



Development of a freeze-dried symbiotic obtained from rice bran

Andressa Neuhaus Ferronato*, Rochele Rossi, Laura Massochin Nunes Pinto, Juliano Garavaglia

NUTRIFOR Research Institute in Food and Health at UNISINOS University, Unisinos Avenue, 950 – São Leopoldo, Rio Grande do Sul, 93022-750, Brazil



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ABSTRACT

This study aimed to assess the growth potential of *Lacidophilus* and *L.plantarum* in rice bran, a co-product from the food industry, and subsequently develop a freeze-dried symbiotic. Furthermore, phytochemicals and antioxidant properties were analysed. The growth was measured using growth kinetics over 72 h. The total phenolic compounds were analysed by the Folin-Ciocalteu method and antioxidant potential by DPPH and ABS methods. Freeze-drying process occurred using a pilot-scale equipment (Liotop LP510), verification and quantification of probiotics occurred through molecular analyses, as DNA extraction and *q*PCR. As a result, there was a good growth in rice bran ($p = 0.04$), suggesting its prebiotic potential. Rice bran also showed significant concentrations of phenolic compounds ($3.69 \text{ mgEAG/mL} \pm 0.04$) and antioxidant activity according ABTS ($8.35 \mu\text{mol ET/mL} \pm 0.106$) and DPPH ($24.71 \mu\text{mol ET/mL} \pm 7.90$) methods. The bacteria concentration decreased significantly when submitted to the freeze-drying process ($p = 0.001$), however, they remained by the minimum concentration required for a product to be considered a symbiotic. Therefore, it was concluded that rice bran and these analysed bacteria proved to be effective for a symbiotic formulation.

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1. Introduction

Gut bacteria's importance, how it interacts with individuals' metabolism and its impact on systemic diseases have been the subject of many studies. Gut microbiota is composed of microorganisms that collectively reside in the intestine, influencing directly and systematically the human organism [1]. However, the gut microbiota is not only composed by beneficial bacteria, but there is a diversity of pathogens interacting in this same environment. It means that a balance between probiotics and pathogens microorganisms impacts positively or negatively in healthy or disease conditions.

World Health Organization (WHO) defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host". Furthermore, symbiotics are defined as a combination of probiotics and prebiotics (nondigestible dietary fibers which are used as substrates for gut bacteria fermentation) in the same product or capsule [2]. Symbiotic intakes have had a greater effect on systemic inflammation when compared to the use of isolated probiotics, both for its ability to increase the number of short-

chain fatty acid-producing bacteria, as for providing substrates for fermentation [3].

However, ensuring the bacteria's survival, both in industrial processes to develop a symbiotic product or even in its passage through the gastrointestinal tract, it is a big challenge [4]. Freeze-drying is a method used to stabilize probiotics in different food matrices. It's because the freeze-drying process can maintain the properties of the substances throughout the process and mainly provides protection for sensitive compounds, where there are nutritional and microbiological losses [5].

Rice is considered a staple food around the world, it is the second main cereal harvest being produced in at least 114 countries with a global production of 645 million tons. However, its industrial processing generates co-products as rice bran (5–8%) which is wasted. In developing countries, rice bran is a co-product allowed only for animal feed or it is discarded as wastage, without considering that its annual global production is around 29.3 million tons [6]. It also is an important co-product of rice processing which has gained great attention due to its composition rich in nutrients, dietary fibers, high antioxidant potential and its promising effects against different metabolic diseases. The bioactive compounds from rice bran, mainly gamma-oryzanol, have been studied for having antioxidant, anti-inflammatory, hypocholesterolemic, antidiabetic and anticancer properties [7].

Although, rice bran in food is not allowed due to the presence of anti-nutritional factors, such as lipases, trypsin inhibitor,

* Corresponding author. Present address: 60 Sunnyside Road, E10 7BB, London, United Kingdom

E-mail address: a.ferronato@qmul.ac.uk (A.N. Ferronato).

hemagglutinins, and phytates. However, the elimination of these undesirable components is viable and well understood in developed countries [8]. The stabilization techniques commonly used are thermal or chemical, such as retained moisture heating, extruded cooking, electric heating or hot air drying. This process is mainly aimed at inactivating lipases or other anti-nutritional factors such that its toxicity does not harm rice bran protein and makes it possible to intake it [6].

For a product to be classified as a symbiotic, it must contain at least between entre 108 a 109 CFU/mL probiotics in the daily portion recommended by the manufacturer [9]. Consequently, due to this mandatory proof, there's an increased interest in molecular techniques for the identification and quantification of microorganisms, as Quantitative Polymerase Chain Reaction (qPCR). This method has a high DNA and RNA detection rate, enabling viable cell quantification when it compares to traditional methods, being fast and efficient in microorganisms quantification. Moreover, qPCR provides a greater reliability of results, ensuring the minimum addition of microorganisms and its classification as a symbiotic product [10].

This study aimed to analyze the grown potential of *L. acidophilus* ATCC® 4356™ and *L. plantarum* ATCC® 8014™ strains in rice bran, developing a freeze-dried symbiotic which maintains the survival rate of these probiotics throughout the process, its cell viability being evaluated by qPCR method before and after lyophilization. Besides, phenolic compounds and antioxidant analyses were also performed to evaluate probiotic interactions with polyphenols contained in rice bran.

2. Materials and methods

2.1. Growth curve analysis

Nutrifor Research Institute developed a fiber-rich product containing rice bran in its formulation which was used in this study to assess whether there is a probiotic strain growth in rice bran. The formulation contained 10 % rice bran, 2 % *Saccharomyces cerevisiae* ATCC 9763, pH 2.0 and 0.5 % glucose which was called FAD. The product was sterilized in an autoclave (121°C, 15 min) for the breakdown of yeast cells and ensuring the growth of probiotic only. Growth kinetics were performed, being point 0 at the moment of inoculation and point 9 after 72 h, under different conditions: only *L. acidophilus*, only *L. plantarum* and a mix with both bacteria within FAD. As a growth control, MRS broth was used. Two washed were performed (centrifugation at 5000 rpm for 2 min, 4°C) and subsequently, reading the optical density in a spectrophotometer at 600 nm [11].

2.2. Total phenolic compounds

The Folin-Ciocalteu method was used as proposed by Singleton et al. [12].

For the standard quantification curve, a gallic acid monohydrate solution was used in five different concentrations: from 0.005 mg/mL to 0.08 mg/mL. For the experiment, 4 aliquots under different conditions were performed: FAD (without bacteria), FAD + *L. acidophilus* only, FAD + *L. plantarum* and the last one with both bacteria inside, FAD + *L. acidophilus* + *L. plantarum*.

2.3. Antioxidant activity

2.3.1. ABTS •+ Method+ method

The determination of antioxidant activity by the ABTS•+ method was carried out by reducing ABTS•+ cation, according to the method described by Re et al. [13], with some modifications. The aliquots used were in the same conditions as the total phenolic

compounds method. After reaction time, absorbances were read on a spectrophotometer (ExpectaMax M5/Molecular Devices, Sunnyvale, CA) in 734 nm. To calculate the antioxidant activity, a standard Trolox curve was constructed and y value in the line equation was replaced by the percentage of inhibition calculated from the absorbance found for each pattern. The antioxidant activity was expressed in μmol of Trolox Equivalent (TE) per mL of the sample ($\mu\text{mol TE/mL}$).

2.3.2. DPPH• Method method

The evaluation of antioxidant activity according to DPPH• consumption was carried out as the methodology described by Brand-Williams et al. [14], with some modifications. The aliquots used were in the same conditions as the previous method (ABTS). The absorbance was read overtime on a spectrophotometer at a wavelength of 517 nm. The standard Trolox curve was performed as in the ABTS method.

2.4. Freeze-drying

Probiotics cultures within FAD were submitted to the freeze-drying process. For this, it was frozen (-45°C for 48 h) and it was dehydrated in a freeze dryer, using pilotscale equipment (Liotop LP510, Liobras, São Paulo, Brazil), under vacuum (0.1 hPa) at a temperature of -30°C . After 50 h, the temperature increased to 0°C (vacuum 0.1 hPa) and this condition was maintained for 15 h. After that, the temperature increased to 10°C , always under vacuum (0.1 hPa).

2.5. DNA extraction and qPCR quantification

For DNA extraction, 3 mL of symbiotic was collected, in triplicate, used The PureLink® Genomic DNA Mini Kit - Invitrogen™ following the protocol provided by the manufacturer. After DNA extraction, the concentration of the samples was verified in SpectraMax Plus 384 Microplate Spectrophotometer equipment, obtaining a final concentration of $2\text{ ng}/\mu\text{L}$. DNA was extracted in three conditions: 0 h point (when probiotics were inoculated), 48 h point (after 48 h of growth) and Liof point (after the freeze-drying process). The DNA was stored at -10°C for further analysis in qPCR. The amplification and detection of each bacteria through qPCR was performed in 96- well microplates, using Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and performed on RT-PCR StepOnePlus system (Applied Biosystems). The samples were analyzed in triplicate and the final reaction volume was 20 μL , containing 0.16 μL of each primer, 10 μL Power SYBR Green Master Mix, 1.0 μL target DNA, completing the final volume with sterile MiliQ water. For each RT-PCR plate used, a positive control (target bacteria) and a control without bacteria DNA were inserted. The primers used to detect the symbiotic product strains were defined based on DNA sequences of the 16S region. For *L. acidophilus*: F - ACAACGATCGCGTAGAAGGT e R - CCAGCAACGACCTGAGTGAA, for *L. plantarum*: F - GGATGCCGCATTTCACCTG e R - TAGGTCGCCTGCTTGAGTTG.

All the experiments were carried out in triplicate.

2.6. Statistical analysis

All data were submitted to analysis of variance (ANOVA), thus evaluating the statically significant differences between the microorganisms quantification before and after the freeze-drying process. Tukey tests ($p < 0.05$) were performed to assess the difference between averages, using the SPSS program (Statistical Package for the Social Sciences) version 21.0.

3. Theory

3.1. Probiotics and gut microbiota

Probiotics are defined as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” (WHO). Probiotic microorganisms have been widely studied due to their performance in different parts of the human body, such as digestive, immunological and even respiratory functions [15]. Animal and human studies have shown that probiotic bacteria, which populate gut microbiota, are associated with body homeostasis, non-digestible carbohydrates fermentation, short-chain fatty acids, and some amino acids synthesis contributing to human physiological and metabolic balance [16].

The role of probiotics in health and disease conditions is increasingly in evidence, including one of its main activities: its role as a therapeutic potential in gastrointestinal disorders, such as Irritable Bowel Syndrome (IBS) and many others conditions [17]. The action mechanisms for the beneficial effects of probiotics are related to the competitive exclusion of pathogenic microorganisms, pathogen adhesion inhibition, antimicrobial substances production, and immune system modulation [18].

Gut microbiota can regulate the host's immune system and moreover, the immune system directly affects gut microbiota composition [19]. The microbiota homeostasis is maintained when the immune system establishes a balance between beneficial microorganisms (probiotics) and opportunistic (pathogenic) microorganisms. This equilibrium condition can only be achieved through communication between the immune system and gut microbiota, and the key to this communication process is a healthy intestinal barrier [20].

3.2. Symbiotic and rice bran

Rice bran is considered an important rice by-product which has gained great huge attention due to its composition rich in

nutrients, dietary fibers, high antioxidant potential and its promising effects against different metabolic diseases. The bioactive compounds from rice bran, mainly gamma-oryzanol, have been studied for having antioxidant, anti-inflammatory, hypocholesterolemic, antidiabetic and anticancer properties [7]. Nutraceutical compounds contained in rice bran, mainly polyphenols, make it a strong candidate to be used as a prebiotic [21].

Symbiotic is defined as a combination of probiotics and prebiotics in the same product or a capsule [2]. However, symbiotics are not only used to improve the survival of probiotics added to food but also to stimulate beneficial bacteria proliferation which already populate the gastrointestinal tract [22]. The first aspect to be taken into consideration when formulating a symbiotic is the appropriate choice of probiotic and prebiotic, ensuring the microorganism growth in that environment, as having a positive effect on the host's health when used separately. Besides, the growth stimulation of other non-probiotic microorganisms must be non-existent (or at least limited) in the prebiotic used [23].

3.3. Freeze-drying

Ensuring probiotic microorganisms survival over time and during digestive processes is a challenge. Thus, new technologies have been developed to maintain probiotics integrity. Freeze-drying is a well-known and frequently used method for food preservation and bioactive compounds protection against various physical-chemical factors [24].

In this method, the samples pass through an initial freeze, reducing the amount of water according to primary (sublimation) and secondary (desorption) drying, reaching values that prevent biological activities and chemical reactions. This process differs from product dehydration because its pressure and temperature conditions are controlled, that is, the previously frozen water

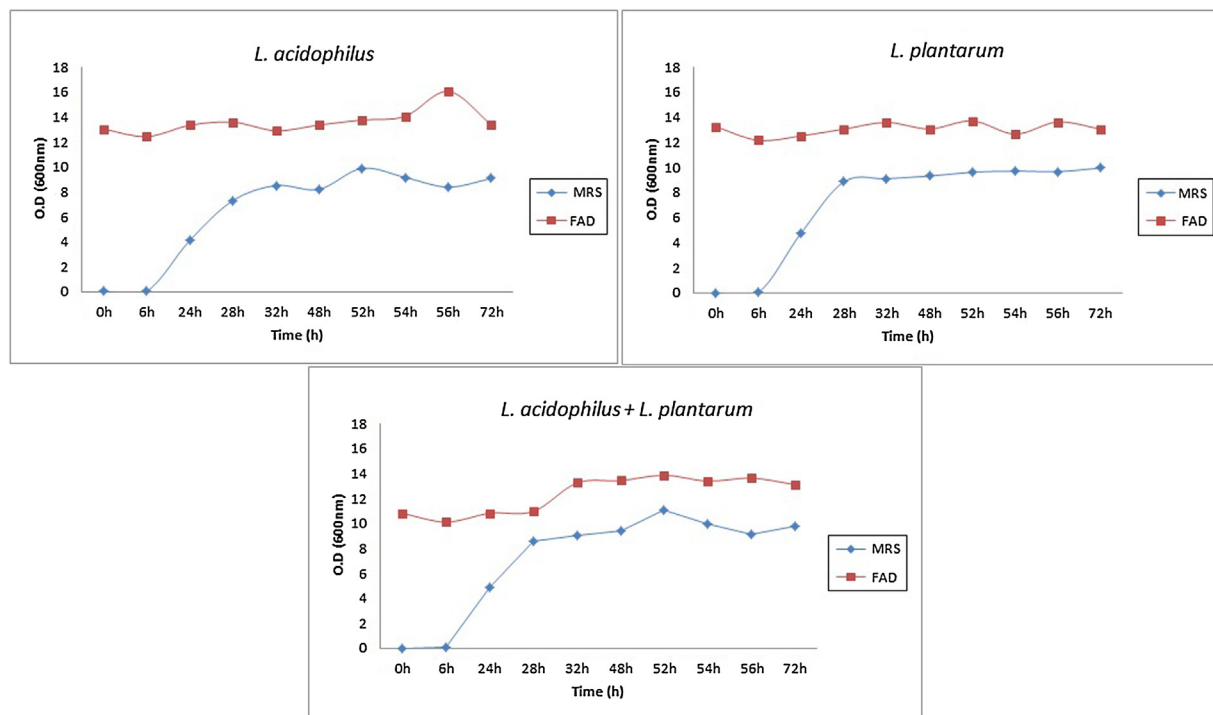


Fig. 1. Growth curve of *L. acidophilus* and *L. plantarum* in FAD and MRS broth over 72 h.

ANOVA test to *L. acidophilus* showed $p = 0.03$; *L. plantarum* showed $p = 0.01$ and *L. acidophilus* + *L. plantarum* showed $p = 0.004$, all indicating that there is a statistically significant difference between the samples

(solid-state) goes directly to the gaseous state, without going through the liquid state – a process known as sublimation [25].

4. Results and discussion

4.1. Growth in rice bran

Fig. 1 showed the growth graphics of *L.acidophilus* and *L.plantarum* within FAD and MRS (control) over 72 h. It was observed that *L.acidophilus* had a better growth in rice bran than in MRS broth ($p = 0.03$). The growth peak occurred at 52 h for MRS and 56 h for rice bran. However, for *L.plantarum* there was a growth peak at 28 h in MRS broth and 32 h in rice bran ($p = 0.01$), maintaining a steady growth over the next 72 h. When the two strains were inoculated together, the growth peak within FAD occurred at 32 h and 52 h for MRS broth. For statistical analysis (ANOVA test) was obtained $p < 0.05$ for three different conditions, indicating that there is a significant difference between the growth of strains in MRS and rice bran.

The results above suggested that rice bran showed a potential prebiotic for the strains analysed, and even better when both strains together. It can be related to the rice bran composition because, despite being considered a co-product of the food industry, it has a high nutritional value, being rich in lipids, proteins, dietary fibres and other food compounds, including minerals such as phosphorus, potassium, magnesium, calcium, manganese, and others [26]. Its main nutrient is dietary fibre, ranging from 20 to 50 %, suggesting a prebiotic potential for the growth of bacteria [7].

Wang et al. [27] showed that polysaccharides can improve the survival of *L. plantarum* during freeze-drying because it can reduce cellular damage and improve cell membrane integrity, which can widely be applied in the food industry. Dimitrellou et al. [28] tested the effects of solutions containing sugars, starch, nitrogenous and whey on the cell viability using freeze-drying when stored under refrigeration, suggesting this technology for the production of probiotic foods.

Saman et al. [29] evaluated the growth of *Lactobacillus plantarum* NCIMB 8826 and *Lactobacillus reuteri* NCIMB 8821 at different degrees of rice bran granulation. They observed that a 3.7 % degree of granule dismemberment resulted in better growth of probiotic strains, suggesting that there is a relation between the granule integrity and its prebiotic potential. Demirci et al. [30] evaluated the growth of *Lactobacillus casei* 431 in a rice bran enriched yogurt. As a result, a better probiotic growth was obtained when 3 % of rice bran was added to the formulation, maintaining the survival rate during storage for up to 21 days.

According to the studies mentioned above, this study chose the rice bran as a growth medium due to its composition contains polysaccharides, proteins and minerals. Moreover, different studies reported the rice bran as an effective alternative due to its prebiotic potential. No other fibre products were used because the aim of this study was to evaluate the growth of two specific bacteria (*L. acidophilus* and *L. plantarum*) in the rice bran, testing if it has a prebiotic potential for those strains selected.

4.2. Total phenolic compounds

Through the analysis of total phenolic compounds (Table 1), it was observed that the pure FAD has 3.69 mgGAE/mL of the product. When only *L.acidophilus* was inoculated, a concentration of 3.71 mgGAE/mL ($p = 0.9$) was obtained and when only *L.plantarum* was inoculated, there was an increase to 4.30 mgGAE/mL ($p = 0.04$). Interestingly, when the strains were inoculated together, there was a decrease in the concentration of total phenolic compounds to 3.97 mgGAE/mL ($p = 0.7$). Statistical tests assessed whether there

Table 1

DNA concentration ($\mu\text{L/g}$) of samples in three different conditions.

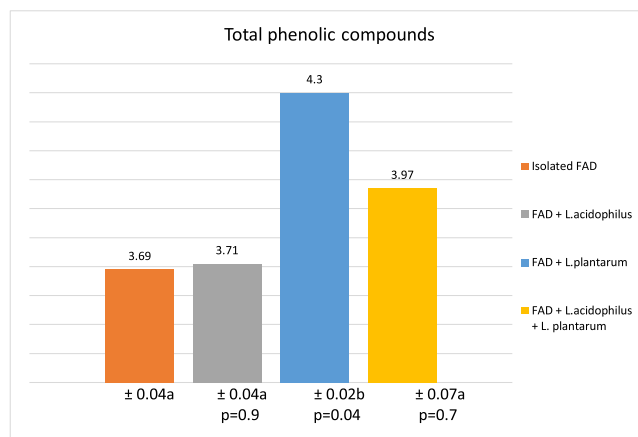
Samples	Conc. <i>L. acidophilus</i> ($\mu\text{L/g}$)	Conc. <i>L. Plantarum</i> ($\mu\text{L/g}$)
FAD 0 h	$1,17 \times 10^{11}$	$1,40 \times 10^9$
FAD 48 h	$1,75 \times 10^{12}$	$2,33 \times 10^9$
	$p = 0,03$	$p = 0,05$
FAD freeze-dried	$6,42 \times 10^{10}$	$4,21 \times 10^7$
	$p = 0,001$	$p = 0,001$

ANOVA test showed p value < 0.05 , appointing that there was a statistically significant difference between the samples.

are significant differences when the strains are inoculated compared to the isolate FAD. The results showed a significant difference only when *L.plantarum* was inoculated, with no differences between pure FAD, FAD only with *L.acidophilus* or FAD containing the two strains together (Graphic 1).

Biological activity and beneficial health effects attributed to polyphenols are mainly due to phenolic metabolites formed in the gastrointestinal tract by microbiota bacteria instead of their original forms present in food [31]. Mantzourani et al. [32] evaluated the total phenolic compounds concentration in a pomegranate juice before and after fermentation with *Lactobacillus plantarum*. The samples that passed through the fermentation process with *L.plantarum* showed higher concentrations of phenolic compounds, even after 4 weeks of storage. Pacheco-Ordaz et al. [33] analyzed the growth of two probiotic bacteria (*Lactobacillus rhamnosus* ATCC 53103 e *Lactobacillus acidophilus* NRRLB 4495) and two pathogenic bacteria (*Escherichia coli* 0157:H7 ATCC 43890 and *Salmonella enterica* serovar *Typhimurium* ATCC 14028) within five different phenolic compounds (catechin and gallic, vanillic, ferulic and protocatechuic acids) present in mango. The results obtained demonstrated that the phenolic compounds only selectively inhibited the pathogenic bacteria and furthermore, positively affected the growth of probiotic bacteria. These data indicate that phenolic compounds act differently and selectively in probiotic and pathogenic bacteria, causing different effects on human health.

Lactic acid bacteria increase the free phenolic acids during cereal fermentation, such as whole grain and oat flours, improving their bioavailability [44]. Randazzo et al. [45] developed six juices (apple, grape, kiwifruit, pomegranate, prickly pear and quince) fermented by probiotics and evaluated the phenolic compounds after fermentation. A general increase of organic compounds was observed, in particular grape, quince and pomegranate. Wu et al.



Graphic 1. Total Phenolic Compounds Concentration in the samples.

ANOVA test showed p value < 0.05 , appointing that there was a statistically significant difference between the samples. Averages followed by the same letter, in the column, do not differ at 5 % probability in the Tukey test.

[43] selected probiotic strains to ferment blueberry and blackberry juices, evaluating the contents of phenolic compounds afterwards. The study showed that the probiotics had remarkable influences on increasing the amount of phenolic acids, and the stronger capacity on metabolism of phenolics and organic acids occurred in samples fermented by *L. plantarum*.

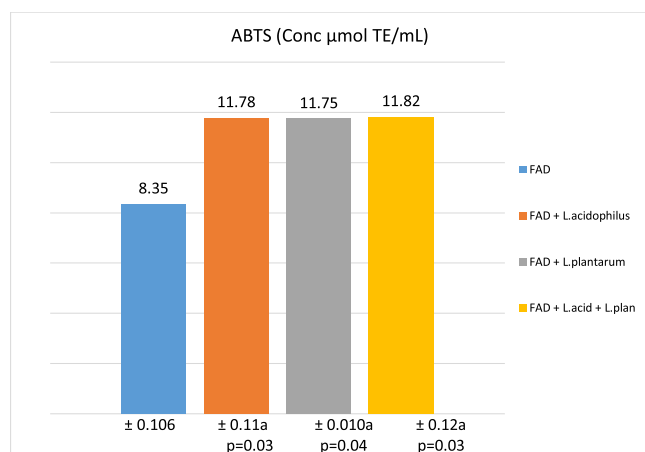
However, it is widely recognized that there is a reciprocal interaction between probiotics and polyphenols: the presence of phenolic compounds improves bacterial growth, as well as the action of bacteria on phenolic compounds, improves their degradation and absorption. Besides, polyphenols are shown to improve the adhesion ability and probiotics survival during exposure to gastrointestinal tract conditions. There is strong evidence in the literature that polyphenol metabolites formed by gut microbiota bacteria exercise a variety of benefits to the host which would not be possible without the presence of these bacteria [37].

4.3. Antioxidants

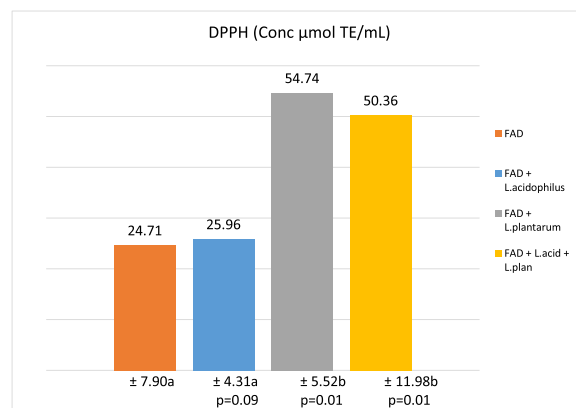
Graphic 2 shows the results found through the capture of ABTS radicals' analysis. FAD obtained 8.35 μmol antioxidant activity for each mL of the sample. When only *L.acidophilus* was inoculated, there was an increase to 11.78 $\mu\text{mol TE/mL}$ ($p = 0.03$) antioxidant activity and when only *L.plantarum* was inoculated there was an increase to 11.75 $\mu\text{mol TE/mL}$ ($p = 0.04$). When the two strains were added to the rice bran together, the antioxidant activity obtained was 11.82 $\mu\text{mol TE/mL}$ ($p = 0.03$). There were no statistically significant differences between the three symbiotic samples, only when compared to control (isolated FAD without probiotics).

To confirm these results, the capture of DPPH radical (Graphic 3) analysis was also performed. It was observed that the sample concentrations necessary for antioxidant activity in DPPH radicals are much higher than those necessary for ABTS radical capture. FAD showed a concentration of 24.71 $\mu\text{mol TE/mL}$, not differing statistically if only *L.acidophilus* was inoculated (25.96 $\mu\text{mol TE/mL}$, $p = 0.09$). However, when only *L.plantarum* was added (54.74 $\mu\text{mol TE/mL}$, $p = 0.01$) or the two strains together (50.36 $\mu\text{mol TE/mL}$, $p = 0.01$), the concentrations necessary to achieve the antioxidant activity of this radical are much higher.

The relation between antioxidants and probiotic bacteria growth is widely recognized in the literature. Demirci et al. [30] analysed the antioxidant capacity of probiotic yogurts containing



Graphic 2. Antioxidant Activity of samples by ABTS methods. ANOVA test showed p value < 0.05 , appointing that there was a statistically significant difference between the samples. Averages followed by the same letter, in the column, do not differ at 5 % probability in the Tukey test.



Graphic 3. Antioxidant Activity of samples by DPPH methods. ANOVA test showed p value < 0.05 , appointing that there was a statistically significant difference between the samples. Averages followed by the same letter, in the column, do not differ at 5 % probability in the Tukey test.

rice bran. As a result, 12.75 % of DPPH radical inhibition was obtained in the samples that added 3% of rice bran when compared to samples without rice bran. Using the ABTS method to confirm the results, 136 mM Trolox/g to 3 % rice bran samples and 0.60 mM Trolox/g to control samples (0 % rice bran) were found. The authors attribute these findings to the antioxidant properties present in rice bran phytochemicals, as the presence of probiotics bacteria.

Gjorgievski et al. [38] studied the antioxidant capacity when the whole milk is fermented by four different probiotics strains and, according to DPPH analyses, *L. acidophilus* showed the highest value of antioxidant capacity. Moreover, the fermentation made molecular changes in the milk, releasing different compounds such as peptides, free amino and fatty acids. De Oliveira et al. [42] developed a goat milk ice cream and they concluded that the inclusion of selected probiotics, such as *L. plantarum*, resulted in an ice cream with greater antioxidant activity during 60 days. However, studies also have shown that these results are not found in dairy products only. Kombucha, the fermented tea, has strong antioxidant properties highly associated with polyphenol content. This activity is varied and depends on the type and composition of the tea, such as the duration of the fermentation [40].

In vivo and in vitro studies indicate that probiotics exhibit antioxidant potential. The consumption of probiotics alone or in food (symbiotics) can reduce oxidative damage, free radicals rate and modulate the fundamental antioxidant enzymes activity in human cells. It because probiotics demonstrate antioxidant activity by secreting enzymes like superoxide dismutase (SOD) and improving glutathione production, enzymes that act as central regulators of reactive oxygen species levels (ROS). Besides the systemic health benefits of probiotics, the incorporation of these bacteria in food can be a good strategy to supply antioxidants on the diet [41].

4.4. Freeze-drying and cell viability via qPCR

After the growth of *L.acidophilus* and *L.plantarum* in FAD, symbiotic samples were frozen and subsequently freeze-dried for 72 h. For the previously frozen rates at points 0 h and 48 h, DNA extraction was performed, as well as the freeze-dried sample to compare the losses in bacterial concentration due to the freeze-drying process. For this, qPCR was performed, one of the most versatile and commonly used methods in molecular biology, being introduced due to its fast, accurate, sensitive and efficient identification and quantification in probiotic bacteria.

Table 3 shows the results of qPCR quantification in the symbiotic before and after the freeze-drying process. The results

showed that there was a statistically significant growth between the inoculation time and after 48 h at 37°C. However, there was also a significant cell death, resulting in decreased probiotics concentration. *Lacidophilus* demonstrated better growth in rice bran when compared to *L.plantarum*. But interestingly, when the strains' survival rates were calculated by dividing the values found at point 48 h and after the freeze-drying process, *L.plantarum* has a survival rate of 55.34 % while *Lacidophilus* had 27.25 %.

The freeze-drying process has been reported as a great technology for preserving compounds and one of the most convenient and successful methods to preserve the bacterial cells, even not all strains survive along the process [42]. Moayyedi et al. [5] evaluated the effects of different drying methods (electrospraying, freeze-drying and spray-drying) on the viability of *L. rhamnosus* and they concluded that freeze-dried microcapsules prolong its survival in digestive system. Li et al. [43] compared the different drying methods on quality, bacterial viability and storage sensibility of a probiotic in apple snacks, and the results showed that freeze-drying followed by microwave drying was the most suitable drying method for the development of the product.

Jofre et al. [44] also reported the importance of the storage under refrigeration. The stability of the cultures was much higher once stored under refrigeration (4 degrees C) when compared to non-refrigerated storage, highlighting the shelf-life as a paramount factor to the industry. All these findings show that freeze-drying is a good alternative for the food industry because the product cannot be developed only, but it must be viable in wide production for the industry.

Nevertheless, despite the significant and expected reduction in cell concentration, even so, the freeze-drying process can be considered an effective method and used for the symbiotic developed from rice bran. According to ANVISA's Technical Regulation [9], the minimum amount of bacteria needed for a product to be considered as a symbiotic is in the range from 10⁸ to 10⁹ CFU in the daily recommendation of portion ready for consumption. After the freeze-drying process, a concentration of 4.21 × 10⁷ µL/g to *L.plantarum* and 6.42 × 10¹⁰ µL/g to *Lacidophilus* was obtained. It means that with only 1 g of freeze-dried symbiotic is obtained an amount higher than the minimum required by ANVISA, making it uses as symbiotic product and being able to receive the claim of a functional product on the label. However, the rice bran which is the main ingredient in the composition is a co-product of the industry and it's not allowed to use it in human food.

5. Conclusions

The chosen bacteria, *Lacidophilus* and *L.plantarum*, had a good growth in rice bran, as well as good interaction with the phenolic compounds present in this industrial co-product. The freeze-drying aimed to improve the conservation of the symbiotic, maintaining the nutritional properties of rice bran and prolonging the survival rate of inoculated bacteria. Although freeze-drying reduced significantly the number of probiotics, sufficient cells were still maintained viable for the product to be considered a symbiotic by legislation. Molecular analysis using qPCR proved to be very efficient for cell quantification, being much faster, more accurate and more reliable than traditional methods as plate count analysis.

Rice bran showed significant amounts of phenolic compounds and antioxidant activities interacting with probiotic bacteria. These findings demonstrate a possible prebiotic potential of high nutritional value. Compiling all the results found that demonstrate the benefits of rice bran and considering the information related to the wastage generated, the reasons that claim its ban on human

food may be of little relevance, even because there are already techniques that inhibit these anti-nutritional factors present in rice bran.

According to all the results presented above, the use of rice bran in human consumption would bring several benefits, both nutritional (mainly due to its antioxidant and prebiotic potential) and industrial (due to wastage). And yet, in a context where the lack of food in the world is a predictable future, it would also bring environmental benefits.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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