Whey protein isolate improves acid and bile tolerances of *Streptococcus thermophilus* ST-M5 and *Lactobacillus delbrueckii* ssp. *bulgaricus* LB-12

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ABSTRACT

Acid tolerance and bile tolerance are important probiotic characteristics. Whey proteins contain branched-chain amino acids, which play a role in muscle building and are popular among athletes. Increasing emphasis is being placed on diets containing less carbohydrate, less fat, and more protein. The effect of incremental additions of whey protein isolate (WPI) on probiotic characteristics of pure cultures is not known. The objective of this study was to determine the influence of added WPI on acid tolerance and bile tolerance of pure cultures of *Streptococcus thermophilus* ST-M5 and *Lactobacillus bulgaricus* LB-12. The WPI was used at 0 (control), 1, 2 and 3% (wt/vol). Assessment of acid tolerance was conducted on pure cultures at 30-min intervals for 2 h of acid exposure and bile tolerance at 1-h intervals for 5 h of bile exposure. Use of 1, 2, and 3% WPI improved acid tolerance of *Strep. thermophilus* ST-M5 and *Lb. bulgaricus* LB-12. The highest counts for acid tolerance of *Strep. thermophilus* ST-M5 and *Lb. bulgaricus* LB-12 were obtained when 3% WPI was used. Use of 2 and 3% WPI improved bile tolerance of *Strep. thermophilus* ST-M5 and *Lb. bulgaricus* LB-12 over 5 h of bile exposure. The use of WPI is recommended to improve acid and bile tolerance of the yogurt culture bacteria *Strep. thermophilus* ST-M5 and *Lb. bulgaricus* LB-12.

Key words: probiotic, culture, whey protein isolate, yogurt

INTRODUCTION

Probiotics are a steadily growing market with global sales of probiotics having reached \$21.6 billion and \$24.23 billion in 2010 and 2011, respectively, with the market expected to reach \$31.1 billion and \$44.9 billion in 2015 and 2018, respectively (Pedretti, 2013). Growth of the probiotic market is due to increased awareness of the health benefits provided by probiotic bacteria. Before probiotics can confer health benefits to their

host, live cells must reach and establish in the lower gastrointestinal (GI) tract (Gerez et al., 2012) before which cells must survive the acidic conditions of the stomach and the bile in the GI tract. Therefore, acid tolerance and bile tolerance are important probiotic characteristics. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* are yogurt culture bacteria that have health beneficial properties but that are affected, to some degree, by the acidic conditions of the stomach and the presence of bile in the GI tract.

Whey protein concentrate (WPC) and whey protein isolate (WPI) are used in food product manufacture and have been reported to have a beneficial influence on viability of probiotic and culture bacteria within the food product. Viability of *Streptococcus thermophilus*, *Lb. delbrueckii* ssp. *bulgaricus* and *Bifidobacterium animalis* in reduced-fat yogurt supplemented with 1.5% WPC was increased up to 1 log cfu/g after 1 wk of storage compared with yogurt with no with WPC (Akalın et al., 2007). When specific AA, such as cysteine, were added to yogurt, a significant increase in viability of culture and probiotic bacteria (*Lb. delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus*, *Bifidobacterium bifidum* BB12, and *Lactobacillus paracasei*) was found (Güler-Akın and Akin, 2007).

Addition of 0.5% WPC and other milk ingredients to replace nonfat dry milk resulted in an increase in counts of *Strep. thermophilus*, *Lb. delbrueckii* ssp. *bulgaricus*, and *B. animalis* after 14 d of storage at 4°C (Marafon et al., 2011). After 28 d of 4°C storage, counts of *Strep. thermophilus* and *B. animalis* were higher in yogurt supplemented with 0.5% WPC than counts in yogurt with added nonfat dry milk (Marafon et al., 2011). Dave and Shah (1998a) reported that after 5 wk of 4°C storage, counts of *Strep. thermophilus* in yogurt supplemented with WPC and casein hydrolysate were up to 0.5 log cfu/mL higher compared with yogurts with no supplementation. They further reported that bifidobacteria counts were 4 log cfu/mL higher in yogurt supplemented with WPC than in yogurt with no WPC supplementation. Fortification of yogurt with up to 4% of whey protein hydrolysates improved the growth of *Lb. acidophilus* by 3 log cycles and enhanced the growth of *Strep. thermophilus* (Lucas et al., 2004).

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Whey proteins are the proteins of choice for athletes and body builders for muscle recovery after weight lifting and workout routines (Tipton et al., 2007). Whey proteins are sources of the branched-chain AA leucine, isoleucine, and valine, 3 essential AA (Sowers, 2009). Branched-chain AA enter the bloodstream through the liver and are oxidized in muscle tissue to provide energy during exercise (Garlick and Grant, 1988; Morifuji et al., 2009; Sowers, 2009).

Prevention of cancer and diabetes, weight loss, and reduction of appetite are linked to low-carbohydrate and high-protein diets (Weigle et al., 2005; Wycherley et al., 2010). Thus, diets containing less carbohydrate, less fat, and more protein are becoming more important (Weigle et al., 2005; Wycherley et al., 2010). Whey protein isolate contains more than 90% protein. Information on the influence of WPI on acid tolerance and bile tolerance is lacking. The objective was to study the effect of incremental addition of WPI on acid tolerance and bile tolerance of *Strep. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*.

MATERIALS AND METHODS

Experimental Design

The treatments consisted of WPI (Grände Ultra 9100; Grände Custom Ingredients Group, Milwaukee, WI) added at 0 (control), 1, 2, and 3% (wt/vol) to de Man, Rogosa, and Sharpe (MRS) broth for *Lb. bulgaricus* and to M17 broth for *Strep. thermophilus*. Acid tolerance of pure cultures (*Strep. thermophilus* ST-M5 and *Lb. bulgaricus* LB-12) was evaluated every 30 min for 2 h, and bile tolerance of pure cultures was determined every 1 h for 5 h. The experiments were conducted and analyzed as a randomized block design with repeated measures. The 3 replications were the blocks.

Preparation of Media

The MRS broth for acid tolerance of *Lb. bulgaricus* was prepared by adding 55 g of MRS broth powder (Difco, Becton, Dickinson and Co., Sparks, MD) to 1 L of distilled water, and M17 broth for acid tolerance of *Strep. thermophilus* was prepared by adding 37.25 g of M17 broth powder (Oxoid, Basingstoke, UK) to 950 mL of distilled water. The MRS and M17 broths were adjusted to pH 2 using 1 N HCl and monitored with a calibrated pH meter (Extech Instruments, Waltham, MA). The MRS and M17 broths were sterilized at 121°C for 15 min. A lactose solution (10% wt/vol) was sterilized and 50 mL/L of lactose solution was aseptically added to previously sterilized M17 broth.

The MRS-THIO broth for bile tolerance of *Lb. bulgaricus* was prepared by adding 55 g of MRS broth powder (Difco), 3 g of bovine bile (oxgall; US Biological, Swampscott, MA), and 2 g of sodium thioglycolate (Acros Organics, Fair Lawn, NJ) to 1 L of distilled water. Oxgall was added to test the bile tolerance of both bacteria; sodium thioglycolate was used as an oxygen scavenger for *Lb. bulgaricus* in MRS broth. The final pH of the oxgall-supplemented MRS-THIO broth was 6.70 ± 0.20 . This MRS-THIO broth was sterilized at 121°C for 15 min.

The M17 broth for bile tolerance of *Strep. thermophilus* was prepared by adding 37.25 g of M17 broth powder (Oxoid) and 3 g of oxgall (US Biological) per 950 mL of distilled water. The final pH of the oxgall-supplemented M17 broth was 7.00 ± 0.20 . This M17 broth was sterilized at 121°C for 15 min. A lactose solution (10% wt/vol) was sterilized and 50 mL was aseptically added to previously sterilized M17 broth.

Lactobacilli MRS agar for pour plating was prepared according to manufacturer's directions and Tharmaraj and Shah (2003). Lactobacilli MRS agar (Difco) was suspended at a concentration of 70 g/L of distilled water. The pH was adjusted to 5.2 with 1 N HCl. The medium was heated to boiling with agitation to completely dissolve the powder and was then sterilized at 121°C for 15 min.

The M17 agar for pour plating was prepared according to the manufacturer's directions by adding 48.25 g of M17 agar powder (Oxoid) per 950 mL of distilled water with agitation and boiling gently. Then, the M17 agar was sterilized at 121°C for 15 min. A lactose solution (10% wt/vol) was separately autoclaved at 121°C for 15 min. This lactose solution was aseptically added at 50°C at a rate of 50 mL to 950 mL of previously sterilized M17 agar.

Peptone water (0.1%) was prepared by dissolving 1 g of peptone powder (Bacto Peptone, Difco) per 1 L of distilled water. Peptone water (99 mL) was autoclaved in dilution bottles at 121°C for 15 min.

Acid Tolerance

Acid tolerance was determined according to the method proposed by Pereira and Gibson (2002), with slight modifications. The M17 and MRS broths contained 0 (control), 1, 2, and 3% (wt/vol) added WPI (Grände Custom Ingredients Group). After addition of WPI, the pH of both broths was adjusted to 2, using a sterilized magnetic stirrer bar for agitation. Freshly thawed pure cultures of *Strep. thermophilus* ST-M5 and *Lb. bulgaricus* LB-12 (Chr. Hansen, Milwaukee, WI) were used to separately inoculate M17 broth (pH 2) and MRS broth (pH 2), respectively. Inoculated broths

(pH 2) containing control and added WPI samples were incubated for 2 h at 43°C for *Lb. bulgaricus* and 37°C for *Strep. thermophilus*.

Inoculated MRS and M17 broths were 10-fold serially diluted in 0.1% (wt/vol) peptone water and pour plated in duplicate every 30 min during the 2 h of incubation. *Lactobacillus bulgaricus* were enumerated by pour plating using previously prepared MRS agar (pH 5.2) and anaerobically incubated at 43°C for 72 h. *Streptococcus thermophilus* were enumerated by pour plating using previously prepared M17 agar and aerobically incubated at 37°C for 24 h (Dave and Shah, 1998a). After the incubation period, the colonies were counted.

Bile Tolerance

Bile tolerance was determined according to the method proposed by Pereira and Gibson (2002) with slight modifications. Both the MRS-THIO broth and the M17 broth contained 0.3% (wt/vol) oxgall. To both these broths, WPI (Grände Ultra 9100; Grände Custom Ingredients Group) was added at 0 (control), 1, 2, and 3% (wt/vol). The MRS-THIO and M17 broths were inoculated with freshly thawed pure cultures of *Lb. bulgaricus* LB-12 and *Strep. thermophilus* ST-M5, respectively. Inoculated broths of the control and WPI samples were incubated for 5 h at 43°C for *Lb. bulgaricus* and 37°C for *Strep. thermophilus*.

Inoculated MRS-THIO containing 0.3% (wt/vol) oxgall and M17 containing 0.3% (wt/vol) oxgall broths were serially diluted in 0.1% (wt/vol) peptone water and pour plated every h for the 5 h of incubation. *Lactobacillus bulgaricus* were enumerated by pour plating using previously prepared MRS agar (pH 5.2) and anaerobically incubated at 43°C for 72 h. *Streptococcus thermophilus* were enumerated by pour plating using previously prepared M17 agar and aerobically incubated at 37°C for 24 h (Dave and Shah, 1998a). After the incubation period, the colonies were counted. Three replications were conducted.

Statistical Analysis

Data were analyzed separately as a randomized block design with repeated measures for acid tolerance of *Strep. thermophilus*, acid tolerance of *Lb. bulgaricus*, bile tolerance of *Strep. thermophilus*, and bile tolerance of *Lb. bulgaricus* using Proc Mixed of SAS (version 9.3, SAS Institute Inc., Cary, NC). The WPI concentration and time of exposure were fixed effects, whereas replicates were random effects. Differences of least squares means were used to determine significant differences at $P < 0.05$ for main effects (WPI concentration and time of exposure) and the interaction effect (WPI con-

centration $\times$ time of exposure). Significant differences ($P < 0.05$) among the main effects were analyzed using Tukey's adjustment.

RESULTS AND DISCUSSION

Acid Tolerance

Acid tolerance of *Strep. thermophilus* ST-M5 as influenced by the addition of WPI over 120 min of incubation is shown in Figure 1. The interaction effect of amount of WPI $\times$ time of acid exposure was significant ($P < 0.0001$). The rate at which the log counts decreased with acid exposure time varied with WPI concentration. For the control, the log count decreased from 9.70 to 5.74 during the first 30 min of acid exposure but only decreased from 5.74 to 3.89 between 30 and 120 min of acid exposure. The log counts for the 1, 2, and 3% WPI levels decreased with time of acid exposure at a slower rate than did the control. For the 3% WPI, the log count only decreased from 10.21 at time 0 to 9.27 at 120 min of acid exposure.

Although we detected no significant ($P > 0.05$) differences in log counts among any of the WPI levels at time 0, the log counts at each WPI level were significantly ($P < 0.05$) different from each other at 60, 90, and 120 min of acid exposure, with log counts increasing as the WPI concentration increased. Addition of 1, 2, or 3% WPI resulted in higher ($P < 0.05$) viable cell counts compared with control samples from 30 to 120 min of incubation. In addition, the 3% WPI showed higher ($P < 0.05$) viable cell counts than did 0, 1, and 2% WPI at 30, 60, 90, and 120 min of incubation. Use of 3% WPI improved survival of *Strep. thermophilus* ST-M5 by about 5 log cfu/mL compared with control.

Acid tolerance of *Lb. bulgaricus* LB-12 as influenced by the addition of WPI over 120 min incubation is shown in Figure 2. The interaction effect of amount of WPI $\times$ time of acid exposure was significant ($P < 0.0001$). Similar to the acid tolerance for *Strep. thermophilus*, the rate at which the log counts of *Lb. bulgaricus* decreased with acid exposure time varied with WPI concentration. In the control, the log count decreased from 8.44 to 3.99 during the first 30 min of acid exposure but only decreased from 3.99 to 3.41 between 30 and 120 min of acid exposure. The 1% WPI treatment followed a similar pattern. We observed a slower decrease in log counts with time of acid exposure for both the 2 and 3% WPI levels, indicating less death. For 3% WPI, the log count only decreased from 8.23 at time 0 to 5.17 at 120 min of acid exposure. Although we detected a <0.3 log difference between the counts among various WPI levels at time 0, the log counts for each WPI level at a given time differed ($P < 0.05$) from each other at 30,

Figure 1. Acid tolerance of *Streptococcus thermophilus* ST-M5 as influenced by different concentrations (0, 1, 2, or 3%) of whey protein isolate over an incubation period of 120 min. Error bars represent SE.

60, 90, and 120 min of acid exposure, with log counts increasing with increasing WPI level. Use of 3% WPI improved survival of *Lb. bulgaricus* LB-12 by about 2 log cfu/mL after exposure to acidic conditions for 30, 60, 90 and 120 min (Figure 2).

According to Conway et al. (1987), *Lb. bulgaricus* and *Strep. thermophilus* are not tolerant to acid environments (i.e., pH 3). Lactobacilli strains have higher proteolytic activity and acid tolerance compared with *Strep. thermophilus* (Shah and Jelen, 1990; Dave and Shah, 1998a,b; Garault et al., 2000). According to

González-Márquez et al. (1997), the optimal pH for growth of lactic acid bacteria is between 5.5 and 7.0, which explains the reduction of viable cells in the control treatment after exposure to acidic conditions for 120 min. According to Nadal et al. (2010), the addition of whey proteins can improve the buffering capacity of a medium, thus reducing the effect of acid environments for the bacterial strain.

According to Lee and Vickers (2008), addition of whey proteins to beverages requires greater amounts of acid to reach a specific acidic pH (<4.5) compared with

Figure 2. Acid tolerance of *Lactobacillus bulgaricus* LB-12 as influenced by different concentrations (0, 1, 2, or 3%) of whey protein isolate over an incubation period of 120 min. Error bars represent SE.

beverages without whey proteins. Polypeptides are generated from milk proteins by the action of proteinases and peptidases present in cell walls of starter culture bacteria (Salminen et al., 2004). The action of peptidases from cell walls provides peptides and free AA used by starter culture bacteria for metabolic processes (Garault et al., 2000; Salminen et al., 2004; Güler-Akin et al., 2009). The addition of WPI to yogurt mixes increases the amount of protein in the yogurt. Pescuma et al., (2007) evaluated the ability of *Strep. thermophilus* CRL 804 and *Lb. bulgaricus* CRL 454 to hydrolyze whey proteins (WPC 89% protein) in a medium. *Streptococcus thermophilus* and *Lb. bulgaricus* were capable of breaking down up to 21% of β -LG and 26% of α -LA into smaller peptides (7 kDa) and free AA (Pescuma et al., 2007). This indicates that the enzymes present in the cell walls of starter culture bacteria have an effect on whey proteins.

An acidic environment kills bacteria by decreasing the cytoplasm pH, affecting internal enzymes, reducing the ability to produce ATP, and making it difficult to produce proteins. Whey protein isolate is a good source of AA needed by bacteria. Amino acid decarboxylases control the pH of the bacterial environment by consuming hydrogen ions as part of the decarboxylation reaction (Cotter and Hill, 2003). Cotter and Hill (2003) reported that glutamate decarboxylase (GAD), which is present in the gram-positive bacteria *Strep. thermophilus* and *Lb. delbrueckii* ssp. *bulgaricus*, operates by combining an internalized AA (glutamate) with a proton and exchanging the resultant product γ -aminobutyrate (GABA) for another AA substrate. Thus, an extracellular AA is converted into an extracellular product but the consumption of an intracellular

proton results in increase in intracellular pH (Cotter and Hill, 2003), leading to enhanced acid tolerance by the use of whey proteins.

Bile Tolerance

Bile tolerance of *Strep. thermophilus* ST-M5 as influenced by the addition of WPI over 5 h of incubation is shown in Figure 3. The interaction effect of amount of WPI $\times$ time of bile exposure was significant ($P < 0.0001$). The change in log counts with bile exposure time varied with WPI concentration. For the control, we detected no significant ($P > 0.05$) differences in log counts after 0, 1, 2, 3, and 4 h of bile exposure, but the log count at 5 h was significantly ($P < 0.05$) lower than each of the previous log counts. The only significant difference for the 1% WPI sample was that the 1-h log count was significantly ($P < 0.05$) higher than the 4-h count. For both the 2 and 3% WPI samples, the respective log counts at time 0 were significantly ($P < 0.05$) lower than the log counts for the remaining times (1, 2, 3, 4, and 5 h), indicating bacterial growth. Moreover, addition of 2 or 3% WPI resulted in higher ($P < 0.05$) viable cell counts than control or 1% WPI at 2, 3, 4, and 5 h of incubation, indicating beneficial effects of higher concentrations of WPI on bile tolerance of *Strep. thermophilus* ST-M5.

Bile tolerance of *Lb. bulgaricus* LB-12 as influenced by the addition of WPI over 5 h of incubation is shown in Figure 4. The interaction effect of amount of WPI $\times$ time of bile exposure was significant ($P < 0.0001$). The counts for all WPI levels decreased over time, and the decrease between 2 and 5 h was greater for the control than for the 1, 2, and 3% WPI levels. Counts at each

Figure 3. Bile tolerance of *Streptococcus thermophilus* ST-M5 as influenced by different concentrations (0, 1, 2, or 3%) of whey protein isolate over an incubation period of 5 h. Error bars represent SE.

Figure 4. Bile tolerance of *Lactobacillus bulgaricus* LB-12 as influenced by different concentrations (0, 1, 2, or 3%) of whey protein isolate over an incubation period of 5 h. Error bars represent SE.

hour were significantly ($P < 0.05$) lower than those at the previous hour for each time for the corresponding WPI level except for the counts at 5 h for the 2% WPI level. At 3, 4, and 5 h of bile exposure, the counts for the 1, 2, and 3% WPI were significantly ($P < 0.05$) higher than counts for the control. At 5 h of incubation, the 2 and 3% WPI samples showed significantly ($P < 0.05$) higher viable cell counts than the control and 1% WPI samples. This indicates that the bacterial death was lower for 2 and 3% WPI compared with 0 and 1% WPI, confirming the protective effect against cell death from bile when using higher concentrations of WPI.

Bile salts normally affect the survival of bacterial cells because of the high susceptibility of the bacterial cell envelope to bile (Jin et al., 1998). Liong and Shah (2005) reported bile tolerance for strains of *Lb. acidophilus* and *Lactobacillus casei* at 2 h of incubation in MRS broth containing 0.3% oxgall. They found the highest cell reduction in MRS broth with 0.3% oxgall compared with the control (MRS broth). In the current study, the presence of 2 and 3% WPI increased the survival of *Lb. bulgaricus* LB-12 up to 1.7 log cfu/mL at 5 h of exposure to bile salts compared with control (Figure 4).

According to Charteris et al. (1998), WPI can protect from gastrointestinal stress by acting as a buffering agent and inhibiting activity of digestive enzymes. A decrease in count is normal because of the susceptibility of phospholipid cell walls of bacteria to bile salts (Jin et al., 1998). Bile works as a detergent that emulsifies and solubilizes lipids (Begley et al., 2005). Higher concentrations of bile dissolve lipids present in the phospholipid cell walls of bacteria, making bacteria susceptible to ion-exchange transport, affecting acid adaptation of

bacteria and causing shrinkage and leakage of intracellular material and eventually bacterial death (Begley et al., 2005).

Bile salts can cross the cell membrane, damage proteins and DNA, and result in leakage of intracellular material (Gunn, 2000; Begley et al., 2005). Whey proteins may slow down the damage of the bacterial cell proteins or facilitate protein repair. In addition, bile emulsifies fats and the lipid membrane of bacterial cells (Gunn, 2000; Begley et al., 2005). Whey proteins may function as a barrier or a partial barrier between the bile and the lipid membrane of the bacterial cell.

CONCLUSIONS

Addition of WPI had a positive effect on 2 probiotic properties of yogurt starter bacteria. Use of 1, 2, and 3% WPI resulted in significantly greater acid tolerance over 120 min of acid exposure and greater bile tolerance over 5 h of bile exposure for pure cultures of *Strep. thermophilus* ST-M5 and *Lb. bulgaricus* LB-12 compared with controls. Use of 3% WPI resulted in the highest counts for acid tolerance of *Strep. thermophilus* ST-M5 and *Lb. bulgaricus* LB-12. Use of 2 and 3% WPI resulted in the highest counts for bile tolerance of *Strep. thermophilus* ST-M5 and *Lb. bulgaricus* LB-12 over 5 h of bile exposure. Thus, WPI improved acid and bile tolerance of culture bacteria *Strep. thermophilus* ST-M5 and *Lb. bulgaricus* LB-12.

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