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Enterococci in the dairy and sausage industry: Is sanitation sufficient to remove biofilm?

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ABSTRACT

Enterococcus spp. were isolated from sausage and yogurt line production. The genomic assay was performed by PCR, and distinct enterococci ($n = 28$) were identified. Testing revealed that 10.7% of the isolates had a resistant phenotype, 7.1% were resistant to erythromycin (*Enterococcus faecium*) and 3.5% were resistant to tetracycline (*Enterococcus gallinarum*). Enterococci cells and biofilm formation in 24 well polystyrene plates and the effect of sanitisation procedures in these biofilms were determined. The sanitisers were chlorinated alkaline H (CAH), chlorinated alkaline A (CAA), quaternary ammonium D (QAD), quaternary ammonium M (QAM), chlorine dioxide (CD), sodium hypochlorite (SH), and peracetic acid (PA). A total of 7 isolates (25%) moderately and the others poorly formed biofilms. The best reduction results were 61 and 55% of the cells with the CAH sanitiser in BHI and water, respectively. The PAA, SH, and CD sanitisers showed low efficiency on *Enterococcus* planctonics, and the other had an effect on cell growth. The sanitisers CAH, QAD, QAM, PAA, and SH showed efficiency in reducing the cell viability of *Enterococcus* in biofilms, and values obtained from CAA and CD suggested low biofilm removal capacity. *Enterococcus* spp. form biofilms and have become a problem in the food industry.

KEYWORDS

E. casseliflavus/flavencens, *E. faecium*, *E. faecalis*, *E. gallinarum*

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1. INTRODUCTION

Due to the ubiquitous status between pathogenic and probiotic strains, *Enterococcus* spp. have been at the heart of this discussion. Strains with probiotic characteristics have been isolated from several foods and are related to health benefits. However, they may also be related to inadequate hygiene practices, and pathogenic strains are investigated deeply for virulence and antibiotic resistance (Amidi-Fazli and Hanifian, 2022; Hammad et al., 2022). The most frequent species are *E. faecium* and *E. faecalis*, but other species have emerged, such as *E. casseliflavus* and *E. gallinarum* (Braïek and Smaoui, 2019).

Enterococcus spp. can adapt to diverse substrates and growth conditions, including the ability to form biofilms (Marinho et al., 2013). Biofilms are a population of one or more microbial species that adhere to a surface forming microcolonies surrounded by an extracellular polymeric substance (EPS) matrix responsible for resistance to adverse conditions (Nikolaev and Plakunov, 2007). When present in the food production line, the cells detach from the biofilm and contaminate the final product, inducing food spoilage.

To remove biofilms in the food industry, the solution of sanitiser must penetrate the EPS and access the microbial cells, inactivate, and remove the biofilm (Brooks and Flint, 2008). Two or more disinfectants are usually used together, *e.g.*, chlorine dioxide, sodium hypochlorite, chlorinated alkaline A/H, quaternary ammonium D/M, and peracetic acid (Pitts et al., 2003; Rather et al., 2021). It is noteworthy that the action of these substances deserves more attention when they are tested on microbial biofilms.

Given the above, the main objective of this study was to evaluate the formation of *Enterococcus* biofilms isolated from the food industry and to compare the action of sanitisers on planktonic cells and biofilms.

2. MATERIALS AND METHODS

2.1. Isolation and identification of *Enterococcus* spp

Isolation of enterococci was performed using a swab (Q-swab) from equipment involved in yogurt and sausage production lines in Paraná State (South/Brazil). The isolation locations were as follows: (1) sausage: stuffing, inox surfaces, PVC tanks, grinder, dough mixer, and spout for filling; (2) yogurt: fermenters, cooling/storage inox, polyvinyl chloride (PVC) molds. Sample collection was carried out before the start of the production process with a swab premoistened in 0.1% peptone water and 0.5% sodium thiosulphate.

All strains were grown on Kanamycin Esculin Azide Agar (KEA-Himedia) at 37 °C for 24 h. Presumptive identification of enterococci was made based on colony morphology, catalase test, and growth in the presence of 6.5% NaCl.

2.2. DNA extraction and molecular identification

The DNA was extracted following the methodology previously described (Furlaneto-Maia et al., 2014a), and PCR was used to identify four clinically relevant species using primers reported in Table 1 (Dutka-Malen et al., 1995).

Table 1. Primers used for identification of *Enterococcus* spp

Identification	Gene	Gene primer sequence (5′- 3′)	Product size (bp)
Enterococcus spp.	*tuf*	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112
E. gallinarum	vanC-1	GGTATCAAGGAAACCTC CTTCCGCCATCATAGCT	822
E. casseliflavus/*E. flavencens*	vanC-2, vanC-3	CTCCTACGATTCTCTTG CGAGCAAGACCTTTAAG	439
E. faecalis	$ddl_{E.faecalis}$	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	941
E. faecium	$ddl_{E.faecium}$	TAGAGACATTGAATATGCC TCGAATGTGCTACAATC	550

PCR amplifications were performed in a final volume of 20 µL containing 1 ρmol of each primer (Forward and Reverse), 0.17 mM dNTPs, 2.5 mM $MgCl_2$, 1 U of Taq DNA polymerase (Invitrogen), a buffer of Taq, and 5 µg mL^{-1} of template DNA. A Thermal Cycler (Techne-Tc3000) was used, and amplicons were analysed by gel electrophoresis in 1.2% agarose stained with ethidium bromide (0.5 g mL^{-1}), observed under UV transillumination, and photographed by L-PIX ST (LOCCUS).

2.3. Susceptibility to antimicrobial

The identified *Enterococcus* spp. were submitted to antimicrobial susceptibility by the disk diffusion method (CLSI, 2016). The antimicrobials vancomycin (30 µg), erythromycin (15 µg), tetracycline (30 µg), ciprofloxacin (5 µg), and chloramphenicol (30 µg) were used (Furlaneto-Maia et al., 2014b). The strains were classified as resistant (R), intermediate resistant (IR), or susceptible (S). *Staphylococcus aureus* ATCC 25923 was used as quality control.

2.4. Quantification of biofilm assay

The biofilm level was determined quantitatively by a crystal violet (CV) assay (Rocha et al., 2019). The 200 µL cells of enterococci (1.5×10^8 CFU mL^{-1}) were inoculated in 24-well polystyrene plates and incubated for 24 h at 37 °C. Sessile cells were fixed with methanol (Synth), and a crystal violet solution (1.0%) was added. The optical density (OD) was measured at a wavelength of 600 nm, and the biofilm was classified as follows: DOS ≤ DO nonbiofilm forming (NBF); DO < DOS ≤ 2× DO poorly biofilm forming (PBF); 2 × DO < DOS ≤ 4 × DO Moderately biofilm forming (MBF); 4 × DO ≤ DOS strongly biofilm forming (SBF); DO = control optical density; and DOS = sample optical density (Stepanovic et al., 2000).

2.5. Susceptibility cells and biofilm to sanitisers

The biocidal action was evaluated under two conditions, in water and BHI medium as organic matter. The 200 µL cells of enterococci (1.5×10^8 CFU mL^{-1}) were inoculated in 24-well polystyrene plates with seven industrial sanitisers (Table 2) and incubated for 24 h at 37 °C. At 0, 15 min, 1 and 3 h, 0.5 mg mL^{-1} XTT (2.3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-2H-

Table 2. Active ingredients and concentration of the sanitisers used against single cells and biofilms of enterococci

Active principle	Concentration
Chlorinated alkaline H (CAH)	2%
Chlorinated alkaline A (CAA)	3%
Quaternary ammonium D (QAD)	10%
Quaternary ammonium M (QAM)	1.5%
Chlorine dioxide (CD)	100 ppm
Sodium hypochlorite (SH)	5 ppm
Peracetic acid (PAA)	0.3%

Producers of sanitisers: Deion (CAH, CAA and SH); Mundial Química (QAD, QAM); AEB Group (PAA); Higex (CD)

tetrazolium-5-carboxanilide – Sigma–Aldrich) was added. The optical density was measured at a wavelength of 490 nm.

The same procedure was followed for the preparation of the biofilm assay. Then, the sanitiser was added, and after 30 min was removed, and XTT was added. The control was the culture *E. faecalis* ATCC 51299 without sanitisers.

2.6. Statistical analysis

The percentage values were calculated using OD values, and differences were assessed by one-way analysis of variance using statistical software (SPSS Inc., Chicago, IL, USA) with a 5% significance level. The experiment was performed in triplicate.

3. RESULTS AND DISCUSSION

3.1. Phenotypic and genotypic identification

A total of 36 swab samplings were performed in the production line of sausages, 40 colonies with enterococci characteristics on KEA, as blackening of the medium around the colony (Fig. 1A)

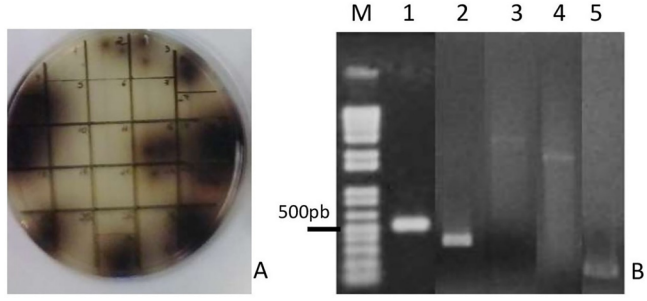

Fig. 1. (A) Characteristics of enterococci colonies on KEA. (B) Amplification gel pictures of PCR amplification. M: Ladder 1 kb plus (Invitrogen); lane 1: *E. faecium* (550 pb); lane 2: *E. casseliflavus/flavencens* (439 pb); lane 3: *E. faecalis* (941 pb); lane 4: *E. gallinarum* (822 pb); lane 5: *Enterococcus* spp. (112 pb)

were selected randomly. A total of 28 (70%) isolates were confirmed by PCR, including 10 (35%) unknown species of *Enterococcus*, 10 (35%) *E. faecium*, 4 (14.2%) *E. gallinarum*, 2 (7.1%) *E. faecalis*, and 2 (7.1%) *E. casseliflavus/flavencens* (Fig. 1B). The highest concentration of isolates was found in stuffing and grinding machines, because they are places with more contact with raw meat. The yogurt production line showed no growth of colonies indicative of enterococci. Overall, the high prevalence of enterococci of raw meat in processed foods can be attributed to their resistance during the processing stages, i.e., resistance to heat, salinity, and fermentation (Maia et al., 2020; Hammad et al., 2022).

Moreover, *Enterococcus* spp. are opportunistic microorganisms in humans, including strains acquired by the consumption of contaminated water and food (Hammad et al., 2022).

3.2. Antibiotic resistance and biofilm formation

A total of 10.7% of the isolates showed a resistant phenotype, with 7.1% resistant to erythromycin (*E. faecium*) and 3.5% resistant to tetracycline (*E. gallinarum*). Resistance to clinically relevant antibiotics, i.e., vancomycin, ciprofloxacin, and chloramphenicol were not found in enterococci. Although *E. casseliflavus/flavescens* are intrinsically vancomycin-resistant (Ben Said et al., 2016), in this study, no resistant phenotype was found. A perusal of the literature revealed varying levels of resistance in enterococci isolated from food (Gaglio et al., 2016; Hammad et al., 2022).

The ability of biofilm formation on abiotic surfaces indicated that none of the enterococci isolates presented strong or nonbiofilm formation, but 7 isolates (25%) showed a moderate ability, and the others poorly formed biofilms on polystyrene plates (Fig. 2), where sessile cells can be protected against various antimicrobials and sanitisers (Castro et al., 2017).

In the industry, biofilms occur within 24 h to 10 days of initial adhesion (Marinho et al., 2013; Castro et al., 2017; Rocha et al., 2019) and could cause corrosion of equipment and transmission of foodborne pathogens or food spoilage bacteria (Meira et al., 2012).

3.3. Sanitiser treatments on *Enterococcus* cells and biofilms

The disinfectants tested in this study were selected based on differences in their antibacterial mechanisms and intended usages. Bacterial growth was measured using XTT, and reductions in the level of viable cells in planktonic forms after treatments are given in Fig. 2.

The mixture of sanitisers was prepared in water and in BHI medium to simulate the presence of organic matter. We observed that the action of chemical agents varied according to the vehicle used as well as the exposure time. The best reduction results were 61 and 55% of the cells with the CAH sanitiser in BHI and water, respectively, at 15 min. After this time, the cells showed growth, with the exception of PAA and CD in water, at 1 h. For some sanitisers (QUAM, PAA, SH, and CD), there were no significant differences ($P > 0.05$) in the decrease of bacterial growth at the times evaluated in BHI broth. Moreover, the sanitisers PAA, SH, and CD, both in water and in BHI, showed low efficiency against *Enterococcus* cells in BHI, and the other sanitisers had an effect on cell growth. These data suggest that cells that were not effectively affected by sanitisers started to grow again at 3 h.

Reductions in the level of biofilm biomass after treatments of 30 min are given in Fig. 3. The quantification of biomass was based on the OD values obtained after the application of XTT, and red–orange colour indicated cell viability. The sanitisers CAH, QAD, QAM, PAA, and SH showed efficiency in reducing the cell viability of *Enterococcus* in biofilms when compared to the

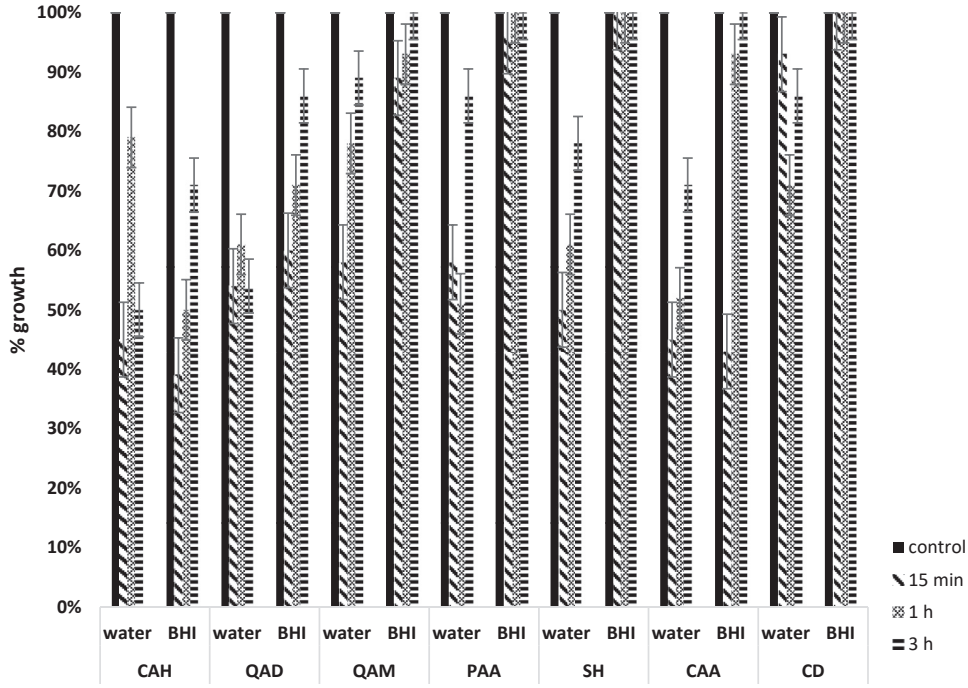

Fig. 2. Percentage of survival of *Enterococcus* cells in sanitisers chlorinated alkaline H (CAH), chlorinated alkaline A (CAA), quaternary ammonium D (QAD), quaternary ammonium M (QAM), chlorine dioxide (CD), sodium hypochlorite (SH), and peracetic acid (PAA) in Brain Heart Infusion media (BHI)

control without sanitisers. Already, according to values from CAA and CD, it is suggested that these sanitizers are not efficient in removing biofilm under the conditions tested in this study. Generally, alkaline detergent may have contributed favorably to the elimination process by dissolving minerals that take part in the biofilm surface interaction.

The efficacy of disinfectants against bacteria can be influenced by concentration, temperature, exposure time, presence of organic matter, and mainly by biofilm-forming microorganisms. Therefore, there is no consensus among authors on the precise action of disinfectants.

Torlak et al. (2015) reported the inefficiency of benzalkonium chloride, peroxyacetic acid, and CD against *Cronobacter* biofilms on polystyrene surfaces. The assumption of this hypothesis reports the dosage recommended by the manufacturer. Pitts et al. (2003) also showed the inefficiency of chlorine dioxide against *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* biofilms on plastic surfaces. Quaternary ammonium presented higher efficiency in *Listeria. monocytogenes* biofilms (Silva et al., 2020), demonstrating the proper use of this compound for sanitisation and corroborating the results obtained in this study.

After formation, bacterial biofilms can have enhanced resistance to the action of sanitisers through the EPS matrix formed, which creates a structure that hinders cleaning sanitiser penetration and does not enable their activity in the inner layers (Castro et al., 2017). It is assumed that planktonic cells or biofilms can be a potential risk for food cross-contamination,

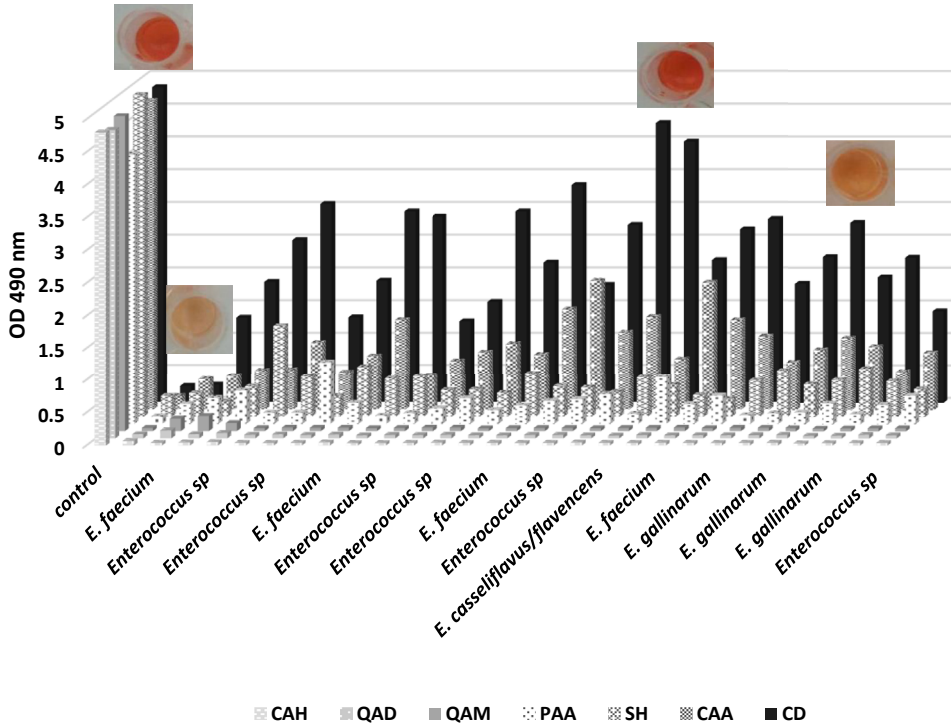

Fig. 3. Effects of sanitiser treatments in *Enterococcus* biofilms; red staining of the well due to action of XTT. CAH: chlorinated alkaline H; QAD: quaternary ammonium D; QAM: quaternary ammonium M; PAA: peracetic acid; SH: sodium hypochlorite; CAA: chlorinated alkaline A; and CD: chlorine dioxide

especially if contamination of the food occurs after an operation or thermal treatment (Meira et al., 2012).

Fernandes et al. (2015) revealed the elimination of biofilms containing *E. faecalis* and *E. faecium* after 1 day of contact with acid-anionic tensioactive, peracetic acid, and chlorinated alcaline; however, the biguanide solution was inefficient. This study differed from our protocol that kept sanitisers for 30 min; perhaps longer exposure to the sanitiser eliminates biofilm formed by *Enterococcus* spp.

To conclude, the results demonstrated that biofilm growth by different *Enterococcus* strains showed different behaviour in relation to the sanitiser used. It is worth mentioning that the structure of the biofilm, associated with the use of any antimicrobial agents, including disinfectants, exerts selective pressure and gives rise to resistant cells (Carlie et al., 2020).

4. CONCLUSIONS

The findings of this research revealed that *Enterococcus* spp. isolated from several surfaces of sausage production line were able to form biofilms on the surface of polystyrene plates.

In addition, planktonic cells in water and organic matter showed different inhibition responses in the presence of sanitisers used in the food industry. Biofilm reduction was also affected by whether the sanitiser was used in the presence of organic matter. None of the tested sanitisers completely eliminated biofilms, evidencing the difficulty of cleaning surfaces after biofilm formation.

AUTHORS' CONTRIBUTION

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Naiele Mücke. The first draft of the manuscript was written by Márcia Cristina Furlaneto and Luciana Furlaneto Maia.

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REFERENCES

Amidi-Fazli, N. and Hanifian, S. (2022). Biodiversity, antibiotic resistance and virulence traits of *Enterococcus* species in artisanal dairy products. *International Dairy Journal*, 129: 105287.

Braïek, O.B. and Smaoui, S. (2019). Enterococci: between emerging pathogens and potential probiotics. *BioMed Research International*, 2019: 5938210. https://doi.org/10.1155/2019/5938210.

Ben Said, L., Klibi, N., Dziri, R., Borgo, F., Boudabous, A., Ben Slama, K., and Torres, C. (2016). Prevalence, antimicrobial resistance and genetic lineages of *Enterococcus* spp. from vegetable food, soil and irrigation water in farm environments in Tunisia. *Journal of the Science of Food and Agriculture*, 30: 1627–1633. https://doi.org/10.1002/jsfa.7264.

Brooks, J.D. and Flint, S.H. (2008). Biofilms in the food industry: problems and potential solutions. *International Journal of Food Science + Technology*, 43: 2163–2176. https://doi.org/10.1111/j.1365-2621.2008.01839.x.

Carlie, S.M, Boucher, C.E., and Bragg, R.R. (2020). Molecular basis of bacterial disinfectant resistance. *Drug Resistance Updates*, 48: 100672. https://doi.org/10.1016/j.drup.2019.100672.

Castro, M.R., Fernandes, M.S., Kabuki, D.Y., and Kuaye, A.Y. (2017). Biofilm formation on stainless steel as a function of time and temperature and control through sanitizers, *International Dairy Journal*, 68: 9–16. https://doi.org/10.1016/j.idairyj.2016.12.005.

Clinical and Laboratory Standards Institute (2016). *Performance standards for antimicrobial susceptibility testing — 26th ed., M100*. CLSI, Wayne, PA, USA.

Dutka-Malen, S., Evers, S., and Courvalin, P. (1995). Detection of glycopeptides resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *Journal of Clinical Microbiology*, 33: 24–27. https://journals.asm.org/doi/10.1128/jcm.33.1.24-27.1995.

Fernandes, M.S., Kabuki, D.Y., and Kuaye, A.Y. (2015). Biofilms of *Enterococcus faecalis* and *Enterococcus faecium* isolated from the processing of ricotta and the control of these pathogens through cleaning and sanitization procedures. *International Journal of Food Microbiology*, 200: 97–103. https://doi.org/10.1016/j.ijfoodmicro.2015.02.004.

Furlaneto-Maia, L., Rocha, K.R., Siqueira, V.L.D., and Furlaneto, M.C. (2014a). Comparison between automated system and PCR-based method for identification and antimicrobial susceptibility profile of clinical *Enterococcus* spp. *Revista do Instituto de Medicina Tropical de São Paulo*, 2: 97–103. https://doi.org/10.1590/S0036-46652014000200002.

Furlaneto-Maia, L., Rocha, K.R., Henrique, F.C., Giazzi, A., and Furlaneto, M.C. (2014b). Antimicrobial resistance in *Enterococcus* sp. isolated from soft cheese in Southern Brazil. *Advances in Microbiology*, 4: 175–181. http://dx.doi.org/10.4236/aim.2014.43023.

Gaglio, R., Couto, N., Marques, C., de Fatima, S.L.M., Moschetti, G., Pomba, C., and Settanni, L. (2016). Evaluation of antimicrobial resistance and virulence of enterococci from equipment surfaces, raw materials, and traditional cheeses. *International Journal of Food Microbiology*, 236: 107–114. https://doi.org/10.1016/j.ijfoodmicro.2016.07.020.

Hammad, A.M., Aly, S.S., Hassan, H.A., Abbas, N.H., Eltahan, A., Khalifa, E., and Shimamoto, T. (2022). Occurrence, phenotypic and molecular characteristics of vancomycin-resistant enterococci isolated from retail raw milk in Egypt. *Foodborne Pathogens and Disease*, 19: 192–198. https://doi.org/10.1089/fpd.2021.0054.

Maia, L.F., Giraldi, C., Terra, M.R., and Furlaneto, M.C. (2020). Vancomycin and tetracycline-resistant enterococci from from raw and processed meats: phenotypic and genotypic characteristics of isolates. *Ciência Animal Brasileira/Brazilian Animal Science*, 21(1). https://revistas.ufg.br/vet/article/view/57674.

Marinho, A.R., Martins, P.D., Ditmer, E.M., d'Azevedo, P.A., Frazzon, J., Van Der Sand, S.T., and Frazzon, A.P. (2013). Biofilm formation on polystyrene under different temperatures by antibiotic resistant *Enterococcus faecalis* and *Enterococcus faecium* isolated from food. *Brazilian Journal of Microbiology*, 44(2): 423–426. https://doi.org/10.1590/s1517-83822013005000045.

Meira, Q.G.S., Barbosa, I.M., Athayde, A.J.A.A., Siqueira-Júnior, J.P., and Souza, E.L. (2012). Influence of temperature and surface kind on biofilm formation by *Staphylococcus aureus* from food-contact surfaces and sensitivity to sanitizers. *Food Control*, 25: 469–475. https://doi.org/10.1016/j.foodcont.2011.11.030.

Nikolaev, Y.A. and Plakunov, V.K. (2007). Biofilm — "City of Microbes" or an analogue of multicellular organisms? *Microbiology*, 76: 125–138. http://dx.doi.org/10.1134/S0026261707020014.

Pitts, B., Hamilton, M.A., Zelver, N., and Stewart, O.S. (2003). A microtiter-plate screening method for biofilm disinfection and removal. *Journal of Microbiological Methods*, 54: 269–276. https://doi.org/10.1016/s0167-7012(03)00034-4.

Rather, M.A., Gupta, K., Bardhan, P., Borah, M., Sarkar, A., Eldiehy, K.S.H., Bhuyan, S., and Mandal, M.J. (2021). Microbial biofilm: a matter of grave concern for human health and food industry. *Journal of Basic Microbiology*, 261: 380–395. https://doi.org/10.1002/jobm.202000678.

Rocha, K.R., Perini, H.F., Souza, C.M., Schueler, J., Tosoni, N.F., Furlaneto, M.C., and Furlaneto-Maia, L. (2019). Inhibitory effect of bacteriocins from enterococci on developing and preformed biofilms of *Listeria monocytogenes, Listeria ivanovii* and *Listeria innocua*. *World Journal of Microbiology and Biotechnology*, 35: 96. https://doi.org/10.1007/s11274-019-2675-0.

Silva, D.A.L., Tavares, R.M., and Nero, L.A. (2020). Interference of sanitizers, NaCl and curing salts on *Listeria monocytogenes* adhesion and subsequent biofilm formation. *Letters in Applied Microbiology*, 71: 438–443. https://doi.org/10.1111/lam.13374.

Stepanovic, S., Vukovic, D., Dakic, I., Savic, B., and Svabic-Vlahovic, M.A. (2000). Modified microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods*, 40: 175–179. https://doi.org/10.1016/s0167-7012(00)00122-6.

Torlak, E., Gökmen, M., and Aydemi, S. (2015). Efficacy of disinfectants against *Cronobacter* biofilm on plastic surfaces. *Quality Assurance and Safety of Crops & Foods*, 7(5): 819–823. https://doi.org/10.3920/QAS2014.0423.