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1 **Diversity and biofilm-forming capability of bacteria recovered from stainless steel**
2 **pipes of a milk processing dairy plant**
3
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Abstract

Bacteria may adhere to and develop biofilm structures onto dairy surfaces trying to protect themselves from adverse conditions such as pasteurization and CIP processes. Thus, biofilms are considered common sources of food contamination with undesirable bacteria. The purpose of this study was to evaluate the diversity of the microbiota attached to stainless steel surfaces in pre- and post-pasteurization pipe lines of a milk processing plant. Seventy Gram-positive isolates were identified as *Enterococcus faecalis* (33), *Bacillus cereus* (26), *Staphylococcus hominis* (8), *Staphylococcus saprophyticus* (2) and *Staphylococcus epidermidis*-*Staphylococcus aureus* (1) species. Fifty five Gram-negative isolates were identified to the species *Escherichia coli* (18), *Klebsiella pneumoniae* (13), *Acinetobacter calcoaceticus* (6), *Serratia marcescens* (6), *Enterobacter* spp. (5), *Pseudomonas aeruginosa* (4), *Escherichia vulneris* (2), and *Proteus mirabilis* (1). Fifty five different strains were detected by the RAPD technique. These were subjected to an *in vitro* assay to evaluate their biofilm-forming capability. *E. faecalis* (7), *A. calcoaceticus* (4), *K. pneumoniae* (3), *St. hominis* (3), and *P. aeruginosa* (2) were the species in which more biofilm producer strains were encountered. The adhered microbiota was also assessed by the PCR-DGGE culture-independent technique. This analysis revealed a greater bacterial diversity than that revealed by culturing methods. In this way, in addition to the bacteria detected by culturing, DNA bands belonging to the genera *Chrysobacterium* and *Streptomyces* were also identified. This study emphasizes that knowledge of attached microorganisms to dairy surfaces may help develop strategies to improve optimal operational parameters for pasteurization and CIP processes in dairy plants.

1. Introduction

Milk is a complex substrate that can support the growth of a wide variety of both Gram-positive and Gram-negative bacteria, as well as that of yeast and moulds (Lafarge et al. 2004; Martins et al. 2006). Milk pasteurization is a common practice for safeguarding consumers from food-borne pathogenic bacteria (Ranieri et al. 2009). This practice is used worldwide in order to increase the shelf-life of this highly perishable food product (Ivy et al. 2012). After pasteurization, cleaning in place (CIP) processes are designed in order to maintain a clean and hygienic environment, including piping and fitting systems (Bayoumi et al. 2012). Bacteria surviving pasteurization and CIP processes could compromise quality and safety of the pasteurized milk and dairy products manufactured with pasteurized milk. In addition, the surviving bacteria can potentially attach to piping and fitting surfaces, where they could promote the development of biofilm structures that enable protection against high temperatures and chemical compounds applied during pasteurization and sanitization procedures. Bacteria within biofilms may attach to tools and equipment at other positions of the plant, thus persisting a longer time in the dairy environment (Brooks and Flint 2008).

Biofilms are bacterial communities embedded in an extracellular matrix composed by proteins, exopolysaccharides, DNA, and/or lipopeptides (Donlan 2002). The presence of biofilms is a widespread phenomenon in many ecosystems, including dairy plants (Latorre et al. 2010). Biofilm development takes place when planktonic cells adhere to a surface in a reversible and non-specific manner due to electrostatic interactions and begin to secrete a complex extracellular matrix. This process is affected by many factors, such as the type of surface, temperature, pH, physical interaction between the constituents, physico-chemical characteristics of bacterial and spore surfaces, etc. (Brooks and Flint 2008; Rickard et al. 2003). The presence of biofilms in

74 equipment and tools of milk processing plants, such as bends in pipes, gaskets, floors,
75 milk handling devices, etc., has been well documented (Brooks and Flint 2008).
76 Biofilms are considered as a source of microbial contamination leading to food
77 spoilage, shelf life reduction, and are also considered as a potential way of pathogen
78 transmission (Brooks and Flint 2008; Latorre et al. 2010). Bacteria embedded in a
79 biofilm have been considered as more resistant to cleaning and sanitising chemicals
80 than the corresponding planktonic cells (Anand and Singh 2013; Peng et al. 2002).
81 Biofilms can further allow attachment of non-biofilm-producing microbial types, thus
82 increasing microbial contamination (Brooks and Flint 2008). Additionally, the
83 development of biofilms creates serious problems in dairy plants due to enhanced
84 corrosion rates of metallic surfaces, reduced heat transfer efficacy, decreased flow of the
85 pipelines and increased fluid frictional resistance; aspects that reduce the
86 microbiological quality of the final products and also lead to economic losses
87 (Mittelman 1998). For all these reasons, the removal of biofilm-embedded
88 microorganisms continues to be a major challenge in dairy industry. A large number of
89 food spoilage and/or pathogenic bacteria, including *Enterococcus faecalis*,
90 *Pseudomonas* sp., *Klebsiella* sp., *Staphylococcus aureus*, *Listeria monocytogenes*,
91 *Bacillus cereus*, and others, have already been associated with biofilms from dairy
92 niches (Brooks and Flint 2008; Latorre et al. 2010; Sharma and Anand 2002).
93 This work aimed to investigate the microbial diversity attached to milk
94 processing surfaces in a dairy plant before and after pasteurization using culturing and
95 culture-independent techniques for a better description of the adherent microbiota. The
96 isolated microorganisms were identified by molecular methods, which included
97 digestion of ribosomal amplicons with restriction endonucleases (ARDRA) and 16S
98 rDNA sequencing and sequence comparison. Additionally, the biofilm forming

99 capability of the identified strains was assessed by analysing their ability to attach onto
100 polystyrene surfaces.
101
102 **2. Material and methods**
103 **2.1. Sampling procedures and isolation of microorganisms**
104 Four to six samples from each sampling point were collected over summer
105 seasons of 2010, 2011 and 2012. Two different positions were sampled along the
106 production pipeline of the milk processing plant. The sampling point A was located
107 before the pasteurizer and receives raw milk. Thus, bacteria recovered from this point
108 represent those able to attach to stainless steel surfaces. The sampling point B was
109 located immediately after the pasteurizer. In addition to be able to attach to the surface
110 of pipes, microorganisms isolated from this sampling point must survive pasteurization.
111 Samples were collected by swabbing a surface of 5 cm^2, following the procedure
112 described by Mattila et al. (1990). Swabs were refrigerated and transferred immediately
113 to the laboratory for analysis.
114 Swabs from each sampling point were used to inoculate in 5 mL of nutrient
115 broth (NB; Fluka), which were subsequently incubated overnight at 37 ºC. Overnight
116 cultures were plated onto nutrient agar plates (NA; Fluka). After an incubation period of
117 24 h at 37 ºC, colonies were picked at random from the plates, purified by subculturing
118 and stored at 4 ºC on the same media. For long-term storage, isolates were cultured in
119 Brain Heart Infusion (BHI, Merck) broth, a 25 % glycerol was added (Merck), and kept
120 frozen at -80 ºC.
121
122 **2.2. Phenotypic identification isolates**

123 Isolates were examined for cell morphology and Gram reaction. According to
124 these, isolates were separated in three groups, Gram-positive rods, Gram-positive cocci
125 and Gram-negative rods.
126

127 **2.3. Molecular identification by ARDRA and 16S rDNA sequencing**

128 DNA extracts of the purified isolates were obtained by suspending a colony in
129 100 μL of molecular-biology-grade water (Sigma-Aldrich), heating at 98 ºC for 15 min,
130 and then treating with the same volume of chloroform. After centrifugation at 16,100 g
131 for 3 min, DNA extracts were harvest from the supernatants and were used directly in
132 PCR reactions. For some isolates in which this procedure did not result in amplification,
133 total genomic DNA was purified using the ATP Genomic Mini Kit (ATP Biotech)
134 following the manufacturer's recommendations. DNA extracts or purified genomic
135 DNA was used as a template to amplify a 1.5 kb DNA fragment of the 16S rRNA gene
136 (16S rDNA) using the universal bacterial primer S-D-Bact0008-a-S-20 27F (5'-
137 AGAGTTTGATYMTGGCTCAG-3') and the universal prokaryotic primer S-*-
138 Univ1492R-b-A-21 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane 1991). The
139 PCR conditions were as follow: one cycle at 95 °C for 5 min, 35 cycles at 94 °C for 30
140 s, 55 °C for 45 s and 72 °C for 2 min, and a final extension cycle at 72 °C for 10 min.
141 For amplified ribosomal DNA restriction analysis (ARDRA), amplicons were purified
142 to remove unincorporated primers and nucleotides using ATP^{TM} Gel/PCR Extraction
143 Kit (ATP Biotech), and subjected to restriction with the restriction enzymes HinfI,
144 HhaI, Sau3AI and HaeIII (Takara), all of them with a short length (4-5 bp) recognition
145 sequence. Digestion fragments were separated in 1% agarose, stained with ethidium
146 bromide (0.5 mg·mL^{-1}) and photographed under UV light.

147 Representative 16S rDNA amplicons of all different ARDRA profiles were
148 sequenced by cycle extension in an ABI 373 DNA sequencer (Applied Biosystems).
149 Approximately 800 bp of sequence was obtained per amplicon; these were compared
150 with those deposited in the GenBank database, using the online BLAST program
151 (http://www.ncbi.nlm.nih.gov/BLAST/), and with those in the Ribosomal Database
152 Project (http://rdp.cme.msu.edu/index.jsp). Sequences sharing a percentage of identity
153 of 97 % or higher to those in databases were considered to belong to the same species
154 (Stackebrandt et al. 2002).
155
156 **2.4. PCR fingerprinting**
157 The intraspecies genetic diversity of the isolates was assessed by independent
158 PCR fingerprinting with primers BOXA2R (5′-ACGTGGTTTGAAGAGATTTTCG-
159 3′), as reported by Koeuth et al. (1995), and M13 (5′-GAGGGTGGCGGTTCT-3′), as
160 described by Rossetti and Giraffa (2005). PCR reaction mixtures contained 5 µL of each
161 DNA extract or purified genomic DNA, 25 µL of Taq master Mix (Ampliqon), 5 µL of
162 primer (10 µmol·L^{-1}) and 15 µL of molecular-biology-grade water in a total volume of
163 50 µL. The PCR conditions for the RAPD analysis were the same as those described
164 above with an annealing temperature of 40 ºC for primer BOXA2R and 42 ºC for primer
165 M13. Reproducibility studies of the PCR fingerprinting technique for the mentioned
166 primers (independent amplification with the same DNA) showed a percentage of
167 similarity of over 90 %. PCR profiles were visualized after 90 min of electrophoresis
168 (75 V) in agarose gels (1.2 %) after staining with ethidium bromide as above. Profiles
169 were clustered using the unweighted pair group method using arithmetic averages
170 (UPGMA) and their similarity expressed by the Sørensen–Dice's coefficient.
171

172 **2.5. Detection and quantification of biofilm production**

173 Biofilm production on polystyrene surface was determined using 96-well
174 microtiter-plates (Nunc), following the quantitative method described by Stepanovic et
175 al. (2000) with minor modifications. In short, 10 µL of an overnight culture at 37 ºC in
176 Tryptic Soy Broth (TSB) supplemented with 0.25 % glucose (for optimal growth of all
177 species) was used to inoculate at 5 % (cell concentration of $\approx 10^6$ $CFU \cdot mL^{-1}$)
178 independent microtiter-plate wells with 200 µL of the same medium. Plates were
179 incubated aerobically for 24 h at 37 ºC. Then, two rounds of vigorous washings with
180 phosphate-buffer saline (PBS) were realized to remove non adhered cells. Microtiter-
181 plates were subsequently dried at room temperature for 15 min prior to staining with a
182 0.1 % crystal violet solution for 15 min. Excess of stain was rinsed off by dipping the
183 microtiter-plates in tap water. After further drying, adherence of the cells was measured
184 as the absorbance released at 595 nm by using an automatic microtiter-plate reader
185 (Bio-Rad) after solubilisation of the dye bound to the plates with a 33 % acetic acid
186 solution. Based upon the absorbance, strains were classified into the four following
187 categories: no biofilm producer ($OD \leq OD_c$), and weak ($ODc < OD \leq 2X\ ODc$),
188 moderate ($2X\ ODc < OD \leq 4X\ ODc$), or strong ($OD > 4X\ ODc$) biofilm producer
189 (Stepanovic et al. 2000), where ODc is the optical density measured for the negative
190 control. Each strain was tested in quadruplicate and average results are presented.
191 Negative (uninoculated broth) and positive (*Staphylococcus epidermidis* B, DG2Ñ and
192 YLIC17, strong biofilm-forming strains) (Delgado et al. 2009) controls were assayed in
193 the same conditions.

194

195 **2.6. Analysis of adherent bacteria by PCR-DGGE**

196 The composition and dynamics of the dominant bacterial populations in the
197 biofilms was analyzed by the culture-independent PCR-DGGE technique. To do this,
198 samples were collected in three consecutive days from identical positions as those
199 analyzed by culturing. Total DNA of the samples was extracted using QIAamp DNA
200 Stool Mini kit (Qiagen) following the manufacturer's instructions. Amplification of the
201 V3 region of the bacterial 16S rRNA gene was performed using two universal primers
202 357F (5'-CCTACGGGAGGCAGCAG-3') and 518R (5'-
203 GTATTACCGCGGCTGCTGG-3'). A GC clamp was attached to the 5' end of the
204 forward primer (CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG),
205 as reported by Muyzer et al. (1993). The PCR conditions were as follow: 95ºC for 5
206 min, 35 cycles at 95 ºC for 30 s, 56 ºC for 30 s, and 72 ºC for 1 min, and a final
207 extension cycle at 72 ºC for 10 min. The DGGE analysis was performed at 60 ºC in 8 %
208 polyacrylamide gels with a formamide-urea denaturing range of 40-60 % using a DCode
209 apparatus (Bio-Rad). Electrophoresis was conducted at 75 V for 17 h and the DGGE
210 patterns were visualized after staining with ethidium bromide as above. The most
211 intense bands were excised from the acrylamide gels and identified after reamplification
212 with the original pair of primers without the GC clamp, DNA purification and
213 sequencing as above.
214
215 **3. Results**
216 **3.1. Culture analysis of pipe-adhered microorganisms**
217 A total of 125 isolates representative of all colony morphologies was selected
218 from the two different sampling points analysed in this study. Isolates were firstly
219 grouped as Gram-negative rods (55 isolates; 44 %), Gram-positive cocci (44 isolates; 35
220 %), and Gram-positive rods (26 isolates; 21 %) (Table 1). Most microbial types were

221 found in both A (after pasteurization) and B (before pasteurization) sampling points. As
222 can be clearly seen, the number of Gram-negative bacteria recovered decreased after
223 pasteurization (from 63 % to 31 %). In contrast, numbers of Gram positive isolates
224 increased after this process (from 37 % to 69 %).

225 The ARDRA profiles obtained after digestion of the amplicons with the
226 restriction enzyme HinfI gave 8 different profiles (Figure 1). Digestion of the amplicons
227 with the restriction enzymes HhaI, Sau3AI and HaeIII further separated some of the
228 profiles to a final total number of 13 different restriction patterns. Representative
229 amplicons of all different profiles were sequenced and their sequences compared with
230 those deposited on databases. Extending the sequencing results to all isolates, 41 out of
231 the 44 Gram-positive cocci isolates were allocated to the species *Enterococcus feacalis*
232 (33) and *Staphylococcus hominis* (8) (Table 1). The other three Gram-positive isolates
233 could not be identified with confidence to the species level. Two of these were
234 identified as members of the *Staphylococcus saprophyticus* species group and one as
235 belonging to the *Staphylococcus epidermidis-Staphylococcus aureus* species group. A
236 single ARDRA profile was obtained from all Gram-positive rods, which was identified
237 by sequencing as belonging to the species *Bacillus cereus* (Table 1). Fifty out of the 55
238 Gram-negative isolates were classified as *Escherichia coli* (18), *Klebsiella pneumoniae*
239 (13), *Acinetobacter calcoaceticus* (6), *Serratia marcescens* (6), *Pseudomonas*
240 *aeruginosa* (4), *Escherichia vulneris* (2), and *Proteus mirabilis* (1) (Table 1). As before,
241 five of the isolates could only be identified to the genus level; they all were assigned to
242 *Enterobacter* spp.

243 Using a threshold of 90% identity obtained in the reproducibility study, 55
244 different RAPD profiles were independently obtained with both M13 and BoxA2R
245 primer (data not shown). As an example, Figure 2 depicts all profiles obtained for the

246 Gram-positive isolates. Therefore, all these 55 profiles were considered to belong to
247 different strains. In this way, 44 Gram-positive cocci isolates resulted in 15 different
248 strains, of which 7 belonged to the species *E. faecalis*, 5 to *St. hominis*, and 2 and 1 to
249 *St. saprophyticus* and *St. epidermidis-St. aureus* species group, respectively. Five
250 profiles were considered among the 26 *B. cereus* isolates. Finally, 35 RAPD strains
251 were found among the 55 Gram-negative isolates, as follows: 9 of *E. coli*, 8 of *K.*
252 *pneumoniae*, 5 of *Enterobacter* sp., 4 of *A. calcoaceticus*, 3 of *P. aeruginosa,* 3 of *S.*
253 *marcescens*, 2 of *E. vulneris,* and 1 of *P. mirabilis*.
254
255 **3.2. Biofilm forming ability of the strains**
256 One strain each of the 55 RAPD profiles was tested for its biofilm forming
257 ability on polystyrene surfaces; this was considered an indirect proof of biofilm
258 production. The results of this assay are summarized in Table 2. Under the study
259 conditions, a majority of the strains (38) were considered as no biofilm producers (27
260 strains) or weak biofilm producers (11 strains). In contrast, 17 strains showed moderate
261 (15) or strong (2) capacity for biofilm formation on polystyrene surfaces. None of the
262 strains of the species *E. coli* (9), *E. vulneris* (2) and *S. marcescens* (3) were able to form
263 biofilm. In contrast, strains of all other species showed at least a certain ability to
264 produce biofilm on the polystyrene surface of the plates. The strongest biofilm
265 producers belonged one strain each to the species *E. faecalis* and *St. hominis.*
266
267 **3.3. Culture independent analysis of pipe-adhered microorganisms**
268 As the pre-enrichment (resuscitation) step could introduce some bias on the
269 microorganisms originally present in the biofilms at the two sampling points analysed, a
270 PCR-DGGE approach was performed to assess by a culture-independent method the

271 composition of the bacterial populations. Three samples in consecutive days were taken
272 from a single site before pasteurization (A1) and from two points after pasteurization
273 (B1 and B2) and analysed by PCR-DGGE (Figure 3). A high bacterial diversity was
274 discovered among samples at the different sampling points, but also between samples
275 from the same sampling point at different dates. Bands at the same level (belonging to
276 the same species) and with similar intensity were only observed occasionally. A total of
277 19 bands were identified, after DNA elution, reamplification, sequencing and sequence
278 comparison against databases. In addition to sequences of the genera identified by the
279 conventional culture approach (*Enterobacteriaceae, Bacillus, Peseudomonas*),
280 sequences belonging to previously undetected genera (*Chryseobacterium,*
281 *Streptomyces*) were also identified.
282
283 **4. Discussion**
284 In this study, pipe-adhered microorganisms from a milk processing dairy were
285 recovered in culture and identified from two different positions, before (sampling point
286 A) and after pasteurization (sampling point B). A varied microbiota composed of both
287 Gram-positive and Gram-negative bacteria belonging to 13 species was isolated from
288 the two positions. This strongly suggests that the pasteurization process does not select
289 for specific bacteria among those present in raw milk. High genetic diversity was found
290 among isolates recovered before and after pasteurization from all bacterial species,
291 which further supports the view that pasteurization does not make a selection for certain
292 genetic profiles. All microbial types identified in this work have already been reported
293 to attach to stainless steel pipe surfaces (Anand and Singh 2013; Mattila et al. 1990;
294 Sharma and Anand 2002). The presence of microorganisms on dairy surfaces in post-

295 pasteurization lines is a cause of concern, as it may cause spoilage of processed dairy
296 products and/or be involved in food safety issues.

297 The bacterial diversity, both at the species and strain levels, was maintained
298 from the raw milk section (A) to the pasteurization section (B), as 11 different species
299 were obtained from both positions. High microbial loads, post-pasteurization
300 recontamination and heat resistance of the strains may all contribute to the presence of
301 high microbial diversity on post-pasteurization pipe line surfaces. However, as concerns
302 the recovery of isolates, those of *E. faecalis* and *B. cereus* increased their numbers after
303 pasteurization. The heat resistance of enterococci from milk and dairy products has been
304 reported before by several authors (Martinez et al. 2003; McAuley et al. 2012).
305 Resistance of *B. cereus* to pasteurization conditions is neither surprising, given the
306 ability of this species to form heat-resistant endospores (Huck et al. 2007). The
307 increasing recovery of these species is maintained even after the CIP process (data not
308 shown), suggesting these bacteria are resistant to different stressful conditions.

309 Resistance to heat (pasteurization) and cleaning (CIP) processes might be linked
310 to the ability of the microorganisms to form biofilms, as has been reported by many
311 authors (Anand and Singh 2013; Flint et al. 2002; Peng et al. 2002). In this context, all
312 *E. faecalis* strains analysed in this work were able to form biofilms on polystyrene
313 surfaces. Similar results have been reported before for enterococci strains from raw milk
314 and fermented meat Jahan and Holley (2014). Similarly, many of the tested
315 *Staphylococcus* strains exhibited a moderate to strong capacity to form biofilms.
316 Staphylococci species have been frequently shown to harbour *ica* genes (Gutierrez et al.
317 2012; Szweda et al. 2012), which are involved in biofilm formation. However,
318 surprisingly, and in contrast to previous reported results (Faille et al. 2001), most *B.*
319 *cereus* strains were found to be no-biofilm producers, even though isolates of these

320 species were a majority population in all samples. The abundance of potential non-
321 biofilm producers strains in all samples argues for attachment of these bacteria to the
322 real biofilm producers forming mixed-species biofilms (Habimana et al. 2010; Lourenco
323 et al. 2011; Simoes et al. 2007) or for these bacteria being attached to inert milk
324 constituents (fat, protein) precipitating on the stainless steel surfaces. This is not
325 surprising since the methodology followed in this study selected for attachment not for
326 biofilm formation. Nevertheless, half of the isolates were shown to produce biofilms in
327 polystyrene plates. In this context, previous studies on biofilm formation on polystyrene
328 surfaces have been found to be positively (Moretro et al. 2003) or negatively (Rivas et
329 al. 2007) correlated with biofilm formation on stainless steel. The ability of the strains
330 of this study to form biofilms onto stainless surfaces has yet to be demonstrated.
331 Different bacterial profiles in samples before (A) and after (B) pasteurization, in
332 different pipe sections (B1 and B2) and at different dates (1, 2 and 3) were revealed by
333 the PCR-DGGE technique. This indicates major changes in the types and numbers of
334 populations in the different sampling points and from consecutive samples of the same
335 point. This strongly suggests that a resident biofilm-forming microbiota was not
336 established in this dairy. It was surprising not to find DNA bands corresponding to *E.*
337 *faecalis*, as it was a dominant species among the cultures. Disagreement between
338 culturing and culture dependent approaches could be due to the pre-enrichment step,
339 which may select for species in good physiological conditions (more thermophilic)
340 and/or those growing faster in the culture conditions of this study. Due to the small 16S
341 rDNA segment amplified for the DGGE analysis, sequences could only be assigned to a
342 genus level; therefore, bands with the same number may belong to different species.
343 The DGGE technique is considered semi-quantitative, as the intensity of individual
344 bands is thought to be an indirect measure of the abundance of their DNA in the

345 population (Muyzer et al. 1993). Though the technique does not distinguish between
346 DNA coming from dead or alive bacteria, the DGGE is considered a valuable tool for
347 the molecular fingerprinting of the microbiota associated to pipes in a dairy plant.

348 In conclusion, culturing and culture independent methods were applied to study
349 the pipe-associated microbiota at different positions in a dairy. A high inter- and intra-
350 species microbial diversity was found among the bacteria recovered from sampled
351 positions. The results suggest that a biofilm-producing microbiota was not established
352 in the analysed dairy plant. Instead, the recovered bacteria can be a reflection of the
353 day-to-day microbial variation of both bacterial types and numbers. In spite of this, the
354 presence of high numbers and types of Gram positive and Gram negative bacteria
355 should be taken into account to implement stronger hygiene routines. The establishment
356 of optimal operational parameters (pasteurization temperature, type and concentration of
357 sanitizers) to improve the overall quality, shelf-life and safety of the milk requires
358 further investigation.

359

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364 **Conflict of interest** The author declare that they have no conflict of interest.

365

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Table 1.- Molecular identification of the isolates recovered in this study from stainless steel pipes of a milk processing plant from samples before (A) and after (B) pasteurization.

Type of microorganisms	**N° of isolates (%)**		**Molecular identification**	**Total**
	Pre-past (A)	Post-past (B)		
Gram-positive cocci	6	27	*Enterococcus feacalis*	33
	5	3	*Staphylococcus hominis*	8
	-	2	*Staphylococcus saprophyticus* species group	2
	1	-	*Staphylococcus epidermidis-St. aureus* species group	1
Gram-positive rods	7	19	*Bacillus cereus*	26
Total Gram-positive	**19**	**51**		**70**
Gram-negative rods	13	5	*Escherichia coli*	18
	4	9	*Klebsiella pneumoniae*	13
	3	3	*Acinetobacter calcoaceticus*	6
	3	2	*Enterobacter* sp.	5
	5	1	*Serratia marcescens*	6
	3	1	*Pseudomonas aeruginosa*	4
	-	2	*Escherichia vulneris*	2
	1/1	-	*Proteus mirabilis*	1
Total Gram-negative	**32**	**23**		**55**
Total	**51**	**74**		**125**

Table 2.- Biofilm forming ability onto a polystyrene surface of bacterial strains isolated from stainless steel pipes from a milk-processing dairy plant.

Species	**Biofilm forming ability**[a]				**Total**
	Negative (-)	Weak (+)	Moderate (++)	Strong (+++)	
Escherichia coli	9	-	-	-	**9**
Klebsiella pneumoniae	2	3	3	-	**8**
Enterococcus faecalis	-	4	2	1	**7**
Bacillus cereus	3	2	-	-	**5**
Enterobacter sp.	3	1	1	-	**5**
Staphylococcus hominis	2	-	2	1	**5**
Acinetobacter calcoaceticus	-	-	4	-	**4**
Pseudomonas aeruginosa	1	-	2	-	**3**
Serratia marcescens	3	-	-	-	**3**
Escherichia vulneris	2	-	-	-	**2**
Staphylococcus saprophyticus species group	1	-	1	-	**2**
Staphylococcus epidermidis-St. aureus species group	1	-	-	-	**1**
Proteus mirabilis	-	1	-	-	**1**
Total	**27**	**11**	**15**	**2**	**55**

[a] Each strain was tested in quadruplicate and average results are presented.

Figure legends

Figure 1.- Differentiation between species using ARDRA profiles obtained after amplification of 16S rDNA and digestion of the amplicons with the restriction enzymes: **a**) HinfI, **b**) HhaI, **c**) Sau3AI and **d)** HaeIII. Lines: **M**, GRS Universal Ladder (Grip), **1**, *K. pneumoniae*; **2**, *A. calcoaceticus*; **3**, *Enterobacter* spp; **4**, *E. coli*; **5**, *E. vulneris*; **6**, *P. aeroginosa*; **7**, *S. marcescens*; **8**, *P. mirabilis*; **9**, *B. cereus*; **10**, *E. faecalis*; **11**, *St. hominis*; **12**, *St. saprophyticus* species group; **13**, *St. epidermidis- St. aureus* species group.

Figure 2.- Representative fingerprinting PCR profiles obtained with primer M13, as follows: **a**) Gram-positive rods isolates **b**) Gram-positive cocci isolates. **M**, Molecular weight marker; Lines **1-5**, *B. cereus* strains; **6-12**, *E. faecalis* strains; **13-17**, *St. hominis* strains; **18-19**, *St. Saprophyticus* group strains; **20**, *St. epidermidis-St. aureus* group strain.

Figure 3.- DGGE profiles of PCR amplicons of the V3 region of the bacterial 16S rDNA representing the biodiversity of the bacterial communities attached to milk pipes in a dairy. Samples were taken in three consecutive days at one position before (A) and at two position after (B1 and B2) pasteurization. Bands with a number were identified at the genus level after DNA isolation, reamplification, sequencing and sequence comparison. Identity of the bands: 1, *Enterobacteriaceae*; 2, *Bacillus* spp.; 3, *Lactobacillus* spp.; 4, *Pseudomonas* spp.; 5, *Shewanella* spp.; 6, *Streptomyces* spp.; 7, *Serratia* spp.; 8, *Chryseobacterium* spp.

Figure 1.

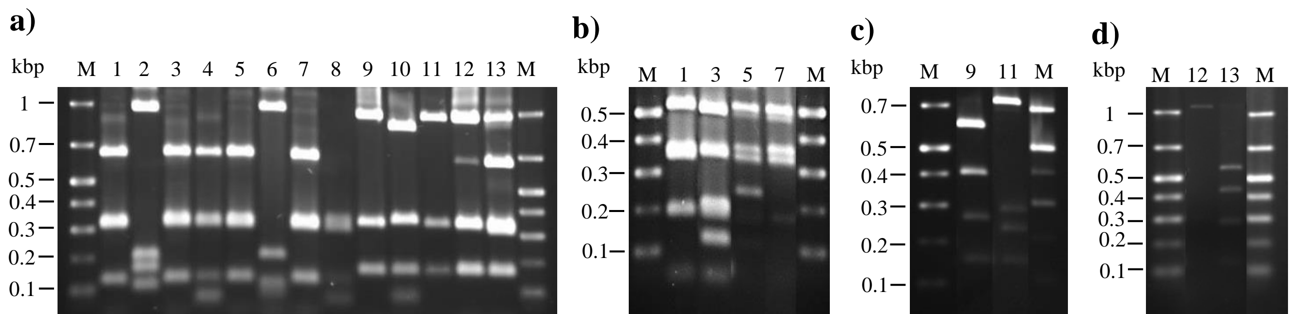
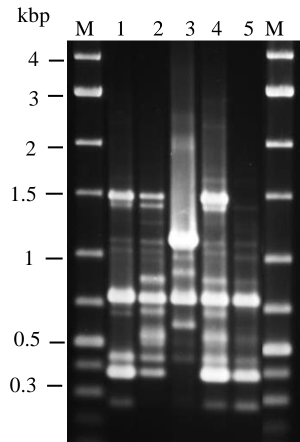
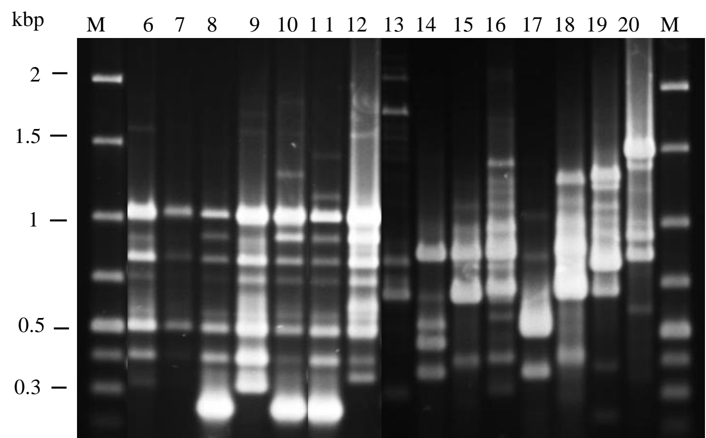


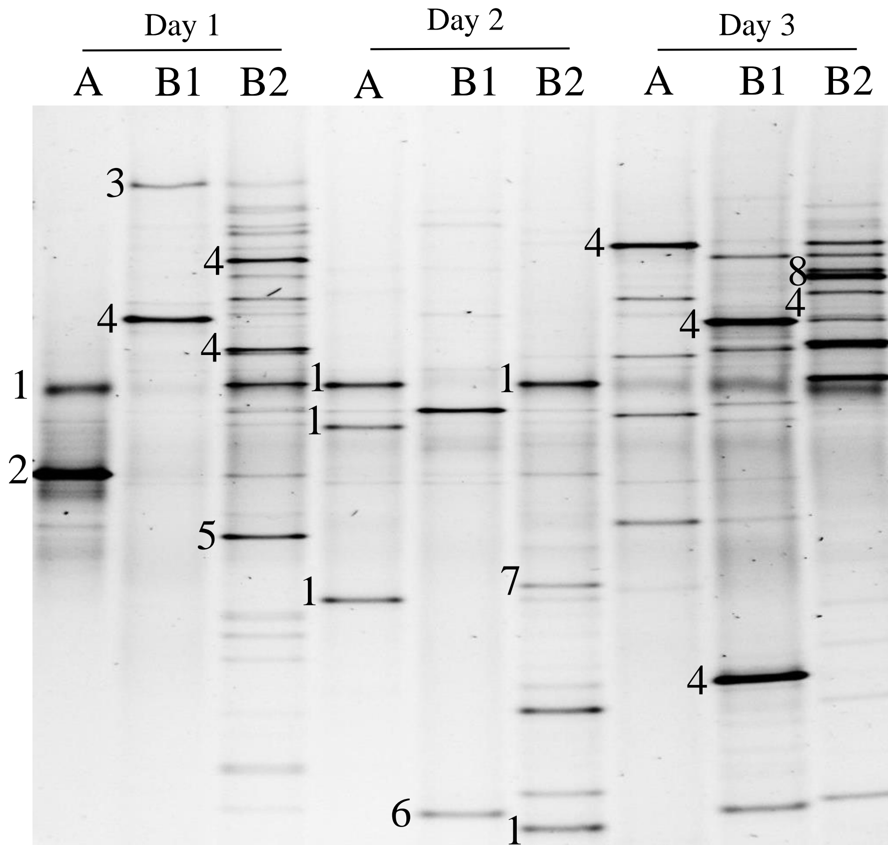
Figure 2.

a)



b)




Day 1
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