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# Impact of Novel Functional Ingredients on *Lactobacillus casei* Viability

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**Abstract:** Nipple fruit (*Solanum mammosum*), teosinte (*Dioon mejiae*), Caesar mushroom (*Amanita caesarea),* and weevil (*Rhynchophorus palmarum*) powders have shown great nutritional content with meaningful dietary applications. This study aspired to investigate the impact of nipple fruit, teosinte, Caesar mushroom, and weevil powders on the bile tolerance, acid tolerance, lysozyme tolerance, gastric juice resistance, and protease activity of *Lactobacillus casei*. Nipple fruit, teosinte, Caesar mushroom, and weevil powders were combined at 2% (wt/vol), whereas the control samples did not include the ingredients. The bile and acid tolerances were analyzed in Difco De Man–Rogosa–Sharpe broth incubated under aerobic conditions at 37 °C. The bile tolerance was investigated by adding 0.3% oxgall, whereas the acid tolerance was studied by modifying the pH to 2.0. The lysozyme tolerance was studied in electrolyte solution containing lysozyme (100 mg/L), while the gastric juice tolerance was analyzed at pH levels of 2, 3, 4, 5, and 7. The protease activity was studied spectrophotometrically at 340 nm in skim milk incubated under aerobic conditions at 37 °C. The results show that nipple fruit increased the counts, whereas Caesar mushroom and weevil powders resulted in lower counts for bile tolerance, acid tolerance, lysozyme resistance, and simulated gastric juice tolerance characteristics. Furthermore, the protease activity increased by adding nipple fruit to skim milk. According to the results, nipple fruit may improve the characteristics of *L. casei* in cultured dairy by-products.

**Keywords:** probiotic characteristics; *Lactobacillus casei*; *Rhynchophorus palmarum*; *Dioon mejiae*; *Amanita caesarea*



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## 1. Introduction

*Lactobacillus casei* strains are autochthonous microorganisms associated with the human host [1]. *L. casei* is a probiotic bacterium that effectively balances intestinal microflora, regulates intestinal disorders, controls the immune system, modulates the cellular immune response, and has a potent antidiarrheal action [2]. Industrially, *L. casei* has applications as probiotics in humans, as an acid-producing starter culture for milk fermentations, and especially as cultures for the intensification and acceleration of flavor development in certain varieties of probiotic yogurt [3].

*L. casei* may regulate gastrointestinal disorders caused by pathogenic bacteria. *L. casei* improves digestion and reactions to milk. The probiotic strain improves lactose digestion and stimulates a healthy gut microbiota, which is critical for digestive health. As a probiotic, *L. casei* improves digestion, reduces symptoms of lactose intolerance, strengthens the immune system, and relieves constipation. In addition, it participates in the production and optimizes the absorption of B and K vitamins. *L. casei* has demonstrated potential in

restoring the intestinal barrier function and intestinal microbiota compromised by anti-inflammatory drugs. Its use also promotes positive results for some diseases related to the digestive tract and chronic infectious diseases, as well as in obesity and patients with depression. *L. casei* supports neutrophilic function and insulin resistance in obese patients, promoting the reduction of metabolic endotoxemia and altering the composition of the intestinal flora and intestinal permeability. *L. casei* alleviates cytokine-induced epithelial barrier dysfunctions in intestinal epithelial cells [3].

It was proposed to carry out a study with the purpose of evaluating whether the continued consumption of milk fermented with *Lactobacillus casei* (DN-114001) showed a beneficial effect on the incidence of the most common infectious disorders in children. The probiotics that have demonstrated the greatest efficacy in treating diarrhea are *L. rhamnosus*, *S. boulardii*, and *Lactococcus lactis*, and those that have demonstrated the greatest efficacy in preventing acute diarrhea are *S. boulardii*, *L. rhamnosus* GG, *B. lactis*, and *S. thermophiles*. The probiotics reduce the duration of symptoms when diarrhea is already established and prevent its onset when there has been recent and close contact with someone who has experienced acute viral diarrhea. Furthermore, optimistic findings have been delivered from some studies on *L. casei* and other lactobacilli probiotics, such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, for treating diarrhea induced by *Clostridium difficile* [4]. However, the WHO has demonstrated that the advantages related to probiotics are strain-dependent; their outcomes rely on the strain utilized. *L. casei* prevents impaired barrier function in intestinal epithelial cells [5]. Other studies have reported that even dead cells can stop the development of intestinal inflammation by maintaining and modulating the intestinal barrier [6].

It has been shown that the quality and quantity of dietary components actively regulate the microbiome of the intestine, with implications on mood, stress, and anxiety and possible therapeutic effects derived from its modulation with probiotics. (*L. acidophilus*, *L. casei*, and *B. Bifidum*) However, more prospective studies in humans are required to plan comprehensive and personalized dietary interventions [6].

The nipple fruit (*Solanum mammosum*) [7], teosinte (*Dioon mejiae*) [8], Caesar mushroom (*Amanita caesarea*) [9], and weevil (*Rhynchophorus palmarum*) were carefully studied for their *L. acidophilus* attributes. The nipple fruit (*Solanum mammosum*) [7], teosinte (*Dioon mejiae*) [8], and Caesar mushroom (*Amanita caesarea*) [9] are ideal fiber, mineral, and protein sources. However, the palm weevil (*Rhynchophorus palmarum L.*) seems to be abundant in polyunsaturated fatty acids such as PUFA n3/omega-3 [10].

There is no research on *L. casei* properties as influenced by these ingredients. *L. casei* is a gram-positive bacteria used as a probiotic for fermented products, and this probiotic enhances the metabolism and improves gut microbiota. As a result, the current study aims to observe the effects of different food sources on *L. casei* attributes.

## 2. Materials and Methods

### *2.1. Plant Material*

Postharvest samples of the nipple fruit (*Solanum mammosum*), teosinte (*Dioon mejiae*), and Caesar mushroom (*Amanita caesarea*) were collected at different times and years in the Guapinol Biological Reserve, Marcovia Municipality, Choluteca Department (Honduras). The plant materials were dried in the shade at room temperature for 20 days and then ground to a powder. Individually, plant material solution (10% wt./wt.) was freeze-dried (LIOTOP model L 101, São Carlos-SP, Brazil) for 48 h at −75 °C and 0.5 Pa. The weevils were dried (Digitronic TFT- Selecta, J.P. SELECTA, Barcelona, Spain) at 50 °C/48 h and were grounded in a Retsch SM 100 knife mill (Retsch GmbH, Haan, Germany) (501–700 mm) [11].

### *2.2. Tolerance to Bile Salt*

A 1% culture of *L. casei* (Danisco, DairyConnection, Madison, WI, USA) was used in peptone water (0.1% w/v) at room temperature (21 °C). The cultures were added to a solution that was prepared with 0.925 g of Difco De Man–Rogosa–Sharpe (MRS) broth,

20 mL of distilled water, 0.05 g of bile salts, and 0.5 g of the ingredients. The control samples did not contain the ingredients. MRS agar was used as a growth medium for the bacteria, and a solution of 82.5 g of MRS, 22.5 g of agar, and 1500 mL of distilled water was prepared. Later, the pH was adjusted to 5.2, and the solution was mixed and boiled (100 °C). A lactose solution of 0.625 g in 25 mL of distilled water was prepared. Finally, the inoculation was carried out using 2.5 mL of culture and 5 mL of lactose in the mother solution to treat the treatments and the control samples. The analysis was carried out in serial solutions, and plating was carried out at hourly intervals (0, 4, and 8 h) to determine the resistance of the bacteria together with the ingredients. The experiment was carried out in three repetitions. Once the hours of incubation were completed (72 h), the bacteria were counted to find the countable plate (25 to 250 CFU) [12].

### *2.3. Acid Tolerance*

For this analysis, the same procedure used for the tolerance to bile salts was used, with small modifications in the broths and the sowing times. For this analysis, bile salts were not used, but the pH of the broths was adjusted to 2 to simulate the acidity of the digestive system. The time intervals were 0, 30 min, and 1 h for *L. casei*. The sowing time intervals were 0, 15, and 30 min [12].

### *2.4. Tolerance to Gastric Juices*

*L. casei* (10% *v*/*v*) was inoculated in an electrolyte solution with $H_2O$, pepsin 0.32% (Sigma-Aldrich, St. Louis, MO, USA), and NaCl 0.2%. The pH was set up with NaOH and HCl. The electrolyte solution was prepared at pH levels of 2, 3, 4, 5, and 7. After the inoculation, the culture media was incubated in anaerobic conditions at 37 °C for 30 min. After 30 min of growth, 1 mL was taken and enumerated in 10-fold diluted peptone water and plated in duplicate with MRS agar. The inoculum was enumerated at 0 and 30 min to determine the colony-forming units by duplicated plate reading [13,14]. All determinations were made in triplicate.

### *2.5. Lysozyme Activity*

The activity of the lysozyme enzyme has the same principle of acid tolerance and tolerance to bile salts in the use of peptone water and agars for each of the bacteria, with certain modifications in the preparation of the samples. For the elaboration of this analysis, 20 mL of distilled water, 0.04 g of calcium chloride, 0.12 g of hydrochloric sodium, 0.03 g of sodium bicarbonate, 0.04 g of hydrochloric potassium, and 0.5 g of ingredients were used; a control containing no percentage of ingredients was also used for this analysis. The solution for the enzyme was prepared in 10 mL of distilled water using 10 mg of the powdered lysozyme. In addition, 2.5 mL of the bacteria plus 2.5 mL of the lysozyme solution were inoculated into the bottles containing the sample and the control [15].

### *2.6. Protease Activity*

To analyze proteolytic activity, 8.8 mL of skim milk (previously sterilized in an autoclave) was placed in a test tube, and 0.2 g of ingredients was added; a control that did not contain the ingredients powder was also used. The o-phthalaldehyde (OPA) reagent was prepared, which was made up of different reagent solutions, starting with the 560 mg OPA solution plus 14 mL of distilled water, then 35 mL of sodium dodecyl sulfate was added, a second 13.38 solution of sodium borate was prepared, and 350 mL of distilled water added. Finally, the solutions and reagents were mixed, and, later, 1.4 mL of 2-mercaptoethanol was added and the OPA reagent was obtained; this reagent was placed in a volumetric flask covered with aluminum foil to prevent its evaporation by light. For this analysis, a reagent was also prepared with 83.53 g of trichloroacetic acid and 1100 mL of distilled water, which was used for the 3 repetitions of the study. In addition, 1 mL of the culture (*L. casei*) was inoculated in the test tubes, and, later, 2.5 mL of the sample, 1 mL of distilled water, and 5 mL of trichloroacetic acid were added to another test tube; this solution was filtered for

10 min using filter paper. Once filtered, 0.15 mL of the sample was placed in a cuvette to which 3 mL of the OPA reagent was previously added, after which the cuvette was placed in the spectrophotometer to obtain the amount of absorbance of the sample. This analysis was carried out in time intervals of 0, 12, and 24 h, in which the same sample preparation procedure was repeated and the absorbance was measured using a spectrophotometer (340 nm) (Nicolet Evolution 100, Thermo Scientific; Madison, WI, USA) [16].

### *2.7. Statistical Analysis*

Data were analyzed using Statistical Analysis Systems (SASs) and examined using the general linear model. Differences of least square means were utilized to examine significant differences for the ingredient effect, time effect, and ingredient × time effect. A Tukey's test was utilized to define the statistical disparities among the ingredient effect, time effect, and ingredient × time effect at $\alpha = 0.05$.

## 3. Results and Discussion

### *3.1. Bile Tolerance*

Bile is a yellow-green liquid essential for digesting fats and removing worn red blood cells and certain toxins from the body. Bile is stored in the gallbladder, and it contains minerals, cholesterol, fats, phospholipids, bile pigments, and bile acids. Bile secretion is synthesized and secreted by the hepatocyte into the bile ducts, which drain into the hepatic duct. Bile performs crucial biological functions, such as promoting the use of fats as a natural emulsifier, activating lipase, improving the digestibility of fats, and protecting the liver. After a meal, bile is secreted into the small intestine [17].

Bile acids have the biological activity of regulating the absorption of fat-soluble vitamins, cholesterol, and lipids. Bile salts make it possible to emulsify fats. It must be remembered that fats are not water-soluble, so bile emulsifies them; that is, it converts them into a suspension of tiny droplets so that the mucous membranes can more easily absorb them.

The most useful components of bile are bile acids (a class of steroids) and lecithin (a phospholipid), which coat dietary fat globules and facilitate their digestion. Micelles are like small droplets made by the liver and carried through the bile duct. The small intestine absorbs fatty acids, monoglycerides, cholesterol, and fat-soluble vitamins (A, D, E, and K). The components fit onto the surface of the absorbing cells of the intestinal wall, discharge their contents into the cells to be processed, and repeat the process until all the fats and nutrients in a meal have been absorbed [17]. Nevertheless, the bile salts can inhibit the growth of probiotics [18]. Figure 1 shows the bile tolerance of *L. casei* over 8 h after incorporating the ingredients. The interaction effect (ingredient concentration × time) was not significant ($p > 0.05$), while the time (hour) effect and ingredient effect were significant ($p < 0.05$) (Tables 1 and 2).

The nipple fruit improved the bile tolerance of *L. casei*, and the growth decreased over time. Caesar mushroom and weevil powders resulted in significantly ($p < 0.05$) lower counts. The growth remained stable until 4 h and significantly ($p < 0.05$) decreased at 8 h. This is a good result since the high antioxidant capacity of this fruit [18] at this concentration does not cause a marked inhibition of the development of *L. casei*. Other reports examined the bile tolerance of *L. acidophilus* and concluded that nipple fruit improved the growth in MRS broth [19]. Furthermore, for other plants, such as carao (*Cassia grandis*), Paz et al. (2022) [20] reported the bile tolerance of *L. bulgaricus* over 8 h of incubation in MRS broth, with it having 0.03% oxgall and improvement with growth. In addition, Aleman et al. (2023) showed that Caesar mushroom and weevil powders inhibit the growth of L. acidophilus under oxgall salt (0.03%) in MRS broth [21]. The bile in the gallbladder originates in the liver. It is a greenish-to-brownish liquid composed of water, cholesterol, phospholipids (a type of fat), bile salts, proteins, and bilirubin. Bilirubin is the product of the breakdown of hemoglobin in the liver and gives bile its yellowish color. The liver eliminates toxins, excess

cholesterol, and impurities of one kind [22]. It is important that functional ingredients can improve the probiotic bile tolerance to improve cultured foods' nutritional profile.

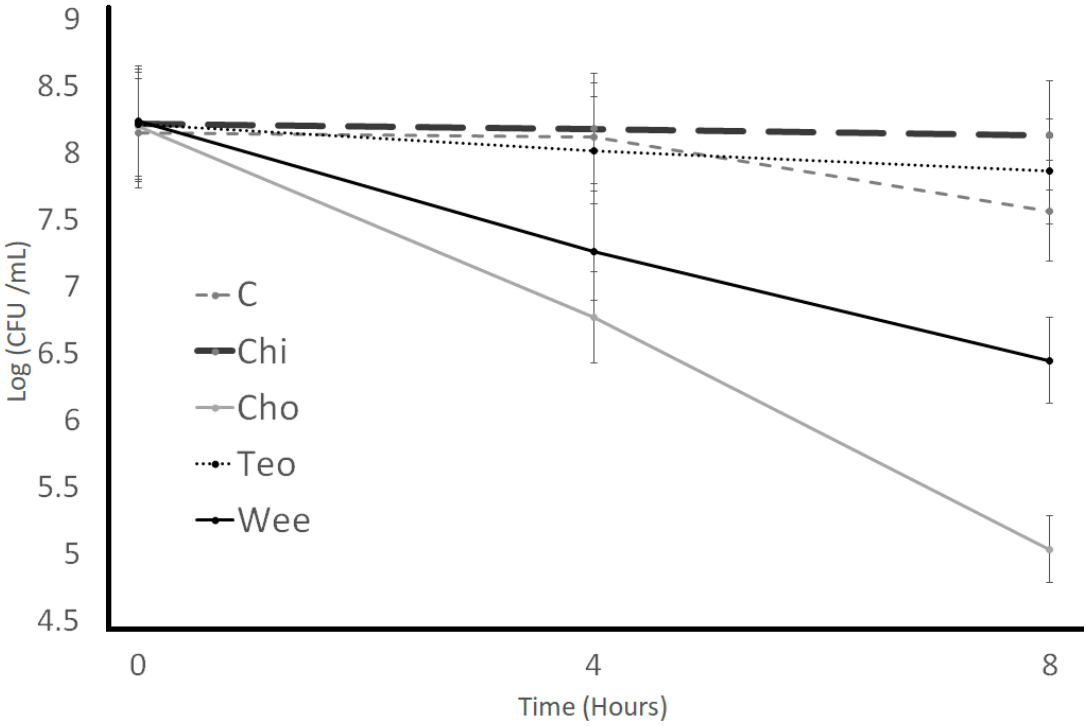


**Figure 1.** Bile tolerance (0.3% oxgalt) of *L. casei* in MRS broth as influenced by ingredients over 8 h. Average of three replicates. Error bars represent standard deviation. C = control, Chi = nipple fruit, Cho = Caesar mushroom, Teo = teosinte flour, Wee = weevil flour.

**Table 1.** The *p*-values 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 | |
| Ingredient | 0.0345 |
| Time (hours) | <0.0001 |
| Ingredient × time | 0.1567 |
| Acid tolerance | |
| Ingredient | 0.0384 |
| Time (minutes) | <0.0001 |
| Ingredient × time | 0.2679 |
| Resistance to gastric juices | |
| Ingredient | 0.0111 |
| pH | <0.0001 |
| Ingredient × pH | 0.1349 |
| Protease activity | |
| Ingredient | 0.0367 |
| Time (hours) | <0.0001 |
| Ingredient × time | 0.2485 |
| Lysozyme resistance | |
| Ingredient | 0.0004 |
| Time (hours) | <0.0001 |
| Ingredient × time | 0.2764 |

**Table 2.** Least squares means for bacterial viability, bile tolerance, acid tolerance, resistance to gastric juices, protease activity, and lysozyme resistance of *Lactobacillus casei*, as influenced by ingredients.

| Test | *L. casei* |
|---|---|
| Bile tolerance | |
| Control | 7.95 $^{B}$ |
| Nipple fruit | 8.31 $^{A}$ |
| Weevil | 7.32 $^{C}$ |
| Caesar mushroom | 6.67 $^{D}$ |
| Teosinte | 8.05 $^{B}$ |
| Acid tolerance | |
| Control | 5.63 $^{B}$ |
| Nipple fruit | 5.94 $^{A}$ |
| Weevil | 4.99 $^{C}$ |
| Caesar mushroom | 4.94 $^{C}$ |
| Teosinte | 5.59 $^{B}$ |
| Resistance to gastric juices | |
| Control | 7.71 $^{A}$ |
| Nipple fruit | 7.41 $^{A}$ |
| Weevil | 7.55 $^{A}$ |
| Caesar mushroom | 7.64 $^{A}$ |
| Teosinte | 7.65 $^{A}$ |
| Protease activity | |
| Control | 0.731 $^{B}$ |
| Nipple fruit | 0.795 $^{A}$ |
| Weevil | 0.747 $^{B}$ |
| Caesar mushroom | 0.749 $^{B}$ |
| Teosinte | 0.726 $^{B}$ |
| Lysozyme resistance | |
| Control | 7.03 $^{B}$ |
| Nipple fruit | 8.27 $^{A}$ |
| Weevil | 6.41 $^{C}$ |
| Caesar mushroom | 6.72 $^{C}$ |
| Teosinte | 6.97 $^{B}$ |

$^{A–D}$ Means within a same column along same test with different letters differ statistically ($p < 0.05$). NS = no significant differences among control and ingredients.

### *3.2. Acid Tolerance and Resistance to Gastrointestinal Juices*

The acid tolerance was studied at a pH of 2 to examine the effect of the ingredients on the log counts of *L. casei*, simulating stomach conditions [23]. The stomach produces hydrochloric acid to digest food. The acidity of gastric juice is due to hydrochloric acid secreted by the stomach's parietal cells. It is essential for activating pepsinogen, which is transformed into pepsin and initiates protein degradation and digestion [24].

Gastric juice is a clear liquid abundantly secreted by numerous microscopic glands scattered throughout the stomach mucosa. These juices are a highly corrosive mixture of various chemicals: hydrochloric acid, water, electrolytes (potassium, calcium, and sodium), and enzymes called pepsins. Its highly corrosive action, and the contraction movements carried out by the stomach make digestion possible. Its function is to help digest proteins. Due to the presence of hydrochloric acid, the pH takes a value between one and two. This acid environment facilitates the breakdown of proteins to convert them into smaller units. Its pepsin and renin enzymes favor the absorption of nutrients in the small intestine because they break down proteins into smaller subunits that are easier to digest. The stomach is capable of secreting gastric juices each day to break down the food and turn it into a pasty substance called chyme, from which the small intestine obtains the necessary nutrients for the body, and then it pours into the bloodstream [24].

The acid tolerance and resistance to gastrointestinal juices of *L. casei* as impacted by the ingredients are shown in Figures 2 and 3. For the acid tolerance and resistance to gastrointestinal juices, the interaction effect (ingredient × time) was not significant ($p > 0.05$), whereas the time (minutes) effect and ingredient effect were significant ($p < 0.05$) (Tables 1 and 2). The nipple fruit improved the acid tolerance of *L. casei*, while Caesar mushroom and weevil powders resulted in significantly ($p < 0.05$) lower counts, and the growth decreased over time. The growth significantly ($p < 0.05$) decreased at 0 to 10 min and remained stable from 10 to 15 min.

Aleman et al. (2023) [19] examined the acid tolerance of L. acidophilus and concluded that nipple fruit improved the growth in MRS broth. In addition, Aleman et al. (2023) [21] showed that Caesar mushroom and weevil powders inhibit the growth of L. acidophilus under acidic conditions. The buffering capacity of potential compounds in the nipple fruit could prevent bacteria decay under acidified conditions. The pH is an essential factor influencing the growth of microorganisms. Some bacteria generally grow at a low pH (3.0), and fungi thrive at a low pH (1.0) [25]. However, the optimum pH range for bacteria is 6.0 to 8.5, and few prefer a pH of 8.5 or higher. Fungi can grow in media with a pH of up to 8.5. Still, most of them like an acidic pH and can alter the pH of an unbuffered medium by the products they generate during their growth, as occurs with some of the bacteria [26]. Acid tolerance is considered a significant property in selecting potential bacteria as probiotics [27]. The results show the prospect of nipple fruit to enhance the tolerance of *L. casei* in the small intestine, where the pH varies from pH 6 to 7.4.

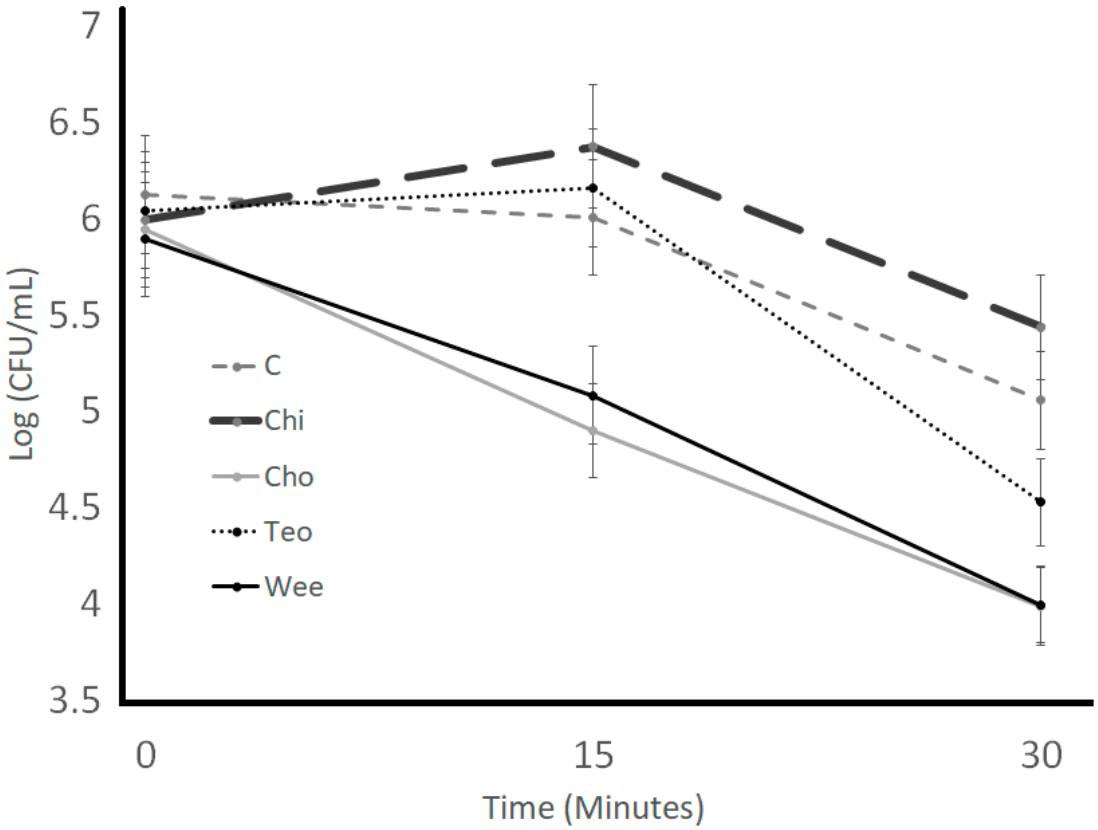


**Figure 2.** Acid tolerance (pH 2) of *L. casei* in MRS broth as influenced by ingredients over 30 min. Error bars represent standard deviation. C = control, Chi = nipple fruit, Cho = Caesar mushroom, Teo = teosinte flour, Wee = weevil flour.

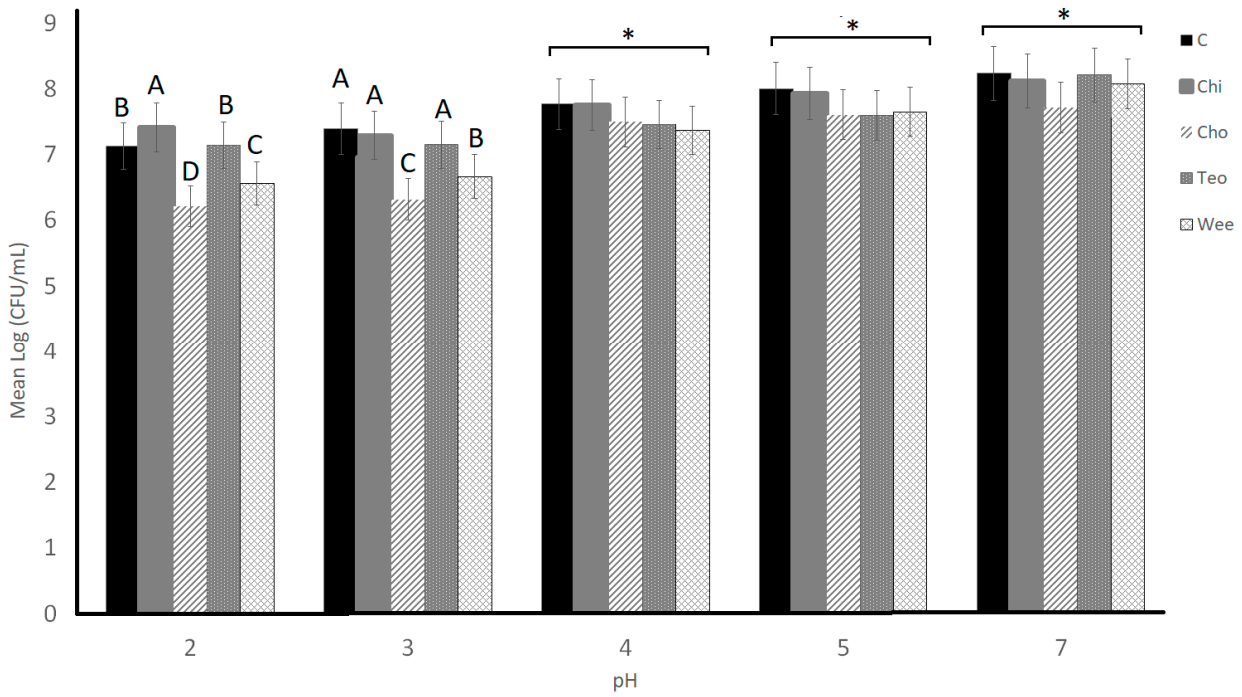


**Figure 3.** Resistance of *L. casei* to simulated gastric juice (pH levels of 2, 3, 4, 5, and 7) in formulated gastric juice solution as influenced by ingredients over different pH conditions. Average of three replicates. Error bars represent standard deviation. * No significant differences among control and ingredients in pH levels of 4, 5, and 7. $^{A–D}$ Means within a same column along same test with different letter differ statistically ($p < 0.05$) among control and ingredients in pH levels of 2 and 3. C = control, Chi = nipple fruit, Cho = Caesar mushroom, Teo = teosinte flour, Wee = weevil flour.

### *3.3. Resistance to Lysozyme*

Lysozyme, or muramidase, is an enzyme found in most mammals, including humans. Lysozyme has a bactericidal function by hydrolyzing peptidoglycan in bacterial cell walls; it primarily acts by weakening the cell wall rather than causing cell damage directly. It facilitates bacterial lysis and can enhance the bacterial susceptibility to other antimicrobial agents or phagocytosis by immune cells. It is found in secretions such as tears, mucus, or saliva; it is also found in large amounts in human breast milk and can be found in organs such as the spleen or lungs, tissues such as bone cartilage, and blood [28]. Antimicrobial enzymes that have been studied are, among others, proteolytic, oxidative, and those with the ability to hydrolyze polysaccharides, including amylases, lyases, Dispersin B, and lysozymes [28]. In particular, the activity of lysozymes against various microorganisms has been widely reported due to their ability to hydrolyze the polymers present in the cell walls of bacteria, specifically those belonging to the gram (+) group [29]. Recent studies have also reported this enzyme's ability to hydrolyze microorganisms' cell walls [30–32]. Likewise, its effectiveness as an antibacterial and antifungal agent has been reported when combined with other active compounds [33–36]. Resistance to lysozyme is shown in Figure 4. The ingredient and time effects were significant ($p < 0.05$), whereas the interaction effect (ingredient × time) was not significant ($p > 0.05$) (Tables 1 and 2). The interaction effect was insignificant ($p > 0.05$), meaning the control and nipple fruit samples followed the same trend. The log counts of the control and nipple fruit samples decreased over time.

For the control and nipple fruit treatments, the log counts decreased from 0 to 60 min and remained stable from 60 to 120 min. The nipple fruit treatments reported a significantly ($p < 0.05$) higher viability than the control samples (Table 2). However, Caesar mushroom and weevil powders resulted in significantly ($p < 0.05$) lower counts. Nipple fruit have considerable amounts of sucrose [21], and this substrate could be used for the survivability of *L. casei*. Furthermore, Aleman et al. (2023) [19] examined the acid lysozyme of *L. acidophilus*

and concluded that nipple fruit improved its growth in MRS broth. Paz et al. (2022) [20] also reported that carao improved the acid and bile tolerance of *L. bulgaricus* in MRS broth. In addition, Aleman et al. (2023) showed that Caesar mushroom and weevil powders inhibit the growth of *L. acidophilus* in an electrolyte solution with lysozyme [21]. Lysozyme destroys the peptidoglycan barrier, an important cytoplasmic membrane element [37]. Lysozymes are enzymes, and they are typically found in the extracellular environment or within certain cells [38]. Lysozymes are involved in various cellular processes [39]. They are in charge of recycling waste cell debris [39].

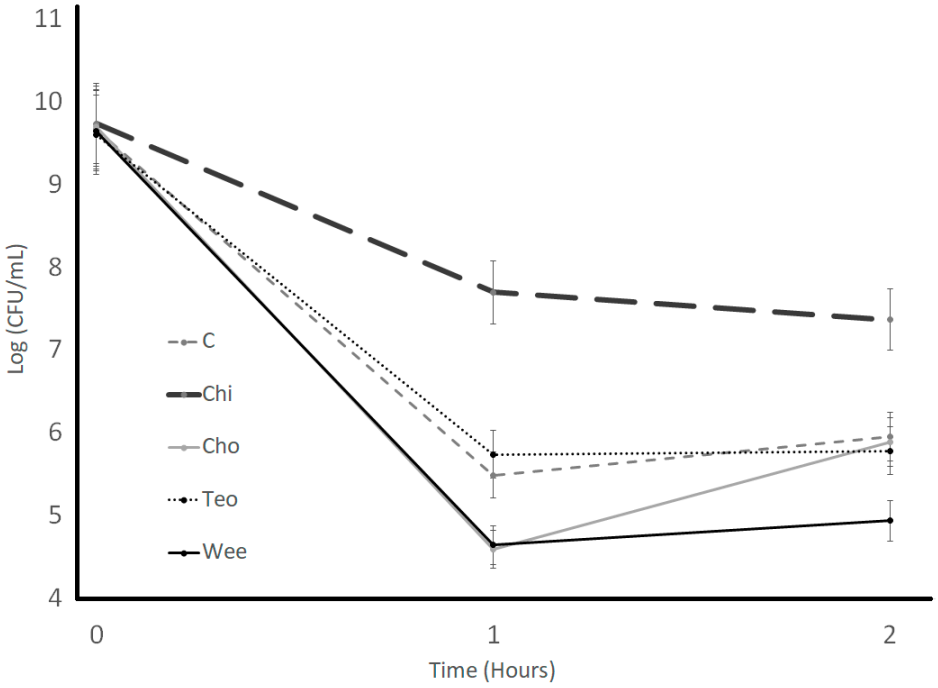


**Figure 4.** Resistance to lysozyme (100 mg/L) of *L. casei* in electrolyte solution as ingredients during incubation time of 2 h. Average of three replicates. Error bars represent standard deviation. C = control, Chi = nipple fruit, Cho = Caesar mushroom, Teo = teosinte flour, Wee = weevil flour.

### *3.4. Protease Activity*

Proteases, present in all living organisms, catalyze the hydrolysis of peptide bonds in proteins. They are grouped according to the amino acid residues of the active site and the mechanisms of action into five groups: serine, cysteine, aspartic, metalloproteases, and peptidases of an unknown catalytic mechanism [40]. The protease activity of *L. casei* is illustrated in Figure 5. The ingredient and time effects were significant ($p < 0.05$), whereas the interaction effect (ingredient × time) was not significant ($p > 0.05$) (Tables 1 and 2), indicating that the nipple fruit samples obeyed a similar trend to control samples. The control and ingredient samples improved the growth over time. The protease activity of *L. casei* improved the growth after 24 h for the control and nipple fruit treatments (Figure 5). All treatments showed no significant difference ($p > 0.05$) from the control samples at 0 h and 12 h, whereas nipple fruit treatments had significantly higher protease activity at 24 h. Nipple fruit has substantial quantities of sucrose, which could improve the growth of *L. casei*, contributing to a more elevated protease activity. The main and general function of proteolysis is to break down the protein molecules in the food that are digested into the smallest amino acids that the body can use. The process is also necessary for the production of active proteins from synthesized polypeptide chains. Proteolysis is also involved in flavor and odor formation and assists in giving desired textural properties [40].

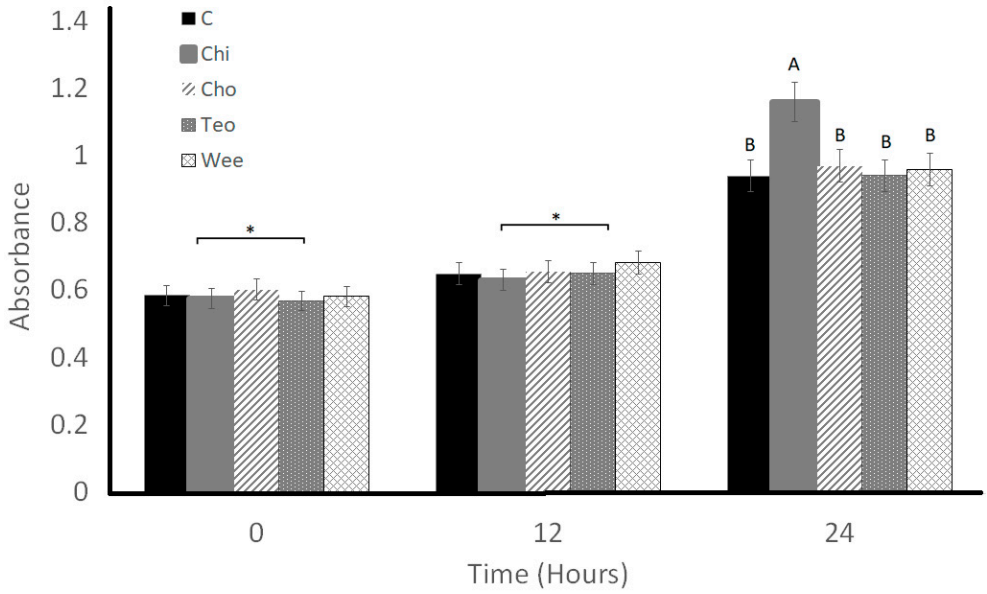


**Figure 5.** The protease activity (37 °C) of *L. casei* in skim milk as influenced by ingredients over an incubation period of 24 h. * Average of three replicates. Values with different letters are significantly different ($p < 0.05$). Error bars represent standard deviation. $^{AB}$ Means with different letter means significant difference ($p > 0.05$) among treatments in 12 h. * Indicates no significant differences ($p > 0.05$) among treatments in 0 h and 12 h. C = control, Chi = nipple fruit, Cho = Caesar mushroom, Teo = teosinte flour, Wee = weevil flour.

## 4. Conclusions

Prebiotics and probiotics are the two most well-known and studied functional food ingredients that can benefit similarly by promoting intestinal health. The probiotic characteristics of *L. casei* were tested and investigated. This study delved into a captivating area of study, focusing on the effects of nipple fruit, teosinte, Caesar mushroom, and weevil powders on the bile tolerance, acid tolerance, lysozyme tolerance, gastric juice resistance, and protease activity of *Lactobacillus casei*. This study's findings reveal that nipple fruit positively impacted the viability of *L. casei*, while Caesar mushroom and weevil powders showed negative effects on bile tolerance, acid tolerance, lysozyme resistance, and simulated gastric juice tolerance characteristics. Consequently, nipple fruit may enhance the characteristics of *L. casei* in cultured dairy by-products. Also, the protease activity was improved by adding nipple fruit to skim milk. Some of these novel food sources, alongside *L. casei*, can be used in developing new fermented dairy products for health purposes. According to the results, nipple fruit may improve the characteristics of *L. casei* in cultured dairy by-products. Further studies, such as on the probiotic properties of nipple fruit in in vivo models, should be carried out to determine the applications of *L. casei* with nipple fruit in cultured products.

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