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REVIEW

Silage and Microbial Performance, Old Story but New Problems

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Abstract

It is generally recognized that inoculants (lactic acid bacteria: LAB) can be used as silage additives to improve the fermentation quality and to enhance animal performance. Especially in Japan, silage-making is a major technique to produce stored feeds and the amount of silage produced in recent years has accounted for about 70% of the amount of roughages produced in farms. Nevertheless in the Japanese environment, commercial inoculants are not always suitable for silage-making. In the present paper, at first, basic aspects of silage-making will be described in Sections 1, 2 and 3, and the problems associated with silage-making for cattle in Japan will be indicated in Sections 4 and 5. For the screening of LAB strains to develop inoculants suitable for silage-making in Japan, the construction of a model system for silage fermentation involving a solid mixed culture consisting of LAB, clostridia and coliform bacteria, referred to as "pouch method", will be presented in Section 6, and the results of screening of LAB by using the pouch method in Japan and in Thailand will be introduced in Section 7. In Section 8, attempts to improve LAB strains isolated through screening by the cell fusion method and electroporation method will be discussed. Finally, aerobic spoilage of silage which is another major problem will be outlined in Section 9.

Discipline: Animal industry
Key words: inoculant, lactic acid bacteria (LAB), silage fermentation, pouch method, aerobic spoilage

Introduction

Lactic acid bacteria (LAB) are Gram-positive, facultative anaerobic bacteria which convert more than 50% of sugar into lactic acid, and consist of 12 genera such as *Lactobacillus, Streptococcus, Lactococcus, Enterococcus, Pediococcus, Leuconostoc,* etc.[26]. In addition, it is generally recognized that the genus *Bifidobacterium* is a member of LAB. In either case, LAB have a long history of effective use for making popular foods such as fermented milk products (cheese, yoghurt), soy sauce "Shoyu", miso, fermented vegetables, etc. Consequently, LAB are considered to be useful and safe microorganisms.

In livestock farming, plant materials such as grasses and crops are transformed into milk and meat by cattle. The plant materials as a staple feed are harvested from the end of spring to autumn and preserved to be administered as feed with a constant nutritive value throughout the year. Usually, silage and hay are common stored feeds. Hay is prepared with a dry matter content of more than 80%. However, hay preparation in Japan is not common because harvest coincides with foggy, rainy and/or typhoon periods which are unsuitable for obtaining a dry matter content of more than 80%. In contrast to hay, silage is derived from materials with a dry matter content

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of 30–40%, which can be easily produced under the Japanese climatic conditions. For that reason, silage-making is a widespread technique for stored feed production in Japan. Presently in Japan, silage-making is a major technique to raise cattle and can not be considered without the performance of LAB. Namely, silage is produced through the process of natural lactic acid fermentation by LAB.

Essentially, the optimum method to raise cattle is grazing that does not compete with humans for food, and we have a long history of cattle grazing. To increase the productivity and the efficiency of production, however, intensive raising and feeding of a large amount of concentrates, instead of grazing, have been widely applied in the last two decades. In more recent years, the development of labor-saving and energy-saving methods as well as low-cost input in livestock industry and dairy farming has been promoted. Thus, there is a growing tendency for the production of roughages in farms and of high quality silage production. From the viewpoints of low cost, high quality and stable production of forage, silage-making is becoming an increasingly important technique in Japan.

Therefore, we describe the basic aspects of silage-making and the problems which have recently arisen in relation to the Japanese environment. In addition, we indicate a future tendency of silage inoculants throughout the introduction of recent results for screening of LAB.

1. What is silage ?

Silage is a kind of stored forage for the winter season in cold and temperate zones. The method of producing silage, ensiling, developed in Europe in the middle of the 19th century originated from a technique depicted in an ancient Egyptian mural. The method was introduced into Japan about 100 years ago and became widespread about 70 years ago along with the encouragement of agriculture coupled with cattle raising. Ensiling is an important technique not only for the winter season in cold and temperate zones but also for the dry season in the tropical zone to make good use of plant materials (sometimes produced in excess) with the highest nutritive value. It is based on natural fermentation whereby LAB ferment sugars to mainly lactic acid under anaerobic environments. The fermentation is performed under anaerobic conditions in a container which is called silo. In such an environment, natural lactic acid fermentation by LAB generates an acidic environment. Namely, with the maintenance of an anaerobic and acidic (about pH 4) environment, silages can be preserved over long periods of time without spoilage[13]. Homo-fermentative lactobacilli are the main LAB used for good quality silage. Particularly, *Lactobacillus plantarum* is recognized as the major microorganism in silage fermentation. Lactococci also contribute to the creation of an acidic environment at the early stage of silage fermentation and lactobacilli become predominant microorganisms for the generation of an acidic environment.

The presence of a tower type construction (silo) near a cowshed is a typical symbol of dairy farms engaged in silage-making. The tower-type silo, which is made of steel with a capacity of about 100–400 t, was introduced from the USA into mainly Hokkaido, Japan, in about 1970, because Hokkaido is the main region of large-scale dairy farming in Japan. However in recent years, many farmers have discontinued the use of tower-type silos because the maintenance of an air-tight structure, the equipment required for taking out silage, etc. are expensive. Presently in Japan, most of the tower-type silos are symbolic relics and some of them were reconstructed as restaurants for the tourists, warehouses, etc. Recently, silos in Japan have become horizontal structures appearing like trenches, bunkers and stacks. The cost to produce silage in these silos and to maintain them is lower than that of the tower-type silos. Furthermore, the roll bale wrapping (RBW) system for silage-making was also introduced into Japan in 1985. In the RBW system, plant materials which are harvested at the time when the nutrient level is highest and which are subsequently dried (dry matter content 40–50%) are arranged in a short column and wrapped with a plastic film. This system can be easily operated by one person, which reduces the manpower cost. Therefore, this system has rapidly become widespread in Japan. One roll of silage made by the RBW system weighs about 1 t and is sufficient to feed 50 head of cattle in one day. However, the fermentation quality of the silage produced by this system is sometimes inadequate due to the wrapping film used to maintain an air-tight environment. In any case, silo should be fundamentally a container to maintain anaerobic (air-tight) conditions to produce good quality silage.

2. LAB found in silages

Microbial changes in the ensiling process of silage fermentation which have been compiled in many books[25,35] are similar to the *Yamahai-moto* of sake fermentation[20]. In these microbial changes, LAB briefly evolve as follows: At the early stage of fermentation, lactococci such as *Lactococcus lactis, Enterococcus faecalis, Pediococcus acidilactici, Leuconostoc mesenteroides*, and lactobacilli such as *Lb. plantarum, Lb. cellobiosus* grow together with aerobic microbes such as yeasts,

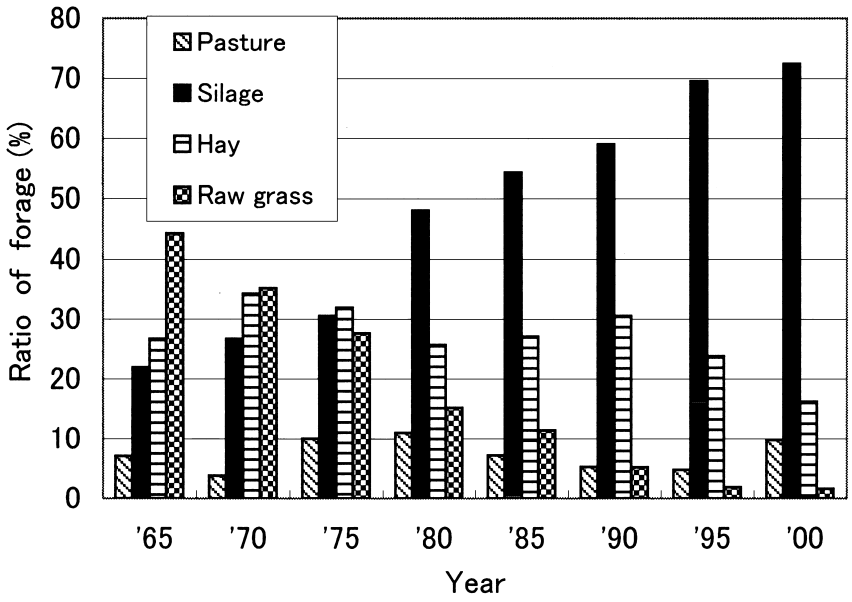

Fig. 1. Ratio of forage production in Japan

fungi and aerobic bacteria in the presence of air between the plant particles. Also, plant respiration is progressing. To promote fermentation, an anaerobic environment is generated and LAB become the predominant microbial population. The LAB flora evolves from lactococci to lactobacilli such as *Lb. plantarum, Lb. pentosus* and *Lb. curvatus*. At the last stage of fermentation, lactobacilli are the predominant microbes due to their tolerance to acidity. Nevertheless, the LAB flora in silage is diversified and depends on the properties of the plant materials, ensiling technology and silo type. The changes from lactococci to lactobacilli do not always occur, since it was even reported that lactococci maybe the predominant LAB at the last stage of silage fermentation[12]. Incidentally, no correlation was reported between the lactobacilli species present at the last stage of fermentation and the materials and/or area for silage-making.

3. Current status of silage and ensiling techniques in Japan

In 2000, the amount of roughages produced in farms in Japan was about 37 million t and silage produced among them accounted for about 70% as shown in Fig. 1. To stabilize milk and meat production of high quality by feeding good quality silage throughout the year, the total amount of silage production and its ratio in roughages have continuously increased. At the same time, it is necessary to solve the problems for the development of ensiling techniques from various food industry wastes (beer waste, tofu waste, etc.), or under unsuitable conditions using grasses with a high moisture content and in a rainy environment. Furthermore, it is also necessary to make provisions for decreasing the forage production cost in anticipation of the liberalization of the international trade of milk products and beef. Therefore, making good quality silage with low cost (inputs) should be one of the major strategies for the survival of the Japanese dairy farming and livestock industry. As part of this strategy plan, the improvement of ensiling techniques based on the development of biological additives such as LAB inoculants and enzymes (through genetic engineering) is widely expected. Obviously, LAB inoculants are also used to improve the preservation efficiency of silage and to enhance cattle performance.

The Japanese climate differs considerably from region to region because the country extends over about 2,000 km from north to south. Therefore, techniques that are suitable for the production of good quality silage in each region are recommended by the National Grassland Research Institute and Prefectural Animal Research Center[17] and are listed in Table 1. The acceleration of lactic acid fermentation by LAB and the inhibition of butyric acid fermentation by butyric acid bacteria (BAB) are essential for the production of good quality silage,

Table 1. Recommended techniques to produce good quality silage in Japan

1. Dry matter content in materials, 35–40%
2. Sugar content in materials, more than 2%
3. High packing density, rapid sealing (air-tight)
4. Storage temperature, less than 25ºC
5. Presence of homo-fermentative LAB

because the fermentation quality of silage can be evaluated based on the contents of lactic acid and butyric acid in silage. Obviously, silage containing large amounts of lactic acid and small amounts of or lacking butyric acid displays a good quality. However, the support of these techniques has not been sufficiently evaluated on a scientific basis.

(1) Dry matter content in the materials

The relationship between the organic acid content and moisture content in grass silages is shown in Fig. 2. The lactic acid content in the silages in which the moisture content ranged from 60 to 80% varied from 7.8 to 4.7% and was not appreciably different (about 40% reduced). In contrast to lactic acid, the butyric acid content in the same silages ranged from 0.1 to 1.8% (18 times increased) and the differences were considerable, namely, butyric acid fermentation was strongly inhibited by a moisture content of 60%. To avoid this problem, grasses are dried under sunshine for one or two days in the field after harvest. However, the amounts of nutrients in grasses decrease by prolonged drying under field conditions characterized by a high humidity level, which leads to the active growth of aerobic bacteria and to plant respiration. For this reason, silages should be produced from materials with high contents of nutrients even if the moisture content is high.

(2) Sugar content in the materials

The first cut of Italian ryegrass, a typical pasture grass in Japan, contains about 2.5–4.0% of sugar (glucose+fructose+fructan, moisture content 70%). This is a sufficient amount for the production of good quality silage. However, the contents of sugars in the grasses grown in the summer season and in the grasses grown under mild climatic or tropical climatic conditions are slightly less than 0.5%. Silages from these materials are not conducive to lactic acid fermentation and lead to poor acidic conditions. Forage crops and grasses cultivated in Japan are diversified because of the variety of the climatic conditions, ranging from the subarctic zone to the subtropical zone. For example, among the gramineous grasses, timothy is typical in Hokkaido, Italian ryegrass and orchard grass are typical in the zone from Honshu to Kyushu, while rhodes grass is typical in Okinawa. Guinea grass is a typical summer grass in the zone from Honshu to Okinawa. Though harvest time and/or regions where a high sugar content can be expected in these grasses are limited, good quality silages from materials with a low sugar content are always required. In this case, some techniques to supplement sugars such as the addition of molasses and/or glucose are necessary. The addition of cellulase to obtain sugars from fibers in the materials is also an effective technique[22].

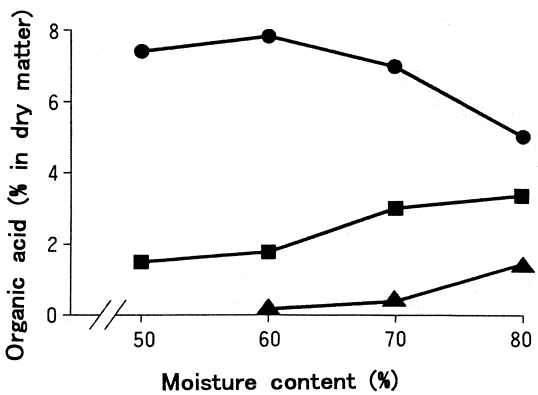

Fig. 2. Relationship between moisture content and acid content in silage
● : lactic acid, ■ : acetic acid, ▲ : butyric acid.

(3) Packing density in a silo and rapid sealing (air-tight)

The gap space between the plant particles left in a silo, when the packing density of the silage materials is low, delays the formation of an anaerobic environment even if the silo is rapidly sealed. As a result, the nutritive value of the materials decreases due to the activity of aerobic microbes and plant respiration. Therefore, high packing density of materials and rapid sealing of the silo are required to enhance anaerobic conditions and promote lactic acid fermentation by LAB.

(4) Storage temperature

The storage temperature is one of the important factors for the fermentation change from butyric acid to lactic acid. Although lactic acid fermentation is delayed at 25ºC, butyric acid fermentation is strongly inhibited, which is one of the reasons why it is very difficult to make good quality silage in the summer season in the southwestern warm district with temperatures above 30ºC for 3 consecutive months. Namely, at more than 30ºC, the growth of BAB is activated, the competitive growth of LAB against various kinds of coexistent bacteria is repressed and consequently less lactic acid is produced by LAB, which yields pH values that are not sufficiently low to inhibit the growth of BAB.

(5) Homo-fermentative LAB

The lactic acid fermentation by LAB is under the control of the homo-fermentation or hetero-fermentation pathway and the pathway is fixed for the LAB species and/or kinds of sugars consumed. The homo-fermentation converts glucose, a typical carbon source

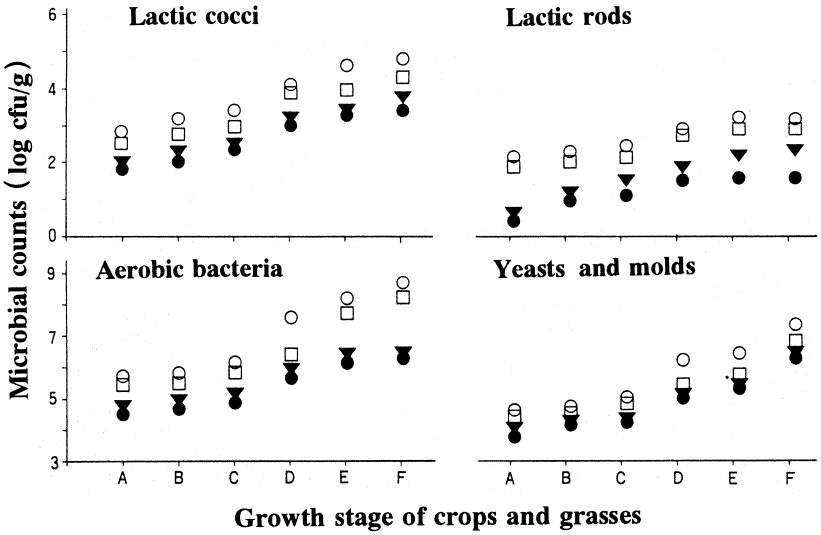

Fig. 3. Distribution of microorganisms in forage crops and grasses at different stages of growth
A: vegetative, B: internode elongation, C: heading, D: flowering, E: milk ripe, F: full ripe.
○ : Corn, □ : Sorghum, ▼ : Guinea grass, ● : Italian ryegrass.

of LAB, almost exclusively to lactic acid, whereas the hetero-fermentation converts glucose to acetic acid/ethanol and carbon dioxide gas together with lactic acid. The molar yield of lactic acid from glucose is about 100 and 50% under the homo- and hetero-fermentation pathways, respectively. The details of lactic acid fermentation by silage LAB are described in the literature[14]. The generation of carbon dioxide gas results in the loss of carbon, namely, nutrient loss from plant materials. Therefore, homo-fermentative LAB such as *Lb. plantarum* are suitable for silage fermentation. The LAB in silages originate from forage crops and grasses. However, the species and the number of LAB vary considerably with plants, fields, seasons, etc. An example of seasonal changes in the microbial flora associated with forage crops and grasses is shown in Fig. 3[1]. It is noteworthy that the number of LAB associated with forage crops and grasses is much lower than that of aerobic bacteria and eukaryotes (yeasts and molds). Furthermore, it is impossible to control the number of homo-fermentative LAB in forage crops and grasses. Therefore, starter LAB strains for efficient lactic acid fermentation have been developed as silage inoculants and are sold in the market. The use of starter LAB leads to stable production of good quality silages, regardless of the activity of natural LAB.

4. LAB inoculants and their suitability for silage-making in Japan

It is well known that starter strains (inoculants) are widely used to control microorganisms in the fermentation industries. The LAB commercial strains have been used as inoculants for silage-making since about 20 years ago, and presently the sales of LAB inoculants are increasing due to the development of a technology for active inoculant preparations[34]. Most of the inoculants are composed of *Lb. plantarum* which is a predominant homo-fermentative strain in silage and a typical strain originating from plant materials. The properties of a bacterial strain vary even within the same species. Not all the commercial LAB inoculants are always suitable for silage-making in the Japanese environment, especially imported inoculants. In addition, the price of LAB inoculants is about ¥500–800 for 1 t of grasses in the market. The total amount of silage produced in Japan in 2000 was about 25 million t. If the LAB inoculants were to be used for all the silages, the potential market of the LAB inoculants could amount to about ¥13–20 billion per year and the market is relatively large. Although the actual gross sales are about ¥3–5 billion per year, it is likely that the market will further develop.

The concept of using LAB inoculants to enhance silage fermentation and the techniques to produce good quality silage in the Japanese environment have been developed in the last 20 years[21]. However, there are conflicting views about the efficiency of LAB inoculants to improve the fermentation quality. This is due to the existence of unfavorable conditions such as the addition of LAB inoculant with less than 10^{5-6} cfu/g in fresh matter or containing less than 2% of sugars in fresh matter[20], suggesting that the usefulness of LAB inoculants may be doubtful. The basic properties of LAB inoculants suit-

able for the Japanese environment and the ability to expect added value are summarized below. The first and fourth items are refer to the first and fourth items in the Table 1, respectively.

(1) Adaptability to materials with a high moisture content

The moisture content of grasses immediately after harvest which is about 90%, decreased to about 60–70% after drying for one or two days in the field. A moisture content of 60–65% is favorable to make good quality silage. However, the harvest season of grasses with the highest nutritive value starts in May in Kyushu and in July in Hokkaido, which coincides with the beginning of the rainy season from Kyushu to Honshu and the heavy fog season in Hokkaido. For these reasons, in some cases, silages are produced from grasses with a moisture content of 80–85% due to the difficulty to expect two or three successive sunny days. Therefore, a LAB strain which accelerates lactic acid fermentation and competitively inhibits butyric acid fermentation under moisture contents of 80–85% could be suitable for making good quality silage in Japan.

(2) Adaptability to the environmental temperature

Feeding of good quality silage throughout the year is an important factor to secure a high milking amount and a good milk quality. However, it is very difficult to make good quality silage during the summer season in the southwestern warm district of Japan, where temperatures are generally above 30ºC for 3 consecutive months. The difficulty seems to be caused by the fact that the competitive growth of native LAB against other microbes is not vigorous enough at such high temperatures. Thus, thermotolerant LAB strains may enable to address the problem mentioned above. On the other hand, silages produced in the autumn season in Hokkaido hardly undergo fermentation and are associated with a low intake. The LAB strains that induce fermentation at low temperatures, could be used under such conditions in Hokkaido. As mentioned above, LAB strains adaptable to the functions of the environmental temperature associated with seasonal and regional changes are required for silage-making in Japan.

(3) Acidity tolerance

Lactobacilli generally display a higher tolerance to low pH than lactococci. Most of the LAB inoculants are composed of *Lb. plantarum* which is a typical *Lactobacillus* species originally isolated from plant materials. The minimum pH for the growth of *Lb. plantarum* is about 4, but the microbes do not survive at less than pH 4. If a LAB strain could survive at about pH 3.5 and maintain a high concentration of living cells, it would competitively inhibit the growth of other microbes and thereby contribute to silage stability.

(4) Bacteriocin production

The homo-fermentative LAB strains are suitable for silage fermentation as they are associated with the nutritive value of silage. The growth of hetero-fermentative LAB strains in silage materials leads to the loss of nutrients and the generation of carbon dioxide. The growth of butyric acid bacteria, clostridia, also leads to nutrient loss. An attempt to inhibit the growth of the pathogenic bacterium, *Listeria monocytogenes*, was challenged by the inoculation of LAB[6]. Bacteriocin-producing LAB are able to inhibit the growth of these spoiling bacteria and pathogenic bacteria, and their application as silage inoculants could be beneficial.

(5) Phage tolerance

It is well known that bacteria are suddenly killed by phage infections in a fermentation tank. Recently, the relationship between phage infection of silage LAB and the fermentation quality has been studied[8,30]. In silages with a moisture content of more than 70%, phages were detected at a high frequency, as shown in Fig. 4, suggesting that phage infection is one of the factors responsible for silage spoilage. A plasmid, pLKS (2.0 Kb), of *Lb. plantarum* NGRI 0101 isolated from silage was found to protect against phage infection, hence the mechanism of phage tolerance in silage LAB was elucidated[3]. Strains to which the phage resistance plasmid had been introduced could be used as silage inoculants, especially when grasses with a high moisture content are used.

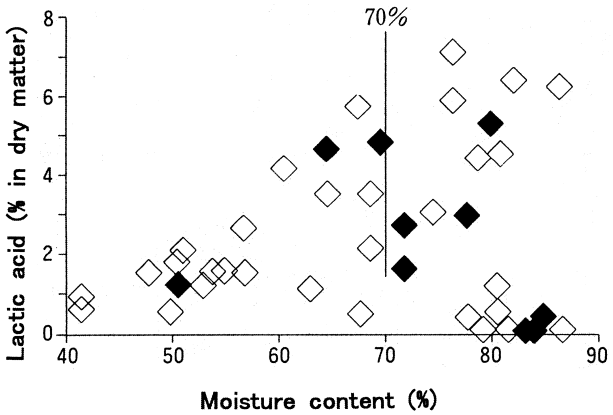

Fig. 4. Plots of phage-positive and -negative silages with moisture content (abscissa) against content of lactic acid (ordinate)

◆ : phage-positive silages,
◇ : phage-negative silages.

(6) Properties as probiotics

Diarrhea in calves is a very serious problem for the farmers due to the delay in calf growth and unexpected death. Though antibiotics are generally used for the treatment, probiotics could also be used. Probiotics are live microbial feed supplements which have a beneficial effect on the host animal by improving its intestinal microbial balance, and some LAB strains act as typical probiotics. For example, the dosage of a *Lb. acidophilus* strain isolated from the digestive tract of cattle suppressed and/or alleviated outbreaks of diarrhea in calves[27]. Silage LAB that could prevent and/or could be used for treating the diarrhea have a potential to reduce calf diarrhea outbreaks.

5. Characteristics of the LAB additive market in Japan

It is well known that silage-making using homofermentative lactobacilli yields a silage with a high nutritive value. Moreover, the use of LAB as additives could be beneficial if the characteristics described in Section 4 were available. Presently in Japan, more than 40 kinds of LAB additives, including imported ones, are in the market for silage-making, and it is usually very difficult to identify the properties of LAB additives from the catalogues. Therefore, the properties of two typical commercial lactobacilli additives were examined, with emphasis placed on the environmental temperature. Fig. 5 shows the relationship between the pH of silage and the storage temperature. According to silage standards[13], the pH of good quality silages is about pH 4, and it is obvious that the quality of silage A (control: glucose was not added) is of medium grade and would not be affected by the storage temperature. While the quality of silage B was improved by the addition of 1% of glucose, compared with silage A, the quality deteriorated in proportion to the increase of the storage temperature. While silage D containing LAB additive B together with 1% of glucose showed the highest quality during storage at 20ºC, the quality deteriorated in proportion to the increase of the storage temperature and became almost similar to that of the control, silage A, when stored at 30ºC. In contrast to silage D, silage C containing LAB additive A together with 1% of glucose exhibited a good quality without any effect of the storage temperature on the fermentation quality. These results suggest that additive A was more suitable for silage-making than additive B at high temperatures. Namely, the choice of inoculants is very important to obtain good results, and the selection of an unsuitable inoculant may result in a poor quality silage as well as waste of investment in silage-making.

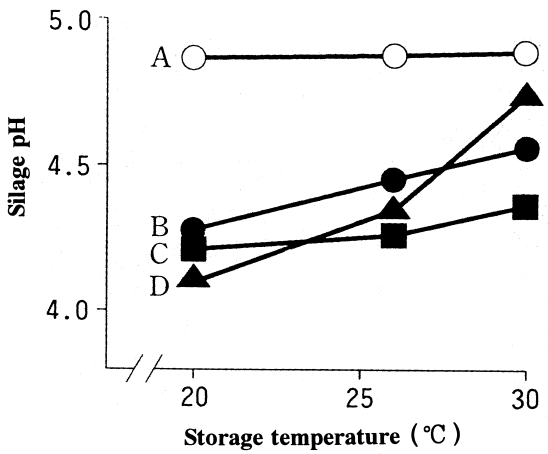

Fig. 5. Effect of environmental temperature on ensiling with some additives

○ : silage without additive, ● : silage with 1% glucose, ■ : silage with inoculant A and 1% glucose, ▲ : silage with inoculant B and 1% glucose.

Under these circumstances, it is necessary to develop LAB additives adaptable to the variations in the weather conditions in Japan and to construct a model system for silage fermentation to screen LAB strains.

6. Construction of a model system for silage fermentation

Culture conditions such as medium composition, inoculum size, culture temperature, aeration, as well as types of microorganisms are essential parameters to be considered for microbial growth. Silage fermentation is a kind of culture of microorganisms, where the culture conditions should be considered. Nevertheless, studies conducted to screen LAB strains for silage-making hardly consider the microbiological culture conditions. In most of the screenings, only one index of lactic acid productivity in liquid culture is generally used. Therefore, strains screened under such conditions are often ineffective to improve the fermentation quality in actual silage-making. In other words, such screening methods in liquid culture do not reflect the silage fermentation of mixed solid culture. On the other hand, the contents of nutrients and microbial flora in crops and grasses are usually different even in the case of the same species because of the differences in the growth environment and fertilizer application due to cultivation on different fields. Consequently, data from experiments on silage production can not be compared to each other and reproducibility of the results can not be expected.

Therefore, it is necessary to construct a model system for silage fermentation with a constant medium composition and microbial flora. This system should reflect

the characteristics of silage fermentation which consists of a solid mixed and non-sterilized culture[31].

(1) Medium

The silage material (medium) should be selected carefully so as to always display constant nutrient contents for various experiments. Alfalfa is a leguminous plant with a high protein content and a large amount of dried alfalfa is fed to cattle. However, the cultivation zone in Japan is limited as alfalfa does not grow well on acidic soils, which cover large areas in Japan. Hence, a large amount of dried alfalfa (alfalfa hay cubes: AHC) is imported from the United States. The same lot of AHC from the United States shows almost constant nutrient contents because the AHC lots are harvested from the same field. Therefore, the same lot of AHC (after powdering: mesh 2 mm, moisture content of 13.4%, glucose + fructose content of 1.8% on a dry matter basis, buffering activity of 72 mg/g dry weight, pH 5.9 in water suspension) was used as a medium (AHC medium) after sterilization with ethylene oxide gas, adjustment of the sugar content by the addition of glucose and adjustment of the moisture content by the addition of water. The sugar content of the original medium was 0.5% when the moisture content was adjusted to 75%.

(2) Silo

Until now, test tubes or wide-mouth glass bottles had been used as silos for ensiling on a laboratory scale[14]. However, it is very difficult to prepare a sufficient number of experimental lots due to the lack of working space. Therefore, bags made from air-tight plastic film were used as a silo instead of test tubes or glass bottles. The plastic film can be easily processed to a free-sized bag by using a heat sealer machine available in the market, and the oxygen permeability is about 1 mL/m^2/day. The AHC medium was put into this bag and incubated after sealing with a vacuum sealer.

(3) Microbial flora

The microbial flora of the model system for silage fermentation consisted of typical silage microbes inoculated onto a gas-sterilized AHC medium. LAB as lactic acid producers and BAB as butyric acid producers were selected as typical silage microbes because the fermentation quality of silage is evaluated based on the lactic acid and butyric acid contents. Although the performance of coliform bacteria (CFB) in silage has not been determined, they are considered to predominate in silage. Therefore, *Lb. plantarum* LP-1 (LAB) as a beneficial microbe, *Clostridium butyricum* HA-1 (BAB) as a harmful microbe and *Klebsiella pheumoniae* G-1 (CFB) as a key microbe, were isolated from silage and were selected as typical silage microbes. The model system for silage fermentation obtained by inoculating these 3 microbes which consists of a solid mixed culture system was designated as "pouch method". Characteristics of the pouch method are summarized in Table 2, compared with the glass bottle method.

(4) Conditions for butyric acid fermentation in the pouch method

The ability to inhibit butyric acid fermentation under conditions suitable for the growth of BAB should be evaluated for a silage inoculant and was defined as the property suitable for silage fermentation. Therefore, the growth properties in a mixed culture of LAB, BAB and CFB using the pouch method, especially the conditions for the initiation of butyric acid fermentation, were studied[28].

① Moisture content of AHC medium: Butyric acid was detected in the culture of BAB using the AHC medium with a moisture content of 85%, while in the AHC medium with a moisture content of 75%, butyric acid was not detected. Namely, the growth of clostridia was inhibited at a low water activity (Aw 0.94)[15]. Therefore, the effect of the inoculum size of BAB and sugar content in the AHC medium on the production of butyric acid was examined by using the AHC medium with a fixed moisture content of 85%.

② Inoculum size: Table 3 shows the relationship between the inoculum size of 3 typical silage microbes

Table 2. Comparison of properties between 2 silage-making methods on a laboratory scale

Items	Glass bottle method	Pouch method
Grass material	Raw	Dried (AHC)
Material packing	Hard work	Easy work
Time required for packing	Slow	Rapid
Fermentation period	About 1 month	About 1 week
Reproducibility of experiments	Impossible	Possible
Sterilization of materials	Impossible	Possible
Control of microbial flora	Impossible	Possible

Table 3. Relationship between inoculum size of silage microbes and organic acids produced

Inoculum size (cfu/g)			Organic acids produced (% in silage)		
CFB	LAB	BAB	Lactic acid	Acetic acid	Butyric acid
0	5×10^3	1×10^6	1.31	0.42	0
1×10^7	5×10^3	1×10^6	0.06	0.35	0.18
1×10^7	5×10^4	1×10^6	0.09	0.39	0.27
1×10^7	5×10^5	1×10^6	0.19	0.47	0.44
1×10^7	5×10^6	1×10^6	1.06	0.41	0
1×10^7	5×10^7	1×10^6	1.52	0.78	0

In AHC medium (moisture content 85%, 1% of glucose added), each pouch was cultured at 37ºC for 4 days, anaerobically.

and the organic acids produced. In the case of a mixed culture of LAB and BAB, no butyric acid was detected even if the inoculum size of BAB became 10^3 times larger than that of LAB. Nevertheless, butyric acid was clearly detected after the inoculation of CFB to the mixed culture of LAB and BAB. When the inoculum size of LAB increased to the same level as that of BAB in the mixed culture, butyric acid was hardly detected. Therefore, it became clear that butyric acid production always occurred when the inoculum size of LAB was less than one-tenth of that of BAB.

③ Sugar content: When 1% of glucose (actual sugar concentration 1.5%) was added to the mixed culture, the number of LAB colonies increased from 10^7 to 10^9 cfu/g, the number of CFB colonies decreased from 10^7 to 10^2 cfu/g and butyric acid was hardly detected. On the contrary, the colony counts of LAB and CFB remained at the same level as the inoculum size and butyric acid was clearly detected when the AHC medium without glucose addition (actual sugar concentration 0.5%) was used for the mixed culture. Namely, there was no inhibition of butyric acid production when the AHC medium containing less than 1.5% of sugar was used. From these results, it was suggested that the LAB strain used inhibited butyric acid production and was a suitable strain for silage-making in high moisture environments when the strain was inoculated with 10^5 cfu/g in AHC medium with a moisture content of 85%, addition of 1% of glucose and inoculation of 10^7 and 10^6 cfu/g CFB and BAB, respectively.

In addition, during silage fermentation, CFB appeared to utilize sugar competitively with other microbes in silage, with a decrease in the amount of sugar available to LAB, causing a pH drop, which in turn created a favorable environment for the growth of BAB. The rapid production of lactic acid in the presence of 1.5% of sugar appears to be necessary because the production of up to 0.6% of lactic acid inhibits the growth of CFB and BAB.

7. Screening of LAB strains suitable for silage-making

LAB strains used as inoculants for silage-making in Japan were screened by applying the pouch method. Suitability for materials with a high moisture content and tolerance to high temperature were the main criteria for this screening. LAB strains were isolated from grass silages produced in Okinawa prefecture, Okinawa main island and Ishigaki island[29,32] and grass silage prepared in Thailand[19].

Table 4. Lactic acid production and specific growth rate of typical isolates

Strain	Specific growth	Lactic acid produced in silage (%)	
(NGRI No.)	rate (h^{-1})	after 6 h	after 168 h
Lactobacillus rhamnosus 0110	0.43	0.01	2.32
Lactobacillus pentosus 0506	0.71	0.23	2.00
Lactobacillus plantarum 0529	0.67	0.23	1.74
Lactobacillus plantarum LP-1	0.53	0.09	2.02

In AHC medium (moisture content 85%, 2% of glucose added), each pouch was cultured at 37ºC, anaerobically.

(1) LAB strains from Okinawa prefecture, Japan

Table 4 shows typical isolates selected at 42ºC for use in the southwestern warm region of Japan. Strain NGRI 0110 produced a larger amount of lactic acid than *Lb. plantarum* LP-1 which was selected as a standard LAB strain by the pouch method, while the former strain exhibited a lower specific growth rate than the latter strain. On the other hand, the amount of lactic acid produced by strains NGRI 0506 and 0529 was lower than that by the LP-1 strain, while the former strains displayed a higher specific growth rate than the latter strain. Fig. 6 shows the effect of lactic acid initially present in the medium on the yield of lactic acid (YLA) in relation to sugar consumption[32]. In strain NGRI 0110, the YLA was maintained in a culture containing 1.2% of lactic acid while in strains NGRI 0205, 0524 and 0529 the YLA decreased to less than 10% in the same culture in a non-competitive way[18]. Strain NGRI 0110 was tolerant to lactic acid and was expected to inhibit the growth of other bacteria due to the maintenance of the cell number at a high level during storage for half a year. Furthermore, the effect of lactic acid production on the multiplication was observed in the mixed culture of acid-tolerant strains with strains with a large specific growth rate. Namely, in the mixed culture of strain NGRI 0110 and strain NGRI 0506 or 0529, the YLA increased 13–23% in comparison with that in each single culture, as shown in Table 5.

Table 6 shows the fermentation quality of Guinea grass silage inoculated with strain NGRI 0110 which showed suitable properties based on the pouch method. Silages inoculated with the LAB strain did not contain butyric acid, showed a pH 4.1–4.2 and a good quality, while silages without LAB inoculation contained butyric acid and were of medium grade. Namely, the inoculation of LAB clearly improved the fermentation quality. Especially, silage inoculated with strain NGRI 0110 contained a small amount of acetic acid and volatile basic nitrogen and its quality was superior to that of the silage inoculated with a commercial strain which was similar to the strain with inoculant A shown in Fig. 5. Therefore, the screening system using the pouch method reflected actual silage-making and enabled to select a strain for practical use.

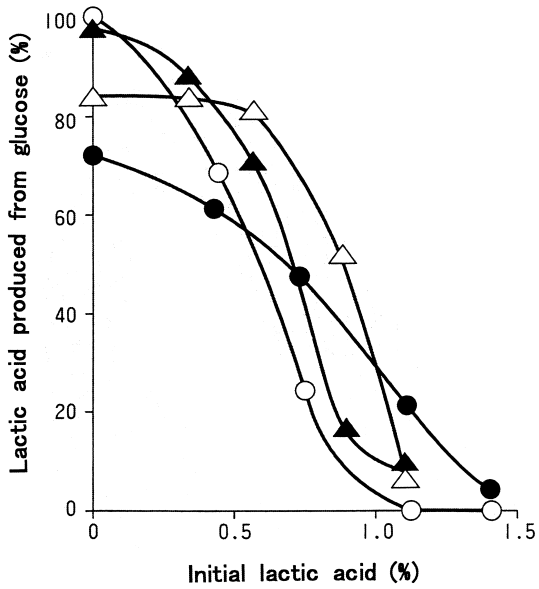

Fig. 6. Effect of initial lactic acid concentration on the yield of lactic acid in relation to the addition of glucose
△ : *Lactobacillus plantarum* NGRI 0529,
○ : *Lactobacillus plantarum* NGRI 0205,
● : *Lactobacillus rhamnosus* NGRI 0110,
▲ : *Lactobacillus pentosus* NGRI 0524.

In addition, strains NGRI 0110 and 0529 were identical with *Lb. rhamnosus* and *Lb. plantarum*, respectively, based on the type of cell wall peptidoglucan, sugar fermentation, growth temperature, and optical isomer of lactic acid produced. This identification was supported by the homology analysis of the 16SrRNA[16].

(2) LAB strains from Thailand

Recently in Thailand, the consumption of fresh milk

Table 5. Lactic acid production of typical isolates

Strain (NGRI No.)	pH	Lactic acid produced in silage (%)	Lactate/Sugar (%)
Lactobacillus rhamnosus 0110	3.8	1.72	66.2
Lactobacillus pentosus 0506	3.9	1.53	78.5
Lactobacillus plantarum 0529	3.9	1.75	81.0
Lactobacillus plantarum 0320	3.9	1.52	77.8
0110 + 0506	3.8	1.95	80.7
0110 + 0529	3.7	2.11	83.1
0110 + 0320	3.8	2.03	83.2

In AHC medium (moisture content 80%, 2% of glucose added), each pouch was cultured at 37ºC for 14 days, anaerobically.

Table 6. Fermentation quality of Guinea grass silage

Items	No inoculant	Inoculated with	
		0110 strain	Marketed strain
		% in silage	
Lactic acid content	0.56	1.66	1.67
Acetic acid content	0.50	0.17	0.50
Butyric acid content	0.03	0	0
Volatile basic nitrogen	0.09	0.04	0.07
pH in silage	5.30	4.10	4.21

Grasses were wilted for one day (moisture content about 70%), cut into segments 2–3 cm long treated with 2% glucose, packed in drum can silos and kept outdoor for 30 days.

has rapidly increased and the production of fresh milk can not satisfy the demand. Also, fresh milk production decreases by about 10–20% in the dry season due to the difficulty in supplying good quality roughage. Producing good quality silage for the dry season should be one of the possible ways to address the problem. Table 7 shows the acid production by typical isolates at various culture temperatures. These isolates exhibited a fermentation change from butyric acid to lactic acid in proportion to the increase of the culture temperature from 37 to 45ºC. Some strains induced lactic acid fermentation at 52ºC. These thermotolerant strains are suitable for areas with a typical continental climate with temperatures of about 40ºC for 4–5 consecutive months. Furthermore, strain NGRI 1007 (I-7) was resistant to the inhibition at 45–52ºC and more than 80 and 30% of lactic acid produced from glucose persisted with the initial presence of 0.5 and 1.0% of lactic acid, respectively, compared with the absence of lactic acid. The tolerance to lactic acid is an important characteristic for the inoculant strain and the strain 1007 could become a suitable material for silage inoculant in tropical regions.

In addition, strain NGRI 1007 was identical with *Streptococcus salivarius* ssp. *thermophilus*, based on the type of cell wall peptidoglucan, sugar fermentation, growth temperature and optical isomer of lactic acid produced. This identification was supported by the homology analysis of 16SrRNA[16].

8. Improvement of LAB inoculants

For the development of silage inoculants adapted to the Japanese environment characterized by a high humidity and high temperature for 3 consecutive months, LAB strains were isolated from silages produced in Okinawa prefecture, Japan. Among them, the use of strain NGRI 0529 which has a large specific growth rate resulted in a high production of lactic acid when it was cultured with the acid- tolerant strain NGRI 0110, as shown in Table 5. However, mixed inoculation is not a stable method since it is influenced by the inoculum size, cell activity, etc. Therefore, it was necessary to improve the LAB strains to obtain more suitable inoculant strains.

(1) Cell fusion method

Characteristics such as specific growth rate, acid tolerance, etc. are controlled by multiple genes. Therefore, it is generally considered that cell fusion could be a

Table 7. Effect of culture temperature on acid production by typical isolates from Thailand

(%)

Strain	at 37ºC		at 45ºC		at 52ºC	
(NGRI No.)	LAP[a)]	BAP[b)]	LAP[a)]	BAP[b)]	LAP[a)]	BAP[b)]
Streptococcus salivarius thermophilus 1007	0	0.11	0.26	0	0.34	0
Streptococcus salivarius thermophilus 1008	0	0.09	0.28	0	0.36	0
Lactobacillus sp. 1015	0	0.12	0.36	0	0.34	0
Pediococcus acidilactici 2009	0	0.12	0.34	0	0.31	0
Pediococcus sp. 2015	0.16	0	0.33	0	0	0

a): Lactic acid produced in silage, b): Butyric acid produced in silage.
In AHC medium (moisture content 80%, 2% of glucose added), each pouch was cultured at 37, 45 and 52ºC for 14 days, anaerobically.

method suitable for introducing such traits. However, there are few studies on cell fusion of lactobacilli and no reports on cell fusion among different lactobacilli species are available, mainly due to the difficulty in protoplast regeneration. An attempt was made to regenerate protoplasts derived from strains NGRI 0110 and 0529 by treatment with a mixture of 300 μg/mL of lysozyme and 10 to 30 μg/mL of N-acetylmuramidase[33]. The type of coagulants and their concentration in the medium strongly influenced the regeneration frequency of the protoplasts. For example, the protoplasts of both strains were regenerated at a high frequency (40–95%) in the medium containing a high concentration of gelatin (300 g/L) and polyvinylpyrrolidone (100 g/L).

(2) Electroporation method

Compared with the cell fusion method, electroporation is a direct and rapid method of transformation. The α-amylase gene from *Lb. amylovorus* was introduced into *Lb. plantarum* for silage inoculant and the the transformant expressed the activity[7]. However, the transformation efficiency was generally very low. Therefore, the method of electroporation with plasmid vectors was tested for application to silage lactobacilli[5]. As a result, it was found that the transformation efficiency depended on the electric field strength, time constant, polyethyleneglycol molecular weight in the electroporation buffer and the incubation temperature after exposure of pulses. Transformation of *Lb. pentosus* NGRI 0225 resulted in a maximum transformation efficiency of 5.7×10^5 transformants/μg DNA of plasmid vector pGK12.

Since phage infection was one of the reasons for the deterioration of silage quality, various kinds of phages were isolated from silages. Furthermore, it was found that phage resistance of *Lb. plantarum* NGRI 0101 could be coded on a 2 Kb plasmid pLKS[3]. The phenotype of phage resistance will be introduced to phage-sensitive strains. Enterocin ON-157, which was a bacteriocin produced by *Ec. faecium* NIAI 157 isolated from a silage produced in Japan, was characterized and its production was coded on a 49 Kb plasmid[23]. Moreover, enterocin SE-K4, which was a bacteriocin produced by a thermophilic strain *Ec. faecalis* K-4 isolated from a silage produced in Thailand, was characterized and its production was coded on a 37 Kb plasmid[2,4]. This genetic information coded on plasmids will be useful for the improvement of LAB inoculants. Although gene expression studies on silage LAB have been recently conducted, it is expected that in the near future LAB strains with multiple properties will be developed.

9. Aerobic spoilage

When the silo is opened for feeding or the anaerobic environment of the silo is replaced by an aerobic environment, silage undergoes changes sometimes referred to as "secondary fermentation", due mainly to the rapid growth of yeasts in the aerobic environment and abundant energy source. Some kinds of yeasts in silage survive under acidic conditions even if lactic acid fermentation proceeds, and they can use lactic acid as an energy source in an aerobic environment[9]. By the consumption of lactic acid, the pH of silage increases to the neutral range and the environment becomes favorable for the growth of various kinds of aerobic bacteria. As a result, not only the nutrient loss from silage but also a reduction of silage intake by cattle occurs. It is well known that some kinds of yeast strains produce killer proteins to inhibit the growth of other yeast strains[24]. Therefore, a killer yeast strain, *Kluyveromyces lactis,* with a wide killing spectrum was used to prevent aerobic spoilage of silage by wild yeasts. This yeast displayed a killing activity against wild yeasts, but assimilated lactic acid with possible silage spoilage. Therefore, the gene coding the key enzyme for gluconeogenesis (phosphoenol- pyruvate carboxykinase) was disrupted to construct a killer strain defective in the ability to grow on lactic acid as a sole carbon source[10]. It is concluded that this genetically modified yeast significantly prolonged the aerobic stability of silages in the model system and actually reduced aerobic spoilage of corn silage in laboratory-scale experiments[11]. However, it will be necessary to develop guidelines for the use of genetically modified microorganisms in the field.

Concluding remarks

Although technologies for the control of microorganisms applied in Japan have reached a high level on a world basis, the application for silage-making started only about 30 years ago. Presently, genetic engineering technology is also being applied for the inoculants of silage-making and the improvement of inoculants for silage-making is making rapid progress. Even though silage-making has a long history of about one hundred years, microbiological aspects require further studies.

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