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Probiotic Characteristics of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* as Influenced by Carao (*Cassia grandis*)

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Abstract: Carao is considered a functional ingredient since its bioactive compounds are meaningful in nutritional, pharmacological, and medicinal applications. The objective of this study was to determine the effects of carao pulp powder on the bacterial viability, acid tolerance, bile tolerance, and protease activity of *S. thermophilus* STI-06 and *L. bulgaricus* LB-12. M17 broth with 0.5% lactose and MRS broth were used for *S. thermophilus* and *L. bulgaricus*, respectively, for determining bacterial viability, acid tolerance, and bile tolerance. Skim milk was used to study the protease activity of both bacteria. The carao was added at 0 (control), 1.3, 2.6, and 5.3 (g/L) into the broths and skim milk. The broths were enumerated for bacterial viability (every 2 h), bile tolerance (every 4 h), and acid tolerance (every 30 min), and the skim milk was analyzed for protease activity (every 12 h). The General Linear Model (PROC GLM) was used to analyze the data. The 2.6 g/L and 5.3 g/L usage level of carao improved the acid tolerance of *S. thermophilus*. Carao did not affect the acid tolerance of *L. bulgaricus*. The usage of 5.3 g/L of carao significantly improved the bile tolerance and protease activity of both bacteria. However, carao did not affect the viability of either bacteria. Overall, 5.3 g/L of carao with these probiotics could be recommended in fermentation processes.

Keywords: yogurt starter culture; carao; acid tolerance; bile tolerance; protease activity; bacterial viability

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1. Introduction

Probiotics are viewed as functional ingredients since they can play a critical role in gut microbiota composition [1]. This group of bacteria can obstruct the colonization of pathogenic bacteria in the intestine by preventing or treating gastrointestinal issues such as diarrhea, irritable bowel syndrome, intestinal inflammation, and allergies. Currently, the food industry is interested in increasing the functionality of food products to obtain nutritional benefits, such as preventing intestinal system disorders, improving intestinal microflora, and increasing the strength of the host immune system [2]. The probiotic strains commonly used to promote host health and control food-borne bacteria are typically lactic acid bacteria (LAB). The *Lactobacillus* species are the most frequently used probiotics, and there are more than 500 probiotic food products that have been launched [3].

Probiotics deliver health benefits beyond regular nutrition, but their application can be limited depending on the type of stress they might face. The number of bacteria present in the digestive system varies widely in the different sections due to the acidic conditions and the high concentration of bile salts. After consumption, probiotics must survive severe stomach and intestinal tract conditions such as high acidity, hydrolytic enzymes, oxygen exposure, bile salts, osmotic stress, and competition with indigenous gut microbes [4,5]. The saliva has the highest microbial load, ranging from 10^7 to 10^9 CFU/mL [6]. On the other

hand, the stomach has the lowest counts of microorganisms, with less than 10^3 CFU/mL, where gastric secretions containing hydrochloric acid exert a microbicidal effect attributed to the low pH (<3) [7]. For these reasons, the World Health Organization (WHO) and the Food and Agriculture Organization of the United Nations (FAO) have recommended that functional food with probiotics should contain at least 10^7 CFU/g [8].

Commonly, yogurt is a source of probiotics and is considered a good vehicle for bioactive compounds sourced from dietary ingredients, such as proteins, vitamins, minerals, polyphenols, and medical herbs and plants. The food industry is also seeking new sources of bioactive compounds.

In South America, *Cassia grandis* (carao) is an exotic plant that has been used by indigenous tribes for centuries for treating anemia, skin ulcers, and diabetes [9]. Fuentes et al. [10] stated that carao has great potential for nutritional, pharmacological, and medicinal applications. Fuentes et al. [11] determined that in the pulp, seeds, and bark, there are high amounts of carotenoids, the precursors of vitamin A, which promote the formation and conservation of dental and bone tissues, mucous membranes, and skin. It also contains minerals such as calcium, magnesium, potassium, sodium, copper, zinc, manganese, phosphorus, sulfur, and iron in high levels, which are relevant to the treatment of anemia. In addition, the proximal composition showed a high concentration of proteins, which could be up to 10.11% in the seeds [11]. For phenolic content, *Cassia grandis* has an abundance of hydroxybenzoic acids, flavonols, flavanols, flavanones, and proanthocyanidins [12]. In addition, carao has been utilized to enhance sensory properties in food systems. Carao gives higher aroma scores in Californian-style black olives, and the masking effect was notable because of its sweet smell. The pleasant odors compounds were methyl-ester-2-propenoic acid, dimethyl silanediol, p-cymene, and diallyl disulfide [13].

Streptococcus thermophilus STI-06 *and Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12 are yogurt starter cultures that provide health benefits, but both microorganisms could be affected, to some degree, by the low gastric pH and the presence of pepsin (an antimicrobial) in the gastrointestinal tract [14]. As a result, the objective was to study the effect of carao on the bacterial viability characteristics, acid tolerance, bile tolerance, and protease activity of *S. thermophilus* and *L. bulgaricus*.

2. Materials and Methods

2.1. Plant Material

The carao fruit was gathered in the Guapinol Biological Reserve, Marcovia Municipality, Choluteca Department (Honduras), between August and September 2021. Carao fruit was manually peeled and the pulp was separated. Later, the pulp was mixed with water (10% *wt./wt.*) and stored frozen (−80 °C) until it was lyophilized (LIOTOP model L 101). The obtained solution was lyophilized at −73 to −76 °C and 0.1–0.3 Pa for 48 h. The lyophilized pulp was ground in a commercial mill (LABOR model SP31). The pulp powder was vacuum-packed in plastic bags.

2.2. Experimental Design

The growth characteristics and acid and bile tolerance of *Streptococcus thermophilus* STI-06 and *Lactobacillus bulgaricus* LB-12 (Chr. Hansen, Milwaukee, WI, USA) were analyzed separately using M17 broth (Oxoid, Basingstoke, UK) containing 0.5% lactose (*S. thermophilus*) and MRS broth (Difco, Becton, Dickinson and Co., Sparks, MD, USA) (*L. bulgaricus*). For the bacterial viability and acid and bile tolerance, counts were enumerated using MRS agar (for *L. bulgaricus*) and *S. thermophilus* agar (for *S. thermophilus*) at various time points. The protease activity of both bacteria was also determined separately in skim milk (Great Value, Leander, TX, USA). In addition to the control (broths without carao), carao was individually added to the broths and the milk at three different concentrations (1.3, 2.65, and 5.3 g/L). For protease activity, the free amino groups were determined by spectrophotometry measurements. All of the experiments were conducted

in triplicate with duplicate readings in the case of microbial growth evaluations and acid and bile tolerance, and with quadruplicate readings for protease activity.

2.3. Bacterial Viability

The bacterial viability of *S. thermophilus* and *L. bulgaricus* was studied according to Loghavi et al. [15] with some changes. Cultures were aseptically inoculated (5% (*v*/*v*)) into sterile MRS broth pH 5.2 (Difco, Becton, Dickinson and Co., Sparks, MD, USA) (*L. bulgaricus*) and M17 broth pH 6.8 (Oxoid, Basingstoke, UK) with lactose (0.5%) (*S. thermophilus*). For the MRS broth, sodium thioglycolate was added as an oxygen scavenger to obtain anaerobic conditions. The broths were incubated aerobically at 37 °C (M17 broth) and anaerobically at 43 °C (MRS broth) for 8 h. The colonies were enumerated at 0, 2, 4, 6, 8, 10, 12, 14, and 16 h as described in the enumeration of *S. thermophilus* and *L. bulgaricus* section.

2.4. Acid Tolerance Test

The acid tolerance of *S. thermophilus* and *L. bulgaricus* was studied according to Pereira and Gibson [16] with some changes. Both MRS and M17 broths were acidified by adjusting the pH to 2.0 with 1N HCl and then autoclaved. After cooling, cultures were inoculated (10% (*v*/*v*)) into sterile MRS broth (*L. bulgaricus*) (Difco, Becton, Dickinson and Co., Sparks, MD, USA) and M17 broth (Oxoid, Basingstoke, UK) with lactose (0.5%) (*S. thermophilus*). The broths were incubated aerobically at 37 °C (M17 broth) and anaerobically at 43 °C (MRS broth) for 2 h. The colonies were enumerated at 0, 30, 60, and 120 min in both of the broths, as described in the enumeration of *S. thermophilus* and *L. bulgaricus* section

2.5. Bile Tolerance Test

The bile tolerance of *S. thermophilus* and *L. bulgaricus* was examined according to Pereira and Gibson [16] with some modifications. Oxgall (bovine bile) (US Biological, Swampscott, MA, USA) was incorporated (0.3% (*w*/*v*)) into both the MRS and M17 broths. In the case of the MRS broth, sodium thioglycolate was added as an oxygen scavenger to obtain anaerobic conditions. Both of the broths were then autoclaved. After cooling, cultures were aseptically inoculated (10% (*v*/*v*)) into sterile MRS broth (Difco, Becton, Dickinson and Co., Sparks, MD, USA) (*L. bulgaricus*) and M17 broth (Oxoid, Basingstoke, UK) with lactose (0.5%) (*S. thermophilus*). The broths were incubated aerobically at 37 °C (M17 broth) and anaerobically at 43 °C (MRS broth) for 8 h. The colonies were enumerated at 0, 4, and 8 h in both of the broths, as described in the enumeration of *S. thermophilus* and *L. bulgaricus* section.

2.6. Enumeration of S. thermophilus and L. bulgaricus

For the preparation of the *S. thermophilus* agar, 10 g of sucrose (Amresco, Solon, OH, USA), 2 g of K_2HPO_4 (Fisher Scientific, Fair Lawn, NJ, USA), 5 g of Bacto yeast extract, and 10 g of Bacto Tryptone (Becton Dickinson and Co., Sparks, MD, USA) were mixed in 1 L of distilled water. The pH of the *S. thermophilus* media was adjusted to 6.8 with 1N HCl before adding 6 mL of 0.5% bromocresol purple solution and 15 g of agar (Fisher Scientific, Fair Lawn, NJ, USA) [17]. For the preparation of the MRS agar, 15 g of agar (Fisher Scientific, Fair Lawn, NJ, USA) and 55 g of *Lactobacilli* MRS broth powder (Becton, Dickinson and Co., Sparks, MD, USA) were mixed in 1 L of distilled water. The pH of the media was adjusted to 5.2 with 1N HCl. The M17 and MRS broths were serially diluted to several dilutions with 99 mL of sterilized peptone water (0.1% (*w*/*v*)) before plating with autoclaved media. The *S. thermophilus* plates were aerobically incubated at 37 °C for 24 h whereas the *L. bulgaricus* plates were incubated anaerobically at 43 °C for 72 h. The colonies were determined by using a Quebec Darkfield Colony Counter (Leica Inc., Buffalo, NY, USA).

2.7. Protease Activity

The protease activity of *S. thermophilus* and *L. bulgaricus* was examined by the Oberg et al. [18] method. *S. thermophilus* and *L. bulgaricus* were inoculated (1% (*v*/*v*))

aseptically into separate bottles of sterile skim milk (Great Value, Leander, TX, USA). The skim milk with *S. thermophilus* was incubated at 37 °C while the skim milk with *L. bulgaricus* was incubated at 43 °C. The protease activity was monitored at 0, 12, and 24 h. After each incubation time, a 2.5 mL sample of the skim milk with culture was vortexed with 1 mL of distilled water in a test tube. The diluted skim milk sample was vortexed after adding 5 mL of 0.75 N trichloroacetic acid (TCA) (Fisher Scientific, Waltham, MA, USA) for protein precipitation and then set for 10 min at room temperature. The precipitated milk was then filtered through Whatman 40 filter paper (Whatman, Clifton, NJ, USA). The TCA filtrate (150 µL) was vortexed with 3 mL of o-phthaldialdehyde solution in a cuvette. The o-phthaldialdehyde solution was prepared by mixing 25 mL of 100 mM sodium borate (Fisher Scientific, Waltham, MA, USA) solution, 2.5 mL of 20% (*w*/*w*) SDS solution (Fisher Scientific, Waltham, MA, USA), 40 mg of o-phthaldialdehyde reagent (Alfa Aesar, Ward Hill, MA, USA) dissolved in 1 mL methanol (Sigma, St. Louis, MO, USA), and 100 µL of β-mercaptoethanol (Sigma, St. Louis, MO, USA) and diluting to 50 mL with distilled water. The absorbance was measured by using a Nicolet Evolution 100 spectrophotometer (Thermo Scientific, Madison, WI, USA) at 340 nm. The o-phthaldialdehyde solution was used as a blank and the non-inoculated sterile skim milk was used as the reference.

2.8. Statistical Analysis

The General Linear Model (PROC GLM) was used to analyze the data in the Statistical Analysis System 9.4 (SAS Institute Inc., Cary, NC, USA). The experiments were analyzed as a two-factor factorial experiment in a randomized block design. To determine significant differences, differences of least square means ($\alpha = 0.05$) were utilized for main effects (carao concentration and time) and interaction effect (carao concentration × time). The significant differences ($p < 0.05$) among the main effects were analyzed using Tukey's adjustment.

3. Results and Discussion

3.1. Bacterial Viability

The growth is shown for *Streptococcus thermophilus* STI-06 (Figure 1) and *Lactobacillus bulgaricus* LB-12 (Figure 2) over 16 h of incubation as influenced by the inclusion of different concentrations of carao. For both bacteria, the carao concentration effect and the carao concentration × hour interaction effect were not significant ($p > 0.05$), whereas the hour effect was significant ($p < 0.05$). For *S. thermophilus*, the log count increased from 11.23 to 11.73 during the first 2 h, and then it declined from 11.73 to 10.78 between 2 and 16 h for control samples. *S. thermophilus* counts for samples containing 1.3 g/L, 2.6 g/L, and 5.3 g/L of carao followed a similar growth tendency. For *L. bulgaricus*, the log count increased from 10.34 to 10.57 during the first 2 h, and then decreased from 10.57 to 10.07 between 2 and 16 h for control samples. Similar results were found for samples with incorporated carao at 1.3 g/L, 2.6 g/L, and 5.3 g/L. In our study, carao did not affect the growth of *S. thermophilus* and *L. bulgaricus* during 16 h of incubation meaning that carao may be utilized at these concentrations with these two bacteria in fermentation processes. Carao has plenty of nutrients, including carbohydrates (71.40%) (particularly sucrose), proteins (7.41%), and minerals (3.14%) [11].

Amirdivani and Baba [19] and Joung et al. [20] noted that herbs and plants such as peppermint (*Mentha piperita*), dill (*Anethum graveolens*), basil (*Ocimum basilicum*), *iospyros kaki* leaf, and *Nelumbo nucifera* leaf improved the metabolic activity of *S. thermophilus* and *L. bulgaricus* in yogurt. Herbal plants may have prebiotic characteristics due to their active ingredients, such as anethole in anise, menthol in peppermint, cinnamaldehyde in cinnamon and may improve gut microflora [21–23].

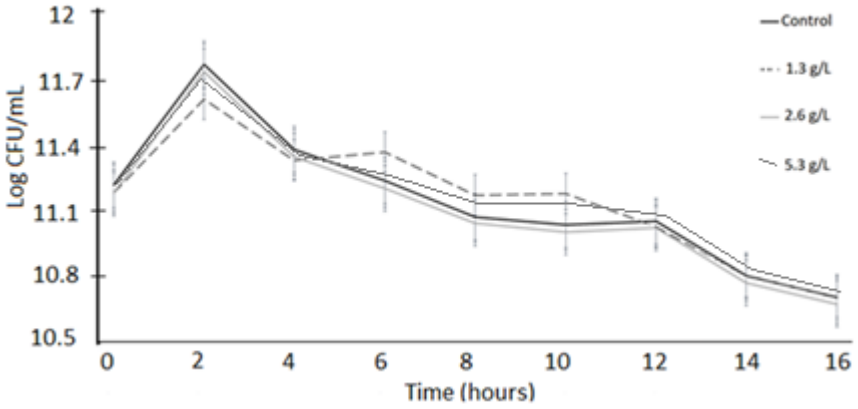

Figure 1. Log counts of *S. thermophilus* as influenced by carao concentration over the incubation period of 16 h.

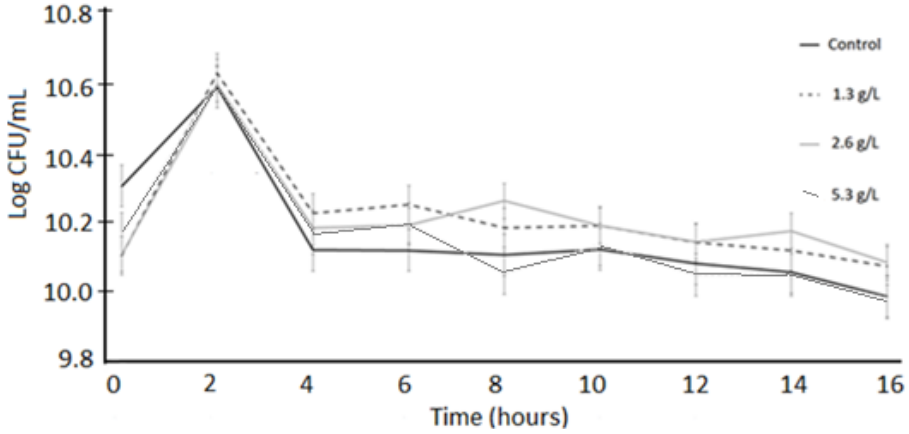

Figure 2. Log counts of *L. bulgaricus* as influenced by carao concentration over the incubation period of 16 h.

S. thermophilus and *L. bulgaricus* have a synergistic effect regarding their growth. The amino acids and small peptides generated by the proteolytic system of *L. bulgaricus* promote the growth of *S. thermophilus* [24–26], while the carbon dioxide, formic acid, folic acid, pyruvic acid, long chain fatty acids, and glutathione produced by *S. thermophilus* facilitate the growth of *L. bulgaricus* [26]. The main lactic acid bacterial metabolites are lactic acid, short-chain fatty acids, γ-amino butyric acid, conjugated linoleic acid, and bacteriocins [26]. An industrial-relevant physiological trait of *S. thermophilus* is its well-established symbiotic relationship with *Lactobacillus delbrueckii ssp. bulgaricus* during milk fermentation which correlates with faster growth and acidification, as well as enhanced aroma compound formation. The interaction between the two species is classified as protocooperation, based on the exchange of metabolites beneficial for optimal growth.

Compared to other LAB, *S. thermophilus* possesses a unique lactose uptake system: LacS, a secondary carrier, which acts primarily as a lactose–galactose antiporter, but also as a lactose–galactose-proton symporter. Following lactose uptake and cleavage by β-galactosidase (LacZ), the glucose moiety is utilized through glycolysis, while galactose is excreted into the medium by most strains [26].

3.2. Acid Tolerance

The acid tolerance of *S. thermophilus* over 120 min of incubation as influenced by the addition of various concentrations of carao is shown in Figure 3. The carao concentration effect, the hour effect, and the carao concentration × hour interaction effect were significant ($p < 0.05$). The log count dropped from 9.18 to 6.73 during the first 30 min and from 6.73 to 3.95 between 30 and 120 min for acidified control broths. The broths incorporating

1.3 g/L of carao had a similar tendency for counts as control broths. The log counts of *S. thermophilus* in broths containing 5.3 g/L of carao decreased with time at a slower rate in an acidic condition.

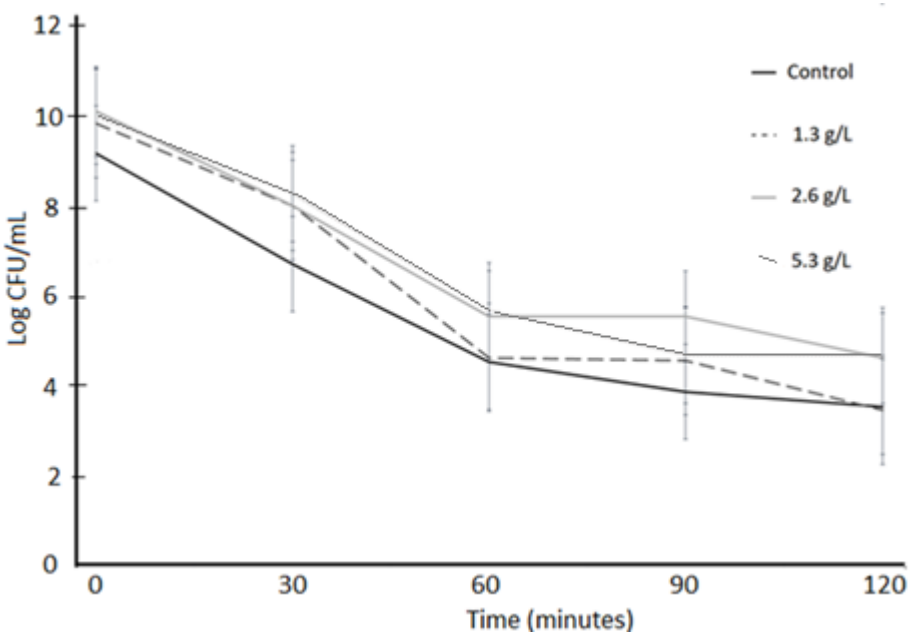

Figure 3. Log counts of *S. thermophilus* to show acid tolerance as influenced by carao concentration over an incubation period of 120 min.

The acid tolerance of *L. bulgaricus* over 120 min of incubation as affected by the inclusion of various concentrations carao is illustrated in Figure 4. The carao concentration effect and the carao concentration × hour interaction effect were not significant ($p > 0.05$), while the hour effect was significant ($p < 0.05$). The log count decreased from 9.07 to 7.78 during the first 30 min and from 7.78 to 3.11 during 30 to 120 min of acid exposure for the control broths. The carao inclusion of 1.3 g/L, 2.6 g/L, and 5.3 g/L resulted in a similar trend as for control broths.

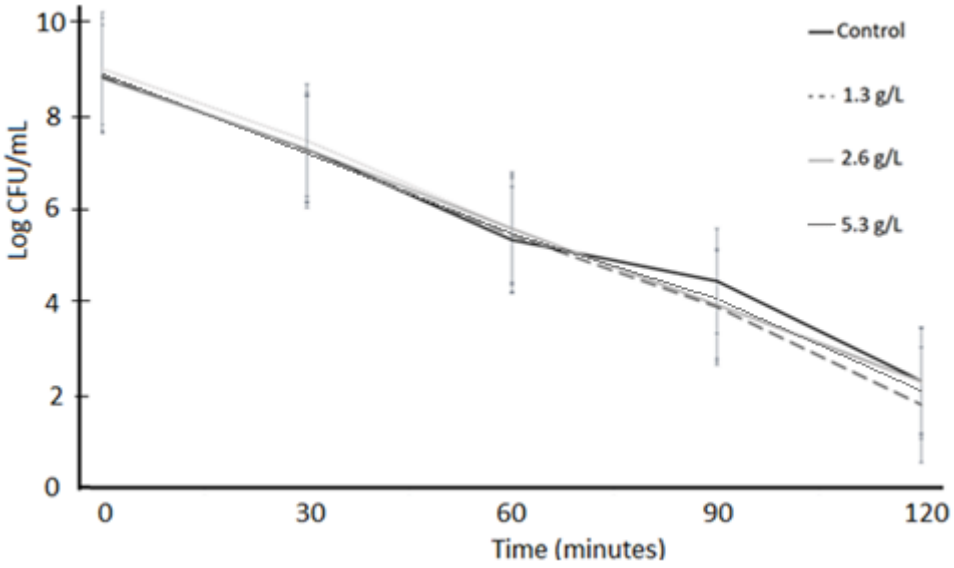

Figure 4. Log counts of *L. bulgaricus* to show acid tolerance as influenced by carao concentration over an incubation period of 120 min.

S. thermophilus requires amino acids, carbohydrates, vitamins, and minerals for growth [27]. It is likely that the microbial nutrients in carao could be used by *S. thermophilus* for growth. In acidified conditions, *S. thermophilus* is not susceptible to proliferating, as the lactic acid bacteria ideally grow between a pH of 5.5 to 7.0 [28]. The acidic conditions inhibit microbial growth by lowering the cytoplasm pH, leading to inactivating critical enzymes, which lessens their capacity to produce ATP, proteins, and essential nutrients for growth [14]. *Lactobacillus* bacteria can survive acidic environments if there is a regulation of enzymatic activities to harmonize the ideal flow of carbohydrates [29]. As a result, *L. bulgaricus* generates rapid molecular responses to restore the damage, and this adaptation can improve the rigidity and impermeability of its membrane [30].

3.3. Bile Tolerance

The bile tolerance of *S. thermophilus* and *L. bulgaricus* over 8 h of incubation as affected by the addition of various concentrations of carao is presented in Figures 5 and 6, respectively. For both probiotics, the carao concentration effect, the hour effect, and carao concentration × hour interaction effect were significant ($p < 0.05$). There were significant ($p < 0.05$) differences in log counts at 0 versus 4 h of bile exposure for a given concentration for both bacteria. Log counts at 8 h were significantly ($p < 0.05$) higher for broth containing 5.3 g/L of carao compared to control broths. For *S. thermophilus*, the log count decreased from 10.27 to 6.59 during 8 h for the control. On the other hand, the log count for the sample containing 5.3 g/L of carao decreased from 10.38 to 6.75 during the 8 h. For *L. bulgaricus*, the log count decreased from 9.02 to 6.27 during 8 h for the control. For the sample containing 5.3 g/L of carao, the log count decreased from 9.05 to 6.51 during the 8 h.

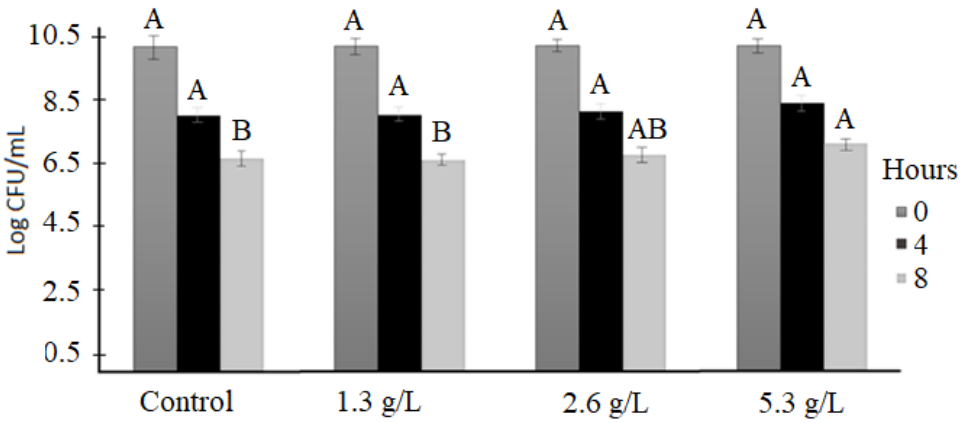

Figure 5. Log counts of *S. thermophilus* to show bile tolerance as influenced by carao concentration and incubation time over 8 h. ^{AB} Values not containing a common letter over various concentrations for a given time are significantly ($p < 0.05$) different.

Similarly, Vargas et al. [14] observed a cell reduction count of *S. thermophilus* (in M17 broth) and *L. bulgaricus* (in MRS broth), respectively, in media containing 0.3% oxgall. Generally, the bacterial cell envelope of probiotics is highly vulnerable to bile salts, and these salts can penetrate the bacteria cell membrane causing shrinkage and leakage of hazardous intracellular material [31]. The bile can emulsify the lipid content from the microbial membrane [32], and this action makes bile hazardous to probiotics. Carao can act as an inhibitor of digestive enzymes such as pancreatic lipase [9]. Carao could possibly function as a barrier between the lipid membrane and the bile to protect these bacteria, leading to higher log counts in broths with carao.

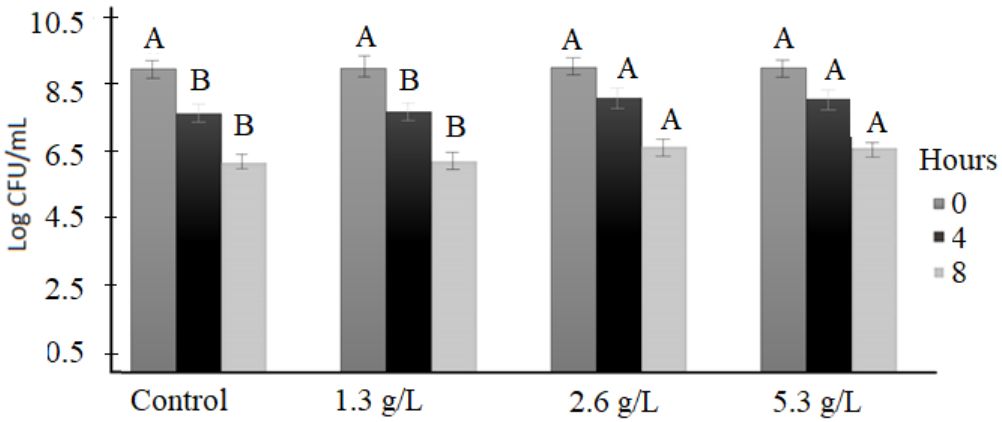

Figure 6. Log counts of *L. bulgaricus* to show bile tolerance as influenced by carao concentration and incubation time over 8 h. [AB] Values not containing a common letter over various concentrations for a given time are significantly ($p < 0.05$) different.

3.4. Protease Activity

The protease activity (absorbance values) of *S. thermophilus* over incubation of 0, 12, and 24 h, as influenced by the addition of various concentrations of carao into skim milk, is displayed in Table 1. The carao concentration effect, the hour effect, and the carao concentration × hour interaction effect were significant ($p < 0.05$). Although there was no significant difference ($p > 0.05$) in absorbance values between various carao concentration at both 0 and 12 h, the inclusion of carao at 5.3 g/L into skim milk showed significantly ($p < 0.05$) higher protease activity than the control at 24 h of incubation. At 24 h, skim milk containing 5.3 g/L of carao had the highest protease activity, while the control samples and samples containing 1.3 g/L of carao had the lowest values.

Table 1. Least square means for protease activity (absorbance) of *Streptococcus thermophilus* STI-06 as influenced by carao over an incubation period of 24 h.

Sample	0 h	12 h	24 h
Control	0.152 ± 0.015 [a]	0.175 ± 0.007 [a]	0.202 ± 0.011 [a]
1.3 g/L	0.152 ± 0.020 [a]	0.170 ± 0.010 [a]	0.211 ± 0.011 [a]
2.6 g/L	0.153 ± 0.017 [a]	0.185 ± 0.015 [a]	0.217 ± 0.014 [ab]
5.3 g/L	0.154 ± 0.023 [a]	0.186 ± 0.027 [a]	0.232 ± 0.008 [b]

[ab] Column means not containing a common letter are significantly ($p < 0.05$) different.

Generally, *S. thermophilus* has a low peptidase activity when compared to other lactic acid bacteria [33]. Depending on the strain, the proteolytic complex of *S. thermophilus* is integrated with peptidases that have distinct properties and specificities compared to other LAB. Although *S. thermophilus* has few amino acid requirements, many of the strains possess the necessary enzymes for casein hydrolysis, uptake of the resulting peptides, and hydrolysis into free amino acids. From an industrial point of view, the proteolytic system of *S. thermophilus* is associated with faster growth and milk acidification, together with increased cell numbers. The proteolytic system in *S. thermophilus* consists of: a serine protease, PrtS, attached to the cell wall by Sortase A and responsible for casein hydrolysis; a group of membrane transporters for oligopeptides or di- and tri-peptide uptake; and an assortment of both intracellular and cell-associated extracellular peptidases responsible for oligopeptide digestion and free amino acid liberation [34]. Carao could possibly enhance the cell density of *S. thermophilus*. Carao has significant amounts of sucrose [11], and using skim milk with sucrose has been shown to increase *S. thermophilus* counts compared to using solely skim milk media [35]. It is expected that a higher cell density could drive increased protease activity [36].

The protease activity (absorbance) of *L. bulgaricus* over incubation of 0, 12, and 24 h, as influenced by the inclusion of various concentrations of carao into skim milk, is illustrated in Table 2. The carao concentration effect, the hour effect, and the carao concentration × hour interaction effect were significant ($p < 0.05$). Similar to the results for *S. thermophilus*, the addition of carao did not significantly ($p > 0.05$) affect initial absorbance values. Therefore, increases in absorbance reading over time can be related to proteolysis. The addition of carao at 5.3 g/L into skim milk showed significantly ($p < 0.05$) higher protease activity than the control at 12 and 24 h of incubation. Similar to *S. thermophilus*, the skim milk containing 5.3 g/L of carao had the highest protease activity, whereas the control samples and samples containing 1.3 g/L of carao had the lowest values at 12 and 24 h. Higher protease activity was detected in *L. bulgaricus* LB-12 than in *S. thermophilus* STI-06.

Table 2. Least square means for protease activity (absorbance) of *Lactobacillus bulgaricus* LB-12 as influenced by carao concentration over an incubation period of 24 h.

Sample	0 h	12 h	24 h
Control	0.161 ± 0.005^{a}	0.313 ± 0.007^{b}	0.417 ± 0.017^{b}
1.3 g/L	0.163 ± 0.005^{a}	0.320 ± 0.015^{b}	0.410 ± 0.025^{b}
2.6 g/L	0.162 ± 0.005^{a}	0.357 ± 0.017^{ab}	0.455 ± 0.019^{ab}
5.3 g/L	0.163 ± 0.007^{a}	0.377 ± 0.013^{a}	0.475 ± 0.013^{a}

[ab] Column means not containing a common letter are significantly ($p < 0.05$) different.

Shah and Jelen [37] reported similar results. Similar to the effect for *S. thermophilus*, carao has significant amounts of sucrose, which could increase the cell density of *L. bulgaricus*, leading to higher protease activity [38]. For achieving maximum survival under stressful conditions, proteolytic activity is a crucial necessity. Therefore, carao addition could be recommended in growing *Streptococcus thermophilus* STI-06 and *Lactobacillus bulgaricus* LB-12.

4. Conclusions

The effect of carao concentration on bacterial viability, acid tolerance, bile tolerance, and protease activity of *S. thermophilus* and *L. bulgaricus* was investigated. For both probiotics, carao did not affect the viability during 16 h of incubation. The broths with 5.3 g/L of carao improved acid tolerance of *S. thermophilus* for 120 min of exposure to acid. On the other hand, carao did not affect the acid tolerance of *L. bulgaricus*. For both bacteria, 5.3 g/L of carao improved bile tolerance during 8 h of exposure and increased protease activity over 24 h of incubation. Overall, carao had some positive effects on probiotic characteristics. Therefore, it is likely that carao at 5.3 g/L concentration may improve the probiotic properties of *S. thermophilus* and *L. bulgaricus* in fermented dairy products.

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