Oat-based Symbiotic Beverage Fermented by *Lactobacillus plantarum, Lactobacillus paracasei* ssp. *casei,* and *Lactobacillus acidophilus*

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ABSTRACT: Oats and probiotics have long been recognized for their health benefits. The objectives of this study were (1) to study the ability of *Lactobacillus plantarum* (B-28), *Lactobacillus paracasei* ssp. *casei* (B-29) isolated from a traditional Bulgarian cereal-based fermented beverage, and *Lactobacillus acidophilus* from Chr. Hansen, Milwaukee, Wis., U.S.A., to remove cholesterol from the media and to adhere to the Caco-2 cell line, (2) to optimize the fermentation conditions to develop a beverage using these probiotics and oats with acceptable sensory and nutritional qualities, and (3) to assess the quality and shelf-life of this beverage and survivability of probiotics in the beverage. *Lactobacillus acidophilus*, B-28, and B-29 were found to remove 70.67% ± 2.35%, 20.26% ± 2.63%, and 16.75% ± 3.83% of cholesterol from media and the percentage of adhesion was 4.69% ± 0.78%, 1.92% ± 0.78%, and 8.36% ± 0.78%, respectively. The blend of oat flour, sugar, inulin, and whey protein concentrate was cooked in water and fermented for 12 h at 37 °C by 2×10^6 colony-forming units (CFU)/mL each of B-28 and B-29 and 2×10^8 CFU/mL of *L. acidophilus*. The beverage had 0.87% ± 0.03% of dietary fiber and had better sensory qualities compared with the commercially available similar product. The probiotics survived for 10 wk of storage at 4 °C, except for *L. acidophilus*, which survived for about 4 wk. The population of B-28 was 1.77×10^6 to 1.29×10^7 CFU/mL and that of B-29 was 7.39×10^7 to 4.49×10^8 CFU/mL throughout the storage period. The oat-based symbiotic beverage is a functional drink providing both probiotics and prebiotics at the same time.

Keywords: symbiotic, adhesion, cholesterol removal, fermentation, viability

Introduction

Functional foods are in great demand at present because of their potential health benefits. For example, dietary oats have been shown to confer a number of significant physiological effects in the prevention or alleviation of disease, and thus may be considered as a multifunctional food (Welch 1995; Behall and others 1997). Dietary fiber of oats has 55% soluble fiber and 45% insoluble fiber. Soluble fiber favors the growth of probiotic bacteria (Johansson and others 1993). Oats contain a high percentage of desirable complex carbohydrates that may reduce the risk of certain cancers and constipation and also promote a good balance of fatty acids. The key cholesterol-lowering ingredient in oats is soluble fiber. It works by binding cholesterol-containing bile acids produced in the liver and speeding their exit from the body. It also helps control diabetes by preventing erratic swings in blood sugar levels (Wursch and Pi-Sunyer 1997). Soluble fiber slows down the absorption of sugar from the intestine into the blood. It also increases the cell's insulin sensitivity and assists the cells in drawing sugar from the blood (Kapica 2001). Due to these benefits, there is a great interest to increase the consumption of products based on oats that contain both soluble and insoluble fibers. Consumption of oat-based products is low, however, mainly due to the lack of acceptable food products based on oats (Salovaara and Backstrom 1991; Valentine 1999).

Fermented foods are more nutritious than their unfermented counterparts because, while growing in the medium, microorganisms synthesize several B-complex vitamins and growth factors, release nutrients locked into plant structures and cells by indigestible materials (splitting of cellulose), and enzymatically split plant materials such as cellulose, hemicellulose, and related polymers normally not digestible by human beings (Farnworth 2003). Fermented nutritional products can be developed using controlled fermentation of native ingredients using probiotic cultures. Probiotics are live microorganisms that, upon ingestion in sufficient numbers, exert health benefits beyond inherent basic nutrition to the host animal by improving its intestinal microbial balance (Guarner and Schaafsma 1998). The most widely used probiotic lactic acid bacteria that can survive in the intestine are *lactobacilli* and *bifidobacteria*. They exert health benefits by improving the host immune system. They possess anticarcinogenic properties and exhibit antitumor activity, in addition to playing important roles in colonization resistance in the intestinal, respiratory, and urogenital tracts, cholesterol metabolism, lactose metabolism, absorption of calcium, and synthesis of vitamins (Fuller 1992).

In the present study, the main idea was to prepare a product having 2 healthy components, dietary fiber of oats and probiotic lactic acid bacteria, wherein the oats are fermented by probiotic bacteria. The objectives of the present study were (1) to study the ability of *Lactobacillus plantarum* (B-28), *Lactobacillus paracasei* ssp. *casei* (B-29) isolated from Bulgarian cereal-based fermented beverage, and *Lactobacillus acidophilus* (Chr. Hansen) to remove cholesterol from the media and to adhere to the Caco-2 cell line, (2) to optimize the fermentation conditions to develop a beverage using these probiotics and oats, with acceptable sensory and nutritional qualities, and (3) to assess the quality and shelf-life of this beverage and survivability of probiotics in the beverage.

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Materials and Methods

Preparation of oats flour

Stone-cut oats were purchased from a local market and were freed from foreign materials. They were milled in a grinder and sieved through 60-mesh sieve. The same batch of oats was milled 3 times so that there is a maximum amount of fiber in the flour. Granulated sugar was purchased from a local market; inulin was obtained from Orafti (Malvern, Pa., U.S.A.); and whey protein concentrate was a gift from NZMP (NZMP, Pa., U.S.A.).

Probiotic cultures

Lactobacillus plantarum (B-28) and *L. paracasei* ssp. *casei* (B-29) isolated from a traditional Bulgarian cereal-based fermented beverage and *L. acidophilus* (Chr. Hansen, Milwaukee, Wis., U.S.A.) were used for the study. *Lactobacillus acidophilus* (ATCC 521), obtained from American type culture collection (ATCC, Manassas, Va., U.S.A.) was used as positive control for adhesion studies only.

Culture media and subculturing

Lactobacillus plantarum (B-28), *L. paracasei* ssp. *casei* (B-29), and *L. acidophilus* were subcultured separately in Difco lactobacilli Man Rogosa Sharpe broth (MRS) (Becton, Dickinson Co., Sparks, Md., U.S.A.) every week, and the cultures were propagated twice in cultivation broth at 37 °C. The transfer inoculum was 1% (v/v) culture grown for 24 to 36 h in a fresh medium under aerobic conditions. The cultures were stored at 4 °C between transfers and were subcultured 1 time before experimental use. Solid media were prepared by the addition of 15 g/L of granulated agar (Fisher Scientific, Fair Lawn, N.J., U.S.A.) to the broths.

Preparation of standard curve for cultures

The fresh cultures were centrifuged at 650 × *g* for 10 min, and the cell pellet was washed twice with 0.9% saline and resuspended in saline solution, and the volume was made up to 10 mL. This stock cell suspension was serially diluted and pour plated in MRS agar media. The optical density of the serially diluted cell suspension was measured at 560 nm in a DU-64 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif., U.S.A.). A standard curve was constructed, giving the relationship between the number of bacteria and optical density (OD). The linear equation was found by fitting straight line. This equation was used to find out the approximate amount of cell suspension to be used for preparing the product.

Analysis of cholesterol removal from media

Cholesterol removal from media by probiotic strains in the present study was studied using the modified method of Kimoto and others (2002). Freshly prepared MRS-THIO broth (MRS broth with 0.2% sodium thioglycollate) was supplemented with 0.2% sodium taurocholate (as a bile salt). Sodium thioglycollate was used as an oxygen scavenger (Gopal and others 1996; Brashears and others 1998; Usman and Hosono 1999). A filter-sterilized cholesterol solution (10 mg/mL in ethanol) was added to the broth to a final concentration of 70 μg/mL. The broth was inoculated with 1% culture and incubated for 24 h at 37 °C. After incubation, cells were removed by centrifugation for 7 min at 5400 × *g* and 4 °C. The cell pellets from centrifugation were resuspended in distilled water to the original volume of the culture and were assayed for cholesterol. The amount of cholesterol in the spent broth and the uninoculated broth was determined by the method of Rudel and Morris (1973), which is explained in next section for convenience. Subtracting the amount in spent broth from that in the uninoculated broth yielded the amount removed by the cells.

For determination of cholesterol, known amount of sample in duplicate was taken in clean test tubes; 3 mL of 95% ethanol was added to each tube, followed by 2 mL of 50% potassium hydroxide. The contents of each tube were mixed thoroughly after addition of each component. Tubes were heated for 10 min in a 60 °C water bath, and after cooling, 5 mL hexane was dispensed into each tube. After mixing thoroughly with a vortex mixer (model 232, Fisher Scientific), 3 mL of distilled water was added, and the mixing was repeated. Tubes were allowed to stand for 15 min at room temperature to allow for separation. Then 2.5 mL of the hexane layer was transferred into a clean test tube. The hexane was evaporated from each tube at 60 °C under the flow of nitrogen gas. *o*-Phthalaldehyde reagent (4 mL) was added to each tube. The reagent contained 0.5 mg of *o*-phthalaldehyde (Sigma Chemical Co., St. Louis, Mo., U.S.A.) per mL of glacial acetic acid. The tubes were allowed to stand at room temperature for 10 min, and then 2 mL of concentrated sulfuric acid was pipetted slowly down the inside of each tube. The contents of each tube were immediately mixed thoroughly on the vortex mixer. After standing at room temperature for an additional 10 min, the absorbance was measured at 550 nm against a reagent blank. The A_{550} was compared with a standard curve to determine the concentration of cholesterol. Results were expressed as micrograms of cholesterol per milliliter. The same procedure was used for the standard curve, except the following amounts of cholesterol (99% standard for chromatography; Sigma Chemical Co.) were assayed in place of the samples: 0, 10, 20, 30, 40, and 50 μg. The A_{550} values were plotted against micrograms of cholesterol. The experiment was carried out 3 times and a mean was calculated.

Adhesion assay

The adhesive ability of probiotic cultures to human Caco-2 colon epithelial cells was examined by using the method of Tuomola and Salminen (1998), with minor modifications. The probiotic glycerol stocks were streaked on MRS agar plates overnight, and a single colony was transferred to 10 mL of MRS broth for 16 h at 37 °C. After incubation, the microorganisms were harvested by centrifugation at 3000 × *g* for 10 min and washed with phosphate buffered saline (PBS, pH 7.4) 3 times. The final concentrations of probiotics were adjusted to 5×10^8 colony-forming units (CFU)/mL.

The Caco-2 human epithelial cell line was obtained from American Type Culture Collection (HTB-37, Rockville, Md., U.S.A.). The cells were cultured in Dulbecco's Modified Eagle's Medium nutrient mixture F-12 Ham (DMEM F-12; Sigma Chemical Co.) supplemented with 20% of fetal bovine serum (FBS, Gibco, PQ, Montreal, Canada) and 2% of antibiotic antimycotic solution (Sigma Chemical Co., catalog nr A9909). 2×10^5 cells were seeded into each well of a 24-well culture plate (Sarstedt, PQ, Montreal, Canada). After confluence, cells were maintained for 2 wk before use in adhesion assays. The media were changed every 2 d.

For the adhesion assay, post-confluence Caco-2 cells were washed twice with PBS (pH 7.4), and 1 mL (about 5×10^7 CFU) of each tested probiotic was added into wells with triplicates for each treatment. After incubation for 1 h at 37 °C, the nonbinding bacteria were washed out with PBS 3 times. Then 0.2 mL of 0.1% trypsin (Gibco) was added. Serial dilutions of the media were made to assess the adhesion percentage by the MRS agar plate counting. The plating method used was the spread plate method. The assay was repeated 5 times, and the data were presented in least squares means. The data were analyzed by analysis of variance (ANOVA) with the mixed model of SAS (SAS Inst. 1999). All the differences were compared by least square means.

Preparation of beverage

Oats flour (5%, w/v), sugar (4%, w/v), inulin (0.2%, w/v), and

whey protein concentrate (0.5%, w/v) were blended in water to make homogenous slurry. The slurry was slowly heated at the rate of 1 °C per min to boiling and boiled for 3 min to break down the starch content of oats. The cooked slurry was sterilized at 121 °C for 15 min. The slurry was then cooled to 37 °C and stirred at intervals to avoid formation of a surface layer.

The fresh cultures were centrifuged at 650 × *g* for 10 min, and the cell pellet was washed twice with 0.9% saline and resuspended in saline solution, and optical density was measured. Based on optical density, the amount of cell suspension to be added to the slurry was calculated using the standard curve for each culture, and added to the slurry. This was mixed well so that the culture was distributed evenly in the slurry. Fermentation was carried out at 37 °C in an aerobic environment.

Optimization of fermentation time and amount of culture for preparing beverage

The slurry was prepared as mentioned in the previous section and distributed into different bottles (100 mL each). The design of the experiment used was factorial. The 2 factors were the amount of culture (inoculation rate) and the fermentation time. Three cultures were added in equal proportion. The 3 different combinations were 2×10^4 CFU/mL of each culture, 2×10^6 CFU/mL of each culture, and 2×10^8 CFU/mL of each culture. Samples were withdrawn at fermentation times: 12, 16, 20, and 24 h. The values of pH were measured, and enumeration was done for each time and culture combination. Also, the product was tasted. The experiment was carried out 3 times, and the results were analyzed using 2-way analysis of variance (SAS system for windows version 8.2). The culture combination and fermentation time were identified, taking into consideration the growth of cultures during fermentation, the population of cultures in the final product, the pH of the product, ability of cultures to remove cholesterol from media, and the ability to adhere to the Caco-2 cell line.

The value of pH was measured using a pH meter (model IQ 240, IQ Scientific Instruments, Inc, San Diego, Calif., U.S.A.). Enumeration of cultures was done using the spread plate method after serial dilution. The colonies of each culture had a different appearance, which was used to count each culture separately. The colonies of *L. plantarum* (B-28) were white, round, smooth, and raised, with moist appearance. *Lactobacillus paracasei* ssp. *casei* (B-29) colonies were flat, irregular in shape, with undulate edges, and white in color, whereas colonies of *L. acidophilus* were flat, matte, rough with irregular edges, and gray to translucent in color.

After the fermentation time and culture combination was finalized, the oat beverage was prepared fresh using the selected culture combination and fermentation time. This was used for chemical analysis, shelf-life, and survivability studies. Fermentation time was 12 h and culture combination was 2×10^6 CFU/mL each of *L. plantarum* (B-28) and *L. paracasei* ssp. *casei* (B-29) and 2×10^8 CFU/mL of *L. acidophilus*. The slurry was prepared as mentioned previously, and culture was added and mixed well. The slurry was distributed into different bottles and incubated at 37 °C for 12 h in an aerobic environment. After fermentation, the product was stored under refrigerated conditions (4 °C) for 10 wk for further analysis. A control sample was prepared similarly, except that the cultures were not inoculated.

Shelf-life and survivability of probiotics

The samples were withdrawn every week, and pH, titratable acidity (%lactic acid), and viscosity were measured, and enumeration was done for 10 wk to find out how long the probiotics can survive in the product. Enumeration was done as mentioned previously.

Lactic acid, reported as titratable acidity, was estimated by titrating a 9 g sample, diluted with 18 mL water with 0.1 *N* sodium hydroxide using phenolphthalein as an indicator (Bradley and others 1992). Viscosity was measured using a Brookefield viscometer (Brookefield Engineering Laboratories, Inc., Middleboro, Mass., U.S.A.) and expressed in mPa. Measurements were made for 15 min at 100 rpm and at 25 ± 2 °C.

Oat beverage was analyzed for chemical composition (total solids, protein, fat, carbohydrate, ash, and dietary fiber) using standard AOAC procedures (Delwiche 2002). Minerals were determined using Inductively Coupled Plasma Atomic Emission Spectroscopy (ICPAES, Leeman Labs Plasma Spec Z.5, Lowel, Mass., U.S.A.) (Guo and others 2001) in an ash solution of the samples, prepared as per AOAC procedure (Delwiche 2002). All values reported are the mean of 3 measurements.

Sensory evaluation

A semi-trained panel of 18 members was engaged for preference, triangle, and difference from control tests to find out the sensory quality of the oat beverage. Three different samples of oat beverage differed in their sugar content and fermentation time. But the inoculation rate (2×10^6 CFU/mL each of *L. plantarum* [B-28] and *L. paracasei* ssp. *casei* [B-29] and 2×10^8 CFU/mL of *L. acidophilus*) remained the same for all 3 samples. The original recipe had 4% sugar and fermented for 12 h (A). This was varied by reducing the fermentation time to 6 h (B). The other variation was with 8% sugar and fermented for 8 h (C). They had a pH of 3.67, 3.89, and 4.15, respectively. The commercial oat beverage was also used for comparison (D). These 4 samples were coded with 3 digits and randomly presented to panelists at the same time, with a volume of 25 mL in every container for the ranking test. The containers were white plastic cups. The serving temperature was room temperature with fluorescent illumination. No booths were used during the evaluation. Panelists were asked to mark which sample they preferred. They were also asked to rate the samples by order of preference from 1 to 4. The sample that had the least rank sum was the most preferred one.

The Triangle test was also conducted to determine whether a sensory difference exists between the test product (most preferred) and the commercial product. Panelists were served with 6 sets of 3 samples each. The samples were coded randomly, and the panelists were instructed that 2 samples were identical and 1 was different. They were asked to taste each product from left to right and select the odd sample. The number of correct replies were counted, and referred to a statistical table for interpretation (Triangle test for difference: critical number [minimum] of correct answers) (Meilgaard and others 1991).

Furthermore, a difference from control test was conducted to know the size of the difference between the test product (most preferred) and the commercial product. Each panelist was presented with a control sample plus 1 test sample, which was the most preferred as determined from the ranking test. They were coded randomly. Panelists were asked to rate the size of the difference between them with reference to intensity of a particular sensory attribute on a scale provided. The results were analyzed using paired t-test (SAS Inst. 1999).

Results and Discussion

Cholesterol removal by *L. plantarum* (B-28), *L. paracasei* ssp. *casei* (B-29), and *L. acidophilus* from media

Earlier studies carried out on *L. plantarum* (B-28) and *L. paracasei* ssp. *casei* (B-29) in our laboratory to find out whether they are probiotic gave us promising results. They were tested for their acid and bile tolerance, antipathogenic activity, and antibiotic resistance and found to survive in the conditions of high oxgall concentration (up to 2%) and low pH (2.0). They also exhibited antimicrobial activity

against pathogens and resistance to many antibiotics (Gotcheva and others 2002). These results suggested that they could reach the small intestine and colon and contribute to the balance of intestinal microflora. Also, they could minimize the negative effects of antibiotic therapy on host bacterial ecosystem. Furthermore, we continued working on their ability to reduce serum cholesterol. It has been reported that a culture of *L. acidophilus* actively taking up cholesterol from laboratory media would function in vivo to exert a hypocholesterolemic effect (Gilliland and others 1985; Danielson and others 1989). So we studied the cholesterol removal by *L. plantarum* (B-28), *L. paracasei* ssp. *casei* (B-29), and *L. acidophilus* from MRS media. The results showed that *L. plantarum* (B-28) and *L. paracasei* ssp. *casei* (B-29) could remove 20.26% ± 2.63% and 16.75% ± 3.83% of cholesterol from media, respectively, whereas *L. acidophilus* removed 70.67% ± 2.35%. The cultures in our study significantly differed from *L. acidophilus* in removing cholesterol from media ($P < 0.0001$). Kimoto and others (2002) reported 72% removal by *L. acidophilus* (ATCC 43121), which is similar to our result. There are not many studies on cholesterol removal by *L. plantarum*. Jones and others (2004), however, showed that microencapsulated *L. plantarum* 80 (pCBH1) cells can efficiently break down and remove conjugated bile acids, glycodeoxycholic acid, and taurodeoxycholic acid with bile salt hydrolase activities, and established a basis for their use in lowering blood serum cholesterol. Tanaka and others (1999) and Pereira and others (2003) also found bile salt hydrolase activity in *L. plantarum*. *Lactobacillus paracasei* ssp. *casei* (B-29) belongs to subspecies casei. *Lactobacillus casei* N19 and E5 and *L. acidophilus* L1 and ATCC 43121 were compared for their ability to deconjugate bile salts and remove cholesterol from MRS broth by Brashears and others (1998). They found out that the strains of *L. casei* differed significantly from *L. acidophilus* in the amount of cholesterol removed. *Lactobacillus casei* strains E5 and N19 removed 8.0 and 10.4 μg of cholesterol/mL, respectively, whereas *L. acidophilus* strains ATCC 43121 and L1 removed 41.6 and 46.9 μg of cholesterol/mL. Cholesterol removal by *L. acidophilus* was due to assimilation, perhaps by the incorporation of cholesterol into the cellular membrane. *Lactobacillus casei* most likely removes cholesterol by means of the destabilization of cholesterol micelles, and the coprecipitation of the cholesterol with the deconjugated bile salts at pH less than 6.0. Our results indicated that even though *L. plantarum* (B-28) and *L. paracasei* ssp. *casei* (B-29) removed significantly less cholesterol from media compared with *L. acidophilus*, they have the ability to reduce serum cholesterol, which supports their potential as probiotics. Probiotic potential of *L. acidophilus* is well established.

Adhesion to the Caco-2 cell line

Adhesion to intestinal epithelial cells is an important prerequisite for colonization of probiotic strains in the gastrointestinal tract, preventing their immediate elimination by peristalsis and providing a competitive advantage in this ecosystem (Alander and others 1997). So we investigated the ability of *L. plantarum* (B-28), *L. paracasei* ssp. *casei* (B-29), and *L. acidophilus* to adhere to Caco-2 cells. Also, we used *L. acidophilus* ATCC 521, which has been shown to have a high adhesion as a positive comparison. Adhesion was expressed as the percentage of bacteria binding relative to the amount of bacteria added to the Caco-2 cell. Figure 1 shows the percentage of adhesion of tested bacterial cultures on human Caco-2 cells. *Lactobacillus paracasei* ssp. *casei* (B-29) was shown to have the significantly best adhesion (8.36%) compared with other 3 bacteria ($P < 0.05$). Moreover, *L. acidophilus* (4.69%) was significantly better than *L. acidophilus* ATCC 521 (2.22%) and *L. plantarum* (B-28) (1.92%) in its adhesive ability. Ouwehand and others (2001) reported about 2% adhesion for *L. casei* 01, 3% for *L. casei* Shirota, 4% for *L. casei* (BIO), 6% for *L. plantarum* ATCC 8014, and 26% for *L. acidophilus* La1 to mucus isolated from adult feces. *Lactobacillus paracasei* F19 had adhesion percentages of 6.6%, 6.0%, 6.2%, and 9.7% to the mucus of newborns, 2-mo-old infants, 6-mo-old infants, and adults, respectively (Kirjavainen and others 1998). Probiotics adhere to intestinal epithelium by different mechanisms. Kleeman and Klaenhammer (1982) reported that 4 *L. acidophilus* strains, BG2FO4, MSO1, MSO2, and MSO4, adhered to human fetal intestinal epithelial cells in a calcium-independent manner. *Lactobacillus acidophilus* ADH adhered to human colonic cells (Conway and others 1987), whereas strain BG2FO4 closely associated with the brush border of the human intestinal cell line HITC FHs0074 (ATCC CCL 241). *Lactobacillus plantarum* 299 and 299v were believed to adhere to human colonic cell line HT-29 by means of a mannose-specific adhesion (Adlerberth and others 1996). The mechanism by which probiotic cultures in the present study adhere to Caco-2 cells needs to be studied.

Fermentation time and inoculation rate

Series of the experiments were carried out to determine the optimal fermentation time and the amount of culture to get an oat beverage with acceptable sensory qualities and probiotic counts. Figure 2 shows the effect of mixed cultures added at different concentrations and fermentation time on their growth. When 3 cultures were added at the rate of 2×10^4 CFU/mL, at 12 h of fermentation, the product had 1.2×10^6 CFU/mL of *L. plantarum* (B-28), 4.2×10^6 CFU/mL of *L. paracasei* ssp. *casei* (B-29), and 1.47×10^7 CFU/mL of *L. acidophilus*. As the fermentation progressed, the number of CFUs of *L. plantarum* (B-28) and *L. paracasei* ssp. *casei* (B-29) decreased slightly up to 16 h, increased up to 20 h, and decreased after that, whereas *L. acidophilus* showed decrease in growth throughout fermentation period. After 12 h of fermentation, the pH was 4.77 and it was decreasing throughout the experiment period. After 24 h, it was 4.17. Here, even though the pH was 4.17, the product tasted sweet and there was no sour taste. When the 3 cultures were added at the rate of 2×10^6 CFU/mL, at 12 h of fermentation, the product had 1.5×10^7 CFU/mL of *L. plantarum* (B-28), 2.2×10^7 CFU/mL each of *L. paracasei* ssp. *casei* (B-29) and *L. acidophilus*.

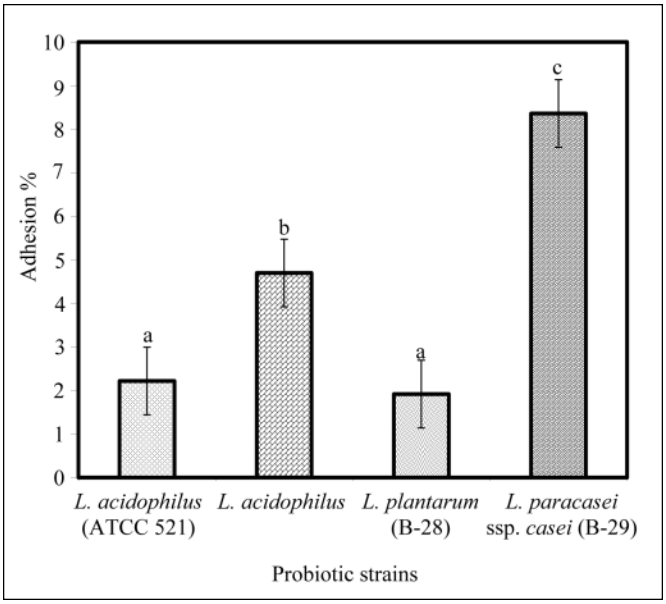

Figure 1—Percentage of adhesion of probiotics on Caco-2 cells (different letters on the bars represent significant difference between the cultures).

The growth of *L. plantarum* (B-28) and *L. paracasei* ssp. *casei* (B-29) increased slightly after 12 h but decreased after 16 h. The growth of *L. paracasei* ssp. *casei* (B-29) increased again, whereas that of *L. plantarum* (B-28) decreased further. The growth rate of *L. acidophilus* remained constant for the 1st 20 h of fermentation but decreased thereafter. The pH of the product after 12 h of fermentation was 3.96 with more balance of sweet and sour and it decreased to 3.86 to 3.89 after 16 h of fermentation with product developing a much more sour taste. When the 3 cultures were added at 2×10^8 CFU/mL, the product had 1.2×10^8 CFU/mL of *L. plantarum* (B-28), 3.5×10^8 CFU/mL of *L. paracasei* ssp. *casei* (B-29), and only 2.3×10^6 CFU/mL of *L. acidophilus*. The growth rate was the same up to 16 h, but after 16 h the growth of *L. plantarum* (B-28) decreased slightly, whereas that of *L. acidophilus* increased. The growth of *L. paracasei* ssp. *casei* (B-29) was the same up to 24 h. The value of pH was 3.79 after 12 h of fermentation and the taste was more sour, with a slight sweetness. The pH decreased to 3.57 at 24 h of fermentation, and the sweet taste disappeared completely.

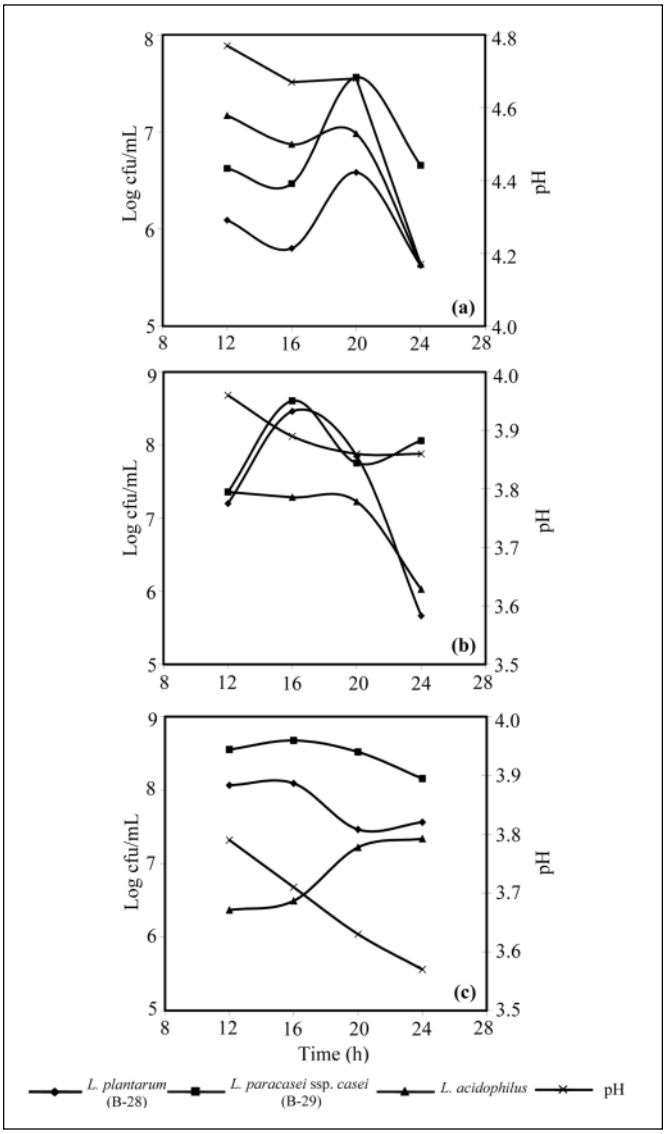

Figure 2—Effect of mixed cultures and fermentation time on their growth in oat beverage: (a) 2×10^4 CFU/mL of each culture; (b) 2×10^6 CFU/mL of each culture, (c) 2×10^8 CFU/mL of each culture.

Analysis of variance for the 2 factors, inoculation rate and fermentation time, showed that both concentration and time significantly affect the growth of *L. plantarum* (B-28), whereas in the case of *L. paracasei* ssp. *casei* (B-29), only concentration has a significant effect on its growth (Table 1). The growth of *L. acidophilus* was not affected by either the concentration or the time. Its number decreased, however, at 12th h of fermentation, and increased just 10-fold after 16 h. The results indicated that there is a lot of interaction between the cultures and competition for the nutrients. When the cultures were added at 2×10^4 CFU/mL, their growth was not consistent, and it did not show a definite trend. They were approximately only 10^7 CFU/mL at any point of fermentation. In contrast to this, when the cultures were added at higher concentration (2×10^8 CFU/mL), we expected them to increase to about 10^{10} or 10^{11} CFU/mL, but the number of *L. plantarum* (B-28) and *L. paracasei* ssp. *casei* (B-29) was the same as the initial number, and the *L. acidophilus* number decreased to 10^6 CFU/mL at 12 h of fermentation. But after 16 h of fermentation, there was a slight decrease in number of *L. plantarum* (B-28) and a slight increase in number of *L. acidophilus*. But this change is not significant. This indicates that *L. paracasei* ssp. *casei* (B-29) can tolerate the growth conditions well and maintain its growth throughout the 24 h of fermentation. At all 3 concentrations and at any time between 12 and 24 h, the population of *L. paracasei* ssp. *casei* (B-29) was highest. Pairwise comparisons (least significant difference [LSD] *t*-tests) for population of cultures, as affected by initial concentration, showed that we could select either 2×10^6 or 2×10^8 CFU of *L. plantarum* (B-28)/mL, 2×10^4 or 2×10^6 CFU of *L. paracasei* ssp. *casei* (B-29)/mL, and any 1 among the 3 concentrations of *L. acidophilus* (Table 2) as initial culture concentrations to be inoculated into the oat slurry to make the beverage. The results indicated that the nutrient content of the beverage was just enough to maintain a probiotic population of about 10^8 CFU/mL. The requirements for nutrients are specific characteristics for each strain of *Lactobacillus* (Kandler and Weiss 1986). The growth capacity of different lactic acid bacteria, isolated from different products, is different based on the nutrient content of the medium in which it is being grown. In the present study, the cultures *L. plantarum* (B-28) and *L. paracasei* ssp. *casei* (B-29) were isolated from the traditional Bulgarian cereal-based beverage and their nutrient requirement, which is out of the scope of this study and may be more than what is provided by the beverage studied. This may be the reason why the beverage in the present study did not have higher than 10^8 CFU of probiotics/mL. The growth capacity of different lactic acid bacteria, isolated from sour dough and cultivated with different amounts of free amino acids, has been tested and shown that the strains of *Lactobacillus* differ in their requirements of amino acids and that, for instance, *Lactobacillus brevis* var *lindneri* required as many as 15 free amino acids, whereas *L. plantarum* required only 4 (Spicher and Schroder 1979). Analysis of variance for pH, as affected by inoculation rate and fermentation time, shows that concentration significantly affected the pH of the product, whereas time did not have any significant effect (Table 1). Pairwise comparisons (LSD t-tests) showed that there was no significant difference between the pH of the product when the inoculation rates were 2×10^6 and 2×10^8 CFU/mL, but it differed significantly and was higher (4.57) when the inoculation rate was 2×10^4 CFU/mL (Table 2). This indicated that we could select the inoculation rates of either 2×10^6 or 2×10^8 CFU/mL. Pairwise comparisons (LSD t-tests) for CFUs and the pH of oat beverage at varied incubation periods showed that we could choose the incubation period of 12 h to make the beverage (Table 2).

Based on the results of the ability to remove cholesterol from media, adhesion assay, interactions between the cultures, final target of probiotic population in a finished product, and the product's pH and sensory attributes, we decided to use 2×10^6 CFU/mL of each of *L. plantarum* (B-28) and *L. paracasei* ssp. *casei* (B-29) and

Table 1—Analysis of variance for *Lactobacillus plantarum* (B-28), *Lactobacillus paracasei* ssp. *casei* (B-29), and *Lactobacillus acidophilus* colony-forming units and pH of oat beverage prepared using mixed culture at various inoculation rates and incubation periods

Source	DF	SS	MSS	F value	Pr > F
L. plantarum (B-28)					
Inoculation rate	2	5.81×10^{16}	2.91×10^{16}	12.46	0.0002
Time	3	8.30×10^{16}	2.77×10^{16}	11.86	<0.0001
Inoculation rate versus time	6	1.01×10^{17}	1.69×10^{16}	7.25	0.0002
L. paracasei ssp. casei (B-29)					
Inoculation rate	2	5.98×10^{17}	2.99×10^{17}	8.44	0.0017
Time	3	2.20×10^{17}	7.33×10^{16}	2.07	0.1309
Inoculation rate versus time	6	2.22×10^{17}	3.70×10^{16}	1.04	0.4218
L. acidophilus					
Inoculation rate	2	2.91×10^{14}	1.46×10^{14}	1.36	0.2748
Time	3	2.46×10^{14}	8.18×10^{13}	0.77	0.5238
Inoculation rate versus time	6	1.77×10^{15}	2.94×10^{14}	2.76	0.0350
pH					
Inoculation rate	2	5.28	2.64	8.39	0.0017
Time	3	0.42	0.14	0.44	0.7248
Inoculation rate versus time	6	0.34	0.06	0.18	0.9791

[a]DF = Degrees of freedom; MSS = Mean sum of squares; SS = Sum of squares.

Table 2—Least significant difference (LSD) *t*-tests for number of colony-forming units (CFU) and pH of oat beverage prepared using mixed culture at 3 inoculation rates and 4 incubation periods[a]

Inoculation rate (CFU/mL)	N	*Lactobacillus plantarum* (B-28) (CFU/mL)	*Lactobacillus paracasei* ssp. Casei (B-29) (CFU/mL)	*Lactobacillus acidophilus* (CFU/mL)	pH
2×10^4	12	1530556b	12052778b	8058333a	4.58a
2×10^6	12	94254444a	149330556b	14997222a	3.90b
2×10^8	12	76500000a	326944444a	10997222a	3.68b
Incubation time (h)					
12	9	44522222b	128033333ab	13288889a	4.18a
16	9	138137037a	293718519a	9970370a	4.09a
20	9	34537037b	141655556ab	14348148a	4.06a
24	9	12517037b	87696296b	7796296a	3.88a

[a]Means with the same letter in the same column are not significantly different at $P = 0.05$.

2×10^8 CFU/mL of *L. acidophilus* and fermenting for 12 h to make the oat beverage. *Lactobacillus acidophilus* had significantly greater ability to remove cholesterol from media compared with *L. plantarum* (B-28) and *L. paracasei* ssp. *casei* (B-29) and adhere to the Caco-2 cell line compared with *L. plantarum* (B-28). In addition, results of earlier studies conducted in our laboratory indicated that *L. acidophilus* could not survive for more than 4 to 5 wk of storage. Therefore, to take advantage of its ability to remove cholesterol and to adhere to the Caco-2 cell line and to compensate for low viability, a higher concentration of *L. acidophilus* was used.

Oat beverage formulation

Oat flour was the main ingredient in the product. We chose to use 5% because less than that will not provide the required amount of dietary fiber, and it was required to add more inulin to meet the dietary fiber requirement. But if more was used, the product would be more viscous, and it would no longer be a beverage. To increase the soluble dietary fiber content to meet the requirement (0.75 g/serving), 0.2% inulin was used (Deis 2001). Inulin acts as a water binder, stabilizer, and texturizer in addition to being a prebiotic (Roberts 2003). Whey protein concentrate was used at the level of 0.5%. This was just enough to make a homogenous product, and more than this would coagulate the slurry when it was autoclaved. Whey protein concentrate acts as a stabilizer that makes the product homogenous (Berry 2002; DMI 2003). Sugar (4%) was added as the energy source for the cultures and to give a balanced taste of sweet and sour to the product. All the ingredients and water were blended in a blender to make a homogenous slurry. The slurry was cooked and autoclaved. Autoclaving was necessary to free the slurry from undesirable microorganisms. When the fermentation was carried out without sterilizing the cooked slurry, the product had an unacceptable flavor and was contaminated with undesirable microorganisms. The contaminant may be *B. cereus,* which is known to be the common spore-forming bacteria found in cereals. The spores are not easily destroyed by heat and will survive cooking of food. The spores will germinate and produce bacteria. So the cooked slurry was sterilized before fermentation.

The beverage was more homogenous and free flowing and had a smooth texture compared with the unfermented slurry. The pH, titratable acidity, and viscosity of the control sample (without fermentation) were 6.3, 0.032%, and 420 mPa, respectively. After fermentation for 12 h, there was a significant change in pH, which reduced to 3.63, titratable acidity, which increased to 0.21%, and viscosity, which decreased to 222 mPa. The product had a good balance of sweet and sour tastes.

The nutrient and mineral composition of the oat beverage is presented in Table 3. The beverage provides 0.80 g of soluble dietary fiber per serving (150 mL), which meets Food and Drug Administration (FDA) requirements (0.75 g per serving). Because this product contains only plant origin raw materials and whey protein concentrate, it can be considered low in saturated fat and cholesterol free. It is also suitable for enrichment with traditional flavors.

Survivability of probiotics during storage

Figure 3 shows the survivability of probiotics during storage for 10 wk in refrigerated condition. Among the 3 cultures, *L. paracasei* ssp. *casei* (B-29) survived very well throughout the storage. Its population ranged from 7.39×10^7 to 4.49×10^8 CFU/mL throughout. In *L. plantarum* (B-28), the population ranged from 1.77×10^6 to 1.29×10^7 CFU/mL. But population of *L. acidophilus* was 6.77×10^7 CFU/mL before storage, and it started decreasing every week. At the 4th wk, it was only 5.55×10^5 CFU/mL, and after that, it decreased every week. Analysis of variance for the probiotic counts showed that there was no significant change in *L. plantarum* (B-28) and *L. paracasei* ssp. *casei* (B-29) populations during storage, whereas, with *L. acidophilus,* there was significant change after the 4th wk, which can be seen in Figure 3. Among the 3 cultures, *L. acidophilus* differed significantly in survivability from the other 2 probiotics. These results, except for *L. acidophilus,* indicate that this product meets the requirements that for a product to be called a probiotic functional food, it should have at least 10^6 CFU/mL of probiotics even after storage.

It is of great importance that when selecting bacteria for their physiological effects, they should stay viable during the whole shelf-life of the food product, that there is no decrease in their resistance to the acidic environment of the food product, and that there is no decrease in their resistance to the acidic environment of the stomach and to bile salts in the small intestine (Sarrela and others 2000). It has also been suggested that the food product

should contain at least 10^6 CFU/g of the probiotic bacterial strain and that it must be possible to eat approximately 300 to 400 g of the product per week (Samona and Robinson 1994).

Changes in viscosity, pH, and titratable acidity during storage

Figure 4 represents the changes in pH, titratable acidity, and viscosity during storage. The product had a pH of 3.63 ± 0.03, titratable acidity of 0.22% ± 0.015%, and viscosity of 222 ± 52 mPa. Analysis of variance showed that there was no significant change in titratable acidity during storage for 10 wk. However, the viscosity of the beverage decreased significantly over the 1st and 2nd wk of storage and then stabilized with no further significant change up to 10 wk of storage. There was also a statistically significant decrease in the pH of the product over the 10-wk storage period. We did not observe any correlation between change in pH and titratable acidity during storage. The significant decrease in viscosity may be due to syneresis. During storage, all the bound water might have leached out, resulting in a decrease in viscosity of the product. The same observation was made in the control sample, even though the viscosity of the control sample was significantly higher than the test sample.

Sensory evaluation

Sensory evaluation was done using a ranking test, a triangle test, and the difference from control test. The results of the ranking test showed that sample C had the least rank sum, indicating that it was the most preferred. The value of the test statistic T was 8.64 and the upper 5% critical value of a chi-square with 3 degrees of freedom was 7.81. Because the value of T at 8.64 is greater than 7.81, the samples were significantly different ($P < 0.05$). The critical value of the multiple comparison (LSD_{rank}) was calculated to determine which samples were significantly different, and the results showed that sample D did not differ significantly from other 3 samples. Samples A and B did not differ significantly from each other, but sample C was significantly different from samples A and B (Meilgaard and others 1991).

Sample C and sample D were subjected to a triangle test to see if the difference could be recognized by the panel between those 2 samples. Results indicated that these 2 samples differed significantly ($P < 0.05$). Furthermore, the difference from the control test was carried out to rate the size of difference between them, with reference to intensity of a particular sensory attribute on a scale provided. The results are presented in Table 4. Analysis of variance showed that there was no significant difference between the 2 samples in appearance, sweetness, bitterness, saltiness, and oat flavor; however, they differed significantly from each other in aroma, sourness, viscosity, and preference. Sample C, which had 8% sugar and was fermented for 8 h, was more acceptable compared with commercial sample D. Increasing the amount of sugar and decreasing the fermentation time increased the acceptability. Sensory evaluation clearly indicates that fermentation improves the sensory quality of the beverage in addition to making it a probiotic drink.

Table 3—Nutrient and mineral composition of oat beverage fermented by *Lactobacillus plantarum* (B-28), *Lactobacillus paracasei* ssp. *casei* (B-29), and *Lactobacillus acidophilus* for 12 h

Nutrients	% (mean ± SD)	Minerals	mg/100 g (mean ± SD)
Total solids	8.85 ± 0.37	Potassium	1.23 ± 0.22
Protein	0.89 ± 0.05	Phosphorous	2.53 ± 0.27
Fat	0.296 ± 0.01	Magnesium	0.66 ± 0.09
Carbohydrate[a]	7.56 ± 0.34	Calcium	1.34 ± 0.13
Ash	0.071 ± 0.01	Sulfur	0.074 ± 0.02
Energy (kJ)[b]	153.19 ± 6.08	Sodium	1.97 ± 0.27
Dietary fiber	—	Iron	0.03 ± 0.004
Insoluble	0.34 ± 0.02	Copper	0.013 ± 0.001
Soluble	0.53 ± 0.02	Zinc	0.031 ± 0.003
Total	0.87 ± 0.03	Aluminum	0.002 ± 0.001
—	—	Manganese	0.018 ± 0.003
—	—	Boron	0.003 ± 0.001

[a]By difference.
[b]Calculated.

Conclusions

The lactic acid bacteria isolated from traditional Bulgarian cereal-based beverages were found to have the ability to remove cholesterol from their growth media and adhere to the Caco-2 cell in culture. These findings suggest that they may aid in lowering

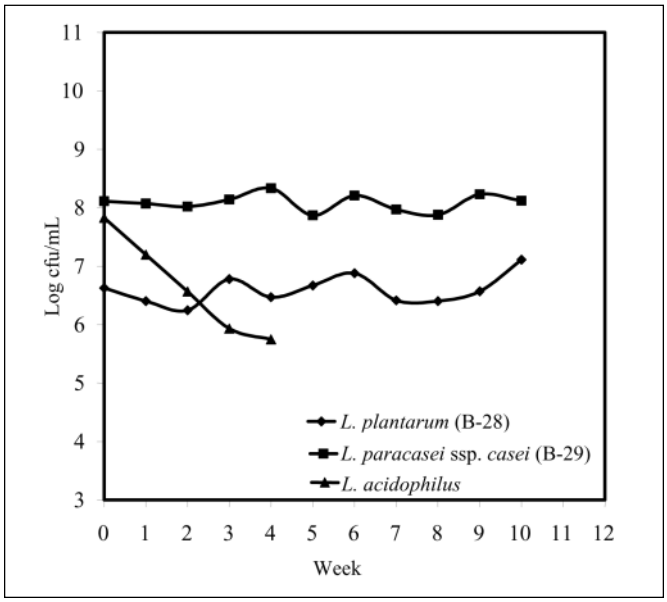

Figure 3—Survivability of probiotics in oat beverage during storage

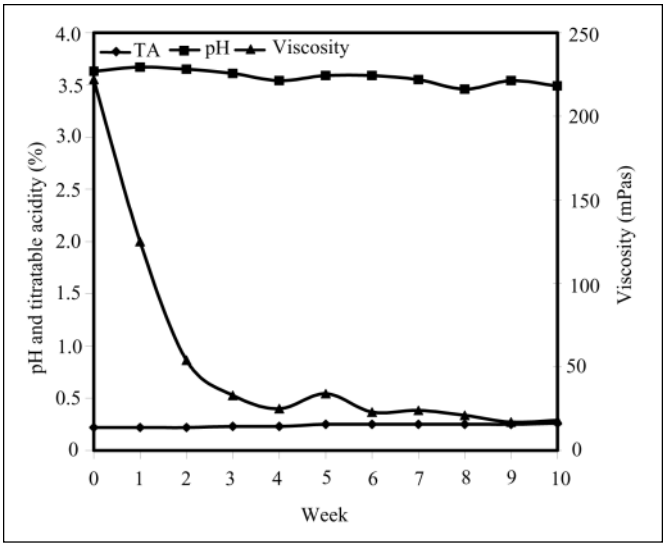

Figure 4—Changes in pH, titratable acidity, and viscosity of oat beverage during storage

Table 4—Paired *t*-test for sensory attributes of oat beverage

	Sample	Mean	*t* value	*P*
Appearance	D (control)	0.15	0.83	0.42[a]
	C (test)	–0.26		
Aroma	D (control)	0.06	–5.17	0.00[b]
	C (test)	2.15		
Sourness	D (control)	–0.26	–7.69	0.00[b]
	C (test)	2.29		
Sweetness	D (control)	–0.15	0.20	0.84[a]
	C (test)	–0.24		
Bitterness	D (control)	0.09	1.51	0.15[a]
	C (test)	–0.38		
Saltiness	D (control)	0	1.74	0.10[a]
	C (test)	–0.5		
Oat flavor	D (control)	–0.12	–0.06	0.96[a]
	C (test)	–0.09		
Viscosity	D (control)	–0.15	–9.07	0.00[b]
	C (test)	2.74		
Preference	D (control)	0.09	–2.21	0.04[b]
	C (test)	1.32		

[a]Not significantly different.
[b]Significantly different.

overall cholesterol in the blood and may be able to colonize the intestinal tract and compete with the resident flora. These cultures were able to ferment oats. This oat-based symbiotic beverage was developed to get the combined benefit of the probiotic property of the cultures, isolated from the traditional Bulgarian cereal-based beverages, and the prebiotic property of dietary fiber of oats. The test beverage had acceptable sensory attributes. *Lactobacillus plantarum* (B-28) and *L. paracasei* ssp. *casei* (B-29) survived in good numbers (10^7 to 10^8 CFU/mL) throughout the storage period in the refrigeration condition; however, *L. acidophilus* only survived at sufficient levels for about 4 wk. Although this oat-based beverage is a nondairy vegetarian product containing no milk, it is an alternative to both dairy and soy beverages and is suited to a healthy lifestyle, whether vegetarian-oriented or not.

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