INFLUENCE OF WHEY PROTEIN HYDROLYSATE ON THE GROWTH OF PROBIOTIC AND TRADITIONAL YOGURT CULTURES IN MILK

BY

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Introduction

Certain probiotic bacteria, primarily, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium* ssp, potentially can provide several health benefits. Among these benefits are: improved lactose utilization, control of serum cholesterol levels, control of intestinal pathogens, and anticarcinogenic actions. Thus, there has been recent focus on incorporating these probiotic bacteria into fermented milk products. However, several problems can be associated with doing this. One problem with probiotic bacteria is possible growth suppression when combined with traditional yogurt cultures, such as *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. Another problem with probiotic bacteria is their lack of proteolytic activity, especially when compared to the traditional yogurt cultures. This limits their growth in milk.

The problems mentioned above can very well affect the viability and performance of probiotics in fermented milk products. In order to receive the desired health benefit, probiotic bacteria must survive and remain viable in adequate numbers during the production, storage, and consumption of the fermented milk products.

Earlier work investigated the effects of various substances on the growth and acid production of several strains of lactobacilli in milk. Researchers discovered substances such as yeast extract, liver extract, various peptones, and corn steep liquor all could stimulate the growth of some strains of lactobacilli in milk. However, these substances are not typically used due to the undesirable flavors that accompany them.

The objective of this study was to determine if supplementing milk with whey protein hydrolysates would enhance the growth of several species of probiotic lactobacilli while at the same time not affecting the growth of traditional starter cultures used in the manufacture of yogurt.

Chapter II

Review of Literature

Health Benefits Associated with Probiotic Cultures

Several potential health benefits have been associated with the consumption of fermented and nonfermented milk products containing probiotic bacteria. These potential health benefits are improved lactose utilization, control of intestinal pathogens, control of serum cholesterol levels, and anticarcinogenic actions. It is not the intent of this review to provide in-depth coverage of each of these potential benefits. However, it is important to include examples of research related to each potential benefit.

Improved Lactose Utilization

Individuals that lack the ability to adequately digest lactose are referred to as lactose maldigestors. The inability of lactose maldigestors to digest lactose is due to insufficient amounts of the enzyme β-galactosidase in the small intestines (Gilliland 1989, Hugh and Hoover 1991). Individuals with this condition experience gastric distress, such as bloating, flatulence, diarrhea, and abdominal pain, when they consume fresh, unfermented dairy products. This gastric distress is due to the formation of hydrogen gas by microbial action on undigested lactose in the gut (Gilliland 1989). Thus, people with lactose maldigestion tend to exclude milk products from their diet. This could result in the lack of these individuals obtaining valuable nutrients, primarily calcium and high quality protein, needed in their diet.

An alternative method for lactose maldigestors to obtain the valuable nutrients mentioned above is to consume milk products containing probiotic cultures, such as *Lactobacillus acidophilus*, *Lactobacillus casei*, or *Bifidobacterium* ssp. One such product currently available is nonfermented acidophilus milk. Kim and Gilliland (1983) investigated the effects of supplementing milk with *L. acidophilus* on lactose utilization in humans. They reported that nonfermented milk supplemented with *L. acidophilus* concentrations of $2.5x10^6$ cfu/ml and $2.5x10^8$ cfu/ml, improved lactose utilization in humans classified as lactose maldigestors. Several reports have suggested that milk containing *L. acidophilus* failed to improve lactose utilization for lactose maldigestors (Payne and others 1981, Savainano and others 1984). However, Gilliland (1989) later noted that those studies contained little information on the cultures used or the procedures used in their production and storage prior to testing.

Gilliland and Kim (1984) reported that yogurt containing viable starter bacteria also could improve lactose utilization for lactose maldigestors. The lactase activity, of the evaluated starter bacteria, increased in the presence of bile. This suggested that even though traditional yogurt cultures do not grow in the gastrointestinal tract, they could provide the needed lactase to hydrolyze lactose in the small intestine.

The use of probiotic or traditional yogurt culture to aid lactose maldigestion is dependent on the culture's lactase activity. Thus, the culture's lactase activity must be maintained during storage and through consumption. Gilliland and Lara (1988) investigated the effects of storage at freezing and subsequent refrigeration temperatures on β-galactosidase activity of *L. acidophilus*. The β-galactosidase activity of one strain was significantly reduced within 7 days of storage at 5°C. Noh and Gilliland (1993)

reported variations in β-galactosidase activity among various strains of *L. acidophilus*. These factors suggest that strains of *L. acidophilus* should be carefully selected for maximum β-galactosidase activity before being incorporated into dairy products to benefit lactose maldigestors.

Control of Intestinal Pathogens

Species of lactobacilli and bifidobacteria can exhibit antagonistic actions toward various intestinal pathogens (Gilliland 1989, Gilliland 1979, Hughes and Hoover 1991). Pathogens inhibited by these organisms include *Salmonella* ssp., *Vibrio* spp., enteropathogenic *Escherichia coli*, *Staphylococcus aureus*, and *Clostridium perfringens*. Researchers believe that the inhibitory action is a result of substances, such hydrogen peroxide, acids or bacteriocins, produced by lactobacilli and bifidobacteria.

Gilliland and Speck (1977) investigated the antagonistic action of *L. acidophilus* toward several intestinal and food borne pathogens in a broth system. The results indicated *L. acidophilus* did exert antagonistic actions toward a variety of pathogens. *Staphylococcus aureus* and *Clostridium perfringes* were more sensitive than were *Salmonella typhimurium* and *Eschericia coli* to the presence of *L. acidophilus*. The researchers noted inhibitory effect of *L. acidophilus* was decreased when catalase was added to the growth media. This suggested that the hydrogen peroxide produced by *L. acidophilus* could be partially responsible for the antagonistic action. They concluded that the antagonistic action of *L. acidophilus* was due to a combination of factors, such as acid, hydrogen peroxide, and other antimicrobial substances.

Several feeding trail studies have shown the benefits of consuming probiotic bacteria. Gonzalez and others (1995) investigated the biotheropeutic role of fermented

milk on infants with post-gastroenterititis syndrome. Hospitalized children suffering from post-gastroenterititis were fed a diet of fermented milk containing *L. acidophilus* and *L. casei*. The researchers reported that symptoms of the disease were eliminated within 4 days after consumption of the fermented milk. They concluded that it is possible to prevent gastrointestinal disorders by consuming milk fermented with a mixture of certain strains of *L. acidophilus* and *L. casei*. Shornikova and others (1997) studied the effects of *L. reuteri* on acute diarrhea in young children. Forty children diagnosed with acute diarrhea were divided into two treatment groups. One group received 10^{10} to 10^{11} cfu/ml of *L. reuteri* and the other group received a placebo. By the second day of treatment, diarrhea persisted in only 26% of the children that received *L. reuteri* compared with 81% of the placebo groups. The researchers concluded that fermented milk containing *L. reuteri* could be effective at controlling acute diarrhea in children.

It is important to mention that different strains of lactobacilli and bifidobacteria produce varying amounts of the inhibitory substances previously mentioned. Thus, care must be taken when selecting strains of lactobacilli and bifidobacteria for the ability to control intestinal or food borne pathogens.

Control of Serum Cholesterol Levels

Another potential health benefit from the consumption of probiotic bacteria is the ability to control serum cholesterol levels. Heart disease is one of the leading causes of death in America (Gilliland 1985). High serum cholesterol levels are associated with the development of coronary heart disease. Several studies have indicated that lactobacilli

and bifidobacteria exhibit the ability to lower serum cholesterol levels (Anderson and Gilliland 1999, Gilliland 1985, Grunewald 1982, Harrison and Peat 1975).

Grunewald (1982) studied the effects of milk fermented with *L. acidophilus* on serum cholesterol levels in rats. Rats were divided into three treatment groups. Treatment groups were classified as the following: (1) rats fed no supplementary milk, (2) rats fed milk, and (3) rats fed milk fermented with *L. acidophilus*. The feeding trial lasted four weeks. The study revealed rats fed milk fermented with *L.* acidophilus had significantly lower serum cholesterol levels than control rats. The researcher concluded that some lactic acid bacteria might play a role in influencing serum cholesterol levels. Harrison and Peat (1975) observed infants fed formulas containing added cells of *L. acidophilus* had significantly lower cholesterol levels by the eighth day of consumption compared to those not receiving the culture.

Two possible mechanisms, for the control of serum cholesterol levels, are the assimilation of cholesterol or the enzymatic deconjugation of bile salts by probiotic cultures. Studies have indicated that certain probiotic bacteria can remove cholesterol from laboratory media (Buck and Gilliland 1994, Gilliland and others 1985). Anaerobic conditions and the presence of bile were required for the organisms to removed cholesterol from the media. Gilliland and Speck (1977) reported that certain strains of lactobacilli could deconjugate bile acids in an anaerobic environment. De Rodas and others (1996) reported that a correlation existed between the reduction of total cholesterol concentrations and total bile concentrations for swine fed *L. acidophilus*. The researchers noted when the cholesterol concentrations were reduced there was also a lowering in the serum bile concentrations.

Brashears and others (1998) evaluated the ability of strains of *L. acidophilus* and *L. casei* on the ability to deconjugate bile salts and remove cholesterol from laboratory media. The results indicated that the removal of cholesterol by *L. acidophilus* was due to assimilation, possibly due to the incorporation of cholesterol in the cellular membrane. For *L. casei*, removal of cholesterol was due to the deconjugation of bile salts. Considerable variations were observed among the cultures tested on cholesterol assimilation and bile salt deconjugation. Thus, strain selection is very important if these cultures are to be used as dietary adjuncts.

Anticarcinogenic Actions

Several studies have indicated that certain strains of lactobacilli and bifidobactia could exert anticarcinogenic actions toward some forms of cancers. This effect may be due to the inhibition of certain bacteria that convert procarcinoges into carcinogens in the intestines.

Hughes and Hoover (1991) reported on the antitumor activity of bifidobacteria. They suggested the anti-tumor activity could be due to the direct removal of procarcinogens, indirect removal of procarcinogens, or the activation of the body's immune system. The researchers reported large numbers of liver tumors developed in mice when an intestinal flora of *E. coli*, *Enterococcus faecalis*, and *Clostridium paraputrificum* was present. However, the number of liver tumors greatly decreased when *B. longum* was introduced. Cell wall fractions of some strains of bifidobacteria contain active antitumor constituents, which could induce activation of the body's immune system.

Gilliland (1989) noted studies where *L. acidophilus* was effective at exhibiting anticarcinogenic actions. Shahani and others (1983) investigated the effects of feeding milk fermented with *L. acidophilus* to rats on tumor cells. The results indicated that feeding rats milk fermented with *L. acidophilus* significantly lowered the numbers of tumor cells.

Viability of Probiotic Bacteria in Dairy Products

The viability of probiotic bacteria in dairy products during storage and at the time of consumption, is an important issue. Studies have been conducted investigating the survival of probiotic bacteria in various dairy food products (Dave and Shah 1997, Hekmat and Mcmahon 1992, Klaver 1993, Nighswonger and others 1996, Ravula and Shah 1998, Shin and others 2000). The results of the studies have been mixed with regard to viability of the probiotics. If the consumer is to receive any of the potential benefits mentioned in the previous section, adequate numbers of viable probiotic bacteria must be present in the product at the time of consumption. It has been suggested that a minimum population level of 10^5-10^6 viable cells per g or ml in fermented and nonfermented products would produce the benefits (Dave and Shah 1997, Kailasapthy and Rybka 1997, Shah and others 1995), although the reason for this level was not apparent. It also has been reported that consumers ideally should ingest a daily dose of 10^9-10^{10} probiotic bacteria to ensure the potential health benefits (Sanders 1999, Sanders and others 1996), again the reason was not clear. The actual numbers required to produce the benefits is still unknown. It likely varies with culture and proposed benefit.

Species of bifidobacteria generally do not grow well in milk and fermented milk products. Possible factors that adversely influence the growth of this organism in such products are sensitivity to low pH, sensitivity to oxygen, and minimal proteolytic activity. Klaver and others (1993) investigated the growth and survival of 17 strains of bifidobacteria in milk. Of the 17 strain tested, 15 did not grow in milk. Their inability to grow was attributed to insufficient proteolytic activity. The study also evaluated the survival of bifidobacteria added to fermented milk during storage at 4°C. Fourteen of the seventeen strains showed a reduction of more than 3 log cycles within 2 weeks of storage. The researchers concluded this was due to the acidic environment created by the yogurt cultures. Samona and Robinson (1994) studied the effects of yogurt cultures on 3 species of bifidobacteria in fermented milk. *B. bifidum*, *B. longum*, and *B. adolescentis* were each grown in milk, either individually or in combination with *L. bulgaricus* and *S. thermophilus*. They noted that the growth of all 3 species of bifidobacteria was suppressed in the presence of yogurt cultures.

Yogurt is possibly the most popular vehicle for delivering probiotic bacteria to the human diet. However, special consideration must be made, with regard to yogurt preparation and culture selection, if probiotic bacteria are to survive the storage process and deliver the appropriate numbers at the time of consumption. Hull and others (1984) investigated the survival of *L. acidophilus* in yogurt. The survival of several strains of *L. acidophilus* was determined at two different steps in the yogurt manufacturing process. *Lactobacillus acidophilus* was added before and after the yogurt fermentation process. Survival of *L. acidophilus* was significantly higher when it was added before the fermentation process. After two weeks of storage at 5°C, 50% of *L. acidophilus*

remained viable. In contrast, *L. acidophilus* added after yogurt fermentation lost viability very rapidly, by 4 days of storage less than 1% survived. These finding were similar to that of Gilliland and Speck (1977). They also observed a rapid decrease in the numbers of *L. acidophilus* when added to yogurt after the fermentation process. They concluded that the decrease in viability for *L. acidophilus* was due to hydrogen peroxide produced by *L. bulgaricus* during the manufacture and storage of the yogurt. This study, however, was limited since only one strain of *L. acidophilus* and one yogurt culture were included. Nighswonger and others (1996) determined the viability of 5 strains of *L. acidophilus* and one strain of *L. casei* added to yogurt during 28 days of storage at 7°C. The strains of *L. acidophilus* and *L. casei* were added to yogurt made with two different yogurt cultures. Three of the five strains of *L. acidophilus* exhibited a significant loss in viability in yogurt made with one yogurt culture. The product prepared using the other yogurt culture caused a significant loss in viability for 4 of 5 strains of *L. acidophilus*. The viability of *L. casei* was not effected in products prepared with either yogurt culture. The variations observed in these studies indicate variations among strains of species of probiotic and yogurt cultures with respect to survival of the probiotic in yogurt.

Unfortunately, little information is currently known with regard to the numbers of viable probiotic bacteria in dairy products in retail outlets. The information currently available is mixed (Micanei and others 1997, Shah and Jelen 1990, Shah 2000). Rybka and Fleet (1997) tested the viability of probiotic bacteria from 50 commercial Australian yogurts, which according to the labels, contained *L. acidophilus* and *Bifidobacterium* ssp. The researchers discovered that *L. acidophilus* and *Bifidobacterium* species exceeded 10^6 cfu/g in only 24% and 14% of the samples, respectively. Shah and others (1995) studied

the viability of *L. acidophilus* and *B. bifidum* from 5 brands of commercial yogurt during refrigerated storage. Populations of viable cultures were determined at 3-day intervals over a 5-week period. Counts of *L. acidophilus* in 3 of 5 samples exceeded 10^6 cfu/g up to 30 days. Initial populations of *B. bifidum* (day 0) in 3 of 5 samples were below 10^6 cfu/g. On the other hand, Shin and others (2000) tested the viability of bifidobacteria in commercial milk and yogurt products obtained from retail outlets during refrigerated storage. The results indicated that the viability of bifidobacteria remained above 10^6 cfu/ml or g until the expiration date for each product.

Providing Adequate Numbers of Probiotics in the Diet

The previous section revealed the potential poor performance or maintenance of viability of some probiotics in various dairy products during storage. Some countries have established standards on probiotic products to address this problem (Sanders and others 1996, Shin and others 2000). In Japan, the Fermented Milks and Lactic Acid Beverages Association require that $\geq 1x10^7$ viable bifidobacteria/ml be present in dairy products that claim to contain bifidobacteria. The Swiss Food Regulation require that such products contain $\geq 1x10^6$ cfu/ml or g. The ingestion of viable probiotic bacteria in the proper amount is vital toward the consumer receiving the marketed health benefits. As a result, special considerations must be addressed before producing a product claiming to contain probiotic cultures. This is a great challenge for the manufactures of dairy products containing a probiotic culture(s). Several approaches can be taken to ensure adequate numbers of probiotics in the human diet. These factors include selection

of acid and resistant strains, minimal antagonistic action of yogurt culture toward the probiotic, and the incorporation of stimulatory substances for probiotic cultures.

Selection of Acid and Bile Resistance Strains

Possibly the most important characteristics for probiotic bacteria are the ability to survive the acidic conditions of the stomach and the bile concentrations in the intestine. Conway (1987) studied the survival of four strains of lactic acid bacteria in human gastric juices. In addition, adhesion of these bacteria to human small intestine cells was studied. The cultures studied included two strains each of *L. acidophilus*, *S. thermophilus*, and *L. bulgaricus*. The results from the study indicated *L. acidophilus* was better able to survive in the human gastric juices and adhere compared to the other lactic acid bacteria. Despite these results, many strains of *L. acidophilus* and *Bifidobacterium* species lack the ability to survive the adverse condition in the gut. As a result, those organisms that do not survive these harsh conditions should not be considered as dietary adjuncts in fermented foods.

Gilliland and others (1984) investigated the effects of bile resistant strains of *L. acidophilus* on the numbers of lactobacilli in the small intestine of calves. Initially, 7 strains of *L. acidophilus* were screened for bile resistance. Then, calves were fed diets supplemented with a high bile resistant strain or low bile resistant strain of *L. acidophilus*. Calves fed a diet containing high bile resistant strains of *L. acidophilus* had larger numbers of lactobacilli in the small intestine than did the ones fed the culture having low bile tolerance.

Lankaputhra and Shah (1995) examined the survival of *L. acidophilus* and *Bifidobacterium* spp in the presence of acid and bile salts. Six strains of *L. acidophilus*

and 9 strains of bifidobacteria were evaluated in the study. The survival varied greatly among strains. The population of 3 of 6 strains of *L. acidophilus* significantly decreased especially at pH 2.5 and lower. Seven of nine strains of bifidobacteria rapidly declined in numbers when placed into acidic conditions. In the presence of bile salts, 3 of 6 and 6 of 9 strains of *L. acidophilus* and bifidobactria, respectively, were significantly reduced.

Other studies have investigated survival of probiotic bacteria under acidic conditions (Laroia and Martin 1991, Shah and Jelen 1990). Shah and Jelen (1990) observed that *L. acidophilus* and its β-galactosidase activity could survive in a pH range of 1.5-3.5. Laroia and Martin (1991) studied the effects of pH on the survival of *B. bifidum* and *L. acidophilus* in frozen dairy desserts. *B. bifidum* did not survive in the low-pH product (pH range 3.9-4.6). The high-pH (pH range 5.6-5.8) products contained high populations of *B. bifidum* and *L. acidophilus*. They noted that the lack of survival was probably due to the low pH levels.

Probiotic and Yogurt Culture Interaction

Traditional yogurt cultures often exhibit a beneficial interaction when grown in the presence of one another. In combination, yogurt cultures tend to grow faster and produce acid more rapidly than, when each are grown separately (Dave and Shah 1998, Gilliland 1985). During fermentation in milk, *L. bulgaricus* can release essential amino acids, which are stimulatory toward *S. thermophilus*. In addition, *S. thermophilus* can produce formic acid and carbon dioxide, which stimulate the growth of *L. bulgaricus*. However, some probiotic bacteria do not share in this beneficial interaction, when grown along with traditional yogurt cultures. During the fermentation and storage of cultured

products, yogurt cultures produce substances, such as lactic acid and hydrogen peroxide, which can suppress the growth of some probiotic bacteria. *Bifidobacterium* spp. are particularly susceptible to the rapid formation of acidic conditions created by yogurt cultures (Buchanan and Gibbons 1974, Ravula and Shah 1998).

Various studies have demonstrated the effects of combining inappropriate probiotic cultures with traditional yogurt cultures in fermented dairy products (Dave and Shah 1997, Landaputhra and others 1996, Nighswonger and others 1996, Shah and others 1995, Samona and Robinson 1994). Dave and Shah (1997) determined the viability of *L. acidophilus* and bifidobactieria in yogurt made from different commercial starter cultures. Viability of probiotic bacteria was measured over a 35-day period. It was discovered that the viability of these organisms during refrigerated storage was dependent on probiotic and yogurt culture interactions. Viable numbers of *L. acidophilus* rapidly declined with yogurts prepared from commercial cultures containing *L. bulgaricus*. Conversely, populations of bifidobacteria were more stable in yogurts containing *L. bulgaricus*. Lankaputhra and others (1996) studied the survival of 9 strains of bifidobacteria during refrigerated storage in the presence of acid and hydrogen peroxide. Six of nine strains of bifidobacteria were adversely effected by the presence of acid and hydrogen peroxide.

Probiotic strains must be carefully selected before they are combined with traditional yogurt cultures to produce a fermented dairy product. The interaction with specific strains of probiotic and yogurt culture should be known before using them together in a particular product.

Stimulatory Substances Used by Lactic Acid Bacteria

As mentioned earlier, the incorporation of stimulatory substances into dairy products could be a method for increasing the numbers of cells of probiotic cultures in the human diet. Most probiotic bacteria tend to grow slowly in milk due to their poor proteolytic activity (Dave and Shah 1998, Ravula and Shah 1998, Shah 2000). All the essential nutrients required for the growth of probiotic bacteria are present in milk (Gilliland 1985). However, they may not be in an easily used forms. For example, some probiotic and lactic acid bacteria have a harder time breaking down proteins into this usable form. As a result, the growth of these organisms can be considerably slower. Therefore, influencing the activity of slow growing starter cultures has been a focus of researchers for many years. One way this has been achieved is by the incorporation of stimulatory substances into the milk or product mixes before inoculation. By increasing the growth rate and acid production of lactic acid bacteria, the fermentation time required for producing cultured dairy products is reduced.

In the 1950's, researchers investigated methods to enhance the growth of slow growing lactic acid bacteria in milk (Anderson and Elliker 1953a, Anderson and Elliker 1953b, Garvie and Mabbitt 1956, Kennedy and Speck 1955, Sandine and others 1956, Speck and others 1958). Most work focused on supplementing milk with materials that could deliver nutrients in a more easily usable form. Through their experiments, various substances where found to stimulate the growth of lactic acid bacteria when supplemented in milk. Sandine and others (1956) reported that pancreas extract could enhance the growth of *L. casei* and *S. lactis*. Speck and others (1958) confirmed that

pancreas extract could simulate the growth some lactic acid bacteria. They conducted a study, which determined the response of *S. lactis* to pancreas, liver, and yeast extracts in milk. The results indicated that each extract, when added to milk, could stimulate the growth of *S. lactis*. The stimulatory effect was attributed to the presence of peptides. Garvie and Mabbit (1956) noted that milk supplemented with peptones were stimulatory toward slow growing strains of *S. lactis*.

Kennedy and Speck (1955) examined the effects of supplementing milk with corn steep liquor to enhance the growth of several lactic acid and spoilage bacteria. Corn steep liquor is a by-product of the corn wet-milling industry (Kennedy 1955). The researchers observed significant stimulation for all the tested lactic acid bacteria grown in milk supplemented with 1% corn steep liquor, while the growth of the spoilage bacteria was unaffected.

Yeast extract, corn steep liquor, peptones, liver and pancreas extracts all can stimulate the growth of various strains of lactic acid bacteria when added to milk. All the substances contain small peptides, which these organisms can utilize easier than proteins for growth. However, their practical use in the dairy industry is very limited due the undesirable flavors and odors that accompany most of them.

Other substances that have been found to be stimulatory toward probiotic bacteria include cysteine and casein hydrolysate (Dave and Shah 1998, Gomes and others 1998, Ravula and Shah 1998, Shah 2000). Ravula and Shah (1998) tested the effects of casein hydrolysate and cysteine on the viability of *L. acidophilus* and bifidobacteria in frozen dairy desserts during a 12 week storage period. Two batches of fermented dairy desserts were made with milk supplemented with either casein hydrolysate or cysteine. The

control batch was made with milk supplemented with 2% skim milk powder. The populations of *L. acidophilus* and bifidobacteria in the control sample decreased to $<10^2$ cfu/g during the 12-week study. On the other hand, *L. acidophilus* and bifidobacteria counts were $>10^5$ cfu/g for samples supplemented with casein hydrolysates or cysteine. These results are similar to the finding of Dave and Shah (1998) who observed significant growth enhancement for *L. acidophilus* and bifidobacteria when yogurt was supplemented with either cysteine or casein hydrolysate. They concluded that growth enhancement was due to nutrients such as peptides and amino acids supplied through these substances.

Use of Whey Proteins to Stimulate the Growth of Lactic Acid Bacteria

Another stimulatory substance that has drawn attention over the years is whey protein. Whey proteins are defined as a heterogeneous mixture of non-casein milk protein (Dybing and Smith 1991, Fox 1992). The whey protein mixture is made up of α-lactalbumin, β-lactoglobulin, bovine serum albumen, several immunoglobins, specific polypeptides, and trace amounts of miscellaneous compounds. Whey is a major by-product of the cheese manufacturing industry. This has created large supplies and a need for alternative uses for this by-product. As a result, whey protein products such as whey powder, whey concentrates, and whey protein hydrolysates are commercially available. Research indicates some of these substances can serve as a source of nutrient rich peptides and amino acid required for the growth of various strains of lactic acid bacteria,

especially the probiotic bacteria (Dave and Shah 1998, Kizer and others 1955, Shah 2000).

Kizer and others (1955) observed that enzymatic hydrolysates of lactalbumin exhibited a growth stimulating ability towards *L. casei* and *S. lactis* when added to milk and to a semi-synthetic medium. Dave and Shah (1998) studied the effects of various supplements on the viability of *L. acidophilus* and bifidobacteria in yogurt. Two ingredient supplements included in this study were whey powder and whey protein concentrates. Viability of the organisms was monitored after the fermentation process and during refrigerated storage (4°C) for 35 days in each yogurt containing the different supplement. The growth of *L. acidophilus* was unaffected by supplementing yogurt with either whey powder or whey protein concentrates. The population of *L. acidophilus* in the two supplemented yogurts where essentially the same as in the control yogurt. This was the case from day 0 through day 35. On the other hand, the growth and viability of bifidobacteria was significantly enhanced in yogurt supplemented with whey protein concentrates. Counts of bifidobacteria were $>10^6$ cfu/g in yogurts supplemented with whey protein concentrates. These counts were maintained throughout the 35-day study. Whey powder had no significant effect on the growth of bifidobacteria.

Poch and Bezkorovainy (1988) reported whey from bovine milk and bovine serum albumin digest were effective as growth stimulates for some but not all of the strains of bifidobacteria tested. Kailasapathy and Supriadi (1996) observed significantly higher numbers of *L. acidophilus* remaining in yogurt supplemented with whey protein concentrates after 21-days of storage (5°C). The varying of results from the reported

studies indicated the importance of proper strain selection in order to obtain the desired response.

Improving the growth of typically slow growing probiotic bacteria in fermented milk products, such as yogurt, is essential for helping assure any potential health and/or nutritional benefit for consumers. However, yogurt usually also contain faster growing more proteolytic organisms, such *S. thermophilus* and *L. bulgaricus*. Incorporating a substance that is stimulatory toward all organisms present in the product could very well further retard the growth of the more acid sensitive probiotics. This in turn would defeat the purpose of incorporating probiotic bacteria as part of the starter culture for making fermented dairy products for their possible health benefits.

Various studies investigated the effects of whey proteins on the growth of traditional starter cultures (Bury and others 1998, Champagne and others 1996, Grieg and Harris 1983, Grieg and Van Kan 1984, Kailasapathy and others 1996, Leh and Charles 1989, Parente and Zottiola 1991). Greig and Van Kan (1984) determined the effects of whey protein concentrates on the fermentation of yogurt. Yogurts were made containing 0%, 5%, 10%, 20%, and 30% whey protein concentrates. The researchers reported no affects on the growth of *S. thermophilus* and *L. bulgaricus* in the yogurts containing whey protein concentrates. Champange and others (1996) similarly observed no stimulatory effects on the growth of *L. bulgaricus,* and *S. thermophilus* by whey protein concentrates. Conversely, Bury and others (1998) reported that adding 1or 2% whey protein concentrates to a whey-based medium significantly increased the growth and acid production of *L. bulgaricus* and *S. thermophilus*.

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CHAPTER III

INFLUENCE OF WHEY PROTEIN HYDROLYSATE ON THE GROWTH OF PROBIOTIC AND TRADITIONAL YOGURT CULTURES IN MILK

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ABSTRACT

Whey protein hydrolysate (WPH) samples were tested for the ability to stimulate the growth of probiotic bacteria in milk. Initially, nine whey protein and WPH samples were screened for the ability to enhance the growth of *L. acidophilus* L-1 in milk. Significant increases in populations were observed only for the whey protein hydrolysate samples. Selected strains of other probiotic bacteria and traditional yogurt cultures were grown in milk supplemented with WPH to determine its effect on their growth. There were varying results, with regards to the probiotic cultures. The growth of *B. longum* S9, *L. acidophilus* O16, and *L. acidophilus* L-1 was significantly higher, when grown in milk supplemented with WPH, compared to the control. However, milk containing WPH had no effect on the growth of *L. acidophilus* NCFM, *L. casei* E5, *L. casei* E10. For traditional yogurt cultures, WPH had no effect on the growth of *L. bulgaricus* 18, *L. bulgaricus* 10442, *S. thermophilus* 1, and *S. thermophilus* 2, while significant increases in growth was observed for *S. thermophilus* 143.

Selected probiotic bacteria were grown in combination with different combinations of the yogurt cultures in milk with and without WPH. No significant effects were observed when *B. longum* S9 was grown in milk containing WPH with the different combinations of yogurt cultures. Significant increases in numbers of *L. acidophilus* O16 and *L. acidophilus* L-1 occurred when grown with the different yogurt cultures in milk supplemented with WPH compared to the control. However, the viability of these cultures was adversely effected during subsequent refrigerated storage. By day 28, the populations of the probiotic cultures in WPH samples were similar or below the control samples.

INTRODUCTION

In recent years, the trend of dairy food manufacturers is to incorporate probiotic bacteria into fermented and nonfermented dairy products. This is primilary due to the potential health benefits attributed to their consumption. Probiotic bacteria by definition refer to live bacteria that beneficially affect the host following ingestion (Kailasapathy and Rybka 1997). There are several problems associated with incorporating probiotic bacteria into the starter used to make cultured dairy products. One problem is slow growth of probiotic bacteria due their low level of proteolytic activity. Another problem is possible growth suppression, of probiotic bacteria, in associative growth with traditional yogurt cultures. In order for probiotic bacteria to deliver their health benefits there must be sufficient numbers present at the time of consumption. Thus, producing a product that contains adequate numbers of probiotics, at the point of consumption in dairy products, is very important. Several countries have established standards for numbers of probiotic bacteria in dairy products (Sanders and others 1996, Shin and other 2000). In Japan, $\geq 1x10^7$ viable bifidobacteria/ml must be present in products claimed to contain bifidobacteria. In addition, the Swiss Food Regulation require that such products contain $\geq 1x10^6$ probiotic organisms per ml or g.

A possible method of ensuring adequate numbers of probiotic bacteria in cultured dairy products is to supplement milk to be fermented with substances stimulatory toward the growth of probiotic bacteria. Substances such as yeast extract, liver extract, peptones, and corn steep liquor when added to milk stimulate the growth of some strains of lactobacilli (Anderson and Elliker 1953, Kennedy and Speck 1955, Kizer and others

1955, Sandine and others 1956, Speck and others 1958). However, use of these substances would cause undesirable flavors in the cultured products.

More recent work has investigated the effects of whey protein and whey protein hydrolysates on the growth of probiotic bacteria in milk (Dave and Shah 1998, Kailasapathy and Supriadi 1996). Some of these substances can serve as a source of nutrient rich peptides and amino acids required for the growth of various strains of lactic acid bacteria and especially the probiotic bacteria.

The objective of this study was to determine if supplementing milk with whey protein hydrolysates would enhance the growth of several species of probiotic lactobacilli and bifidobacteria while at the same time not affecting the growth of traditional starter culture bacteria used in the manufacture of yogurt.

MATERIALS AND METHODS

Source and Maintenance of Cultures

Three strains of *Lactobacillus acidophilus* (L-1, O16, and NCFM), two of *Lactobacillus casei* (E5 and E10), two of *Lactobacillus delbrueckii ssp. bulgaricus* (18 and 10442), one of *Bifidobacterium longum* (S9), and three of *Streptococcus thermophilus* (1, 2, and 143) were used in this study. Cultures were obtained from the stock culture collection of the Food Microbiology Laboratory at Oklahoma State University. The identity of all strains studied was confirmed by testing fermentation patterns and Gram stain.

Cultures were maintained by weekly subculturing using 1% inocula and 18 hour incubation at 37°C in lactobacilli MRS broth (Difco Laboratories, Detroit Michigan) for all cultures except *Bifidobacterium longum* which was maintained in lactobacilli MRS broth supplemented with 0.1% thioglycolic acid. All cultures were incubated for 18 hours at 37°C. The cultures were stored at 5°C between transfers. Stock cultures were stored in lactobacilli MRS agar stabs, followed by a monthly subculture into fresh MRS agar stabs. The cultures were subcultured three times in the appropriate broth medium prior to each experiment.

Bacterial Growth Media

Lactobacilli MRS agar was utilized to measure the total numbers of probiotic and traditional yogurt cultures. The lactobacilli MRS agar medium was made by adding 1.5% agar to MRS broth (Difco Laboratories, Detroit Michigan). Bile resistant lactobacilli and

bifidobacteria were enumerated using Lactobacillus Selection (LBS) agar supplemented with 0.15% oxgall (LBSO agar). The medium was prepared from individual ingredients according to the manufacturer's formulation (Becton Dickinson and Company, Cockeysville MD) and dispensed into sterile bottles. The nonfat milk (NFM) was prepared by reconstituting nonfat dried milk in water at 10% (w/v), pasteurizing at 85°C or 100°C for 30 min, and holding at 5°C until inoculation. It was prepared fresh either the day of or evening before the experiment.

Enumeration of Bacteria

To measure the total numbers of *L. acidophilus, L. casei, B. longum*, *L. delbrueckii ssp. bulgaricus* and *S. thermophilus*, appropriate dilutions were prepared according to methods described in the *Compendium of Methods for the Microbiological Examination of Foods* (Vanderzant and Splittsloesser 1990) and plated using the pour plate method with lactobacilli MRS agar. The plates were overlayed with the same medium and incubated at 37°C for 48 hour. To selectively enumerate the bile resistant probiotic bacteria (*L. acidophilus*, *L. casei*, and *B. longum*) in the fermented milk samples, appropriate dilutions were prepared and plated on LBSO agar. Plates were placed in plastic bags, sealed, flushed with CO_2 for 30 sec, and incubated at 37°C for 48 hour. A Quebec colony counter (Darkfield - Model 3325, Buffalo New York) was used to count the colonies to allow determination of numbers of colony forming units per gram.

Initial Screening of Whey Protein Samples

Nine whey protein samples (from commerical suppliers) were tested for their influence on the growth and acid production of *L. acidophilus* L-1 in NFM. The NFM (10% w/v) was prepared and dispensed in 20mL volumes into test tubes each containing 0.2g of one of 9 whey protein or whey protein hydrolysate (WPH) sample. Samples were mixed and heated in a boiling water bath for 30 minutes. After cooling, samples were inoculated with 1% of a freshly prepared MRS broth culture of *L. acidophilus* L-1 and incubated for 16 hr at 37°C. Following incubation, samples were placed in an ice-water bath to stop growth and acid production. Total numbers of lactobacilli were enumerated by plating on MRS agar. In addition, to plating the pH of each sample was measured using a pH meter (Fisher Scientific AR25).

Influence of WPH-1 on Probiotic and Traditional Yogurt Cultures

The effects of supplementing milk with whey protein hydrolysate (WPH-1) were determined for six probiotic and five traditional cultures. Nonfat milk was prepared and dispensed in 20mL volumes for each culture to be tested into test tubes containing the desired concentration of WPH-1 and a test tube without WPH-1 (Control). Samples were mixed and heated at 85°C for 30 minutes. After cooling, one tube of each milk sample was inoculated with 1% of a freshly prepared MRS broth culture of the organism to be tested and incubated for 16 hr at 37°C. Following incubation, samples were placed in an ice-water bath to stop growth and acid production. Samples were appropriately diluted and plated on MRS agar. The acidity (pH) of each sample was measured.

Influence of Different Concentrations of WPH-1 on the Growth of Probiotic and Yogurt Cultures in Milk

The growth of individual probiotic and traditional yogurt cultures was evaluated in milk containing different concentrations of WPH-1. Nonfat milk was prepared and dispensed in 20mL volumes into test tubes containing the desired concentrations of WPH-1. Samples were mixed and heated at 85°C for 30 minutes. Once heated, the samples were cooled to 37°C and inoculated (1%) with a freshly prepared probiotic or yogurt culture. Samples were mixed and incubated for 16 hr at 37°C. Following incubation, samples were placed in an ice-water bath to stop growth and acid production. Samples were plated on MRS agar and the pH values were measured.

Influence of WPH-1 on Probiotic Cultures when Combined with Traditional Yogurt Cultures

The effects of WPH on the growth of individual probiotic bacteria in milk containing different combinations of *L. delbrueckii ssp. bulgaricus* (18 and 10442) and *S. thermophilus* (1 and 143) were evaluated. Reconstituted NFM was prepared and separated into two containers. Milk was added in 100ml volumes to one container containing (0.5%) WPH and to another without WPH (control). The two containers were heated to 85°C for 30 minutes and placed in a refrigerator overnight at 5°C. Each sample was inoculated with 0.5% of a freshly prepared MRS broth probiotic culture and 0.1% of freshly prepared MRS broth yogurt cultures and incubated in a 37°C waterbath. The pH of each sample was measured hourly. When each sample reached pH 4.80, it was placed in an ice-water bath to stop growth and acid production. Samples were then plated on LBSO agar to enumerate the bile resistant probiotic organisms.

Effects of WPH on the Shelf Stability of Probiotic Culture in Yogurt

The effects of WPH on the viability of individual probiotic bacteria in milk containing different combinations of traditional yogurt cultures were evaluated over 42 days of storage at 5°C. Samples were prepared as in the previous section. Following sample preparation and incubation, the samples were dispensed in 10g volumes into test tubes and stored at 5°C. Samples were plated on days 0, 3, 7, 14, 21, 28, 35, 42 on LBSO agar. Following each plating, pH measurements were recorded for each sample.

Statistical Methods

Analysis of variance for each set of data was conducted as a factorial arrangement of treatments in a randomized complete block design to determine whether significant differences existed. Each replication was a block. For the shelf stability experiment, the set of data was conducted as a split plot in a randomized complete block design. Each replication was a block, milk treatment was the main unit treatment, and days of storage was the subunit treatment. The SAS PROC GLM procedure with LSMEANS and Least significant difference statements were used to compare means for significant differences at the 5% level of confidence.

RESULTS

Initial Screening of Whey Protein Samples

Nine whey protein samples were evaluated for their ability to influence the growth and acid production of *L. acidophilus* L-1. Significant differences ($P<0.05$) were found among samples for the ability to enhance the growth and acid production of *L. acidophilus* L-1 (Table 1). Whey protein samples WPH-1 and WPH-3 significantly ($P<0.05$) increased the growth and acid production of *L. acidophilus* L-1, when compared to the control. There was over a one-log cycle increase in total numbers of *L. acidophilus* L-1 when WPH-1 or WPH-3 was added to the milk. Whey protein sample WPH-1 appeared to have the greatest influence on the growth of *L. acidophilus* L-1. In addition, the pH values dropped over 1.5 units more when either whey protein hydrolysate sample was added to milk, than in the control. Numbers of *L. acidophilus* L-1 were significantly ($P<0.05$) lower in milk supplemented with whey protein sample WP-2, compared to the control. The remaining whey protein samples had no significant ($P<0.05$) influence on the growth of *L. acidophilus* L-1. Based on these results, whey protein sample WPH-1 was used for the remainder of the study.

Influence of WPH-1 on Probiotic and Traditional Yogurt Cultures

The influence of WPH-1 on six strains of probiotic and five strains of traditional yogurt bacteria were evaluated. For the stains of probiotic bacteria, supplementing milk with WPH-1 had varying results (Table 2). The addition of 1% WPH-1 significantly ($P<.05$) increased the growth of *L. acidophilus* O16 and *L. acidophilus L-1*. For both

organisms, the growth was increased over one log cycle when grown in milk supplemented with WPH-1 compared to the control. In addition, the growth of *B. longum* S9 was significantly (P<.05) stimulated by WPH-1. However, WPH-1 had no effect (P<.05) on the growth of *L. acidophilus* NCFM, *L. casei* E5, and *L. casei* E10.

Supplementation of milk with 1% WPH-1 had a slight to no effect on the growth of several traditional yogurt cultures used in this study (Table 2). The growth of *L. delbrueckii* ssp. *bulgaricus* 10442 and *L. delbrueckii* ssp. *bulgaricus* 18 showed slight enhancement of growth, although not significant (P<.05), due to the addition of WPH-1, when compared to the control. Furthermore, the growth of *S. thermophilus* 1 and *S. thermophilus* 2 was not significantly (P<.05) effected. However, the growth of *S. thermophilus* 143 was significantly (P<.05) increased in milk supplemented with WPH-1 compared to the control. The total numbers of *S. thermophilus* 143 were over half a log cycle higher in milk supplemented with WPH-1, than in the control.

Influence of Differing Concentrations of WPH-1 on the Growth of Probiotic and Yogurt Cultures

The growth of probiotic and traditional yogurt cultures in nonfat milk supplemented with different concentrations of WPH-1 was evaluated. Significant differences (P<.05) existed for several strains of probiotic bacteria, when grown in milk supplemented with differing concentrations of WPH-1 (Table 3). The growth of *L. acidophilus* L-1 was significantly (P<.05) increased when grown in milk supplemented with .5%, and .2% WPH-1 compared to the control. In addition, the population of *L. acidophilus* O16 was significantly higher in milk supplemented with .5%, .2%, .1%, and

.05% WPH-1 compared to the control. The growth of *B. longum* S9 was significantly increased in milk containing .5%, .2%, and .1% WPH-1, when compared to the control. However, neither strain of *L. casei* was effected by the addition of different WPH-1 concentrations.

With the exception of *S. thermophilus* 143, the supplementation of milk with different concentrations of WPH-1 had little to no effect on the growth of any of the traditional yogurt cultures tested (Table 4). No significant differences ($P<.05$) were observed for *L. delbrueckii* ssp. *bulgaricus* 18, *L. delbrueckii* ssp. *bulgaricus* 10442, *S. thermophilus* 1,and *S. thermophilus* 2, when grown in milk supplemented with different WPH-1 concentrations. The population of *S. thermophilus* 143 significantly ($P<.05$) increased by each level of WPH-1 tested (.5%, .2%, .1%, .05%, and .01%) compared to the control (Table 4).

Another lot sample of WPH-1 (WPH-1a) was obtained from the same manufacturer and tested. Only those probiotic and traditional cultures that were stimulated by WPH-1 were tested in WPH-1a. Similar results were obtained (See appendix tables 22 and 23).

Influence of WPH-1 on Probiotic Cultures when Combined with Traditional Yogurt Culture

While there appeared to be some variations among yogurt cultures, the growth of both strains of *L. acidophilus* was significantly ($P<.05$) increased in the milk supplemented with WPH-1 compared to the controls (Tables 5and 6). The growth of *L. acidophilus* O16 appeared to be effected greatest when *L. delbrueckii* ssp. *bulgaricus* 18 and *S. thermophilus* 1 were the traditional yogurt cultures. There was roughly a one log

cycle increase in the population of *L. acidophilus* O16 in milk supplemented with WPH when grown in the presence of *L. delbrueckii* ssp. *bulgaricus* 18 and *S. thermophilus* 1, compared to the control. When *L. acidophilus* O16 was grown in the presence of the remaining combinations of yogurt cultures, the populations were closer to a half log cycle increase (Table 5). The population for *L. acidophilus* L-1 increase approximately a half a log cycle in each of the different combinations of yogurt cultures when compared to the control (Table 6).

No Significant ($P<.05$) effects on the growth of *B. longum* S9 was observed with any of the combinations of traditional yogurt cultures in milk supplemented with WPH-1, when compared the control (Table 7). Total numbers of *B. longum* S9 in milk containing WPH-1 were very similar to the control in all the different combinations of yogurt cultures.

Effects of WPH-1 on the Shelf Stability of Probiotic Cultures in Yogurt

The viability of probiotic cultures during storage at 5°C decreased over time. Initially, strains of *L. acidophilus* were significantly ($P<.05$) higher in nonfat milk supplemented with WPH-1 (Table 8). However, over time the positive effects on the growth of probiotic cultures in milk supplemented with WPH-1 diminished. The survival during storage of *L. acidophilus* O16 was adversely effected in milk containing WPH-1 and the differing yogurt cultures over time (Table 8). By day 28, the total numbers of *L. acidophilus* O16 present in milk containing WPH-1 were significantly ($P<.05$) lower than total numbers of *L. acidophilus* O16 in the control samples, for all but one, of the different combinations of yogurt cultures. *L. acidophilus* O16 was especially susceptible

when grown and stored in milk containing WPH-1, using the *L. delbrueckii* ssp. *bulgaricus* 18 and *S. thermophilus* 1 or *L. delbrueckii* ssp. *bulgaricus* 18 and *S. thermophilus* 143 combinations. The decline in total numbers was not as severe for *L. acidophilus* L-1. At 28 days of storage, no significant differences ($P<.05$) existed in population between *L. acidophilus* L-1 grown and stored in milk containing WPH-1 and the control in any of the different combinations of yogurt cultures.

proteins. However, the growth and viability of bifidobacteria was significantly enhanced in yogurt supplemented with whey proteins.

In the initial screening portion of this study seven whey protein samples and two whey protein hydrolysate samples were evaluated. The experiment indicated that the two whey protein hydrolysate samples were the only samples to significantly enhance the growth of *L. acidophilus* L-1. This was not surprising since a factor reported to limit growth of probiotic bacteria in milk is that they have low levels of proteolytic acitivity (Klaver and others 1993, Dave and Shah 1998). The WPH apparently provided a more readily available source of peptides or amino acids needed for growth of the probiotic cultures.

The experiment testing the effects of WPH-1 on the growth of probiotic and traditional yogurt cultures presented varying results. The growth of *B. longum* S9, *L. acidophilus* O16, and *L. acidophilus* L-1were significantly increased by the incorporation of WPH-1 in milk. Growth of only one strain (*S. thermophilus* 143) of the yogurt cultures listed was stimulated by WPH suggesting that the WPH could be added to milk being cultured by a combination of *L. acidophilus* or *B. longum* to enhance their growth without influencing growth of the traditional yogurt cultures. Of course these results show the importance of selecting the yogurt culture not influenced by the WPH if either of these two species of probiotic bacteria are being included in the starter culture. Unfortunately, neither strain of *L. casei* benefited from the WPH. Thus, WPH supplementation would offer no advantage for this species of *Lactobacillus*.

Significant increases in total numbers of *L. acidophilus* O16 were observed in milk containing WPH-1 concentrations of .5%, .2%, .1%, and .05%. The growth of *L.*

acidophilus L-1 was significantly increased at WPH-1 concentrations of .5% and .2%. This suggests that strain L-1 has a higher requirement for one or more components of WPH, than does strain O16. The results further suggest that *B. longum* S9 also is more demanding than *L. acidophilus* O16. Milk supplemented with the higher concentrations of WPH-1 had a larger effect on the stimulation of all three probiotic cultures. The growth of each of these cultures was increased over one log cycle, when grown in milk supplemented with .5% WPH-1, compared to the control. Even though not compared in the same experiments none of the cultures grown in milk containing 1% WPH-1 grew better than in milk containing 0.5% WPH. This indicates that using a WPH-1 concentration level of 0.5% would work just as well in effectively stimulating the growth of probiotic cultures in milk, compared to a concentration level of 1%. Thus, it would be more cost effective to use the lower concentration level (0.5%) in order to achieve the same results.

To confirm that supplementing milk with WPH would stimulate growth of probiotic bacteria growing in association with traditional yogurt cultures, the influence of WPH on growth of various combinations in milk was evaluated. Growth of *L. acidophilus* L-1 and *L. acidophilus* O16 was significantly increased by the WPH, when grown in nonfat milk with each of the different combinations of yogurt cultures. However, it is important to note that the magnitude of the enhancement effect of WPH on these probiotic cultures was not nearly as high when grown in the presence of traditional yogurt cultures, compared to when grown individually in nonfat milk containing WPH. Moreover, the stimulatory effect of WPH on *B. longum* S9 when grown individually in milk was completely lost with the addition of the different combinations of yogurt

cultures. This may be due to sensitivity of *B. longum* to acid conditions created by the yogurt cultures.

It has been documented that traditional yogurt cultures can suppress the survival of some probiotic bacteria during refrigerated storage (Gilliland 1985, Samona and Robinson 1994, Nighswonger and others 1996, Ravula and Shah 1998). During fermentation, yogurt cultures produce substances, such as lactic acid and hydrogen peroxide, which can be anatagonistic toward some strains of probiotic bacteria (Samona and Robinson 1994, Lankaputhra and others 1996, Dave and Shah 1997). Supplementing milk with WPH yielded varying effects on the shelf stability of *L. acidophilus* O16 and *L. acidophilus* L-1, during storage at 5°C. Initially, milk containing WPH had significantly higher populations of probiotic bacteria compared to the control. However, over time the populations of probiotic bacteria decreased. In the case of *L. acidophilus* O16, WPH exhibited an adverse effect. By day 28, the total numbers were significantly lower in milk supplemented with WPH compared to the control for 3 of the 4 different yogurt culture combinations evaluated (Table 10). The viability of *L. acidophilus* L-1 was more stable than *L. acidophilus* O16 over time. By day 28, the populations of *L. acidophilus* L-1 grown with WPH were slightly higher than the control for each different yogurt combination. Various studies have reported poor survival of probiotic bacteria in dairy food products containing yogurt cultures (Dave and Shah 1997, Gilliland and Speck 1977, Rybda and Fleet 1997). The researcher concluded that the survival of the probiotic bacteria was effected by the yogurt cultures due to the accumulation of acid and hydrogen peroxide. In contrast, some studies have reported that the viability of some probiotic bacteria remained high over time (Hull and others 1984, Shah and others 1995,

Micanei and others 1997). The results from the shelf stability study and published reports stress the importance of careful probiotic culture selection before they are combined with traditional yogurt cultures to produce a fermented dairy product.

Table 1. Influence of different whey proteins on the growth and acid production of *Lactobacillus acidophilus* L-1 in 10% nonfat milk.

Sample[1]	**pH**	**Log_{10} cfu/g**[2]
Control	5.8[a]	7.78[a]
WPH-1	4.1[b]	9.30[b]
WP-2	5.3[c]	7.30[c]
WPH-3	4.2[b]	8.95[b]
WP-4	5.8[a]	7.91[a]
WP-5	5.8[a]	7.93[a]
WP-6	5.8[a]	7.94[a]
WP-7	5.8[a]	8.13[a]
WP-8	5.8[a]	7.84[a]
WP-9	5.8[a]	8.03[a]

[1]WP and WPH = (1%) Whey Protein sample + 20mL 10% NFDM; Control = 20mL 10% NFDM without whey proteins.
[2]Measurements made after 16 hr growth. Each value is a mean from 3 replicate experiments; cfu = colony forming units.
[abc]Values with no common superscript letters differ significantly ($P<0.05$); SEM = 0.048.

Table 2. Influence of whey protein hydrolysate[1] (WPH-1) on the growth of probiotic and traditional yogurt cultures in nonfat milk.

Type of culture	Species	pH[2]		Log_{10} cfu/g[2]	
		Control	WPH-1	Control	WPH-1
Probiotic	*L. acidophilus* L-1	5.8[a]	4.3[b]	7.75[a]	9.20[b]
	L. acidophilus O16	6.2[a]	4.3[b]	7.99[a]	9.76[b]
	L. acidophilus NCFM	4.4[a]	4.0[b]	9.01[a]	9.03[a]
	L. casei E5	6.4[a]	6.4[a]	7.95[a]	7.95[a]
	L. casei E10	6.5[a]	6.5[a]	7.78[a]	7.78[a]
	B. longum S9	5.9[a]	4.5[b]	8.47[a]	9.53[b]
Traditional yogurt	*L. bulgaricus* 18	4.3[a]	4.1[b]	8.78[a]	8.92[a]
	L. bulgaricus 10442	4.3[a]	4.0[b]	8.67[a]	9.03[a]
	S. thermophilus 1	4.3[a]	4.3[a]	9.21[a]	9.23[a]
	S. thermophilus 2	4.1[a]	4.1[a]	9.15[a]	9.26[a]
	S. thermophilus 143	4.6[a]	4.3[b]	8.43[a]	9.08[b]

[1]WP-1; added (1%) to yogurt mix prior to heating.
[2]Measurements made after 16 hr growth. Each value is a mean from 3 replicate experiments; cfu = colony forming units.
[ab]Means in same row, for each parameter measured, without common superscript letter differ significantly ($P<0.05$); SEM = 0.044.

Table 3. Influence of different concentrations of whey protein hydrolysate (WPH-1) on growth of probiotic bacteria in nonfat milk.

Organism	WPH-1 Concentration	pH[1]	Log_{10} cfu/g[1]
	Control	5.73[a]	8.06[a]
	0.01%	5.48[a]	8.23[a]
L. acidophilus L-1	0.05%	5.2[b]	8.36[a]
	0.1%	4.95[b]	8.36[a]
	0.2%	4.78[b]	8.57[b]
	0.5%	4.36[b]	8.97[b]
	Control	6.07[a]	7.93[a]
L. acidophilus O16	0.01%	5.97[a]	7.98[a]
	0.05%	5.67[a]	8.61[b]
	0.1%	5.47[b]	8.92[b]
	0.2%	5.27[b]	9.25[b]
	0.5%	4.83[b]	9.48[b]
	Control	6.50[a]	7.75[a]
	0.01%	6.50[a]	7.74[a]
L. casei E5	0.05%	6.52[a]	7.73[a]
	0.1%	6.52[a]	7.76[a]
	0.2%	6.49[a]	7.78[a]
	0.5%	6.48[a]	7.75[a]
	Control	6.38[a]	7.86[a]
	0.01%	6.37[a]	7.94[a]
L. casei E10	0.05%	6.42[a]	7.94[a]
	0.1%	6.35[a]	8.05[a]
	0.2%	6.31[a]	8.07[a]
	0.5%	6.23[a]	8.11[a]
	Control	5.83[a]	8.36[a]
	0.01%	5.34[b]	8.41[a]
B. longum S9	0.05%	5.4[b]	8.61[a]
	0.1%	5.12[b]	8.89[b]
	0.2%	4.98[b]	9.01[b]
	0.5%	4.74[b]	9.19[b]

[1]Measurements made after 16 hr growth. Each value is a mean from 3 replicate experiments; cfu= colony forming units.

[ab]Means in the same column for each organism, for each parameter measured, without common superscript letter differ significantly ($P<0.05$); SEM = 0.037.

Table 4. Influence of different concentrations of whey protein hydrolysate (WPH-1) on the growth of traditional yogurt cultures in nonfat milk.

Organism	WPH-1 Concentration	pH[1]	Log_{10} cfu/g[1]
	Control	4.69[a]	8.79[a]
	0.01%	4.58[a]	8.69[a]
L. bulgaricus 18	0.05%	4.44[a]	8.53[a]
	0.1%	4.33[b]	8.61[a]
	0.2%	4.33[b]	8.59[a]
	0.5%	4.35[b]	8.70[a]
	Control	4.37[a]	8.13[a]
L. bulgaricus 10442	0.01%	4.31[a]	8.02[a]
	0.05%	4.25[a]	8.11[a]
	0.1%	4.20[a]	8.33[a]
	0.2%	4.14[a]	8.39[a]
	0.5%	4.10[a]	8.55[a]
	Control	4.49[a]	9.24[a]
	0.01%	4.46[a]	9.24[a]
S. thermophilus 1	0.05%	4.51[a]	9.25[a]
	0.1%	4.54[a]	9.29[a]
	0.2%	4.52[a]	9.23[a]
	0.5%	4.47[a]	9.27[a]
	Control	4.37[a]	8.88[a]
	0.01%	4.44[a]	9.24[a]
S. thermophilus 2	0.05%	4.37[a]	8.91[a]
	0.1%	4.30[a]	8.97[a]
	0.2%	4.25[a]	9.03[a]
	0.5%	4.18[a]	9.00[a]
	Control	4.56[a]	7.86[a]
	0.01%	4.45[a]	8.37[b]
S. thermophilus 143	0.05%	4.40[a]	8.62[b]
	0.1%	4.3[a]	8.86[b]
	0.2%	4.2[a]	9.10[b]
	0.5%	4.16[b]	9.06[b]

[1]Measurements made after 16 hr growth. Each value is a mean from 3 replicate experiments; cfu= colony forming units.

[ab]Means in the same column for each organism, for each parameter measured, without common superscript letter differ significantly ($P<0.05$); SEM = 0.079.

Table 5. Influence of WPH -1 on the growth of *Lactobacillus acidophilus* O16 when grown in the presence of yogurt cultures in nonfat milk.

Yogurt cultures[1]	**Log_{10} cfu/g on LBSO agar[2]**	
	Control	**WPH-1**
L. bulgaricus 18 & *S. thermophilus* 1	7.50[a]	8.45[b]
L. bulgaricus 18 & *S. thermophilus* 143	8.24[a]	8.96[b]
L. bulgaricus 10442 & *S. thermophilus* 1	7.70[a]	8.28[b]
L. bulgaricus 10442 & *S. thermophilus* 143	7.45[a]	8.28[b]

[1]Different yogurt cultures combined with *L. acidophilus* O16 grown in 10% NFDM with and without WPH-1, until pH 4.80 was reached, incubated at 37°C.
[2]Measurements made once samples reached pH 4.80. LBSO agar selectively enumerates *L. acidophilus*. Each value is a mean from 3 replicate experiments; cfu = colony forming units.
[ab]Values with no common superscript letters differ significantly ($P<0.05$); SEM = 0.163.

Table 6. Influence of WPH-1 on the growth of *Lactobacillus acidophilus* L-1 when grown in the presence of yogurt cultures in nonfat milk.

Yogurt cultures[1]	**Log_{10} cfu/g on LBSO agar[2]**	
	Control	**WPH-1**
L. bulgaricus 18 & *S. thermophilus* 1	7.76[a]	8.10[b]
L. bulgaricus 18 & *S. thermophilus* 143	8.06[a]	8.57[b]
L. bulgaricus 10442 & *S. thermophilus* 1	7.66[a]	8.04[b]
L. bulgaricus 10442& *S. thermophilus* 143	8.01[a]	8.62[b]

[1]Different yogurt cultures combined with *L. acidophilus* L-1 grown in 10% NFDM with and without WPH-1, until pH 4.80 was reached, incubated at 37°C.
[2]Measurements made once samples reached pH 4.80. LBSO agar selectively enumerates *L. acidophilus*. Each value is a mean from 3 replicate experiments; cfu = colony forming units.
[ab]Values with no common superscript letters differ significantly ($P<0.05$); SEM = 0.163.

Table 7. Influence of WPH-1 on the growth of *Bifidobacterium longum* S9 when grown in the presence of yogurt cultures in nonfat milk.

Yogurt cultures[1]	**Log_{10} cfu/g on LBSO agar[2]**	
	Control	**WPH-1**
L. bulgaricus 18 & *S. thermophilus* 1	7.26[a]	7.56[a]
L. bulgaricus 18 & *S. thermophilus* 143	7.30[a]	7.41[a]
L. bulgaricus 10442 & *S. thermophilus* 1	6.93[a]	7.12[a]
L. bulgaricus 10442 & *S. thermophilus* 143	7.22[a]	7.11[a]

[1]Different yogurt cultures combined with *B. longum* S9 grown in 10% NFDM with and without WPH-1, until pH 4.80 was reached, incubated at 37°C.
[2]Measurements made once samples reached pH 4.80. LBSO agar selectively enumerates *L. acidophilus*. Each value is a mean from 3 replicate experiments. cfu = colony forming units.
[ab]Values with no common superscript letters differ significantly ($P<0.05$); SEM = 0.163.

Table 8. Influence of WPH-1[1] on the shelf-stability of two strains of *Lactobacillus acidophilus* grown in the presence of different traditional yogurt culture combinations in nonfat milk.

Culture Combination[2]			Log_{10} cfu/g on LBSO agar[3]			
			Control		WPH-1	
L. acidophilus	*L. bulgaricus*	*S. thermophilus*	Day 0	Day 28	Day 0	Day 28
O16	18	1	7.68[a]	7.02[c]	8.46[b]	5.97[d]
	18	143	8.24[a]	6.37[c]	8.96[b]	5.31[d]
	10442	1	7.70[a]	6.04[c]	8.28[b]	5.68[d]
	10442	143	7.95[a]	7.29[c]	8.44[b]	7.02[c]
L-1	18	1	7.79[a]	6.13[c]	8.48[b]	6.36[c]
	10442	1	7.89[a]	6.20[c]	8.16[b]	6.27[c]
	10442	143	7.77[a]	7.17[c]	8.47[b]	7.36[c]

[1]Whey protein hydrolysate (WPH-1); added 0.5% to yogurt mix prior to heating.
[2]Different culture combinations, between each *L. acidophilus* strain and yogurt cultures, used to make the fermented milk. For each experiment, 0.5% of *L. acidophilus* and 0.1% of each yogurt culture were used to inoculate each milk treatment.
[3]Colony forming units for the culture combinations grown to a pH of 4.80 at 37°C and after 28 days of storage at 5°C. LBSO agar selectively enumerates
L. acidophilus. Each value is a mean from 3 replicate experiments.
[ab]Means in the same row, for each milk treatment, without common superscript differ significantly ($P<0.05$); SEM = 0.127.

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APPENDIX A

IDENTITY OF PROBIOTIC AND TRADITIONAL YOGURT CULTURES

API BIOCHEMICAL TEST

Procedure

1. Culture the select strain three times in MRS broth (medium). In order to obtain a stabilization of the biochemical tests.
2. Incubate at 37°C for 24 hours.
3. Transfer the culture into a sterilized centrifuge tube.
4. Centrifuge the culture for 10 minutes at 10000 RPM.
5. Remove the supernatant.
6. Wash the cells (pellet) with 10mL of CHL broth depending on the size of the pellet.
7. Go back to steps 6 and 7.
8. Add 10mL of CHL broth into the pellet and vortex.
9. Using a sterilized pipette, distribute the bacterial suspension into the tubes of the api 50CH strips.
10. Incubate the stripes at 37°C for 24 hours under anaerobic conditons.

CHL MEDIUM

• Polypeptone	10.00g
• Yeast extract	5.00g
• Tween 80	1.00mL
• Dipotassium phosphate	2.00g
• Sodium acetate 3 H_2O	5.00g
• Diammonium citrate	2.00g
• Magnesium sulfate 7 H_2O	0.20g
• Manganese sulfate 4 H_2O	0.05g
• Bromcresol purple	0.17g
• Distilled water	1000mL

Table 9

Confirmation of identity of cultures of *Lactobacillus acidophilus*

Test[1]	La[2]	La-L1	La-O16
Amygdalin	+	+	+
Arabinose	-	-	-
Esculin	+	+	+
Fructose	+	+	+
Galactose	+	+	+
Gluconate	-	-	-
Glucose	+	+	+
Lactose	+	+	+
Maltose	+	+	+
Mannitol	-	-	-
Mannose	+	+	+
Melezitose	-	-	-
Melibiose	+/-	-	-
Raffinose	+/-	+	-
Rhamnose	-	-	-
Ribose	-	-	-
Salicin	+	+	+
Sorbitol	-	-	-
Surcose	+	+	+
Trehalose	+/-	+	+
Xylose	-	-	-

[1]All cultures were Gram + rods; catalase negative; and did not grow at 15°C
[2]La=*Lactobacillus acidophilus*; reactions as listed in the 9th Edition of Bergey's Manual of Systematic Bacteriology.

Table 10

Confirmation of identity of cultures of *Lactobacillus casei*

Test[1]	Lc[2]	Lc-E5	Lc-E10
Amygdalin	+	+	+/-
Arabinose	-	-	-
Cellobiose	+	+	+
Esculin	+	+	+
Fructose	+	+	+
Galactose	+	+	+
Gluconate	+	-	-
Glucose	+	+	+
Lactose	+/-	+	+
Maltose	+	+	+
Mannitol	+	+	+
Mannose	+	+	+
Melezitose	+	+	+
Melibiose	-	-	-
Raffinose	-	-	-
Rhamnose	-	-	-
Ribose	+	+	+
Salicin	+	+	+/-
Sorbitol	+	+	+
Sucrose	+	+	+
Trehalose	+	+	+
Xylose	-	-	-

[1]All cultures were Gram + rods; catalase negative; and grew at 15°C
[2]Lc=*Lactobacillus casei*; reactions as listed in the 9th Edition of Bergey's Manual of Systematic Bacteriology.

Table 11

Confirmation of identity of *Bifidobacterium longum*

Test[1]	Bl[2]	Bl-S9
Arabinose	+	+/-
Cellobiose	+	-
Fructose	+	+/-
Galactose	+	+
Gluconate	-	-
Inuline	-	-
Lactose	+	+
Maltose	+	+
Mannitol	-	-
Mannose	+/-	+/-
Melezitose	+	-
Melibiose	+	+
Raffinose	+	+
Salicin	-	-
Sorbitol	-	-
Sucrose	+	+
Trehalose	-	-
Xylose	+/-	+/-

[1]Culture was Gram + irregularly shaped rods; fructose-6 phosphate-phosphoketolase positive; catalase negative.
[2]Bl=*Bifidobacterium longum*; reactions as listed in the 9th Edition of Bergey's Manual of Systematic Bacteriology.

Table 12

Confirmation of identity of *Lactobacillus delbrueckii* subsp. b*ulgaricus*

Test[1]	Lb[2]	Lb-18	Lb-10442
Amygdalin	-	-	-
Arabinose	-	-	-
Cellobiose	-	-	-
Esculin	-	-	-
Fructose	+	+	+
Galactose	-	-	-
Glucose	+	+	+
Gluconate	-	-	-
Lactose	+	+	+
Maltose	-	-	-
Mannitol	-	-	-
Melezitose	-	-	-
Melibiose	-	-	-
Raffinose	-	-	-
Rhamnose	-	-	-
Ribose	-	-	-
Salicin	-	-	-
Sorbitol	-	-	-
Sucrose	-	-	-
Trehalose	-	-	-
Xylose	-	-	-

[1]All cultures were Gram + rods; catalase negative.
[2]Lb=*Lactobacillus delbrueckii* subsp *bulgaricus*; reactions as listed in the 9th Edition of Bergey's Manual of Systematic Bacteriology.

Table 13

Confirmation of identity of cultures of *Streptococcus salivarius* subsp. *thermophilus*

Test[1]	St[2]	St-1	St-2	St-143
Arabinose	-	-	-	-
Fructose	+	-	-	+
Galactose	+/-	-	-	-
Glucose	+-	+	+	+
Glycerol	-	-	-	-
Inulin	-	-	-	-
Lactose	+	+	+	+
Maltose	+/-	-	-	-
Mannitol	-	-	-	-
Mannose	+	-	-	-
Rhamnose	-	-	-	-
Salicin	-	-	-	-
Sorbitol	-	-	-	-
Sucrose	+	+	+	+
Xylose	-	-	-	-

[1]All cultures were Gram + cocci in pairs or chains; catalase negative.
[2]St=*Streptococcus salivarius* subsp. *thermophilus*; reactions as listed in the 9th Edition of Bergey's Manual of Systematic Bacteriology.

APPENDIX B

WHEY PROTEIN SCREENING EXPERIMENT
RAW DATA

Table 14. Influence of different whey proteins on the growth and acid production of *Lactobacillus acidophilus* L-1 in 10% nonfat milk.

Sample	pH			Log_{10} cfu/g			
	Rep1	Rep2	Rep 3	Rep 1	Rep 2	Rep 3	AVG
Control	6.0	5.7	5.8	7.82	7.57	7.96	7.78
WPH-1	4.2	4.0	4.0	9.23	9.18	9.48	9.30
WP-2	5.5	5.2	5.2	7.32	7.11	7.46	7.30
WPH-3	4.4	4.1	4.0	8.97	8.76	9.11	8.95
WP-4	6.0	5.7	5.8	7.99	7.64	8.11	7.91
WP-5	6.0	5.7	5.8	8.00	7.71	8.08	7.93
WP-6	6.0	5.7	5.8	7.96	7.74	8.11	7.94
WP-7	6.0	5.6	5.8	8.18	7.99	8.23	8.13
WP-8	6.0	5.7	5.8	8.08	7.34	8.11	7.84
WP-9	6.0	5.7	5.8	8.11	7.90	8.08	8.03

APPENDIX C

INDIVIDUAL PROBIOTIC AND TRADITIONAL YOGURT CULTURES RAW DATA

Table 15. Influence of whey protein hydrolysate (WPH-1) on the growth and acid production of strains of *Lactobacillus acidophilus, Lactobacillus casei*, and *Bifidobacterium longum* in nonfat milk.

Organism	Sample	pH			Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	AVG
L. acidophilus	Control	5.8	5.7	5.8	7.89	7.83	7.53	7.75
L-1	WPH-1	4.4	4.2	4.3	9.20	9.11	9.30	9.20
L. acidophilus	Control	6.2	6.2	6.2	7.68	8.08	8.20	7.99
O16	WPH-1	4.4	4.2	4.4	9.89	9.65	9.75	9.76
L. acidophilus	Control	4.6	4.2	4.3	9.04	9.08	8.92	9.01
NCFM	WPH-1	4.0	3.9	4.0	8.93	9.11	9.04	9.03
L. casei E5	Control	6.4	6.2	6.5	7.90	7.90	8.04	7.95
	WPH-1	6.4	6.4	6.5	7.82	7.99	8.04	7.95
L. casei E10	Control	6.4	6.4	6.6	7.80	7.74	7.80	7.78
	WPH-1	6.4	6.4	6.6	7.82	7.72	7.81	7.78
B. longum S9	Control	5.7	5.9	5.8	8.18	8.73	8.49	8.47
	WPH-1	4.4	4.4	4.6	9.49	9.63	9.48	9.53

Table 16. Influence of whey protein hydrolysate (WPH-1) on the growth and acid production of strains of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in nonfat milk.

Organism	Sample	pH			Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	AVG
L. bulgaricus	Control	4.4	4.3	4.2	8.46	8.94	8.95	8.78
18	WPH-1	4.1	4.1	4.0	8.62	9.04	9.11	8.92
L. bulgaricus	Control	4.2	4.4	4.2	8.48	8.71	8.83	8.67
10442	WPH-1	3.8	4.0	4.0	8.84	9.15	9.11	9.03
S. thermophilus	Control	4.2	4.4	4.4	9.08	9.34	9.20	9.21
1	WPH-1	4.0	4.4	4.4	9.08	9.32	9.28	9.23
S. themophilus	Control	4.0	4.2	4.2	9.18	9.20	9.08	9.15
2	WPH-1	4.0	4.2	4.2	9.11	9.30	9.20	9.26
S. thermophilus	Control	4.6	4.6	4.6	8.11	8.57	8.61	8.43
143	WPH-1	4.2	4.4	4.4	8.94	9.23	9.08	9.08

APPENDIX D

DIFFERENT CONCENTRATIONS OF WPH-1 AND WPH-1a EXPERIMENTS RAW DATA

Table 18. Influence of different whey protein hydrolysate (WPH-1) concentrations on the growth and acid production of *Bifidobacterium longum* in nonfat milk.

Strain	Concentration	pH			Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	AVG
S9	Control	5.62	5.74	6.14	8.77	8.43	7.87	8.36
	0.5%	4.81	4.64	4.78	9.26	9.11	9.20	9.19
	0.2%	5.01	4.78	5.15	9.11	8.95	8.97	9.01
	0.1%	5.20	4.84	5.33	8.92	8.97	8.78	8.89
	0.05%	5.34	5.22	5.64	8.81	8.72	8.30	8.61
	0.01%	5.17	5.53	6.03	8.64	8.56	8.04	8.41

Table 19. Influence of different whey protein hydrolysate (WPH-1) concentrations on the growth and acid production of *Lactobacillus casei* in nonfat milk.

Strain	Concentration	pH			Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	AVG
E-5	Control	6.47	6.50	6.54	7.77	7.75	7.74	7.75
	0.5%	6.47	6.46	6.52	7.83	7.79	7.64	7.75
	0.2%	6.48	6.49	6.49	7.77	7.80	7.78	7.78
	0.1%	6.52	6.50	6.53	7.71	7.79	7.69	7.73
	0.05%	6.52	6.50	6.54	7.70	7.72	7.76	7.73
	0.01%	6.46	6.49	6.54	7.78	7.74	7.71	7.74
E-10	Control	6.49	6.47	6.19	7.71	7.79	8.08	7.86
	0.5%	6.48	6.38	5.82	7.81	7.96	8.56	8.11
	0.2%	6.52	6.40	6.01	7.86	7.94	8.40	8.07
	0.1%	6.53	6.43	6.10	7.74	7.87	8.41	8.01
	0.05%	6.53	6.47	6.27	7.82	7.81	8.18	7.94
	0.01%	6.48	6.46	6.17	7.75	7.84	8.23	7.94

Table 20. Influence of different whey protein hydrolysate (WPH-1) concentrations on the growth and acid production of *Lactobacillus dellbrueckii ssp. bulgaricus* in nonfat milk.

Strain	Concentration	pH			Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	AVG
18	Control	4.40	4.51	5.17	9.04	8.83	8.49	8.79
	0.5%	4.32	4.33	4.41	8.83	8.87	8.41	8.70
	0.2%	4.23	4.25	4.50	8.79	8.82	8.15	8.59
	0.1%	4.19	4.22	4.58	8.85	8.79	8.18	8.61
	0.05%	4.18	4.34	4.80	8.77	8.83	8.00	8.53
	0.01%	4.37	4.42	4.96	8.75	8.79	8.54	8.69
10442	Control	4.33	4.15	4.64	7.72	8.74	7.94	8.13
	0.5%	4.35	4.02	3.94	8.10	8.99	8.56	8.55
	0.2%	4.19	4.10	4.13	8.04	8.71	8.41	8.39
	0.1%	4.18	4.17	4.24	7.86	8.60	8.41	8.29
	0.05%	4.21	4.21	4.34	7.64	8.70	8.00	8.11
	0.01%	4.24	4.22	4.46	7.62	8.63	7.82	8.02

Table 21. Influence of different whey protein hydrolysate (WPH-1) concentrations on the growth and acid production of *Streptococcus thermophilus* in nonfat milk.

Strain	Concentration	pH			Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	AVG
1	Control	4.48	4.45	4.53	9.20	9.23	9.28	9.24
	0.5%	4.47	4.48	4.46	9.28	9.30	9.23	9.27
	0.2%	4.54	4.51	4.52	9.20	9.30	9.20	9.23
	0.1%	4.57	4.50	4.55	9.26	9.32	9.28	9.29
	0.05%	4.55	4.46	4.52	9.30	9.26	9.20	9.21
	0.01%	4.38	4.45	4.54	9.18	9.26	9.28	9.24
2	Control	3.97	4.93	4.22	8.97	8.97	8.70	8.88
	0.5%	4.01	4.31	4.21	9.08	8.92	9.00	9.00
	0.2%	4.02	4.43	4.29	9.00	9.11	8.97	9.03
	0.1%	4.04	4.57	4.28	8.92	8.99	9.00	8.97
	0.05%	4.05	4.70	4.37	8.85	8.98	8.90	8.91
	0.01%	4.05	4.89	4.37	8.92	9.00	8.80	8.91
143	Control	4.57	4.58	4.54	7.74	7.95	7.89	7.86
	0.5%	4.17	4.17	4.15	9.00	9.11	9.08	9.06
	0.2%	4.21	4.22	4.17	9.04	9.15	9.11	9.1
	0.1%	4.32	4.33	4.25	8.83	8.85	8.91	8.86
	0.05%	4.41	4.43	4.37	8.54	8.62	8.70	8.62
	0.01%	4.46	4.48	4.42	8.11	8.34	8.65	8.37

Table 22. Influence of different concentrations of whey protein hydrolyate (WPH-1a) on the growth of probiotic bacteria in nonfat milk.

Organism	WPH-1 Concentration	PH[1]	Log_{10} cfu/g[1]
	Control	5.52[a]	7.84[a]
	0.01%	5.41[a]	8.12[a]
L. acidophilus L-1	0.05%	5.11[a]	8.19[a]
	0.1%	4.87[b]	8.26[a]
	0.2%	4.58[b]	8.30[b]
	0.5%	4.16[b]	8.69[b]
	Control	6.09[a]	8.18[a]
L. acidophilus O16	0.01%	5.89[a]	8.36[a]
	0.05%	5.72[a]	8.59[a]
	0.1%	5.54[b]	8.90[b]
	0.2%	5.29[b]	9.06[b]
	0.5%	4.93[b]	9.25[b]
	Control	5.52[a]	8.62[a]
	0.01%	5.36[a]	8.79[a]
B. longum S9	0.05%	5.2[a]	8.80[a]
	0.1%	5.12[a]	8.90[a]
	0.2%	4.99[b]	9.03[b]
	0.5%	4.71[b]	9.14[b]

[1]Measurements made after 16 hr growth. Each value is a mean from 3 replicate experiments; cfu= colony forming units.

[ab]Means in the same column for each organism, for each parameter measured, without common superscript letter differ significantly ($P<0.05$).

Table 23. Influence of different concentrations of whey protein hydrolyate (WPH-1a) on the growth of traditional yogurt cultures in nonfat milk.

Organism	WPH-1 Concentration	pH[1]	Log_{10} cfu/g[1]
L. bulgaricus 18	Control	4.37[a]	8.65[a]
	0.01%	4.07[a]	8.71[a]
	0.05%	4.13[a]	8.98[a]
	0.1%	4.20[a]	8.74[a]
	0.2%	4.27[a]	8.70[a]
	0.5%	4.27[a]	8.92[a]
L. bulgaricus 10442	Control	3.97[a]	8.81[a]
	0.01%	3.93[a]	8.88[a]
	0.05%	3.92[a]	8.85[a]
	0.1%	3.94[a]	8.85[a]
	0.2%	3.94[a]	8.88[a]
	0.5%	3.96[a]	8.87[a]
S. thermophilus 1	Control	4.37[a]	9.13[a]
	0.01%	4.53[a]	9.11[a]
	0.05%	4.17[a]	9.21[a]
	0.1%	4.10[a]	9.14[a]
	0.2%	4.30[a]	9.11[a]
	0.5%	4.10[a]	9.16[a]
S. thermophilus 143	Control	4.59[a]	8.07[a]
	0.01%	4.51[a]	8.43[a]
	0.05%	4.42[a]	8.49[a]
	0.1%	4.33[a]	8.99[b]
	0.2%	4.24[a]	9.11[b]
	0.5%	4.22[a]	9.12[b]

[1]Measurements made after 16 hr growth. Each value is a mean from 3 replicate experiments; cfu= colony forming units.

[ab]Means in the same column for each organism, for each parameter measured, without common superscript letter differ significantly ($P<0.05$).

Table 24. Influence of different whey protein hydrolysate (WPH-1a) concentrations on the growth and acid production of select strains of *Lactobacillus acidophilus* in nonfat milk.

Strain	Concentration	pH			Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	AVG
L-1	Control	5.51	5.50	5.54	7.78	7.89	7.86	7.84
	0.5%	4.35	4.04	4.10	8.18	8.93	8.95	8.69
	0.2%	4.79	4.46	4.50	7.90	8.45	8.56	8.30
	0.1%	4.87	4.87	4.87	7.78	8.57	8.43	8.26
	0.05%	5.09	5.13	5.10	7.92	8.34	8.32	8.19
	0.01%	5.37	5.39	5.47	8.04	8.20	8.11	8.12
O16	Control	6.07	6.0	6.2	8.26	8.11	8.18	8.18
	0.5%	4.98	4.88	4.92	9.26	9.23	9.26	9.25
	0.2%	5.26	5.0	5.6	9.00	9.08	9.11	9.06
	0.1%	5.53	5.5	5.6	8.85	8.91	8.93	8.90
	0.05%	5.67	5.8	5.7	8.67	8.28	8.83	8.59
	0.01%	5.88	5.8	6.0	8.48	8.40	8.20	8.36

Table 25. Influence of different whey protein hydrolysate (WPH-1a) concentrations on the growth and acid production of *Bifidobacterium longum* in nonfat milk.

Strain	Concentration	pH			Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	AVG
S9	Control	5.30	5.75	5.52	8.78	8.45	8.64	8.62
	0.5%	4.55	4.85	4.72	9.15	8.97	9.30	9.14
	0.2%	4.89	5.09	5.00	9.15	8.83	9.11	9.03
	0.1%	4.97	5.25	5.14	8.93	8.77	9.00	8.90
	0.05%	5.04	5.46	5.10	8.90	8.62	8.95	8.82
	0.01%	5.31	5.50	5.27	8.96	8.60	8.81	8.79

Table 26. Influence of different whey protein hydrolysate (WPH-1a) concentrations on the growth and acid production of *Lactobacillus dellbrueckii ssp. bulgaricus* in nonfat milk.

Strain	Concentration	pH			Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	AVG
18	Control	4.4	4.3	4.4	8.70	8.62	8.62	8.65
	0.5%	4.2	4.2	4.4	8.76	9.40	8.62	8.92
	0.2%	4.4	4.2	4.2	8.74	8.73	8.64	8.70
	0.1%	4.2	4.2	4.2	8.46	9.08	8.67	8.74
	0.05%	4.2	4.2	4.0	8.80	9.11	9.04	8.98
	0.01%	4.0	4.2	4.0	8.99	8.27	8.88	8.71
10442	Control	3.82	3.80	4.3	9.04	9.00	8.38	8.81
	0.5%	3.80	3.78	4.31	8.97	8.99	8.65	8.87
	0.2%	3.75	3.76	4.31	9.04	9.08	8.52	8.88
	0.1%	3.79	3.74	4.3	9.08	9.04	8.42	8.85
	0.05%	3.74	3.73	4.28	9.11	9.00	8.45	8.85
	0.01%	3.82	3.76	4.22	9.04	9.08	8.51	8.88

Table 27. Influence of different whey protein hydrolysate (WPH-1a) concentrations on the growth and acid production of *Streptococcus thermophilus* in nonfat milk.

Strain	Concentration	pH			Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	Rep 1	Rep 2	Rep 3	AVG
1	Control	4.2	4.5	4.4	9.04	9.04	9.30	9.13
	0.5%	4.2	3.8	4.3	9.11	9.15	9.23	9.16
	0.2%	4.2	4.4	4.3	9.00	9.08	9.25	9.11
	0.1%	4.2	3.8	4.3	9.08	9.15	9.20	9.14
	0.05%	4.2	4.0	4.3	9.18	9.18	9.26	9.21
	0.01%	4.2	5.1	4.3	9.18	9.04	9.11	9.11
143	Control	4.54	4.60	4.63	7.96	8.11	8.15	8.07
	0.5%	4.16	4.22	4.22	9.00	9.18	9.18	9.12
	0.2%	4.23	4.25	4.24	9.11	9.04	9.18	9.11
	0.1%	4.30	4.37	4.31	8.93	8.95	9.08	8.99
	0.05%	4.37	4.41	4.48	8.34	8.54	8.59	8.49
	0.01%	4.49	4.49	4.54	8.26	8.50	8.52	8.43

APPENDIX E

PROBIOTIC AND TRADITIONAL YOGURT CULTURE EXPERIMENTS RAW DATA

Table 28. Influence of whey protein hydrolysate (WPH-1) on the growth of *Lactobacillus acidophilus* O16 combined with yogurt cultures in milk at pH 4.80.

Yogurt Cultures	Sample	Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	AVG
L. bulgaricus 18 &	Control	7.74	7.32	7.45	7.50
S. thermophilus 1	WPH-1	8.60	8.38	8.38	8.45
L. bulgaricus 18 &	Control	8.15	8.38	8.20	8.24
S. thermophilus 143	WPH-1	8.92	9.00	8.97	8.96
L. bulgaricus 10442 &	Control	7.71	7.80	7.59	7.70
S. thermophilus 1	WPH-1	8.15	8.38	8.30	8.28
L. bulgaricus 10442 &	Control	7.32	7.45	7.57	7.45
S. thermophilus 143	WPH-1	8.26	8.51	8.08	8.28

Table 29. Influence of whey protein hydrolysate (WPH-1) on the growth of *Lactobacillus acidophilus* L-1 combined with yogurt cultures in milk at pH 4.80.

Yogurt Cultures	Sample	Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	AVG
L. bulgaricus 18 &	Control	7.76	7.75	7.78	7.76
S. thermophilus 1	WPH-1	7.88	8.23	8.18	8.10
L. bulgaricus 18 &	Control	8.11	8.08	8.00	8.06
S. thermophilus 143	WPH-1	8.56	8.61	8.54	8.57
L. bulgaricus 10442 &	Control	7.72	7.53	7.74	7.66
S. thermophilus 1	WPH-1	7.97	7.94	8.20	8.04
L. bulgaricus 10442 &	Control	8.04	8.00	8.00	8.01
S. thermophilus 143	WPH-1	8.65	8.58	8.63	8.62

Table 30. Influence of whey protein hydrolysate (WPH-1) on the growth of *Bifidobacterium longum* S9 combined with yogurt cultures in milk at pH 4.80.

Yogurt Cultures	Sample	Log_{10} cfu/g			
		Rep 1	Rep 2	Rep 3	AVG
L. bulgaricus 18 &	Control	7.78	6.95	7.04	7.26
S. thermophilus 1	WPH-1	7.95	7.41	7.32	7.56
L. bulgaricus 18 &	Control	7.40	7.28	7.23	7.30
S. thermophilus 143	WPH-1	7.34	7.20	7.70	7.41
L. bulgaricus 10442 &	Control	7.18	6.64	6.98	6.93
S. thermophilus 1	WPH-1	7.34	6.92	7.11	7.12
L. bulgaricus 10442 &	Control	7.34	7.23	7.08	7.22
S. thermophilus 143	WPH-1	7.18	7.11	7.04	7.11

APPENDIX F

SHELF STABILITY EXPERIMENTS
RAW DATA

Table 31. The shelf stability of *Lactobacillus acidophilus* O16 in the presence of *Lactobacillus bulgaricus* 18 and *Streptococcus thermophilus* 1 in yogurt supplemented with and without whey protein hydrolysate (WPH-1).

Replication	Day	pH		Log_{10} cfu/g on LBSO agar	
		C	WPH	C	WPH
1	0	4.80	4.80	7.74	8.60
	3	4.90	4.86	7.65	7.96
	7	4.77	4.76	7.49	7.20
	14	4.78	4.70	7.56	6.57
	21	4.68	4.63	7.28	5.96
	28	4.40	4.44	7.20	6.11
	35	4.60	4.70	7.18	5.96
	42	4.35	4.40	7.11	6.04
2	0	4.81	4.81	7.85	8.40
	3	4.89	4.85	7.76	7.77
	7	4.78	4.74	7.57	6.92
	14	4.71	4.63	7.34	6.51
	21	4.49	4.51	7.41	6.51
	28	4.40	4.40	7.15	5.91
	35	4.65	4.70	7.11	6.26
	42	4.40	4.37	6.99	5.89
3	0	4.80	4.80	7.45	8.38
	3	4.81	4.78	7.58	7.65
	7	4.73	4.67	6.81	7.54
	14	4.29	4.36	7.11	6.46
	21	4.52	4.54	7.15	6.60
	28	4.49	4.52	6.72	5.88
	35	4.55	4.60	6.79	5.96
	42	4.51	4.53	5.94	5.18

Table 32. The shelf stability of *Lactobacillus acidophilus* O16 in the presence of *Lactobacillus bulgaricus* 18 and *Streptococcus thermophilus* 143 in yogurt supplemented with and without whey protein hydrolysate (WPH-1).

Replication	Day	pH		Log_{10} cfu/g on LBSO agar	
		C	WPH	C	WPH
1	0	4.80	4.79	8.15	8.92
	3	4.76	4.70	8.15	8.34
	7	4.82	4.74	7.66	6.53
	14	4.80	4.78	6.89	6.00
	21	4.66	4.61	6.38	5.56
	28	4.58	4.60	6.40	5.15
	35	4.82	4.75	5.46	4.90
	42	4.77	4.75	5.18	4.30
2	0	4.79	4.80	8.38	9.00
	3	4.81	4.86	8.23	8.20
	7	4.73	4.72	7.95	6.43
	14	4.76	4.77	7.54	5.67
	21	4.61	4.60	6.81	5.42
	28	4.63	4.63	6.89	5.90
	35	4.77	4.78	6.34	4.59
	42	4.72	4.59	6.30	4.60
3	0	4.81	4.80	8.20	8.97
	3	4.79	4.81	8.11	7.91
	7	4.80	4.73	7.48	6.26
	14	4.77	4.68	6.78	5.58
	21	4.78	4.73	6.34	5.28
	28	4.70	4.65	5.82	4.88
	35	4.90	4.80	5.58	4.30
	42	4.83	4.78	5.26	4.00

Table 33. The shelf stability of *Lactobacillus acidophilus* O16 in the presence of *Lactobacillus bulgaricus* 10442 and *Streptococcus thermophilus* 1 in yogurt supplemented with and without whey protein hydrolysate (WPH-1).

Replication	Day	pH		Log_{10} cfu/g on LBSO agar	
		C	WPH	C	WPH
1	0	4.78	4.77	7.81	8.26
	3	4.99	4.87	7.88	7.76
	7	5.10	4.94	7.15	7.49
	14	4.53	4.39	6.57	6.64
	21	4.73	4.51	6.40	6.32
	28	4.52	4.39	5.98	5.76
	35	4.52	4.36	5.79	5.46
	42	4.45	4.34	5.69	5.11
2	0	4.79	4.78	7.61	8.46
	3	4.97	4.85	7.67	7.71
	7	5.01	4.94	7.46	7.04
	14	4.40	4.32	6.97	6.18
	21	4.51	4.38	6.34	6.08
	28	4.39	4.28	5.99	5.79
	35	4.46	4.36	5.97	5.70
	42	4.37	4.28	5.70	5.53
3	0	4.79	4.78	7.67	8.11
	3	4.78	4.82	7.77	7.64
	7	5.02	4.93	7.65	7.04
	14	4.49	4.40	6.72	6.26
	21	4.53	4.39	6.73	6.15
	28	4.42	4.33	6.15	5.48
	35	4.50	4.41	5.92	5.62
	42	4.40	4.31	5.70	5.46

Table 34. The shelf stability of *Lactobacillus acidophilus* O16 in the presence of *Lactobacillus bulgaricus* 10442 and *Streptococcus thermophilus* 143 in yogurt supplemented with and without whey protein hydrolysate (WPH-1).

Replication	Day	pH		Log_{10} cfu/g on LBSO agar	
		C	WPH	C	WPH
1	0	4.80	4.79	7.99	8.81
	3	4.84	4.93	8.04	8.34
	7	4.85	4.88	7.92	7.60
	14	4.70	4.75	7.76	7.52
	21	4.77	4.86	7.84	7.08
	28	4.44	4.59	7.61	6.86
	35	4.53	4.68	6.96	6.72
	42	4.54	4.68	7.38	5.75
2	0	4.79	4.80	7.95	7.88
	3	4.71	5.02	8.08	7.95
	7	4.94	4.78	8.00	7.91
	14	4.72	4.65	7.83	7.94
	21	4.57	4.75	7.54	7.64
	28	4.45	4.66	7.28	7.45
	35	4.65	4.82	7.18	7.30
	42	4.46	4.61	6.71	6.68
3	0	4.79	4.80	7.91	8.64
	3	4.67	4.99	7.04	7.18
	7	4.75	4.79	7.92	7.34
	14	4.71	4.71	7.82	7.20
	21	4.60	4.75	7.71	6.75
	28	4.70	4.72	6.99	6.75
	35	4.33	4.72	7.20	6.66
	42	4.43	4.61	6.79	6.58

Table 35. The shelf stability of *Lactobacillus acidophilus* L-1 in the presence of *Lactobacillus bulgaricus* 18 and *Streptococcus thermophilus* 1 in yogurt supplemented with and without whey protein hydrolysate (WPH-1).

Replication	Day	pH		Log_{10} cfu/g on LBSO agar	
		C	WPH	C	WPH
1	0	4.79	4.79	7.41	8.51
	3	4.99	4.89	6.93	7.65
	7	4.46	4.41	5.88	7.45
	14	4.40	4.36	6.59	7.45
	21	4.34	4.27	4.00	5.15
	28	4.27	4.25	6.98	6.40
	35	4.16	4.10	5.30	5.81
	42	4.17	4.16	4.43	6.26
2	0	4.80	4.80	7.98	8.52
	3	4.98	4.93	7.36	7.59
	7	4.43	4.37	7.28	7.45
	14	4.47	4.35	7.26	7.36
	21	4.43	4.27	4.84	4.90
	28	4.34	4.27	6.00	6.08
	35	4.19	4.14	5.04	6.70
	42	4.13	4.10	4.77	6.26
3	0	4.79	4.80	7.98	8.40
	3	4.94	4.95	6.74	7.52
	7	4.40	4.41	6.75	7.43
	14	4.41	4.36	6.58	7.28
	21	4.37	4.28	4.30	5.15
	28	4.30	4.25	5.40	6.61
	35	4.22	4.12	6.15	6.67
	42	4.07	4.06	3.78	5.67

Table 36. The shelf stability of *Lactobacillus acidophilus* L-1 in the presence of *Lactobacillus bulgaricus* 10442 and *Streptococcus thermophilus* 1 in yogurt supplemented with and without whey protein hydrolysate (WPH-1).

Replication	Day	pH		Log_{10} cfu/g on LBSO agar	
		C	WPH	C	WPH
1	0	4.80	4.80	7.92	8.20
	3	4.66	4.58	7.28	7.46
	7	4.57	4.51	6.36	6.49
	14	4.52	4.47	6.38	6.48
	21	4.35	4.33	6.85	6.43
	28	4.26	4.23	6.54	5.79
	35	4.22	4.17	5.74	5.32
	42	4.32	4.29	6.04	4.53
2	0	4.74	4.80	7.86	8.18
	3	4.50	4.53	7.40	7.79
	7	4.45	4.48	6.41	6.90
	14	4.37	4.37	6.58	6.81
	21	4.26	4.27	6.80	6.66
	28	4.13	4.20	6.58	6.51
	35	4.07	4.13	5.38	5.79
	42	4.16	4.22	4.26	5.30
3	0	4.77	4.75	7.90	8.11
	3	4.48	4.51	7.43	7.77
	7	4.42	4.44	6.57	6.85
	14	4.39	4.38	6.43	6.66
	21	4.27	4.23	7.15	6.97
	28	4.17	4.17	5.49	6.51
	35	4.14	4.12	6.30	5.85
	42	4.22	4.26	5.26	5.11

Table 37. The shelf stability of *Lactobacillus acidophilus* L-1 in the presence of *Lactobacillus bulgaricus* 10442 and *Streptococcus thermophilus* 143 in yogurt supplemented with and without whey protein hydrolysate (WPH-1).

Replication	Day	pH		Log_{10} cfu/g on LBSO agar	
		C	WPH	C	WPH
1	0	4.75	4.75	7.85	8.49
	3	4.68	4.63	7.74	8.38
	7	4.82	4.74	7.74	8.18
	14	4.76	4.74	7.78	8.04
	21	4.50	4.31	7.60	7.72
	28	4.47	4.20	7.26	7.20
	35	4.49	4.27	7.23	7.04
	42	4.44	4.22	6.15	6.18
2	0	4.77	4.76	7.69	8.41
	3	4.75	4.65	7.66	8.23
	7	4.73	4.58	7.60	8.25
	14	4.66	4.47	7.66	7.96
	21	4.76	4.72	7.41	7.78
	28	4.51	4.28	6.97	7.53
	35	4.51	4.35	6.18	6.60
	42	4.50	4.27	5.00	6.04
3	0	4.80	4.78	7.78	8.52
	3	4.82	4.66	7.78	8.08
	7	4.89	4.70	7.73	8.04
	14	4.82	4.66	7.74	8.15
	21	4.70	4.58	7.57	7.65
	28	4.46	4.28	7.28	7.36
	35	4.71	4.46	6.72	6.48
	42	4.48	4.27	5.51	5.83

VITA

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