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Effects of Weevil (*Rhynchophorus palmarum*), Teosinte (*Dioon mejiae*) and Caesar's Mushroom (*Amanita caesarea*) on the Properties of *Lactobacillus acidophilus* LA-K

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Abstract: Weevil (*Rhynchophorus palmarum*) powder, teosinte (*Dioon mejiae*) and Caesar's mushroom (*Amanita caesarea*) flour have bioactive compounds with significant nutritional applications. This paper aimed to examine the influence of weevil, teosinte, and mushroom powders on the protease activity, acid tolerance, bile tolerance, lysozyme tolerance, and gastric juice resistance ability of *Lactobacillus acidophilus* LA-K. Acid tolerance was determined by adjusting the pH of MRS broth to 2.0 for *L. acidophilus* incubated under aerobic conditions at 37 °C. Bile tolerance was determined by incorporating 0.3% of oxgall. Protease activity was determined spectrophotometrically at 340 nm. Resistance to 100 mg/L of lysozyme in an electrolyte solution was also determined. All ingredients were incorporated at 2% (*w*/*v*), while the control had no added ingredients. Acid and lysozyme tolerance were examined at 0, 30, 60, 90, and 120 min of incubation, whereas bile tolerance was analyzed at 0, 4, and 8 h. Gastric juice tolerance was determined at pH 2, 3, 4, 5, and 7 during 0 and 30 min of incubation, while protease activity was evaluated at 0, 12, and 24 h. Use of weevil flour, and Caesar's mushroom powder resulted in significantly ($p < 0.05$) lower counts for bile tolerance, acid tolerance, lysozyme resistance and simulated gastric juice tolerance characteristics. Protease activity increased with the use of teosinte flour. As such, this probiotic bacterium can be used alongside certain novel food sources at 2% concentration in the manufacture of fermented products such as yogurt.

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

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1. Introduction

The human gastrointestinal tract (GIT) harbors a complex and dynamic microbial ecosystem, the gastrointestinal microbiome, which is estimated to include more than 400 different bacterial species [1], and is responsible for essential functions, including metabolic activities, trophic effects on the intestinal epithelium, and interactions with the host's immune system [2]. Inflammatory bowel disease (IBD) is a chronic relapsing disorder in which an abnormal interaction of the intestinal flora and the host is observed. IBD patients have an increased risk of developing colorectal cancer. Recently, probiotics have been proposed to restore intestinal microflora (eco-medicine) and reduce inflammation. Probiotics improve the nutritional and microbiological balance of GIT. They act as vectors

that deliver their active components to various target sites of the GIT, and whose fate and effects differ between strains. Most effects occur only when live microorganisms are ingested, but sometimes this may not be necessary to achieve the desired benefits [3].

Probiotics have been used in numerous studies in animal models with promising results. In human studies, the use of a multistrain probiotic (VSL#3, which contains four strains of lactobacilli, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus delbrueckii* sp. *Bulgaricus*, and *Lactobacillus plantarum*; strains of *bifidobacterial*, *Bifidobacterium longum*, *Bifidobacterium infantis*, and *Bifidobacterium breve*; and *Streptococcus salivarius* sp. *Thermophilus*) has been shown, with a high level of evidence, to decrease the activity (prevention of onset and maintenance of remission) of pouchitis (also known as *pouchitis*—a non-specific inflammation of the ileal pouch) in ulcerative colitis (UC) after ileal anastomosis [2]. Nevertheless, the International Dairy Federation believes that consuming a product containing approximately 10^6–10^7 of CFU per gram of viable bacteria is necessary for therapeutic benefits, meaning that the cultured products must be loaded with probiotics. As a result, prebiotics, a group of nutrients utilized for benefit by gut microbiota, are used to improve the probiotic characteristics [4].

One of the most common ways to apply probiotics is to use it with other ingredients such as dietary fibers, vitamins, and minerals [5] to improve valuable food sources. Thus, efforts to use new prebiotic sources is desirable [6]. *Dioon mejiae*, also named teosinte, is a growing fossil tree discovered in Honduras. It is native to the Department of Olancho, with the largest population being observed in Rio Grande and Saguay municipalities. This teosinte is a cycad, a species group that is heavy or bulky before the separation between monocots and dicotyledons, which is utilized for its nutritional properties, including its grains; it is not linked to the variety of Zea mays, which is also recognized by the common name, teosinte. Seeds of this plant are used to prepare flour for typical foods. The starch content of this seed, called sago, is so high that it is used as a nutritional booster [7]. Sago is known for being a good energy source, gluten-free source, blood pressure regulator, digestion modulator, weight regulator, and a nervous system booster [7].

Amanita caesarea, popularly recognized as Caesar's mushroom, is an edible fungus. It features an eye-catching orange crown, yellow gills, and a stem. Its importance has been recognized since the earliest days of the Romans [8]. Caesar's mushroom (*Amanita caesarea*) is an excellent source of protein, fiber, and mineral. Furthermore, it includes many phytochemical constituents, such as phenolics, tocopherols, ascorbic acid, and carotenoids. As a result, *Amanita caesarea* is a healthy item to include in a regular diet [9]. On the other hand, insects play an essential role as a source of proteins, fats, and vitamins in the diet of indigenous peoples worldwide. Insects contain polyunsaturated fats, some of which are easily digestible. The palm weevil (*Rhynchophorus palmarum* L.) seems to be rich in polyunsaturated fatty acids of the n-3 series (PUFA n3/omega-3) [10].

L. acidophilus is a Gram-positive bacteria used as a probiotic for fermented products to enhance metabolism and improve gut microbiota. Given the aforementioned context, the current study aims to observe the effects of using extracts of weevil (*Rhynchophorus palmarum*), teosinte (*Dioon mejiae*), and Caesar's mushroom (*Amanita caesarea*) on the properties of *L. acidophilus*.

2. Materials and Methods

2.1. Plant Material

Teosinte and Caesar's mushroom were collected from the Guapinol Biological Reserve, Marcovia Municipality, Choluteca Department (Honduras), around May and June 2022. Teosinte (*Dioon mejiae)* and Caesar's mushroom (*Amanita caesarea)* were individually placed in water solution (10% *w*/*w*), and then held at −80 °C temperature to freeze-dry in an Labconco (model L 101, Kansas City, MO, USA) lyophilizer for 48 h at −75 °C and 0.5 Pa. After lyophilization, the extracts were ground and stored in vacuum-pack plastic bags for further use.

Adult weevils were obtained from the entomology laboratory at the National University of Agriculture (UNAG). Weevils were dehydrated in a convection oven (Digitronic TFT-Selecta, J.P. SELECTA, Barcelona, Spain) at 50 °C/48 h, and were grounded using a knife mill Retsch SM 100 (Retsch GmbH, Haan, Germany) (501–700 mm) [11].

2.2. Experimental Design

Weevil, teosinte, and Caesar's mushroom were evaluated for their antioxidant capability, total phenolic compounds, and contents of carotenoids, sugar, and organic acid. Their viability properties were examined on *L. acidophilus* (Danisco, DairyConnection, Madison, WI, USA), and enumerated using MRS agar. Three ingredients at 2% concentration (*w*/*w*) (weevil, teosinte, and Caesar's mushroom) were investigated and compared with the control (no ingredient). *L. acidophilus* LA-K was inoculated at 10% *v/w* in all the tests. Different tests were applied in MRS broth, including viability examination (microbial growth), pH 2 (acid tolerance), and oxgall salt (0.3%) (bile tolerance). Also, the culture was examined in an electrolyte solution with lysozyme (100 mg/L) and simulated gastric juice containing pepsin (0.32%) and NaCl (0.2%). Protease activity was analyzed in skim milk. Different time points were evaluated among each test (detailed in sections below). All experiments were repeated and triplicated with duplicate readings [12].

2.3. Total Polyphenols

Total phenolic compounds (TPCs) were analyzed using the Folin–Ciocalteu technique with slight modification. Briefly, 10 g of the dry and pulverized material of each plant material (weevil, teosinte, and Caesar's mushroom) was macerated in 80% aqueous methanol in an amber bottle at room temperature for 24 h with occasional shaking; it was then immediately filtered, and the residue of this filtration was extracted two more times in the same manner. The extract evaporates most of the solvent without heating beyond 40 °C. The evaporation product was lyophilized. Briefly, 2 mg of each lyophilized extract was placed in an Erlenmeyer flask, to which 50 mL of distilled water was added, and was then shaken. Next, 0.5 mL of an aliquot of solution was mixed with 0.75 mL of Folin–Ciocalteu reagent, leaving it to stand at room temperature for 5 min, and then, 0.75 mL of 20% 1 N sodium bicarbonate (Na_2CO_3) was added, which was let to rest for 90 min at room temperature. A Lambda 35 UV/Vis spectrophotometer (Perkin Elmer Instruments, Norwalk, CT, USA) was used to test the blue complex's absorbance at 750 nm. A standard solution of gallic acid was prepared from 0.1 g/L, in which 25 mg was gallic acid, placed in a 25 mL volumetric flask, and brought to volume with distilled water. The total phenolic content was estimated as mg gallic acid equivalent/g dry weight (mg GAE/g) [13].

2.4. Radical-Scavenging Activity Assay

Briefly, 0.1 mL of samples were dissolved with an additional 0.4 mL of 80% methanol and transferred to a 1.5 mL centrifuge tube. Next, 20 µL was added to a 150 µmol/L solution of DPPH (1, 1-diphenyl-2-picrylhydrazyl) to determine the antioxidant activity of the plant extracts. All reactions were carried out for 30 min at room temperature in 96-well microplates protected from light. Then, the absorbance was measured at 520 nm using an Elisa Versa Max Tunable Spectrometer Microplate Reader (Perkin Elmer Instruments, Norwalk, CT, USA). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was utilized as the reference [14].

The antioxidant activity is expressed as inhibition percentage, which corresponds to the amount of DPPH radical neutralized by the extract at a certain concentration according to the following equation:

$$\boldsymbol{I}\% = \frac{\boldsymbol{blank\ absorbance - sample\ absorbance}}{\boldsymbol{blank\ absorbance}} \times 100 \quad (1)$$

The extracts were substituted using 80% methanol, and another 80% methanol mixture without DPPH was used. The antioxidant potential was determined using a reference calibration created with Trolox.

2.5. Total Carotenoids Content

Carotenoids were extracted from weevil, teosinte, and Caesar's mushroom powder using the method recommended by Cano et al. (2019) [15] with minor modifications. Briefly, 1 g of the lyophilized material was mixed with 0.5 g of magnesium carbonate and 50 µL of (all-E)-β-apo-8′-carotenal (0.40 mg/mL). For the extraction, 20 mL of tetrahydrofuran (THF) prepared with 0.1% (*w*/*v*) butylated hydroxytoluene (BHT) was used. The solution was combined and homogenized using an Omnimixer (OMNI Macro S®, OMNI International, Kennesaw, GA, USA) at 3000 g for 3 min before being placed in an ultrasonic water bath (J.P. Selecta S.A., Barcelona, Spain) for 30 min at 50–60 Hz and 360 W.

The obtained solution was centrifuged at 15,000× *g* for 10 min at 4 °C. Briefly, 20 mL of acetone was mixed into the pellet, and the sample was extracted 3 times. The supernatant was mixed using 20 mL of diethyl ether at the end to obtain a colorless pellet. When an emulsion appeared, 20 mL of NaCl solution (30% (*w*/*v*)) was applied. Using 2.5 g of anhydrous sodium sulfate, the organic phase was dried for 10 min at 25 °C. The sample was filtered using Whatman No. 1 filter paper, and the supernatant was washed using a rotating evaporator at 30 °C. The extract was diluted to 2 mL with a MeOH/MTBE/H_2O (45.5:52.5:2, *v*/*v*/*v*) solution and purified via a 0.45 µm filter. A Lambda 35 UV/Vis spectrophotometer was used to measure the total carotenoid concentration at 450 nm (Perkin Elmer Instruments, Waltham, MA, USA). The hexane solution was used as the control. The total carotenoid content was estimated using Equation (2).

$$\textbf{Total carotenoid content}\left(\frac{\boldsymbol{\mu g}}{\boldsymbol{g}}\right) = \frac{(\boldsymbol{A}\times\boldsymbol{V})}{\boldsymbol{E}1\%1\boldsymbol{cm}\times\boldsymbol{P}\times 100}\times 10^6 \quad (2)$$

where *A* is the absorption at 450 nm, *V* is the total volume of absorption (mL), *E1*%1 cm is the absorption coefficient of the carotenoids mixed in hexane (ξ = 2500 dL/g cm [16]), and *P* is the amount of the plant tissue extraction (g).

2.6. Organic Acid

Organic acids (citric, tartaric, L-malic, quinic, and succinic) (Sigma, St. Louis, MO, USA) with 0.5% *w/v* was used as an internal standard [17]. A Model 160 UV sensor (214 nm) was utilized. The sensor was set at 0.100 AU, and the calibration curve of the pulse was connected to a Vista 401 integrator (Varan Assoc., Sunnyvale, CA, USA) (attenuation of 4 and a chart speed of 0.5 cm/min). The injector and column (Bio-Rad Laboratories, Hercules, CA, USA) were inserted with a 40 × 4.6 mm ion exclusion filter capsule filled with Aminex HPX-85H resin and a 2-µ£ Rheodyne Model 7302 column inlet filter (Rheodyne, Cotati, CA, USA). The Degassed 0.0008 N H2S04 fluid was used as a mobile phase at 0.7 mL/min of flow velocity.

2.7. Sugar Profile

Sugars (fructose, maltose, glucose, and sucrose) (Sigma, St. Louis, MO, USA) with 0.5% *w/v* were used as an internal standard. Briefly, 10 g of plant material powder was combined and blended in 80% ethanol using a Vitris 45 homogenizer for 1 min. The obtained solution was boiled for 15 min, and filtered via a 0.2 µm pore size syringe filter (Whatman™ Puradisc, Buckinghamshire, UK). The samples were injected in a Beckman series 340 liquid chromatography coupled with a model 112 pump, an (model 210) injector coupled with a 20/IL sample loop, and a model 156 index refraction sensor. The HPLC system was coupled with a 300 mm × 7.8 mm id. column with Aminex HPX-87C resin, which was used at 75 °C and with a speed of 0.5 cm/min. The mobile phase was HPLC grade at a fluid rate of 1.2 mL/min [18].

2.8. Bile Tolerance Test

The bile tolerance of *L. acidophilus* was evaluated using Pereira and Gibson's 2002 [19] method with slight changes. Culture (10 *v*/*w*) was inoculated in an MRS broth supplemented with sodium thioglycolate (1% *w*/*v*), lactose (0.5% wt/*v*), and bile salt (oxgall salt 0.3%). The freeze-dried plant material was also added at 2% *w*/*v*. The cultured broths were incubated at 37 °C for several periods (0, 4, and 8 h). The samples were enumerated in 10-fold diluted peptone water, and plated in duplicate with MRS agar.

2.9. Acid Tolerance Test

The acid tolerance of *L. acidophilus* was evaluated using Pereira and Gibson's 2002 [19] method with slight changes. Culture (10 *v*/*w*) was inoculated in acidified MRS broth supplemented with sodium thioglycolate (1% *w*/*v*) and lactose (0.5% *wt*/*v*) (1N HCl, pH 2.0). The freeze-dried plant material was also added at 2% *w*/*v*. The acidified cultured broths were incubated at 37 °C for several periods (0, 5, 10, and 15 min). The samples were enumerated in 10-fold diluted peptone water and plated in duplicate with MRS agar.

2.10. Protease Activity

Protease activity of *L. acidophilus* was evaluated using Oberg et al.'s (1991) method [20]. Culture (10 *v*/*w*) was inoculated in skim milk. The freeze-dried plant material was also added at 2% *w*/*v*. The cultured broths were incubated at 37 °C for several periods (0, 12, and 24 h). Briefly, 2.5 mL of cultured milk was mixed with 10 mL of 0.75 N trichloroacetic acid (TCA) and 1 mL of distilled water. The obtained solution was filtered using a Whatman Number 2 filter paper. The TCA filtrate (150 uL) was mixed with an OPA reagent (3 mL), and the absorbance was measured utilizing a spectrophotometer at 340 nm (Nicolet Evolution 100, Thermo Scientific; Madison, WI, USA).

2.11. Viability Test

The acid tolerance of *L. acidophilus* was evaluated using Marcia et al.'s (2023) method [21]. Culture (10 *v*/*w*) was inoculated in an MRS broth supplemented with sodium thioglycolate (1% *w*/*v*) and lactose (0.5% *wt*/*v*). The freeze-dried plant material was also added at 2% *w*/*v*. The cultured broths were incubated at 37 °C for several periods (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h). The samples were enumerated in 10-fold diluted peptone water, and plated in duplicate with MRS agar.

2.12. Tolerance to Simulated Gastric Juice

The simulated gastric juice (SGJ) tolerance of *L. acidophilus* was evaluated as García-Ruiz et al. (2014) [22] and Liao et al. (2019) [23]. Culture (10 *v*/*w*) was inoculated in simulated gastric juice formulated with H_2O, pepsin 0.32% (Sigma-Aldrich, St. Louis, MO, USA), NaCl 0.2%, NaOH, and HCl for pH adjudication. The freeze-dried plant material was also added at 2% *w*/*v*. The simulated gastric juice was adjusted to five concentration gradients (pH 7.0, 5.0, 4.0, 3.0, and 2.0) with 1 M HCl and 1 M NaOH. The cultured simulated gastric juice was incubated at 37 °C for 30 min. The samples were enumerated in 10-fold diluted peptone water and plated in duplicate with MRS agar. Samples were counted at 0 and 30 min.

2.13. Lysozyme Tolerance Test

L. acidophilus resistance to lysozyme was evaluated using Zago et al.'s (2011) method [24]. Culture (10 *v*/*w*) was inoculated in an electrolyte solution formulated with 0.22 g/L $CaCl_2$, 6.2 g/L NaCl, 2.2 g/L KCl, and 1.2 g/L $NaHCO_3$ in the presence of lysozyme (100 mg/L) (Sigma-Aldrich). The freeze-dried plant material was also added at 2% *w*/*v*. The electrolyte solution was incubated at 37 °C for 0, 30, 60, 90, and 120 min. The samples were enumerated in 10-fold diluted peptone water, and plated in duplicate with MRS agar.

2.14. Enumeration of L. acidophilus

The samples were enumerated in 10-fold diluted peptone water, and plated in duplicate with MRS agar. The MRS agar was formulated with 1 L of distilled water, 55 g of MRS broth powder (Difco, Becton, Dickinson and Co., Sparks, MD, USA), and 12 g of agar powder. The MRS agar was adjusted to pH 5.2, and sterilized at 121 °C for 15 min (Vargas et al., 2015). The plates were incubated at 37 °C for 72 h. A Quebec Darkfield Colony Counter was used to estimate the colony forming units (Leica Inc., Buffalo, NY, USA) [25].

2.15. Statistical Analysis

The chemical analysis was conducted with an analysis of variance (ANOVA). Data regarding bile, acid, gastric juice, lysozyme tolerance and protease activity were examined using the General Linear Model (PROC GLM). Differences of least square means were used to determine significant differences at $p < 0.05$ for the main effects (ingredients and time) and interactions effects (ingredients*time). Tukey's test was used to determine the statistical differences ($p < 0.05$) among the main and interaction effects at $\alpha = 0.05$. Data were processed in Statistical Analysis Systems (SAS).

3. Results and Discussion

3.1. Chemical Compositions and Antioxidant Activity of Different Food Sources

Table 1 compares the results of TPC, TCC, and antioxidant activity of weevil, teosinte, and Caesar's mushroom powder. Weevil and Caesar's mushroom powder have the highest TPC, TCC, and antioxidant activity. Teosinte flour was low in TPC content, antioxidant activity, and TCC.

Table 1. Total phenolic content, total carotenoid content, and antioxidant activity of different plants.

Sources	TPC (μg GAE/mL)	TCC (mg Q/mL)	Antioxidant Activity (%)
Weevil	333.22 ± 10.67 [a]	7.65 ± 0.32 [a]	41.56 ± 2.77 [a]
Caesar's mushroom	305.73 ± 9.05 [a]	8.65 ± 0.45 [a]	38.78 ± 2.05 [a]
Teosinte	45.09 ± 2.65 [b]	3.04 ± 0.21 [b]	14.43 ± 1.02 [b]

Means with different letters within the same column represent significant differences at $p < 0.05$.

Tables 2 and 3 show the sugar and acid contents (g/100 g) of weevil, teosinte, and Caesar's mushroom powder. Caesar's mushroom powder had the highest amount of maltose compared to weevil and teosinte. Also, maltose was not detected in weevil and teosinte. Weevil contained the highest amount of significant ($p < 0.05$) quinic acid, but low amounts of other acids (equal or less than 0.05 g/100 g) compared to the other sources.

Table 2. Sugar profile of food sources.

Food Sources	Sucrose (g/100 g)	Fructose (g/100 g)	Glucose (g/100 g)	Maltose (g/100 g)
Weevil	0.04 ± 0.01 [a]	Not detected	Not detected	Not detected
Caesar's mushroom	0.05 ± 0.02 [a]	0.09 ± 0.02 [a]	0.27 ± 0.03 [a]	1.37 ± 0.19
Teosinte	0.06 ± 0.02 [a]	0.10 ± 0.03 [a]	0.22 ± 0.04 [a]	Not detected

Means with different letters within the same column represent significant differences at $p < 0.05$.

Table 3. Organic acid profile of different food sources.

Food Sources	Citric (g/100 g)	Tartaric (g/100 g)	L-Malic (g/100 g)	Quinic (g/100 g)	Succinic (g/100 g)
Weevil	0.07 ± 0.03 [a]	0.05 ± 0.01 [b]	Not detected	3.48 ± 0.06 [b]	0.10 ± 0.04 [b]
Caesar's mushroom	Not detected	1.31 ± 0.18 [a]	2.05 ± 0.21 [a]	Not detected	2.44 ± 0.24 [a]
Teosinte	0.04 ± 0.01 [a]	0.02 ± 0.00 [b]	0.88 ± 0.07 [b]	1.79 ± 0.10 [a]	0.05 ± 0.01 [b]

Means with different letters within the same column represent significant differences at $p < 0.05$.

Teosinte is traditionally used for food and beverage that indicate high polyphenol content, and according to El Gendy et al. (2022) [26], it has 83.00 ± 4.1% of inhibition in the ABTS method and a lower TPC (37.14 µg GAE/mg) compared to the obtained results. Teosinte is rich in nutritional values and calories due to the presence of carbohydrates such as starch with high amylopectin, essential amino acids, dietary fibers, and highly unsaturated fatty acids. Glucose and fructose were reported at 0.12 ± 0.01 g/100 g and 0.24 ± 0.01 g/100 g [7] in teosinte, respectively, while glucose content of the current study was higher (0.27 ± 0.03 g/100 g) and fructose content was lower (0.09 ± 0.02 g/100 g).

According to Zhu et al. (2016) [27], Caesar's mushroom showed 89.74% radical scavenging ability at 6.0 mg/mL of concentration, while at 0.5 mg/mL concentration, 14.43 ± 1.02% of antioxidant activity was evaluated. The antioxidant ability of Caesar's mushrooms might be due to the presence of flavonoids, phenolic acids, and tannins. Monosaccharides of Caesar's mushroom mainly include D-glucose and α-D-lyxose; the backbone was made from 1,4 linked α-D-glucose and 1,3,6-linked α-D-glucose with branches from 1-linked α-D-lyxose [27]. In our study, weevil powder had low sugar content and significant amounts of quinic acid. Palm weevil (*Rhynchophorus palmarum* L.) appears to be abundant in PUFA n3/omega-3 and high-quality proteins [10].

3.2. Viability Test and Bile Tolerance

The viability of *L. acidophilus* over 10 h of incubation after adding the power of the three ingredients is shown in Figure 1. The ingredient effect and the interaction effect (ingredient × hour) were not significant ($p > 0.05$), whereas the hour effect was significant ($p < 0.05$) (Table 1). The interaction effect was not significant ($p > 0.05$). All treatments showed a similar viability growth as the control samples (Tables 4 and 5), and the control and broths with ingredients increased in log counts over time. Muramalla and Aryana (2011) [28] studied the growth of *L. acidophilus* in MRS broth and observed that viability increased in the first 3 h of incubation. Marcia et al. (2023) [21] and Aleman et al. (2023) [12] also noted that Carao and nipple fruit did not affect the growth of *Lactobacillus acidophilus* LA-K.

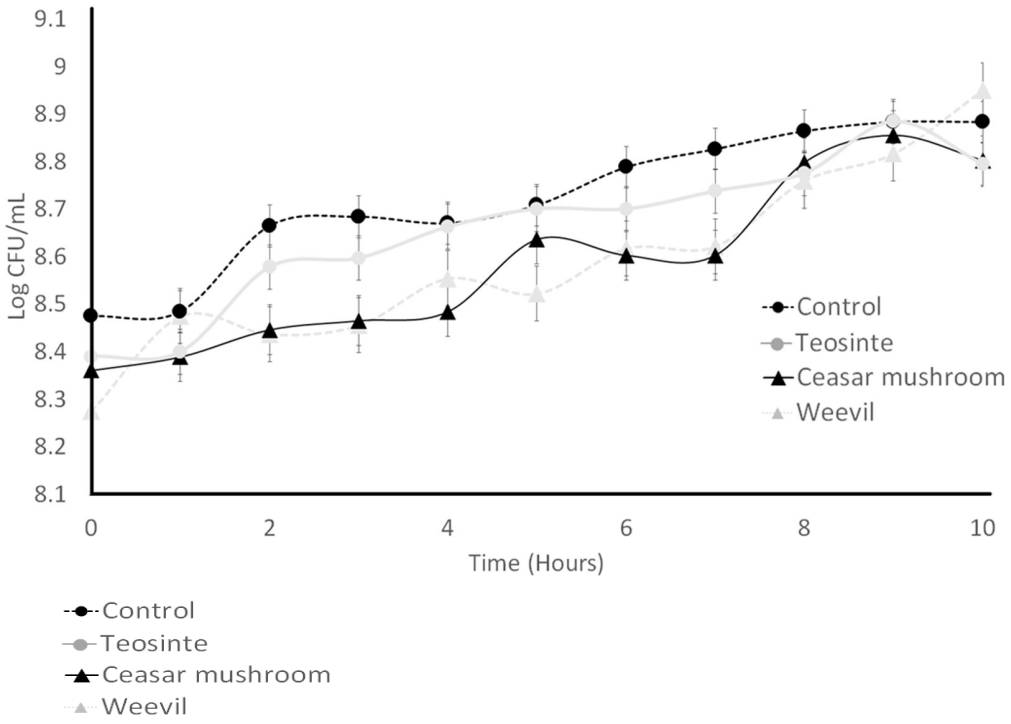

Figure 1. Viability (37 °C) of *L. acidophilus* LA-K in MRS broth as influenced by the ingredients over 10 h. Average of three replicates. Error bars represent SE.

Table 4. 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 acidophilus* LA-K.

Effect	*L. acidophilus* LA-K
Viability	
Ingredient	0.1529
Time (Hours)	<0.0001
Ingredient × time	0.3913
Bile tolerance	
Ingredient	0.0345
Time (Hours)	<0.0001
Ingredient × time	0.2567
Acid tolerance	
Ingredient	0.0193
Time (Minutes)	<0.0001
Ingredient × time	0.2663
Resistance to gastric juices	
Ingredient	0.0102
pH	<0.0001
Ingredient × pH	0.0119
Protease activity	
Ingredient	0.0107
Time (Hours)	<0.0001
Ingredient × time	0.1943
Lysozyme resistance	
Ingredient	0.0043
Time (Hours)	<0.0001
Ingredient × time	0.2871

Table 5. Least squares means for bacterial viability, bile tolerance, acid tolerance, resistance to gastric juices, protease activity, and lysozyme resistance of *Lactobacillus acidophilus* LA-K as influenced by the ingredients.

Test	*L. acidophilus* LA-K
Bacterial viability	
Control	NS
Weevil	NS
Caesar's mushroom	NS
Teosinte	NS
Bile tolerance	
Control	7.54 ^{A}
Weevil	6.10 ^{C}
Caesar's mushroom	6.04 ^{C}
Teosinte	7.05 ^{B}
Acid tolerance	
Control	5.02 ^{A}
Weevil	4.62 ^{B}
Caesar's mushroom	4.81 ^{B}
Teosinte	5.05 ^{A}
Resistance to gastric juices	
Control	8.34 ^{A}
Weevil	7.25 ^{B}
Caesar's mushroom	7.44 ^{B}
Teosinte	8.11 ^{A}
Protease activity	
Control	0.319 ^{B}
Weevil	0.422 ^{A}
Caesar's mushroom	0.303 ^{B}
Teosinte	0.454 ^{A}
Lysozyme resistance	
Control	6.25 ^{B}
Weevil	7.07 ^{A}
Caesar's mushroom	6.31 ^{B}
Teosinte	6.92 ^{A}

$^{A–C}$ Means within a same column along same test with different letter differ statistically ($p < 0.05$). NS = not significant differences among control and ingredients.

Bile tolerance is one of the essential characteristics of probiotic bacteria, as it determines the bacteria's capacity to prevail in the small intestine, and therefore, the ability to recreate their active role as a probiotic [29,30]. The bile tolerance of *L. acidophilus* over 8 h of incubation after incorporating weevil, teosinte, and Caesar's mushroom is shown in Figure 2. The interaction effect (ingredient × time) was not significant ($p > 0.05$), whereas the time (hour) and ingredient effects were significant ($p < 0.05$) (Table 4). Teosinte flour did not affect the bile tolerance of *L. acidophilus*, and the growth decreased over time, whereas weevil and Caesar's mushrooms reported lower counts when compared to the control samples (Table 5). The growth stayed steady until 4 h, and significantly ($p < 0.05$) reduced at 8 h.

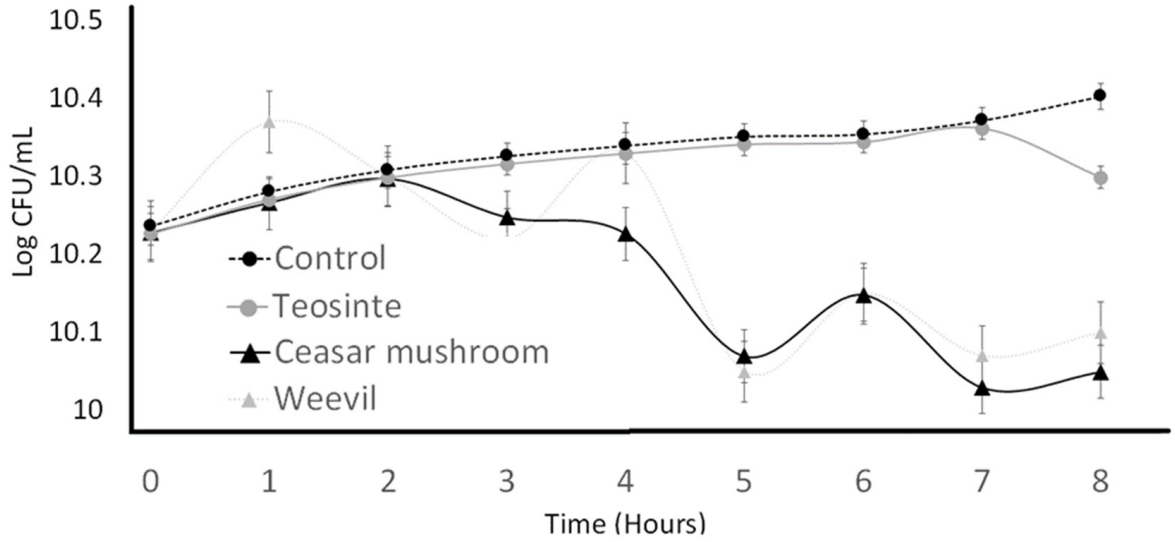

Figure 2. Bile tolerance (0.3% oxgall) of *L. acidophilus* LA-K in MRS broth as influenced by the ingredients over 8 h. Average of the three replicates. Error bars represent SE.

Other investigations noted similar counts for *L. acidophilus* inoculated in MRS broth [31,32]. Generally, the bacterial cell wall is appreciatively powerless to bile salts, which can harm the cell wall generating leakage of destructive intracellular material [33]. Effects of Weevil and Caesar's mushrooms on bile resistance were not comparable with other treatments' effects on probiotic growth, suggesting that weevil and Caesar's mushrooms might negatively affect probiotic resistance. Insects (Saadoun et al., 2022) [34] and mushroom (Smolskaitė et al., 2015) [35] ingredients have shown antimicrobial properties. On the other hand, teosinte flour did not affect the bile tolerance and lipid solubilization of *L. acidophilus* cells membranes at 2%.

3.3. Acid Tolerance and Resistance to Gastrointestinal Juices

Acid tolerance and resistance to gastrointestinal juices are severe acid stresses that simulate stomach conditions [36]. The acid tolerance and resistance to gastrointestinal juices of *L. acidophilus* over 15 min of incubation as affected by weevil, teosinte and Caesar's mushroom is illustrated in Figure 3. The interaction effect (ingredient × time) was not significant ($p > 0.05$), whereas the time (hour) and ingredient effects were significant ($p < 0.05$) (Table 4). Teosinte flour did not affect the acid tolerance of *L. acidophilus,* and the growth decreased over time, whereas weevil and Caesar's mushrooms reported lower counts when compared to the control samples (Table 5). For gastric juice resistance, the interaction effect (ingredient concentration and pH), pH effect, and ingredient effect were significant ($p > 0.05$) (Table 4), meaning that the control and some ingredients did not follow the same trend. At pH 5 and 7, lower log counts were found in weevil and Caesar's mushrooms compared to the control samples, whereas teosinte flour did not affect the gastric juices. When pH levels were lower, log counts were lower. The gastrointestinal

system comprises a vital barrier where the esophagus, small intestine, and large intestine present different stresses such as acids, digestive enzymes including pepsin and lipase, and salts such as NaCl and bile salts [37]. Insects [34] and mushroom [35] ingredients have shown antimicrobial properties. Probiotics must prevail over the acidic environment to colonize the small intestine, and ingredients with addition must, at least, not influence the probiotics' survivability in acidic conditions.

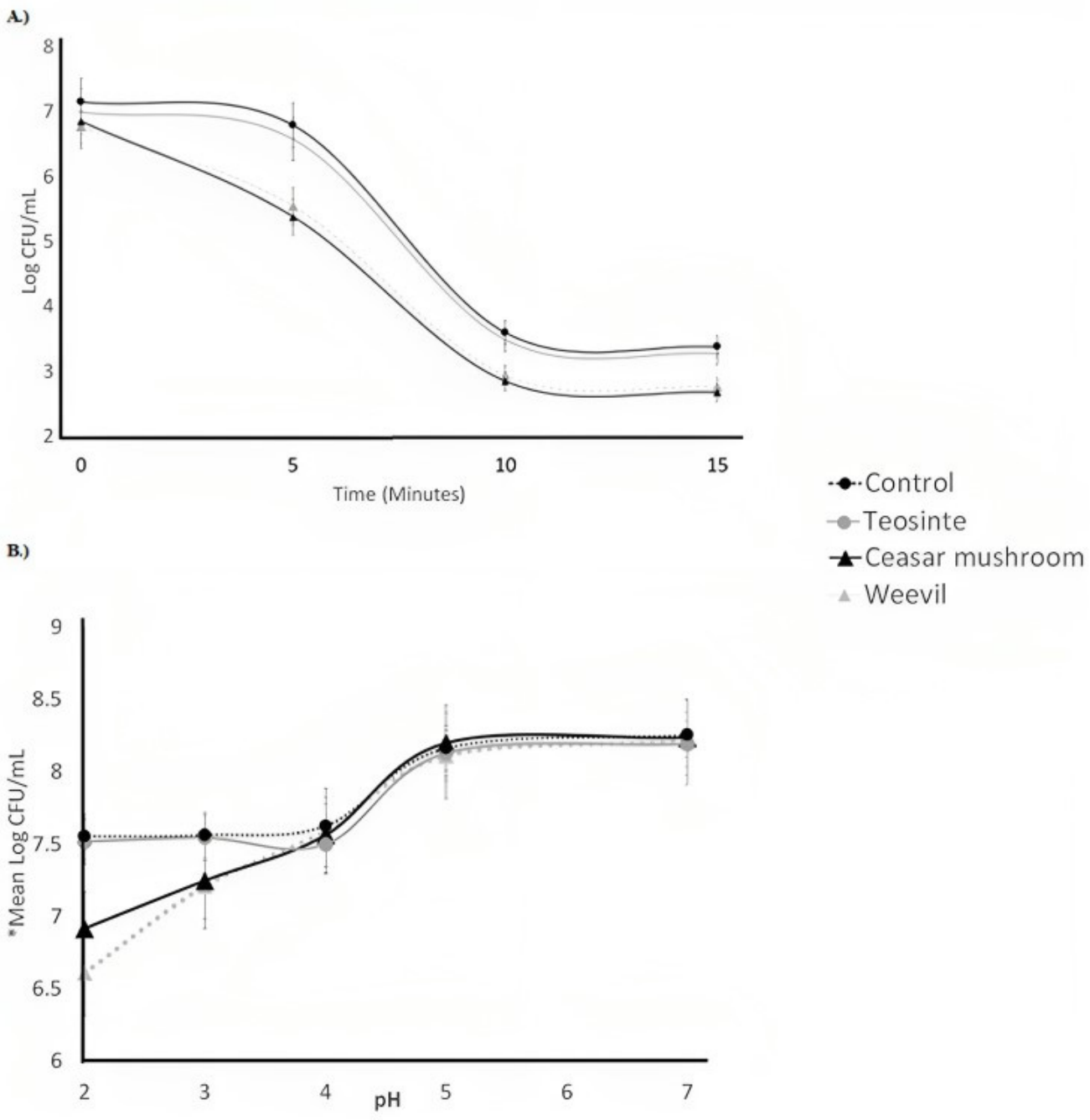

Figure 3. (**A**) Acid tolerance (pH 2) of *L. acidophilus* LA-K in MRS broth as influenced by the ingredients over 15 min. (**B**) Resistance of *L. acidophilus* LA-K to simulated gastric juice (pH 2, 3, 4, 5, and 7) in formulated gastric juice solution as influenced by the ingredients over different pH conditions. * Average of the three replicates. Error bars represent SE.

3.4. Resistance to Lysozyme

The saliva and mouth are the first obstacles that probiotics must undergo when consuming cultured products. Lysozyme is a crucial antibacterial component in saliva, and is essential to the nonspecific immune defense mechanism [38]. Lysozyme saliva partakes in maintaining the constant equilibrium of the oral cavity as a nonimmune defense against bacteria [38]. Resistance of the three ingredients and control 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 ($p < 0.05$) (Table 4). Caesar's mushrooms did not affect the lysozyme resistance of *L. acidophilus*, and the growth decreased over time, whereas weevil and teosinte flour reported higher counts when compared to the control samples (Table 5). Alginate–milk microsphere encapsulates have been shown to increase the survivability of some *Lactobacillus* bacteria, such as *L. bulgaricus*, in simulated gastrointestinal conditions [39]. Insect [34] proteins and teosinte [7] starch can protect the bacteria from harmful enzymatic activity.

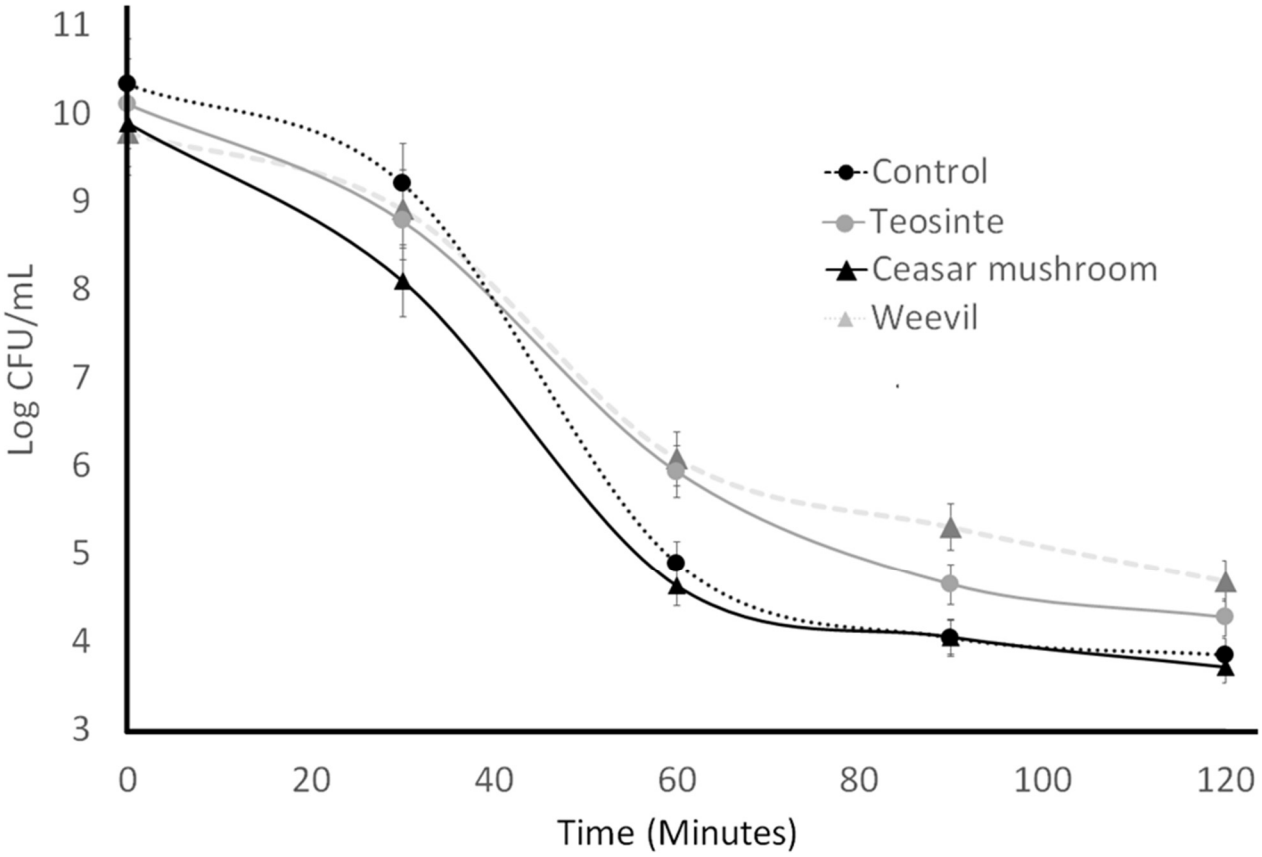

Figure 4. Resistance of *L. acidophilus* LA-K to lysozyme (100 mg/L) in an electrolyte solution as influenced by the ingredients during an incubation time of 2 h. Average of the three replicates. Error bars represent SE. Chi = *Solanum*.

It is possible that proteins from weevil and starch from teosinte could have an encapsulating effect on *L. acidophilus*, resulting in enhanced resistance to lysozyme. Nevertheless, the mechanisms of action regarding lysozyme inhibition by weevil and starch from teosinte must be studied in-depth to understand how these ingredients inhibit lysozyme's enzymatic action in *L. acidophilus*.

3.5. Protease Activity

Lactic acid bacteria assist in the digestion and catabolism of proteins [40]. They catalyze the hydrolysis of peptide bonds and transform them into amino acids, which are then absorbed and used for their growth [40]. Protease activity of *L. acidophilus* is shown in Figure 5. The ingredient and time effects were significant ($p > 0.05$), whereas the interaction effect (ingredient × time) was not significant ($p < 0.05$) (Table 4). Control and teosinte samples had no significant ($p > 0.05$) difference in protease activity (Table 5), whereas weevil powder increased the protease activity, and Caesar's mushroom decreased the activity after 12 h and 24 h. *L. acidophilus* is known to synthesize serine proteinase [41]. Weevil powder protein content [10] could be used as a substrate to increase the protease activity of *L. acidophilus* in skim milk. Protease activity is essential for growth, and weevil addition into skim milk could be recommended to improve the growth of *L. acidophilus*.

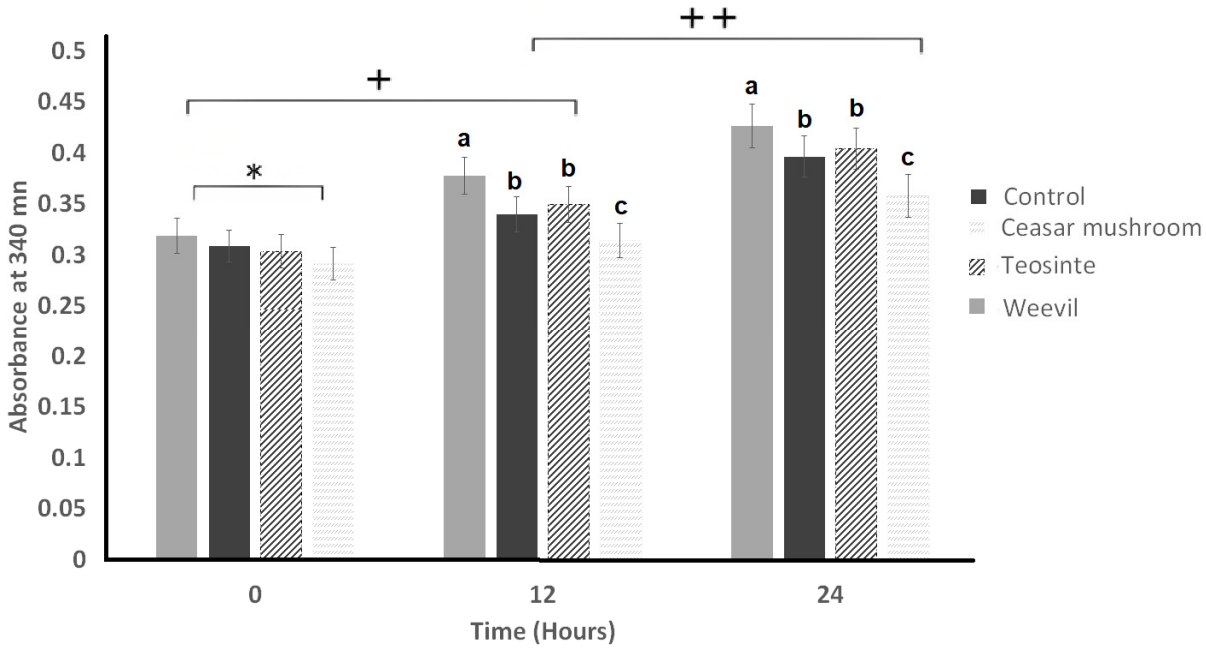

Figure 5. The protease activity (37 °C) of *L. acidophilus* LA-K in skim milk as influenced by the ingredients over an incubation period of 24 h. Average of the three replicates. Values with different letters are significantly different ($p < 0.05$). Error bars represent SE. ^{abc} Means with different letter means significant difference ($p > 0.05$) among treatments in 4 h and 8 h. * Indicates not significant difference ($p > 0.05$) among treatments in 0 h. + Indicates significant difference ($p < 0.05$) between 0 and 4 h. ++ Indicates significant difference ($p < 0.05$) between 4 and 8 h.

4. Conclusions

The synergistic effects of weevil, Caesar's mushroom, and teosinte on the characteristics of L. acidophilus such as acid tolerance, lysozyme tolerance, bile tolerance, and gastric juice resistance were studied. Overall, teosinte flour did not affect viable counts in all the tests. In contrast, weevil and Caesar's mushrooms had lower bile, acid, and gastric juice tolerance counts compared to the control samples.

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