

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

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26 Abstract

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

30 A combination of phenotypic (selective M17 medium, 42°C as an optimum growth temperature, ability to grow

31 on hostile media) and genotypic approaches (16S rRNA, ITS genes sequencing and MLST technique) allowed to

32 identify four genetically distinct strains of *S. macedonicus*.

- 33 These four strains and two references, Streptococcus thermophilus LMD-9 and Lactobacillus rhamnosus GG
- 34 (LGG), were tested for their capacity to survive during two hours at low pH values, and at different
- 35 concentrations of an equimolar bile salts mixture (BSM). Two different cell lines, Caco-2 TC7 and HT29-MTX,
- 36 were used for the adhesion study.
- 37 The results show that *S. macedonicus* strains selected constitute a distinct genetic entity from the Greek strain *S.*
- 38 macedonicus ACA-DC-198. They were able to survive up to pH 3 and can tolerate high concentrations of bile
- 39 salts (10 mM), which is not the case for LMD-9 and LGG strains. Our strains also display *in vitro* adhesion
- 40 similar to the LGG strain on Caco-2 TC7 and higher adhesion than the LMD-9 strain to Caco-2 TC7 and HT29-
- 41 MTX cell models.
- 42 This first characterization allows considering *S. macedonicus* as a potential candidate for possible probiotic
- 43 effects that need to be investigated.

45 Key word: *Streptococcus macedonicus*; Multi Locus Sequence Typing MLST; gastrointestinal tract; bile salts;
46 adhesion; epithelial cells.

47 Introduction

Fermented milks are widely produced and consumed over the world since Antiquity. These traditional products, initially manufactured to allow the storage of milk, have persisted and sometimes evolved over the centuries. *Lben* and *Rayeb* designate the main fermented products in the Maghreb countries (Kurmann et al. 1992). These products are mostly obtained by spontaneous fermentation of bovine milk at room temperature. Milk coagulation results in a curd, which can be consumed as such (*Rayeb*) or is further subjected to a churning with addition of water to aggregate the butter grains. After butter extraction, the residual buttermilk is called *Lben* (Bensalah et al., 2009).

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

Furthermore, several *in vivo* studies performed either in humans or in animal models have reported that different LAB are able to exert probiotic effects on the host health (Fijan 2014; Nagpal et al. 2012). However, in order to be qualified as probiotic, bacterial strains must meet certain selection criteria. These criteria include, ecological origin of bacteria (human or food origin with Generally Recognized As Safe GRAS status), their tolerance to the hostile conditions of the stomach and the small intestine, and their ability to adhere, even transiently, to intestinal surfaces (Chae et al. 2013; Uroić et al. 2016). The survival ability to the harsh conditions of the digestive tract is considered as an important criterion since it allows the bacteria to reach its target and exert biological activities. Indeed, several studies have shown that the survival ability of LAB to low pH and to bile salts stress conditions was highly variable depending on the species and even the strain (Junjua et al. 2016; Reale et al. 2015). 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

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).

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

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

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

Furthermore, the genomes of *S. macedonicus* published up to date all contain bacteriocin-related sequences. This
corroborates previous publications on the ability of *S. macedonicus* strains to produce antimicrobial peptides
directed against the alteration flora and the pathogenic species (Anastasiou et al. 2009; Georgalaki et al. 2002;
Georgalaki et al. 2013; Grazia et al. 2017; Papadelli et al. 2007; Pieterse et al. 2008; Zoumpopoulou et al. 2013). *S. macedonicus* strains exhibit up to 10 genes related to bacteriocins and ribosomally synthesized antimicrobial
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.

Finally, *S. macedonicus* seems to have a good immunomodulatory activity (Cho et al. 2010; 2012) and produce
exopolysaccharides, which could not only be of technological interest but also display bioactivities (Tiwari and
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.

107 2017; Vendramin et al. 2014).

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

1 Material and methods

2 Samples collection

Forty traditional fermented cow milk samples "*Lben*" and "*Rayeb*" (Fig. 1) were obtained from 12 farms and 13 milk sellers situated in Constantine city center and suburbs, North-East Algeria. All milk samples were collected aseptically, from June to September 2013, in 200 mL screw-cap bottles, kept at 4-8°C and transported in cool boxes within hours for analyses.

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

118 Serial dilutions (10⁻¹ to 10⁻⁷) were made for each milk sample in peptone water and 0.1 mL of the appropriate

dilution were plated on LM17 agar medium (Terzaghi and Sandine 1975) and incubated at 42°C for 24 to 48 h.

120 Gram-positive, catalase-negative and facultative anaerobic bacteria were purified by re-streaking on LM17 agar.

Salt tolerance was determined using LM17 broth containing 6.5% (w/v) NaCl with incubation for 48 h at 37°C.

Growth at pH 9.6 was determined in LM17 with the pH adjusted by using NaOH. Esculin Bile Azide (EBA)

(Oxoid, Dardilly, France) was used for the presumptive identification of enterococci. Thermophilic lactic

4 streptococci strains identified and displaying general phenotypic characteristics of *S. macedonicus* (no growth

5 on: EBA, high salt content, and alkaline pH) were stored at -20 °C in reconstituted skim milk (10%, w/v).

42 126 Genotypic identification

127 Genomic DNA extraction

128 After 24 hours growth in LM17medium, *S. macedonicus* strainswere recovered by centrifugation and genomic

129 DNA was extracted as previously described (Colmin et al., 1991).

130 Amplification

The 16S ribosomal RNA (16S rRNA) gene was amplified by Polymerase Chain Reaction (PCR) using the
universal primers SSUfor (5' –TGCCAGCAGCCGCGGTA-3') and SSUrev (5' –

133 GACGGGCGGTGTGTACAA-3') (Dufour et al., 2008). The 16S–23S internal transcribed spacer (ITS)

- 134 sequences were obtained using ITS16S (5'- TTGTACACACCGCCCGTCA-3') and ITS23S (5'-1 135 GGTACCTTAGATGTTTCAGTTC-3') primers (Dufour et al. 2008; Kostman et al. 1992). 2 3 136 PCR reactions were carried out in a Mastercycler pro Thermocycler apparatus (Eppendorf, Hamburg, Germany) 4 5 137 б with a final volume of 20 µL containing 100 ng of genomic DNA, 1X PCR buffer, 3 mM MgCl₂, 0.2 mM dNTP, 7 138 0.5 µM of each primer and 0.5 U of Taq polymerase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, 8 9 10 139 USA). Thirty cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s), and extension (72°C, 1 min) steps were 11 12 140 repeated to amplify the 16S rRNA. For ITS, hybridization temperature was 54°C and extension time 45s. 13 14 141 Finally, PCR products were checked on an agarose gel 1% (w/v) beside EZ load Molecular Ruler 100 bp (Bio-15 16 142 Rad). 17 18 143 Sequencing 19 20 144 Amplified PCR products were purified with the High Pure PCR Product purification kit (Roche Applied Science, 21 22 145 Meylan, France) according to the manufacturer's recommendations. DNA sequencing was performed by the 23 24 146 Beckman Coulter Genomics society 25 26 147 (https://myproject.beckmangenomics.com/myp/home?siteid=col&mapid=home). 27 28 148 Multi Locus Sequence Typing (MLST) 29 30 149 The Multi Locus Sequence Typing study was adapted from Delorme et al. (2007; 2010); El-Sharoud et al. 31 32 150 (2012); Junjua et al. (2016) and Shibata et al. (2014). Primers used for amplification of internal fragments of 33 34 151 ddlA, pepO, glcK, ilvC, thrS, tkt, dnaE, pyrE and serB genes were specifically designed for S. macedonicus with 35 36 152 Primer3Plus program (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi), based on S. 37 38 153 macedonicus ACA-DC 198 genome (Papadimitriou et al. 2012), and were synthesized by the Eurogentec 39 ⁴⁰ 154 society. These primers sequences are listed in Table 1. PCR and gel electrophoresis conditions were those 41 42 155 previously described by Delorme et al. (2007). Genes dpr, gmk, rpoD, parC, pta, pyrC and recN were amplified 43 44 156 using Shibata's primers and PCR conditions (Shibata et al. 2014). The Sanger sequencing of each fragment on 45 46 157 both strands was realized by Beckman Coulter Genomics Society. A concatenated sequence of the 10 variable 47 48 158 housekeeping genes (pyrC, ilvC, pta, recN, ddlA, dpr, gmk, parC, rpoD and serB) was created and the 49 50 159 phylogenetic tree was built with Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 51 52 160 1993) (bootstrap: 1000 replicates) on the MEGA 7 software. Sequences of the genes from S. gallolyticus strains 53 54 161 55 56 162 57 58 59 60 61 62 63 64 65

 - UCN34, ATCC43143 and ATCCBAA-2069 were used to root the tree.
 - 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

and KU873025 (*ilvC* fragment), KU873031 (*ddlA* fragment), KU873032 (*dpr* fragment), KU873033 (*gmk*

165 fragment), KU873035 (*rpoD* fragment), KU873034 (*parC* fragment), KU873026 and KU873027 (*pyrC*

fragment), KU873028 and KU873029 (*pta* fragment), KU873030 (*recN* fragment), and KU873036 (*serB*fragment).

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

70 Acid and bile salts tolerance

S. thermophilus LMD-9 (ATCC® BAA-491[™]; GenBank accession: CP000419) and *L. rhamnosus* GG (LGG)
(ATCC® 53103[™]; NCBI Reference Sequence: NC_013198) were included in this study and two independent

experiments were performed in duplicate for each strain.

174 Acidity survival assay

Tolerance of S. macedonicus strains to low pH was carried out according to Thirabunyanon et al. (2009) with some modifications. S. macedonicus strains were pre-cultured twice in skim milk supplemented with bactotryptone (1%, w/v) (Sigma Aldrich, St Louis, MO, USA) and incubated overnight at 42°C. LMD-9 and LGG strains were pre-cultured in LM17 at 42°C and MRS at 37°C, respectively. Cultures were inoculated in LM17 and incubated at 42°C for Streptococcus genus and in MRS at 37°C for L. rhamnosus. After 14 h of incubation, cultures were centrifuged ($1878 \times g$ for 15 min at 37° C) and bacterial pellets were washed with Hank's Balanced Salt Solution (HBSS, Fisher Scientific). Afterwards, bacterial cells were re-suspended in HBSS at a final concentration of about 10¹⁰ CFU/mL (CFU: colony forming unit). A volume of 0.2 mL bacterial suspension was added to 1.8 mL HBSS. Potential of Hydrogen was adjusted using hydrogen chloride (HCl) 1 M ₄₃ 184 to pH 7 (control), pH 4, pH 3 and pH 2. Bacteria were then incubated at 37°C for 2 hours. The viable bacteria 45 185 were determined by plate count of CFU and results were expressed as Log₁₀ CFU/mL.

186 Bile salts survival assay

The survival of *S. macedonicus* strains to high concentrations of bile salts was carried out according to the
method described previously by Iyer et al. (2010) and Kebouchi et al. (2016)with some modifications. Briefly,
after an overnight culture (14 h), bacterial pellets were washed with HBSS and re-suspended in LM17 and MRS
for *Streptococcus* genus and *L. rhamnosus*, respectively, at a final concentration of 10¹⁰ CFU/mL. Bacterial
suspensions were exposed for 2 h at 37°C to an equimolar bile salts mixture (BSM) containing sodium
taurocholate, sodium cholate, and sodium deoxycholate (Sigma Aldrich®) (Kebouchi et al. 2016) at different

concentrations (0, 4, 6 and 10 mM). After plating serial dilutions on LM17 and MRS agar and incubating of plate
dishes for 48 h at 42°C for streptococci strains and 37°C for LGG, the number of viable bacteria was determined
by enumeration of CFU and results were expressed as Log₁₀ CFU/mL.

Bacterial adhesion to intestinal epithelial cells (IEC)

197 Cell culture conditions

Two cells lines Caco-2 TC7 and HT29-MTX were used to study the *in vitro* adhesion ability of *S. macedonicus* strains. The cell culture conditions of Caco-2 TC7 (UMR 7274, Laboratoire Réactions et Génie des Procédés, Nancy, France) and mucus secreting cells HT29-MTX (INSERM UMR S 938, Paris, France) (Lesuffleur et al. 1990) were performed using the method previously described by Kebouchi et al. (2016). For adhesion assay, the cell lines were seeded in six well culture plates (Dominique DUTSCHER) with an effective area of 9.7 cm² at a concentration of 11.10⁶ cells per well. All kind of cells were cultured in Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM, Glutamax, Fisher Scientific) supplemented with glucose, heat-inactivated fetal calf serum (FCS), antibiotics (penicillin/streptomycin) and eventually non-essential amino-acids (medium composition available in Kebouchi et al. (2016).

All cells were maintained in culture medium with antibiotics at 37°C in a humidified atmosphere with 10% CO₂

for 21-25 days. Two days before the adhesion assays, cells were kept in culture medium without antibiotics.

209 Adhesion assay

The in vitro adhesion ability of S. macedonicus strains, LMD-9 and LGG strains to cultured cell lines was studied. This study was performed according to Kebouchi et al. (2016). Succinctly, bacterial cells from an overnight culture (14 h) were collected and the bacterial pellets were washed with HBSS and re-suspended at a final concentration of 10¹⁰ CFU/mL in culture medium free of antibiotics. In order to have the bacterial cell to epithelial cell ratio of 1000 to 1, two milliliters of each bacterial suspension were added per well to cell monolayers, which were previously washed with HBSS to remove dead cells and cellular debris. The six-well plates containing the bacterial cells on the cell monolayers were incubated during 2 h at 37°C in a humidified atmosphere with 10% CO₂. Supernatants were then collected and epithelial cells were washed thoroughly to recover non-adherent bacteria. Adherent bacteria were obtained by scraping cell wells with Triton[®] X-100. Adhesion was evaluated by enumeration of adherent and non-adherent bacteria after plating of serial dilutions on 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

223 (ANOVA) followed by comparison of multiple averages with Tukey correction. Results were expressed as

means \pm SEM. Differences were considered statistically significant when *P* value < 0.05.

Results

 $^{2}_{2}$ 223 $^{3}_{4}$ 224

5 Identification of S. macedonicus strains

7 Isolation and phenotypic identification

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

4 Genotypic identification

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

Phylogeny

Sixteen housekeeping genes were partially sequenced in four *S. macedonicus* strains. No differences were found for 6 genes: *glcK*, *pepO*, *thrS*, *tkt*, *pyrE* and *dnaE*, with 100% sequence identity between the four strains isolated and the Greek strain ACA-DC 198 (Papadimitriou et al. 2012). These last genes were remote in the MLST study. The nucleotide sequences of 363 to 842 bp internal fragments were determined for the ten other loci (*ddlA*, *ilvC*, *serB*, *dpr*, *gmk*, *rpoD*, *parC*, *pta*, *pyrC* and *recN*). The sequence diversity within the *S. macedonius* genes allows distinguishing of two to three alleles per locus; the maximal percent nucleotide sequence divergence within *S. macedonicus* ranges from 0.2% to 1.5% (Table 4). Among these genes, *PyrC* have the highest number (7 sites) and the highest proportion (1.5%) of polymorphic sites, followed by *ddlA* (6 sites, 0.7%). Genes *rpoD* and *serB* are the least polymorphic ones (2 sites, 0.2%). Table 5 summarizes the allelic profiles of the five *S. macedonicus* strains used in this study. Phylogenetic analysis of the concatenated sequences (total length of 5126 bp) yielded

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

Acid and bile salts tolerance

Survival to acid stress

The viability of strains S. macedonicus PA, LMD-9 and LGG was not affected after exposure to pH 4 during 2 h (data not shown). At pH 3 (Fig. 3a), the PA strain was also more resistant than other S. macedonicus strains with a loss of viability lower than 1 log and the same results were obtained for LMD-9 strain, whereas no susceptibility of LGG strain was observed. The three other S. macedonicus strains P2, LK13 and LS5 were more sensitive to an exposure at this pH with a survival decrease higher than 1.5 log. Moreover, no S. macedonicus strain was detected after an exposure during 1 h at pH 2, contrariwise to LMD-9 and LGG strains which were more resistant (data not shown).

Survival to bile salts stress

The four S. macedonicus strains were more resistant than LGG and LMD-9 strains. Furthermore, no viable bacteria were detected at 10 mM and 4 mM of BSM for LGG and LMD-9 strain, respectively.

At the concentration of 6 mM BSM (Fig. 3b), the four S. macedonicus strains underwent a significant loss of 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 sensitive strain P2. Furthermore, except the P2 strain, the three S. macedonicus conserved viability after 2 h exposure to 10 mM BSM (Fig. 3b) and no significant effect was detected at 4 mM BSM (data not shown).

Bacterial adhesion to IEC

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

Strains PA, P2 and LK13 of S. macedonicus studied have an adhesion capacity to Caco2-TC7 cells similar to

that LGG strain (Fig. 4a). LS5 strain has a lower adhesion capacity than the other S. macedonicus strains. These values were even higher than those of LMD-9 strain.

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

280 Discussion

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

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

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

We chose pH values close to the physiological conditions of the GIT varying from 1 to 2 when the stomach is
empty and up to 4 - 5 during meals (Chen et al. 2009). Our results indicate that *S. macedonicus* strains tolerate
acidity up to pH value of 3, with a progressive reduction in viability between pH 4 and 3 depending on the
strains (data not shown). According to Ripamonti et al. (2011), a strong reduction in the viability of fecal origin
strains *S. macedonicus* SB230 and SB330 was observed after exposure to pH 3 (Table 6). Furthermore, although *S. macedonicus* ACA-DC 198 strain does not survive after 1 hour exposure to pH 2.5 (Zoumpopoulou et al.

(Maragkoudakis et al. 2009). Therefore, the S. macedonicus strains should be resistant enough to cross the GIT 311 in a food matrix context. 312 The majority of survival studies to bile salts used the extract of bile whose exact composition is not well-known. 313 We preferred to use a bile salts mixture containing conjugate and free bile salts according to the study of Kebouchi et al. (2016). The physiological bile salt concentrations vary from more than 40 mM to less than 1 mM (Ruiz et al. 2013) with a concentration of 10 mM in small intestine (Bajor et al. 2010), which justifies the concentrations adopted in this study. Our strains, except strain P2, seem to well tolerate high concentrations of BSM, up to 10 mM. According to the literature, the behavior of bacteria towards bile salts is strain-dependent. Indeed, S. macedonicus strains ACA-DC 198, LC743, SB230, SB330 and ACA-DC 208 resist quite well to bile salts (Cho et al. 2010; Ripamonti et al. 2011; Zoumpopoulou et al. 2008,2017). However, the ten strains of S. macedonicus tested by Blaiotta et al. (2013) display very different levels of resistance to bile salts (from 1% to 100% of survival). LAB can counteract bile salts by expressing a bile salt hydrolase (BSH). Although, the bsh gene was shown in silico to be a pseudogene in the ACA-DC 198 genome (Papadimitriou et al. 2014), partial enzyme activity of BSH type was observed in vitro for this strain (Zoumpopoulou et al. 2008). In fact, bile salt resistance is not always correlated with BSH activity, as reported for Lactobacillus salivarius (Fang et al. 2009). Other mechanisms, unknown to date in S. macedonicus, are developed by bacteria to respond to bile stress, such as cell wall composition, induction of stress protein or production of exopolymers. Concerning our four S. macedonicus strains, they are more tolerant to BSM than LGG (used as a positive control in several studies) or S. 328 thermophilus LMD-9 and their integration in a food matrix could certainly allow them to cross the GIT. 329 The in vitro adhesion ability of the four S. macedonicus strains to human intestinal epithelial cell lines Caco-2

2008), it has been isolated from mouse stools at a concentration close to that of the oral administration

TC7 and HT29-MTX was investigated. Caco-2 cells are able to differentiate spontaneously in vitro culture 331 forming a cell monolayer expressing apical and basolateral proteins as enterocytes of the human small intestine 332 whereas HT29-MTX cells differentiate into polarized goblet cells and secrete massive amounts of mucus mainly 333 composed with MUC5AC mucins (stomach origin) (Kebouchi et al. 2016; Lesuffleur et al. 1990) and Pinto et al. 334 1983). To our knowledge, there are few studies on the adhesion capacity of S. macedonicus strain to intestinal 335 epithelial cells. Our results revealed that S. macedonicus strains, except one, adhere at the same level as LGG to 336 Caco-2 TC7 cells. Regarding HT29-MTX, PA strain was characterized by its moderate adhesion capacity 337 compared to LGG strain. (Boleij et al. 2011) reported that S. macedonicus CIP105865T (SM) strain adheres 338 moderately to Caco-2 and HT29 cells, however a weak adhesion to the same cells was observed for S.

macedonicus strains tested by Zoumpopoulou et al. (2017). The adhesion of bacteria to epithelial cells are generally related to the bacterial cell surface structures, among which pili and other surface proteins (Boleij et al. 2009; Jans et al. 2015). In S. gallolyticus (phylogenetically close to S. macedonicus), pil3 contributes to the adhesion to HT29-MTX mucus since it binds specifically to mucin MUC5AC (Martins et al. 2015). The analysis of the genome of S. macedonicus ACA-DC 198 revealed the presence of the operon pil3 (Papadimitriou et al. 2014) which seems to have been preserved in S. macedonicus along evolution. An heterogeneity in the level of pili encoding-gene expression in different strains may explain the affinity of strain PA for HT29-MTX cells. To conclude, we isolated four new S. macedonicus strains from traditional Algerian fermented milks, differentiable on the genetic level from the Greek strain ACA-DC 198 (whole genome available on databases). These strains have a sufficient survival capacity to gastric acidity and bile salts in the context of a food bowl. Their in vitro adhesion ability to Caco-2 TC7 is significantly higher than the one of LMD-9 strain. A moderate adhesion ability to HT29-MTX is obtained with one particular strain (PA). This first characterization encourages the exploration of their probiotic effects in animal models. **Conflicts of interest** The authors declare that they have no competing interests. **Ethics statement** This article does not contain any studies with human participants or animals performed by any of the authors.

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Tables

glcK-for glcK-rev ddlA-for ddlA-rev pepO-for pepO-rev ilvC-for ilvC-rev thrS-for :hrS-rev :kt-for	GGGATTGACCTTGGTGGGAC AAGACTTGCTGCACCGATGA CTGAGAGTGTCATGCGTGCG ATGGCTTTCGCGTTTTTCAA CCACATTGGGTCTCCTGCTT GGCGCCAAATGCAAGAAGAA TGGTTATGGCTCACAAGGTCA TGAATGCGTCGTCATCGTTT TGGACCGTAAAAGGCTGCTT	<i>Glucose kinase</i> D-alanine D-alanine ligase Endopeptidase Ketol acid-reductoisomerase
ddlA-for ddlA-rev pepO-for pepO-rev ilvC-for ilvC-rev thrS-for thrS-rev :kt-for	CTGAGAGTGTCATGCGTGCG ATGGCTTTCGCGTTTTTCAA CCACATTGGGTCTCCTGCTT GGCGCCAAATGCAAGAAGAA TGGTTATGGCTCACAAGGTCA TGAATGCGTCGTCATCGTTT TGGACCGTAAAAGGCTGCTT	D-alanine D-alanine ligase Endopeptidase Ketol acid-reductoisomerase
pepO-for pepO-rev ilvC-for ilvC-rev thrS-for thrS-rev :kt-for	CCACATTGGGTCTCCTGCTT GGCGCCAAATGCAAGAAGAA TGGTTATGGCTCACAAGGTCA TGAATGCGTCGTCATCGTTT TGGACCGTAAAAGGCTGCTT	Endopeptidase Ketol acid-reductoisomerase
pepO-rev ilvC-for ilvC-rev thrS-for thrS-rev :kt-for	TGGTTATGGCTCACAAGAAGAA TGAATGCGTCGTCACAAGGTCA TGAATGCGTCGTCATCGTTT TGGACCGTAAAAGGCTGCTT	Ketol acid-reductoisomerase
thrS-for thrS-rev thrS-rev	TGAATGCGTCGTCATCGTTT TGGACCGTAAAAGGCTGCTT	
tkt-for	CCTGACCACGAAGATGCACT	Threonyl-tRNA synthetase
tkt-rev	CAGGACATGGTTCTGCCCTT CGTGAGTTGGTCCGTCTTCA	Transketolase
pyrE-for pyrE-rev	TCACAAATTGCATCAGATTTGCT TCACGCCAAGTTTCTTGATTTTCT	Orotate phosphoribosyltransferas
dnaE-for dnaE-rev	GTCAGACCTCTATCCACGCG CGCAAATAGACTCCCGAGCT	DNA polymerase III
serB-for serB-rev	TGGTAAGGCGCTTCTTTTCG TGGATGTGGACTCTACCCTCA	Phosphoserine phosphatase
dpr-for dpr-rev	CCAAGCGGTTGCTGATTTGTC ATTTGATTCCTGGTGCTTGTCC	Peroxide resistance protein
gmk-for gmk-rev	CCGTTTCTATGACAACACGCC CGTCCGATAACACGATCAACA	Guanylate kinase
rpoD-for rpoD-rev	CGGACAAAGAAGGAAATCCATC GTTTCAACCATGTGAACAGGA	RNA polymerase sigma factor
parC-for parC-rev	TGGCTTGAAGCCTGTTCAAC TCAATGAGGTAAACAACCGC	DNA topoisomerase IV subunit A
pta-for pta-rev	TGTTTGCTGACTGTGCGATT CCATCAATAGCTTGAGCAGCT	Phosphate acetyltransferase
pyrC-for pyrC-rev	GGCTTTGATCAGGTTGCAGA GGTCTTCTTCGTGAACGGCAA	Dihydrotase
recN-for recN-rev	AGGGAAATTCAATCGTGATGGC TCTTCAAAAGTTCGCGAGCTTG	DNA repair protein
	InaE-for InaE-rev ierB-for ierB-rev Ipr-for Ipr-rev gmk-for gmk-rev poD-for poD-rev barC-for barC-rev bta-for ota-rev byrC-for pyrC-rev recN-for recN-rev	InaE-forGTCAGACCTCTATCCACGCGInaE-revCGCAAATAGACTCCCGAGCTierB-forTGGTAAGGCGCTTCTTTCGierB-revTGGATGTGGACTCTACCCTCAlpr-forCCAAGCGGTTGCTGATTTGTCipr-revATTTGATTCCTGGTGCTTGTCCgmk-forCCGTTTCTATGACAACACGCCgmk-revCGTCCGATAACACGATCAACApoD-forCGGACAAAGAAGGAAATCCATCpoD-forCGGCTTGAAGCCTGTTCAACpoD-forTGGCTTGAAGCCTGTTCAACparC-forTGGCTTGAAGCTGTGCGATTota-revCCATCAATAGCTTGAGCAGCAota-revCCATCAATAGCTTGAGCAGCToyrC-forGGCTTTGATCAGGTTGCAGAoyrC-forAGGGAAATTCAATCGTGATGGCrecN-forAGGGAAATTCAATCGCGAGCTTG

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

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

No.	Isolate	Fermentee	d milk	Orig	in	RI	NAr 16S		ITS	16S-23S		
1 2 3	LK13 LS5 PA	Lben (Lbl Lben (Lbs Rayeb (Rl	K1) S) p1FP)	Milk Milk Farm	seller 7 sell. 11 19	S.	macedor	nicus	S. n	nacedonic	us	
4	P2	Rayeb (Rl	o2FP)	Farm	19							
5	LF9	Lben (LbI	Fb2)	Milk	sell. 3				S. th	hermophil	us ITS I	
6	LA14	Lben (Lb)	A)	Milk	sell. 12	S.	thermop	hilus	S. th	hermophil	us ITS I	[
ITS type	es were att	ributed acco	rding to (N	Aora et a	al. 2003)).						
Table 4	. Genetic d	liversity at S	. macedon	<i>icus</i> loc	i							
Locus	Leng	th of	No. of v	ariable	No	o. of	Maxin	nal % di	ivergenc	e within	-	
	seque	ence (bp)	sites		alle	eles	S. mac	edonicı	us		-	
pyrC		477		7		3			1.5			
ilvC		776		3		3			0.4			
pta		466		2		3			0.4			
recN		400		3		2			0.7			
ddlA		842		6		2			0.7			
apr		303 415		2		2			0.5			
gmK manC		415		2		2			0.4			
rnoD		402		2 1		2			0.4			
serB		433		1		2			0.2			
serb		490		1		2			0.2		_	
Table 5	. MLST da	ata of the 4 S	. macedon	<i>icus</i> stra	ains isola	ated in t	his study	and the	e ACA-I	DC 198 st	rain	
		1 0010										
(Papadi	mitriou et a	al. 2012)										
(Papadin	Origi	n 2012)					Alle	le no.				
(Papadi n	mitriou et a Origi	n	pyrC	ilvC	pta	recN	Alle ddlA	le no. dpr	gmK	parC	rpoD	se
(Papadin n	Origi Raye	n b (Rb1FP)	pyrC	ilvC 2	pta 1	<i>recN</i>	Alle ddlA 2	le no. dpr 2	gmK 2	parC 2	rpoD 2	se
(Papadin n	Origi Raye Raye	n b (Rb1FP) b (Rb2FP)	<i>pyrC</i> 1 2	<i>ilvC</i> 2 2	<i>pta</i> 1 2	<i>recN</i> 1 1	Alle ddlA 2 2	le no. <u>dpr</u> 2 2	<i>gmK</i> 2 2	<i>parC</i> 2 2	<i>rpoD</i> 2 2	se
(Papadi:	Origi Origi Raye Raye Lben	n b (Rb1FP) b (Rb2FP) (LbS)	<i>pyrC</i> 1 2 2	<i>ilvC</i> 2 2 1	<i>pta</i> 1 2 2	<i>recN</i> 1 1 2	Alle ddlA 2 2 2 2	le no. <i>dpr</i> 2 2 2 2	<i>gmK</i> 2 2 2	<i>parC</i> 2 2 2	<i>rpoD</i> 2 2 2	SE
(Papadii n	Origi Origi Raye Raye Lben Lben	n b (Rb1FP) b (Rb2FP) (LbS) (LbK1)	<i>pyrC</i> 1 2 2 2	<i>ilvC</i> 2 2 1 2	<i>pta</i> 1 2 2 2	<i>recN</i> 1 1 2 1	Alle ddlA 2 2 2 2 2	le no. <u>dpr</u> 2 2 2 2 2	<i>gmK</i> 2 2 2 2	<i>parC</i> 2 2 2 2	<i>rpoD</i> 2 2 2 1	Se
(Papadi n 3 -DC 198	Origi Origi Raye Raye Lben Lben Kass	n b (Rb1FP) b (Rb2FP) (LbS) (LbK1) eri cheese	<i>pyrC</i> 1 2 2 3	<i>ilvC</i> 2 2 1 2 3	<i>pta</i> 1 2 2 3	<i>recN</i> 1 1 2 1 2	Alle ddlA 2 2 2 2 1	le no. <u>dpr</u> 2 2 2 2 1	<i>gmK</i> 2 2 2 2 1	<i>parC</i> 2 2 2 2 1	<i>rpoD</i> 2 2 2 1 2	Se
(Papadii n 3 -DC 198	mitriou et a Origi Raye Raye Lben Lben Kasse	n b (Rb1FP) b (Rb2FP) (LbS) (LbK1) eri cheese	<i>pyrC</i> 1 2 2 2 3	<i>ilvC</i> 2 2 1 2 3	<i>pta</i> 1 2 2 3	<i>recN</i> 1 1 2 1 2	Alle <u>ddlA</u> 2 2 2 2 1	le no. dpr 2 2 2 2 2 1	<i>gmK</i> 2 2 2 2 1	<i>parC</i> 2 2 2 2 1	<i>rpoD</i> 2 2 2 1 2	Se

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

587 macedonicus

Source	Methods	Results
Acid tolerance		
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 ₃ , 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; on two strains with a survival rate highe 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.
Bile salt tolerance		
Zoumpopoulou et al. (2008)	Strain ACA-DC 198 in PBS solution, pH 8 with 1% or 2% Oxgall, incubation at 37 °C for 1, 2 and 4 h.	Resistante: 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% Oxgall 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% Oxgall 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% Oxgall, incubation at 37°C for 24-48 h.	From 1 to 100% of survival: only tw 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 IB	EC	
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.</i> <i>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 11.10^6 cells/well into DMEM at 37°C with 10% CO ₂ 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).

590 Figure legends

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

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

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

595 gallolyticus strains

596 The tree was based on concatenated sequences of ten housekeeping genes (*pyrC*, *ilvC*, *pta*, *recN*, *ddlA*, *dpr*, *gmk*,

parC, *rpoD* and *serB*). The tree was constructed using the Maximum Likelihood method based on the Tamura-

Nei model (Tamura et al. 1993). The percentage of trees in which the associated taxa clustered together is shown

599 next to the branches. Scale bar = 0.002 nucleotide substitution per site. Evolutionary analyses were conducted in

600 MEGA7

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

The survival at pH 3 was evaluated for 1 and 2 h compared to the negative control (pH 7) and expressed as Log_{10}

504 CFU/mL (a). The survival to 6 mM and 10 mM BSM concentrations was evaluated for 2 h compared to the

negative control (0 mM BSM) and expressed as Log_{10} CFU/mL (b). Means \pm SEM of two independent

experiments conducted in duplicate are presented. Mean values with different letters are significantly different at

p < 0.05

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

41 610 The data were expressed as the number of adherent bacteria (CFU/mL) per Caco-2 TC7 (a) and HT29-MTX

43 611 cells (b). Means ± SEM of two independent experiments conducted in duplicate are presented. Mean values with

45 612 different letters are significantly different at p < 0.05





gallolyticus strains



Fig. 3 Survival of S. macedonicus, S. thermophilus LMD-9 and L. rhamnosus GG (LGG) strains after exposure





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)