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## **Aerococcus sp., a promising genus as a source of anti-Salmonella bioprotective agents for the dairy industry revealed by a miniaturised screening method**

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1 ***Aerococcus* sp., a promising genus as a source of anti-*Salmonella* bioprotective agents for**  
2 **the dairy industry revealed by a miniaturised screening method**

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ABSTRACT

Biopreservation is a promising technology for insuring safety of food. This study evaluated the anti-*Salmonella* potential of 1450 dairy isolates from 54 genera, in conditions mimicking dairy matrices, to find new bioprotective strains. Among the screened isolates, 6.4% exhibited an antagonistic activity towards at least one of the two *Salmonella* Mbandaka and Montevideo reference strains. *Lactococcus* sp. generated the highest number of bioprotective candidates, followed by *Aerococcus* sp. from which seven isolates were able to inhibit at least 80% of 20 *Salmonella* strains in milk synthetic medium, and/or in cheese synthetic medium. Inhibition by *Aerococcus* sp. was characterised by the production of hydrogen peroxide (30 to 100 mg L<sup>-1</sup>) and acetic acid (0.25 to 0.34 g L<sup>-1</sup>). *Aerococcus* sp. appears to be a new promising source of bioprotective agents against *Salmonella* in dairy matrices, along with the well-described *Lactococcus* genus.

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## 40 1. Introduction

41

42 In Europe, ~ 500,000 tons of raw milk cheeses are produced each year (IDF, 2016).  
43 The use of raw milk leads to undeniable advantages for milk producers, cheese makers, as  
44 well as for consumers. Because raw milk cheeses are high added value products, the price of  
45 the raw milk collected to produce them is also higher than that of raw milk destined to the  
46 production of pasteurised products (Casabianca & Jeanneaux, 2020). The organoleptic  
47 qualities of raw milk cheeses are also recognised to be stronger than for processed cheeses  
48 thanks to an abundant native microbiota (Montel et al., 2014). However, raw milk cheeses,  
49 mainly semi-soft and semi-hard cheeses, are more susceptible to the presence and growth of  
50 some bacterial pathogens and can be potential vehicles for food-borne outbreaks. Regarding  
51 the latter issue among the zoonotic pathogens that the dairy industry must face, the most  
52 recorded are pathogenic *Campylobacter* sp., *Escherichia coli*, *Listeria monocytogenes*,  
53 *Salmonella enterica* and enterotoxin-producing *Staphylococcus aureus* (Jourdan-da Silva et  
54 al., 2018).

55 *Salmonella* is one of the food-borne pathogens responsible for the highest number of  
56 human cases. It was associated in 2018 in Europe to 1581 food-borne outbreaks that were  
57 reported by the EFSA (EFSA, 2019) leading to 11,581 human cases. In foods and animals,  
58 *Salmonella* Enteritidis, *Salmonella* Typhimurium, monophasic *Salmonella* Typhimurium,  
59 *Salmonella* Infantis and *Salmonella* Derby are the most encountered serovars in Europe.  
60 These serovars are also considered to be the most reported in human cases (ESFA, 2019), but  
61 their occurrence is country-dependent, food-dependent, and tends to fluctuate over time.

62 Preventing the occurrence of *Salmonella* in raw milk cheeses is of utmost importance  
63 for sanitary and economic reasons. On farms, diverse sanitary measures can be implemented  
64 to reduce the risk of *Salmonella* contamination during milking. Nevertheless, these preventive

65 practices are not always effective enough, and complementary approaches have to be used  
66 (Schlüsselhuber et al., unpublished).

67         Among such approaches, biopreservation has received an increased interest over the  
68 last decade. Biopreservation has been defined as the use of natural microflora and/or their  
69 antimicrobial compounds to extend food shelf-life and enhance food safety (Stiles, 1996).  
70 Using microorganisms (whether they originate or not from the natural microflora) and their  
71 products to preserve food has been practiced in many areas (Ross, Morgan, & Hill, 2002).  
72 The microflora of most fermented foods is mainly composed of lactic acid bacteria (LAB).  
73 LAB are known for their high biopreservation potential and are generally recognised as safe  
74 for human. These bacteria are able to produce a large variety of antimicrobial substances such  
75 as organic acids, hydrogen peroxide, biosurfactants (Sharma, 2016) and bacteriocins (Gálvez,  
76 Abriouel, López, & Omar, 2007). When used as bioprotective cultures, they can contribute to  
77 a number of ways to control pathogens (Ben Said, Gaudreau, Dallaire, Tessier, & Fliss, 2019)  
78 and to extend the shelf-life of fermented foods (Leyva Salas et al., 2017).

79         Microorganisms other than LAB can also be used for biopreservation. One strain of  
80 *Brevibacterium linens*, a bacterial species commonly used for cheese ripening, was shown to  
81 have a protective effect against *Listeria* sp. in red smear cheese (Eppert, Valdés-Stauber,  
82 Götz, Busse, & Scherer, 1997) and predicted bacteriocin biosynthesis genes were present in  
83 most of the *Brevibacterium* strains previously investigated (Pham et al., 2017). In soft cheese,  
84 a *Staphylococcus equorum* strain was able to inhibit *Listeria monocytogenes* growth by  
85 producing a bacteriostatic substance (Carnio et al., 2000). Staphylococcal bacteriocins can  
86 also be used to control *Staphylococcus aureus* in cheese (Miceli de Farias, dos Santos  
87 Nascimento, Cabral da Silva Santos, & de Freire Bastos, 2019). In addition to bacteria, yeasts  
88 isolated from smear ripened cheeses can also have an inhibitory action against pathogens like

89 *L. monocytogenes* (Dieuleveux, Van Der Pyl, Chataud, & Gueguen, 1998; Goerges, Aigner,  
90 Silakowski, & Scherer, 2006).

91 To select antagonistic bacterial isolates, screening methods can be employed such as  
92 the commonly used agar diffusion assay (Abraham et al., 1941). This method allows to detect  
93 the inhibition due to acidification (in unbuffered medium) and/or to the production of non-  
94 specific (e.g., hydrogen peroxide) and specific (e.g., bacteriocin) inhibitory compounds.  
95 Despite the common use of this method, it cannot be applied when using opaque media.  
96 Therefore, classical nutrient agar media are often used, but their composition do not reproduce  
97 the composition of complex food matrices such as dairy products.

98 The aim of this study was to select microbial isolates exhibiting inhibitory activity  
99 towards *Salmonella*, through the production of chemical compounds, in conditions  
100 encountered during semi-soft cheese making process. A new miniaturised screening method  
101 was designed and used to screen a collection of dairy microbial isolates. The most promising  
102 bioprotective strains were identified, and the mechanisms involved in their activity were  
103 investigated.

104

## 105 **2. Materials and methods**

106

### 107 *2.1. Microbial strains and growth conditions*

108

109 This study is part of a project targeting serovars encountered specifically in Normandy  
110 region. The most encountered serovars in Normandy in 2012–2017 were *Salmonella enterica*  
111 subsp. *enterica* serovar Mbandaka (hereinafter referred to as *S. Mbandaka*) and *S. enterica*  
112 subsp. *enterica* serovar Montevideo (hereinafter referred to as *S. Montevideo*). Two reference  
113 strains from the Institut Pasteur (Paris, France), *S. Mbandaka* CIP 105.859 and *S. Montevideo*

114 CIP 104.583, were initially used as primary targets for a pre-screening procedure. Then, ten *S.*  
115 Mbandaka and ten *S.* Montevideo strains, isolated from various farms in Normandy (France)  
116 were further used for screening assays (Table 1). All the strains were routinely stored in 15%  
117 glycerol at  $-80\text{ }^{\circ}\text{C}$ .

118 *Salmonella* strains were grown at  $30\text{ }^{\circ}\text{C}$  for 24 h in Luria Bertani (LB) broth pH 7.5  
119 made with  $10\text{ g L}^{-1}$  casein pancreatic peptone (Biokar),  $5\text{ g L}^{-1}$  yeast extract (Biokar) and  $10\text{ g}$   
120  $\text{L}^{-1}$  NaCl (Fisher). A total of 1,450 bacterial and yeast isolates from the UCMA collection  
121 (Université de Caen Normandie, France), collected between 1978 and 2016 in dairy  
122 environments from the Normandy region, were screened for inhibitory activities towards  
123 *Salmonella* strains. All the screened isolates were routinely grown in brain heart infusion pH  
124 7.4 (BHI, Conda), for 24 h at  $30\text{ }^{\circ}\text{C}$  in Deepwell® from one colony. This medium was chosen  
125 as it allows growth of most microbial groups investigated in this study, including Gram  
126 positive and Gram negative bacteria, and yeasts.

127

## 128 2.2. *Development of a miniaturised method to screen microbial isolates for anti-* 129 *Salmonella activities*

130

131 A miniaturised method based on the agar diffusion plate method was developed in 96-  
132 well microtiter plates (Fig. 1). The screened microbial isolates were cultivated in Deepwell®  
133 plates in conditions mentioned above. After incubation, a replicator was used to transfer a few  
134 microliters ( $2\text{--}3\text{ }\mu\text{L}$ ) from the Deepwell® plate cultures onto the surface of the agar media  
135 used as the first layer in 96-well microtitre plates (agar layer of about  $100\text{ }\mu\text{L}$ ). Three different  
136 media were used as first layer: BHI agar (commonly used in literature for bacterial screening,  
137 as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2004) milk agar,  
138 or cheese agar. The two later were used to mimic the nutritional and physicochemical

139 conditions encountered during two processing steps in the manufacture of semi-soft cheese.  
140 Milk agar pH 6.3 was used to mimic the end of the milk maturation step, just before renneting,  
141 and was composed of 100 g L<sup>-1</sup> of skim milk powder (Difco) and 12 g L<sup>-1</sup> of bacteriological  
142 agar (Conda). Cheese agar was used to mimic cheese ripening and was prepared as previously  
143 described by (Guichard & Bonnarme, 2005) using 2.4% NaCl, 60% of unsalted Camembert  
144 cheese curd and 1.7% agar (Biokar). To allow the growth of acid-sensitive isolates,  
145 particularly belonging to the *Actinobacteria* class, pH was adjusted to 6.8.

146         Microtitre plates containing inoculated BHI agar and milk agar were then incubated at  
147 30 °C for 24 h, and those containing inoculated cheese agar were incubated at 15 °C for 48 h.  
148 After incubation, the resultant growth was killed by UV irradiation (0.124 J cm<sup>-2</sup>) for 15 min  
149 (Daly et al., 2010). In each well, a 70 µL overlay of soft LB agar (0.8% agar) inoculated with  
150 an overnight culture of *Salmonella* was added (final concentration of 10<sup>6</sup> cfu mL<sup>-1</sup>). All the  
151 plates were incubated at 30 °C for 24 h (Fig. 1).

152         After the 24h-incubation step, *Salmonella* viability was revealed using a blue-dye  
153 resazurin test. Resazurin dye was prepared extemporaneously in phosphate sodium buffer (pH  
154 7.4) at a concentration of 0.05% (w/v) and sterilised using PES filter (0.22 µm pore size). In  
155 each well, 20 µL of resazurin solution were deposited onto the agar surface. After 5 min at  
156 room temperature, the coloration of the entire upper layer in each well was recorded and  
157 compared with a positive control (*Salmonella* growth in LB agar) and a negative control  
158 (sterile LB agar overlay). A pink colour revealed the viability of *Salmonella* whereas a blue  
159 colour indicated its inhibition (Fig. 1). All the microbial isolates showing an antagonistic  
160 activity (blue coloration of the well) were tested in duplicate to confirm the inhibition of  
161 *Salmonella*.

162 All the isolates were tested for their activity against the two *Salmonella* reference  
163 strains. The isolates inhibiting of one or both reference strains were then tested, in duplicate,  
164 against twenty *Salmonella* field strains (Table 1).

165

### 166 2.3. Identification of the inhibition mechanisms of selected antagonist isolates

167

#### 168 2.3.1. Detection of anti-*Salmonella* compounds in cell-free supernatants of antagonistic 169 isolates

170 The isolates exhibiting antagonistic activity towards at least 16 of the 20 (80%)  
171 *Salmonella* strains (Table 1) were selected to investigate the mechanisms of inhibition. This  
172 arbitrary inhibition threshold of 80% was intended to select the isolates with the widest  
173 spectrum of inhibitory activity towards *Salmonella*.

174 Liquid cheese synthetic medium (CSM, pH 6.8) was prepared as previously described  
175 (Mansour, Beckerich, & Bonnarme, 2008). Liquid milk synthetic medium (MSM, pH 6.3)  
176 was prepared from CSM with modifications: lactose and sodium lactate concentrations were  
177 adjusted to 48.5 g L<sup>-1</sup> and 1 g L<sup>-1</sup>, respectively. Isolates were grown in 5 mL of media from  
178 one colony for 72 h at 150 rpm at either 30 °C (MSM) or 15 °C (CSM). Cells from 5 mL of  
179 culture were pelleted by centrifugation at 4150 × g for 10 min at room temperature, and the  
180 cell-free supernatants (CFS) were obtained by filtering through 0.22 µm PES filters (Nalgene)  
181 and then stored at -20 °C until use. *Salmonella* Mbandaka CIP 105.859 was cultured in 5 mL  
182 of LB from one colony and incubated 24 h at 37°C. *Salmonella* concentration was then  
183 adjusted at 5×10<sup>6</sup> cfu mL<sup>-1</sup> in LB broth twice concentrated.

184 CFS were tested against *S. Mbandaka* CIP 105.859 to assess the contribution to the  
185 detected anti-*Salmonella* activities of the potential production of organic acids, hydrogen  
186 peroxide, proteinaceous compounds and biosurfactants. Four treatment conditions were

187 applied to CFS, i.e., non-treated, neutralised, neutralised treated with catalase, and neutralised  
188 treated with pronase. The CFS pH was neutralised to pH 7 with 6 N NaOH. The neutralised  
189 CFS were treated with catalase (100 mg.mL<sup>-1</sup>, Sigma) at 30 °C for 30 min, or pronase (100  
190 mg.mL<sup>-1</sup>, Serva) at 37 °C for 2 h. One hundred microlitres of treated (neutralised, catalase-  
191 treated, or pronase-treated) or non-treated CFS were mixed with 100 µL *S. Mbandaka* CIP  
192 105.859 LB culture, into microplate wells, reaching a final *Salmonella* concentration of  
193 2.5×10<sup>6</sup> cfu mL<sup>-1</sup>. The microplates were incubated at 30 °C for 24 h. After incubation,  
194 *Salmonella* inhibition was evaluated by comparing OD<sub>600nm</sub> in control wells (*S. Mbandaka*  
195 without CFS) and in assay wells containing both *S. Mbandaka* and CFS. Inhibition was  
196 expressed by the OD<sub>600nm</sub> ratio between assay and control. Non-treated CFS were considered  
197 active against *Salmonella* when at least 80% inhibition were observed. The activity of treated  
198 CFS was assessed in triplicate by comparing with the activity of non-treated CFS.

199 For the rapid screening of biosurfactant production, non-treated CFS were subjected to  
200 a drop-collapse assay (Walter, Syldatk, & Hausmann, 2010). Briefly, 5 µL of non-treated CFS  
201 were mixed with 1 µL of methylene blue to improve visualisation before a drop assay onto a  
202 hydrophobic surface (Parafilm®). The stability of the drop was checked and compared with a  
203 negative control (sterile medium). If CFS contains biosurfactants, the drop spreads because  
204 the interfacial tension between the drop and the hydrophobic surface is reduced.

205 Hydrogen peroxide production in active non-treated CFS was quantified using H<sub>2</sub>O<sub>2</sub>  
206 Dosatest strip® (0 to 100 mg mL<sup>-1</sup>, VWR).

207

### 208 2.3.2. *Detection and quantification of major organic acids by high performance liquid* 209 *chromatography*

210 Reverse phase HPLC (Waters Alliance HPLC system with 2695 pump and 2998 PDA  
211 detector) was performed at 0.6 mL min<sup>-1</sup> and 40 °C on a Carbomix HNP5 8% cross-linking

212 7.8 × 300 mm (Sepax). Elution was performed in an isocratic mode for 30 min using 5 mM  
213 H<sub>2</sub>SO<sub>4</sub> buffer as mobile phase and monitored at 210 nm. Active CFS of selected strains grown  
214 in MSM medium were two fold diluted with mobile phase before injection of 10 µL. For  
215 quantification of lactic and acetic acids in each CFS, acid standards were injected in  
216 concentrations ranging from 2 mM to 64 mM (Sigma). Several standards of organic acids were  
217 also injected at 64 mM to compare elution time with produced molecules in active CFS: citric  
218 (Panreac), fumaric (Acros), formic (Sigma), butyric (Fluka), benzoic (Panreac), succinic  
219 (Sigma) and phenyllactic acids (Sigma).

220

### 221 3. Results

222

#### 223 3.1. Antagonistic activities of microbial isolates towards two *Salmonella* reference strains

224

225 The miniaturised screening method developed in the current study was used to assess  
226 the anti-*Salmonella* activity of 1450 isolates against two reference *Salmonella* strains, *S.*  
227 *Mbandaka* CIP 105.859 and *S. Montevideo* CIP 104.583, in BHI, milk agar and cheese agar  
228 (Table 2). This method was validated using strains previously screened using agar diffusion  
229 assays and showing antagonism, through production of chemical compound, toward one  
230 *Salmonella* strain used in this study. Around one-third of the screened isolates belonged to the  
231 *Lactococcus* genus.

232 Among the 1450 isolates, 93 (6.4%) were able to inhibit at least one *Salmonella*  
233 reference strain in BHI. Seventy-three (5%) isolates inhibited *S. Mbandaka* CIP 105.859  
234 whereas 55 (3.85%) inhibited *S. Montevideo* CIP 104.583 strain in this medium. Thirty-five  
235 (2.4%) isolates inhibited both *Salmonella* strains in these conditions.

236 In milk medium, 119 isolates (8.2%) inhibited *S. Mbandaka* CIP 105.859 and 135  
237 (9.3%) inhibited *S. Montevideo* CIP 104.583. In this medium, 61 isolates (4.2%) displayed an  
238 antagonistic activity against the two *Salmonella* strains.

239 In cheese medium, results were similar to those obtained in milk medium. A total of  
240 118 (8.1%) isolates were active against *S. Mbandaka* CIP 105.859 and 132 (9.1%) against *S.*  
241 *Montevideo* CIP 104.583 (Table 2). Forty-four isolates (3%) inhibited both *Salmonella*  
242 strains. Antagonistic isolates were different depending on the medium and on the *Salmonella*  
243 strains.

244 As shown in Table 2, isolates displaying an anti-*Salmonella* activity belonged  
245 predominantly to the *Lactococcus* genus, followed by, in descending order, *Aerococcus* sp.,  
246 *Lactobacillus* sp., and *Leuconostoc* sp. Some antagonistic isolates were also found among  
247 other bacterial genera, including *Alcaligenes* sp., *Brachybacterium* sp., *Hafnia* sp.,  
248 *Pseudomonas* sp., *Raoultella* sp., *Staphylococcus* sp., and *Weissella* sp., and among yeasts  
249 (*Candida* sp. and *Yarrowia* sp.). The genus *Aerococcus* was associated with a noticeable  
250 inhibitory activity towards *Salmonella* strains, since more than half of the isolates were active  
251 (Table 2). Over the 31 screened *Aerococcus* strain, 29 (93%) were able to inhibit at least one  
252 *Salmonella* strain in at least one medium.

253

254 3.2. *Screening of selected isolates against 20 Salmonella strains originating from dairy*  
255 *environments.*

256

257 Based on the previous results, 49 isolates were selected according to their taxonomic  
258 affiliation and their inhibitory activity towards the reference *Salmonella* strains in either milk  
259 agar or cheese agar (Table 3). They were screened using the miniaturised method with milk

260 medium and cheese medium against twenty *Salmonella* isolates collected from farms'  
261 environment (Table 1).

262 Thirty-one out of the 49 isolates inhibited at least 16 out of the 20 *Salmonella* strains  
263 in one or in both media (Table 3). Among them, three *Lactococcus* sp., two *Aerococcus* sp.,  
264 one *Alcaligenes* sp., one *Leuconostoc* sp., and one *Raoultella* sp. isolates displayed inhibition  
265 only in milk medium. Eleven isolates (six *Lactococcus* sp., three *Aerococcus* sp., one  
266 *Pseudomonas* sp., and one *Streptococcus* sp.) inhibited *Salmonella* strains only in cheese  
267 medium. Ten *Lactococcus* sp., and two *Aerococcus* sp. isolates were active in both milk and  
268 cheese media. None of the tested field *Salmonella* strains was resistant to all the screened  
269 isolates.

270

### 271 3.2.1. Inhibition mechanisms of the active isolates in milk and cheese media

272 The 49 isolates selected on the previous step were grown either in MSM, CSM or both  
273 media (according to their previous activities through the miniaturised method in milk  
274 medium, cheese medium or both) to test CFS activities against *S. Mbandaka* CIP 105.859.  
275 Similar results were obtained for both *Salmonella* strains so only the results obtained for *S.*  
276 *Mbandaka* CIP 105.859 strain are shown (Table 3). After 24 h incubation, the ratio between  
277 OD<sub>600</sub> obtained from *Salmonella* grown in the presence of CFS and OD<sub>600</sub> obtained in pure  
278 culture was calculated. CFS activity was considered as inhibitory toward *Salmonella* strain  
279 when values  $\leq 0.20$  (corresponding to 80% inhibition) were obtained. Twenty CFS were  
280 tested in MSM, and among them, seventeen non-treated CFS inhibited the reference strain,  
281 with at least 80% of inhibition. All the tested *Aerococcus* strains (n = 4) were active against  
282 both *Salmonella* strains. After CFS neutralisation, only these *Aerococcus* strains still inhibited  
283 *Salmonella* growth (96–99% of inhibition). None of the CFS inhibited *Salmonella* growth in  
284 CSM (data not shown).

285 All the active CFS, which pH ranged between 4.5 and 6.5, lost their inhibitory activity  
286 against the pathogen after pH neutralisation except for the four *Aerococcus* isolates.  
287 According to these results, the inhibition mechanism of these isolates could be related to the  
288 production of organic acids combined with a pH effect. For the four *Aerococcus* sp. UCMA  
289 5972, UCMA 18036, UCMA 18039 and UCMA 6078 strains, CFS was still active with  
290 pronase treatment, but not with catalase treatment. This suggest that the anti-*Salmonella*  
291 activity was mainly due to the production of hydrogen peroxide. This production was  
292 confirmed by H<sub>2</sub>O<sub>2</sub> Dosatest® strips, with quantities produced ranging from 30 to 100 mg L<sup>-1</sup>  
293 depending on the supernatant. After pronase treatment, all neutralised CFS kept their  
294 inhibitory activity. Therefore, none of the antagonistic activities were related to the presence  
295 of proteinaceous compounds such as bacteriocins. A drop-collapse assay was performed on  
296 non-treated CFS to reveal the production of biosurfactants. None of the strains appeared to  
297 produce such molecules.

298

### 299 3.2.2. Assessment of organic acids involved in *Aerococcus* anti-*Salmonella* activity

300 Due to the anti-*Salmonella* activity of their CFS, the production of organic acids by  
301 *Aerococcus* strains was studied. After 72 h of incubation in MSM, the four *Aerococcus* active  
302 CFS (Table 3) were collected and analysed using HPLC with UV detection at 210 nm.  
303 Chromatograms of *Aerococcus* CFS were compared with chromatograms of sterile MSM. No  
304 production of lactic acid was observed, while acetic acid was produced at concentration  
305 ranging from 0.23 to 0.34 g L<sup>-1</sup>, depending on the strain. Additionally, an unknown molecule  
306 was produced and eluted at 9.1 min. However, none of organic acids standards injected were  
307 found to correspond (data not shown).

308

## 309 4. Discussion

310

311           Classic nutritive media such as MRS, M17, and BHI agar are well-used for spot-on-  
312 lawn assays to detect microbial antagonistic activities. BHI agar has the advantage to allow  
313 the growth of a large panel of microorganisms thanks to its nutrient-rich composition. For  
314 LAB-only screening, MRS agar is commonly used. These non-opaque media are easy to use  
315 but their composition is far from that of real matrices encountered during dairy food making  
316 processes. It is known that antimicrobial activities revealed when using classic nutritive media  
317 can be lost in real dairy matrix conditions.

318           In this study, a new screening method was developed and applied for the detection of  
319 microbial antagonistic activities towards pathogenic bacteria using dairy-like media. The  
320 method developed has the advantage of using conditions mimicking semi-soft cheese making  
321 process, from milk maturation to cheese ripening steps, in terms of nutritional content for  
322 microorganisms, pH and temperature. It also has the advantage of overcoming difficulties  
323 associated to opacity of dairy-like media. The method was also designed to manage a large  
324 number of isolates in 96-well microplates, therefore cell concentrations of the isolates to be  
325 screened were not adjusted when inoculating the first agar layer. The consequence is that the  
326 cellular levels of the screened dairy isolates in microplate wells may vary depending on the  
327 microbial species or strains investigated, and on the trials. As the anti-*Salmonella* activity of  
328 the isolates was confirmed in a second experiment, it was considered as a minor drawback of  
329 our method. Additionally, strains belonging to 18 different genera, including fastidious ones  
330 (e.g., *Lactobacillus* sp), showed inhibitory activities with this method, suggesting that it was  
331 of broad use. Mainly members of the *Actinobacteria* class were not detected as active in this  
332 experiment, but some of them exhibited activity toward a *S. Newport* strain (data not shown).

333           Over the 1450 microbial isolates tested in the current study, most of them were LAB  
334 (1004 out of 1450) and especially of the *Lactococcus* genus (37%). Interestingly, a higher

335 number of antagonistic strains were found in “milk” and “cheese” agar compared with  
336 classical BHI agar. It appeared that the physicochemical properties of milk and cheese media  
337 (e.g., lower pH, and concentrations of citrate, free fatty acids and minerals) were more  
338 suitable for revealing the production of inhibitory molecules by this bacterial group. This was  
339 consistent with the study of Matevosyan, Bazukyan, and Trchounian (2019a), where LAB  
340 antibacterial activities were more important in milk agar than in classic medium such as MRS.  
341 Another study showed that calcium and magnesium ions in combination were involved in the  
342 increase in LAB antibacterial activity (Matevosyan et al., 2019a). Besides compositions,  
343 microbial antagonistic activities are also highly dependent on culture conditions (Matevosyan,  
344 Bazukyan, & Trchounian, 2019b). For example, activities of antimicrobial substances were  
345 shown significantly influenced by temperature, NaCl and glucose concentrations  
346 (Rohmatussolihat, Lisdiyanti, Yopi, Widyastuti, & Sukara, 2018). This corroborates the  
347 importance of using media as close as possible to the food matrix for the selection of  
348 bioprotective agents that may then still be active when applied to real food processes.

349         The screening performed against 20 field *Salmonella* strains from different serovars, in  
350 the same conditions as those used with the two *Salmonella* reference strains, showed that  
351 *Lactococcus* and *Aerococcus* were the major antagonistic genera. None of the 20 field  
352 *Salmonella* were resistant to all of the 31 tested isolates. Most isolates were not able to inhibit  
353 all *Salmonella* strains in our conditions whatever the serovar. This indicates that the inhibitory  
354 activities of the screened isolates were strain-dependent.

355         LAB, especially *Lactococcus*, *Lactobacillus* and *Leuconostoc*, are well-known for  
356 their antagonistic activities against pathogens (El Kheir et al., 2018; Lindgren & Dobrogosz,  
357 1990) and were confirmed as such in this study. Additionally, some isolates belonging to the  
358 *Enterococcus*, *Pediococcus*, *Streptococcus* and *Weissella* genera exhibited a predictable  
359 inhibitory activity. The anti-*Salmonella* activity of a large proportion of LAB isolates

360 belonging to the *Aerococcus* genus was more surprising, as published reports on the  
361 antimicrobial potential of this genus are scarce to our knowledge. Although the four  
362 investigated *Aerococcus* strains had a similar behaviour toward the *Salmonella* strains, they  
363 were probably not clones because they originated from different substrates (air versus raw  
364 milk) and from different farms.

365 CFS were investigated to decipher the mechanisms involved in *Salmonella* inhibition.  
366 An inhibitory activity was associated to CFS from isolates grown in MSM, whereas none was  
367 found for CFS from CSM. This could be explained by several factors leading to a limited  
368 growth of the tested strains and/or to a lower production of inhibitory molecules in CSM than  
369 in MSM. Indeed, CSM was incubated at 15 °C whereas MSM was incubated at 30 °C, and  
370 CSM had a pH of 6.8 pH whereas MSM pH was of 6.3. The lactose concentration in CSM  
371 was of 20 g L<sup>-1</sup> compared with 48.5 g L<sup>-1</sup> in MSM, and lactic acid concentration was of 18 g  
372 L<sup>-1</sup> in CSM and of 1 g L<sup>-1</sup> in MSM. This could have impacted the growth of the tested  
373 microbial isolates.

374 It is well documented that most of the LAB inhibition activities are notably related to  
375 the production of organic acids, mostly lactic acid (Liu, 2003) but also acetic acid,  
376 phenyllactic acid (Gerez et al., 2013) and other organic acids (Garnier, Torres, Font de  
377 Valdez, & Rollán, 2020). Many primary substrates for organic acid production are found in  
378 dairy matrices: carbohydrates, amino acids (Stiles, 1996), and unsaturated free fatty acids  
379 through epoxide and hydroperoxide formation (Molimard & Spinnler, 1996). In this study, all  
380 the *Lactococcus* sp. tested showed an important production of lactic acid and produced also  
381 acetic acid, as reported in other studies (Özcelik, Kuley, & Özogul, 2016; Røssland,  
382 Langsrud, Granum, & Sørhaug, 2005).

383 Interestingly, *Aerococcus* strains were not associated with the production of lactic  
384 acid. Mechanisms of action were different for the four *Aerococcus* strains mainly

385 investigated in the current study, compared with the other LAB strains. Indeed, the  
386 *Aerococcus* antagonist activity seemed to be related, at least in part, to the production of  
387 organic acids (acetic acid) and hydrogen peroxide. This is consistent with a previous study  
388 showing a production of hydrogen peroxide by this genera (Stepanskyi et al., 2017). In the  
389 *Aerococcus* genus, the decarboxylation of pyruvate due to the action of a pyruvate oxidase  
390 allows the formation of hydrogen peroxide through the reduction of dioxygen in *Aerococcus*  
391 *viridans* (Yanze Kontchou & Blondeau, 1990). The enzyme glycerophosphate-oxidase  
392 (GPO), isolated from *A. viridans*, also contributes to the formation of hydrogen peroxide  
393 (Streitenberger, López-Mas, Sánchez-Ferrer, & García-Carmona, 2001). This enzyme has  
394 been described in other LAB genera such as: *Leuconostoc* (Emi, Kojima, & Ando, 1984),  
395 *Pediococcus* (Šůchová, Demnerová, Bond, & Králová, 1992) and *Streptococcus* (Esders &  
396 Michrina, 1979). In this study, the concentration of hydrogen peroxide recorded in CFS ranged  
397 between 30 and 100 mg L<sup>-1</sup>. Such concentrations are similar and potentially higher than those  
398 recorded for strains belonging to *Lactobacillus acidophilus*, and which ranged between 1.7  
399 and 50 mg L<sup>-1</sup> (Hertzberger et al., 2014; Strus, Brzywczy-Włoch, Gosiewski, Kochan, &  
400 Heczko, 2006).

401 An unknown molecule eluted at 9.1 min, and which was not produced by the other  
402 tested strains, could be related to the observed inhibition. The identification and assessment of  
403 the potential anti-*Salmonella* activity of this molecule remain to be performed.

404 The *Aerococcus* genus is composed of eight species: *Aerococcus christensenii*,  
405 *Aerococcus sanguinicola*, *Aerococcus suis*, *Aerococcus urinae*, *Aerococcus urinaehominis*,  
406 *Aerococcus viridans*, *Aerococcus urinaeequi* and *Aerococcus vaginalis* (Tohno et al., 2014).  
407 Some species of this genus, such as *A. viridans*, appear to be strongly associated to dairy  
408 environments. They have been found in air, soil, and dairy environments (Ruoff, 2011).  
409 *Aerococcus* genus has frequently been detected as a dominant flora in raw milk samples

410 collected on Normandy dairy farms (Mallet, 2012). *Aerococcaceae* was also the major taxon  
411 found in milk and air dust samples collected on farms located in Okayama and Hiroshima  
412 (Japan) using automatic milking systems (Wu et al., 2019). In sixteen French farms, the  
413 *Aerococcus* genus was also found in milk, ambient air, air from the milking area, dust and on  
414 teat skin (Vacheyrou, 2011).

415 In another study, the bacterial diversity of cows' teat skin was investigated. Among  
416 the major phylum encountered (Firmicutes) *Aerococcus* sp., and especially *A. viridans*, were  
417 found (Verdier-Metz et al., 2012). The microbial diversity in bovine faeces was also  
418 investigated to evaluate their importance as microorganisms' source (Kagkli, Vancanneyt,  
419 Hill, Vandamme, & Cogan, 2007). *Aerococcus viridans* was identified in 62 of the 76 samples  
420 collected from one farm, and accounted for 83% of the total isolates.

421 The *Aerococcus* genus has been detected in raw goat milk and in Pélardon cheese, a  
422 raw goat milk cheese (Penland et al., 2018). In Mexican cheeses, *Aerococcus* sp. was found in  
423 high proportion in Cotija (a Mexican handcrafted product made from raw cow milk) and  
424 Adobera-de-mesa cheeses (Murugesan et al., 2018). In Cotija cheese, this bacterium was  
425 detected at the surface and in the core of the product (Chombo-Morales, Kirchmayr,  
426 Gschaedler, Lugo-Cervantes, & Villanueva-Rodríguez, 2016). In this study all strains  
427 belonged to *Aerococcus urinaeequi*.

428 Among *Aerococcus* species, some are known to be involved in rare human clinical  
429 cases, especially *A. urinae* and *A. sanguinicola*, associated to urinary tract infections.  
430 However, regarding species described in milk and cheese (*A. viridans/urinaeequi*), no case of  
431 contamination and no clinical case linked to the consumption of dairy products have been  
432 reported so far.

433 Because of the anti-*Salmonella* activity of *Aerococcus* strains and their natural  
434 presence in dairy products, this genus appears to be an unexpected promising candidate to

435 control *Salmonella* in dairy products. Further investigations will be conducted to determine if  
436 the production of hydrogen peroxide can potentially affect the growth of dairy starter cultures.

437

## 438 **5. Conclusions**

439

440 In this study, we developed a new agar-based screening method allowing the detection  
441 of microbial antagonistic activities against *Salmonella* in dairy-like opaque media. It allows  
442 the screening of a large number of isolates at the same time. Starting from microbial cultures  
443 grown in Deepwell®, this screening method can be used to inoculate simultaneously diverse  
444 opaque media incubated in different conditions, mimicking various physicochemical and  
445 nutritional conditions encountered during the processing steps of a food matrix. This is of  
446 particular interest for the dairy industry, as this method allows a screening adapted to different  
447 steps of cheese making processes. This method may also be transferred for the screening of  
448 microbial isolates against other pathogens than *Salmonella*.

449 While some isolates were able to inhibit all the tested *Salmonella* strains, the inhibition  
450 potential was found, as expected, to be essentially strain-dependent. Combinations of  
451 antagonistic isolates with various mechanisms of anti-*Salmonella* actions should be  
452 considered for insuring a protection from the beginning to the end of the cheese making  
453 process. Associations of strains displaying different inhibition mechanisms could bring a  
454 larger spectra of *Salmonella* inhibition and prevent the emergence of resistance mechanisms  
455 from the pathogens.

456 From this study, the *Aerococcus* genus, and especially *Aerococcus urinaeequi*, seems  
457 to be a promising source of bioprotective strains to be used in addition to dairy starters.  
458 Compared with other LAB genera, little is known about *Aerococcus*, and especially about  
459 *Aerococcus* species encountered in dairy environments. Therefore, further investigations

460 dealing with antibiotic resistance, the production of biogenic amines and the presence of  
461 virulence factors need to be evaluated before further consideration of their use as protective  
462 cultures.

463

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465

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470

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631

## Figure legend

**Fig. 1.** Miniaturised screening method developed in the current study; the cylinder represents one well of a 96-well microplate.

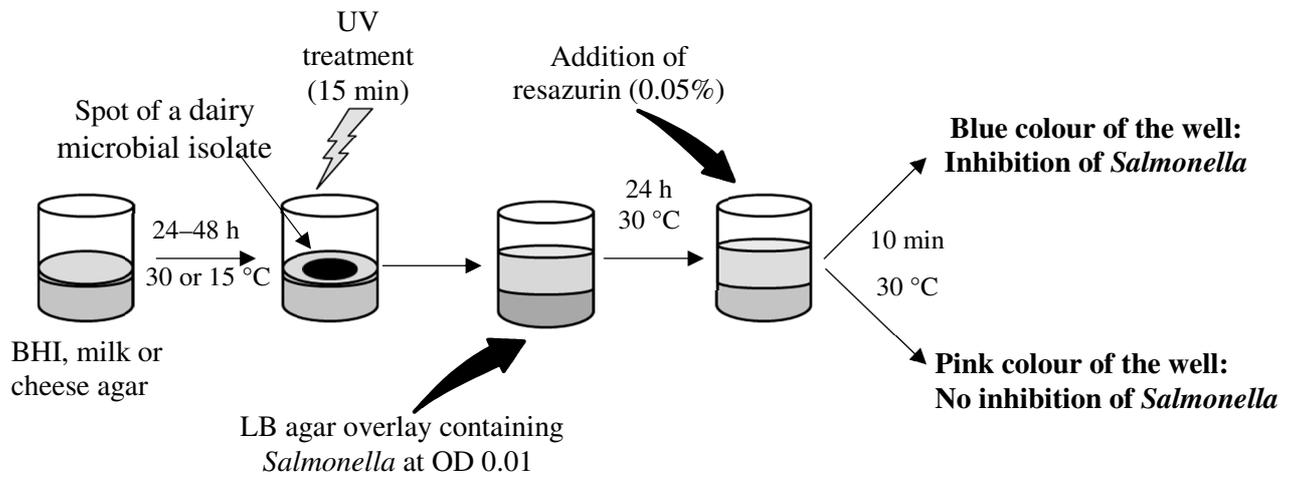


Figure 1

**Table 1**

*Salmonella enterica* ssp. *enterica* strains used in this study.

Serotype	Strain number	Origin
Mbandaka	UCMA 17913	Milk
	UCMA 19835	Manure
	UCMA 19837	Raw milk
	UCMA 19842	Raw milk
	UCMA 19851	Plastic around fodder
	UCMA 19892	Faeces
	UCMA 19906	Feeding area
	UCMA 19909	Raw milk
	UCMA 19917	Raw milk
	UCMA 20264	Milk
	CIP 105.859	Faeces
Montevideo	UCMA 19832	Raw milk
	UCMA 19834	Manure
	UCMA 19840	Water
	UCMA 19867	Faeces
	UCMA 19870	Puddle
	UCMA 19876	Feeding mix
	UCMA 19889	Faeces
	UCMA 19904	Tractor wheel
	UCMA 20260	Raw milk
	UCMA 20266	Milk
	CIP 104.583	Monkey

**Table 2**Antagonistic activities of the 1450 screened dairy isolates towards two *Salmonella* reference strains. <sup>a</sup>

Presumed genus	Number of screened isolates	Number (%) of antagonistic isolates					
		<i>Salmonella</i> Mbandaka CIP 105.859			<i>Salmonella</i> Montevideo CIP 104.583		
		BHI	Milk	Cheese	BHI	Milk	Cheese
<i>Aerococcus</i>	31	19 (61.3)	17 (54.8)	20 (64.5)	14 (45.2)	19 (61.2)	18 (58.1)
<i>Alcaligenes</i>	3	1 (33.3)	1 (33.3)	0	2 (66.6)	1 (33.3)	0
<i>Candida</i>	9	6 (66)	1 (11.1)	0	3 (33.3)	1 (11.1)	0
<i>Enterococcus</i>	30	1 (3.3)	4 (13.3)	3 (10)	0	7 (23.3)	8 (26.6)
<i>Geotrichum</i>	4	2 (50)	0	2 (50)	2 (50)	0	0
<i>Hafnia</i>	8	1 (12.5)	0	0	1 (12.5)	0	0
<i>Kluyveromyces</i>	9	2 (22.2)	0	1 (11.1)	2 (22.2)	0	0
<i>Lactobacillus</i>	322	4 (1.2)	14 (4.3)	9 (2.7)	3 (0.93)	11 (3.4)	8 (2.4)
<i>Lactococcus</i>	536	19 (3.5)	57 (10.6)	59 (11)	12 (2.2)	70 (13.1)	66 (12.3)
<i>Leuconostoc</i>	102	1 (0.9)	14 (13.7)	16 (15.6)	0	8 (7.8)	20 (19.6)
<i>Pediococcus</i>	3	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)
<i>Pichia</i>	3	0	0	0	0	1 (33.3)	0
<i>Pseudomonas</i>	34	3 (8.8)	4 (11.7)	1 (2.9)	2 (5.8)	2 (5.8)	1 (2.9)
<i>Raoultella</i>	4	2 (50)	1 (25)	0	0	1 (25)	0
<i>Staphylococcus</i>	96	4 (4.1)	0	1 (1.1)	4 (4.1)	2 (2.1)	3 (3.3)
<i>Streptococcus</i>	10	1 (10)	1 (10)	1 (10)	0	3 (30)	1 (10)
<i>Weissella</i>	8	0	0	1 (12.5)	1 (12.5)	0	2 (25)
<i>Yarrowia</i>	3	1 (33.3)	1 (33.3)	0	1 (33.3)	1 (33.3)	0
No identification	66	5 (7.5)	3 (4.5)	3 (4.5)	7 (10.6)	7 (10.6)	4 (6.1)
Other isolates	169	0	0	0	0	0	0
Total isolates	1450	73 (5.0)	119 (8.2)	118 (8.1)	55 (3.8)	135 (9.3)	132 (9.1)

<sup>a</sup>Other isolates were: *Acinetobacter* (7), *Arthrobacter* (17), *Brachybacterium* (3), *Brevibacillus* (1), *Brevibacterium* (12), *Carnobacterium* (6), *Corynebacterium* (23), *Cryptococcus* (1), *Curtobacterium* (1), *Cutaneotrichosporon* (1), *Debaryomyces* (2), *Dietzia* (1), *Escherichia* (1), *Ewingella* (1), *Exiguobacterium* (1), *Galactomyces* (2), *Gordonia* (1), *Halomonas* (1), *Jeotgalicoccus* (9), *Kocuria* (10), *Leifsonia* (2), *Leucobacter* (8), *Macroccoccus* (11), *Microbacterium* (13), *Microcaccaceae* (4), *Micrococcus* (3), *Obesumbacterium* (2), *Ochrobactrum* (3), *Pantoea* (1), *Pseudoclavibacter* (2), *Psychrobacter* (8), *Renibacterium* (1), *Rhodococcus* (4), *Rothia* (2), *Stenotrophomonas* (5), and *Trichosporon* (1).

**Table 3**

Dairy isolates exhibiting inhibitory activity towards field *Salmonella* strains in milk medium and/or cheese medium and characterisation of the inhibition mechanism associated to cell-free supernatant from MSM cultures. <sup>a</sup>

UCMASt rain number	Identity	Percentage of field <i>Salmonella</i> isolates inhibited		Selected isolate	<i>Salmonella</i> Mbandaka growth inhibition (%) by cell- free supernatant			
		Milk	Cheese		Non-treated	Neutralised	Catalase- treated	Pronase- treated
1676	<i>Leuconostoc citreum</i>	90	25	Yes	99	0	0	0
3915	<i>Raoultella planticola</i>	95	35	Yes	99	0	0	0
4132	<i>Alcaligenes faecalis</i>	95	0	Yes	0	0	0	0
4441	<i>Lactococcus lactis</i>	80	30	Yes	61	0	0	0
5972	<i>Aerococcus urinaeequi</i>	85	20	Yes	99	97	0	99
13205	<i>Lactococcus lactis</i>	90	75	Yes	98	0	0	0
18022	<i>Lactococcus lactis</i>	85	40	Yes	99	0	0	0
18039	<i>Aerococcus urinaeequi</i>	85	65	Yes	99	98	0	99
1049	<i>Lactococcus lactis</i>	75	80	Yes	-	-	-	-
1495	<i>Lactococcus lactis</i>	30	90	Yes	-	-	-	-
4512	<i>Lactococcus raffinolactis</i>	45	85	Yes	-	-	-	-
6472	<i>Lactococcus lactis</i>	55	90	Yes	-	-	-	-
6731	<i>Aerococcus urinaeequi</i>	40	90	Yes	-	-	-	-
6761	<i>Aerococcus urinaeequi</i>	30	95	Yes	-	-	-	-
7402	<i>Pseudomonas lundensis</i>	55	100	Yes	-	-	-	-
9110	<i>Lactococcus lactis</i>	5	85	Yes	-	-	-	-
18010	<i>Aerococcus urinaeequi</i>	70	85	Yes	-	-	-	-
21104	<i>Lactococcus raffinolactis</i>	60	85	Yes	-	-	-	-
21105	<i>Streptococcus parauberis</i>	15	100	Yes	-	-	-	-
6078	<i>Aerococcus urinaeequi</i>	85	95	Yes	98	98	0	99
1151	<i>Lactococcus lactis</i>	80	100	Yes	100	0	0	0
1044	<i>Lactococcus lactis</i>	100	100	Yes	98	0	0	0
1053	<i>Lactococcus lactis</i>	95	90	Yes	99	0	0	0
1068	<i>Lactococcus lactis</i>	90	90	Yes	99	0	0	0
1594	<i>Lactococcus lactis</i>	80	90	Yes	99	0	0	0
4571	<i>Lactococcus lactis</i>	90	95	Yes	80	0	0	0
6360	<i>Lactococcus lactis</i>	85	80	Yes	99	0	0	0
8483	<i>Lactococcus lactis</i>	95	95	Yes	99	0	0	0
8501	<i>Lactococcus lactis</i>	90	100	Yes	99	0	0	0
9863	<i>Lactococcus lactis</i>	90	95	Yes	68	0	0	0
18036	<i>Aerococcus urinaeequi</i>	90	85	Yes	99	99	0	99
3066	<i>Lactobacillus plantarum</i>	0	0	No	-	-	-	-
4459	<i>Leuconostoc mesenteroides</i>	60	60	No	-	-	-	-
4460	<i>Leuconostoc mesenteroides</i>	60	55	No	-	-	-	-
4514	<i>Staphylococcus xylosus</i>	35	45	No	-	-	-	-
4515	<i>Weissella hellenica</i>	55	10	No	-	-	-	-
4546	<i>Leuconostoc mesenteroides</i>	35	25	No	-	-	-	-
4935	<i>Lactococcus lactis</i>	0	75	No	-	-	-	-
5042	<i>Lactococcus lactis</i>	5	40	No	-	-	-	-
5744	<i>Aerococcus urinaeequi</i>	75	65	No	-	-	-	-
7340	<i>Lactococcus lactis</i>	75	55	No	-	-	-	-
11210	<i>Yarrowia lipolytica</i>	0	15	No	-	-	-	-
11417	<i>Candida anglica</i>	25	0	No	-	-	-	-
12627	<i>Raoultella planticola</i>	65	15	No	-	-	-	-
12716	<i>Hafnia alvei</i>	5	0	No	-	-	-	-
18046	<i>Aerococcus urinaeequi</i>	50	55	No	-	-	-	-
21106	<i>Enterococcus faecalis</i>	35	15	No	-	-	-	-
21107	<i>Lactococcus lactis</i>	75	75	No	-	-	-	-
21108	<i>Streptococcus gallolyticus</i>	40	20	No	-	-	-	-

<sup>a</sup> Isolate selected when able to inhibit at least 80% of field *Salmonella* strains - Non tested condition.