

THE TESTING OF SANITIZERS EFFICACY TO ENTEROCOCCI ADHERED ON GLASS SURFACES

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ABSTRACT

The aim of this work was to test the ability of 6 strains of enterococci to adhere on glass surfaces in environment with different content of milk residues and then to evaluate efficacy of 2 commercial sanitizers (alkaline and acidic) used in milk production. Tested enterococci were isolated from milk, dairy products and from rinse water after sanitation milking machine. Suspension of enterococci ($8 \log \text{CFU} \cdot \text{mL}^{-1}$) was prepared in phosphate buffered saline (PBS), PBS with content 0.1% and 1% of skimmed reconstituted milk. Glass plates were immersed into bacterial suspension for 1 h at 37 °C. The number of enterococci adhered on glass surface in PBS achieved an average value $3.47 \log \text{CFU} \cdot \text{mm}^{-2}$, in PBS with 0.1% of milk $2.90 \text{CFU} \cdot \text{mm}^{-2}$, in PBS with 1% of milk $2.63 \text{CFU} \cdot \text{mm}^{-2}$. Differences between the tested files were not statistically significant ($p > 0.05$). In the second part of work the glass plates with adhered enterococci were exposed to the effect of alkaline sanitizer (on basis of NaOH and NaClO), respectively acidic sanitizer (on basis of H_3PO_4). Sanitation solutions were prepared and tested according to manufacturer recommendations (concentration 0.25%, contact time 20 min, temperature 20 °C). Alkaline sanitation solution was 100% effective against all tested enterococci regardless to content of milk residues in environment. Acidic sanitation solution was 100% effective only against *E. faecalis*_D (isolated from rinse water after sanitation). Average value of reduction of enterococci with acidic sanitation solution, which were on glass plates in environment PBS was $2.84 \text{CFU} \cdot \text{mm}^{-2}$, in PBS with 0.1% of milk was $2.45 \text{CFU} \cdot \text{mm}^{-2}$ and in PBS with 1% of milk was $2.16 \text{CFU} \cdot \text{mm}^{-2}$. It can be concluded, that increase of milk residues in environment decrease the adhesion of enterococci on glass surface, but also effectiveness of acidic sanitation solution.

Keywords: enterococci; sanitation; glass; biofilm

INTRODUCTION

Traditionally enterococci are considered as part of the lactic acid bacteria. Like most other lactic acid bacteria, some enterococcal strains are used as starter or protection cultures or feed supplements as well as probiotics (Klein, 2003). Enterococci are present in the microbial association of a variety of fermented foods such as cheeses (Koluman et al., 2009) or meat products (Barbosa et al., 2010).

The positive influence of enterococci on cheeses, respectively on other fermented foods, seems due to specific biochemical traits such as proteolytic, lipolytic activity, citrate utilisation, and production of aromatic volatile compounds (Giraffa, 2003; Foulquie Moreno et al., 2006). Some strains of enterococci are used in “food technology” because of their ability to produce bacteriocins and to act as a starter in fermented product (Settanni and Moschetti, 2010).

Another important characteristic of genus *Enterococcus* is that enterococci are not considered “generally recognised as safe” due to its use as an indicator of faecal contamination (Foulquie Moreno et al., 2006; Cassenego et al., 2011), because they are part of humans and animals intestinal microbiota. For enterococci is typical also intrinsic resistance to some antimicrobial agents

commonly prescribed for Gram-positive cocci such as cephalosporin, lincomycin, cotrimoxazole, and low levels of penicillin and aminoglycosides (Marinho et al., 2013; Medeiros et al., 2014). Several investigations showed the occurrence of vancomycin resistant enterococci also in food of animal origin (Klein, 2003; Kročko et al., 2011; Ducková et al., 2014a). Enterococci also exhibit resistance to a wide variety of other antimicrobials, by acquisition of resistance genes via transposons or plasmids (Marinho et al., 2013; Medeiros et al., 2014).

Several studies have also shown that enterococci posse virulence determinants. Although enterococcal virulence factors are found more frequently among clinical strains, they are also detected in food isolates. Over the years several virulence factors have been identified in food enterococci which include: aggregation substances (agg), cytolysin (cyt), gelatinase (gelE), enterococcal surface protein gene (esp), cell wall adhesions (efaAfm and efaAfs) (Valenzuela et al., 2009; Barbosa et al., 2010; Jahan and Holley, 2014). One of the main factors of enterococci virulence is also the biofilm formation (Necidová et al., 2009). Biofilm production can promote increase resistance to antibiotic and other antimicrobials (Tsikrikonis et al., 2012).

The formation of biofilm creates major problem in the food industry since it may represent an important source of contamination for materials or foodstuffs coming into contact with them, so leading to food spoilage or transmission of diseases (Hamadi et al., 2013). Biofilm can be defined as matrix-embedded bacterial population adhered to a surface or to each other (Jahan and Holley, 2014). The process of bacterial biofilm formation is occurring in four depended stages. The adhesion of bacteria to surface is the first and essential stage in the formation of biofilm. The bacterial adhesion stage is associated with the production of exopolysaccharides, DNA and proteins. The initial stage of bacterial adhesion was reported to be a reversible because of the weakness of the interactions between bacteria and surfaces however this stage becomes irreversible as a result of anchoring by appendages and/or production of extracellular polymers mainly exopolysaccharides. This adhesion depends on both physicochemical properties of cell surface, and also on characteristics of the surrounding medium (Hamadi et al., 2013; Ouali et al., 2014).

Milk, the main raw material dealt with at dairies, is very good growth medium for bacteria. According to the literature, biofilm problems in the dairy process have been found in air-handling systems, cooling systems, milk transfer lines, on conveyors, in packaging machines, in heat exchangers, on ultra-filtration surfaces, in mixers, tanks and other equipment, on floor and in drains (Salo et al., 2006). It has also been found that biofilm cells of bacteria were more resistant than planctonic cells to disinfectants containing e.g. chlorine, iodine, quarternary ammonium and anionic acid compounds (Wirtanen and Salo, 2004; Salo et al., 2006).

The aim of this study was therefore to test the ability of enterococci to adhere on glass surface in environment with different content of milk residues and then to evaluate efficacy of 2 commercial sanitizers (alkaline and acidic) used in milk production.

MATERIAL AND METHODOLOGY

Tested strains of enterococci were isolated from different sources – *E. faecalis*_A and *E. faecalis*_B from traditional Slovak bryndza cheese, *E. faecalis*_C and *E. faecalis*_D from rinse water after milking machine sanitation, *E. faecium*_I from sheep milk and *E. faecium*_{II} from rinse water after milking machine sanitation.

The adhesion of enterococci to glass was determined modified method described by Carballo and Araújo (2012). Overnight cultures of enterococci (37 °C) in Tryptone Soy Broth (TSB) (HiMedia, India) were pelleted by centrifugation (4000 rpm, 20 min). Separated bacteria were washed three times with phosphate buffered saline (PBS) and then were suspended in PBS. Bacterial cell density was adjusted with PBS to 8 log CFU.mL⁻¹ by spectrophotometer. Except enterococcal suspension in PBS, were also prepared enterococcal suspensions in PBS with content of 0.1% and 1% of skimmed reconstituted milk. Glass was cut in plates 10 x 25 x 1 mm, washed and sterilized (160 °C, 4 h).

Glass plates were immersed into bacterial suspensions (4 mL) for 1 h at 37 °C. After incubation, plates were rinsed twice with 4 mL of PBS and immersed in 4 mL

TSB. Adhered enterococci were immediately released from glass plates with ultrasonic probe UP 100 H (Hielscher ultrasound technology, Germany) (30 W, 20 s). Ten-fold serial dilutions of TSB in saline were made. After 48 h incubation at 37 °C on Slanetz-Bartley agar (HiMedia, India) the number of enterococci was counted. Each experiment was performed three times.

In the second part of this work, efficacy of two commercial sanitizers was tested on enterococci adhered on glass plates. The sanitation solutions were prepared and tested according to manufacturer recommendations (concentrations 0.25%, contact time 20 min, temperature 20 °C). Both tested sanitizers are commonly used for sanitation of machine and equipments processing milk. Alkaline sanitizer contained NaOH and NaClO, acidic sanitizer contained H₃PO₄.

The glass plates with adhered enterococci, obtained as explained previously, were immersed into 4 mL of each sanitation solution for 20 min. Then the plates were washed with PBS (4 mL, twice) and the number of surviving enterococci was determined as already explained.

RESULTS AND DISCUSSION

Numbers of enterococci adhered on glass plates in environment with different content of milk residues are in Table 1.

Carballo and Araújo (2012) found out similar results with ours. They determined numbers of *Salmonella* strains attached on stainless steel after one hour incubation in TSB at room temperature in the range from 3.9 to 4.7 log CFU.mm⁻² and numbers of *Listeria monocytogenes* strains were higher in the range from 5.1 to 5.5 log CFU.mm⁻².

The biofilm forming capability of *Staphylococcus aureus* on stainless steel and glass surface verify Marques et al. (2007). Their results obtained after 15-day incubation showed biofilm formation on both surfaces with bacterial count in the order of 10⁷ CFU.mm⁻² and 10⁸ CFU.mm⁻² on stainless steel and glass surfaces, respectively.

Necidová et al. (2009) monitored the capability of enterococci to form biofilm. These authors determined biofilm formation potential in glass tubes containing suspension of tested stains (35 °C, 2 days) and after staining glass tubes by safranin solutions. The capability of forming biofilm was detected in 28% of *Enterococcus* spp. strains. Higher number of biofilm forming strains of the *Enterococcus faecium* (33%) than *Enterococcus faecalis* (28%) has been registered.

Table 1 shows effect of different concentrations of milk residues in environment to adhesion of enterococci on glass surface. It can be concluded that increase of milk residues in environment, paradoxically decreased the adhesion of enterococci on glass surface. The differences between compared numbers of enterococci were not statistically significant ($p > 0.05$).

Comparable results (average values 3.36, 2.73 and 2.52 log CFU.mm⁻² in PBS, in PBS with 0.1% of milk and in PBS with 1% of milk respectively) for the same strains enterococci adhered on stainless steel plates have been previously published by Ducková et al. (2014b).

The role of milk or milk components in inhibiting bacterial adhesion was reported previously by several works.

Barnes et al. (1999) reported that adhesion to the milk treated stainless steel varied with the organism used. With *Staphylococcus aureus*, *Listeria monocytogenes*, *Serratia marcescens* cells, attachment was reduced to levels $\leq 20\%$ of clean surface values. In contrast, *Escherichia coli* and *Pseudomonas fragi* cells adhered in small numbers to the clean stainless steel surface, with less than 1 organisms per held of view, making any effect of protein film difficult to assess.

Also **Hamadi et al. (2013)** reported that milk reduces *Staphylococcus aureus* adhesion and the level of this reduction depends on contact time. The adhesion results were interpreted in terms of hydrophobicity and electron donor/electron acceptor properties of both surfaces (cell surface, stainless steel surface).

Dat et al. (2010) and **Srey et al. (2013)** explain lower bacterial adhesion on surfaces with milk residues with repulsion between negatively charged milk proteins and equally charged surfaces of bacterial cells. Another explanation of mentioned results is the lack of nutritional substances in the environment, because according **Mah and O'Toole (2001)**, initiation of biofilm formation is the natural behavior of bacteria in nutrient deficient environment.

Tables 2 and 3 show the effect of alkaline and acidic sanitation solutions, respectively, on enterococci adhered on glass plates.

Alkaline sanitation solution containing NaOH and NaClO was 100% effective against all tested strains enterococci, which were adhered on glass plates regardless to content of milk residues in environment.

Acidic sanitation solution containing H_3PO_4 was 100% effective only against *E. faecalis_D* (isolated from rinse water after sanitation). Average value of reduction of enterococci with acidic sanitation solution, which were on

glass plates in environment PBS was 2.84 CFU.mm^{-2} , in PBS with 0.1% of milk was 2.45 CFU.mm^{-2} and in PBS with 1% of milk was 2.16 CFU.mm^{-2} . The values of reduction of enterococci adhered on glass after application of acidic sanitation solution decrease with increase of milk residues content in environment which glass plates were in contact. Effectiveness of acidic sanitation solution obviously decreased presence of milk residues.

Ducková et al. (2014b) found out similar results for the same strains of enterococci adhered on stainless steel plates. Alkaline sanitation solution containing NaOH and NaClO was 100% effective against all tested strains enterococci adhered on stainless steel. Average value of reduction of enterococci with acidic sanitation solution, which were on stainless steel plates in environment PBS was 2.76 CFU.mm^{-2} , in PBS with 0.1% of milk was 2.37 CFU.mm^{-2} and in PBS with 1% of milk was 1.97 CFU.mm^{-2} .

Trachoo and Frank (2002) reported also similar results. They found out that sanitizers containing chlorine are more effective than acidic sanitizers based on peracetic acid or mixture of peracetic and peroxyoctanoic acid against *Campylobacter jejuni* in biofilms.

The efficiency of sanitizers: hydrogen peroxide, sodium dichloroisocyanurate and peracetic acid on formation of biofilm by *Staphylococcus aureus* on stainless steel and glass surfaces tested **Marques et al. (2007)**. Peracetic acid was the most efficient in removing adhered cells, presenting 5.26 and 4.5 decimal reduction for adhered cells on stainless steel and glass surfaces, respectively.

Carballo and Araújo (2012) reported that by the manufacturer recommended concentrations of sanitation solutions (quaternary ammonium compounds, alquyldiethylenediamineglycine and di-alquyldiamineethylglycine) were not effective to kill *Listeria monocytogenes* and *Salmonella* spp., especially they were adhered to surfaces.

Krebs-Artimová et al. (2010) tested effectiveness of

Table 1 Numbers ($\log \text{CFU.mm}^{-2}$) of adhered enterococci released from glass plates after 1 h cultivation at 37 °C in different environments.

Strains of enterococci	Initial numbers of enterococci in suspension ($\log \text{CFU.mL}^{-1}$)	Adhered enterococci ($\log \text{CFU.mm}^{-2}$)		
		in PBS	in PBS with 0.1% of milk	in PBS with 1% of milk
<i>E. faecalis_A</i>	8.74	4.31	3.48	3.14
<i>E. faecalis_B</i>	8.71	3.63	3.35	3.34
<i>E. faecalis_C</i>	7.61	2.44	1.79	1.55
<i>E. faecalis_D</i>	8.71	3.46	2.90	2.65
<i>E. faecium_I</i>	8.69	3.51	3.06	2.74
<i>E. faecium_{II}</i>	8.66	3.49	2.84	2.37
<i>x</i>	8.52	3.47	2.90	2.63
x_{\min}	7.61	2.44	1.79	1.55
x_{\max}	8.74	4.31	3.48	3.34

Table 2 Effectiveness of alkaline sanitation solution (concentration 0.25%, temperature 20 °C, 20 min) against enterococci adhered on glass surface in environment with different content of milk residues

Strains of enterococci	Adhered enterococci (log CFU.mm ⁻²) on glass surface					
	in PBS		in PBS with 0.1% of milk		in PBS with 1% of milk	
	before sanitation	after sanitation	before sanitation	after sanitation	before sanitation	after sanitation
<i>E. faecalis</i> _A	4.31	-	3.48	-	3.14	-
<i>E. faecalis</i> _B	3.63	-	3.35	-	3.34	-
<i>E. faecalis</i> _C	2.44	-	1.79	-	1.55	-
<i>E. faecalis</i> _D	3.46	-	2.90	-	2.65	-
<i>E. faecium</i> _I	3.51	-	3.06	-	2.74	-
<i>E. faecium</i> _{II}	3.49	-	2.84	-	2.37	-

Table 3 Effectiveness of acidic sanitation solution (concentration 0.25%, temperature 20 °C, 20 min) against enterococci adhered on glass surface in environment with different content of milk residues.

Strains of enterococci	Adhered enterococci (log CFU.mm ⁻²) on glass surface					
	in PBS		in PBS with 0.1% of milk		in PBS with 1% of milk	
	numbers before sanitation	reduction after sanitation	numbers before sanitation	reduction after sanitation	numbers before sanitation	reduction after sanitation
<i>E. faecalis</i> _A	4.31	2.40	3.48	1.95	3.14	0.80
<i>E. faecalis</i> _B	3.63	2.45	3.35	3.30	3.34	3.32
<i>E. faecalis</i> _C	2.44	2.42	1.79	1.78	1.55	1.54
<i>E. faecalis</i> _D	3.46	3.46*	2.90	2.90*	2.65	2.65*
<i>E. faecium</i> _I	3.51	3.50	3.06	2.62	2.74	2.52
<i>E. faecium</i> _{II}	3.49	2.83	2.84	2.17	2.37	2.15
<i>x</i>	3.47	2.84	2.90	2.45	2.63	2.16
<i>x</i> _{min}	2.44	2.40	1.79	1.78	1.55	0,80
<i>x</i> _{max}	4.31	3.50	3.48	3.30	3.34	3.32

* total elimination

sanitary detergents against enterococci in planktonic form. They found out that alkaline sanitary detergent on chlorine base applied as 0.75% was sufficiently effective on enterococci damage, also at conditions with reduced temperature (40°C), in presence of organic matters (0.1% of milk) and also with water hardness 45°. Acidic sanitary detergent on base of phosphoric acid applied as 0.75% solution in combination with 40 °C temperature had 100% of effectiveness on enterococci damage only in the environment without organic matters regardless of water hardness.

Also Lavová et al. (2011) found out that presence of organic loads (1% of milk) and lower temperature decreased the sanitation effect of the sanitary detergents on

the base of NaClO or H₃PO₄ against enterococci in planktonic form. They found also a weaker powerful of acidic sanitation solution in comparing with alkaline.

CONCLUSION

It may be concluded that obtained results contribute to the better understanding of enterococci adhesion as initial phase of forming biofilm. Results also indicate that adhered enterococci can survive sanitation process, especially by using acidic sanitation solutions and in environment with residues of milk. In food and especially in dairy industry it is necessary to prevent biofilm formation and the contamination of food undesirable microorganisms by thorough cleaning and sanitation

process. The risks of enterococci biofilm formation not consist only in food contamination but also in possibility of antibiotic resistance genes transfer or other virulence factors.

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