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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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^{-1}) and acetic acid (0.25 to 0.34 g L^{-1}). *Aerococcus* sp. appears to be a new promising source of bioprotective agents against *Salmonella* in dairy matrices, along with the well-described *Lactococcus* genus.

1. Introduction

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

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

Preventing the occurrence of *Salmonella* in raw milk cheeses is of utmost importance for sanitary and economic reasons. On farms, diverse sanitary measures can be implemented 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 (Schlusselhuber 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 *Staphyloccoccus 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 *Staphyloccoccus 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

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

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

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

2. Materials and methods

2.1. Microbial strains and growth conditions

This study is part of a project targeting serovars encountered specifically in Normandy region. The most encountered serovars in Normandy in 2012–2017 were *Salmonella enterica* subsp. *enterica* serovar Mbandaka (hereinafter referred to as *S*. Mbandaka) and *S. enterica* subsp. *enterica* serovar Montevideo (hereinafter referred to as *S*. Montevideo). Two reference 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 °C.
118 *Salmonella* strains were grown at 30 °C for 24 h in Luria Bertani (LB) broth pH 7.5
119 made with 10 g L^{-1} casein pancreatic peptone (Biokar), 5 g L^{-1} yeast extract (Biokar) and 10 g
120 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 °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 (2–3 µL) 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 µL). 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

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

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

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

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

2.3. Identification of the inhibition mechanisms of selected antagonist isolates

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

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

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

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

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

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

Hydrogen peroxide production in active non-treated CFS was quantified using H_2O_2 Dosatest strip® (0 to 100 mg mL^{-1}, VWR).

2.3.2. Detection and quantification of major organic acids by high performance liquid chromatography

Reverse phase HPLC (Waters Alliance HPLC system with 2695 pump and 2998 PDA detector) was performed at 0.6 mL min^{-1} 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_2SO_4 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.

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

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

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

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

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

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

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

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

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

All the active CFS, which pH ranged between 4.5 and 6.5, lost their inhibitory activity against the pathogen after pH neutralisation except for the four *Aerococcus* isolates. According to these results, the inhibition mechanism of these isolates could be related to the production of organic acids combined with a pH effect. For the four *Aerococcus* sp. UCMA 5972, UCMA 18036, UCMA 18039 and UCMA 6078 strains, CFS was still active with pronase treatment, but not with catalase treatment. This suggest that the anti-*Salmonella* activity was mainly due to the production of hydrogen peroxide. This production was confirmed by H_2O_2 Dosatest® strips, with quantities produced ranging from 30 to 100 mg L^{-1} depending on the supernatant. After pronase treatment, all neutralised CFS kept their inhibitory activity. Therefore, none of the antagonistic activities were related to the presence of proteinaceous compounds such as bacteriocins. A drop-collapse assay was performed on non-treated CFS to reveal the production of biosurfactants. None of the strains appeared to produce such molecules.

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

Interestingly, *Aerococcus* strains were not associated with the production of lactic 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^{-1}. 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^{-1} (Hertzberger et al., 2014; Strus, Brzychczy-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.

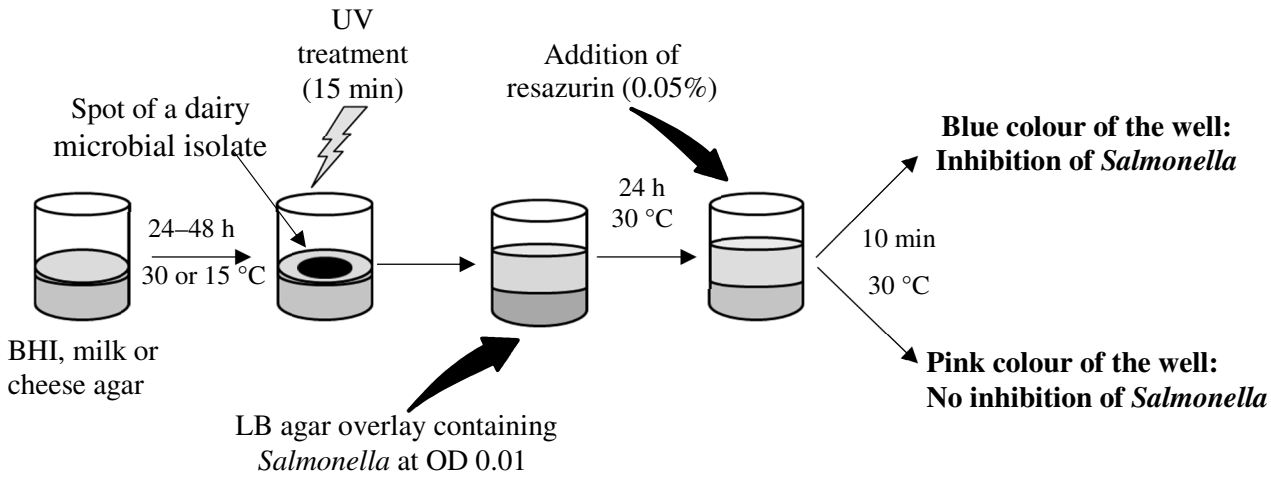
UV
treatment
(15 min)
Addition of
resazurin (0.05%)
Spot of a dairy
microbial isolate
Blue colour of the well:
Inhibition of *Salmonella*
24 h
30 °C
24–48 h
30 or 15 °C
10 min
30 °C
BHI, milk or
cheese agar
Pink colour of the well:
No inhibition of *Salmonella*
LB agar overlay containing
Salmonella at OD 0.01

Figure 1

Table 1

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

Serotype	Strain number	Origin
	UCMA 17913	Milk
Mbandaka	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. [a]

Presumed genus	Number of screened isolates	Number (%) of antagonistic isolates					
		Salmonella Mbandaka CIP 105.859			*Salmonella* Montevideo CIP 104.583		
		BHI	Milk	Cheese	BHI	Milk	Cheese
Aerococcus	31	19 (61.3)	17 (54.8)	20 (64.5)	14 (45.2)	19 (61.2)	18 (58.1)
Alcaligenes	3	1 (33.3)	1 (33.3)	0	2 (66.6)	1 (33.3)	0
Candida	9	6 (66)	1 (11.1)	0	3 (33.3)	1 (11.1)	0
Enterococcus	30	1 (3.3)	4 (13.3)	3 (10)	0	7 (23.3)	8 (26.6)
Geotrichum	4	2 (50)	0	2 (50)	2 (50)	0	0
Hafnia	8	1 (12.5)	0	0	1 (12.5)	0	0
Kluyveromyces	9	2 (22.2)	0	1 (11.1)	2 (22.2)	0	0
Lactobacillus	322	4 (1.2)	14 (4.3)	9 (2.7)	3 (0.93)	11 (3.4)	8 (2.4)
Lactococcus	536	19 (3.5)	57 (10.6)	59 (11)	12 (2.2)	70 (13.1)	66 (12.3)
Leuconostoc	102	1 (0.9)	14 (13.7)	16 (15.6)	0	8 (7.8)	20 (19.6)
Pediococcus	3	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)	1 (33.3)
Pichia	3	0	0	0	0	1 (33.3)	0
Pseudomonas	34	3 (8.8)	4 (11.7)	1 (2.9)	2 (5.8)	2 (5.8)	1 (2.9)
Raoultella	4	2 (50)	1 (25)	0	0	1 (25)	0
Staphylococcus	96	4 (4.1)	0	1 (1.1)	4 (4.1)	2 (2.1)	3 (3.3)
Streptococcus	10	1 (10)	1 (10)	1 (10)	0	3 (30)	1 (10)
Weissella	8	0	0	1 (12.5)	1 (12.5)	0	2 (25)
Yarrowia	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)

[a] Other isolates were: *Acinetobacter* (7), *Arthrobacter* (17), *Brachybacerium* (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), *Macrococcus* (11), *Microbacterium* (13), *Micrococcaceae* (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. [a]

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

[a] Isolate selected when able to inhibit at least 80% of field *Salmonella* strains - Non tested condition.