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Role of *Lactobacillus reuteri* DSM 17938 in survival of *Artemia franciscana*

Development of a new experimental model for probiotic studies

Tiziana Racca

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Department of Molecular Sciences, SLU

Supervisor: Stefan Roos

Co-supervisor: Ludwig Lundqvist

To my parents, who simply made it possible

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1. POPULAR SCIENCE SUMMARY

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The potential of *Artemia franciscana* as experimental model

When we refer to animal testing, mice are reasonably the first animals we think of. They are in fact largely used in research since they are relatively closely related to humans, thus likely to better reflect human physiology than other organisms. However, many ethical issues arise when it comes to experiments on vertebrates in general, and very strict animal welfare regulations are in place in order to avoid them unnecessary suffering. *Artemia franciscana* is brine shrimp and it represents to some extent a potential, ethically acceptable alternative to mice. In this project, *Artemia* was evaluated as an experimental model for probiotic studies, with survival rate of second larval stage (instar II metanauplii)being the main output to be measured. Protective effects of *Lactobacillus reuteri* DSM 17938 were initially supposed to be assessed in the infection model *Artemia* challenged with Enterotoxigenic *Escherichia coli* (ETEC), but it wasn't possible due to inconsistencies observed in the response of *Artemia* upon infection; on the other hand, *L. reuteri* DSM 17938 exerted an unexpected positive effect on *Artemia* reared in standard conditions which should have been optimal but instead caused spontaneous death of *Artemia*. It was therefore hypothesized that metanauplii were exposed to some kind of stress that caused premature death of *Artemia* and, based on this assumption, investigations on the benefit provided by *L. reuteri* DSM 17938 to *Artemia* in this setting were carried out in order to further explore probiotic functionality and characterize *Artemia* as experimental model.

L. reuteri showed to be more effective when killed, rejecting the hypothesis that it needed to be metabolically active to exert its action on *Artemia*. In particular, bacterial components of *L. reuteri* were heat-treated at 90°C, 60°C or they were smashed following addition of beads to bacteria and subsequent agitation, and interestingly the last variant provided the highest benefit to the metanauplii. Besides this, it was possible to trace the amelioration in survival rate exerted by the so-called bead-beaten bacteria back to a pool of molecules including proteins, genomic DNA and polysaccharides. At the same time, the solution obtained when washing plate-grown *L. reuteri* was lethal for *Artemia* unless administered in low doses.

The results create a number of new questions and there are still many knowledge gaps to fill. One example is the immune response of *Artemia* before and after administration of *L. reuteri*; its investigation may increase the understanding of whether *Artemia* is actually exposed to a stress and how the probiotic contributes to improvement of survival. According to the results gathered so far, it is anyways possible to speculate that *Artemia* increased mortality over time is due to the long storage time of its cysts and that the protective effects conferred by *L. reuteri* demonstrate that it can be used as an aging model for probiotic studies.

2. ABSTRACT

The crustacean *Artemia franciscana* represents a potential experimental model for human studies. The aim of the project was to assess whether *Artemia* could be used as a model for investigation of probiotic properties. For all the experiments, *Artemia* cysts were rehydrated, decapsulated and allowed to hatch for 28 hours before the developed larvae (nauplii) were challenged and/or treated with probiotics; measurement of survival rate occurred after further 24 and 48 hours. Interestingly, administration of *L. reuteri* 17938 during an initial study showed that the probiotic rescued *Artemia* from spontaneous death occurring in the absence of any established and chosen challenge in a dose-dependent manner. Assuming that *Artemia* death was related to some kind of stress, the mode of action by means of which *L. reuteri* provided protective effects was investigated. Heat-treated and bead-beaten *L. reuteri* positively affected *Artemia* survival rate to a greater extent than live *L. reuteri*, with the highest improvement occurring during administration of bead-beaten *L. reuteri*. In particular, administration of different fractions of the bead-beaten *L. reuteri* cultures to Artemia allowed for identification of the molecule responsible for amelioration of *Artemia* fitness in bacterial components ranging between 3 and 300 kDa in weight. Supernatants obtained from exudates of plate-grown bacteria instead revealed itself to be lethal when added in amounts corresponding to 10^7 CFU/ml of the bacterial culture or higher. Further studies on a genetic and immunological level on *Artemia* are required in order to elucidate the complex interactions establishing among the host, the probiotic and the environment. However, based on the data gathered, it is possible to suggest that *Artemia* can be used as a potential chronic stress model, for example as aging or starvation models. Besides this, *L. reuteri* shows to provide an overall protective effect against this stress, although differences in the extent of the protection were observed according to the growth protocol and the killing mode of bacterial cultures. Also, the lethality of the supernatants demonstrates once again that the probiotic activity is the result of multiple and intertwined biological pathways, sometimes in contrast to one another.

Key words: germ-free *Artemia*, probiotics, *Lactobacillus reuteri* DSM 17938, experimental model, survival rate

3. INTRODUCTION

3.1 Probiotics

According to the definition by WHO and FAO in 2001, probiotics are live microorganisms that confer a health benefit to the host when administered in adequate amounts[1]. The probiotic concept can be traced back to the beginning of the twentieth century, when Elie Metchnikoff claimed that introduction of sour milk containing "Bulgarian Bacillus" into the diet was beneficial, although the term "probiotic" itself was introduced only in 1965 by Lilly and Stillwell[2]. It is important to distinguish probiotics from prebiotics and symbiotics. The former are in fact indigestible food ingredients that selectively promote the growth or activity of beneficial bacteria thereby benefiting the host, while the latter are combinations of probiotics and prebiotics designed to improve the survival of ingested microorganisms and their colonization of the intestinal tract, thus conferring benefits upon host health[1,3]. Postbiotics as well must be mentioned: they are non-viable microbial cells which, when administered in sufficient amounts, confer benefits to the host[1,4,5]; In other words, postbiotics include microbial cells, cell constituents and metabolites. They are the newest member of the biotics family since they started gaining relevance in scientific literature only in the past two decades[1]. Interest in probiotics and prebiotics has instead gradually increased since the 1980s, during a historical period when antimicrobial resistance and limited pharmaceutical research and development started representing a big issue[6]. Back in 2017, a search on PubMed for human clinical trials showed that over 1500 trials had been published on probiotics and close to 350 on prebiotics. Although these studies were heterogeneous with regard to strain(s), prebiotics tested, and populations included, accumulated evidence supports the view that benefits are measurable across many different outcomes[3].

The most common genera used as probiotics are *Lactobacillus* and *Bifidobacterium*, but the yeast *Saccharomyces boulardii* and some *E. coli* and *Bacillus* species are also used[3]. In order to be considered a probiotic, a strain must have specific properties and characteristics[7]. First of all, it must be safe, which entails being not pathogenic, non-allergenic, non-cancerogenic[8] and lacking transferable antibiotic resistance genes[9]. For the same reason, but also to guarantee reproducibility of the product, the strain must be stable genotype- and phenotype-wise[7]. It might also be an advantage if the strain has high bile and acid resistance, because it must be able to pass through the gastrointestinal tract without being severely impaired; in particular, it is important that the strain is able to survive and grow during gastrointestinal transit[10]. The probiotic microbes should confer a health benefit and must by that have properties that are important for the recovery from disorders for which a treatment is being studied[11]. In the case of an infection for example, the probiotic might be either responsible for exclusion or reduction of pathogenic adherence or capable of producing acids, hydrogen peroxide and bacteriocins antagonistic to pathogen growth[12]. Usually probiotics exert their beneficial effects through a combination of different molecular mechanisms, which are diverse and sometimes strain specific. These modes of action can be grouped in three main categories: competitive exclusion, immune modulation and enhancement of the barrier function[7]. However, there are many aspects that have yet to be clarified in this regard, as the interactions probiotic-host (which don't limit themselves to the intestine but also to multiple organs) and probiotic-microbiota are extremely complex. It is thus challenging to export findings coming from the laboratory into clinical trials, where multiple factors like genetics, microbial diversity, diet and environment come in.

3.2 *Lactobacillus reuteri*

Lactobacillus reuteri is a rod-shape Gram-positive bacterium belonging to the genus *Lactobacillus* which colonizes the digestive tract of a broad range of mammals and birds including humans[13]. It was first isolated in 1962 by the microbiologist Gerhard Reuter, but was recognized only in 1980 as an individual species. The abundance of *L. reuteri* in the gut fluctuates throughout historical time and among different geographical areas[14]. With the establishment of a westernized diet in the last few decades the detection of this species within the human population has become increasingly rare, yet its presence in people coming from non-industrialized areas like Papua New Guinea is still nowadays predominant[15]. Notably, the decreased microbial diversity, and in particular of the *L. reuteri* abundance in the digestive tract which characterizes the western population, is directly correlated to the increase in incidence of inflammatory diseases, which suggests a potential role of *L. reuteri* in the treatment of such pathologies[16].

L. reuteri is one of the most clinically studied probiotic species because of some of its characteristics that makes it suitable for the use[14]. On one side, *L. reuteri* is an optimal candidate because it is one of the few bacterial species of human origin that has co-evolved with humans since the beginning[17]. Moreover, its ability to tolerate and survive the low pH conditions, characterizing the stomach and the bile salts released in the small intestine, makes its oral administration feasible and successful[7].

L. reuteri has been shown to exhibit several health-promoting benefits that result in the maintenance of the gut homeostasis[16]. First of all, the solely adhesion of the probiotic on the intestinal mucosa limits nutrients availability to other bacteria and restricts pathogen access to the epithelium[7,18]. In addition, some strains of *L. reuteri* can secrete different bioactive metabolites and compounds that have multiple effects for hindering colonization of pathogens and remodeling the commensal microbiota in the gastrointestinal tract. Some of them were summarized by Shokoufeh Karimi in her doctoral thesis *Exploring Probiotics-Host Interactions.Intestinal Immune and Defence Responses to Lactobacillus reuteri in Health and Disease*[7]. She mentioned reutericyclin, which disturbs the membrane of many Gram-positive bacteria; reuterin, which derives from glycerol dehydration and promotes *L. reuteri* growth by regenerating NAD^+ upon its reduction; vitamin B12, which is involved in the conversion of glycerol into reuterin; and histamine, which has anti-inflammatory effects. Indeed *L. reuteri* might play an important role also in immune modulation, by downregulating inflammatory cytokines and boosting regulatory T cell development and function. Finally, it contributes to reinforcement of the intestinal barrier by improving mucosal barrier and permeability; although the mechanism is not fully understood, *in vivo* and *in vitro* studies have shown that probiotics can upregulate genes of the tight junction complex.

The most studied strain of *L. reuteri* in the company BioGaia is *L. reuteri* DSM 17938, a derivative of *L. reuteri* ATCC 55730. Unlike the mother strain *L. reuteri* ATCC 55730, *L. reuteri* DSM 17938 lacks unwanted plasmid-borne resistance against tetracycline and lincomycin[19]. Its most important applications are found in the treatment of infant colic[20–22] and diarrhoea in children[23–25], but *L. reuteri* DSM 17938 has also been observed to be beneficial in adult patients suffering from *Helicobacter pylori* infections[26,27].

It is essential to further investigate the mode of action of *L. reuteri* DSM 17938, which is not fully understood yet, in order to be able to empower its bioactivity and consequently its probiotic activity. As above-mentioned, *L. reuteri* like many other probiotics are currently used mainly for the treatment

of functional gastrointestinal disorders. A better understanding of the mechanisms behind its probiotic activity could not only lead to improvement of already existent therapies but also lead to evaluation of potential applications in the treatment of other pathologies which have been recently associated to a microbial imbalance.

3.3 *Artemia franciscana*

Studies about probiotics usually involve *in vitro* studies with cellular cultures and *in vivo* studies followed by clinical trials. In this project we used quite a unique model, *Artemia franciscana*, which is a key organism in aquaculture[28,29]. *Artemia franciscana* is a filter-feeding crustacean belonging to the class *Branchiopoda*, also known as *Artemia salina* in north America[30]. Its life cycle is described clearly by C. Drewes[30]. Sexes are separated and adults are sexually dimorphic. They are oviparous and lay eggs that can be of two types according to the environmental conditions; the "summer eggs" continue developing and hatch quickly, while the "winter eggs" are thicker and the development of the embryos that they contain is arrested at the gastrula stage. The latter are in a dried and encysted form which can survive in a metabolically inactive stage for many years. When encysted eggs are exposed to more favorable conditions, such as rehydration, the eggs swell and rapid development of the embryos resumes, resulting in completion of the nauplius (larval) stage. In laboratory, where the conditions are optimal, hatching occurs in 24h. For the first few hours nauplii stay within a hatching membrane that hangs beneath the cyst shell and this phase is called umbrella stage. When the hatching is completed, instar I nauplii (first larval stage) detach definitely from the cysts; their digestive tract is not mature yet and they still feed on stored yolk. Within few hours they molt into Instar II metanauplii (second larval stage) and start filter-feeding; their food consists mainly of microalgae, bacteria, and detritus. *Artemia franciscana* undergoes several other molts before reaching the adult stage (after around 30 days), which can last around four months.

As artificial feed formulations are not available yet, live prey is still the main feed for fish in aquaculture and *Artemia franciscana*, together with rotifers, is the main representative[29]. Because of convenience in production and their biochemical composition, the brine shrimp *Artemia* is in fact the most frequently used live food in the larviculture of economically important crustaceans and fish[31]. *Artemia* has many characteristics that make it suitable for the industry. First of all, it is consistently available as it is an off-shelf food in the form of dormant cysts. In addition, they are very tolerant to various culture environments, they resist even rough handling, and may be disinfected resulting in a biologically uncontaminated live feed[32]. Finally, a crucial aspect of *Artemia* is that it can be used as a carrier for components which are otherwise difficult to administer to fish and crustacean larvae and that instead can be previously incapsulated in *Artemia* itself, such as essential nutrients, pigments, prophylactics, and therapeutics[32]. This last advantage has recently been reevaluated in regard to probiotic administration to fish. Intensive culture of fish has in fact always suffered from mass mortality which has both nutritional and bacteriological explanations. In particular, microbiological problems are mainly due to opportunistic pathogens that are harbored on/in the live feed, *Artemia* included[28,33,34]. To tackle this issue, pouring of antimicrobials in the water has been the most common counteraction until recently. However, this measure unsurprisingly led to promotion and selection of antibiotic-resistant bacteria rendering the antibiotic treatment increasingly ineffective over time[35] and now many alternative strategies are being evaluated[36–38]. As previously stated, probiotics are gaining increasing interest and they currently constitute one of the alternative options considered to solve antimicrobial resistance[28,33,35,39–41].

In the last decades, *Artemia franciscana* has proven itself to be an ideal alternative experimental model for many reasons. The main one is that *Artemia* can be reared in gnotobiotic conditions, which allows full control over host-associated microbial communities. Practical factors here also come into play: its culture is relatively cheap and doesn't require much space, and its cysts are dormant so they can be stored and used when necessary. All the developmental stages of *Artemia* are well characterized and established molecular techniques are already available. Last but not least, the rapid generation cycle of this crustacean makes it possible to get results from experiments very fast and, according to the results themselves, change and/or adjust the setting of the experiments promptly[42]. Initially used for toxicity tests[43,44], today *Artemia* is also used extensively to assess potential protective effects of different chemicals against marine pathogens in aquaculture[42,45]. In addition, a 2014 study showed that it is a good infection model organism to study bacterial virulence[46]; *Artemia* has in fact a very good resilience and it is halophilic since it lives in water of high salinity: it is thus ideal for running biological toxicity assays of marine pathogens or human pathogens that are salt-tolerant, like *Pseudomonas aeruginosa* or *Staphylococcus aureus*[46].

In this project we explored *L. reuteri* DSM 17938 activity in *Artemia franciscana*. With the initial purpose to study its potential protective effects in an infection model, enterotoxigenic *E. coli* was initially used as a challenge for *Artemia*, but due to difficulties faced in developing the model *Vibrio parahaemolyticus* was used as a substitute. However, *L. reuteri* turned out to be beneficial for *Artemia* even without any challenge, which encouraged us to abandon the infection model and focus exclusively on the interaction *Artemia-L.reuteri*, whose characteristics we have tried to elucidate in the report.

4. AIM

The aim of the project was to:

- evaluate to what extent *Artemia franciscana* could be considered a suitable experimental model for probiotic studies;
- investigate molecular mechanisms underlying protective effects of *L. reuteri* DSM 17938 on instar II *Artemia* metanauplii in stressful conditions.

5. ETHICAL CONSIDERATIONS

To date crustaceans are not included for protection under EU animal welfare legislation, so no ethical permits to carry out experiments with *Artemia franciscana* were required. Nevertheless, crustacean ability to feel pain is largely debated within the scientific community[47]. The main issue is the definition of animal pain, whose criteria valid for humans cannot be applied in this field. Unlike nocireception, which consists on involuntary protective reflexes in response to noxious stimuli, pain is something relative to the emotional state, which is hard to measure in invertebrates in general[47,48]. Sneddon proposed a definition of animal pain which included a list of anatomical prerequisites such as presence of nocireceptors[49]. However, he was blamed by many scientists for lowering the evidential bar for pain too low to establish any real confidence that the alleged "pain behaviours" in crustaceans are truly related to anything in any way analogous to the definition and use of the word pain that we are familiar with as humans[48]. Many studies have tried to demonstrate that crustaceans can feel pain[50–53], but recent studies have shown that conclusions were based on not replicable experiments or overinterpretation of behavioral responses. Hence, there is lack of scientifically valid evidence of pain in crustaceans at this time[48] and by that the *Artemia* model doesn't have the same issues as vertebrate models.

6. MATERIALS AND METHODS

6.1 Bacterial strains

Lactobacillus reuteri strain DSM 17938 (a kind gift of BioGaia AB, Stockholm, Sweden) was used as the main probiotic bacterium. *Lactococcus lactis* MG 1363 (lab strain) and *L. reuteri* ATCC PTA 6475 (a kind gift of BioGaia AB) were used for comparison. All the strains were stored in glycerol stocks at -80°C.

The porcine pathogenic enterotoxigenic *Escherichia coli* (ETEC) and *Vibrio parahaemolyticus* were used in challenge tests. Both of them were stored at -80°C, having *V. parahaemolyticus* in Marine Broth 2216 (Difco Laboratories, Detroit, MI. USA) with 40% sterile glycerol.

6.2 Bacterial cultures of *L. reuteri* DSM 17938, *L. lactis* MG 1363 and *L. reuteri* ATCC PTA 6475

L. lactis MG 1363 and *L. reuteri* ATCC PTA 6475 were cultivated only in liquid De Man, Rogosa, Sharp (MRS) (Oxoid, Blasingstoke, UK) broth, *L. reuteri* DSM 17938 was cultivated both in liquid broth and on agar.

Regarding the liquid cultures, around 25 µl of each strain was taken from the respective stock vials (10^{10} CFU/ml) transferred in 10 ml of MRS broth and allowed to grow for 24h at 37°C.

L. reuteri DSM 17938 was also cultivated on agar. In every single plate were transferred 100 µl of *L. reuteri* collected from the stock vial. Bacteria were then spread evenly with loops on plates and allowed to grow for 48h at 37°C in anaerobic conditions in anaerobic jars with anaerocult pouches inside (Merck Millipore Anaerocult™ A) .

6.3 Bacterial cultures of ETEC and *V. parahaemolyticus*

Around 25µl of ETEC collected by the stock vial were transferred to 10 ml Luria Bertani (LB) broth and allowed to grow on the shaker at 120 rpm overnight at 37°C. Two hundred µl of the growing bacteria were re-incubated in 10 ml of fresh LB broth for 4h on the shaker at 120 rpm at 37°C before using them as challenge on *Artemia* ; in this way it was assured that ETEC was added to *Artemia* while it was in the exponential phase and its activity on *Artemia* was optimal.The cultivation was carried out under safety cabinet.

Ten µl of *V. parahaemolyticus* were transferred from the stock solution into 15 ml Marine broth and allowed to grow on the shaker at 115 rpm overnight at 28°C. One hundred µl of growing bacteria were re-incubated for 6h before using them as challenge on *Artemia* on the shaker at 115 rpm at 28°C for the same reasons of ETEC. The cultivation was carried out under laminar flow hood to avoid contamination. The concentration used for all the experiments was 10^7 CFU/ml.

6.4 Separation of MRS broth from bacterial cells in liquid cultures

The MRS medium was washed away from bacterial cells grown in liquid culture before they were used for the treatment of *Artemia* in order to relate them to any beneficial/detrimental effect with certainty and exclude any potential role of MRS broth in the experiment outcome. Liquid cultures were centrifugated at 4000 rpm for 15 minutes. The supernatant containing MRS broth was then removed and 10 ml of PBS were added. The culture was centrifugated again with the same setting

and supernatant was removed again. This process was repeated twice and finally the pellet was resuspended in 10 ml of new PBS.

6.5 Collection of plate-grown bacteria and separation of bacterial cells from the supernatant

Each plate was washed with 1 ml of PBS in order to collect colonies. The solution was transferred in a falcon tube and centrifugated at 4000 rpm for 15 minutes. The supernatant was collected and stored, the pellet was centrifugated again with the same setting and the newly formed supernatant was added to the one previously stored. Ten ml of PBS were then added to the pellet which underwent the same process of washing described for the liquid cultures (section 6.4).

6.6 Killing of bacteria

L. reuteri was both heat-treated and bead-beaten.

Bacteria from liquid cultures were separately heated at 90°C for 10 minutes and 60°C for 30 minutes after being washed with PBS. Bacteria collected from plates were heated at 90°C for 10 minutes after being separated from the supernatant and washed with PBS.

Bacteria from liquid cultures alternatively were washed and then transferred in the bead beater machine. Bacteria were beated for 45 seconds for 3 times at a speed of 4.0 m/s; 5 minutes of rest were between each cycle.

6.7 Fractionation of bead-beaten *L. reuteri* DSM 17938

Bacterial components from bead-beaten *L. reuteri* DSM 17938 culture were separated using VivaSpin centrifugal (Sartorious, Goettinger, Germany) units which were centrifuged at 4000 rpm for 15 minutes. Cut off values were 300 000 Da and 3000 Da.

6.8 Axenic hatching of *Artemia*

Axenic, meaning germ-free, instar II *Artemia* metanauplii were obtained following decapsulation and hatching process as previously described by Marques in 2005 with slight changes[33]. Briefly, 50 mg of *Artemia* cysts were hydrated in 10 ml milliQ water for 1h. During the hydration process, gentle aeration was provided continuously through a 0.22 μm filter. Further, 0.33 ml NaOH (32 %) and 5 ml NaOCl (13 %) were added. The reaction was stopped after about 2 minutes by adding 5 ml of autoclaved $Na_2S_2O_3$ (10 g/l). During this entire process, aeration was provided continuously in order to facilitate proper mixing. After decapsulation, cysts were washed several times with filtered autoclaved seawater (10 g/l) (FASW) and collected over a 50 μM sterile sieve. This entire procedure was carried out under laminar flow hood to avoid contaminations. The collected decapsulated cysts were subsequently transferred to Falcon tubes containing 35 ml of FASW. The Falcon tubes were placed on rotors in an incubation room at 28°C for 28h with continuous illumination (2000 lux). The incubation and hatching processes were continued for 28h after which sterile instar II *Artemia* metanauplii were collected for the experiments.

6.9 Verification of axenity of hatched *Artemia*

Axenity of the *Artemia* metanauplii was verified by transferring 500 µl of the hatching water into 15 ml of Marine broth followed by incubation at 28°C for 5 days on the shaker at 115 rpm[54]. Experiments started with non-axenic metanauplii were retrospectively discarded.

6.10 *Artemia* survival studies

Different types of experiments were set up with *Artemia*. All the processes were exerted under the laminar flow hood with autoclaved equipment to avoid contamination; throughout the duration of the experiments *Artemia* were placed on rotors in an incubation room at 28 °C with continuous illumination (2000 lux). All the experiments were repeated two or three times and each treatment/challenge in every experiment was done in five replicates. Each replicate contained 20 *Artemia* in 10 ml FASW 10 g/L. The OD of any bacterial cultures to be added to *Artemia* was measured at 550 nm in order to calculate the amount of µl required to reach the wanted CFU/ml within the system.

First of all, in order to assess whether *Artemia* could be a good infection model when challenged with ETEC, different concentrations of ETEC (10^5, 10^6,10^7 and 10^8 CFU/ml) were tested. Negative control consisted on *Artemia* in FASW; survival rate was measured 48h after the challenge.

Potential detrimental effects of *L. reuteri* DSM 17938, *L. lactis* MG 1363 and *L. reuteri* ATCC PTA 6475 on *Artemia* were evaluated. 10^7 and 10^8 CFU/ml of each probiotic strain from liquid cultures were tested on *Artemia*. Negative control consisted on *Artemia* in FASW; survival rate was measured 48h after the treatment. Following this experiment all the probiotics tested were administered at a concentration of 10^8 CFU/ml.

Potential protective effects of both live and heat-treated (90°C) *L. reuteri* DSM 17938 against *V. parahaemolyticus* infection were studied by comparing survival rates of *Artemia* after administration of either live or dead *L. reuteri* DSM 17938 in either presence or absence of the challenge with *V. parahaemolyticus*. The probiotic and the postbiotic obtained from the liquid cultures and the pathogen were added at the same time at the beginning of the experiment. *Artemia* in FASW was used as negative control, *Artemia* challenged with *V. parahaemolyticus* without any probiotic added was used as positive control. Survival rate was measured 24h and 48h post exposure.

L. reuteri DSM 17938 was killed in different ways and postbiotics resulting from these different protocols were tested on *Artemia* to start investigating the mechanisms involved behind the beneficial effects exerted by heat-treated (90°C) *L. reuteri* DSM 17938. Following growth in liquid culture, *L. reuteri* DSM 17938, heat-treated *L. reuteri* DSM 17938 (60°C) and bead-beaten *L. reuteri* DSM 17938 were provided; the supernatant and the pellet containing either the live or heat-treated (90°C) bacterial cells were collected from plate-grown cultures. All these variants were tested on *Artemia*. *Artemia* in FASW was used as negative control, heat-treated (90°C) *L. reuteri* DSM 17938 from liquid culture was used as positive control. Survival rate was measured 24h and 48h post exposure to the probiotic/postbiotics.

Potential toxic effects of the chemicals used for the decapsulation process on *Artemia* were evaluated by assessing the survival rate of this model after decapsulation lacking selective steps in relation to the standard protocol (section 6.8). *Artemia* cysts were collected after the sole rehydration, after

addition of chemicals and after being washed with FASW and allowed to hatch for 28h at 28°C. The latter represented the negative control. *Artemia* collected after rehydration was also challenged with *V. parahaemolyticus* after the hatching period. Survival rate was assessed after 52h and 76h from the decapsulation process (24h and 48h after challenge with *V. parahaemolyticus*).

Potential role of proteins in lethality of the supernatant deriving from plate-grown *L. reuteri* DSM 17938 was evaluated by administering the supernatant previously heated at 90°C for 10 minutes. *Artemia* challenged with supernatant collected directly from the plates was used as positive control, *Artemia* in FASW was used as negative control. Survival rate was measured after 24h and 48h from the treatment.

Dose-response curves of the supernatant deriving from plate-grown *L. reuteri* DSM 17938 were obtained by assessing survival rate of *Artemia* challenged with the supernatant in amounts corresponding to 10^5, 10^6,10^7 and 10^8 CFU/ml of the bacterial cultures. *Artemia* in FASW was used as negative control. Measurements were done after 24h and 48h from the challenge.

Investigation of bead-beaten *L.reuteri* DSM 17938 effect on *Artemia* was carried out by testing different bacterial components separated according to molecular weight through fractionation. Components larger than 300 kDa, ranging between 300 and 3 kDa and smaller than 3 kDa were given to *Artemia*. *Artemia* in FASW was used as negative control, *Artemia* treated with bead-beaten *L. reuteri* DSM 17938 were used as positive control. Survival rate was measured 24h and 48h after the treatment.

6.11 Statistical analyses

a) Standard deviations were calculated for each treatment of every experiment except for the assessment of chemical toxicity.
b) T-tests were carried out for assessment of statistically significant differences between:
   i) negative control and *L. reuteri* DSM 17938 (10^8 CFU/ml) after 48h (section 7.2)
   ii) negative control and supernatant/heat-treated supernatant/plate-grown culture after 24h (section 7.6)
   iii) bead-beaten *L. reuteri* DSM 17938 and the fraction 3-300 kDa of bead-beaten *L. reuteri* DSM 17938 after 48h (section 7.8)
c) Anova and subsequent Tukey Kramer post-hoc were carried out to assess statistically significant differences in survival rate of *Artemia* after administration of live *L. reuteri* DSM 17938 or 90°C heat-treated *L. reuteri* DSM 17938 either in the presence or absence of *V. parahaemolyticus* (section 7.3)
d) Anova and subsequent Tukey post-hoc were carried out for assessment of statistically significant differences among:
   i) *L. reuteri* DSM 17938 killed in different ways and in relation to the negative control (section 7.4)
   ii) Different concentrations of supernatant and in relation to the negative control (7.7)
   iii) Different fractions of bead-beaten *L. reuteri* DSM 17938 and in relation to the negative control (section 7.8)

For T-tests and Anova analyses α-value = 0.05

7. RESULTS

7.1 Evaluation of ETEC in an infection model was unsuccessful.

10^5, 10^6, 10^7 and 10^8 CFU/ml of ETEC were added to *Artemia* to evaluate whether the increase of bacterial concentration was related to a consistent trend in *Artemia* survival rate reduction. However, nothing could be concluded according to the results, which fluctuated as presented (**Fig. 1**); statistical analyses also supported inconsistency of results, since rather high standard deviations were measured.

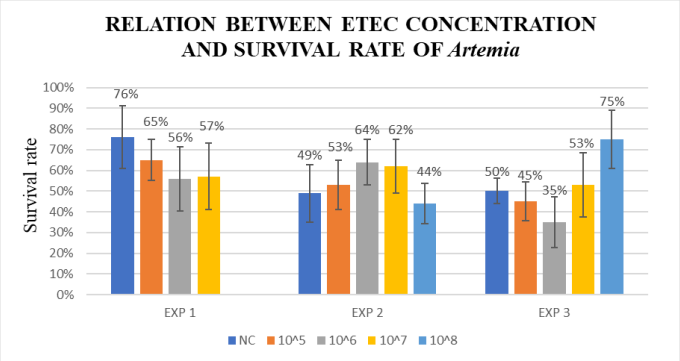


Fig.1. Trends of survival rate of *Artemia* challenged with increasing concentrations of ETEC. *EXP1*, *EXP2* and *EXP3* are three replicates of the same experiment. *NC*:negative control. Measurements were taken 48h after challenge. Error bars indicate standard deviations.

7.2 Probiotics exert beneficial effects on *Artemia*.

Different concentrations of *L. reuteri* DSM 17938, *L. reuteri* ATCC PTA 6475 and *L. lactis* MG 1363 were tested in order to assess any beneficial/detrimental effects on *Artemia*. Compared to the negative control, which presented a survival rate of 53% after 48h from the beginning of the experiment, all three strains/species of probiotics exerted a positive effect on *Artemia* **(Fig. 2)**. Specifically, their administration at the concentration of 10^8 CFU/ml to the system exerted a stronger effect that the one obtained at the concentration of 10^7 CFU/ml.

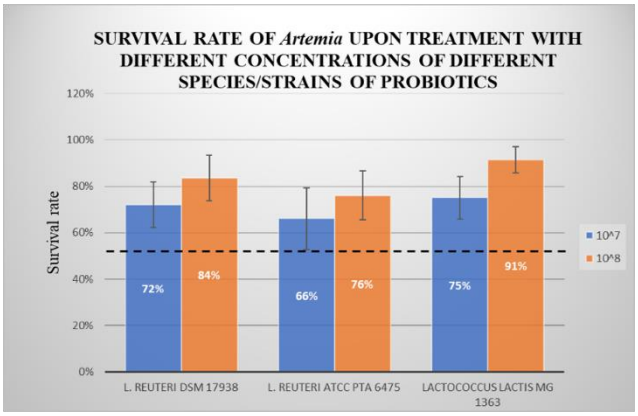
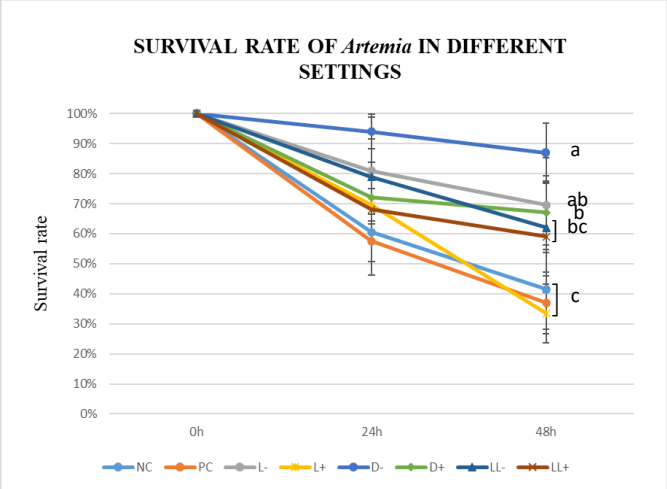


Fig.2. Impact of different probiotics (*L. reuteri* DSM17938, *L. reuteri* ATCC PTA 6475 and *L. lactis MG* 1363) on *Artemia* survival rate. *Dashed line*: negative control. Measurements were taken after 48h from treatments. Error bars indicate standard deviations (average standard deviation is 9,6%). P value (NC, *L. reuteri DSM 17938* 10^8)=0,00026

7.3 90°C heat-treated *L. reuteri* confers the highest fitness to *Artemia* both in the presence and absence of *V. parahaemolyticus*.

In order to explain the molecular mechanism behind the positive effect produced by *L. reuteri* DSM 17938, *Artemia* was treated with either live or 90°C heat-treated *L. reuteri* at 10^8 CFU/ml both with and without challenge provided by administration of *V. parahaemolyticus*. Live *L. lactis* MG 1363 effectiveness was tested as well in the presence or absence of *V. parahaemolyticus* as control for live *L. reuteri* DSM 17938 (**Fig. 3**).

Fig.3. Survival rate of Artemia over time in different conditions. *NC*: negative control, i.e. *Artemia* without any treatment; *PC*: positive control, i.e. *Artemia* challenged with *V. parahaemolyticus*; L-: *Artemia* treated with *L. reuteri* 17938; *L+*: *Artemia* challenged with *V. parahaemolyticus* and treated with *L. reuteri* DSM 17938; *D-*: *Artemia* treated with *L. reuteri* 17938 killed at 90°C for 10 minutes; *D+*: *Artemia* challenged with *V. parahaemolyticus* and treated with *L. reuteri* 17938 killed at 90°C for 10 minutes; *LL-*: Artemia treated with *L. lactis MG* 1363 ; *LL+*: Artemia challenged with *V. parahaemolyticus* and treated with *L. lactis MG* 1363. Error bars indicate standard deviations. Anova and Tukey-Kramer post-hoc were carried out; significant differences among the treatments are indicated by different letters (p<0,05).



No differences between negative and positive control were observed, which didn't allow us to determine the actual role of *V. parahaemolyticus* in *Artemia* survival rate. Both live and dead *L. reuteri* improved survival rate of *Artemia* in a statistically significant way, although dead bacteria were more beneficial. Drop in survival rate of challenged *Artemia* was restrained by dead *L. reuteri*, while live *L. reuteri* intriguingly showed no protective effects in presence of *V. parahaemolyticus*.

7.4 *L. reuteri* killed in different ways improve *Artemia* survival rate slightly differently, albeit always to a greater extent compared to live *L. reuteri*. Bead-beaten *L. reuteri* provides the highest benefit to *Artemia*.

L. reuteri DSM 17938 grown in liquid medium was both heat-treated at 90°C and 60°C and bead-beaten. Plate grown *L. reuteri* DSM 17938 was introduced to identify differences in the effectiveness of the probiotic in relation to different growth methods; bacterial components in plate-grown cultures were used either alive or heat- treated at 90°C as treatment as well as the supernatant from which they were separated from.

Results showed that regardless of the way *L. reuteri* was killed, it exerted a better action than the live bacteria **(Fig. 4)**. In particular, the best performance was given by *Artemia* treated with bead-beaten *L. reuteri*, which was the only one to show a statistically significant difference with live *L. reuteri*.

L. reuteri heat-treated at 90°C and 60°C affected positively and similarly *Artemia* survival rate, whose amelioration relative to the negative control was comparable to the one provided by both live and 90°C heat-treated bacterial components from the

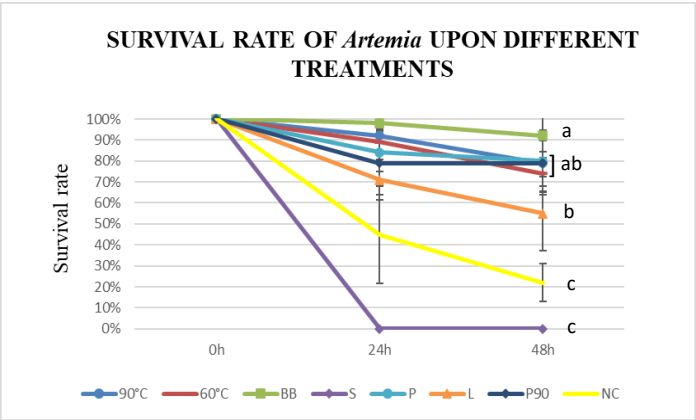


Fig.4. Survival rate of *Artemia* over time upon administration of different treatments. *90°C*: *L. reuteri* DSM 17938 killed at 90°C for 10 minutes; *60°C*: *L. reuteri* DSM 17938 killed at 60°C for 30 minutes; *BB*: bead-beaten *L. reuteri* DSM 17938; *S*: supernatant of plate-grown *L. reuteri* DSM 17938; *P*: pellet of plate-grown *L. reuteri* DSM 17938; *L*: live *L. reuteri* DSM 17938; *P90*: pellet of plate-grown *L. reuteri* DSM 17938 heated at 90°C for 10 minutes; *NC*: negative control, i.e. *Artemia* without any treatment. Error bars indicate standard deviations. Anova and Tukey post-hoc were carried out; significant differences among the treatments are indicated by different letters (p<0,05).

plate grown cultures. Surprisingly, the supernatant revealed itself to be lethal for the nauplii after already 24h of incubation.

7.5 Chemicals used for decapsulation of *Artemia* cysts are toxic but they are not responsible for low survival rate of negative control.

The survival rate of *Artemia* observed after 48h lower than it should be, thus different steps of the decapsulation process have been examined to understand whether one of them was critical. *Artemia* was hatched according the standard protocol, after the addition of the chemicals or without the addition of chemicals at all. Besides this, given the high survival rate that could potentially be measured in *Artemia* hatched in physiological conditions, these latter were challenged with *V. parahaemolyticus* to assess the extent of its detrimental effects on the metanauplii (**Fig. 5**).

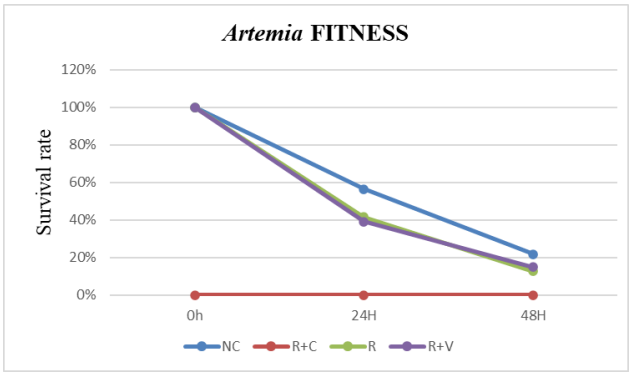


Fig.5. Survival rate of *Artemia* decapsulated with different methods. *NC*: negative control, i.e. *Artemia* decapsulated according the standard protocol (section 5.1); *R+C*: *Artemia* collected after addition of chemicals for disinfection of cysts during decapsulation; *R*: *Artemia* collected after rehydration; *R+V*: *Artemia* collected after rehydration and challenged with *V. parahaemolyticus*.

Artemia treated with chemicals that were not successively washed away didn't hatch at all, so it was impossible to measure their survival rate over 48 hours. In relation to this outcome, it was inferred that chemicals were not responsible for low survival rate of the negative controls (decapsulated with the standard protocol), since the same survival rate was measured for *Artemia* hatched without chemicals. The survival rate of *Artemia* hatched in physiological conditions after 48 hours was still low, and conclusions on *V. parahaemolyticus* action could not be drawn.

7.6 Proteins in the supernatant of plate-grown cells are likely not responsible for *Artemia* death.

Since supernatant of plate-grown bacteria turned out to be lethal for *Artemia*, both 90°C heat-treated supernatant and cultures collected directly from the plates were administered to the *Artemia* to evaluate possible differences in the outcome (**Fig. 6**). None of the metanauplii survived any of the interventions, which suggested that proteins in the supernatant, denatured at 90°C, were not involved and that the presence of bacterial cells didn't prevent *Artemia* survival from being affected by the supernatant itself.

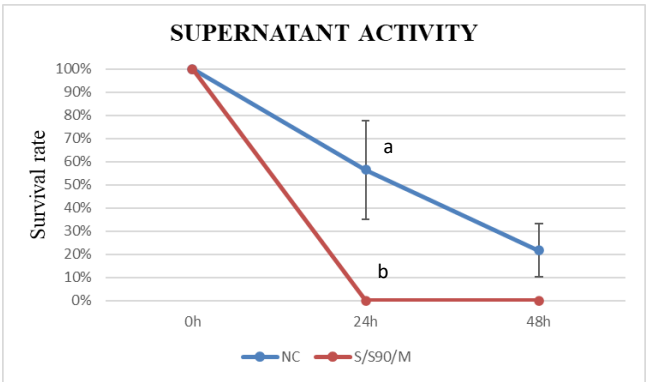
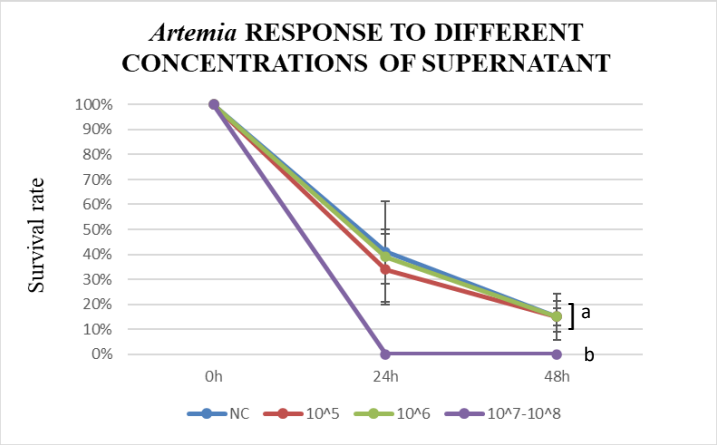


Fig.6. Survival rate of *Artemia* over time upon administration of plate-grown *L. reuteri* DSM 17938 components. *NC*: negative control, i.e. *Artemia* without any treatment; *S*: supernatant of plate-grown *L. reuteri* DSM 17938; *S90*: supernatant of plate-grown *L. reuteri* DSM 17938 heated at 90°C for 10 minutes; M: plate-grown *L. reuteri* DSM 17938. Error bars indicate standard deviations. P value (24h) $=7{,}34*10^{-6}$.

7.7 The lethality of supernatant is dose dependent.



supernatant. *NC*: negative control, i.e. *Artemia* without any treatment. Error bars indicate standard deviations. Anova and Tukey post-hoc were carried out; significant differences among the treatments are indicated by different letters ($p<0,05$).

Different amounts of supernatant (corresponding to 10^5, 10^6, 10^7 and 10^8 CFU/ml of the bacterial culture) were tested on *Artemia* in order to draw a dose-response curve (**Fig. 7**). As it can be clearly seen from the graph, the supernatant was lethal for *Artemia* only in concentrations higher than 10^7 CFU/ml, while survival rate measured when the supernatant was present in lower doses was comparable to the one of the negative control. No statistically significant differences were observed among negative control, 10^5 and 10^6 treatments.

7.8 The molecule from the bead-beaten *L. reuteri* DSM 17938 culture responsible for improvement of *Artemia* survival ranges between 3 and 300 kDa in weight.After being bead-beaten the *L. reuteri* culture was fractionated in order to narrow down the pool of molecules that could have an active role in improvement of *Artemia* survival. Cut off sizes were 3000 kDa and 3 kDa and the three obtained fractions were added to *Artemia*. The fraction containing bacterial components ranging between 3 and 300 kDa exerted a positive action on *Artemia* which was basically identical to the one exerted by the bead-beaten *L. reuteri* original culture while the other two fractions didn't affect survival rate which was comparable to the one measured for the negative control. Dramatic differences in effectiveness between the intermediate fraction and the two extreme ones could thus be observed.

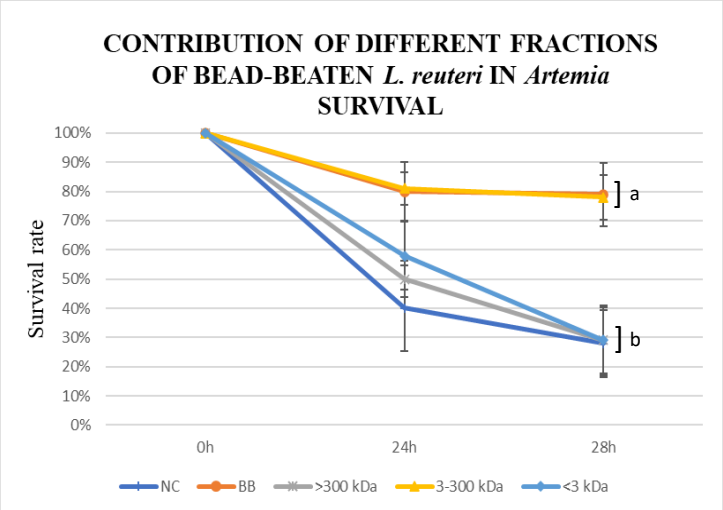


Fig.8. Survival rate of *Artemia* over time upon administration of different fractions of bead-beaten *L. reuteri* DSM 17938. *NC*: negative control, i.e. *Artemia* without any treatment; *BB:* bead-beaten *L. reuteri* DSM 17938; *>300kDa*: fraction of bead-beaten *L. reuteri* DSM 17938 containing molecules bigger than 300 kDa in weight; *3-300 kDa*: fraction of bead-beaten *L. reuteri* DSM 17938 containing molecules ranging between 3 and 300 kDa in weight; *<3 kDa*: fraction of bead-beaten *L. reuteri* DSM 17938 containing molecules smaller than 3 kDa in weight. Error bars indicate standard deviations. Anova and Tukey post-hoc were carried out; significant differences among the treatments are indicated by different letters ($p<0,05$).

8. DISCUSSION

In the present study Enterotoxigenic *Escherichia coli* (ETEC) was initially chosen as pathogen to develop an infection model with *Artemia franciscana* and investigate potential protective effects exerted by *Lactobacillus reuteri* DSM 17939. This choice was supported by evidence of effectiveness of *L. reuteri* DSM 17938 in reducing detrimental effects of ETEC in culture cell experiments [55]; also, *Artemia* had already been proven to be a good host model for virulence analysis not only for marine, but also for human disease-causing pathogens[46]. However, we weren't able to reproduce an infection model, since no pattern was observed in survival rate of *Artemia* upon administration of increasing concentrations of pathogen. Intriguingly, increase in survival rate occurred when *L. reuteri* was given to *Artemia* instead. Due to the relatively low survival rate of the negative control, it was hypothesized that *Artemia* was being exposed to some kind of stress during hatching and throughout the experiments, and that *L. reuteri* was able to counteract this. Its protective effect towards *Artemia* was however lost when added together with *Vibrio parahaemolyticus*, a well-known *Artemia* opportunistic pathogen. Since administration of *L. reuteri* occurred in parallel with addition of the pathogen, it was not surprising that live *L. reuteri* didn't provide any protection for *Artemia*; in fact, many other studies have demonstrated probiotics to be protective against pathogens only when administered to the host prior than the microbial challenge[28,41]. Conversely, it was striking that 90°C heat-treated *L. reuteri* exerted a benefit on challenged *Artemia* and was shown to be even more beneficial than live *L. reuteri* in absence of *V. parahaemolyticus*, which was in disagreement with other studies were administration of dead bacteria didn't confer any protection to *Artemia*[41].

Regardless of the mode of killing (90°C and 60°C heat-treated, bead-beaten) dead *L. reuteri* always improved survival rate of *Artemia* to a greater extent than the live bacteria. Fractionation of bead-beaten bacteria allowed for identification of the weight range of the molecule responsible for the benefit provided to *Artemia*, namely 3 to 300 kDa, and administration of the exudate of plate-grown bacteria led to total *Artemia* death. Based on these findings, both low molecular weight metabolites and membrane vesicles were excluded from being involved and it was hypothesized that the very same molecule was likely to be localized either within the bacterial cells or on the surface of cell walls. At this point however it is important to note that the supernatant administered to *Artemia* derived from plate-grown bacteria, since supernatant from cultures grown in liquid medium is discarded when bacterial cells are resuspended in PBS. It is known that the properties of *L. reuteri* grown on plate might differ from the one observed for *L. reuteri* grown in broth, likely as consequence of biofilm formation. Furthermore, enzymes that are common to both cultures are found to be super activated in plate-grown bacteria to an extent up to 100-fold[56]. It was therefore hypothesized that lethality of the supernatant, observed also in other studies with other probiotic species[39], could be ascribed to an overdose of some kind of molecule. Proteins, however, were temporarily excluded from being responsible for *Artemia* death because administration of 90°C heat-treated supernatant had the same detrimental effect as the non-treated supernatant. Supernatant of plate-grown *L. reuteri* was administered to *Artemia* with increasing concentrations in order to construct a dose-response curve. Survival rate wasn't affected by this supernatant as long as it was in low doses, but it was observed to drop drastically when the supernatant concentration corresponded to 10^7 CFU/ml, which supported the initial hypothesis.

Based on the results obtained, some conclusions can be drawn. First of all, *L. reuteri* confers a benefit to *Artemia* exposed to some kind of stress during the sensitive period of hatching and subsequent

development. In particular, dead bacteria improved the survival rate of *Artemia* to an extent even greater than live ones, supporting the recent findings about the effectiveness of lactic acid bacteria extracts in stimulation of cytokine production by macrophages and possibly other immune cells[5].

Postbiotics represent a good alternative to probiotics since they are more stable storage-wise and might increase accessibility of beneficial molecules for receiving hosts. Besides this, concerns about safety of probiotics have recently been raised. Despite extensive evidence of their positive effects, probiotics are still live bacteria and can represent a risk for the pre-term infants and people either with underlying conditions or immunosuppression[57]. Although knowledge about possible adverse effects of probiotics is still limited, the main concern is development of bacteremia and/or sepsis following administration[58]. No clinical trials have reported cases of sepsis so far, but some cases of sepsis attributable to probiotics, especially following administration of *S. boulardii*, have been reported. They are very few and they affected mainly people belonging to risk groups; nevertheless, it is something to take into account. Other theoretical risks associated with probiotic administration are the acquisition/transmission of resistance genes, excessive immune response in susceptible individuals and prevention of healthy gut bacterial colonization in neonates[57,58].

All these issues can be overcome by postbiotics, as they are non-viable bacterial cells and/or bacterial constituents. Inactivation of probiotics can be achieved by different methods, including heat, chemicals (e.g., formalin), gamma or ultraviolet rays, and sonication, with heat treatment being the method of choice for inactivation of probiotic strains in most cases[57]. Of course, different structural bacterial components are affected depending on the method used, which might be responsible for the different biological activity of resulting postbiotics. For this project it was therefore decided to test *L. reuteri* obtained by using two different inactivation methods, heat treatment and bead beating, in order to detect possible differences in effectiveness. It was observed that both provided a benefit to *Artemia*, but bead-beaten bacteria improved survival rate to a slightly higher degree. This indicates that there are a bacterial component in both types of postbiotics that positively influences *Artemia* performance; but also that the two methods affect those bacterial components in different ways, which determines the different levels of protection conferred by bead-beaten and heat-treated *L. reuteri* to *Artemia*.

The fractionation of bead-beaten *L. reuteri* culture allowed us to narrow down the pool of possible molecules responsible for the improvement of survival rate, but further studies are needed to identify the specific compound. The potential molecules that are going to be mentioned below and that are likely to be involved in *Artemia* protection have been shown to have an immunomodulatory action mediated mainly by Toll-like or NOD-like receptors (TLR and NLR respectively) and have been isolated from different bacterial species and strains[5]. It must therefore be considered that activation of the innate immune system is strain-specific and that different biological pathways underlie beneficial effects provided by different strains. For this reason, comparison of molecular mechanisms responsible for beneficial effects provided by dead *L. reuteri* DSM 17938 and dead *L. lactis* MG 1363 for *Artemia* could be very interesting.

Bacterial cell wall components are likely to be involved in immunomodulation, since both treatments affect cell wall stability; in particular, as a Gram-positive bacterium *L. reuteri* might activate *Artemia*'s immune system through components that are released from the cell wall after its killing, such as peptidoglycan and teichoic acids[5]. Peptidoglycan is the main component of the Gram-positive cell walls and is characterized by an extensive network of cross-links among its peptide chains which define its tridimensional structure; NOD1 and NOD2 are the pattern recognition receptors specialized

in recognition of this polymer[59]. Teichoic acids instead are phosphodiester polymers of glycerol or ribitol, and they can be covalently linked to either peptidoglycan (wall teichoic acids) or the cytoplasmic membrane[5]; teichoic acids from different *Lactobacillus* species have shown to elicit immune response through interaction with TLR2[60].
Some probiotic lactobacilli have been shown to interact with the immune system of the host also through other molecules which are not constitutive of the bacterial cell wall[5]. S-layer for example is a monomolecular crystalline envelope produced by the self-assembly of protein or glycoprotein subunits on the outer cell surface commonly found in prokaryotes; a study from 2008 demonstrated that S-layer proteins play an important role in activation of Th2 cells through cross-talk with dendritic cells[61]. Another group of molecules that has been found to interact with the innate immune system and that could explain the protective action of dead *L. reuteri* are the surface-associated lipoproteins, surface heat-shock proteins and genomic DNA, whose unmethylated CpG motifs are proposed to be involved[5,62].

Identification of the exact molecular mechanism behind the protective effect of *L. reuteri* on *Artemia* should be coupled to clarifications about the probiotic pharmacokinetics in the model, which is related to bacterial uptake, proliferation and colonization of the gastrointestinal tract in *Artemia*. Encapsulation ability of *Artemia* varies in fact between nauplii and adults[38], and it also depends on type of bacteria used, time of exposure, and status (live or dead) of the bacteria[33]. These variabilities must be taken into account when the functionality of a probiotic is being investigated, since it is important to distinguish between the intrinsic ability of the strain to positively influence the host and its ability to reach and maintain itself in the location where the effect is to be exerted[35]: if the probiotic is naturally able to provide a benefit to the host but it does not colonize, its activity will be just temporary and will ceases soon after its administration is interrupted[38,63]. Quantification and/or detection through fluorescent microscopy of the bioencapsulated bacteria in *Artemia* are experiments carried out in other studies with the exact purpose of clarifying all these aspects[63,64] and they could also be applied to *L. reuteri* DSM 17938.

The nature of the interaction between *L. reuteri* DSM 17938 and *Artemia* was analyzed from the probiotic point of view in the project, trying to identify the molecule responsible for the protective effect exerted by *L. reuteri* itself. One could also investigate the interaction from an *Artemia* point of view, monitoring alterations in gene expression and immune response in *Artemia* upon administration of probiotics; real time PCR and immuno-assays like ELISA and western-blot are just some of the possible alternatives to achieve that. *Artemia* immune system is relatively simple since it depends completely on innate system[65]. The prominent immune responses of invertebrates are in fact Toll-like receptor-mediated antimicrobial peptide production, hemolymph coagulation and melanin formation. In addition, being aerobic organisms, they have developed a set of antioxidant defense systems to protect them from ROS accumulation, including antioxidant enzymes such as superoxide dismutase (SOD), glutathione (GSH) redox cycle enzymes and glutathione S-transferase (GST). Protection against pathogens has been ascribed to the cooperation between the melanin formation pathway (known also as prophenoloxidase (Pro-PO) activating system) and antioxidant enzymes. Particular interest has been addressed to heat-shock protein 70 (Hsp70), which has been shown to be responsible for Pro-PO system activation and its upregulation has been associated to protection against several type of abiotic and biotic stresses in *Artemia*[41,66–68]. The protective function of the Hsp70 is documented to be due to its chaperone activity maintaining protein homeostasis by protecting nascent

polypeptides from misfolding, facilitating co- and post-translational folding, assisting in assembly and disassembly of macro-molecular complexes, and regulating translocation[42,69]. Since its expression is not affected by sampling or handling, Hsp70 is being evaluated as indicator for environmental stressor at the moment, despite the fact that its reliability can anyway be biased by background pathology of the host[70]. In relation to probiotics, it has been observed that *L. reuteri* (although not the strain DSM 17938) enhanced gut barrier function through cytoprotective induction of Hsp analog to Hsp70 in a porcine intestinal epithelial cell line model[71]. For all these reasons, Hsp70 expression is the most reasonable parameter to measure over time during the experiments: the data gathered will give information about whether and how *L. reuteri* DSM 17938 affects *Artemia* immune system improving its fitness.

It is noteworthy that results obtained in this study relative to probiotic activity were always compared to other studies that used *Artemia* as infection model, whereas in this case it is not known what *L. reuteri* is protective against. It was initially speculated that *Artemia* was exposed to some kind of environmental stressor, such as salinity or pH, but survival rate of *Artemia* didn't change when *Artemia* was reared in seawater with different salt concentrations and pH turned out to be within the optimal range for *Artemia* development. Of course, other variabilities such as water qualities could be assessed, however the cause of low survival rate is probably to be sought in *Artemia* cysts. At the beginning of the project the *Artemia* survival rate measured was around 50-60 %, which was already lower than the expected 80-90%. Rearing conditions were kept unvaried during the whole period of study, but survival rate gradually decreased over time and the most recent experiments showed a survival rate of 20-30%. Analyses were done to evaluate potential mistakes in the decapsulation/hatching procedure, but no faulty methods could be pinpointed. The two possible alternatives that explain this trend are decreased cysts viability or increased mortality rate of nauplii. Decreased embryos viability was in fact observed with increased storage time also from Clegg in 1976, although no biological explanation was given for this correlation[72]. On the other hand, a recent study from 2016 associated increased mortality rate of nauplii to decreased cysts buoyancy (tendency to float), which was in turn caused by constant selective pressure against floating cysts over time exerted by human harvesting[73]. If low survival rate of *Artemia* were ascribed to either reason, then it would be possible to speculate that *Artemia* worked as either aging or starvation model. In the first case, increased storage time might lead to a progressive molecular impairment which could be correlated to the aging process. In the second case, cyst yolk represents the link between buoyancy and mortality rate; the yolk constitutes in fact the energy reserve for the developing encysted embryos and newly hatched nauplii, and decreased buoyancy is a direct result of alteration in yolk composition. For this reason, increased mortality rate of nauplii could be due to insufficient energy availability and amelioration of survival rate following administration of probiotics/postbiotics might be a feeding effect by providing energy or other type of nutrients.

Characterization of *Artemia* is fundamental in order to define it as experimental model for further studies in the probiotic field. So far, mice are mainly used as experimental models, but *Artemia* represents a valid support for the research without running into ethical issues. Differences between humans and brine shrimps however mustn't ever be underestimated. With regard to probiotic studies, germ-free *Artemia* is revealing itself to be a promising model to investigate the mechanisms through which probiotics influence the innate immune system and contribute to the integrity of the gastrointestinal tract. Nevertheless, it is important to consider that probiotics can have effects on a

systemic level in humans, and whether the treatments tested on *Artemia* have side effects in a more complex organism cannot be excluded. On the other hand, use of *Artemia* reared in physiological conditions as a model organism is still premature, since the intestinal microbiota would have a relevant part in the outcome of the experiments[74]. It is known that gut microbiota has a huge impact on human health, but the molecular mechanisms by which its alteration increase susceptibility to diseases have just started to be identified. Elements that make this research challenging are the fact that there is an intra-personal variability of the microbial diversity and that this diversity can be influenced by a broad range of factors, like diet or stress. Using a model such as *Artemia* retaining its natural microbiota for studying probiotics would be controversial for several reasons. First of all, the relative abundance of the phyla composing the microbiota is different from the human one, with a prevalence of *Proteobacteria*. Secondly, being an aquatic organism, *Artemia*'s microbiota is largely influenced by the bacteria present in the environment and it is characterized by a transient bacterial community[35]. Translational relevance in humans of the results obtained based on all these considerations would be reasonably questionable and would require very strong evidence.

This issue of course doesn't pose itself when the research on probiotics is related to aquaculture. Because of the increasing spread of antimicrobial resistance in fish due to misuse of antibiotics, probiotics have been suggested as a preventive measure, but there are some factors that limit their use, including the short survival of probiotics in seawater and risk of microbiological pollution[28]. A good alternative could be provided by *Artemia*, one of the main live feeds currently used in the fish industry[32] due to the many advantages it has in terms of availability, culture and storage for the culturist. *Artemia* (nauplii or adults) is being evaluated as a carrier for components that are otherwise difficult to administer to fish and crustacean larvae, among which are probiotics. Indeed, more and more studies are showing that they represent an excellent form of prophylaxis against opportunistic infections[28]. This project could pave the way for further investigations about its use in management of environmental stressors.

In conclusion, the results presented in the report suggest that *Artemia* is a promising model in probiotic research that could complement mice in laboratory. Furthermore, findings in this field could be exploited in aquaculture as well, where probiotics could have a more direct application.

9. ACKNOWLEDGMENTS

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