



Full Length Article

Studies on the effect of *Lactococcus garvieae* of dairy origin on both cheese and Nile tilapia (*O. niloticus*)Eman N. Abdelfatah^{a,*}, Heba Hassan H. Mahboub^b^a Department of Food Control, Faculty of Veterinary Medicine, Zagazig University, Zagazig City 44511, Sharkia, Egypt^b Department of Fish Diseases and Management, Faculty of Veterinary Medicine, Zagazig University, Zagazig City 44511, Sharkia, Egypt

ARTICLE INFO

Keywords:

Bacteriocin
Dairy products
Lactic acid bacteria
Lactococcus garvieae
Oreochromis niloticus
Staphylococcus aureus

ABSTRACT

The aims of this study were to evaluate the activity of *Lactococcus garvieae* of dairy origin against pathogenic bacteria during cheese manufacture and its suitability and safety as a probiotic on Nile tilapia (*Oreochromis niloticus*). For these purposes, *Lactococcus garvieae* isolated from raw cow milk was tested to control the growth of *Staphylococcus aureus* in artificially contaminated cheese during storage under refrigeration. Also a feeding experiment was conducted on 120 *Oreochromis niloticus* using a diet containing *Lactococcus garvieae* as a probiotic bacteria against pathogenic *S. aureus*. The findings of this study showed that *Lactococcus garvieae* of dairy origin produced inhibitory substances against pathogenic microorganisms. The selected strain had a good inhibitory activity against *Staphylococcus aureus* in artificially contaminated cheese during refrigerated storage. Concerning fish experiment, it showed no evidence of disease in fish that were fed a diet containing *Lactococcus garvieae*, and showed a higher survival rate than others. Further investigations for purification of the produced inhibitory substance and confirming that is a bacteriocin-like substance are needed. Nonetheless, it is the first report of using *L. garvieae* of dairy origin as a probiotic for controlling the pathogenic *Staphylococcus aureus* in *Oreochromis niloticus*.

1. Introduction

Lactococcus garvieae (*L. garvieae*) is one of the genus *Lactococcus* species [1]. In the past, the species of this genus were known as the lactic acid producing members of streptococci. They are not pathogenic for human or even animals [2], except *L. garvieae*, which is considered the only pathogenic *Lactococcus* species. It causes a septicemic process called lactococcosis, that was defined in rainbow trout in Japan for the first time [3]. Since then, *L. garvieae* has been identified as the main cause for many outbreaks in other fish species in several countries [4]. Currently, it was discovered that *L. garvieae* is not limited to aquatic species; as it has been caused mastitis in cows and has been found in some dairy products such as goat cheese and raw cow milk [5]. Additionally, *L. garvieae* is considered also an emerging zoonotic pathogen [6]. Thus, the importance of *L. garvieae* is increasing in all fields of life either in human or in animals, but the available data for this new pathogen in foods other than fish products are still very scarce [5].

In contrast, the bacteriocins produced by lactococci have been studied extensively; these substances are antimicrobial peptides synthesized by the bacterial ribosome that act mainly against closely related

species [7]. Nisin is possibly the most important known bacteriocin. It is produced by *Lactococcus lactis* strains and used as a food preservative [8]. During a survey of Lactic Acid Bacteria (LAB) for other bacteriocins, antimicrobial substances produced by *L. garvieae* strain were identified and termed garviecin L1-5 [9]. Then in the last ten years, several new bacteriocins from *L. garvieae* have been reported including, garvicin ML [10], garvieacin Q [11], garvicin A [12], and garvicin KS [13]. Likewise, there are other non-purified bacteriocins produced by *L. garvieae* detected by Suneel and Kaliwal [14].

L. garvieae isolated from raw milk and dairy products have been reported to inhibit indicator strains due to the production of bacteriocin [15], while Alomar [16] suggested that hydrogen peroxide may play a role in the inhibition of *Staphylococcus aureus* (*S. aureus*) by *L. garvieae*. The efficiency of *L. garvieae* for inhibition of *S. aureus* may also depend on the interactions of both these organisms with the raw milk microflora [17].

Staphylococcus aureus is one of the most prevalent enterotoxin producing microbes, and it is considered the main cause for staphylococcal food poisoning and gastroenteritis worldwide. Enterotoxin production by this strain at levels hazardous to public health has been reported in

Peer review under responsibility of Faculty of Veterinary Medicine, Cairo University.

* Corresponding author.

E-mail address: emannabil1982@yahoo.com (E.N. Abdelfatah).<https://doi.org/10.1016/j.ijvsm.2018.11.002>

Received 31 July 2018; Received in revised form 7 November 2018; Accepted 8 November 2018

Available online 17 November 2018

2314-4599/ © 2018 Faculty of Veterinary Medicine, Cairo University. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

different cheese varieties [18]. Cheese manufacturing from raw milk can lead to staphylococcal outbreaks, especially when the curd is insufficiently acidified, or when the cheese manufacture occurred under poor hygienic conditions [18]. New biopreservation strategies based on using the inhibitory effect of some bacterial strains, including some strains of microbial communities of raw milk such as LAB, could help in the control of pathogenic *S. aureus* strain in cheese by several ways including, bacteriocin production, lower pH [19], and H₂O₂ [20]. On the other hand, Staphylococci are not part of the normal fish microflora and its presence on fish is an indicator for a disease [21,22]. Currently, *S. aureus* has been recorded recently in *Oreochromis niloticus* (*O. niloticus*) causing high mortalities with different histopathological changes [23]. Also, it causes a health hazard for fish handlers and consumers [24]. For these reasons, we choose *S. aureus* as an indicator to test the effects of the *L. garvieae* bacteriocin. It is worth mentioning that the administration of probiotics during tilapia fish farming through feed can improve their feed conversion ratio (FCR) and can reduce mortality among the fish by 20% [25]. Furthermore, in finfish, immune responses can be increased using many probiotics through the stimulation of innate and cellular immunity [26].

Therefore, the aims of the present study were evaluation of the ability of *L. garvieae* strain to control the pathogenic effects of *S. aureus* during cheese manufacture, and assessment its activity against pathogenic *S. aureus* in *O. niloticus*. Also this study aimed to assess its safety and its potential use as a probiotic in dairy products and fish. Finally, we evaluated the ability of this strain to produce inhibitory substances to explain the possible cause of the inhibitory effect on pathogenic bacteria.

2. Materials and methods

2.1. Bacterial strains and culture conditions

A lyophilized stock culture of pathogenic *S. aureus* strain (ATCC 6538) was obtained as a reference pathogenic strain from the Microbiology Department in the Faculty of Veterinary Medicine at Zagazig University, Egypt. The culture was grown in broth (brain heart infusion; Oxoid) at 37 °C for 18–24 h. *L. garvieae* strain was obtained from our previous work (we chose the strain that carry only *Fbp* gene) [27] in which it was isolated, identified, and stored frozen at –80 °C in broth culture contained 15% (w/v) glycerol. Then it was cultured on M17 medium anaerobically at 37 °C for 48 h.

2.2. Assessment of the antimicrobial activity of *L. garvieae* during cheese manufacturing

2.2.1. Preparation of cultures [28]

The used bacterial strains were preserved at –80 °C in frozen broth with 15% glycerol. *S. aureus* and *L. garvieae* strains were subcultured for two times and then exposed to decimal serial dilutions, after which they were plated on specific agar media to determine the viable cell numbers, on Baird Parker agar at 37 °C for 24–48 h and M17 agar anaerobically at 30 °C for 24 h, respectively.

Table 1

Counts of *S. aureus* in cheese during refrigerated storage over 10 days.

Cheeses containing (CFU/g)	Storage time (days)					
	0	2	4	6	8	10
(A) Cheese without added <i>L. garvieae</i> that was experimentally contaminated with <i>S. aureus</i>	3.35 ± 0.04	3.35 ± 0.05	3.51 ± 0.02	3.73 ± 0.02	4.77 ± 0.01	5.97 ± 0.06
(B) Cheese containing <i>L. garvieae</i> that was experimentally contaminated with <i>S. aureus</i>	3.66 ± 0.02	3.49 ± 0.04	3.48 ± 0.03	3.53 ± 0.04	3.84 ± 0.02	3.90 ± 0.02
(C) Control cheese that contained no added cultures	< 1	< 1	< 1	< 1	< 1	< 1

2.2.2. Preparation of cheeses containing *L. garvieae*

For cheese preparation, we must use only milk free from the used cultures thus, pasteurized milk was first bacteriologically tested for the presence of *S. aureus* and *L. garvieae* using Baird Parker agar at 37 °C for 24 h, and M17 agar at 30 °C for 24 h, respectively. The cheese was then manufactured according to the method previously described by Abou-Donia [29], with adding 10⁸ colony forming units (CFU)/mL of the *L. garvieae* strain. To contaminate cheese with *S. aureus* culture, 10³ CFU/g of it was added during the agitation step to the salted curd. Overall, we prepared three different batches of fresh cheese:

- Cheese made from Pasteurized milk that was experimentally contaminated with *S. aureus* only.
- Cheese made from Pasteurized milk that was experimentally contaminated with *S. aureus* and containing *L. garvieae*.
- Control cheese that was prepared with no added cultures.

2.2.3. Microbial examination of the prepared cheeses

The experimentally contaminated cheeses, and non-contaminated control cheese were subjected to counts of *S. aureus* and *L. garvieae*, at day zero and then every two days, till the end of the experiment duration at day ten. For this purpose, 25 g of each cheese batch were resuspended with 225 mL of peptone water at concentration 0.1% and were then subjected to several serial dilutions in the same broth, followed by spread plating (0.1 mL) on Baird Parker agar at 37 °C for 48 ± 2 h and on M17 agar at 30 °C for 24 h to enumerate *S. aureus* and *L. garvieae*, respectively. Growing colonies were enumerated, and the results were expressed as CFU/g. The experiments were repeated three times in separated occasions.

2.3. Assessment of pathogenicity of the examined *S. aureus* in fish

2.3.1. Assessment of pathogenicity of the examined *S. aureus* in fish

A total number of 60 *O. niloticus* that seemed healthy with an average body weight of 50 ± 5 g were randomly selected and divided in six glass fish tanks (80 × 60 × 30 cm) containing 80 L of water, resulting in a stocking rate of ten fish per tank. The fish tanks were provided with everyday refreshed dechlorinated tap water with the temperature maintained at 22 ± 2 °C during the experimental period, and an air pump for continuous aeration. The fish were adapted in this environment for 14 days and were provided with a basic food two times a day. A bacterial suspension was prepared by culturing the bacterium for 24 h in tryptic soy broth (TSB). One mL of inoculums contained approximately 10¹⁰ CFU/mL was diluted in 1 L distilled water to get a final adjustment to the bacterial culture to 10⁷ CFU /mL [23]. We classified the fish into 2 groups (each with three replicates “N = 10”). The first group of fish was inoculated intraperitoneally (I.P) with 0.5 mL of the prepared bacterial suspension, the inoculation dose was selected according to a biological test (data not shown). The second group of fish was served as a negative control by inoculating fish I.P with 0.5 mL of sterile saline solution. The fish groups were checked regularly, and mortalities were recorded for 14 days (Table 2). Dead fish were examined bacteriologically for bacterial re-isolation.

Table 2
Mortality rates record in pathogenicity experiment with *S. aureus* and in feeding experiment using *L. garvieae*.

Experiments	No. of fish	Mortalities within							No of dead fish	Mortality rate %
		48 h	72 h	96 h	5 days	week	10 Days	2 weeks		
Pathogenicity experiment with <i>S. aureus</i>										
Group 1	30	2	3	5	4	5	6	5	30	100%
Group 2	30	0	0	0	0	0	0	0	0	0
Feeding experiment using <i>L. garvieae</i>										
Group 1*	30	0	0	0	0	0	0	0	0	0
Group 2*	30	0	0	0	0	0	0	0	0	0
Group 3*	30	2	2	4	3	5	7	4	27	90%
Group 4*	30	1	3	4	2	3	2	–	15	50%

Group 1, fish inoculated IP with 0.5 mL of *L. garvieae*, Group 2, fish inoculated IP with 0.5 mL of saline solution, Group 1* fish fed an ordinary diet, Group 2*, fish fed a diet containing *L. garvieae*, Group 3* fish infected with *S. aureus* and fed an ordinary diet and Group 4*, fish infected with *S. aureus* and fed a diet containing the probiotic bacterium.

Table 3
Immunological parameters following the feeding experiment.

Groups	Parameters		
	IgM (mg/mL)	Lysozyme (µg/mL)	Nitric Oxide(µg/mL)
Gp. (1)	0.57 ^b	0.37 ^b	35.38 ^b
Gp. (2)	0.96 ^a	0.66 ^a	49.60 ^a
Gp. (3)	0.26 ^d	0.17 ^c	20.71 ^c
Gp. (4)	0.64 ^b	0.39 ^b	36.52 ^b
SEM ¹	0.07	0.05	3.08
P value	0.000	0.000	0.000

SEM¹, Standard error of the mean.

Means bearing different superscripts within the same column are significantly different ($P < .05$). Highest value is represented by the letter (a), followed by the letter (b), then (c) and letter (d) represent the lowest value.

Group (1), control group; Group (2), fish fed a diet containing the probiotic bacterium; Group (3), fish infected with *S. aureus*, and Group (4), fish infected with *S. aureus* that were fed a diet containing the probiotic bacterium

2.3.2. Re-isolation of *S. aureus* from morbid fish

We examined the newly dead or moribund fish. Bacteria were isolated under aseptic conditions from some internal organs. Samples were inoculated on Tryptone Soya Broth, then incubated at 37 °C for 24 h. Loopfuls were taken from the broth media and streaked on the surface of Baird parker agar plates, then incubated at 37 °C for 24 h. The grown colonies on the plates were identified using biochemical tests [30]. Gram staining, Oxidase and Catalase tests were performed to confirm the cause of morbidity or mortality [31].

2.4. Assessment of the effect of *L. garvieae* on fish

2.4.1. Assessment of the effect of *L. garvieae* on fish in vivo to ensure its safety

A total number of 60 *O. niloticus* that seemed healthy with an average body weight of 50 ± 5 g were randomly selected and divided them in six glass fish tanks (80 × 60 × 30 cm) containing 80 L of water, resulting in a stocking rate of ten fish per tank. The fish were adapted for 14 days, and then we put the fish into 2 groups (each with three replicates). The first group was inoculated I.P with 0.5 mL of the suspension containing 10⁷ *L. garvieae* [32], and the second group was served as a negative control by inoculating it I.P with 0.5 mL of sterile saline solution. The fish groups were checked regularly, and the living and dead fish numbers were recorded for 14 days. Dead fish were examined bacteriologically for bacterial re-isolation.

2.4.2. Assessment of the probiotic activity of *L. garvieae* in fish in vivo [33]

2.4.2.1. Preparation of feed with probiotic. *L. garvieae* cells were prepared by inoculating the bacterium in TSB and incubating for 48 h at 30 °C. The culture was centrifuged at 3000 rpm for 30 min, then the

bacteria were cleaned with sterile saline solution for two times. The final bacterial concentration in this saline suspension was adjusted to 10⁷ cells/mL as mentioned before [32]. For the feeding experiment, the bacterial suspension containing *L. garvieae* isolate was added to the marketed food using an automatic mixer to give 1 × 10⁷ bacterial cells/g. The pellets were subjected to air for 24 h at room temperature to dry and then refrigerated till using (4 °C). Then the viability of *L. garvieae* in the stored feed was evaluated twice during the period of the experiment (once every week) according to Irianto and Austin [34].

2.4.2.2. Feeding experiment. A total of 120 *O. niloticus* that seemed healthy with an average body weight of 50 ± 5 g were distributed in four equal groups, each with three replicates (10 fish per replicate), which were divided in three glass fish tanks (80 × 60 × 30 cm), resulting in stocking rate 10 fish per tank. The fish were adapted for 2 weeks during which they were fed an artificial diet, and supplied with continuously aerated dechlorinated water with the temperature kept up at 20 ± 2 °C. Fish in the first and third groups were fed on a diet without bacterial supplementation during the feeding experiment. Fish in the second and fourth groups were received a diet containing 10⁷ *L. garvieae* bacterial cells/g at 5% biomass/day two times a day. After 14 days of the feeding experiment, the fish in the first and second groups were served as controls, so they were injected I.P with 0.5 mL of sterile saline solution. We made I.P injection for the third and fourth groups with 0.5 mL of the *S. aureus* suspension contained 10⁷ bacterial cells/mL which was prepared according to Gaafar et al. [23]. The injected fish were observed regularly during the period of the experiment, and the mortality rate was recorded; dead fish were examined bacteriologically for bacterial re-isolation. At the end of the experimental period, blood samples were collected from the caudal blood vessel of each fish in each group [35]. The blood samples were centrifuged at 3000 g for 15 min and the supernatant serum was collected and stored at –20 °C until used for biochemical factors include Lysozyme, IgM and immunoglobulin.

2.4.2.3. Humoral immunological studies. Serum Lysozyme activity was measured using a modified turbidimetry method described by Ellis [36]. Nitric Oxide Assay: The nitric oxide (NO) level in each tested serum sample was measured using the method described by Ragaraman et al. [37]. Immunoglobulin M (IgM) was determined by nephelometry method (MININEPH TM Human Kit, the binding Site Ltd, Birmingham, UK).

2.5. Preparation of *L. garvieae* culture supernatant

Isolated *L. garvieae* bacterial strain was inoculated into 100 mL of M17 broth and incubated at 37 °C for 24 h. Then, centrifugation of the broth was applied at 12,000 rpm for 20 min, where the cell residues were discarded, giving rise to a clear supernatant free from cells.

Adjustment of the supernatant pH to 5.0 was made with 1 N NaOH, and it was then evaporated using rotary flash evaporation; sterilized with 0.22- μm filter paper (Millipore, India). This solution was used to assess the antimicrobial activity of *L. garvieae*.

2.6. Assessment of the activity of the inhibitory substances produced by *L. garvieae* in vitro

The activity of the supernatant was evaluated via the agar well diffusion method [38]. Lyophilized stock cultures of *S. aureus* were used as an indicator strain. Briefly, molten M17 agar media (45 °C) was first injected (1% v/v) with a standardized suspension of *S. aureus*, then this medium was rapidly distributed into sterile Petri dishes. After solidification of the medium, 3 wells of 6 mm diameter for each were made into the agar. These wells were filled with different amount of previously produced BLS as, 15 μL , 30 μL , and 45 μL , where the effects of organic acids as antimicrobial were excluded by adding 1 N NaOH to make an adjustment to pH until reach 6.5 [39]. The plates were allowed to diffuse for 2 h at 4 °C, then they were incubated at 37 °C as it was the best condition for the indicator strain growth and examined after 24 h [40]. The zones of inhibition were detected in millimeters by the scale used for zone interpretation (HiMedia, Mumbai).

2.7. Statistical analysis

All data were analyzed using SPSS software (v. 16). Analysis of data was performed using one-way analysis of variance (ANOVA). Tukey's honest significant difference (HSD) multiple comparison test was used to check the significance of differences between the mean values. The alpha level for the determination of significance was set to .05. Means in the same column followed by different letters are significantly different, and the highest value is represented by the letter (a).

3. Results

Microbiological examination of the Pasteurized milk used in cheese manufacture showed undetectable levels of *S. aureus* and *L. garvieae*. As shown in Table 1, *S. aureus* could be grown in cheeses injected with 10^3 CFU/g of the strain during refrigerated storage as the log count of *S. aureus* was 5.97 ± 0.06 CFU/g at the end of the experiment refrigeration time. When the cheeses were prepared with both, the *L. garvieae* and *S. aureus* strains, inhibition of *S. aureus* growth was detected, and the average counts of this pathogen at the end of the experiment refrigeration time were lower than that of the other category of cheese where the *L. garvieae* strain was absent (3.90 ± 0.02 CFU/g, Fig. 1). These results showed that the inhibition of *S. aureus* in cheese

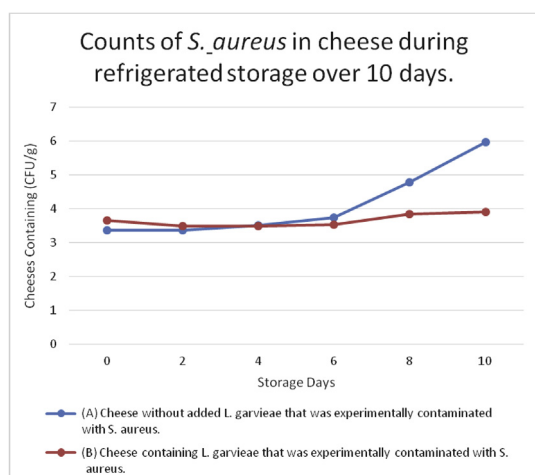


Fig. 1. Counts of *S. aureus* in cheese during refrigerated storage over 10 days.

might have occurred due to the production of inhibitory substances.

On the other hand, as it was shown in (Table 2), the used *S. aureus* strain gave a mortality rate in the second day post-inoculation. Then increased gradually to reach 100% at the end of the experiment in the group of fish which were injected I.P with this bacterium. While no mortality occurred in the control group. These results indicated that this strain was extremely pathogenic to *O. niloticus*. Postmortem lesions were enlargement and congestion of kidneys, spleen, and liver. After re-isolation of bacteria from internal organs (kidneys, liver, and spleen) of fish, *S. aureus* was isolated and identified as yellow halo colony surrounding the yellow zone on mannitol salt agar media. The gram staining of these colonies revealed typical gram positive cocci in grape like clusters, Oxidase negative and catalase positive.

Additionally, the examined *L. garvieae* strain after I.P injection gave no signs of disease or caused mortality, thus we can evaluate *L. garvieae* as harmless to *O. niloticus*, and it was therefore considered safe for use in these fish.

Our results also showed no evidence of mortality or signs of disease in the second group of fish that received a diet containing *L. garvieae* bacteria during the period of the feeding experiment, and the survival rate of this group was 100%, unlike the control group. While the survival rate was 10% in the third group which fed on an ordinary diet and injected with *S. aureus* and was 50% in the fourth group which fed on a diet containing *L. garvieae* and injected with *S. aureus*. Re-isolation of *S. aureus* was done from internal organs of morbid fish in the third and fourth groups as mentioned before. Table 3 showed that, group 2, which received a diet containing *L. garvieae*, showed a highly significant increase in IgM levels, lysozyme activity, and nitric oxide levels, however, these levels were highly significantly decreased in group 3, which was infected with *S. aureus*. Group 4, which was infected with *S. aureus* and fed a diet containing the probiotic bacterium was higher in Lysozyme activity, IgM and nitric oxide compared to the control group which fed an ordinary diet. So, these results confirmed the role of *L. garvieae* in improving the fish defense mechanism by elevating the immunological parameters and show the bad effect of *S. aureus* through decreasing the immunological parameters of *O. niloticus*.

To know the possible cause of the inhibitory activity of *L. garvieae* against a pathogenic *S. aureus* strain in vitro, we prepared the culture supernatant, then assessed the antimicrobial activity using the agar well diffusion method. The *L. garvieae* strain was found to have antimicrobial activity. As the supernatant derived from it showed moderate zones of inhibition with different amounts; for example, with 15 μL , 30 μL and 45 μL of supernatant, zones of inhibition were 18 mm, 20 mm and 23 mm, respectively, formed against *S. aureus*. This inhibition may be occurred due to proteinous substances produced mainly by LAB. Inhibition due to other causes including, hydrogen peroxide or organic acids was prevented through culturing the producer strain under anaerobic condition, and neutralizing the culture supernatant before applying the antimicrobial activity.

4. Discussion

Over the last few years, members of the genus lactococcus such as *Lactococcus lactis* and other strains producing antimicrobial substances have been isolated from various products, including meat, meat products, vegetable products, dairy products, and raw milk [41,42]. They are considered as a technological control method for controlling pathogenic bacteria like *S. aureus* in spite of the traditional chemical one. Also, many authors have been reported *L. garvieae* as a component of the natural microbiota of different dairy products manufactured from raw milk [43–45].

Regarding the first part of our work in cheese, the results cleared that the average counts of *S. aureus* in cheese contained both *S. aureus* and *L. garvieae* strains, at the end of the experimental refrigeration time, were lower than that of the cheese in which the *L. garvieae* strain was absent. Other studies applied on the bacteriocins producing *L. garvieae*

suggest that their effects on *S. aureus* in cheese can vary in accordance with the type of cheese and the bacteriocin-producing strain. *S. aureus* increased by 1.8 log CFU/g in Manchego cheese when it was made from milk without bacteriocin-producing bacteria [46]; however, this increase was lower than the 2 log CFU/g, and 3 log CFU/g in case of Feta cheese, and camembert-type goat cheese [18,47] respectively. Conversely, other authors found that *L. garvieae* had a bacteriostatic effect on *S. aureus* in both shaken and static buffered BHI cultures [17]. Others found that the addition of bacteriocinogenic strains of Lactic Acid Bacteria to milk during cheese manufacture ended with only slight inhibition of *S. aureus* [19].

Effects of other bacteriocins as nisin on *S. aureus* in cheese have been previously reported in cheese manufacturing [48–50].

Several previous works have been applied on *L. garvieae*, where bacteriocins have been detected and purified. The bacteriocin, termed garvicin KS (GarKS), is produced by *L. garvieae* strains isolated from raw milk and it has a wide inhibitory spectrum against important pathogens belonging to the genera *Staphylococcus*, *Bacillus*, *Listeria*, and *Enterococcus* [9]. Also, Garvicin L1-5 is a small bacteriocin, with a molecular mass of about 2.5 kDa, produced by *L. garvieae* L1-5 isolated from a raw cow's milk sample. It inhibits bacteria from the *Lactococcus*, *Listeria*, *Enterococcus*, and *Clostridium* genera [13]. *L. garvieae* IPLA 31405, isolated from among the normal microbiota of a raw-milk cheese [51]. It lacks hemolysin and gelatinase activities, and produces a bacteriocin active against food-borne pathogens [5]. Bacteriocins can be produced also from *L. garvieae* of nondairy origin and they have different antimicrobial activities against bacteria [10–12].

However, a slight genetic relation between dairy isolates and fish isolates of *L. garvieae* was detected [52]. The strains of dairy origin were evaluated to have a weak lactose acidifying capacity and a low incidence of known virulence factors. While those of fish origin have not any acidifying properties and have many virulence factors [53]. Although, *L. garvieae* might contribute to improve physical properties of dairy products, and no reports have been recorded about the association between consumption of raw-milk cheese and *L. garvieae* infections in human [5,52,53]. The safety of *L. garvieae* of dairy origin should be detected before using it as a biopreservative in food.

On the other hand, the experimental challenge with *S. aureus* showed its higher pathogenicity to *O. niloticus* because of the high mortality rates recorded (Table 2). These results are coordinated with Gaafar et al. [23] who recorded remarkable mortalities in *O. niloticus* after applying pathogenicity test with *S. aureus*.

Regarding the fact that the diseases have been spread in the aquatic environments every year, it clears that it is preferable to protect fish against the infection with pathogenic bacteria to prevent losses of fish. This can be achieved by adding a probiotic bacteria to fish diet to increase the resistance against the disease and to minimize damages or losses. In our work, we used *L. garvieae* of dairy origin to evaluate its effect on *S. aureus*. The results showed that *L. garvieae* had an inhibitory effect on *S. aureus* *in vivo* with no disease signs or mortality after I.P injection into *O. niloticus*. These results are similar to those detected by some authors [54–56] as they recorded a new *L. garvieae* subspecies strain has a probiotic activity. Also, they mentioned that *L. garvieae* is able to produce a novel bioactive peptide and a volatile phenol compound that can be used as food additives to improve food safety due to their antifungal and antioxidant properties.

In our work, there were no signs of disease on fish fed a diet supplemented with *L. garvieae* after being challenged with *S. aureus* and we also observed a higher survival rate in these fish. Similar findings were reported by Robertson et al. [57], who observed a higher survival rate in the fish group fed probiotics for a period of time in spite of the presence of pathogenic bacteria. Furthermore, other studies reported the same effect of feed supplemented with probiotics in fish challenged with other microbes such as *Aeromonas hydrophila* [33]. On the other hand, dietary supplementation with *L. garvieae* appeared to elevate the serum lysozyme activity, immunoglobulins, and nitric oxide, thus it

improved the *O. niloticus* immune status. Also, fish group infected with *S. aureus* and fed a diet containing the *L. garvieae* bacterium had a higher Lysozyme activity, IgM and nitric oxide compared to the control group, so *L. garvieae* has an immunological role in reducing the infectivity with *S. aureus*. Similar results were recorded by Nikoskelainen et al. [58], who reported an improvement in the immunity of rainbow trout through using probiotics by stimulating phagocyte activity, production of immunoglobulin, and complement-mediated bacterial killing. Also, it was found that the serum lysozyme activity in fish was elevated after adding *Bacillus subtilis* and *Lactobacillus acidophilus* to their diet [59]. Moreover, *Lactococcus lactis* was used as a probiotic treatment against *A. hydrophila* in tilapia fish and resulted in elevations of respiratory burst activity, lysozyme activity and superoxide dismutase [60]. However, the fish group which infected only with *S. aureus* strain, showed the lowest levels of the measured immunological parameters. This can be explained by the presence of extraordinary numbers of virulence factors that allow *S. aureus* to resist extreme conditions, and affect host cell [61].

As known before *L. garvieae* had an inhibition effect on some types of bacteria especially, coagulase-positive bacteria due to nutritional competition or H₂O₂ production. Hydrogen peroxide has a destructive effect on the microbial populations as it may cause a rapid bacteriostatic or even bactericidal effect especially gram-negative bacteria [62], but in our work we excluded this effect as discussed before. So, we tried to detect and obtain the inhibitory substances produced by *L. garvieae* strain isolated from raw dairy products. For this purpose, we prepared the culture supernatant. This solution was used to assess the antimicrobial activity of *L. garvieae*, using the agar well diffusion method.

Our results revealed the production of moderate zones of inhibition with different amounts of supernatant containing the inhibitory substance produced by *L. garvieae*. These results are in line with the fact shows that bacteriocins are expressed at low levels, due to the interactions between bacteriocins and milk components, and the availability of nutrients necessary for bacterial growth and the manufacture of these materials [63]. This moderate inhibitory effect can be influenced by different factors exist in food, inactivation by food pH or enzymes, poor solubility, unequal distribution in the food matrix. Additionally, low stability during food shelf life, the diversity, and sensitivity to the microbial load of food also play an important role [64]. Our results are nearly similar to that detected by Suneel and Kaliwal [14]. Another author found that, the antimicrobial substance produced by *L. garvieae* strain was active against closely related species, tested Gram-positive bacteria, and Gram-negative strains [15]. Finally, we can detect that our work can be considered the first one that demonstrates the great potential of using *L. garvieae* of dairy origin in the treatment of diseases in fish caused by pathogenic bacteria like *S. aureus*.

5. Conclusions

L. garvieae of dairy origin can produce inhibitory substance which can control pathogenic *S. aureus* during cheese manufacture. Further studies on purification of this substance and applying tests to confirm it as a bacteriocin are needed. Also, more studies to investigate the activity of pure one on pathogenic microorganisms are recommended to open new possibilities for its application on the improvement of dairy product industry. This study is the first report of using *L. garvieae* of dairy origin as a probiotic for controlling the pathogenic *S. aureus* in *O. niloticus*, as the results demonstrate the great potential of using this *L. garvieae* strain for the treatment of diseased fish. Other works in the future for applying it as an alternative to the existing antibiotics used in treatment of fish diseases are also recommended.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interests

None declared.

Ethical approval

This study was approved by the Committee of Animal Welfare and Research Ethics, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Acknowledgements

The authors thank the participants in this work. This work was performed using the facilities of the Faculty of Veterinary Medicine, Zagazig University.

References

- Cai Y, Yang J, Pang H, Kitahara M. *Lactococcus fujiensis* sp. Nov, a lactic acid bacterium isolated from vegetable matter. *Int J Syst Evol Microbiol* 2011;61:1590–4. <https://doi.org/10.1099/ijs.0.025130-0>.
- Ruoff KL. *Leuconostoc, Pediococcus, Stomatococcus, and miscellaneous gram-positive cocci that grow aerobically*. In: Murray PR, Baron EJ, Pfaller MA, Tenoer FC, Tenover RH, editors. *Manual of clinical microbiology*. 6th ed. Washington, D.C.: American Society for Microbiology; 1995. p. 315–23. ISBN: 9781555810863.
- Vendrell D, Balcázar JL, Ruiz-Zarzuola I, de Blas I, Gironés O, Múzquiz JL. *Lactococcus garvieae* in fish: a review. *Comp Immunol Microbiol Infect Dis* 2006;29:177–98. <https://doi.org/10.1016/j.cimid.2006.06.003>.
- Evans JJ, Klesius PH, Shoemaker CA. First isolation and characterization of *Lactococcus garvieae* from Brazilian Nile tilapia, *Oreochromis niloticus* (L.), and pintado, *Pseudoplatystoma corruscans* (Spix & Agassiz). *J Fish Dis* 2009;32:943–51. <https://doi.org/10.1111/j.1365-2761.2009.01075.x>.
- Fernández E, Alegria A, Delgado S, Mayo B. Phenotypic, genetic and technological characterization of *Lactococcus garvieae* strains isolated from a raw milk cheese. *Int Dairy J* 2010;20:142–8. <https://doi.org/10.1016/j.idairyj.2009.11.004>.
- López-Campos G, Aguado-Urda M, Mar Blanco M, Gibello A, Cutuli MT, López-Alosa V, et al. *Lactococcus garvieae*: a small bacteria and a big data. *Health Inform Sci Syst* 2015;3:1–9.
- Cotter PD, Hill C, Ross RP. Bacteriocins: developing innate immunity for food. *Nat Rev Microbiol* 2005;3:777–88. <https://doi.org/10.1038/nrmicro1273>.
- Delves-Broughton J. Nisin and its uses as a food preservative. *Food Technol J* 1990;100:106–12. <https://doi.org/10.1111/j.1471-0307.1990.tb02449.x>.
- Villani F, Aponte M, Blaiotta G, Mauriello G, Pepe O, Moschetti G. Detection and characterization of a bacteriocin, garviecin L1–5, produced by *Lactococcus garvieae* isolated from raw cow's milk. *J Appl Microbiol* 2001;90:430–9. <https://doi.org/10.1046/j.1365-2672.2001.01261.x>.
- Borrero J, Brede DA, Skaugen M, Diep DB, Herranz C, Nes IF, et al. Characterization of garvicin ML, a novel circular bacteriocin produced by *Lactococcus garvieae* DCC43, isolated from mallard ducks (*Anas platyrhynchos*). *Appl Environ Microbiol* 2011;77:369–73. <https://doi.org/10.1128/AEM.01173-10>. Epub 2010 Nov 5.
- Tosukhowong A, Zendo T, Viessangan W, Roytrakul S, Pumpuang L, Jaresthitkunchai J, et al. Garvieacin Q a novel class II bacteriocin from *Lactococcus garvieae* BCC 43578. *Appl Environ Microbiol* 2012;78:1619–23. <https://doi.org/10.1128/AEM.06891-11>.
- Maldonado BA, Cárdenas N, Martínez B, Ruiz-Barba JL, Fernández-Garayzábal JF, Rodríguez JM, et al. Novel class II d bacteriocin from *Lactococcus garvieae* that inhibits septum formation in *L. garvieae* strains. *Appl Environ Microbiol* 2013;79:4336–46. <https://doi.org/10.1128/AEM.00830-13>.
- Ovchinnikov KV, Chi H, Mehmeti I, Holo H, Nes I, Diep DB. Novel group of leaderless muropeptide bacteriocins from gram-positive bacteria. *Appl Environ Microbiol* 2016;82:5216–24. <https://doi.org/10.1128/AEM.01094-16>.
- Suneel D, Kaliwal B. Identification and characterization of *Lactococcus garvieae* and antimicrobial activity of its bacteriocin isolated from cow's milk. *Asian J Pharm Clin Res* 2013;6:104–8. ISSN – 0974-2441.
- Villani F, Aponte M, Blaiotta G, Mauriello G, Pepe O, Moschetti G. Detection and characterization of a bacteriocin, garviecin L1–5, produced by *Lactococcus garvieae* isolated from raw cow's milk. *J Appl Microbiol* 2001;90:430–9.
- Alomar J. Study of physiological properties of *Lactococcus lactis* and *Lactococcus garvieae* for the control of *Staphylococcus aureus* in technology Cheese [PhD. dissertation]. Nancy: National Polytechnic Institute of Lorraine; 2007.
- Delbes-Paus C, Dorchie G, Chaabna Z, Callon C, Montel MC. Contribution of hydrogen peroxide to the inhibition of *S. aureus* by *Lactococcus garvieae* in interaction with raw milk microbial community. *Food Microbiol* 2010;27:924–32. <https://doi.org/10.1016/j.fm.2010.05.031>.
- Meyrand A, Boutrand-Loei S, Ray-Guenot S, Mazuy C, Gaspard CE, Jaubert G. Growth and enterotoxin production of *Staphylococcus aureus* during the manufacture and ripening of Camembert-type cheeses from raw goats' milk. *J Appl Microbiol* 1998;85:537–44. <https://doi.org/10.1046/j.1365-2672.1998.853531.x>.
- Arqués JL, Rodríguez E, Gaya P, Medina M, Guamis B, Nuñez M. Inactivation of *S. aureus* in raw milk cheese by combinations of high pressure treatments and bacteriocin-producing lactic acid bacteria. *J Appl Microbiol* 2005;98:254–60. <https://doi.org/10.1111/j.1365-2672.2004.02507.x>.
- Otero M, Nader-Macias ME. Inhibition of *S. aureus* by H₂O₂ – producing *Lactobacillus gasseri* isolated from the vaginal tract of cattle. *Anim Reprod Sci* 2006;96:35–46. <https://doi.org/10.1016/j.anireprosci.2005.11.004>.
- Atyah MA, Zamri-Saad M, Siti-Zahrah A. First report of methicillin-resistant *Staphylococcus aureus* from cage-cultured tilapia (*Oreochromis niloticus*). *Vet Microbiol* 2010;144:502–4. <https://doi.org/10.1016/j.vetmic.2010.02.004>.
- Hammad AM, Watanabe W, Fujii T, Shimamoto T. Occurrence and characteristics of methicillin-resistant and -susceptible *Staphylococcus aureus* and methicillin-resistant coagulase-negative staphylococci from Japanese retail ready-to-eat raw fish. *Int J Food Microbiol* 2012;156:286–9.
- Gaafar AY, Soliman MK, Ellakany HF, Affr NA, Elbially AK, Mona SZ, et al. Comparative pathogenicity of methicillin-resistant *Staphylococcus aureus* (MRSA) in Nile tilapia (*Oreochromis niloticus*) and *Tilapia zilli*. *Life Sci J* 2015;12:186–94.
- Hafsat AG, Yaqub AG, Galadima BG, James AA, Abubakar S. Methicillin resistant *S. aureus* (MRSA) and methicillin resistant coagulase negative Staphylococci (MRCoNS) isolated from fish and fish handlers in Maiduguri, Nigeria. *Adv J Food Sci Technol* 2015;9:494–502. <https://doi.org/10.19026/ajfst.9.1954>.
- Aly SM, Mohamed MF, John G. Effect of probiotics on the survival, growth and challenge infection in Tilapia nilotica (*Oreochromis niloticus*). *Aquacult Res* 2008;39:647–56. <https://doi.org/10.1111/j.1365-2109.2008.01932.x>.
- Heo WS, Kim YR, Kim EY, Bai SC, Kong I. Effect of dietary probiotic, *Lactococcus lactis* subsp. *lactis* I2, supplementation on the growth and immune response of olive flounder (*Paralichthys olivaceus*). *Aquaculture* 2013;376:20–4. <https://doi.org/10.1016/j.aquaculture.2012.11.009>.
- Abdelfatah EN, Tahoun AB. Identification of lactic acid bacteria in raw milk and kariesh cheese with special reference to *Lactococcus garvieae*. *J Food Nutr Sci* 2015;3:203–8. <https://doi.org/10.11648/j.jfns.20150306.11>.
- Arqués JL, Rodríguez E, Gaya P, Medina M, Guamis B, Nuñez M. Inactivation of *S. aureus* in raw milk cheese by combinations of high pressure treatments and bacteriocin-producing lactic acid bacteria. *J Appl Microbiol* 2005;98:254–60. <https://doi.org/10.1111/j.1365-2672.2004.02507.x>.
- Abou-Donia SA. Egyptian Domiat soft white pickled cheese. *Review. New Zealand J Dairy Sci Technol* 1986;21:167–90.
- Noga EJ. Fish disease: diagnosis and treatment. John Wiley & Sons; 2010. p. 20–62. In our work, fish fed a diet supplemented with *L. garvieae* showed no evidence of disease.
- Buller NB. Bacteria from fish and other aquatic animals: a practical identification manual. CABI 2004:114–60.
- Chang PH, Plumb JA. Histopathology of experimental *Streptococcus* sp. infection in tilapia, *Oreochromis niloticus*(L.), and channel catfish, *Ictalurus punctatus* (Rafinesque). *J Fish Dis* 1996;19:235–41. <https://doi.org/10.1111/j.1365-2761.1996.tb00130.x>.
- Aly SM, Abd-El-Rahman AM, John G, Mohamed MF. Characterization of some bacteria isolated from *Oreochromis niloticus* and their potential use as probiotics. *Aquaculture* 2008;277:1–6. <https://doi.org/10.5897/AJB11.1871>.
- Irianto A, Austin B. Probiotics in aquaculture. *J Fish Dis* 2002;25:633–42. <https://doi.org/10.1046/j.1365-2761.2002.00422.x>.
- Ostrander GK. The laboratory fish. Book. 1st ed. Academic Press/Elsevier; 2000. p. 516–8. ISBN: 0125296509.
- Ellis AE. Lysozyme assays: lysozyme assay. In Stolen JS, Fletcher EC, Aderson DP, Robertson DS, van Muiswinkel WB, editors. *Techniques in fish immunology*. USA: SOS publications. p. 101–103.
- Rajaraman V, Nonnecke BJ, Franklin ST, Hammel DC, Horst RL. Effect of vitamins A and E on nitric oxide production by blood mononuclear leukocytes from neonatal calves fed milk replacer. *J Dairy Sci* 1998;81:3278–85. [https://doi.org/10.3168/jds.S0022-0302\(98\)75892-8](https://doi.org/10.3168/jds.S0022-0302(98)75892-8).
- Pal VijVijai, Jamuna M, Jeevarathnam K. Isolation and characterization of bacteriocin producing lactic acid bacteria from a south indian special dosa (Appam) batter. *J Cult Collect* 2005;4:53–60. ISSN: 1310-8360.
- Savado A, Ouattara CAT, Bassole IHN. Antimicrobial activity of lactic acid bacteria strains isolated from Burkina Faso fermented milk. *Pakistan J Nutr* 2004;3:174–9. <https://doi.org/10.3923/pjn.2004.174.179>.
- Schillinger U, Lucke FK. Identification of lactobacilli from meat and meat products. *Food Microb* 1987;4:199–208. [https://doi.org/10.1016/0740-0020\(87\)90002-5](https://doi.org/10.1016/0740-0020(87)90002-5).
- Vanden-Berg DJC, Smits A, Pot B, Ledebor AM, Kersters K, Verbekal JMA, et al. Isolation, screening and identification of lactic-acid bacteria from traditional food fermentation processes and culture. *Food Biotechnol* 1993;7:189–205. <https://doi.org/10.1080/08905439309549857>.
- Yildirim Z, Johnson MG. Detection and characterization of a bacteriocin produced by *Lactococcus lactis* subsp. *cremoris* R isolated from radish. *Letl Appl Microbiol*. 1998;26:297–304. <https://doi.org/10.1046/j.1472-765X.1998.00335.x>.
- Alegria A, Alvarez-Martín P, Sacristán N, Fernández E, Delgado S, Mayo B. Diversity and evolution of microbial populations during manufacture and ripening of Casín, a traditional Spanish, starterfree cheese made from cow's milk. *Int J Food Microbiol* 2009;136:44–51. <https://doi.org/10.1016/j.ijfoodmicro.2009.09.023>.
- El-Baradei G, Delacroix-Buchet A, Ogier J-C. Bacterial diversity of traditional Zabady fermented milk. *Int J Food Microbiol* 2008;121:295–301. <https://doi.org/10.1016/j.ijfoodmicro.2007.11.014>.
- Foschino R, Picozzi C, Borghi M, Cerliani MC, Cresci E. Investigation on the microflora of Caprino Lombardo cheese from raw goat milk. *Ital J Food Sci* 2006;18:33–49.
- Nuñez M, Bautista L, Medina M, Gaya P. *Staphylococcus aureus*, thermostable nuclease and staphylococcal enterotoxins in raw ewes milk Manchego cheese. *J Appl Bacteriol* 1988;65:29–34. <https://doi.org/10.1111/j.1365-2672.1988.tb04313.x>.

- [47] Erkmen O. Behavior of *S. aureus* in Turkish Feta cheese during manufacture and ripening. *J Food Prot* 1995;58:1201–5. <https://doi.org/10.4315/0362-028X-58.11.1201>.
- [48] Abdalla OM, Davidson PM, Christen GL. Survival of selected pathogenic bacteria in white pickled cheese made with lactic acid bacteria or antimicrobials. *J Food Prot* 1993;56:972–6. <https://doi.org/10.4315/0362-028X-56.11.972>.
- [49] Zottola EA, Yezzi TL, Ajao DB, Roberts RF. Utilization of cheddar cheese containing nisin as an antimicrobial agent in other foods. *Int J Food Microbiol* 1994;24:227–38. [https://doi.org/10.1016/0168-1605\(94\)90121-X](https://doi.org/10.1016/0168-1605(94)90121-X).
- [50] Rodríguez E, Arqués JL, Gaya P, Tomillo J, Nuñez M, Medina M. Behaviour of *S. aureus* in semi-hard cheese made from raw milk with nisin-producing starter cultures. *Milchwissenschaft* 2000;55:633–5.
- [51] Flórez AB, Reimundo P, Delgado S, Fernández E, Alegría Á, Guijarro JA, et al. Genome sequence of *Lactococcus garvieae* IPLA 31405, a bacteriocin producing, tetracycline-resistant strain isolated from a raw-milk cheese. *J Bacteriol* 2012;194:5118–9.
- [52] Foschino R, Nucera D, Volponi G, Picozzi C, Ortoffi M, Bottero MT. Comparison of *Lactococcus garvieae* strains isolated in northern Italy from dairy products and fishes through molecular typing. *J Appl Microbiol* 2008;105:652–62. <https://doi.org/10.1111/j.1365-2672.2008.03780.x>.
- [53] Fortina MG, Ricci G, Foschino R, Picozzi C, Dolci P, Zeppa G. Phenotypic typing, technological properties and safety aspects of *Lactococcus garvieae* strains from dairy environments. *J Appl Microbiol* 2007;103:445–53. <https://doi.org/10.1111/j.1365-2672.2006.03265.x>.
- [54] Varsha K, Priya S, Devendra L, Nampoothiri K. Control of spoilage fungi by protective lactic acid bacteria displaying probiotic properties. *Appl Biochem Biotechnol* 2014;172:1–12. <https://doi.org/10.1007/s12010-014-0779-4>.
- [55] Varsha KK, Devendra L, Shilpa G, Priya S, Pandey A, Nampoothiri KM. 2,4-Di-tert-butyl phenol as the antifungal, antioxidant bioactive purified from a newly isolated *Lactococcus* sp. *Int J Food Microbiol* 2015;211:44–50. <https://doi.org/10.1016/j.ijfoodmicro.2015.06.025>. Epub 2015 Jul 2.
- [56] Varsha KK, Nampoothiri KM. *Lactococcus garvieae* subsp. bovis subsp. nov., lactic acid bacteria isolated from wild gaur (*Bos gaurus*) dung, and description of *Lactococcus garvieae* subsp. garvieae subsp. nov. *Int J Syst Evol Microbiol* 2016;66:3805–9. <https://doi.org/10.1099/ijsem.0.001268>. Epub 2016 Aug 4.
- [57] Robertson PAW, O'Dowd C, Burrells C, Williams P, Austin B. Use *Carnobacterium* sp. as probiotic for Atlantic salmon (*Salmo salar* L) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Aquaculture* 2000;185:235–43. [https://doi.org/10.1016/S0044-8486\(99\)00349-X](https://doi.org/10.1016/S0044-8486(99)00349-X).
- [58] Nikoskelainen S, Ouwehand AC, Bylund G, Salminen S, Lilius EM. Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria (*Lactobacillus rhamnosus*). *Fish Shellfish Immunol* 2003;15:443–52. [https://doi.org/10.1016/S1050-4648\(03\)00023-8](https://doi.org/10.1016/S1050-4648(03)00023-8).
- [59] Aly SM, Ahmed YAG, Ghareeb AAA, Mohamed MF. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. *Fish Shellfish Immunol* 2008;25:128–36. <https://doi.org/10.1016/j.fsi.2008.03.013>.
- [60] Zhou X, Tian Z, Wang Y, Li W. Effect of treatment with probiotics as water additives on tilapia (*Oreochromis niloticus*) growth performance and immune response. *Fish Physiol Biochem* 2010;36:501–9.
- [61] Liu GY. Molecular pathogenesis of *Staphylococcus aureus* infection. *Pediatric Res* 2010;65:71R–7R. <https://doi.org/10.1203/PDR.0b013e31819dc44d>.
- [62] Bjorck L, Rosen CG, Marshall V, Reiter B. Antibacterial activity of the lactoperoxidase system in milk against pseudomonads and other gram-negative bacteria. *Appl Environ Microbiol* 1975;30:199–204.
- [63] Furtado DN, Todorov SD, Landgraf M, Destro MT, Franco BD. Bacteriocinogenic *Lactococcus lactis* subsp. *lactis* DF04Mi isolated from goat milk: application in the control of *Listeria monocytogenes* in fresh Minas-type goat cheese. *Braz. J Microbiol* 2015;46:201–6. <https://doi.org/10.1590/S1517-838246120130761>.
- [64] Gálvez A, López RL, Abriouel H, Valdivia E, Omar NB. Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. *Crit Rev Biotechnol* 2008;28:125–52. <https://doi.org/10.1080/07388550802107202>.