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Note

Intracellular Enzyme Activities and Autolytic Properties of *Lactobacillus Acidophilus* and *Lactobacillus Gasseri*

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A number of fermented milk products containing *Lactobacillus acidophilus* and *Lactobacillus gasseri* are now available as probiotic products. The proteolytic, lipolytic and autolytic properties of human-derived *L. acidophilus* (5 strains) and *L. gasseri* (7 strains) were evaluated, as these factors are closely related to cell viability and flavor development in the products. All *L. gasseri* strains showed higher intracellular protease activities than the *L. acidophilus* strains; in contrast, lipase activities in *L. gasseri* were mostly lower than those of *L. acidophilus.* The many strains of *L. gasseri* were shown to have a greater tendency than *L. acidophilus* to autolyze in dispersed solution; this liability was more distinct at lower pH values. These properties should be taken into account when bacteria are selected for production of probiotic products.

Keywords: *L. acidophilus, L. gasseri,* Intracellular enzymes, Autolysis, Probiotics, Protease, Lipase

Introduction

A probiotic bacterium is defined as "a microorganism that brings useful functions for the host by improving the balance of bacterial flora in the host intestine" (Fuller, 1989; Fuller, 1992). Probiotic bacteria include human-derived *Bifidobacteria, Lactobacillus casei, Lactobacillus rhamnosus,* and *Lactobacillus acidophilus* (which has been classified into six species, *L. acidophilus, L. crispatus, L. amylovorus, L. gallinarum, L. gasseri,* and *L. johnsoni,* often called the *L. acidophilus* group) (Shah, 2000). The health effects and functional properties of milk that has been fermented using these probiotic lactic acid bacteria are gradually being recognized by the consumer, and products fermented using various kinds of probiotic bacteria have already appeared on the market.

Although *L. acidophilus* has been reported to possess many advantages and health-giving properties compared to *Bifidobacteria,* the growth of *L. acidophilus* in milk is slow (Itoh *et al.,* 1991). Many commercialized fermented milk products containing *L. acidophilus*- group lactic acid bacteria are made in combination with conventional yogurt starters or by adding a concentrate of live *L. acidophilus* cells propagated and collected in advance from another broth. There were concerns that the survival rate of *L. acidophilus* cells in such products is reduced by the effect of the acid produced during the fermentation process, thus resulting in decreased probiotic effects when consumed. Up to now, many reports concerning the acid tolerance, bile tolerance and viability of *L. acidophilus* group strains in milk have attempted to select *L. acidophilus* strains for yogurt or cheese-making which have a greater possibility of reaching the intestine as viable cells (Prasad *et al.,* 1998; Conway *et al.,* 1987; Gilliland *et al.,* 1984; Holcomb *et al.,* 1991; Ibrahim and Bezkorovainy, 1993; Lankpauthra and Shah, 1995; Chou and Weimer, 1999; Masuda *et al.,* 2005). There have been few reports on the effect of the enzyme activities of *L. acidophilus* strains on the flavor of fermented dairy products. If the selected strain exhibits high enzyme activity, for example by protease or lipase, the degraded protein and lipid products which are gradually generated may affect the sensory properties of the fermented products. Intracellular enzymes exuded via autolytic rupture of the cells may also cause such flavor alterations. The autolytic properties of *L. gasseri* were demonstrated by Yokoi *et al.,* (2004).

In the present investigation, the intracellular protease and lipase activities and autolytic properties of human-derived *L. acidophilus* and *L. gasseri* strains, which have been widely used in the production of probiotic fermented products in Japan, were examined.

Materials and methods

Bacterial strains and culture conditions The test strains were *L. acidophilus* JCM1028, JCM1034, JCM1132, JCM1229, and JCM11047, and *L. gasseri* JCM1025, JCM 1130, JCM1131, JCM8788, JCM8789, JCM8790, and JCM 11657. All of these originated from the human intestine and were obtained from the Japan Collection of Microorganisms (Wako, Japan). All cultures were maintained in

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MRS broth (Difco Laboratories, Detroit, USA) and stored at −80℃. The stock cultures were subcultured twice in MRS broth before use and stored at 4℃.

The stock cultures were inoculated into 1000 ml of MRS broth at 5% (v/v) and incubated at 37℃ for 18 h. Cells were harvested by centrifugation at 5,000 rpm for 5 minutes and washed twice in about 80 ml of sterile distilled water. The washed cells were freeze-dried using a FDU-1000 freeze dryer (Tokyorika, Japan).

Intracellular protease activity Freeze-dried cells (15 mg) of the test strains were suspended in 1.5 ml of 100 mM Tris HCl buffer solution (pH 7.0) containing 0.4% sodium azide, and were disrupted by ultrasonication using a Sonifier 250 at 4℃ (20 min; 2 min×10 times with one-minute intervals; BRANSON, USA). The crude enzyme solution (0.4 ml) from which the cell debris had been removed by centrifugation (12,000 rpm, 15 min) was mixed with 0.4 ml of fluorescein isothiocyanate (FITC)- labeled casein solution (0.5% w/w) of pH 7.0 and allowed to react at 37℃ for 2 hours with rotation. FITC-labeled casein was prepared according to the Twining method (Twining, 1984). After the reaction, an equal amount of 14% trichloroacetic acid solution was added and mixed, and the fluorescence of the supernatant was measured using a fluorophotometer (EX: 490 nm, EM: 522 nm; Shimadzu Corporation, Japan). A unit of protease activity was defined as the amount (μg) of casein hydrolyzed by 1 mg of dry cells per hour.

Intracellular lipase activity A fat emulsion was prepared as follows: butter oil (3% of the total volume) was mixed with 50 ml of distilled water containing Tween 80 (1.5% v/v), then homogenized at 12,000 rpm for 10 min using an AM-7 homogenizer (NISSEI, Japan). Freeze-dried bacterial cells (50 mg), prepared as above, were suspended in 20 ml of 0.02 M phosphate buffer (pH 7.0), and disrupted by ultrasonication. Three ml of the centrifuged supernatant was mixed with 15 ml of the fat emulsion and kept at 37℃ for 18 hours with mild shaking. An ether-ethanol (1: 1) mixture (10 ml) was added to stop the reaction, and the liberated free fatty acids were titrated with 0.02 M NaOH, using three drops of 0.1% phenolphthalein solution as an indicator. A unit of lipase activity was defined as the amount (μg) of free fatty acid liberated by 1 mg of dry cells per hour.

Autolytic properties The cells of the test strains were harvested by centrifugation (3,000 rpm, 15 min, 5℃) from MRS broth after incubation at 37℃ for 8 h, and washed twice by centrifugation with sterile distilled water. The washed cells were redispersed in 5 ml of 0.05 M phosphate buffer (pH 5.0 or pH 7.0) containing 0.01% sodium azide so that turbidity (at an optical density of 620 nm, 1 cm path length) was achieved around 0.9–1.0. The solution was kept in a water bath at 37℃, and changes in turbidity was measured periodically after 2, 4, 6, 8 and 24 hours.

Results

The intracellular protease activities of the tested strains are compared in Fig. 1. All *L. gasseri* strains showed higher protease activities than the *L. acidophilus* strains. In contrast, the intracellular lipase activities of *L. gasseri* strains, as shown in Fig. 2, were mostly lower than those of *L. acidophilus*.

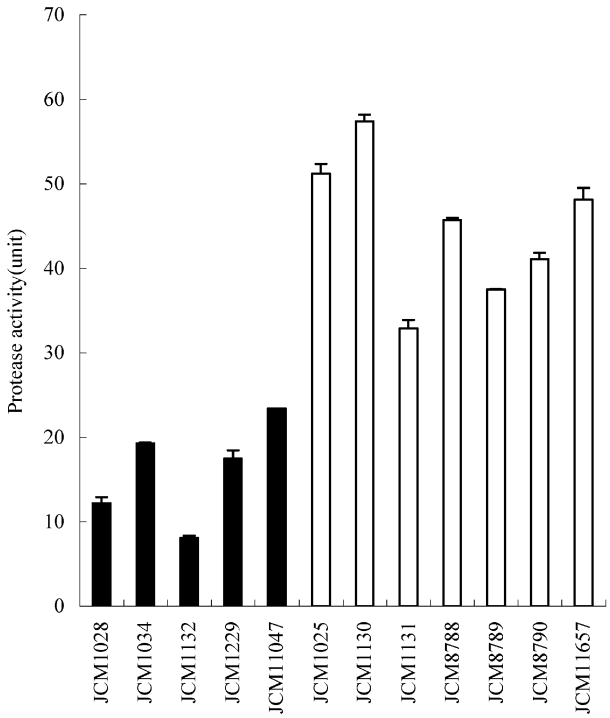

Fig. 1. Intracellular protease activities of *L. acidophilus* and *L. gasseri* using FITC-labeled casein as a substrate. ■, *L. acidophilus* strains; □, *L. gasseri* strains. Data are expressed as means (±SE) of triplicate measurements.

The changes in turbidity of cells dispersed in solutions of different pH values are shown in Figs. 3 and 4. The turbidity loss reflects the decrease in cells numbers by destruction through autolysis. Gradual decreases in turbidity were observed with the passage of time in all strains, and the decreases were generally more significant in *L. gasseri* strains than in *L. acidophilus* strains. Autolysis in *L. gasseri* strains was more distinct at pH 5.0 than at pH 7.0.

The results suggested that there are a number of *L. gasseri* strains that are liable to autolyze in dispersed conditions, in particular under conditions of lower pH.

Discussion

Over the last few years in Japan, the number of fermented milk products containing *L. acidophilus* group lactic acid bacteria has increased. The growth of human-derived *L. acidophilus* group bacteria in milk is usually slow, and the flavor of products made with this strain alone is unsatisfactory. For this reason, products are often made by co-culture with conventional yogurt starter bacteria or by addition of sufficient viable cells of *L. acidophilus* group lactic acid bacteria cultured and harvested from a separate broth at the start of fermentation by yogurt bacteria. Proteolytic and lipolytic functions of co-existing probiotic bacteria affect the flavor of the products. In the case of protease, the output of exocellular protease in the early stage of fermentation was

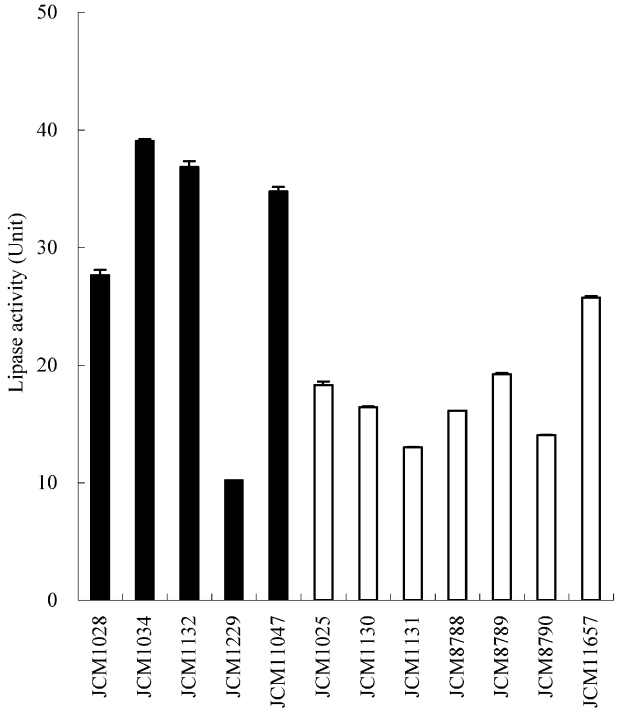

Fig. 2. Intracellular lipase activities of *L. acidophilus* and *L. gasseri* using butter oil as a substrate. ■, *L. acidophilus* strains; □, *L. gasseri* strains. Data are expressed as means (±SE) of triplicate measurements.

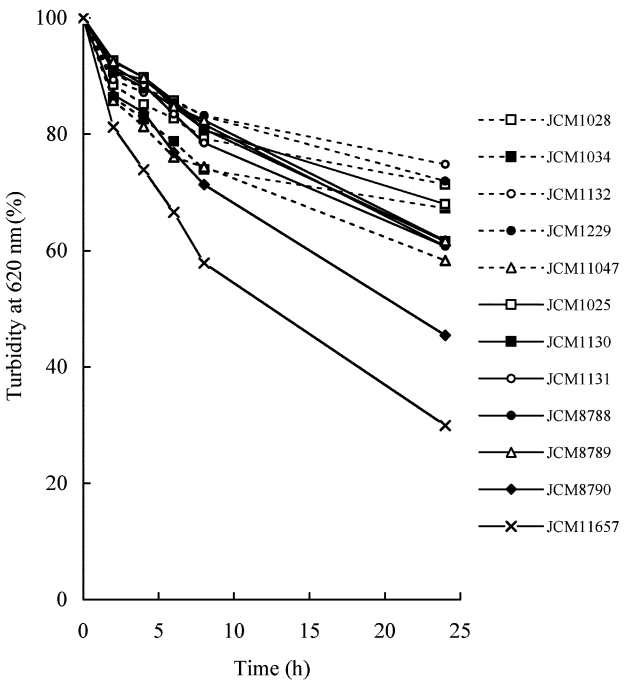

Fig. 3. Turbidity of *L. acidophilus* strains (---) and *L. gasseri* strains (—) dispersed in pH 7.0 phosphate buffer at 37℃. The bacterial cells were harvested from MRS broth after incubation at 37℃ for 8 h. The results are indicated in percentage of the initial turbidity. Each point represents the mean of triplicate measurements.

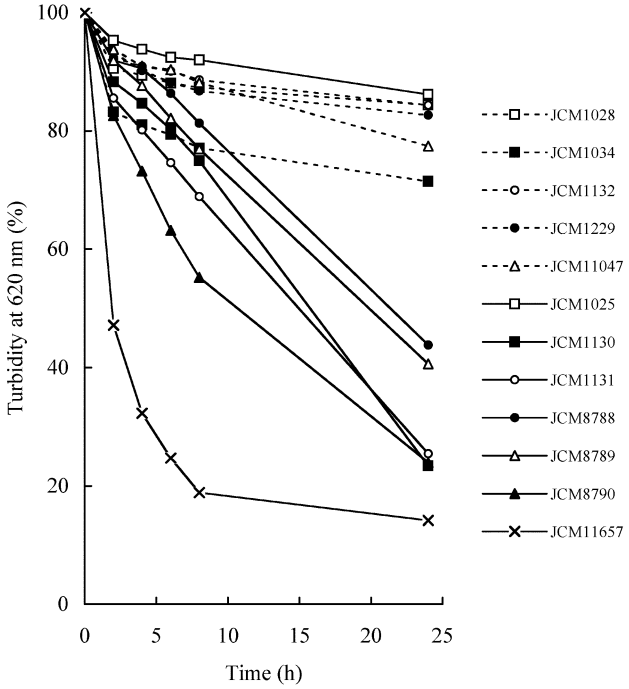

Fig. 4. Turbidity of *L. acidophilus* strains (---) and *L. gasseri* strains (—) dispersed in pH 5.0 phosphate buffer at 37℃. The bacterial cells were harvested from MRS broth after incubation at 37℃ for 8 h. The results are indicated in percentage of the initial turbidity. Each point represents the mean of triplicate measurements.

shown to be a deciding factor in whether *L. acidophilus* or *L. gasseri* could grow well in milk (Masuda *et al.,* 2003). The release of intracellular proteases from cells will be concerned with the alternation of the flavor of the product during further fermentation and storage. Intracellular protease activities, which are compared in Fig. 1, were shown to be generally higher in *L. gasseri* than in *L. acidophilus*. In a previous study, we attempted to incorporate viable cells of *L. acidophilus* JCM11047 and JCM 1132 and *L. gasseri* JCM11657 into fresh cheese, with the aim of creating a new probiotic delivery food (Masuda *et al.,* 2005). Different rates of protein hydrolysis were observed during storage of the products. These results indicate that intracellular protease activities should be taken into account when strains of probiotic bacteria are selected.

The lipolytic activity of lactic acid bacteria is especially important for aroma formation in fermented products. Lipase activities are known to be generally low or negligible in lactobacilli (Vogel *et al.,* 1998; Kenneally *et al.,* 1998). The intracellular lipase activities of the tested strains shown in Fig. 2 were strain-specific, but most of the *L. acidophilus* strains showed higher lipase activities than the *L. gasseri* strains, contrary to the results obtained for protease. Lipase activity is not high in either species, but is measurable in both. This should be taken into account in the consideration of flavor formation in the product.

Autolysis is observed in a variety of bacteria. The autolytic properties of lactococci used for cheese-making

have been thoroughly investigated (Hannon *et al.,* 2003), because the enzymes released play a key role in the development of cheese flavor. In cheese-making, cells that have a tendency to autolyze are favored, since the release of enzymes will accelerate ripening. For preparation of viable probiotics, in contrast, autolysis is unfavorable. The autolytic properties of the *L. acidophilus* group have been demonstrated by several researchers (Fernandez *et al.,* 1994; Fernandez *et al.,* 1995; Ohmiya and Sato, 1975). The likelihood of autolysis was found to vary at different pH values and depending on the conditions of the dispersed solutions. The results shown in Figs. 3 and 4 indicate the presence of strains that are remarkable easy to autolyze in *L. acidophilus* and *L. gasseri*. Recently, Yokoi *et al.,* (2004) reported that autolysis was induced in *L. gasseri* JCM1130 and JCM1131 by infection with a prophage. We did not confirm the relationship between prophage and autolysis in the strains used. In our work, both strains showed greater autolysis at lower pH values, in contradiction of the results of Yokoi *et al.,* (2004); however, the autolytic response at different pH values may be affected by the composition of the dispersed solution.

In conclusion, proteolytic and lipolytic properties, together with autolytic properties, should be evaluated when selecting strains of *L. acidophilus* and *L. gasseri* for probiotic purposes, because there is a close relationship between autolytic properties and the viability of the bacteria, and because enzymes released during storage affect the quality of the product.

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