Influence of decapsulation and probiotics in the microbiome of *Artemia franciscana*

Pedro Bernardino Quintela Vale

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Orientador
Professora Auxiliar Olga Maria Lage, Faculdade de Ciências

U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

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O Presidente do Júri,

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Abstract

In aquaculture, the bottleneck of the production is often the larvae stage. In this stage reared animals are commonly fed with live *Artemia franciscana*. Adding bacterial supplements as probiotics can be used to diminish mortality. One way of delivering the probiotics is through *Artemia*, but this delivery system and associated microbiome changes are not thoroughly studied. The objectives of this thesis were to study: 1) the effect of decapsulation and probiotics on the microbiome of *Artemia franciscana* using the molecular technique denaturing gradient gel electrophoresis (DGGE) and 2) the effects of these supplements on the crustacean growth and mortality. To achieve these objectives a commercial brand of probiotics (AquaStar® Growout) as well as the scarcely studied planctomycete *Rhodopirellula rubra* strain LF2 were assayed.

Non-decapsulated (normal) and decapsulated *Artemia* were reared in 4 treatments: 1) control (no bacteria supplementation); 2) probiotic (supplementation with *Bacillus*, *Lactobacillus*, *Pediococcus* and *Enterococcus*); 3) planctomycetes 4) a mixture of the two. Studies on *Artemia's* microbiome through DGGE and on *Artemia's* growth and mortality were performed and analysed. DGGE data was analysed statistically with cluster analysis, nMDS, SIMPER and ANOSIM. Growth and mortality data were searched for significant differences with Tukey and Dunnett tests.

Results indicate that decapsulation plays a major role in microbiome changes. The exposure to the different treatments showed that one hour was sufficient to impose differences in the *Artemia*'s microbiome. This was also observed, over time, within each specific treatment and among different treatments. Independently of decapsulation process, the cysts always clustered together in the DGGE gel analyses, indicating similarities between their microbiome. Normal *Artemia* microbiomes became more dissimilar than decapsulated ones did during the different stages of *Artemia*'s life cycle. Probiotics had a stabilizing effect in the microbiome. In *Artemia* growth assays, no significant differences in adult sizes among treatments were found. Control and probiotic plus algae treatments had significantly less mortality than the other treatments. Probiotic and planctomycetes do not serve as food for *Artemia* as 100 % mortality was attained. Planctomycetes affect the viability of *Artemia*. These studies showed that *Artemia*'s bacterial community is a dynamic and changeable one.

Keywords: Aquaculture · Larvae feed · Live feed · *Artemia franciscana* · Probiotic · Planctomycetes · Bacterial community · Microbiome · DGGE · Mortality · Cluster analysis · nMDS analysis · SIMPER · ANOSIM

Resumo

Em aquacultura, o estado larvar é o fator limitante da produção, nesta fase o animal a ser produzido é normalmente alimentado com o alimento vivo: *Artemia franciscana*. Adicionar um suplemento bacteriano como probiótico pode ser usado para diminuir a mortalidade. Uma maneira de fornecer esse probiótico é através de *Artemia*, mas este sistema de entrega e as mudanças no microbioma associado não estão bem estudados. Os objetivos desta dissertação foram estudar: 1) o efeito da descapsulação e probióticos no microbioma de *Artemia franciscana* usando a técnica molecular eletroforese em gel com gradiente desnaturante (DGGE) e 2) os efeitos desses suplementos no crescimento e mortalidade do crustáceo. Para atingir estes objectivos uma marca comercial de probióticos (AquaStar® Growout) tal como o pouco estudado planctomycete (*Rhodopirellula rubra* strain LF2) foram ensaiados.

Artémias não descapsuladas (normais) e descapsuladas foram incubadas em 4 tratamentos: 1) controlo (sem suplementação de bactérias); 2) probiótico (suplementação com *Bacillus*, *Lactobacillus*, *Pediococcus* e *Enterococcus*); 3) planctomycetes; 4) uma mistura dos dois. Foram realizados e analisados estudos do microbioma da *Artemia* por DGGE, e estudos do crescimento e mortalidade da *Artemia*. Os dados do DGGE foram analisados estatisticamente com clustering, nMDS, SIMPER e ANOSIM. Nos dados de crescimento e mortalidade procuraram-se diferenças significativas com os testes de Tukey e Dunnett.

Os resultados indicam que o descapsulamento desempenha um papel importante nas mudanças do microbioma. A exposição aos diferentes tratamentos mostraram que uma hora era suficiente para impor diferenças no microbioma da *Artemia*. Isto também foi observado, ao longo do tempo, dentro de cada tratamento específico e entre os diferentes tratamentos. Independentemente do processo descapsulação, os cistos foram sempre agrupados nas análises do gel de DGGE, indicando semelhanças entre seu microbioma. O microbioma das artémias normais tornou-se mais dissimilar do que o das descapsuladas durante as diferentes fases do ciclo de vida da *Artemia*. Os probióticos tiveram um efeito estabilizador no microbioma. Nos ensaios de crescimento de *Artemia*, não foram encontradas diferenças significativas nos tamanhos dos adultos entre os tratamentos. Os tratamentos controlo e probiótico mais algas tiveram significativamente menor mortalidade do que os outros tratamentos. Probióticos e planctomycetes não servem como alimento para Artemia, dado que foi obtida uma mortalidade de 100 %. Planctomycetes afeta a viabilidade da

Artemia. Estes estudos mostraram que a comunidade bacteriana da *Artemia* é dinâmica e alterável.

Palavras-chave: Aquacultura · Alimentação larvar · Alimento vivo · *Artemia franciscana* · Probiótico · Planctomycetes · Comunidade Bacteriana · Microbioma · DGGE · Mortalidade · Clustering · Análise nMDS · SIMPER · ANOSIM

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Abbreviations list

AD - Decapsulated adults

AD+A - Decapsulated adults fed algae

AD+M - Decapsulated adults plus planctomycetes

AD+MA - Decapsulated adults plus planctomycetes fed algae

AD+P - Decapsulated adults plus probiotic

AD+PA - Decapsulated adults plus probiotic fed algae

AD+PM - Decapsulated adults plus probiotic plus planctomycetes

AD+PMA - Decapsulated adults plus probiotic plus planctomycetes fed algae

AN - Normal adults

AN+A - Normal adults fed algae

AN+M - Normal adults plus planctomycetes

AN+MA - Normal adults plus planctomycetes fed algae

AN+P - Normal adults plus probiotic

AN+PA - Normal adults plus probiotic fed algae

AN+PM - Normal adults plus probiotic plus planctomycetes

AN+PMA - Normal adults plus probiotic plus planctomycetes fed algae

ANOSIM - Analysis of Similarity

APS - Ammonium Persulfate

CD - Decapsulated cysts

CD+M - Decapsulated cysts plus planctomycetes

CD+P - Decapsulated cysts plus probiotic

CD+PM - Decapsulated cysts plus probiotic plus planctomycetes

CN - Normal cysts

CN+M - Normal cysts plus planctomycetes

CN+P - Normal cysts plus probiotic

CN+PM - Normal cysts plus probiotic plus planctomycetes

DGGE - Denaturing Gradient Gel Electrophoresis

ND - Decapsulated nauplii

ND+M - Decapsulated nauplii plus planctomycetes

ND+P - Decapsulated nauplii plus probiotic

ND+PM - Decapsulated nauplii plus probiotic plus planctomycetes

NMDS – Non-metric Multidimensional Scaling

NN - Normal nauplii

NN+M – Normal nauplii plus planctomycetes

NN+P - Normal nauplii plus probiotic

NN+PM - Normal nauplii plus probiotic plus planctomycetes

PCR - Polymerase Chain Reactions

SIMPER - Similarity Percentage Analysis

TAE - Tris-acetate-EDTA

TEMED – Tetramethylethylenediamine

1. Introduction

Over the last 20 years fisheries' productivity has stabilized at 90 million annual tons (Fig. 1) with changes in species caught and fishing areas used to do so. Meanwhile, the decline and overexploitation of marine species continues with more and more species being fully exploited (FAO, 2012). Monfort (2010) states that the European market, to which Portugal belongs, is characterized by a high fish deficit, making fish imports reach, in 2008, 30 billion Euros. The European Union has duplicated fish importation, making the import quota rise from 7% to 15% between the 90´s and 2007. Fishing alone cannot supply the expanding human population need for fish, so aquaculture's fish production fills in the global demand. In fact, aquaculture food production has increased almost 12 times in the last 30 years (Fig. 1) (FAO, 2012; Huysveld et al., 2013).

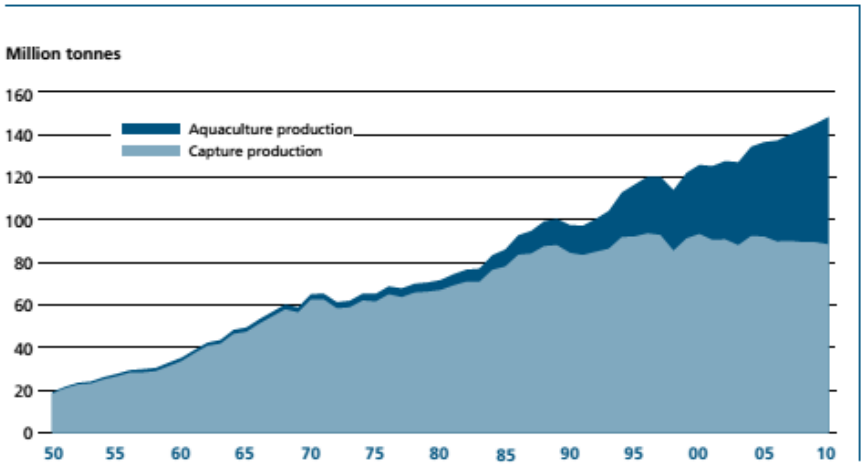


Figure 1 - Capture fisheries and aquaculture production worldwide in million tonnes (FAO, 2012).

Aquaculture is the rearing of any aquatic organism and initially it did not include the fish's whole life cycle, with fry being caught from nature and fattened. However, in the last decades, most fish production reproduces the whole life cycle (Lavens et al., 1996).

1.1. Fish Larvae Feeding

The cultivation of fish larvae involves the use of different raising, feeding and microbiological control protocols because fish larvae are very small, fragile, not yet with fully developed digestive and sensorial systems and with low mobility. Three limiting factors to the supply of feed arise from the characteristics referred: 1) the small size of the fish larvae, which limits physically the feed they can ingest; 2) the digestive system

is incomplete in the beginning of exogenous food intake, without a functional stomach and with few enzymes; and 3) the sensory systems (vision, smell, palate, touch), needed for detection and capture of feed/food aren't fully developed. Thus, when choosing the feed for aquaculture, one should consider the following: it should be easily digested, have enzymes which allow autolysis, be formed of small particles and contain the necessary nutrients to fish larvae in excess and it should be engaging to the senses. Live feed, contrary to artificial feed, addresses many of these requirements such as: having a bigger contrast with the water, movement and good distribution in the water column. To be an adequate diet the feed should: be available year round, have a good cost-benefit relationship, be simple and easy to use (Lavens et al., 1996). Currently, some of the more used live feed are microalgae, rotifer *Brachionus plicatilis* and the brine shrimp *Artemia* sp. (Fig. 2) (Honnens et al., 2013). Fish larvae's inability to feed on artificial feed justifies the need to use of these live feeds (González et al., 2011).

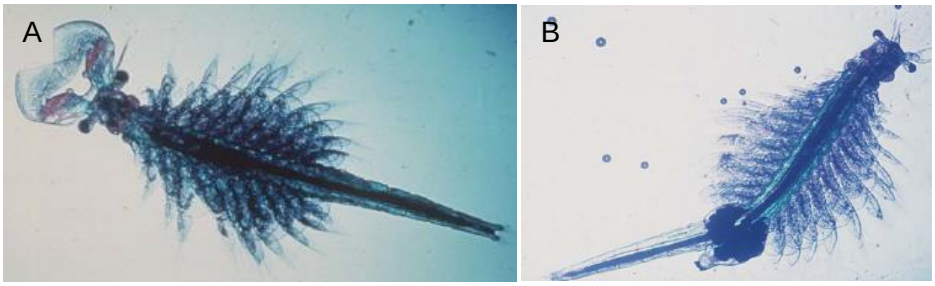


Figure 2 - *Artemia* sp. male (A) and female (B) (Lavens et al., 1996)

1.2. Brine shrimp

Among the live feed mentioned brine shrimp (*Artemia* sp.), a genus of crustacean of the Branchiopoda class which makes up of a complex group of species, are the most widely used. Their broad use is justified by their unique capacity to form metabolic inactive embryos, called cysts, that won't develop if kept dry. The cysts have all the requirements needed to be a good diet: they are found all year round in large quantity on margins of costal hypersaline lakes, lagoons and solar saltworks all over the world; and after 24 hour of incubation in salt water, free swimming nauplius are obtained that can be directly supplied, as feed, to fish larvae. This makes brine shrimp the most convenient, simplest and easiest live feed to use in aquaculture. Another advantage of this feed is the quick spawning time under good conditions. They can reach sexual maturity, in 8 days, and produce 300 nauplii each 4 days. The cysts can also be used as live feed if previously decapsulated. *Artemia* started to be used in aquaculture in the 1930's. In 1951, the exploitation of one of the biggest natural

reservoirs of *Artemia*, the Great Salt Lake in Utah began. The species found in the Great Salt Lake is *Artemia franciscana*, which is a bisexual species. Besides bisexual, *Artemia* can be parthenogenetic. Soon the demand for brine shrimp surpassed supply, prices rose, but shortly world-wide productions appeared, stabilizing prices (Lavens et al., 1996). However, the origin of most cysts is still *Artemia franciscana* from the Great Salt Lake (Endebu et al., 2013).

Female *Artemia* (Fig. 2 - B) can switch between oviparity and ovoviviparity. Under appropriate conditions, they lay fertilized eggs, but under extreme conditions (such as high salinity or low levels of oxygen) the embryos only develop up to gastrula stage, form a thick shell and enter diapause. Subsequently they are released, forming cysts that float in hypersaline waters. The shell is made up of 3 layers (Fig. 3): 1) the alveolar layer that protects against physical damage and from ultraviolet (UV) radiation, 2) the outer cuticular membrane that protects from the entry of unwanted molecules and 3) the embryonic cuticle that becomes the hatching membrane (Lavens et al., 1996).

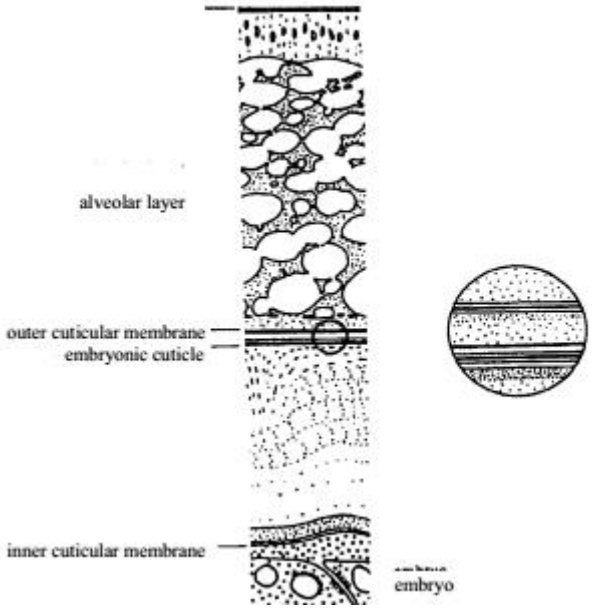


Figure 3 - Schematic structure of *Artemia* cysts (Lavens et al., 1996)

The cyst bursts when submerged in water between 12 and 20 hours and the embryo appears, surrounded by a membrane that will rupture and lead to the free nauplii. In the first larval stage, the nauplius has three appendices: first antennae with sensory function, second antennae with motor and feeding functions and labrum (mouthpart) in the ventral region (Brusca & Brusca, 1990; Lavens et al., 1996). However the digestive system is not functional yet. Eight hours after the first larval

stage of the brine shrimp, a moult occurs and a second larval stage is reached. In this stage, the nauplius already has a functional digestive system and already feeds on food particles between 1 and 50 µm which are filtered by the second antennae. More fifteen moults occur in which the *Artemia* develops complex eyes, by the tenth moult the antennae suffer sexual differentiation and lose motor functions, creating sexual dimorphism (Fig. 2). The female *Artemia*'s antennae become sensory organs and male's become hooks. The thorax's appendices differentiate themselves into appendices with motor, filter feeding and breathing functions (Lavens et al., 1996). *Artemia*'s feeding strategies include filtering, grazing, cannibalism and coprophagy (Tkavc et al., 2011).

As already stated, *Artemia* taxonomy is complex, formed by numerous species and strains. The cysts' diameter, growth speed and heat resistance are specific characteristics and are relatively constant within a strain. Between strains there are variations of size, energetic content, hatch quality (percentage, rate and efficiency), growth speed, temperature and salinity tolerance, reproductive capacity and nutritional value. The latter also varies between yields. It is, however, possible to bio-encapsulate the nutrients desired and needed by the fish larvae, as is the case with highly unsaturated fatty acids (HUFA) to overcome these differences. This bio-encapsulation process can also be called *Artemia* enrichment and it is a way to deliver compounds and supplements to fish larvae, such as HUFA, or vitamins, chemotherapeutics, vaccines and probiotics. Doing so, it improves fishes' rates of survival and growth, the success of moult and pigmentation, resistance to stress and reduces malformations. (Dehghan et al., 2011; Lavens et al., 1996).

1.3. Probiotics

The term probiotic literally means "for life" and its discovery is attributed to Eli Metchnikoff in 1907, who stated that "the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (FAO & WHO, 2001). This term was then defined by Fuller in 1989 as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance". Verschuere in 2000 redefined the term, in an attempt of better represent the interactions possible in aquatic environments, as "a live microbial adjunct that has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response toward disease, or by improving the quality of its ambient environment."

The role played by each different microorganism as a probiotic is not yet fully understood, however several action mechanisms were proposed:

- Colonization of the intestinal epithelium by the probiotics;
- Competition for resources like energy and nutrients;
- Interference in *quorum-sensing* (communication between bacteria);
- Production of inhibitory substances such as bacteriocines by the probiotics;
- Stimulation of the immune system;
- Nutritional contribution, helping to digest food by producing organic compounds, such as enzymes (Tinh et al., 2007).

These mechanisms hinder and avoid the colonization and growth of the pathogenic microorganisms and promote their elimination, as well as improve immune response and better food use (Tinh et al., 2007).

Although the native genera more often found in fish are *Clostridium*, *Bacteroides*, *Porphorymonus*, and *Fusobacterium* (Burr & Gatlin III, 2005), some of the common bacteria used as probiotics are *Bacillus* and lactic acid bacteria, such as *Lactobacillus*, *Pediococcus* and *Enterococcus* (Avella et al., 2010; Klein et al., 1998; Suzer et al., 2008). This happens, not only, because these bacteria are used as probiotics, already, in land animals, but also because it is known that they possess some of the action mechanisms mentioned above or have proven to be beneficial to the host. For example Bacillus and Lactobacillus have shown the capacity to increase fish' immune response (Burr & Gatlin III, 2005; Fuller, 1989).

Planctomycetes are common environmental strains that can be found naturally in *Artemia* (Høj et al., 2009). Their ecological role is not yet completely known, namely their importance as food source or their interactions with higher trophic levels.

1.4. Microbial Community of *Artemia*

Artemia's cysts contain bacteria, archaea and fungi (Lavens et al., 1996; Riddle et al., 2013). Most studies of *Artemia* are performed on laboratory raised crustaceans and the ones about its microbial community are frequently based on culture-dependent identification (Riddle et al., 2013; Tkavc et al., 2011). It has been postulated that *Vibrio parahaemolyticus*, spirochetes, fungi and microsporidia can be *Artemia*'s pathogens, while *Acinetobacter* and *Flexibacter* can have beneficial effects on the growth and survival rate of the crustacean. Furthermore, certain bacteria can have beneficial

impacts on the protein and amino acids of *Artemia* turning them into more appealing food source to fish larvae (Tkavc et al., 2011). Proteobacteria are the most frequently isolated bacteria in laboratory studies with *Brevundimonas*, *Micrococcus*, *Pseudomonas, Rhizobium* and *Sphingomonas* found in homogenized newly hatched *Artemia,* spread plated in marine agar (Høj et al., 2009). In *Artemia* enriched with microalgae and spread plated in thiosulfate-citrate-bile salts-sucrose agar it was found bacterial strains of *Pseudomonas* and *Vibrio* (Høj et al., 2009; Tkavc et al., 2011). In Høj et al., 2009 the culture independent method: denaturing gradient gel electrophoresis (DGGE) was performed and the bacterial strains obtained indicated that the majority of the community belonged to uncultured strains of Gammaproteobacteria (*Thioalkalivibrio*, *Oleiphilus*, *Beggiatoa* and *Vibrio*) and Planctomycetales (*Pirellula*).

In natural environments *Artemia* presents *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes* in descending quantity. The number of bacterial operational taxonomic units associated with *Artemia* decreases with increasing salinity in natural environments (Tkavc et al., 2011). Another molecular study identified 37 bacterial and 48 archeal sequences from adult *Artemia* collected from the Great Salt Lake. The bacterial strains were identified as *Vibrio*, *Halolactibacillus*, *Halomonas*, *Roseovarius*, *Alkalilimnicola*, and *Caulobacter* and the archeal strains as *Haloterrigena*, *Haloarcula*, *Halogeometricum* and *Halovivax* (Riddle et al., 2013).

1.5. Denaturing gradient gel electrophoresis (DGGE)

To allow the analysis of the *Artemia*'s microbiome we decided to use DGGE. According to Muyzer & Smalla, 1998, DGGE is a molecular technique, more specifically a genetic fingerprinting technique, providing a profile of the diversity of the analysed microbial community. One of the problems of classic microbiology is that several bacteria are nonculturable. As a molecular technique, DGGE doesn't present this problem. It also allows the study of the structure and dynamic of the community, of the influence of external factors in the community and of alterations over time. The latter is an advantage over cloning methods and makes DGGE ideal to monitor community profiles over time, even if this variation is very complex. DGGE (Fig. 4) separates DNA or RNA with different sequences since double stranded molecules are partially denatured by the gradient reducing their mobility. With this technique, one is able to distinguish 50% of the different sequences and this value can be raised to 100% when a GC-clamp (addition of a rich GC sequence to one of the sides of the DNA strand) or when ChemiClamp (connect covalently a photoactive chemical by

activating it with UV) are used (Muyzer & Smalla, 1998). The second has disadvantages, such as DNA damaging by the UV and the inability for some bands to be amplified after this treatment. To visualize the DNA band in the gel, ethidium bromide, which causes background staining, SYBR Green I (green florescent dye) or silver staining, although this one also stains single stranded DNA, can be used. DGGE is widely applied to the microbial ecology field using the structural genes of 16S ribosomal RNA, although functional genes can also be used. This makes it into a powerful, easy and fast tool to use in the analysis of the structure and activity of microbial communities, thus, allowing the analysis of large numbers of isolates, while at the same time distinguishing between distantly phylogenetic related bacteria (Muyzer & Smalla, 1998).

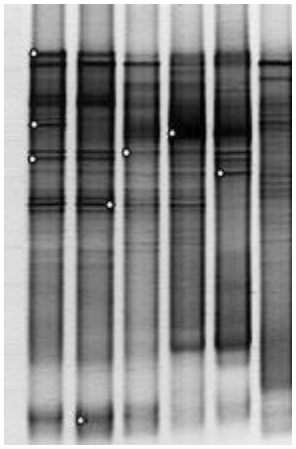


Figure 4 - Example of DGGE profiles of ribosomal DNA stained with ethidium bromide (Muyzer & Smalla, 1998).

1.6. Objectives

The use of probiotics in aquaculture has been growing and with it there is been a subsequent rise in interest in studies of the effects of probiotics in the intestinal microbiome of fishes.

Since fish larvae mortality is one of the limiting factors to the expansion of aquaculture right now, the supply of probiotics increases the success and performance of the production of this fish food. However, it is important to know if the method of delivery of those probiotics, namely by *Artemia* enrichment, actually works. Studies in the field of fish larvae's feeding are of great economic and social importance because they remove the biggest obstacle to the production of aquatic animal protein (Bricknell & Dalmo, 2005; Tinh et al., 2007; Verschuere et al., 2000).

The objectives of this thesis are to study: 1) the effect of decapsulation and probiotics on the microbiome of *Artemia franciscana* using the molecular technique denaturing gradient gel electrophoresis (DGGE) and 2) the effects of these supplements on the crustacean growth and mortality. To achieve these objectives a commercial brand of probiotics as well the scarcely studied planctomycetes bacteria will be assayed.

2. Materials and methods

2.1. *Artemia* rearing, experiments and sampling

According to Sorgeloos et al. (1977), the removal of the hard external layer that encysts the *Artemia* embryo causes the disinfection of the latter. To test this, a set of INVE Aquaculture - EG *Artemia* cysts were decapsulated with an adapted version of Sorgeloos et al. (1977) and Lavens et al, (1996) protocols. Briefly, the cysts were hydrated with water; hypochlorite was added to make up a concentration of 2.12 % (v/v), after 10 minutes the layers external to the outer cuticle membrane dissolved. The cyst were then collected with a filter and washed profusely with sterilized sea water, then immersed in a 0.3% (v/v) choric acid solution to remove traces of hypochlorite and washed again. Afterwards the decapsulated cysts were transferred to 2 sterile bottles with saturated brine solution causing dehydration and kept at 4°C. For each treatment, cysts from 11 mL of decapsulated cysts/brine solution were transferred to 5 mL of sterile seawater.

Figure 5 – *Artemia* rearing system in the greenhouse.

A hatching protocol was prepared based on Lavens et al. (1996). One g of *Artemia* cysts or 5 mL of decapsulated cysts solution were inoculated in 500 mL of sterilized sea water at 25° C and under a continuous 20 µmol/ m^2/ s light regiment. To provide oxygen and keep the *Artemia* floating aeration, from the bottom, was achieved

with Nitto Kohki Co. Ltd pump. The 2 L containers with conical bottoms used for the assays were disinfected with 50 % commercial bleach and 70 % alcohol. The incubation system (Fig. 5) was placed in a Coldkit's Matrix greenhouse to obtain the desired temperature and light conditions.

A total of 4 experiments were performed to determine the effects of different supplies of probiotics. These were the AquaStar® and planctomycetes which were prepared as follows: (1) 150 mg of probiotic AquaStar® Growout (which contains *Bacillus*, *Lactobacillus*, *Pediococcus* and *Enterococcus*) were added to 1 L of sterile seawater; (2) cultures of the planctomycetes *Rhodopirellula rubra* strain LF2 were progressively upscaled, starting from a culture in modified M13 (Lage & Bondoso, 2011) solid medium to a final volume of 2.4 L of culture in the same medium. Afterwards the culture was centrifuged and a total of 35 mL of pellet was obtained. The pellet was added to 1.8 L of sterile seawater resulting in a final absorbance of 0.302 when measured at 600 nm. To feed *Artemia* in some of the treatments, non-axenic *Nannochloropsis* sp., was cultured in an adequate sea water based medium at room temperature and natural lighting until stationary phase was attained. Absorbance of the algae was read at 678 nm and conversion to cell concentration was done in order to obtain a final concentration in the 500 mL *Artemia* cultures of 5×10^5 cells/ml.

In the experiments the supplements were added after 0 and 24 hours of *Artemia* incubation: **(1)** 150 mL of AquaStar® probiotic suspension; **(2)** 75 mL planctomycetes suspension; **(3)** or a mixture of the two, half of the volume of each one. In the control experiment **(4)** no supplement (bacteria) was added. In each experiment were assayed: (a) cysts that were not decapsulated or fed with algae; (b) decapsulated cysts not fed with algae; (c) cysts that were not decapsulated but were fed with algae at 24^{th} hour of incubation and (d) decapsulated cysts fed with algae.

To verify the existence of changes in the microbiome of *Artemia* on different stages and under different treatments, two samples of 10 mL were taken after 1, 22 and 48 hours of incubation from all the treatments. The *Artemia* were filtered through a disinfected net (50 µm mesh), rinsed abundantly with sterilized seawater, re-suspended in sterilized seawater and 20 individuals captured for classic microbiologic analysis and 100 to 200 were captured for DNA extraction. Both were re-suspended in 500 µl sterilized seawater.

2.2. Classic Microbiology

The classic microbiology assays were performed to count the number of bacterial colony forming units and have an idea of the species involved in the microbiome of *Artemia*. For the assay, twenty individuals of each phase (cysts, nauplii and adults) were homogenized in sterile sea water, with a disinfected SilentCrusher M (Heidolph) homogenizer at 12000 rpm for 5 minutes, which had been previously disinfected with alcohol and bleach. Serial decimal dilutions were made with sterilized seawater. One hundred µl of each dilution were spread plated in several selective solid media: Difco™'s marine agar (MA), Difco™'s thiosulfate-citrate-bile salts-sucrose agar, Difco™'s Reasoner´s 2A agar (R2A) with seawater, Difco™'s Man, Rogosa and Sharpe and modified M13 (Lage & Bondoso, 2011). The cultures were incubated aerobically at 25° C and colony forming units were counted at 1, 2, 7 and 14 days after incubation.

2.3. DNA extraction and amplification

The samples of *Artemia* individuals (100 to 200) were stored for molecular analysis. DNA was then extracted with 3 different kits: E.Z.N.A. Tissue Kit, Bio-Rad Quantum Prep AquaPure Genomic DNA Tissue Kit and PowerSoil® DNA Isolation Kit, according to the manufacturers' instructions. However in the latter, the following alterations were introduced to allow better yield: in step 2, vortex was done for 15 minutes, in 5 minutes sets with 1 minute of rest between sets; in step 4, solution C1 was added and then the whole sample plus the solution were placed at 70°C for 10 min; in step 20, instead of 100 µl of solution C6, 30 µl were added twice and centrifuged each time.

To amplify the microbial DNA retrieved from the *Artemia* samples, polymerase chain reactions (PCR) were prepared with the 16S rDNA enconding primers GC-358F (5′- CCT ACG GGA GGC AGC AG -3′ with a GC-clamp at the 5´ end) and 907RM (5′-CCG TCA ATT CMT TTG AGT TT -3′) in 50 µl of PCR mixture (1x Green GoTaq® Flexi Buffer, 1.5 mM $MgCl_2$, 0.2 µM of each deoxynucleotide triphosphates, 0.5 µM of each primer and 0.75 units of GoTaq® Flexi DNA Polymerase). Depending on the samples' initial DNA concentration the necessary quantity DNA template varied between 5 µl and 12 µl. MyCycler™ thermal cycler was programmed for a touchdown to an initial denaturation temperature of 94° C for 5 minutes, 10 cycles of 1 minute at 94° C, 1 minute at decreasing temperature with each cycle starting at 65° C and ending at 55° C and 3 minutes at 72° C, 20 cycles of 1 minute at 94° C, 1 minute at 55° C and 3

minutes at 72° C; and final extension for 10 min at 72° C. The touchdown allows for a wider range of different nucleotides sequences to be amplified, obtaining a representative amplification of most of the different microbial DNA.

Results were observed by electrophoresis in a 1.5 % agarose gel in 1x Tris-acetate-EDTA (TAE) buffer. The ladder used was λ DNA / EcoR1+HindIII from MBI in order to quantify the PCR products. Images were obtained with a GenoPlex from VWR and DNA bands quantified in GenoSoft also from VWR. When more than one band appeared in the gel the band of interest, the product with 600 bp approximately, was removed and purified with the DNA extraction kit illustra GFX PCR DNA and Gel Band Purification Kit following manufactures instructions.

2.4. DGGE studies

Two stock solutions of acrylamide were prepared: 0 % denaturant (15 mL 40 % acrylamide/bis Bio-Rad; 5 mL 20x TAE pH 7.4; 75 mL ultra-pure H_2O) and 80 % denaturant (15 mL 40 % acrylamide/bis Bio-Rad; 5 mL 20x TAE pH 7.4; 33.6 g urea, 32 mL deionised formamide; completed to 100 mL with ultra-pure H_2O). Both were filtered through a glass microfiber filter. Then, to obtain an acrylamide gel with 80 to 40 % gradient, 80, 40 and 0 % solutions were made by adding the respective amounts of 80 and 0% denaturant solution, tetramethylethylenediamine (TEMED) and 10% ammonium persulfate (APS) (Table 1). The gel was poured between the two plates and let to solidify for 2 hours.

Table 1 – Solutions composition to make an acrylamide gel with 80 to 40 % denaturant gradient.

	80% Denaturant	0% Denaturant	TEMED	APS
80 %	8.75 ml	0 ml	8 µl	45 µl
40 %	4.4 ml	4.4 ml	8 µl	45 µl
0 %	0 ml	5 ml	5 µl	40 µl

Using the data from the DNA quantification, 800 ng of DNA from the microbiome of *Artemia* subjected to different treatments were loaded into the gel with a syringe. The gel was done in 1 x Tris-acetate-EDTA (TAE) buffer, ran at 100 V and 60°C for 16 hours. In order to stain the gel, a solution of 20 mL of TAE and 3 µl of SYBRGold, was poured over the gel and stained for 1 hour in the dark. The image was acquired with the Bio-Rad Gel Doc™ XR hardware and Bio-Rad Quantity One – Chemidoc software. With the Quantity One software, bands and band types (bands in different lanes in the same level) were defined and a presence/absence matrix was formed along with the DNA relative quantity (with preferences set to % of Bands in

Lane). With those reports, matrices with relative quantity for each lane and band type, presence and absence of treatment and time of treatment were made.

2.5. Band extraction and sequence analysis

DGEE bands were extracted with a scalpel, placed in microcentrifuge tubes with 20 µl of ultra-pure H_2O, kept at 4°C overnight and then conserved at -20°C. Afterwards the dissolved bands were amplified using the same primers (without GC clamp) and PCR program as above. The PCR products with sufficient amount of DNA were sequenced by Sanger in MacroGen. Sequences were manually cleaned in Chromas 2.12 and consensuses were matched in ProSeq 2.91. The consensuses were blasted against the whole NCBI database. The closest sequences were then aligned in MEGA 6 and a maximum likelihood tree was constructed.

2.6. Supplementation assays

To achieve our second objective, a 17 days supplementation assay was designed. Twenty mL of sterile seawater were placed in 50 mL test-tubes, each with 10 already hatched crustaceans. Three replicas were made for each treatment. Treatments consisted in the addition to the cultures every 2 days of: 1) 125 µl algae, (control); 2) 1.5 mL of planctomycetes; 3) 750 µl of planctomycetes and 65 µl of algae 4) 3 mL of probiotic and 5) 1.5 mL of probiotic and 65 µl of algae. Probiotic concentration values were calculated to be the same as in the DGGE aimed treatments. The *Artemia* growth conditions were similar to the ones for the DGGE assay with a continuous 20 µmol/ m^2/ s light source and 22 to 27° C. The lids were left unlocked so that the renewal of air was possible. After the first week, water was changed and the number of deaths was counted. At the end of the assay (17 days), mortality was again evaluated; surviving individuals were killed with chloroform at 10 % (v/v) and measured from the furca to the middle of eyes in a microscope.

2.7. Statistical analysis

2.7.1. DGGE studies

Based on the matrix reports the following statistical analysis were done in PAST 2.17 (Hammer et al., 2001): cluster analysis, non-metric multidimensional scaling (nMDS), similarity percentage analysis (SIMPER) and analysis of similarity (ANOSIM), all based in Bray-Curtis coefficient (Bray & Curtis, 1957).

Cluster and nMDS are ordination methods and group samples based on their similarity. For the nMDS, besides the DNA relative quantity matrix, the presence or absence of the various treatments was included as binary variable (0/1) and when time was a factor it was normalized with [(value – mean)/(standard deviation)]. Treatments (decapsulation, probiotic and planctomycetes) and time were included as vectors in the plots. The bigger the vector the better the correlation with the samples, the more believable it is that the factor justifies the changes in the microbiome. However the presence of samples that were not subject to a certain treatment and appeared in the positive range of the vector makes it less correlated with the samples distribution. A good correlating factor will have the biggest vector and samples that were affected by the factor in its positive range. NMDS plots also present stress levels that indicate how well the graphical representation characterizes the real differences among *Artemia*'s microbiome profiles. Stress values above 0.2 mean the distribution is close to random, below 0.2 mean the distribution is a good representation of the samples distribution and finally below 0.1 means that there is no chance for misinterpretation of the distribution (Clarke, 1993).

For SIMPER and ANOSIM, non-decapsulated (normal) and decapsulated, time-based and treatment-based groups were compared pair-wise. SIMPER values were summarized in tables that contain: the groups being compared, average dissimilarity, number of bands types necessary to achieve 50% of the average dissimilarly, band type that contributed to the dissimilarly and its contribution for the dissimilarity. ANOSIM presents R values of the groups being compared, if the value is between 0 and 1, the groups differed more between groups than within group, if the value varied between -1 and 0, the groups differed more within their own group than between groups. ANOSIM also informs of the differences significance between those groups, with a 95 % confidence ($p < 0.05$) (Rees et al., 2004).

2.7.2. Supplementation assays

The statistical significance (0.05) of the mortality and growth data was evaluated under Tukey and Dunnett tests in IBM's SPSS (Dunnett, 1955; Tukey, 1949).

3. Results and discussion

3.1. Classic Microbiology

The attempt to grow bacteria from *Artemia* in solid media was met with hard challenges. Like in a previous study, the homogenization of *Artemia* was difficult and provided inconsistent and therefore unusable results. Factors that may contributed to this are mainly the small size of the crustacean, inappropriate homogenization system or system disinfection difficulties.

3.2. Optimization of DNA extraction and amplification

DNA from *Artemia* (cysts, nauplii and adults) associated microbiome was extracted with 3 different kits: E.Z.N.A. Tissue Kit, Bio-Rad Quantum Prep AquaPure Genomic DNA Tissue Kit and PowerSoil® DNA Isolation Kit. Further amplification of the 16S rDNA allowed to assess which was the one enabling a better yield (Fig. 6).

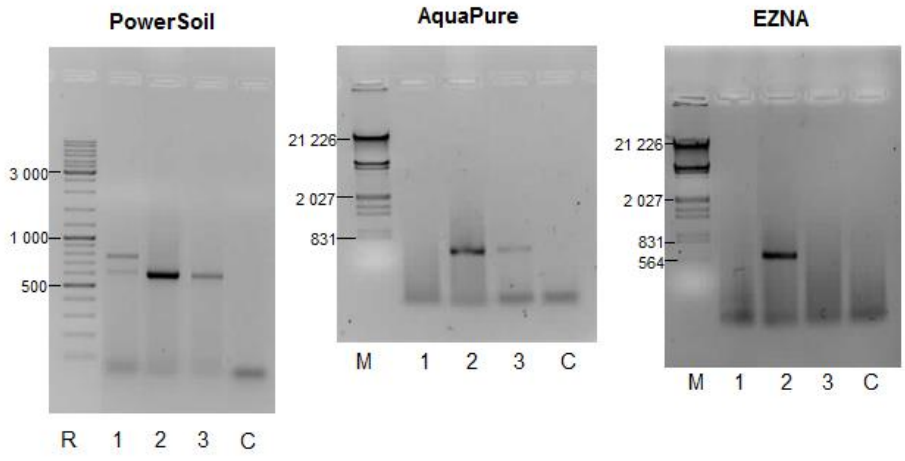


Figure 6 – Comparison of gels with PCR products from 3 different DNA extraction kits. R – Gene Ruler™ DNA Ladder Mix; M - λ DNA / EcoR1+HindIII Ladder; 1 - Normal cysts; 2 - Normal nauplii; 3 - Normal adults; C – Negative control.

Because PowerSoil® DNA Isolation Kit was the only capable of extracting DNA from all the types of samples: cysts, nauplii and adults, it was the one chosen for subsequent work.

Except for the sample 22, decapsulated adults plus planctomycetes, it was possible to amplify the 16S rDNA from all the sample's microbiomes (Fig. 7). The lack of amplification of sample 22 might have been related to the high mortality of *Artemia* that occurred when these cultures were supplemented with planctomycetes (see results below) with a consequent reduced number of bacteria in the microbiome.

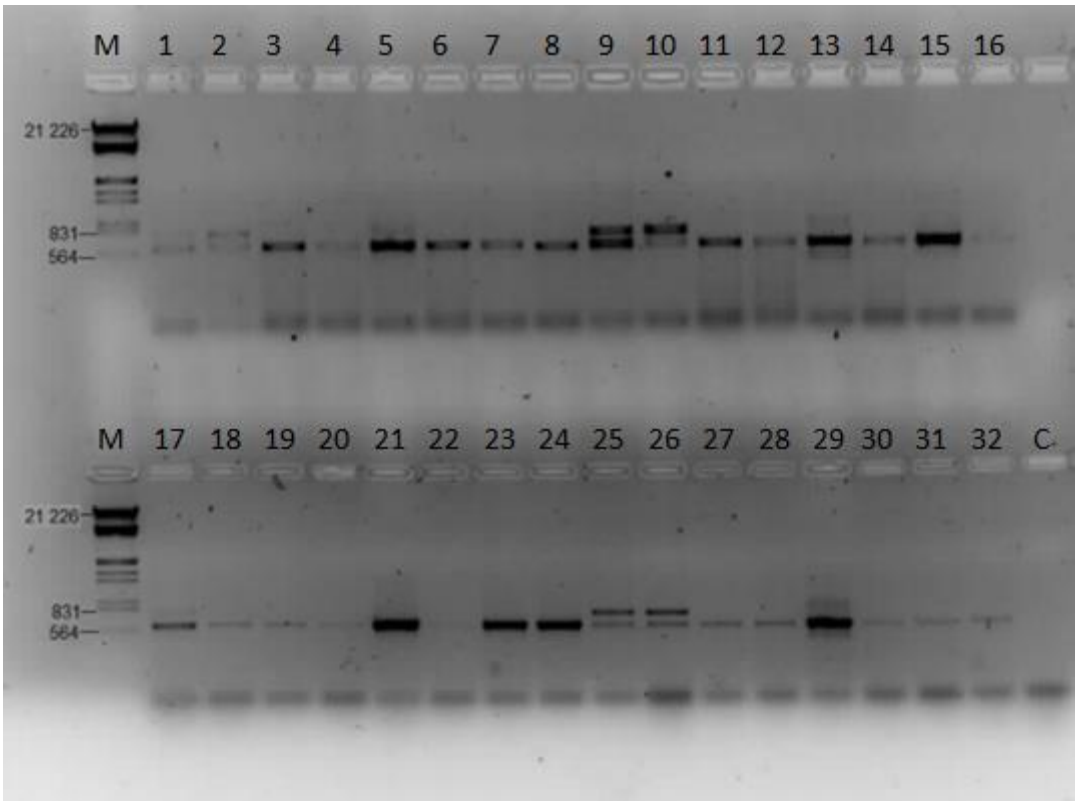


Figure 7 – 16S rDNA PCR amplifications, in agarose gel, of all the samples of the *Artemia* microbiome.

1	Normal cysts	9	Normal cysts plus probiotic	17	Normal cysts plus planctomycetes	25	Normal cysts plus probiotic plus planctomycetes
2	Decapsulated cysts	10	Decapsulated cysts plus probiotic	18	Decapsulated cysts plus planctomycetes	26	Decapsulated cysts plus probiotic plus planctomycetes
3	Normal nauplii	11	Normal nauplii plus probiotic	19	Normal nauplii plus planctomycetes	27	Normal nauplii plus probiotic plus planctomycetes
4	Decapsulated nauplii	12	Decapsulated nauplii plus probiotic	20	Decapsulated nauplii plus planctomycetes	28	Decapsulated nauplii plus probiotic plus planctomycetes
5	Normal adults	13	Normal adults plus probiotic	21	Normal adults plus planctomycetes	29	Normal adults plus probiotic plus planctomycetes
6	Decapsulated adults	14	Decapsulated adults plus probiotic	22	Decapsulated adults plus planctomycetes	30	Decapsulated adults plus probiotic plus planctomycetes
7	Normal adults fed algae	15	Normal adults plus probiotic fed algae	23	Normal adults plus planctomycetes fed algae	31	Normal adults plus probiotic plus planctomycetes fed algae
8	Decapsulated adults fed algae	16	Decapsulated adults plus probiotic fed algae	24	Decapsulated adults plus planctomycetes fed algae	32	Decapsulated adults plus probiotic plus planctomycetes fed algae
M	λ DNA / EcoR1+HindIII Ladder	C	Negative control				

Unexpectedly, the microbiome from the cysts (Fig. 7 – [lanes 1, 2, 9, 10, 25, 26]) amplified two bands instead of only one, one characteristic of the 16S rRNA gene (about 600 bp) and another with higher molecular weight. The reason for this is unknown. However one possibility is that even thought we were using primers specific for the 16S rRNA gene, potential amplification of a random sequence from a non-

bacterium organism, that was unable to survive in the other *Artemia* life phases, can justify the presence of the unexpected band. Furthermore, when the extracted band of interest (600 bp) was electrophoresed in a gradient gel (DGGE) no DNA band could be observed or it was degraded (Fig. 8 – [lanes 1, 2]). Therefore, we stopped extracting the band and used the whole PCR product of cysts.

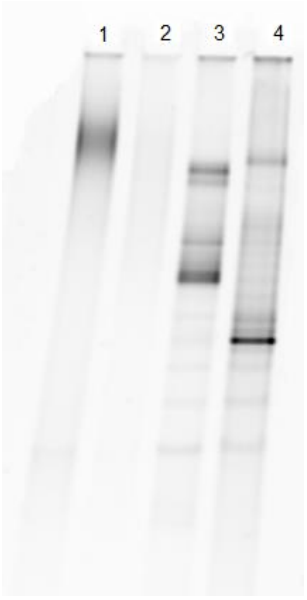


Figure 8 – DGGE of *Artemia* microbiome showing lanes of the cyst bands extracted from agarose gel in comparison to nauplii lanes. 1 – Normal cysts (degraded DNA); 2 – Decapsulated cysts (no DNA evident); 3 – Normal nauplii; 4 – Decapsulated nauplii.

3.3. DGGE studies

The 16S rRNA gene profiles of the communities were analysed by DGGE (Figs 11 - 16 A) and relative quantity matrices were created and used to do cluster analysis' dendograms (Figs 11 - 16 B) as well as nMDS plots (Figs 11 - 16 C). Each profile represents the bacterial community of a certain treatment and sampling time. To simplify nomenclature, cysts that did not suffer decapsulation process will be referred to as "normal" and the ones that did will be referred to as "decapsulated". Sampling times are also referred as stages in *Artemia* life cycle: 1 hour – cysts; 22 hours – nauplii; 48 hours – adults. All the statistical analysis (cluster, nMDS, SIMPER and ANOSIM) were done based on Bray-Curtis similarity (Bray & Curtis, 1957).

Firstly, the treatments (control, probiotic, planctomycetes and probiotic plus planctomycetes) will be analysed by comparing each sampling time (corresponding to cysts, nauplii and adults). Afterwards each sampling time will be analysed by comparison among treatments.

3.3.1. Temporal comparison within the same treatment

DGGE profiles of *Artemia*'s microbiomes from different sampling times are compared within the same treatment as to ascertain if time is an important factor in the acquisition of microbiome differences. This study represents a total of 4 gels: 1) control (Fig. 9), 2) probiotic (Fig. 10), 3) planctomycetes and 4) probiotic plus planctomycetes (Fig. 11).

3.3.1.1. Artemia's control treatment

In the control treatment, *Artemia* were not supplemented with bacteria (probiotics or planctomycetes). The DGGE results of *Artemia*'s microbiome in the control treatment are presented in Figure 9.

The cluster analysis represented graphically by the dendrogram (Fig. 9 B) and the non-metric multidimensional analysis scaling (nMDS) (Fig. 9 C) show that, in general, there is a higher similarity between the normal individuals within themselves than with the decapsulated ones and that the reverse is also true (Fig. 9 B, C). This is visible by the clustering of the normal *Artemia* microbiome's profiles 3, 5 and 7, and of the decapsulated profiles 2, 4, and 6 (Fig. 9 B, C). This result is even reinforced in the nMDS where decapsulated nauplii and adults are placed in the direction of the decapsulation vector and normal nauplii and adults are on the opposite side of it. Rather than a grouping based on development/incubation time the ordinations seems

to point to a dichotomy between decapsulated and normal individuals. This is further established by the results in the similarity percentage analysis (SIMPER) (Table 2) where the comparison normal *vs* decapsulation has the highest dissimilarity value (0.826). Band type 63 (Fig 9 A) was the band that had the biggest influence in the dissimilarity of the groups. The separation between *Artemia* microbiome into normal and decapsulated profiles is only disturbed by two cases.

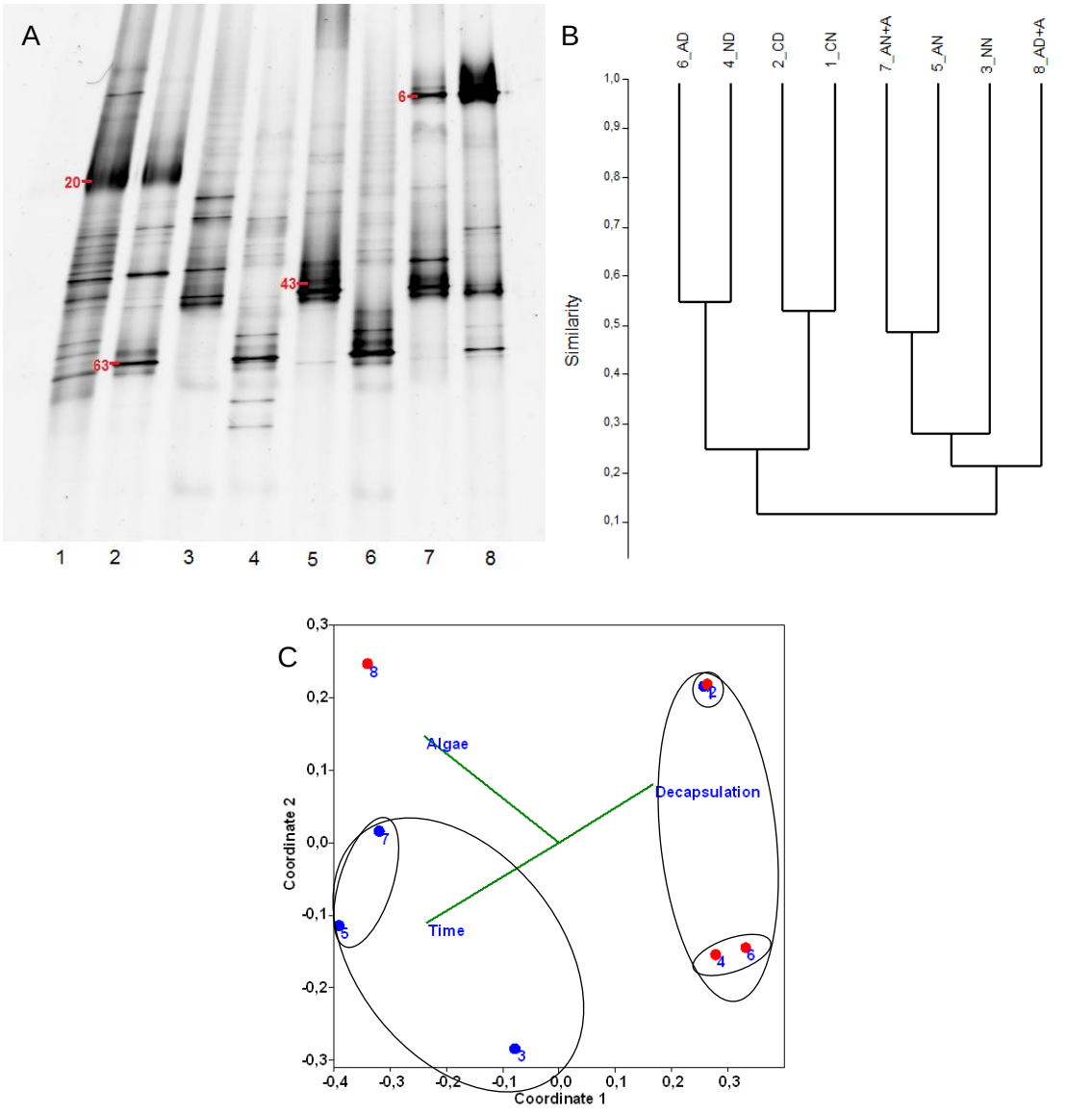


Figure 9 - (A) DNA DGGE gel profiles of microbiome of *Artemia* control treatment (no bacteria supplementation) with band types numbers. 1 – CN - normal (no decapsulation) cysts; 2 - CD- decapsulated cysts; 3- NN – normal nauplii; 4 - ND- decapsulated nauplii; 5 – AN - normal adults; 6 – AD - decapsulated adults; 7 – AN+A – normal adults fed with algae; 8 AD+A – decapsulated adults fed with algae. (B) Dendrogram of DGGE profiles of the control treatment, based on Bray-Curtis similarity. (C) Non-metric multidimensional analysis scaling (nMDS) plot (stress: 0.198) based on Bray-Curtis similarity with vectors showing the correlation of the 3 different factors and circles represent the groups formed in the dendrogram.

Table 2 – Similarity percentage (SIMPER) analysis between control treatment groups, groups that are based on common characteristic – decapsulation process or sampling time. Results of the comparison of the groups show average dissimilarity, number of bands that contributed to 50% of the dissimilarity, the band that had most influence and its contribution to the dissimilarity.

Groups being compared	Average dissimilarity	# Bands contributed 50% dissimilarity	Main band	
			Band type	% Contribution
normal *vs* decapsulation	0.826	11	63	7.31
adults fed algae *vs* adults	0.795	8	6	13.64
adults with nauplii	0.719	10	43	6.22
adults *vs* cysts	0.818	10	20	15.48
nauplii *vs* cysts	0.820	10	20	15.44

Table 3 - R values from analysis of similarity (ANOSIM) between control treatment groups, groups that are based on common characteristic – decapsulation process or sampling time. Positive values indicate that profiles differed more between the groups than within the group.

R values	Decapsulated
Normal	0,2708

R values	Algae	Adults	Nauplius
Algae	-	-0,25	1
Adults	-0,25	-	-0,5
Nauplius	1	-0,5	-
Cysts	1	0	1

The first one is profile 1 that represents *Artemia* normal cysts' microbiome and is grouped in the decapsulated group with profile 2 - microbiome of decapsulated cysts (Fig. 11 - B, C). This means that the cysts present more similarity between themselves independently of decapsulation process they suffer. This may be because the DNA extracted from microbiome of the cysts presented an extra band above the 600bp band (Fig. 7 – [lanes 1, 2, 9, 10, 24, 25]). This is reinforced by the SIMPER analysis (Table 2). When the microbiome of cysts was compared to the other life stages, band type 20 was the most influential. We speculate that this band might be the band above the 600 bp since it appears in all and only the cysts. The cysts were grouped in the decapsulated side but the similarity between them was higher than with the other members of the decapsulated group. The cysts similarity with normal individuals (nauplii and adults) is a little over 10 % (Fig. 8).

The decapsulation process may justify why there is a less diverse microbiome between moults in the decapsulated group comparatively to the normal group which is visible through the higher similarity and lower scattering of decapsulated nauplii and adults (Fig. 11 B, C). This is due to the elimination of bacteria during the decapsulation

process which makes the microbiome of decapsulated *Artemia* less varied resulting in less evolution of the microbiome over time. The higher dispersion of the microbiome's profiles of normal *Artemia* is reinforced, in the normal *vs* decapsulated comparison, by the low R value obtained in the analysis of similarity (ANOSIM, 0.2708 - Table 3) and the high value obtained in the SIMPER (0.826), meaning that these groups are the most dissimilar and also the ones with more variation within themselves.

In SIMPER (Table 2), cysts were shown to have the second and third highest dissimilarity values when compared to nauplii (0.820) and adults (0.818) respectively, while ANOSIM shows that cysts are a different group from nauplii and adults fed algae (Table 3).

The second case that disturbs the normal/decapsulated dichotomy concerns profile 8 – decapsulated adults fed with algae and can be justified by the presence of algae which makes profile 8 group up with the normal samples probably due to its similarity with profile 7 – normal adults fed with algae. The non-axenic algae supplementation seems to have a strong effect in the bacterial community. This is reinforced by the nMDS plot (Fig 11 C) since the algae vector is the biggest. In the nMDS we can see the same groups that appeared in the dendrogram. Furthermore the nMDS has a stress value below 0.2 (0.198), which means that this 2D representation is an accurate representation of the real differences among *Artemia*'s microbiome profiles. It provides a clearer idea of the distance of profile 8 in relation to the other profiles and also evidences the effect of the algae in profiles 7 and 8. Profile 5 and 7 have the same treatment except 7 was fed with algae; this affects the profile moving it closer to profile 8. In SIMPER analysis (Table 2), adults fed with algae *vs* adults was the comparison that needed the smaller amount of bands (8) but highly impactful ones to achieve half of the dissimilarity. Algae appear to be the factor with more impact in the control treatment, because the culture of *Nannochloropsis* sp. was non-axenic and their associated microorganisms seem to have influenced the microbiome of *Artemia* more than decapsulation or time.

As no comparison between development phases had a similarity value of 100 % (which would mean the presence of the same microbiome), it is clear that time had an effect on the microbiome communities. The highest similarity is below 60% (Fig. 9 B). In the nMDS (Fig. 9 C) we do see that the time vector is well correlated with normal treatments, as normal treatments with longer incubation times (nauplii and adults) are in the more positive side of the vector while both cysts are in the negative side of the vector. Furthermore, SIMPER shows high average dissimilarity values between the

development phases (0.719 - 0.820), and ANOSIM (Table 3) shows that nauplii and cysts are two different groups, but not that adults and nauplii are.

3.3.1.2. *Artemia*'s probiotic treatment

When the probiotic was added to *Artemia* cultures a rather analogous scenario to the control was obtained. As in the control, none of the samples of different times has the same microbiome (Fig. 10 B, C) or dissimilarity of 0 (Table 4). The dendrogram and nMDS plot (stress: 0.177) of the probiotic treatment (Figs 10 B, C) show normal individuals forming one cluster (similarity > 50 %) and decapsulated forming another (similarity > 40 %), with the similarity between them being below 20%. The size of the decapsulation vector in the nMDS plot (Fig. 10 C) together with the positioning of the normal and decapsulated groups show how impactful the decapsulation treatment was in the definition of the microbiome. Attesting to this, SIMPER dissimilarity values (Table 4) and ANOSIM R values (Table 5) are the highest for the normal *vs* decapsulation comparison (0.880 and 0.802). Furthermore, ANOSIM shows that the differences between normal and decapsulated groups are significant ($p < 0.05$). Decapsulation appears to be the factor with more impact in the probiotic treatment.

As well as in the control treatment, cysts were grouped together (Fig. 10 B, C) and more closely to the decapsulated group disturbing the normal/decapsulated dichotomy (Fig. 10 B). This is further established by the SIMPER and ANOSIM results (Table 4 and 5). In SIMPER, cysts have the second and third highest values with nauplii (0.867) and adults (0.848), respectively. ANOSIM showed that cysts differed more when compared to nauplii (0.5) and adults (0.75) groups than when within their own group. However, contrary to the control, the probiotic plus algae treatments were in their respective normal or decapsulated groups. This is shown by the similarity between adults fed and not fed with algae treatments, which is higher than 50% in both cases, and higher than with the nauplius of their respective normal/decapsulated group (Fig. 10 B, C). SIMPER (Table 4) dissimilarity is also the lowest between the non-fed and fed with algae adults treatments (0.651) and ANOSIM (-0.5, Table 5) reporting that the differences between these two groups are smaller than within them. Comparing the decapsulation vector with the algae vector (Fig. 10 C), this one was much smaller and much less correlated to the samples correspondent distribution. This situation is opposite to what happened in control treatment and evidences that it was the probiotic that influenced the microbiome of the adult *Artemia* and not the algae (Fig. 5 B). The fact that the probiotic plus algae are in their expected groups instead of grouped together could mean that the probiotic had a stabilizing effect on the microbiome even

when algae are given to *Artemia*, making less noticeable the changes that algae can make to the microbiome. This stabilization may be due to the creation of a more stable and cohesive bacterial community across development cycle of *Artemia* by inserting the 4 bacterial species of the probiotic and regulating the ones present. It is also evident that nauplii were more dissimilar with all of the adults microbiome (Figs 10 B, C and Table 4).

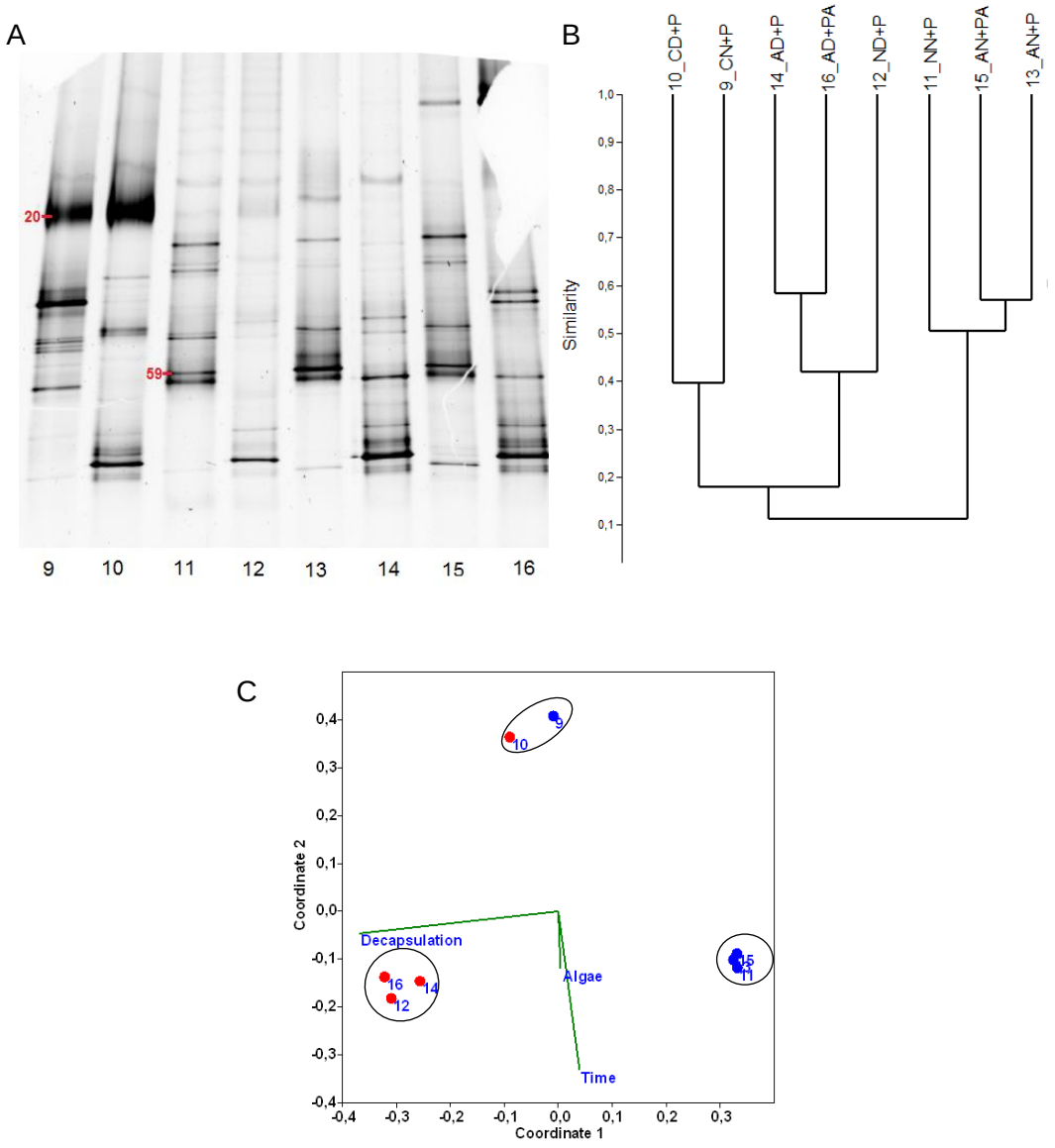


Figure 10 - (A) DNA DGGE gel profiles of microbiome of *Artemia* subjected to the probiotic treatment with band types numbers. 9 – CN+P - normal cysts plus probiotic; 10 – CD+P - decapsulated cysts plus probiotic; 11 – NN+P – normal nauplii plus probiotic; 12 – ND+P -decapsulated nauplii plus probiotic; 13 – AN+P - normal adults plus probiotic; 14 – AD+P - decapsulated adults plus probiotic; 15 – AN+PA - normal adults fed algae plus probiotic; 16 – AD+PA - decapsulated adults fed algae plus probiotic. (B) Dendrogram of DGGE profiles of the probiotic treatment, based on Bray-Curtis similarity. (C) Non-metric multidimensional analysis scaling (nMDS) plot (stress: 0.177) based on Bray-Curtis similarity with vectors showing the correlation of the 3 different factors and circles represent the groups formed in the dendrogram.

Table 4 – Similarity percentage (SIMPER) analysis between probiotic treatment groups, groups that are based on common characteristic – decapsulation process or sampling time. Results of the comparison of the groups show average dissimilarity, number of bands that contributed to 50% of the dissimilarity, the band that had most influence and its contribution to the dissimilarity.

Groups being compared	Average dissimilarity	# Bands contributed 50% dissimilarity	Main band	
			Band type	% Contribution
normal *vs* decapsulation	0.880	11	20	9.722
adults fed algae *vs* adults	0.651	12	59	8.182
adults *vs* nauplii	0.694	11	59	7.672
adults *vs* cysts	0.848	6	20	25.77
nauplii *vs* cysts	0.867	8	20	25.2

Table 5 - R values from analysis of similarity (ANOSIM) between groups, groups that are based on common characteristic – decapsulation process or sampling time. Positive values indicate that profiles differed more between the groups than within the group.

R values	Decapsulated
Normal	0,802

R values	Algae	Adults	Nauplii
Algae	-	-0,5	-0,25
Adults	-0,5	-	-0,25
Nauplii	-0,25	-0,25	-
Cysts	0	0,75	0,5

In the nMDS plot (Fig. 10 C) cysts are in the negative side of the time vector, contrary to nauplii and all of the adults. Dissimilarity in SIMPER analysis (Table 4) between adults and nauplii is below 0.7, which means that they are more similar than in the control treatment. Furthermore, scattering in both normal and decapsulated microbiome profiles is small, contrary to the control treatment (Figs 12 B, C). Both the similarities between nauplii and adults and normal and decapsulated microbiome profiles could be explained by the probiotics stabilizing effects in the microbiome.

SIMPER analysis (Table 4) showed that band types 59 and 20 were the most influential to the microbiome differences.

3.3.1.3. *Artemia*'s planctomycetes treatment

Unfortunately the gel that compared the microbial samples in different times of the planctomycetes treatment was torn and the remnant DNA was insufficient to perform another gel.

3.3.1.4. *Artemia*'s probiotic plus planctomycetes treatment

The dendrogram (Fig. 11 B) of the profiles of *Artemia* microbiome in the probiotic plus planctomycetes treatment shows that once again cysts were grouped

together (> 60% similarity) and in the opposite side of the nMDS plot (stress: 0.171) time vector (Fig 11 C). In both the dendrogram and the nMDS plot (Figs 11 B, C) the most disparate profile is profile 29 – normal adults which may be due to the smear observed (Fig. 11 A). In the dendrogram, the decapsulated profiles were grouped together, but decapsulated cysts were further apart of the decapsulated group that in previous treatments (Fig. 9 B to 11 B). In the dendrogram (Fig. 11 B), normal profiles present a rather erratic distribution, not allowing for a clear separation between normal and decapsulated profiles. Contrary to the dendrogram, nMDS plot (Fig 11 C) shows a separation between decapsulated and normal treatments, with normal being distributed in the negative side of the decapsulation vector and with decapsulated adults being in the positive side. In SIMPER and ANOSIM (Table 6 and 7) the normal *vs* decapsulation comparisons have high values (0.818 and 0.417, respectively). Also, the differences between normal and decapsulated groups were significant ($p < 0.05$), thus confirming what we saw in the nMDS plot and reinforcing the role of decapsulation in the development of the microbiome.

Since planctomycetes are the only new varying factor in this treatment comparatively to the previous probiotic gel analysed, it is reasonable to assume that they had an effect in the microbiome, especially on that of normal *Artemia*. Planctomycetes may affect the *Artemia* microbiome because they caused the death of this crustacean as it was observed in the supplementation assays (see below). This fact may lead to a big shift in the microbial community and to an overall diminished quantity of microbial DNA. The shift in the microbiome may be due to the creation of conditions favourable to bacteria that specialized in decomposing organic matter.

In the nMDS (Fig. 11 C), adults are further on the positive side of the time vector and treatments with algae are closer to the algae vector. This shows that these vectors can explain well the reason for the differences, since the vectors correlate well with the distribution of the profiles. Furthermore, the SIMPER and ANOSIM (Table 6 and 7) show that high values in the comparisons of the cyst microbiomes to the ones of other life phases, indicating that time influenced in the distribution and therefore in the microbiome. Like in the control treatment, in the probiotic plus planctomycetes treatment, the normal group had a higher dispersion than the decapsulated groups.

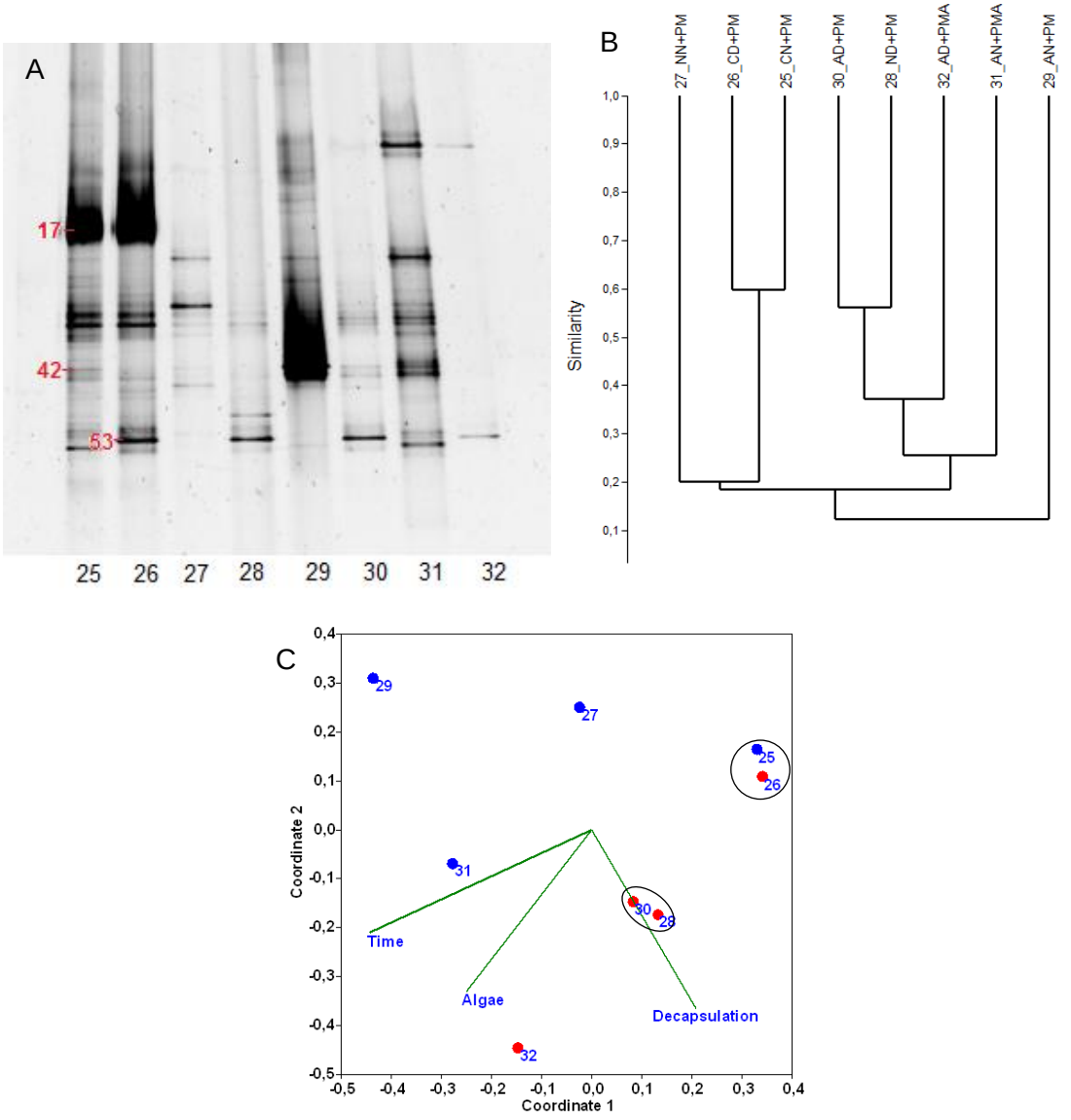


Figure 11 - (A) DNA DGGE gel profiles of microbiome of *Artemia* subjected to the probiotic plus planctomycetes treatment with band types numbers. 25 – CN+PM - normal cysts plus probiotic plus planctomycetes; 26 – CD+PM - decapsulated cysts plus probiotic plus planctomycetes; 27 – NN+PM – normal nauplii plus probiotic plus planctomycetes; 28 – ND+PM -decapsulated nauplii plus probiotic plus planctomycetes; 29 – AN+PM - normal adults plus probiotic plus planctomycetes; 30 – AD+PM - decapsulated adults plus probiotic plus planctomycetes; 31 – AN+PMA - normal adults fed algae plus probiotic plus planctomycetes; 32 – AD+PMA - decapsulated adults fed algae plus probiotic plus planctomycetes. (B) Dendrogram of DGGE profiles of the probiotic plus planctomycetes treatment, based on Bray-Curtis similarity. (C) Non-metric multidimensional analysis scaling (nMDS) plot (stress: 0.171) based on Bray-Curtis similarity with vectors showing the correlation of the 3 different factors and circles represent the groups formed in the dendrogram.

Table 6 – Similarity percentage (SIMPER) analysis between probiotic plus planctomycetes groups, groups that are based on common characteristic – decapsulation process or sampling time. Results of the comparison of the groups show average dissimilarity, number of bands that contributed to 50% of the dissimilarity, the band that had most influence and its contribution to the dissimilarity.

Groups being compared	Average dissimilarity	# Bands contributed 50% dissimilarity	Main band	
			Band type	% Contribution
normal *vs* decapsulation	0.818	9	53	10.56
adults fed algae *vs* adults	0.781	8	42	10.93
adults with nauplii	0.738	8	42	11.25
adults *vs* cysts	0.824	6	17	23.69
nauplii *vs* cysts	0.744	5	17	26.23

Table 7 - R values from analysis of similarity (ANOSIM) between probiotic plus planctomycetes groups, groups that are based on common characteristic – decapsulation process or sampling time. Positive values indicate that profiles differed more between the groups than within the group.

R values	Decapsulated
Normal	0,417

R values	Algae	Adults	Nauplius
Algae	-	-0,25	0,25
Adults	-0,25	-	-0,25
Nauplius	0,25	-0,25	-
Cysts	1	0,25	0,25

The differences in *Artemia* microbiome across the different life phases times are consistent with previous studies (Riddle et al., 2013; Tkavc et al., 2011). Riddle et al. (2013) verified that cysts had bacteria from the genera *Idiomarina* and *Salinivibrio*, while the adults had *Halomonas* sp. and *Vibrio* sp. Tkavc et al. (2011) observed that the diversity of the adult *Artemia*'s intestinal bacteria is lower than the diversity of the microbiome of the nauplii.

3.3.2. Comparison between different treatments

In the gels and analyses subsequently presented, the different treatments across samples of the same time frame/development phase are evidenced in a total of 3 gels: cysts (1 hour), nauplius (22 hours) and adults (48 hours, both fed and non-fed with algae).

3.3.2.1. Cysts

Although cysts were only exposed for one hour to the different treatments before sampling, it was enough to create changes and differences among the microbiome profiles (Fig. 12 and Table 8). As seen before, cysts were always grouped together in temporal analyses (Fig 9 - 11 B), meaning that the differences between decapsulated and normal treatments are less important in cysts than in other life

stages. Also worth to mention is that their bacterial communities might not differentiate as much as the other life stages or be in concentration enough to be detected.

In the DGGE gel profiles of *Artemia* cyst microbiomes (Fig. 12 A) there is a noticeable thick band present in all of them. It is possible that this band is caused by the extra band above the desired 600bp band (Fig. 7 – [lanes 1, 2, 9, 10, 24, 25]). However, with no evidence to support this, the band was accounted for.

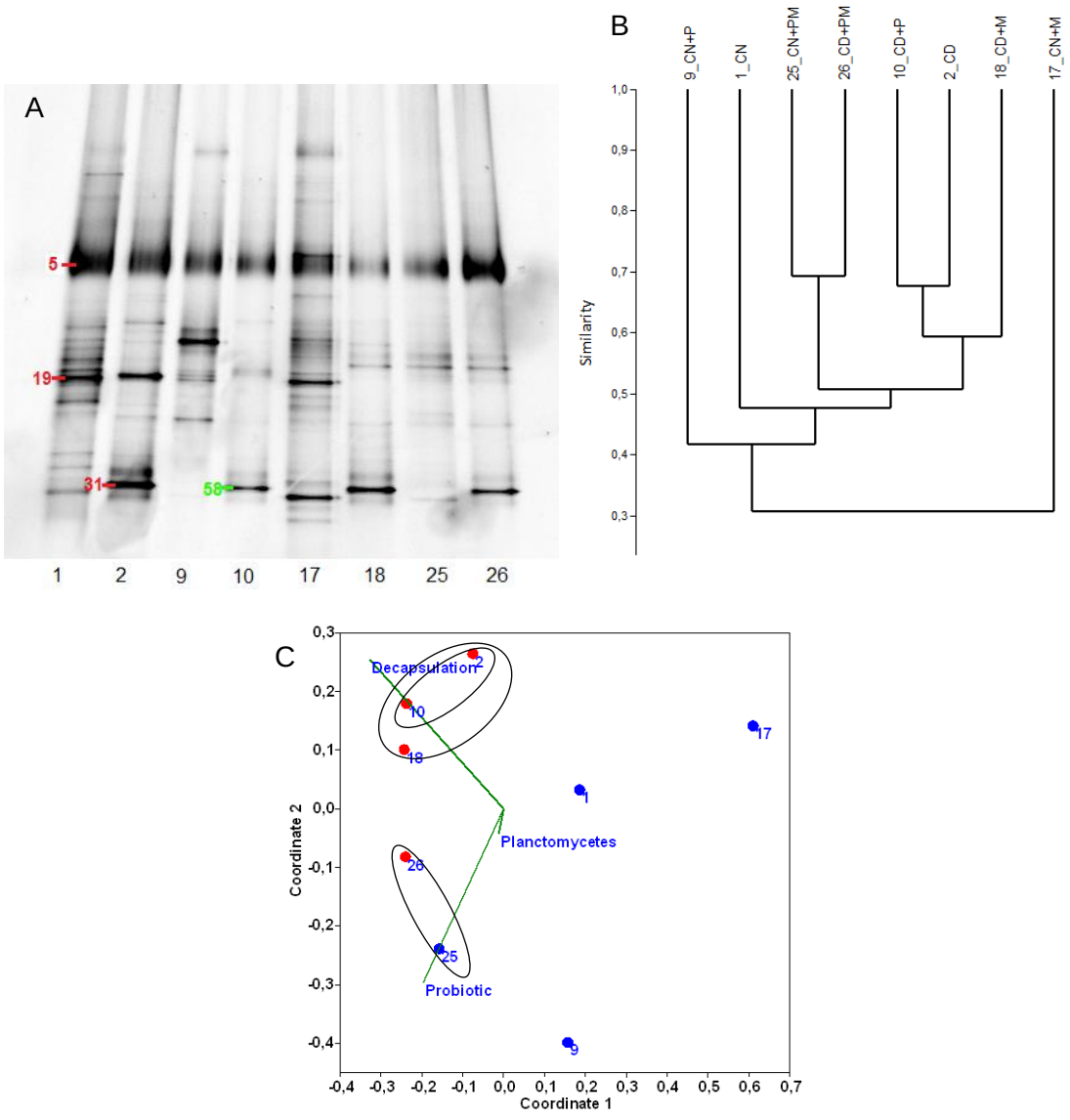


Figure 12 - (A) DNA DGGE gel profiles of microbiome of *Artemia* cysts (1 hour) with band types numbers (red) and extracted band (green). 1 – CN - normal (no decapsulation) cysts; 2 – CD - decapsulated cysts; 9 – CN+P – normal cysts plus probiotic; 10 – CD+P - decapsulated cysts plus probiotic; 17 – CN+M - normal cysts plus planctomycetes; 18 – CD+M - decapsulated cysts plus planctomycetes; 25 – CN+PM – normal cysts plus probiotic plus planctomycetes; 26 - CD+PM – decapsulated cysts plus probiotic plus planctomycetes. (B) Dendrogram of DGGE profiles of cysts, based on Bray-Curtis similarity. (C) Non-metric multidimensional analysis scaling (nMDS) plot (stress: 0.149) of DGGE profiles of cysts, based on Bray-Curtis similarity with vectors showing the correlation of the 3 different factors and circles represent the groups formed in the dendrogram.

Table 8 – Similarity percentage (SIMPER) analysis between cyst groups, groups that are based on common characteristic – decapsulation process or treatment. Results of the comparison of the groups show average dissimilarity, number of bands that contributed to 50% of the dissimilarity, the band that had most influence and its contribution to the dissimilarity.

Groups being compared	Average dissimilarity	# Bands contributed 50% dissimilarity	Main band	
			Band type	% Contribution
normal *vs* decapsulation	0.592	7	31	14.43
control *vs* probiotic	0.522	7	19	12.44
control *vs* planctomycetes	0.519	8	31	11.81
control *vs* probiotic + planctomycetes	0.555	6	5	18.49

Table 9 - R values from analysis of similarity (ANOSIM) between cyst groups, groups that are based on common characteristic – decapsulation process or treatment. Positive values indicate that profiles differed more between the groups than within the group.

R values	Decapsulated
Normal	0,4688

R values	Control	Probiotic	planctomycetes
Control	-	0,25	-0,25
Probiotic	0,25	-	0
Planctomycetes	-0,25	0	-
Probiotic + Planctomycetes	1	0,25	0,5

Cysts' microbiome treated with probiotic plus planctomycetes (PM) (profiles 25 and 26) have the highest similarity and are clustered together (similarity > 65%), evidencing a strong influence of this supplementation treatment in the microbiome, which is even stronger than the decapsulation process. Other than profile 26, all of the other decapsulated cysts' microbiomes (profiles 2, 10 and 18) are clustered together (similarity > 60%) showing a great influence of the decapsulation process. Normal cysts (profiles 1, 9 and 17), expect profile 25, are the ones further apart and have a minimum similarity above 30%. However, in the nMDS plot (Fig. 12 C – stress: 0.149) the separation between normal and decapsulated microbiome's profiles is evident.

The decapsulation vector has nearly the same size as the probiotics vector (Fig. 12 C) and correlates really well with the samples that suffered that process, while samples that did not are in the opposite side of the vector, pointing to this as the main factor that justifies the distribution of the samples and therefore the microbiome changes. This is further established by the SIMPER and ANOSIM results (Table 8 and 9), in the normal *vs* decapsulation comparison the average dissimilarity is the highest (0.592), the groups differed more between groups than within group and the differences between groups were significant ($p < 0.05$).

Profile 26 (CD+PM) is not in the same quadrant as the other decapsulated profiles, but that is due to the similarity with profile 25 (CN+PM). Furthermore SIMPER (Table 8) showed that cysts with PM (probiotic plus planctomycetes) is the treatment with the smaller amount of bands (6) necessary to reach 50% of dissimilarity with control (decapsulated cysts); ANOSIM R values (Table 9) report that the microbiomes of both cysts with PM (profiles 25 and 26) are the only ones that compared to other supplementation treatment groups differed more between groups than within group (> 0.25, Table 9). The greater effect in change in the microbiome may be due to this specific higher microbial diversity that got in contact with the cysts.

Planctomycetes vector is the smallest (Fig. 12 C) and only correlates with profiles 25 (CN+PM) and 26 (CD+PM) but not with the planctomycetes treatment (profiles 17 and 18). Although this vector cannot justify the distribution of the samples, planctomycetes had a great impact, since profile 17 (CN+M) is the most dissimilar one.

The probiotic vector (Fig. 12 C) is correlated with profile 9 (CN+P), 25 (CD+PM) and 26 (CD+PM), which had probiotic in their treatments. Profile 10 (CD+P) is not in the positive range of the probiotic because decapsulation process had more impact than the treatment given afterwards. However, probiotic seemed to a greater effect in cysts, reinforced by the vector size and positive ANOSIM R value (0.25, Table 9).

3.3.2.2. Nauplii

Figure 15 A shows DGGE gel of the nauplius microbiome in which *Artemia* were sampled with 22 hours of incubation. Different treatments are compared with cluster analysis (Fig. 13 B) and with nMDS (Fig. 13 C).

The dendrogram and the nMDS plot (Fig. 13 B, C) present a clear separation between the normal and decapsulated group. The stress of the nMDS plot is below 0.1 (0.043) and, as such, there is no prospect of misinterpretation of nMDS plot (Clarke, 1993). The decapsulation vector is the biggest (Fig. 13 C). The vector correlates really well with the localization of the profiles. The decapsulated profiles are on the positive side of the vector and the normal profiles in the negative side. SIMPER and ANOSIM have their highest values in the normal *vs* decapsulation comparison (0.827 and 1, respectively). Furthermore ANOSIM shows that the differences are significant ($p < 0.05$). The previous statements point to decapsulation as the responsible factor for the modification of the bacterial community.

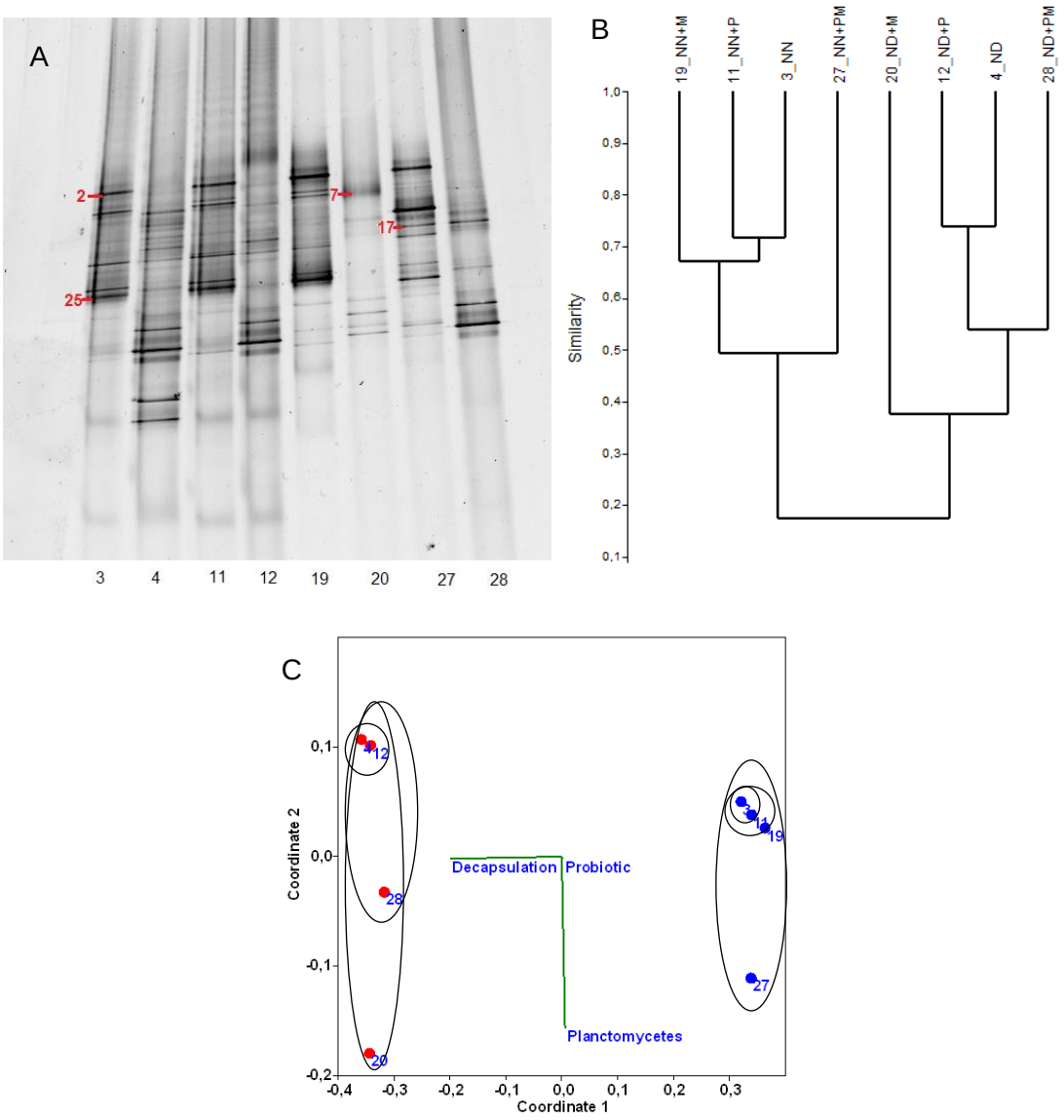


Figure 13 - (A) DNA DGGE gel profiles of microbiome of *Artemia* nauplii (22 hours) with band types numbers. 3 – NN - normal (no decapsulation) nauplii; 4 – ND - decapsulated nauplii; 11 – NN+P – normal nauplii plus probiotic; 12 – ND+P - decapsulated nauplii plus probiotic; 19 – NN+M - normal nauplii plus planctomycetes; 20 – ND+M - decapsulated nauplii plus planctomycetes; 27 – NN+PM – normal nauplii plus probiotic plus planctomycetes; 28 - ND+PM – decapsulated nauplii plus probiotic plus planctomycetes. (B) Dendrogram of DGGE profiles of nauplii, based on Bray-Curtis similarity. (C) Non-metric multidimensional analysis scaling (nMDS) plot (stress: 0.043) of DGGE profiles of nauplii, based on Bray-Curtis similarity with vectors showing the correlation of the 3 different factors and circles represent the groups formed in the dendrogram. Note that the biggest vector is the decapsulation one.

Adults plus planctomycetes treatment (profile 19 and 20) is more divergent in the decapsulated group than in the normal group (Fig. 13 B, C). Planctomycetes do seem to have created a strong change in the microbiome, since nauplius treated with planctomycetes (profiles 20, 27 and 28) with the exception of profile 19 (normal nauplius plus planctomycetes) are in the positive side of the planctomycetes vector, which has an appreciable length. This demonstrates that planctomycetes had an influence over the microbiome, which is further establish by having the second highest value of dissimilarity in SIMPER (0.687 - Table 10).

The probiotic vector is the smallest, apparently non-existent (Fig. 13 C), meaning that this factor did not correlate well with the distribution of the profiles and thus is not a good justification for the differences seen between treatments in nauplius. Further attesting to this, both in normal as in decapsulated profiles we see that control and probiotic profiles are the ones more similar (Figs 13 B, C and Table 10; 0.539).

Table 10 – Similarity percentage (SIMPER) analysis between nauplius groups, groups that are based on common characteristic – decapsulation process or treatment. Results of the comparison of the groups show average dissimilarity, number of bands that contributed to 50% of the dissimilarity, the band that had most influence and its contribution to the dissimilarity.

Groups being compared	Average dissimilarity	# Bands contributed 50% dissimilarity	Main band	
			Band type	% Contribution
normal *vs* decapsulation	0.827	9	2	7.985
control *vs* probiotic	0.539	9	25	8.04
control *vs* planctomycetes	0.687	8	7	16.46
control *vs* probiotic + planctomycetes	0.680	9	17	6.543

Table 11 - R values from analysis of similarity (ANOSIM) between nauplius groups, groups that are based on common characteristic – decapsulation process or treatment. Positive values indicate that profiles differed more between the groups than within the group.

R values	Decapsulated
Normal	1

R values	Control	Probiotic	planctomycetes
Control	-	-0,5	-0,5
Probiotic	-0,5	-	-0,5
Planctomycetes	-0,5	-0,5	-
Probiotic + Planctomycetes	-0,25	-0,25	-0,5

3.3.2.3. Adults

Figure 14 A shows the DGGE gel of adult *Artemia* microbiome, in which the crustaceans were sampled with 48 hours of incubation both fed (at the 24th hour) or non-fed with algae. Decapsulated adults plus planctomycetes (sample 22) was not included in the gel because of low yield of DNA (Fig. 7). The different treatments were then compared with cluster analysis (Fig. 14 B) and with nMDS (Fig. 14 C), in these comparisons normal adults (profile 5) were not included because the tear and the smile effect made impossible to determine the type of the bands.

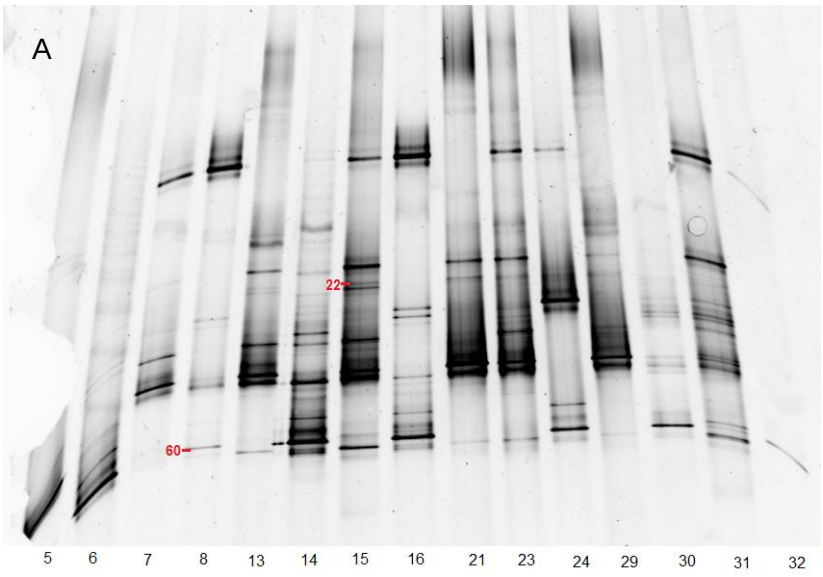
In the dendrogram (Fig. 14 B) we can see the usual separation between normal and decapsulated groups. However, profile 30 (AD+MP) appeared in the normal group.

This may be caused by the lower amount of DNA, as lane 30 is one of the faintest ones. The normal *vs* decapsulation clustering is supported by the average dissimilarity obtained from SIMPER (Table 12) which is the second highest (0.850), by ANOSIM (Table 13) in which R values are in the positive range (0.478) and differences were significant ($p < 0.05$). Once again decapsulation is behind the distribution of the samples and therefore of the microbiome changes.

In the decapsulated cluster, all algae treatments grouped together with the exception of profile 32 (AD+MPA), fact that can be justified by the smile effect on the gel and by the low amount of DNA of this sample. The similarity between decapsulated adults fed with algae cluster and the decapsulated adults is below 20%. In the normal dominant cluster algae do not seem to have a distinctive distribution between adults non-fed and fed with algae microbiome profiles (Fig. 14 B).

When nMDS plot was attempted with all of the microbiome profiles the stress was over 0.2, and thus close to a random ordination (Clarke, 1993) because of this, separated plots of samples of treatments with (Fig. 14 C) and without (Fig. 14 D) algae were done, obtaining stresses of 0.098 and 0.162, respectively. In both nMDS plots (Fig. 14 C and D) the separation between normal and decapsulated profiles is clearer than in the dendrogram, with the normal profiles positioned in the negative side of the decapsulation vector and decapsulated profiles in the positive side. Even profile 30, clustered together with normal samples in the dendrogram (Fig. 14 B), is in the correct decapsulated side.

In the non-fed algae plot (Fig. 14 C) the decapsulation and the planctomycetes vectors have nearly the same size, while the probiotic one is very small. The profiles are in positive range of their respective treatment vectors, with the exception of profile 14 (AD+P) and 30 (AD+MP). These results point to decapsulation and planctomycetes as the best correlated factors to the distribution of the profiles and therefore the microbiome changes. Profiles 6 (AD) and 14 (AD+P) are the ones closer together of the decapsulated profiles, reinforcing the small role of probiotics had in changing the microbiome. Also supporting this idea, the average dissimilarity is the lowest in the control *vs* probiotics comparison (0.740).



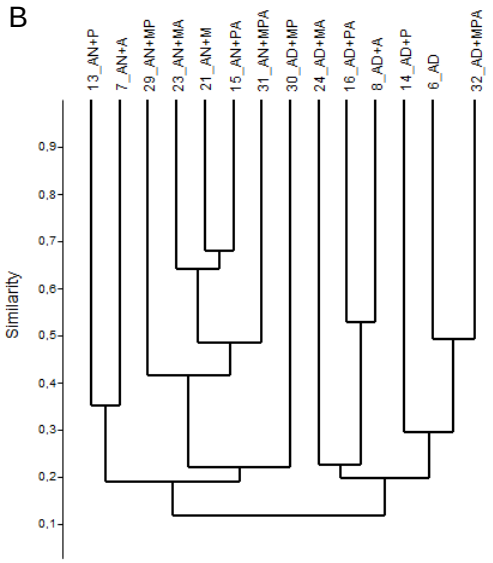
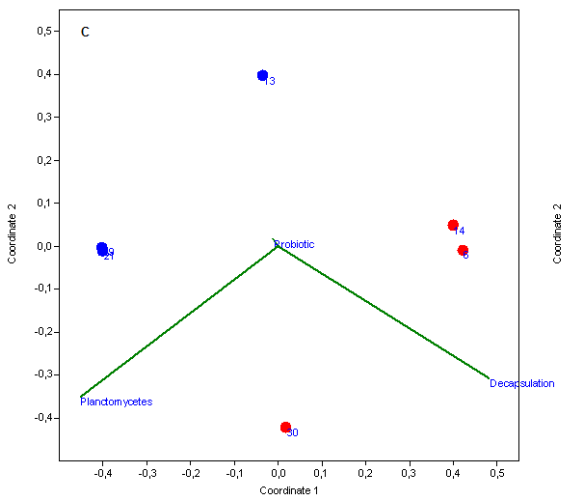


Figure 14 - (A) DNA DGGE gel profiles of microbiome of *Artemia* adults (48 hours) with band types numbers. 5 – AN - normal adults; 6 – AD - decapsulated adults; 13 – AN+P – normal adults plus probiotic; 14 – AD+P - decapsulated adults plus probiotic; 21 – AN+M - normal adults plus planctomycetes; 29 – AN+PM – normal adults plus probiotic plus planctomycetes; 30 - AD+PM – decapsulated adults plus probiotic plus planctomycetes. (B) Dendrogram of DGGE profiles of adults, based on Bray-Curtis similarity. (C) Non-metric multidimensional analysis scaling (nMDS) plot (stress: 0.098) of DGGE profiles of adults, based on Bray-Curtis similarity with vectors showing the correlation of the 3 different factors. (D) – Plot of nMDS (stress: 0.162) of DGGE profiles of adults fed algae, based on Bray-Curtis similarity with vectors showing the correlation of the 3 different factors.



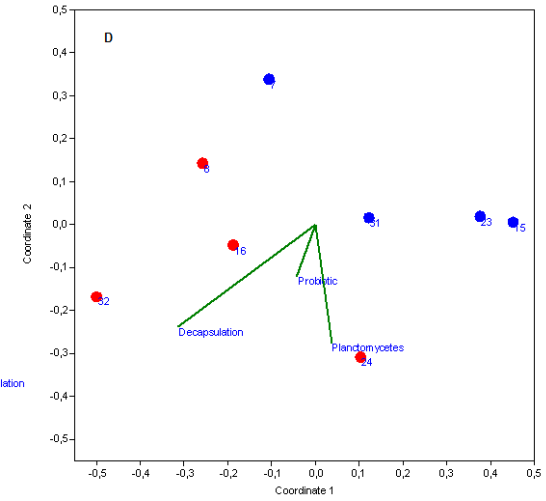


Table 12 – Similarity percentage (SIMPER) analysis between adult groups, groups that are based on common characteristic – decapsulation process or treatment. Results of the comparison of the groups show average dissimilarity, number of bands that contributed to 50% of the dissimilarity, the band that had most influence and its contribution to the dissimilarity. Note: As sample 22 and profile 5 were not analysed, some samples were removed to keep logical pair-wise comparisons.

Groups being compared	Average dissimilarity	# Bands contributed 50% dissimilarity	Main band	
			Band type	% Contribution
normal *vs* decapsulation	0.850	11	60	11.32
non-fed *vs* fed algae	0.796	11	60	11.56
control *vs* probiotic	0.740	10	60	10.12
control *vs* planctomycetes	0.860	9	22	8.256
control *vs* probiotic + planctomycetes	0.797	8	60	16.61

Table 13 - R values from analysis of similarity (ANOSIM) between adult groups, groups that are based on common characteristic – decapsulation process or treatment. Positive values indicate that profiles differed more between the groups than within the group.

R values	Decapsulated
Normal	0.478

R values	Fed algae
Non-fed algae	0.171

R values	Probiotic	Planctomycetes	Probiotic + Planctomycetes
Control	-0.370	1	-0,037

In the algae treatments nMDS plot, decapsulation and planctomycetes vectors are now smaller and probiotics is bigger than in the non-fed treatments nMDS plot. This means that algae homogenised *Artemia*'s microbiome with their own microbial load. The impact of algae addition in *Artemia* microbiome seems big, which is reinforced by an average dissimilarity value of 0.796 (Table 12) and a positive R value of 0.171 (Table 13).

Band type 60 is present in all decapsulated profiles. When the treatments were compared to the control, band type 60 (Table 12) was the distinguishing band type, indicating that the treatments can influence the native microbiome. This is true to all but one group, control *vs* planctomycetes. This discrepancy is however explained by the absence of sample 22 (AD+M) which led to a comparison of only algae treatments for the control *vs* planctomycetes comparison and thus making the R value for the control *vs* planctomycetes positive, since there is less dispersion.

The control *vs* PM (probiotic plus planctomycetes) comparison was the one that needed the less amount of band types to achieve half of the value of the average dissimilarity. It also has the second highest dissimilarity (Table 12). This indicates a

stronger effect in the microbiome when PM is supplied comparatively to other treatments.

The differences in *Artemia* microbiome across different treatments are concordant with Verschuere et al. (1999). In this study, the introduction of 9 bacterial strains in *Artemia*'s rearing water induced drastic influence on the microbial communities of *Artemia* (Verschuere et al., 1999).

The differences in *Artemia* microbiome caused by algae are concordant with Olsen et al. (2000). In this study the addition of algae to the *Artemia* cultures modified the structure and quantity of the associated bacterial community (Olsen et al., 2000).

Decapsulation disinfects the cysts (Sorgeloos et al., 1977) thus having a great effect in the microbiome as it was seen in the results obtained in the present study, in which the comparison between normal and decapsulated was significantly different for all but the control treatment.

3.4. Band extraction and sequence analysis

A total of 132 bands were excised from the DGGE gels, of which 10 were assayed for amplification and only one amplified, band 58 from cysts comparison gel (Fig. 12 – green number). This one was sequenced, and after cleaning and consensus, a maximum-likelihood tree (Fig. 15) was constructed.

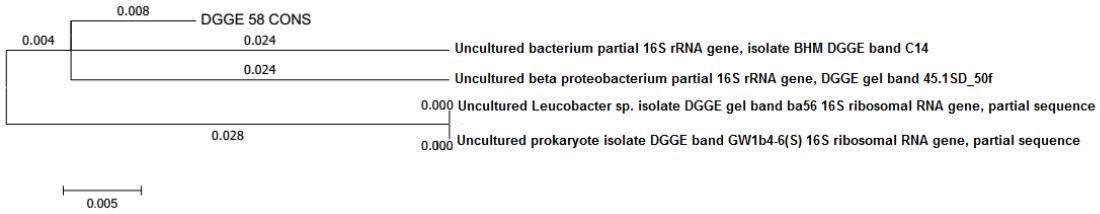


Figure 15 – Maximum-likelihood tree of 16S rRNA gene sequence extracted from DGGE band 58.

The closer taxonomic group to the extracted band was an uncultured *Betaproteobacteria*. All of the isolates in the phylogenetic tree (Fig. 15) were from DGGE samples. The "uncultured bacterium partial 16S rRNA gene, isolate BHM DGGE band C14" is from a marine oil-degrading microbial consortia (Gertler et al., 2012). The "uncultured beta proteobacterium partial 16S rRNA gene, DGGE gel band 45.1SD_50f" is from bacterial communities involved in rocks colonization and soil formation in high mountain environments (Esposito et al., 2013).

3.5. Supplementation assays

It is important to verify what are the effects that bacteria supplementation induces in *Artemia* because it may allow to understand if bacteria (1) are being ingested / attach to *Artemia*'s surface, (2) can be used as food supply for *Artemia* rearing, (3) help them to have better survival rates and/or (4) are even noxious to the crustacean.

Five treatments were performed, using 10 brine shrimps reared with: 1) algae - control; 2) planctomycetes; 3) planctomycetes and algae; 4) AquaStar® probiotic and 5) AquaStar® probiotic and algae.

3.5.1. Mortality studies

The mortality results are shown in Figure 16. In the first week no *Artemia* mortality was observed in the following treatments: algae (control) and probiotic with algae, these were significantly different from the other treatments. The highest mortality was in the planctomycetes treatments with and without algae, 83.3±11.5 % and 93.3±11.5 % respectively, and the treatments were not significantly different. This seems to point out, not only that *Artemia* cannot feed on planctomycetes, but also that planctomycetes is lethal to *Artemia*, since in the algae treatment the shrimp had a food source but still died. Possible explanations for this may be that, as planctomycetes tend to form clusters, these could get caught on their gills causing *Artemia* to suffocate. Also planctomycetes may produce some substance that is noxious to *Artemia* and/or consume too much oxygen. The treatment with only probiotic had a mortality rate of 33.3±5.8 %, already showing that this probiotic cannot be used for *Artemia* as food supply.

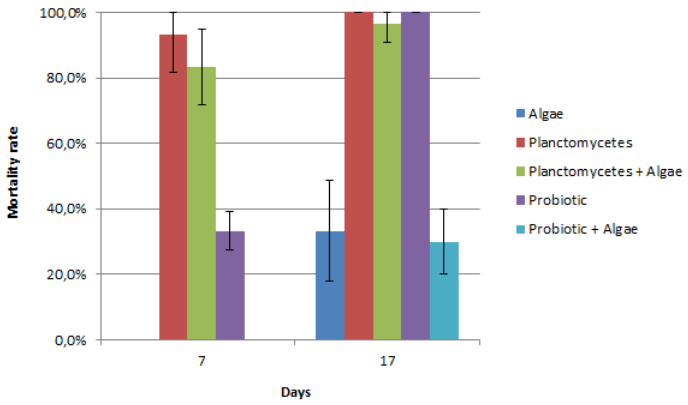


Figure 16 – *Artemia*'s mortality rate at 7 and 17 days of supplementation with different supplies.

Mortality occurred in all treatments after 17 days, with probiotic plus algae having the lowest 30.0±10.0 %, followed by the control (algae only treatment) with a mortality of 33.3±15.3 %. This difference, although not significant, could be due to the beneficial qualities of the probiotic. Treatments with no algae addition had 100 % mortality after 17 days and were significantly different from the control and probiotic with algae treatments, thus showing that these bacteria cannot be the only sustenance delivered to the crustaceans on the long term, not being useful as feed. Planctomycetes plus algae treatment had only one survivor in the 3 replicas (mortality: 96.7±5.8 %). This survivor may indicate a non-noxious reason for *Artemia*'s death when planctomycetes are supplied.

Although the main objective of the supply of the probiotics to *Artemia* is to use *Artemia* as a delivery system of the probiotic to fish, we decided to check the effects on *Artemia* itself to understand if the probiotic affected *Artemia* growth and mortality. *Artemia* mortality results showed that the planctomycete *R. rubra* is lethal and that AquaStar® probiotic plus algae did not affect mortality in relation to the control. In Neu et al. (2014) several bacteria with antibacterial and antivirulence activity, and therefore promising probiotics, in fact were lethal to *Artemia*. In Patra & Mohamed (2003) state that *Saccharomyces boulardii* increased the survival of *Artemia* when subjected to a pathogenic *Vibrio* (*Nannochloropsis* sp. was used to feed *Artemia*). In Marques et al. (2005) dead bacteria diminished *Artemia* mortality, establishing the nutritional value and not the probiotic effect as the influential factor in survival of *Artemia* in their study. In Lamari et al. (2013) six lactic acid bacteria protected *Artemia* from *Vibrio*. None of

the strains used in these papers were the same as we used so direct comparison is not possible. However, it is acceptable to say that because a wide range of results derived of the supply of bacterial supplements and probiotic tests may occur, our result are reliable.

3.5.2. Growth

At the end of the assay the size of the ones that remained alive was measured and is shown in Figure 18. The mean size ranged from 1.5 – 2.0 mm and a broad dispersion of values was obtained in all treatments. Consequently, there are no significant differences among the replicas of each treatment or between different treatments. So in terms of growth no improved performance seemed to occur with probiotic or planctomycetes.

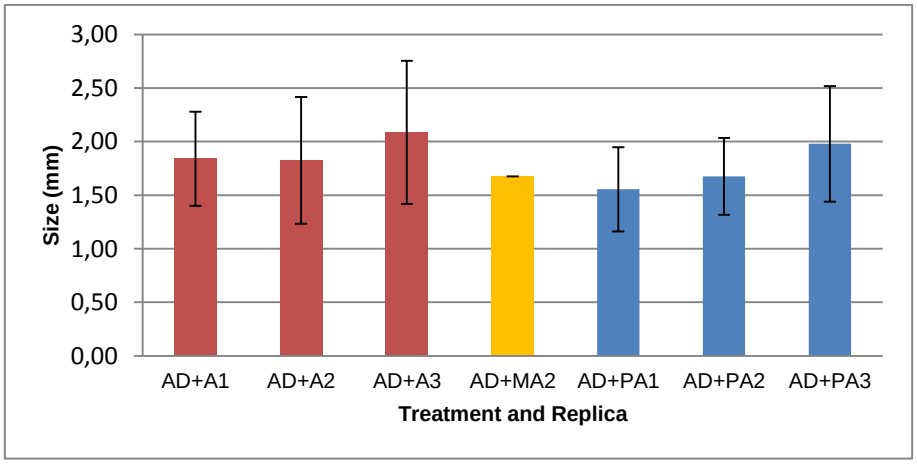


Figure 17 – Decapsulated *Artemia* size in the assays: AD+A - the control treatment, only algae added; AD+MA – fed algae and supplied planctomycetes; AD+PA - fed algae and supplied probiotic. Numbers represent the replica.

Contrary to the results of Marques et al. (2005) and Lamari et al. (2013) the supply of our probiotic did not create significant differences in growth performance. In Marques et al. (2005), *Cytophaga* spp. improved the growth performance of *Artemia*. In Lamari et al. (2013), six lactic acid bacteria were used and all improved growth. However, none of the strains used in these papers were the same as we used so direct comparison is not possible (Lamari et al., 2013; Marques et al., 2005).

4. Conclusions

These studies showed that *Artemia* bacterial community is a dynamic and changeable one.

In the temporal analysis a modification of the *Artemia* microbiome is visible as along the different life phases. The change in the microbiome is stronger in *Artemia* that did not suffer the decapsulation process (normal *Artemia*). As in normal *Artemia* the community diversity was not lessened by the decapsulation, process that eliminates some of the bacteria, the microbiome is given the chance to change more than the decapsulated *Artemia.* The variation over time of the microbiome is seen no matter what treatment, as *Artemia* microbiome profiles never have a similarity of 100 % among different sampling times. This could be important for the aquaculture production, since different microbiomes could benefit more different species and/or in different life cycle phases than others. This would be achieved by providing the different microbiome of *Artemia* to the animal being produced. It could also allow for a better microbiological control, supplying *Artemia* in a determined life stage so it does not have a high quantity of a certain bacteria that would affect negatively the production.

All treatments had different impacts in the microbiome creating different profiles. However, none of them were significantly different from the control and as such the efficiency of the supplements is in question and further studies would be needed. This means that the supplementation of probiotic and/or planctomycetes had an effect in the microbiome in all the life phases, which indicates that the supply of probiotics should be started in the cyst phase. Also, the cysts show differences between treatments which means they could be supplied to the animal of interest if its mouth size can only handle that.

Even though planctomycetes are part of the natural microbiome of *Artemia* (Høj et al., 2009), the supply of *Rhodopirellula rubra* strain LF2 interfered with the viability of *Artemia* as seen by the mortality obtained in the supplementation assays. Planctomycetes also created major differences in DGGE studies in the comparison between treatments, with microbiome profiles, where these bacteria were supplied, being sometimes the more dissimilar ones.

Decapsulation is truly a strong modifying factor in all of the studies. The ANOSIM analysis showed that normal *vs* decapsulated comparisons were always

significantly different ($p < 0.05$), except for the control. It also credited as a major influence in the distribution in all of the temporal and treatments nMDS analyses.

Mortality studies show that control treatment (algae supplied) and probiotic plus algae treatment are not significantly different between each other, but are significantly different from the other treatments. This points to probiotic as being a benefit or having no effect in the survival. Growth of *Artemia,* evaluated by its size, was not affected by the treatments.

4.1. Future and prospects

As one of the expanding food producing industries, aquaculture research should be a priority to the scientific community.

Although *Artemia* is one of the most used live feeds in aquaculture, studies about its microbiome are scarce and none of them focuses on statistical analysis of the microbiome variations after the addition of bacteria, as it was done in this study. We think this type of study is of the utmost importance since we need to know if the bio-encapsulation of probiotics in *Artemia* is actually delivering the probiotics to the animal being reared. Therefore, further studies would need to be developed.

One of the most obvious follow up studies would be the delivery of the probiotics bio-encapsulated in *Artemia* to the animal being reared and see if it impacts its intestinal microbiome, while having other treatments where no probiotic would be added and another where probiotic would be added directly to the animal's tank.

Another quite interesting one would be to repeat the studies we did to try to retrieve viable DNA from the band types from the DGGE gel and sequence them to have a better idea of which species specifically are changing.

Nevertheless, further investigation and research in *Artemia*'s microbiome is indispensable to better understand how we can use *Artemia* to deliver the probiotics to the animal being reared in an effective way.

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