

## 学位論文の要旨

### Abstract of Thesis

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学位論文題目 Title of Thesis (学位論文題目が英語の場合は和訳を付記)

Culture-dependent and culture-independent analysis of microbiota associated with anaerobic storage and aerobic deterioration of alfalfa silage

(培養法および非培養法によるアルファルファサイレージの嫌氣的貯蔵および好氣的変敗に関わる微生物フローラの解析)

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Forages are more often preserved as silage than hay in temperate and cold regions. Understanding of silage microbiota is important for better management and hygiene control. In addition to lactic acid bacteria (LAB), species of the Enterobacteriaceae family, *Bacillus* sp., *Clostridium* sp., and some other microorganisms may be involved in the anaerobic storage and aerobic spoilage process. Modern ensiling typically utilizes a sugar-rich crop, wilted crop, and homo-fermentative LAB inoculant; hence, concentrations of lactic acid and remaining sugars at silo opening may be greater than the past, increasing the risk of aerobic spoilage by supplying sufficient substrates for growth of the microorganisms. It is difficult to simultaneously pursue proper preservation in a silo and high stability after silo opening.

Differences exist between crop species in terms of susceptibility or resistance to aerobic deterioration. Corn silage is known to spoil easily in the presence of air, and significant heating will often be seen 1–2 days after silo opening. Grass silage occasionally shows a resistance to aerobic deterioration, but such stability is not promising and hard to predict, and accordingly the aerobic stability is difficult to control without the use of additives. In contrast, alfalfa silage can remain unheated for more than a week, and does not spoil despite lactic acid predomination over the fermentation. Because published results indicated that ensiling fermentation is necessary for inhibition of spoilage, factors and substances involved in the anaerobic storage and aerobic deterioration of alfalfa silage are worth examining.

In this study, both laboratory-scale experiments and practical survey were conducted to understand microbiota of alfalfa silage. Plate-culture and culture-independent denaturing gradient gel electrophoresis analysis was employed to clarify the association of conventional and non-conventional bacteria.

In the first step, two laboratory-scale experiments were made using direct-cut and wilted alfalfa silage. In experiment 1, silage was prepared with and without addition of molasses (M),

homo-fermentative LAB inoculant (*Lactobacillus rhamnosus*, L), or M+L, and aerobic stability was determined 60 days after ensiling. Additives were used to attenuate aerobic stability after silo opening. In experiment 2, only M was used to modify fermentation and aerobic stability, and silage was opened 5, 10, and 60 days after packaging. Both direct-cut and wilted silages had lactic acid as the predominant fermentation product, whereas acetic acid content in direct-cut silage increased due to long storage. Lactic acid fermentation was enhanced by M and L treatment. No spoilage was seen in 60-day silage regardless of additive use. Short-stored wilted silage deteriorated after exposure to air for 7 days, but this deterioration did not occur in M-treated wilted silage. As LAB species involved in the high aerobic stability of alfalfa silage, *Lactobacillus buchneri* and *Weissella cibaria* (experiment 1) and *Lactobacillus sakei* and *Lactobacillus garvieae* (experiment 2) were demonstrated. However, alfalfa silage could resist deterioration even without inhabitation of these LAB species.

Secondly, two follow-up laboratory-scale experiments were performed. Wilted alfalfa silage was prepared with and without M addition, and aerobic spoilage test was conducted 5, 10, and 60 day after ensiling. To understand details of the changes in fermentation products and microbiota, frequent sampling was made 1, 3, 5 and 7 days after silo opening. Untreated wilted silage resisted deterioration for at least 5 days, even with marginal amount of lactic and acetic acids at silo opening. Great improvement of aerobic stability was seen in M-treated silage, with significant increase of acetic acid content in the presence of air observed. *Lactobacillus fructivorans* was shown to be involved in this stability improvement, and this LAB species was successfully isolated by plate-culture using liver infusion sake medium. Subsequently, inoculation experiment was made using wilted Italian ryegrass (IR) and whole crop corn (WC) and inhibitory activity of *L. fructivorans* against aerobic deterioration was evaluated. Improvement of aerobic stability was seen for wilted IR silage at  $10^4$ ,  $10^5$ , and  $10^6$  cfu/g inoculation levels, whereas benefits of *L. fructivorans* were marginal in WC silage even at  $10^6$  cfu/g inoculation level. Inhabitation of *L. fructivorans* was not considered decisive feature in the high aerobic stability of M-treated alfalfa silage.

For the third step, a practical survey was performed to ascertain if microbiota for laboratory-scale silo can be seen for large-scale practical silo. Alfalfa silage and corn silage samples were collected from 2 large-scale farms in China; one of these farms used stack silos and the other used bunker silos. Three samples were collected from the upper and lower layers of each silo. Thus, 6 samples were examined for each of alfalfa-stack silo, alfalfa-bunker silo, corn-stack silo, and corn-bunker silo. Lactic acid was the main fermentation product except for alfalfa-bunker silo, in which acetic acid and butyric acid were predominant. *Lactobacillus plantarum*, *Weissella paramesenteroides*, and *Pediococcus pentosaceus* were found exclusively in alfalfa silages. Differences between stack and bunker silos were apparent in alfalfa silage with regard to fermentation products and DGGE band patterns.

Despite of the objective to identify bacteria associated with ensiling fermentation and spoilage inhibition, large year-to-year and harvest-to-harvest variations were shown and few straightforward results were demonstrated in this study. However, integration of culture-dependent and culture-independent microbiota analysis enabled detection of *L. fructivorans* and evaluation of its roles in fermentation and aerobic stability. Research is worth continuing to clarify substance(s) produced with combination of alfalfa substrate and growth of the identified and yet unidentified bacteria.