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Short Communication

Biofilm formation on polystyrene under different temperatures by antibiotic resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from food

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Abstract

The ability of antibiotic resistant *E. faecalis* and *E. faecium* isolated from food to form biofilm at different temperatures in the absence or presence of 0.75% glucose was evaluated. A synergistic effect on biofilm at 10 °C, 28 °C, 37 °C and 45 °C and glucose was observed for *E. faecalis* and *E. faecium*.

Key words: enterococci, resistant, food, temperatures, biofilm, polystyrene.

Biofilms are a community of microorganisms attached on biotic or abiotic surface. In food industry, biofilms are a potential source of product contamination and may lead to food spoilage and serious fouling problems in equipment. The ability of bacteria to attach to surfaces and develop into a biofilm depends on many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, CO_2, pH, osmolarity and temperature. Bacteria in the biofilms are able to promote genetic exchange and are protected from the immune system, antibiotics and biocides (Fisher and Phillips, 2009).

Enterococci are Gram-positive cocci, part of humans and animals intestinal microbiota, and have been commonly isolated from food and water (Fisher and Phillips, 2009; Riboldi *et al.*, 2009; Cassenego *et al.*, 2011). *Enterococcus faecalis* and *Enterococcus faecium* are the most frequently species isolated from clinical and food samples (d'Azevedo, *et al.*, 2006; Fisher and Phillips, 2009). This genus has the ability to survive adverse environmental conditions, such as extreme temperatures (10-45 °C), pH-values (4.5-10.0) and salinity (Fisher and Phillips, 2009). These characteristics may contribute to the spread and persistence of enterococci in a remarkable array of environmental (Fisher and Phillips, 2009). In food, enterococci develop important role as start cultures or as probiotics, however, they are not considered "generally recognized as safe", due to its use as an indicator of fecal contamination (Cassenego *et al.*, 2011).

Another important characteristic of this genus is the intrinsic resistance to some antimicrobials agents commonly prescribed for Gram-positive cocci such as cephalosporin, lincomycin, cotrimoxazole, and low levels of penicillin and aminoglycosides. Enterococci also exhibit resistance to a wide variety of other antimicrobials, by acquisition of resistance genes via transposons or plasmids. Antibiotic resistant strains have been isolated from clinical, environment and food samples. Resistant bacteria may be transferred to humans through the food chain, and colonize the gastrointestinal tracts and/or may be able to transfer resistance genes to the resident microflora (d'Azevedo, *et al.*, 2006; Gomes *et al.*, 2008; Cassenego *et al.*, 2011; Fisher and Phillips, 2009; Frazzon *et al.*, 2009; Riboldi *et al.*, 2009).

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Although it is known that enterococci are able to grow over a wide range of temperatures, until today there are no studies examining the effects of temperature on biofilm formation by antibiotic resistant *Enterococcus* sp. isolated from food. So, the objective of this study was to investigate the ability of antibiotic resistant *E. faecalis* and *E. faecium* isolated from food in South Brazil to form biofilm at different temperatures in the absence or presence of 0.75% glucose was evaluated.

A total of 86 antibiotic resistant enterococci (65 *E. faecalis* and 21 *E. faecium*) isolated from vegetables, meat and dairy products collected during 2006 to 2009 in Porto Alegre, RS (Brazil), were used in this study. All the strains have already been identified at genus and species level and screened for antibiotic resistance profile by disc-diffusion method (Table 1) (Riboldi *et al.*, 2009; Frazzon *et al.*, 2009). Biofilm formation on polystyrene microplates was quantified with the crystal violet staining method with some improvements (Stepanovic *et al.*, 2000). Briefly, bacteria were grown at 35 °C in tryptic soy broth (TSB) for 18 h. The wells of sterile 96-well flat-bottomed polystyrene microplates were filled with 180 μL of TSB or TSB supplemented with 0.75% glucose (TSBG) and 20 μL of bacterial suspension containing approximately 10^8 cfu/mL. Afterwards, the plates were incubated for 18 h at four different temperatures (10 °C, 28 °C, 37 °C and 45 °C). The optical density (O.D.) was measured at 492 nm (OD_{492}) in spectrophotometer (Anthos 2010 Microplates Reader) (Vogel *et al.*, 2000). The cut-off O.D. (O.D.c) was defined as three standard deviations above the mean O.D. of the negative control. All strains were separated into categories using the O.D. measurement of bacterial films, as commonly used by Stepanovic *et al.* (2000), as follows: O.D. ≤ O.D.c = non-adherent, O.D.c < O.D. ≤ (2x O.D.c) = weakly adherent, (2x O.D.c) < O.D. ≤ (4x O.D.c) = moderately adherent and (4x O.D.c) < O.D. = strongly adherent. Experiments for biofilm formation in TSB and TSBG media at different temperatures were performed at least three times for each strain. The negative control wells contained TSB or TSBG medium. *Staphylococcus epidermidis* American Type Culture Collection 35984 was used as the positive control, because it is classified as a strong adherent and has been used successfully in researches studies of biofilm formation by enterococci (Stepanovic *et al.*, 2000; Hufnagel *et al.*, 2004). The gelatinase activity was also performed at 10 °C, 28 °C, 37 °C and 45 °C, following the protocol described by Marra *et al.* (2007).

Significance of association between biofilm formation, culture media and temperature was determined by Studen's *t*-test and one-way ANOVA. Statistical analysis was performed in Statistic Package of the Social Science software (SPSS) 13th edition and a p value < 0.05 was considered significant.

The capacity of antibiotic resistant *E. faecalis* and *E. faecium* isolated from food to form biofilm *in vitro* at different incubation temperatures in the absence or presence of 0.75% glucose was conducted to understand how *E. faecalis* and *E. faecium* isolated from food can produce biofilm in a variety of environmental factors, including temperature and sugar, which are common in foods, food-processing and clinical environments. Among the 86 enterococci antibiotic resistant isolates from food, only one *E. faecalis* (1.16%) displayed a non-adherent classification under all tested conditions. The addition of 0.75% glucose in TSB increased the power of bacteria to adherent on microplates at 10 °C, 28 °C, 37 °C and 45 °C when compared to TSB (Table 2). A significant difference was observed between *E. faecalis* strains at 37 °C (p = 0.003) and 45 °C (p = 0.001) in TSBG. The results are presented in Table 2. The association of the glucose in the medium and the capacity of biofilm formation have been reported for several bacterial species (Stepanovic *et al.*, 2000; Sousa *et al.*, 2008).

Despite the fact of *Enterococcus* survive at low temperatures, only 20% *E. faecalis* and 33.3% *E. faecium* isolates were able to form biofilm in TSB at 10 °C. An increment of 6.2% and 9.6% in the adherence was observed to *E. faecalis* and *E. faecium,* respectively, in the presence of glucose (Table 2). All resistant enterococci biofilm producers at 10 °C, were classified as weakly adherent and this property was more frequently reported in strains isolated from meat and dairy products. This fact could be correlated with the adaptive response of these strains to growth at low temperature, since that low positive temperature is used to store food products. Pagedar *et al.* (2010) also observed the ability of *Staphylococcus aureus* to survive within preformed biofilm at temperatures established in dairy industries. Thammavongs *et al.* (1996) showed that the *E. faecalis* JH2-2 strain exhibit a mechanism of adaptive responses to low positive temperatures. The membrane struc-

Table 1 - Antimicrobial resistance profile of *Enterococcus faecalis* and *Enterococcus faecium* isolated from food.

	Number of strains showing resistant to[a]										
Species	AMP	ST	BAC	CIP	GEN	RIF	CLO	NIT	TET	ERI	VAN
E.faecalis	5	4	27	8	13	1	7	2	25	30	2
E.faecium	4	1	6	2	6	0	4	2	5	13	0

[a]Antibiotics: ampicillin (AMP), streptomycin (ST), bacitracin (BAC), ciprofloxacin (CIP), gentamicin (GEN), rifampicin (RIF), chloramphenicol (CLO), vancomicyn (VAN).

Table 2 - Adherence of antimicrobial-resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from food growing in TSB or TSBG at different temperatures.

Species	TSB (%)					TSBG[1] (%)			
	T[2] (°C)	NA[3]	WA[3]	MA[3]	SA[3]	NA[3]	WA[3]	MA[3]	SA[3]
E. faecalis (n = 64)	10	80.0	20.0	0.0	0.0	73.8	26.2	0.0	0.0
	28	13.8	53.8	23.1	9.3	4.6	53.8	30.8	10.8
	37	10.8	50.8	26.2	12.3	1.5*	38.5 *	32.3 *	27.7 *
	45	32.3	60.0	7.7	0.0	27.7 *	53.8 *	17.0 *	1.5 *
E. faecium (n = 21)	10	66.7	33.3	0.0	0.0	57.1	42.9	0.0	0.0
	28	14.3	38.1	19	28.6	9.5	23.8	23.8	42.9
	37	14.3	23.8	38.1	23.8	0.0	23.8	19	57.1
	45	28.6	33.3	23.8	14.3	0.0	42.9	33.3	9.5

1: TSBG: supplemented with 0.75% of glucose; 2: T: temperature; 3NA: non- adherence; WA: weak adherence; MA: moderate adherence; SA: strong adherence; *Statistically significant difference.

tures, such as lipids and fatty acid, have been related with temperature resistance in enterococci (Fisher and Phillips, 2009).

At temperature 28 °C, temperature found in Brazilian Food and Nutrition Services, 86.15% and 95.38% of *E. faecalis* were biofilm producer, in TSB and TSBG, respectively. On the other hand, the amount of biofilm produced by *E. faecium* strains was not significantly affected by the presence of the glucose at the same temperature. Baldassarri *et al.* (2001) noticed that glucose had a positive effect in *E. faecalis* isolated from clinical and environmental sampling, but not in *E. faecium.*

At 37 °C, the optimal temperature for enterococci, 98.46% *E. faecalis* and 100% *E. faecium* isolates antibiotic resistant growing in TSBG, were able to form biofilm on polystyrene. It is not surprising that bacteria form more biofilm at 37 °C than in other temperatures tested. At this temperature bacteria grow best and, consequently the cells number increase, and as a result, the cell mass facilitates the sedimentation, resulting in a higher degree of initial attachment (Dewanti and Wong 1995). The glucose in the TSB decreases the frequency of non-adherent. The ability to onset of biofilm in *E. faecalis* strains was strongly influenced by the presence of 0.75% glucose at 37 °C ($p = 0.003$) when compared with TSB at the same temperatures. The presence of glucose in the blood and urine, may be one reason why these microbes are frequently found in urinary tract, wounds, bloodstream, and endocardium infections (Hufnagel *et al.*, 2004; Marra *et al.*, 2007; Fisher and Phillips, 2009). Perhaps, the presence of the BopD protein, a homologous to a sugar-binding transcriptional regulator, may well be connected with biofilm formation and glucose in *E. faecalis* and *E. faecium* strains (Bourgogne *et al.*, 2006).

The temperature of 45 °C was chosen regarding the maximum temperature (47.8 °C) used to grow *Enterococcus* sp. (Fisher and Phillips, 2009). At 45 °C, 67.7% *E. faecalis* and 71.4% *E. faecium* antibiotic resistant isolates growing in TSB medium were able to produce biofilm. A 4.6% and 9.5% percentage increment was observed to *E. faecalis* and *E. faecium,* respectively, in TSBG. The classification more often detected in the strains was weakly adherent. A significant difference on biofilm formation and glucose ($p = 0.001$) was exhibited by *E. faecalis* at 45 °C. Stressing conditions during growth are responsible for qualitative and quantitative changes in the bacterial membrane fatty acid profile. The modification in the composition of the membrane lipids affects principally the fluidity of the cellular membrane. With the increase of membrane fatty content and the decrease of saturated fatty acid levels at higher temperatures, the enterococci membrane has demonstrated to be less resilient (Fisher and Phillips, 2009). However, the mechanisms by which the growth temperature modifies enterococci heat resistance are poorly understood.

We examined the association between the gelatinase activity, biofilm formation and temperatures, and at 10 °C 26.5% of the antibiotic resistant strains were classified as weakly adherent were gelatinase negative, on the other hand, at 45 °C 20.93% strains classified as non-adherent were gelatinase positive These results agree with Gomes *et al.* (2008), and Mohamed and Murray (2005), that established no relationship between biofilm formation and gelatinase activity.

In conclusion, this study demonstrated a synergistic effect between glucose and biofilm formation by antibiotic-resistant *E. faecalis* and *E. faecium* isolated from food at 10 °C, 28 °C, 37 °C and 45 °C. No association was detected between gelatinase, biofilm production and temperatures. The capacity of resistant enterococci isolated from food to form biofilm is alarming, since the biofilm formation contributes to survival, persistence and dissemination of resistant enterococci and/or resistance genes in diverse environmental conditions. Further studies are still needed to determine other factors that contribute to biofilm formation by enterococci isolated from food, and research should focus on methods of inactivation and removal of biofilm in

order to reduce the risk of enterococci contamination in the food industry. To our knowledge, there are no other studies that examined the biofilm forming abilities of antibiotic resistant *Enterococcus* sp. isolated from food exposed to different temperature conditions.

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