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## **Streptococcus macedonicus strains isolated from traditional fermented milks: resistance to gastrointestinal environment and adhesion ability**

Tedj El Moulouk Khaldi, Mounira Kebouchi, Claire Soligot, Mohamed Amine Gomri, Karima Kharroub, Yves Le Roux, Emeline Roux

### ► To cite this version:

Tedj El Moulouk Khaldi, Mounira Kebouchi, Claire Soligot, Mohamed Amine Gomri, Karima Kharroub, et al.. Streptococcus macedonicus strains isolated from traditional fermented milks: resistance to gastrointestinal environment and adhesion ability. Applied Microbiology and Biotechnology, 2019, 103 (6), pp.2759-2771. 10.1007/s00253-019-09651-z . hal-02111693

**HAL Id: hal-02111693**

**<https://hal.univ-lorraine.fr/hal-02111693v1>**

Submitted on 17 Mar 2022

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1 **Authors:** Tedj El Moulouk Khaldi<sup>a</sup>, Mounira Kebouchi<sup>b</sup>, Claire Soligot<sup>b</sup>, Mohamed Amine Gomri<sup>c</sup>, Karima

2 Kharroub<sup>c</sup>, Yves Le Roux<sup>b</sup> and Emeline Roux<sup>d\*</sup>

3

4 **Title:** *Streptococcus macedonicus* strains isolated from traditional fermented milks: resistance to

5 gastrointestinal environment and adhesion ability

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7

8 a: Laboratoire Alimentation, Nutrition et Santé (ALNUTS), Institut de la Nutrition, de l'Alimentation et des

9 Technofologies Agro Alimentaires (INATAA), Université Frères Mentouri Constantine 1 (UFMC1), Route de Ain

10 El Bey, 25000 Constantine, Algérie

11 b: Université de Lorraine, INRA, URAFPA, F-54000 Nancy, France

12 c: Laboratoire Biotechnologie et Qualité des Aliments (BIOQUAL), Equipe Métabolites des Extrémophiles

13 METEX, Institut de la Nutrition, de l'Alimentation et des Technologies Agro Alimentaires (INATAA), Université

14 Frères Mentouri Constantine 1 (UFMC1), Route de Ain El Bey, 25000 Constantine, Algérie

15 d: Université de Lorraine, CALBINOTOX, F-54000 Nancy, France

16

17 \* Corresponding author at: Emeline Roux

18 E-mail address: [emeline.roux@univ-lorraine.fr](mailto:emeline.roux@univ-lorraine.fr)

19 Telephone number: +33 3 72 74 70 01

20

21 Mohamed Amine Gomri ORCID: 0000-0003-0347-3412

22 Karima Kharroub ORCID: 0000-0002-6167-993X

23 Yves Le Roux ORCID: 0000-0001-6819-9114

24 Emeline Roux ORCID: 0000-0003-3402-0555

25

## 26 **Abstract**

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2  
3 27 In this study, *Streptococcus macedonicus* (*S. macedonicus*) strains were identified from food products: Algerian  
4  
5 28 traditional fermented milks (*Lben* and *Rayeb*). Important prerequisites of probiotic interest such as acidity, bile  
6  
7 29 salts tolerance, and adhesion ability to epithelial cells were investigated.

8  
9 30 A combination of phenotypic (selective M17 medium, 42°C as an optimum growth temperature, ability to grow  
10  
11 31 on hostile media) and genotypic approaches (16S rRNA, ITS genes sequencing and MLST technique) allowed to  
12  
13 32 identify four genetically distinct strains of *S. macedonicus*.

14  
15 33 These four strains and two references, *Streptococcus thermophilus* LMD-9 and *Lactobacillus rhamnosus* GG  
16  
17 34 (LGG), were tested for their capacity to survive during two hours at low pH values, and at different  
18  
19 35 concentrations of an equimolar bile salts mixture (BSM). Two different cell lines, Caco-2 TC7 and HT29-MTX,  
20  
21 36 were used for the adhesion study.

22  
23 37 The results show that *S. macedonicus* strains selected constitute a distinct genetic entity from the Greek strain *S.*  
24  
25 38 *macedonicus* ACA-DC-198. They were able to survive up to pH 3 and can tolerate high concentrations of bile  
26  
27 39 salts (10 mM), which is not the case for LMD-9 and LGG strains. Our strains also display *in vitro* adhesion  
28  
29 40 similar to the LGG strain on Caco-2 TC7 and higher adhesion than the LMD-9 strain to Caco-2 TC7 and HT29-  
30  
31 41 MTX cell models.

32  
33 42 This first characterization allows considering *S. macedonicus* as a potential candidate for possible probiotic  
34  
35 43 effects that need to be investigated.

36  
37 44  
38  
39 45 **Key word:** *Streptococcus macedonicus*; Multi Locus Sequence Typing MLST; gastrointestinal tract; bile salts;  
40  
41 46 adhesion; epithelial cells.

## 47 **Introduction**

48 Fermented milks are widely produced and consumed over the world since Antiquity. These traditional products,  
49 initially manufactured to allow the storage of milk, have persisted and sometimes evolved over the centuries.

50 *Lben* and *Rayeb* designate the main fermented products in the Maghreb countries (Kurmann et al. 1992). These  
51 products are mostly obtained by spontaneous fermentation of bovine milk at room temperature. Milk coagulation  
52 results in a curd, which can be consumed as such (*Rayeb*) or is further subjected to a churning with addition of  
53 water to aggregate the butter grains. After butter extraction, the residual buttermilk is called *Lben* (Bensalah et  
54 al., 2009).

55 This traditional process, depending on the environmental conditions (geographical, climatic, etc.), leads to a  
56 fermented milk containing atypical strains of Lactic Acid Bacteria (LAB) with potential new technological and  
57 biological properties. Indeed, it has been shown that pure strains isolated from traditional fermented products  
58 exert metabolic activities that are highly different from commercial strains (Prashant et al. 2009).

59 Furthermore, several *in vivo* studies performed either in humans or in animal models have reported that different  
60 LAB are able to exert probiotic effects on the host health (Fijan 2014; Nagpal et al. 2012). However, in order to  
61 be qualified as probiotic, bacterial strains must meet certain selection criteria. These criteria include, ecological  
62 origin of bacteria (human or food origin with Generally Recognized As Safe GRAS status), their tolerance to the  
63 hostile conditions of the stomach and the small intestine, and their ability to adhere, even transiently, to intestinal  
64 surfaces (Chae et al. 2013; Uroić et al. 2016). The survival ability to the harsh conditions of the digestive tract is  
65 considered as an important criterion since it allows the bacteria to reach its target and exert biological activities.

66 Indeed, several studies have shown that the survival ability of LAB to low pH and to bile salts stress conditions  
67 was highly variable depending on the species and even the strain (Junjua et al. 2016; Reale et al. 2015).  
68 Another criterion for probiotic selection concerns the evaluation of the adhesion ability of strains to the intestinal  
69 mucosa. This property is important since it allows the bacterial colonization in the intestines and therefore  
70 exerting some beneficial actions on the host (Kumar and Kumar 2015). Therefore, this property is widely sought  
71 after in LAB, especially those used extensively in the dairy industry (Turpin et al. 2012).

72 Among the dairy products containing LAB with probiotic potential, Algerian fermented milks presents an  
73 important source of these bacteria. Collected at high temperature in summer, these products may contain new  
74 non-pathogenic strains of thermophilic LAB. Indeed, some strains of atypical *Lactococcus*, resistant to relatively  
75 high temperature have been identified in an Algerian *Lben*, usually known for its mesophilic flora (Bensalah et

76 al. 2009). *Streptococcus thermophilus* (*S. thermophilus*), widely used in dairy industry, is presently the sole  
77 species among the *Streptococcus* genus to have the GRAS status obtained from the American Food and Drug  
78 Administration (FDA 2002,2012) and the Qualified Presumption of Safety (QPS) status from the European Food  
79 Safety Authority (EFSA). This bacterium is recognized for its beneficial effects such as the prevention of chronic  
80 gastritis (Rodríguez 2010) or the alleviation of lactose intolerance (EFSA Panel on Dietetic Products, Nutrition  
81 and Allergies 2010; Savaiano 2014).

82 The beneficial effects are not restricted to this bacterium since other species belonging to the *Streptococcus*  
83 genus and which have been isolated from dairy products, could also exert positives actions on the host after  
84 consumption as well as on the flavor of fermented milks. Among them, *S. macedonicus* was first isolated from  
85 Greek Kasserli cheese (Tsakalidou et al. 1994) and later from Italian cheeses (Lombardi et al. 2004). It is a  
86 thermophilic bacterium and homofermentative (Tsakalidou et al. 1998) derived from the sub-group  
87 *Streptococcus gallolyticus*(Schlegel et al. 2003). However, it is generally considered non-pathogenic  
88 (Maragkoudakis et al. 2009) and has even been reported to have a “food grade” status (De Vuyst and Tsakalidou  
89 2008). Nevertheless, genomic analysis based on *S. macedonicus* genomes allow to establish its non-pathogenic  
90 status (Papadimitriou et al. 2012, 2016).

91 Furthermore, the genomes of *S. macedonicus* published up to date all contain bacteriocin-related sequences. This  
92 corroborates previous publications on the ability of *S. macedonicus* strains to produce antimicrobial peptides  
93 directed against the alteration flora and the pathogenic species (Anastasiou et al. 2009; Georgalaki et al. 2002;  
94 Georgalaki et al. 2013; Grazia et al. 2017; Papadelli et al. 2007; Pieterse et al. 2008; Zoumpopoulou et al. 2013).  
95 *S. macedonicus* strains exhibit up to 10 genes related to bacteriocins and ribosomally synthesized antimicrobial  
96 peptides. In contrast, only a few sequenced strains of *S. thermophilus* possess those bacteriocins-related  
97 sequences (Treu et al. 2017), making *S. macedonicus* of great technological interest.

98 Finally, *S. macedonicus* seems to have a good immunomodulatory activity (Cho et al. 2010; 2012) and produce  
99 exopolysaccharides, which could not only be of technological interest but also display bioactivities (Tiwari and  
100 Misra 2008; Vincent et al. 2001). Additionally, *S. macedonicus*, in co-culture with other LAB, leads to naturally  
101 folate bio-enriched fermented milk (Laiño et al. 2015). All these results indicate that *S. macedonicus* could  
102 potentially be a good candidate as probiotic.

103 Nevertheless, little information is available on prerequisites of *S. macedonicus* probiotic interest such as the  
104 tolerance to the hostile conditions of gastrointestinal tract and the adhesion ability to the intestinal mucosa. Up to  
105 date, only one complete assembled genome is available in databases (strain ACA-DC 198, Papadimitriou et al.

106 2012) and six other genomes are published as scaffolds or contigs (Papadimitriou et al. 2016; Treu et al.  
1 2017; Vendramin et al. 2014).

3  
4 108 The aim of this study is i) the identification of different *S. macedonicus* strains isolated from Algerian traditional  
5  
6 109 fermented milks and ii) the characterization *in vitro* of some prior properties required for probiotics such as  
7  
8 110 resistance to low pH and bile salts stress, as well as the adhesion ability to intestinal epithelial cells.  
9

## 10 111 **Material and methods**

### 11 12 112 **Samples collection**

13 113 Forty traditional fermented cow milk samples “*Lben*” and “*Rayeb*” (Fig. 1) were obtained from 12 farms and 13  
14  
15 114 milk sellers situated in Constantine city center and suburbs, North-East Algeria. All milk samples were collected  
16  
17 115 aseptically, from June to September 2013, in 200 mL screw-cap bottles, kept at 4-8°C and transported in cool  
18  
19 116 boxes within hours for analyses.  
20  
21  
22

### 23 117 **Isolation and phenotypic identification of *S. macedonicus* strains**

24  
25 118 Serial dilutions ( $10^{-1}$  to  $10^{-7}$ ) were made for each milk sample in peptone water and 0.1 mL of the appropriate  
26  
27 119 dilution were plated on LM17 agar medium (Terzaghi and Sandine 1975) and incubated at 42°C for 24 to 48 h.  
28  
29 120 Gram-positive, catalase-negative and facultative anaerobic bacteria were purified by re-streaking on LM17 agar.  
30  
31 121 Salt tolerance was determined using LM17 broth containing 6.5% (w/v) NaCl with incubation for 48 h at 37°C.  
32  
33 122 Growth at pH 9.6 was determined in LM17 with the pH adjusted by using NaOH. Esculin Bile Azide (EBA)  
34  
35 123 (Oxoid, Dardilly, France) was used for the presumptive identification of enterococci. Thermophilic lactic  
36  
37 124 streptococci strains identified and displaying general phenotypic characteristics of *S. macedonicus* (no growth  
38  
39 on: EBA, high salt content, and alkaline pH) were stored at -20 °C in reconstituted skim milk (10%, w/v).  
40  
41

### 42 126 **Genotypic identification**

#### 43 44 127 **Genomic DNA extraction**

45  
46 128 After 24 hours growth in LM17 medium, *S. macedonicus* strains were recovered by centrifugation and genomic  
47  
48 129 DNA was extracted as previously described (Colmin et al., 1991).  
49

#### 50 130 **Amplification**

51  
52 131 The 16S ribosomal RNA (16S rRNA) gene was amplified by Polymerase Chain Reaction (PCR) using the  
53  
54 132 universal primers SSUfor (5' -TGCCAGCAGCCGCGGTA-3') and SSUrev (5' -  
55  
56 133 GACGGGCGGTGTGTACAA-3') (Dufour et al., 2008). The 16S-23S internal transcribed spacer (ITS)  
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65

134 sequences were obtained using ITS16S (5'- TTGTACACACCGCCCGTCA-3') and ITS23S (5' –  
135 GGTACCTTAGATGTTTCAGTTC-3') primers (Dufour et al. 2008; Kostman et al. 1992).  
136 PCR reactions were carried out in a Mastercycler pro Thermocycler apparatus (Eppendorf, Hamburg, Germany)  
137 with a final volume of 20 µL containing 100 ng of genomic DNA, 1X PCR buffer, 3 mM MgCl<sub>2</sub>, 0.2 mM dNTP,  
138 0.5 µM of each primer and 0.5 U of Taq polymerase (Invitrogen, Thermo Fisher Scientific, Waltham, MA,  
139 USA). Thirty cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 1 min) steps were  
140 repeated to amplify the 16S rRNA. For ITS, hybridization temperature was 54°C and extension time 45s.  
141 Finally, PCR products were checked on an agarose gel 1% (w/v) beside EZ load Molecular Ruler 100 bp (Bio-  
142 Rad).

### 143 **Sequencing**

144 Amplified PCR products were purified with the High Pure PCR Product purification kit (Roche Applied Science,  
145 Meylan, France) according to the manufacturer's recommendations. DNA sequencing was performed by the  
146 Beckman Coulter Genomics society  
147 (<https://myproject.beckmangenomics.com/myp/home?siteid=col&mapid=home>).

### 148 **Multi Locus Sequence Typing (MLST)**

149 The Multi Locus Sequence Typing study was adapted from Delorme et al. (2007; 2010); El-Sharoud et al.  
150 (2012); Junjua et al. (2016) and Shibata et al. (2014). Primers used for amplification of internal fragments of  
151 *ddlA*, *pepO*, *glcK*, *ilvC*, *thrS*, *tkt*, *dnaE*, *pyrE* and *serB* genes were specifically designed for *S. macedonicus* with  
152 Primer3Plus program (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>), based on *S.*  
153 *macedonicus* ACA-DC 198 genome (Papadimitriou et al. 2012), and were synthesized by the Eurogentec  
154 society. These primers sequences are listed in Table 1. PCR and gel electrophoresis conditions were those  
155 previously described by Delorme et al. (2007). Genes *dpr*, *gmk*, *rpoD*, *parC*, *pta*, *pyrC* and *recN* were amplified  
156 using Shibata's primers and PCR conditions (Shibata et al. 2014). The Sanger sequencing of each fragment on  
157 both strands was realized by Beckman Coulter Genomics Society. A concatenated sequence of the 10 variable  
158 housekeeping genes (*pyrC*, *ilvC*, *pta*, *recN*, *ddlA*, *dpr*, *gmk*, *parC*, *rpoD* and *serB*) was created and the  
159 phylogenetic tree was built with Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei  
160 1993) (bootstrap: 1000 replicates) on the MEGA 7 software. Sequences of the genes from *S. gallolyticus* strains  
161 UCN34, ATCC43143 and ATCCBAA-2069 were used to root the tree.

### 162 **Nucleotide sequence accession numbers and strain accession number**

163 The sequences of all alleles have been deposited in the GenBank database under accession numbers KU873024  
164 and KU873025 (*ilvC* fragment), KU873031 (*ddlA* fragment), KU873032 (*dpr* fragment), KU873033 (*gmk*  
165 fragment), KU873035 (*rpoD* fragment), KU873034 (*parC* fragment), KU873026 and KU873027 (*pyrC*  
166 fragment), KU873028 and KU873029 (*pta* fragment), KU873030 (*recN* fragment), and KU873036 (*serB*  
167 fragment).

168 The *S. macedonicus* PA strain was deposited in the French collection of food bacteria (CIRM-BIA, Rennes,  
169 France) under the accession number CIRM-BIA 2314.

### 170 **Acid and bile salts tolerance**

171 *S. thermophilus* LMD-9 (ATCC® BAA-491™; GenBank accession: CP000419) and *L. rhamnosus* GG (LGG)  
172 (ATCC® 53103™; NCBI Reference Sequence: NC\_013198) were included in this study and two independent  
173 experiments were performed in duplicate for each strain.

### 174 **Acidity survival assay**

175 Tolerance of *S. macedonicus* strains to low pH was carried out according to Thirabunyanon et al. (2009) with  
176 some modifications. *S. macedonicus* strains were pre-cultured twice in skim milk supplemented with  
177 bactotryptone (1%, w/v) (Sigma Aldrich, St Louis, MO, USA) and incubated overnight at 42°C. LMD-9 and  
178 LGG strains were pre-cultured in LM17 at 42°C and MRS at 37°C, respectively. Cultures were inoculated in  
179 LM17 and incubated at 42°C for *Streptococcus* genus and in MRS at 37°C for *L. rhamnosus*. After 14 h of  
180 incubation, cultures were centrifuged (1878 × g for 15 min at 37°C) and bacterial pellets were washed with  
181 Hank's Balanced Salt Solution (HBSS, Fisher Scientific). Afterwards, bacterial cells were re-suspended in HBSS  
182 at a final concentration of about 10<sup>10</sup> CFU/mL (CFU: colony forming unit). A volume of 0.2 mL bacterial  
183 suspension was added to 1.8 mL HBSS. Potential of Hydrogen was adjusted using hydrogen chloride (HCl) 1 M  
184 to pH 7 (control), pH 4, pH 3 and pH 2. Bacteria were then incubated at 37°C for 2 hours. The viable bacteria  
185 were determined by plate count of CFU and results were expressed as Log<sub>10</sub> CFU/mL.

### 186 **Bile salts survival assay**

187 The survival of *S. macedonicus* strains to high concentrations of bile salts was carried out according to the  
188 method described previously by Iyer et al. (2010) and Kebouchi et al. (2016) with some modifications. Briefly,  
189 after an overnight culture (14 h), bacterial pellets were washed with HBSS and re-suspended in LM17 and MRS  
190 for *Streptococcus* genus and *L. rhamnosus*, respectively, at a final concentration of 10<sup>10</sup> CFU/mL. Bacterial  
191 suspensions were exposed for 2 h at 37°C to an equimolar bile salts mixture (BSM) containing sodium  
192 taurocholate, sodium cholate, and sodium deoxycholate (Sigma Aldrich®) (Kebouchi et al. 2016) at different



193 concentrations (0, 4, 6 and 10 mM). After plating serial dilutions on LM17 and MRS agar and incubating of plate  
194 dishes for 48 h at 42°C for streptococci strains and 37°C for LGG, the number of viable bacteria was determined  
195 by enumeration of CFU and results were expressed as Log<sub>10</sub> CFU/mL.

## 196 **Bacterial adhesion to intestinal epithelial cells (IEC)**

### 197 **Cell culture conditions**

198 Two cells lines Caco-2 TC7 and HT29-MTX were used to study the *in vitro* adhesion ability of *S. macedonicus*  
199 strains. The cell culture conditions of Caco-2 TC7 (UMR 7274, Laboratoire Réactions et Génie des Procédés,  
200 Nancy, France) and mucus secreting cells HT29-MTX (INSERM UMR S 938, Paris, France) (Lesuffleur et al.  
201 1990) were performed using the method previously described by Kebouchi et al. (2016). For adhesion assay, the  
202 cell lines were seeded in six well culture plates (Dominique DUTSCHER) with an effective area of 9.7 cm<sup>2</sup> at a  
203 concentration of 11.10<sup>6</sup> cells per well. All kind of cells were cultured in Dulbecco's Modified Eagle's Minimal  
204 Essential Medium (DMEM, Glutamax, Fisher Scientific) supplemented with glucose, heat-inactivated fetal calf  
205 serum (FCS), antibiotics (penicillin/streptomycin) and eventually non-essential amino-acids (medium  
206 composition available in Kebouchi et al. (2016).

207 All cells were maintained in culture medium with antibiotics at 37°C in a humidified atmosphere with 10% CO<sub>2</sub>  
208 for 21-25 days. Two days before the adhesion assays, cells were kept in culture medium without antibiotics.

### 209 **Adhesion assay**

210 The *in vitro* adhesion ability of *S. macedonicus* strains, LMD-9 and LGG strains to cultured cell lines was  
211 studied. This study was performed according to Kebouchi et al. (2016). Succinctly, bacterial cells from an  
212 overnight culture (14 h) were collected and the bacterial pellets were washed with HBSS and re-suspended at a  
213 final concentration of 10<sup>10</sup> CFU/mL in culture medium free of antibiotics. In order to have the bacterial cell to  
214 epithelial cell ratio of 1000 to 1, two milliliters of each bacterial suspension were added per well to cell  
215 monolayers, which were previously washed with HBSS to remove dead cells and cellular debris. The six-well  
216 plates containing the bacterial cells on the cell monolayers were incubated during 2 h at 37°C in a humidified  
217 atmosphere with 10% CO<sub>2</sub>. Supernatants were then collected and epithelial cells were washed thoroughly to  
218 recover non-adherent bacteria. Adherent bacteria were obtained by scraping cell wells with Triton® X-100.  
219 Adhesion was evaluated by enumeration of adherent and non-adherent bacteria after plating of serial dilutions on  
220 LM17 and MRS agar. Two independent experiments were performed in duplicate.

### 221 **Statistical analysis**

222 Experimental data for acid, bile salts survival and bacterial adhesion were analyzed using analysis of variance  
1  
2 223 (ANOVA) followed by comparison of multiple averages with Tukey correction. Results were expressed as  
3  
4 224 means  $\pm$ SEM. Differences were considered statistically significant when  $P$  value  $< 0.05$ .

## 6 225 **Results**

### 9 226 **Identification of *S. macedonicus* strains**

#### 11 227 **Isolation and phenotypic identification**

13 228 First of all, the selection scheme adopted (Fig. 1) led to the recovery of 670 isolates -growing at 42°C on LM17  
14  
15 229 medium, being cocci Gram positive and catalase negative - from the 40 products (23 *Rayeb* and 17 *Lben*)  
16  
17 230 provided by 12 farmers and 13 retailers (Table 2). In a second time, the isolates were selected for their non-  
18  
19 231 ability to grow on hostile medium (NaCl 6.5% (w/v) and pH 9.6) and to hydrolyze esculin. Only 6 fermented  
20  
21 232 products had strains responding to those criteria and were further investigated by molecular biology techniques  
22  
23 233 to confirm the presence of *S. macedonicus* strains.

#### 25 234 **Genotypic identification**

27 235 Amplification and sequencing of 16S rRNA gene (partial sequence around 900 bp) (Table 3) indicated the  
28  
29 236 presence of four *S. macedonicus* strains having 100% identity with ACA-DC 198 and two *S. thermophilus*  
30  
31 237 strains (Lf9 having 100% identity with *S. thermophilus* JIM8232 or MN-ZLW-002; LA14, 100% identity with *S.*  
32  
33 238 *thermophilus* LMD-9). These results were comforted by amplification and sequencing of ITS 16S-23. The *S.*  
34  
35 239 *thermophilus* strain Lf9 has an ITS type I and the LA14 strain type II.

#### 37 240 **Phylogeny**

39 241 Sixteen housekeeping genes were partially sequenced in four *S. macedonicus* strains. No differences were found  
40  
41 242 for 6 genes: *glcK*, *pepO*, *thrS*, *tkl*, *pyrE* and *dnaE*, with 100% sequence identity between the four strains isolated  
42  
43 243 and the Greek strain ACA-DC 198 (Papadimitriou et al. 2012). These last genes were remote in the MLST study.  
44  
45 244 The nucleotide sequences of 363 to 842 bp internal fragments were determined for the ten other loci (*ddlA*, *ilvC*,  
46  
47 245 *serB*, *dpr*, *gmk*, *rpoD*, *parC*, *pta*, *pyrC* and *recN*). The sequence diversity within the *S. macedonius* genes allows  
48  
49 246 distinguishing of two to three alleles per locus; the maximal percent nucleotide sequence divergence within *S.*  
50  
51 247 *macedonicus* ranges from 0.2% to 1.5% (Table 4). Among these genes, *PyrC* have the highest number (7 sites)  
52  
53 248 and the highest proportion (1.5%) of polymorphic sites, followed by *ddlA* (6 sites, 0.7%). Genes *rpoD* and *serB*  
54  
55 249 are the least polymorphic ones (2 sites, 0.2%). Table 5 summarizes the allelic profiles of the five *S. macedonicus*  
56  
57 250 strains used in this study. Phylogenetic analysis of the concatenated sequences (total length of 5126 bp) yielded  
58  
59

251 the tree shown in Fig. 2. It revealed a clear separation of the species *S. macedonicus* and *S. gallolyticus*,  
1 252 supported by significant bootstrap values and a clear separation between Algerian and Greek strains of *S.*  
2 253 *macedonicus*.

## 6 254 **Acid and bile salts tolerance**

### 8 255 **Survival to acid stress**

10 256 The viability of strains *S. macedonicus* PA, LMD-9 and LGG was not affected after exposure to pH 4 during 2 h  
11  
12 257 (data not shown). At pH 3 (Fig. 3a), the PA strain was also more resistant than other *S. macedonicus* strains with  
13  
14 258 a loss of viability lower than 1 log and the same results were obtained for LMD-9 strain, whereas no  
15  
16 259 susceptibility of LGG strain was observed. The three other *S. macedonicus* strains P2, LK13 and LS5 were more  
17  
18 260 sensitive to an exposure at this pH with a survival decrease higher than 1.5 log. Moreover, no *S. macedonicus*  
19  
20 261 strain was detected after an exposure during 1 h at pH 2, contrariwise to LMD-9 and LGG strains which were  
21  
22 262 more resistant (data not shown).

### 24 263 **Survival to bile salts stress**

26 264 The four *S. macedonicus* strains were more resistant than LGG and LMD-9 strains. Furthermore, no viable  
27  
28 265 bacteria were detected at 10 mM and 4 mM of BSM for LGG and LMD-9 strain, respectively.  
29  
30 266 At the concentration of 6 mM BSM (Fig. 3b), the four *S. macedonicus* strains underwent a significant loss of  
31  
32 267 viability ranging from 1 log for the LS5 and LK13 strains, 2.5 log for the PA strain to 4.6 log for the most  
33  
34 268 sensitive strain P2. Furthermore, except the P2 strain, the three *S. macedonicus* conserved viability after 2 h  
35  
36 269 exposure to 10 mM BSM (Fig. 3b) and no significant effect was detected at 4 mM BSM (data not shown).

### 39 270 **Bacterial adhesion to IEC**

41 271 The ability of *S. macedonicus* strain (PA, P2, LS5 and LK13) to adhere to intestinal epithelial Caco2-TC7 and  
42  
43 272 HT29-MTX cells (IEC) *in vitro* is shown in Fig. 4. The LMD-9 and LGG strains were also included as  
44  
45 273 references.

47 274 Strains PA, P2 and LK13 of *S. macedonicus* studied have an adhesion capacity to Caco2-TC7 cells similar to  
48  
49 275 that LGG strain (Fig. 4a). LS5 strain has a lower adhesion capacity than the other *S. macedonicus* strains. These  
50  
51 276 values were even higher than those of LMD-9 strain.

53 277 However, among *S. macedonicus* strains, only PA strain have shown a significant high adhesion ability to  
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55 278 mucus-secreting cells HT29-MTX (around 13 bacteria per cell) (Fig. 4b). Low adhesion values were obtained  
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57 279 with other *S. macedonicus* strains (P2, LS5 and LK13).

## 280 Discussion

1  
2 281 The first objective of this work was to identify different strains, in particular of *S. macedonicus*, in raw milks  
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4 282 fermented spontaneously (*Lben* and *Rayeb*). The majority of the isolates from these raw milks was enterococci  
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6 283 and was consequently eliminated from the selection. In fact, enterococci were frequently isolated from  
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8 284 traditional dairy products (Cogan et al. 1997) and their predominance in Algerian traditional dairy products was  
9  
10 285 confirmed by Bensalah et al. (2009). *S. thermophilus* and *S. macedonicus* share the same characters targeted by  
11  
12 286 the phenotypical identification protocol. Only genetic analysis of 16S rRNA gene and ITS sequences allowed  
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14 287 separating the species. Thus, from the 40 traditional fermented milks subject of this work, we could only identify  
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16 288 four strains of *S. macedonicus* and two strains of *S. thermophilus*. These results show a presence of low  
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18 289 frequency of *S. macedonicus* in traditional fermented milks.

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20 290 The MLST technique was often used to discriminate bacterial species: *Streptococcus bovis*/*Streptococcus*  
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22 291 *equinus* complex (SBSEC) (Jans et al. 2016; Shibata et al. 2014). Our strains, sharing the same geographical  
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24 292 surface present a small degree of genetic variability. This low polymorphic character of *S. macedonicus* strains  
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26 293 was highlighted by Jans et al. (2016): they detected by MLST a very low but visible genetic diversity on the  
27  
28 294 phylogenetic tree of *S. macedonicus* strains isolated from various origins (Greek, Somali, Ivorian and Malian). In  
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30 295 the same way, our four Algerian strains constitute a distinct genetic entity from the Greek strain *S. macedonicus*  
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32 296 ACA-DC-198.

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34 297 In the second part of this study, we examined *in vitro* some properties that probiotic strains should possess. In  
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36 298 addition, two strains, *S. thermophilus* LMD-9 and LGG were used as references. LGG strain is recognized as a  
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38 299 probiotic and it is known to have a good adhesion capacity and a high tolerance to the harsh conditions of the  
39  
40 300 digestive tract. Regarding LMD-9 strain, it was used as a reference because it is part of streptococci and also for  
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42 301 its best adhesion capacity and survival to acid and bile salt stresses among other *S. thermophilus* strains tested  
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44 302 (Junjua et al. 2016).

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46 303 We chose pH values close to the physiological conditions of the GIT varying from 1 to 2 when the stomach is  
47  
48 304 empty and up to 4 - 5 during meals (Chen et al. 2009). Our results indicate that *S. macedonicus* strains tolerate  
49  
50 305 acidity up to pH value of 3, with a progressive reduction in viability between pH 4 and 3 depending on the  
51  
52 306 strains (data not shown). According to Ripamonti et al. (2011), a strong reduction in the viability of fecal origin  
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54 307 strains *S. macedonicus* SB230 and SB330 was observed after exposure to pH 3 (Table 6). Furthermore, although  
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56 308 *S. macedonicus* ACA-DC 198 strain does not survive after 1 hour exposure to pH 2.5 (Zoumpopoulou et al.  
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309 2008), it has been isolated from mouse stools at a concentration close to that of the oral administration  
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2 310 (Maragkoudakis et al. 2009). Therefore, the *S. macedonicus* strains should be resistant enough to cross the GIT  
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4 311 in a food matrix context.  
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6 312 The majority of survival studies to bile salts used the extract of bile whose exact composition is not well-known.  
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8 313 We preferred to use a bile salts mixture containing conjugate and free bile salts according to the study of  
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10 314 Kebouchi et al. (2016). The physiological bile salt concentrations vary from more than 40 mM to less than 1 mM  
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12 315 (Ruiz et al. 2013) with a concentration of 10 mM in small intestine (Bajor et al. 2010), which justifies the  
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14 316 concentrations adopted in this study. Our strains, except strain P2, seem to well tolerate high concentrations of  
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16 317 BSM, up to 10 mM. According to the literature, the behavior of bacteria towards bile salts is strain-dependent.  
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18 318 Indeed, *S. macedonicus* strains ACA-DC 198, LC743, SB230, SB330 and ACA-DC 208 resist quite well to bile  
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20 319 salts (Cho et al. 2010; Ripamonti et al. 2011; Zoumpopoulou et al. 2008,2017). However, the ten strains of *S.*  
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22 320 *macedonicus* tested by Blaiotta et al. (2013) display very different levels of resistance to bile salts (from 1% to  
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24 321 100% of survival). LAB can counteract bile salts by expressing a bile salt hydrolase (BSH). Although, the *bsh*  
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26 322 gene was shown *in silico* to be a pseudogene in the ACA-DC 198 genome (Papadimitriou et al. 2014), partial  
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28 323 enzyme activity of BSH type was observed *in vitro* for this strain (Zoumpopoulou et al. 2008). In fact, bile salt  
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30 324 resistance is not always correlated with BSH activity, as reported for *Lactobacillus salivarius* (Fang et al. 2009).  
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32 325 Other mechanisms, unknown to date in *S. macedonicus*, are developed by bacteria to respond to bile stress, such  
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34 326 as cell wall composition, induction of stress protein or production of exopolymers. Concerning our four *S.*  
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36 327 *macedonicus* strains, they are more tolerant to BSM than LGG (used as a positive control in several studies) or *S.*  
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38 328 *thermophilus* LMD-9 and their integration in a food matrix could certainly allow them to cross the GIT.  
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40 329 The *in vitro* adhesion ability of the four *S. macedonicus* strains to human intestinal epithelial cell lines Caco-2  
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42 330 TC7 and HT29-MTX was investigated. Caco-2 cells are able to differentiate spontaneously *in vitro* culture  
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44 331 forming a cell monolayer expressing apical and basolateral proteins as enterocytes of the human small intestine  
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46 332 whereas HT29-MTX cells differentiate into polarized goblet cells and secrete massive amounts of mucus mainly  
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48 333 composed with MUC5AC mucins (stomach origin) (Kebouchi et al. 2016; Lesuffleur et al. 1990) and Pinto et al.  
49  
50 334 1983). To our knowledge, there are few studies on the adhesion capacity of *S. macedonicus* strain to intestinal  
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52 335 epithelial cells. Our results revealed that *S. macedonicus* strains, except one, adhere at the same level as LGG to  
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54 336 Caco-2 TC7 cells. Regarding HT29-MTX, PA strain was characterized by its moderate adhesion capacity  
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56 337 compared to LGG strain. (Boleij et al. 2011) reported that *S. macedonicus* CIP105865T (SM) strain adheres  
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58 338 moderately to Caco-2 and HT29 cells, however a weak adhesion to the same cells was observed for *S.*  
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339 *macedonicus* strains tested by Zoumpopoulou et al. (2017). The adhesion of bacteria to epithelial cells are  
1  
2 340 generally related to the bacterial cell surface structures, among which pili and other surface proteins (Boleij et al.  
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4 341 2009; Jans et al. 2015). In *S. gallolyticus* (phylogenetically close to *S. macedonicus*), pil3 contributes to the  
5  
6 342 adhesion to HT29-MTX mucus since it binds specifically to mucin MUC5AC (Martins et al. 2015).The analysis  
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8 343 of the genome of *S. macedonicus* ACA-DC 198 revealed the presence of the operon pil3 (Papadimitriou et al.  
9  
10 344 2014) which seems to have been preserved in *S. macedonicus* along evolution. An heterogeneity in the level of  
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12 345 pili encoding-gene expression in different strains may explain the affinity of strain PA for HT29-MTX cells.  
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14 346 To conclude, we isolated four new *S. macedonicus* strains from traditional Algerian fermented milks,  
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16 347 differentiable on the genetic level from the Greek strain ACA-DC 198 (whole genome available on databases).  
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18 348 These strains have a sufficient survival capacity to gastric acidity and bile salts in the context of a food bowl.  
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20 349 Their *in vitro* adhesion ability to Caco-2 TC7 is significantly higher than the one of LMD-9 strain. A moderate  
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22 350 adhesion ability to HT29-MTX is obtained with one particular strain (PA). This first characterization encourages  
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24 351 the exploration of their probiotic effects in animal models.

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30 354 **Conflicts of interest**

31  
32 355 The authors declare that they have no competing interests.

33  
34 356 **Ethics statement**

35  
36 357 This article does not contain any studies with human participants or animals performed by any of the authors.

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569 **Table 1.** Oligonucleotide primers for *S. macedonicus* (Multilocus Sequence Typing Method)

Locus	Primer	Sequence (5' - 3')	Gene function
<i>glcK</i>	glcK-for glcK-rev	GGGATTGACCTTGGTGGGAC AAGACTTGCTGCACCGATGA	<i>Glucose kinase</i>
<i>ddlA</i>	ddlA-for ddlA-rev	CTGAGAGTGTATGCGTGCG ATGGCTTTCGCGTTTTTCAA	D-alanine D-alanine ligase
<i>pepO</i>	pepO-for pepO-rev	CCACATTGGGTCTCCTGCTT GGCGCCAAATGCAAGAAGAA	Endopeptidase
<i>ilvC</i>	ilvC-for ilvC-rev	TGGTTATGGCTCACAAGGTCA TGAATGCGTCGTCATCGTTT	Ketol acid-reductoisomerase
<i>thrS</i>	thrS-for thrS-rev	TGGACCGTAAAAGGCTGCTT CCTGACCACGAAGATGCACT	Threonyl-tRNA synthetase
<i>tkt</i>	tkt-for tkt-rev	CAGGACATGGTTCTGCCCTT CGTGAGTTGGTCCGTCTTCA	Transketolase
<i>pyrE</i>	pyrE-for pyrE-rev	TCACAAATTGCATCAGATTTGCT TCACGCCAAGTTTCTTGATTTTCT	Orotate phosphoribosyltransferase
<i>dnaE</i>	dnaE-for dnaE-rev	GTCAGACCTCTATCCACGCG CGCAAATAGACTCCCCGAGCT	DNA polymerase III
<i>serB</i>	serB-for serB-rev	TGGTAAGGCGCTTCTTTTCG TGGATGTGGACTCTACCCTCA	Phosphoserine phosphatase
<i>dpr</i>	dpr-for dpr-rev	CCAAGCGGTTGCTGATTTGTC ATTTGATTCCTGGTGCTTGTC	Peroxide resistance protein
<i>gmk</i>	gmk-for gmk-rev	CCGTTTCTATGACAACACGCC CGTCCGATAACACGATCAACA	Guanylate kinase
<i>rpoD</i>	rpoD-for rpoD-rev	CGGACAAAGAAGGAAATCCATC GTTTCAACCATGTGAACAGGA	RNA polymerase sigma factor
<i>parC</i>	parC-for parC-rev	TGGCTTGAAGCCTGTTCAAC TCAATGAGGTAAACAACCGC	DNA topoisomerase IV subunit A
<i>pta</i>	pta-for pta-rev	TGTTTGCTGACTGTGCGATT CCATCAATAGCTTGAGCAGCT	Phosphate acetyltransferase
<i>pyrC</i>	pyrC-for pyrC-rev	GGCTTTGATCAGGTTGCAGA GGTCTTCTTCGTGAACGGCAA	Dihydratase
<i>recN</i>	recN-for recN-rev	AGGGAAATTCAATCGTGATGGC TCTTCAAAGTTTCGCGAGCTTG	DNA repair protein

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572 **Table 2.** Isolation of thermophilic lactic cocci (growth at 42°C, Gram+, catalase-)

No.	Product code	Fermented milk	Origin	Isolates number
1	LB1	<i>Lben</i>	Milk seller 1	10
2	Rb1	<i>Rayeb</i>	Milk seller 2	11
3	LbFb2	<i>Lben</i>	Milk seller 3	17
4	RbFb1	<i>Rayeb</i>	Milk seller 3	18
5	LbFb1	<i>Lben</i>	Milk seller 4	18
6	RbFb2	<i>Rayeb</i>	Milk seller 4	16
7	LbG1	<i>Lben</i>	Milk seller 5	18
8	RbG1	<i>Rayeb</i>	Milk seller 5	17
9	LbG2	<i>Lben</i>	Milk seller 6	19
10	RbG2	<i>Rayeb</i>	Milk seller 6	16
11	LbK1	<i>Lben</i>	Milk seller 7	18
12	Rbk1	<i>Rayeb</i>	Milk seller 7	17
13	Lbk2	<i>Lben</i>	Milk seller 8	16
14	Rbk2	<i>Rayeb</i>	Milk seller 8	17
15	Lbk3	<i>Lben</i>	Milk seller 9	18
16	Rbk3	<i>Rayeb</i>	Milk seller 9	16
17	Lbk4	<i>Lben</i>	Milk seller 10	17
18	Rbk4	<i>Rayeb</i>	Milk seller 10	18
19	LbS	<i>Lben</i>	Milk seller 11	19
20	RbS	<i>Rayeb</i>	Milk seller 11	16
21	LbA	<i>Lben</i>	Milk seller 12	13
22	RbA	<i>Rayeb</i>	Milk seller 12	16
23	LbW	<i>Lben</i>	Milk seller 13	17
24	LbN1	<i>Lben</i>	Farm 1	16
25	RbN1	<i>Rayeb</i>	Farm 1	18
26	LbN2	<i>Lben</i>	Farm 2	15
27	RbN2	<i>Rayeb</i>	Farm 2	17
28	LbN3	<i>Lben</i>	Farm 3	15
29	RbN3	<i>Rayeb</i>	Farm 3	18
30	LbN4	<i>Lben</i>	Farm 4	16
31	RbN4	<i>Rayeb</i>	Farm 4	19
32	Lbchek	<i>Lben</i>	Farm 5	17
33	RbMR1	<i>Rayeb</i>	Farm 6	18
34	RbMR2	<i>Rayeb</i>	Farm 7	18
35	RbFz	<i>Rayeb</i>	Farm 8	19
36	Rb1FP	<i>Rayeb</i>	Farm 9	18
37	Rb2FP	<i>Rayeb</i>	Farm 9	17
38	RbJ	<i>Rayeb</i>	Farm 10	18
39	RbM	<i>Rayeb</i>	Farm 11	16
40	RbC	<i>Rayeb</i>	Farm 12	17
<b>Total</b>	40	<i>Rayeb</i> (23), <i>Lben</i> (17)	Farms (12), Milk sellers (13)	670

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574 **Table 3.** Genotypic identification of presumptive *S. macedonicus* and *S. thermophilus* isolates

575 (no growth on BEA, NaCl (6,5%) and pH 9,6)

No.	Isolate	Fermented milk	Origin	RNAr 16S	ITS 16S-23S
1	LK13	<i>Lben</i> (LbK1)	Milk seller 7		
2	LS5	<i>Lben</i> (LbS)	Milk sell. 11	<i>S. macedonicus</i>	<i>S. macedonicus</i>
3	PA	<i>Rayeb</i> (Rb1FP)	Farm 9		
4	P2	<i>Rayeb</i> (Rb2FP)	Farm 9		
5	LF9	<i>Lben</i> (LbFb2)	Milk sell. 3		
6	LA14	<i>Lben</i> (LbA)	Milk sell. 12	<i>S. thermophilus</i> ITS II	

576 ITS types were attributed according to (Mora et al. 2003).

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578

579 **Table 4.** Genetic diversity at *S. macedonicus* loci

Locus	Length of sequence (bp)	No. of variable sites	No. of alleles	Maximal % divergence within <i>S. macedonicus</i>
<i>pyrC</i>	477	7	3	1.5
<i>ilvC</i>	776	3	3	0.4
<i>pta</i>	466	2	3	0.4
<i>recN</i>	400	3	2	0.7
<i>ddlA</i>	842	6	2	0.7
<i>dpr</i>	363	2	2	0.5
<i>gmK</i>	415	2	2	0.4
<i>parC</i>	462	2	2	0.4
<i>rpoD</i>	435	1	2	0.2
<i>serB</i>	490	1	2	0.2

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582

583 **Table 5.** MLST data of the 4 *S. macedonicus* strains isolated in this study and the ACA-DC 198 strain

584 (Papadimitriou et al. 2012)

Strain	Origin	Allele no.										
		<i>pyrC</i>	<i>ilvC</i>	<i>pta</i>	<i>recN</i>	<i>ddlA</i>	<i>dpr</i>	<i>gmK</i>	<i>parC</i>	<i>rpoD</i>	<i>serB</i>	
PA	<i>Rayeb</i> (Rb1FP)	1	2	1	1	2	2	2	2	2	2	
P2	<i>Rayeb</i> (Rb2FP)	2	2	2	1	2	2	2	2	2	2	
LS5	<i>Lben</i> (LbS)	2	1	2	2	2	2	2	2	2	2	
LK13	<i>Lben</i> (LbK1)	2	2	2	1	2	2	2	2	1	2	
ACA-DC 198	Kasseri cheese	3	3	3	2	1	1	1	1	2	1	

585

586 **Table 6.** Synthetic table representing some studies about acid and bile salt tolerance and adhesion to IEC of *S.*

587 *macedonicus*

Source	Methods	Results
<b>Acid tolerance</b>		
Zoumpopoulou et al. (2008)	<i>S. macedonicus</i> ACA-DC 198 in PBS solution pH 2.5; incubation at 37°C for 0.5, 1, 2 and 3 h.	No survival after exposure 1 h at pH 2.5.
Cho et al. (2010)	LC743 strain in solution at pH 2, 3 and 4; incubation at 37°C for 1, 2 and 3 h.	No effect at pH 4. Slight decrease after 3 h at pH 3 (7.14 to 7.05 log-value); at pH 2, decrease from 7.14 to 5.33 log.
Ripamonti et al. (2011)	SB230 and SB330 strains in artificial gastric fluid (NaCl, 0.72 g/L; KCl, 0.05 g/L; NaHCO <sub>3</sub> , 0.37 g/L; pepsin, 0.3 g/L) pH 3 and 7 for 0, 90 or 180 min.	At pH 7, decrease from 8.58 to 8.36 log-value after 8 h. At pH 3, no viability after 90 min.
Blaiotta et al. (2013)	Ten <i>S. macedonicus</i> strains in solution with NaCl 0.85% at pH 2.5 and 6 for 30 min at 37°C.	Highly variable (from 5 to 95% survival) depending on the strain; only two strains with a survival rate higher than 60%
Zoumpopoulou et al. (2017)	<i>S. macedonicus</i> strains in PBS solution pH 2.5; incubation at 37°C for 2 h.	Strong reduction in viability (decline > 3 log)
Our study	Four <i>S. macedonicus</i> strains in HBSS solution pH 7, 4, 3 and 2 for 2 h at 37°C.	Our strains tolerate acidity until pH value of 3.
<b>Bile salt tolerance</b>		
Zoumpopoulou et al. (2008)	Strain ACA-DC 198 in PBS solution, pH 8 with 1% or 2% Oxfall, incubation at 37 °C for 1, 2 and 4 h.	Resistant: no viability loss after exposure 4 h to bile salts.
Cho et al.(2010)	LC743 strain in MRS broth with 0.05% L-cysteine and 0.3% Oxfall for 7 h at 37°C.	Good bile salts tolerance (decrease from 9 to 8.4 log-value).
Ripamonti et al. (2011)	3 h incubation in artificial gastric fluid pH 7, then 3 h in artificial intestinal fluid (0.1% pancreatin and 0.3% Oxfall bile salts, pH 8).	Reduction of population from 8.36 to 7.63 log-value.
Blaiotta et al. (2013)	Ten <i>S. macedonicus</i> strains on MRS or M17 agar plates with 0.3% Oxfall, incubation at 37°C for 24-48 h.	From 1 to 100% of survival: only two strains out of 10, survived at 100% and 60%; the 8 other strains less than 46%.

Zoumpopoulou et al. (2017)	<i>S. macedonicus</i> strains in PBS solution, pH 8 with 1% (w/v) bile salts, incubation at 37 °C for 3 h.	Good bile tolerance for one only strain (ACA-DC 208) (viability loss < 1,5 log ).
Our study	Four <i>S. macedonicus</i> strains for 2 h at 37°C with equimolar BSM at 0, 4, 6 and 10 mM.	Three strains tolerate concentrations of BSM up to 10 mM.

#### Bacterial adhesion to IEC

Boleij et al. (2011)	Caco-2 and HT-29 cells cultured in well plates to $1.10^6$ cells/well into DMEM. Incubation 2 h at 37°C with <i>S. macedonicus</i> CIP105865T.	Moderate (20%) adherence to Caco-2 and HT-29 cells.
Zoumpopoulou et al. (2008)	Caco-2 and HT-29 cells cultured in well plates to $1.10^5$ cells/well into DMEM. Incubation 2 h at 37°C with <i>S. macedonicus</i> strains	Weak adhesion to Caco-2 (<1%) and HT-29 cells (<4%).
Our study	Caco-2 TC7 and HT29-MTX in well plates to $1.10^6$ cells/well into DMEM at 37°C with 10% CO <sub>2</sub> for 21-25 h. Incubation 2 h with bacteria at 37°C.	Similar adhesion ability as LGG strain to Caco-2 TC7. One strain with moderate adhesion capacity to HT29-MTX cells (half of LGG).

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590 **Figure legends**

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2 591 **Fig. 1** Selection scheme of *S. macedonicus* and *S. thermophilus* strains

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4 592 The diagram summarizes the steps of selection of *S. macedonicus* and *S. thermophilus* strains from the fermented  
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6 593 milk samples to the strain, (-) no growth

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8 594 **Fig. 2** Phylogenetic tree of five *S. macedonicus* strains (LK13, P2, LS5, PA and ACA-DC 198) and three *S.*  
9  
10 595 *gallolyticus* strains

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12 596 The tree was based on concatenated sequences of ten housekeeping genes (*pyrC*, *ilvC*, *pta*, *recN*, *ddlA*, *dpr*, *gmk*,  
13 597 *parC*, *rpoD* and *serB*). The tree was constructed using the Maximum Likelihood method based on the Tamura-  
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15 598 Nei model (Tamura et al. 1993). The percentage of trees in which the associated taxa clustered together is shown  
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17 599 next to the branches. Scale bar = 0.002 nucleotide substitution per site. Evolutionary analyses were conducted in  
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19 600 MEGA7

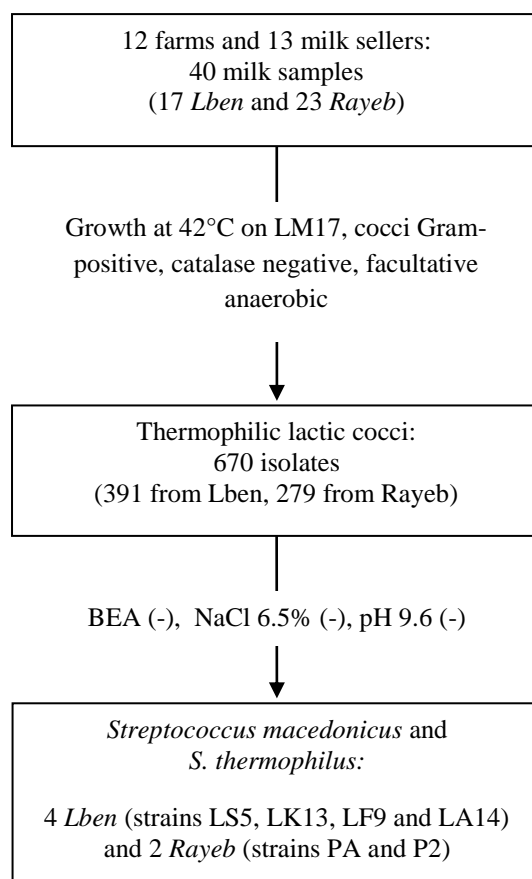
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21 601 **Fig. 3** Survival of *S. macedonicus*, *S. thermophilus* LMD-9 and *L. rhamnosus* GG (LGG) strains after exposure  
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23 602 to pH 3 (a) and to BSM concentrations of 6 mM and 10 mM (b)

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25 603 The survival at pH 3 was evaluated for 1 and 2 h compared to the negative control (pH 7) and expressed as Log<sub>10</sub>  
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27 604 CFU/mL (a). The survival to 6 mM and 10 mM BSM concentrations was evaluated for 2 h compared to the  
28  
29 605 negative control (0 mM BSM) and expressed as Log<sub>10</sub> CFU/mL (b). Means ± SEM of two independent  
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31 606 experiments conducted in duplicate are presented. Mean values with different letters are significantly different at  
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33 607 p < 0.05

34  
35 608 **Fig. 4** Adhesion of *S. macedonicus*, *S. thermophilus* LMD-9 and *L. rhamnosus* GG (LGG) *S.* strains to Caco-2  
36  
37 609 TC7 (a) and to HT29-MTX cells (b)

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39 610 The data were expressed as the number of adherent bacteria (CFU/mL) per Caco-2 TC7 (a) and HT29-MTX  
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41 611 cells (b). Means ± SEM of two independent experiments conducted in duplicate are presented. Mean values with  
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43 612 different letters are significantly different at p < 0.05

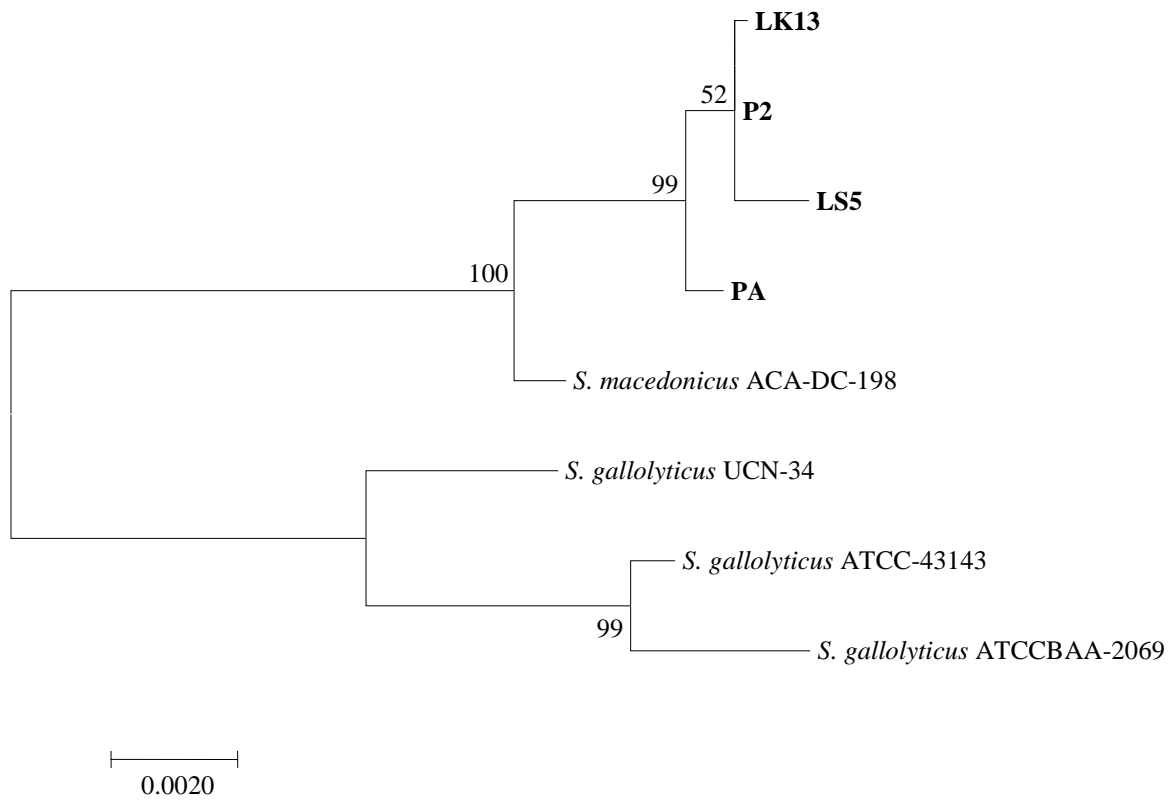
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**Fig. 1** Selection scheme of *S. macedonicus* and *S. thermophilus* strains

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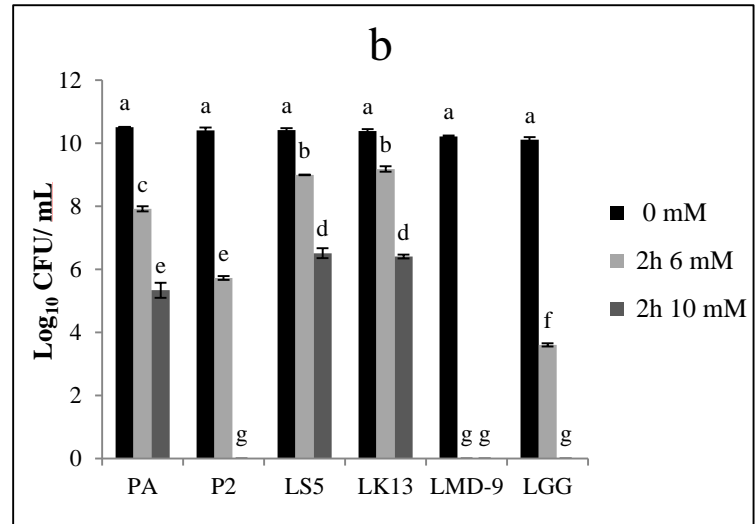
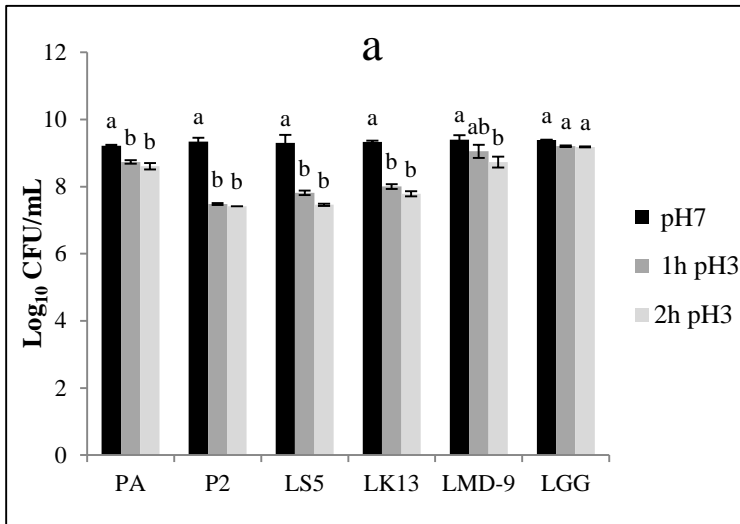


22

23 **Fig. 2** Phylogenetic tree of five *S. macedonicus* strains (LK13, P2, LS5, PA and ACA-DC 198) and three *S.*

24 *gallolyticus* strains

25



26

27 **Fig. 3** Survival of *S. macedonicus*, *S. thermophilus* LMD-9 and *L. rhamnosus* GG (LGG) strains after exposure  
 28 to pH 3 (a) and to BSM concentrations of 6 mM and 10 mM (b)

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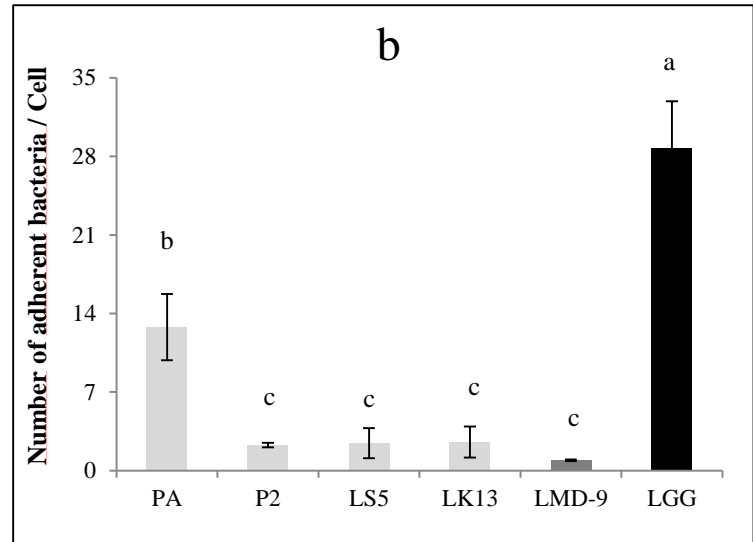
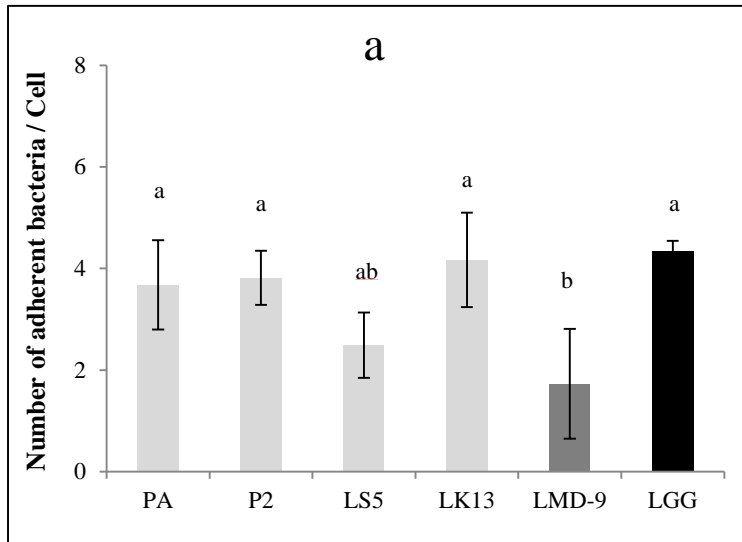
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42 **Fig. 4** Adhesion of *S. macedonicus*, *S. thermophilus* LMD-9 and *L. rhamnosus* GG (LGG) strains to Caco-2 TC7

43 (a) and to HT29-MTX cells (b)