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Impact of Carao (*Cassia grandis*) on *Lactobacillus plantarum* Immunomodulatory and Probiotic Capacity

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Abstract: *Lactobacillus plantarum* has beneficial effects on the reduction of symptoms of poor lactose digestion and hypercholesterolemia, removal of the duration and severity of diarrheal processes, improvement of the intestinal permeability barrier, prevention of some types of cancer by adsorption or inactivation of genotoxic agents, increased resistance to intestinal and extraintestinal infections, attenuation of inflammatory bowel disease, and prevention of allergies (especially food). On the other hand, carao (*Cassia grandis*) has shown remarkable nutritious content with influential dietary applications. As a result, this investigation aimed to explore the effect of *Cassia grandis* pulp on viability of *Lactobacillus plantarum* under gastrointestinal conditions, immunomodulatory capacity, and probiotic potential. Adding carao to the medium under different experimental conditions, including rich and minimal culture media and a gastrointestinal digestion process of skimmed milk, did not substantially affect Lactobacillus plantarum's growth but prolonged its viability. The administration of *Lactobacillus plantarum* with carao in mice did not induce a proinflammatory response at a systemic level. Still, it did cause an increase in the production of immunoregulatory cytokines. Also, the viability of TSB broth was improved by adding carao. Carao improved the growth of acid tolerance, bile tolerance, growth in TSB broth, and NaCl resistance. According to the results, carao may enhance the characteristics of *L. plantarum* when enriching fermented dairy products.

Keywords: probiotic characteristics; *Lactobacillus plantarum*; immunomodulatory capacity; *Cassia grandis*

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1. Introduction

The human gastrointestinal tract hosts a complex and dynamic microbial community, which is specific for each person depending on the environment and genetic factors. Each tract harbors around 1.5 kg of bacteria exceeding 60% of the fecal mass. This microbial community contains hundreds of species that vary throughout the gastrointestinal tract, with the lowest concentration found in the stomach (10^3–10^4 cfu/g) and the highest in the colon (10^{11}–10^{12} cfu/g). This intestinal microbiota plays a vital role in human health due to its participation in digestion and critical function in developing the intestinal mucosa and the immune system [1]. Gut microbiota is crucial in beneficial health effects attributed to probiotic microorganisms. *Lactobacillus plantarum* has improved lactose digestion and reduced flatulence and discomfort. Lactose intolerance is when colonic fermentation of undigested lactose produces gastrointestinal effects such as abdominal pain, bloating,

borborygmi, and diarrhea. Many populations worldwide include many adults who cannot digest lactose. In humans, and indeed in all mammals, the expression of the lactase enzyme is downregulated in adulthood. Some probiotic strains can compensate for the lack of endogenous lactase in the human intestine. This improvement is evidenced by a decrease in hydrogen levels in respiration since this increases when undigested carbohydrates reach the colon and are fermented. This improved digestibility reduces symptoms related to lactose intolerance in lactose-intolerant people [2–4].

The beneficial health effects attributed to probiotic microorganisms identify them as possible components of the so-called "functional foods" and "nutraceuticals" [5]. Several main factors have led to research on nutraceuticals and functional foods becoming an expanding area, and one of the most active in the health sciences: these factors include the tendency of consumers, recipients of increasing health information, to develop self-care; the concern of medicine to prevent common chronic diseases in aging populations and to deal not only with the treatment of pathologies but also with increasing well-being (which is a form of prevention); technological advances; and the growth of the health products market [6]. Research trends are leading to the examination of new delivery systems and formulations that add new food ingredients and sources for fermented dairy products [7].

Marcia et al. (2022) [8] determined that the carao fruit can be used as a bioactive ingredient due to its content of macronutrients and micronutrients and its antioxidant capacity. It is a precursor of vitamin A (retinol) due to its carotenoid content. More than 500 species of *Cassia* are reported worldwide; among them is *Cassia grandis*, commonly known as carao. This plant is considered endemic to Central America, and its fruit is traditionally used in popular medicine. Various studies validate the functional and nutraceutical potential of *C. grandis* globally. It is regarded as a potential antidiabetic and anti-anemic agent [9].

Furthermore, due to its chemical quality, it can be used to develop food formulations. This fruit contains 47 bioactive molecules from the flavonoid group, highlighting its potential medicinal use in human health. Due to its phytochemical quality, carao is a functional ingredient that could be useful for people with special diets. Its use in intestinal cells at the in vitro level determined that it prevents inflammation and cell death, being attributed to the discovery of a new potential pharmaceutical effect [10]. Carao is a plant that has been widely studied from a chemical point of view, allowing the identification of flavonoids. These compounds have been shown to possess antioxidant and anti-inflammatory properties that could benefit human health. Carao has been shown to have interesting functional properties that make it suitable for use in food products, mainly as a prebiotic in fermented beverages [10]. Another notable aspect is its pharmaceutical potential to prevent inflammation and cell death in vitro, which suggests that it could have applications in the pharmaceutical industry. Carao has demonstrated its importance as a versatile ingredient with possible health benefits and functional and pharmaceutical applications [10]. However, more research needs to be carried out to fully understand the extent of its uses in the food and pharmaceutical industries. Exploring its properties and applications will allow us to take full advantage of carao's benefits in different applications. There is no research on *L. plantarum* characteristics as impacted by carao. As a result, the viability under different conditions, immunomodulatory capacity, and probiotic potential of *L. plantarum* as affected by carao were tested and investigated.

2. Materials and Methods

2.1. Plant Material

Postharvest Carao (*Cassia grandis*) fruit samples were collected in the Guapinol Biological Reserve, Marcovia Municipality, Choluteca Department (Honduras) by the Institute of Biotechnology of the National University of Agriculture, located in Olancho, Honduras. The carao pulp was sterile and manually separated from the fruit, dried (Digitronic TFT-Selecta, J.P. SELECTA, Barcelona, Spain), and ground (Retsch GmbH, Haan, Germany) [11].

2.2. Tolerance to Bile Salt

The strain (*L. plantarum* LP299v) was inoculated (1% culture) on MRS broth and with bile salts (DIBICO®, Cuautitlán Izcalli, Mexico) in concentrations of 0.3% (*w*/*v*). The pH of the medium was adjusted to 7.0. Once the hours of incubation were completed, the bacteria were counted to find the countable plate (25 to 250 CFU) at 4 h intervals of incubation (0, 4, and 8 h). The culture was incubated at 37 °C for 24 h. After this, the viable cell count was carried out using CFU/mL, thus considering microbial survival and resistance to bile salts [12].

2.3. Tolerance to pH

The strain (*L. plantarum* LP299v) was inoculated (10% culture) on MRS broth and plates were prepared with MRS agar, adjusting the pH of the culture medium to 2. The survival and resistance of the bacteria to the pH change were quantified after incubation at 37 °C for 24 h and compared with viable microorganisms from the inoculum. Furthermore, MRS broths were set up at 2 to mimic the pH of the gastric acids in the stomach, and the times of measurement were 0 min, 30 min, and 1 h [13].

2.4. Tolerance to NaCl

The strain (*L. plantarum* LP299v) was inoculated (10% culture) in three different liquid culture media (MRS) with a pH of 6, adding different concentrations of NaCl (2%, 4%, 6%, 8%, and 10%), and incubated at 37 °C for 24 h. After incubation, microbial viability and growth were determined at high concentrations of NaCl.

2.5. Viability of Lactobacillus plantarum

The growth of *L. plantarum* was examined in the presence and absence of carao. The growth kinetics of *L. plantarum* were evaluated in Tryptic Soy Broth (TSB), and *Lactobacillus plantarum* specific medium (LPSM) broth supplemented with carao at levels of 2% and 5% and without supplementation (Control samples). The composition per liter of LPSM was Bacto proteose peptone (Difco, Detroit, Michigan) (10 g), Bacto beef extract (Difco) (10 g), Bacto yeast extract (Difco) (5 g), d -sorbitol (Sigma, Kawasaki City, Kanagawa) (20 g), ciprofloxacin (Sigma) (4 mg), sodium acetate (5 g), ammonium citrate (2 g), potassium phosphate (2 g), magnesium sulfate (0.1 g), and manganese (II) sulfate (0.05 g). The initial inoculum was made from a suspension in sterile PBS adjusted for turbidity equivalent to the 0.5 MacFarland standard (approximately 1.5×10^8 bacteria/mL), which was subsequently counted on MRS agar. The media with and without carao were incubated for 72 h in both aerobiosis and anaerobiosis, taking aliquots at 2, 6, 12, 24, 36, 48, and 72 h for counts [14]. Sucrose was used as the positive control, and broth without carao was considered the negative control.

2.6. Viability of Lactobacillus plantarum under Gastrointestinal Digestion

In vitro digestion was carried out in the gastric and intestinal stages, using sterile skimmed milk in the presence and absence of 5% carao. The culture of *L. plantarum* was subjected to in vitro digestion to evaluate the possible growth of *L. plantarum* during digestion. For the gastric stage, 10 mL of sterile skimmed milk with and without carao were taken to which 10 mL of gastric enzyme solution (NaCl 2.75 g/L, KCl 0.82 g/L, and pepsin 1 g/L) were added in 50 mL tubes. Next, the mixture was inoculated with *L. plantarum* (approximately 1.5×10^8 bacteria/mL) and the indicator bacteria, and the pH was adjusted between 3 and 3.5 with 1N HCL. These mixtures were incubated at 37 °C for 2 h aerobically, the pH was recorded, and aliquots were taken to perform post-digestion counts on MRS agar. Once the gastric stage was completed, the intestinal stage was continued, for which 10 mL of intestinal enzyme solution (pancreatin 1 g/L and bile 2 g/L, pH 6.20) was added. The resulting mixture was adjusted to a pH between 7 and 7.5 with 1N $NaHCO_3$ and incubated under anaerobic conditions for 20 h. Aliquots were taken for counting at 4, 16, and 20 h on MRS agar [15].

2.7. Effect of Carao on Intestinal Infection with Y. enterocolitica O9 In Vivo

2.7.1. Animals

The animal experiment guidelines established by the Declaration of Helsinki were used as a reference for the animal models, which were approved by the Ethics Committee in Food Research at the Honduran Association of Medicine and Nutrition (ASOHMENU) with form number AS-ASHOMENU-0013-2022. Thirty-six pathogen-free female BALB/c mice, 6 weeks old, from Janvier Labs (Saint Berthevin, France) were used. Six groups of six animals each were made and placed under standard barrier conditions in cages with steel mesh floors in the Animal Experimentation Unit of the University of Agriculture. All animal experiments were carried out according to the guidelines of the Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and supervised by the University Bioethics Committee from Honduras.

2.7.2. Preparation of Fermented Milk Added with *L. plantarum*

Fermented milk was supplemented with *L. plantarum* that was supplied by the Department of Nutrition of the University of Agriculture in Honduras and was manufactured according to the procedure described by Bergillos-Meca et al. (2015) [16]. The preparation process was as follows: raw goat milk was skimmed, concentrated, and heated at 80 °C for 30 min. Once cooled, it was inoculated with the traditional starters used to manufacture yogurt (*Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*) and *L. plantarum*. The inoculum was prepared from a suspension in phosphate-buffered saline at a known concentration, which was subsequently centrifuged to be added to the milk. Milk and inocula were incubated at 37 °C for 6 h. Inoculation of the animals occurred as two groups (1 and 2) received a dose of 10^9 viable bacteria in 100 µL of fermented milk once a day intragastrically for 19 days. This administration was performed with a stainless-steel feeding needle and a 1 mL syringe. Two other groups (3 and 4) received 100 µL of fermented milk without *L. plantarum*, and groups (5 and 6) received 100 µL of water under the same conditions. To evaluate the actual number of viable bacteria, dilutions of the inoculum were plated on LPSM agar, and the number of CFU was recorded after 24 h of incubation at 37 °C.

2.7.3. Experimental Infection with *Y. enterocolitica* O9 IP383

Y. enterocolitica strain IP383 was grown in TSA at 25 °C for 24 h, harvested, washed, and resuspended in sterile water. The actual number of IP383 bacteria was evaluated by plating the decimal dilutions prepared on CIN agar and incubated at 37 °C for 24 h. Mice were challenged with a single dose of 10^8 viable bacteria via the intragastric route in 100 µL of water [17].

2.7.4. Measurement of Cytokines and IgA

Mice were anesthetized with a mixture of ketamine and xylazine before blood was obtained through cardiac puncture. Whole blood was collected into EDTA tubes to separate plasma for cytokine assays. The mice were then sacrificed, and the cecal contents were removed, weighed, and homogenized in PBS. Cytokines and total IgA were determined in cecal homogenates using commercial ELISA kits (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Statistical Analysis

Data were examined utilizing SAS systems and studied employing the general linear model. Differences of least square means were utilized to investigate significant differences for the carao concentration, time, and carao concentration × time effect. Tukey's test was utilized to determine the considerable discrepancies among the effects at $\alpha = 0.05$.

3. Results

3.1. Bile Tolerance

Lipids are nonpolar and require bile salts to stabilize the emulsion and to facilitate contact between enzyme and substrate, which allows the metabolization of lipids and their absorption in the intestinal wall. Figure 1 illustrates the bile resistance of *L. plantarum* over 8 h after adding carao at different bile concentrations. The carao concentration × time effect was not significant ($p > 0.05$), whereas the main effects (hour and carao concentration effect) were significant ($p < 0.05$) (Table 1).

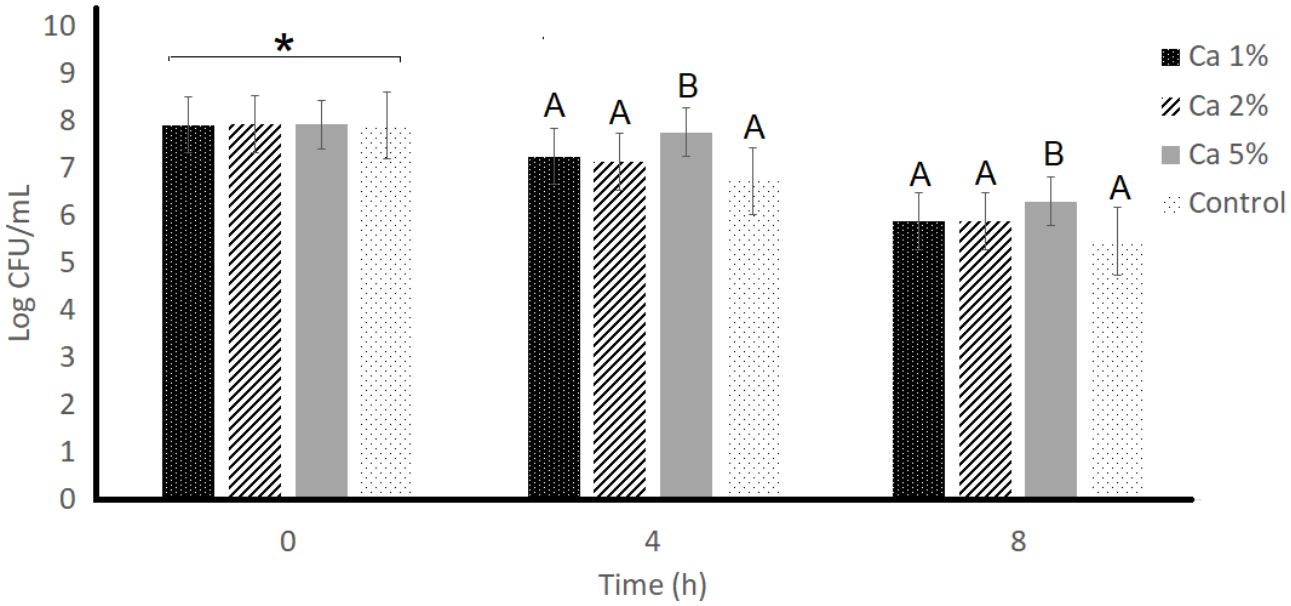

Figure 1. Bile tolerance (0.3% oxgalt) of *L. plantarum* in MRS broth as influenced by carao concentration over 8 h. Ca = carao concentration at 1%, 2%, and 5%. * Treatments were not stadistical different.

Table 1. The *p*-value of ingredient, time or pH, and their interaction for bacterial viability, bile tolerance, acid tolerance, resistance to gastric juices, protease activity, and lysozyme resistance of *Lactobacillus casei*.

Effect	*L. casei*
Bile tolerance	
Carao concentration	0.0432
Time (Hours)	<0.0001
Carao concentration × time	0.2587
Acid Tolerance	
Carao concentration	0.0254
Time (Minutes)	<0.0001
Carao concentration × time	0.1543
Microbial growth on TSB	
Carao concentration	0.0201
Time (Hours)	<0.0001
Carao concentration × time	0.1349
Microbial growth on LPSM	
Carao concentration	0.0765
Time (Hours)	<0.0001
Carao concentration × time	0.1674
Microbial growth under gastrointestinal digestion	
Carao concentration	0.0980
Time (Hours)	<0.0001
Carao concentration × time	0.1245
Tolerance to NaCl	
Carao concentration	0.0284
NaCl concentration	<0.0001
Carao concentration × time	0.0723

3.2. Acid Tolerance

Parietal cells in the stomach's proximal two thirds (body) secrete acid. Gastric acid aids digestion by creating the optimal pH for pepsin and gastric lipase and stimulating pancreatic bicarbonate secretion. The acid tolerance and resistance to pH of *L. plantarum* as affected by carao are shown in Figure 2. The carao concentration × time effect was not significant ($p > 0.05$), whereas the main effects (hour and ingredient effect) were significant ($p < 0.05$) (Table 1).

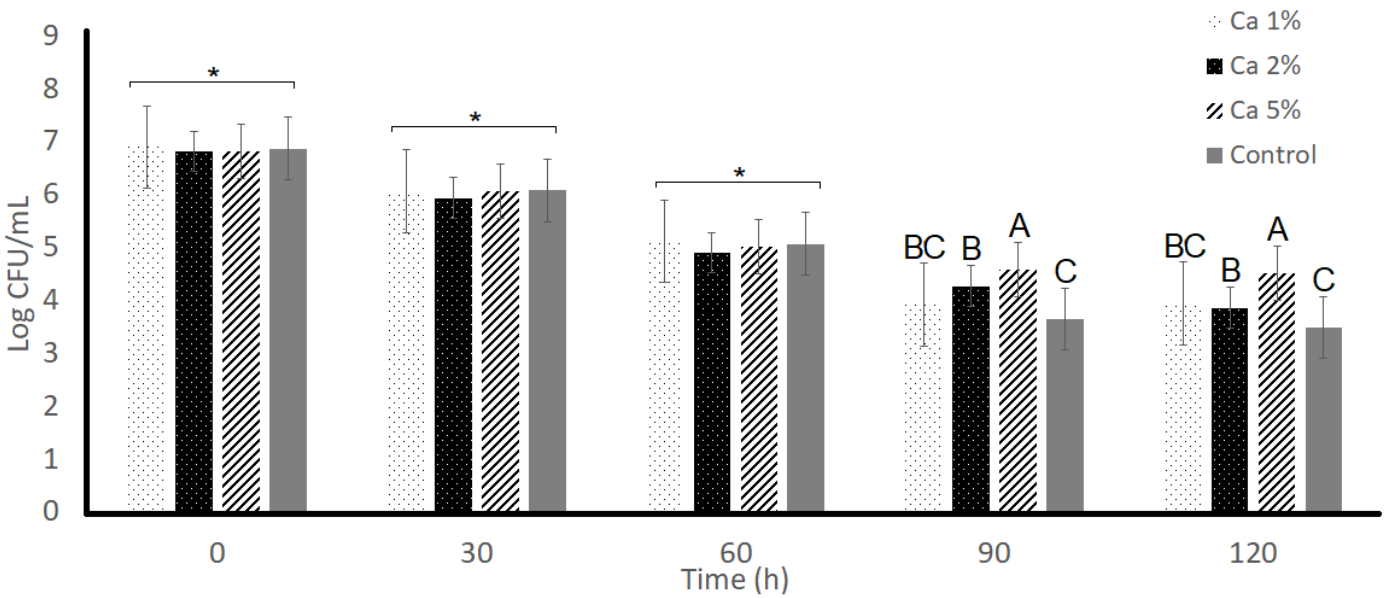

Figure 2. Acid tolerance (pH 2) of *L. plantarum* in MRS broth as influenced by ingredients over 30 min. Error bars represent standard deviation. C = control, Ca = carao con-centration at 1%, 2%, and 5%. * Treatments were not stadistical different.

3.3. Resistance to NaCl

Many studies have suggested that a high-salt diet increases sodium accumulation in the skin of mice, thereby increasing their immune response to a parasite that infects the skin. The findings thus suggest that salt in the diet could have therapeutic potential against microbial infections. Nevertheless, probiotics are also inhibited by NaCl. The NaCl tolerance of *L. plantarum* as affected by carao is shown in Figure 3 The carao concentration × time effect and the carao concentration effect were not significant ($p > 0.05$), whereas the time effect was significant ($p < 0.05$) (Table 1). In this study, a low level of tolerance to salt (NaCl) was observed.

3.4. L. plantarum Viability Characteristics

The carao concentration × time effect and the carao concentration effect were not significant ($p > 0.05$), whereas the time effect was significant ($p < 0.05$) (Table 1). Initially, the growth of *L. plantarum* in TSB was examined in the absence and presence of carao, which was incorporated at concentrations of 2% and 5%. The cultures were incubated anaerobically. The results are presented in Figure 4 and show that supplementation of a relatively nutrient-rich medium with carao had little effect on growth: viable bacterial counts were similar on all three media during the exponential growth phase (established approximately between 2 and 24 h), and only at 48 h was a significant difference observed in favor of the medium supplemented with the highest amount of carao.

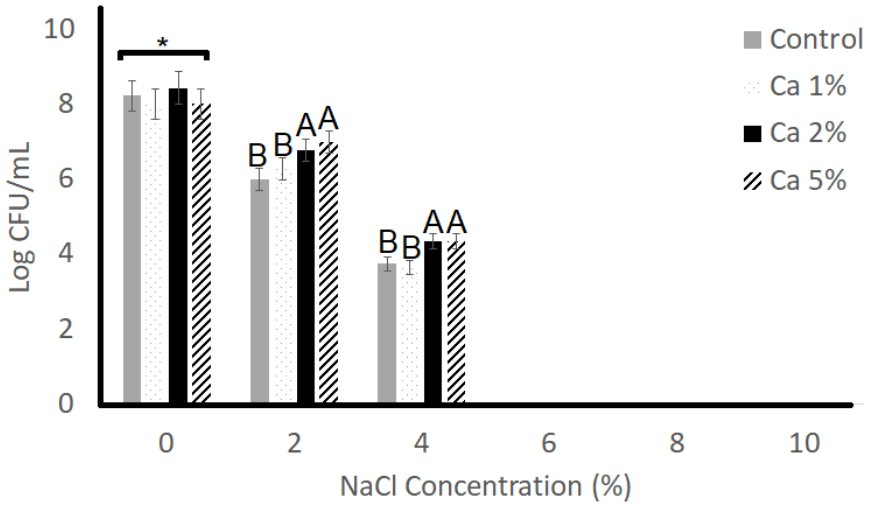

Figure 3. Resistance to NaCl of *L. plantarum*. * Average of three replicates. Ca = carao con-centration at 1%, 2%, and 5%.

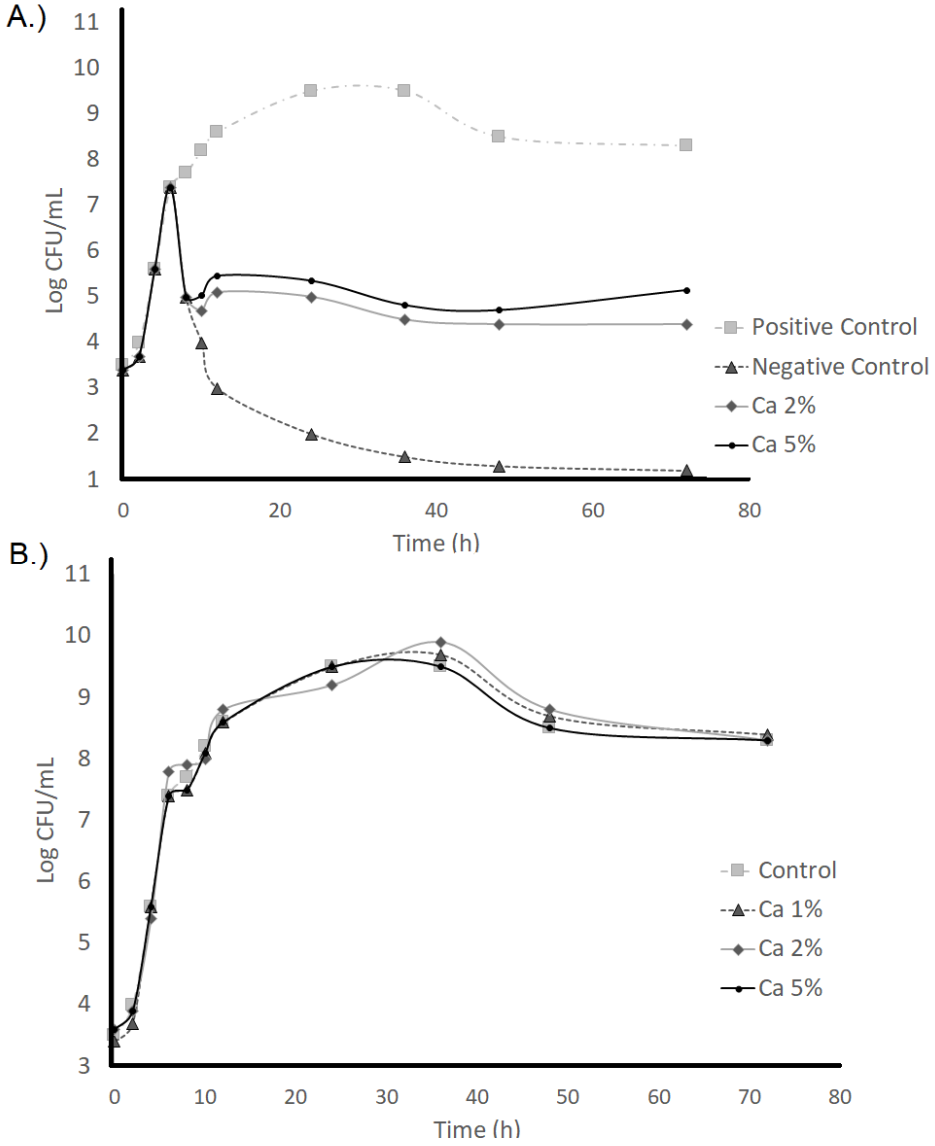

Figure 4. The growth of *L. plantarum* in TSB (**A**) and LPSM (**B**).

3.5. L. plantarum Resistance to the Gastrointestinal Digestion Process In Vitro: Carao Influence

The results are presented in Figure 5 and show that the bacteria perfectly endure the gastrointestinal conditions without the presence of carao influencing this significantly. In other words, carao did not influence the growth of *L. plantarum* under gastrointestinal digestion. On the contrary, in our current essays, using inoculars of the same order of magnitude, no significant reduction was observed after 3 h of gastric digestion. Two factors can explain this resistance of *L. plantarum*: the pH difference, which in current trials was never less than 3, suggesting that between values 3 and 2.6 a resistance limit can be located; and the presence of milk proteins, which was already revealed in the aforementioned preliminary experiments, in which the damping power of skim milk preserved the viability of *L. plantarum* in the presence of HCL, although at pH values greater than 3.

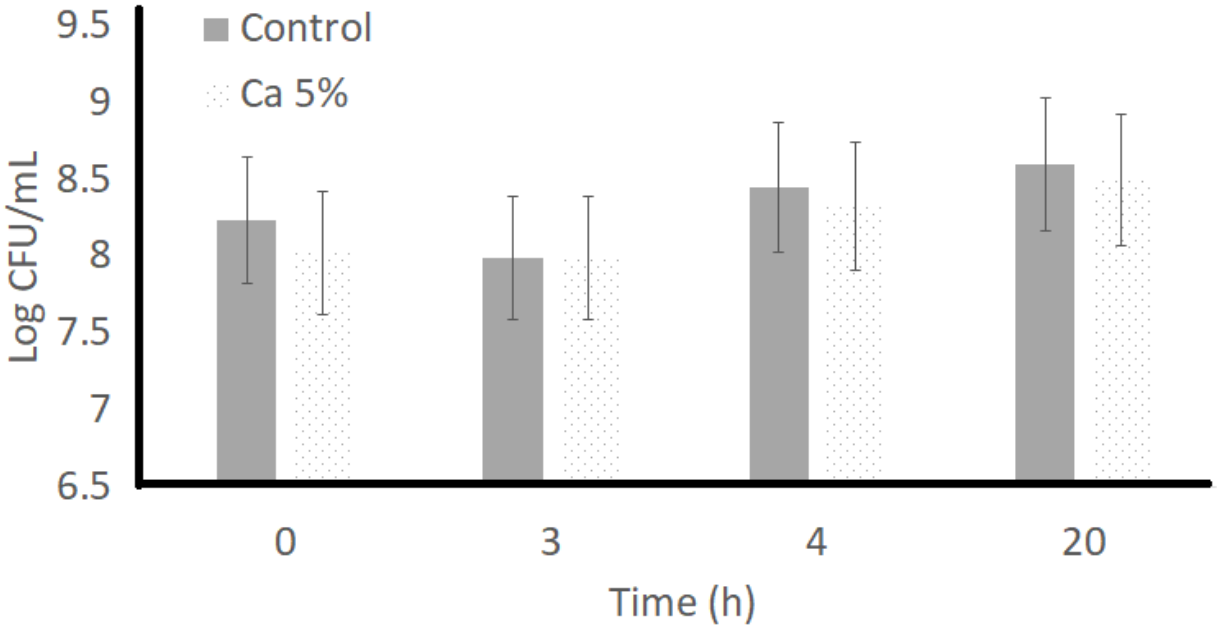

Figure 5. Survival of *L. plantarum* in a gastrointestinal digestion simulation test in the absence and presence of carao. No statistical difference was found between control samples and carao samples.

3.6. Effect of Treatment with L. plantarum with Carao on Experimental Infection with Y. enterocolitica O9 in Mice

Mice received *L. plantarum* with carao in fermented milk once a day until the day of sacrifice, while control mice only received fermented milk. On day 10, animals were challenged with IP383. Two groups of infected mice (untreated control and carao-treated mice, n = 6 in each group) were sacrificed at various times after infection (days 14, 18, 22, 26, and 30) to detect *Y. enterocolitica* in the PP. *L. plantarum* with carao treatment could not shorten the persistence of *Y. enterocolitica* in the intestinal lumen. However, the results are shown in Figure 5 and demonstrate that PP cultures from carao-treated mice became negative earlier than those from untreated mice, and the difference was not statistically significant ($p > 0.05$). This suggests that carao did not promote the removal of IP383 in mouse Peyer's patches.

As shown in Figure 6, infection with IP383 significantly increased the level of the proinflammatory cytokine TNF-α (Figure 6) in control mice ($p < 0.05$), in mice receiving fermented milk ($p < 0.05$), and in those treated with carao with *L. plantarum*. ($p < 0.05$). Interestingly, the cecal level of TNF-α (Figure 6) was significantly higher in infected mice receiving carao with *L. plantarum* than in infected mice receiving fermented milk alone ($p < 0.05$).

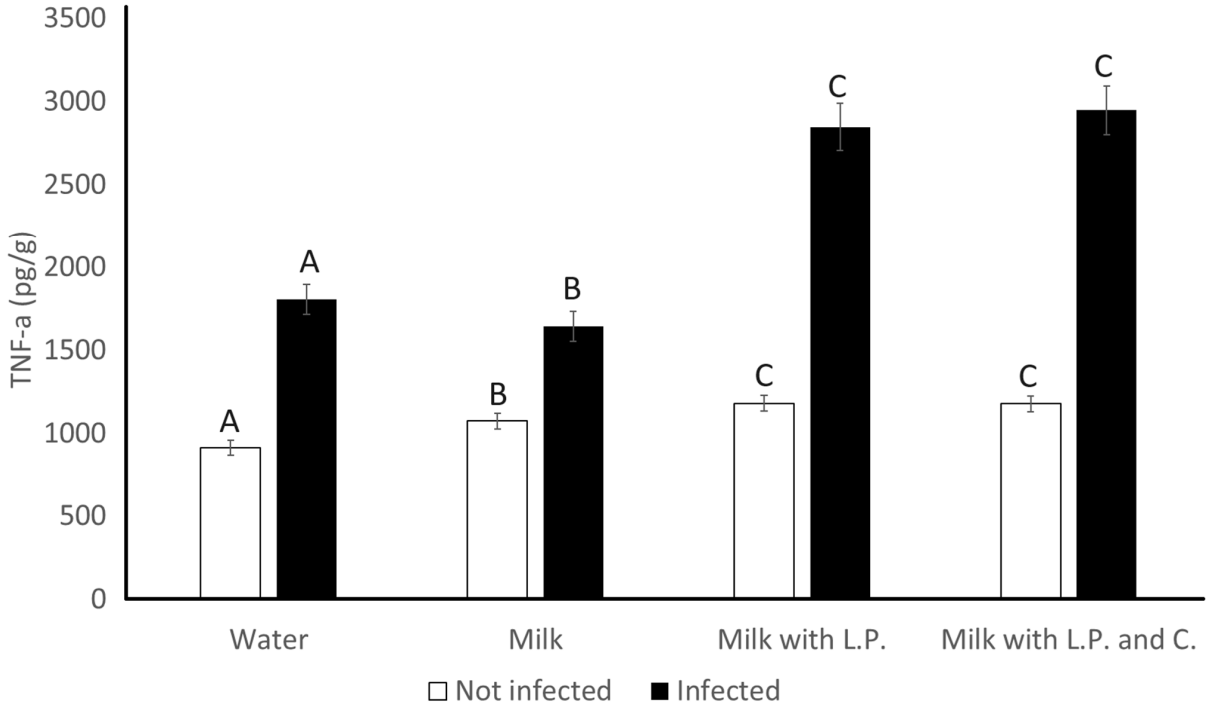

Figure 6. Levels of TNF-a in the cecal contents of uninfected mice (white bars) and mice infected with *Y. enterocolitica* (black bars). L.P. = *Lactobacillus plantarum;* C. = Carao (*Cassia grandis*).

3.7. Effect of Treatment with L. plantarum with Carao on Intestinal Cytokine and IgA Production in Uninfected Mice and Y. enterocolitica O9-Infected Mice

To examine the effects of *L. plantarum* with carao on the intestinal response to IP383 infection and to differentiate them from possible effects of the fermented milk vehicle, mice were randomly divided into three groups (each group of 12 mice): One group of mice received *L. plantarum* with carao, another group of mice received *L. plantarum* suspended in fermented milk for 19 consecutive days, another group received fermented milk, and a third group (untreated control) received water for the same period. Half of the animals in each group were exposed to IP383 on day 17. Cecal samples were taken at the end of the experiment, on day 20.

Figure 7 shows that the anti-inflammatory cytokine IL-10 was significantly decreased in the cecal contents of animals treated with fermented milk ($p < 0.05$) and in those receiving fermented milk containing *L. plantarum* with carao ($p < 0.05$). Infection with IP383 reduced the level of IL-10 in control mice ($p < 0.05$), but this cytokine was not affected in animals treated with *L. plantarum* with carao or fermented milk alone. Determinations of total IgA in cecal contents are presented in Figure 8. Only in the group of animals treated with *L. plantarum* with carao or *L. plantarum* alone was an infection with IP383 associated with a significant increase in IgA production ($p < 0.05$). Furthermore, mice treated with *L. plantarum* with carao and infected with IP383 had higher total IgA levels compared to mice infected with IP383 in the untreated control group ($p < 0.05$) and in the group treated with the dairy vehicle alone ($p < 0.05$).

3.8. Effects of Treatment with L. plantarum with Carao on Plasma Cytokine Levels in Uninfected Mice and Y. enterocolitica O9-Infected Mice

To better understand the immunomodulatory activity of *L. plantarum* with carao at the systemic level, we used the same experimental design described above to examine the concentrations of TNF-α (Figure 9), IL-10 (Figure 10) and IFN-α (Figure 11) in the plasma of control mice, of mice treated with fermented milk, and those treated with *L. plantarum* with carao, both those infected and uninfected with IP383. Figure 9 shows that the concentration of TNF-α was not significantly modified by the treatments with *L. plantarum* with carao or

the milk vehicle alone. Furthermore, the concentration of this cytokine was not affected by exposure to IP383. As shown in Figure 10, plasma levels of IL-10 were significantly higher only in the group receiving *L. plantarum* with carao and IP383 compared with the uninfected control group ($p < 0.05$) and the vehicle-treated group uninfected dairy ($p < 0.05$). Finally, Figure 11 shows plasma IFN-α values. Whether infected or not, *L. plantarum* with carao-treated mice showed higher plasma concentrations of IFN-α than the uninfected control group ($p < 0.05$ and $p < 0.05$, respectively), the infected control group ($p < 0.05$ and $p < 0.05$), and the group that received the dairy vehicle alone ($p < 0.05$ and $p < 0.05$).

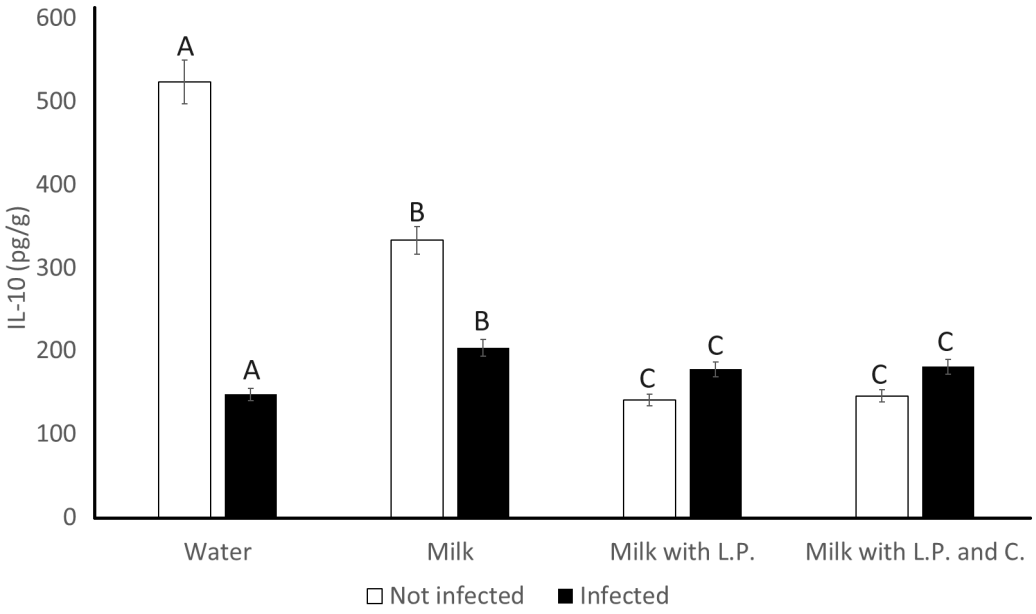

Figure 7. Levels of IL-10 in the cecal contents of uninfected mice (white bars) and mice infected with *Y. enterocolitica* (black bars). L.P. = *Lactobacillus plantarum*; C. = Carao (*Cassia grandis*).

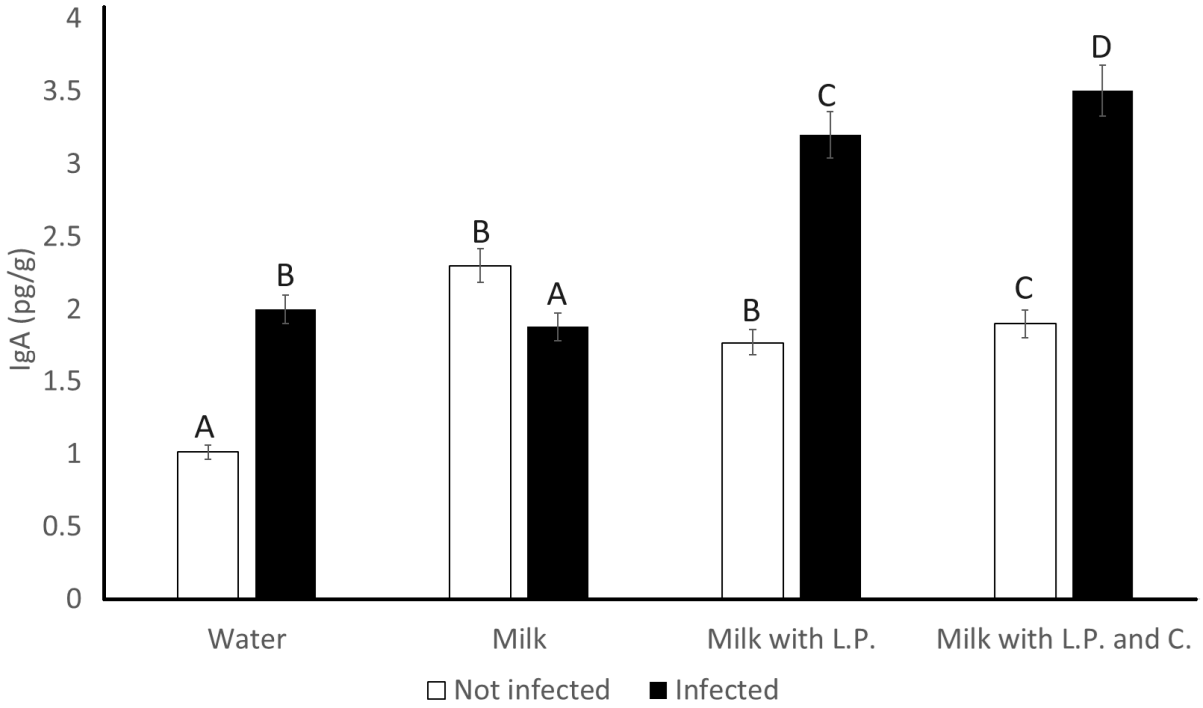

Figure 8. Levels of IgA in the cecal contents of uninfected mice (white bars) and mice infected with *Y. enterocolitica* (black bars). L.P. = *Lactobacillus plantarum;* C. = Carao (*Cassia grandis*).

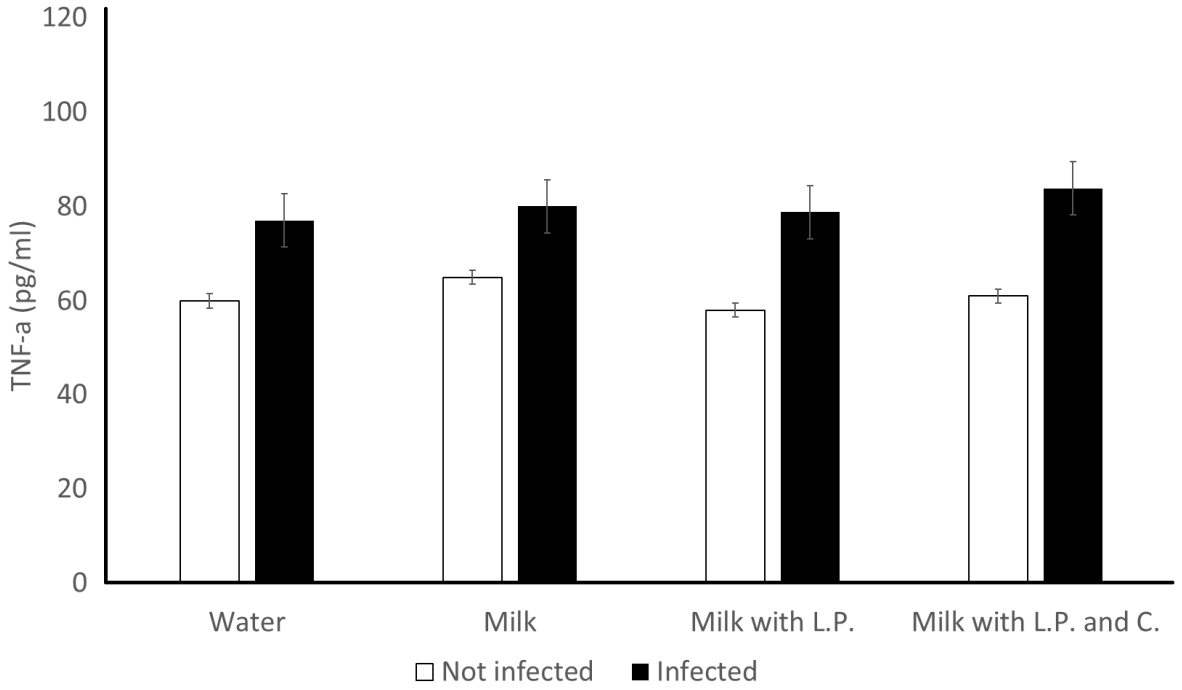

Figure 9. Levels of TNF-a in the plasma contents of uninfected mice (white bars) and mice infected with *Y. enterocolitica* (black bars). L.P. = *Lactobacillus plantarum;* C. = Carao (*Cassia grandis*). No statistical difference was found between control samples and carao samples.

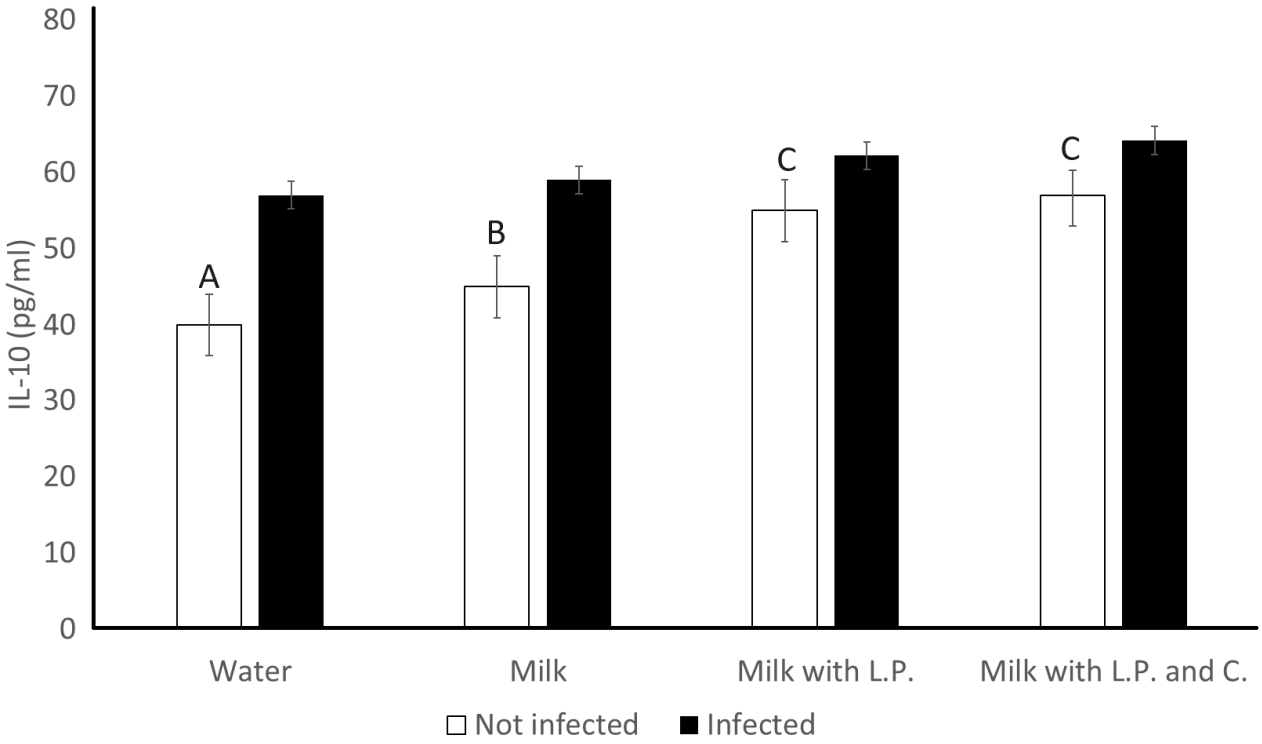

Figure 10. Levels of IL-10 in the plasma contents of uninfected mice (white bars) and mice infected with *Y. enterocolitica* (black bars). L.P. = *Lactobacillus plantarum*; C. = Carao (*Cassia grandis*). No statistical difference was found between control samples and carao samples among infected mice.

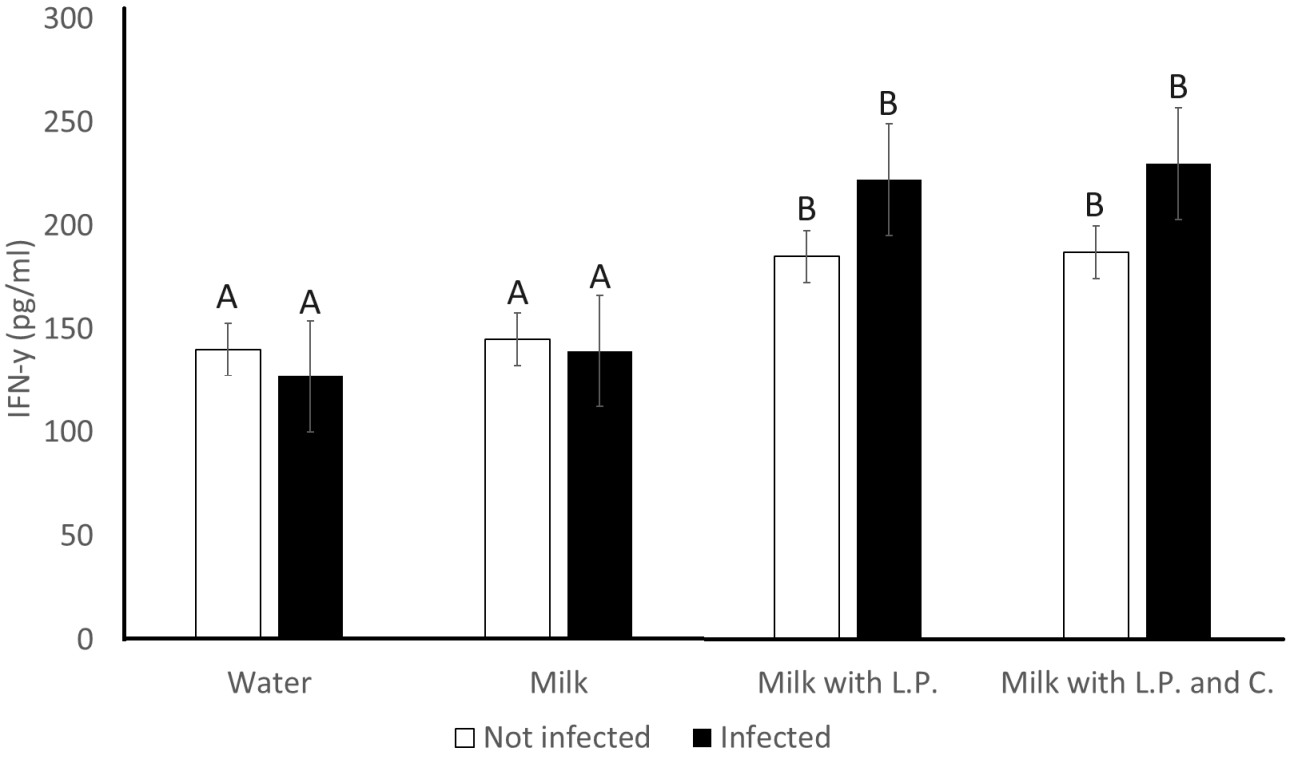

Figure 11. Levels of INF-y in the plasma contents of uninfected mice (white bars) and mice infected with *Y. enterocolitica* (black bars). L.P. = *Lactobacillus plantarum;* C. = Carao (*Cassia grandis*).

4. Discussion

L. plantarum can tolerate bile salts at 0.3% (*w*/*v*). According to Ruiz et al. (2016) [18], bacteria that survive certain concentrations of bile salts have metabolic activity that will allow colonization of the intestine since they come into direct contact with bile salts in the small intestine. The carao improved the bile tolerance of *L. plantarum*, and its growth declined over time. These results are good since this fruit has a high antioxidant capacity. Plant extracts are desirable substrates for human microbiota as nutrients. Subtreatments can be used to improve the balance of these microorganisms. Other reports have shown that carao improves bile tolerance in *L. acidophilus* [11]. Bile salts have two main tasks: one is to emulsify large fat molecules into smaller, simpler fats; the other is to make fats more soluble in water by forming micelles, aggregate droplets of fatty acids, cholesterol, and monoglycerides (those simple fats) dissolved in water to facilitate absorption by the intestine. The transformation of fats into bile salts in the duodenum is also important to prepare fat-soluble vitamins to pass through the lining of the intestine into the bloodstream.

The carao improved the tolerance to pH 2. The samples stored at room temperature resisted pH changes for 1 h, showing greater growth at values at 30 min than the control samples when compared to carao samples, with increased growth, thus demonstrating their survival in acidic conditions. Similar characteristics were obtained by Lara and Burgos (2012) [19], who indicated that lactic acid bacteria with probiotic potential must survive and resist pH changes (neutral to acidic). Generally, probiotics that resist very acidic pH (<4) belong to the *Lactobacillus* species [20]. The buffering capacity of potential compounds in carao could prevent bacteria from disintegrating under acidified conditions. Extreme pH affects the structure of all macromolecules. The hydrogen bonds that hold DNA strands together break at very low pH. The proton motive force responsible for ATP production in cellular respiration depends on the H+ concentration gradient across the plasma membrane (see Cellular respiration. The protein structure is the most pH-sensitive component in the cell. Moderate changes in pH modify the ionization of amino acid functional groups and

disrupt hydrogen bonds, promoting changes in the molecule's folding, denaturation, and destroying activity [21].

The culture media supplemented with 2% and 4% (*w*/*v*) of salt presented greater bacterial growth with the carao treatments. This property is necessary for the probiotic strains since gastric juice contains this salt (2% to 4%), which is the first physiological barrier of the digestive tract and which also corresponds to low pH values along with the action of proteolytic enzymes [22].

The fact that this difference was also manifested in the death phase of the cultures aligns with the observation that carao has a prolonged effect on viability in the late stages of cultures of a strain of *Lactobacillus* species [23]. The relative richness of TSB, especially its dextrose content, can minimize the effect of supplementation with a complex energy source such as carao. Goh et al. (2007) [24] have described diauxic growth of *L. paracasei* when grown in a medium with dextrose and carao: dextrose is consumed first, and the operon responsible for carao metabolization was only expressed when glucose had been consumed. The tests were repeated using a minimal medium, whose only organic source was yeast extract. As a comparison term, LPSM broth without ciprofloxacin or an indicator was chosen since it is a suitable medium to support *L. plantarum* development equal to or greater than that achieved in TSB. If Figure 4A,B is compared, it is observed that the growth in LPSM was even higher than that achieved in TSB, and the exponential phase was prolonged for about 12 h longer. The results in Figure 5 show that the minimal medium, without a carbohydrate carbon source, allowed the growth of *L. plantarum*, although at much lower levels than those obtained in the LPSM broth, which has a high sorbitol content. Kaplan and Hutkins (2000) [25], to explain the growth of *Lactobacillus* and *Bifidobacterium* strains in MRS medium devoid of carbohydrates, adduced the possible presence of contaminating sugars in the medium itself or the carao supplement.

Furthermore, it must be kept in mind that both in Tryptic Soy Broth and in the *Lactobacillus plantarum*-specific medium used in the present work, there are other sources of organic carbon, including amino acids, and that *lactobacilli* are capable of using amino acids such as histidine or arginine to obtain energy by phosphorylation at the substrate level. Supplementation with carao significantly prolonged the cultures' viability of the values of the minimal medium (Figure 5). However, no appreciable differences were observed between media supplemented with 2% and 5% carao. For this reason, and given that the dissolution of 5% carao in one liter of water poses technical problems, supplementation with 2% was chosen for further study.

Preliminary studies were carried out to evaluate the probiotic potential of *L. plantarum*, its ability to resist the acidic pH, and the action of bile salts [26]. In this work, this study was extended, examining the behavior of *L. plantarum* in the experimental conditions of an in vitro approach to a real situation of ingestion of the probiotic: inoculum not less than 10^8 viable bacteria per mL, suspension in skim milk, and simulation of digestion, with a gastric stage of 3 h and another intestinal stage of 20 h, carrying out viable bacteria counts at the end of each stage and after 4 h of the intestinal stage was initiated. In the preliminary studies cited, the incubation for 30 min at pH 2.6 in saline solution reduced the viable count in three logarithmic units [27] and decreased the persistence of *Y. enterocolitica* in Peyer's patches [28].

Our previous studies that aimed at characterizing the probiotic potential *of L. plantarum* with carao demonstrated that this strain could not modify the course of intestinal infection with *Y. enterocolitica* in mice when measured by fecal excretion of *Y. enterocolitica*. However, they conferred partial protection against *Salmonella enterica* serovar. Typhimurium, suggesting that an immunomodulation mechanism was involved [27]. In the present study, we found that the presence of *Y. enterocolitica* in the Peyer's patches of experimentally infected mice was significantly shortened by *L. plantarum* with carao pretreatment, which was associated with the modulation of the host biological response. *Y. enterocolitica* O9 is a bacterium well adapted to the intestinal environment, as evidenced by its ability to remain in the intestine for long periods. Pretreatment with *L. plantarum* with carao could

not shorten the persistence of viable *yersinia* in the intestinal contents, which excludes any antibiosis mechanism of *L. plantarum* with carao against *Y. enterocolitica*. Indeed, the in vitro studies demonstrated that the inhibitory action of *L. plantarum* against enteropathogenic bacteria is due to acid production in dextrose-rich media but not in fecal samples [27]. However, the ability of *L. plantarum* with carao to accelerate bacterial clearance in PPs suggests an immunological mechanism. The proinflammatory cytokine TNF-α is a key molecule in innate immunity to infection [29]. The results presented in this study indicate that intestinal infection with *Y. enterocolitica* induces a local TNF-α response. This is consistent with reports showing that TNF-α production is required to restrict a primary *Y. enterocolitica* infection in mice [30,31]. Our data demonstrated that pretreatment with *L. plantarum* with carao increased TNF-α production in response to infection, while pretreatment with the fermented milk vehicle alone had no effect. Therefore, pretreatment with *L. plantarum* with carao could exert a protective effect against *Y. enterocolitica* infection by enhancing the TNF-α response. This protective effect of *L. plantarum* with carao could extend to other intestinal pathogens whose elimination is mediated by TNF-α. However, along with their defensive role, proinflammatory cytokine responses can be detrimental to the host; therefore, regulatory mechanisms exist to limit the extent of collateral damage to host tissues.

5. Conclusions

The viability under different conditions, immunomodulatory capacity, and probiotic potential of *L. plantarum* as affected by carao were tested and investigated. The results indicate that carao did not affect the immunomodulatory capacity of *L. plantarum*. *Carao* increased the counts of acid tolerance, bile tolerance, growth on TSB broth, and NaCl resistance. According to the results, carao may improve the attributes of *L. plantarum* when fortifying fermented dairy products. According to the results, carao may enhance the characteristics of *L. plantarum* when enriching fermented dairy products.

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