*Journal of Microbiology and Biotechnology*
Vol. 6. No. 6, 461 - 467, 1996

# Screening of Bacteriocinogenic Lactic Acid Bacteria and Their Antagonistic Effects in Sausage Fermentation

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Four strains of lactic acid bacteria (LAB), that lower the pH of sausage $\leq$4.2 within 24 h of incubation at 37°C, were screened from 57 bacteriocin producing LAB which were isolated from kajamie shikhae and natural fermented sausages. The proteinaceous nature of the bacteriocin was confirmed by losing antimicrobial activity after pronase treatment. Inhibitory activity against pathogens, times of bacteriocin production and sensory tests were compared between 4 isolates and 3 commercial starters. Especially, strain NFS #8-1, screened from natural fermented sausage and identified as *Pediococcus acidilactici*, antagonized a large number of foodborne pathogens including *Listeria monocytogenes*, *Aeromonas hydrophila*, *Bacillus cereus*, *Clostridium perfringens*, *Salmonella typhimurium* and *Staphylococcus aureus*. Production of bacteriocin by strain NFS #8-1 was early in the growth phase (mid log phase) and its sensory acceptance was high. The feasibility of using strain NFS #8-1 as a starter for the production of microbiologically safe fermented sausage is envisaged.

Lactic acid bacteria (LAB) play an important role in sausage fermentation. They produce mainly lactic acid, that imparts the unique tangy flavor to the product, lowers the pH of meat, denatures the meat protein and releases the moisture uniformly. Moreover, it prevents the proliferation of health-threatening microorganisms, especially foodborne pathogens. This indicates that the use of an excellent acid producer dictates not only the quality of sausage but also influences the safety. *Listeria monocytogenes*, *Staphylococcus aureus*, and *Aeromonas hydrophila* are major spoilage bacteria of concern in sausage fermentation. Low pH, spices, nitrate and high salt concentration are essential to prevent their proliferation in sausage; however, many strains are resistant to these hostile environment. This demands the urgent introduction of more "barriers" or "hurdles" to achieve the microbial safety of sausage. In other words, acid production alone is not enough to guarantee the safety of fermented sausage (1).

Although it is not a common phenomenon, some LAB are known to produce a proteinaceous antimicrobial compound, "bacteriocin". Due to their proteinaceous nature, these LAB have a high potential as food biopreservatives (14). Many of them inhibit food poisoning, spoilage, and in some cases, pathogenic bacteria. This strongly implies that bacteriocinogenic LAB might be useful as a sausage starter when they produce lactic acid rapidly as well (5, 18).

The goal of this research is to screen bacteriocin producing LAB from fermented foods and to determine their antimicrobial activity against spoilage microorganisms and, finally, to develop them as sausage starters for use here in Korea. These could then replace the imported starter cultures in the near future.

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Key words: bacteriocin, sausage starter, antagonistic effect, foodborne pathogens

## MATERIALS AND METHODS

### Bacterial Strains and Culture Condition

The microorganisms used in this experiment were maintained at -70°C deep freezer before use. To obtain working cultures, cells were thawed and grown in appropriate broth 3 times. When anaerobic incubation was needed, an anaerobic jar (BBL) was used.

### Isolation of Bacteriocinogenic Lactic Acid Bacteria

Kajamie shikhae (fermented *Verasper variegatus* with millet) were purchased from a local market. The initial pH's of the samples ranged pH 5.2-4.3 when they were obtained. Beaker-scale natural fermented sausages were made according to the following formula: 120 g ground beef, 23 g ground fat, 1.5 g dextrose, 4.3 g NaCl, 0.001 g $NaNO_3$, 0.5 g white pepper, 0.5 g mustard, 0.2 g nutmeg, 0.9 g coriander, 0.2 g allspice, 0.5 g garlic, 0.5 g ginger, 0.5 g liquid smoke, and 20 ml water. All meat ingredients were stuffed into 5 cm cellulose casing by

hands. Sausage was incubated at 40°C for 24 h. One hundred g of sample and sterile 400 ml phosphate buffer (pH 6.5) were mixed and blended in a Stomacher Lab-Blender (U.K.) for 2 min. For the isolation of bacteriocin producers, a modified sandwich method or triple agar layer method developed by Kim *et al.* (15) was used. Samples were serially diluted and 0.1 ml was pour plated in 5 ml tempered (50°C) MRS agar, solidified, and 5 ml MRS soft agar (0.75%) was overlaid on it. Five ml MRS soft agar seeded with fresh indicators (*L. sake* KFRI 166 or *L. plantarum* NCDO 955) were finally overlaid. The number of indicator was ca. $5\times10^6$ cfu. After incubation (37°C for 24 h), the plates were flipped, *viz*, exposing the bottom layer to the top, and the colonies showing a distinct halo were carefully dug out and purified by streaking on MRS agar.

**Confirmation of Bacteriocin**

The proteinaceous nature of bacteriocin was verified by proteolytic enzyme treatment. Tentative bacteriocin producers were stabbed on MRS agar and anaerobically incubated overnight at 37°C to rule out the pseudo-effect by $H_2O_2$. One hundred μl of Pronase IV (1 mg/ml, Sigma) was loaded adjacent to the colony, and the plates were incubated at 30°C for 90 min for the diffusion of enzyme. Plates were overlaid with 5 ml MRS soft agar inoculated with $5\times10^6$ cfu *L. plantarum* NCDO 955. Confirmation of bacteriocin was decided by observing the disappearance of halo around colonies.

**Selection of Fast Acid Producers**

Screening strategy was set to select bacteriocin producing LAB that lower the pH equivalent to the pH of commercial starters within 24 h at 37°C. Bacteriocin producers were grown overnight in MRS broth, centrifuged, and added (ca. $5\times10^7$ cfu/g) to sausage formula. After incubation at 37°C for 24 h, 100 g of sausage was blended with 4× vol. of distilled water and pH was measured. The same experiment was done with 3 imported commercial starters (SPX, SL, and FF-1, Christian Hansen, Denmark). To make the conditions the same as for the bacteriocin producers, commercial starters were grown in MRS broth 3 times prior to use and the same number of cells were added to the sausage formula.

**Antilisterial and Antistaphylococcal Effect**

The antimicrobial effects of bacteriocinogenic fast-acid producers and commercial starters in sausage fermentation were separately tested against *L. monocytogenes* (hemolytic) and *S. aureus* (enterotoxin B producer). The number of inoculated starters, both selected strains and commercial starters, were ca. $5\times10^7$ cfu/g. A minimum number of *S. aureus* to cause food poisoning is ca. $5\times10^5$-$10^6$ cfu/g. To simulate the right conditions, almost an equal number (ca. $5\times10^5$ cfu/g) of *S. aureus* was inoculated. The numbers of inoculated *L. monocytogenes* was ca. $10^5$ cfu/g. Initial numbers of each pathogen in the sausage was counted and sausages were incubated at 37°C for 24 h. After incubation, 100 g of sausage was blended by adding 400 ml of phosphate buffer (pH 6.5), serially diluted and plated on selective agar. The selective media used for the enumeration of *L. monocytogenes* and coagulase positive *S. aureus* were PALCAM Listeria Selective Agar (Merck) and Vogel Johnson Agar (Difco), respectively, and they were prepared by adding appropriate supplements. Experiments were triplicated and the mean number of each pathogen was calculated. The log reduction (log initial #/log final #) of each pathogen was obtained.

**Antimicrobial Spectrum**

Selected strains and commercial starters were stabbed on MRS agar, sealed with MRS soft agar and anaerobically incubated for 24 h at 37°C. Fresh test microorganisms (ca. $5\times10^6$ cfu) were seeded into their appropriate 5 ml of soft agar and incubated for 24 h in an appropriate environment. The strength of the bacteriocinogenic activity of each culture was expressed by the diameter (mm) of the halo.

**Production of Bacteriocin in Broth and on Agar**

Both deferred and well diffusion methods (15) were used to detect bacteriocin activity in broth as well as on solid agar. *L. plantarum* NCDO 955 was used as an indicator.

**Detection of Bacteriocin during Growth**

The initiation time for bacteriocin production during growth was assayed in MRS broth. Strains of interest were inoculated in MRS broth at 1% inoculum level (ca. $5\times10^6$ cfu/ml) and were incubated at 37°C. Every 2 h, pH of the broth was measured and cell numbers in the culture broth was enumerated by surface plating. At the same time, culture broth was centrifuged (10,000 rpm) and 4× vol. of chloroform was added to supernatant culture broth. The whole mixture was vortexed for 30 sec, set at room temperature for 5 min, and residual chloroform was evaporated by placing it in a 40°C heat block. Sixty μl of chloroform treated broth was loaded in the well that was bored in a soft agar lawn of *L. plantarum* NCDO 955 to see the formation of a halo.

**Sensory Evaluation**

Sausages, that were prepared by adding selected strains and commercial starters, were tasted by 26 panelists (11 men and 15 women). The sum of scores obtained from questions regarding taste, texture, flavor, and overall acceptability were expressed.

**Identification of Selected Strains**

Selected strains were identified by their Gram staining, morphology, a catalase test, an oxidase test, and by the use of API 50 CHL kit (BioMereux, France).

## RESULTS

**Isolation of Bacteriocinogenic LAB**

A total of 57 bacteriocin producing strains were isolated: 41 strains from natural fermented sausages and 16 strains from kajamie shikhae. The proteinaceous nature of bacteriocin was confirmed by observing the disappearance of halo after Pronase treatment (data not shown).

**Selection of Fast Acid Producers**

Out of 57 bacteriocinogenic LAB, a total of 4 strains, designated as NFS #4, 6-6, 8-1, and S #3, were selected. Final pH of sausages made by the selected strains and commercial starters, and the source of isolation are listed in Table 1.

**Identification of Selected Strains**

Physiological and morphological characteristics of 4 selected strains are summarized in Table 2. They were tentatively identified as *L. plantarum* (2 strains), *L. paracasei* subsp. *paracasei* (1 strain) and *P. acidilactici* (1 strain).

**Antimicrobial Spectrum**

The bacteriocinogenic antimicrobial spectra of selected strains and commercial starters are listed in Table 3. Selected strains antagonized many test bacteria, while only a few were inhibited by commercial starters.

***In situ* Inhibition of *S. aureus* and *L. monocytogenes***

Anti-staphylococcal and -listerial effect of 4 selected strains and 3 commercial starters are listed in Table 4 and 5, respectively. Strain NFS #8-1 (*P. acidilactici*) showed the highest value in reduction (3.7 log cycle), and strain NFS #6-6 comes second (1.6 log cycle). Strain FF-1, a commercial starter, showed lowest value (0.16 log cycle). All 4 selected bacteriocinogenic strains reduced the number of *L. monocytogenes* ranging log 2.6-2.99 cycle which was similar to the data for commercial starters.

**Production of Bacteriocin on Agar or in Broth**

Not all strains produce bacteriocin in broth as well as on agar. Only strain NFS #8-1 showed bacteriocin activity under the two conditions, *i.e.*, agar and broth.

Strain NFS #8-1 started producing bacteriocin after

**Table 1.** pH's of the sausages[a] developed by selected strains and imported starters[b].

| Strains | Origin | pH |
|---|---|---|
| NFS #4 | natural fermented sausage | 4.2 |
| NFS #6-6 | natural fermented sausage | 4.24 |
| NFS #8-1 | natural fermented sausage | 4.18 |
| S #3 | natural fermented sausage | 4.5 |
| SPX | Christian Hansen | 4.23 |
| SL | Christian Hansen | 4.11 |
| FF-1 | Christian Hansen | 4.23 |

[a]Approximately $5 \times 10^7$ cfu/g of cells were inoculated and were incubated at 37°C for 24 h. [b]SPX (*Staphylococcus xylosus* + *Pediococcus pentosaceus*), SL (*Staphylococcus carnosus* + *Lactobacillus pentosus*), FF-1 (*Staphylococcus xylosus* + *Pediococcus pentosaceus*).

**Table 2.** Morphological and physiological[a] characteristics of selected strains.

| Test items | NFS #4 | NFS #6-6 | NFS #8-1 | S #3 |
|---|---|---|---|---|
| catalase | − | − | − | − |
| oxidase | − | − | − | − |
| morphology | bacillus | bacillus | coccus tetrad | bacillus |
| Gram staining | + | + | + | + |
| glycerol | − | − | − | − |
| erythritol | − | − | − | − |
| D-arabinose | − | − | − | − |
| L-arabinose | − | − | + | + |
| ribose | − | + | + | + |
| D-xylose | − | − | + | − |
| L-xylose | − | − | − | − |
| adonitol | − | − | − | − |
| β-methylxyloside | − | − | − | − |
| galactose | + | + | + | + |
| D-glucose | + | + | + | + |
| D-fructose | + | + | + | + |
| D-mannose | + | + | + | + |
| L-sorbose | − | − | − | − |
| rhamnose | − | + | − | + |
| dulcitol | − | + | − | − |
| inositol | − | − | − | − |
| mannitol | − | + | − | + |
| sorbitol | − | + | − | + |
| α-methyl-D-mannoside | − | − | − | − |
| α-methyl-D-glucoside | − | − | − | − |
| N-acetylglucosamine | + | + | + | + |
| amygdaline | + | + | +/− | + |
| arbutine | + | + | − | + |
| esculine | − | + | − | − |
| salicine | + | + | − | + |
| cellobiose | + | + | + | + |
| maltose | + | + | − | + |
| lactose | + | + | − | + |
| melibiose | − | + | − | +/− |
| saccharose | + | + | + | + |
| trehalose | − | + | − | + |
| inuline | − | − | − | − |
| melezitose | + | − | − | + |
| D-raffinose | − | + | − | − |
| amidon | − | − | − | − |
| glycogen | − | − | − | − |
| xylitol | − | − | − | − |
| β-gentibiose | + | + | +/− | + |
| D-turanose | − | + | − | + |
| D-lyxose | − | − | − | − |
| D-tagatose | + | − | + | − |
| D-fucose | − | − | − | − |
| D-arabitol | − | + | − | − |
| L-arabitol | − | − | − | − |
| gluconate | − | + | − | + |
| 2-cetogluconate | − | − | − | − |
| 5-cetogluconate | − | − | − | − |
| identification | *L. paracasei* subsp. *paracasei* | *L. plantarum* | *P. acidilactici* | *L. plantarum* |

[a]Data obtained by API 50 CHL kit. +, -, and +/-: positive, negative, and weak reaction, respectively.

**Table 3.** Antimicrobial spectra[a] and bacteriocin strength[b] of selected strains and commercial starters.

| Strains | NFS #4 | NFS #6-6 | NFS #8-1 | S #3 | SPX | SL | FF-1 |
|---|---|---|---|---|---|---|---|
| *Lc. diacetylactis* 185 | – | – | – | – | – | – | – |
| *Lb. acidophilus* 507 | – | – | – | 11 | – | – | – |
| *Lb. brevis* 353 | – | – | – | – | – | – | – |
| *Lb. confusus* 653 | 9 | – | 11 | 8 | – | – | – |
| *Lb. plantarum* 464 | – | – | – | – | – | – | – |
| *Lb. plantarum* NCDO955 | – | 17 | 22 | 20 | – | 14 | 22 |
| *Lb. reuteri* 661 | – | 12 | 15 | – | – | – | – |
| *Lb. sake* 816 | 9 | – | 11 | 8 | – | – | – |
| *Lb. sake* 166 | 10 | 11 | 23 | 10 | – | – | 18 |
| *Lb. bulgaricus* 425 | – | – | – | 11 | – | – | – |
| *Lb. casei* 196 | – | – | – | – | – | – | – |
| *Lb. delbrueckii* 149 | – | – | 11 | – | – | – | – |
| *Lb. fermentum* 164 | – | – | 11 | – | – | – | – |
| *Lb. gasseri* 658 | – | – | – | 14 | – | – | – |
| *Lb. helveticus* 659 | – | – | 12 | – | – | – | – |
| *Lb. pentosus* 481 | – | – | – | – | – | – | – |
| *Ped. pentosaceus* 167 | – | – | 16 | 8 | – | – | – |
| *Ped. acidilactici* 443 | – | 8 | – | 8 | – | – | – |
| *Ped. cerevisiae* 438 | – | – | 12 | – | – | – | – |
| *Leu. mesenteroides* 817 | – | – | 24 | – | – | – | – |
| *Pro. freudenreichii* 668 | – | – | – | – | – | – | – |
| *B. cereus* 437 | – | – | – | – | – | – | – |
| *B. coagulans* 841 | 7 | – | 24 | – | – | 11 | – |
| *Mic. luteus* 454 | 10 | 16 | 22 | 18 | – | – | – |
| *Ent. faecalis* 354 | – | – | 12 | 11 | – | – | – |
| *Cl. perfringens* 752 | – | – | 11 | – | – | – | – |
| *Lis. monocytogenes* 220 | – | 10 | 15 | – | – | – | – |
| *Stph. aureus* 219 | – | 9 | 11 | – | – | – | – |
| *Sal. typhimurium* 191 | – | – | – | – | – | – | – |
| *Aer. hydrophila* 461 | – | – | 9 | – | – | – | – |
| No. of sensitive cells | 5 | 7 | 18 | 12 | 0 | 2 | 2 |

[a]Deferred assay was used. [b]Number in the column indicate diameter (mm) of halo.

**Table 4.** Changes in log cfu/g of *Staphylococcus aureus*[a] and pH's in sausages before and after fermentation.

| Strains | Initial # | Final # | Final pH | Log reduction |
|---|---|---|---|---|
| NFS #4 | 5.7 | 4.3 | 4.25 | 1.4 |
| NFS #6-6 | 5.7 | 4.11 | 4.28 | 1.59 |
| NFS #8-1 | 5.7 | 2.0 | 4.23 | 3.7 |
| S #3 | 5.7 | 4.4 | 4.59 | 1.3 |
| SPX | 5.7 | 4.3 | 4.7 | 1.4 |
| SL | 5.7 | 4.34 | 4.16 | 1.36 |
| FF-1 | 5.7 | 5.54 | 4.28 | 0.16 |

[a]Approximately $5 \times 10^5$ cfu/g and $5 \times 10^7$ cfu/g of *Staphylococcus aureus* and starters were coinoculated, respectively.

**Table 5.** Changes in log cfu/g of *Listeria monocytogenes*[a] and pH's in sausages before and after fermentation.

| Strains | Initial # | Final # | Final pH | Log reduction |
|---|---|---|---|---|
| NFS #4 | 5.2 | 2.21 | 4.24 | 2.99 |
| NFS #6-6 | 5.2 | 2.24 | 4.26 | 2.96 |
| NFS #8-1 | 5.2 | 2.6 | 4.21 | 2.6 |
| S #3 | 5.2 | 2.48 | 4.59 | 2.72 |
| SPX | 5.2 | 2.67 | 4.65 | 2.53 |
| SL | 5.2 | 2.08 | 4.17 | 3.12 |
| FF-1 | 5.2 | 2.36 | 4.27 | 2.84 |

[a]Approximately $10^5$ cfu/g and $5 \times 10^7$ cfu/g of *Listeria monocytogenes* and starters were coinoculated, respectively.

**Table 6.** Sensory evaluation[a] of sausages produced by selected strains and imported starters.

| Items | NFS #4 | NFS #6-6 | NFS #8-1 | S #3 | SPX | SL | FF-1 |
|---|---|---|---|---|---|---|---|
| taste | 2 (126) | 6 (114) | 1 (130) | 7 ( 99) | 5 (123) | 4 (124) | 2 (126) |
| overall | 4 (480) | 5 (473) | 3 (501) | 7 (449) | 6 (455) | 1 (512) | 2 (512) |

[a]Numbers in parentheses indicate the sum of scores and the one preceding parentheses indicate the order of acceptability.

8 h incubation which corresponds to the late log phase of growth (data not shown).

**Sensory Evaluation**

There was not much difference in the quality of taste, flavor, color and overall acceptability between sausages made by the selected strains and commercial starters (Table 6).

## DISCUSSION

When we wish to isolate bacteriocin producing food grade LAB, the habitat or source of the bacteria should be food. Likewise, the key to successful experiments is the use of an appropriate indicator bacteria. A large number of bacteriocins have a narrow antibacterial spectrum. Thus, the indicator must be closely related to the bacteria that are indigenous to the foods of concern. In this respect, it appears that the choice of foods and indicator, *i.e.*, natural fermented sausage, kajamie shikhae, *L. sake* and *L. plantarum*, was pertinent to the initial isolation step.

To prevent the proliferation of health threatening microorganisms, the United States Department of Agriculture (USDA) recommends that the pH of fermented sausage should be less than pH 5.2. In this experiment, the pH's of sausages produced by commercial starters was pH < 4.2. Therefore, selection of fast acid producing strains will build more hurdles for pathogenic bacteria and enhance the safety of sausage (7).

Of particular interest, strain NFS #8-1, tentatively identified as *P. acidilactici*, exhibited the widest spectrum in which *A. hydrophila* is included. *A. hydrophila* is a Gram negative psychrotrophic opportunistic pathogenic bacteria that cause serious concern in refrigerated foods. Inhibition of Gram negative bacteria by LAB bacteriocin is a rare phenomenon. In most cases, Gram negative bacteria are insensitive to bacteriocins of LAB. However, several reports have indicated that some lactic bacteriocins inhibit a limited number of Gram negative bacteria including *A. hydrophila* (2, 3, 16, 22, 25). The pseudo effect by $H_2O_2$ was ruled out as the cells were anaerobically incubated. We do not know at this moment whether it is mediated by bacteriocin or by the synergistic effect of organic acid and sublethal injury (13) imposed on *A. hydrophila*. Additional experiments are being conducted with purified bacteriocin from strain NFS #8-1.

To the best of our knowledge, a 3.7 log cycle reduction of *S. aureus* by strain NFS #8-1 is superior to any other data reported so far (5, 17, 19, 20). The minimum inhibitory pH to control the growth of *S. aureus* is pH 4.0, however, the pH of strain NFS #8-1 (pH 4.23) is a little bit higher than minimum inhibitory pH. Moreover, the pH of strain SL, an imported starter, is even lower than that of strain NFS #8-1, but its log reduction is only 1.36 (Table 4). An antimicrobial spectrum also indicates both strains (NFS #8-1 and 6-6) inhibited *S. aureus*, while commercial starters did not. Thus, pH alone may not have been the sole factor responsible for controlling *S. aureus* and bacteriocin might have been an additional one. To confirm the direct linkage of bacteriocin, comparison with an isogenic bacteriocin negative variant should be followed.

Inhibition of bacteriocinogenic LAB against *L. monocytogenes* is not a surprise at this time because *Listeria* are taxonomically related to *Lactobacillaceae* (26). Of particular interest would be rather the degree of inhibition or inhibitory strength. Reduction of *L. monocytogenes* by selected strain, ranging ca. 2.6-2.99 log cycle, is somewhat superior to commercial starters and any other reported data (7, 9-12, 21). In order to produce a hygienically safe sausage, the initial load of microorganisms in raw meat should be either low or the starter must have a strong antimicrobial activity. Unfortunately, hygienic conditions for the slaughtering process in Korea are unlikely to be as good as in developed countries. Initial numbers of contaminants including *S. aureus* and *L. monocytogenes* on the surface of raw meat might be high. Use of selected strains, especially strain NFS #8-1, could eliminate the aforementioned problem.

There have been several reports that stated bacteriocin was produced only on solid media (4, 8, 15, 24). So was the case for strains NFS #6-1, 4 and S #3. However, the lack of bacteriocin production in broth might not cause problem in sausage fermentation, which is a solid system. It is not clear whether bacteriocin production is inductive for those strains. Saucier *et al.* (23) and Eijsink *et al.* (6) have reported that the inducing factor might account in part for differences in bacteriocin production in broth and on solid media.

Early production of bacteriocin by starters during sausage fermentation will provide their ecological niche, and also it will contribute to the safety of sausage by inhibiting food spoilage bacteria. However, a detection

method of bacteriocin during the fermentation of sausage has yet to be developed. Therefore, MRS broth, in lieu of actual meat, was employed. After 8 h incubation, which corresponds to the mid log phase, strain NFS #8-1 initiated bacteriocin production at which the pH of the broth was 4.7 (data not shown).

In conclusion, strain NFS #8-1 possess many desirable characters as a sausage starter, *i.e.*, fast acid production, early production of bacteriocin which antagonizes a large number of bacteria including foodborne pathogens, and excellent sensory acceptance. In addition, bacteriocin produced by this strain could be an excellent candidate as a future biopreservative for foods.

## Acknowledgement

The help of Dr. Seong Kwan Cha and Miss Joo Sun Lee in identification of the isolates is gratefully acknowledged. The author's sincere thanks are also extended to Dr. Bibek Ray (Animal Science Department, University of Wyoming, Laramie, Wyoming 82071, USA) for his kind donation of *Lactobacillus plantarum* NCDO955.

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**(Received September 18, 1996)**