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# *In vitro* and *in vivo* safety evaluation of the bacteriocin producer *Streptococcus macedonicus* ACA-DC 198

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ABSTRACT

*Streptococcus macedonicus* ACA-DC 198, a bacteriocin producer isolated from Greek Kasseri cheese, was used in a series of *in vitro* and *in vivo* experiments in order to evaluate its pathogenic potential. The strain was examined *in vitro* for haemolytic activity, antibiotic resistance and presence of pathogenicity genes encountered in *Streptococcus pyogenes*. Subsequently, the strain was orally administered to mice (8.9 log cfu daily), continuously over a period of 12 weeks, in order to ascertain the effects of its long term consumption on animal health and gastric inflammation. *S. macedonicus* ACA-DC 198 was found to be non-haemolytic and sensitive to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, streptomycin, tetracycline, and vancomycin, with the only resistance observed against kanamycin. PCR amplification and DNA–DNA hybridization did not reveal the presence of any of the *S. pyogenes* pathogenicity genes examined, namely *emm*, *scpA*, *hasA*, *speB*, *smez2*, *speJ*, *sagAB*, *hylA*, *ska*, *speF*, *speG*, *slo*, *hylP2* and *mga*. In the mouse study, no detrimental effects were observed in the behaviour, general well being, weight gain and water consumption of the animals receiving *S. macedonicus* ACA-DC 198. Histologic analysis showed no evidence of inflammation in the stomach of the animals receiving *S. macedonicus* ACA-DC 198, while faecal microbiological analysis revealed that the strain retained its viability passing through the mouse gastrointestinal tract. Finally, no evidence of translocation to the liver, spleen and mesenteric lymph nodes was observed. In conclusion, none of the examined virulence determinants were detected in *S. macedonicus* ACA-DC 198 and its long term, high dosage oral administration did not appear to induce any pathogenic effect in mice.



## 1. Introduction

Lactic acid bacteria (LAB) constitute the global majority of commercial starter cultures, in both volume and value, with the largest part being applied in the dairy industry (Hansen, 2002). *Streptococcus thermophilus*, in particular, is one of the main industrial starter cultures used in the dairy sector, with over $10^{21}$ live cells annually consumed (Bolotin et al., 2004). *S. thermophilus* has been granted a "Generally Recognised As Safe" (GRAS) status by the Food and Drug Administration (FDA) in the U.S.A. and is included within the list of safe organisms of the "Qualified Presumption of Safety" (QPS) system of the European Food Safety Authority (EFSA, 2004), even though it belongs to the genus *Streptococcus* that includes important human pathogens like *Streptococcus pyogenes* and *Streptococcus pneumoniae*.

Apart from *S. thermophilus*, another dairy *Streptococcus* has been described, *Streptococcus macedonicus*, isolated from Kasseri, a traditional Greek semi-hard protected designation of origin (PDO) cheese (Tsakalidou et al., 1998). Since its first isolation and characterization, *S. macedonicus* has been encountered in all cheese varieties (soft, semi-hard and hard), in both commercial and artisanal products. The particular species is common in South European countries like Greece (Georgalaki et al., 2009), Italy (Lombardi et al., 2004; Pacini et al., 2006; Aponte et al., 2008) and France (Callon et al., 2004), where the per capita cheese consumption is the highest in the world. In addition, *S. macedonicus* has also been detected in Pozol, a Mexican fermented maize beverage (Diaz-Ruiz et al., 2003).

Several studies so far have demonstrated that *S. macedonicus* possesses multifunctional properties that can be exploited in the food industry. Peptidase activity (Georgalaki et al., 2000; Lombardi et al., 2004) and production of exopolysaccharides (Vincent et al., 2001) are included in the interesting technological properties of *S. macedonicus*.

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In particular, *S. macedonicus* ACA-DC 198 produces the lantibiotic macedocin, which apart from inhibiting pathogenic streptococci and *Clostridium perfringens* (Zoumpopoulou et al., 2008; Maragkoudakis et al., 2009), has been found active also against food spoilage bacteria such as *Clostridium tyrobutyricum* and *Brochothrix* sp. (Georgalaki et al., 2002; Maragkoudakis et al., 2009). In addition, *S. macedonicus* ACA-DC 198 induced specific pro-inflammatory cytokine responses in human peripheral blood cells (Zoumpopoulou et al., 2008). Finally, in the field of dairy technology, the strain has been successfully used as a cheese adjunct starter culture (Anastasiou et al., 2007) and as a cheese protective culture against *C. tyrobutyricum* (Anastasiou et al., 2009).

The potential application of *S. macedonicus* in the food industry, however, requires the adequate demonstration of its safety. As already mentioned, *S. macedonicus* is regularly consumed, since it belongs to the natural microflora of cheese, indicating a possible GRAS/QPS status of the species. However, it has been classified in the *Streptococcus bovis* group (Tsakalidou et al., 1998), which includes mainly zoonotic species that can also cause opportunistic infection in humans (Segura and Gottschalk, 2004). So far, only preliminary, *in vitro* results have been published on the safety of *S. macedonicus*, dealing with haemolytic activity and antibiotic resistance (Lombardi et al., 2004; Zoumpopoulou et al., 2008).

The aim of this study was to evaluate in more depth important safety parameters of *S. macedonicus* ACA-DC 198. Apart from the standard phenotypic safety parameters, haemolysis and antibiotic resistance, the presence of multiple common streptococcal virulence genes has also been examined. In addition, the effects of a long term oral administration of *S. macedonicus* ACA-DC 198 have been evaluated *in vivo* using a murine model.

## 2. Materials & methods

### 2.1. Bacterial strains and culture conditions

*S. macedonicus* ACA-DC 198, isolated from traditional Greek Kasseri cheese, was obtained from the ACA-DC collection of the Agricultural University of Athens. For routine use the strain was cultured in M17 (Oxoid, UK) broth and agar (1.5% w/v), for 18 and 48 h respectively, at 37 °C. *S. pyogenes* LMG 21599$^T$, isolated from human with scarlet fever, was cultured in BHI (Biokar Diagnostics, France) broth and agar (1.5% w/v), for 18 and 48 h respectively, at 37 °C. *Enterococcus faecalis* ACA-DC 3339 was cultured in M17 (Oxoid) broth, for 18 h at 37 °C. Routine enumeration was carried out using the dilution plate count method. The strains were stored at −80 °C in cryovials with the appropriate broth supplemented with 20% (v/v) glycerol.

### 2.2. Phenotypic safety assessment

*S. macedonicus* was tested for haemolytic activity using Columbia agar (Oxoid) containing 5% (v/v) whole human or sheep blood, after 48 h of microaerobic incubation (37 °C, 10% $CO_2$ in a modified atmosphere incubator), as described previously (Zoumpopoulou et al., 2008). Strain *E. faecalis* ACA-DC 3339 was used as a positive control for β-haemolysis.

The assay for antibiotic resistance was performed in 96-well plates, as described previously (Maragkoudakis et al., 2006), with antibiotic concentrations ranging from 1 to 1024 μg/ml. The following antibiotics were tested: ampicillin, chloramphenicol, ciprofloxacin, erythromycin, kanamycin, streptomycin, tetracycline and vancomycin (Sigma-Aldrich, USA). The growth of the strain was evaluated visually and by optical density (OD at 600 nm) measurements for a growth period of 24 h. The susceptibility to each antibiotic was expressed as the minimum inhibitory concentration (MIC, μg/ml) necessary for growth inhibition. The choice of antibiotics and the characterization of sensitivity/resistance were performed according to the *S. thermophilus* relevant guidelines and breakpoints of the European Scientific Commitee for Animal Nutrition (SCAN, 2002) and the European Food Safety Authority (EFSA, 2005). Strains previously studied with the same method (Maragkoudakis et al., 2006; Zoumpopoulou et al., 2008) were used as controls.

### 2.3. Determination of virulence genes in S. macedonicus ACA-DC 198

For the detection of common streptococcal virulence factor genes in the genome of *S. macedonicus* ACA-DC 198, a PCR approach was applied in conjunction with Southern DNA–DNA hybridization. Specifically, chromosomal DNA was isolated from *S. macedonicus* ACA-DC 198 and *S. pyogenes* LMG 21599$^T$ using the Wizard Genomic DNA purification Kit (Promega, USA), according to the instructions of the manufacturer. The isolated genomic DNA from both species was used as template for PCR with primers designed according the nucleotide sequence of several *S. pyogenes* virulence factors genes, which are listed in Table 1. The design of primers was based on the genome sequence of *S. pyogenes* M1 GAS (GenBank accession number AE004092, Ferretti et al., 2001). In order to identify conserved regions in the virulence genes of the above strain that could be used as PCR primers, the genes were aligned with the respective ones from other streptococci (e.g. *Streptococcus dysgalactiae*, *Streptococcus canis* and *S. pneumoniae*).

Each 50-μl PCR-reaction mixture contained 1 X PCR buffer, 200 μM of each dNTP, 3 mM $MgCl_2$, 0.2 mM of each primer, 1 U of Dynazyme polymerase (Finnzymes, Finland) and 1–2 μl of sample. PCR conditions were optimised on the basis of theoretical calculations of the melting temperatures of the primer pairs used and on the results of several amplification experiments. The conditions used included initial denaturation at 94 °C for 2 min, followed by 30 cycles of melting at 94 °C for 30 s, annealing at various temperatures depending on the primer pair used (Table 1) for 1 min, elongation at 72 °C for 1 min, and a final extension at 72 °C for 5 min. Amplified PCR products were cloned to the PCR 2.1 vector, included in the TA-Cloning Ligation Kit (Invitrogen, USA) and sequenced by the Lark Technologies Inc. (UK).

The PCR products amplified from *S. pyogenes* LMG 21599$^T$ DNA were digoxigenin labeled according to the instructions of the DIG-System manufacturer (Roche Diagnostics, Germany) and then used as probes for Southern hybridizations on *Hind*III-digested chromosomal DNA from the above two strains. The hybridizations were performed at low stringency conditions (40 °C in the presence of 25% v/v formamide) that allowed approximately 30% mismatch for all the probes used, according to calculations performed based on the equations suggested by the DIG-System manufacturer (Roche Diagnostics).

### 2.4. Animals and housing conditions

Specific-pathogen-free (SPF) 6–8 week old inbred male C57BL/6 mice were obtained from the Central Animal Facility of the Hellenic Pasteur Institute (HPI). They were housed in the animal facilities of the HPI according to the relevant Greek national legislation, fed a commercial diet and given water *ad libitum*, except otherwise stated.

### 2.5. Administration of S. macedonicus ACA-DC 198 to mice

Mice were divided into the control ($n = 15$) and the study ($n = 10$) groups. Control group animals received sterile M17 broth in their drinking water for the duration of the study. In the study group, a *S. macedonicus* ACA-DC 198 culture, grown overnight in M17 broth, was mixed daily with the animal drinking water for a period of 12 weeks. Final bacterial population in the animal drinking water was adjusted at 8.0 log cfu/ml, while its viability in water was measured before and during the course of the experiment. Water consumption, weight gain and general health status (hair loss, behavioural changes,

**Table 1**
Primers used and amplicons' size obtained in PCR amplification of streptococcal virulence genes from *S. pyogenes* LMG 21599$^T$ and *S. macedonicus* ACA-DC 198 genome.

| Virulence determinant | Gene | Set of primers | Primer sequence (5′→3′) | $T_{an}$ (°C) | *S. pyogenes* amplicons used as probes (bp) | *S. macedonicus* amplicon size (bp) | Reference |
|---|---|---|---|---|---|---|---|
| M-protein | *emm* | emmF | TATTSGCTTAGAAAATTAA | 50 | 1200 | – | Burton et al. (2006) |
| | | emmR | GCAAGTTCTTCAGCTTGTTT | | | | |
| C5a peptidase | *scpA* | scpAF | CGGGTATCATGGGACTGTTGC | 52 | 1250 | – | Burton et al. (2006) |
| | | scpAR | TTGCCGATGTTGCGACTTC | | | | |
| *Hyaluronan synthase* | *hasA* | hasAF | GTGTGTTAGCACAGACCTATCC | 50 | 720 | – | This study |
| | | hasAR | CTTCGAAAATAGTCCATAAGGC | | | | |
| Pyrogenic exotoxin (Cysteine protease) SpeB | *speB* | speBF | TGACGCTAACGGTAAAGAAAACA | 52 | 800 | – | Burton et al. (2006) |
| | | speBR | GCCGCCACCAGTACCAAGAGC | | | | |
| Pyrogenic exotoxin Smez2 | *smez2* | smez2F | GGACGAATATGCAGCCAATGA | 50 | 330 | – | Burton et al. (2006) |
| | | smez2R | GTATGAAAAACCAGTCTACCAC | | | | |
| Pyrogenic exotoxin SpeJ | *speJ* | speJF | AATAATCTTTCATGGGTACGGAAG | 52 | 500 | – | This study |
| | | speJR | TTGATCGCAATTTCTAATTGCCC | | | | |
| Streptolysin S | *sagAB* | sagAF | ATTGAGCTAGCCTTGTCCTTGT | 52 | 1150 | – | Burton et al. (2006) |
| | | sagAR | GTATTCCGCAAAATCTCTAACG | | | | |
| Hyaluronate lyase (chromosomally encoded) | *hylA* | hylAF | ATGAAATTGGAACTCCTCGTGC | 50 | 500 | – | This study |
| | | hylAR | CGAGTCATATCCATCATTTCTCC | | | | |
| Streptokinase (Ska) | *ska* | skaF | CGATCTAACATCACAACCTGC | 50 | 460 | Non specific (1300) | This study |
| | | skaR | GATGTGATGGTGTCACCGATAGC | | | | |
| Pyrogenic exotoxin SpeF | *speF* | speFF | GCTCTTGTATCAGCCACAATGGC | 50 | 450 | Non specific (900, 1300) | This study |
| | | speFR | GCATCTCCACCGAGACTATCTGC | | | | |
| Pyrogenic exotoxin SpeG | *speG* | speGF | CTATGGAAGTCAATTAGCTTATGC | 50 | 500 | Non specific (850, 1500) | This study |
| | | speGR | CCGATGTATAACGCGATTCCG | | | | |
| Streptolysin O | *slo* | sloF | CTGGGCTACTGACGGCAGC | 50 | 900 | Non specific (700) | This study |
| | | sloR | CGGAGCTTCATTGCTGACACC | | | | |
| Hyaluronate lyase (bacteriophage encoded) | *hylP2* | hylP2F | GCTACGAGTCCAATTTAAGCGG | 50 | 400 | Non specific (300) | This study |
| | | hylP2R | GTATACATCACCATAGCAGCACC | | | | |
| Virulence factors' regulator Mga | *mga* | mgaF | CATGCAATTTATGAAGGAAGTGGG | 50 | 500 | Non specific (500, 1000, 1200) | This study |
| | | mgaR | TGTCGTAGACGGCAGAGTGTCC | | | | |

–: Absence of amplicon, $T_{an}$: annealing temperature.

diarrhoeas) of the animals were monitored for the course of the experiment, while faecal samples were collected at 1, 3, 6 and 12 weeks post administration and examined for presence of viable *S. macedonicus* cells.

### 2.6. Evaluation of S. macedonicus-associated gastritis and tissue translocation

At the selected time points of 0, 6 and 12 weeks animals were sacrificed by cervical dislocation ($t=0$, $n=5$ animals, control group; $t=6$ and 12 weeks, $n=5$ animals per time point and group). The stomach, liver, spleen and mesenteric lymph nodes were aseptically excised for further analysis. Histopathological examination of the associated gastric inflammation in gastric tissues was performed as previously described (Sgouras et al., 2004). Polymorphonuclear (PMN) and lymphocyte infiltration in the lamina propria was evaluated according to the updated Sydney system (Dixon et al., 1996). The spleen, liver and mesenteric lymph nodes were examined for presence of viable *S. macedonicus* cells, as described below.

### 2.7. Detection of S. macedonicus ACA-DC 198 in faecal and tissue samples

All faecal, spleen, liver and mesenteric tissue samples collected were weighted and homogenized in Maximum Recovery Diluent (MRD, Oxoid). The samples were then diluted in MRD using the standard serial decimal dilution method and were subsequently plated on the SM chromogenic medium (Pacini et al., 2006), selective for *S. macedonicus*. Following aerobic incubation for 48 h at 37 °C, colonies with morphology and colour resembling *S. macedonicus* were examined microscopically and collected for a rapid lysis colony-PCR

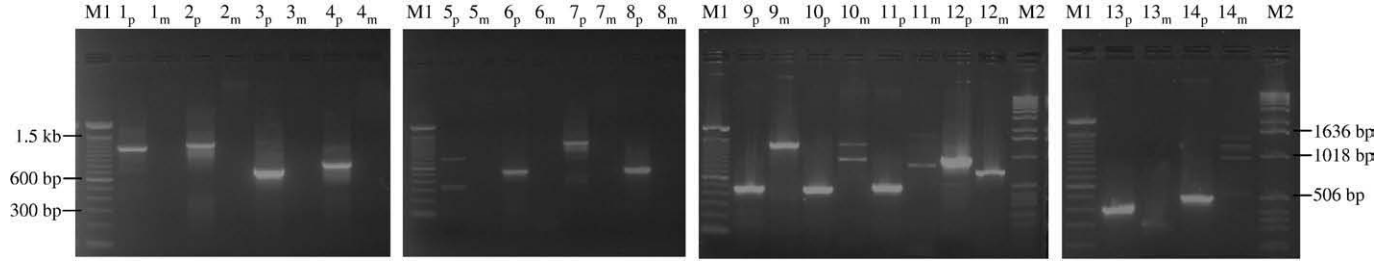


**Fig. 1.** PCR products amplified from *S. macedonicus* ACA-DC 198 (lanes numbered with the subscript $_m$) and *S. pyogenes* LMG 21599$^T$ (lanes numbered with the subscript $_p$) genome using specific primers for the virulence genes *emm* (lanes $1_p$, $1_m$), *scpA* (lanes $2_p$, $2_m$), *hasA* (lanes $3_p$, $3_m$), *speB* (lanes $4_p$, $4_m$), *smez2* (lanes $5_p$, $5_m$), *speJ* (lanes $6_p$, $6_m$), *sagAB* (lanes $7_p$, $7_m$), *hylA* (lanes $8_p$, $8_m$), *ska* (lanes $9_p$, $9_m$), *speF* (lanes $10_p$, $10_m$), *speG* (lanes $11_p$, $11_m$), *slo* (lanes $12_p$, $12_m$), *hylP2* (lanes $13_p$, $13_m$), and *mga* (lanes $14_p$, $14_m$). Lanes M1 and M2, 100 bp and 1 kb DNA ladder respectively (Invitrogen).

method (Veyrat et al., 1999). The *S. macedonicus* species-specific PCR was carried out using the primers 16MAC and BSR534/18, as previously described (Papadelli et al., 2003).

### 2.8. Statistical analysis

The mice water consumption, weight gain, as well as the faecal and tissue bacteria populations were compared using the ANOVA multiple sample comparison. Statistical evaluation of chronic inflammatory infiltration and activity of chronic gastritis was conducted by the Wilcoxon rank sum test. All statistical analyses were performed with the StatGraphics Centurion XV software (StatPoint Inc.).

## 3. Results

### 3.1. Phenotypic safety assessment

No haemolytic activity was recorded when *S. macedonicus* ACA-DC 198 was grown on blood agar plates. According to the MIC values obtained and the breakpoints (BP) used, the strain was found sensitive to ampicillin (≤1 μg/ml, BP 4), chloramphenicol (4 μg/ml, BP 8), erythromycin (≤1 μg/ml BP 4), streptomycin (4–8 μg/ml, BP 16), tetracycline (≤1 μg/ml, BP 4), and vancomycin (≤1 μg/ml, BP 4). The only antibiotic to which resistance was observed was kanamycin (128–256 μg/ml, BP 8). No breakpoints have been suggested for *S. thermophilus* and ciprofloxacin, but the MIC value obtained (2 μg/ml) is low and under the limit suggested for lactobacilli, pediococci and enterococci (BP 4, 4 and 16, respectively).

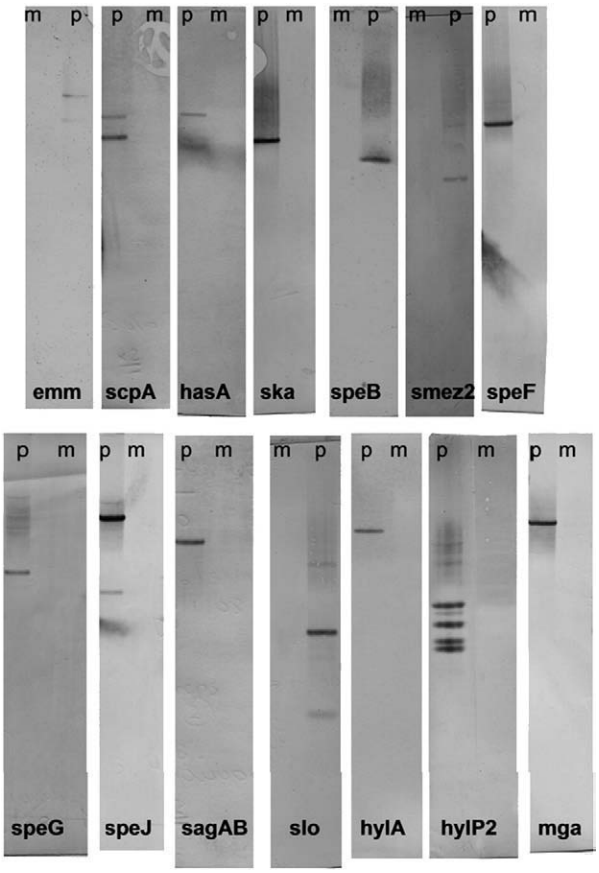


**Fig. 2.** Southern blots hybridized with amplicons of the streptococcal virulence genes listed in Table 1. p: lanes with *S. pyogenes* LMG 21599$^T$ *Hind*III-digested DNA, m: lanes with *S. macedonicus* ACA-DC 198 *Hind*III-digested DNA.

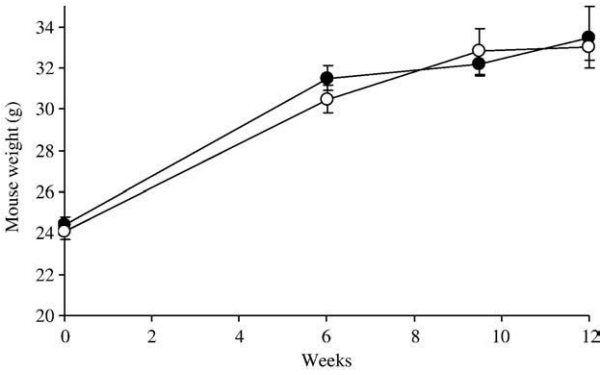


**Fig. 3.** Mean mice weight gain (±SD) throughout the course of the study in the control group (o, $N=15$), receiving sterile broth, and the study group (●, $N=10$), receiving the *S. macedonicus* ACA-DC 198 culture.

### 3.2. Determination of virulence determinant genes in *S. macedonicus* ACA-DC 198

The application of PCR with primers specific for 14 common *S. pyogenes* virulence genes led to the amplification of products of the expected size from the genome of *S. pyogenes* LMG 21599$^T$ (Fig. 1, Table 1). The correspondence of these products to the respective virulence genes was confirmed by sequence analysis. In the case that additional products of different size were amplified (e.g. with *smez2* or *sagAB* primers, Fig. 1) the nucleotide sequence was confirmed only for the product with the expected size, before its use as a probe.

As far as the genome of *S. macedonicus* ACA-DC 198 is concerned, 8 out of 14 sets of primers used did not amplify any product (Fig. 1, Table 1). In the cases that amplification occurred (6 out of 14 sets of primers), the amplicon size, except in one case (*mga*), did not correlate with the size of products amplified from *S. pyogenes* genome. Furthermore, sequence analysis of all amplified products confirmed that they were non-specific, revealing no homology with any virulence genes (data not shown). Therefore, none of the selected virulence determinant genes were detected in *S. macedonicus* ACA-DC 198 by PCR.

The use of amplicons obtained from *S. pyogenes* LMG 21599$^T$ as probes to detect possible respective virulence genes in the genome of *S. macedonicus* ACA-DC 198 did not result in any hybridization signal for any of the genes tested (Fig. 2). The low stringency conditions of hybridizations applied allowed approximately 30% mismatch for all probes. Therefore, the negative result of Southern hybridizations indicated that there is no gene in the genome of *S. macedonicus* ACA-DC 198 with more than about 70% homology to the 14 streptococcal virulence genes tested in this study.

**Table 2**
Chronic inflammatory infiltration and activity of reactive gastritis in the antrum of control and study group mice.

| Weeks | Group | Chronic inflammatory infiltration[a] | | | | Activity of reactive gastritis[b] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Number of mice[c] in each grade[d] | | | | | | | |
| | | 0 | 1 | 2 | 3 | 0 | 1 | 2 | 3 |
| 0 | Control | 5 | 0 | 0 | 0 | 5 | 0 | 0 | 0 |
| 6 | Control | 5 | 0 | 0 | 0 | 5 | 0 | 0 | 0 |
| | Study | 3 | 2 | 0 | 0 | 5 | 0 | 0 | 0 |
| 12 | Control | 3 | 2 | 0 | 0 | 3 | 2 | 0 | 0 |
| | Study | 1 | 4 | 0 | 0 | 3 | 2 | 0 | 0 |

[a] Lymphocyte infiltration in antrum.
[b] Polymorphonuclear infiltration in antrum.
[c] Five mice examined at each time point for each study group.
[d] Histopathology grades assigned according to the updated Sydney system, as follows: normal, 0; mild, 1; moderate, 2; marked, 3.

**Table 3**
Microbiological analysis of tissues excised from mice of the control and study groups.

| Tissue | Control group | | | Study group | | | |
|---|---|---|---|---|---|---|---|
| | Mice[a] | | Microbial load[b] (log cfu/g) | Mice[a] | | | Microbial load[b] (log cfu/g) |
| | 6 weeks | 12 weeks | | 0 weeks | 6 weeks | 12 weeks | |
| Mesenteric lymph nodes | 2/5 | 3/5 | 2.7 ± 0.17 | 2/5 | 3/5 | 2/5 | 2.5 ± 0.13 |
| Liver | 2/5 | 2/5 | 2.7 ± 0.42 | 1/5 | 3/5 | 1/5 | 2.6 ± 0.35 |
| Spleen | 2/5 | 1/5 | 2.3 ± 0.21 | 2/5 | 2/5 | 2/5 | 2.2 ± 0.11 |

[a] Number of mice where microbial growth was recovered from tissues at 6 and 12 weeks respectively.
[b] Mean microbial load (±SD) recovered from the examined tissues throughout the study.

### 3.3. Administration of *S. macedonicus* ACA-DC 198 to mice

*S. macedonicus* ACA-DC 198 survived well in the drinking water of the mice (<1.0 log cfu/ml reduction in 48 h). The mean daily water consumption per animal for the control and study groups was calculated at 7.9 ± 1.2 and 7.7 ± 2.9 ml [mean ± standard deviation, (SD)] respectively, while the mean *S. macedonicus* daily uptake per mouse was calculated at approximately 8.9 log cfu. No animal deaths, abnormal physiology or unexpected behavioral effects, such as movement impediment or aggressiveness, were observed macroscopically in any of the animal groups during the course of the study. Similarly, no differences in animal weight (Fig. 3) were recorded between the two groups over the whole experimental period.

### 3.4. Evaluation of *S. macedonicus*-associated gastritis

Lymphocytic as well as polymorphonuclear cell infiltration in the gastric mucosa, as a result of *S. macedonicus* administration, is depicted in Table 2. Specifically, at 6 weeks, there was a mild lymphocytic infiltration (score 1) in the antrum of two of the animals in the study group compared to the control group. At 12 weeks, 4 animals were found to develop mild gastritis in the study group, compared to two in the control group ($P>0.05$, Wilcoxon rank sum test). No polymorphonuclear cell infiltration was observed in the antrum of any of the animals in the study or control group at 6 weeks. However, mild infiltration was observed in only two animals in both groups after 12 weeks. Histology of all fundus samples appeared normal and without evidence of reactive chronic gastritis. Similarly, no atrophy or intestinal metaplasia was observed in the animals as well as complete absence of lymphoid follicle formation.

### 3.5. Detection of *S. macedonicus* ACA-DC 198 in faecal and tissue samples

*S. macedonicus*-like colonies were successfully isolated on the selective SM agar from faecal samples of the study group in population levels of 7.5 ± 0.2 log cfu/g (mean ± SD) and their identity was confirmed by species-specific PCR, as described above. Colony morphology resembling that of *S. macedonicus* was not encountered in the faecal flora of the control group, while PCR examination of the recovered colonies of the control group yielded negative results.

Concerning the tissue samples, bacterial colonies were recovered in a number of samples in both study and control group animals (Table 3). All colony types were examined with PCR and none of them was found to be *S. macedonicus*. The sole exception was observed on a mesenteric lymph node sample in one out of the five mice sacrificed from the study group ($t=3$ weeks), in which about half of the colonies (2.2 log cfu/g) from the total recovered flora (2.5 log cfu/g) were confirmed as *S. macedonicus* using PCR. However, the bacterium was not detected in the spleen or liver samples of the same animal, or, as mentioned above, in any other tissue samples of the study or control groups throughout the course of the experiment.

## 4. Discussion

This is the first reported study on the safety evaluation, based on a phenotypic, genotypic and *in vivo* investigation, of *S. macedonicus*, a dairy streptococcal species with potential probiotic and protective applications.

The lack of haemolytic activity is among the *in vitro* criteria proposed by FAO/WHO (2002) for the evaluation of potential probiotic bacteria. Lactic acid bacteria in general are usually non-haemolytic. Although the *Streptococcus* genus in particular includes strongly haemolytic and pathogenic species, such as *S. pneumoniae* and *S. pyogenes*, *S. macedonicus* ACA-DC 198 was not found to possess any haemolytic activity.

The absence of transmissible antibiotic resistance is considered a key safety prerequisite for both FAO/WHO (2002) probiotic selection guidelines and the EFSA QPS system for food and feed additives (EFSA, 2004). In this study *S. macedonicus* ACA-DC 198 was found sensitive to all the antibiotics tested, with the exception of kanamycin, using the EFSA breakpoints suggested for *S. thermophilus*, which is considered by the GRAS and QPS systems as safe (EFSA, 2008). Various antibiotic resistance profiles for LAB have been reported so far in the literature (Temmerman et al., 2002; Danielsen and Wind, 2003; Mathur and Singh, 2005), that are, however, subject to the method and media variation. Although species variation plays an important role in LAB antibiotic resistance, a general sensitivity to penicillin G, ampicillin, tetracycline, erythromycin and chloramphenicol, and an intrinsic resistance to aminoglycosides, quinolones and glycopeptides has been observed (Hummel et al., 2007).

As far as the genotypic investigation is concerned, this is the first systematic study for the presence of genes associated with virulence in the genome of *S. macedonicus* ACA-DC 198. The use of PCR and/or hybridization method for virulence factors detection has been widely applied not only for streptococci (Podbielski et al., 1991; Dewinter et al., 1999) but also for other bacterial species (Thoerner et al., 2003; Gerrish et al., 2007). In the present work, the application of PCR and Southern hybridization indicated that *S. macedonicus* ACA-DC 198 lacks genes homologous to *S. pyogenes* virulence determinants. The virulence factors genes examined include known genes for major virulence factors of Group A streptococci, which have been recently reviewed (Hynes, 2004), while some of them are also regarded as virulence factors for Group B streptococci (Liu and Nizet, 2004), for streptococci associated with animal diseases (Segura and Gottschalk, 2004), as well as for the pathogen *S. pneumoniae* (Jedrzejas, 2004). The set of primers used managed to amplify the respective virulence genes from the genome of *S. pyogenes* LMG 21599$^T$, while no virulence gene relative product was amplified from the genome of *S. macedonicus* ACA-DC 198. The results of this rapid PCR-detection method were confirmed by the Southern hybridization approach, which demonstrated that there is no gene in the genome of the above bacterium with more than about 70% homology to the streptococcal virulence genes tested. The positive hybridization resulted from the genome of *S. pyogenes* LMG 21599$^T$ verified that the probes detected the relative virulence associated area in genomic DNA of the pathogenic strain. Similar findings were recently reported by Burton et al. (2006) who applied PCR in conjunction to hybridization for the safety assessment of another streptococcal strain of probiotic interest, namely *Streptococcus salivarius* K12.

The present data does not rule out the possible presence of similar proteins with low (<70%) nucleotide homology. The whole genome sequencing or phenotypic screening for each gene product is required in order to conclude with certainty that these virulence factors are absent or inactive in *S. macedonicus*. *S. thermophilus* genome analysis has demonstrated that the most streptococcal virulence-related genes are either inactivated or absent unless they code for proteins performing basic cellular functions (Bolotin et al., 2004). The absence of a complete genome sequence of *S. macedonicus* does not allow a

complete virulence assessment. Nevertheless, the lack of genes with significant nucleotide homology to streptococcal virulence determinants provides evidence for the non-pathogenic status of *S. macedonicus*.

Apart from the necessary *in vitro* safety examinations, the safety of a strain intended for probiotic use must be also demonstrated *in vivo* (FAO/WHO, 2002). Oral toxicity studies have already been utilised for the evaluation of the safety of various promising strains. In most studies live bacteria are administered at high doses (8.0–11.0 log cfu/animal daily), while the duration of the administration ranges from short or few-days trials (Shu et al., 1999; Zhou et al., 2000a; Lara-Villoslada et al., 2007a; Fernández et al., 2005), to longer periods 1 month or more (Zhou et al., 2000b; Bernardeau et al., 2002; Huang et al., 2003; Park et al., 2005; Lara-Villoslada, 2007b). Food and water intake as well as body weight gain and macroscopic observation of the animals were the general health status indicators monitored, with no detrimental effects being reported due to LAB administration. In the present work, the long (12 weeks), continuous oral administration of high doses (~9.0 log cfu/mouse/day) of *S. macedonicus* ACA-DC 198 did not result in the death of any of the mice in the study group. In addition, macroscopic observation did not reveal any detrimental effects in the study group, such as diarrhoea, hair loss, movement impediment or abnormal behaviour. Moreover, no statistical significant differences were observed between the mean water consumption and animal weight of the study and control groups, at 0, 3, 6 and 12 weeks of study.

Histological examination of the excised stomach samples showed no significant difference in the *S. macedonicus*-associated chronic active inflammatory infiltration compared to the control animal group. Furthermore, histopathology did not reveal any formation of lymphoid follicles, glandular atrophy or intestinal metaplasia in any of the animals. Consequently, no significant gastric inflammation was found to be associated with the administration of *S. macedonicus* ACA-DC 198.

Bacterial translocation is the passage of bacteria from the intestinal tract to extra-intestinal sites and organs and is considered as the first step in the developing bacterial infections from intestinal pathogens (Liong, 2008). Therefore, it constitutes an important safety parameter for candidate probiotics that has been examined so far in the literature. Shu et al. (1999) and Zhou et al. (2000a, b) investigated the effect of administration of probiotic *Lactobacillus rhamnosus*, *Lactobacillus acidophilus* and *Bifidobacterium lactis* strains in mice. Although in both cases bacteria were recovered from the animal tissues examined, none of the isolated colonies belonged to the administered cultures, and differences in the recovered microbial populations of the control and study groups were not significant. Similar findings were reported by Park et al. (2005) and Lara-Villoslada et al. (2007a,b). On the other hand, Fernández et al. (2005), apart from not finding the administered organism, reported that all tissue samples examined were free of any microbial population. In the present work, low-level bacterial loads were recovered from the animal tissues, with no statistical difference ($P>0.05$) between the populations found in the control and study groups. The administered *S. macedonicus* ACA-DC 198 strain was found only in a mesenteric lymph node sample of one out of the ten animals of the study group. Taking into account the absence of the microorganism in other tissue samples and animals, the isolation of the strain could be a result of contamination, probably due to a rupture of the small intestine during the excision of the mesenteric lymph node tissue sample. Therefore, according to our findings, the orally administered *S. macedonicus* ACA-DC 198 strain does not appear to possess the potential to translocate to the examined tissues.

*S. macedonicus* ACA-DC 198 was steadily recovered from the faeces of the animals of the study groups in numbers of 7.5 log cfu/g, approximately 1.0–1.5 log lower than the administered dose, indicating a successful passage through the mouse GI tract. This finding confirms that viable bacteria cells in relatively high populations did in fact reach the small intestine but did not translocate. Since the survival of lactic acid bacteria in the low-pH and proteolytic stomach environment of the host organism is by no means guaranteed and so confirming the bacterial presence in faeces is crucial in order to prove that bacteria were indeed present in the intestine and thus the results obtained on translocation are valid.

The daily dosage of *S. macedonicus* used in this study amounted to 8.9 log, which for a 12 week old mouse of 32 grams corresponds to 7.4 log cfu per gram of body weight. Compared to an adult human with an average weight of 75 Kg that consumes 100 g of cheese with an *S. macedonicus* population of approximately 6.0 log cfu/g (Georgalaki et al., 2009), the dose amounts to 8.0 log cfu, i.e. 3.1 log cfu per gram of body weight. As a result, the dosage of *S. macedonicus* used in this experiment, without causing any detrimental effect whatsoever, was approximately 20,000-fold more than the one a human would consume through his daily diet.

*S. macedonicus* belongs to a genus of known pathogens, but this should not necessarily be an obstacle for its consideration as a safe strain, as is the case for *S. thermophilus*. In addition, several *Bacillus* species are included in the QPS list of safe strains (EFSA, 2008), even though the *Bacillus* genus includes important pathogens. So far, *S. macedonicus* has been isolated in two cases (Herrero et al., 2002; Malkin et al., 2008) from patients with endocarditis. However, even GRAS status species, including known probiotic bacteria, have been associated with endocarditis cases and are characterized as opportunistic pathogens. A characteristic example is that of *L. rhamonusus*, which, although it has been isolated on various occasions from endocarditis cases (EFSA, 2008), it remains in the QPS list of EFSA, due to the rarity of the associated cases and the general safe history of the strain.

The evidence presented in this study, in conjunction with the widespread presence of *S. macedonicus* in cheese, strongly indicates that it is a safe bacterium. However, complete genome analysis, investigating the presence or absence of virulence determinants, will provide further evidence and confirm the safety of this species.

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