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Growth of Facultatively Heterofermentative Lactobacilli on Starter Cell Suspensions

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The growth of facultatively heterofermentative lactobacilli (FHL) on cell suspensions of the homofermentative *Lactobacillus helveticus* was investigated. Osmotic lysis of *L. helveticus* led to a significant increase of ribose. It decreased steadily in parallel with the growth of FHL, strongly suggesting that the bacteria used ribose as a growth substrate.

Facultatively heterofermentative lactobacilli (FHL) predominate in the nonstarter microfloras of diverse cheese varieties. The influence of this adventitious flora on cheese quality is controversial. Its positive impact on cheese quality is mainly in the areas of improvements in flavor acceptability (12), the prevention of secondary fermentation (7), and the suppression of undesired adventitious nonstarter organisms (13). However, cheese can be affected by ripening problems due to high numbers of undesired strains. According to Kleter (10), they are not necessary for a good ripening process and their presence may even cause taste and flavor defects.

FHL occur in very small numbers in the cheese milk, presumably originating from the raw milk and the cheese-making environment. Usually, they reach counts up to 10^8 CFU/g in ripe cooked cheeses (4), in cheddar cheese (16), and in Swiss-type cheese (3). Obviously, they develop well under the highly selective conditions of ripening cheese, which implies the presence of available growth substrates. Lactose and galactose are usually metabolized after primary fermentation by the starter cultures. Other possibly available energy sources for nonstarter bacteria are citrate, lactate, milk components, microbial metabolites, and microbial cell lysis products. Further potential substrates include sugars released from starter nucleic acids (ribose), sugars released from cell walls (*N*-acetylglucosamine), free amino acids and peptides, gycerol released by lipolysis, fatty acids, and glycoproteins and glycolipids of the milkfat globule (for a review see references 5 and 16).

Thomas (17) showed that lactobacilli are able to grow in suspensions of cheese starter bacteria; however, attempts to detect the growth substrates were unsuccessful. By contrast, Lane et al. (11) suggested that starter cell lysates are not a major source of growth substrates due to the faster growth of FHL in experimental cheddar made with slow-lysing starter cultures than in cheese made with fast-lysing starter cultures.

The aim of this study was to evaluate the growth of different FHL strains on starter cells and to prove the presence of free sugars in the supernatants of the cultures.

Lactobacillus helveticus (KK1 commercial culture; Federal Research Station of Alpine Dairying, Rotholz, Austria) was grown for 20 h at 37°C in 700 ml of MRS broth. The cells were harvested by centrifugation (4,420 × *g*; 10 min; 4°C), washed three times in 100 ml of quarter-strength Ringer's solution, and resuspended in 25 ml of phosphate buffer (50 mM KH_2PO_4, 50 mM $Na_2HPO_4 \cdot 2H_2O$, pH 7.2). The FHL strains (*Lactobacillus rhamnosus* ATCC 7469, *Lactobacillus paracasei* subsp. *paracasei* ATCC 27216, *L. paracasei* subsp. *paracasei* ATCC 25598, *L. paracasei* subsp. *paracasei* ATCC 11974, *Lactobacillus casei* ATCC 393, and *L. paracasei* subsp. *tolerans* ATCC 25599) were grown in 20 ml of MRS broth for 20 h at 30°C. Twenty milliliters of the washed cell suspension was inoculated with 1 ml of the diluted FHL cultures so that initial counts of about 100 CFU/ml were reached. This suspension was incubated in Erlenmeyer flasks at 30°C for 7 days. Sampling was carried out every 24 h for the microbiological analysis and every 48 h or only after 7 days for sugar analysis. The data presented are the means from two repetitions.

The following methods were used to differentiate the microflora: *L. helveticus*, MRS agar, pour plate method, with anaerobic incubation at 37°C for 72 h (1); FHL, FHL agar (6), surface method, with microaerophilic incubation (GEBbox microaer; bioMérieux no. 96125) at 38°C for 72 h.

The filter-sterilized supernatants of the cell suspension served as a basis for sugar and amino sugar analyses. Maltose, lactose, glucose, galactose, rhamnose, arabinose, ribose, glycerine, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine were detected by high-performance liquid chromatography (HP-1090; Hewlett-Packard, Waldbroun, Germany) and model 156 refractive index detector (Beckman, Berkeley, Calif.). The separation was carried out with two Nucleogel Ion 300 OA columns mounted in parallel (Macherey-Nagel, Düren, Germany) at 50°C. Double-distilled water (on-line degassing) (HP 1050; Hewlett-Packard) was used as the mobile phase.

While viable counts of *L. helveticus* decreased during the week of incubation, the FHL strains under study showed exponential growth from approximately 100 inoculated cells/ml to at least 10^7 CFU/ml (Fig. 1) during the incubation period. Later, the FHL counts remained more or less at this level. The only type strain which did not grow in the cell suspension was ATCC 25599. No viable bacteria were detected within 24 h for this strain. Initial counts of *L. helveticus* in the cell suspensions ranged between 10^9 and 10^{10} CFU/ml. The decrease in counts in the blank during incubation is illustrated in the inset of Fig. 1. The pH in the cell suspensions remained stable throughout the incubation period.

In the cell suspensions, ribose proved to be the only detectable sugar. The lysis of *L. helveticus* led to an increase of ribose from 0.12 to 0.75 mg/ml during the 1-week incubation (Fig. 2). Ribose concentrations in cultures with ATCC 25599 adjuncts were comparable to those of the blank samples. By contrast, in

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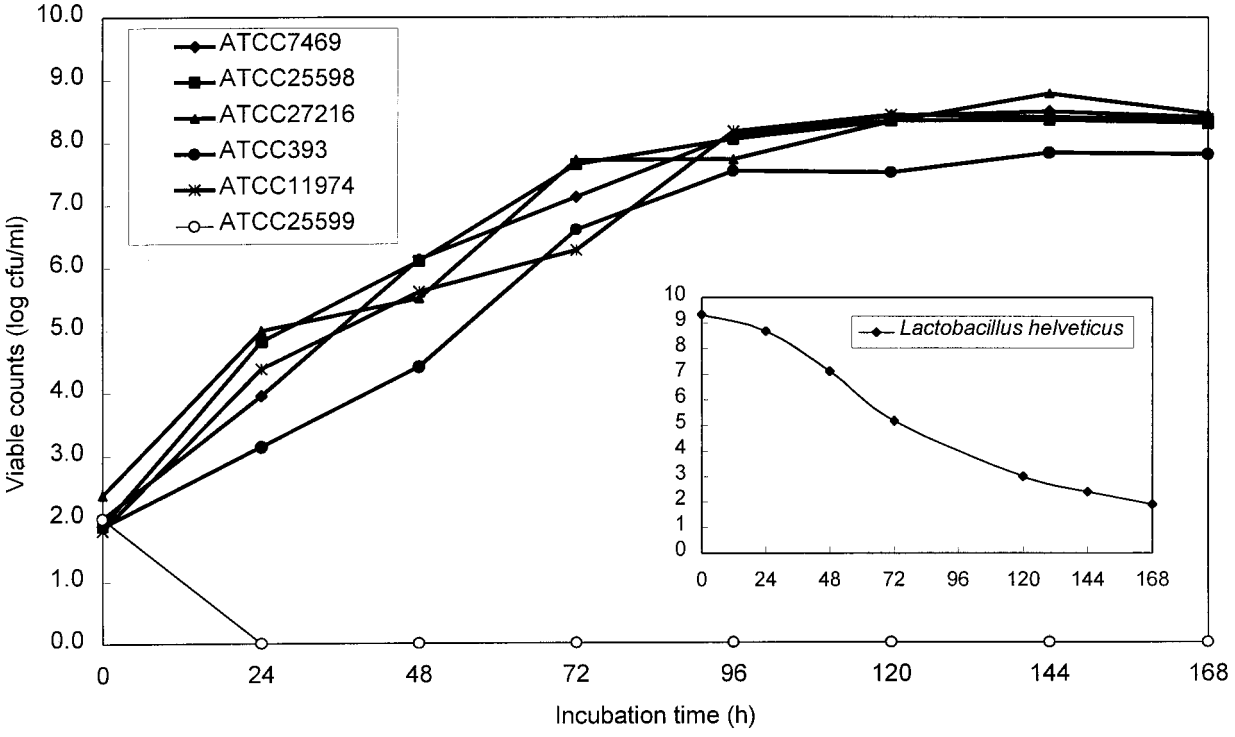

FIG. 1. Development of facultatively heterofermentative lactobacilli in cell suspensions of *L. helveticus*. (Inset) Decrease of *L. helveticus* cells in the blank.

all samples inoculated with any other strain, the initial ribose concentration (0.15 mg/ml) decreased steadily. ATCC 27216 deviated from the other strains: after the initial decrease, a second increase and a further decrease followed.

L. helveticus provided sufficient carbon source to support growth of FHL to above 10^7 CFU/ml. The decrease of free ribose clearly paralleled the exponential growth of the ribose-fermenting lactobacillus strains. Therefore, we suggest that the FHL used ribose as an energy source, with the exception of ATCC 25599, which is described as ribose negative (8). The second increase of ribose in cultures inoculated with ATCC 27216 may be attributed to lysis of the strain itself. Except with

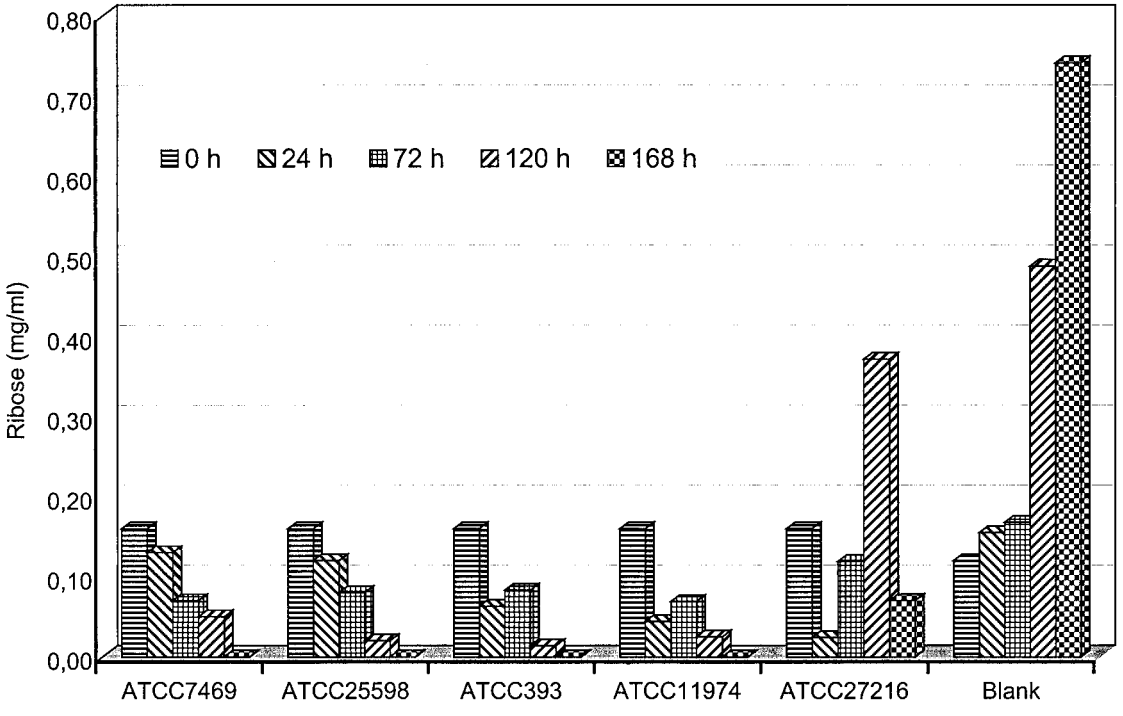

FIG. 2. Decrease of ribose in *L. helveticus* suspensions inoculated with facultatively heterofermentative lactobacilli and increase of ribose in the blank.

this strain a continuous consumption of ribose was observed. The fact that no sugars except ribose were detected does not prove their general absence. Their concentrations might be just under the detection limit. Nevertheless, they could be able to support the growth of FHL. This is also suggested by Thomas (17), who explained the lack of detectable free sugars in his investigations by their continued consumption by the nonstarter microorganisms, so that their levels remained under the detection limit. Additionally, it must be supposed that the amount of released free sugars is organism specific.

The lysis of starter bacteria plays a significant role in cheese ripening, especially influencing the rate of secondary proteolysis (2, 9, 14, 15). Valence et al. (18) first demonstrated the autolysis of *L. helveticus* in Swiss cheese, which significantly influenced ripening by the release of intracellular enzymes. Thus, the extent of autolysis should be taken into consideration in starter selection. For practical application, one should consider that the rate of starter cell lysis might influence the growth of secondary microflora. Ribose may not be the only energy source for FHL, but the fact that it is primarily ribose-fermenting species that predominate in the secondary floras of a variety of cheeses leads to the assumption that ribose metabolism is of utmost importance.

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