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Analysis of Different Protocols for Multiplication of Commercial Probiotics in Aquaculture Systems

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Abstract

Probiotics can be defined as non-pathogenic living organisms used to improve the health of the host and act against the development of pathogens. Shrimp farms have used artisanal strategies to multiply the bacteria of commercial probiotics and thus decrease the costs with the commercial product. The study was conducted at the Marine Station of Aquaculture, FURG, Brazil. The three experiments used homemade protocols of different shrimp farms. Water collections were performed at 00 h and 24 h to quantify bacteria. The experiments were (I) 25 g of Pro-W probiotic, 25 g of powdered milk, 50 mL of molasses, and 25 g of calcium carbonate, (II) 25 g of Pro-W probiotic, 50 g of rice bran, 33.33 g of crystal sugar and 12.5 g of sodium bicarbonate and (III) 25 g of Pro-W probiotic, 25 g of Bokashi Bran, 50 mL of molasses and 12.5 g of sodium bicarbonate. The protocols were based on the traditional practice of shrimp farms in Latin America. Each experiment had 6 treatments and 3 repetitions. The treatments were carried out in freshwater and saltwater. T1: Control in freshwater (only probiotic); T2: Control in saltwater (only probiotic); T3: Negative control in freshwater (only fertilizers); T4: Negative control in saltwater (only fertilizers); T5: Protocol in freshwater (probiotic + fertilizers); T6: Protocol in saltwater (probiotic + fertilizers). The three experiments showed growth of *Bacillus*, coccoid and filamentous bacteria in all treatments, however the results indicate that the protocols used could not contribute significantly to the replication of commercial probiotics.

1. Introduction

The intensification of marine shrimp farming increased productivity, however, it has resulted in a higher occurrence of different diseases. Research for pathogen control and prevention management has become fundamental for the success of the production. Commonly, producers use antibiotics for the treatment of bacterial diseases [1]. However, their use is not recommended since it enables the selection of pathogens resistant to these products [2] and the negative impact on the final consumer [3, 4]. Through genetic selection and mutation, pathogenic bacteria are resistant to antibiotics, making them inefficient. In addition, they may contaminate the soil, effluent, and the environment itself. [2, 5]. As an alternative, producers have used ecologically viable tools such as intensive and super-intensive production systems and application of bacterial probiotics to increase aquaculture production and reduce waste and contamination.

Unlike traditional systems, intensive systems use high aeration, specific feed, and animal and water health products. This is done to increase productivity and restrict the spread of diseases by increasing biosafety and by reducing water exchange and effluents using small areas for culture [6]. Intensive systems require greater disease control, control over excess organic matter, and nutrient management. Thus, probiotics are widely used as an advantageous alternative to antibiotics due to their mechanisms of action and possible practical benefits in culture systems [7, 8]. Probiotics are non-pathogenic living organisms used to improve host health [9]. They are specific strains of beneficial bacteria and yeasts, which have mechanisms of action in the colonization of the gastrointestinal tract of cultured animals, acting against the development of pathogens [10, 11]. Moreover, probiotic bacteria can compete and eliminate pathogenic microorganisms, which, in turn, can overlap groups or eliminate them by competitive exclusion, preventing their development in the aquatic environment or sediment [12, 13].

Probiotics can prevent the proliferation of pathogenic bacteria like *Vibrio* (gram-negative bacteria) that are responsible for several diseases in the shrimp culture system [14], as well as improve water quality and control

the onset of other diseases [15]. Several authors have found results that corroborate the efficiency of using probiotics to reduce *Vibrio* sp. For example, [16] showed better survival rates and less severe lesions from *Vibrio* sp. in the tissues of shrimp in treatments using probiotics. [17] observed greater survival and efficacy in protecting shrimp against Vibrio parahaemolyticus in the commercial probiotic treatment. [18] identified a lower number of *Vibrio* spp. in the intestinal microbiota of *L. vannamei* using a diet supplemented with *Lactobacillus plantarum* in addition to increasing animal survival. [19] showed that shrimp fed without probiotics and challenged with the presence of *V. parahaemolyticus* and *V. alginolyticus* obtained less significant growth than those with added *Bacillus cereus* in the feed.

Besides the *Vibrio* group, other groups of bacteria can cause problems for cultured animals. Shrimp diseases caused by filamentous bacteria are known worldwide [20]. [21] showed that *Flexibacter maritimus* filamentous bacteria have pathogenic potential in shrimp, perceiving clinical signs in *L. vannamei* larvae, such as tissue necrosis and changes in the gills, impairing breathing and causing massive animal mortality.

Despite being costly, knowledge on the application of commercial probiotics and their use linked to the prevention and/or treatment of diseases is well evidenced in shrimp farming [8, 9, 22]. However, producers want the benefits of probiotics with low economic investment. However, as producers want the benefits of probiotics with low economic investment, they often carry out homemade multiplication practices of commercial probiotics using empirical protocols instead of using them as indicated by the manufacturers to increase product volume and reduce costs These homemade protocols use the most varied ingredients and methods, which can cause severe risks to the aquaculture system. There are several possibilities of contamination since the ingredients used, such as vegetable and animal meal, may either contain, or favor the growth of pathogenic microorganisms [23]. The water used in the farms is also a potential transmitter of several pathogenic bacteria, protozoa, and viruses [24], and some microorganisms may be resistant to different disinfection techniques [25].

Currently, there is a lack of scientific information establishing which microorganisms grow in cultures using these protocols. This technique, widely used in shrimp farming in Latin American countries, may be responsible for the spread of diseases, disease development, and, consequently, economic losses. The objective of this study was to contribute information on the action of empirical protocols on the multiplication of a commercial probiotic and its potential for the development of probiotic bacteria and possibly pathogenic bacteria in the cultured media.

2. Materials And Methods

This study was developed at the Marine Station of Aquaculture, Institute of Oceanography, Federal University of Rio Grande (EMA - FURG), Rio Grande, Rio Grande do Sul State, Brazil. The experiments were carried out in an experimental room with controlled air and temperature. Three different multiplication protocols of a commercial probiotic were tested. The protocols and treatments are described in Table 1.

Table 1
Description of the products used in each multiplication experiment of the commercial probiotic Sanolife ProW® (INVE Aquaculture) and treatments used in experiments I, II, and III.

Experiment	Fertilizers	Probiotic	Treatments					
			T1	T2	T3	T4	T5	T6
I	25g of milk powder 50mL of molasses 25g calcium carbonate	25g of ProW®	FW Control	SW Control	FW Negative Control	SW Negative Control	FW Protocol	SW Protocol
II	50g of rice bran 33.33g crystal sugar 12.5g sodium bicarbonate	25g of ProW®	FW Control	SW Control	FW Negative Control	SW Negative Control	FW Protocol	SW Protocol
III	25g of bokashi bran 50mL of molasses 12.5g sodium bicarbonate	25g of ProW®	FW Control	SW Control	FW Negative Control	SW Negative Control	FW Protocol	SW Protocol
Legend of treatments: FW = Freshwater; SW = Saltwater; Control = only probiotic; Negative control = only fertilizers; Protocol = probiotic + fertilizers.								

The commercial probiotic used was Sanolife ProW® (INVE Aquaculture), composed of *Bacillus subtilis* (2.75 x 10^{10} CFU/g) and *Bacillus licheniformis* (2.25 x 10^{10} CFU/g) at a dosage of 1 g/L. The experiments were performed in tanks with 25 L of useful volume, freshwater, and chlorinated saltwater (40 ppm of chlorine). Dechlorinated saltwater was added after 6 hours (1 ppm of acid ascorbic). Three treatments with saltwater and three treatments with freshwater, with three repetitions each, randomly distributed.

Each experimental unit was equipped with 300 W heaters (Roxin) and with a constant air supply using a 2 HP blower (IBRAM) coupled with a cylindrical airstone (5 cm each) to keep the temperature between 28–30ºC. The dissolved oxygen (DO) and temperature monitoring was performed with a previously disinfected multiparametric probe YSI 556 MPS (YSI® Inc., Yellow Springs, USA) at 00 h, 12 h, and 24 h. The pH was measured with a bench pH meter (Mettler Toledo, FEP20, Brazil) at 00 h, being 7.41 ± 0.04 for fresh water and 7.73 ± 0.13 for marine water. Each experimental study lasted 24 h. Tap water was used for the freshwater

treatment and the measured salinity in each experiment was 24.8 (experiment I), 25.7 (experiment II), and 24.7 (experiment III).

2.1. Microorganisms

Samples containing 20 ml were collected at 00 h (after inclusion of the products in water) and 24 h with a sterile pipette and processed at the Phytoplankton Ecology and Marine Microorganisms Laboratory and the Laboratory of Ecology of Microorganisms Applied to Aquaculture - IO / FURG. Prior to the preparation of the blade, each sample was subjected to sonication (Qsonica Sonicators) with a 60 Hz wave amplitude for 40 seconds to break down microorganisms and organic matter. To determine the abundance of bacteria, the collected samples were filtered through polycarbonate membrane filters (Nucleopore, 0.2 µm pore and 2.5 mm in diameter) previously darkened with Irgalan Black and later stained with 1% Acridine Orange in the concentration 1 µg/mL [26]. Bacteria were photographed using a camera coupled to an epifluorescence microscope (Axioplan-Zeiss), with 1000X magnification, and counting was performed by selecting 30 random fields per photograph. Bacteria were classified based on their morphotype, such as *Bacillus* (rods), coccoid, filamentous, and *Vibrios*.

2.3. Statistical analysis

The data were subjected to analysis of normality of data distribution (Shapiro-Wilk test) and homoscedasticity of variances (Levene test). When the assumptions were not met, statistical transformations were carried out, and the data were subjected to one-way analysis of variance (ANOVA) ($\alpha = 0.05$). When statistical differences were detected ($p < 0.05$), the Post-hoc Tukey test with a 95% confidence level [27] was applied.

3. Results

Throughout the experiments, the growth of the bacillus morphotype, coccoid and filamentous bacteria was verified. No *Vibrio* bacteria were observed in any of the experiments. The mean DO values in Experiments I, II, and III are compiled in Table 2. Data were collected at 00 h, 12 h, and 24 h.

Table 2
Mean values of dissolved oxygen (DO) in mg/L over 24 h. Different overwritten letters represent statistical differences ($p < 0.05$) between treatments.

Experiment	Time	Treatments					
		T1	T2	T3	T4	T5	T6
I	00	8.88 ± 0.4^{a}	6.93 ± 0.3^{b}	8.44 ± 0.2^{a}	6.71 ± 0.1^{b}	8.24 ± 0.1^{a}	7.04 ± 0.2^{b}
	12	2.92 ± 2.3^{a}	0.67 ± 0.7^{a}	0.12 ± 0.05^{a}	0.08 ± 0.01^{a}	1.04 ± 1.1^{a}	0.08 ± 0.01^{a}
	24	4.91 ± 1.4^{a}	4.69 ± 0.8^{a}	0.11 ± 0.02^{b}	0.10 ± 0.04^{b}	0.08 ± 0.01^{b}	0.06 ± 0.02^{b}
II	00	8.9 ± 0.8^{a}	6.33 ± 0.6^{a}	8.07 ± 0.1^{a}	6.99 ± 0.5^{a}	8.55 ± 0.5^{a}	6.46 ± 0.1^{a}
	12	2.02 ± 1.6^{a}	3.93 ± 0.7^{a}	1.05 ± 0.6^{a}	1.74 ± 1.2^{a}	0.1 ± 0.02^{a}	0.37 ± 0.4^{a}
	24	5.18 ± 0.1^{a}	4.54 ± 0.4^{a}	2.89 ± 0.7^{a}	1.34 ± 0.9^{a}	0.1 ± 0.01^{a}	0.06 ± 0.01^{b}
III	00	8.6 ± 0.3^{a}	5.2 ± 0.1^{b}	8.1 ± 0.2^{a}	5.7 ± 0.4^{b}	7.9 ± 0.4^{a}	5.48 ± 0.3^{b}
	12	0.48 ± 0.3^{a}	1.1 ± 0.8^{a}	2.3 ± 1.3^{a}	1.1 ± 0.8^{a}	0.04 ± 0.03^{a}	0.06 ± 0.01^{a}
	24	2.8 ± 1.7^{a}	1.0 ± 1.2^{ab}	0.2 ± 0.05^{ab}	0.49 ± 0.3^{a}	0.1 ± 0.02^{b}	0.22 ± 0.1^{ab}

3.1 Experiment I – Milk Powder/Molasses/Calcium carbonate

There were significant differences ($p < 0.05$) between treatments at 00 h and 24 h for DO values (Table 2). Treatments T1 and T2 maintained DO above 0.5 mg/L at 24 h, with values statistically different from the other treatments, where the obtained levels were close to zero at the end of the experiment. The initial average temperature was measured at 27.6 ± 0.58 ºC and the final at 30.1 ± 0.87 ºC.

The *Bacillus* group showed growth in all treatments from the initial time (00 h) to the final (24 h), differing statistically. When comparing the values between treatments for the 24 h period, no statistical differences ($p > 0.05$) were identified due to the similar growth rates of bacteria in the different treatments (Fig. 1). In treatments T3 and T4, where there were no commercial probiotics, the growth was similar to the other treatments.

The growth of coccoid bacteria (Fig. 2) showed statistical differences ($p < 0.05$) between the initial and final time, except for T3, which showed no significant differences (initial $1.0x10^{6}$ and final $9.11x10^{6}$). The treatments T1 (initial $4.37x10^{4}$ and final $3.68x10^{6}$) and T2 (initial $2.75x10^{4}$ and final $5.57x10^{6}$) presented the lowest coccoid values at the end of the experiment. Their growth was statistically similar to the other treatments. The treatments that obtained the most significant final growth were the treatments T4 ($7.65x10^{6}$), T5 ($8.84x10^{6}$), and T6 ($7.77x10^{6}$).

3.2. Experiment II – Rice Bran/Crystal sugar/Sodium bicarbonate

The DO behavior showed no significant differences ($p < 0.05$) between treatments in the initial period (Table 2). At the end of the experimental period, T6 was significantly different from the other treatments, obtaining the lowest DO value of the experiment, while T1 and T2 maintained the highest levels of this parameter after 24 h ($p < 0.05$).

The initial mean temperature was 30.5 ± 0.8 ºC, and the final was 30.4 ± 0.8. In Experiment II, for the growth of the *Bacillus* morphotype, there were significant differences ($p<0.05$) for all treatments between initial and final time, where all treatments showed significant growth of bacteria. T5 and T6 had higher values for initial and final bacterial counts. As shown in Figure 4, T3 was the only treatment to present differences compared to other treatments at the beginning of the experiment ($p<0.05$). There was no significant difference ($p>0.05$) between treatments for the final growth, although all treatments showed significant growth when compared to the initial time (Fig. 4).

Statistical differences were observed for the final growth of the coccoid morphotype compared to the initial one (Fig. 5). After 24 h, the highest final count was observed in T3, with $1.76x10^7$. The lowest final count was observed in the T1 ($9.79x10^6$) and T2 ($5.42x10^6$) treatments.

The filamentous bacteria also grew during Experiment II (Fig. 6), showing less total growth for T1 (initial $9.57x10^2$ and final $6.10x10^5$) and T2 (initial $1.93x10^4$ and final $5.36x10^5$). The most significant growth of filamentous was observed in treatments T5 ($3.72x10^6$) and T6 ($2.62x10^6$) in the final time; both treatments used the protocol (probiotic + fertilizers).

3.3. Experiment III – Bokashi Bran/Molasses/Sodium bicarbonate

The DO values differed significantly ($p < 0.05$) between 00 h and 24 h (Table 2). The lowest oxygen value of the final time was observed at T5. All treatments showed a decrease in oxygen availability in the water compared to the initial period. The initial average temperature was 30.3 ± 1.2 ºC, and the final average temperature was 31.8 ± 1.8 ºC.

Significant differences ($p < 0.05$) in the quantification of the Bacillus morphotype were observed between the initial and the final experimental time. All treatments had significant growth of this type of bacteria over the 24 h of the experiment. The lowest count for this bacterium at the start time was observed in T3 and T4, both negative treatments (only fertilizers). However, the count at the end time was statistically equal for all treatments.

As shown in Fig. 7, treatments T1 and T2 obtained similar initial *Bacillus* growth despite the different salinities (0.25 and 24.7). At the end of the experiment, the highest Bacillus growth was observed in treatment T3, although it is not statistically different from the other treatments. Treatments T5 and T6 obtained significant growth when compared to the initial time.

The development of coccoids can be seen in Fig. 8. There were significant differences ($p < 0.05$) from the initial time to the final time in the growth of bacteria, where the lowest initial count of coccoid was detected in treatments T3 ($1.56x10^5$) and T4 ($8.53x10^4$).

For the quantification of filamentous bacteria in Fig. 9, we observed statistical differences ($p < 0.05$) in the beginning and the end times for each treatment. The development of this bacterium was similar to the end time and without statistical differences ($p > 0.05$) between treatments.

4. Discussion

4.1. Experiment I – Milk Powder/Molasses/Calcium carbonate

The significant differences ($p < 0.05$) in the initial DO may be related to water sources. Freshwater (T1, T3, and T5) contains a higher DO concentration than saltwater due to the number of dissolved salts (salinity). For the final DO, the values of T1 and T2 were statistically higher when compared with the other treatments. This difference may be associated to the higher consumption of DO in treatments T3, T4, T5, and T6, including the experiments with powdered milk and molasses. These products are organic carbon sources used by microorganisms to feed, grow, and multiply. Thus, the rapid development of bacteria using the available carbon sources may have affected DO availability [28].

Significant differences ($p < 0.05$) were observed for the growth of *Bacillus* between the beginning and the end of the experimental period. Within the conditions tested, all treatments showed the same growth trend, regardless of water salinity. The negative treatments (T3 and T4), which did not use the commercial probiotic, were as efficient in the development of *Bacillus* bacteria as the treatments that contemplate the application only of the probiotic (T1 and T2) and the treatments that rely on the complete application of the protocol (T5 and T6). After 24 h, the growth of *Bacillus* was statistically similar between treatments. This can be related to the isolated and controlled environment where the experiment was carried out or to the nutritional difference between tested ingredients. The short duration of the experiment may also have affected the appearance of possible statistical differences.

The growth of the group of coccoid bacteria obtained significant differences ($p < 0.05$) during the experiment, where T1 and T2 treatments showed lower values at time 00 h. This behavior may be related to the lack of available nutrients since only the commercial probiotic was included in those treatments. However, at the end of the experimental period, the growth of coccoid was equal in all treatments, without significant differences.

Filamentous bacteria grew in smaller numbers when compared to *Bacillus* and coccoid, but in some cases, they may have the pathogenic potential for the cultivated species. Statistical differences ($p < 0.05$) between the initial and final time for each treatment, showing the lower growth of this group in treatments T1 and T2, may be related to the suppression made by bacteria from commercial probiotics on other microorganisms. Thus, it is noteworthy that its growth was more significant in freshwater treatments. All treatments showed significantly higher growth in the final time when compared to the initial time. Despite this behavior, no statistical differences were observed at the end of the experiment ($p < 0.05$); that is, the filamentous growth was equal in all treatments, regardless of the products used.

4.2. Experiment II – Rice Bran/Crystal sugar/Sodium bicarbonate

There were no statistical differences for DO at times 00 h and 24 h. However, there were significant differences ($p < 0.05$) in the final time for oxygen consumption at T6, which differed from the other treatments, recording the lowest mean concentration (0.06 ± 0.01b).

Although we did not identify statistical differences between the other treatments, the lowest final DO concentration was observed in treatment T5 (0.1 ± 0.01a). The levels of DO play an essential role during bacterial processes [29]. Moreover, the fact that the T5 and T6 treatments have the lowest levels may be related to the more significant amount of organic matter developed in the environment, as both had the addition of the complete protocol. The recorded temperature was about 30 ºC, similar to temperature conditions often found in shrimp culture farms.

The growth of the *Bacillus* group differed statistically from the initial time to the final time, demonstrating that there was growth in all treatments. The count was lower in saltwater treatments T2 and T4 than in the other treatments for the initial time. These differences were not noticed in the final time, where the average growth was statistically equal for all treatments.

Despite the absence of the probiotic in the negative treatment, *Bacillus* bacteria grew. The protocol contemplates rice bran, which can stimulate the appearance of several groups of microorganisms that make up the primary productivity in the aquatic environment [30] due to the amount of organic carbon released.

Bacteria of the coccoid morphotype also grew in this experiment presenting similar growth in the initial and final time between all treatments. Thus, in the control treatments T1 and T2, where the dosage of commercial probiotic was high, the rapid domination of the medium by *Bacillus* from the commercial product may have hindered their growth. Probiotic bacteria of the genus *Bacillus* spp. produce bacteriocins capable of suppressing and killing a variety of microorganisms [31]. No *vibrio*s were identified during the experiment.

In this experiment, with an average temperature of 30 ºC and an available carbon source, the filamentous also occupied and colonized the water. There was no significant difference in the final time, although T5 and T6 showed significant growth, and the DO reached levels close to zero. The water can be quickly colonized when quickly degraded products, such as sugar, are used, making carbon available to microorganisms.

4.3. Experiment III – Bokashi Bran/Molasses/Sodium bicarbonate

Oxygen consumption was significantly higher in treatment T5 (0.1 ± 0.02) in the final time, followed by T6 (0.22 ± 0.1). Both treatments have the highest total bacterial counts, T5 ($7.89X10^7$) and T6 ($6.65x10^7$), which may explain the higher overall consumption of DO.

Treatments T3 and T4 had the lowest initial *Bacillus* count, which may be explained by the fact that these treatments do not include commercial probiotics. Through the quantification of *Bacillus*, we can observe that probiotics in treatments T1 and T2 were similar to those in treatments where the product was not included,

which is the case of negative treatments, T3 and T4. Thus, our findings show that the protocol does not increase the development of *Bacillus* in treatments with the commercial product.

Bokashi Bran is a compound, acquired through a mixture of bran of vegetable origin (rice bran, wheat,), bran of animal origin (bone meal, meat), energy source (sugar, molasses, etc.), and oilseeds [32]. Commonly used as a fertilizer for growing fruit, it is now used in aquaculture, although it does not yet have a standardized commercial formula. This experiment demonstrates the ability of organic fertilization with Bokashi Bran to favor the appearance of *Bacillus* bacteria, although its final count is statistically equal between treatments.

Coccoid and filamentous morphotype bacteria showed statistical differences ($p < 0.05$) at the end of 24 h, obtaining similar growth between all treatments. Oxygen reached rates close to zero near the middle of the experimental period, with no recovery of DO rates, even with a constant mechanical supply of blown air. The abundant presence of microorganisms may have collaborated with the excessive consumption of DO and the increasing amount of organic matter in the medium, which need oxygen to grow and multiply.

4.4. General discussion

The development of microorganisms in water depends on several factors such as available nutrients, temperature, DO, pH, and growing time. Each group of bacteria has its preferences; *Bacillus spp.* are mesophilic, developing between temperatures of 10–48 ºC. Bacteria with a morphotype of coccoid and filamentous can develop in the most varied temperature ranges. Temperature is considered the most critical environmental factor for microbiological growth [31].

The bacteria present in commercial probiotics are of the genus *Bacillus spp*. (*B. subtilis* and *B. licheniformis*), classified as gram-positive, sporulation and mandatory or facultative aerobics may occur [31]. During the growth and multiplication of aerobic microorganisms, the introduction of mechanically blown air is traditional as the DO is consumed so quickly that the diffusion of atmospheric oxygen cannot supply the microorganism's respiratory demand. Despite the fact that the experiments had a constant supply of air blown, the microorganisms consumed oxygen, and DO levels dropped to close to 0.00 mg/L between 12 h and 24 h. This increased DO consumption may have been due to the rapid proliferation of different bacteria, which need oxygen to grow and multiply [31]. These results indicate that adding the fertilizer to the protocols may significantly decrease the DO of the shrimp production systems, which may affect their growth or cause their death, depending on the amount added.

The pH can also change during cell growth due to the metabolic reactions of microorganisms that can consume or produce acid or base substances. *B. subtilis* can produce, for example, acetic acid [33]. The addition of buffer substances is necessary for pH maintenance, such as calcium carbonate and sodium bicarbonate, both added to the tested protocols. The tested protocols showed differences in the composition of their ingredients, thus causing the development of different amounts of bacteria in each experiment. Despite the product differences, the growth behavior of groups of bacteria was similar between experiments I, II, and III. All experiments showed significantly higher *Bacillus* growth in the final time when compared to the initial time. Coccoid and filamentous bacteria were also quantified, with significantly higher growth at the end of the 24 h period.

There was no identification and quantification of bacteria from the *Vibrio* group throughout the experiments. The dosage of the commercial probiotics used in this study (1 g/L) was at least 1000x higher than the

recommended dosages for nursery phases (1 mg/L) in shrimp cultures, resulting in 50 million of *Bacillus* per mL. As a hypothesis, the pressure of *Bacillus* abundance may have excluded the appearance of *Vibrios*, or the values may have been below the detection limit. This absence can be explained by the total time of the experimental period, which may have been insufficient to allow the appearance of bacteria of this genus. Santos (2020) quantified the appearance of *Vibrios* on the fifth experimental day, in a culture of *L. vannamei* in the Aquamimicry System, while the present study lasted only 1 day.

Water temperature of all experiments (I, II, and III) remained within the ideal range of 28–30 ºC for the growth of *L. vannamei*. This study aimed to maintain temperatures at levels similar to those found on production farms, based on empirical protocols. Different salinities, with fresh and saltwater salinity close to 0 mg/L and 25 mg/L, respectively, also aimed to simulate practices seen on the farms. In some shrimp farms, using fresh water to carry out the multiplication of probiotics is usual; on the other hand, saltwater is often used directly from the culture ponds, without prior disinfection.

When comparing all the experiments, the experiment that presented the most significant total growth of *Bacillus* was Experiment III (treatment T3), where the base of the protocol was the Bokashi Bran, reaching a value of $1.08x10^8$. Even with the differences between the products used, the development of *Bacillus* was similar in all control treatments, regardless of water salinity, temperature, and DO.

The closed and controlled environment may have influenced the growth dynamics of bacteria and protozoa. [34] quantified a more significant presence of *Bacillus*, coccoid and filamentous bacteria, flagellates, ciliates, amoebas, and rotifers in treatments exposed to light compared to treatments with light restriction. The lack of light exposure in the experiments in this study may have harmed the development of microorganisms. There were no exchanges between indoor and outdoor environments, and no interactions with farm animals. The multiparameter probe and the pipette were disinfected during the experimental period before each measurement and water sampling.

The growth of the bacteria population may also have been limited by the total multiplication time of the protocols. After 24 h, the diversity of microorganisms that developed was not expressive compared to more extended cultures. By carrying out this study for more than 24 h, statistical differences could be evidenced in the quantification of microorganisms between treatments due to the possibility of more significant interactions between microorganisms such as predation and pasture as the natural evolution of the community.

However, the duration of the experiment (24 h) is based on the standard practices of farms from which the empirical protocols were designed. The commercial probiotic multiplication protocols are carried out daily. At the end of 24 h period, the multiplication product is divided among the culture units and included directly in the water or mixed with the feed supplied to the animals.

The development of *Bacillus* in all treatments can demonstrate the ability of home protocols to collaborate with their growth in water within 24 h. Nonetheless, these products are not able to increase *Bacillus* quantification when acting along with commercial probiotics. Thus, this confirms that *Bacillus* bacteria develop regardless of the products added to the water, as there were no statistical differences in the final time between treatments. The structure of microorganisms was similar in all experiments, demonstrating that the development of the microbial loop was similar regardless of salinity and products added to water. The initial abundance of

microorganisms, at 00 h, right after the inclusion of the products in the water, may be linked to the possibility that the disinfection technique used was not efficient in eliminating all the microorganisms present in natural water. The optimal salinity ranges used by most bacteria can be quite broad, with natural waters being an important inoculum [35] that helps form a diverse microbial community capable of assimilating the available nutrients.

Our findings show that the protocols observed in cultivation farms, carried out empirically, do not demonstrate scientific efficacy. The bacterial counts demonstrated that increasing the volume of probiotic bacteria present in the commercial product using various products (powdered milk, molasses, rice bran, bokashi bran, sugar) to provide a food source for the bacteria present in the commercial product was not effective. However, this emphasizes the importance of understanding the action mechanisms of commercial probiotics and investigating the effectiveness of empirical practices so that the producer does not waste financial resources on techniques that do not work.

Validating scientifically and seriously the practices used in different stages of animal production guides the decision-making process of the producer and gives assurance. Ultimately, other empirical protocols are carried out on shrimp farms, but the effectiveness of their applications will be undefined until scientifically tested.

5. Conclusion

Our findings allow us to conclude that the empirical protocols used in shrimp farms could not reproduce the bacteria present in the commercial probiotic, as there was no statistically superior increment of *Bacillus* bacteria at 24 h, both for fresh and saltwater, in any treatment. However, it cannot be affirmed that the appearance of *Bacillus* in the experiments comes exclusively from the commercial product. Furthermore, the protocols in question did not favor the appearance of bacteria of the *Vibrio* genus.

Declarations

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Author contribution: Flávia Banderó Hoffling: Methodology, Investigation, Data Curation, Formal analysis, Writing - Original Draft, Visualization. **Paulo Cesar Oliveira Vergne de Abreu:** Writing - Review & Editing, Supervision, Funding acquisition. **Missileny de Jesus Xavier:** Conceptualization,Methodology, Investigation. **José Maria Monserrat:** Conceptualization,Methodology. **Wilson Wasielesky Jr:** Supervision, Funding acquisition. **Dariano Krummenauer:** Conceptualization, Validation, Writing - Original Draft, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Conflict of interest: The authors declare no competing interests.

Data Availability: All data generated or analyzed during this study are included in this published article.

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Figures

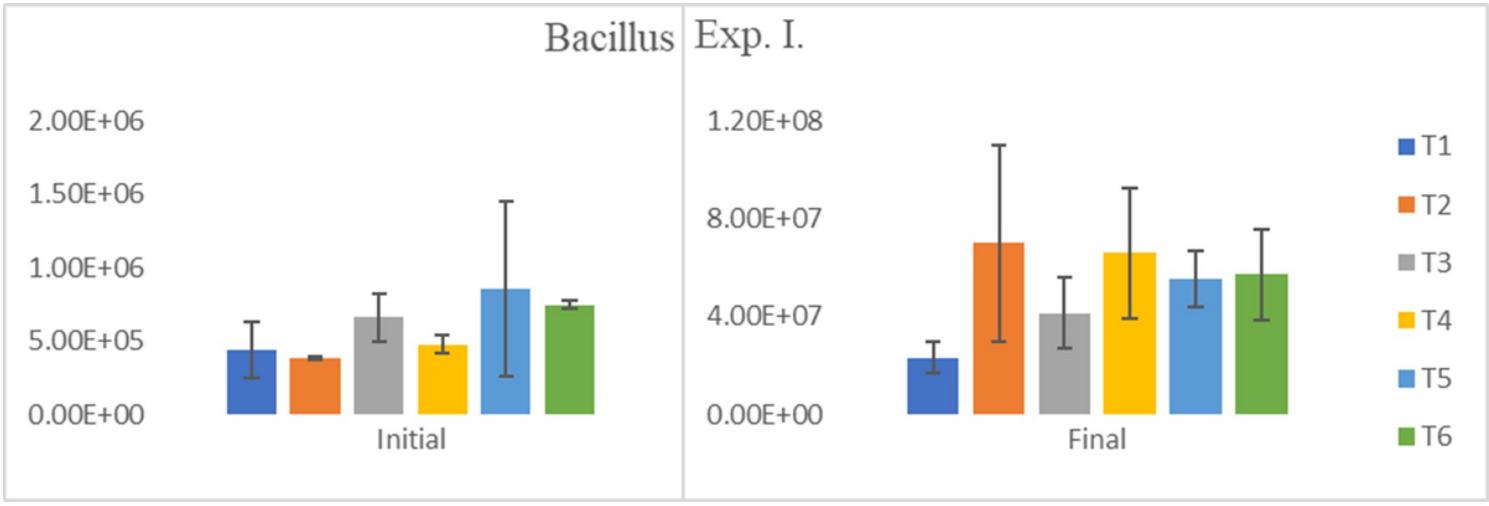

Figure 1

Quantification of *Bacillus* per mL (means ± standard deviation) at the beginning and the end of Experiment I. Note differences in the scales between the beginning and the end of the experiment

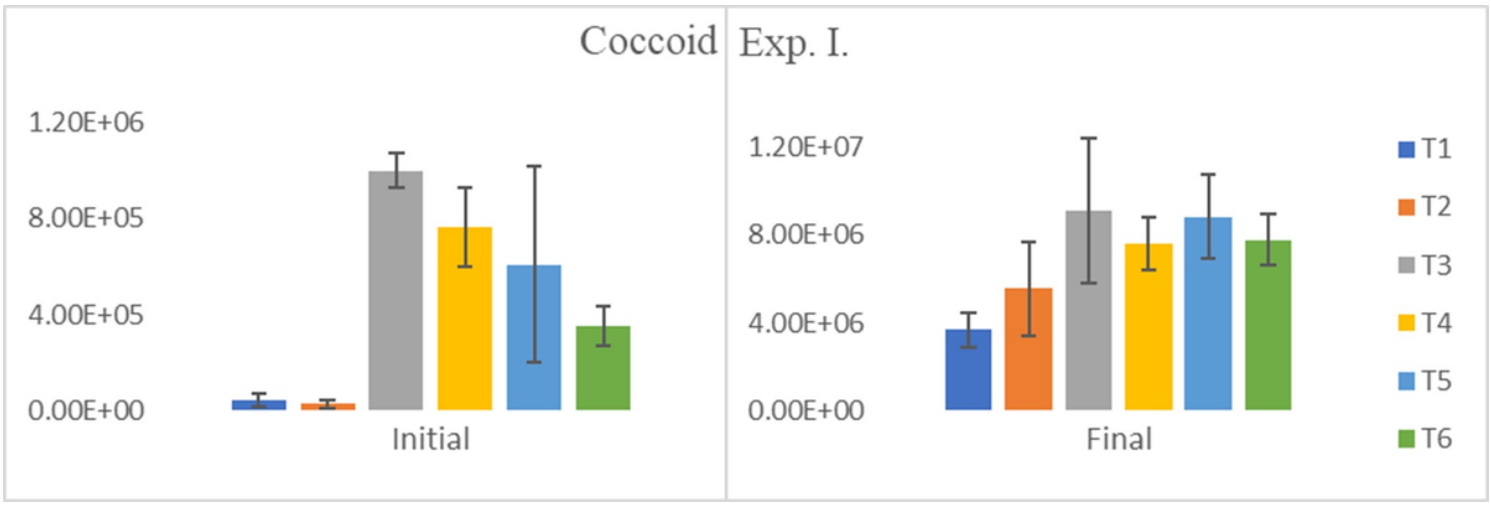

Figure 2

Quantification of the coccoid bacteria group per mL (means ± standard deviation) at the beginning and the end of Experiment I. Note differences in scales between the beginning and the end of the experiment

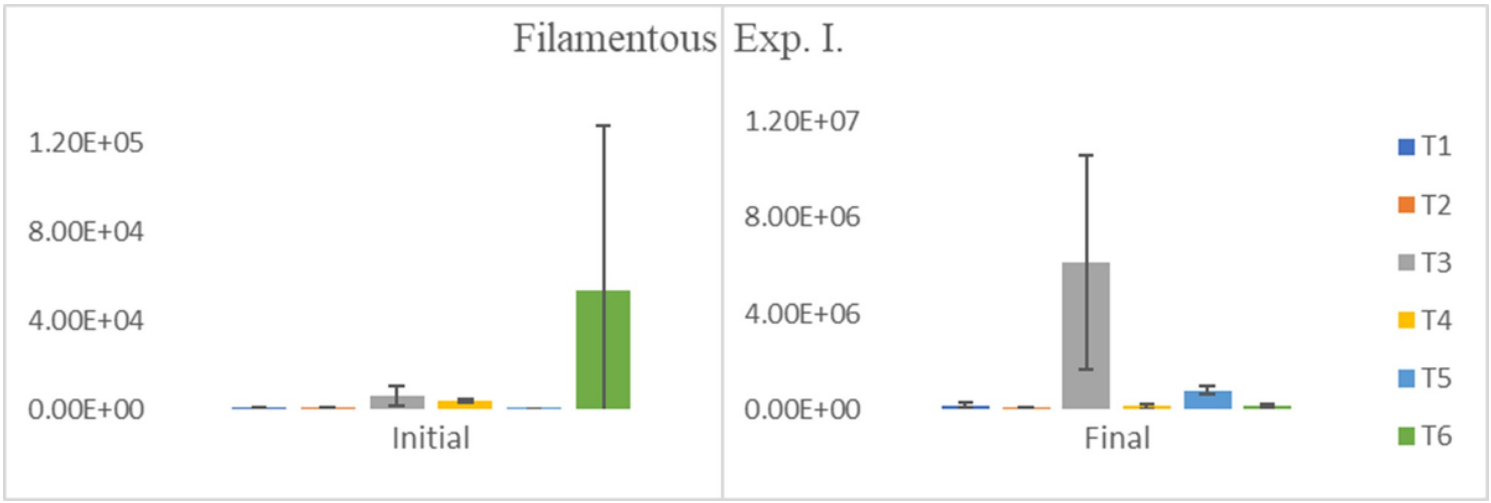

Figure 3

Quantification of the group of filamentous bacteria per mL (means ± standard deviation) at the beginning and the end of Experiment I. Note differences in the scales between the beginning and the end of the experiment

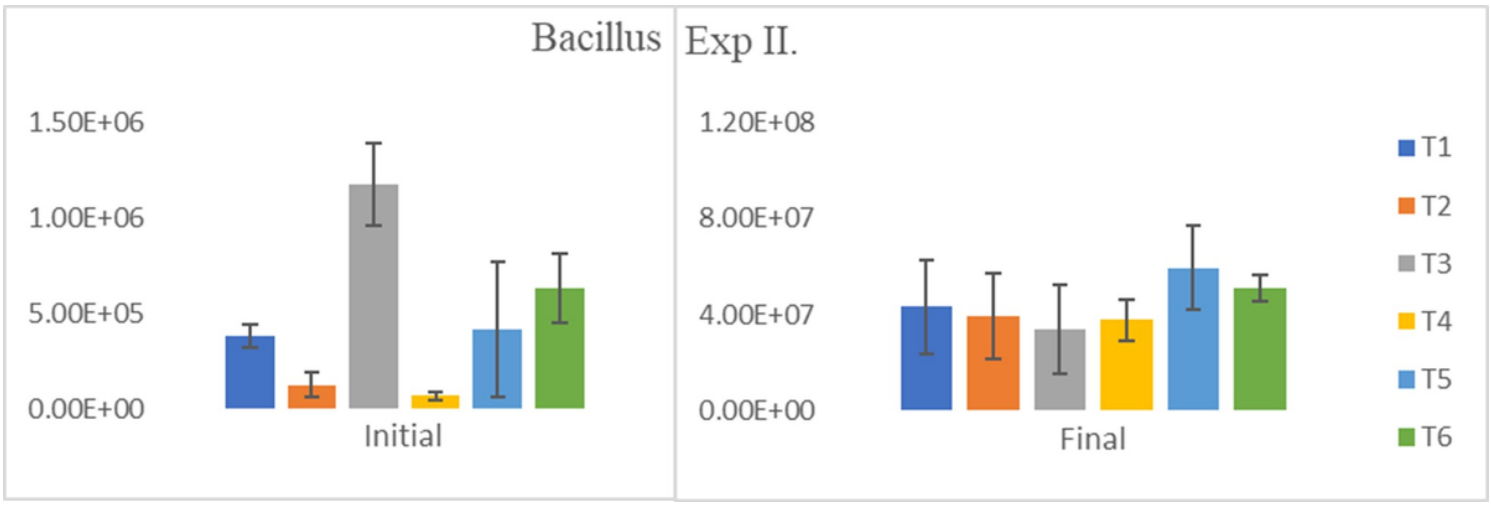

Figure 4

Quantification of the *Bacillus* bacteria group per mL (means ± standard deviation) at the beginning and the end of Experiment II. Note differences in scales between the beginning and the end of the experiment

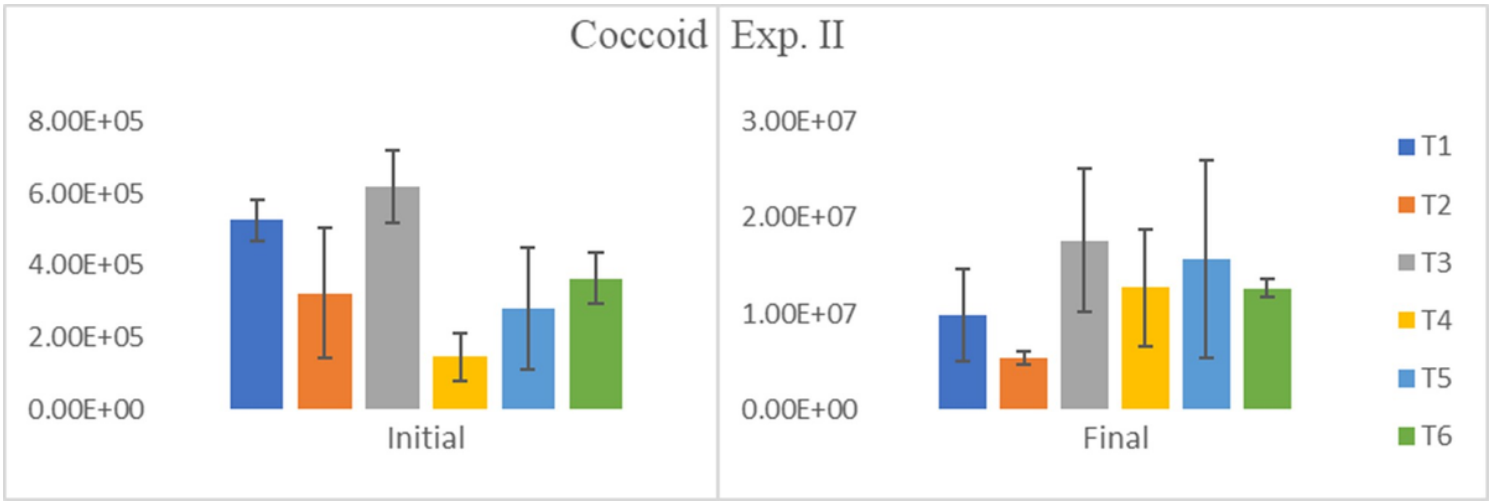

Figure 5

Quantification of coccoid bacteria per mL (means ± standard deviation) at the beginning and the end of Experiment II. Note differences in scales between the beginning and the end of the experiment

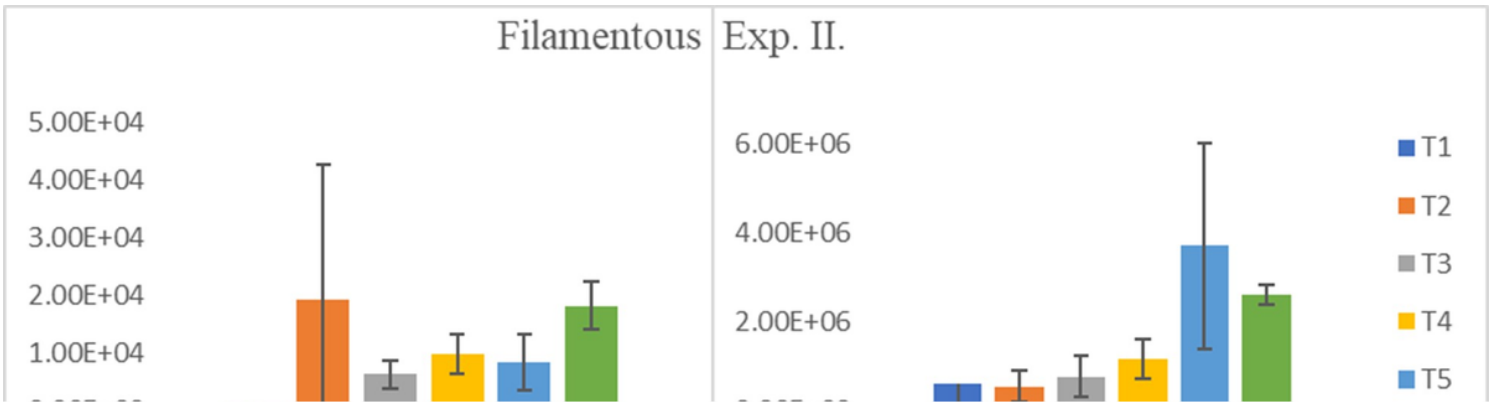

Figure 6

Quantification of the group of filamentous bacteria per mL (means ± standard deviation) at the beginning and the end of Experiment II. Note differences in scales between the beginning and the end of the experiment

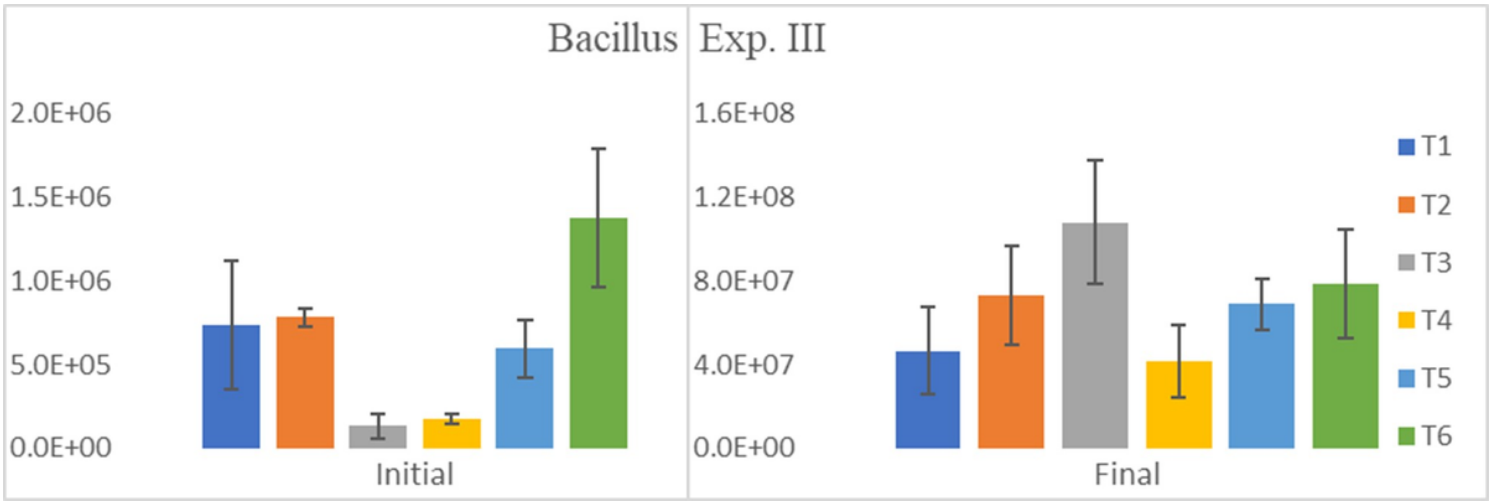

Figure 7

Quantification of the *Bacillus* bacteria group per mL (means ± standard deviation) at the start and end time of Experiment III. Note differences in scales between the beginning and end of the experiment

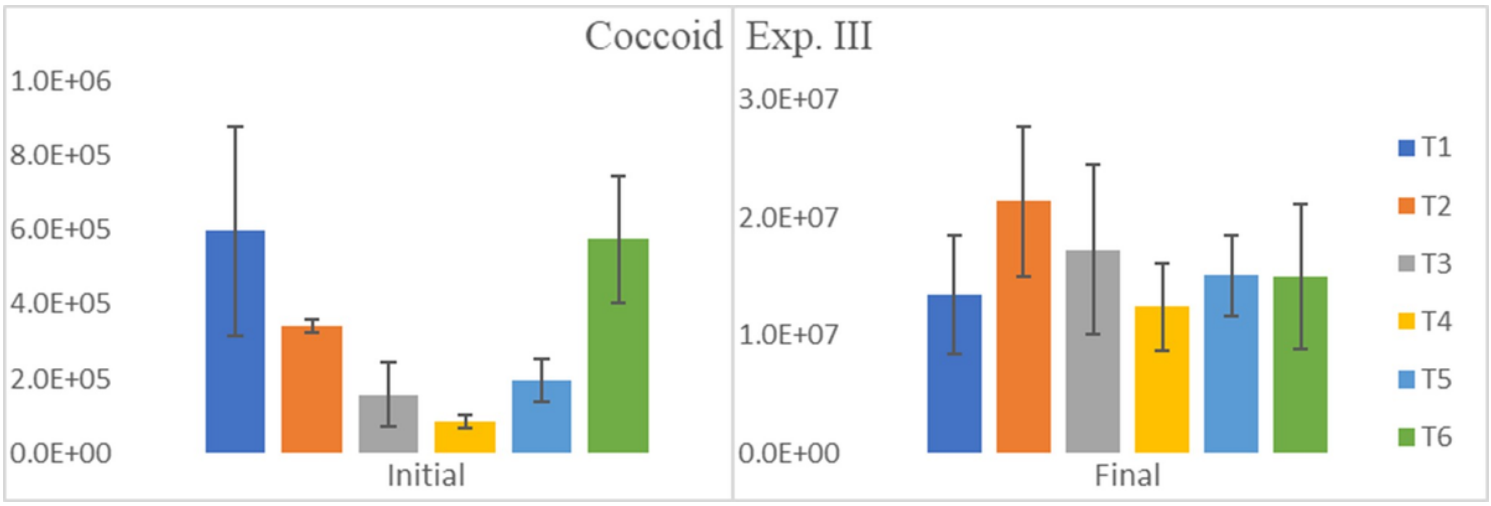

Figure 8

Quantification of the group of coccoid bacteria per mL (means ± standard deviation) at the beginning and the end of Experiment III. Note differences in scales between the beginning and the end of the experiment

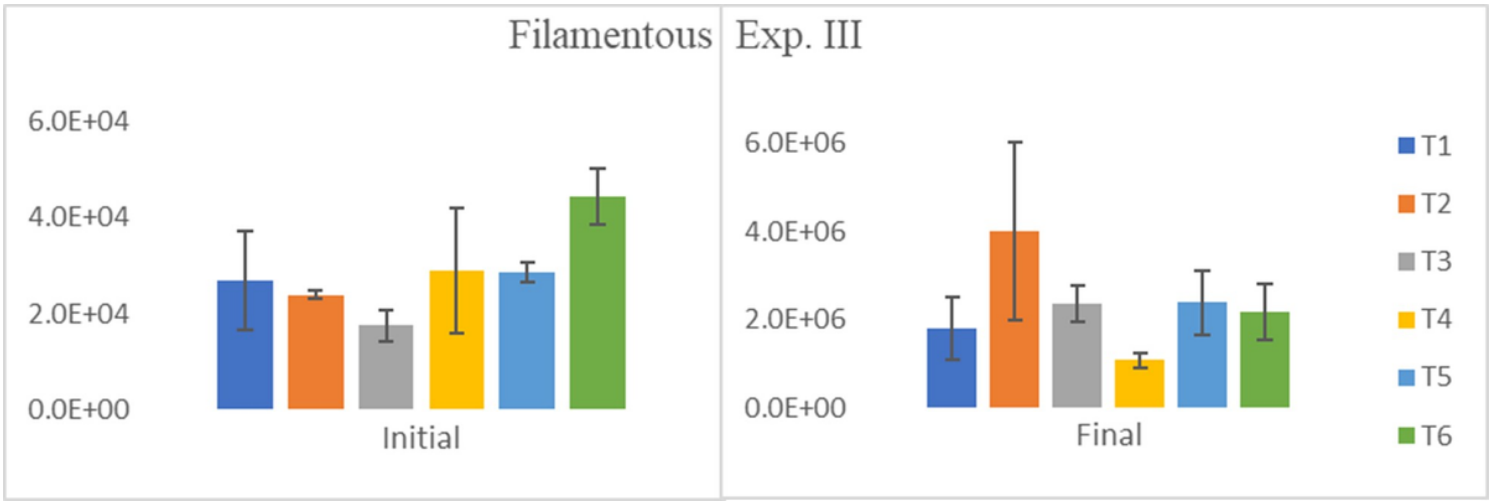

Figure 9

Quantification of the group of filamentous bacteria per mL (means ± standard deviation) at the beginning and the end of Experiment III. Note differences in the scales between the beginning and the end of the experiment