The 3rd ISTAP, Faculty of Animal Science, Gadjah Mada University, 2002 ISBN : 979 – 97243 – 1 - 7

MICROBIOLOGICALLY FEED CONSERVATION: THE ROLE OF LACTIC ACID BACTERIA IN THE SILAGE FERMENTATION

Yimin Cai[1] and Zaenal Bachrudin[2]

Introduction

Silage is now the most common preserved cattle feed in many countries (Cai, 2001; McDonald, 1991). It is well established that lactic acid bacteria (LAB) play an important role in silage fermentation. LAB is a major component of the microbial flora which lives in various types of forage crops (Cai et al., 1994, 1998). The LAB commonly grows with other plant-associated microorganisms during silage fermentation, and they generally determine the fermentation characteristics of silage. Moist dairy farm silage is based on natural lactic acid fermentation (Cai and Kuamai, 1994). The epiphytic LAB transforms the water-soluble carbohydrates into organic acids during the ensiling process. As a result, the pH is reduced and the forage is preserved. However, due to the low numbers of LAB, especially lactobacilli present in forage, the amounts of lactic acid produced are usually not sufficient to yield significantly low pH values, which allows the growth of clostridia and results in poor quality silage (Cai, 1999, 2001). Therefore, it is necessary to use some bacterial inoculants to control microbes in silage fermentation. The addition of LAB inoculants at ensiling is intended to ensure rapid and vigorous fermentation that results in faster accumulation of lactic acid, lower pH values at earlier stages of ensiling, and inhibition of growth of some harmful bacteria (Cai et al., 1999a, 1999b; Zhang et al., 2000). In this paper, identification of LAB isolated from forage and their application for silage preparation, quality and aerobic stability of silage treated with lactobacilli, and development of a new method for preparation and conservation of tea grounds silage are described.

Identification of Pediococci Isolated from Forage by 16S Rrna Sequence Analysis and DNA-DNA Hybridization

Physiological and biochemical properties

Pediococci are often found living in association with plant material, dairy products and foods produced by LAB (Cai et al. 1998; Gashe, 1985, 1987; Lin et al., 1991, 1992) and several papers have reported pediococci as the dominant microbial

[1] National Institute of Livestock and Grassland Science, Nishinasuno, Tochigi 329-2793, Japan, cai@affrc.go.jp

[2] The Faculty of Animal Science Gadjah Mada University Yogyakarta, 55281, Indonesia bachrudin@lycos.com

population on forage crops and silage. Some isolates from forage crops and silage have been identified as *P. acidilactici* and *P. pentosaceus* (Lin et al., 1991, 1992). However, as available phenotypic procedures are difficult to assign isolates to known species because it is difficult to differentiate easily and clearly between species of LAB (Judicial, 1996; Nigatu et al., 1998; Tanasupawat et al., 1993). In order to determine the taxonomic status of pediococci, a total of forty-one strains were isolated from the forage crops. Carbohydrate fermentation patterns of *Pediococcus* species are shown in Table 1. These strains were divided into two groups on the basis of their carbohydrate fermentation patterns. All isolates were easily distinguished from the type strains of *Pediococcus* species. Characteristics of strains LA 3 and LS 5 are shown in Table 2. Strains LA 3 and LS 5 were homofermentative and Gram-positive tetrad cocci that formed L(+) and D(-) lactic acid. Strains LA 3 grew at low pH (3.5) and high temperature (50°C) conditions. Strain LS 5 did not grow below pH 4.0 or above 45 °C. These properties show that these strains belong to the genus *Pediococcus*. Strains in Group A and B were different from the type strain of *P. acidilactici* and *P. pentosaceus* in some carbohydrate fermentation patterns, and could not be identified to the species level on the basis of phenotypic characteristics.

16S rRNA sequence

The genetic interrelationships of members of the LAB have been studied extensively using 16S rRNA sequence and DNA-DNA hybridization experiments and new genera and species have been added (Collins et al., 1989, 1990, 1993). Recent results clearly indicated that the genera *Pediococcus, Enterococcus, Leuconostoc, Weissella* and *Lactococcus* exhibit a high degree of sequence similarity with each other and form a phylogenetically coherent group that is separate from other bacteria (Collins et al., 1989, 1990, 1993).

The phylogenetic tree shown in Fig. 1, was constructed from evolutionary distances by the neighbor-joining methods. Following phylogenetic analysis, representative strains LA 3 and LS 5 were placed in the clusters comprising the genus *Pediococcus*. This cluster was recovered in 100% of bootstrap analysis. *P. acidilactici* JCM 8797^T and *P. pentosaceus* JCM 5890^T were the most closely related species with the strains LA 3 and LS 5 in the phylogenetic tree, and they showed the high sequence homology value (>98%) with each other, respectively. Therefore, representative strains LA 3 and LS 5 were placed in the genus *Pediococcus* in the phylogenetic tree, confirming that these strains belong to the genus *Pediococcus* and that they are most closely related species to *P. acidilactici* or *P. pentosaceus*.

DNA-DNA hybridization

The results for DNA base composition and DNA-DNA hybridization analyses are shown in Table 3. Representative strains LA 3 and LS 5 had a G+C content 40.5 mol% and 38.6 mol%. Strains LA 3 had higher levels of DNA relatedness (>91.8%)

to the type strains of *P. acidilactici.* Strain LS 5 was 88.8% or 97.5% homologous with the type strains of *P. pentosaceus.* The DNA-DNA hybridization results demonstrated that strains LA 3 could be assigned to *P. acidilactici*, and LS 5 would be assigned to *P. pentosaceus.*

Application of Pediococci for Silage Preparation

The addition of LAB inoculants at ensiling is intended to ensure rapid and vigorous fermentation that results in faster accumulation of lactic acid, lower pH values at earlier stages of ensiling, and inhibition of growth of some pathogenic bacteria (McDonald et al., 1991). Many studies (Sebastian et al., 1996; Sharp et al., 1994) have shown the advantage of such inoculants. Generally, moist dairy farm silage is based on natural lactic acid fermentation. The epiphytic LAB transforms the water-soluble carbohydrates into organic acid in the ensiling process. As a result the pH is reduced and the forage is preserved. However, LAB, especially lactobacilli, are present in forage in very low numbers (Cai et al., 1997, 1998). When LAB fails to produce sufficient lactic acid during fermentation to reduce the pH and inhibit the growth of clostridia, the resulting silage will be of poor quality. Generally, low numbers of lactobacilli ($<10^3$ cfu g^{-1} FM) and high numbers of aerobic bacteria ($>10^5$ cfu g^{-1} FM) were present in the material and poor quality silage resulted. The factors involved in assessing fermentation quality include the chemical composition of the silage material and physiological properties of epiphytic bacteria. In our study, alfalfa and Italian ryegrass has relatively low water-soluble carbohydrate content and low numbers of lactobacilli. During silage fermentation, the lactobacilli could not produce sufficient lactic acid to inhibit the growth of harmful bacteria, the resulting silage was of poor quality. Therefore, it is necessary to use some bacteria inoculants to control microbes in silage fermentation.

The changes in temperature during silage fermentation are well known. Generally, fermentation heating in the silage was consistently correlated with microorganism development and plant respiration. The temperature rises rapidly in the early stage of the ensiling processes and reaches above 45°C. In addition the growth of some lactobacilli would be inhibited by the high temperature conditions. As shown in Table 4, in stored at 25 °C, silages inoculated with *P. acidilactici*, *P. pentosaceus* and *L. casei* were well preserved with significantly (P <0.05) reduced fermentation loss compared with the control in alfalfa and Italian ryegrass silages. The most plausible explanation lies in the physiological properties of LAB. These strains were homofermentative LAB which grew well at 25 °C and at low pH (3.5) conditions. Therefore, inoculation with these LAB may result in beneficial effects by promoting the propagation of LAB and by inhibiting the growth of clostridia and aerobic bacteria, as well as by decreasing the amount of gas-production and DM loss. On the other hand, when stored at 48°C, silage inoculated with *P. acidilactici* was also well preserved, with a significantly (P <0.05) lower pH, butyric acid, ammonia nitrogen content, gas production and DM loss, and significantly (P <0.05)

higher lactate content than the control. However, silages inoculated with *P. pentosaceus* and *L. casei* were of poor quality and were of similar quality to the control in the two kinds of silage. These results reflect the observation that strains *P. acidilactici* could grow at 50°C, but *P. pentosaceus* and *L. casei* did not grow at this temperature and may die above 45°C. Therefore, during silage fermentation, *P. acidilactici* improved silage quality and reduced fermentation loss at high temperatures. While strains *P. pentosaceus* and *L. casei* were unable to grow and ferment WSC to produce sufficient lactic acid resulting in the pH value of silage not falling to less than 4.2, and so allowing the butyric acid fermentation by clostridia to occur.

The results confirmed that *P. acidilactici* was considered suitable as potential silage inoculants, and it was more effective in improving silage quality than *P. pentosaceus* and inoculant strain *L. casei* under high temperature (48°C) conditions.

Quality and Aerobic Stability of Silage Treated with Lactobacilli

L. casei and *L. plantarum* are usually found living in association with forage crops and silage, many studies have been reported on lactobacilli presention as dominant microbial population on forage crops and farm silage (Cai and Kumai, 1994; Cai et al., 1994). The lactobacilli play a more important role in fermentation processes and effectively promoted lactic acid fermentation for a longer time than lactic acid-producing cocci, e.g. enterococci, streptococci, leuconostocs, Weissella and pediococci. Generally, when the lactobacilli reach a level of at least 10^5 cfu/g FM, silage can be well preserved (Hellings, 1985). However, as shown in Table 5, the low number of LAB ($<10^3$ cfu/g FM) and high numbers of aerobic bacteria present in these materials suggested the need to control the microbes during the silage fermentation.

The addition of LAB inoculants at ensiling is intended to ensure rapid and vigorous fermentation that results in faster accumulation of lactic acid, lower pH values at earlier stages of ensiling and improved forage conservation. Many studies have shown the advantage of such inoculants (Cai et al., 1997, 1999a, 1999b; Sebastian, 1996). Cai et al. (1999) reported that two selected strains, *Lactobacillus casei* FG 1 and *L. plantarum* FG 10 isolated from forage crops were used as additives at 1.0×10^5 cfu/g to alfalfa, Italian ryegrass and sorghum, and their effect on fermentation characteristics and aerobic deterioration of silage were studied. As shown in Table 6, these strains were homofermentative lactobacilli which are able to grow at low pH conditions. The fermentation quality and DM loss are shown in Table 7, inoculation with these LAB may result in beneficial effects by promoting the propagation of LAB and by inhibiting the growth of clostridia and aerobic bacteria, as well as by decreasing the amount of gas-production, DM loss and improving silage quality.

In some experiment, the addition of LAB impaired the aerobic stability of silage (Weinberg et al., 1993). This was in contrast to the results of Ohyama et al. (1975)

and Sebastian et al. (1996), which indicated improved aerobic stability of inoculated silages. Weinberg et al. (1993) used LAB as a silage additive for wheat, ledysarum, corn and sorghum, and reported that the some inoculated silages that spoiled upon exposure faster than the control silage. Regression analysis indicated that aerobic deterioration of inoculated silages was associated with high levels of residual WSC and lactic acid and lack of volatile fatty acids. This is in agreements with part of our results, the LAB treatment in sorghum silages contained relatively high level of WSC and lactic acid, and these silages suffered aerobic deterioration faster than that of the alfalfa and guinea grass silages. As shown in Table 8 and Table 9, the LAB treated silages were also found to be more susceptible to aerobic exposure than the respective control silages. The numbers of total yeast were high in the LAB treated silages and they increased during aerobic incubation. Most yeast strains isolated from deteriorated silage had a high tolerance to lactic acid, but low tolerance to butyric acid. These yeasts were able to grow at low pH conditions and utilize lactic acid and WSC for growth, but are inhibited by low concentrations of butyric acid and propionic acid. Results showed that the yeasts would grow vigorously after the opening of the silo and lead to the aerobic deterioration in the LAB treated silages. The relatively high level of butyric acid and propionic acid produced in the control silages could explain the great stability observed in these silages. The results confirmed that *L. casei* and *L. plantarum* improved fermentation quality but did not inhibit the growth of yeast and prevent aerobic deterioration of the silage.

Development of a New Method for Preparation and Conservation of Tea Grounds Silage

With rapid growth of population and industrialization during the last two decades, there has been a tremendous increase in the generation of domestic as well as industrial wastes. Growing public awareness about health and environmental issues urges the industries to increase efficiency and reduce wastes generated. In the beverage industry, wastes from tea grounds are of particular concern given their rapid increase in recent years. Though a small part is converted into raw compost material, wastes generated from tea grounds are generally incinerated. With the aim of achieving self-sufficiency in feeding and reducing industrial wastes, a new technique for the preparation and storage of silage from tea grounds was developed (Cai et al., 2001, 2002).

As shown in Fig.2, tea grounds contained about 10^6 (cfu/g of fresh matter) aerobic bacteria, 10^3 to 10^4 mould and yeast, but lactic acid bacteria counts were below the limit of detection (<10 cfu/g of fresh matter). Water-soluble carbohydrates were consistently at or below the detectable level (0.01g/kg of dry matter). *Lactobacillus plantarum* FG1, a strain selected from forage, and *Lactobacillus rhamnosus* SN1, from a commercial inoculant, were used along with acremonium cellulase (AUS) as additives to tea grounds for silage preparation. The quality and

chemical composition of tea grounds silage were shown in Table 10 and Table 11. After 125 days of fermentation, silages treated with AUS and strain FG1 were well preserved and exhibited low pH values and high content of lactic acid, protein, tannin, caffeine, carotene and vitamin E. The results showed that tea grounds are a potential new resource for livestock feed.

The practical application of this technology would probably mean zero emission in the tea beverage production. Clearly, the construction of large scale ensiling entities requires the involvement and investment from various parties. To achieve that goal, we are working in close cooperation with universities and private sector.

Reference

Cai, Y. 1999. Identification and characterization of *Enterococcus* species isolated from forage crops and their influence on silage fermentation. J. Dairy Sci. 82: 2466-2471.

Cai, Y. 2001. The role of lactic acid bacteria in the preparation of high fermentation quality. Glassl. Sci. 47: 527-533.

Cai, Y., N. Masuda, Y. Fujita, H. Kawamoto and S. Ando. 2001. Development of a new method for preparation and conservation of tea grounds silage. Anim. Sci. J. 72: J536-J541.

Cai, Y. and S. Kumai. 1994. The proportion of lactate isomers in farm silage and the influence of inoculation with lactic acid bacteria on the proportion of L-lactate in silage. Jpn. J. Zootech. Sci. 65:788-795.

Cai, Y., S. Kumai, J. Zhang, and Y. Benno. 1999a. Comparative studies of lactobacilli and enterococci associated with forage crops as silage inoculants. Animal. Sci. J. 70: 188-194.

Cai, Y., S. Kumai, M. Ogawa, Y. Benno, and T. Nakase 1999b. Characterization and identification of Pediococcus species isolated from forage crops and their application for silage preparation. Appl. Environ. Microbiol. 65: 2901-2906.

Cai, Y., S. Ohmomo, and S. Kumai. 1994. Distribution and lactate fermentation characteristics of lactic acid bacteria on forage crops and grasses. Journal of Japanese Society of Grassland Science 39:420-428.

Cai, Y., S. Ohmomo, M. Ogawa, and S. Kumai. 1997. Effect of NaCl-tolerant lactic acid bacteria and NaCl on the fermentation characteristics and aerobic stability of silage. J. Appl. Microbiol. 83: 307-313.

Cai, Y., Y. Benno, M. Ogawa, and S. Kumai 1999. Effect of applying lactic acid bacteria isolated from forage crops on fermentation characteristics and aerobic deterioration of silage. J. Dairy Sci. 82: 520-526.

Cai, Y., Y. Benno, M. Ogawa., S. Ohmomo, S. Kumai, and T. Nakase. 1998. Influence of *Lactobacillus* spp. from an inoculant and of *Weissella* and *Leuconostoc* spp. from forage crops on silage fermentation. Appl. Environ. Microbiol. 64: 2982-2987.

Cai, Y., Y. Fujita, T. Sato, N. Masuda, T. Nishita and M. Ogawa (2002) Preparation and conservation of barley tea grounds silage and its fermentation quality. Anim. Sci. J. 73: 283-289.

Collins, M. D., A. M. Williams, and S. Wallbanks. 1990. The phylogeny of *Aerococcus* and *Pediococcus* as determined by 16S rRNA sequence analysis: description of *Tetragenococcus* gen. nov. FEMS Microbiol. Lett. 70, 255-262. 1990.

Collins, M. D., C. Ash, J. A. Farrow, S. Wallbank, and A. M. Williams. 1989. 16S ribosomal ribonucleic acid sequence analyses of lactobacilli and related taxa. Description of *Vagococcus fluvialis* gen. nov., sp. nov. J. Appl. Bacteriol. 67: 453-460.

Collins, M. D., J. Metaxopoulos, and S. Wallbanks. 1993. Taxonomic study on some leuconostoc-like organisms from fermented sausages: Description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. J. Appl. Bacteriol. 75: 595-603.

Gashe, B. A. 1985. Involvement of lacitic acid bacteria in the fermentation of Tef (*Eragrostis tef*), an Ethiopian fermented food. J. Food. Sci. 50: 800-801.

Gashe, B. A. 1987. Kocho fermentation. J. Appl. Bacteriol. 62: 473-474.

Hellings, Ph., G. Bertin, and M. Vanbelle. 1985. Effect of lactic acid bacteria on silage fermentation. Pages 932-933 in Proc. 15th Int. Grassland Congr. Kyotosyuppan. Kyoto. Japan.

Judicial Commission. 1996. Opinion 68. Designation of strain B213c (DSM 20284) in place of strain NCDO 1859 as the type strain of *Pediococcus acidilactici* Lindner 1887. Int. J. Syst. Bacteriol. 46: 835.

Lin, C., K. K. Bolsen, B. E. Brent, and D. Y. C. Fung. 1992. Epiphytic lactic acid bacteria succession during the pre-ensiling and ensiling periods of alfalfa and maize. J. Appl. Bacteriol. 73: 375-387.

Lin, C., K. K. Bolsen, B. E. Brent, R. A. Hart, J. T. Dickerson, A. M. Feyerherm, and W. R. Aimutis. 1991. Epiphytic microflora on alfalfa and whole-plant corn. J. Dairy Sci. 75: 2484-2493.

McDonald, P., N. Henderson, and S. Heron. 1991. The Biochemistry of Silage. 2nd ed. p. 48-249. Chalcombe Publications. Great Britain.

Nigatu, A., S. Ahrne, B. A. Gashe, and G. Molin. 1998. Randomly amplified polymorphic DNA (RAPD) for discrimination of *Pediococcus pentosaceus* and *Pediococcus acidilactici*

Ohyama, Y., S. Masaki and S. Hara 1975. Factors influencing aerobic deterioration of silages and changes in chemical composition after opening silos. J. Sci. Food Agric. 26:1134-1147.

Sebastian, S., L. E. Phillip, V. Fellner, and E. S. Idziak. 1996. Comparative assessment of bacterial inoculated corn and sorghum silages. J. Anim. Sci. 71: 505-514.

Sharp, R., P. G. Hooper, and D. J. Armstrong. 1994. The digestion of grass silages produced using inoculants of lactic acid bacteria. Grass Forage Sci. 49: 42-53.

Tanasupawat, S., S. Okada, M. Kozaki, and K. Komagata. 1993. Characterization of *Pediococcus pentosaceus* and *Pediccocuus acidilactici* strains and replacement of the type strains of *P. acidilactici* with the proposed neotype DSM 20284. Request for an opinion. Int. J. Syst. Bacteriol. 43: 860-863.

Weinberg, Z. G., G. Ashbell, Y. Hen, and A. Azrieli. 1993. The effect of applying lactic acid bacteria at ensiling on the aerobic stabiling of silages. J. Appl. Bacteriol. 75:512-518.

Zhang J., Y. Cai, R. Kobayashi and S. Kumai. 2000. Characteristics of lactic acid bacteria isolated from forage crops and their effects on silage fermentation. J.