

Estimation of lactic acid bacterial cell number by DNA quantification

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Summary

Lactic acid bacteria are provided by fermented foods, beverages, medicines, and supplements. Because the beneficial effects of medicines and supplements containing functional lactic acid bacteria are related to the bacterial cell number, it is important to establish a simple method for estimating the total number of lactic acid bacterial cells in the products for quality control. Almost all of the lactic acid bacteria in the products are dead, however, making it difficult to estimate the total number of lactic acid bacterial cells in the products using a standard colony-counting method. Here we estimated the total lactic acid bacterial cell number in samples containing dead bacteria by quantifying the DNA. The number of viable *Enterococcus faecalis* 0831-07 cells decreased to less than 1×10^{-8} by 15 min of heat treatment at 80°C. The amount of extracted DNA from heat-treated cells was 78% that of non-heated cells. The number of viable *Lactobacillus paraplantarum* 11-1 cells decreased to 1×10^{-4} after 4 days culture. The amount of extracted DNA of the long-cultured cells, however, was maintained at 97%. These results suggest that cell number of lactic acid bacteria killed by heat-treatment or long-term culture can be estimated by DNA quantification.

Keywords: Lactic acid bacteria, medicines, supplements, DNA quantification, total cell number estimation

1. Introduction

Lactic acid bacteria have various functions, such as digestive functions and immunostimulation, which are considered to be beneficial for maintaining human health (1). Functional lactic acid bacteria are provided as fermented foods, such as yogurt, pickles, and fermented juices, as well as in medicines and supplements (2,3). We previously screened functional lactic acid bacteria using a unique evaluation system utilizing silkworms as experimental animals (4-8).

During the course of those studies, we realized that the development of a simple method for estimating the total number of lactic acid bacterial cells in samples is important for validation of foods and supplements containing functional lactic acid bacteria. The total number of lactic acid bacterial cells in samples is the sum of viable and dead cells. Measuring the number of

viable cells is relatively easy by spreading appropriately diluted samples on agar plates, incubating the plates, and counting the resulting colonies. The number of viable cells is substantially decreased, however, by long-term culture or sterilization during the manufacturing process. Therefore, a simple method for determining the total cell number of lactic acid bacteria is desired. The use of a dye that stains lactic acid bacteria (9) or an antibody against lactic acid bacteria was proposed for measuring the number of lactic acid bacteria (10,11). These methods, however, may be problematic, requiring special instruments and complicated techniques, and having limited application to specific lactic acid bacteria species.

DNA quantification has been proposed for estimating the total number of bacterial cells in samples containing both viable and dead cells (12). A possible problem in applying this method to lactic acid bacteria is a decrease in the amount of DNA due to its degradation during long-term culture or sterilization. In the present study, we show that the amount of DNA from lactic acid bacteria is still maintained under conditions in which viable cells were decreased. Our results suggest that the total bacterial number of lactic acid bacteria can be estimated

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by DNA quantification.

2. Materials and Methods

2.1. Lactic acid bacteria used in this study

Leuconostoc paraplantarum 11-1 (4) and *Enterococcus faecalis* 0831-07 are the lactic acid bacteria isolated in our laboratory.

2.2. Preparation of lactic acid bacteria

Glycerol stocks of lactic acid bacteria kept at -80°C were thawed at room temperature and spread on MRS (de Man, Rogosa, Sharpe) agar plates, followed by culture at 30°C . The emerged colonies were cultured in MRS medium at 30°C in a bottle. Cells were heat-treated by placing full-growth cell cultures at 80°C for 15 min.

2.3. Measurement of viable cell number

A 100- μL aliquot of lactic acid bacteria culture was diluted in saline and spread on an MRS agar plate. After incubation at 30°C , the number of colonies was counted, and number of viable cells in the sample was calculated.

2.4. DNA quantification

Bacterial cells were harvested from 1.5 mL of culture, washed with 1 mL saline, and pelleted by centrifugation, and then the cell pellets were suspended in 0.6 mL of DNA extraction buffer (20 mM Tris-HCl (pH 8.0), 25 mM EDTA, 250 mM NaCl, 1% SDS). The suspension was mixed with 0.5 mL zirconium oxide beads (Yasui Kikai Corporation, Osaka, Japan) and the cells were homogenized using a bead-type homogenizer (Yasui Kikai Corporation, Osaka, Japan) at 2,500 rpm for 5 min. The samples were mixed with 0.5 mL phenol/chloroform/isoamyl alcohol, shaken vigorously, and centrifuged at 12,000 rpm for 5 min. The aqueous layer was collected and washed with 0.5 mL of chloroform, followed by ethanol precipitation. Precipitates were dissolved in 100 μL Tris-EDTA buffer and treated with 50 $\mu\text{g}/\text{mL}$ RNaseA (NIPPON GENE CO., LTD, Toyama, Japan). Samples were extracted with phenol/chloroform/isoamyl alcohol followed by ethanol precipitation. The sample was dissolved in 100 μL of Tris-EDTA buffer. The amount of DNA was determined by fluorometric determination (QubitTM dsDNA HS Assay Kit, Invitrogen) based on the detection of DNA-specific fluorescence.

3. Results

3.1. Relationship between amount of DNA and viable cell number of exponentially growing cells

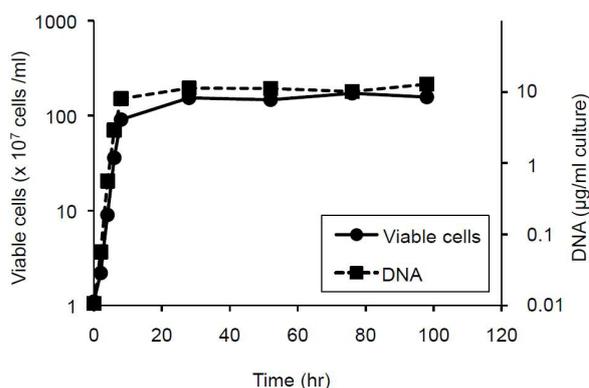


Figure 1. Time-course of the changes in colony-forming units and amount of DNA of lactic acid bacteria *Enterococcus faecalis* 0831-07. *E. faecalis* 0831-07 was cultured in MRS liquid media and sampled periodically. Viable bacterial number was calculated by counting the colonies on agar plates on which appropriately diluted samples had been spread and incubated. The amounts of DNA extracted from the bacterial samples were determined by a fluorometric method as described in the Materials and Methods section.

We cultured an *Enterococcus faecalis* strain (0831-07) in liquid medium and periodically sampled the culture over 5 days. DNA was extracted from the bacterial samples and quantified as described in the Materials and Methods section. During the exponentially growing phase (4-8 h), the amount of DNA increased along with the number of viable cells (Figure 1). At the stationary phase, in which the number of viable bacteria did not increase, the amount of DNA also did not increase (Figure 1). The ratios between the amount of DNA ($\mu\text{g}/\text{mL}\cdot\text{culture}$) and the number of viable cells ($\times 10^7$ cells/mL) were almost constant (0.059-0.088) for 4 to 100 h after inoculation. Therefore, when lactic acid bacteria cells proliferate exponentially or even at the stationary phase, it is possible to predict the number of cells on the basis of the amount of DNA.

3.2. Quantification of DNA in heat-treated lactic acid bacteria

In most cases, the lactic acid bacteria used for supplements is killed due to heat-treatment or long-term culture during the production process. Therefore, under these conditions, the total number of bacterial cells in the samples is much different from the viable number of cells. The number of viable *E. faecalis* 0831-07 cells cultured at 30°C for 28 h was 1.6×10^9 cfu/mL. After treatment at 80°C for 15 min, the number of viable cells decreased to less than 10 cfu/mL (Figure 2A). The amount of DNA extracted from the heat-treated cells was 78% that extracted from non-heat-treated samples (Figure 2B). Heat treatment at 80°C for 15 min remarkably decreased the number of viable cells, whereas the amount of DNA remained at the same level as before the heat treatment. The size of chromosomal DNA extracted from the heat-treated

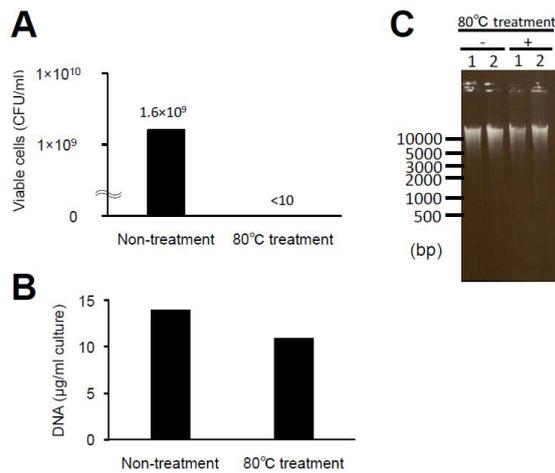


Figure 2. Changes in colony-forming units and amount of DNA after heat-treatment of *E. faecalis* 0831-07. *E. faecalis* 0831-07 was cultured in MRS liquid media for 28 h and heat-treated at 80°C for 15 min. Viable bacterial cell number per milliliter of culture media was calculated by counting the colonies on agar plates on which the samples had been spread and incubated (A). DNA was extracted from the sample and analyzed by DNA quantification (B) or agarose gel electrophoresis (C).

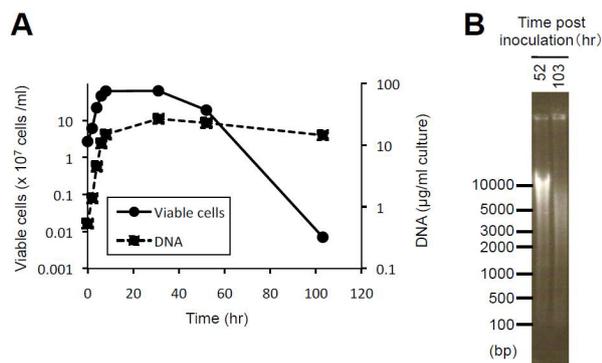


Figure 3. Time-course of the changes of colony-forming units of lactic acid bacteria *Lactobacillus paraplantarum* 11-1, amount of DNA, and degradation of DNA after long-term culture. *Lactobacillus paraplantarum* 11-1 was cultured in MRS liquid media and sampled periodically. Viable bacterial cell number was calculated by counting the colonies on agar plates on which the samples had been spread and incubated. The amount of DNA extracted from the bacterial samples was determined by a fluorometric method (A). DNA extracted from *L. paraplantarum* 11-1 was analyzed by agarose gel electrophoresis followed by staining with ethidium bromide. Photography was taken on a UV transilluminator (B).

sample was indistinguishable from that extracted from the non-heat-treated samples when analyzed by agarose gel electrophoresis (Figure 2C), suggesting that the heat treatment did not degrade the DNA.

3.3. The amount of DNA in lactic acid bacterial cells after long-term culture

L. paraplantarum 11-1 is a lactic acid bacterium with high innate immunity stimulating activity (4). This bacterium is useful for manufacturing of healthy foods. When overnight culture of *L. paraplantarum* 11-1 in stationary phase was cultured for an additional 4 days, the number of viable bacteria decreased to 1/10,000 that of the stationary phase culture (Figure 3A). Analysis by agarose gel electrophoresis revealed that the DNA in the long-term cultured *L. paraplantarum* 11-1 degraded into small fragments (Figure 3B). The amount of DNA from the long-term cultured *L. paraplantarum* 11-1, however, was 97% that of the stationary phase culture (Figure 1). This indicates that the number of the heat-killed lactic acid bacteria can be estimated by quantifying the DNA.

3.4. Estimation of total cell number of lactic acid bacteria by DNA determination

We next attempted to estimate the total number of lactic acid bacterial cells from the amount of DNA extracted from the cells (Table 1). The sizes of the *E. faecalis* and *L. paraplantarum* genomes are 3.2 and 3.1 Mbp, respectively, which means the amount of one genome of DNA is 3.3 fg and 3.2 fg, respectively (13,14) (Table 1). The concentration of the DNA harvested from overnight (28 h, stationary phase) cultures of *E. faecalis* and *L. paraplantarum* was 11 and 27 µg/mL of culture, respectively. The ratio of the total cell number calculated from the DNA amount and the viable cell count was 2.3 and 13, respectively (Table 1). The cell number calculated from the DNA amount was much larger than the viable cell number calculated from the colony count. This is probably caused by the presence of unculturable bacteria (including dead cells) in the samples. Therefore, it is important to note that the total

Table 1. Total cell number of *Enterococcus faecalis* 0831-07 and *Lactobacillus paraplantarum* #11-1 cells estimated by DNA quantification

Species	Strain	Genome size (Mbp)	Calculated amount of genome DNA/cell (fg)	Determined amount of DNA (µg/mL culture)	Cell number calculated from DNA amount (× 10 ⁹ CFU/ml culture)	Viable cell number (× 10 ⁹ CFU/mL culture)	Cell number calculated from DNA amount/Viable cell number
<i>Enterococcus faecalis</i>	0831-07	3.2	3.3	11	3.4	1.5	2.3
<i>Lactobacillus paraplantarum</i>	#11-1	3.1	3.2	27	8.3	0.63	13

The cells were cultured in MRS medium at 30°C for 28 h. The amount of genome DNA in cells was calculated from the genome sizes of *E. faecalis* and *L. paraplantarum*. The DNA amounts were determined as described in the Materials and Methods section. Molecular weight of one pair of deoxynucleotides is 616. Avogadro's constant is 6.02×10^{23} .

cell number estimated by DNA quantification is always much larger than that of viable cells.

4. Conclusion

In the present study, we showed that the amount of DNA in lactic acid bacteria was maintained at a certain level, even though almost of the cells were killed by heat-treatment or by long-term culture. This indicates that the number of lactic acid bacteria can be estimated on the basis of the amount of DNA in fermented foods or supplements.

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