on dairy biofilm population.** The effect of *Lc. lactis* was assessed by different methods. Firstly, enumeration of viable cells after 48 h of *Lc. lactis* application, revealed a significant decrease of the microbial population (Fig. 1). Indeed, adhesion of *Lc. lactis* on biofilm-covered silicone resulted in a decrease of the gram-positive bacteria by up to six orders of magnitude after 48 h of contact (Fig. 1a). Interestingly, adhered thermophilic and mesophilic *Bacillus* strains were totally removed after 24 h of bio adhesion (Fig. 1a). The numbers of gram-negative bacteria decreased by seven orders of magnitude after 48 h (Fig. 1b). Although the, fungal population decreased by five orders of magnitude, it was more resistant than the gram-positive and gram-negative bacteria and adhered cells were observed after 48 h of *Lc. lactis* adhesion (Fig. 1c). These results showed that *Lc. lactis* was able to adhere to silicone surface and produce a biofilm which obviously provided for a significant reduction of adhered cells after 48 h of contact. In fact, our result was corroborated with García-Almendárez et al. [8] who showed the competitive exclusion of *Listeria* by *Lc. lactis* UQ2 after 72 h and observed that this was probably the ability of the strain to form biofilms on stainless steel. Moreover, according to ESEM observations, this bio adhesion favored a decrease of the biofilm thickness (Fig. 2b) and successful removal of attached biofilm after 48 h. ESEM micrographs revealed a haze-like film covering the microcolonies on silicone after 48 h that probably corresponded to *L. lactis* biofilm (Fig. 2b). Consequently, the ability of *Lc. lactis* to produce biofilms probably affected the final population of the biofilm on the surface, resulting from competitive removal. In fact, we suggest that *Lc. lactis* adhesion could probably induce the insufficiency of oxygen supply to the silicone interface, resulting in removal of the biofilm. Indeed, Mahdavi et al. [27] reported that an air-liquid interface appears to be very important in enhancing the attachment of bacteria to surface, and biofilm development was found to be sensitive to oxygen and nutrients availability. These findings were also consistent with the results of Habimana et al. [28] which evidenced that multiplication of an undesirable organism may be inhibited by competition for nutrients or by synthesis of antagonistic compounds such as acids, bacteriocins, or surfactants, which possess good anti adhesive properties. Indeed, recent accurate studies by Rodriguez et al. [5] determined the significant factors that may lead to an optimized factor enhanced adhesive and antimicrobial activity of *Lc. lactis* such as the strategy of silicone surface coating by bio surfactant of *Lc. lactis* able to modify the surface properties and make it simultaneously anti-adhesive and give it antimicrobial activity. Thus, it may be a step in developing the strategies to prevent microbial colonization of silicon-rubber.

**PCR-DGGE fingerprinting patterns after *Lc. lactis* bio adhesion.** Diversity of the biofilm bacterial communities and the effect of *Lc. lactis* bio adhesion on their dynamics were assessed by denaturing gradient gel electrophoresis. Indeed, many studies have reported DGGE to be one of the few techniques that allows for highly reproducible visual comparisons of the profiles from microbial communities to be derived and has been successfully applied to a wide variety of microbial ecosystems [29, 9, 30, 10]. DGGE profiles

showed that the dairy biofilm were dominated by only a few species of bacterial populations (Fig. 3a). In order to determine the dominant groups of microorganisms in the biofilm and the impact of *Lc. lactis* bio adhesion on the biofilm communities, 15 selected DNA bands were excised from DGGE gel, sequenced and subjected to BLAST GeneBank analysis. The highest similarity results are summarized in Table 2. The dendrogram for the biofilm samples showed two distinct groups (Fig. 3b). The first contained DGGE profiles of the biofilm formed on silicone after 2, 7, 15, 27, 42, and 55 h. The second contained DGGE profiles of the impact of *Lc. lactis* adhesion on biofilm communities adhered on silicone after 2, 6, 24, and 48 h. After 42 h of biofilm formation, the dominant bands in samples F2 to F42 were affiliated to the phylum *Firmicutes* (e.g., bands 1, 2, 3 and 4 corresponding to *Lactococcus lactis, Bacillus cereus, Bacillus licheniformis*, and *Pediococcus*, respectively, Fig. 3 and Table 2). Significant changes in the bacterial community composition were observed after 48 h (samples F55), when other bands became visible, affiliated to *Ochrobactrum, Stenotrophomonas maltophilia*, and *Burkholderia* sp. (e.g., corresponding to bands 5, 8 and 11 respectively, Fig. 3 and Table 2). Irrespective of the feeding regime employed, both facultative anaerobes and aerobic species became rapidly established, while strictly anaerobic species, such as the gram-negative anaerobes, took up more time to establish. This finding was consistent with the previous study of Mc Bain et al. [31], who reported that aerobic bacteria were rapidly adhered to oral plaque. After application of *Lc. lactis* on silicone, changes and different bacterial population dynamics was observed in the samples B2 to B48 (Fig. 3a). The group that contained DGGE profiles of samples B2 and B6 was characterized by a low number of bands distributed over the gel gradient corresponding to *Gordonia* sp., *Pediococcus pentosaceus*, and *Bacillus thuringiensis* (e.g., bands 6, 7, and 12, respectively, Fig. 3 and Table 2). Major changes in the DGGE band composition after 24 and 48 h of *Lc. lactis* adhesion could be observed in samples B24 and B48. Reduction in the number of bands in these samples was followed by the disappearance of most of the bandes in sample s B24 and B48 with increased intensity of the bands 9, 14, and 15 corresponding to the *Lc. lactis* strain (Fig. 3, Table 2). Indeed, bio adhesion might be favorable for development of the anaerobiosis at the interface and may decrease the proliferation of aerobic bacteria [30].

This study resulted in better understanding of dairy biofilm formation under dynamic flow which deeply affects its structure. Moreover, we demonstrated that *Lc. lactis* is able to produce a biofilm which obviously exhibited an immediate bactericidal activity and was able to remove adhered cells from the substrate. Nevertheless, protective biofilms of *Lc. lactis* on industry workshop surfaces may be beneficial and their presence may modify the physico-chemical properties of the substrates, reduce adhesion of the undesirable microorganisms and favor the antimicrobial coating of devices making it simultaneously anti-adhesive and give it antimicrobial activity.

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