



## Full length article

# Probiotic properties and immunomodulatory activity of gastrointestinal tract commensal bacterial strains isolated from the edible farmed snail *Cornu aspersum maxima*

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## ABSTRACT

The aim of this study was to determine the *in vitro* probiotic properties as well as the immunomodulatory activity of bacterial strains isolated from the gastrointestinal tract of the edible-farmed land snail *Cornu aspersum maxima*. Forty lactic acid bacterial strains (named Sgs1-40) were isolated from the intestinal tract and eight strains (named SgmA-H) from the oesophagus-crop of snails. Several criteria were used to examine whether they may be applied as snail-specific for the screening of the presumptive probiotic bacterial strains. Principal Component Analysis using criteria such as the tolerance of these strains to the pedal mucus, gastric mucus, gastric juices and low pH, as well as the expression of the cell surface traits of hydrophobicity, biofilm formation and autoaggregation capacity revealed discrimination of twelve strains exhibiting presumptive *in vitro* probiotic properties. Injection of eight of these strains, which were identified as *Lactobacillus plantarum*, in snail haemocoel increased the recruitment and phagocytic activity of amoebocytes in snail haemolymph. The Sgs14 and SgmB strains, exhibiting the highest immunostimulatory activity in haemolymph, were FITC-labelled and orally administered to snails for ten days. The Sgs14 strain was able to adhere to intestinal mucosa of snails and stimulate the chemotactic and phagocytic activity of amoebocytes in haemolymph as well as the bactericidal activity of haemolymph serum. These responses are potentially mediated by the regulation of TLRs expression in the gut mucosa. These data indicate that the determination of properties such as snail mucus and gastric juice tolerance, cell surface traits for adhesion as well as increased chemotactic and phagocytic activity in snail haemolymph are eligible criteria to screen for snail-specific probiotics. To the best of our knowledge, this is the first work that investigates the probiotic properties of gastrointestinal microflora of the terrestrial farmed snail *Cornu aspersum maxima*.

## 1. Introduction

Beneficial host-microbe interactions leading in symbiosis are well established showing the important role of the gastrointestinal microflora in the health status of man and animals. Gut microflora is a key player in the function and integrity of the gastrointestinal tract, maintenance of immune homeostasis and metabolism [1,2]. However, the disruption of the normally occurring gut microflora, known as dysbiosis, could lead to infectious diseases, metabolic and immune disorders [3,4]. Rebiosis of the affected microflora may be achieved by probiotic administration. Until now, the majority of the studies on such issues have been performed mainly on vertebrates and certain molluscs [5,6], while little is known about the interactions of the gut microbiota with the gastrointestinal and innate immune system of snails.

Furthermore, in the process of screening for snail probiotic strains there are not established bacterial phenotypic, biochemical and cellular markers which could be used for the prediction of their capacity to colonize the snail gut or to exhibit immunomodulatory activity.

Snails are ecologically as well as economically very important animals and are used as food and medicine. Snails are a rich source of proteins containing high amounts of essential amino acid, calcium and low fat [7]. Also snail's pedal mucus has pharmaceutical application [8]. The wild snail populations have decreased in their natural habitat and some of the edible species are under imminent threat of extinction as a result of the intensive collection [9]. Thus there is an increasing interest for the snail farming. However, a lot of difficulties occur in snail farming operations because snails are extremely susceptible animals in climate changes and infections and their physiology has not been

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adequately explored.

Although invertebrates lack an adaptive immunity, they have evolved a series of defence mechanisms and a sophisticated innate immunity that enable them to respond to the invading pathogens and more importantly establish and maintain a beneficial symbiotic microbiota in the gut [10]. External and internal physical and chemical barriers, such as pedal mucus which cover the snail's body and exhibit antimicrobial activity [8] as well as gastric mucus and gastric acidic juices [11], may foster host specific microenvironments that are compatible with the growth of their symbiots but inhibit the growth and colonization of other invaders in the gastrointestinal tract.

Amoebocytes are the main cellular components of the innate immune system of *Cornu aspersum*, as they constitute 85% of the haemocyte population and are responsible for several functions such as cellular recognition, phagocytosis, encapsulation and killing of the invading microorganisms [12,13]. The innate immunity responses of invertebrates are mainly mediated by Pattern Recognition Receptors (PRRs) that recognize Microbe-Associated Molecular Patterns (MAMPs) and activate the haemocytes [14] as well as the production of a vast diversity of haemolymph factors and antimicrobial peptides [15]. However, the majority of them have not been identified in snails and the mechanism of the cross talk between the gut symbiots and the innate immune system of snails remain poorly understood [16].

On vertebrates, the health benefits of the probiotic microorganism are associated with their capacity to resist the harsh gastrointestinal tract conditions and adhere to intestinal epithelium of the host [17,18]. The interactions between probiotics and immune system are mediated through the Toll-like receptors (TLRs) present on intestinal epithelial, dendritic and macrophage cells. These interactions lead to the stimulation of the cellular immune responses such as the chemotaxis and phagocytosis of macrophages as well as the regulation of the proinflammatory and antiinflammatory immune responses to maintain host homeostasis [19,20]. In addition, *in vitro* cell surface traits of probiotic strains such as the cell surface hydrophobicity, autoaggregation and biofilm formation have been associated with their colonization and immunomodulatory capacity and are used for the screening of novel presumptive probiotic bacteria strains for vertebrates [21,22]. However, similar studies have not been performed for invertebrates such as snails.

Thus, it was of interest to determine whether pedal mucus, gastric mucus, acidic pH and gastric juices, as well as the cell surface hydrophobicity, autoaggregation and biofilm formation ability in association with immunomodulatory activity such as chemotaxis and phagocytosis may be applied as snail-specific criteria for the screening of presumptive probiotic intestinal bacteria.

## 2. Materials and methods

### 2.1. Animals

Adult *Cornu aspersum* maxima snails (diameter 38–40 mm, shell high 38–40 mm, live body weight 13–15 g) were obtained from the snail farm 'DomaineVolvi' situated in Nea Apollonia, Thessaloniki, Greece. Animals were kept in ventilated glass chambers under aseptic conditions. They were acclimatized to laboratory conditions (20 ± 2 °C, 70–90% humidity, 12 h light/dark) and fed with sterilized snail commercial food. The chambers were re-moistened with sterilized water every day and cleaned on a weekly basis.

### 2.2. Isolation of lactic acid bacterial strains (LABs)

Isolation of LABs was performed from five snails that were kept under starvation for ten days immediately after arrival. The shells of snails were sterilized with 70% ethanol and removed aseptically. Snails were then dissected and the intestinal tracts as well as the oesophagus-crop were isolated and homogenized in phosphate buffered saline (PBS)

[23]. Bacterial isolates were obtained from MRS agar (PanReac, Barcelona, Spain) after cultivation at 25 °C for 48 h. Distinct catalase and oxidase negative colonies that consisted of Gram-positive cells were picked and maintained at –80 °C in MRS broth with 25% (v/v) glycerol. All strains were cultured at least three times in MRS broth at 25 °C prior to experimental use. The growth kinetic of strains was performed by inoculating 100 µl of the bacterial cultures (5 × 10<sup>7</sup> CFU/ml) in 10 ml of MRS broth. The Optical Density (O.D. 600 nm) was determined every 2 h for a total period of 24 h and the results were used to identify the exponential and stationary growth phase of bacterial isolates. For tolerance tests and *in vivo* assays, bacterial cells were harvested from broth cultures at exponential growth phase during the maximum growth rate.

### 2.3. Species identification

Species identification was carried out by a Polymerase Chain Reaction (PCR) amplification of 16S rRNA with *Lactobacillus* group-specific PCR primer pairs, as previously described, (S-G-Lab-0159-a-S-20: GGA AAC AGA TGC TAA TAC CG and S-G-Lab-0677-a-A-17: CAC CGC TAC ACA TGG AG) [24]. The PCR products were purified and the sequencing was carried out by VBC-Biotech & Eurofins Genomics (Vienna, Austria). Sequences were compared and aligned with those from the GenBank database using the BLASTn algorithm of the NCBI network server.

### 2.4. Tolerance to snail gastrointestinal barriers

Pedal mucus was collected from the surface of five snails by prodding the extended foot [25]. Gastric juices and gastric mucus were collected from 10 snails. Gastric juices were collected by syringe from the crop and stomach. The salivary glands, oesophagus and crop of snails were isolated, homogenized in PBS solution (10 g tissue/ml) and centrifuged at 13,000 × g for 10 min to collect gastric mucus. For the tolerance activity, 5 µl of the bacterial cultures containing 5 × 10<sup>7</sup> CFU/ml were incubated in 20 µl of pedal mucus or gastric mucus or gastric juices or MRS broth (control samples) for 4 h at 25 °C. After incubation, 2 µl of each sample were inoculated on MRS agar plates and incubated at 25 °C for 24 h. The growth of bacterial isolates in the presence of pedal mucus, gastric mucus and gastric juices were compared to the corresponding control samples. Bacterial growth at acidic pH was screened by inoculating 2 µl of 5 × 10<sup>7</sup> CFU bacterial cells on MRS agar with pH 3.0 and incubating plates at 25 °C for 24 h. Also a combined test was performed by incubating bacterial strains for 4 h in gastric mucus followed by inoculation on MRS agar with pH 3.0. Three independent experiments were performed.

### 2.5. Cell surface traits

Biofilm formation was determined based on bacterial ability to adhere on abiotic surfaces and was expressed as the O.D. at 570 nm as previously described [26]. Autoaggregation capacity and cell surface hydrophobicity were performed according to Kotzamanidis et al. [21]. Autoaggregation was measured on the basis of their sedimentation characteristics over a period of 5 h and was expressed as: 1 – (At/A0) × 100, where At represents the absorbance at time t = 0–5 h and A0 the absorbance at t = 0 at 600 nm. Cell surface hydrophobicity was assessed based on the bacterial ability to adhere to hydrocarbons. Bacterial suspensions were mixed with xylene to perform a biphasic system consisting of an aqueous hydrophilic phase and a xylene hydrophobic phase. Hydrophobicity (H %) was expressed by the decrease in the absorbance of the aqueous phase and calculated using the formula: H % = (1 – A1/A0) × 100. At least three independent experiments were performed for the determination of the cell surface traits at exponential (16 h) as well as at stationary (24 h) growth phase of the bacterial isolates.

## 2.6. Animal treatments

Animals were injected with  $5 \times 10^8$  bacterial cells suspended in 100  $\mu$ l Snail Saline (3.7 mM NaOH, 36 mM NaCl, 2 mM KCl, 2 mM MgCl $_2$ ·2H $_2$ O, 4 mM CaCl $_2$ ·2H $_2$ O, pH 7.8) into the cephalopodal sinus of the upper sub-epithelial region of the head-foot [27] or were fed with 1 g/day of commercial snail-food containing  $5 \times 10^8$  CFU bacterial isolates for ten consecutive days. Haemolymph was collected 3 h post injection or 10 days post feeding from the pericardial cavity of snails [28] and was centrifuged at  $300 \times g$  for 10 min to separate serum from haemolymph cells. Haemolymph serum or cells were used immediately in the appropriate experiments. All groups included in the present study consisted of five animals and each experiment was performed at least twice.

## 2.7. Determination of chemotactic activity in haemolymph

Determination of chemotactic activity in snail haemolymph was performed in snails injected with  $5 \times 10^8$  gastrointestinal bacterial isolates or in snails fed with 1 g/day of commercial food containing  $5 \times 10^8$  CFU of gastrointestinal bacterial isolates for a total period of ten days. At the indicated time, haemolymph was collected and haemolymph cells were then counted using haemocytometer. Cell viability was determined by trypan blue exclusion. Haemolymph serum was used to opsonise yeast cells.

## 2.8. Phagocytic activity of haemolymph amoebocytes

Heat-killed baker's yeast cells of *Saccharomyces cerevisiae* ( $2 \times 10^4$  cells) were opsonised in haemolymph serum for 30 min at 25 °C. Opsonised yeast cells were mixed with haemolymph containing  $2 \times 10^5$  amoebocytes and phagocytosis was performed for 30 min at 25 °C. At the indicated time post interval phagocytosis of yeast cells was determined as the number of amoebocytes containing yeast cells as described by Kourelis et al. [29].

## 2.9. Bactericidal activity of haemolymph serum

Antibacterial activity of the haemolymph serum was determined against *Escherichia coli* (NCIMB 8879), *Staphylococcus aureus* (NCIMB 9518) and *Bacillus subtilis* (NCIMB 3610). A nutrient broth *E. coli* culture containing  $3 \times 10^6$  CFU/ml was mixed with haemolymph serum isolated from control or bacteria-fed snails and was incubated for 4 h at 25 °C. Samples were then inoculated in nutrient broth and the *E. coli* growth was determined by measuring the O.D. at 600 nm every hour for a total of 10 h.

## 2.10. Interaction of bacterial strains with intestinal mucosa

Snails were fed with 1 g/day for ten days with commercial snail-food containing  $5 \times 10^8$  CFU of bacterial strains labelled with Fluorescein Isothiocyanate (FITC) (Sigma, St Louis, MO, USA). FITC-labelling of bacterial strains was performed as previously described [30]. Intestines from control or treated snails were removed after a 24 h starvation period and dissected into small pieces. Intestinal pieces were fixed in 4% paraformaldehyde in PBS at 4 °C for 48 h and then embedded in paraffin blocks according to Sainte-Marie [31]. Sections of 3  $\mu$ m thickness were examined by fluorescence light microscopy.

## 2.11. Determination of TLRs in snail intestine and haemolymph

Expression of TLRs was determined on haemolymph cell smears and intestinal sections. Haemolymph samples containing  $10^5$  cells were cytocentrifuged at  $300 \times g$ , for 5 min at 4 °C. Cells were fixed with 4% formaldehyde in PBS for 10 min and stored in 70% ethanol solution at 4 °C. Intestinal sections from control or treated snails were prepared as

described above. Cells and intestinal sections were rehydrated in PBS and blocked by incubation with 5% (v/v) Fetal Bovine Serum in PBS for 30 min at room temperature. TLR2 and TLR4 expression was determined by a direct immunofluorescence assay by incubating the samples with FITC-conjugated anti-mouse TLR2 and TLR4 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in the dark, for 2 h at room temperature. The expression of TLR6 was examined by an indirect immunofluorescence assay. The samples were incubated for 2 h at room temperature with the primary anti-mouse TLR6 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) washed in PBS and incubated with the secondary anti-goat IgG FITC conjugated antibody (Sigma, St Louis, MO, USA) for 1 h at room temperature. After repeated washing with PBS, samples were examined by fluorescence light microscopy to evaluate the expression of Toll-like receptors on haemolymph cells of snails. The expression of TLRs in intestinal sections was quantified by measuring the mean fluorescent intensity (Digital Units/pixels $^2$ ) produced by FITC in 10 randomly chosen areas (32.384 pixels) per field using Image J software [32]. Thirty fields at  $400 \times$  magnification were used for each measurement.

## 2.12. Statistical analysis

Multiple comparisons between means were performed by one-way Analysis of Variance (ANOVA) followed by Tukey's test. Mann-Whitney test was used to compare the differences of the fluorescence intensity of treated and control group. P values lower than 0.05 were considered to be statically significant (IBM SPSS Statistics 23). Bacterial properties examined in this study were analyzed by Principal Component Analysis (PCA) using the Minitab software (Release 18, trial version; Minitab Inc.).

## 2.13. Key resources table

Resource	Source	Identifier
Antibodies		
TLR6 antibody (S-14)	Santa Cruz Biotechnology	SC-5661
Anti-goat IgG (whole molecule)-FITC	SIGMA-ALDRICH	F7367
TLR2 antibody (TL2.1)	Santa Cruz Biotechnology	SC-21759
TLR4 antibody (MTS510)	Santa Cruz Biotechnology	SC-13591
Chemical		
CaCl $_2$ ·2H $_2$ O	APPLICHEM PANREAC	A4689, CAS:10035-04-8
ethanol absolute	Scharlau	ET 00052500, CAS:64-17-5
Fluorescein Isothiocyanate	SIGMA_ALDRICH	F 7250, CAS:3326-32-7
Glycerol	APPLICHEM PANREAC	A1123, CAS:56-81-5
MgCl $_2$	SIGMA_ALDRICH	M8266, CAS:7786-30-3
Paraformaldehyde	APPLICHEM PANREAC	A3813, CAS:30525-89-4
Trypan Blue (C.I. 23850)	APPLICHEM PANREAC	A0668, CAS:72-57-1
Xylene	APPLICHEM PANREAC	131769, CAS: 1330-20-7

## 3. Results

### 3.1. Lactic acid bacterial strains

Of about 200 colonies formed after the cultivation of the intestinal or oesophagus-crop exudates on MRS agar, forty-eight colonies were characterized as LABs based on their morphology and biochemical characteristics (small, round, opaque and white colonies, rod shaped, Gram-positive, catalase negative and oxidase negative). Forty LABs named Sgs1-40 were isolated from the intestinal tract while eight LABs named SgmA-H were isolated from the oesophagus-crop. According to

**Table 1**  
In vitro probiotic properties.

STRAINS	SOURCE	A. Tolerance to GIT-barriers <sup>a</sup>				B. Cell surface traits <sup>b</sup>			
		pH 3.0		pH 3.0		AYTOAGGREGATION 16 h		HYDROPHOBICITY 16 h	
		GASTRIC MUCUS	GASTRIC MUCUS & pH 3.0	PEDAL MUCUS	GASTRIC JUICES	AYTOAGGREGATION 24 h	BIOFILM 16 h	BIOFILM 24 h	HYDROPHOBICITY 24 h
<b>INTESTINAL TRACT</b>									
Sgs 29	-	-	-	-	++	++	++	++	+
Sgs 37	-	-	-	-	++	++	++	++	-
Sgs 17	-	-	-	-	++	++	++	++	-
Sgs 39	-	-	-	-	++	++	++	++	-
Sgs 32	-	-	-	-	++	++	++	++	-
Sgs 12	-	-	-	-	++	++	++	++	-
Sgs 35	-	-	-	-	++	++	++	++	+
Sgs 26	-	-	-	-	++	++	++	++	-
Sgs 28	-	-	-	-	++	++	++	++	-
Sgs 36	-	-	-	-	++	++	++	++	-
Sgs 11	-	-	-	-	++	++	++	++	+
Sgs 10	-	-	-	-	++	++	++	++	+
Sgs 38	+	+	+	+	++	++	++	++	-
Sgs 19	+	+	+	+	++	++	++	++	-
Sgs 34	+	+	+	+	++	++	++	++	++
Sgs 2	+	+	+	+	++	++	++	++	-
Sgs 16	+	+	+	+	++	++	++	++	-
Sgs 18	+	+	+	+	++	++	++	++	+
Sgs 20	+	+	+	+	++	++	++	++	-
Sgs 24	+	+	+	+	++	++	++	++	+
Sgs 33	++	++	++	++	++	++	++	++	-
Sgs 27	++	++	++	++	++	++	++	++	-
Sgs 15	++	++	++	++	++	++	++	++	-
Sgs 25	++	++	++	++	++	++	++	++	+
Sgs 30	++	++	++	++	++	++	++	++	-
Sgs 31	++	++	++	++	++	++	++	++	-
Sgs 40	++	++	++	++	++	++	++	++	+
Sgs 21	++	++	++	++	++	++	++	++	+
Sgs 9	++	++	++	++	++	++	++	++	+
Sgs 23	++	++	++	++	++	++	++	++	+
Sgs 22	++	++	++	++	++	++	++	++	+
Sgs 1	++	++	++	++	++	++	++	++	+
Sgs 4	++	++	++	++	++	++	++	++	+
Sgs 7	++	++	++	++	++	++	++	++	+
Sgs 8	++	++	++	++	++	++	++	++	+
Sgs 13	++	++	++	++	++	++	++	++	+
Sgs 14	++	++	++	++	++	++	++	++	+
Sgs 5	++	++	++	++	++	++	++	++	+
Sgs 3	++	++	++	++	++	++	++	++	+
Sgs 6	++	++	++	++	++	++	++	++	+
<b>OESOPHAGUS-CROP</b>									
Sgm A	++	++	++	++	++	++	++	++	++
Sgm B	++	++	++	++	++	++	++	++	++
Sgm C	++	++	++	++	++	++	++	++	++
Sgm D	++	++	++	++	++	++	++	++	-
Sgm E	++	++	++	++	++	++	++	++	-
Sgm F	++	++	++	++	++	++	++	++	-
Sgm G	++	++	++	++	++	++	++	++	-
Sgm H	++	++	++	++	++	++	++	++	-

<sup>a</sup> Tolerance to gastrointestinal (GIT) barriers: no (-), poor (+), moderate (++) and high (+++) tolerance.

<sup>b</sup> Autoaggregation, biofilm formation and hydrophobicity values expressed as no (-) low (+), moderate (++) and high (+++) capacity. Autoaggregation%: 25–30% (+), 30–40% (++) and 40–66% (+++). BiofilmAbsorbance: 0.5–0.75 (+), 0.75–0.9 (++) and 1.0–1.5 (+++). Hydrophobicity%: 10–25% (-), 25–35% (+), 35–45% (++) and 45–60% (+++).

bacterial growth curves, all the strains reached the maximum growth rate from 14 h to 18 h (exponential phase 8 h – 18 h) and the stationary phase at 24 h.

### 3.2. Tolerance to snails' gastrointestinal tract physical and chemical barriers

The tolerance capacity of bacterial strains to low pH, pedal mucus, gastric mucus and gastric juices was categorized into four classes: high (+++), moderate (++) , poor (+) and no (–) tolerance and are presented in Table 1 arranged in an increasing order according to their tolerance to pH 3.0. Twenty of the Sgs strains exhibited high or moderate acid tolerance capacity in association with high or moderate resistance to pedal mucus, gastric mucus, gastric juices and to the combined conditions of gastric mucus and pH 3.0. Bacterial Sgm strains showed a distinct tolerance pattern as all these strains exhibited moderate tolerance to pH 3.0, gastric mucus and gastric juices and no or poor tolerance to pedal mucus.

### 3.3. Cell surface traits

Both Sgs and Sgm strains at the exponential growth phase exhibited increased cell surface traits such as hydrophobicity, autoaggregation and biofilm formation ability. However, most of these strains lose this phenotype at the stationary growth phase suggesting discrimination among these strains. Most of the strains exhibiting increased cell surface traits at both growth phases exhibit also increased resistance to the gastrointestinal physical and chemical barriers. For instance, at both growth phases, bacterial strains Sgs1, Sgs4, Sgs7, Sgs8, Sgs13 and Sgs14 exhibited moderate hydrophobicity values and high autoaggregation and biofilm formation capacity in association with moderate tolerance capacity to the gastrointestinal acidic pH, gastric mucus, pedal mucus, gastric juices and to the combined conditions of gastric mucus and pH 3.0.

### 3.4. Principal Component Analysis

PCA was carried out to demonstrate the potential correlations among *in vitro* probiotic properties and select the most promising presumptive probiotic strains. The PCA results presented in Fig. 1, show that two components which account for 65.4% of the variability of the original dataset have been extracted. According to the first principal component values, the ability of bacterial strains to tolerate the gastrointestinal tract barriers of snails such as the acidic pH, gastric mucus, gastric mucus-pH 3.0, pedal mucus and gastric juices are highly associated with each other as well as are positively correlated with their hydrophobicity, autoaggregation and biofilm formation ability at stationary growth phase. Considering the screening process, strains SgmA-H were separated from bacterial strain Sgs1-40 according to their *in vitro* properties, indicating that the isolation site of the strains may influence their tolerance and cell surface properties. SgmA-H strains were characterized by moderate tolerance to gastrointestinal tract conditions (GIT-resistance) and low to moderate values on the cell surface properties (GIT-adherence) in contrast to the Sgs strains that includes bacterial strains with a wide range of tolerance capacity. The Sgs1, Sgs3, Sgs4, Sgs5, Sgs6, Sgs7, Sgs8, Sgs9, Sgs13 and Sgs14, as well as SgmA and SgmB strains have been distinguished from the rest Sgs and Sgm strains, since they exhibited the highest values in comparison to the rest Sgs and Sgm strains, respectively. The above strains were selected and further *in vivo* investigated.

### 3.5. Chemotaxis and phagocytic capacity of haemolymph cells

The *in vivo* immunomodulatory activity of Sgs1, Sgs3, Sgs4, Sgs5, Sgs6, Sgs7, Sgs8, Sgs9, Sgs13 and Sgs14, as well as SgmA and SgmB was studied by determination of the chemotaxis and phagocytic activity of

haemolymph accumulated cells, 3 h post of their injection into the cephalopodal sinus of snails. The injection of Sgs1, Sgs4, Sgs5, Sgs6, Sgs7, Sgs9, Sgs14 and SgmB strains significantly ( $P < 0.01$ ) increased the number of the amoebocyte population in snail haemolymph (Fig. 2) in association with increase of their phagocytic activity in comparison to the control group (Fig. 3). At 5 min post-initiation, 30% phagocytosis was detected reaching maximum 49% at 30 min. The strains Sgs3, Sgs8, Sgs13 and SgmA exhibiting low chemotactic activity induced also low phagocytic activity, indicating that the chemotaxis of amoebocytes is associated with their phagocytic activity.

### 3.6. Molecular identification of presumptive probiotic strains

The bacterial strains Sgs1, Sgs4, Sgs5, Sgs6, Sgs7, Sgs9, Sgs14 and SgmB that exhibited increased immunomodulatory activity as well as the SgmA strain were identified to species level by partial sequencing of the 16S rRNA genes. All the strains showed up to 99% identification with the strain *Lactobacillus plantarum* CIP 1303151(NR104573.1), indicating that the potency of the *Lactobacillus plantarum* strains to modulate the snail immune responses is a strain specific characteristic. The 16S rRNA sequences of the nine strains were submitted to GenBank (MK760567-MK760576).

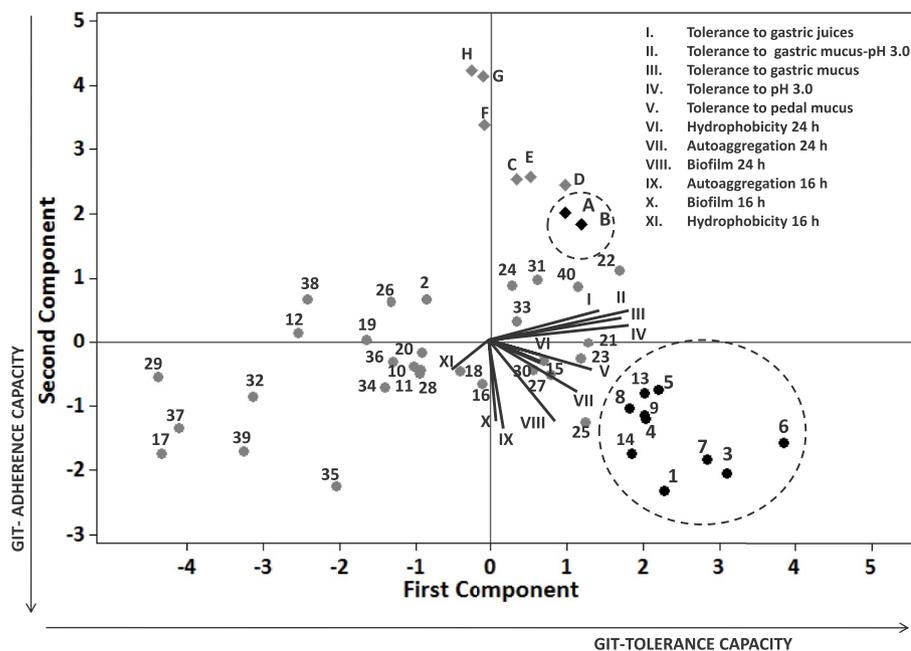
### 3.7. Interaction of bacterial strains with intestinal mucosa

The strains Sgs14 and SgmB that exhibited the highest chemotaxis and phagocytic activity were further studied for their ability to interact with the intestinal mucosa of snails. By fluorescent microscopy we observed differential interaction of the Sgs14 and SgmB strains. The FITC-labelled Sgs14 cells or their antigens were observed to interact with the snail intestine. This interaction is site-specific since positive fluorescent signals were detected only on the edges of the internal ridges (Fig. 4A). In addition, Sgs14 interaction with snail intestinal cells is tissue specific since the FITC-labelled SgmB strain that isolated from snail oesophagus-crop failed to interact with snail intestinal epithelium (Fig. 4B).

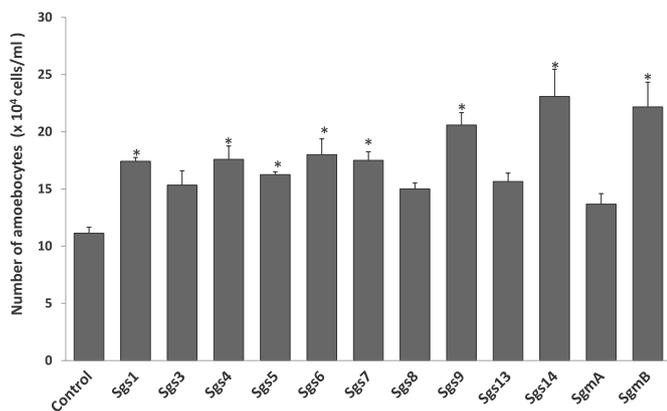
### 3.8. Determination of the immunomodulatory activity in haemolymph and gut of Sgs14-fed snails

To examine whether Sgs14 strain interaction with the gut mucosa is associated with systemic cellular immune responses, snails were fed with 1 g/day sterilized snail food containing  $5 \times 10^8$  CFU/g for ten consecutive days and chemotaxis and phagocytic capacity of haemolymph cells was determined. The data in Fig. 5 demonstrate that the Sgs14 strain significantly ( $P < 0.01$ ) increased the number of amoebocytes in snail haemolymph (Fig. 5A) as well as their phagocytic activity in comparison with control (Fig. 5B). Increased phagocytosis in Sgs14-fed snails is not due to haemolymph factors since haemolymph isolated from Sgs14 fed snails or control snails induce the same level of phagocytosis in normal snail amoebocytes (Fig. 6A). However, the bactericidal activity against *E. coli* of haemolymph serum isolated from Sgs14-fed snails is statistically ( $P < 0.01$ ) increased in comparison with the serum of unfed snails (Fig. 6B). Similar data of bactericidal activity were obtained against *S. aureus* (Fig. S1A) and *B. subtilis* (Fig. S1B). All the above data indicate that the Sgs14 cells or their antigens directly activate chemotaxis and phagocytosis of haemolymph cells as well as their activation to produce antimicrobial factors.

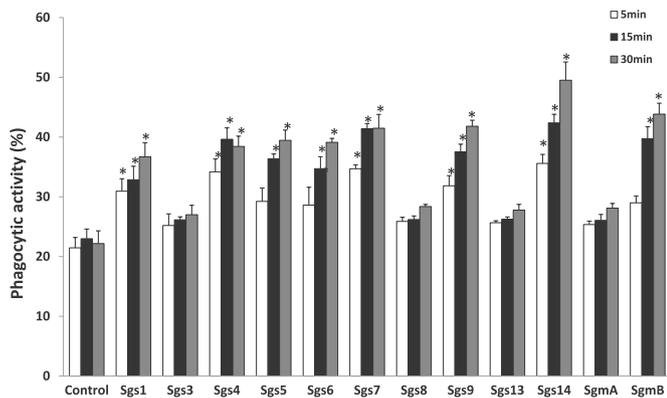
To further investigate Sgs14 cells interaction with gut and haemolymph cells we performed immunofluorescence analysis to detect TLRs expression on snail gut paraffin sections and haemolymph cells smears. The data in Fig. 7 indicate that all haemolymph cells of normal or Sgs14-fed snails express TLR-2 (Fig. 7A), TLR-4 (Fig. 7B) and TLR-6 (Fig. 7C). Furthermore, fluorescence analysis revealed increased expression of TLR-2, TLR-4 and TLR-6 in gut paraffin sections of Sgs14-fed snails (Fig. 8). These data indicate that Sgs14 strain may exhibits its



**Fig. 1.** PCA based on the *in vitro* probiotic properties (I-XI). PC1 is mostly associated with the bacterial tolerance capacity to the gastrointestinal tract barriers (GIT-resistance). PC2 represents the *in vitro* adherence ability of strains (GIT-adherence) since the higher contributions arise from the biofilm formation and autoaggregation ability. The arrows indicate the direction in which bacterial strains are distributed according to their values on PC1 and PC2, from the lowest to the highest values. Strains in the circles exhibited the highest *in vitro* tolerance and adherence capacity in comparison to the rest Sgs and Sgm strains, respectively.



**Fig. 2.** Determination of the amoebocytes accumulated in snail haemolymph by 3 h post bacterial injection. Each histogram bar represents the mean values of 5 snails  $\pm$  SEM. (\*) Statistically significant differences in comparison with the control group,  $P < 0.01$ .



**Fig. 3.** Determination of the phagocytic capacity of amoebocytes accumulated in haemolymph by 3 h post bacterial injection. Each histogram bar represents the mean values  $\pm$  SEM of three independent experiments. (\*) Statistically significant differences in comparison with the control group,  $P < 0.01$ .

immunomodulatory activity by affecting TLR expression.

#### 4. Discussion

In this study we have demonstrated that the surface traits such as hydrophobicity, autoaggregation and biofilm formation of snail gut LABs in association with their capacity to tolerate snail pedal mucus, gastric mucus, gastric juices and acidic conditions as well as their immunomodulatory activity on haemolymph cells may be used as criteria to characterize them as presumptive probiotics. To the best of our knowledge, this is the first research on the isolation of commensal bacterial strains from the intestinal tract of snails and determination of their host-specific probiotic potential. Our study provides new evidence that gut commensal lactobacilli strains interact with the gastrointestinal mucosa of snails and enhance the cellular immune responses such as chemotaxis and phagocytosis, as well as antimicrobial activity of haemolymph factors.

Forty lactic acid bacterial strains were isolated from the intestinal tract (Sgs1-40) of the snail *Cornu aspersum maxima* and eight strains (SgmA-H) were isolated from the oesophagus-crop, where harsh gastrointestinal conditions occur due to the acidic gastric juices and mucus. Defence barriers of snails, such as the pedal mucus, gastric acidity, gastric juices and gastric mucus serve as restrictive barriers to the food-administrated microbes and shape the composition of gut microflora in snails. Thus, snail presumptive probiotic strains have to tolerate these chemical and physical barriers to colonize the intestinal mucosa of snails and exert their probiotic health benefits. Our findings demonstrate that the bacterial tolerance capacity to snail pedal mucus, acidic pH, gastric mucus and gastric juices of snails is strain specific as the degree of tolerance varied among the tested LABs. The acidic pH was shown to be the major restrictive barrier as it inhibited the growth of thirteen of the forty Sgs strains, while gastric juices were considered as the less strict barrier as most of the strains shown high viability in the presence of gastric juices. Considering the pedal mucus tolerance, although pedal mucus has been shown to exhibit antibacterial activity against Gram-negative and Gram-positive bacteria [33] in this study most of the examined Sgs strains were found to be resistant. The antimicrobial activity of pedal mucus is known to be stimulated by agglutinating molecules that have also been identified as opsonic factors in haemolymph of snails and participate in self/non-self recognition in

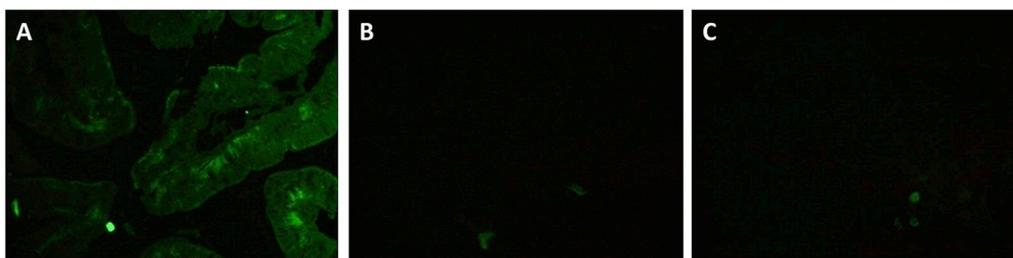


Fig. 4. Fluorescence microphotographs showing the interaction of the FITC-labelled (A) Sgs14 and (B) SgmB in intestinal mucosa of bacteria-fed snails and (C) Fluorescence microphotography of unfed snails.

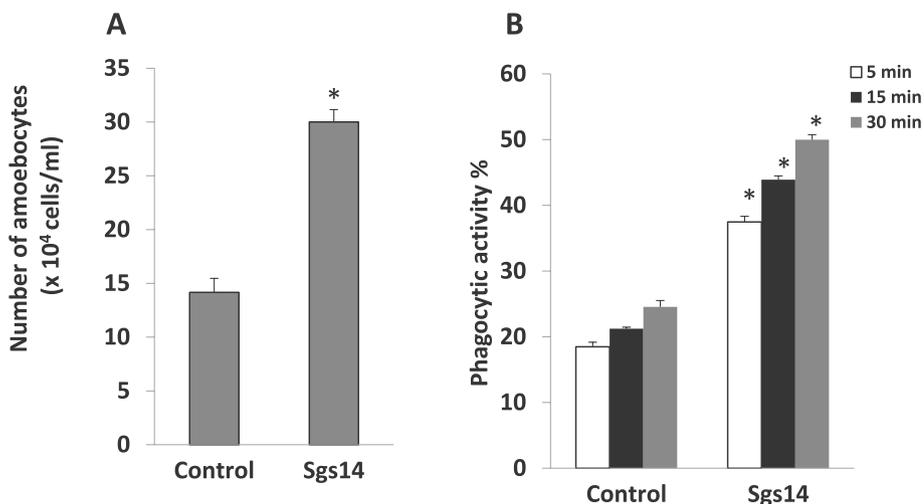


Fig. 5. Determination of the amoebocytes recruitment and their phagocytic activity in snail haemolymph of the Sgs14-fed snails. (A) Determination of the amoebocytes accumulated in snail haemolymph. Each histogram bar represents the mean values of 5 snails ± SEM. (B) Determination of the phagocytic capacity of amoebocytes. Each histogram bar represents the mean values ± SEM of three independent experiments. (\*) Statistically significant differences in comparison with the control group, P < 0.01.

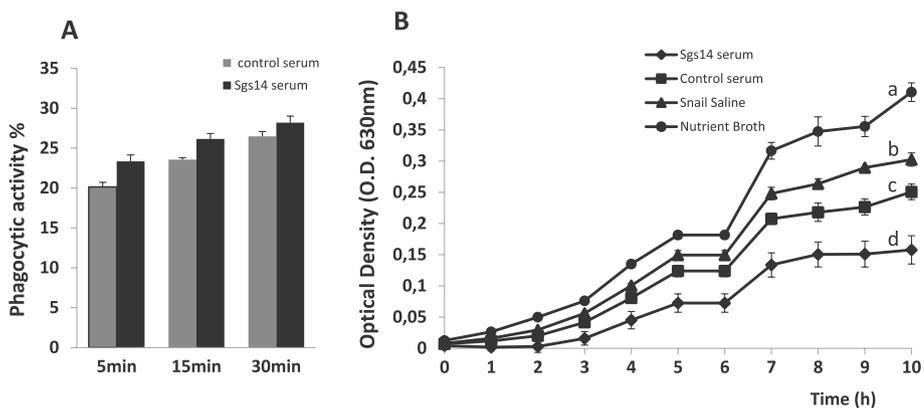


Fig. 6. Alterations in Sgs14-fed snail haemolymph. (A) Amoebocytes from untreated snails were incubated with haemolymph serum that was obtained from Sgs14-fed snails (Sgs14-serum) and untreated snails (control serum). Each histogram bar represents the means of phagocytic activity of amoebocytes ± SEM of three independent experiments. (B) Bactericidal activity of haemolymph serum obtained from Sgs14-fed (Sgs14-serum) and untreated snails (control serum) against the *E. coli* growth during a 10 h time interval in comparison with the growth of *E. coli* in Snail Saline and Nutrient Broth. Lines with different superscripts (a, b, c, d) differ significantly, P < 0.01.

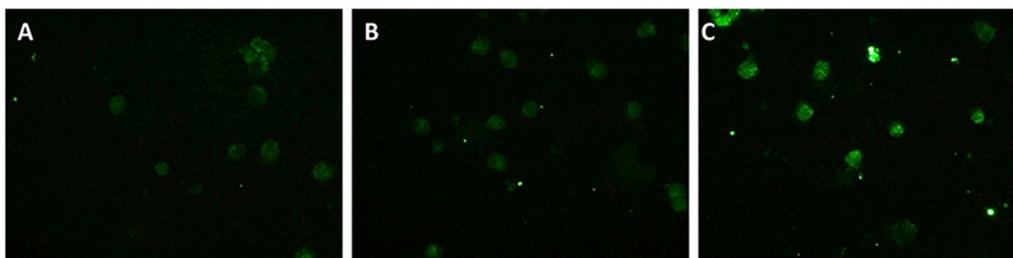
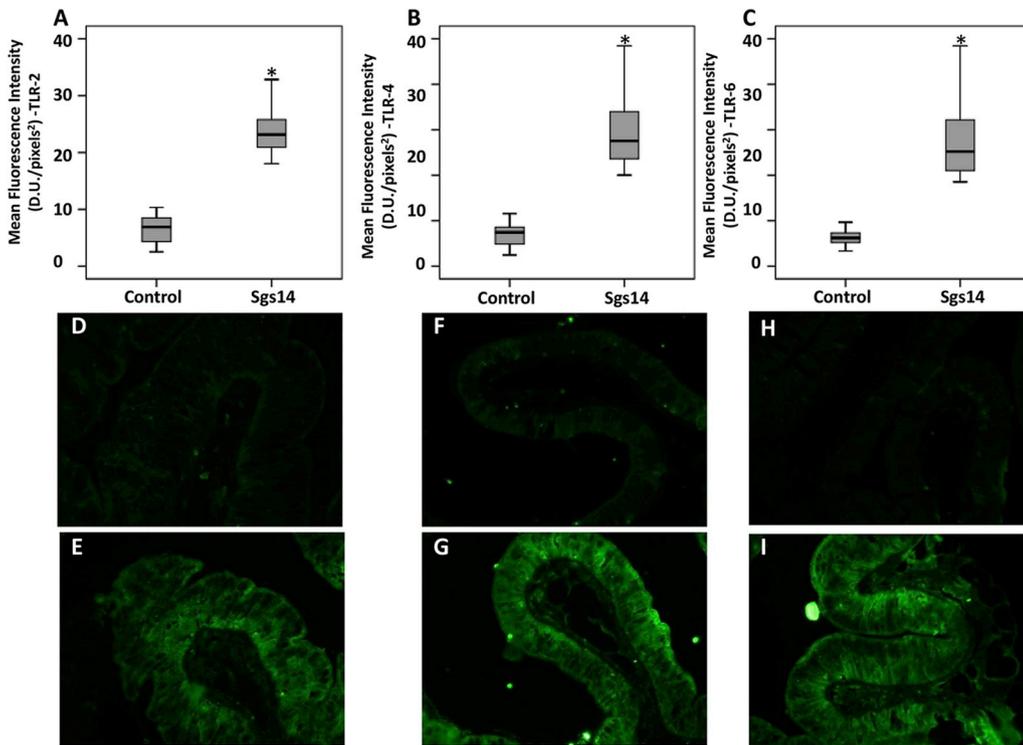


Fig. 7. Immunofluorescence microphotographs showing (A) TLR2, (B) TLR4 and (C) TLR6 positive cells in slides of haemolymph samples of untreated snails.

invertebrates [34,35], indicating that the external and internal defence factors could be in tight communication in order to respond to the different bacterial antigens that trigger them. According to the overall *in vitro* tolerance results, the tolerance of Sgs strains to acidic pH is positively correlated with tolerance to pedal mucus, gastric mucus,

gastric mucus-pH 3.0 and gastric juices. An interesting exception was observed since the Sgs10, Sgs11, Sgs12, Sgs26, Sgs28, Sgs32, Sgs35, Sgs36 strains, that showed no resistance to pH 3.0, were able to grow in the presence of gastric mucus as well as under the combined conditions of gastric mucus and pH 3.0, indicating that mucus could aid the



**Fig. 8.** Effect of oral Sgs14 strain administration on the expression of (A) TLR2, (B) TLR4 and (C) TLR6 in the intestinal epithelium of snails. Box plot diagrams display the full range of variation of the mean fluorescence intensity (Digital Units/pixels<sup>2</sup>) as measured in 10 areas/field for a total of 30 fields obtained from immunofluorescence slides of Sgs14-fed and untreated snails. (\*) Statistically significant differences in comparison with the (control) untreated group,  $P < 0.001$ . Immunofluorescence microphotographs showing the expression profile of (D and E) TLR2, (F and G) TLR4 and (H and I) TLR6 in the intestinal mucosa of (D, F, H) untreated and (E, G, I) Sgs14-fed snails.

survival of bacterial strains in acidic environments. In contrast, SgmA-H strains that were isolated from the oesophagus-crop, showed a different tolerance pattern as they were characterized as moderate tolerant to low pH, gastric mucus and gastric juices and no or poor tolerant to pedal mucus, indicating that the isolation site influence the bacterial tolerance properties.

Most of the tested bacterial strains Sgs and Sgm exhibited high cell surface hydrophobicity in association with high autoaggregation and biofilm formation capacity at exponential growth phase. However, the expression level of the above cells surface traits was affected by the growth phase of bacterial strains and only twelve of these strains were able to maintain their high values also at stationary growth phase. It is demonstrated that hydrophobicity, autoaggregation and biofilm formation are strain specific properties and their variations could depend on the physiological state of bacteria cells [36,37], environmental and genetic factors [22] as well as on the structural organization of the cell surface glyco-proteinaceous materials [38]. In addition, PCA revealed positive correlation between the tolerance capacity to pedal mucus, gastric mucus, acidic pH and gastric juices and the expression of the cell surface properties at stationary phase. The bacterial strains that auto-aggregate and form biofilms could be tolerant to the harsh gastrointestinal conditions as they are covered with an extracellular matrix of polysaccharides which protects them from the environmental stresses including low pH and gastric juices [39,40].

The tolerance capacity of the snail gut bacterial strains to pedal mucus, gastric mucus, gastric juices and acidic pH in combination with the cell surface traits proved to be efficient for the discrimination of the gut commensal LABs. The Sgs and Sgm strains were successfully separated into two distinct groups, indicating that the *in vitro* properties are linked to the isolation site, while the Sgs1, Sgs3, Sgs4, Sgs5, Sgs6, Sgs7, Sgs8, Sgs9, Sgs 13, Sgs14 as well as the SgmA and SgmB strains were distinguished from the rest bacterial strains as they exhibited higher *in vitro* probiotic properties than the rest Sgs and Sgm strains, respectively. However, eight of these strains also exhibited immunomodulatory activity since their injection in snail haemocoel resulted in a significant increase of amoebocyte accumulation in snail haemolymph in association with increase of their phagocytic activity. The Sgs14 and SgmB

strains exhibited the highest immunomodulatory activity as they doubled the population of amoebocytes in haemolymph as well as their phagocytic activity in comparison to the control group. Although, both the Sgs14 and SgmB strains exhibited high immunomodulatory activity in haemolymph, only the Sgs14 strain was able to colonize snail gastrointestinal tract. This interaction is characterized as site specific since Sgs14-positive fluorescent signals were detected only on the edges of the internal ridges as well as tissue specific since the FITC-labelled SgmB strain isolated from snail oesophagus-crop failed to interact with snail gut. The SgmB strain exhibited poor tolerance capacity to pedal mucus and poor biofilm formation ability in comparison to Sgs14 and this may explain the inability of this strain to interact with the gut. The entrance of the bacterial strain SgmB to the gastrointestinal tract could have been inhibited by the antimicrobial activity of pedal mucus or SgmB was unable to adhere to intestinal mucosa of snail as it was unable to form biofilm, which is consider to be the first step during adherence of bacterial cells on host surfaces [41,42]. As we have previously showed [21] several presumptive probiotic lactobacilli strains although they were not able to adhere to Caco-2 intestinal epithelial cells, exhibited high immunostimulatory activity such as increased chemotaxis, phagocytosis and cytokine production in mice in association with hydrophobicity and autoaggregation capacity. All the above data indicate that the tolerance and cell surface properties as well as the adhesion ability may not be the only criteria linked to the immunomodulatory activity of LABs but alternative and important components of a complex mechanism which enable a microorganism to interact with and modulate the innate immune responses of the snail.

The interaction of the Sgs14 strain with the gut mucosa of snail resulted in increased recruitment and phagocytic activity of amoebocytes in snail haemolymph demonstrating that *Lactobacillus* adherence capacity is associated with immunomodulatory activities on snail gut epithelium similarly to vertebrates [26]. Amoebocytes are the most important cellular factors of snails as they participate in defense reactions, such as phagocytosis and the production of antimicrobial peptides as well as in wound healing, shell regeneration and nutrient transport [43,44]. Furthermore, oral administration of the Sgs14 strains in snails results in increased antibacterial activity of haemolymph

serum against *E. coli*, *S. aureus* and *B. subtilis* in comparison to the control group. Antibacterial activity of serum has already been demonstrated in untreated *Achatina achatina* [45] and *Helix lucorum* [46] indicating the presence of antimicrobial factors in the serum of snails. The data in this study suggest that the *Lactobacillus plantarum* Sgs14 strain could enhance the production of the antimicrobial factors in haemolymph and increase the bactericidal activity of snail serum against potential pathogens. The Sgs 14 cells or their antigens may directly activate the chemotaxis and phagocytosis of haemolymph cells as well as their activation to produce antimicrobial factors by affecting TLR expression. Our immunofluorescence analysis by using specific anti-mouse TLR2, TLR4 and TLR6 antibodies revealed positive signals in both intestine and haemolymph cells of snails suggesting homology among TLRs in snails and mammals. TLR genes relative to mammals and insects have been reported in various mollusks such as oysters [47], scallops [48] as well as in the freshwater *Biomphalaria glabrata* snails [49]. The expression of TLR2, TLR4 and TLR6 on intestinal epithelium was increased by two to four folds after the oral administration of Sgs14. In contrast no alteration on phagocytic activity was observed when haemolymph from Sgs14-fed snails was incubated with amoebocytes from normal snails, indicating that the increased phagocytosis in Sgs14-fed snails is not mediated by haemolymph factors but by a direct interaction of Sgs14 strain with the haemolymph cells and the gut mucosa. Taking into consideration that the administration of *L. plantarum* Sgs14 results in increased accumulation of amoebocytes, the increased expression of TLR-2, TLR-4 and TLR-6 on Sgs14-fed snails' intestines could be associated either with an increased accumulation of amoebocytes or epithelium cells that express the above TLRs on snails' intestines or with an increased expression of TLR genes and a subsequently increase of TLR proteins on the surface of amoebocytes or epithelium cells on intestines. However, further investigation is needed including the identification of the TLRs gene of *Cornu aspersum* at molecular level as well as the investigation of the differential transcriptional modulation of TLRs gene in different tissues such as haemocytes and intestinal tract upon immune challenge.

In conclusion, in this study we have demonstrated criteria that enable the discrimination of snail intestinal bacteria that exhibit probiotic activity. The *Lactobacillus plantarum* Sgs14 strain exhibited immunostimulatory activity in haemolymph and gut mucosa of snails that is characterized by an increase in chemotaxis and phagocytic activity of amoebocytes in haemolymph, TLR2/TLR4/TLR6 expression on gut mucosa and bactericidal activity of haemolymph serum. This response occurs in association with the capacity of the Sgs14 strain to tolerate the acidic pH, pedal mucus, gastric mucus and gastric juices as well as with high cell surface hydrophobicity, autoaggregation and biofilm formation ability, indicating that the above *in vitro* properties are appropriate for the screening of the most promising presumptive probiotic strains for snails. Thus, the rebiosis by the establishment of a permanent beneficial microflora on the gastrointestinal tract of snails by using commensal gut symbiots such as the Sgs14 strain is suggested as a novel strategy to eliminate dysbiosis and improve snail health and farming.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2019.06.061>.

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