Administration of the probiotic Lactobacillus rhamnosus IMC 501 as a strategy for the control of Vibrio bacteria in the brine shrimp Artemia

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Significance and Impact of the Study: Probiotics have renowned potential as sustainable alternatives to antibiotics in aquaculture, but only a limited number are available for commercial uses. This study shows that the commercial probiotic strain Lactobacillus rhamnosus IMC 501® is efficiently incorporated by Artemia metanauplii, the most used live food in aquaculture, and that they drive a reduction in the incidence of the pathogen bacteria from the Vibrionaceae family within the Artemia. Our results, thus, highlight the potential of L. rhamnosus for its use as probiotic in aquaculture in order to prevent/reduce the spreading of outbreak diseases in aquaculture facilities.

Keywords
aquaculture, Artemia, Lactobacillus rhamnosus, probiotics, Vibrionaceae.

Abstract
The present study aimed to address the capability of the probiotic bacterium Lactobacillus rhamnosus IMC 501® to survive in seawater and the ability of Artemia metanauplii to incorporate it, as well as to analyse the potential effect of the probiotic as a control agent for potentially pathogenic Vibrionaceae bacteria in Artemia. The results demonstrate the ability of L. rhamnosus IMC 501® to survive in seawater for up to 30 h. They also advocate their capability to be efficiently incorporated into Artemia metanauplii at concentrations of $10^4$ CFU per Artemia after 30 min of suspension in probiotic solution, thereby promoting a 1-log reduction in Vibrionaceae levels after 3 h. These low levels of Vibrio bacteria were maintained for about 30 min after transfer into clear seawater, a sufficient time for Artemia to be ingested by aquatic organisms. These results contribute to broaden the knowledge on the suitability of probiotics as sustainable alternatives for the prevention/reduction of diseases in aquaculture facilities.

Introduction
Aquaculture outputs of commercial species have expanded as a partial alternative to overfishing of wild populations. Due to the high densities managed in production systems, the fish manipulation and the not always optimal water quality in intensive farms, the incidence of viral, bacterial and fungal infections may increase, leading to potential economic losses and worsening environmental conditions (Olafsen 2001; Bondad-Reantaso et al. 2005). Considering bacterial infections, some Vibrio sp., Pseudomonas sp. and Aeromonas sp. strains have been reported to be the main cause of several disease outbreaks in marine fish farms (Gatesoupe 1994, 1999; Carnevali et al. 2004). The reduction in the incidence of such pathogens by administering probiotics as food supplement has been largely investigated in the last decades (Gatesoupe 2002; Villamil et al. 2003; Touraki et al. 2013; Giarma et al. 2017) due to the high demand for sustainable alternatives to chemotherapeutics in aquaculture systems. According to the FAO/WHO definition, probiotics are ‘live microorganisms that, when administered in adequate amounts, confer a health benefit on the host’. In aquatic environments, the concept of probiotics includes also micro-organisms that exert the beneficial effect not only by colonizing the host but also...
by being present in the water. For the aquaculture sector, a definition has been proposed by Merrifield et al. (2010) as ‘a probiotic organism can be regarded as a live, dead or component of a microbial cell, which is administered via the feed or to the rearing water, benefiting the host by improving disease resistance, health status, growth performance, feed utilization, stress response or general vigour, which is achieved at least in part via improving the hosts microbial balance or the microbial balance of the ambient environment’. Lactic acid bacteria (LAB) have been identified as an indigenous component of fish gut microbiota (Merrifield et al. 2013). They are fast-growing micro-organisms, widely used as probiotics in land-based food production and agriculture industries. LAB have also been used in aquaculture as antagonist to pathogens in order to improve fish health (Villamil et al. 2003; Gatesoupe 2008), to modify microbiota in prey (Villamil et al. 2003) and larvae (Gatesoupe 1994; Carnevali et al. 2004) and to improve larval growth and survival (Dagá et al. 2013; Loh et al. 2015). To allow the probiotic bacteria to reach a host and maintain viability, probiotics are commonly added to the rearing environment (water) or administered through food (live prey or dry feed) (Makridis et al. 2000; Villamil et al. 2003; Carnevali et al. 2004; Touraki et al. 2013; Pintado et al. 2014).

The brine shrimp, Artemia sp., is the most used prey for the feeding of marine larvae in fish farms (Zmora and Shpigel 2006) thanks to its ability to produce cysts and capability to be reared and maintained at high densities. The brine shrimp is a nonselective filter feeder capable of ingesting a variety of particles usually used for its enrichment, such as microalgae and/or lipid emulsions. However, it is well known that the enrichment of Artemia sp. leads to an increase in organic matter, making them carriers for potential pathogens or opportunistic bacteria, particularly Vibronaeaceae, which can reach values of up to $10^4$ per Artemia (Pintado et al. 2014). The incorporation of LAB into Artemia by suspending nauplii in a liquid solution supplemented with the probiotic for a certain time has been proposed as an effective method to improve the microbiota balance of farmed fish and shrimps (Loh et al. 2015).

The probiotic strain Lactobacillus rhamnosus IMC 501®, isolated from human samples (Verdenelli et al. 2009), has demonstrated, through in vitro and in vivo tests, an inhibitory effect against potentially pathogenic microorganisms such as Escherichia coli, Staphylococcus aureus, Candida albicans, Clostridium perfringens and Streptococcus mutans (Verdenelli et al. 2009). The positive effect of this L. rhamnosus strain is mainly due to the production of certain metabolites, such as acid acetic, lactic acid, diacetyl or bacteriocins (Verdenelli et al. 2009). Results obtained in fish, using this probiotic delivered via Artemia sp. or added to the rearing water, demonstrated an improvement in larval development in false percula clownfish (Amphiprion ocellaris), a promotion in oocyte maturation and backbone calcification in zebrafish (Danio rerio) and an enhancement of gonadosomatic index, fecundity and embryonic survival in killifish (Fundulus heteroclitus) (Avella et al. 2010; Giorgini et al. 2010; Lombardo et al. 2011; Carnevali et al. 2012; Gioacchini et al. 2012; Avella et al. 2012). The promotion of biomineralization process (Maradonna et al. 2013) and the modulation of the gut microbiome in zebras (inducing transcriptional down-regulation of genes involved in cholesterol and triglycerides metabolism) have also been demonstrated (Falcinelli et al. 2015). In addition, an in vitro inhibitory activity towards Gram-positive and Gram-negative pathogenic bacterial strains has also been demonstrated for L. rhamnosus IMC 501 (Coman et al. 2014). However, antagonism towards Vibrio sp. has not yet been investigated.

Considering the ability of LAB to produce extracellular products inhibiting the growth of potential pathogens in Artemia cultures, such as Vibrio (Villamil et al. 2003; Lamari et al. 2014), we hypothesize that L. rhamnosus IMC 501 incorporated in Artemia brine shrimps might reduce the concentration of some potentially pathogenic or opportunistic bacteria belonging to the Vibronaeaceae family in that prey.

The present study focussed on the use of the probiotic L. rhamnosus IMC 501 aiming to (i) assess the survival time of the probiotic in seawater, (ii) evaluate the efficiency of the brine shrimp Artemia metanauplii to incorporate the probiotic and (iii) estimate the residence time of the probiotic in Artemia metanauplii and its antagonistic effect on Vibronaeaceae levels in Artemia.

Results and discussion
To examine the capacity of the probiotic bacteria L. rhamnosus IMC 501 to survive in seawater, an experiment was conducted by suspending the probiotic (1g l$^{-1}$) in sterilized seawater during 30 h at 26°C. The counting of the colony forming units (CFU) per ml was performed in the selective agar for lactic acid bacteria (MRS, Man, Rogosa and Sharpe; ITW Test & Measurement GmbH Company, Düsseldorf, Germany) and in MRS Agar supplemented with 1% Aniline Blue (MRSA-B Agar). L. rhamnosus IMC 501 was able to survive in seawater for the entire experimental period (30 h) and maintain high CFU levels in the suspension media for at least 6 h. The levels of probiotic in seawater decreased by about 4-log units from 6 to 24 h, even though CFU were not counted in the intermediate time. No significant differences
(\(P > 0.05\)) were observed between MRS and MRSA-B Agar, \(L.\ rhamnosus\) IMC 501 being able to grow similarly in both media, as shown in Table 1.

We then examined the capacity of \(Artemia\) to incorporate the probiotic bacteria \(L.\ rhamnosus\) IMC 501. To ensure an efficient incorporation of the probiotic in the \(Artemia\) gut, it is important to ascertain that the brine shrimps are at the developmental stage of Instar III, which they attain 24 h after hatching, as the mouth is not open in earlier stages (Planas and Cunha 1999). Accordingly, the incorporation of \(L.\ rhamnosus\) IMC 501 was assessed in \(Artemia\) megalopae by suspending the brine shrimp in seawater with a single dose (1 g l\(^{-1}\)) of probiotic \(L.\ rhamnosus\) IMC 501 during 24 h. The counting of CFU per ml was performed for total marine bacteria in Marine Agar (MA), for lactic acid bacteria in MRS Agar and for \(Vibrio\) bacteria in Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) Agar. As shown in Table 2, the levels of total marine bacteria remained rather constant in both the treated \(Artemia\) (AP) and the nontreated control \(Artemia\) (AC) during the all period. Regarding lactic acid bacteria, highest levels of probiotic were observed after 30 min in AP, when compared with AC. Similar results were obtained by Makridis et al. (2000) by suspending \(Artemia\) in a probiotic solution of \(10^7\) bacteria per ml. According to these authors, a 20–30 min lapse corresponds to the passage of the probiotic through the gut of the brine shrimp.

The concentration of \(Vibrio\) bacteria changed significantly over the duration of the experiment, with a major decrease in Vibrionaceae levels reported from 30 min to 3 h in the AP treatment. However, the difference between the two treatments was not significant (\(P > 0.05\)).

To assess the residence time of \(L.\ rhamnosus\) in \(Artemia\) megalopae and its antagonist level against Vibrionaceae, the brine shrimp was suspended in seawater with one dose (1 g l\(^{-1}\)) of probiotic for 30 min. Afterwards, the \(Artemia\) was maintained in probiotic-free seawater for 3 h, and \(L.\ rhamnosus\) concentration (CFU per \(Artemia\), MRS) was measured. At the start of the experiment, the brine shrimp was able to accumulate probiotic levels in the order of \(4.98 \pm 2.65 \times 10^7\) CFU per \(Artemia\), being able to maintain these concentrations stable for about 2 h. Interestingly, Vibrionaceae concentration (CFU per \(Artemia\), TCBS Agar) decreased by 1-log unit after 30 min in AP treatment, although differences were not significant (\(P > 0.05\)). As observed, \(L.\ rhamnosus\) IMC 501 was able to induce a temporary change in \(Artemia\) microbiota by transiently diminishing Vibrionaceae bacteria, which increased again as the probiotic levels decreased. The probiotic was no longer detected in MRS Agar beyond 180 min (Fig. 1), suggesting, therefore, that probiotic concentrations in \(Artemia\) were also reduced at undetectable levels.

The antagonistic ability of LAB on the reduction in \(Vibrio\) sp. concentrations in brine shrimp \(Artemia\) due to its capability to produce bacteriocin or organic acids has been widely described (Ringø et al. 2003; Dimitrijević et al. 2009; Sarika et al. 2010; Lamari et al. 2014; Pintado et al. 2014). In this regard, the reduction in \(V.\ alginolyticus\), \(V.\ anguillarum\) and \(V.\ harveyi\) concentrations has been tested by using LAB strains of \(L.\ casei\), \(L.\ plantarum\), \(L.\ lactis\) and \(L.\ rhamnosus\) GP1. However, there are no reports on the effect of \(L.\ rhamnosus\) IMC 501 on Vibrionaceae bacteria. In the present study, \(L.\ rhamnosus\) IMC

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**Table 1** Survival time of the probiotic \(Lactobacillus\ rhamnosus\) IMC 501 in seawater. Concentrations are expressed as means ± standard deviations.

| Time (hours) | MRS \(\log_{10}\) CFU per ml | MRSA-B \(\log_{10}\) CFU per ml |
|-------------|-----------------------------|---------------------------------|
| 0           | 7.84 ± 0.06                 | 7.23 ± 0.21                    |
| 0.25        | 7.54 ± 0.07                 | 7.53 ± 0.06                    |
| 0.5         | 7.63 ± 0.12                 | 7.66 ± 0.13                    |
| 1           | 7.22 ± 0.06                 | 7.54 ± 0.06                    |
| 3           | 6.96 ± 0.21                 | 7.12 ± 0.21                    |
| 6           | 7.10 ± 0.02                 | 7.30 ± 0.00                    |
| 24          | 3.33 ± 0.10                 | 3.38 ± 0.25                    |
| 30          | 2.99 ± 0.01                 | 3.38 ± 0.21                    |

**Table 2** Incorporation of the probiotic \(Lactobacillus\ rhamnosus\) IMC 501 into the brine shrimp \(Artemia\) sp. colony-forming units (CFU) is expressed as means ± standard deviations.

| Time (hours) | AC   | CFU (log\(_{10}\) CFU per ml) | AP   | CFU (log\(_{10}\) CFU per ml) |
|-------------|------|-------------------------------|------|-------------------------------|
| 0           | MA   | 3.05 ± 1.29                   | MA   | –                             |
|             | MRS  | 0.00 ± 0.00                   | MRS  | –                             |
|             | TCBS | 1.17 ± 1.23                   | TCBS | –                             |
| 0.25        | MA   | 3.76 ± 0.96                   | MA   | 3.94 ± 0.67                   |
|             | MRS  | 0.00 ± 0.00                   | MRS  | 2.23 ± 0.16                   |
|             | TCBS | 1.17 ± 0.18                   | TCBS | 2.07 ± 0.67                   |
| 0.5         | MA   | 4.33 ± 0.25                   | MA   | 4.00 ± 0.06                   |
|             | MRS  | 0.00 ± 0.00                   | MRS  | 4.29 ± 0.05                   |
|             | TCBS | 2.14 ± 0.09                   | TCBS | 0.70 ± 0.31                   |
| 1           | MA   | 3.90 ± 0.43                   | MA   | 3.23 ± 0.91                   |
|             | MRS  | 0.00 ± 0.00                   | MRS  | 3.83 ± 0.54                   |
|             | TCBS | 1.45 ± 0.35                   | TCBS | 0.70 ± 0.99                   |
| 3           | MA   | 3.91 ± 0.19                   | MA   | 3.83 ± 0.00                   |
|             | MRS  | 0.00 ± 0.00                   | MRS  | 3.58 ± 0.14                   |
|             | TCBS | 1.58 ± 1.25                   | TCBS | 0.25 ± 0.36                   |
| 6           | MA   | 4.16 ± 0.67                   | MA   | 3.99 ± 0.77                   |
|             | MRS  | 0.00 ± 0.00                   | MRS  | 3.96 ± 0.54                   |
|             | TCBS | 1.00 ± 0.74                   | TCBS | 0.18 ± 1.14                   |
| 24          | MA   | 4.80 ± 0.88                   | MA   | 4.54 ± 0.1                   |
|             | MRS  | 0.00 ± 0.00                   | MRS  | 2.95 ± 0.74                   |
|             | TCBS | 3.90 ± 0.39                   | TCBS | 3.87 ± 0.25                   |
501 did not show antagonistic activity against *V. anguillarum* in *in vitro* well diffusion trials (not shown). Nevertheless, our *in vivo* tests results suggest a 1-log reduction in initial Vibrionaceae CFU levels in AP treatment during the first 30 min of maintenance in clear seawater. These findings support the hypothesis that *L. rhamnosus* IMC 501 incorporated into *Artemia* might antagonize, in that prey under its culture conditions, the concentration of some potentially pathogenic or opportunistic bacteria belonging to the Vibrionaceae family. As reported by Dimitrijević et al. (2009), a second-class bacteriocin was isolated from *L. rhamnosus* 68, with bacteriostatic effect. These authors argue that bactericidal or bacteriostatic effect may vary depending on the probiotic concentration, growth phase or overall experimental conditions, among others, differing this feature among bacteria strains. Therefore, whether the reduction in Vibrio bacteria was due to competition of *L. rhamnosus* IMC 501 for resources rather than antagonism should be investigated in further studies.

One of the major limitations nowadays concerning the applicability of probiotics as food supplement in aquaculture is their viability and availability for commercial uses, mainly due to incompatibility with industrial processes (Chuphal et al. 2021). This study shows that the commercial probiotic strain *L. rhamnosus* IMC 501° was efficiently incorporated by *Artemia* metanauplii, the most used live food in aquaculture, and that its use can drive a reduction in the incidence of pathogenic bacteria from the Vibrionaceae family within the *Artemia*. Therefore, our results highlight the potential of *L. rhamnosus* for its use as aquafeed product, mainly at early stages of aquaculture productions, such as fish, shellfish or shrimp hatcheries, in order to prevent / reduce the spreading of diseases and to enhance growth and survival at early stages of development.

**Materials and methods**

**Strain activation conditions**

The commercial product of lyophilized *L. rhamnosus* IMC 501° (Batch n° C025396A) was kindly provided by Synbiotec S.r.l (Camerino, Italy) (Verdenelli et al. 2009) at a concentration of 10¹¹ colony forming units (CFU) per gram. The activation of probiotic was performed following the recommendations given by the manufacturer, by suspending 1 g l⁻¹ of commercial product powder in sterilized marine water with gentle aeration at room temperature (20°C).

**Survival of Lactobacillus rhamnosus IMC 501 in seawater**

The ability of the bacterium to survive in saltwater (35 salinity) was tested after suspending 50 mg of the commercial product in 50 ml sterile seawater (1 g l⁻¹) (Fig. 2). That concentration would correspond to actual levels of 10⁸ g l⁻¹. The suspension was maintained for 30 h at 26°C under gentle aeration. Samples (1 ml) were taken in duplicate at 0, 0.25, 0.5, 1, 3, 6, 24 and 30 h, serially diluted and spread on selective lactic acid MRS (Man, Rogosa and Sharpe; ITW Company) and MRSA-B Agar (MRS Agar supplemented with 1% Aniline Blue for the staining of lactic acid colonies). The plates were incubated for 7 days at 30°C in the dark before CFU counting.

**Incorporation of Lactobacillus rhamnosus IMC 501 in Artemia**

The decapsulation of *Artemia* cysts was performed using sodium hypochlorite (6.7 mmol l⁻¹ of active hypochlorite per gram of cysts) and subsequent chloride neutralization with sodium thiosulphate (0.1 N). After decapsulation,
Artemia cysts (INVE) were incubated for 24 h at 29°C and 35 salinity, under strong light and continuous aeration. Freshly hatched nauplii were concentrated on a 125 µm mesh, rinsed and maintained for 30 min in running tap water. Afterwards, the nauplii were transferred (about 100 nauplii per ml) to 5 l buckets containing 4 l of seawater and 1 l (10⁶ cells per ml) culture of the marine microalga Phaeodactylum tricornutum. The nauplii were fed on a mixture of lipid emulsion Red Pepper (1 g l⁻¹) (Bernaqua, Belgium) and freeze-dried Spirulina (0.1 g l⁻¹) (Iberfrost, Tomiño, Pontevedra, Spain) and maintained for 24 h at 27°C with gentle aeration (Fig. 2).

The incorporation of L. rhamnosus IMC 501 was carried out on the following day in Artemia treated metanauplii (AP Artemia), while standard enriched Artemia metanauplii were used as control (AC Artemia). For each condition, 30 000 24-h-enriched metanauplii were collected in a 125 µm mesh, gently washed and transferred to a jar containing 300 ml of seawater and incubated at 20°C under moderate aeration. In AP treatment, a single dose of 1 g l⁻¹ L. rhamnosus IMC 501 was added at the start of the experiment. Artemia samples were taken at 0, 0.25, 0.5, 1, 3, 6 and 24 h.

At each sampling time, 1000 metanauplii were collected in 50 µm mesh, gently washed with seawater to remove the excess of foam generated, transferred to an Eppendorf tube and homogenized using a micropistil. Homogenates were filled up to 1 ml with sterile seawater and serial dilutions of homogenates were spread on MA, Man Rogosa and Sharpe Agar (MRS) and in TCBS. The plates were incubated in the dark at 30°C for 7 days, for the quantification of total marine bacteria, lactic acid and Vibrio bacteria respectively. Both treatments (AP and AC) were performed in duplicate.

Residence time of Lactobacillus rhamnosus IMC 501 in Artemia metanauplii

For AP conditions, 25 000 24-h-enriched Artemia metanauplii were transferred to a bucket containing 300 ml of seawater and a single dose of the probiotic L. rhamnosus IMC 501 (1 g l⁻¹), and maintained for 30 min (Fig. 2). After the incorporation period, the metanauplii from AP and AC treatment were collected in a 50 µm sterile mesh, mildly washed with sterile seawater and transferred to buckets containing 300 ml of clear seawater under gentle aeration. Samples were taken at 0, 0.5, 1, 2 and 3 h for microbial analysis. For that, 1000 metanauplii were taken from each bucket and homogenized with a micropistil in an Eppendorf tube. The homogenates were filled up to 1 ml with sterile seawater and serial dilutions were spread on MA, MRS and in TCBS. The plates were
incubated in the dark at 30°C for 7 days, for the quantification of total marine bacteria, lactic acid and Vibrio bacteria respectively. Both treatments (AP and AC) were performed in duplicate.

**Statistics and data analysis**

Data are provided as mean ± standard deviation. Differences between control (AC) and probiotic (AP) treatments were determined by one-way ANOVA tests, assuming as significant level an alpha value of 0.05. Statistical analyses were performed using the free R software version 3.5.0.

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**Conflict of Interests**

No conflict of interests declared.

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