UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Possible Immunostimulant Effect of Fibers to Treat Vibriosis in Shrimp

Trabajo de integración curricular presentado como requisito para la obtención del título de Ingeniería Biomédica

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Urcuquí, Agosto 2019

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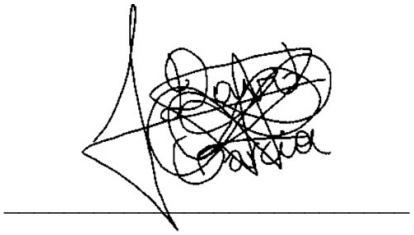

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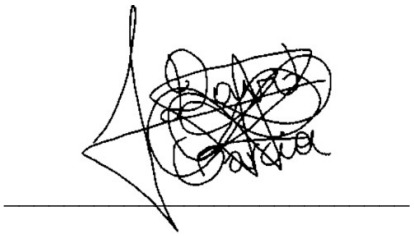

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DEDICATION.

To my parents and my brother, who have been a fundamental part throughout my university career, always supporting me and giving me all their love, pushing me to keep going, get up if I fall and teaching me to be a better person.

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TOPIC: "Possible immunostimulant effect of fibers to treat vibriosis in shrimp"

ABSTRACT

The shrimp industry in Ecuador have increased in the last years to the point of becoming the second export product in Ecuador. The Shrimp´s exportation generated 3 million of dollars approximately in 2017. However, the appearance of diseases that attack shrimp has been an obstacle for the shrimp industry. The immune system of shrimp is different from that of human because they do not have an immune system of memory, this means that vaccines to treat the diseases do not work and antimicrobial drugs remain a significant safety concern. It is estimated that when a disease attacks the shrimp, only 60-70% will survive. Prebiotics extracted from natural sources such as beta glucans in the form of fibers emerges as a possible solution to strengthen the immune system of the shrimp, thus preventing it from getting sick and generating large demands for shrimp farms. We hypothesized that this fibers can stimulate the immune system to prevent shrimp diseases like vibriosis. In this project we will focus on extracting the fibers from vegetal sources in Ecuador and we will characterize those using common techniques as XRD, SEM, and then we will prove the effect of fibers as immunostimulants for shrimp through in vivo and in vitro assays.

Keywords: Fibers, beta glucan, immunostimulant, prebiotics.

TÍTULO: "Posible effecto immunoestimulante de fibras para tratar vibriosis en camarones"

RESUMEN

La industria del camarón en Ecuador ha aumentado en los últimos años hasta el punto de convertirse en el segundo producto de exportación en Ecuador. La exportación del camarón generó 3 millones de dólares aproximadamente en 2017. Sin embargo, la aparición de enfermedades que atacan al camarón ha sido un obstáculo para la industria camaronera. El sistema inmune de los camarones es diferente al de los humanos porque no tienen un sistema inmune de memoria, esto significa que las vacunas para tratar las enfermedades no funcionan y los medicamentos antimicrobianos siguen siendo un problema de seguridad significativo. Se estima que cuando una enfermedad ataca a los camarones, el 60-70% muere. Los prebióticos extraídos de fuentes naturales como los betas glucanos en forma de fibras emergen como una posible solución para fortalecer el sistema inmunológico de los camarones, evitando así que se enferme y generando grandes demandas de granjas de camarones. Presumimos que estas fibras pueden estimular el sistema inmunitario para prevenir enfermedades del camarón como la vibriosis. En este proyecto nos enfocaremos en extraer las fibras de fuentes vegetales en Ecuador y las caracterizaremos usando técnicas comunes como XRD, SEM, y luego probaremos el efecto de las fibras como inmunoestimulantes para camarones a través de ensayos in vivo e in vitro.

Palabras claves: Fibras, beta glucano, immunoestimulante, prebióticos.

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PROBLEM STATEMENT.

The economy of Ecuador is mainly maintained by the petroleum, the agriculture production and aquaculture production. Of all the examples mentioned before, the aquaculture is one of the most important activities in Ecuador. This activity is mainly maintained by the shrimp production due to about the 95% of aquaculture activity is related with shrimp industry. This industry has gained relevance due to the social impact that produces and the earnings generated by exports. Currently, in Ecuador there are about 210.000 hectares dedicated to shrimp culture, being Santa Elena the province that is more dedicated to this activity (specifically to larvae production), followed by El Oro, Esmeraldas, Manabi and Guayas. In the country exists 506 companies that develop activities related with Exploitation of Shrimp Farms (shrimp farms) and Shrimp larvae hatcheries (Shrimp Larval Laboratories). These companies generates, directly or indirectly, about 180000 jobs (Espinoza, Figueroa, Laínez, & Malavé, 2017). The fact that the companies and the area destined to shrimp production increase in number due to the profitability have turned shrimp into the second product that generates the most profit from exports, only behind oil. Over the time, the money generated by exports as well as the exported pounds have increased. According to the National Chamber of Aquaculture, in 2016 the exported kilograms was around 800.000, generating about 2.5 billions of dollars. For the year 2017, the kilograms was approximately 940.000 with an earnings of more than 2.86 billions of dollars (National Chamber of Aquaculture, 2018). If the trend continues, it is expected that these parameters will continue to increase in the following years.

All the data exposed previously highlight the importance and impact of the shrimp industry in Ecuador. Although this industry is very profitable, one of the biggest challenges facing this industry is the appearance, spread and control of diseases, such as vibriosis, that attack shrimp and generate losses, both economic and production. Vibriosis provoke that the global shrimp production falls 19% annually, generating losses about 1 billion of dollars (Giudicelli, 2017). The methods to eradicate vibriosis consist of in the employment of antibiotics but its indiscriminate use have generated a drug resistance. In a study performed in Ecuador in 2017, the researchers found that 92,5 % of the Vibrio strains are resistance to ampicillin and 53,1% are resistance to tetracycline (Sperling, Alter, & Huehn, 2015). More important is the fact the utilization of antibiotics have a direct impact in the quality and the flavor of the shrimp which affect its

commercialization to USA and Europe where the controls for the shrimp products are very rigorous due to for the customers the sanitary quality is very important. Probiotics are very good solutions but its limitations, among which are the reduced efficacy by a single probiotics and the little adaptation of the strains to the marine environment, have caused the need to find a solution that is efficient, that is easy to manufacture and that adapts to the needs.

OBJECTIVES

OVERALL:

Extracting and characterizing fibers extracted from different species of plants, vegetal and fruits to immunostimulate the shrimp *Penaeus vannamei* immune system.

SPECIFICS:

- Extracting fibers from different species of vegetables, plants and fruits.
- Characterizing the fibers, using different techniques of characterization, to obtain its properties (morphology, topology, composition, crystallinity)
- Proving the immunostimulant effect of the fibers in vitro and in vivo tests.

CHAPTER I

INTRODUCTION

Around the world, shrimp industry is one of the most relevant aquaculture activities due to its high sustainability and profit-earning capacity, which have a direct impact in the economic and social aspects of the main producers shrimps countries, which are China, Thailand, Indonesia, India, Vietnam, Brazil, Ecuador and Bangladesh. These countries represents the about the 55% of the global shrimp production (World Wildlife Fund, 2008). Ecuador have seen how the shrimp production have increased in the last 10 years, remaining as top 3 exportation products in Ecuador. Furthermore, shrimp industry became in the main non petroleum exportation income of Ecuador (National Chamber of Aquaculture, 2018). However, one of the principal drawbacks that all the shrimp producers face is the appearance and propagation of diseases that affect the shrimps and generate large monetary losses. This diseases can be produced by parasites, fungi, virus and bacteria (Lio Po & Leaño, 2016). In this context, vibriosis is one of the most common and prevalent disease that can spread rapidly in the culture and it could decrease about the 60% of the total shrimp production. This is caused by bacteria of the Vibrio genus and it can affect both species of cultured penaeid shrimps: *P.monodon* and *P.vannamei,* being the latter the most cultivated species in Ecuador. Currently, more than 14 different species of Vibrio bacteria represent a risk for the shrimp. Among the symptoms that the disease can cause are lethargy, body malformation, slow growth, and tissue and appendage necrosis (Novriadi, 2016).

To destroy pathogenic vibrio is very common to employ antibiotics but its frequent and prolonged use have provoked a drug resistance, which means that the efficacy of the antibiotics to combat the disease have decreased. Other problem that entails the use of antibiotics is the accumulation of residues in the tissue of the shrimps that is dangerous for the human health (Rueda Escobar, 2018). Also, due to the above, the shrimp exportation to other countries is negatively affected, especially to European countries (European Comission, n.d.). European Union is very strict with the requirements for the shrimp exportation. These requirements are related with the presence of waste in the shrimp due to the use of antibiotics. Even more, the shrimp need to have a sustainability certified that ensure that the shrimps are 100% organic, which means that it is free for antibiotics (CBI, 2018). The recent efforts have focused on look alternative solutions to avoid the use of antibiotics. The employment of probiotics and prebiotics to improve the

immune system have been reported in the literature, obtaining very good results. In previous studies carried out in fishes, specifically in tropical gar juveniles, the addition of some prebiotics, which are compounds that that stimulates the immune system, as Mannan Oligosaccharides in the balanced diet showed an improvement in the immune system, the size and the weight of the fishes besides avoiding the appearance of bacterial diseases (Nájera-Arzola et al., 2018). Probiotics, such as *Lactobacillus sp.* and *Bacillus sp.,* have been widely used due to its ability to improve the immune response, increase the extracellular enzyme production, enhance the reproduction and combat pathogens in farming fishes. Also, the probiotics can maintain the quality of the water in which the fishes are raised (Banerjee & Ray, 2017). These studies have served as a base to understand the modulation, response and behavior of the immune system in marine species but there is many important differences between the immunity system of fishes and shrimps. Shrimp have only an innate immunity that is mediated only by one type of immune cells, called hemocytes. Hemocytes are classified, due the number and size of the granules present in the cytoplasm, into granular, semi granular and hyaline. These cells are responsible of carrying out all the immunological process in shrimp that includes non-specific humoral response and non-specific cellular response. The cellular response involve the process of fagocytosis, node formation and encapsulation (Jiravanichpaisal, Lee, & Söderhäll, 2006). Hyaline hemocytes and, in little proportion, semi granular hemocytes are mainly responsible for these cellular response (Giulianini, Bierti, Lorenzon, Battistella, & Ferrero, 2007). The humoral response involve process like the formation of cytokines (Aguirre Guzman, Sanchez Martinez, Campa Cordova, Luna-González, & Asencio, 2011), the creation of antimicrobial peptides, and the generation of phenoloxidase and O_2 radical (Holmblad & Söderhäll, 1999). The granular and semi granular hemocytes carry out these process (Johansson & Soderhall, 1989). Especially important are the phenol activity and the generation of super oxide anion O_2 to destroy the pathogens. In contrast, fishes like other vertebrates possess both innate and acquired immunity. The specific humoral response is mainly mediated by immunoglobulins and the specific cellular response involved lymphoid B and T cells (Rubio-Godoy, 2010). This is one of the main reasons that why vaccines do not work in shrimps. Because of these differences, appear the necessity of carrying out studies in shrimps to evaluate the effect of probiotics, complex carbohydrates such prebiotics, nutritional factors as vitamins, and other molecules from animal source as chitin/chitosan or from vegetal source (Apines-Amar & Amar, 2015) that serve as inmunostimulants in the improvement

of the immune system and the control of Vibriosis in shrimps. Probiotics have been widely used in shrimp culture to enhance the immune response of this crustacean. The first definition of probiotics was an organism or substances that contribute to intestinal microbial balance (Parker, 1974). Nowadays, according to the Food and Agriculture organization and the World Health Organization, probiotics are live microorganisms which, when administered in an appropriate amount provide health benefits to the host (FAO / WHO, 2001). In a study carried out in 2013 by Gonzalez *et.al,* the effect of a microbial mixture composed of lactic acid bacteria, which is a probiotic microorganism, and yeast was evaluated. The mixture was added to the feed in juvenile of *L.vannamei.* The results showed a survival of 100% of the individuals due to the increase of the hemocytes in the hemolymph of this specie (Luna-González et al., 2013). Also, Sotomayor *et.al* found that the utilization of the probiotic organism *V. alginolyticus* inhibit the growth of pathogens of the *Vibrio* genus in shrimp culture (Sotomayor & Balcázar, 2003). Probiotics demonstrate to work well nut its instability and its poor adaptation to marine environment is still a problem to its application. As a solution, prebiotics have been attracting attention in the recent years as immunostimulants for shrimp. Prebiotics are carbohydrates which can be classified according to molecular size or degree of polymerization. Examples of prebiotics can be fructo-oligosaccharides, mannanoligosaccharide, inulin and Beta glucans (Akhter, Wu, Memon, & Mohsin, 2015). Probiotics are used as immunostimulants due to its capability to interact with many patterns recognition receptors (PRRs) found in immune cells. Even more, prebiotics can interact with receptors in pathogens, called microbial associated molecular patterns (MAMPs), like peptidoglycan triggering an immune response (Bron, Van Baarlen, & Kleerebezem, 2012). Beta glucan are polysaccharides (biopolymer) of high molecular weight naturally present in the cell wall of various living organism such as bacterias, yeasts, fungus and plants (Pizarro, Ronco, & Gotteland, 2014). In plants, beta glucans are present in the form of 1-4 beta glucans and 1-3 beta glucans. This beta glucan can interact with a specific plasma protein found in the hemolymph in shrimp called beta glucan binding protein (BGBP). Then, this plasma protein join to the surface of the hemocyte and triggering an immune response (Vargas-Albores & Yepiz-Plascencia, 2000). Grains and cereals contain a lot of quantity of beta glucans in its cell wall. The major source of beta glucans in the nature are oat, bran, wheat and barley (Ahmad, Anjum, Zahoor, Nawaz, & Dilshad, 2012). In general, barley has an amount of beta glucan of 41,6 ± 0,6 g/kg, oat has a content of 34,9 ± 0,8 g/kg, and wheat has a quantity of 4,8 ± 0,2 g/kg

(Havrlentová & Kraic, 2006). Fibers present in plants, especially in cereal grains, are an abundant source of beta glucans. These beta glucans can be used in shrimp culture to improve its immune system. In this study we evaluated the utilization of the vegetal fibers, a very common prebiotic, as a source of beta glucans as an immunostimulant. We use several fibers both extracted from different natural sources and commercially available in order to test the immunostimulant effect. The results shows that are a close relationship between the immunological activity of the fibers and the survival of the shrimps with the physic-chemical properties of the fibers.

CHAPTER II

METODOLOGY

Physic-Chemical characterization of the fibers.

The characterization of the fibers was performed in ESPE University, located in Sangolqui, Ecuador. The SEM analysis was carried out using a MIRA 3 (TESCAN, CZ) field emission scanning microscope (FEG-SEM) in order to know the morphology and the surface structure of the fibers. FTIR analysis were carried out in a Spectrum Spotlight 200 FT-IR instrument (Perkin Elmer, USA). A gold-plated sample was used as a background, and then the spectra of the samples were recorded. The wavelength range for the analysis was between 4000 to 500 cm^{-1} with a total number of scans of 36 and a 4 cm^{-1} wavelength resolution. For the XRD analysis were used a EMPYREAN diffractometer (PANalytical, NL) in a Bragg-Brentano configuration at 40kV and 45A and monochromatic X Rays of Cu K-α wavelength (λ = 1.541 Å) using a Ni filter to obtain the XRD patterns. The crystallinity indexes (CrI) for the extracted fibers were calculated following the method described by Segal *et.al*, employing the next equation:

$$CrI(\%) = \frac{I_{002} - I_{am}}{I_{002}} x100\%$$

where I_{002} is the maximum intensity of the 002 lattice diffraction peak and I_{am} is the intensity exhibited by the amorphous part of the fibers samples (Segal, Creely, Martin, & Conrad, 1959). Samples of fibers previously dried by a lyophilization process were mounted on a zero-background substrate. Scans were obtained from 5 to 90 degrees in 0,01 degree steps, 10 seconds per step, 16 spinner revolution time by minute, repeated eight times. For the calculation of CrI, it was used height ratio of the (200) peak and the height of the minimum between the 200 and 110 peaks (Tyagi, Zhang, Shan, Yan, & Surampalli, 2009).

Evaluation of immunostimulant effect of fibers by in vivo and in vitro assays.

In vitro and in vivo assays was carried out in CENAIM, located in Santa Elena province. It is important to note this analysis are part of another complementary work to this. In a first step, a screening was carried out in vitro in order to evaluate the immunostimulant effect of fibers. For this purpose, we use two well-stablished test to measure the immune activity in shrimps. On the one hand, we use the superoxide anion (O_2^-) test in primary cultures of shrimp hemocytes. On the other hand, we employ the phenoloxidase (PO) test

in shrimp hemocyte extract. For both test, we use fibers at four doses 10, 1, 0.5 and 0.1 mg/ml. This first screening allow us to identify 6 fibers with immunostimulant activity. In a second screening, the fibers was tested at 2 doses 1 and 0.5 mg/ml in three different assays in order to verify the immunostimulant effect of the selected fibers. The fibers was exposure 2 hours in shrimp hemocytes cultures. Commercial beta glucan and phorbol 12-myristate 13-acetate (PMA) was used as positive control group and hemocytes without treatment as negative control group for superoxide anion test. Commercial beta glucano and Laminina was used as positive control group and hemocytes as negative control group in the phenoloxidase test.

The two fibers with the best results (F4 and C101) were selected for in vivo test in P. vannamei shrimp. The bioassay was carried out in 225 shrimp with a weight of 1.93 ± 0.66g that were distributed in 15 aquariums. The aquariums contained 40 L of filtered and UV sterilized sea water (15 shrimp juveniles per experimental unit). Continuous aeration was provided, and the temperature was set at 31.2 ± 0.6 C^o. The fibers was mixed with balanced meal in a proportion of 100mg/Kg of balanced meal. The shrimp were fed with this mix by 49 days, only from Monday to Friday, to avoid the possible negative effect of the immunostimulant effect of the fibers in the growth. The amount of food was the 5% of the biomass. Each treatment had 5 replicates. At the end of the test, 15 shrimp by treatment were collected to analyze and evaluate the immune activity by superoxide anion test and phenoloxidase test.

Measurement of super oxide anion.

Super oxide anion was measured by the method described by Munoz (Munoz & Alfaro, 1999), which consist in the reduction of nitro blue tetrazolium (NBT). It was used a hemocyte concentration of $2x10^5$ h/well in a 96-well microtiter plate. To activate the primary culture of shrimp hemocytes, it was treated with Hank's salts for 75 min and then it was exposed to the six fibers for 2 hours and incubated for 2 hours with 0.3 mg/ml of NBT. The results were expressed in rates, dividing the optical density values (OD) of the samples stimulates by the sample without stimulation. The results were interpreted as follow: rate values smaller than 1 means a lack of immune activity, rate values between 1 and 1.5 means low immune activity and rate values between 1.5 and 2 means a good immune activity.

Measurement of phenoloxidase test.

Phenolxidase test was carried out according to the method described by Leonard (Leonard, Söderhäll, & Ratcliffe, 1985). A hemocyte pellet was obtained and then it was resuspended in a Cacodylate buffer solution with a concentration of 10^6 h/ml. The solution was centrifuged by 3 minutes in order to obtain the supernatant to use as a sample. We placed 50 µL of each simple in a 96-well microplate by triplicate using Cacodylate buffer and Laminarin as elicitor. The microplate with the sample was incubated by 70 minutes. After that, phenoloxidase activity was measured through a spectrophotometer using L-3,4 dihydroxyphenylalanine as the substrate. The values smaller than 200 means a low immune activity, the values between 200 and 350 means a normal immune activity and the values in the range of 350 to 500 indicate a high immune activity.

Statistical analysis.

Statistical analysis were carried out in order to know the veracity of the results from the in vitro and in vivo tests. It was used two statistical tests: one-way ANOVA test and Dunnett post hoc test. One-way ANOVA test was performed to reject or accept the null hypothesis that is H_0: $\mu_1 = \mu_2 = \mu_3 = \mu_k$ where µ are the means of the groups. An alpha value of 0,05 was used for this tests. A p-value (probability) is calculated by the ANOVA test. If p-value is lower than alpha value, the null hypothesis is rejected meaning that means of at least one group is different. In contrast, if p-value is higher than alpha value, the null hypothesis is accepted meaning that the means of all the groups are equal (Kent State University Libraries, 2019). Assumptions of equal variances was taken. In order to do a multiple comparison between the group treatments and the control group, a Dunnett's test was performed. For this test, a value for a t critical ($t_{Dunnett}$) is founded looking the Dunnett-critical value table that depends of the alpha value (0,05), the sample size and the degrees of freedom form the ANOVA "within groups" output for each analysis. Then, it applied the following formula:

$$D_{Dunnett} = t_{Dunnett}\sqrt{\frac{2MS_{S/A}}{n}}$$

where $D_{Dunnett}$ is the critical distance between means, $t_{Dunnett}$ is the critical value of t, MS is the mean square of the "within group" in the ANOVA test and n is the sample size.

After we calculate the $D_{Dunnett,}$ it is necessary to find the value of the difference between means by each treatment by the next formula:

$$\boldsymbol{Difference\ between\ means} = |\boldsymbol{Mean_{control}} - \boldsymbol{Mean_{treatment}}|$$

If this value is equal of higher than the $D_{Dunnett}$, the treatment group is statistically different from the control group. This test is carried out comparing individually each treatment group with the control group (Dunnett, 1955).

CHAPTER III

RESULTS AND DISCUSSION

Characterization results of natural fibers.

Fibers extracted from natural sources, named as F1, F4, and F6 and commercial fibers, named as C20, C50 and C101 were characterized in order to know its physic-chemical properties (composition, morphology, topology and crystallinity) and the relationship between these and the immune activity. The methods used to comply with this were Fourier-transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and X-ray diffraction (XRD).

XRD was used to know the crystallinity properties of the fibers. The six samples (Fig.1) present similar diffractograms, in which stand out two prominent and characteristics peaks. In the literature we found that the first peak around 18 degrees at 2θ° represent the amorphous region while the second peak around 22-24 degrees at 2θ° belong to the (200) crystallographic plane and correspond to the fibers crystalline (Park, Baker, Himmel, Parilla, & Johnson, 2010; Terinte, Ibbett, & Schuster, 2011).

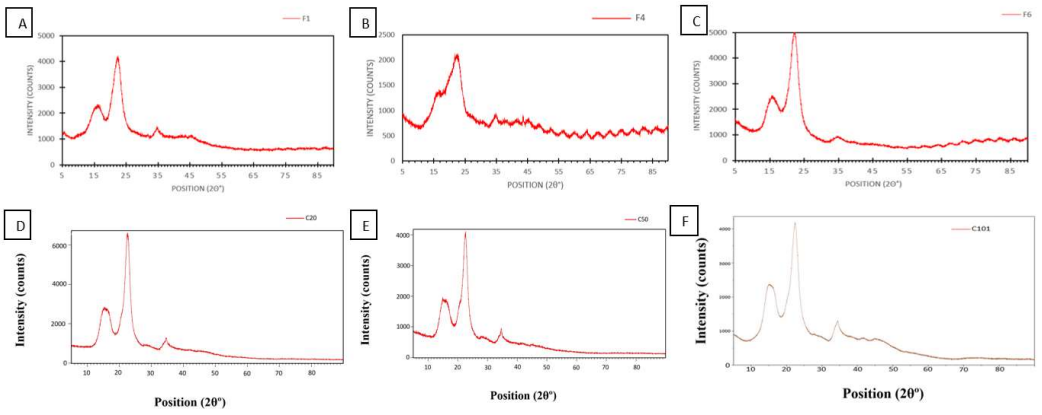

Figure 1. XRD analysis of the six samples of fibers A) F1 fiber, B) F4 fiber, C) F6 fiber, D) C20 fiber, E) C50 fiber and F) C101 fiber.

From the XRD analysis we obtain the crystallinity indexes for each of the fibers. The values are presented in the next table.

Table 1. Crystallinity indexes of the six samples of fibers.

Fiber Sample	Crystallinity Index
F1	57,8%
F4	34,6%
F6	44,5%
C20	77,7%
C50	73,4%
C101	78,1%

The degree of crystallinity of the natural fibers are lower than the degree of crystallinity of the commercial fiber, which suggest that the natural fibers are more amorphous than the commercial ones (Table 1). Fibers, as all the prebiotics, need to pass through the intestinal tract without being degraded in order to eject its immunostimulant properties (Roberfroid, 2018). The high crystallinity of fiber is a critical parameter that protect the fiber of the enzymatic degradation (Poletto, Pistor, Santana, & Zattera, 2012).

FTIR analysis was carried out in order to verify the composition of the fibers (Fig.2). As we can see in the figure 2, there is a strong broad band around 3400 cm^{-1} due to the presence of different O-H stretching modes. Furthermore, it can observed two bands around 2920 and 2850 cm^{-1}which is assigned to asymmetric and symmetric methyl and methylene stretching groups. These two bands are more visible in the spectra of F1 fiber (Fig. 2A) and F6 fiber (Fig. 2C). In the fingerprint region we can see some bands at ~1595, ~1510 and ~1270 cm^{-1}. These bands are present due to C=C, C-O stretching or bending vibrations of lignin and its different groups. C20 (Fig. 2D) and C50 (Fig. 2E) exhibit better these bands. The bands around 1425, 1335, 1220 and 1110 cm^{-1} are assigned to C-H, C-O deformation, bending or stretching vibrations of many groups presented in lignin and carbohydrates. Finally, there is bands around 1060 and 1030 cm^{-1} that can be due to C=O, C-H, C-O-C and C-O deformation or stretching vibrations present in carbohydrates (Poletto, Ornaghi Júnior, & Zattera, 2014). These results show that the composition of the natural fibers and the commercial fibers are very similar.

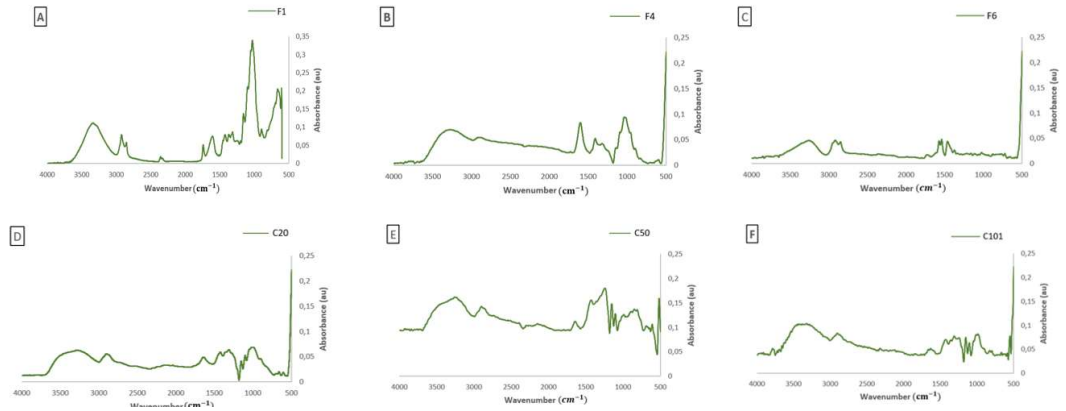

Figure 2. FTIR spectra of the six samples of fibers A) F1 fiber, B) F4 fiber, C) F6 fiber, D) C20 fiber, E) C50 fiber and F) C101 fiber.

SEM images give us a description of the morphology and topology of the samples (Fig.3). F1 fiber (Fig. 3A), C20 fiber (Fig. 3D), C50 fiber (Fig. 3E) and C101 fiber (Fig. 3F) show a tube-like structures. But F1 fiber (Fig. 3A), F4 fiber (Fig. 3B) and F6 fiber (Fig. 3C) exhibit a roughness and porous surface. These structures are not present in the commercial fibers, which present a smooth surface.

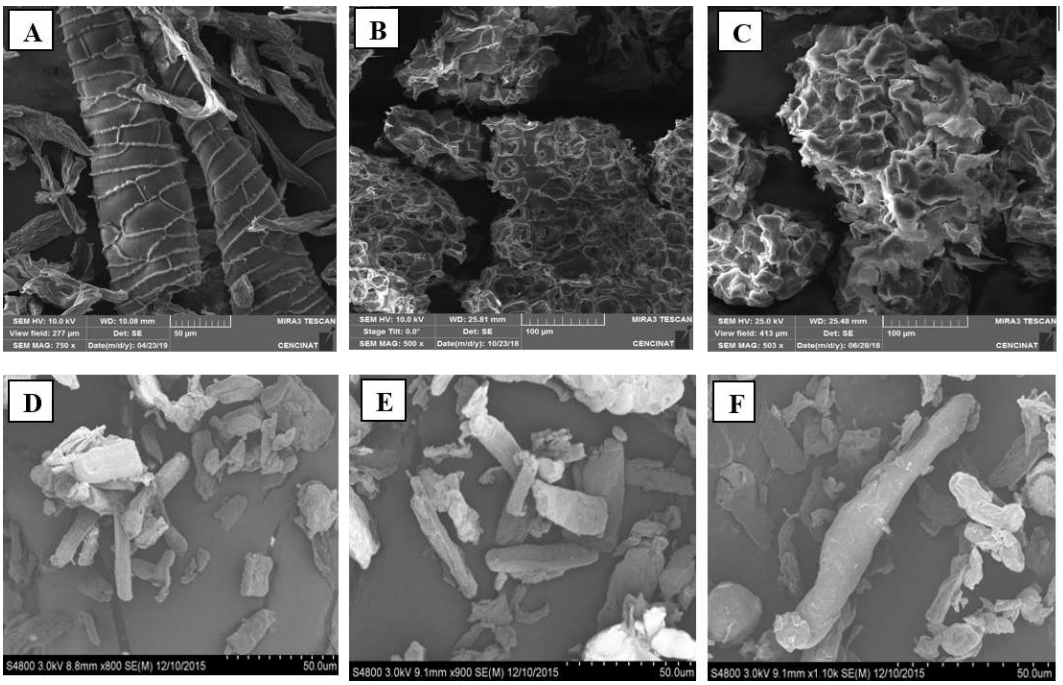

Figure 3. SEM images from the six fibers A) F1 fiber, B) F4 fiber, C) F6 fiber, D) C20 fiber, E) C50 fiber and F) C101 fiber.

The characterization results showed that the fibers extracted from natural way have the same composition as the commercial fibers, but they differ in the degree of crystallinity and in the structure. These characteristics bring to the natural fibers an opportunity of producing a biological activity.

Immunological results for in vitro test.

In vitro tests was performed in the six fibers extracted from natural sources in order to obtain a previous overview of the immunostimulant effect of the fibers samples in the shrimps, through the modulation of the hemocytes.

The immune activity was measured by the NBT test and by the phenoloxidase test. The fibers were evaluated in two different concentrations (1 mg/ml and 0,5 mg/ml). The NBT test showed that F4, F6, C20, C50 and C101 improved the immune activity at a concentration of 1 mg/ml since these showed a rate between 1,5 and 2, standing out among these F4 and C101. F4 obtained a rate of 3,13±0,31 at a concentration of 1 mg/ml and C101 obtained a rate of 1,89±0,21 at a concentration of 1 mg/ml. In contrast, only F4 and C101 showed to have positive effect in the immune activity at a concentration of 0,5 mg/ml. F4 obtained a value of 1,84±0,14 and C101 had a value of 1,79±0,26 (Table 2).

Table 2. NBT rates using two different concentrations of the six fibers resulted from the in vitro test.

	F1	**F4**	**F6**	**C20**	**C50**	**C101**	**β Glucan**	**PMA**
1 mg/ml	1,16±0,20	3,13±0,31	1,66±0,23	1,50±0,12	1,69±0,26	1,89±0,21	1,89±0,27	1,97±0,12
0,5 mg/ml	1,02±0,08	1,84±0,14	1,33±0,08	1,23±0,12	1,53±0,35	1,79±0,26	1,49±0,16	

There is a significant difference in all the treatments at both concentrations, according to $p<0,05$ given by one-way ANOVA test. In the same way, the Dunnett's test revealed F1, F4, C20 and C101 are statistically different from the control groups PMA and Beta glucan at both concentrations.

In the same way, the phenoloxidase test served to quantify the immune activity given by the presence of the fibers. In this sense, all the fibers showed to have an excellent ability to improve the immune activity of the hemocytes at two concentrations (1mg/ml and 0,5

mg/ml). C101 had a value of 500±14,95, which is the highest value for the phenol activity for a concentration of 1 mg/ml. C50 with a value of 496±19,30, C20 with a value of 469±25,5 and F4 with a value of 433±41,85 exhibit very good ability to improve the immune activity. For a concentration of 0,5 mg/ml, the fiber with the best result is C50. This fiber has a value of phenol activity of 455±15,83. Also, C20 with a value of 444±8,59, C101 with a value of 430±5,56 and F4 with a value of 405±11,08 showed very good results (Table 2)

Table 3. Phenol activity of the six fiber samples and control groups at two different concentrations.

	F1	**F4**	**F6**	**C20**	**C50**	**C101**	**β Glucan**	**Laminin**	**Base**
1 mg/ml	397± 8,54	433± 41,85	410±9,11	469±25,53	496± 19,30	500± 14,93	433±11,14	396± 12,25	304± 26,15
0,5 mg/ml	388± 13,11	405± 11,08	393±14,15	444±8,59	455± 15,83	430± 5,56	404±15,53		

All the treatments show to be significantly different to $p<0,05$ at both concentrations, based on the one-way ANOVA test. Dunnett's test confirmed F4, C20, C50 and C101 treatments are statistically different from negative control group, Beta Glucan control group and Laminin control group at both concentrations

Immunological results for bioassay.

In the same way, we evaluate the immune response of the shrimp in the presence of fibers through NBT test and phenoloxidasse test.

NBT test revealed that F4 and C101 effectively improved the immune activity of the hemocytes in the shrimp feed with these fibers in comparison with the shrimp that was not treated with the fibers, which are the control group. The rate NBT of F4 was 2,38±0,29 and the rate NBT of C101 was 2,14±0,23. The control group obtained a rate of 1,28±0,06 (Table 3).

Table 4. Results from the immunological tests performed in bioassay.

Treatments	NBT rates	Phenol activity	
		Base	Stimulated
F4	2,38±0,29	48,33±4,62	242,17±40,78
C101	2,14±0,23	47,56±4,91	216,83±16,26
Control	1,28±0,06	49,33±7,86	252,06±18,47

The results of the NBT test in all the treatments are significant different to $p<0,05$, based on one-way ANOVA test. Dunnett's test confirmed that F4 and C101 are statistically different from the control group.

The phenol activity was measured by the phenol oxidase test in shrimp feed with F4 and C101 fibers and control group (without treatment) at the beginning of the bioassay (base) and at the final of the same (stimulated). Shrimp treated with F4 and C101 exhibited a normal immune activity as well as the shrimp that did not received any treatment because the values of the phenol activity are in the range of 200 and 350 (Table 3).

The results obtained in this test are not significant different to the $p>0,05$ given by the one-way ANOVA test. Dunnett's test showed F4 and C101 treatments are not statistically different from the control group. These results suggest that F4 and C101 did not improve maintaining the immune activity of the shrimp.

CHAPTER IV

CONCLUSIONS.

- Natural fibers present similar composition as commercial fibers but different crystallinity, morphology and topology.
- According to in vitro and in vivo test, F4 demonstrated the best immunostimulant effect.
- F4 fiber and C101 fibers exhibited excellent immunostimulant properties.

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