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Korrespondenzadresse:
asmaa.cherifanntar@
univ-tlemcen.dz

[1])Laboratory of Food, Biomedical and Environmental Microbiology (LAMAABE), Abou Bakr Belkaid University, Tlemcen, Algeria; [2])Departamento de Microbiología y Bioquímica, Instituto de Productos Lácteos de Asturias (IPLA-CSIC), Paseo Río Linares s/n, 33300 Villaviciosa, Asturias, Spain; [3])Laboratoire universitaire de biodiversité et écologie microbienne, LUBEM Université de Bretagne Occidentale, Brest, France; [4])Institute of Applied Sciences and Techniques "ISTA", University of Tlemcen, Alegria; [5])Department of Biology, Faculty of Science, Amar Telidji University, Laghouat, Algeria

Assessment of the efficiency of cleaning and disinfection protocols against *Enterococcus faecalis* biofilms recovered from milk pipes

Bewertung der Wirksamkeit von Reinigungs- und Desinfektionsprotokollen gegen Enterococcus faecalis-Biofilme die Milchleitungen entnommen wurden

Asmaa Cherif Anntar[1,4]), Mohamed Salih Barka[1,4]), Ibrahim Benamar[1,5]), Nahida Bendimered[1,4]), Karima Boumediene[1]), Baltasar Mayo[2]), Ivan Leguirinel[3]), Buomedine Moussa Boudjemaa[1,4])

Summary

The formation of bacterial biofilm in dairy plant is the main source of milk and related dairy products contamination which is commonly related to improper Cleaning and Disinfection (C&D). Cleaning-In-Place (CIP) is the conventional process described to clean and remove on milk residues and bacterial contaminants from dairy processing lines. In this study, effectiveness of 3, 5 and 7 steps CIP protocols was evaluated against *Enterococcus faecalis (E. faecalis)* mature biofilms formed on stainless steel (SS) coupons. The results revealed that the most effective treatment was the 7 step protocol based on 2% sodium hydroxide (NaOH) cleaning combined with 3% enzyme mixture at 50°C for 20 minutes followed by 1% nitric acid (HNO_3) at 55°C for 20 minutes and completed by 0.5% quaternary ammonium at 25°C for 20 minutes showed a significant difference in the number of recovered cells between untreated and treated coupons with a value of 5.32 log cfu/cm^2 which was a very satisfying reduction level followed by CIP 2 with 4 log reduction value and CIP 1 presented by 3.14 log reduction. Even with 5 log reduction, a complete *E. faecalis* biofilm removal was not reached, showing persistence of mature biofilm to C&D protocols.

Keywords: biofilm, cleaning in place, dairy industry, *Enterococcus faecalis,* stainless steel

Introduction

Biofilm is a complex community enclosed in a self-produced organized matrix, mainly composed of extracellular polymeric substances (EPS) formed on biotic or abiotic surfaces (Costerton, 2004). The biofilm formation is one of the common ways adapted by bacteria to develop coordinated three dimensional structures that promote and increase their tolerance, resistance and persistence to sanitizers (Satpathy et al., 2016; Flemming and Wingender, 2010).

Various spoilage and disease-causing bacteria can attach and form biofilms in milk processing lines (Weber et al., 2019; Cherif-Antar et al., 2016; Srey et al., 2013; Malek et al., 2012; Sharma and Anand, 2002). Biofilm formation is favoured by both milk residues and environmental conditions during milk processing (Flint et al., 2015). Dairy biofilms can act as a harbour and/or substrate for other microorganisms' weakly biofilm producers, increasing the probability of pathogens survival and further spread during milk processing. They may also lead to post-pasteurisation contamination and cross contamination affecting the quality, functionality and safety of dairy products. Bacterial biofilm has become one of the most worrisome food hygiene problems, threatening human health and causing great economic losses (Gupta and Anand, 2018; Møretrøand Langsrud, 2017; Murpy et al., 2016).

C&D in dairy industry are very important to ensure microbial food quality and safety. C&D have been incorporated into the CIP protocols in food manufacturing industries (Romney, 1990; Zottola andSasahara, 1994). It is well known that milk residues are composed of organic and inorganic substances such as protein, butterfat, calcium and iron which may promote the bacterial adhesion (Mittelman, 1998; Flint et al., 1997). Based on this deposits nature, the standard CIP system used for dairy processing lines is as follow: 1. a pre-rinse with cold water to remove gross residues; 2. the circulation of alkaline and acid detergent (separated or dual phases) to remove fat, protein and mineral remaining residues; 3. an intermediate cold water rinse to flush out detergent; 4. the circulationof disinfectant to inactivate and reduce the surface population of viable cells remaining after cleaning thus preventing their growth on surfaces before production restarts; 5. the process is finished with a final cold water rinse to flush out disinfectant (Møretrø et al., 2012; Vlková et al., 2008; Chisti, 1999).

C&D studies have focused on removal of spoilage and foodborne pathogenic biofilms from food manufacturing including *Listeria monocytogenes, Bacillus cereus, Staphylococcus* spp., *Campylobacter jejuni, Salmonella* spp. and *Pseudomonas aeroginosa* (Fagerlund et al., 2020; Kocotet al., 2020; García-Sánchez et al., 2019; Wang et al., 2016; Lee et al., 2010).

E. faecalis is frequently isolated from clinical and food samples (Cherif-Antar et al., 2016; Kibi et al., 2013; Fisher and Phillips, 2009; d'Azevedo et al., 2006). In food, enterococci species play an important role as a start cultures or as probiotics (Cassenegoet al., 2011). It is well known that various strains of this genus isolated from clinical, environmental and food samples are resistant to several antimicrobial agents and form biofilms on biotic and abiotic surfaces (Marinho et al., 2013). They are also used as an indicator of faecal contamination (Moreno et al., 2006; Franz et al., 2003). However, the European Food Safety Authority (EFSA) does not recommend them in the qualified presumption of safety approaches (EFSA, 2008). It is difficult to find in the literature references about effective cleaning protocols against *E. faecalis* biofilms isolated from food processing plants.

The aim of this study was to optimize the effectiveness of C&D protocols to determine the most appropriate combination of parameters (chemical agent concentration, temperature and treatment time) of the highest effective protocol (3, 5 and 7steps programs) in reducing *E. faecalis* biofilms developed on SS surfaces.

Materials and methods

Strain origin

E. faecalis (lamaabe4-31) strain used in this study was isolated from dairy post-pasteurization pipe after CIP application. The strain was identified by Amplified Ribosomal DNA Restriction Analysis (ARDRA) and 16S rDNA sequencing. Then, their capacity to form biofilm was evaluated (Cherif-Antar et al., 2016).

Experimental system used for biofilm development

The experimental system used in this study for *E. faecalis* biofilm development shown in fig. 1 was inspired from systems used by Gram et al. (2007) and Bagge et al. (2001). It consists of two SS circles (AISI, 304), in which 12 SS coupons are held in a vertical and a radial position. The SS coupons are 2.5 cm long and 1 cm wide and have a thickness of 1 mm. The whole system was placed in a sterile beaker and covered with aluminum foil. Before starting the bacterial cell adhesion step and biofilm formation, the experimental SS system was previously cleaned and sterilized according to the method described by Rossoni and Gaylarde (2000). First, it was cleaned with Acetone 100%, washed by immersion in alkaline detergent [NaOH 1% (w/v), pH 13.2] for 1 hour, rinsed with sterilized water, dried and cleaned with alcohol 70% (v/v). After that, they were washed with sterile water, dried for 2 hours at 60°C and autoclaved at 121°C for 15minutes.

Protocol used for biofilm development

An overnight culture of *E. faecalis* strain was obtained from the stock on glycerol stored at –80°C and inoculated into Brain Heart Infusion (BHI) broth (Merck) at 37°C for 18 h reaching approximately 9 log cfu/mL. Volume of 200 mL of BHI broth (Merck) previously sterilized and 50 mL

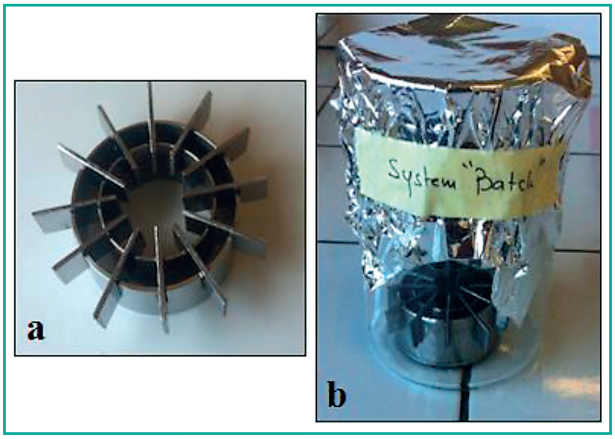

FIGURE 1: *The experimental system used for the biofilm formation of E. faecalis (a) SS device: two circles on which 12 coupons are arranged vertically and (b) the device inside the sterile beaker.*

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of BHI broth (Merck) containing the bacterial culture were added to the beaker at a final concentration of approximately 2.10^8 log cfu/mL. The whole was incubated at 37°C under 100 rpm agitation. The experiment lasted for 7 days in conditions without renewal of the medium.

Effectiveness of C&D protocols

To investigate the effectiveness of caustic detergent, acid detergent, enzyme mixture and disinfectant combined or alone in reducing the number of attached cells to SS surfaces, a 7 days *E. faecalis* biofilm was developed. Several protocols were applied with the combination of concentration, temperature and time of treatment value according to 3, 5 and 7 steps protocols shown in table 1, 2 and 3, respectively. Based on results from 3, both 5 and 7 steps protocols were established. The cleaning chemicals used were NaOH and HNO_3 while the disinfectant was quaternary ammonium. The multi-enzyme mixture tested was composed of Alpha Amylase, Protease (subtilisin), Lipase and Mannanase (Mannan endo-1,4-beta-mannosidase). All chemical products used in this study were provided by Henkel-Algeria.

To recover the attached cells remaining after treatment, each SS coupon was rinsed in 0.1% peptone water for removal planktonic cells, and then immersed into 10 mL of saline solution and sonicated at 100 Hz for 30 sec using an ultrasonic apparatus (Wise Clean-Ultrasonic Cleaner Set, D06H), then vortexed for 30 sec, this step was triplicated. The resulting suspension was diluted in saline solution and plated in triplicateon plate count agar (PCA) (Fluka). Plates were incubated at 37°C for 48 h. Three replicates were performed for each treatment. The control coupons did not receive treatment, and their counts were used to calculate the number of decimal reductions (log N cfu/cm²) due to the C&D protocols.

Statistical analysis

Analysis of variance (ANOVA) were conducted on the log transformed data to determine if any significant differences ($p < 0.05$) lay between the protocols treatments exist using Matlab, The Mathworks software.

Results

A total of 9 cleaning and disinfection protocols were applied on *E. faecalis* mature biofilms formed on SS coupons. To evaluate the effectiveness of chemical agents against biofilms NaOH, HNO_3, enzymes and quaternary ammonium were used. The basic sequence of operations is based on 3, 5 and 7 steps CIP. For each program, concentration of chemical agent, temperature and treatment time were studied.

Effectiveness of caustic 3 steps CIP protocols

The effectiveness of 3 steps CIP protocols using 1 and 2% NaOH was determined against 7 days *E. faecalis* biofilm. After 15 minutes of treatment at 90°C, 1% caustic cleaning achieved 3.98 log cfu/cm². A close value (3.82 log cfu/cm²) was obtained at 70°C after 15 minutes. On the other hand, at 80°C, the values of the log

TABLE 1: *Description of the steps and combinations applied in the CIP protocols 3 STEPS.*

Protocols/ Steps	Description			
	Chemical agents	Concentration (%:w/v)	Temperature (°C)	Time (min)
1. Water 2. Caustic detergent 3. Water	NaOH	1–2	70–80–90	5–10–15–20
1. Water 2. Acid detergent 3. Water	HNO_3	1–1.5	55–65–75	5–10–15–20
1. Water 2. Caustic detergent +Enzymes 3. Water	NaOH at 2%+ Enzymes mixture	0.5–1–2–3	30–50–70	5–10–15–20

TABLE 2: *Description of the steps and combinations applied in the CIP protocols 5 STEPS.*

Protocols/ Steps	Description			
	Chemical agents	Concentration (%:w/v)	Temperature (°C)	Time (min)
1. Water 2. Caustic detergent 3. Water 4. Acid detergent 5. Water	NaOH HNO_3	2 1.5	80 65	10 10
1. Water 2. Caustic detergent 3. Water 4. Acid detergent 5. Water	NaOH HNO_3	2 1	90 55	20 20
1. Water 2. Caustic detergent+ +Enzymes 3. Water 4. Acid detergent 5. Water	NaOH at 2%+ Enzymes mixture HNO_3	3 1	50 55	20 20

TABLE 3: *Description of the steps and combinations applied in the CIP protocols 7 STEPS.*

Protocols/ Steps	Description			
	Chemical agents	Concentration (%:w/v)	Temperature (°C)	Time (min)
1. Water 2. Caustic detergent 3. Water 4. Acid detergent 5. Water 6. Disinfectant 7. Water	NaOH HNO_3 Quaternary ammonium	2 1.5 0.5	80 65 25	10 10 20
1. Water 2. Caustic detergent 3. Water 4. Acid detergent 5. Water 6. Disinfectant 7. Water	NaOH HNO_3 Quaternary ammonium	2 1 0.5	90 55 25	20 20 20
1. Water 2. Caustic detergent +Enzymes 3. Water 4. Acid detergent 5. Water 6. Disinfectant 7. Water	NaOH at 2%+ Enzymes mixture HNO_3 Quaternary ammonium	3 1 0.5	50 55 25	20 20 20

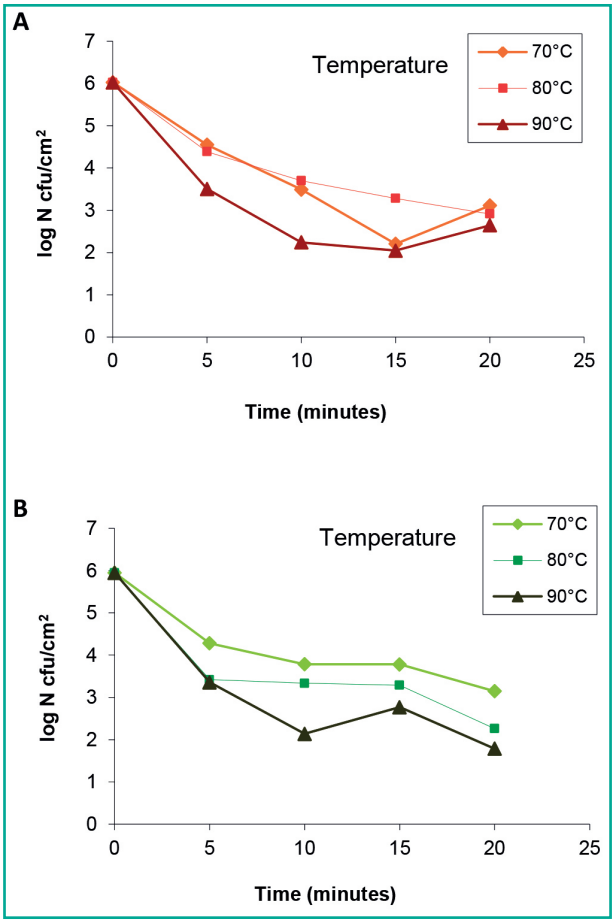

FIGURE 2: *The effectiveness of 3 steps CIP protocols using 1 (A) and 2% (B) NaOH on 7 days E. faecalis biofilm formed on SS surfaces.*

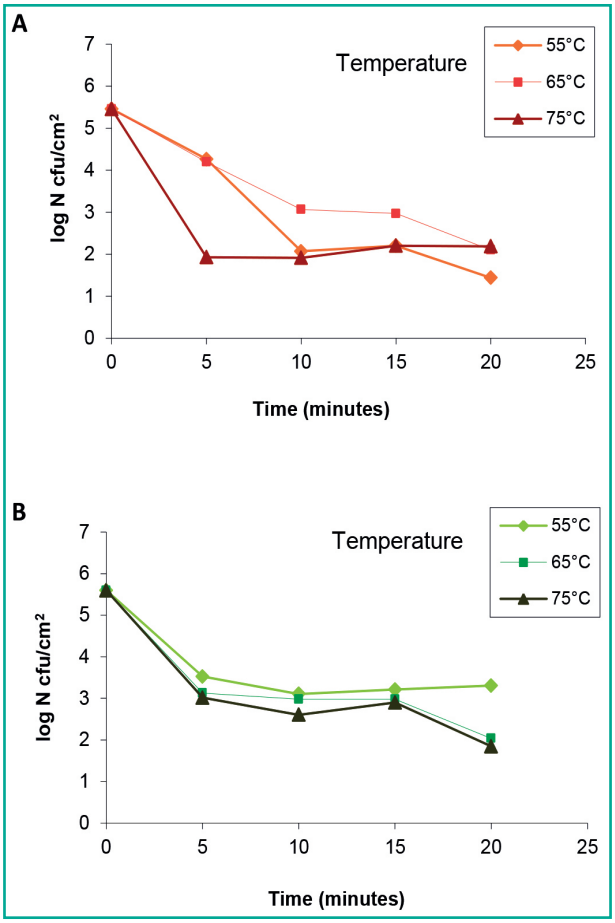

FIGURE 3: *The effectiveness of 3 steps CIP protocols using 1 (A) and 1.5% (B) HNO_3 on 7 days E. faecalis biofilm formed on SS surfaces.*

reduction varied between 1.64 and 3.11 log cfu/cm². The application of 2% NaOH protocol increased the log reduction value to 4.16 log cfu/cm² after 20 minutes at 90°C.

The kinetics of NaOH action at different temperatures on SS coupons were followed for 20 minutes (Fig. 2). It seems that the kinetic inactivation has the same convex shape in which the inactivation rate was the fastest in the first 5 minutes of contact, and then the inactivation rate decreased. Kinetics observation and the associated analysis of variance showed that increasing the NaOH concentration from 1% to 2% did not significantly affect either the rate of inactivation or the rate of reduction after 20 minutes of treatment. However, after 20 minutes of treatment, increasing the temperature from 70°C to 90°C significantly increased the inactivation rate and the fractional reduction rate.

Effectiveness of acid 3 steps CIP protocols

HNO_3 was tested at two different concentrations 1 and 1.5% on *E. faecalis* biofilms. The highest recorded log reduction (4.01 log cfu/cm²) was obtained after 1% HNO_3 treatment for 20 min at 55°C, while, treatment at 65 and 75°C reduced *E. faecalis* biofilms formed on SS coupons but not as much as coupons treated at 55°C.

The inactivation kinetics showed that the number of survival bacteria decreased rapidly during the first 5 minutes followed by a level leading to an average number of 3.4 log cfu/cm² during 20 minutes (Fig. 3). Increasing the concentration of HNO_3 from 1% to 1.5% did not significantly affect the inactivation of bacterial biofilm formed on SS coupons. However, although the effect of temperature was not very marked, it had a significant effect on the final decimal reduction numbers for the two concentrations studied. So, for E. faecalis biofilm inactivation, the effectiveness of HNO_3 treatment for the levels studied was only slightly affected by concentration and treatment temperature.

Effectiveness of caustic combined enzyme mixture 3 steps CIP protocols

The study of the impact of the enzymatic mixtures concentrations on the inactivation of 7 days *E. faecalis* biofilm formed on SS coupons was carried out under alkaline conditions in the presence of 2% NaOH. For all the kinetics obtained shown in Fig. 4 and 5, inactivation was observed during the first 5 minutes of exposure then the surviving population no longer decreased significantly. The treatment temperature strongly affects the effectiveness of the antimicrobial activity of the mixture enzyme associated with the caustic treatment. Whatever the enzyme concentration, its effect was optimal at 50°C. At 70°C, the enzyme concentration only slightly affects the inactivation of bacteria, only the effect of NaOH inactivated bacteria; it was the same at 30°C.

On the other hand, at 50°C the number of decimal reductions increased with the enzyme concentration, it was 5.3 log cfu/cm² for a concentration of 3% after 20 minutes of treatment.

The temperature activity of the enzyme mixture was optimum at 50°C, beyond or below this optimum enzyme addition temperature did not increase the effectiveness of the alkaline treatment with NaOH.

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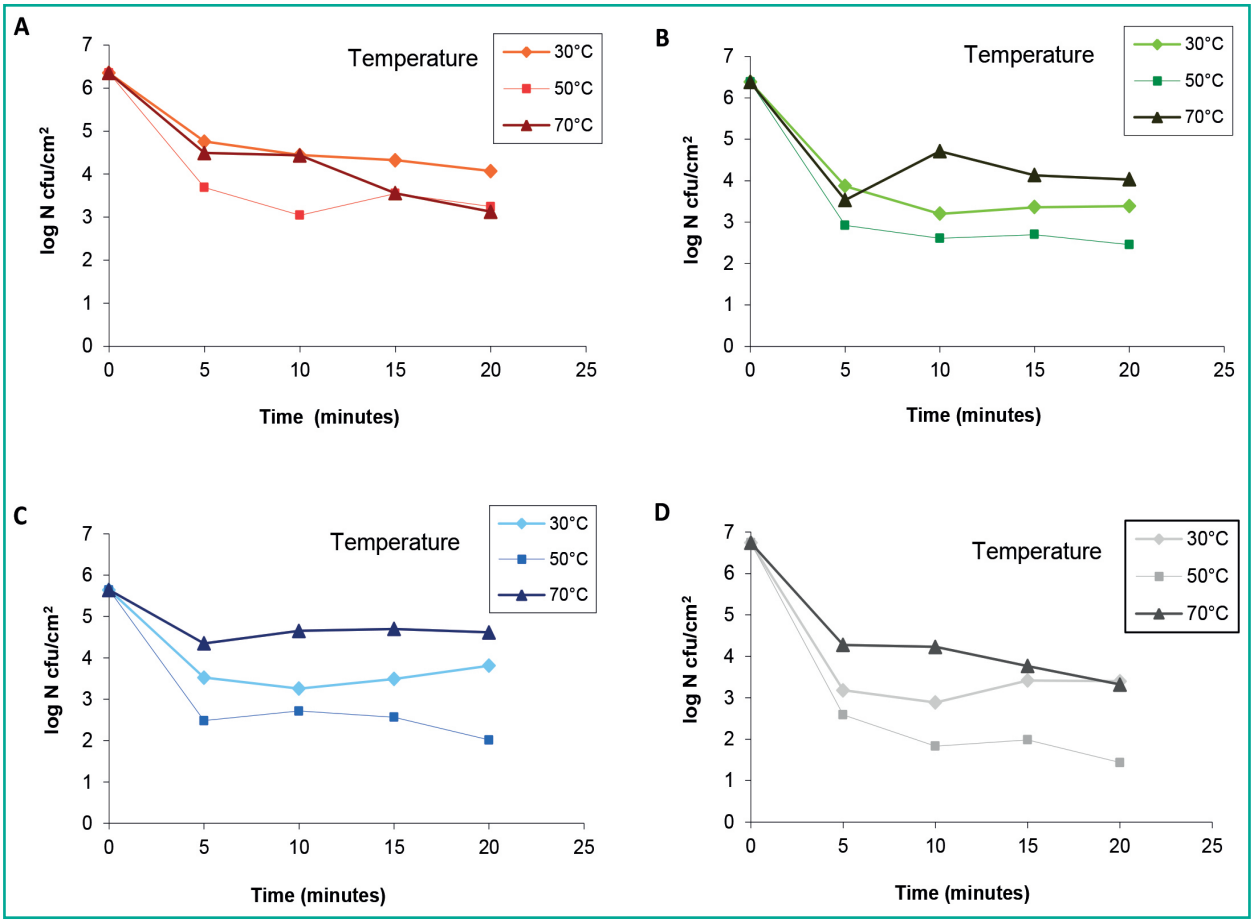

FIGURE 4: *Effect of treatment temperature of 3 steps CIP protocols based on 2% NaOH combined to enzyme mixture at different concentration A) 0.5%; B) 1%; C) 2% and D) 3% on 7 days E. faecalis biofilm formed SS surfaces.*

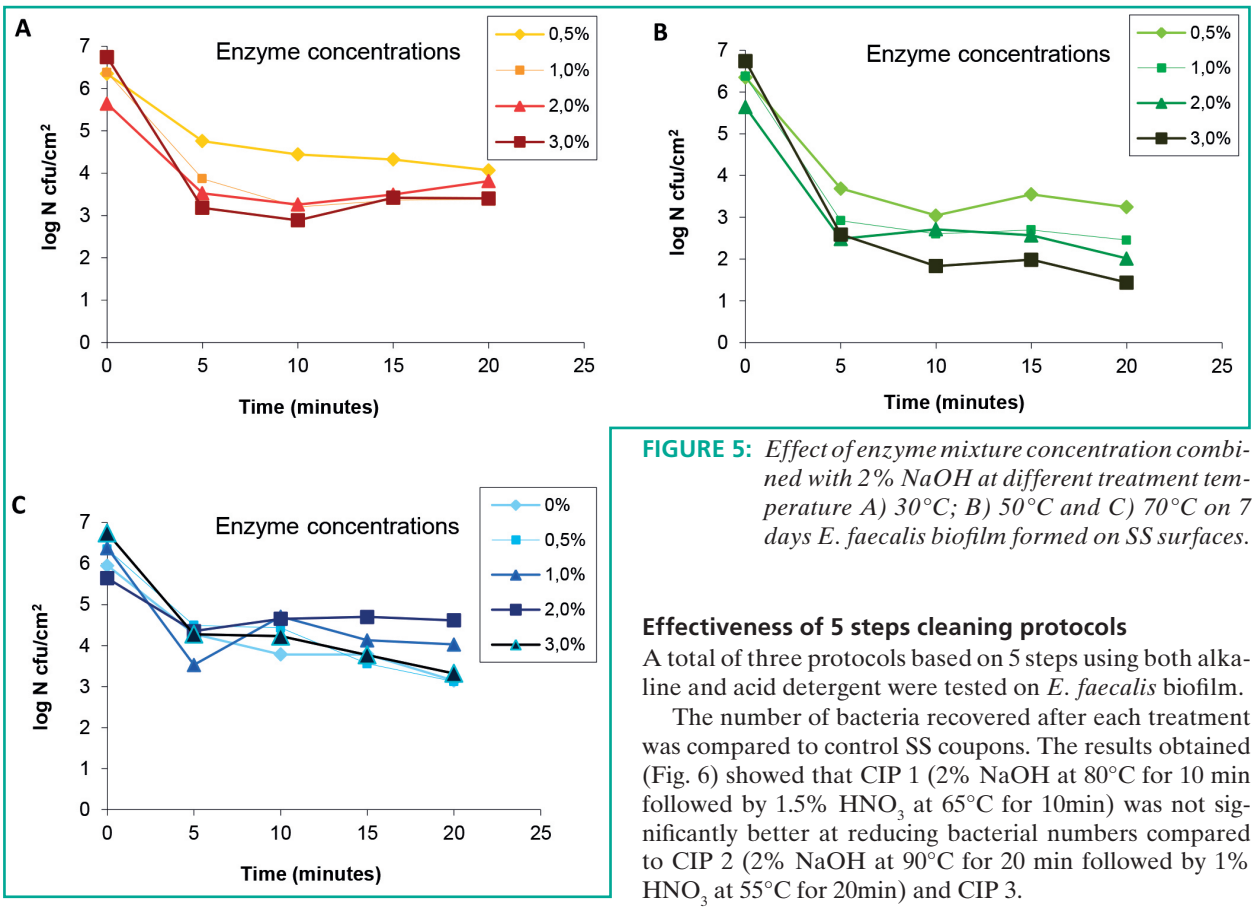

FIGURE 5: *Effect of enzyme mixture concentration combined with 2% NaOH at different treatment temperature A) 30°C; B) 50°C and C) 70°C on 7 days E. faecalis biofilm formed on SS surfaces.*

Effectiveness of 5 steps cleaning protocols

A total of three protocols based on 5 steps using both alkaline and acid detergent were tested on *E. faecalis* biofilm.

The number of bacteria recovered after each treatment was compared to control SS coupons. The results obtained (Fig. 6) showed that CIP 1 (2% NaOH at 80°C for 10 min followed by 1.5% HNO_3 at 65°C for 10min) was not significantly better at reducing bacterial numbers compared to CIP 2 (2% NaOH at 90°C for 20 min followed by 1% HNO_3 at 55°C for 20min) and CIP 3.

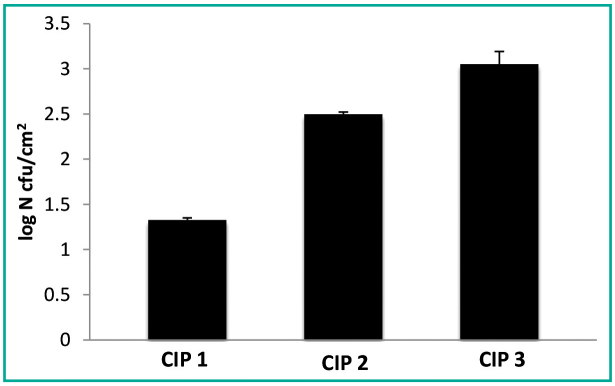

FIGURE 6: *Effectiveness of 5 Steps CIP protocols on 7 days E. faecalis biofilm formed on SS surfaces. CIP 1: 2% NaOH at 80°C for 10 min followed by 1.5% HNO_3 at 65°C for 10 min. CIP 2: 2% NaOH at 90°C for 20 min followed by 1% HNO_3 at 55°C for 20 min. CIP 3: 2% NaOH combined with 3% enzyme mixture at 50°C for 20 min followed by 1% HNO_3 at 55°C for 20 min.*

The highest log reduction with a value of 3.05 log cfu/cm² was obtained after 5 steps CIP protocol provided by enzyme mixture addition at 3% concentration composed of Alpha Amylase, Protease (subtilisine), Lipase et Mannanase (Mannan endo-1,4-beta-mannosidase).

Effectiveness of 7 steps CIP protocols

The effectiveness of incorporating disinfection steps into 7 steps CIP protocols was testing against a 7 days *E. faecalis* biofilm formed on SS coupons based on 5 steps established CIP protocols. The disinfectant tested was quaternary ammonium at 0.5% for 20 min at 25°C.

This step had increased the log reduction of attached cells compared to 5 steps CIP protocols. CIP 3 (2% NaOH combined with 3% enzyme mixture at 50°C for 20 min followed by 1% HNO_3 at 55°C for 20 min completed with 0.5% quaternary ammonium with a value of 5.32 log cfu/cm² which was a very satisfying reduction level followed by

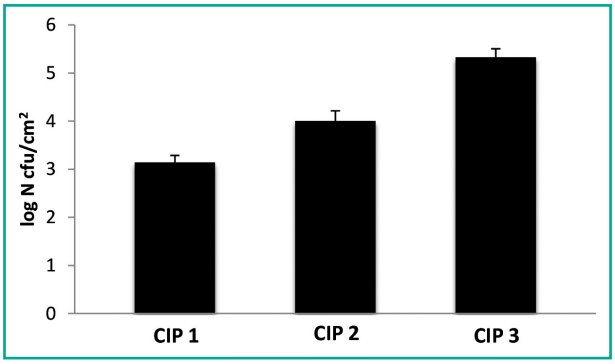

FIGURE 7: *Effectiveness of 7 Steps CIP protocols on 7 days E. faecalis biofilm formed on SS surfaces. CIP 1: 2% NaOH at 80°C for 10 min followed by 1.5% HNO_3 at 65°C for 10 min completed with 0.5% quaternary ammonium for 20 min at 25°C. CIP 2: 2% NaOH at 90°C for 20 min followed by 1% HNO_3 at 55°C for 20min completed with 0.5% quaternary ammonium for 20 min at 25°C. CIP 3: 2% NaOH combined with 3% enzyme mixture at 50°C for 20 min followed by 1% HNO_3 at 55°C for 20 min completed with 0.5% quaternary ammonium for 20 min at 25°C.*

CIP 2 with 4 log reduction value and CIP 1 presented by 3.14 log cfu/cm² compared to control (6.76 log).

Discussion

Bacterial adhesion to food contact surfaces and biofilm formation in dairy processing lines is alarming since they adversely affect the quality and safety of dairy products (Cappitelli et al., 2014; Vlková et al., 2008).

This study provides evidence of optimizing CIP protocols effect against dairy biofilms. It is difficult to find references in the literature about effective cleaning protocols for *E. faecalis* biofilm in dairy plant. Most studies on *E. faecalis* biofilms have focused on clinical strains (Keun Oh et al., 2021; Tan et al., 2019; Zheng et al., 2018).

In the present study, *E. faecalis* tested was isolated from dairy post-pasteurization pipe after CIP application. The biofilm formed on SS coupons submitted to different antimicrobial agents was a 7 days mature because the resistance of biofilm cells to C&D increases with the age of the biofilm (Fernandes et al., 2015; Srey et al., 2013; Rushdy and Othman, 2011).

Despite the development of emerging and eco-friendly strategies based on phages (O'Sullivan et al., 2019) ultrasound (Shu et al., 2021) and quorum sensing inhibitors (Lillicrap et al., 2016), a conventional CIP remains the most widely used method to mitigate undesirable biofilms (Rosado Castro et al., 2021; Fernandes et al., 2015).

Dairy biofilms are predominated by EPS and milk residues, mostly proteins and calcium phosphate. These residues may adhere to the surface and act as a conditioning film on which bacterial adherence could be promoted. Their elimination needs the use of alkaline and acid detergent (Chisti, 1999; Dunsmore et al., 1981). In our study, NaOH, HNO_3, enzyme mixture and quaternary ammonium arranged in 3, 5 and 7 steps CIP protocols were tested. The obtained results showed that 3 steps CIP protocols based on NaOH and HNO_3 reached 4.16 and 4.01 log cfu/cm², respectively. These results are similar to those obtained by Fernandes et al. (2015) showing that the application of an anionic surfactant detergent removed 4.28 log of *E. faecium* biofilm and 3.93 log of *E. faecalis* biofilm. Bremer et al. (2006) reported the inefficacy of a standard CIP protocol (water rinse, 1% NaOH at 65°C for 10 min, 1% HNO_3 for 10 min, water rinse) to remove bacteria attached to surfaces.

While the reduction rate was increased by using a successive combination of alkaline detergent with enzyme mixture composed of Alpha Amylase, Protease (subtilisin), Lipase and Mannanase (Mannan endo-1,4-beta-mannosidase) reaching 5.30 log cfu/cm² reduction of *E. faecalis* biofilm. This reduction level obtained indicates that enzymes allow to alkaline detergent more effectiveness than using alone by facilitating the chemical agent's penetration and diffusion into the biofilms and further inactivation of bacterial cells (Mnif et al., 2020; Meireles et al., 2016).

The combination of both alkaline and acid detergent in 5 steps CIP protocols has not given satisfying results until the addition of 3% enzymatic mixture to 2% NaOH at 50°C for 20 min followed by 1% HNO_3 at 55°C for 20 min.

A recent study showed when an enzymatic cocktail of Protease, lipase, cellulase, α-amylase and DNase was applied to *Macrococcus caseolyticus* dairy biofilm removal was more efficient compared with the use of NaOH (2.5%, w/v) and HNO_3 (2%, v/v) (Mnif et al., 2020).

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Molobela et al. (2010) showed that activity of Proteases and amylases on *P. fluorescens* biofilms was more effective in removing biofilms and degrading EPS when it's accompanied by other treatments. Parkar et al. (2004) has also found an improvement in cleaning effectiveness by combining the enzymatic action with detergents on *B. flavothermus* biofilm formed on SS coupons.

This study showed that the effect of added enzymes to alkaline detergent was not sufficient until the application of disinfection step. Quaternary ammonium applied gave a maximum log reduction of 5.32 log cfu/cm^2 obtained at only 0.5 % after 20 minutes. This step was preceded by 2% NaOH cleaning combined with 3% enzyme mixture at 50°C for 20 min followed by 1% HNO_3 at 55°C for 20min. The significant value obtained suggests that disinfection is essential to complement cleaning processes in dairy industry. Quaternary ammonium tested has been shown to be effective against *E. faecalis* biofilms. It is a cationic surfactant commonly used in the food industry because of their hard-surface cleaning, odour, removal and antimicrobial properties. It is active on several bacteria and can be used over a wide temperature range. Quaternary ammonium damages the outer layers of bacteria, thereby promoting the release of intracellular constituents. Besides killing bacteria, the chemical nature of quaternary ammonium can cause modifications on the properties of abiotic surfaces, decreasing their tension and therefore preventing attachment of microorganisms (Ferreira et al., 2011; Simões et al., 2005).

Our study shows the reduction of 7 days *E. faecalis* biofilm formed on SS coupons but not a complete removal which means the resistance of mature biofilms to sanitation process. Schlegelová et al., have shown in 2010 that *Listeria monocytogenes, Salmonella* spp., *Bacillus cereus, Staphylococcus* spp., *Enterococcus* spp., and *Escherichia coli* persist in biofilm on food contact surfaces after sanitation.

The capacity of resistant enterococci isolated from food to form biofilm is worrisome since the biofilm formation contributes to survival, persistence and dissemination of resistant enterococci and/or resistance genes in diverse environmental conditions (Marinho et al., 2013).

Conclusion

Biofilm installed into dairy processing lines can cause a serious problem for dairy industry due to fragility of milk and dairy products. This study investigated the effectiveness of different CIP protocols testing several concentration, temperature and treatment time. *E. faecalis* selected for biofilm formation was isolated from post-pasteurization pipe after CIP application. Despite the significant reduction of the 7 steps complete protocol, the total removal of the mono-species *E. faecalis* biofilm was not achieved revealing the persistence of mature biofilms. That leads us to think about a deeper research on microbial mix biofilm with milk flow for biofouling to reproduce the original conditions of dairy processing lines and the application of optimizing CIP cycles in a pilot plant.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Address of corresponding author:
Dr. Asmaa Cherif Anntar
Laboratory of Food, Biomedical and Environmental Microbiology (LAMAABE)
Abou Bakr Belkaid University
Tlemcen
Algeria
asmaa.cherifanntar@univ-tlemcen.dz