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Intrinsic Properties of So-Called Dormant Probiotic Bacteria, Determined by Flow Cytometric Viability Assays

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Plate counting and four culture-independent flow cytometric assays were used to determine the viability and intrinsic properties of three probiotic strains during storage. The strains showed reduction in plate counts but were able to maintain esterase activity, intact cytoplasmic membrane, and pH gradient. The apparently uncultivable probiotic cells were active and stress resistant.

Our knowledge of the complex nature of bacterial viability has increased (7). The ability of cells to duplicate is generally considered the critical parameter between bacterial life and death and has traditionally been measured using plate counts, often leading to the generalization that viability means the same as in vitro cultivability. However, in vitro cultivability is a limited marker of cell viability. "Uncultivable" cells may retain several characteristics typical of living cells and have been proposed to be able to regain cultivability, a process referred to as resuscitation (10). Whether the reason for observed uncultivability is a programmed survival state of the cells or merely a sublethal injury, it seems that in many cases, plate counts yield limited and sometimes misleading information on viability statuses of stressed bacteria. Most studies involving dormant or active but noncultivable cells have focused on environmental and medical samples, but probiotic bacteria have been studied to lesser extent (1, 3, 9). We investigated the intrinsic properties of probiotic bacteria during storage and compared different markers of viability and their applicability to probiotics. The aim was to assess how the changes in these properties related to the viability and activity of the cells and to determine which methods are suitable for assaying the viability and activity of probiotic bacteria.

Bifidobacterium longum 46 (BL46; DSM 14583), *Bifidobacterium lactis* Bb-12 (Bb-12; Chr. Hansen, Denmark), and *Lactobacillus acidophilus* La-5 (La-5; Chr. Hansen) were fermented in sterile skim milk and reinforced clostridial medium anaerobically at 37°C until late exponential phase. Products were stored at 4°C and subjected to weekly viability assays. Prior to the assays, milk samples were purified from solids as described previously (8). Plate counts were obtained by plating BL46 and Bb-12 on brucella agar and La-5 on de Man-Rogosa-Sharpe agar supplemented with 50 mM L-cysteine. Membrane integrity was assessed using a LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes). To measure esterase activity, cells were incubated with 50 μM 5 (and 6)-carboxyfluorescein diacetate (cFDA; Molecular Probes) for 10 min at 30°C, using 30 μM propidium iodide (PI) as a counterstain. In addition, esterase activity was measured using cells incubated (15 min, 30°C) with 1 μM succinimidyl ester of cFDA (cFDA-SE; Molecular Probes). The cFDA-SE-stained cells were also used for measurement of intracellular pH by modifying methods described previously (2, 5). The minimum intracellular pH required for cell growth was determined for each strain (5), and the cells with internal pHs higher than the minimum intracellular pH were identified as viable in subsequent analyses. Calibration curves were obtained by adjusting cells to different internal pH values ranging from 4 to 7 (5). All stained cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson). Cells stained with LIVE/DEAD or cFDA-PI were plotted on an FL-3/FL-1 dot plot, and the ratios for stained and counterstained cells were determined. The cFDA-SE-stained cells were enumerated by calculating the ratio of FL-1-positive cells to the total number of cells. To measure intracellular pH, the samples were plotted on FL-1/FL-2, Counts/FL-1, and Counts/FL-2 histograms. The FL-1/FL-2 ratios were linear between pH 5.75 and 7.0. Analysis was performed with CellQuest Pro (Becton Dickinson). The pH measurements were analyzed with Ratio Calculator, courtesy of Perttu Terho, Turku Centre for Biotechnology, Finland (available at http://www.btk.fi/cic/ratiocalculator/). The results were converted to cells/ml using the plate counts and LIVE/DEAD results for the fresh samples. Prior to statistical analysis, an average for the duplicate samples was calculated and converted to log units. Student's t test was used to test the significance of the changes in cell counts. P values of ≤ 0.05 were considered statistically significant.

The plate counts for all strains decreased during storage (Fig. 1). The counts for BL46 and La-5 decreased below the detection limit by the end of storage, while the counts for Bb-12 decreased by approximately 1 log CFU/ml. The plate counts differed significantly from the results of all other viability assays. No significant changes in LIVE/DEAD counts were observed during storage, suggesting that the cells were able to maintain cytoplasmic membrane integrity. The results of the cFDA-PI assay indicated a significant decrease in the

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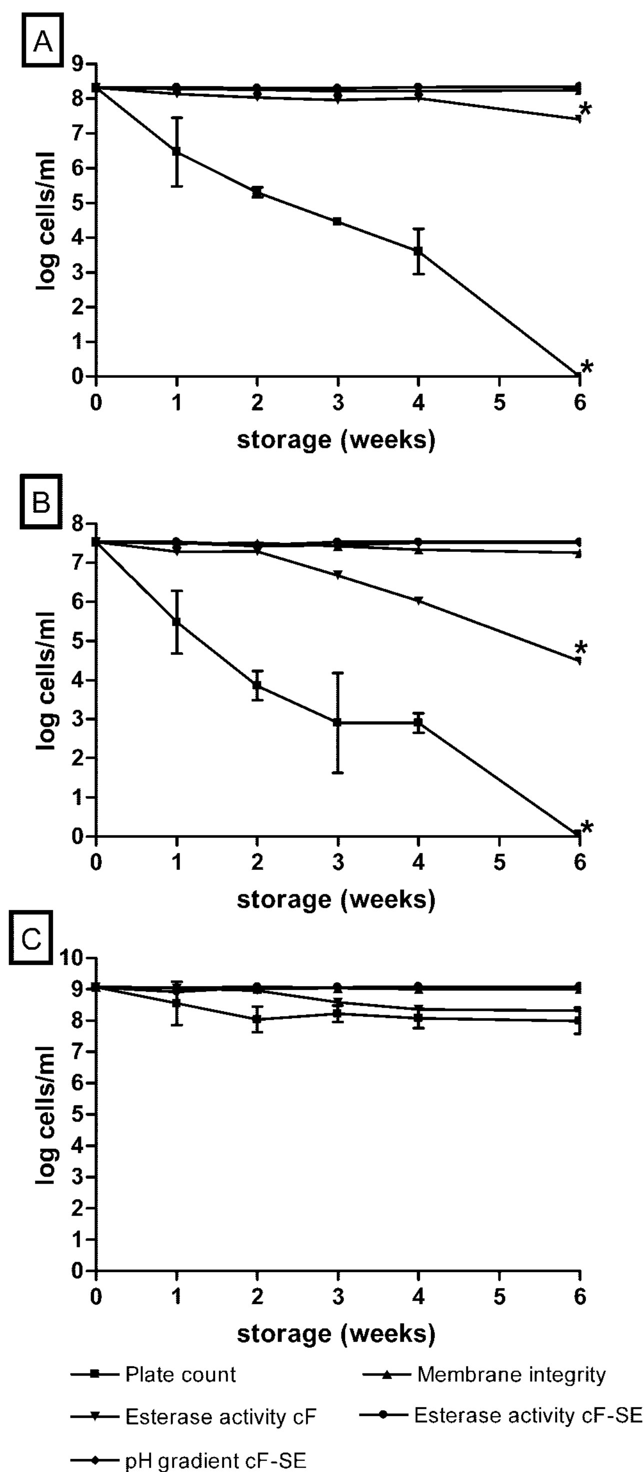

FIG. 1. Changes in the intrinsic properties of *Bifidobacterium longum* 46 (A), *Lactobacillus acidophilus* La-5 (B), and *B. lactis* Bb-12 (C) during storage in fermented milk. The cell counts are presented as averages for duplicate samples. *, statistically significant change ($P \leq 0.05$). cF, cFDA.

enzyme activities of BL46 and La-5 during storage, but such decrease was not observed with the cFDA-SE assay, even though both assays measure esterase activity. The observed difference is likely due to the succinimidyl group of cFDA-SE, which binds to intracellular proteins, resulting in long-term retention of the dye inside cells. The reduction of green fluorescence in cFDA-PI-stained cells may be due to energy-dependent efflux of carboxyfluorescein (4). The results indicate that cFDA-SE is a more consistent marker of esterase activity than cFDA. All strains were capable of maintaining pH gradients between intracellular and extracellular matrices. Throughout the study, a certain number of cells (from 0.5% to 7.7%) were not able to maintain intracellular pHs above the minimum pH required for growth, but the number of these cells did not change during storage. The viability assay results for the cells stored in fermented milk and nutrient medium were comparable to each other. The results for the duplicate samples did not differ from each other.

Viable bacteria are thought to be required for most health benefits of probiotics (13, 14), but it is difficult to address which functions of viable cells are critical for the desired health effects. Cell division itself is not a health effect, although it is a prerequisite for colonization in the gastrointestinal tract. In many cases, the proposed mechanisms for the health effects of probiotics are based on metabolic activity rather than cell division (12). The growing evidence for the existence probiotic bacteria in the dormant or active but uncultivable state questions the status of in vitro proliferation assaying as the only method applied for determination of the probiotic viability or quality of probiotic products. Moreover, proliferation capacity in laboratory conditions may not always be a good representation of proliferation capacity in the intestine, most often the natural niche of probiotic bacteria.

The so-called dormant state may be common among probiotics and is relevant to commercially used strains, such as La-5, which has been reported to decline in viability during storage (11, 15, 16). There is an urgent need for regulation of the microbial quality of probiotic products (6). Here, we demonstrate that probiotic bacteria that fail to grow on nutrient agar may possess several properties typical of viable cells. These results are significant for probiotic research, as the microbial quality of a probiotic product is attributed to the type, quantity, and viability of the probiotic bacteria it contains. A product that seems sterile based on plate counts may contain high levels of viable and active bacteria, making enumeration complicated. While accepting cultivation as the most important method for determining viability of probiotic cells in foods, it should be emphasized that cultivation-based methods provide only a limited view of the physiological statuses of cells. Culture-independent methods, such as flow cytometry, provide means of detecting so-called dormant cells. Thus, the limited knowledge on the life cycle of probiotic cells and the possible health effects of non-readily cultivable bacteria should be taken into consideration when further developing regulatory guidelines in this area.

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