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ORIGINAL ARTICLE

Surface properties and exopolysaccharide production of surface-associated microorganisms isolated from a dairy plant

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Abstract

Purpose The purpose of this study was to isolate the surface-associated microorganisms from the dairy plant surfaces with a high probability of biofilm formation and determine the most adhesive strains in terms of surface properties and exopolysaccharide production.

Methods Four hundred and ninety-five surface-associated microorganisms were isolated from potential biofilm-forming surfaces of a dairy plant. One hundred and seventy of these were isolated after cleaning/disinfection of the pasteurized milk, white cheese and butter tank, yogurt and ice cream filling unit, ice cream air pressing, and condensed milk pipe. It is noteworthy that some isolates might cause post-production contamination, food infection, and intoxication. Selected 42 isolates were identified by Gram staining, physiological and biochemical tests, and 16S rRNA gene sequencing. Then, surface properties and exopolysaccharide production of 10 selected isolates were determined. To evaluate the surface properties, microbial adhesion to hydrocarbons, static water contact angle, salt aggregation, and surface zeta potential tests were performed.

Result The microbial adhesion to hydrocarbons (MATH) test exhibited the lowest standard deviations, and the most consistent results between the replicates. The highest hydrophilic characteristics and exopolysaccharide production were exhibited by Gram-negative *Pseudomonas aeruginosa*, followed by Gram-positive *Bacillus toyonensis*. Also, a significant diversity of neutral sugar was determined in their alditol acetate forms by using gas chromatography–mass spectrometry. In this context, it is believed that the determination of the EPS content of the isolates would contribute to establishing an effective cleaning/disinfection procedure for dairy plants.

Conclusion This study indicated that microbial adhesion is still a common problem in the dairy industry. Because of this situation, dairy plants should be organized and constructed to be suitable for hygiene and sanitary applications.

Keywords Dairy plant · Adhesion · Identification · Surface property · Exopolysaccharide production · Neutral sugar content

Introduction

Biofilm is a microbial-derived cell community located in a matrix that contains extracellular polymeric substances, which allows microorganisms to bind irreversibly to living and non-living surfaces or irreversibly to each other. Microorganisms in biofilms have a different phenotype in terms of their reproductive rate and gene transcription profile compared with planktonic types (Donlan and Costerton 2002). These microorganisms are typically more virulent, more easily adaptable to changing environmental factors, and are able to develop resistance to antibiotics (Watnick and Kolter 2000).

Numerous examples of the microorganisms are isolated from dairy plants in literature (Cherif-Antar et al. 2016; Soares et al. 2011). The microorganisms, separated from the surfaces by various methods, are then isolated and counted by cultural methods following inoculation into selective and/or non-selective media. Various methods can be used for the isolation of biofilm-forming microorganisms from surfaces. These methods include the use of swabs (Marques et al. 2007; Valeriano et al. 2012), humidified swabs (Lortal et al.

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2009; Waines et al. 2011), gauze (Tang et al. 2011), scraping (Frank and Koffi 1990), sponges (Knight and Craven 2010), sonication and centrifugation (Bjerkan et al. 2009; Kajiyama et al. 2009), surface washing, immersion in a washing solution, agar sausage (Harrigan 1998), cutting (Lortal et al. 2009), and vortexing (Mustapha and Liewen 1989).

Identification of the microorganisms is generally carried out by morphological, physiological, and biochemical tests. Biochemical tests usually include API test kits (BioMérieux, France) (Bağcı 2012; Brolazo et al. 2011; Palmer et al. 2010). Traditional microbiological techniques require a long time and a lot of chemicals, media, and labor. However, these techniques may also be insufficient for identifying species when used alone. In addition to these tests, microorganisms can be identified by molecular microbiological techniques such as determination of DNA base composition (using PCR techniques), DNA hybridization tests, FISH (fluorescence in situ hybridization), and 16S rRNA gene sequencing (Palmer et al. 2010; Waines et al. 2011).

The surface properties of microorganisms are important factors in the mechanisms of adhesion. The term macroscopic hydrophobicity refers to the wettability of a surface in an air environment (Ukuku and Fett 2002). Microbial hydrophobicity is an important factor that affects adhesion to a surface, i.e., biofilm formation. Microbial hydrophobicity can be determined by methods such as microbial adhesion to hydrocarbon test or through hydrophobic interaction chromatography (Rijnaarts et al. 1995).

Studies have shown that both adherence-cohesion interactions on a surface and the ability of the microorganisms to produce extracellular polymeric substances (EPS) are important for adhesion (Chen and Stewart 2002; Drenkard 2003). It has also been shown that EPS production is a precondition for supporting the formation of biofilms on surfaces and enhancing adhesion. Therefore, it is of great importance to understand the ability of surface-adhering microorganisms to produce EPS and to understand the content of EPS they produce.

The aim of this study was first to determine the surfaces having a potential risk of biofilm formation in dairy plants. For this purpose, microorganisms were isolated from a dairy plant in Ankara. Isolation was applied from the surfaces with a high probability of biofilm formation by using general and selective media. The selected isolates were identified by morphological, physiological, and biochemical tests and 16S rRNA gene sequencing. The selected isolates were evaluated based on their ability to produce EPS and their morphological structure. The surface properties of the microorganisms were assessed using the microbial adhesion to hydrocarbons (MATH) test, the static water contact angle test, the salt aggregation test, and the surface zeta potential test. EPS were isolated from microorganisms and their total sugar, uronic acid, and neutral sugar contents were determined.

Materials and methods

Isolation and identification of microorganisms from a dairy plant

Microbiological samples were taken from 13 sampling points from a dairy plant in Ankara, Turkey, in order to isolate surface-associated microorganisms. The sampling points were: (1) raw milk tank, (2) pasteurized milk tank, (3) starter tank, (4) yogurt-filling unit (350 g), (5) yogurt filling unit (1500 g), (6) white cheese tank, (7) white cheese-pressing cloth, (8) kashar maturing bench, (9) old kashar maturing bench, wood, (10) butter tank, (11) ice cream filling unit, (12) ice cream air pressing pipe, and (13) condensed milk pipe. Sampling was generally repeated twice, before and after the cleaning/disinfection step. Samples were assigned a two-digit code, the first digit indicating the sampling point, and the second digit indicating whether sanitation has been applied or not, with 1 and 2 representing that the sample was taken either before or after the sanitation step, respectively.

Microbiological sampling was carried out by rubbing a moistened swab (with 0.1% buffered peptone water) strongly in different directions on the sampling surface. The swabs were then immersed in tubes containing 2 mL of Tryptic Soy Broth (TSB, Sigma-Aldrich), and the tubes were vortexed for 2 min to allow the passage of microorganisms into the broth (Marques et al. 2007; Valeriano et al. 2012; Waines et al. 2011).

The agar media and incubation temperatures used for the isolation of microorganisms are shown in Table 1.

Microbiological cultivation was carried out using the spread plate technique as two replicates (Harrigan 1998; Temiz 2010). Inoculated Petri dishes were incubated at the optimum growth temperature of the target microorganisms (Table 1). The incubation times were 24–48 h for the bacteria and 24–96 h for the yeasts. After incubation, the colonies developed on the agar media were examined to assess their morphological characteristics, and colonies showing different morphological characteristics were selected and their pure cultures were obtained. In order to obtain pure cultures, the individual colonies firstly re-streaked onto the same selective media which they were isolated from, and then the colonies which were grown on the selective media were re-streaked onto the nutrient agar (Merck) plates. Isolated pure cultures were maintained as stock cultures at − 80 °C in a brain heart infusion (BHI, Merck) broth medium containing 20% glycerol (*v*/*v*) for further analysis. Intermediate stock cultures were prepared using nutrient agar slant from the stock cultures, and they were stored in a refrigerator at 0–5 °C with regenerating every 3 months. From these intermediate stock cultures, 24-h cultures were obtained in a nutrient broth (Merck) medium and they were used for further analysis.

Table 1 The agar media and incubation temperatures used for the isolation of the target microorganisms

The agar media	Target microorganisms	Incubation temperature (°C)
Violet red bile dextrose agar (VRBDA, Merck)	*Enterobacteriaceae* members	37 ± 1
Fluorocult violet red bile agar (Merck)	*E. coli*	37 ± 1
Baird parker agar (BPA, Oxoid)	*Staphylococcus* spp.	37 ± 1
Pseudomonas agar base (PA, Sigma-Aldrich) with CFC (cetrimide, fucidin, cephalosporin) supplement	*Pseudomonas* spp.	37 ± 1
Lactobacillus agar acc. to De Man, Rogosa and Sharpe (MRS agar, Sigma-Aldrich)	*Lactobacillus* spp.	30 ± 1
M17 agar (Oxoid)	*Lactococcus* spp.	37 ± 1
Chromogenic *Listeria* agar (OCLA, Oxoid)	*Listeria* spp.	37 ± 1
Dextrose casein-peptone agar (DCPA, Merck)	*Bacillus* spp.	37 ± 1
Brilliant green phenol red lactose sucrose agar (BPLS Agar, Merck)	*Salmonella* spp.	37 ± 1
Yeast extract agar (YEA, Sigma-Aldrich)	Yeasts	30 ± 1

The microscopic morphology of each pure culture was determined and an API test kit (bioMérieux, France) was used to identify them at the species level. The API test kits used were as follows**:** API 20 E for *Enterobacteriaceae* members; API 20 NE for *Pseudomonas* spp.; API Staph for *Staphylococcus* spp.; API *Listeria* for *Listeria* spp.; API 50CHB for *Bacillus* spp.; API 50CHL for *Lactobacillus* spp.; API Strep for *Lactococcus* spp.; and API 20C AUX for yeasts.

API test kits do not always give consistent results for the identification of lactic acid bacteria (Brolazo et al. 2011; Martín et al. 2010). To ensure the discrimination of lactic acid bacteria, some basic morphological, physiological, and biochemical tests (Gram staining, catalase activity, gas production from growth in glucose, growth at 10 °C and 45 °C, growth in 2%, 4%, and 6% salt-containing media, growth at pH 9.6, arginine hydrolysis, and an hemolysis test) were also used to assess suspected isolates of lactic acid bacteria from the MRS agar and M17 agar media.

To identify pure cultures at the species level, the isolates were also subjected to 16S rRNA gene sequencing using universal primers (Sanger et al. 1977). DNA extraction was performed using Qiagen DNeasy Blood & Tissue Kit (Qiagen Inc., Valencia, CA). The 16S rRNA genes were amplified using the primer pairs of 27F (5′-AGA GTT TGA TCC TGG CTC AG) and 907R (5′-CCC CGT CAA TTC ATT TGA GTT T). The PCR mix (50 μL) was prepared from 1 μL dNTP, 5 μL 10× buffer, 0.3 μL 27F primer, 0.3 μL 907R primer, 0.3 μL Taq polymerase (Boehringer GmbH, Mannheim, Germany), and 2 μL $MgCl_2$. Five hundred nanograms of DNA extract was amplified with the PCR mix. Polymerase chain reaction amplification was performed with the following thermal conditions: 94 °C for a 2-min step, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and finished by a 72 °C for a 5-min step. PCR purification was applied with Promega PCR Purification kit (Promega, Madison, WI, USA). Sequence results were evaluated using the NCBI BLAST program.

Surface properties of microorganisms

Microbial adhesion to hydrocarbons, static water contact angle, salt aggregation, and surface zeta potential test measurements were performed in triplicate as described below.

Microbial adhesion to hydrocarbons test

The cell surface hydrophobicity of the identified microorganisms was determined using the microbial adhesion to hydrocarbons test and reported as % H. The absorbance values of 24-h cell cultures before and after the application of n-decane (BDH Chemicals, UK) were measured at 400 nm using a spectrophotometer (Thermo Scientific, iCE 3000 Series Atomic Absorption Spectrometers, USA) (Rosenberg et al. 1980). In this method, the absorbance value before the n-decane application (A_0) was firstly measured by the spectrophotometer. Then 1 mL of n-decane was added onto 3 mL culture suspension. The suspension was then vortexed for 2 min and allowed to stand for 15 min to remove the hydrocarbon. The absorbance value after the n-decane application (A) was measured by the spectrophotometer from the underlying phase of the two-phase mixture. Surface hydrophobicity (% H) was determined using the following formula.

$$\%H = [(A_0 – A)/A_0] \times 100$$

A_0 The absorbance value before the n-decane application
A The absorbance value after the n-decane application

Static water contact angle test

The static water contact angle values of the identified microorganisms were determined using the sessile drop method described by Absolom et al. (1983). Three replicates were studied for each measurement using a goniometer (Krüss DSA 100, Germany) at room temperature.

Salt aggregation test

Eighty microliters 24-h culture was transferred to 80 μL ammonium sulphate solution at different concentrations (between 0.01 and 4 M) in each well of a cavity slide. Crystal violet was added to make the agglutinates visible. The salt aggregation test (SAT) value was obtained by observing the lowest concentration of ammonium sulfate that produces visible agglutinates (Styriak et al. 1999).

Surface zeta potential test

Before measurements, the 24-h cultures (viable cell number: 1.5×10^8 CFU/mL) of test microorganisms were prepared. Measurements were then made using a Zetasizer (3000HSA, Malvern Instruments, UK).

Isolation of exopolysaccharides

The microorganism was inoculated into 10 mL of sterile skimmed milk, and incubated at 37 °C for 24 h. One milliliter of 4% trichloroacetic acid (*v*/*v*) was then added to each culture to precipitate proteins and cells, and the mixture was incubated in a shaking water bath at 37 °C for 2 h. The suspension was centrifuged for 35 min at 10,000×*g* at 4 °C, and the supernatant was separated and filtered through a 0.45-μm membrane filter (Millipore, USA). Following this, 96% cold ethyl alcohol (*v*/*v*) was added to the supernatant at ratios of 1:1, 1:2, and 1:3 followed by centrifugation at 5000×*g* for 15 min at 4 °C after each treatment. The resulting EPS were dissolved in 1 mL of water and filtered through a 0.45-μm membrane filter. Finally, the EPS were obtained in dry form by lyophilization using a freeze dryer (ALPHA 1–4 LDplus, Christ, Germany) (Yang 2000).

Determination of total sugar

The total sugar was determined spectrophotometrically using the phenol sulfuric acid method described by DuBois et al. (1956).

Determination of uronic acid

The uronic acid content of the isolated EPS was determined spectrophotometrically following conversion to galactonic acid (Blumenkrantz and Asboe-Hansen 1973).

Determination of neutral sugar

The neutral sugars predominant in the EPS derived from each microorganism were identified using GC-MS. EPS (10–100 μg) were first treated with 0.5 N NH_4OH solution (100 μL) and incubated at room temperature for 10–15 min in a closed tube. Next, 1 mg of $NaBH_4$ was added and the solution was kept closed for 10 min at 100 °C. At this stage, aldoses are reduced to alditols. The tube contents were dried at 55 °C. Excess $NaBH_4$ was cleaved by the addition of 100 μL of 2 M trifluoroacetic acid. Then, 100 μL of methanol was added and the solution was dried at 55 °C to remove the cleavage products (repeated two times). The residue was dissolved with 0.5 M HCl (200 μL) in methanol, and the solution was kept closed at 100 °C for 15 min and dried at 55 °C. During this step, the alditols are derivatized to methyl ester alditol forms. These methyl ester alditols were acetylated by adding a mixture of pyridine-acetonitrile (200 μL; 1:1, *v*/*v*) and incubated at 100 °C for 30 min in a closed tube. The percentage distribution of neutral sugars in the EPS solution isolate was quantitatively determined using GC-MS with a TRACE DSQII (Thermo Fisher Scientific, Waltham, MA, USA) (Sassaki et al. 2008).

The conditions used for the GC-MS analysis were as follows: injector, split mode; injection volume 2 μL; detector MS DSQII; column: 7HG-G006–11 Zebron ZB-1701 capillary GC column (30 m × 0.25 mm × 0.25 μm, Phenomenex); carrier gas: helium (1 mL/min); mass range 35–500; temperature program: 60 °C for 5 min, 60 °C–270 °C at 10 °C/min, 270 °C for 15 min; ion source temperature: 230 °C; transfer line temperature: 270 °C.

Results

Isolation and identification of microorganisms from the dairy industry

Totally, 495 isolates were obtained using the different agar media. Among these, 36 were isolated from VRBDA, 38 from FVRBA, 34 from BPA, 19 from PA, 55 from MRS agar, 78 from M17 agar, 17 from OCLA, 66 from DCPA, 20 from BPLS agar, and 132 from YEA media. Of the 495 colonies isolated, there were 163 typical and 332 atypical colonies. Totally, 170 isolates were obtained after the sanitation steps in the dairy plant. These microorganisms were isolated after the cleaning/disinfection processes of the tanks of pasteurized

milk, white cheese and butter, the filling units of yogurt and ice cream, and the pipes of ice cream air pressing and condensed milk. Eighty-six out of 170 isolates produced mucous colonies.

The isolated microorganisms were grouped according to diversity in their macroscopic (colony shape and structure, typical or atypical colony formation, etc.) and microscopic morphologies, Gram-staining reactions (for bacteria), physiological and biochemical test results, behavior in the dairy industry (saprophyte or fecal contamination indicator), their pathogenicity, and toxicity. Among these, 42 isolates were selected for identification at the species level using API test kits and 16S sequence analysis. These 42 isolates, identified by API and 16S rRNA gene sequencing, were re-grouped according to diversity in their macroscopic (colony shape and structure, typical or atypical colony formation, etc.) and microscopic morphologies, Gram-staining reaction (for bacteria), the results of physiological and biochemical tests, API tests and 16S rRNA gene sequencing, pathogenicity and toxicity potential, spoilage-forming potential in milk and dairy products, and whether they were microorganisms indicative of fecal contamination. Following this, 10 isolates were selected from 42 isolates considering the isolate re-groups mentioned above and studied further. The identity of these isolates is shown in Table 2 along with their isolation surface, isolate codes, isolation media, and their identity obtained through 16S rRNA gene sequencing at the species level. Some of the microorganisms mentioned in Table 2 were isolated from selective media that are used for the selection of other microorganisms instead of from their own selective media.

API test kits usually produce accurate results when defining many bacterial groups. In this study, the results obtained with the API test kits were found to be consistent with the results obtained with 16S rRNA gene sequencing, but there were differences at the species level. For *Staphylococcus* species, the identification results obtained using 16S rRNA gene sequencing were more consistent with the API results at the species level compared with the identification of other bacteria and yeasts.

Surface properties of microorganisms

In these experiments, *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 strains were used as standard control microorganisms, in addition to the 10 test microorganism isolates.

Microbial adhesion to hydrocarbons test

The MATH test surface hydrophobicity values of the 10 selected isolates and the controls are shown in Table 3. Among the surface tests, the MATH test exhibited the lowest standard deviations, and the most consistent results between the replicates.

The MATH test is one of the criteria used to evaluate the surface adhesion potential of microorganisms. A value greater than 70% indicates that a test microorganism is hydrophobic, values from 30 to 70% indicate it is weakly hydrophobic, and values less than 30% indicate it is hydrophilic (Abasolo-Pacheco et al. 2015; Kwaszewska et al. 2006). It is thought that hydrophilic MATH values of microorganisms increase surface adhesion. Besides the microorganisms, surfaces also have hydrophilic or hydrophobic characteristics. In the food industry, stainless steel surfaces are frequently used, and the hydrophilic property of these stainless steel surfaces is an important factor in biofilm formation (Frank 2001).

In general, the surface hydrophobicity values of the test microorganisms were positive. However, some test microorganisms (*K. variicola*, *E. coli*, *P. mirabilis*, and *E. coli* ATCC 25922) had negative values. In general, the literature reports that the surface hydrophobicity values of microorganisms are positive. It has however also been reported in the literature that some microorganism strains have negative values. For example, the surface hydrophobicity values of *E. coli* strains by using dodecane have been reported as being −2.0% and −6.0% by Saini (2010). This is because some hydrocarbons can

Table 2 Microorganisms selected for further study

Isolation surface	Isolate code	Identity
Condensed milk pipe	YEA 13.2.1	*Enterococcus faecalis*
Yogurt filling unit, 1500 g	BPA 5.2.2	*Staphylococcus epidermidis*
Raw milk tank	M17 1.1.1	*Lactococcus garvieae*
Condensed milk pipe	M17 13.1.2	*Lactococcus garvieae*
Yogurt filling unit, 1500 g	YEA 5.2.6	*Bacillus toyonensis*
Raw milk tank	FVRBA 1.1.5	*Pseudomonas aeruginosa*
White cheese pressing cloth	VRBDA 7.1.1	*Escherichia coli*
White cheese pressing cloth	FVRBA 7.1.2	*Klebsiella variicola*
Yogurt filling unit, 350 g	MRS 4.2.1	*Candida parapsilosis*
Raw milk tank	VRBDA 1.1.1	*Proteus mirabilis*

Table 3 Surface test values of the isolates

Isolate Code	Microorganism	MATH Test surface hydrophobicity (%)*	Static water contact angle (°)*	SAT (Molar)*	Surface Zeta Potential (mV)*
FVRBA 1.1.5.	*Pseudomonas aeruginosa*	10.6 ± 0.5**	26.0 ± 5.1	2.5	−5.9 ± 4.1**
M17 1.1.1.	*Lactococcus garvieae*	25.1 ± 0.7	0.0 ± 0.0	1.5	−16.9 ± 3.8
M17 13.1.2.	*Lactococcus garvieae*	29.2 ± 0.7	12.0 ± 3.3	<0.01	−15.6 ± 4.6
YEA 5.2.6.	*Bacillus toyonensis*	30.4 ± 0.2	33.0 ± 4.5**	3.0 **	−8.8 ± 0.2
YEA 13.2.1.	*Enterococcus faecalis*	35.1 ± 1.1	24.0 ± 1.9	<0.01	−11.5 ± 3.0
MRS 4.2.1.	*Candida parapsilosis*	45.7 ± 0.1	0.0 ± 0.0	2.0	−7.9 ± 0.7
BPA 5.2.2.	*Staphylococcus epidermidis*	74.4 ± 0.6	25.0 ± 7.2	<0.01	−13.1 ± 4.2
ATCC 25923	*Staphylococcus aureus*	94.2 ± 0.2	6. 0 ± 4.5	<0.01	−13.7 ± 6.0
FVRBA 7.1.2.	*Klebsiella variicola*	− 10.6 ± 0.0	22.0 ± 2.0	0.4	−11.4 ± 4.8
ATCC 25922	*Escherichia coli*	− 8.9 ± 0.1	21.0 ± 3.0	<0.01	−18.4 ± 1.5
VRBDA 7.1.1.	*Escherichia coli*	− 7.5 ± 0.4	18.0 ± 7.4	2.5	−7.4 ± 2.3
VRBDA 1.1.1.	*Proteus mirabilis*	− 5.9 ± 0.3	0.0 ± 0.0	2.5	−11.0 ± 4.4

*Measurements were performed in triplicate, and results are presented as mean ± standard deviation

**The best result for each test in terms of adhesion capability

diffuse in water, resulting in a higher final absorbance value (A) than the initial absorbance value (A_0). However, in such cases, evaluation can be made using other different hydrocarbons like octane (Saini 2010).

When the negative values were ignored, *P. aeruginosa* and both of the *L. garvieae* isolates were the most hydrophilic microorganisms isolated. These isolates were followed by *B. toyonensis*, which had a relatively lower hydrophilic characteristic. The most hydrophobic isolates were *C. parapsilosis* and *S. epidermidis*. The control *S. aureus* ATCC 25923 strain was the most hydrophobic microorganism, giving a 94.2 ± 0.2% value, whereas the control *E. coli* ATCC 25922 strain gave the most negative surface hydrophobicity value.

Surface property values may vary strain to strain. On the other hand, the MATH values obtained for the tested microorganisms are generally consistent with the results reported in the literature for the same species (Hamadi and Latrache 2008; Li and McLandsborough 1999; Minagi et al. 1986). Since information about the MATH values for *L. garvieae* and *B. toyonensis* is not available in the literature, the MATH values for *L. garvieae* and *B. toyonensis* were compared with those of *L. lactis* and *B. subtilis*, respectively. In the literature, the MATH value for *L. lactis* was reported as 34.0% (Marín et al. 1997), whereas the MATH value for *B. subtilis* was reported as 28.0% (Abasolo-Pacheco et al. 2015).

Surface hydrophobicity values of the microorganisms vary greatly depending on the strain. For example, the surface hydrophobicity values of the *P. aeruginosa* strains were reported to lie within the range 12.0–84.0% (Vanhaecke et al. 1990), whereas the surface hydrophobicity values of *S. epidermidis* strains were reported to range between 22.0 and 81.0% (Jones et al. 1996). In another report, the surface hydrophobicity values of *S. epidermidis* strains were reported to be 4.0, 7.0, 8.0, and 55.0% (Hanlon et al. 1999).

In this study, the control *E. coli* ATCC 25922 strain and the test *E. coli* strain gave similar results, and similar results were observed between the control *S. aureus* ATCC 25923 strain and the test *S. epidermidis* strains.

Static water contact angle

The static water contact angles of the 10 selected isolates and the controls are shown in Table 3.

The surface adhesion potential of microorganisms can be evaluated by measuring the static water contact angle. In the literature, it is reported that there is a positive correlation between the static water contact angle and the ability of microorganisms to adhere to the surface (Boonaert et al. 2001; Li and Logan 2004).

When the static water contact angle values of the isolates were examined, *B. toyonensis* and *P. aeruginosa* exhibited the highest, whereas *C. parapsilosis* and *P. mirabilis* exhibited the lowest values (Table 3). It can be said that *B. toyonensis* and *P. aeruginosa* have the highest adhesion capability based on static water contact angle results.

The surface properties may vary on the strain basis. For example, the static water contact angle values of *P. aeruginosa* strains have been reported to be 36° (Pasmore et al. 2001), between 21 and 85°, generally between 65 and 85° (Triandafillu et al. 2003). Moreover, the static water contact angle values in present study are generally consistent with the literature for the same species (Feng et al. 2009; Hamadi and Latrache 2008; van Merode et al. 2008). Because of the lack of information in the literature about the static water contact angle values for *B. toyonensis*, the static water contact angle

values for *B. toyonensis* were compared with those for *B. subtilis* or *B. cereus*. The static water contact angle values for *B. subtilis* have been reported as being between 33 and 59°, generally 40°, for the vegetative forms, and between 20 and 45°, generally 30°, for the spore forms (Ahimou et al. 2001). The static water contact angle value of *B. cereus* has been reported as being 25° (Bernardes et al. 2010).

Salt aggregation test

The SAT values of the 10 selected isolates and the controls are shown in Table 3.

The salt aggregation test gives information about the surface adhesion of microorganisms. Cultures with SAT values of 0.01 to 0.2 M are considered highly hydrophobic, while those of 0.2 to 1.5 M are considered hydrophobic, and cultures with SAT values greater than 1.5 M are considered hydrophilic (Styriak et al. 1999). Microorganisms with hydrophilic SAT values demonstrate a greater ability to adhere surfaces. Hence, *B. toyonensis*, *P. aeruginosa*, *E. coli*, *P. mirabilis*, and *C. parapsilosis* were found to be hydrophilic, *L. garvieae* (M17 1.1.1.) and *K. variicola* were hydrophobic, and *E. faecalis*, *S. epidermidis*, and *L. garvieae* (M17 13.1.2.) were highly hydrophobic. The control strains *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were also highly hydrophobic (Table 3). It can be said that *B. toyonensis*, *P. aeruginosa*, *E. coli*, and *P. mirabilis* have the highest adhesion capability, whereas *E. faecalis*, *S. epidermidis*, and *L. garvieae* (M17 1.1.1.) have the lowest adhesion based on salt aggregation test results.

The surface properties may vary even in different strains of a species. For example, the SAT values of *P. aeruginosa* strains have been reported to be within the range 0.0–4.0 M (Vanhaecke et al. 1990). The SAT values of *S. aureus* strains have been reported to be < 0.1 M (Ljungh et al. 1985) or between 0.025–2.0 M (Ljungh and Wadström 1995). Otherwise, considering the same species, SAT results are mostly consistent with the literature (Arana et al. 1999; Marín et al. 1997). In as much as there is no information about the SAT values for *B. toyonensis* in the literature, they were compared with those for *B. thuringensis*, *B. licheniformis*, or *B. cereus*. These latter species have been reported as having SAT values of 2.0 M, 1.8 M, and 0.2 M, respectively (Obuekwe et al. 2009).

Surface zeta potential test

The surface zeta potential values of the 10 selected isolates and the controls are shown in Table 3.

The surface zeta potential is used to assess the potential of microorganisms to adhere various surfaces. The value of the surface zeta potential depends on ionic strength. As the ionic strength increases, the surface zeta potential value for a microorganism increases, which causes its hydrophobicity to increase. Because of this increase in hydrophobicity, the ability of the microorganism to adhere to surfaces decreases. The surface zeta potential value is determined using the absolute value of the data. Microorganisms with small absolute surface zeta potential values have a high surface binding ability. In other words, the absolute surface zeta potential value and the ability of microorganisms to adhere to surfaces are inversely proportional (Li and Logan 2004).

When the absolute surface zeta potential values of the isolates were examined, *P. aeruginosa* and *E. coli* exhibited the lowest, whereas *L. garvieae* (M17 1.1.1. and M17 13.1.2.) and *S. epidermidis* exhibited the highest values (Table 3). It can be said that *P. aeruginosa* and *E. coli* have the highest adhesion capability, whereas *L. garvieae* (M17 1.1.1. and M17 13.1.2.) and *S. epidermidis* have the lowest adhesion based on absolute surface zeta potential results.

Even a different strain of a species may have different surface properties. For example, the surface zeta potential values of *P. aeruginosa* strains have been reported to be − 9.0 and − 16.0 mV (Gómez-Suárez et al. 2002). The surface zeta potential values of the *S. epidermidis* strains have been reported as being between − 6.0 and − 10.0 mV (Gallardo-Moreno et al. 2009). On the other hand, surface zeta potential results were obtained in accordance with the literature (Li and McLandsborough 1999; Wang et al. 2012). The *B. toyonensis* surface zeta potential values were compared with those of *B. subtilis* or *B. licheniformis* because there is not any study about this. The surface zeta potential values of *B. subtilis* have been reported as being between − 15.0 and − 50.0 mV (Ahimou et al. 2001). The *B. licheniformis* surface zeta potential values have been reported as being between − 16.0 and − 43.0 mV (Li et al. 2009).

Exopolysaccharide composition

Total sugar and uronic acid

The total sugar and uronic acid contents of the EPS produced by the 10 selected isolates are shown in Table 4.

In literature, the results for the total sugar of the isolates are generally consistent with those for total uronic acid content (Strathmann et al. 2002). The results of the present study were also consistent in terms of total sugar and uronic acid content. *P. aeruginosa* showed the highest sugar- and uronic acid–producing abilities followed by *S. epidermidis*. Both the *L. garvieae* isolates showed the lowest sugar-producing ability, followed by *E. coli*. However, these two *L. garvieae* isolates were followed by *P. mirabilis* in terms of the lowest uronic acid content. *B. toyonensis* showed relatively lower sugar- and uronic acid–producing abilities compared with *P. aeruginosa*.

The ability to produce EPS by a microorganism, as well as the EPS content, is thought to be major contributors to

Table 4 Total sugar and uronic acid contents

Isolate code	Microorganism	Total sugar (μg/10^9 cells)	Uronic acid (μg/10^9 cells)
FVRBA 1.1.5.	*Pseudomonas aeruginosa*	1900	108
BPA 5.2.2.	*Staphylococcus epidermidis*	1560	89
VRBDA 1.1.1.	*Proteus mirabilis*	1368	62
FVRBA 7.1.2.	*Klebsiella variicola*	1357	65
MRS 4.2.1.	*Candida parapsilosis*	1333	69
YEA 5.2.6.	*Bacillus toyonensis*	1227	63
YEA 13.2.1.	*Enterococcus faecalis*	1050	72
VRBDA 7.1.1.	*Escherichia coli*	1012	66
M17 13.1.2.	*Lactococcus garvieae*	837	58
M17 1.1.1.	*Lactococcus garvieae*	790	52

adhesion and biofilm formation on surfaces. Uronic acid is the most abundant acidic sugar found in the EPS. Therefore, assessing the EPS production ability of the test microorganisms, and measuring the total sugar and uronic acid, may be most predictive of the ability of a microorganism to adhere surface. As mentioned earlier, *P. aeruginosa* and *B. toyonensis* exhibited the best results based on the surface tests. When these two bacteria were assessed in terms of total sugar and uronic acid in their EPS, *P. aeruginosa* was found to have a much better results than *B. toyonensis*.

In one study, the total carbohydrate content of *P. aeruginosa* was found to be 705–749 μg/10^9 cells in the biofilm and 535–512 μg/10^9 cells in the EPS. Moreover, the uronic acid content was found to be 408–450 μg/10^9 cells in the biofilm and 354–381 μg/10^9 cells in the EPS (Strathmann et al. 2002). In another study, the total carbohydrate content in *P. aeruginosa* was found to be 1006 μg/10^9 cells in the biofilm and 767 μg/10^9 cells in the EPS. In this same study, the uronic acid content was found to be 474 μg/10^9 cells in the biofilm and 403 μg/10^9 cells in the EPS (Wingender et al. 2001).

Neutral sugar content

The percentage distribution of neutral sugars extracted from each isolate is shown in Table 5.

The neutral sugar content of the EPS may be an important contributing factor for adhesion of microorganisms (Yang 2000). As expected, there were large variations in the neutral sugar content in EPS produced by the different isolates (Table 5).

Glucose and mannitol were the two most abundant two neutral sugars produced by *P. aeruginosa*, with mannose and glucose being two most abundant two neutral sugars produced by *K. varriicola*, sorbitol and mannose by *P. mirabilis*, glucose and sorbitol by *L. garvieae* (M17 13.1.2.), glucose and mannose by *B. toyonensis*, sorbitol and glucose by *S. epidermidis*, mannose and sorbitol by *L. garvieae* (M17 1.1.1.), mannose and sorbitol by *E. faecalis*, glucose and ribose by *C. parapsilosis*, and sorbitol and mannose by *E. coli* (Table 5).

The following sugars have been demonstrated to be present in the EPS from different microorganisms: ribose, arabinose, mannose, glucose, and galactose in the EPS from the *Bacillus* spp. (Fox 1999); glucose, xylose, and rhamnose in the EPS from the *P. aeruginosa* (Yokota et al. 1987); glucose, galactose, and rhamnose in the EPS from the *Enterococcus* spp. (Mozzi et al. 2006); fucose and galactose in the EPS from the *L. lactis* subsp. *lactis* (Suzuki et al. 2013); glucose, mannose, galactose, and arabinose in the EPS from the *C. albicans* (Kiran et al. 2015); and mannose, galactose, glucose, galactonic acid, arabinose, fucose, rhamnose, and xylose in the EPS from the *S. epidermidis* and *E. coli* (Bales et al. 2013).

EPS are microbial substances that have important contributions in the formation of biofilm. EPS are critical to the maturation of the biofilm structure as well as the initial binding stage of microorganisms (Marshall 1992), (Sutherland 1982). The EPS protect the bacteria from dehydration by holding water and are drying very slowly (Ophir and Gutnick 1994), (Roberson and Firestone 1992). EPS are also important for the survival of microorganisms in adverse environmental conditions (Rinker and Kelly 1996). Moreover, the EPS are effective in keeping the nutrients for the biofilm structure to mature and protecting the cells against antimicrobial agents.

In order to eliminate microorganisms in the biofilm structure, the biocides must penetrate to the EPS structure and reach the microorganism cells which are in the inner layers (Meyer 2003). Since the EPS composition differs according to the biofilm type, different methods are used for each biofilm structure. For example, oxidation agents such as peracetic acid and chlorine are preferred for the elimination of biofilm layers formed by *Pseudomonas* and *Listeria* on stainless steel surfaces (Jang et al. 2006). Active chlorine is preferred due to its ability to remove microorganisms in the biofilm structure as well as to remove EPS on the surface (Meyer 2003). Ozone is a strong oxidizing agent and has been successfully applied to

Table 5 Percentage distribution of neutral sugars extracted from each isolate

Analytical type	% distribution of neutral sugars									
	Pseudomonas aeruginosa	*Klebsiella variicola*	*Proteus mirabilis*	*Lactococcus garvieae* (M17 13.1.2.)	*Bacillus toyonensis*	*Staphylococcus epidermidis*	*Lactococcus garvieae* (M17 1.1.1.)	*Enterococcus faecalis*	*Candida parapsilosis*	*Escherichia coli*
Monosaccharides										
D-Glucose	30.25	17.37	24.87	27.62	28.04	24.26	11.37	11.65	37.82	14.56
α-D-Talose	8.42	4.06	0.02	0.10	0.21	0.16	5.79	2.04	5.12	1.55
α-D-Galactose	0.79	2.83	0.78	1.38	1.32	2.14	3.51	1.75	8.88	–
β-D-Mannose	5.31	4.69	0.30	0.26	1.40	0.75	6.80	2.79	4.50	1.43
α-D-Mannose	9.10	29.34	15.16	18.31	22.94	15.85	16.91	21.85	4.16	18.57
α-D-Altrose	–	1.34	–	–	–	–	–	–	1.13	–
D-Fructose	–	0.02	0.01	0.02	–	–	0.03	–	–	–
α-D-Ribose	–	–	0.38	–	0.67	–	2.55	1.61	13.12	2.12
Disaccharides										
Maltose	–	0.36	–	0.11	–	–	0.29	0.30	–	1.04
Kojibiose	–	–	–	–	–	–	0.07	–	–	0.21
Aldopentoses										
Xylose	7.77	–	0.02	–	–	–	1.27	–	–	1.06
D-Arabinose	1.30	–	0.39	–	–	–	–	–	2.07	0.39
Sugar alcohols										
D-Glucitol	3.27	0.58	0.06	0.02	0.09	–	0.53	0.45	1.87	2.29
Galactitol	3.99	12.26	13.29	13.07	15.75	14.12	11.89	13.76	8.57	9.20
D-Mannitol	22.10	3.56	–	0.05	3.21	0.37	14.95	10.47	11.44	11.15
Sorbitol	7.70	17.28	30.91	25.96	20.23	28.24	15.56	20.59	–	22.56
Iditol	–	3.63	13.82	13.10	6.14	14.12	8.17	12.17	–	13.52
D-Xylitol	–	2.70	–	–	–	–	0.39	0.58	1.32	0.54

"–" means not detected

remove biofilms from oligotrophic water systems (Barnes and Caskey 2002).

Bacteriocins are also used to minimize the biofilm formation of foodborne pathogenic microorganisms. It was stated that the use of plantaricin 423, pediocin PD-1, and nisin was effective against the biofilm structure formed by *Oenococcus oeni* (Nel et al. 2002). Moreover, EPS also increase the resistance of microorganisms to cleaning agents. It was reported that the catalase enzyme in biofilm structure of *P. aeruginiosa* was effective in reducing the efficacy of hydrogen peroxide-containing disinfectants (Stewart et al. 2000).

In the present study, the EPS-producing ability of surface adhesive microorganisms in a dairy plant and the content of EPS they produce were determined. The data about EPS content of the isolates shed some light on the determination of the chemicals which should be preferred for effective cleaning/disinfection in a dairy plant.

Discussion

In this study, several microorganisms were isolated from different sampling points in a dairy plant. The microorganisms were isolated after cleaning/disinfection to a similar degree as before cleaning/disinfection of the sampling points in the dairy plant. The microorganism isolates included microorganisms that may be pathogenic or are opportunistic pathogens, or could potentially affect other microorganisms and cause a deterioration of the milk and dairy products. Some sampling points drew attention as being processing points that may cause post-production contamination.

Stainless steel is the most preferred material on the surface of the equipment and materials used in the food industry, and the hydrophilic property of these surfaces is an important factor in biofilm formation (Frank 2001). In literature, it was shown that the critical surface tension value promotes the attachment of microorganisms to various surfaces (Boulange-Petermann et al. 1993), (Bryers 1987). As the free surface energy and the wettability of the surface increase, the binding of the bacterial cells to the surface approaches the maximum level. The surfaces with high free surface energy such as stainless steel and glass are more hydrophilic. These surfaces cause higher bacterial binding and thus cause more biofilm formation compared with hydrophobic surfaces such as Teflon, nylon, and rubber (Blackman and Frank 1996), (Hyde et al. 1997), (Mafu et al. 1990), (Sinde and Carballo 2000). In a study by Smoot and Pierson (Smoot and Pierson 1998), it was noted that *Listeria monocytogenes* attaches faster but stronger to stainless steel surfaces than rubber surfaces.

In this study, numerous microorganisms were isolated after cleaning and disinfection of the pasteurized milk tank, yogurt filling unit, white cheese tank, ice cream filling unit, ice cream air pressing pipe, butter tank, and condensed milk pipe. All these units came into direct contact with the final dairy product. This is important in terms of food hygiene and food quality, so further precautions should be taken in this particular dairy plant.

API test kits and 16S rRNA gene sequencing results were consistent at the genus level, but there were some differences at the species level. The species level identification was most consistent for *Staphylococcus* but varied significantly for lactic acid bacteria and yeasts.

Within the scope of this study, the surface properties of 10 selected isolates were determined to assess their ability for surface adhesion. Among the surface tests, the MATH test exhibited the lowest standard deviations, and the most consistent results between the replicates. It can be said that, MATH test is the most useful test in determining the surface properties of microorganisms with respect to surface adhesion. The hydrophilic character of microorganisms provides higher surface adhesion potential. *P. aeruginosa*, a Gram-negative isolate, and *B. toyonensis*, a Gram-positive isolate, exhibited the best results in terms of surface test. The ability of the test bacteria to produce exopolysaccharides, which are a major contributor to adhesion, is thought to be related more to biofilm formation on the surface than on the cell's ability to adhere to the surface. In addition, the total sugar and uronic acid content have great importance during the assessment of surface adhesion. When the exopolysaccharides from these two bacteria were assessed in terms of total sugar and uronic acid, *P. aeruginosa* was indicated to have better results than *B. toyonensis*. Moreover, neutral sugar was determined in their alditol acetate forms by using gas chromatography–mass spectrometry. A wide variety of neutral sugar content was determined for each of the isolates. *P. aeruginosa* had richer neutral sugar content in its exopolysaccharide than that of *B. toyonensis*. As a result, it is believed that determination of the EPS content would contribute to establishing of the effective cleaning/disinfection procedure for dairy plants.

In the next step of the study, the biofilm formation will be investigated with these two test bacteria (*P. aeruginosa* and *B. toyonensis*) by using stainless steel plates in batch and flow growth media.

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Compliance with ethical standards

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

Research involving human participants and/or animals This article does not contain any studies with human participants or animals performed by any of the authors.

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