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Study of Biofilm Formation from Lactobacillus Fermentum S Cultivated on Different Carbohydrates.

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ABSTRACT

Bacterial biofilms are a source of many chronic infections. Biofilms and their inherent resistance to antibiotics are attributable to a range of health issues. Microorganisms can interact with the different surfaces differently. Most often they form biofilms on contacting surfaces, but can also react with various metals to form complex compounds. Yet bacterial biofilms remain poorly understood and strategies for their control remain underdeveloped. The aim of the present study is to analyzed the structure of the biofilms developed by EPS of the species Lactobacillus fermentum S, cultivayed on different carbohydrates by using different techniques including Congo Red Agar method (CRA), scanning electron microscopy (SEM) and, atomic force microscopy (AFM). When cultivated in a media with high content of saccharides such as 10% solutions, strain L. fermentum S synthesizes exopolysaccharides. The different black staining is caused by different mechanisms for synthesis of EPS of the species depending on the presence of different carbohydrates – into and extracellular. A biofilm formed on the steel surface which is an indicator of the good adhesive capacity of L. fermentum S type. Altogether, our data suggest that different techniques have to be used to clarify the process influenced by microorganisms.

Keywords: probiotic bacteria; microbial biofilms, congo red metod, scanning electron microscopy, atomic force microscopy

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INTRODUCTION

Although many bacteria can grow in a free-living, 'planktonic' state, it is quite common for them to adhere to surfaces by producing extracellular polysaccharide or in some cases by means of specialised structures termed holdfasts. The adherent bacteria produce microcolonies, leading to the development of biofilms. The biofilms initially may be composed of only one bacterial type, but frequently develop to contain several bacteria living in a complex community. Microorganisms growing in a biofilm are associated with chronic and recurrent human infections and are highly resistant to antimicrobial agents. Biofilms are defined as microbially derived sessile communities characterized by the cells that are irreversibly attached to a substratum or to each other. They are embedded in a matrix of extracellular polymeric substances (EPS) that have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription [1]. Microbial exo polysaccharides (EPSs) are high-molecular-mass polymers primarily produced by bacteria, fungi and blue-green algae [2]. The generally recognized food-grade lactic acid bacteria (LAB) can produce different EPSs including homo polysaccharides and hetero polysaccharides based on their monosaccharide composition [3]. Recently, the EPSs from LAB have also been received with increased attention by virtue of their antibacterial [4,5,6], antioxidant [7], immune modulatory [8] and antitumor [9] activities. Production of slime factor also plays an important role in antibiotic resistance and it has been reported that slime producing strains are more resistant to antibiotics than non-slime producing strains [10]. All microbes like Gram positive and Gram negative bacteria have capacity to synthesized biofilm. Multidrug-resistant organisms have been reported worldwide and are now recognized as one of the most difficult healthcare-associated infections to control and to treat [11]. Study of microbial biofilms has received significance attention over the past decades. Therefore, studies and diagnostic methods identifying virulent bacterial strains, i.e., strains with a capacity for slime production. Consequent biofilm formations are necessary to develop effective strategies for biofilm control and improvement of patient care.

There are various methods to detect biofilm production like Tissue Culture Plate (TCP), Tube method (TM), Congo Red Agar method (CRA), bioluminescent assay, piezoelectric sensors, and fluorescent microscopic examination. Although EPSs produced by LAB have been studied extensively, molecular modification of the EPSs and the resulting changes in molecular characteristics and functionalities of the polymers are poorly studied.

The aim of the present study is to analyzed the structure of the biofilms developed by EPS of the species L. fermentum S, cultivated on different carbohydrates by using different techniques including Congo Red Agar method (CRA), scanning electron microscopy (SEM) and, atomic force microscopy (AFM).

MATERIALS AND METHODS

Place and duration of the study: The study was conducted at the Department of Biology, University of Shumen, Bulgaria, from January 2016 to June 2016.

Strain: L. fermentum S was obtained from Collection of Department of Biology, Shumen University. The strain cultivated in media of MRS (de Mann Rogosa Sharpe, Biolife 272-20128, Milano, Italia). Molecular analysis in LAB (lactic acid bacteria) was performed by molecular identification (16S rRNA gene sequencing) in GeXP Genetic Analysis System (Beckman Coulter, USA) [12]. The pH of media was adjusted to 6.5 with 1M NaOH. The basic media was sterilized by autoclaving at 121°C for 20 min.

Biofilm detection was done by the following methods:

Media for study of EPS sintesis: The strain cultivated in media of MRS (de Mann Rogosa Sharpe, Biolife 272-20128, Milano, Italia). The basic media was sterilized by autoclaving at 121°C for 20 min, and carbohydrates supplemented were sterilized using 0.22 µM filters (Manisart®). The basic MRS broth was supplemented with 10% glucose,10% sucrose, 10% fructose, 10% galactose and 10% maltose to be tested [12-16].

Media for study of microbial biofilm with congo red agar CRA: The strain cultivated in media of MRS (de Mann Rogosa Sharpe, Biolife 272-20128, Milano, Italia) with 10% glucose,10% sucrose, 10% fructose, 10% galactose, 10% lactose and 10% maltose and congo red. CRA plates were inoculated with test organisms and

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incubated at 37° C for 24 h aerobically. Black colonies with a dry crystalline consistency indicated biofilm production [19]. The experiment was performed in triplicate and repeated three times.

Study of bacterial adhesion: Before the assays, the strain L. fermentum S were twice pre-cultured in MRS broth, for 24 h at 37°C. Exponential culture in both were used as inoculum for the adhesion analysis.

Preparation of the metal samples: The steel plates made of low carbon steel are weighed with an allowance of 0,0001g with an assay-balance. They are put sterilely in a liquid ambient which contains a L. fermentum S. The samples were incubated at 37°C for 24 h. The structure of the layer over the metal plates was analyzed by SEM (scanning electron microscopy) JSM 5510 and atomic force microscopy (AFM) . All experiments were performed in triplicate [17].

Analysis by means of AFM microscopy:

An AFM Anfatec Instruments AG, Germany was used for characterization of surface topology. The measurements were realized in non-contact mode when the tip was scanning over the studied surface at a distance of few nanometers. A silicon nitride tip with a curvature radius of about 10 nm and force constant about 43 N/m was used. The three-dimensional images of scanned samples were created with ANFATEC PRESENT software.

RESULTS

Therefore, the presence of a translucent or creamy material involving a mucoid colony is indicative of EPS production potential. When cultivated in a media with high content of saccharides such as 10% glucose solution, 10% sucrose solutions, 10% fructose solutions, and 10% maltose solutions, strain L. fermentum S synthesizes exopolysaccharides (Fig. 1).

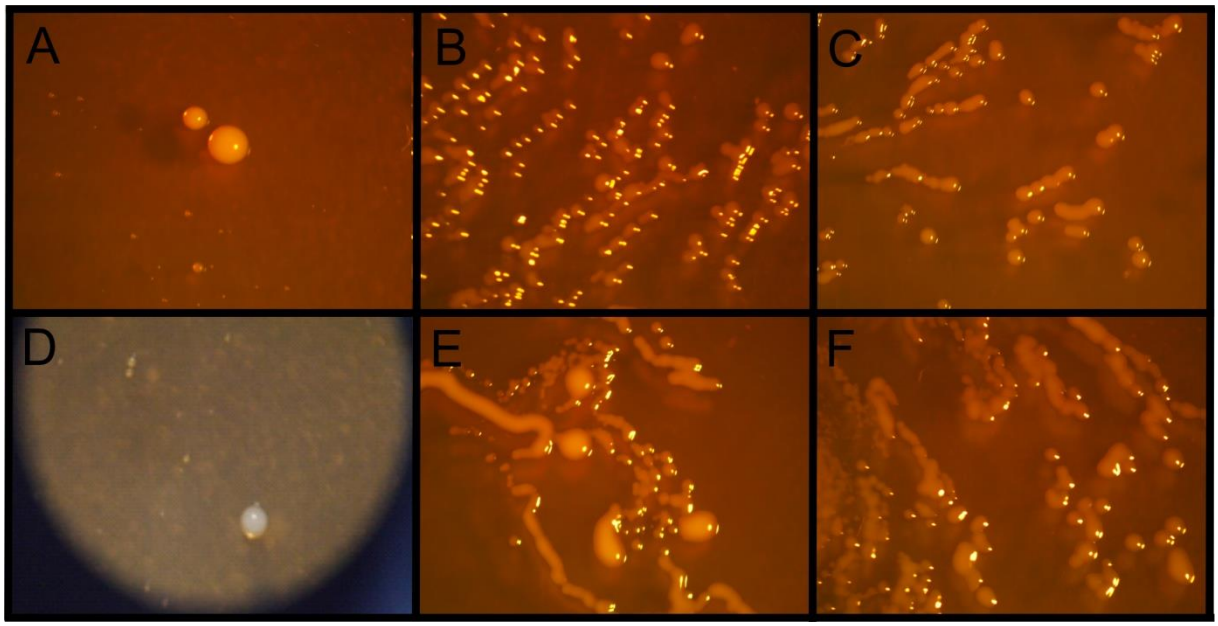

Figure 1: EPSs (exopolysaccharides) produced by L. fermentum S cultivated in a media containing 10% carbohydrate, which are secreted in the culture medium: A: 10% glucose;B 10%fructose;C10%sucrose; D10%galactose; E10%lactose; F10%maltose.

The pictures were taken using stereomicroscope OPTIKA (Italy).

Only by cultivation in media with 10% galactose solution, the species did not formed mucoid colonies (Fig. 1 D).

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For chemical analysis of the formed microbial biofilm was used congo red agar CRA method and the results are represented on Figure 2.

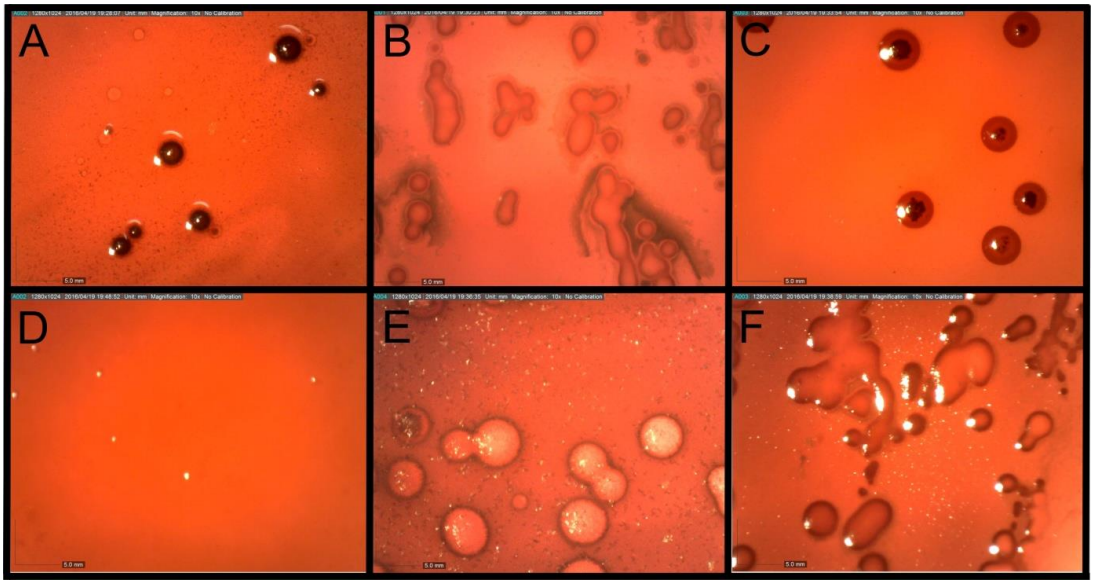

Figure 2: EPSs (exopolysaccharides) produced by L. fermentum S Congo red agar method: A: 10% glucose;B 10%fructose;C10%sucrose; D10%galactose; E10%lactose; F10%maltose. Black colonies shows biofilm formation of LAB, cultivated in media contained different carbohydrates. The pictures were taken using stereomicroscope OPTIKA (Italy).

In the presence of high concentrations of sugars (as in our case 10%) lactic acid bacteria synthesize extracellular exopolysaccharide (Figure 1A,B,C,E and F), which is displayed as mucoid colonies on agar medium. By adding the staining congo red, the exopolysaccharides produced by lactic acid bacteria are displayed in black (Figure 2A,B,C,E and F). We hypothesis, that the different black staining is caused by different mechanisms for synthesis of EPS of the species depending on the presence of different carbohydrates – into and extracellular. By using the method, we demonstrate that by presence of galactose, the species did not formed mucoid colonies (Figure 2 D).

The structure of the layer over the steel plates was analyzed by Scanning electron microscopy. The results from this procedure are shown in figure 3.

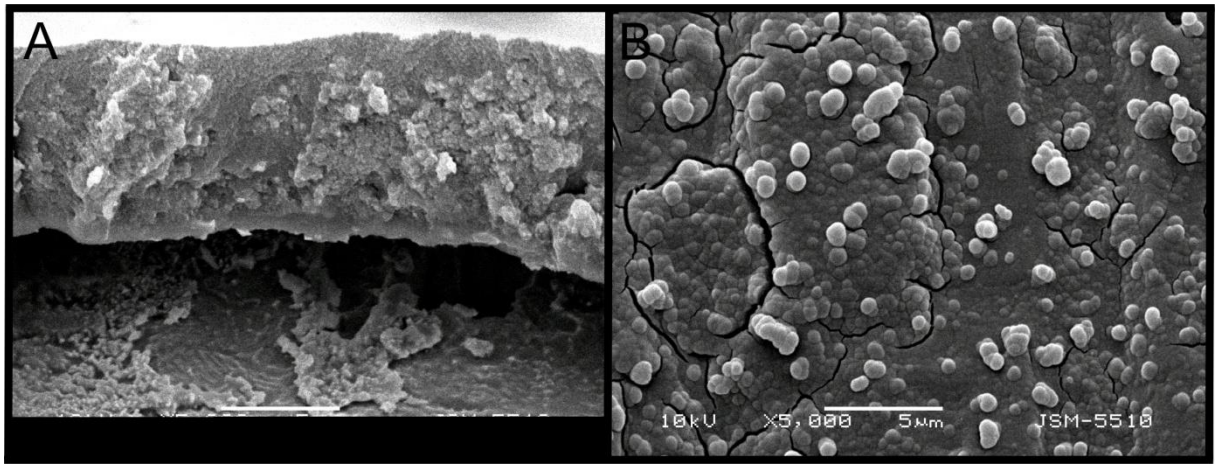

Figure 3: Biofilm formed by L. fermentum S on the surface of mild steel, visualized using SEM. A) Biofilm formed by EPS from lactic acid bacteria cultivated with 10% maltose; B) Biofilm formed by lactic acid bacteria cultivated with 10% maltose.

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Microscope techniques provide information about the morphology of microbial cells and colonies, their distribution on the surface and the nature of corrosion products (crystalline or amorphous). They can also reveal the type of attack (e.g. pitting or uniform corrosion) by visualizing changes in microstructure and surface features after removal of the covering and corrosion products (figure 3).

The pictures in Fig. 3B show that there's a biofilm formed on the steel surface which is an indicator of the good adhesive capacity of L. fermentum S type. Microorganisms can interact with the metal surfaces differently. Most often they form biofilms on contacting surfaces, but can also react with various metals to form complex compounds. For this reason we think that different techniques have to be used to clarify the biofilm formed by microorganisms. Scanning electron microscopy is a very powerful tool to study surface morphology of macromolecules which helps to know about its common physical properties.

AFM imaging of biopolymer as polysaccharides was generally conducted in air or under a liquid in order to avoid excessive dehydration. The topographical AFM images of L. fermentum S EPS were shown in Fig.4.

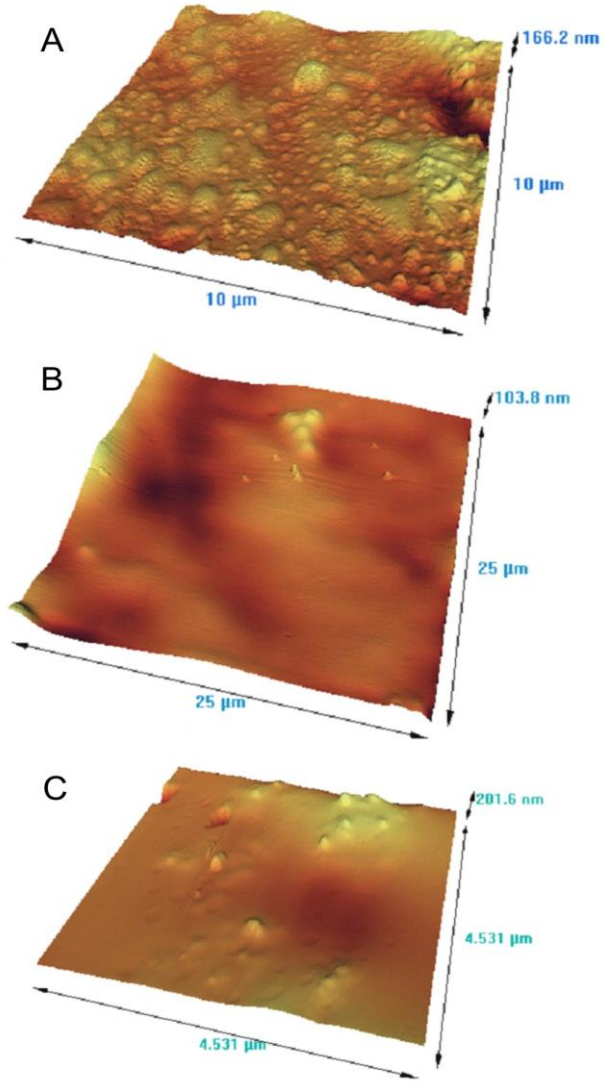

Figure 4: Atomic force microscopy (AFM) images of molecular structure of L. fermentum S EPS. A) Biofilm formed by lactic acid bacteria cultivated with 10% glucose; B) Biofilm formed by lactic acid bacteria cultivated with 10% fructose; C) Biofilm formed by lactic acid bacteria cultivated with 10% maltose.

L. fermentum S EPS deposited from 10 g/mL aqueous solution, roundness lumps and chains can be seen (Fig. 4 A,B and C).

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DISCUSSION

The presence of EPS associated with bacterial cells can be recognized by the formation of colonies in mucous solid medium [12,13]. In our previous studies [12-16], it was shown that at the presence of high concentration of lactose (5% to 15%), high concentration of sucrose 4%, mixed sucrose 4% and 2% maltose and mixed sucrose 5% and 5% maltose, mixed 5% sucrose and 5% fructose and mixed 5% sucrose and 5% fructose the strains Lactobacillus delbrueckii B5, L. delbrueckii K27, L. delbrueckii B8, L. delbrueckii O43, L. delbrueckii K3, L. delbrueckii K17, and L. delbrueckii K15 synthesized exopolysaccharides which have inhibitory properties. It is well known that some lactobacillus strains such as genus Leuconostoc secreted trans glucosidases after cultivation in the presence of sucrose. Similar experiments have also been demonstrated by other authors [17,18]. Homo polysaccharides produced by GRAS (Generally Recognised as Safe) lactic acid bacteria are often synthesised by a single extra-cellular sucrose enzyme, using only sucrose as substrate [18]. They can be produced in largest quantities (bulk scale). Moreover, their structure can be modified allowing optimisation of their physicochemical properties.

Our studies also show that the use of sugar supplementation (carbochidrates was normally used though similar results were obtained using 10%) is essential for the detection of slime production using the Congo red medium. The Congo red method is rapid sensitive and reproducible and has the advantage that colonies remain viable on the medium" [19].

Congo Red Agar allows for the direct analysis of the colonies and the identification of slime-forming strains (which appear as black colonies on the red agar) and non-slime-forming strains (yelow-coloured colonies). This is not a quantitative assay because it is based on a subjective chromatic evaluation. The strains that score positive during the test have black spikes on red colonies which remain unchanged in colour.

The SEM images of the EPS showed a stacked flakes with relatively uniform shapes Fig.3A. Similar porous web microstructure of EPS was reported earlier with the EPSs produced by Streptococcus thermophilus GST-6 [20] and Lactobacillus plantarum strains [2,21]. Chen M., and colleagues [4] showed that after sulphonation the appearance of polysaccharide fragments without uniform size could be changed into regular even structure similar to tile. This was in agreement with the similar micro structural change described above for the EPS from strain L. fermentum S of this study. However, Zhang and colleagues [23] demon strated a polyphasic convoluted structure by SEM of a sulphated persimmon polysaccharide. Sulphonation of polysaccharides, involving both dehydrolysis and hydrolytic degradation [24], could change appearance of the polymers. These reactions resulting in solubility change of the polysaccharides [25], together with the etch effect of hydrolysis during sulphonation were thought to be important factors affecting appearance of sulphated polysaccharides. However, the molecular mechanism of how sulphonation affecting micro structure of EPS needs to be further studied.

The AFM-based single-molecule force spectroscopy (AFM–SMFS) technology is a powerful tool to characterize the force-induced conformational transitions, the dynamics, and super molecular structures of polysaccharides at the molecular level [26,27,28].

The maximal height of lumps at 10% solution of glucose is 166,2 nm, at 10% solution fructose is 193nm and 10% solution maltose is 201,6nm. This result suggested that L. fermentum S EPS could combine water in the aqueous. Furthermore, it showed pseudo plastic behavior because the strong interaction between water molecules and the hydroxyl groups (−OH) of L. fermentum S EPS. A similar experiment was reported about an acidic polysaccharide from Mesona blumes gum [21,29]. The AFM images of Mesona blumes gum showed different shapes, spherical lumps and worm, respectively in low and high concentration. The reduction in viscosity could also be attributed to polymer degradation due to the cleavage of glycosidic bonds with in the polysaccharide structure [30]. These results showed a potential choice for L. fermentum S EPS to be selected as biothickener and stabilizer agent. And strain L. fermentum S can be chosen as the starter to overcome the problem of syneresis in yoghurt industry.

The microorganisms, in order to survive and to form stable microbial population, create a microbial biofilm, which however makes them much more stable against antibiotics compared to when they are in a free state. So arise biomaterial-centered infections (BCI). The ability of the microorganisms for adhesion to different surfaces is determined on the one hand to the species and their metabolism and on the other by the

type and elemental composition of the material itself. A good possibility for the removal of BCI's eventual use of nanocover of "good bacteria"-probiotics. The search for biomaterials that are able to provide for the optimal resistance to the infection can be based only on the deep understanding of the interactions between bacteria and biomaterials.

According to a public announcement by the US National Institutes of Health, “Biofilms are medically important, accounting for over 80% of microbial infections in the body”. Yet bacterial biofilms remain poorly understood and strategies for their control remain underdeveloped. Standard antimicrobial treatments typically fail to eradicate biofilms, which can result in chronic infection and the need for surgical removal of afflicted areas. The need to create effective therapies to counter biofilm infections presents one of the most pressing challenges in anti-bacterial drug development. The research on microbial exopolysaccharides is attracting increased attention. By the analysis of the microbial biofilm we recommend to use all methods in combination, as every of them provids different information for the type and the mechanisms of synthesis of the biofilm and the EPS. In this study, the EPS producing strains L. fermentum S was identified. It suggested from the SEM and AFM images that L. fermentum S EPS has potential ability to produce the biofilm. Further work is needed to investigate the applications and function in vitro of this EPS.

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