Anti-bacterial and anti-adherence activity of *Enterococcus* spp. against *Staphylococcus aureus* CECT 976

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Abstract. Lactic acid bacteria (LAB) have been associated with dairy products and are generally considered as beneficial microorganisms. They are used to improve the safety and the quality of food products by the production of many substances, such as organic acids, diacetyl, hydrogen peroxide and bacteriocins. *Enterococcus* spp. are among LAB group widely found in Mediterranean dairy products and they have been used as probiotics and bioprotective cultures in fermentative processes to control the growth of pathogenic bacteria. Moreover, they had the ability to form biofilms offering barriers against the colonization by other bacteria on biotic and abiotic surfaces. However, to our knowledge no study on the use of *Enterococcus* strains as a positive biofilm to control pathogen's adhesion on abiotic surfaces was reported. The objective of this study was to use, on one hand, *Enterococcus* spp. as protective culture in the dairy industry. On the other hand, the use of *Enterococcus* spp. as positive biofilm against adhesion of *Staphylococcus aureus 976* on Stainless steel. The results showed that *E. faecium and E. faecalis* tested had a large spectrum against *Staphylococcus aureus 976*, and their abilities to reduce the adhesion of *Staphylococcus aureus 976* were more than 2 log UFC/cm².

Keywords. *Enterococcus* spp. - Adhesion - Biofilm - *Staphylococcus aureus* CECT 976 - Stainless steel 316L.

Activité antibactérienne et anti-adhérence d'Enterococcus spp. à l'encontre de* Staphylococcus aureus *CECT 976

Résumé. *Les bactéries lactiques (LAB) isolées à partir des produits laitiers sont considérées depuis longtemps comme des micro-organismes bénéfiques. Ils sont utilisés pour améliorer la qualité et la sureté des produits alimentaires en produisant de nombreuses substances telles que: les acides organiques, le diacétyle, le peroxyde d'hydrogène et les bactériocines. Enterococcus spp. fait partie du groupe des bactéries lactiques largement répandu dans les produits laitiers méditerranéens. Ils ont été utilisés comme probiotiques et comme cultures bio-protectrices dans les processus de fermentation destinés à contrôler la croissance des bactéries pathogènes. En outre, ils étaient capables de former des biofilms offrant des barrières contre la colonisation par d'autres bactéries sur des surfaces biotiques et abiotiques. Cependant, à notre connaissance, aucune étude n'a été reportée sur l'utilisation de souches d'Enterococcus comme biofilm positif afin de contrôler l'adhésion sur l'acier inoxydable de bactéries pathogènes. L'objectif de cette étude était d'une part, l'utilisation d'Enterococcus spp. comme culture bio-protectrice dans l'industrie laitière. D'autre part, comme biofilm positif à l'encontre de l'adhésion de Staphylococcus aureus CECT 976 sur l'acier inoxydable. Les résultats ont montré que E. faecium et E. faecalis testés présentent un spectre large contre Staphylococcus aureus 976 et que leur capacité à réduire l'adhésion de Staphylococcus aureus 976 était supérieure à 2 unités log UFC / cm².*

***Mots-clés*.** *Enterococcus spp – Adhésion – Biofilm* - Staphylococcus aureus *CECT 976 - Acier inoxydable 316L.*

I - Introduction

Antibiotic resistance as a growing problem across the world stimulates wide efforts for finding safe and natural antibiotic alternative agents such as probiotics. Recently, the use of probiotics as natural live microorganisms against antibiotic-resistant and food-spoilage microorganisms has been reconsidered as an alternative to antibiotics and certain chemical preservatives. Moreover, the Adhesion of pathogenic microorganisms to equipment materials used in dairy industry and biofilm development are sustainable sources of contaminations that are responsible for food-related illness and important economic losses. This lifestyle confers protection to bacterial cells and decreases the efficiency of cleaning and disinfection procedures, because of their ability to persist in surfaces. Biofilms are aggregates microorganisms attached to each other's and embedded in a matrix of extracellular polymeric substances (EPS), which allowed their elimination more difficult from food processing environment. *Staphylococcus aureus* is well admitted for its ability to adhere and form biofilm in food surfaces (Hamadi *et al*., 2005; Oulahal *et al*., 2008) representing an important risk for food industries. Contamination of food during preparation, caused by the presence of bacteria in dairy surfaces is one of the main sources of foodborne outbreaks. Moreover, it can be a source of contamination for other foods and surfaces (Teixeira *et al*., 2007; Silva *et al*., 2003). In order to avoid contamination and biofilm formation by Staphylococcus aureus, it's important to eliminate or control its biofilm. Most workers had focused on the use of chemical treatment to eliminate the adhesion and biofilm formation (Muazu *et al.,* 2015; Emanuel *et al.,* 2010). Hence, the elimination of biofilm could not be effective to prevent them. Therefore, strategies using biofilms produced by the competitive exclusion microorganisms to inhibit foodborne pathogens in the food processing environments are of major importance. Enterococcus spp. are an important part of the natural Lactic acid bacteria consortium. They have been used as positive biofilm to control spoilage and pathogenic bacteria in sessile states (Ait Meddour *et al*., 2015; Li *et al*., 2015).

This study aims to use food isolated *Enterococcus strains* as bioprotective cultures in the dairy industry, and as positive biofilm against adhesion of *Staphylococcus aureus* CECT 976 on Stainless steel 316L.

II - Materials and Methods

1. Bacterial strains, growth and culture conditions

Enterococcus spp. used in this study were isolated by Elmoslih *et al*. (2017) and Achemchem *et al*. (2005) from different type of milk. The strain pathogen was *Staphylococcus aureus* 976 (CECT*)* from the Spanish Type Culture Collection. Strains of *Enterococcus* spp. were grown in MRS broth (Man, Rogosa, and sharpe) and *Staphylococcus aureus* 976 in BHI for 16h at 30°C.

The cells were harvested by centrifugation at 4000×g for 20 min at 4°C, then the supernatants were removed. Finally, the pellets were dissolved in a sterile saline solution and adjusted to 10^8 CFU/ml. The suspensions were used for further experiments.

2. Antibacterial activity of *Enterococcus* spp.

The inhibitory activity spectrum was obtained using the agar spot test against *S. aureus* 976 (Casla *et al*., 1996). 5 µl aliquots of an overnight culture of each producing strain was spotted onto unbuffered appropriate agar plates and incubated for approximately 24h. After that the plates, were then overlaid with 6 ml of soft agar medium (0.75% agar) seeded with against *S. aureus* 976, and then incubated. The sensitivity of the strain was evaluated by checking for clear zones around spots.

A. Surface preparation

The material used was stainless steel 316L. The material was cut into 1cm² squares (10/10/2 mm coupons-tests), and these surfaces were cleaned by soaking them in ethanol 95% for 15 min. and were rinsed three times with distilled water. Finally, the substrate was autoclaved for 20 min/120°C. (Hamadi *et al*., 2014; Akbas *et al*., 2016).

3. Effect of *Enterococcus* spp. biofilm against bacterial adherence

The surfaces have been already colonized by *Enterococcus* spp. were rinsed three times with sterilized distilled water. Then, ten milliliter of *S. aureus* 976 suspension was deposited on AISI 316L for 3 hours at 30°C. After the contact time, the surfaces were then rinsed three times to remove non adhering bacteria. To detach the bacteria cells adhered to surfaces, this latter were immersed in a test tube containing 20 ml of physiological water and sonicated for 10 min, and then the count was determined using the serial dilution technique with cultured bacteria in chapman for *Staphylococcus aureus*, for 24hours at 30°C with triplicate repetition for each strain. (Speranza *et al*., 2009).

A. Statistical analysis

Data analysis was performed using the Software STATISTICA version 6. Newman-keuls test was used to compare between bacteria (P-Values < 0.05 were considered significant). Different letters (a, b, c, and d) indicates significant differences among the strains studied.

III - Results and discussion

1. Antibacterial activity of Enterococcus spp.

The strains studied showed an inhibitory activity against *S. aureus* 976 in a plat assay (Table 1). The twelve strains isolated showed a strong inhibitory activity toward *S. aureus* 976. These results were in agreement with different research which showed that *Enterococcus* spp. have the ability to inhibit bacteria by different mechanisms such as the production of enterocins (Onda *et al*., 2002; Ait Meddour *et al*., 2015).

Table 1. Anti-bacterial activity of twelve *Enterococcus* spp. strains against *Staphylococcus aureus* 976.

Code	Species	*S. aureus* 976
A1	*Enterococcus faecium*	+++
A7	*Enterococcus faecalis*	+++
A8	*Enterococcus faecium*	+++
A9	*Enterococcus faecalis*	+++
A10	*Enterococcus faecium*	+++
A11	*Enterococcus faecium*	+++
A12	*Enterococcus faecalis*	+++
A13	*Enterococcus faecium*	+++
A14	*Enterococcus faecium*	++
A15	*Enterococcus faecium*	+++
F58	*Enterococcus faceium*	++
F420	*Enterococcus hirae*	++

Sensitivity was expressed as the size of inhibition zones: (-): <1 mm; +: 1-10; ++: 10-20 mm; +++: >20 mm;

2. Anti-adherence of Staphylococcus aureus using *Enterococcus* spp. biofilm

The anti-adhesion of *S. aureus 976* by a group of 10 days of Enterococci strains on AISI 316L showed a significant difference between Enterococci (Figure 1). The treatment of AISI 316L with *Enterococcus* spp. reduce *S. aureus* 976 adhesion ~ 2 log CFU/ml for A12 and A11. And a moderate reduction adhesion ~ 1.3 log CFU/ml for other strains except for A15 which showed a low reduction of *S. aureus* 976 by reaching 0.91 log CFU/ml reduction. Our results strongly showed the efficacy of *Enterococcus* spp. biofilms in reducing *S. aureus* 976 adhesion. In line with another finding (Amel, Farida et al. 2015) demonstrated that *Enterococcus durans* impair the adhesion and biofilm formation of pathogenic bacteria on plastic and stainless steel. This adhesion's reduction could be explained by the ability of Enterococcus spp. to produce EPS, which invasive the surfaces and reduce the adhesion of S. aureus 976. Moreover, by the production of bacteriocins, that's decrease the adhesion of pathogens.

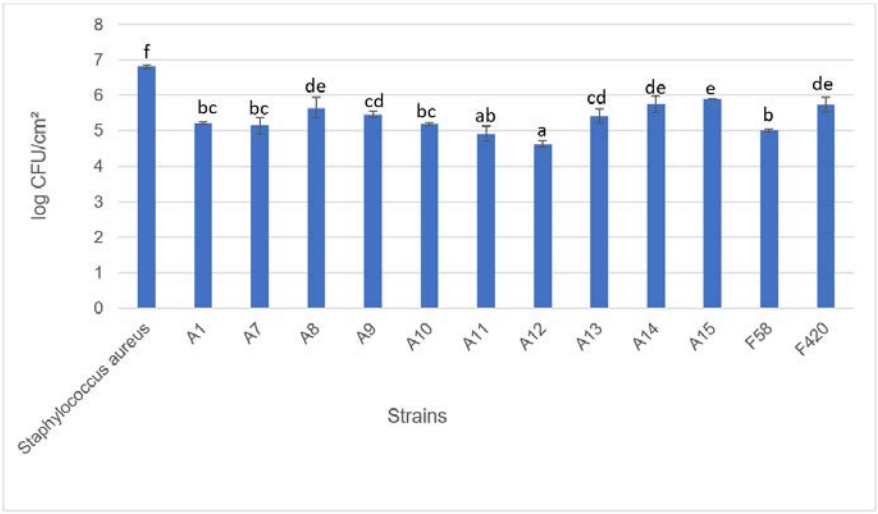

Figure 1. Adhesion inhibition of *Staphylococcus aureus 976* by *Enterococcus* spp. on AISI 316L.

IV - Conclusions

The use of *Enterococcus* spp. as positive biofilm revealed to reduce adhesion of *S. aureus* 976 approximately 2 log CFU/cm² for some strains. This work unveiled the use of *Enterococcus* spp. tested as potential probiotics and also as strategy for preventing bacteria adhesion and biofilm formation on AISI 316L to mitigate contamination of dairy product. The use of exclusion method: treatment of surfaces by *Enterococcus* spp. would represent more realistic approach on industries than other methods such as displacement and competition since preventing them is better than remove them.

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