

ORIGINAL RESEARCH PAPER

**STUDY OF PHYSIOLOGICAL PROPERTIES OF SOME PROBIOTICS IN
MULTIPLE CULTURES WITH MESOPHILIC LACTIC ACID BACTERIA
BY FLORA DANICA CH. HANSEN COMMERCIAL STARTER**

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The aim of this study was to establish the growth ability and stability of probiotic strains *Lactobacillus acidophilus* (commercial code La-5[®]), *Lactobacillus casei* ssp. *paracasei* (commercial code *L. casei* 431[®]) and *Bifidobacterium bifidus* (commercial code BB-12[®]) in multiple cultures with mesophilic lactic bacteria, *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *diacetylactis* and *Leuconostoc mesenteroides* spp. *cremoris*, as Flora Danica Chr. Hansen commercial starters.

Under the controlled fermentative conditions described below, a good starter combination, for the high rate of cells multiplication and for the good viability during storage, was identified in the mixture of *L. casei* 431[®], BB-12[®] and Flora Danica, in ratio of 1:1:1 (9 log CFU/mL for each starter culture).

Keywords: Probiotics, Christian Hansen commercial starters, La-5[®], BB-12[®], *L. casei* 431[®], Flora Danica, multiple starter cultures

Introduction

“Probiotic” means “for life” in Greek. Probiotics are live bacteria with health benefits. The first scientist to discover health benefits of probiotics was the immunologist Dr. Eli Metchnikoff who was awarded the Nobel Prize in 1908. “Probiotics” are defined as “live microorganisms which, when administered in adequate amounts, confer a health benefit on the host” (FAO/WHO, 2001). Numerous reports have suggested that probiotics confer some forms of health benefits to humans. For instance, they have been shown in various extents to produce antimicrobial compounds, modulate host immune system, inhibit *Helicobacter pylori*, alleviate lactose intolerance, assimilate cholesterol, prevent autoimmunity, and exhibit anti mutagenic properties (Canducci, 2000; Armuzzi, 2001; Kailaspathy and Chin, 2000).

Why should we consume probiotics in foods? The natural balance in our intestinal system can be disturbed by: bacteria infections, stress, antibiotic treatment, travelling. This may result in intestinal disturbances such as diarrhoea, constipation, etc. Taking probiotics through food or supplements can help to balance the microbiota in the gut. This is important due to the fact that the gastrointestinal tract is the largest immune organ in the human body.

In order to provide the consumer with the most of these putative health benefits, it is understandable that a sufficient amount of viable probiotics must reach the intestines. Thus, along with the innate health-promoting capability, their viability in the products has been cited as an important prerequisite for achieving beneficial health effects (Arunachalam, 2000; Galdeano and Perdigón, 2006). Hence, different forms of delivery ways (vehicles) should be studied and optimized to ensure that probiotics are viable and delivered in sufficient numbers before the expiration date (Godward *et al.*, 2000).

For probiotics delivered through foods, additional amounts of cells are likely required prior to processing to account for the loss of cells during the processing and/or storage phases.

Maintaining a high level of viable probiotic cell count in fermented foods, during the shelf life of the dairy fermented product, is not a simple task. Many factors influence the viability of probiotics in fermented foods: strain variation, acid accumulation, interaction with starter cultures, level of dissolved oxygen and hydrogen peroxide (H₂O₂), and storage condition (Gilliland, 2002). Nevertheless, several studies reported that some commercially available dairy products contain an insufficient number of viable probiotics (as defined by < 10⁶ CFU/g or mL before the final time for shelf life), thereby diminishing the potential health benefits granted by these products (Tharmaraj and Shah, 2003). Thus, understanding the survival of probiotics and developing methods to maintain and/or to promote their viability throughout the product shelf life continues to be an important subject of research in this field.

Probiotics were first commercialized via yogurts: Yakult was introduced in Japan in 1935, followed by Activia introduced in France in 1987. After 2006, the interest in probiotic products increased rapidly in the world (USA, Europe and Asia) and other fermented dairy products, as well as other foods, became food vectors to deliver probiotics to consumers. Among dairy products, yogurt is likely the most recognized product containing probiotics (Lourens-Hattingh and Viljoen, 2001). But in some countries, there are also other fermented types of milk which are very popular and may be used as carriers for probiotics. Many previous studies focused on the viability of particular probiotic strains in yoghurts. But there are almost no studies related to the viability of probiotic strains in fermented dairy products with mesophilic strains.

The aim of this study was the evaluation of the physiologic behavior of three probiotic Christian Hansen strains La-5®, *L. casei* 431® and BB-12®, in multiple cultures with Flora Danica, a mesophilic aromatic culture, type LD, used to produce fermented product named Sana, containing strains *Lactococcus lactis* subsp. *cremoris*, *Lactococcus lactis* subsp. *lactis*, *Leuconostoc mesenteroides*

subsp. *cremoris* and *Lactococcus lactis* subsp. *diacetylactis*, in order to establish the ability of multiplication and stability during preservation. Enhancing the understanding of different probiotic strains survivability in “Sana” may provide an opportunity for diversification of probiotic products with added value (to lead to a more effective delivery of probiotic-associated health benefits via fermented dairy products).

Materials and methods

Starter cultures and chemicals

Probiotic cultures, *Lactobacillus acidophilus* (La-5[®], La), *Lactobacillus casei* ssp. *paracasei* (L. casei 431[®], Lc), *Bifidobacterium bifidus* (Bb-12[®], Bb) were provided by Chr. Hansen, Denmark, as freeze-dried commercial starters. Mesophilic lactic bacteria Flora Danica (FD), containing strains *Lactococcus lactis* ssp. *cremoris*, *Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *diacetylactis* and *Leuconostoc mesenteroides* spp. *cremoris* were provided by Chr. Hansen, Denmark, as freeze-dried commercial starters. The storage and maintenance of the cultures were carried out following the recommendation of the manufacturer. The chemicals were pure grade and purchased from Merck KGaA, Darmstadt, Germany, Sigma-Aldrich Chemie GmbH, Germany, Bayer AG, Germany

Preparation of samples

The freeze-dried cultures were reactivated in 3.5% fat UHT milk (Prodlacta Brasov, Romania) and incubated for 15 minutes, at 30°C, in aerobic conditions. The number of cells in the inoculum was 9 log CFU/mL for all types of cultures, mixed in equal ratio. The samples were obtained by the combination of Flora Danica starter with culture of probiotic bacteria as double cultures: La+FD, Lc+FD and Bb+FD or in multiple cultures in combinations: La+Lc+FD, Lc+Bb+FD, La+Bb+FD or La+Lc+BB+FD. After inoculation, the samples were incubated at 37°C, till pH 4.6 was reached. Measurement of the pH of each sample was performed at all times with pH-meter (Portamess[®] 911, Switzerland) disinfected periodically with ethanol at 90%. After fermentation, the samples were stored at 4°C, for 21 days, representing the target shelf life. The cell viability was analyzed initially, after 7, 14 and 21 days respectively.

Enumeration of probiotic bacteria and mesophilic lactic bacteria

Viable cell counts were performed by preparing serial decimal dilutions into 0.1% (w/v) peptone water.

The Flora Danica strains were subsequently counted by plating (in duplicate) into MRS agar (Merck) (ISO 8261 IDF122:2001) using double-layer medium. The same conditions of cultivation were used for counting *L. casei* 431[®]. The plates were incubated aerobically for 48 h, at 37°C.

The probiotics were counted by cultivation on selective media. The medium for La-5[®] was MRS agar with clindamycin (Sigma) and ciprofloxacin (Bayer) (BS ISO 20128:2006). Clindamycin and ciprofloxacin stock solution, prepared by dissolving

2 mg and 20 mg antibiotic respectively into 10 mL distilled water, filter-sterilized and then added into the melted and tempered MRS agar at a concentration of 0.5 ml/l.

The media for BB-12[®] was a medium based on MRS agar with the addition of L-Cysteine hydrochloride (Merck) and mupirocin (ISO 29981: 2010). L-Cysteine hydrochloride stock solution, prepared by dissolving 10 g into 100 ml of distilled water, autoclaved at 121°C, for 15 minutes and added at a concentration of 5 ml/l into the melted and tempered MRS agar. Mupirocin stock solution (1% w/v filter sterilized) was added at a concentration of 2.5 ml/l into the melted and tempered MRS agar before pouring in the plates.

For La-5[®] and BB-12[®], the plates were incubated in anaerobic conditions into Anaerobic jar (Merck) by using Anaerocult[®] A reagent (Merck), for 48 h, at 37°C. All plate counts were carried out in duplicates. The results were recorded as colony forming units (CFU) per ml of fermented product. The data was expressed as means from three independent experiments with two replicates.

Results and discussions

The multiplication ability of probiotics in multiple cultures in combination with Flora Danica

The pH is an essential factor with influence upon probiotics multiplication and physiological activity. The pH was measured and registered hourly, during a period of 4 h. The probiotic strains were counted immediately after inoculation, and at the end of fermentation which took place at 37°C for 4 h.

In the samples where La-5[®] was monitored as starters, the pH evolution during fermentation was variable. The fastest pH decrease was registered in samples where the starter combinations were La-5[®] and Flora Danica and La-5[®] with *L. casei* 431[®] and Flora Danica (Figure 1). The slowest reduction of pH was registered in samples where the BB12 was present in multiple cultures.

In the samples in which *L. casei* 431[®] was monitored as starter, the decrease to 4.6 was obtained in 4 h, when the combination of cultures *L. casei* 431[®] (Lc), BB-12[®] (Bb) and Flora Danica (FD) was used (Figure 2).

When the behaviour of BB-12[®] was evaluated as probiotic strain, the combination of cultures that ensures the pH reduction to the value of 4.6 in 4 hours was BB-12[®], La-5[®] and Flora Danica (Figure 3). The fastest pH reduction was recorded for the combination BB-12[®] and Flora Danica.

The growth of bacteria is correlated with the pH evolution, many probiotic strains being negatively influenced by lowering the pH under the value of 5.0. The growth, recorded for all the tested strains, varied with the probiotic cultures and with the combinations used. The rate of probiotic multiplication in multiple starter cultures is presented in Figure 4.

The best rate of multiplication in multiple cultures, both for La-5[®] (La) and for *L. casei* 431[®] (Lc) was registered in the combination La-5[®] (La), *L. casei* 431[®] (Lc) and Flora Danica. This appeared to be the best combination used for optimal time of fermentation which was correlated with the pH reduction.

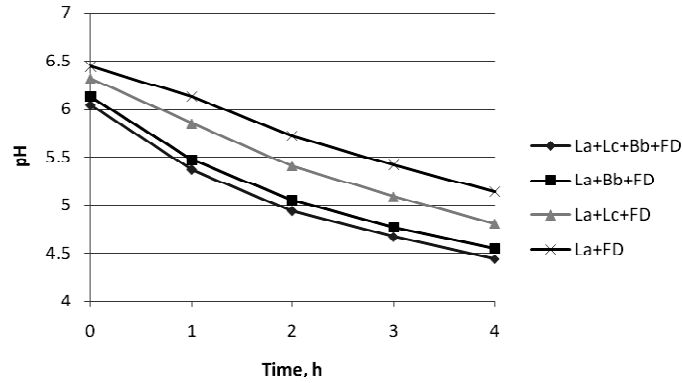


Figure 1. The pH evolution during fermentation by monitoring La-5[®] as probiotic starter in combination with *L. casei* 431[®] (Lc), BB-12[®] (Bb) and Flora Danica (FD)

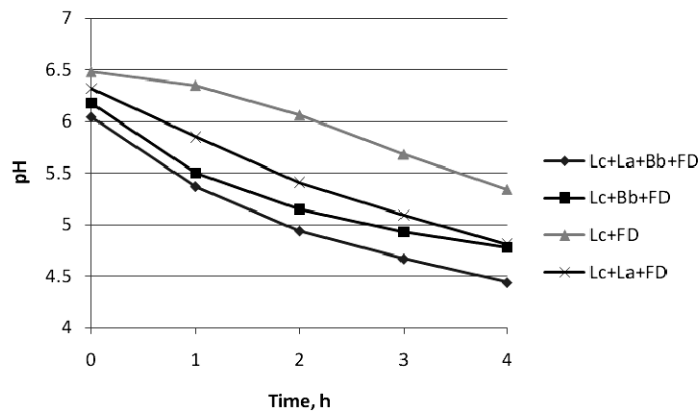


Figure 2. The pH evolution during fermentation by monitoring *L. casei* 431[®] (Lc) starter in combination with La-5[®], BB-12[®] and Flora Danica

The viability of probiotics in multiple cultures with Flora Danica during the storage of fermented food

The viability evaluation of the three probiotic strains from the probiotic starter cultures *Lactobacillus acidophilus* (La-5[®]), *Lactobacillus casei* ssp. *paracasei* (*L. casei* 431[®]) and *Bifidobacterium bifidus* (BB-12[®]) in multiple cultures combinations with the mesophilic LD mixt starter culture, Flora Danica, during 3 weeks of storage in refrigeration conditions, has indicated the differences in survival ability of the species of probiotics tested in this study.

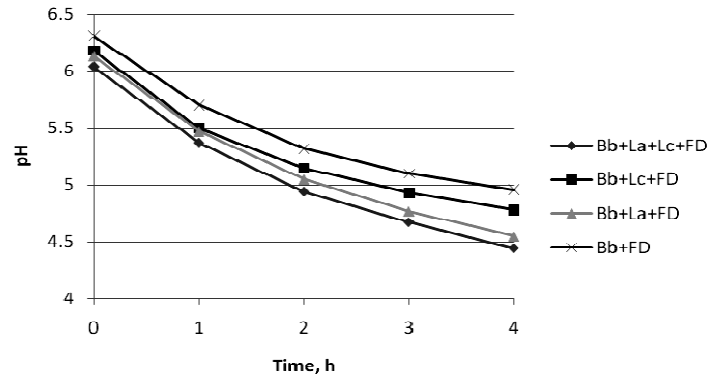


Figure 3. The pH evolution during fermentation by monitoring BB-12[®] (BB) starter in combination with La-5[®] (La), *L. casei* 431[®] (Lc) and Flora Danica (FD)

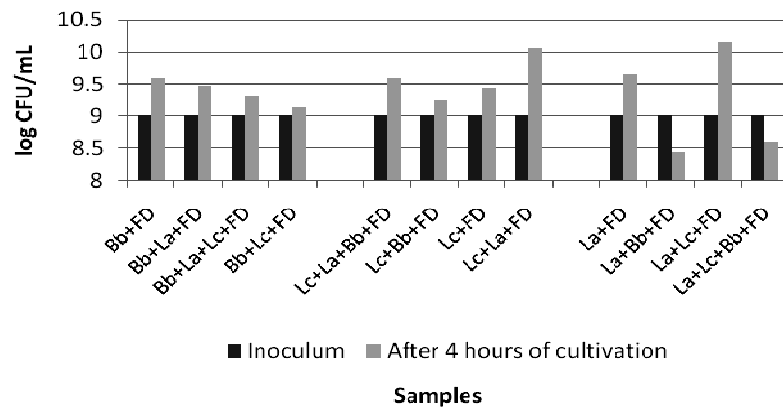


Figure 4. The multiplication of probiotic starters in multiple cultures

The *Lactobacillus acidophilus* (La-5[®]) demonstrated a different surviving in the same combined samples, in comparison with other two probiotic strains. Thus, the probiotic strain, La-5[®], remained at a stable level of viability during three weeks in the milk sample fermented with cultures combination La-5[®], BB-12[®] and Flora Danica. In this combination of cultures, the probiotic strain *Lactobacillus acidophilus* has started from an initial number higher than $1.8 \cdot 10^{10}$ CFU/mL, after 4 hours of fermentation, and then viable cells decreased at a final population of $3.8 \cdot 10^7$ CFU/ml (Figure 5). This evolution is positively correlated with the pH evolution in the sample. A similar evolution of *Lactobacillus acidophilus* viability

during refrigeration storage period for fermented foods was reported by Stanton *et al.* (1998).

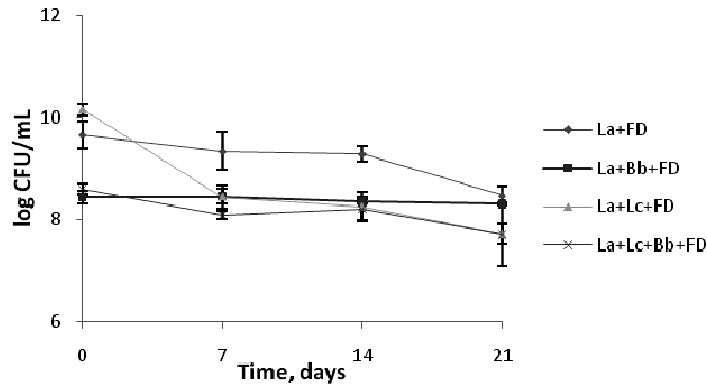


Figure 5. Dynamics of *Lactobacillus acidophilus* viability, in multiple cultures, during fermented product storage at 4°C

The best combination of cultures to ensure the *Lactobacillus casei* ssp. *paracasei* stability during fermented products storage, at 4°C, as shown in Figure 6, is obtained when this probiotic strain is combined with Flora Danica, as double starter culture, and in combination with *Bifidobacterium bifidum* and Flora Danica.

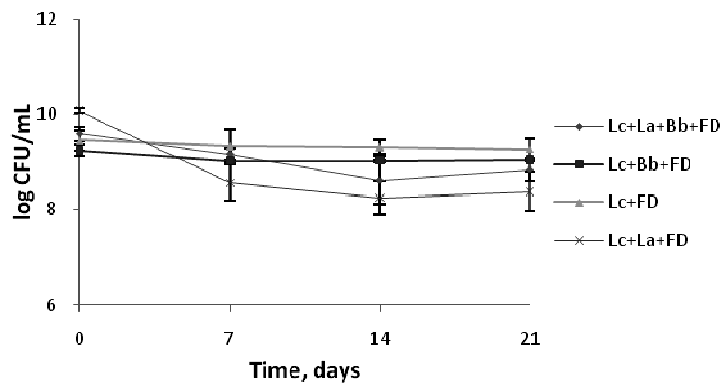


Figure 6. The *Lactobacillus casei* ssp. *paracasei* viability, in multiple cultures, during fermented products storage at 4°C

Starting from an initial concentration of *Lactobacillus casei* ssp. *paracasei* viable cells of $2 \cdot 10^9$ CFU/ml, after the fermentation time, and reaching at the end of the

storage period (21 days) a level of $1.15 \cdot 10^9$ CFU/ml when the combinations of starter culture, *L. casei* 431[®], BB-12[®] and Flora Danica were used.

The presence of *Lactobacillus acidophilus* in the combination of cultures with *Lactobacillus casei* ssp. *paracasei* affects negatively their viability during fermented storage. Thus, in combination of cultures *L. casei* 431[®], La-5[®] and Flora Danica, starting from an initial number of viable cells before preservation at 4°C, of $1.2 \cdot 10^{10}$ CFU/ml, the *L. casei* 431[®] decreased with 2 log CFU after 14 days and with 1 log CFU after 21 days.

In the combination *L. casei* 431[®], La-5[®], BB-12[®] and Flora Danica, the viability of *Lactobacillus casei* ssp. *paracasei* decreased after 14 days from $4.1 \cdot 10^9$ CFU/ml to $5.8 \cdot 10^8$ CFU/ml. After 21 days, a reduced tendency of growth at $7.3 \cdot 10^8$ CFU/ml was registered. A similar tendency was reported by Fitzsimmons *et al.* (2001) for a probiotic *Lactobacillus casei* strain, after 14 days to 21 days, during the storage in refrigeration conditions of a dairy product.

When the viability of *Bifidobacterium bifidus* (BB-12[®]) strain was studied in combination with La-5[®] and *L. casei* 431[®] and Flora Danica, it was observed that after 14 days and 21 days respectively, in all samples the probiotic strain BB-12[®] showed a drastic decrease of the number of viable cells at the level of 10^6 CFU/ml (Figure 7).

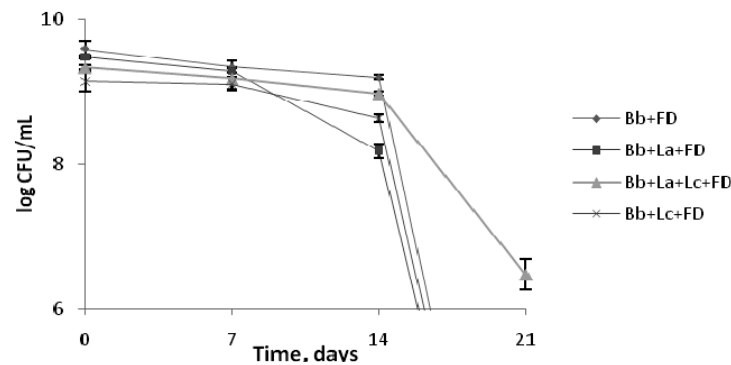


Figure 7. *Bifidobacterium bifidus* (BB-12[®]) viability evolution, in multiple cultures, during storage of fermented food at 4°C

In all other samples, the number of viable cells of BB-12[®] decreased to values much lower than 10^6 CFU/ml, which is the minimum recommended level of this strain in fermented probiotic foods.

In some dairy products, like yoghurt, the addition of combinations of probiotic strains as *Lactobacillus acidophilus* La-5[®], *Lactobacillus casei* ssp. *paracasei* 431[®] and *Bifidobacterium bifidus* BB-12[®] enjoys a good recognition and acceptance from the consumers (Phillips *et al.*, 2006).

Only few studies describe the behavior of the probiotics in combination with mesophilic lactic acid bacteria. The results of the present study confirm that probiotic strains may be used in other dairy products than yoghurts, like in sour milk or kefir, where combinations with mesophilic cultures are used, while still keeping their probiotic properties.

Conclusions

The probiotic studied strains in multiple combinations with Flora Danica showed different behavior and survival.

The multiplication of probiotic strains tested in multiple cultures with Flora Danica varies in function of the probiotic strain and in function of the combinations they are used in. The best combination, with a significant growth of probiotic strains, was *Lactobacillus acidophilus* (La-5[®]), *Lactobacillus casei* ssp. *paracasei* (*L. casei* 431[®]) and Flora Danica. The rate of cells multiplication is positively correlated with the pH level during the fermentation process.

Regarding the viability of probiotic strains during 21 days of storage at 4°C, the combinations with the highest viability of above 8 log CFU/ml and 9 log CFU/ml were: *Lactobacillus casei* ssp. *paracasei* (*L. casei* 431[®]), *Bifidobacterium bifidus* (BB-12[®]) and Flora Danica for *Lactobacillus casei* ssp. *paracasei* best viability preservation; or *Lactobacillus acidophilus* (La-5[®]), *Lactobacillus casei* ssp. *paracasei* (*L. casei* 431[®]) and Flora Danica for *Lactobacillus acidophilus* best viability preservation.

These are identified as good combinations recommended in further research in order to obtain dairy fermented products, as Sana, with mesophilic lactic acid bacteria and probiotic starter cultures, in order to ensure a good viability of the probiotics during storage.

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