Studies on utilization of tropical grasses in dry-aged beef production toward establishing an Okinawan food brand

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CONTENTS

Abbreviations

Basilisk, *Urochloa decumbens* (Stapf) R.D. Webster (syn. *Brachiaria decumbens* Stapf) cv. Basilisk

MG5, *Urochloa brizantha* (syn. *Brachiaria brizantha*) cv. MG5 Vitória

Katambora, *Chloris gayana* cv. Katambora

Transvala, *Digitaria eriantha* cv. Transvala

DM, dry matter

CP, crude protein

LAB, lactic acid bacteria

GCMS, gas chromatography-mass spectrometry

SPME, solid-phase micro extraction

Chapter 1

General introduction

In Okinawa, the southernmost part of Japan, the beef industry accounted for 24.5% of gross agricultural production in 2019 and sales of calves during the past 10 years ranked fourth throughout Japan. According to the official figures of the Okinawa prefectural government, more than 3 million foreign tourists visited Okinawa in 2018, the highest number recorded in any year at that stage, and 10 million of domestic and international tourists were recorded in 2019. There is an urgent need to boost the production of Okinawa's famous unique beef brands such as 'IshigakiGyu', 'YamashiroGyu' and 'MiyakoGyu' to cope with this increase. Feeding high quality grass is necessary for breeding cows to be healthy with high reproductive rates and for growing and fattening animals to achieve high levels of production. Okinawa has a subtropical climate and warm season so perennial grasses can be grown successfully. The many small islands comprising the Prefecture of Okinawa where cattle are raised often suffer from drought, so introduced forage species should be drought-tolerant. Therefore, introduction of new species suitable for the climate and the situation in Okinawa would be a significant advance towards Okinawan beef brand. Some species of the genus *Brachiaria*, which are now recognized as species of the genus *Urochloa*, introduced from Africa, are of considerable economic importance in the tropics due to their adaptation to low-fertility soils (Rao et al. 1996), their drought-tolerance (Gayalin et al. 1994; Guenni et al. 2002) and good nutritive value (Lascano et al. 1996). In Brazil, *Urochloa* pastures extend over almost 100 million hectares (Jank et al. 2014). *Urochloa decumbens* (Stapf) R.D. Webster (syn. *Brachiaria decumbens* Stapf) cultivar Basilisk (referred to subsequently as Basilisk), originally from Uganda, is known for its drought tolerance (Oram 1990; Miles et al. 1996). Actually, Basilisk is sown in Queensland, Australia and since the 1970s has been grown in Brazil, well-adapted to infertile acid soils in the Brazilian savanna, while showing high

productivity and persistence (Kissmann 1977). A cultivar of one species, *Urochloa brizantha* (syn. *Brachiaria brizantha*) cv. MG5 Vitória (referred to subsequently as MG5), in tropical America also known under the cultivar names 'Toledo' and 'Xaraés' (Cook et al. 2020), has been shown to have high nutritive value in studies with growing cattle in Okinawa and has performed comparably with *Chloris gayana* cv. Katambora and *Digitaria eriantha* cv. Transvala (Nakanishi et al. 2006, 2008). It was considered that Basilisk and MG5 could be possibly more productive than grasses currently grown in Okinawa so investigated yield of dry matter, digestible dry matter and crude protein of Basilisk and MG5 in comparison with those of recommended grass varieties of Okinawa Prefecture and other species of the genus *Urochloa*. Actually, seed production and seed viability of MG5 in Okinawa are low (Kouki et al. 2007; 2009) and due to phytosanitary considerations (contamination of commercial seed lots with soil particles), it is difficult to import seeds of MG5 and Basilisk from other countries such as Brazil (Kouki and Ebina 2009). Therefore, a study was conducted in Okinawa to assess 2 methods of propagating MG5 vegetatively. By the way, silage is one of the way to feed cow and it can be stored. In order to improve the fermentation quality of silage using tropical grasses, it is necessary to understand the characteristics of silages stored in Okinawa and the fermentation processes which occur. To shed light on this issue, it was analyzed the fermentation characteristics of silage and identified lactic acid bacteria (LAB) involved in the fermentation process in silages made of tropical grasses and other crops grown in the Main Island and Ishigaki Island, Okinawa.

Following the grass researches towards Okinawan beef brand, there is also an urgent need to boost the production and awareness of Okinawan unique food brands because Okinawa is a popular sightseeing spot from foreign tourists. Dry-aging, an aging method, is believed to improve beef meat quality in terms of certain characteristics and which is also connected to Okinawan beef brand. It is generally believed that dry-aged beef originated

from New York, with dry-aged products being sold at exclusive restaurants and delicacy stores in Manhattan and recognized as a luxury food. Now, it has spread all over the world, such as in Japan, Korea, Singapore, Taiwan and Hong Kong, and is not only restricted to Western countries. Actually, businesses related to dry-aged beef have already started in Okinawa. Thus, I hope to create an Okinawan food brand of dry-aged beef by adding value to Okinawan beef or imported beef. In general, the dry-aging process is performed under aerated conditions, whereas the wet-aging process essentially involves vacuuming and packaging, which means that the conditions do not involve aeration. However, there is little thing known about specific differences by scientific approach between beef aged by these two methods. To shed light on this issue, in this study, scientific data, such as free amino acids and hardness, were analyzed in the round of Okinawan delivered cow beef during dry-aging and wet-aging processes along with a comparison with beef imported from Australia (hereinafter referred to as Australian beef). In major cities such as New York, most of the popular dry-aged beef steak products are believed to be aged with mold. Actually, isolating an excellent mold strain with robust growth on meat at low temperatures such as 2 °C was succeeded. This strain released an odor similar to that of nuts and fried potato and was later identified as *M. flavus*. In this study, it was analyzed the hardness and free amino acid content of beef while it was dry-aging with this mold, and its characteristics with those of the meat aged in the absence of any mold was compared. Additionally, this study analysed volatile aroma compounds related to savory flavor from dry-aged beef with the mold using gas chromatography-mass spectrometry (GCMS) with solid-phase micro extraction (SPME).

Chapter 2

Forage production and quality of *Urochloa decumbens* cultivar 'Basilisk' and *Urochloa brizantha* cv. MG5 Vitória in Okinawa, Japan

2-1 Introduction

The Prefecture of Okinawa consists of many small islands where cattle are raised on pasture, and droughts are experienced on some islands. However, these islands are essential in terms of beef production in Okinawa. Okinawa experiences a subtropical climate so tropical perennial grasses can be grown successfully. Growing highly productive grasses would remove the need for farmers to depend on imported forage. Introduction of new species suitable for grazing by cattle would be a significant advance and drought tolerance would be an added benefit. Some species of the genus *Brachiaria*, which are now recognized as species of the genus *Urochloa*, introduced from Africa, are of considerable economic importance in the tropics due to their adaptation to low-fertility soils (Rao et al. 1996), their drought-tolerance (Gayalin et al. 1994; Guenni et al. 2002) and good nutritive value (Lascano et al. 1996). In Brazil, *Urochloa* pastures extend over almost 100 million hectares (Jank et al. 2014). *Urochloa decumbens* (Stapf) R.D. Webster (syn. *Brachiaria decumbens* Stapf) cultivar 'Basilisk', originally from Uganda, is known for its drought tolerance (Oram 1990; Miles et al. 1996). Actually, 'Basilisk' is sown in Queensland, Australia and since the 1970s has been grown in Brazil, well-adapted to infertile acid soils in the Brazilian savanna, while showing high productivity and persistence (Kissmann 1977). A cultivar of one species, *Urochloa brizantha* (syn. *Brachiaria brizantha*) cv. MG5 Vitória (referred to subsequently as MG5), in tropical America also known under the cultivar names 'Toledo' and 'Xaraés' (Cook et al. 2020), has been shown to have high nutritive value in studies with growing cattle in Okinawa and has performed comparably with *Chloris gayana* cv. Katambora and *Digitaria*

eriantha cv. Transvala (Nakanishi et al. 2006, 2008). This cultivar also proved to be more tolerant of drought than all other *Urochloa* species and cultivars tested (Kudaka et al. 2010b). It was considered that 'Basilisk' and 'MG5' could be possibly more productive than grasses currently grown in Okinawa so investigated yield of dry matter, digestible dry matter and crude protein of Basilisk and MG5 in comparison with those of recommended grass varieties of Okinawa Prefecture and other species of the genus *Urochloa*.

2-2 Materials and Methods

2-2-1 Research location and period

The research was conducted during 2 periods (2002–2005 and 2006–2008) at Okinawa Livestock Research Center (Nakijin, Okinawa, Japan) (26º68' N, 127º94' E; 90 masl). Soils of the experimental area were Kunigami Merge, a red acidic Acrisol (Miyagi and Kondo 1990) and are composed of 32.8% clay (0.2 mm) (Oshiro 2007). Chemical characteristics are: pH 4.7, total carbon, 1.33%; total nitrogen, 0.12%; organic matter, 2.2%; cation exchange capacity, 13.5 meq/100 g soil; Ca, 74.4 meq/100 g soil; Mg, 40.6 meq/100 g soil; and K, 14.0 meq/100 g soil. Specific gravity is relatively high. Main clay mineral is kaolinite. Climatic conditions during the 2 experimental periods (2002–2005 and 2006–2008) are illustrated in Figures 1-1 and 1-2, indicating a cold and dry winter season from November to March. In Experiment 1, between June 2002 to April 2005 performance of Basilisk was compared with that of the other *Urochloa* species and cultivars as well as cultivars from other genera, while in Experiment 2 from December 2006 to December 2008 comparisons were made with other species within the genus *Urochloa* as well as Katambora.

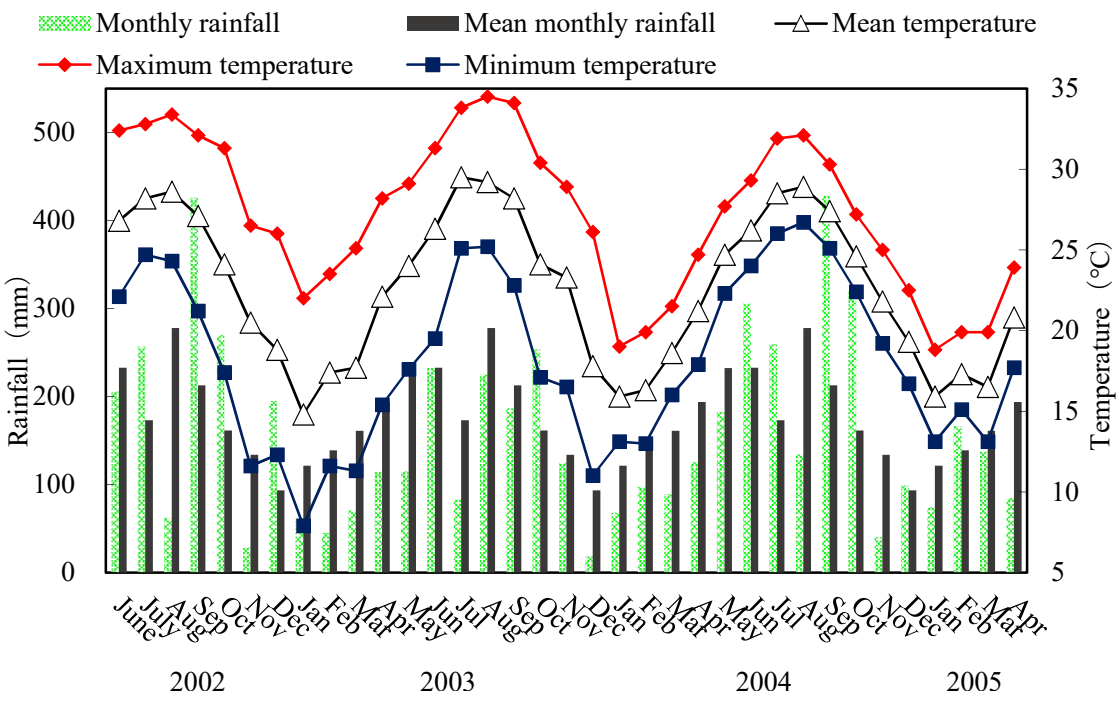

Figure 2-1. Average, maximum and minimum temperatures, monthly rainfall from June 2002 to April 2005 and mean monthly rainfall.

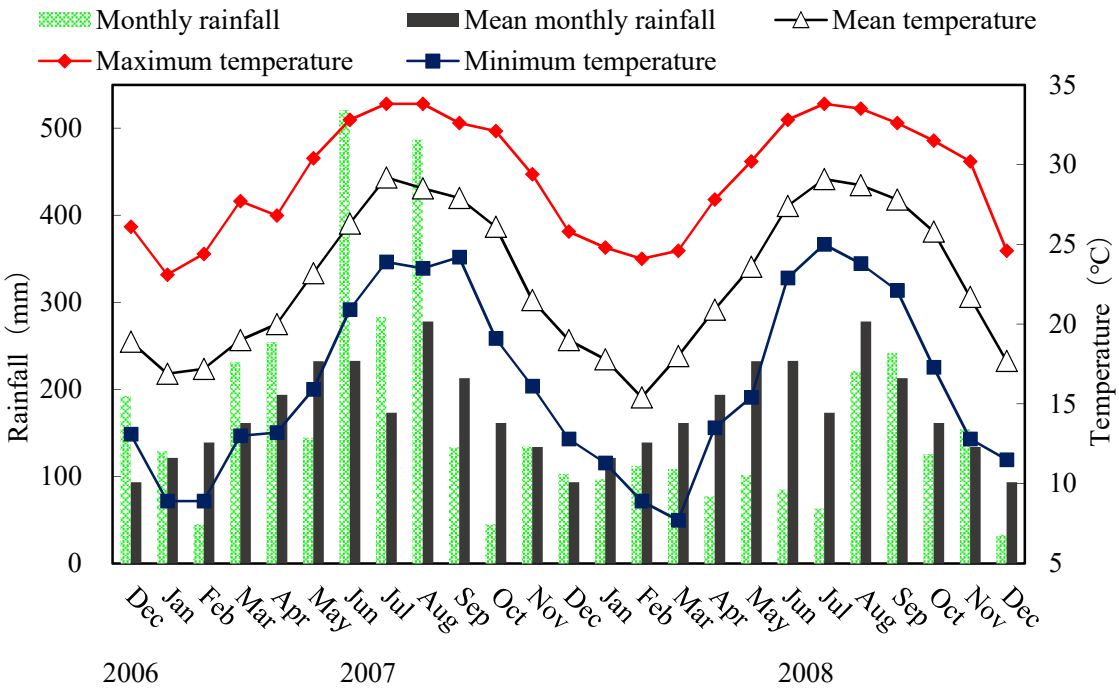

Figure 2-2. Average, maximum and minimum temperatures, monthly rainfall from December 2006 to December 2008 and mean monthly rainfall.

2-2-2 Experiment 1

The design was a complete randomized block with 7 grasses and 3 replications. Seeds of selected grasses were sown at 27.8 kg/ha on 11 October 2001, except for *Digitaria eriantha* cultivar Transvala and *Cynodon nlemfuensis* (Table 2-1), which were planted vegetatively using stolons at 4 stolons/m^2 on 29 October 2001. Seed of Katambora was sown on 22 April 2002 to increase the number of cultivars involved. Plot size was 2 × 3 m = 6 m^2. Basal fertilizer of N:P:K at 50:13:25 kg/ha was applied at planting and further N:P:K at 70:17:45 kg/ha was applied as maintenance fertilizer after each harvest. Measurement of species performance commenced in June 2002, when original plantings had become established following a few preliminary harvests. The previous harvest was in April 2002, while Katambora was harvested for the first time in June 2002. Harvesting occurred at locally used intervals, i.e. approximately every 40 days from April to October (summer season) and approximately every 55 days from November to March (winter season) in each year, ceasing in April 2005.

2-2-3 Experiment 2

The design was again a complete randomized block with 7 grasses and 3 replications. On 18 October 2005 seeds of grasses were sown at 41.7 kg/ha, while *Urochloa mutica* was planted with 4 stolons/m^2 (Table 2-1). Basal fertilizer of N:P:K at 80:14:40 kg/ha was applied at planting and the same quantities were applied as maintenance fertilizer after each harvest. Again plants were allowed to establish before observations commenced on 18 December 2006. The previous harvest was on 7 November 2006. Harvests occurred approximately every 55 days from November to March and every 40 days from April to October as described above until December 2008.

Table 2-1. List of grass varieties investigated in Experiments 1 and 2.

Grass varieties investigated in 2002–2005 (Experiment 1)	Planting	Grass varieties in 2006–2008 (Experiment 2)	Planting
Urochloa decumbens cv. Basilisk	Seeds	*Urochloa decumbens* cv. Basilisk	Seeds
Urochloa humidicola	Stolons	*Urochloa brizantha* cv. Marandu	Seeds
Chloris gayana cv. Callide	Seeds	*Urochloa humidicola*	Seeds
Chloris gayana cv. Katambora	Seeds	*Urochloa brizantha* cv. MG5	Seeds
Cynodon nlemfuensis	Seeds	*Urochloa ruziziensis*	Seeds
Digitaria eriantha cv. Transvala	Stolons	*Urochloa mutica*	Stolons
Megathyrsus maximus cv. Gatton	Seeds	*Chloris gayana* cv. Katambora	Seeds

2-2-4 Dry matter yield

In both studies, forage on each plot was harvested (6 m^2) at approximately 10 cm from ground level and forage samples (500 g) for each plot were collected and dried at 70 ℃ for 48 h to determine dry matter (DM) yield. The dried samples were ground with a mill and the powder was sieved through a 1 mm mesh for analyzing for nitrogen concentration for crude protein concentration and in vitro DM digestibility.

2-2-5 In vitro dry matter digestibility and crude protein analysis

Grass samples were analyzed for in vitro DM digestibility according to the pepsin-cellulase assay (Goto and Minson 1977). Crude protein (CP) concentration was determined by the microKjeldahl method using an Auto Analyzer.

2-2-6 Statistical analysis

One-way analysis of variance (ANOVA) was used for the statistical analysis of DM yield, in vitro DM digestibility, digestible DM yield, CP concentration and CP yield in each experiment using the RStudio Version 1.4.1717. Tukey's test was used to identify the differences between Basilisk or MG5 and other grass varieties.

2-3 Results

2-3-1 Experiment 1

2-3-1-1 Dry matter

In Experiment 1 from 2002 to 2005, there was no significant difference in DM yield between Basilisk and other varieties in 2002 (Table 2-2). However, in 2003 DM yield of Basilisk (46.7 t/ha) was significantly greater than that of *U. humidicola* (33.1 t/ ha; P = 0.02), *C. nlemfuensis* (33.8 t/ha; P = 0.03) and Transvala (30.2 t/ha; P = 0.004). As a result, total DM yield of Basilisk (119.5 t/ha) from 2002 to 2005 was significantly greater than that of Transvala (87.4 t/ha; P = 0.01).

Table 2-2. Dry matter yield (t/ha) of forage of a range of tropical grasses in Okinawa (Experiment 1 – Mochizuki et al. 2005).

Grass variety	2002 S4h[1]	2003 S7h	2004 S8h	2005 S2h	Total
Urochloa decumbens cv. Basilisk	21.6 ± 1.1	46.7 ± 1.4	45.7 ± 0.7	5.5 ± 0.2	119.5 ± 0.5
Urochloa humidicola	17.1 ± 1.6	33.1 ± 3.5*	43.4 ± 1.4	5.1 ± 0.5	98.7 ± 6.9
Chloris gayana cv. Callide	20.2 ± 0.7	36.9 ± 0.7	38.5 ± 1.8	5.3 ± 0.4	100.9 ± 3.3
Chloris gayana cv. Katambora	18.5 ± 0.5	35.4 ± 3.1	37.0 ± 3.6	4.4 ± 0.8	95.3 ± 7.6
Cynodon nlemfuensis	19.6 ± 0.8	33.8 ± 0.9*	38.6 ± 2.2	3.2 ± 0.3	95.1 ± 2.7
Digitaria eriantha cv. Transvala	16.7 ± 0.5	30.2 ± 1.7**	36.5 ± 1.5	4.1 ± 0.2	87.4 ± 3.0*
Megathyrsus maximus cv. Gatton	21.4 ± 0.9	42.2 ± 0.9	43.7 ± 0.6	4.9 ± 0.4	112.1 ± 1.0

Within columns, * and ** indicate significant differences ($P < 0.05$) and ($P < 0.01$) compared with Basilisk; [1]Sum of n harvest.

2-3-1-2 Digestibility

Mean DM digestibility of Basilisk (56.7%) from 2002 to 2005 was significantly greater than that of *U. humidicola* (54.3%; P = 0.02), Callide (54.5%; P = 0.03), Katambora (51.4%; P < 0.001) and *C. nlemfuensis* (52.9%; P < 0.001) (Table 2-3). For digestible DM yield, there was no significant difference between Basilisk and other varieties in 2002 and 2005, while in 2003 that of Basilisk (25.5 t/ha) was significantly greater than those of *U. humidicola* (17.4 t/ha; P = 0.006), Katambora (17.7 t/ha; P = 0.008), *C. nlemfuensis* (17.0 t/ha; P = 0.004) and Transvala (16.1 t/ha; P = 0.001) (Table 2-4), and in 2004 that of Basilisk (23.8 t/ha) was significantly greater than those of Katambora (17.1 t/ha; P = 0.01) and *C. nlemfuensis* (18.1 t/ha; P = 0.03). Over the complete study, total digestible DM yield of Basilisk (64.8 t/ha) was significantly higher than that of *U. humidicola* (51.2 t/ha; P = 0.04), Katambora (46.8 t/ha; P = 0.006), *C. nlemfuensis* (47.2t/ha; P = 0.007) and Transvala (46.3 t/ha; P = 0.005).

Table 2-3. Mean dry matter digestibility (%) of forage of a range of tropical grasses in Okinawa (Experiment 1 – Hanagasaki et al. 2006).

Grass varieties	2002 S4h[1]	2003 S7h	2004 S8h	2005 S2h	Overall mean
Urochloa decumbens cv. Basilisk	54.4 ± 0.2	56.5 ± 0.5	54.3 ± 0.2	72.8 ± 1.0	56.7 ± 0.1
Urochloa humidicola	53.2 ± 0.1	53.8 ± 0.7*	51.3 ± 0.2*	71.6 ± 0.2	54.3 ± 0.3*
Chloris gayana cv. Callide	52.8 ± 0.2	54.0 ± 0.3	50.9 ± 0.2**	74.7 ± 0.1	54.5 ± 0.2*
Chloris gayana cv. Katambora	48.3 ± 0.3***	50.7 ± 0.1***	48.1 ± 0.5***	74.4 ± 0.4	51.4 ± 0.3***
Cynodon nlemfuensis	50.7 ± 0.7*	53.0 ± 0.5**	48.8 ± 0.8***	74.6 ± 0.4	52.9 ± 0.7***
Digitaria eriantha cv. Transvala	51.3 ± 0.4	56.5 ± 0.5	53.5 ± 0.3	76.5 ± 0.6*	56.1 ± 0.1
Megathyrsus maximus cv. Gatton	51.0 ± 1.0*	54.8 ± 0.2	53.7 ± 0.5	76.2 ± 0.6*	55.5 ± 0.5

Within columns, *, ** and *** indicate significant differences ($P < 0.05$), ($P < 0.01$) and ($P < 0.001$) compared with Basilisk; [1]Mean of n harvest.

Table 2-4. Digestible dry matter yield (t/ha) of forage of a range of tropical grasses in Okinawa (Experiment 1 – Hanagasaki et al. 2006).

Grass varieties	2002 S4h[1]	2003 S7h	2004 S8h	2005 S2h	Total
Urochloa decumbens cv. Basilisk	11.4 ± 0.6	25.5 ± 0.4	23.8 ± 0.1	4.2 ± 0.1	64.8 ± 0.3
Urochloa humidicola	8.9 ± 0.8	17.4 ± 1.9**	21.2 ± 0.5	3.8 ± 0.3	51.2 ± 3.5*
Chloris gayana cv. Callide	10.3 ± 0.5	19.8 ± 0.3	18.9 ± 1.0	4.2 ± 0.3	53.2 ± 1.9
Chloris gayana cv. Katambora	8.6 ± 0.1	17.7 ± 1.6**	17.1 ± 1.8*	3.5 ± 0.6	46.8 ± 4.0**
Cynodon nlemfuensis	9.6 ± 0.5	17.0 ± 0.6**	18.1 ± 0.7*	2.5 ± 0.3	47.2 ± 1.2**
Digitaria eriantha cv. Transvala	8.3 ± 0.2	16.1 ± 0.8**	18.7 ± 0.7	3.2 ± 0.2	46.3 ± 1.5**
Megathyrsus maximus cv. Gatton	10.6 ± 0.6	21.7 ± 0.5	22.2 ± 0.4	3.8 ± 0.3	58.3 ± 0.9

Within columns, * and ** indicate significant differences ($P < 0.05$) and ($P < 0.01$) compared with Basilisk; [1]Sum of n harvest.

2-3-1-3 Crude protein

Mean CP concentration (13.1%) of Basilisk was significantly higher than that of Callide and Katambora ($P < 0.05$) (Table 2-5). Regarding CP yield, that of Basilisk (5.1 t/ha) was significantly higher than that of *U. humidicola* (3.6 t/ha; $P = 0.03$) and Transvala (3.3 t/ha; $P = 0.01$) in 2003, while in 2005 that of Basilisk (1.0 t/ha) was significantly higher than that of Katambora (0.6 t/ha; $P = 0.04$) and *C. nlemfuensis* (0.5 t/ha; $P = 0.02$) (Table 2-6). Total CP yield of Basilisk (13.7 t/ha) was significantly higher than that of Katambora (10.1 t/ha; $P = 0.02$) and Transvala (9.5 t/ha; $P = 0.008$).

Table 2-5. Mean crude protein concentration of forage of a range of tropical grasses in Okinawa (Experiment 1 – Hanagasaki et al. 2006).

Grass varieties	2002 S4h[1]	2003 S7h	2004 S8h	2005 S2h	Overall mean
Urochloa decumbens cv. Basilisk	13.0 ± 0.2	12.8 ± 0.1	12.2 ± 0.1	17.9 ± 0.7	13.1 ± 0.1
Urochloa humidicola	13.3 ± 0.4	12.8 ± 0.4	11.2 ± 0.2	16.6 ± 0.6	12.7 ± 0.3
Chloris gayana cv. Callide	11.9 ± 0.3	11.5 ± 0.2	10.7 ± 0.0	13.9 ± 0.2**	11.5 ± 0.1*
Chloris gayana cv. Katambora	11.6 ± 0.3	11.6 ± 0.3	10.6 ± 0.5*	13.5 ± 0.5***	11.4 ± 0.3*
Cynodon nlemfuensis	14.0 ± 0.4	13.6 ± 0.4	12.0 ± 0.3	17.1 ± 0.1	13.5 ± 0.3
Digitaria eriantha cv. Transvala	13.4 ± 0.3	12.6 ± 0.3	10.9 ± 0.2	15.2 ± 0.2*	12.4 ± 0.1
Megathyrsus maximus cv. Gatton	13.6 ± 0.4	13.7 ± 0.1	11.4 ± 0.2	16.5 ± 0.5	13.1 ± 0.1

Within columns, *, ** and *** indicate significant differences ($P < 0.05$), ($P < 0.01$) and ($P < 0.001$) compared with Basilisk; [1]Mean of n harvest.

Table 2-6. Crude protein yield (t/ha) of forage of a range of tropical grasses in Okinawa (Experiment 1 – Hanagasaki et al. 2006).

Grass varieties	2002 S4h[1]	2003 S7h	2004 S8h	2005 S2h	Total
Urochloa decumbens cv. Basilisk	2.6 ± 0.2	5.1 ± 0.1	4.9 ± 0.1	1.0 ± 0.0	13.7 ± 0.2
Urochloa humidicola	2.2 ± 0.1	3.6 ± 0.4*	4.3 ± 0.1	0.8 ± 0.1	10.9 ± 0.7
Chloris gayana cv. Callide	2.2 ± 0.2	3.8 ± 0.1	3.8 ± 0.2	0.7 ± 0.1	10.5 ± 0.4
Chloris gayana cv. Katambora	2.0 ± 0.1	3.8 ± 0.4	3.7 ± 0.5	0.6 ± 0.1*	10.1 ± 1.1*
Cynodon nlemfuensis	2.5 ± 0.2	4.1 ± 0.2	4.3 ± 0.1	0.5 ± 0.0*	11.5 ± 0.4
Digitaria eriantha cv. Transvala	2.0 ± 0.1	3.3 ± 0.2*	3.6 ± 0.1	0.6 ± 0.0	9.5 ± 0.4**
Megathyrsus maximus cv. Gatton	2.6 ± 0.2	5.0 ± 0.1	4.5 ± 0.1	0.8 ± 0.1	12.9 ± 0.2

Within columns, * and ** indicate significant differences ($P < 0.05$) and ($P < 0.01$) compared with Basilisk; [1]Sum of n harvest.

2-3-2 Experiment 2

2-3-2-1 Dry matter

In Experiment 2 from 2006 to 2008, DM yield of Basilisk (2.6 t/ha) was significantly lower than that of *U. brizantha* cv. MG5 (3.9 t/ha; P = 0.007) in 2006 but was significantly higher than that of *U. mutica* (46.3 vs. 37.9 t/ha; P = 0.01) in 2007 and overall (93.0 vs. 78.6 t/ha; P = 0.008) (Table 2-7). DM yield of MG5 (3.9 t/ha) was significantly higher than that of *U. humidicola* (2.9 t/ha; P =0.03), *Urochloa ruziziensis* (2.1 t/ha; P < 0.001), *U. mutica* (2.0 t/ha; P < 0.001) and Katambora (2.8 t/ha; P = 0.03) in 2006 and was significantly higher than that of *U. mutica* (46.8 vs. 37.9 t/ha; P = 0.008 and 46.6 vs. 38.7 t/ha; P = 0.005) in 2007 and 2008. Total DM yield of MG5 (97.2 t/ha) was significantly higher than that of *Urochloa brizantha* cv. Marandu (85.5 t/ha; P = 0.03) and *U. mutica* (78.6 t/ha; P < 0.001).

Table 2-7. Dry matter yield (t/ha) of forage of *Urochloa* cultivars and *Chloris gayana* cv. Katambora in Okinawa (Experiment 2 – Kudaka et al. 2010a).

Grass variety	2006 1 harvest	2007 S8h[1]	2008 S8h	Total
Urochloa decumbens cv. Basilisk	2.6 ± 0.1	46.3 ± 2.4	44.1 ± 1.4	93.0 ± 3.7
Urochloa brizantha cv. Marandu	3.1 ± 0.2	42.1 ± 0.9	40.3 ± 0.2^{+}	85.5 ± 1.3^{+}
Urochloa humidicola	2.9 ± 0.0^{+}	44.7 ± 0.4	43.0 ± 0.3	90.5 ± 0.4
Urochloa brizantha cv. MG5	3.9 ± 0.2**	46.8 ± 0.6	46.6 ± 1.3	97.2 ± 0.9
Urochloa ruziziensis	2.1 ± 0.2^{+++}	44.4 ± 1.4	47.4 ± 0.9	94.0 ± 2.2
Urochloa mutica	2.0 ± 0.2^{+++}	37.9 ± 0.6*$^{++}$	38.7 ± 0.6^{++}	78.6 ± 0.9**$^{+++}$
Chloris gayana cv. Katambora	2.8 ± 0.1^{+}	44.1 ± 0.6	48.3 ± 1.2	95.3 ± 1.7

Within columns, * and ** indicate significant differences ($P < 0.05$) and ($P < 0.01$) compared with Basilisk; $^{+}$, $^{++}$ and $^{+++}$ indicate significant differences ($P < 0.05$), ($P < 0.01$) and ($P < 0.001$) compared with MG5; [1]Sum of n harvest.

2-3-2-2 Digestibility

Mean DM digestibility of Basilisk (54.8%) from 2006 to 2008 was significantly higher than that of Katambora (52.8%; P = 0.02) (Table 2-8). That of MG5 (53.1%) was significantly lower than that of *U. ruziziensis* (56.4%; P < 0.001). Digestible DM yield of Basilisk in 2007 and 2008 (23.6 and 23.5 t/ha) was significantly greater than those of *U. mutica* (19.4 t/ha; P = 0.03 and 19.6 t/ha; P = 0.01) (Table 2-9). Total digestible DM yield of Basilisk (48.8 t/ha) was also significantly greater than that of *U. mutica* (40.3 t/ha; P = 0.004). Also, digestible DM yield of MG5 in 2007 and 2008 (23.9 and 24.2 t/ha) were significantly greater than those of *U. mutica* (P = 0.01 and P = 0.002). Total digestible DM yield of MG5 (50.3 t/ha) was also significantly greater than that of *U. mutica* (P < 0.001).

Table 2-8. Mean dry matter digestibility (%) of forage of *Urochloa* cultivars and *Chloris gayana* cv. Katambora in Okinawa (Experiment 2 – Kudaka et al. 2010a).

Grass variety	2006 1 harvest	2007 M8h[1]	2008 M8h	Overall mean
Urochloa decumbens cv. Basilisk	62.7 ± 1.0	54.4 ± 0.6	54.2 ± 0.1	54.8 ± 0.4
Urochloa brizantha cv. Marandu	61.2 ± 0.9^{++}	55.3 ± 0.2	53.5 ± 0.8	54.8 ± 0.4
Urochloa humidicola	61.2 ± 0.5^{++}	53.7 ± 0.3	54.2 ± 1.0	54.4 ± 0.5
Urochloa brizantha cv. MG5	54.9 ± 0.5***	53.5 ± 0.1	52.4 ± 0.1	53.1 ± 0.1
Urochloa ruziziensis	63.2 ± 0.2^{+++}	56.6 ± 0.2*$^{+++}$	55.2 ± 0.3^{+++}	56.4 ± 0.1^{+++}
Urochloa mutica	63.7 ± 0.4	54.2 ± 0.2	51.5 ± 0.3	53.5 ± 0.2
Chloris gayana cv. Katambora	60.9 ± 0.8^{++}	53.3 ± 0.3	51.3 ± 0.1	52.8 ± 0.1*

Within columns, * and *** indicate significant differences ($P < 0.05$) and ($P < 0.001$) compared with Basilisk; $^{++}$ and $^{+++}$ indicate significant differences ($P < 0.01$) and ($P < 0.001$) compared with MG5; [1]Mean of n harvest.

Table 2-9. Digestible dry matter yield (t/ha) of forage of *Urochloa* cultivars and *Chloris gayana* cv. Katambora in Okinawa (Experiment 2 – Kudaka et al. 2010a).

Grass variety	2006 1 harvest	2007 S8h[1]	2008 S8h	Total
Urochloa decumbens cv. Basilisk	1.6 ± 0.1	23.6 ± 1.4	23.5 ± 0.8	48.8 ± 2.2
Urochloa brizantha cv. Marandu	1.9 ± 0.1	22.2 ± 0.5	21.2 ± 0.2	45.3 ± 0.4
Urochloa humidicola	1.7 ± 0.0	22.2 ± 0.2	22.9 ± 0.4	46.8 ± 0.2
Urochloa brizantha cv. MG5	2.1 ± 0.1	23.9 ± 0.4	24.2 ± 0.7	50.3 ± 0.4
Urochloa ruziziensis	1.4 ± 0.1^{++}	23.8 ± 0.8	25.3 ± 0.4	50.5 ± 1.1
Urochloa mutica	1.3 ± 0.1^{++}	19.4 ± 0.4*$^{+}$	19.6 ± 0.3*$^{++}$	40.3 ± 0.6**$^{+++}$
Chloris gayana cv. Katambora	1.7 ± 0.0	21.5 ± 0.2	24.0 ± 0.6	47.3 ± 0.7

Within columns, * and ** indicate significant differences ($P < 0.05$) and ($P < 0.01$) compared with Basilisk; $^{+}$, $^{++}$ and $^{+++}$ indicate significant differences ($P < 0.05$), ($P < 0.01$) and ($P < 0.001$) compared with MG5; [1]Sum of n harvest.

2-3-2-3 Crude protein

Mean CP concentration (12.7%) and total CP yield (10.4 t/ha) of Basilisk were not significantly different from those of other *Urochloa* cultivars (Tables 2-10 and 2-11). Mean CP concentration (12.9%) of MG5 was not significantly different from that of other *Urochloa* cultivars but total CP yield (10.9 t/ha) of MG5 was significantly higher than that of *U. mutica* (8.9 %; P = 0.006).

Table 2-10. Mean crude protein concentration (%) of forage of *Urochloa* cultivars and *Chloris gayana* cv. Katambora in Okinawa (Experiment 2 – Kudaka et al. 2010a).

Grass variety	2006 1 harvest	2007 M8h[1]	2008 M8h	Overall mean
Urochloa decumbens cv. Basilisk	18.2 ± 0.5	12.5 ± 0.3	12.2 ± 0.2	12.7 ± 0.1
Urochloa brizantha cv. Marandu	18.0 ± 0.7	13.9 ± 0.4	13.0 ± 0.3	13.7 ± 0.4
Urochloa humidicola	17.1 ± 0.2	12.4 ± 0.3	11.7 ± 0.1	12.4 ± 0.2
Urochloa brizantha cv. MG5	16.1 ± 0.3	13.2 ± 0.2	12.3 ± 0.2	12.9 ± 0.2
Urochloa ruziziensis	18.9 ± 0.4^{+}	13.5 ± 0.3	12.3 ± 0.3	13.2 ± 0.1
Urochloa mutica	20.6 ± 0.4^{+++}	13.9 ± 0.1	11.1 ± 0.2	12.9 ± 0.2
Chloris gayana cv. Katambora	17.0 ± 0.3	12.3 ± 0.2	11.3 ± 0.2	12.1 ± 0.2

+ and +++ indicate significant differences ($P < 0.05$) and ($P < 0.001$) compared with MG5; [1]Mean of n harvest.

Table 2-11. Crude protein yield (t/ha) of forage of *Urochloa* cultivars and *Chloris gayana* cv. Katambora in Okinawa (Experiment 2 – Kudaka et al. 2010a).

Grass variety	2006 1 harvest	2007 S8h[1]	2008 S8h	Total
Urochloa decumbens cv. Basilisk	0.5 ± 0.0	5.1 ± 0.3	4.8 ± 0.2	10.4 ± 0.5
Urochloa brizantha cv. Marandu	0.6 ± 0.0	5.2 ± 0.1	4.8 ± 0.0	10.6 ± 0.1
Urochloa humidicola	0.5 ± 0.0	4.9 ± 0.1	4.4 ± 0.0	9.8 ± 0.1
Urochloa brizantha cv. MG5	0.6 ± 0.0*	5.4 ± 0.2	4.9 ± 0.2	10.9 ± 0.2
Urochloa ruziziensis	0.4 ± 0.0^{++}	5.1 ± 0.3	4.5 ± 0.1	10.0 ± 1.4
Urochloa mutica	0.4 ± 0.0^{++}	4.6 ± 0.0	3.9 ± 0.1^{+}	8.9 ± 0.1^{++}
Chloris gayana cv. Katambora	0.5 ± 0.0^{+}	4.7 ± 0.1	4.9 ± 0.2	10.1 ± 0.2

Within columns, * indicate significant differences ($P < 0.05$) compared with 'Basilisk'; + and ++ indicate significant differences ($P < 0.05$) and ($P < 0.01$) compared with 'MG5'; [1]Sum of n harvest.

2-4 Discussion

This forage production study has provided valuable information on the relative performance of a range of tropical grasses including *U. decumbens* cv. Basilisk and *U. brizantha* cv. MG5 over a number of years in Okinawa. Basilisk and MG5 performed as well as all other cultivars evaluated in most years and outperformed some cultivars in some years. While rainfall in 2003 was well below the long-term mean for the area (Figure 2-1), Basilisk maintained a high level of production and had higher DM yield than *U. humidicola*, *C. nlemfuensis* and *D. eriantha* cv. Transvala, supporting claims that this cultivar has a good level of tolerance of drier conditions. However, even in this 'dry' year, rainfall received was 1,530 mm, which should be adequate to support good DM yields given that >100 mm was registered in 7 of the 12 months. As well as having good DM production, Basilisk showed CP concentration as high as all other cultivars tested with mean values of 13.1% and 12.7% in the 2 experiments. The level of *U. decumbens* (14%) was within the range expected for immature leaves in Costa Rica (Lascano and Euclides 1996). Basilisk would provide an excellent diet for grazing ruminants, especially given their ability to select a better quality diet than the mean of total feed on offer. DM digestibility of 56.7% and 54.8% in the 2 experiments would ensure that there was an adequate supply of available energy. In Okinawa, area of grass pasture covers more than 95% of the area with 4 major grass varieties recommended, i.e. *Ch. gayana*, *D. eriantha*, *M. maximus* and *C. nlemfuensis* in the past 15 years. *D. eriantha* cv. Transvala has become the major grass sown in Okinawa and represented 27.9% of total area sown to grass in Okinawa in 2020 because of its perceived excellent characteristics and its suitability for local conditions (Hanagasaki 2022). However, Basilisk was obviously superior to Transvala in Experiment 1, in terms of yields of DM, digestible DM and CP, suggesting that it could be an acceptable substitute. In addition, Basilisk performed significantly better than *U. mutica*, which is also a recommended grass variety for sowing in Okinawa

Prefecture. In fact, Basilisk is a high yielding species, particularly if nitrogen fertilizer is provided. Up to 30 t DM/ha/yr can be obtained on fertile soils in Vanuatu, and the same biomass production is possible under coconut plantations in the Solomon Islands (Cook et al. 2020). The average yield is, however, generally lower at about 10 t DM/ha/yr. Basilisk yielded 4 t DM/ha/yr without fertilizer in Colombia (FAO 2016). In studies in northeast Brazil (Rodrigues et al. 2014), total forage production of Basilisk (8.9 t DM/ha) and MG5 (11.6 t DM/ha) over 2 years was significantly higher than Koronivia grass (*Brachiaria humidicola*) and Gamba grass (*Andropogon gayanus*). In addition, milk yield of cows grazing Basilisk (8 kg/cow/d) was greater than on *Brachiaria dictyoneura* (now: *Urochloa humidicola*) (6 kg/cow/d) in Colombia (Lascano and Euclides 1996). *U. decumbens* has an allelopathic effect, inhibiting germination of seeds of other plants (Barbosa et al. 2008). In our study, it prevented invasion by other grasses throughout the 10 years since Basilisk was planted (see bottom photo in Figure 2-3). Before it can be sown widely a reliable source of seed will be needed. However, Basilisk seed production in Okinawa has been insufficient to meet demand (Kouki et al. 2009), despite the fact Basilisk seed production is a success at 17–22 °S and elevations of 600–1,000 masl in Australia and Brazil (Hare et al. 2005). Unless seed production can be increased successfully in Okinawa, efforts will be needed to source supplies of seed from countries where seed is already grown successfully if its potential is to be realized in the area.

Figure 2-3. Appearance of *Urochloa decumbens* cv. Basilisk cultivated in Okinawa.

2-5 Abstract

Two studies were conducted to assess forage growth and nutritive value of *Urochloa decumbens* (syn. *Brachiaria decumbens*) cv. Basilisk in comparison with other grass species grown in Okinawa during 2002–2005 and 2006–2008. Harvests were performed every 40 days from April to October and every 55 days from November to March. In Experiment 1, total dry matter (DM) yield of Basilisk (119.5 t/ha) was significantly higher than that of *Digitaria eriantha* cv. Transvala (87.4 t/ha; $P = 0.01$), one of the most popular recommended grass varieties in Okinawa Prefecture. Mean DM digestibility of Basilisk was 56.7%, significantly higher than that of other recommended grass varieties (54.5–51.4%). In addition, total digestible DM yield (64.8 t/ha) and crude protein (CP) yield (13.7 t/ha) of Basilisk were significantly higher than those of other varieties including Transvala ($P < 0.01$). In Experiment 2, total DM yield of Basilisk and MG5 during 2006–2008 was 93.0 t/ha and 97.2 t/ha and significantly higher only than that of *Urochloa mutica* (syn. *Brachiaria mutica*) (78.6 t/ha; $P < 0.01$ and $P < 0.001$), whereas mean DM digestibility (54.8%) of Basilisk was significantly higher only than that of *Chloris gayana* cv. Katambora (52.8%; $P < 0.05$). Total digestible DM yield of Basilisk (48.8 t/ha) and MG5 (50.3 t/ha) were significantly higher only than that of *U. mutica* (40.3 t/ha; $P < 0.01$ and $P < 0.001$) while their total CP yield (10.4 t/ha and 10.9 t/ha) were similar to those of other *Urochloa* cultivars ($P > 0.05$).

Chapter 3

Studies with *Urochloa brizantha* cv. MG5 Vitória in Okinawa, Japan: Vegetative propagation and a tractor tyre stress test

3-1 Introduction

Seed production and seed viability of MG5 in Okinawa are low (Kouki et al. 2007; 2009) and due to phytosanitary considerations (contamination of commercial seed lots with soil particles), it is difficult to import seeds of MG5 from other countries such as Brazil (Kouki and Ebina 2009). As a result, MG5 is still uncommon in Okinawa and mechanisms and strategies for increased usage should be developed. Besides, forage is usually used for hay production which involves mowing, aerating and baling using heavy tractors, 5 or 6 times per year while cattle are grazed in some areas of the Prefecture. Consequently, a forage cultivar such as MG5 must be resistant to tractor tyre stress and produce acceptable growth under this regime. To address these issues we investigated methods of vegetative propagation of MG5 and production of MG5 for 2 years, while being harvested by tractors.

3-2 Materials and Methods

The research was conducted at Okinawa Livestock Research Center (Nakijin, Okinawa, Japan) (26º41' N, 127º56' E; 90 masl).

3-2-1 Study 1: Vegetative propagation

3-2-1-1 Raising plantlets

A tray comprising 55 cells, each 4.5 × 4.5 × 4.5 cm, was filled with a 50:50 mixture of potting compost (TAKII & Co. Ltd., Kyoto, Japan) and red ball earth 1 (TAKII & Co. Ltd.). For raising plantlets, soil in the trays was kept moist by sprinkling with 3.7 mm water per day in a glass-house. Cuttings were taken from a mature pasture stand of MG5

of 70–90 cm height. Two methods were compared to obtain material for planting. For the first method (Method 1: higher cutting and root formation), grass stems (culms) were cut above the third joint (node) from the base. While the cut plant portion was removed and discarded, the uncut stem portion stayed in the field for two weeks (Figure 3-1). During this time adventitious roots start to develop from the lowest node. The stem was then cut to retain 2 nodes including the rooting node and was inserted to a depth of 3 cm into soil in the trays. For the second method (Method 2: lower cutting and direct planting), MG5 stems were cut at about 10 cm from ground level (Figure 3-1) and the lowest node was inserted immediately into the soil in the trays to a depth of 3 cm. Acceptably formed plantlets were identified about 2 months later by counting those rooted cuttings where, if lifted by the stem, soil did not fall away from the stem as roots were completely attached to the soil.

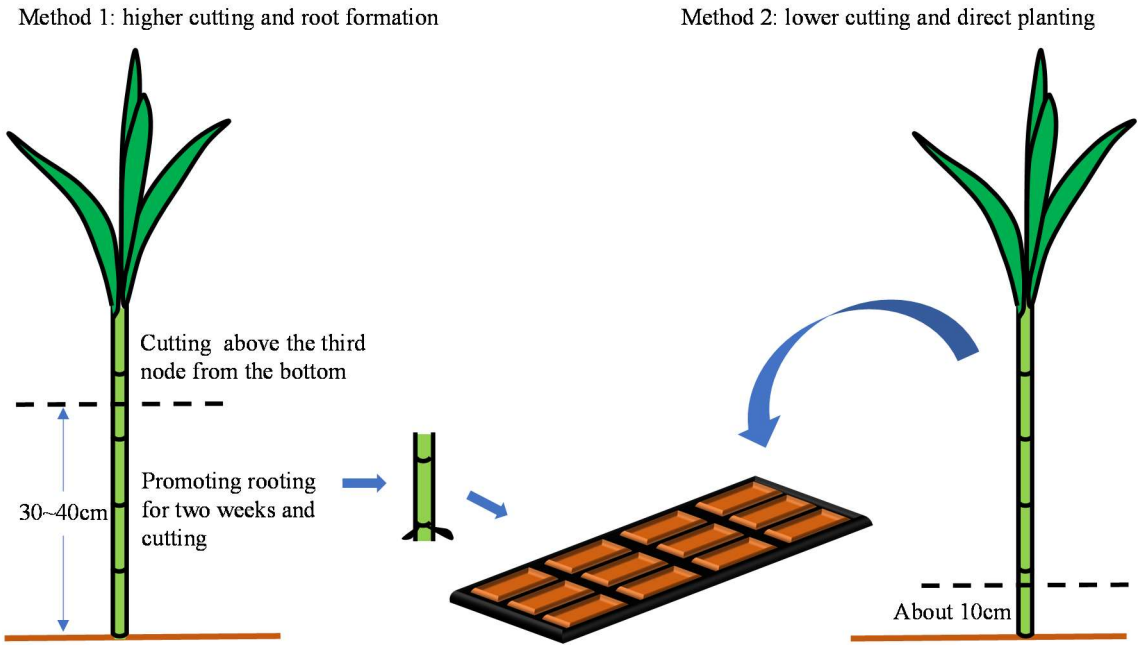

Figure 3-1. Method 1 – Higher cutting and root formation; and Method 2 – Lower cutting and direct planting.

3-2-1-2 Transplanting plantlets into the ground

Plantlets obtained by vegetative propagation and about 21 weeks of age, were transplanted into the field of Kunigami merge. The merge is a fine-grained red soil sometimes containing gravel but with low organic matter concentration. Two transplanting methods were compared in terms of time necessary for planting a given area: using a vegetable transplanter machine (Yanmar Holdings Co. Ltd., Osaka, Japan); and by means of a manual planting tool with 2 handles (Figure 3-2). In the former, plantlets were fed into a hopper on the machine and were drawn down into the ground while in the latter, a plantlet was placed in the bottom of the tool, the jaws at the bottom were inserted into the soil, and the soil was opened by forcing the levers at the top apart. While press wheels compacted the soil around the plantlet for the vegetable planter, soil was pressed down with the foot for the planting tool method. Both methods involved 3 people and their working time was recorded. For each method, 110 plantlets were planted 18 cm apart in 8 furrows 36 cm apart, giving an area of about 50 m^2 for each method.

Figure 3-2. Mechanical and non-mechanical transplanting of MG5 plantlets.

3-2-2 Study 2: Test for tractor tyre stress

Seeds of *Chloris gayana* cv. Katambora and MG5 were each sown at 30 kg/ha on 27 September 2005. For each grass, an area of 47.6 m^2 (14 × 3.4 m) replicated 4 times was used to assess the impacts of tractor tyre stress or no tractor stress (Control) on plant growth. Both grasses were mown by hand 3 times, the last one on 27 June 2006, before the investigation commenced. To impose tractor tyre stress on appropriate plots, a tractor (98 PS, about 3 tonnes weight, gear M-2, Iseki Co. Ltd., Ehime, Japan) was driven over the whole of each plot 3 times evenly every harvest: 16 August, 05 October and 19 December in 2006, and 21 May, 15 August and 29 October in 2007, to simulate mowing, aerating for drying and collecting in round bales as for a conventional harvest. To determine DM yield, 3 different fixed areas (1 × 1 m) in each plot were cut by hand for each treatment and the harvested material was dried for 48 h at 72 ºC.

3-2-3 Statistical analysis

Statistical treatment of the tyre stress test was by two-way analysis of variance (ANOVA) with a Fisher's least significant difference test for the 2 factors, grass species and tractor tyre stress, regarding DM yield for each date and total yield.

3-3 Results

3-3-1 Study 1: Vegetative propagation

3-3-1-1 Raising plantlets

Cutting stalks near the base and directly inserting them into the soil (Method 2: lower cutting and direct planting) resulted in nearly 77% of seedlings produced. This compares with about 67% success rate with high cutting and planting after roots appear (Method 1: higher cutting and root formation).

3-3-1-2 Transplanting plantlets into the ground

Time involved in transplanting plantlets showed that machine planting took 107 seconds per 20 m and 39 seconds for a change of direction. In contrast, manual planting took 287 seconds per 20 m. Thus, manual planting of 1,000 m^2 took twice as much time as machine planting (Table 3-1). MG5 generally grew rapidly with both methods of transplanting (Figure 3-3).

Table 3-1.Vegetative propagation and transplanting of *Urochloa brizantha* cv. MG5: A) Percentage of rooted cuttings (plantlets) produced with 2 methods. B) Time for transplanting rooted cuttings in a 1,000 m^2 plot by 2 methods.

Method	A) Percentage of rooted plantlets after 2 months	B) Time for transplanting
Higher cutting and root formation	66.7% (1,115)[1]	Not applicable
Lower cutting and direct planting	76.7% (648)	Not applicable
Machine planting	Not applicable	6.3 hours
manual planting	Not applicable	12.9 hours

[1]Numbers in parenthesis present the number of stems inserted in a compost-soil mixture.

Figure 3-3. The growth of MG5 several months after transplanting plantlets.

3-3-2 Study 2: Test for tractor tyre stress

There was no major difference between the 2 grasses regarding Grand total DM yield over 2 years (Table 3-2). However, tyre stress depressed ($P < 0.05$) Total DM yield in 2007 and Grand total DM yield over 2 years in MG5, while Katambora was generally unaffected by tyre stress.

Table 3-2. Effects of tractor tyre stress on dry matter yield of 2 tropical grasses during 2 years (kg/10a).

Date		*Urochloa brizantha* cv. MG5		*Chloris gayana* cv. Katambora	
		Control	Tyre stress	Control	Tyre stress
2006	16-Aug	750ab	660bc	792a	643c
	5-Oct	484a	483a	503a	476a
	19-Dec	330a	312b	423a	405a
Total 2006		1564ab	1455b	1718a	1524ab
	21-May	735a	420b	893a	828a
	15-Aug	1255a	871b	812b	796b
	29-Oct	723a	436b	556b	491b
Total 2007		2683a	1727c	2261b	2115bc
Grand total		4247a	3182c	3979ab	3639bc

Means followed by different letters within each row differ significantly according to Fisher's least significant difference test ($P < 0.05$).

3-3-3 Conclusion

It appears that under the conditions of Okinawa, cutting stems of MG5 low to the ground and inserting them immediately into a soil-compost mixture in a glass-house will result in successful production of plantlets. While tractor tyre stress did not influence DM yields of Katambora severely, impact of tractor tyres markedly lowered yields of MG5, especially in the second year. However, total DM yield of MG5 under tractor tyre stress for the 2 years was not significantly different from that of Katambora, which indicates the production capacity of MG5.

3-4 Discussion

The standard method for vegetative propagation of grasses in Okinawa is to cut stems (culms) to retain 2 joints (nodes), the cuttings then being inserted into a mixture of soil and potting mix (Mochizuki et al. 2005). With MG5 this method normally results in a success rate of about 10% of plantlets being produced (T. Hanagasaki unpublished data). Results have been similar when a commercial rooting accelerator (TGG010S or TGG020S, both from the auxin group; Tokai Global Greening Co. Ltd., Gifu, Japan) was used, indicating that treatment with that plant hormone had no effect on rooting of MG5 cuttings. In a comparison trial, success rate for MG5 (18%) has been lower than those for other *Urochloa* species and cultivars (*U. brizantha* cv. Marandu at 31%, *U. decumbens* at 28%, *U. ruziziensis* at 52% and *U. humidicola* at 56%) (T. Hanagasaki unpublished data). However, in the current study both methods to produce rooted cuttings resulted in a satisfactory percentage of plantlets. And MG5 generally grew rapidly with both methods of transplanting. While this methodology is acceptable for small areas, for planting large areas there is a need to locate a source of commercial seed free of contamination by soil particles and thus can be safely imported. Considering that MG 5 is a fairly erect-growing tussock grass with short rhizomes and Katambora is stoloniferous (although it can attain an erect growth habit in a dense pasture), this finding is in general agreement with the observations of Honda and Yamanobe (1958), who reported that tractor tyre stress generally markedly suppressed growth of erect grasses but could have favorable impact on sod-forming grasses, if subjected to stress on only few occasions separated by reasonable intervals. Hosono et al. (1965) reported that forage yield of Italian ryegrass decreased as the number of transits increased (0, 1, 3 and 5 times). Tractor tyre stress could be a concern where material is harvested as hay using heavy tractors and balers but it would not be a significant issue under cut-and-carry or grazing systems. Furthermore, in a practical situation the impact would probably be reduced as the total area of pasture

is not normally affected by each operation in the haymaking process. However, soil compaction, which was not considered in this study, should also be taken into account.

3-5 Abstract

Feeding of high quality grass is critical to ensure breeding cows remain healthy with high reproductive rates and growing and fattening cattle achieve good growth rates. The Brazilian grass cultivar, *Urochloa brizantha* cv. MG5 Vitória, is highly nutritious and is known for its drought tolerance. In view of its low seed production potential in subtropical Japan and of phytosanitary problems (contamination with soil particles) of imported seed, a study was conducted in Okinawa to assess 2 methods of propagating this cultivar vegetatively. Cutting stems (culms) at about 10 cm from ground level and inserting them 3 cm into a 50:50 compost:soil mixture produced a 77% success rate in terms of rooted plantlets in a glasshouse compared with 67% for cutting the culm at 3 nodes from the base, subsequently allowing 2 weeks for adventitious roots to form on the lowest node, then cutting below the node where roots emerged and planting the rooted propagule in the same mixture. It seems that the simple process of cutting stems at about 10 cm from ground level and inserting them into a suitable mixture of soil and compost should result in an acceptable yield of plantlets for establishment of an MG5 forage crop. However, locating a source of high-quality seed free of phytosanitary problems would seem to be a better solution to increase the areas in Okinawa planted to MG5. In the tractor tyre stress trial conducted over 2 years, an MG5 forage crop established from seed showed depressed yields on the treatment subjected to tractor tyre pressure but performed as well as *Chloris gayana*, a much-used forage grass in Okinawa.

Chapter 4

Identification and characterization of lactic acid bacteria associated with tropical grass silage produced in Okinawa

4-1 Introduction

Farmers produce and store silage to feed cows during times when pasture growth is slow, such as the winter season in Okinawa. Lactic acid bacteria (LAB) are the key to producing high quality silage (Cai 2001) because good preservation depends on the production of sufficient organic acids to inhibit the growth of undesirable microorganisms, such as spoilage bacteria, food-borne pathogens, yeasts and molds, under anaerobic conditions (Li and Nishino 2011; Dunière et al. 2013). In general, silages made from tropical grasses have higher pH than silages from cool temperate grasses due to lower lactic acid or higher acetic acid and butyric acid concentrations (Panditharatne et al. 1986). This could result from low water-soluble carbohydrate (WSC) concentrations and limited populations of LAB in the silage (Cai 2001). In order to improve the fermentation quality of silage, it is necessary to understand the characteristics of silages stored in Okinawa and the fermentation processes which occur. Then steps can be taken to modify the fermentation process. It is obvious that LAB play a critical role in producing good quality silages with low pH values (Cai 2001). Moreover, LAB influence not only the quality of silage but also nutrition and metabolism in cattle because some LAB can play an important role as probiotics with beneficial outcomes for farm animals (Perdigon et al. 1995). In fact, it is well established that some LAB improve the intestinal microflora and promote the growth and health of animals (Mitsuoka 1990; Perdigon et al. 1995). The environment in the Prefecture of Okinawa, consisting of many islands in the subtropical region, is unique and microflora in the area could also be unique. Consequently, creating good silages in Okinawa could also be a unique process, different from other regions. As such,

determining the types of microorganisms native to Okinawa and their culturing for silage making are really important. However, at present, little is known about LAB related to silages made of tropical grass grown in Okinawa (Hanagasaki and Cai 2009). To shed light on this issue, we analyzed the fermentation characteristics of silage and identified LAB involved in the fermentation process in silages made of tropical grasses and other crops grown in the Main Island and Ishigaki Island, Okinawa.

4-2 Materials and Methods

Our study was based on 2 types of silage: (i) silage prepared in the field (round bale silage); and (ii) silage prepared in the laboratory.

4-2-1 Round bale silage preparation

Round bale silages from: (i) Transvala grass; (ii) a mixture of Rhodes grass and Para grass; (iii) sorghum; and (iv) corn were obtained in the field at several sites in the Okinawa Prefecture (Figure 4-1).

4-2-1-1 Transvala.

Forage of *Digitaria eriantha* cv. Transvala was obtained from an experimental field at Okinawa Livestock Research Center (Nakijin, Okinawa, Japan) on 9 May 2007 (Figure 4-1). The material was drawn from the third harvest in 2007 and the grass had regrown for 40 days following the second harvest. The process of producing round bale silage was as follows: Transvala forage was mowed, then aerated using a tedder rake and allowed to dry for 1 day. The wilted material was then converted into approximately 580 kg round bales using a roll baler (Rollant 46, CLAAS, Harsewinkel, Germany) according to manufacturer's instructions. Bales were then wrapped with white polyethylene film and stored for 37 days (Silage A) or 61 days (Silage B) upright on their flat base. On each of

15 June and 9 July 2007, a single bale was opened and 500 g samples for analysis were collected from the top, middle and bottom of the bale. Bales were about 100 cm tall and samples were drawn at about 25 cm from the top, 50 cm from the top and 25 cm from the bottom in about the center of the bale, by removing outer layers of silage and extracting material close to the center.

4-2-1-2 Grass mix

A mixed pasture of Rhodes grass (*Chloris gayana*) and Para grass (*Urochloa mutica*, syn. *Brachiaria mutica*) (1:1) on a farm in Ishigaki Island (Tonoshiro, Ishigaki) (Figure 4-1), which had regrown for 60 days from the previous harvest, was mowed, aerated using a tedder rake and allowed to dry for 1 day. The height of grass was about 80 cm and vegetative growth was virtually complete but pasture was not wilting. Round bale silage was produced using a roll baler (RF130, VICONJAPAN, Saitama, Japan) according to manufacturer's instructions, including wrapping in white polyethylene film. Bales were stored upright for 105 days, until 8 August 2007, when a bale was opened and a 500 g sample for analysis was collected from the center of the bale at about 50 cm from the top.

4-2-1-3 Sorghum

Sorghum (*Sorghum vulgare*, BMR Sweet) on a farm in Ishigaki Island (Ohama, Ishigaki) (Figure 4-1) was mowed at 79 days after sowing, aerated using a tedder rake and allowed to dry for 2 days. Round bale silage was produced using a roll baler (Rollant 46, CLAAS) according to manufacturer's instructions, including wrapping in white polyethylene film. Bales were stored upright for 43 days until 9 August 2007, when a bale was opened and a 500 g sample for analysis was collected from the center of the bale at about 50 cm from the top.

4-2-1-4 Corn

Corn (*Zea mays*) on a farm in Ishigaki Island (Ohama, Ishigaki) (Figure 4-1) was mowed at 84 days after sowing, aerated using a tedder rake and allowed to dry for 2 days. Roll bale silage was produced using a roll baler (Rollant 46, CLAAS) according to manufacturer's instructions including wrapping in white polyethylene film. Bales were stored upright for 38 days until 9 August 2007, when a bale was opened and a 500 g sample for analysis was collected from the center of the bale at about 50 cm from the top.

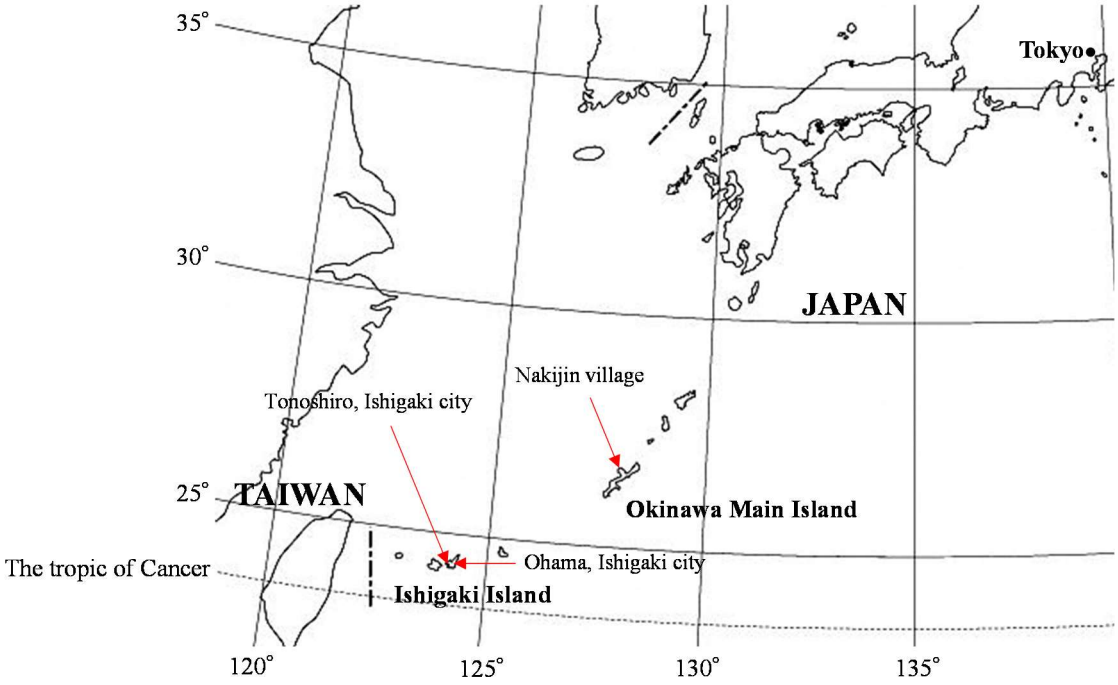

Figure 4-1. Map of Okinawa indicating where the field silage used in this study was produced.

4-2-2 Laboratory silage preparation

Laboratory silages were prepared using a small-scale system of fermentation (Tanaka and Ohmomo 1994) with material from a third grass harvest in 2007 (40 days following the second harvest). Approximately 100 g portions of material of 2 grasses, Transvala and *Panicum maximum* (now *Megathyrsus maximus*) cv. Natsuyutaka, were chopped into about 20 mm length and packed into 3 nylon and polyethylene bags (Hiryu KN type, 180 × 260 cm; Asahi Kasei Corp., Tokyo, Japan). Air was withdrawn and bags were sealed with a vacuum sealer (BH 950; Matsushita Co. Ltd., Tokyo, Japan). After 4, 9 and 30 days of storage at 25 °C, a bag was opened and 3 samples per storage day treatment were taken for chemical and microbiological analysis. Table 4-1 presents a summary of the different silages used in the study.

Table 4-1. Summary of silages produced for analysis in this study.

Silage	Round bale silage									Laboratory silage						
Material	Transvala						Grass mix[1]	Sorghum	Corn	Transvala			Natsuyutaka			
Mowing place	Nakijin						Tonoshiro	Ohama		Nakijin						
Storage days	37[2]			61[3]			105	43	38	4	9	30	4	9	30	
Sampling part[4]	T	M	B	T	M	B		M		All in the pack						

[1]Mixture of Rosegrass and Paragrass (1:1); [2]Silage A in the text; [3]Silage B in the text; [4]T = Top, M = Middle, B = Bottom.

4-2-3 Chemical analysis

The DM concentration of fresh material and silages was determined by the removal of moisture using toluene distillation with ethanol correction (Dewar and McDonald 1961). The organic acid concentrations were measured by high-performance liquid chromatography (JASCO Corp., Tokyo, Japan) using Shodex Rspak KC-811 column (8 × 300 mm; Showa Denko K.K., Tokyo, Japan). Concentration of ammonia-nitrogen (ammonia-N) was determined by the Kjeltec system (Kjeltec Auto Sampler System 1035 Analyzer, Tecator, Hoganas, Sweden).

4-2-4 Microbiological analysis

Numbers of microorganisms were measured by the plate count method (Yamazato et al. 1986). Samples (10 g) of silage were blended with 90 mL of sterilized distilled water and 10^{-1} to 10^{-8} serial dilutions were made in sterilized distilled water. Analyses on samples were performed in triplicate. From each dilution, 0.1 mL of suspension was spread on agar plates. LAB were counted on MRS agar plates (DIFCO Laboratories, Michigan, USA) after incubating in an anaerobic box (TE-HER Hard Anaerobox, ANX-1; Hirasawa Ltd., Tokyo, Japan) at 30 °C for 2 days. Aerobic bacteria and *Escherichia* spp. were counted on nutrient agar and blue light agar plates (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan), respectively, after incubating at 30 °C for 2 days. Yeast and mold were counted on potato dextrose agar plates (Nissui Pharmaceutical Co. Ltd.) with pH 3.5 by adding 10% (w/v) tartaric acid, after incubating at 30 °C for 2 days. Molds were distinguished from yeast by colony appearance and cell morphological observation. For bacilli, the suspension was heated at 75 °C for 15 min before spreading on nutrient agar plates, which were incubated at 30 °C for 2 days, when bacilli were counted. For clostridia, the suspension was heated at 75 °C for 15 min before spreading on clostridia count agar plates (Nissui Pharmaceutical Co. Ltd.) and incubated in an anaerobic box at 30 °C for 2 days,

when clostridia were counted. Colonies were counted as viable numbers of microorganisms [log colony-forming units (log cfu)/g fresh matter (FM)]. For the next test for LAB, the cultivated LAB, following the counting described above, were purified on another MRS agar plate.

4-2-5 Morphological, physiological and biochemical tests

Morphology (strain shape of rod or cocci) and Gram stain were examined after 24 h of incubation on MRS agar plates. Catalase activity and gas production from glucose (hetero- or homo-fermentative) were determined according to Kozaki et al. (1992). The isomers of lactic acid that bacteria formed from glucose were determined by enzymatic analysis using a UV method (F-kit, D-lactic/L-lactic acid; Boehringer Manheim GmbH, Mannheim, Germany). Carbohydrate assimilation and fermentation of 49 different compounds (plus a Control) were conducted using API 50 CH strips (bioMerieux Japan Ltd., Tokyo, Japan).

4-2-6 16S ribosomal DNA (rDNA) sequencing

Cells grown for 8 h in MRS broth at 30 °C were used for DNA extraction and purification (Saitou and Miura 1963). Genome DNA was extracted from cells after enzymatic and detergent digestion. The 16S rDNA region was amplified by polymerase chain reaction (PCR) performed in a PCR Thermal Cycler (GeneAmp PCR System 9700; Applied Biosystems, Waltham, USA) using the prokaryotic 16S rDNA universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′- GGTTACCTTGTTACGACTT-3′) with a Takara Taq PCR Kit (Takara Shuzo Co. Ltd, Shiga, Japan) by the PCR method according to Suzuki et al. (1969). Sequencing was performed twice on both strands by the dideoxy method (Sanger et al. 1977), using a PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) in combination with an Applied

Biosystems model 310A automated sequencing system.

4-2-7 Sequence alignments and phylogenetic inference

Sequence similarity searches were performed in the GenBank data library using the BLAST program. Nucleotide substitution rates (Knuc values) were calculated (Kimura and Ohta 1972) and the phylogenetic tree was constructed by the neighbor joining method (Saitou and Nei 1987) using the CLUSTAL W software program (Hitachi Software Engineering Co. Ltd., Tokyo, Japan) for assembly and alignment. The topologies of trees were evaluated by bootstrap analysis of the sequence data with CLUSTAL W software based on 100 random resamplings (Thompson et al. 1994).

4-3 Results

4-3-1 Fermentation quality and microbiological analysis

The DM concentration average for Silage A was about 35% but values for Silage B differed for different sampling points (Table 4-2), decreasing from top (42.9%) to bottom (33.6%) of the bale, i.e. moisture drained to the bottom of the bale. The pH values for all samples of Silage A were 4.77, while values for Silage B were 5.08–5.28. Lactic acid concentrations of Silage A and Silage B were 0.27–0.39% and 0.03–0.22% of fresh matter (FM), respectively. Ammonia-N concentrations of Silage A and Silage B were 0.44–0.53% and 0.48–1.06% of FM, respectively, while butyric acid concentrations of Silage A and Silage B were 0.05–0.08% and 0.16–0.19%. Silage A had 6.7–6.9 log cfu LAB/g FM, more than Silage B with 4.8–6.8 log cfu LAB/g. Clostridia were detected in Silage B but not in Silage A. There were nd–4.8 log cfu yeast/g, 3.9–4.5 log cfu aerobic bacteria/g, 2.3–3.9 log cfu bacilli/g, nd–2.5 cfu *Escherichia*/g and no mold in both silages. Regarding the 3 round bale silages in Ishigaki, grass silage had the highest ammonia-N and the lowest lactic acid concentrations, while corn silage had the lowest ammonia-N and the highest lactic acid concentration (Table 4-3). LAB concentrations were highest in corn silage and lowest in grass silage. There were nd–5.5 log cfu yeast/g, 3.1–4.6 log cfu aerobic bacteria/g, 2.5–3.6 log cfu bacilli/g and nd–1.5 log cfu *Escherichia*/g but no mold and no clostridia in the 3 silages. Laboratory silages had high ammonia-N and low lactic acid concentrations (Table 4-4). Lactic acid concentration was 0.09% FM by 9 days of storage but could not be detected at 30 days, while ammonia-N continued to increase during storage. This means that the quality of both laboratory silages deteriorated during storage for 30 days. However, LAB populations increased from about 3 log cfu/g to about 7 log cfu/g in only 4 days and then decreased slightly to about 6 log cfu/g at 30 days of storage. There were nd–3.2 log cfu yeast/g, 4.9–7.5 log cfu aerobic bacteria/g, 4.0–5.8 log cfu bacilli/g, 4.5–6.9 log cfu *Escherichia*/g, 1.3–3.0 log cfu mold/g and nd–1.0 log

cfu clostridia/g in both laboratory silages.

Table 4-2. Fermentation quality and microbiological analysis of Transvala round bale silages in Nakijin.

	Fresh material	SilageA stored for 37 days			SilageB stored for 61 days		
		Top	Center	Bottom	Top	Center	Bottom
Fermentation quality							
DM (% FM[1])	25.8	35.1	35.0	35.3	42.9	40.9	33.6
pH	5.56	4.77	4.77	4.77	5.21	5.08	5.28
Lactic acid (% FM)	nd[2]	0.39	0.27	0.36	0.21	0.22	0.03
Acetic acid (% FM)	nd	0.15	0.14	0.11	0.22	0.04	0.18
Propionic acid (% FM)	nd	nd	nd	nd	0.05	nd	0.05
Butyric acid (% FM)	nd	0.05	0.08	0.05	0.16	0.19	0.18
Ammonia N (% FM)	0.04	0.53	0.49	0.44	1.06	0.48	0.67
Microorganism composition (log colony-forming units per gram of FM)							
Lactic acid bacteria	3.00	6.90	6.86	6.76	4.83	6.79	5.38
Clostridia	nd	nd	nd	nd	1.78	1.48	3.20
Escherichia	6.68	nd	2.00	1.00	nd	2.45	nd
Mold	3.90	nd	nd	nd	nd	nd	nd
Yeast	3.78	4.83	4.00	4.59	nd	4.67	3.50
Aerobic bacteria	7.04	4.48	4.30	4.46	3.90	4.15	4.51
Bacilli	4.83	2.90	2.78	2.85	3.43	2.34	3.89

[1]FM: fresh matter (green forage or silage); [2]nd: not detected.

Table 4-3. Fermentation quality and microbiological analysis of round bale silages made from grass, sorghum and corn in Ishigaki.

	Grass mix[1]	Sorghum	Corn
Fermentation quality			
DM（% FM[2]）	46.5	34.9	36.5
pH	5.25	4.68	4.43
Lactic acid（% FM）	0.14	0.42	0.60
Acetic acid（% FM）	0.06	0.09	0.06
Propionic acid（% FM）	nd[3]	nd	nd
Butyric acid（% FM）	nd	nd	nd
Ammonia N（% FM）	0.44	0.11	0.04
Microorganism composition（log colony-forming units per gram of FM）			
Lactic acid bacteria	4.59	5.23	6.28
Clostridia	nd	nd	nd
Escherichia	1.48	nd	nd
Mold	nd	nd	nd
Yeast	nd	4.38	5.48
Aerobic bacteria	3.08	4.60	4.36
Bacilli	2.48	3.26	3.59

[1]Mixture of Rhodes grass and Para grass (1:1); [2]FM: silage fresh matter; [3]nd: not detected.

Table 4-4. Fermentation quality and microbiological analysis of fresh grass and laboratory silage.

	Transvala from Nakijin				Natsuyutaka from Nakijin			
	Fresh material	Laboratory silage (storage day)			Fresh material	Laboratory silage (storage day)		
		4	9	30		4	9	30
Fermentation quality								
DM (% FM[1])	25.8	25.5	23.2	24.5	24.9	25.1	25.5	23.6
pH	5.56	5.68	5.49	5.67	6.60	5.97	5.95	5.41
Lactic acid (% FM)	nd[2]	0.08	0.09	nd	nd	0.05	nd	nd
Acetic acid (% FM)	nd	0.10	0.17	0.22	nd	0.20	0.33	0.67
Propionic acid (% FM)	nd	nd	0.01	0.03	nd	nd	0.01	0.09
Butyric acid (% FM)	nd	nd	0.07	0.39	nd	nd	0.07	0.37
Ammonia N (% FM)	0.04	0.25	0.33	0.77	0.20	0.60	0.79	1.07
Microorganism composition (log colony-forming units per gram of FM)								
Lactic acid bacteria	3.00	7.30	7.30	6.20	3.53	7.04	7.30	6.08
Clostridia	nd	1.00	nd	1.00	nd	nd	nd	nd
Escherichia	6.68	6.85	6.08	5.38	5.30	7.23	7.34	4.45
Mold	3.90	2.41	2.70	2.48	3.48	3.04	2.28	1.30
Yeast	3.78	1.70	nd	3.23	3.61	nd	nd	nd
Aerobic bacteria	7.04	7.11	6.26	6.04	6.90	7.38	7.46	4.93
Bacilli	4.83	5.77	4.00	4.85	4.00	4.40	4.81	4.18

[1]FM: fresh matter (grass or silage); [2]nd: not detected.

4-3-2 Physiological properties of isolated LAB

The physiological properties of 37 of the presumptive LAB strains isolated in this study are shown in Tables 4-5, 4-6 and 4-7. Okn1 to Okn8 were isolated from Silage A and Okn9 to Okn14 from Silage B, while Okn15 was isolated from fresh Transvala and Okn23 to Okn26 from fresh Natsuyutaka. Okn16 to Okn22 were isolated during storage of the Transvala laboratory silage and Okn27 to Okn29 during storage of the Natsuyutaka laboratory silage; Okn30 to Okn32 were isolated from the grass mix silage, Okn33 to Okn35 from sorghum silage and Okn36 and Okn37 from corn silage. All isolates were Gram-positive strains and most were catalase-negative, but Okn4, Okn6, Okn23, Okn27, Okn28 and Okn33 were catalase-positive. According to morphological and biochemical characters as well as isolation sources, the 37 strains were divided into 16 groups (A–P). Strains in Groups A, E and F were homo-fermentative rods, which did not produce gas from glucose and formed DL-lactic acid. In contrast, strains in Groups C, G and H were hetero-fermentative cocci, which produced gas from glucose and formed D(-)-lactic acid. Strains in Groups D and K were homo-fermentative cocci, which did not produce gas from glucose and formed L(+)-lactic acid, while the strain in Group B was homo-fermentative rods, which did not produce gas from glucose and formed L(+)- lactic acid. Strains in Groups I and J were homo-fermentative cocci, which did not produce gas from glucose and formed L(+)-lactic acid. Strains in Groups L and N were homo-fermentative rods, which did not produce gas from glucose, while strains in Groups M and O were hetero-fermentative rods, which did produce gas from glucose, and the strain in Group P was homo-fermentative cocci, which did not produce gas from glucose. All representative strains sequenced with 16S rDNA in each group showed high-sequence homology values (100% or almost 100%) with the type LAB strain.

Table 4-5. Characteristics and 16S rDNA sequence similarity with each type strain of lactic acid bacteria isolated from Transvala round bale silages in Nakijin

Characteristics	Group A				Group B	Group C							Group D	
	Okn1	Okn2	Okn9	Okn10	Okn3	Okn4	Okn5	Okn6	Okn7	Okn11	Okn12	Okn13	Okn8	Okn14
Source	Silage A	Silage A	Silage B	Silage B	Silage A	Silage A	Silage A	Silage A	Silage A	Silage B	Silage B	Silage B	Silage A	Silage B
Shape	Rod	Rod	Rod	Rod	Rod	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Gram stain	+[1]	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase activity	-[2]	-	-	-	-	+	-	+	-	-	-	-	-	-
Fermentation type	Homo	Homo	Homo	Homo	Homo	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Hetero	Homo	Homo
Optical isomer of lactate	DL	DL	DL	DL	L (+)	D (-)	D (-)	D (-)	D (-)	D (-)	D (-)	D (-)	L (+)	L (+)
16S rDNA sequence similarity[4]	100	na[3]	100	na	100	99.6	na	na	99.9	99.8	na	na	100	100
Identified species	*Lactobacillus plantarum*				*Lactobacillus acidipiscis*	*Weissella paramesenteroides*							*Pediococcus pentosaceus*	

[1]+: positive reaction; [2]-: negative reaction; [3]na: not analyzed; [4]Sequence similarity with each type strain (%).

Table 4-6. Characteristics and 16S rDNA sequence similarity with each type strain of lactic acid bacteria strains isolated from fresh material and the laboratory silages

Characteristics	Group E	Group F			Group G		Group H	Group I					Group J		Group K
	Okn16	Okn23	Okn27	Okn28	Okn24	Okn17	Okn20	Okn15	Okn18	Okn19	Okn25	Okn21	Okn26	Okn29	Okn22
Source of grass	Tran[1]	Nats[2]	Nats	Nats	Nats	Tran	Tran	Tran	Tran	Tran	Nats	Tran	Nats	Nats	Tran
Storage day	4	0	4	9	0	4	9	0	4	4	0	9	0	9	9
Shape	Rod	Rod	Rod	Rod	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Gram stain	+[3]	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase activity	-[4]	+	+	+	-	-	-	-	-	-	-	-	-	-	-
Fermentation type	Homo	Homo	Homo	Homo	Hetero	Hetero	Hetero	Homo	Homo	Homo	Homo	Homo	Homo	Homo	Homo
Optical isomer of lactate	DL	DL	DL	DL	D(-)	D(-)	D(-)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)	L(+)
16S rDNA sequence similarity[6]	100	99.8	100	na[5]	99.7	100	99.9	100	na	na	99.9	na	100	na	100
Identified species	*Lactobacillus plantarum*	*Lactobacillus paraplantarum*			*Weissella paramesenteroides*		*Weissella kimchii*	*Lactococcus lactis* subsp. lactis					*Lactococcus garvieae*		*Pediococcus pentosaceus*

[1]Tran: Transvala; [2]Nats: Natsuyutaka; [3]+: positive reaction; [4]-: negative reaction; [5]na: not analyzed; [6]Sequence similarity with each type strain (%).

Table 4-7. Characteristics and 16S rDNA sequence similarity with each type strain of lactic acid bacteria isolates isolated from the round bale silages in Ishigaki

Characteristics	Group L		Group M	Group N			Group O	Group P
	Okn30	Okn33	Okn31	Okn34	Okn35	Okn36	Okn37	Okn32
Source	Grass[1] silage	Sorghum silage	Grass silage	Sorghum silage	Sorghum silage	Corn silage	Corn silage	Grass silage
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Cocci
Gram stain	+[2]	+	+	+	+	+	+	+
Catalase activity	-[3]	+	-	-	-	-	-	-
Fermentation type	Homo	Homo	Hetero	Homo	Homo	Homo	Hetero	Homo
16S rDNA sequence similarity[4]	99.9	99.9	99.9	100	100	99.9	99.9	100
Identified species	*Lactobacillus plantarum*		*Lactobacillus brevis*	*Lactobacillus casei*			*Lactobacillus fermentum*	*Pediococcus pentosaceus*

[1]Mixture of Rhodes grass and Para grass (1:1); [2]+: positive reaction; [3]-: negative reaction; [4]Sequence similarity with each type strain (%).

4-3-3 Carbohydrate fermentation assays using API 50 CH strips

From the result of API 50 CH strips, all strains produced acid from glucose but groups displayed a distinct carbohydrate fermentation pattern. Groups A, E and L had almost the same fermentation patterns, especially using L-arabinose and melezitose (Tables 4-8, 4-9 and 4-10). Group F used α-methyl-D-glucoside, lactose and melibiose. Groups C and G had a similar pattern, especially using α-methyl-D-glucoside and saccharose. Groups D, K and P had the same pattern, especially using L-arabinose, β-gentiobiose and D-tagatose. Group B used glycerol, mannitol and α-methyl-D-glucoside, Group H used amygdalin and gluconate, Group I used D-xylose and β-gentiobiose, Group J used β-gentiobiose and D-tagatose, Group M used D-xylose, β-methyl-xyloside and α-methyl-D-glucoside, Group N used arbutine and β-gentiobiose and Group O used L-sorbose and D-tagatose.

Table 4-8. API50 CH fermentation patterns of lactic acid bacteria strains isolated from Transvala round bale silages in Nakijin

Carbohydrate	Group A				Group B	Group C							Group D	
	Okn1	Okn2	Okn9	Okn10	Okn3	Okn4	Okn5	Okn6	Okn7	Okn11	Okn12	Okn13	Okn8	Okn14
Glycerol	-[1]	-	-	-	w[3]	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+[2]	+	+	+	+	+	+	+	+	+	+	+	+	+
Ribose	+	+	+	+	+	w	+	+	+	w	+	+	+	+
D-Xylose	-	-	-	-	-	+	+	+	+	+	+	+	+	-
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	+	+	w	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	w	+	w	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	-	-	+	-	-	-	-	-	-	-	-	+
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	+	+	+	+	-	-	w	-	-	-	-	-	-
Sorbitol	+	+	+	+	-	-	-	-	-	-	-	-	-	-
α-Methyl-D-mannoside	+	+	+	+	+	-	-	-	-	-	-	-	-	-
α-Methyl-D-glucoside	-	-	-	-	+	+	+	+	w	+	+	+	-	-
N-Acetyl-glucosamine	+	+	+	+	+	w	w	w	+	+	+	+	+	+
Amygdaline	+	+	+	+	+	-	-	-	-	-	-	-	+	+
Arbutine	+	+	+	+	+	-	-	-	-	-	-	-	+	+
Esculine	+	+	+	+	+	-	-	-	-	-	-	+	+	+
Salicine	+	+	+	+	+	w	-	w	-	-	-	w	+	+
Cellobiose	+	+	+	+	+	+	-	+	-	w	w	+	+	+
Maltose	+	+	+	+	w	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	-	-	-	-	-	-	-	+	-
Melibiose	+	+	+	+	-	+	+	+	w	+	+	+	+	-
Saccharose	+	+	+	+	-	+	+	+	w	+	+	+	+	-
Trehalose	+	+	+	+	+	+	+	+	w	+	+	+	-	+
Inuline	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	+	+	+	+	-	-	-	-	-	-	-	-	-	-
D-Raffinose	-	w	+	+	-	-	-	-	-	-	-	-	+	-
Amidon	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycogene	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Gentiobiose	w	w	-	+	w	w	-		-	w	w	w	w	w
D-Turanose	+	+	+	-	-	+	-	+	-	+	w	+	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-	+	+
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	w	-	-	w	-	+	-	w	w	-	-	-
2-keto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5-keto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1]-: negative reaction; [2]+: positive reaction; [3]w: weakly positive

Table 4-9. API50 CH fermentation patterns of lactic acid bacteria strains isolated from grass material and the laboratory silages

Carbohydrate	Group E		Group F		Group G		Group H	Group I					Group J		Group K
	Okn16	Okn23	Okn27	Okn28	Okn24	Okn17	Okn20	Okn15	Okn18	Okn19	Okn25	Okn21	Okn26	Okn29	Okn22
Glycerol	-[1]	w[3]	w	w	-	-	-	-	-	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabinose	+[2]	-	-	-	+	+	w	-	+	+	+	-	-	-	+
Ribose	+	+	+	+	w	+	-	+	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	-	+	w	+	+	+	+	+	-	-	+
L-Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Methyl-xyloside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Galactose	+	+	+	+	w	+	-	+	+	+	+	+	w	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	w	+	+	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	w	+	+	+	+	+	+	+	+	+	+
L-Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rhamnose	w	w	w	w	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol	+	-	+	+	-	-	-	-	-	+	+	+	+	+	-
Sorbitol	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-
α-Methyl-D-mannoside	w	w	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Methyl-D-glucoside	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-
N-Acetyl-glucosamine	+	+	+	+	w	+	w	+	+	+	+	+	+	+	+
Amygdaline	+	+	+	+	-	-	w	+	+	+	+	+	+	+	+
Arbutine	+	+	+	+	-	-	w	+	+	+	+	+	+	+	+
Esculine	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
Salicine	+	+	+	+	-	-	w	+	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	-	-	-	w	+	+	+	w	-	-	-
Melibiose	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
Saccharose	+	+	+	+	+	w	+	-	+	+	+	+	-	-	-
Trehalose	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
Inuline	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Melezitose	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Raffinose	+	+	+	+	w	-	-	-	-	-	-	-	-	-	-
Amidon	-	-	-	-	-	-	-	-	w	w	+	w	w	-	-
Glycogene	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Gentiobiose	w	w	w	w	-	w	w	w	+	+	w	+	w	+	w
D-Turanose	+	-	-	-	w	-	-	-	-	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-	-	-	-	-	-	-	w	+	+
D-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D-Arabitol	w	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate	-	-	w	w	w	-	w	-	w	w	w	w	-	-	-
2-keto-gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5-keto-gluconate	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-

[1]-: negative reaction; [2]+: positive reaction; [3]w: weakly positive

Table 4-10. API50 CH fermentation patterns of lactic acid bacteria strains isolated from the round bale silages in Ishigaki

Carbohydrate	Group L		Group M	Group N			Group O	Group P
	Okn30	Okn33	Okn31	Okn34	Okn35	Okn36	Okn37	Okn32
Glycerol	-[1]	w[3]	-	-	-	-	-	-
Erythritol	-	-	-	-	-	-	-	-
D-Arabinose	-	-	-	-	-	-	-	-
L-Arabinose	+[2]	+	+	-	-	+	-	+
Ribose	+	+	+	+	+	+	+	+
D-Xylose	-	-	+	-	-	+	-	-
L-Xylose	-	-	-	-	-	-	-	-
Adonitol	-	-	-	+	+	-	-	-
β-Methyl-xyloside	-	-	+	-	-	-	-	-
Galactose	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	w	+	+
D-Mannose	+	+	-	+	+	-	+	+
L-Sorbose	-	-	-	+	+	-	+	-
Rhamnose	w	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-
Inositol	-	-	-	w	w	-	-	-
Mannitol	+	+	-	+	+	-	+	-
Sorbitol	+	+	-	+	+	-	+	-
α-Methyl-D-mannoside	-	+	-	-	-	-	-	-
α-Methyl-D-glucoside	-	-	+	w	w	-	w	-
N-Acetyl-glucosamine	+	+	w	+	+	+	+	+
Amygdaline	+	+	-	+	+	-	+	+
Arbutine	+	+	-	+	+	+	+	+
Esculine	+	+	-	+	+	-	+	+
Salicine	+	+	-	+	+	w	+	+
Cellobiose	+	+	-	+	+	+	+	+
Maltose	+	+	-	+	+	w	+	+
Lactose	+	+	-	-	-	-	-	-
Melibiose	+	+	w	-	-	w	-	-
Saccharose	+	+	-	w	w	w	+	-
Trehalose	+	+	-	+	+	+	+	+
Inuline	-	-	-	-	-	-	+	-
Melezitose	+	+	-	+	+	-	+	-
D-Raffinose	+	+	-	-	-	+	-	-
Amidon	-	-	-	-	-	-	-	-
Glycogene	-	-	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-	-	-
β-Gentiobiose	w	w	-	+	+	w	+	w
D-Turanose	-	+	-	+	+	-	+	-
D-Lyxose	-	-	-	-	-	-	-	-
D-Tagatose	-	-	-	+	+	-	+	+
D-Fucose	-	-	-	-	-	-	-	-
L-Fucose	-	-	-	-	-	-	-	-
D-Arabitol	-	-	-	-	-	-	-	-
L-Arabitol	-	-	-	-	-	-	+	-
Gluconate	-	w	-	-	-	-	w	w
2-keto-gluconate	-	-	-	-	-	-	-	-
5-keto-gluconate	-	-	-	-	-	-	-	-

[1]-: negative reaction; [2]+: positive reaction; [3]w: weakly positive

4-3-4 Phylogenetic tree based on 16S rDNA sequence

In an effort to identify Okn strains at the species level, molecular phylogenetic analysis was conducted and phylogenetic trees were produced based on the 16S rDNA sequences from evolutionary distances by the neighbor joining method (Figures 4-2 and 4-3). Following phylogenetic analysis, Okn1 and Okn9 (representative of Group A), Okn16 (Group E), Okn23 and Okn27 (Group F), Okn30 and Okn33 (Group L), Okn31 (Group M), Okn3 (Group B), Okn34, Okn35 and Okn36 (Group N) and Okn37 (Group O) were placed in a cluster, making up the genus *Lactobacillus*. Okn4, Okn7 and Okn11 (Group C), Okn17 and Okn24 (Group G) and Okn20 (Group H) made up the genus *Weissella*. Okn15 and Okn25 (Group I) and Okn26 (Group J) made up the genus *Lactococcus*, while Okn8 and Okn14 (Group D), Okn22 (Group K) and Okn32 (Group P) made up the genus *Pediococcus*. Closest related species for different Okn strains were as follows: Okn1 and Okn9 (Group A), Okn16 (Group E) and Okn30 and Okn33 (Group L) – *Lactobacillus plantarum*; Okn23 and Okn27 (Group F) – *L. paraplantarum*; Okn31 (Group M) –*L. brevis*; Okn3 (Group B) – *L. acidipiscis*; Okn34, Okn35 and Okn36 (Group N) – *L. casei*; Okn37 (Group O) – *L. fermentum*; Okn4, Okn7 and Okn11 (Group C) and Okn17 and Okn24 (Group G) – *Weissella paramensenteroides*; Okn20 (Group H) – *W. kimchii*; Okn15 and Okn25 (Group I) – *Lactococcus lactis* subsp. *lactis*; Okn26 (Group J) – *Lactococcus garvieae*; Okn8 and Okn14 (Group D), Okn22 (Group K) and Okn32 (Group P) – *Pediococcus pentosaceus*. According to a BLAST search, all representative strains in each group showed high-sequence homology values (100% or almost 100%) with the most closely related species in the phylogenetic tree. Therefore, strains in Groups A, E and L were identified as *Lactobacillus plantarum*, strains in Group F as *L. paraplantarum*, the strain in Group M as *L. brevis*, the strain in Group B as *L. acidipiscis*, strains in Group N as *L. casei*, the strain in Group O as *L. fermentum*, strains in Groups C and G as *Weissella paramesenteroides*, the strain in Group H as *W. kimchii*, strains in Group I as

Lactococcus *lactis* subsp. *lactis*, strains in Group J as *Lactococcus garvieae* and strains in Groups D, K and P as *Pediococcus pentosaceus*.

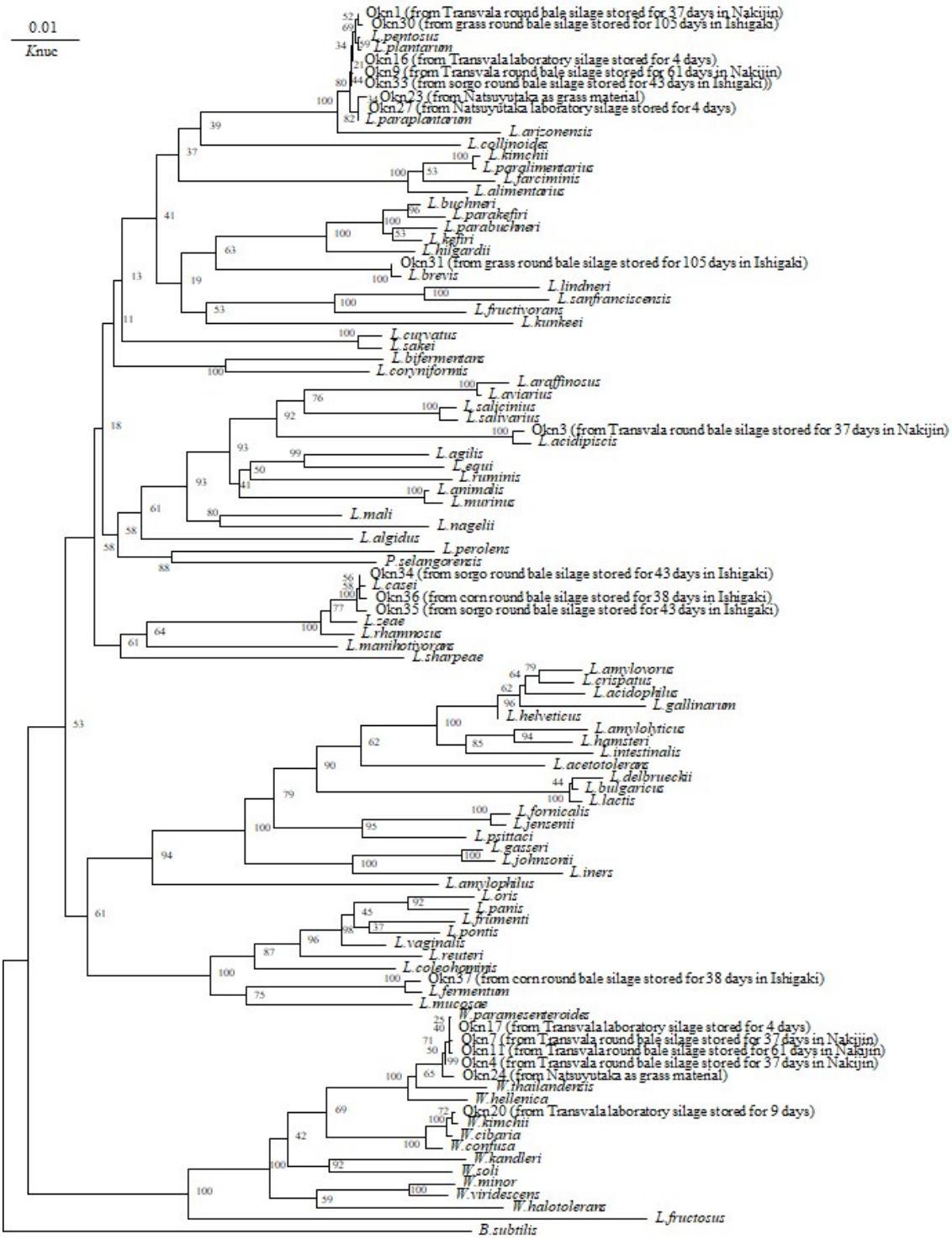

Figure 4-2. Phylogenetic tree showing the relative positions of isolates as inferred by the neighbor joining method of complete 16S rDNA sequence.

Bootstrap values for a total of 100 replicates are shown at the nodes of the tree. *Bacillus subtilis* is used as an outgroup. The bar indicates 1% sequence divergence. *L. Lactobacillus*; *W. Weissella*; Knuc, nucleotide substitution rate.

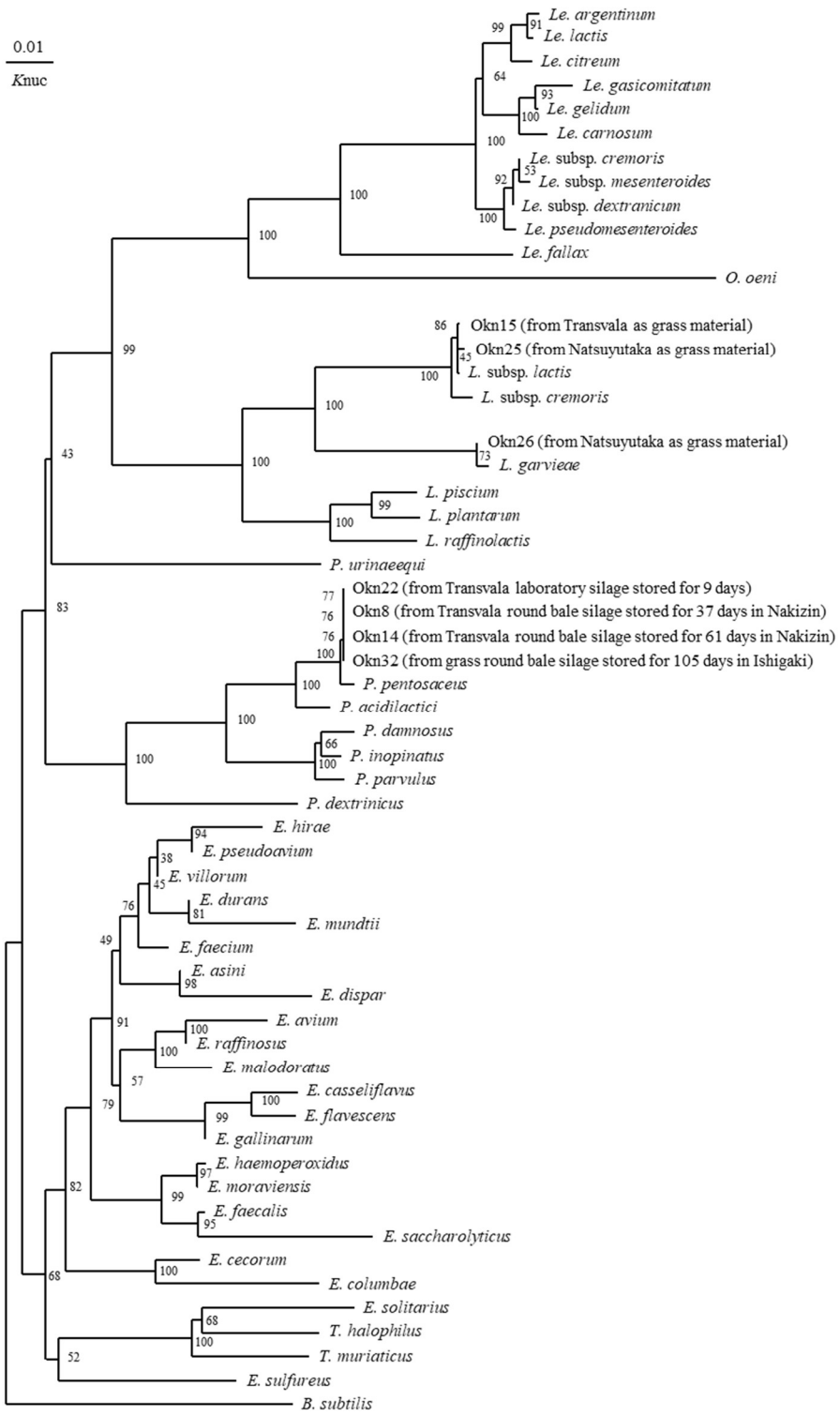

Figure 4-3. Phylogenetic tree showing the relative positions of isolates as inferred by the neighbor joining method of complete 16S rDNA sequence.

Bootstrap values for a total of 100 replicates are shown at the nodes of the tree. *Bacillus subtilis* is used as an outgroup. The bar indicates 1% sequence divergence. *Le. Leuconostoc*; *L. Lactococcus*; *P. Pediococcus*; *E. Enterococcus*; Knuc, nucleotide substitution rates.

4-4 Discussion

This study has revealed that silages made in Okinawa contain a very wide range of species of bacteria with differing fermentation characteristics. Silage A made of Transvala smelled really good even though the data for fermentation quality were not as good as for silages made of cool temperate grasses grown in the colder regions in Japan. In fact, lactic acid concentration in Italian ryegrass silage was twice that in Silage A (0.68 vs. 0.34% FM) and acetic acid concentration was two-and-a-half times that in Silage A (0.33 vs. 0.13% FM) (Cai et al. 1999a). Nevertheless, Silage A was considered to be of better quality than conventional silages made in subtropical regions. Tamaki et al. (2007) reported that Transvala was highly nutritious and could be a replacement for oats. The pH value of Silage A was lower than that of Silage B and the laboratory silages. Moreover, lactic acid in Silage B was lower than that in Silage A and the number of LAB in Silage B was scattered with 4.8–6.8 log cfu/g FM. The laboratory silages were low in quality and had an offensive smell like that of Silage B. Possible reasons were that evacuation of bags for these silages at ensiling was incomplete and the grass was not sufficiently dried. It is known that effective wilting of forage before ensiling may be the most important aspect in making good silage and ideal dry matter concentration of forage at ensiling is a minimum of 30% (Romero et al. 2015). On the contrary, in the case of over 40% DM, it is difficult to maintain anaerobic conditions and fermentation can be lower in quality (Romero et al. 2015). This rule could apply to Silage B with 33.6–42.9% DM resulting in unacceptable quality. When moisture concentration in forage is too high at ensiling, many kinds of microorganisms can proliferate before LAB grow sufficiently to reduce pH. In the laboratory silages, lactic acid concentration was low even though high numbers of LAB (about 7 log cfu/g) were detected. Furthermore, LAB detected in the laboratory silages were mainly cocci. LAB cocci are very important in the initial stages of fermentation, because they maintain an acidic environment, which is then colonized by

predominantly *Lactobacillus* (Tjandraatmadja et al. 1991; Ohmomo et al. 2002). While *Lactococcus* were present, they were unable to create an acidic environment and *Lactobacillus* was not the predominant flora during storage. Cai et al. (1999a) reported that, when *Lactobacillus* reaches a level of at least 5 log cfu/g FM, silage can be well preserved. *Lactobacillus* was obviously the predominant flora in Silage A, sorghum silage and corn silage, all of which were of acceptable quality. Many species in the genus *Lactobacillus*, e.g. *L. plantarum* (Cai 2001; Ohmomo et al. 2002), have been found in silages. In fact, *Lactobacillus* plays a more important role in fermentation processes and promotes effective lactic acid fermentation for longer than lactic acid-producing cocci (Cai et al. 1998; 1999b). Additionally, *L. plantarum* was the predominant species and was still active 100 days after ensiling tropical grass in work reported by Tjandraatmadja et al. (1991). In fact, *L. plantarum* 'Chikusou1gou' is produced on a commercial basis in Japan. Accordingly, it is necessary that *Lactobacillus* is present in significant numbers as early as possible during storage for producing high quality silages made of subtropical grasses. Consequently, research into the fermentation effects of *L. plantarum* isolated in Silage A and sorghum silage seems warranted. In addition, *L. brevis* and *L. fermentum*, hetero-fermentative bacteria, have been reported in large numbers at the end of the ensiling process (Tjandraatmadja et al. 1991). Actually, *L. brevis* was present in grass silage and *L. fermentum* in corn silage in Ishigaki. *Pediococcus pentosaceus* was isolated from good quality silages in this study. EFSA (2014) reported that a mixture of *P. pentosaceus* and *L. plantarum* showed potential to improve preservation of nutrients in silage. In a study by Soundharrajan et al. (2019), addition of *P. pentosaceus* and *L. brevis* at ensiling produced a marked improvement in silage quality, which was attributed to their high antibacterial and probiotic properties. A combination of *P. pentosaceus* and *Lactobacillus* isolated in this study may also have the ability to improve Okinawan silage. Therefore, to increase the possibility of stimulating proliferation of these beneficial LAB

as early as possible, wilting grass sufficiently to below 70% moisture seems critical. In addition, compacting fresh forage effectively and wrapping silage properly to minimize the amount of air in the silage should enhance the chances that the environment in the silage would be suitable for rapid proliferation of LAB. We plan to investigate the effects of inoculating fresh forage with these strains at ensiling in an endeavor to improve the quality of silage produced. There have been very few reports that *L. acidipiscis* exists at ensiling, e.g. Hanagasaki and Cai (2009); Shokryazdan et al. (2018). It was originally isolated from fermented fish (Tanasupawat et al. 2000) and Greek Kopanisti cheese (Kazou et al. 2017) and when recently isolated from mulberry silage was shown to have antiproliferative and antioxidant properties (Shokryazdan et al. 2018). In addition, *L. acidipiscis* was shown to effectively absorb and expel dietary lead (Pb) from gastrointestinal tracts of chickens (Jahromi et al. 2017). Based on these findings and its isolation from Silage A, there seems merit in assessing the benefits of inoculating forage with this product at ensiling with the aim of improving silage quality in subtropical areas like Okinawa and providing nutritional benefits when fed to livestock. This study has revealed new data on catalase activity of LAB, which were generally believed to be catalase-negative. We have shown that the 2 strains identified as *W. paramenseteroides*, the strain identified as *L. plantarum* and all 4 strains identified as *L. paraplantarum* have catalase activity. This indicates a possibility that LAB present in Okinawan silages have unique characteristics. Further study is needed to elucidate each LAB strain's characteristics and identify the LAB strains most suitable for use in making silage in Okinawa.

4-5 Abstract

In Okinawa, rate of increase in gross agricultural production during 2011–2016 was the highest in Japan and sales of calves ranked fourth throughout Japan. Raising cattle by feeding high quality silage is beneficial both nutritionally and economically. However, little is known about lactic acid bacteria (LAB) present in silages made from tropical grass in Okinawa. To improve understanding of fermentation processes in silages, the LAB present in a range of silages (*Digitaria eriantha*, *Megathyrsus maximus*, *Chloris gayana*, *Urochloa mutica*, *Sorghum* sp. and *Zea mays*) were identified. All isolates were Gram-positive and mainly catalase-negative bacteria. According to morphological and biochemical characters, 37 isolates were divided into 16 groups and on the basis of 16S rDNA sequence analysis, 7 were identified as *Lactobacillus plantarum*, 3 as *L. paraplantarum*, 1 as *L. brevis*, 1 as *L. acidipiscis*, 3 as *L. casei*, 1 as *L. fermentum*, 9 as *Weissella paramesenteroides*, 1 as *W. kimchii*, 5 as *Lactococcus lactis* subsp. *lactis*, 2 as *Lactococcus garvieae* and 4 as *Pediococcus pentosaceus*. Some of this wide variety of LAB in Okinawan silage could be beneficial for improving quality of silages and further studies are planned to determine benefits of inoculating forage with particular strains at ensiling.

Chapter 5

Changes in free amino acids and hardness in round of Okinawan delivered cow beef during dry- and wet-aging processes

5-1 Introduction

In Okinawa, the livestock industry is a major part of the Okinawan economy and there are numerous dams, some of which have finished giving birth and thus, are termed as Okinawan delivered cows. The turnover for Okinawan delivered cows is approximately 5000 heads per year; these slaughtered cows are generally sold at a lower price than fattened cattle and heifers. The reason for them being less valuable is their less tender meat. However, there is significant potential for adapting these cows' meat and increasing their value. Dry-aging is one aging method that is said to improve meat with respect to some characteristics. During the dry-aging process, juices are absorbed into the meat and chemical breakdown of protein occurs, giving a more intense nutty and beefy flavor (Dashmaa et al. 2016). Moreover, during aging, the endogenous enzymes break down myofibrillar proteins in the muscle, which leads to more tender beef (Baird 2008; Campbell et al. 2001). Because of this, it is considered that lean meat with low fat content is more suitable for dry aging and has higher potential to undergo a change in its quality. Specifically, round of beef (i.e., from the rear leg of the cow) is most likely to be appropriate for dry-aging. Indeed, dry-aging products using round of beef have already been commercialized in Okinawa. In general, the dry-aging process is performed under aerated conditions, whereas the wet-aging process essentially involves vacuuming and packaging, which means that the conditions do not involve aeration. It was reported that a survey on consumer preferences between dry-aged and wet-aged beef in an executive summary of the National Cattlemen's Beef Association's Center in the United States of America (hereinafter referred to as "USMEF") (Savell 2008). There is only a little thing

known about specific differences by scientific approach between beef aged by these two methods. The result that dry-aged steaks had significantly higher beefy and brown/roasted flavor intensities than the unaged or vacuum-aged steaks, whereas vacuum-aged steaks had significantly higher bloody/serumy and sour flavor intensities than the unaged or dry-aged steaks was reported (Warren and Kastner 1992). It was stated that wet-aged beef had significantly greater percentages of acids than dry-aged beef (King et al 1995). In terms of hardness, rib and loin steaks from their wet-aging treatment were significantly more tender than the rib and loin steaks from their dry-aging treatment (Parrish Jr. et al. 1991). However, both vacuum aging and dry-aging for 11 days resulted in tenderness scores that were significantly higher than the unaged controls (Warren and Kastner 1992). To shed light on this issue, in this study, scientific data, such as free amino acids and hardness, were analyzed in the round of Okinawan delivered cow beef during dry-aging and wet-aging processes along with a comparison with beef imported from Australia (hereinafter referred to as Australian beef).

5-2 Materials and Methods

5-2-1 Animals and muscle samples

Two types of beef were used for the aging experiments: one from Okinawan delivered cows that were > 10 years old and thus, had already finished giving birth and the other one Australian beef. Type of Okinawan delivered cow beef was a kind of Japanese cow called black-haired Japanese cow. Type of Australian beef was crossbreed thought to be in the family lineage of black cattle mainly, which was raised in pastures. With regard to Okinawan delivered cow beef, two chunks of meat (approximately 6 kg/each) from the round in the same position of both right and left sides of the carcass from one individual were used for both dry-aging and wet-aging experiments. The same approach was also applied to two other individuals, so there were three pieces for each aging experiment.

With regard to Australian beef, six chunks of meat (approximately 6 kg/each) from the round of each individual were grouped into dry-aging and wet-aging experiments, namely, three in each group. Each chunk of meat was divided into five pieces by cutting for experiments involving 0, 1, 2, 3, or 4 weeks of aging.

5-2-2 Aging environment

The dry-aging environment was created in a refrigerator (Showa Denko K.K.) in Okinawa Industrial Technology Center at a temperature of 2 °C. Dry boxes were placed in the refrigerator for the dry-aging experiment. Three pieces of meat were put in the dry box without a fan for each week under maintained conditions of approximately 80% relative humidity and no air flow. The pieces of meat for the wet-aging experiment, which were vacuumed and packaged, were placed in a refrigerator set at 2 °C during the aging process, the same as that used for the dry-aging experiment.

5-2-3 Measurement of moisture and trim losses

Moisture loss is the weight of water lost from meat and is determined by measuring the difference in meat weight between before and after it has been subjected to dry aging. Trim loss is weight of the trimming part of meat that is discolored and dehydrated. Productive loss is the sum of moisture and trim losses.

5-2-4 Quantitative analysis of amino acids

First, sampling for the dry-aging experiment for 2, 3, and 4 weeks was performed to a depth of over 1 cm from the surface of the edible part, after it had been trimmed. The sampling for the dry-aging experiment for 0 and 1 week and the wet-aging experiment for 0, 1, 2, 3, and 4 weeks was performed to a depth of over 1 cm from the surface of the meat without trimming. These were cut and homogenized. Extract solutions were

obtained from these homogenized samples after protein was removed with acetonitrile and perchlorate and fat was removed with hexane. Sample solutions for LC/MS were prepared after extract solutions had been filtered. Sample solutions were injected onto an Intrada Amino Acid column (3 × 100 mm, Imtakt Corp., Kyoto, Japan) at a flow rate of 0.6 ml/min. The separation was performed with a two-pump gradient. Solvent A was acetonitrile/tetrahydrofuran/25 mM ammonium formate/formic acid (9/75/16/0.3, v/v/v/v). Solvent B was acetonitrile/100 mM ammonium formate (20/80, v/v). The gradient program was as follows: 0, A 100%; 2.75, A 100%; 7.75, A 83%; and 7.76 min, A 0%. Analyses were monitored in the positive-ion mode using an ESI source at 350 °C and MRM. Amino acids were sorted according to their features into four groups. Glycine, alanine, threonine, serine and proline were classified as sweet-tasting amino acids. Aspartic acid, glutamic acid, glutamine and asparagine were classified as umami-tasting amino acids. Methionine, lysine, isoleucine, leucine, phenylalanine, tyrosine, valin, histidine, arginine and cystine were classified as savory-tasting amino acids. Finally, carnosine, anserine, taurine, ornithine and GABA were classified as functional amino acids.

5-2-5 Measurements of drip and cooking losses

Samples for these measurements were cut to a size of approximately 2.5 cm (length) × 2.5 cm (width) × 1 cm (height) at a depth of over 1 cm from the surface of the meat and were frozen until subsequent analyses. Prior to analyses, these frozen samples were kept at a normal temperature for 2.5 h and weighed before their drip was removed. The weight of these samples was measured again after their drip had been removed. The weight difference was calculated and was denoted as a ratio relative to the initial weight. The percentage of drip loss was calculated in this way. In terms of cooking loss, samples that had already undergone drip loss measurement were put into a plastic bag and incubated

at 70°C in a water bath for 1 h. After they had been cooled and their drip had been removed, the weight of these samples was measured. The weight difference between before and after incubation was determined as a ratio relative to the weight before incubation (Muramoto et al. 2014).

5-2-6 Rheological properties

Breaking stress, shearing stress, and other rheological properties of each meat sample were measured using a rheometer RE-3305S (Yamaden Co. Ltd., Tokyo, Japan) and a breaking strength analyzer BAS-33005-16 (Yamaden Co. Ltd.). Samples for these measurements were those that had already undergone both drip and cooking loss measurements as described above. With regard to the measurement of breaking stress, this was performed on a sample of approximately 1 cm in height using a rheometer with a plunger No. 5 stick-type at a speed of 1 mm/s. Approximately seven runs were performed for each measurement and the average was calculated. The measurement of shearing stress was performed in an almost similar manner. Specifically, it was performed on samples of a size of approximately 1 cm (width) × 1 cm (height) using a rheometer with a plunger No. 21 knife-type at a speed of 1 mm/s. These samples were cut vertically on muscle fiber. Approximately five runs were performed for each measurement and the average was calculated.

5-2-7 Statistical analysis

Two-way analysis of variance (ANOVA) was used for statistical analysis of dry-aged and wet-aged beef with the program JMP® 13. Tukey's test was used for identifying differences ($P < 0.05$) between in the same beef sample during the same aging process for each experiment. P-values < 0.05 were considered to be statistically significant.

5-3 Results

5-3-1 Changes in productive loss

It was thought that moisture loss occurred immediately after the initiation of the dry-aging process, whereas trim loss occurred approximately 10 days after the dry-aging process had started. Levels of both losses increased in both beef types as the number of days of dry aging increased (Figure 5-1). In Australian beef, drip loss significantly decreased and cooking loss ($P = 0.086$) showed a decreasing trend during the dry-aging process. In addition, drip loss ($P = 0.07$) showed a decreasing trend and cooking loss significantly increased during the wet-aging process. In Okinawan delivered cow beef, drip loss significantly decreased and cooking loss ($P = 0.42$) appeared to remain unchanged during the dry-aging process (Figure 5-2).

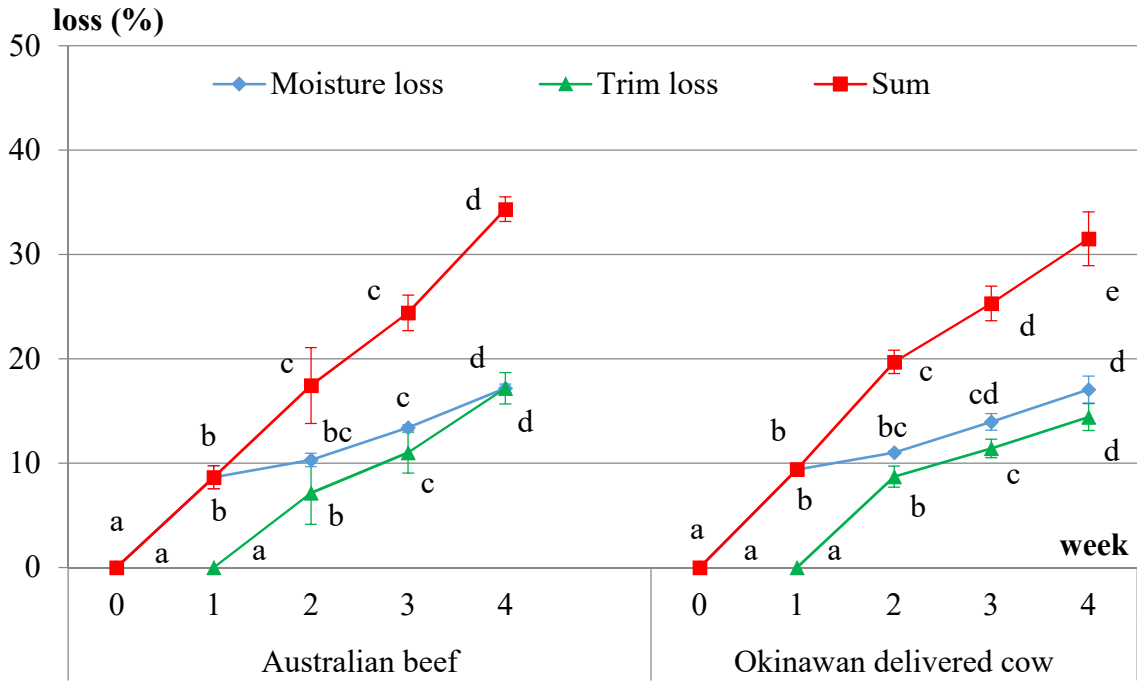

Figure 5-1. Changes in moisture and trim losses during the dry-aging process.
Different letters in the same group indicate significant differences.

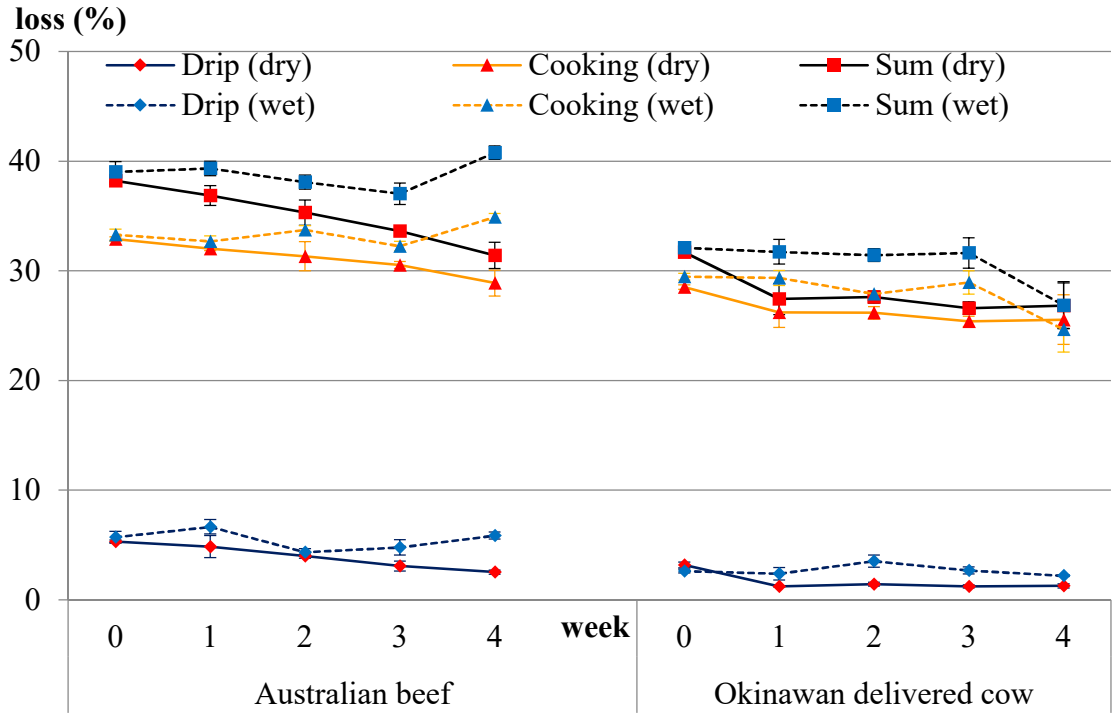

Figure 5-2. Changes in drip and cooking losses during both dry- and wet-aging processes.

5-3-2 Changes in free amino acid content

The results also showed that sweet-, savory- and umami-tasting amino acids increased in both beef types during dry- and wet-aging processes (Figure 5-3 and Table 5-1). Their sum reflects the level of proteinogenic amino acids, which also increased. However, levels of functional amino acids did not increase in both beef types during dry- and wet-aging processes, but rather decreased in Okinawan delivered cow beef. Total amino acids, including functional amino acids, in both beef types significantly increased during dry- and wet-aging processes (data not shown); this increase was almost the same as that observed for proteinogenic amino acids. GABA and cystine were not detected at all in all samples. Representative sweet-, savory- and umami-tasting amino acids, namely, alanine, leucine and glutamic acid, respectively, are shown (Figure 5-4 and Table 5-2). Levels of leucine and glutamic acid increased stably in both beef types during both aging processes. In terms of alanine, it showed no increase in Okinawan delivered cow beef after dry- and wet-aging processes. In terms of ornithine, it was initially at a low level and remained constant in both beef types during both aging processes.

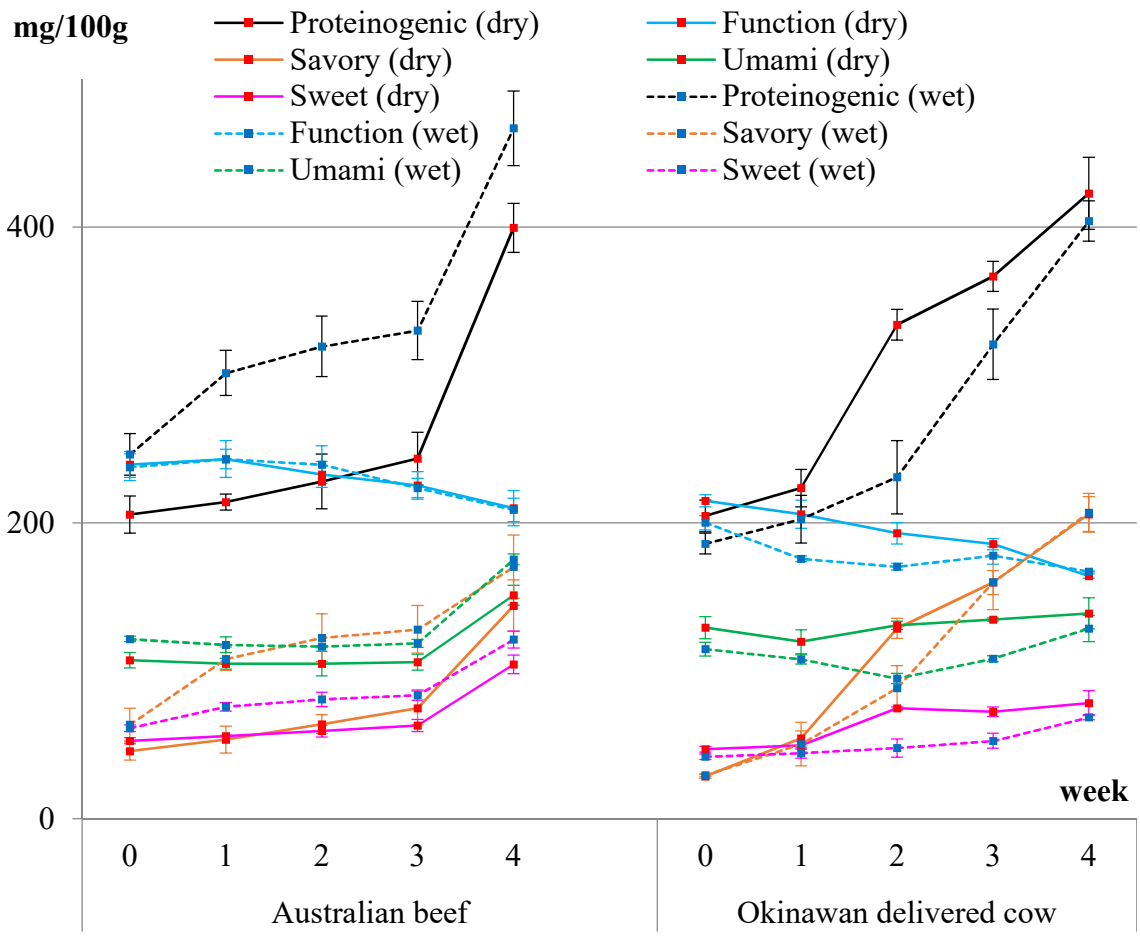

Figure 5-3. Change in levels of each amino acid group in both types of beef during both dry- and wet-aging processes.

Amino acid groups are classified as sweet-, umami- and savory-tasting and functional. The sum of sweet-, umami- and savory-tasting amino acids constitute the proteinogenic category. Changes in their levels during the dry-aging process are shown by solid lines. Changes in their levels during the wet-aging process are shown by dotted lines.

Table 5-1. Results of the average value ± standard deviation and Tukey's test for each amino acid group listed in Figure 5-3.

Australian beef											
	week	0		1		2		3		4	
Proteinogenic	dry	206±22	a	214±9	a	228±32	a	243±31	a	399±29	b
	wet	246±24	a	301±26	a	319±36	a	330±34	a	467±44	b
Function	dry	239±15	a	243±22	a	233±15	a	225±16	a	210±21	a
	wet	238±16	a	243±11	a	239±22	a	224±11	a	209±14	a
Umami	dry	107±9	a	105±6	a	105±15	a	106±9	a	151±12	b
	wet	121±3	a	118±9	a	116±1	a	119±5	a	175±6	b
Sweet	dry	53±4	a	56±1	a	59±7	a	63±7	a	104±11	b
	wet	61±4	a	76±5	ab	81±9	b	84±6	b	121±10	c
Savory	dry	46±10	a	53±16	a	64±11	a	75±20	a	144±30	b
	wet	64±19	a	108±14	ab	122±28	ab	128±28	ab	170±37	b
Okinawan delivered cow											
	week	0		1		2		3		4	
Proteinogenic	dry	205±19	a	223±22	a	334±18	b	367±18	bc	423±42	c
	wet	186±12	a	202±28	a	230±43	a	321±41	b	404±24	b
Function	dry	215±7	a	206±16	ab	193±12	ab	186±6	bc	164±2	c
	wet	200±8	a	176±3	b	170±4	b	178±10	b	167±0	b
Umami	dry	129±13	a	120±14	a	131±5	a	135±2	a	139±18	a
	wet	115±8	ab	108±5	ab	95±6	a	108±4	ab	129±16	b
Sweet	dry	47±3	a	50±4	a	75±2	b	72±6	b	78±15	b
	wet	42±3	a	44±6	a	48±11	a	53±9	ab	69±3	b
Savory	dry	29±2	a	54±9	a	128±12	b	160±14	b	206±21	c
	wet	29±2	a	50±25	a	88±26	a	160±32	b	207±22	b

Different letters in the same group represent significant differences ($P < 0.05$).

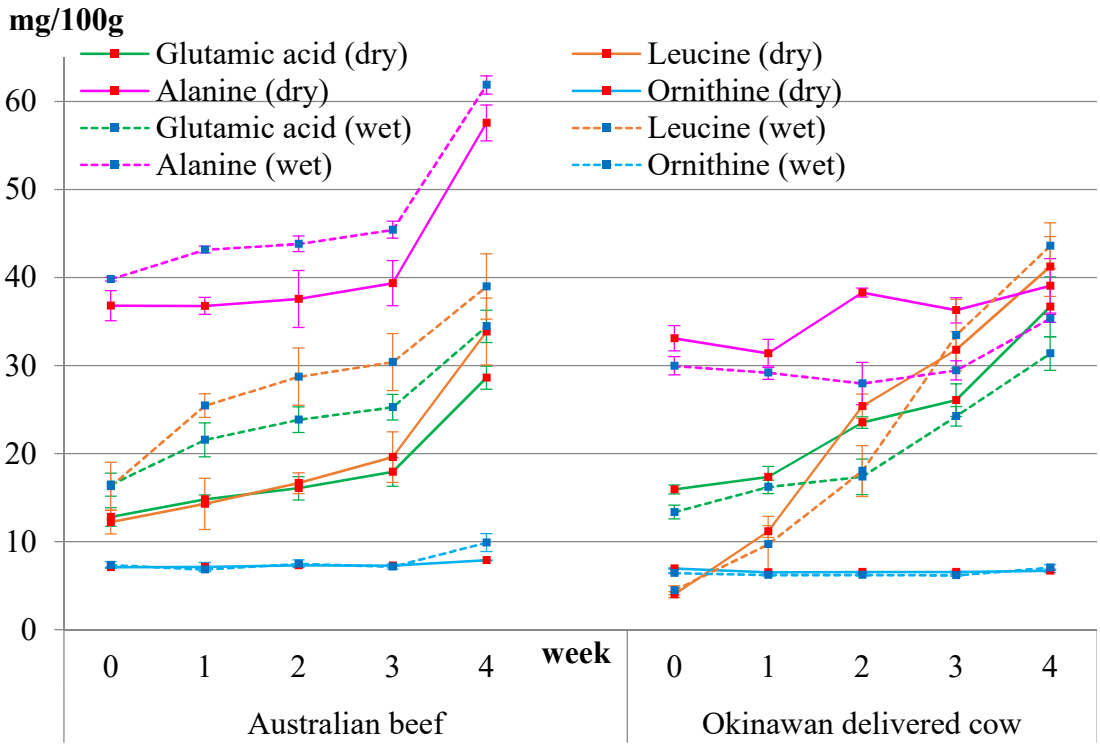

Figure 5-4. Changes in levels of alanine, glutamic acid, leucine and ornithine in both types of beef during both dry- and wet-aging processes.

Changes in their levels during the dry-aging process are shown by solid lines. Those during the wet-aging process are shown by dotted lines.

Table 5-2. Results of the average value ± standard deviation and Tukey's test for each amino acid listed in Figure 5-4.

		Australian beef									
	week	0		1		2		3		4	
Alanine	dry	37±3	a	37±2	a	38±6	a	40±4	a	58±4	b
	wet	40±0	a	43±1	ab	44±2	b	45±2	b	62±2	c
Glutamic acid	dry	13±2	a	15±1	ab	16±2	ab	18±3	b	29±2	c
	wet	16±2	a	22±3	ab	24±3	ab	25±3	b	34±3	c
Leucine	dry	12±2	a	14±5	a	17±2	a	20±5	a	34±7	b
	wet	16±5	a	25±2	ab	29±6	ab	30±6	b	39±6	b
Ornithine	dry	7±0	a	7±1	a	7±0	a	7±0	a	8±0	a
	wet	7±1	ab	7±0	a	7±1	ab	7±1	a	10±2	b
		Okinawan delivered cow									
	week	0		1		2		3		4	
Alanine	dry	33±2	ab	32±3	a	38±1	ab	36±2	ab	40±4	b
	wet	30±2	ab	29±1	ab	28±4	a	29±2	ab	35±1	b
Glutamic acid	dry	16±1	a	17±2	a	24±1	ab	26±3	b	38±5	c
	wet	13±1	a	16±1	a	17±4	a	24±2	b	31±3	c
Leucine	dry	4±1	a	11±1	a	25±2	b	32±4	b	42±5	c
	wet	5±1	a	10±5	ab	18±5	b	33±7	c	44±5	c
Ornithine	dry	7±0	a	7±0	a	7±0	a	7±0	a	7±0	a
	wet	6±0	ab	6±0	a	6±0	a	6±0	a	7±1	b

Different letters in the same group represent significant differences ($P < 0.05$).

5-3-3 Changes in hardness

There were approximately decreasing trends in breaking stress [dry aging of Australian beef: $P < 0.05$, wet aging of Australian beef: $P = 0.09$, dry aging of Okinawan delivered cow beef: $P < 0.05$, and wet aging of Okinawan delivered cow beef: $P < 0.05$] (Figure 5-5). In addition, there were approximately decreasing trends in sharing stress [dry aging of Australian beef: $P = 0.1$ and wet aging of Australian beef: $P < 0.05$ and dry aging of Okinawan delivered cow beef: $P = 0.086$ and wet aging of Okinawan delivered cow beef: $P = 0.136$] (Figure 5-6). Probability values for the strain regarding breaking point and shearing point were also determined (breaking point in Australian beef during dry- and wet-aging processes: $P = 0.52$ and $P = 0.7$, respectively; shearing point in Australian beef during dry- and wet-aging processes: $P = 0.8$ and $P = 0.1$, respectively; breaking point in Okinawan delivered cow beef during dry- and wet-aging processes: $P = 0.054$ and $P = 0.059$, respectively; and shearing point in Okinawan delivered cow beef during dry- and wet-aging processes: $P = 0.055$ and $P = 0.658$, respectively). Australian beef has a decreasing trend about only shearing point during wet-aging process, contrary to the case for Okinawan delivered cow beef (Figures 5-5 and 5-6).

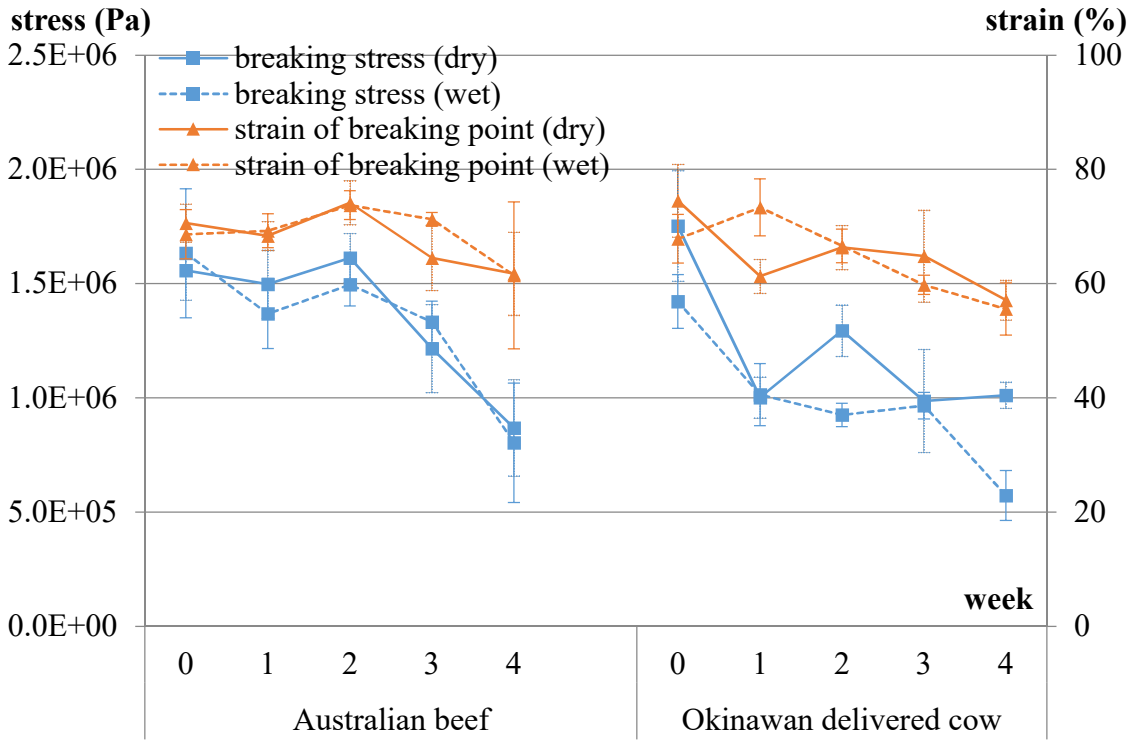

Figure 5-5. Changes in breaking stress and strain of breaking point in both types of beef during both dry- and wet-aging processes.

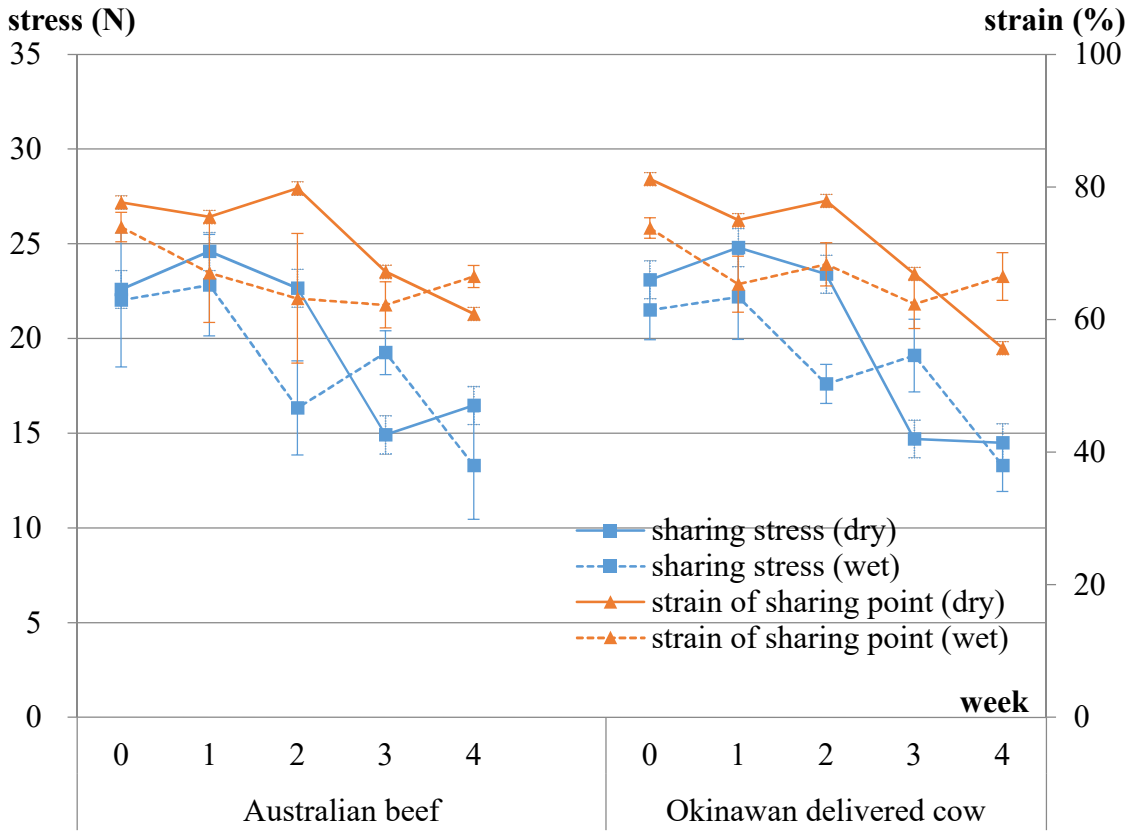

Figure 5-6. Changes in shearing stress and strain of shearing point in both types of beef during both dry- and wet-aging processes.

5-3-4 Changes in drip and cooking losses

Upon comparison of drip loss, cooking loss and the sum of these between beef types and between aging methods (Table 5-3), there were significant differences ($P < 0.01$) between beef types for all these variables, which was also the case ($P < 0.01$) between the aging methods.

Table 5-3. Comparison of drip loss, cooking loss and their sum between beef types and between aging methods.

	Beef type		Aging method		SEM†	*P* values in ANOVA‡	
	Australian	Okinawan	wet-aging	dry-aging		Beef type	Aging method
Drip loss	4.7	2.2	4.1	2.8	0.1	**	**
Cooking loss	32.3	27.2	30.7	28.8	0.3	**	**
Sum	37.0	29.4	34.8	31.6	0.4	**	**

Values are least-square means (n = 3). †Pooled standard error of the mean. ‡Asterisks indicate **P < 0.01 in analysis of variance.

5-3-5 Statistical analysis

In the comparison of each amino acid group between beef types and between aging methods (Table 5-4), there were significant differences between beef types regarding function ($P < 0.05$), sweetness ($P < 0.01$) and total ($P < 0.05$). However, there was no significant difference between the aging methods for all studied variables even for all amino acids (data of amino acids not shown). Comparison of rheological properties between beef types and between aging methods showed there was a significant difference between beef types regarding breaking stress alone (Table 5-5).

Table 5-4. Comparison of levels of each amino acid group between beef types and between aging methods.

	Beef type		Aging method		SEM†	*P* values in ANOVA‡	
	Australian	Okinawan	wet-aging	dry-aging		Beef type	Aging method
Function	230.3	185.5	204.4	211.4	3.3	*	ns
Sweet	61	42.9	52.7	51.2	2.5	**	ns
Savory	97.4	111.2	112.7	95.9	10.6	ns	ns
Umami	122.3	120.7	120.3	122.7	3.7	ns	ns
Proteinogenic amino acids	295.4	289.6	300.8	284.2	16.1	ns	ns
Total amino acids	525.7	475	505.2	495.6	14.9	*	ns

Values are least-square means (n = 3). †Pooled standard error of the mean. ‡Asterisks indicate **P < 0.01 and *P < 0.05 in analysis of variance.

Table 5-5. Comparison of rheological properties between beef types and between aging methods.

	Beef type		Aging method		SEM†	*P* values in ANOVA‡	
	Australian	Okinawan	wet-aging	dry-aging		Beef type	Aging method
Breaking stress (Pa)	1.3E+06	1.1E+06	1.2E+06	1.3E+06	6.9E+04	*	ns
Strain of breaking point (%)	68.4	64.7	66.8	66.3	1.6	ns	ns
Sharing stress (N)	20.6	19.5	19.7	20.5	9.6E+05	ns	ns
Strain of sharing point (%)	74.5	69.4	72.8	71.1	2	ns	ns

Values are least-square means (n = 3). †Pooled standard error of the mean. ‡Asterisks indicate *P < 0.05 in analysis of variance.

5-4 Discussion

The present study demonstrated the effects of both dry- and wet-aging processes on Okinawan delivered cow beef and Australian beef. Productive loss increased as the number of days of dry-aging increased. However, there was no productive loss, such as moisture and trim losses, during the wet-aging process. This shows that the price of dry-aged beef must be higher than that of wet-aged beef to compensate for this loss. In our study, productive loss from both beef types increased to > 30% under conditions of approximately 80% relative humidity for 4 weeks. Many of the compounds responsible for flavor are concentrated by the dry-aging process, according to USMEF (Savell 2008). In other words, the distinguishing effect of the dry-aging process on beef is that it concentrates the flavor (Dashmaa et al. 2016; Savell 2008; Warren and Kastner 1992). Therefore, it is considered that amino acids in beef are also concentrated during the dry-aging process. On the contrary, as described in Introduction, wet-aged steaks had significantly higher sour flavor than dry-aged steaks (Warren and Kastner 1992) and wet-aged beef had significantly greater percentages of acids than dry-aged beef (King et al 1995). Free amino acid is one kind of acids and possibly contributes to sour flavor of wet-aged beef. In our study, the change in levels of amino acids during the dry-aging process was compared with that during the wet-aging process. The meat from Okinawan delivered cow beef seemed to have a higher increase in proteinogenic amino acids during the dry-aging process than during the wet-aging process, particularly in the middle of the aging process, contrary to the case for Australian beef. However, finally, 4 weeks after aging, these increases during both aging processes were about the same in each type of beef. On ANOVA, there was no significant difference between aging methods for all studied variables related to free amino acids. It is known that there are many steps in the degradation of proteins to produce free amino acids. The way to produce free proteinogenic amino acids assumes to be from short peptide not directly from protein.

Therefore, it is difficult to elucidate the difference in the underlying mechanism of amino acid production between dry- and wet-aging processes by molecular biological techniques. As such, in the current study, we cannot draw definitive conclusions that the dry-aging process can concentrate amino acids to a greater extent than the wet-aging process and vice versa. The results showed that glutamic acid increased stably, which is essential for the umami taste of meat, for beef during both types of aging. It was reported that meat from Japanese brown cattle have high levels of glutamic acid and leucine, which increase stably during the aging process (Sugioka et al. 2015). However, alanine did not increase but fluctuated sharply and non-proteinogenic amino acids also did not increase during the aging process. The results in our study were very similar to these results. In particular, the results in Okinawan delivered cow beef were exactly the same as these. The trend of a decrease in hardness during the dry-aging process is almost the same as that during the wet-aging process. It is difficult to analyze a non-uniform tissue, such as a piece of meat, using a hardness test because of its scattered value of measurement. At this point, the only assertion that can be safely made is that there were similar decreasing trends in hardness during both aging processes. However, the tender I bit them is the clear difference between both dry- and wet-aged beef. Upon chewing wet-aged beef, its tenderness seemed to remain for longer than that of dry-aged beef, whereas dry-aged beef was much easier to cut with the teeth. The hardness of dry-aged beef gradually decreases as the number of days of aging increases (Tsuchiya et al. 2013). In addition, it was reported that for both the Prime and Choice comparisons, Warner-Bratzler shear force values did not differ between the dry- and wet-aged steaks (Sitz et al. 2006). These are similar to the results in our study. From the tests of the strain regarding breaking point and shearing point, deformation process of meats in both type beefs while being pressed seem to be totally different. The positive effects, such as an increase in free amino acids and a decrease in hardness, in dry-aged beef were almost the same as those found in wet-aged

beef in our study. In fact, there were no significant differences between the two aging methods for all studied variables, except drip, cooking and productive losses. On the other hand, some significant differences between the beef types were identified on ANOVA, particularly regarding free amino acids. However, it is difficult to study why these differences occurred because these types of beef have different genetic factors and have undergone different fattening methods and for different periods since slaughter. In conclusion, there was no significant difference between dry- and wet-aging methods for all studied variables related to free amino acids or hardness in this study.

5-5 Abstract

Aging trials were conducted to determine characteristics associated with dry- and wet-aging processes of beef from delivered cows grown in Okinawa, i.e., dams that had finished giving birth (Okinawan delivered cow beef). Changes in free amino acids, hardness and other factors were analyzed in round of Okinawan delivered cow beef during dry- and wet-aging processes along with a comparison with characteristics of beef imported from Australia. Functional amino acids did not increase during both dry- and wet-aging processes. However, proteinogenic amino acids increased significantly ($P < 0.05$) and hardness tended to decrease during both dry- and wet-aging processes. On comparison between dry- and wet-aging processes by analysis of variance, drip and cooking losses were significantly lower during the dry-aging process than during the wet-aging process. However, there was no significant difference in free amino acids or hardness in this comparison. As a result, there was no significant difference between dry- and wet-aging methods for all studied variables related to free amino acids or hardness in this study.

Chapter 6

Changes in free amino acid content and hardness of beef while dry-aging with *Mucor flavus*

6-1 Introduction

The primary factors that determine the quality of dry-aged products include the length of aging, storage temperature, relative humidity, and air flow (Dashmaa et al. 2016; Savell 2008). Another important factor is the choice of intentionally incorporating certain microorganism in the process or not; if yes, then determining the type of microorganisms to be used and their culturing are also important. Owing to such factors, each company in Japan has their own method of dry-aging products. In major cities such as New York, most of the popular dry-aged beef steak products are believed to be aged with mold. These steaks are served at high prices in top-end steakhouses and exclusive restaurants (Neil 2012). For example, “mellow and intense” and “earthy and nutty” are the types of phrases commonly used to describe the flavor characteristics of dry-aged beef (Savell 2008), and these result from the type of mold used. In general, the beef’s natural enzymes break down the proteins and connective tissue in the muscle, which leads to more tender beef (Baird 2008; Campbell et al. 2001). Moreover, reportedly, certain molds produce enzymes that enable them to penetrate into the meat, wherein they release proteases and collagenolytic enzymes that break down muscle and connective tissues (Dashmaa et al. 2016). There is a possibility that proteases and collagenolytic enzymes increase free amino acids. In fact, it was reported that meat from Japanese brown cattle have high levels of some free amino acids, which increase stably during the aging process (Sugioka et al. 2015). Therefore, I hypothesized that mold-treated, dry-aged beef would exhibit increased free amino acid content and decreased meat hardness. The main mold genera associated with the dry-aging of beef are *Mucor*, *Thamnidium* and *Rhizopus* (Dashmaa et

al. 2016). We succeeded in isolating an excellent mold strain with robust growth on meat at low temperatures such as 2 °C. The mold strain can add unique savory flavor, not originally from meat and then it is possible to create a new Okinawan brand using the mold, attracting tourists. In this study, we analyzed the hardness and free amino acid content of beef while it was dry-aging with this mold, and we compared its characteristics with those of the meat aged in the absence of any mold.

6-2 Materials and Methods

6-2-1 Animals and muscle samples

The meat used for the experiments were beef lumps imported from Australia. Type of Australian cow beef was crossbreed thought to be mainly in the family lineage of black cattle, which was raised in pastures. Two types of aging method were applied in each aging experiment: dry aging with mold and dry aging without mold. Six chunks of meat (approximately 6 kg of lump cut from six different animals) from the same type were grouped into the dry-aging with mold or dry-aging without mold group (further referred to as mold-aged and normal-aged, respectively), with three chunks of meat in each group. Each chunk of meat was cut and divided into five pieces for experiments involving 0, 1, 2, 3, and 4 weeks of aging.

6-2-2 Mold identification

6-2-2-1 Culture and morphological observations

A mold isolate was incubated in petri dishes with potato dextrose agar (PDA; Becton Dickinson, New Jersey, USA) at 15 °C for 1–3 weeks in dark condition. Microscopic slides were prepared from the portions of the colonies grown on the PDA plates by mounting them in lactophenol (with/without cotton blue). Microscopic examinations were performed with a SMZ800 stereomicroscope (Nikon Corp., Tokyo, Japan) and a

BX51 microscope (Olympus Corp., Tokyo, Japan) with Nomarski interference contrast at magnifications of up to × 1500. All micrographs were captured with a digital camera (DS-Fi2-L3; Nikon Corp.).

6-2-2-2 DNA sequencing analysis

The genomic DNA of our mold strain was extracted by physical disruption using beads (Nippon Gene Co., Ltd., Tokyo, Japan). The primers used included ITS5 and ITS4 (White et al. 1990) for the nuclear ribosomal internal transcribed spacer (ITS) regions, which include ITS1, 5.8S and ITS2. Polymerase chain reactions (PCRs) were performed with PrimeSTAR HS DNA polymerase (Takara Bio Inc., Shiga, Japan). The sequencing primers ITS5, ITS3 and ITS4 (White et al. 1990) were used for the amplification of ITS. The sequences were assembled with ChromasPro 1.7 (Technelysium Pty, Ltd., South Brisbane QLD, Australia). Multiple alignments were performed with CLUSTALW (Julie et al. 1994), and the final alignments were manually adjusted. Ambiguous positions and alignment gaps were excluded from the analysis. The neighbor joining (Saitou and Nei 1987) phylogenetic tree with the Kimura two-parameter model (Kimura 1980) was constructed using the TechnoSuruga Lab Microbial Identification database (TechnoSuruga Laboratory, Shizuoka, Japan). A bootstrap test with 1000 iterations was used to assess the reliability of the branches (Felsenstein 1985). The positions with gaps and the regions of uncertain nucleotide alignment were excluded from the phylogenetic analyses. DNA extraction, PCR amplification, DNA sequencing and molecular phylogenetic analyses were all performed by TechnoSuruga Laboratory Co., Ltd. (Shizuoka, Japan).

6-2-3 Aging environment

Aging environment was established in a refrigerator (Showa Denko K.K.) in Okinawa

Industrial Technology Center at a temperature of 2 °C. Accordingly, dry boxes were placed in the refrigerator. Further, three pieces of meat were placed in the boxes for each week under maintained conditions of approximately 80% relative humidity. For the mold-aging experiments, our mold strain was cultured on PDA plates (Merck Ltd., Tokyo, Japan) for a week and then allowed to contact each piece of meat, and these were later used for the experiments involving 1, 2, 3 or 4 weeks of aging.

6-2-4 Measurement of moisture and trim losses

The same as 5-2-3

6-2-5 Quantitative analysis of amino acids

The same as 5-2-4

6-2-6 Measurements of drip and cooking losses

The same as 5-2-5

6-2-7 Rheological properties

The same as 5-2-6

6-2-8 Statistical analysis

One-way analysis of variance (ANOVA) was used for the statistical analysis of productive loss using the JMP 13 (SAS Institute Inc.). Tukey's test was used to identify the differences between each week of the same aging process for each experiment. P-values < 0.05 were considered to be statistically significant

6-3 Results

6-3-1 Identification of the mold strain

A BLAST search showed that the ITS sequence of our mold strain had the highest sequence homology (88.2–99.5% similarity) with that of *M. flavus* in the DNA database (GenBank/DDBJ/EMBL). A neighbor joining phylogenetic tree was constructed with the ITS sequences of our mold strain and the closely related strains according to the database. Using phylogenetic analysis, the ITS sequence of our mold strain was found to form a cluster with the *M. flavus* complex (Walther et al. 2013) (Figure 6-1). Morphological observation of the mold culture colonies on PDA showed yellow to white colonies with a floccose appearance at 15 °C (Figure 6-2a). Sporangiophores with multispored sporangia had erectly formed from the vegetative mycelium (Figure 6-2b and c). These morphological characteristics matched those previously reported for *M. flavus* (Schipper 1975). Based on its ITS sequence and morphology, our mold strain was identified as *M. flavus.*

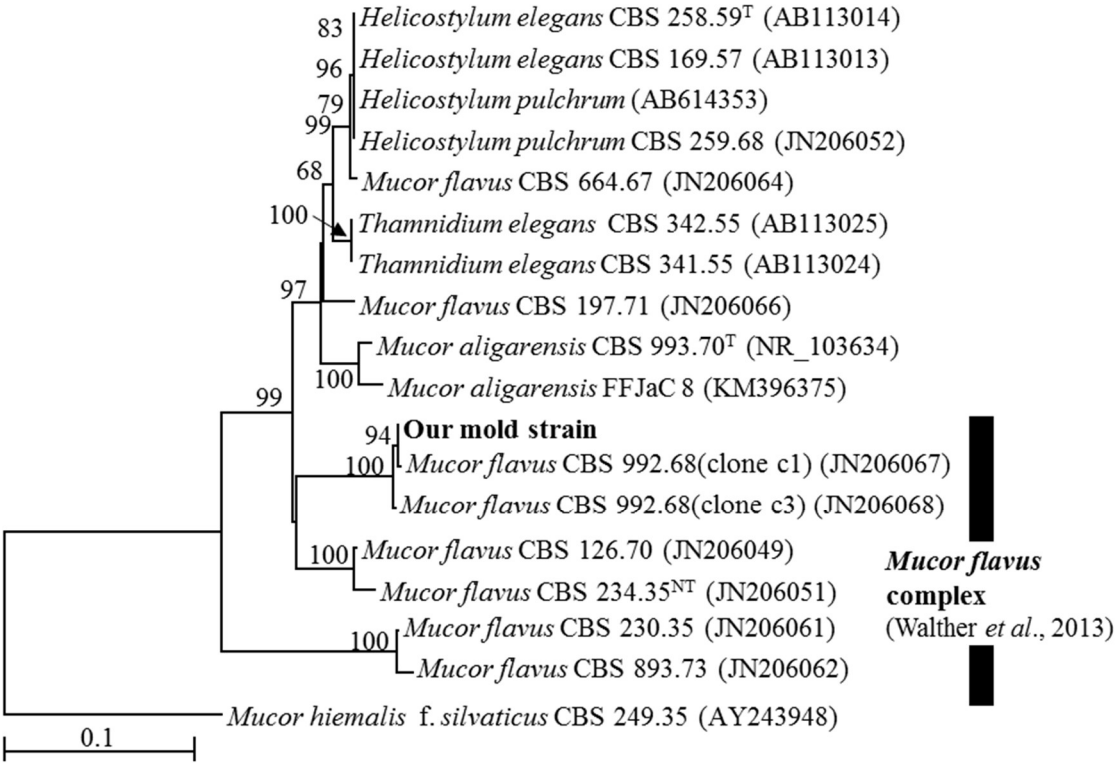

Figure 6-1. Phylogenetic relationships between our mold strain and *Mucor* species based on neighbor joining analysis of their ITS sequences.

The values on the branch nodes represent bootstrap support values (%) from 1000 iterations. Bootstrap values > 50% are indicated. The superscripts T and NT indicate ex-type and ex-neotype strains, respectively. The scale bar indicates 0.1 nucleotide substitutions/site.

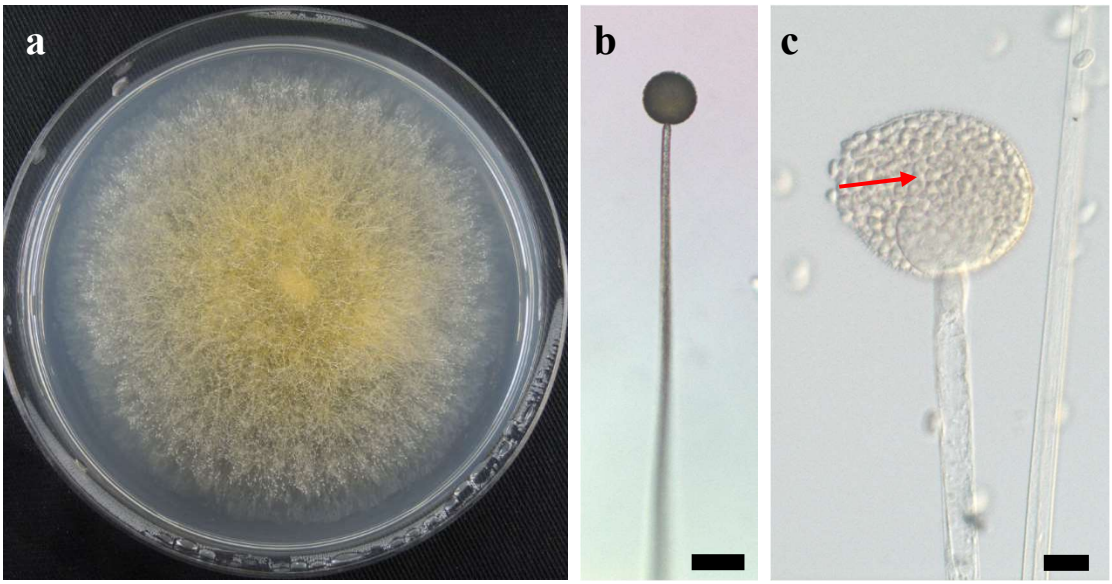

Figure 6-2. Morphological characteristics of our mold strain.

a Colony appearance on potato dextrose agar after 1 week incubation at 15 °C. **b** and **c** Sporangiophores and sporangia with columella (red arrow). The scale bars represent 50 μm (**b**) and 10 μm (**c**).

6-3-2 Changes in productive loss

Productive loss is the sum of moisture and trim losses. For both dry-aging processes (i.e., in the presence/absence of our mold strain), moisture loss occurred immediately after the initiation of dry aging, whereas trim loss occurred approximately 10 days after the initiation. In normal-aged and mold-aged beef, the moisture and trim losses increased every week ($p < 0.001$), but with no increase observed between weeks 3 and 4 for mold-aged beef (Figure 6-3). Moreover, the overall productive loss after 4 weeks of aging was significantly lower for mold-aging compared with that for normal-aging ($p < 0.01$) in despite of no significant difference from 1 to 3 weeks of aging between both aging methods. The values of productive loss in normal-aged and mold-aged beef were 0, 7.0, 21.3, 26.4, 31.7 and 0, 7.2, 20.8, 28.6, 27.2 from 0, 1, 2, 3 and 4 weeks of aging.

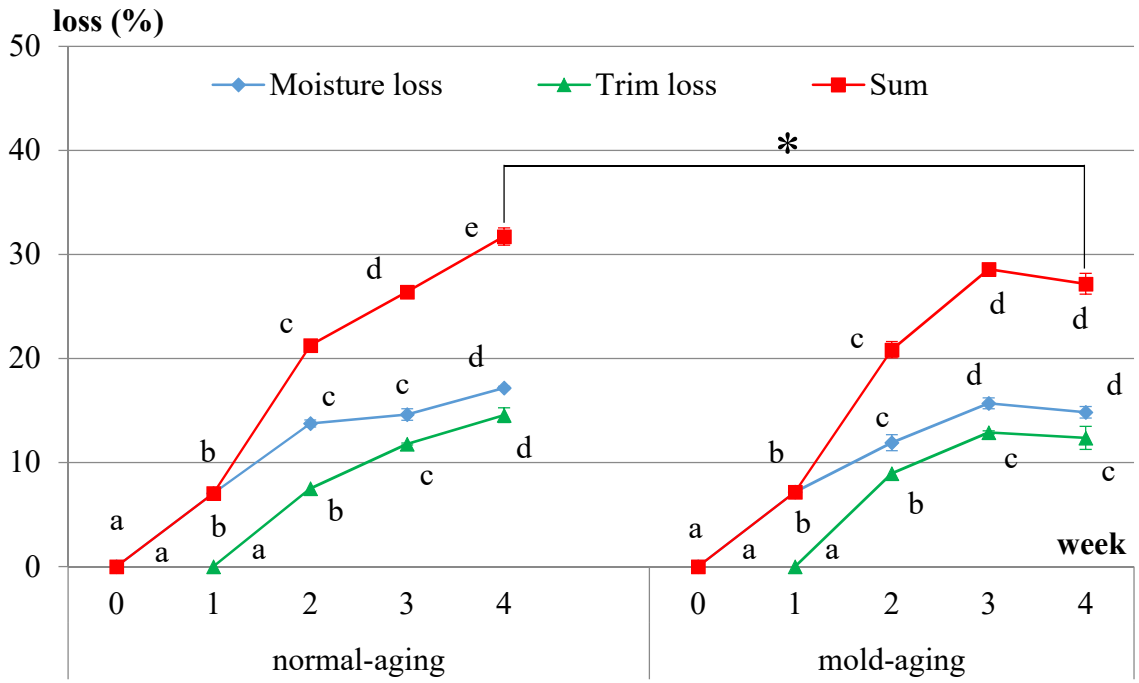

Figure 6-3. Changes in moisture and trim losses during normal- and mold-aging processes. Different letters in the same aging process represent significant differences ($p < 0.05$) between weeks. *$P < 0.01$ represents for the total productive loss between the two aging methods after 4 weeks of aging (one-way ANOVA).

6-3-3 Changes in free amino acid content

Internal meat samples showed that the levels of umami-tasting amino acids decreased with both aging methods (Figure 6-4). The level of functional amino acids did not increase but remained constant throughout both dry-aging methods. The level of sweet-tasting amino acids remained constant during the normal-aging process but decreased between weeks 2 and 3 of the mold-aging process. As for the savory-tasting amino acids, no significant changes were observed for either aging method. Additionally, aspartic acid, cystine, glycine and GABA were not detected in any of the internal meat samples. Surface meat sampling showed that the level of sweet-, savory- and umami-tasting amino acids (proline, histidine, aspartic acid and GABA, respectively) and functional amino acids dramatically increased during the mold-aging process ($p < 0.0001$), whereas it did not increase during the normal-aging process (Figures 6-5 and 6-6). Proline or GABA was not detected on the surface meat of the normal-aged beef (Figure 6-6), whereas cystine was not detected in the surface meat from either dry-aging method.

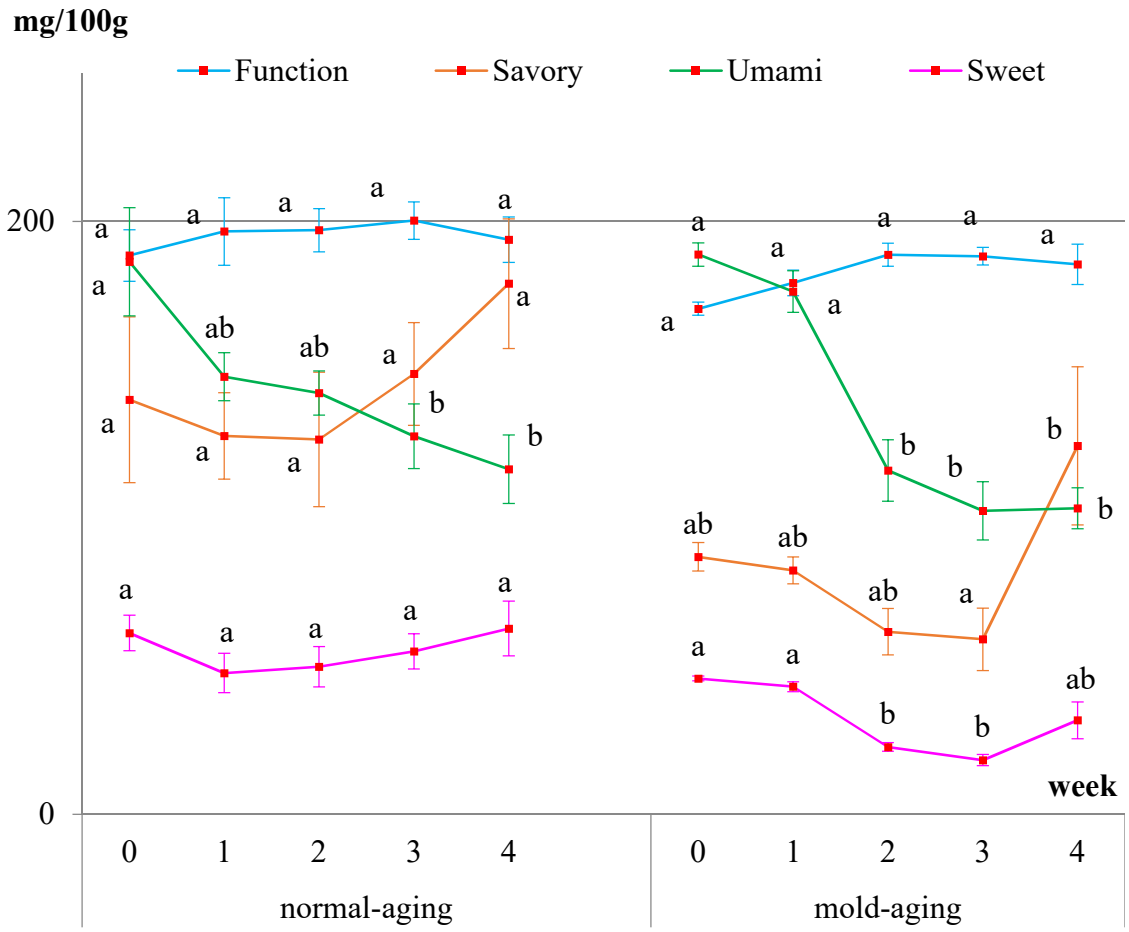

Figure 6-4 Changes in the levels of each amino acid group inside the meat during the normal- and mold-aging processes.

Amino acid groups were classified as functional, sweet-, umami- or savory-tasting. Different letters in the same aging process represent significant differences ($p < 0.05$) between weeks.

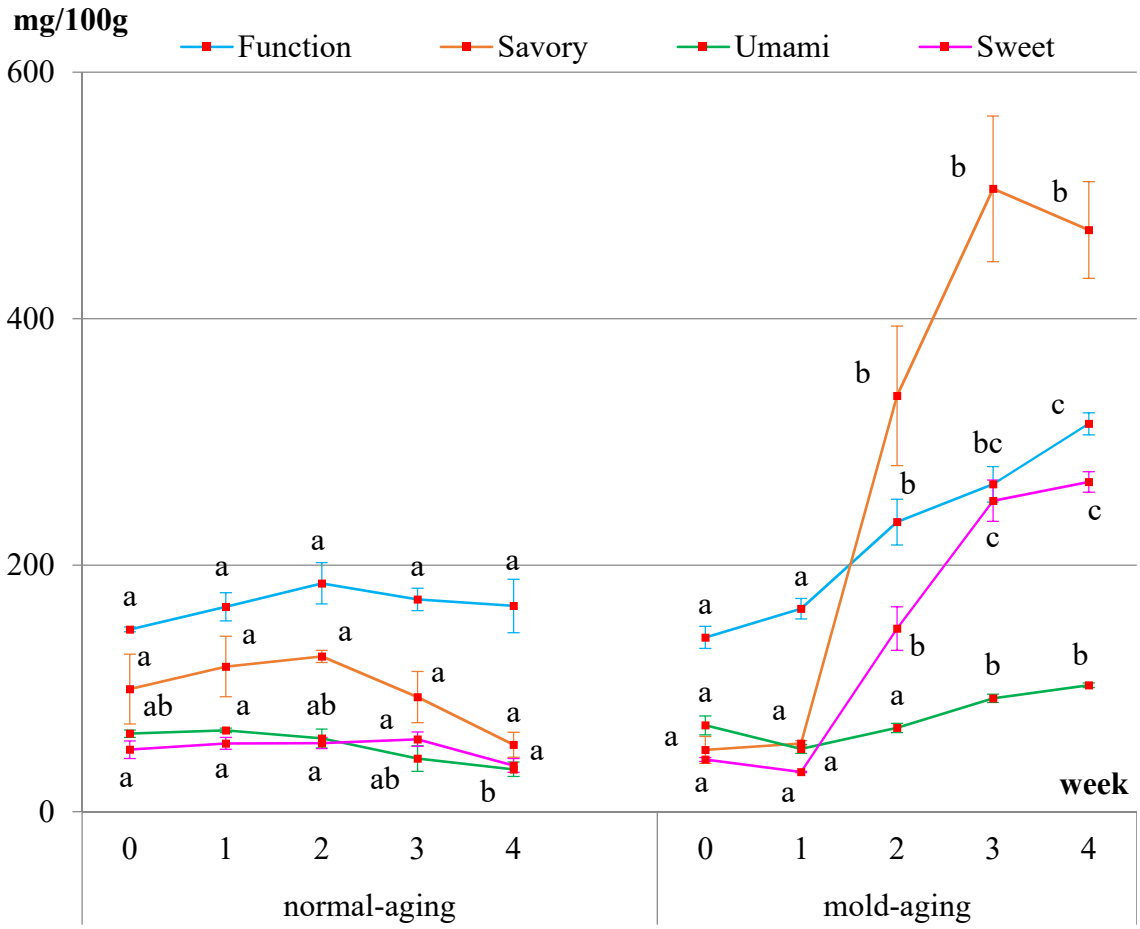

Figure 6-5. Changes in the levels of each amino acid group in surface meat during the normal- and mold-aging processes.

Amino acid groups are classified as functional, sweet-, umami- or savory-tasting. Different letters in the same aging process represent significant differences ($p < 0.05$) between weeks.

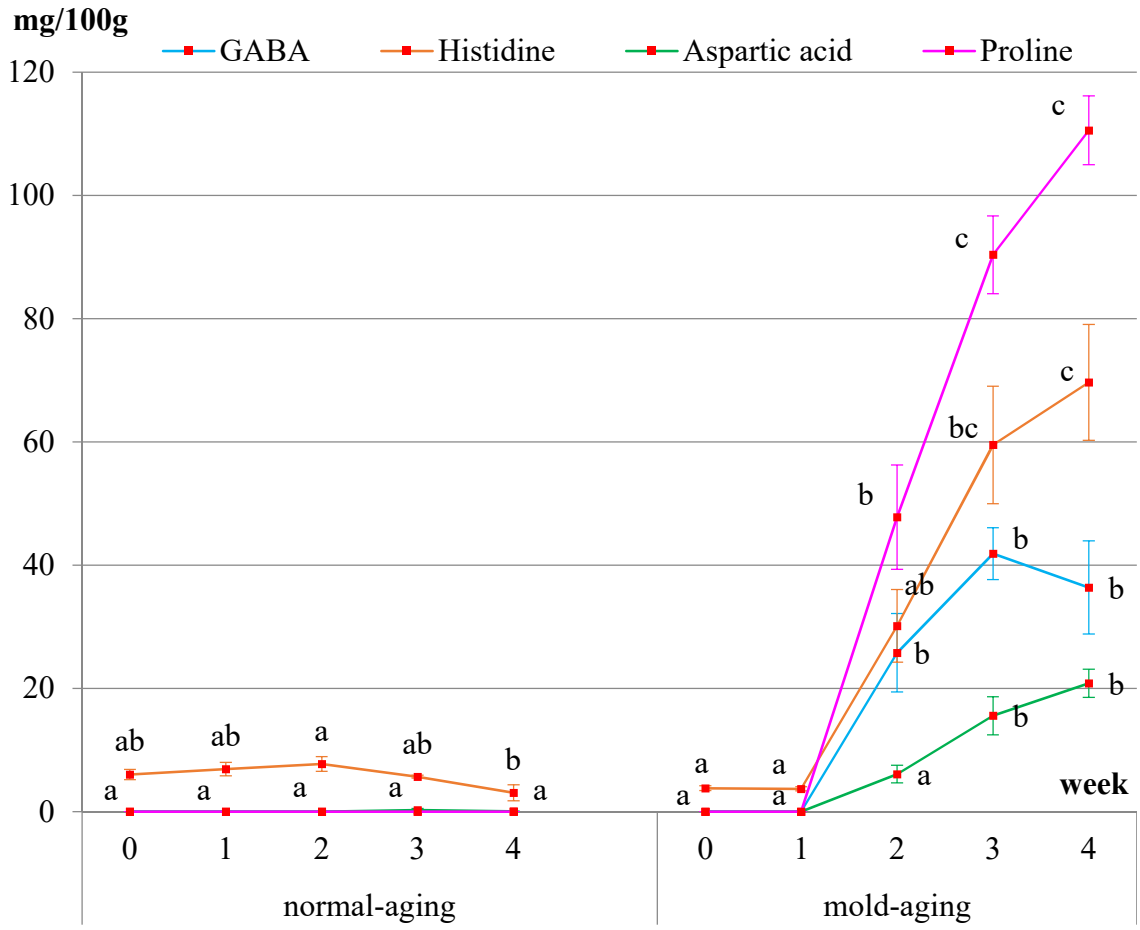

Figure 6-6. Changes in the levels of proline, aspartic acid, histidine and GABA in surface meat during normal- and mold-aging processes.

Different letters in the same aging process represent significant differences ($p < 0.05$) between weeks. GABA and aspartic acid were not detected in normal-aged beef.

6-3-4 Changes in drip and cooking losses

In normal-aged beef, drip loss decreased [1.9, 1.8, 1.1, 0.9, and 0.7 (weeks 0, 1, 2, 3 and 4), $p = 0.021$] and cooking loss showed a decreasing tendency [31.4, 30.5, 28.9, 27.9, and 28.2 (weeks 0, 1, 2, 3 and 4), $p = 0.089$] during the aging process. In mold-aged beef, drip loss [3.4, 4.3, 1.3, 0.7, and 0.8 (weeks 0, 1, 2, 3 and 4), $p = 0.001$] and cooking loss [32.3, 33.6, 30.1, 27.2, and 28.3 (weeks 0, 1, 2, 3 and 4), $p = 0.013$] decreased significantly during the aging process (Figure 6-7).

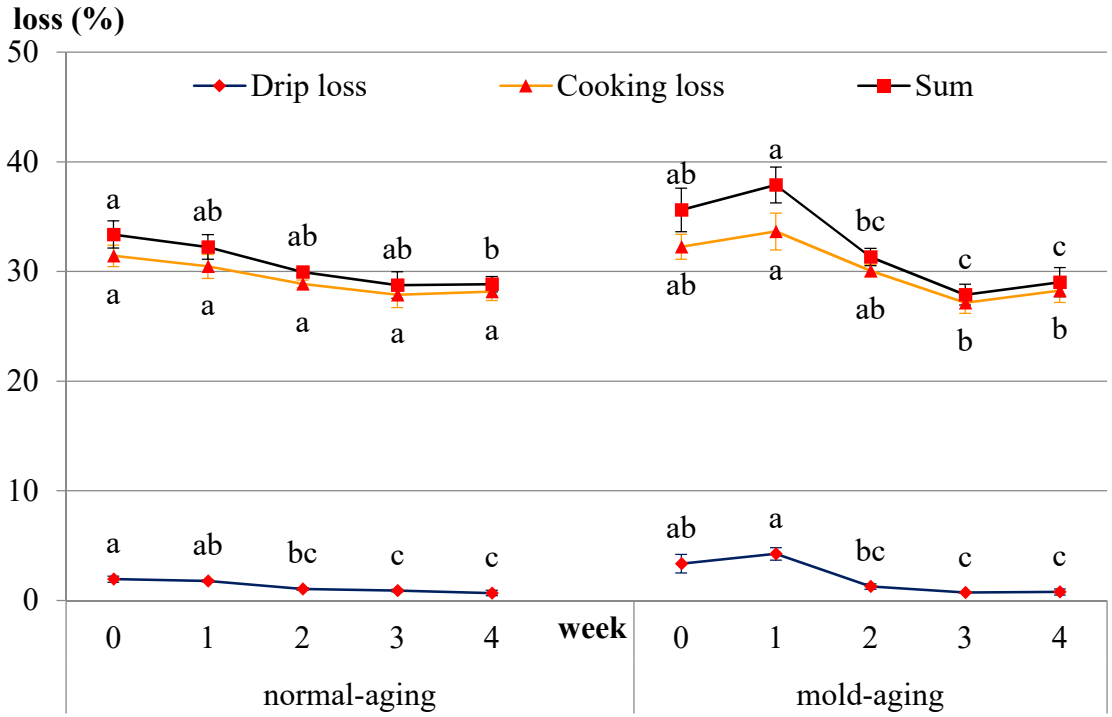

Figure 6-7. Changes in drip and cooking losses during normal- and mold-aging processes. Different letters in the same aging process represent significant differences ($p < 0.05$) between weeks.

6-3-5 Changes in hardness

Breaking stress in the normal-aged beef during the aging process did not significantly decrease over time [1.51E + 06, 1.49E + 06, 1.46E + 06, 1.28E + 06 and 1.01E + 06 (weeks 0, 1, 2, 3 and 4), $p = 0.24$], whereas for mold-aged beef, it did significantly decrease [1.61E + 06, 1.47E + 06, 1.50E + 06, 1.24E + 06 and 1.03E + 06 (weeks 0, 1, 2, 3 and 4), $p = 0.008$] (Figure 6-8). The strain of the breaking point had the same decreasing tendency in both groups but was only significant for the mold-aged beef [normal-aging: 75.9, 76.7, 73.0, 69.0 and 63.1 (weeks 0, 1, 2, 3 and 4), $p = 0.1693$; mold-aging: 81.1, 77.1, 69.6, 63.2 and 57.2 (weeks 0, 1, 2, 3 and 4), $p = 0.0003$]. The breaking stress values were similar at the endpoints of the two aging methods.

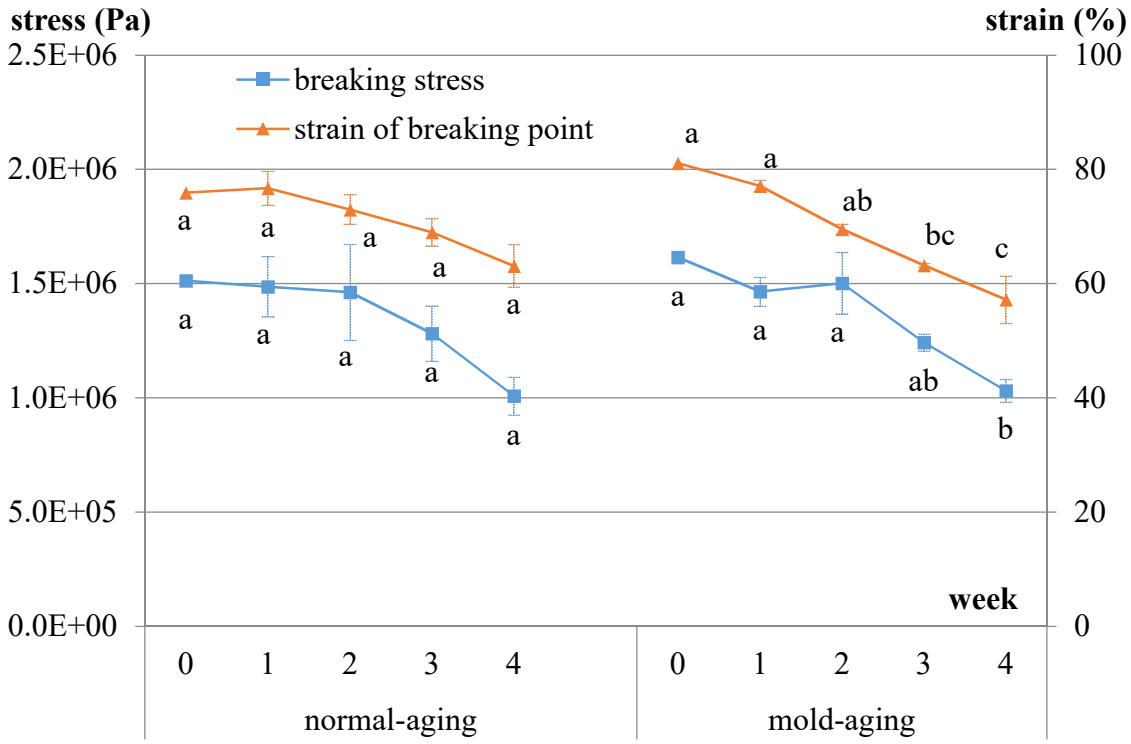

Figure 6-8. Changes in breaking stress and the strain of breaking point in beef during normal- and mold-aging processes.

Different letters in the same aging process represent significant differences ($p < 0.05$) between weeks.

6-4 Discussion

Our mold strain was found to belong to the *Mucor* genus, which is used for preparing various food products, including sausage, salami, cheese, tofu and vegetables, worldwide (Stephanie et al. 2017). Therefore, *Mucor* is a relatively safe mold genus, and its fermentation effects are expected to improve the quality of meat in terms of certain aspects. In the present study, during mold-aging process, the mold mycelium was observed to sparsely spread on the meat during the first week and to completely cover it after 2 weeks, producing light gray-colored meat and the mold grew stably even after that (Figure 6-9). While it grew, productive loss did not increase between the weeks 3 and 4 of aging and did not exceed 30%, a significantly lower level than that in normal-aged beef. This was an interesting finding, and the mold strain possibly prevented moisture evaporation from the meat and trim once it had completely covered the meat surface. Substantial yield losses and increased processing times that are associated with the dry aging process require to increase prices for the sale and distribution of dry-aged product (Laster 2007). Decreasing productive loss of dry-aged beef is very important because it directly influence the retail segment of the beef industry. Under the circumstances, there is a possibility that covering the meat by the mold increases retail yield and it may be the key of dry-aging process. The amino acid content of the interior meat samples did not stably increase during either aging process. Instead, umami-tasting amino acid levels were observed to decrease because glutamine which accounts for a large percentage of umami-tasting amino acid, levels decreased sharply during the aging process. It was reported that many of the compounds responsible for flavor are concentrated by the dry-aging process (Savell 2008). Namely, the distinguishing effect of the dry-aging process on beef is that it concentrates the flavor (Dashmaa et al. 2016; Savell 2008; Warren and Kastner 1992). Contrary to expectation, the present result didn’t show that free amino acids concentrate during the aging process as reported above. It, however, is difficult to

conclude the changes in each amino acid based on only the dry-aging process considering the different lengths of time between slaughter and the initiation of dry-aging for the different pieces of meat; this would greatly influence the concentration of each amino acid present. It is particularly interesting that the amino acids increased on the meat surface during the mold-aging process but not during the normal-aging process. GABA, one of the functional amino acids, that is famous for its sedative and other effects (Abdou et al. 2006) significantly increased after 2 weeks of mold-aging, when the mold completely covered the surface meat. To the best of our knowledge, there have been no previous reports of GABA content in beef meat. It is, therefore, highly possible that our mold strain itself produced GABA, proline and aspartic acid during growth. The hardness of mold-aged beef gradually decreased during the aging process and had significantly decreased after 4 weeks of aging. Proteases and collagenolytic enzymes produced by certain molds have previously been shown to break down the muscle and connective tissues of meat (Dashmaa et al. 2016). In contrast, the hardness of normal-aged beef did not significantly decrease. However, a definitive conclusion that only mold-aged beef became more tender cannot be drawn from the study findings. No significant decrease was observed during the normal-aging process due to the large error margins in the data. It is possible that normal-aged beef becomes softer as the natural enzymes in beef have been reported to break down proteins and connective tissue in the muscle, leading to more tender beef (Baird 2008; Campbell et al. 2016). Notably, the breaking stress values were similar at the endpoints of the two aging methods in our study. The present study showed that our isolated mold strain mainly had an effect on the free amino acid content and beef hardness. During the aging process, the mold was observed to also produce savory odors, which are important factors in increasing meat flavor and its appeal. Therefore, further studies are needed to determine the chemical compounds conferring this odor to mold-aged beef.

Figure 6-9. Changing appearance of mold-aged beef.
The photographs of mold-aged beef following two (left) and three (right) weeks of aging.

6-5 Abstract

A mold strain thought to be suitable for dry-aging process was isolated. The information about the scientific aspects of molds related to dry-aged beef is scarce. We, therefore, conducted aging trials to determine the characteristics of the isolated mold strain associated with dry-aging process. Specifically, during the dry-aging of beef with the mold strain, the changes in the free amino acid content, hardness, productive loss, drip and cooking loss were analyzed. These characteristics were compared with those obtained while dry-aging in the absence of a mold. Based on results in the current study, the isolated mold strain was identified as *Mucor flavus*. The free amino acid content of the interior meat sample in the mold-aging beef decreased or remained constant during the aging process. However, that in the trimming sections of the beef dramatically increased in the presence of mold. In addition, hardness of mold-aging beef gradually decreased during the aging process and finally decreased significantly. As a result, amino acids such as GABA (gamma-aminobutyric acid), proline, and aspartic acid were produced by our mold strain, *M. flavus* during its growth on beef meat, and the mold conferred savory odors to the dry-aged beef.

Chapter 7

Effect of dry-aging with *Mucor flavus* on beef taste and aroma

7-1 Introduction

For example, ‘mellow and intense’ and ‘earthy and nutty’ are the types of phrases commonly used to describe the flavor characteristics of dry-aged beef (Savell, 2008), and these result from the type of mold used. Okinawa Industrial Technology Center succeeded in isolating the mold strain, *Mucor flavus*, creating savory odors (Chapter 6). Several companies produce dry-aged beef products in Okinawa, using the isolated mold strain. Okinawa had over 10 million tourists in 2018, and there is an urgent need to develop new food content. The mold strain has distinctive characteristics (Chapter 6) and then it is possible to create a new Okinawan brand using the mold, attracting tourists. By the way, regarding free amino acids inside meats, we did not draw definitive conclusions that the mold aging process can concentrate amino acids, although mold itself produced many kinds of amino acids (Chapter 6). However, the surface meat after aging and trimming is expected to have more amino acids because the moisture content is relatively low compared to the interior meat. Therefore, amino acids in the meat surface should be analysed to confirm the increase. Besides, the mold strain can add unique savory flavor, not originally from meat, on dry-aged beef (Chapter 6), but the details of aroma compounds are yet unclear. The present study analysed volatile aroma compounds related to savory flavor from dry-aged beef with the mold using gas chromatography-mass spectrometry with solid-phase micro extraction.

7-2 Material and Methods

7-2-1 Beef material

The meat used for the experiments were beef sirloins imported from New Zealand. The

cow beef was a crossbreed between Angus and Hereford, rose in pastures.

7-2-2 Aging environment

The aging environment was established in a refrigerator (Showa Denko K.K.) in the Okinawa Industrial Technology Centre. Accordingly, dry boxes were placed in the refrigerator. Further, three pieces of meat were placed in the boxes for 3 weeks under-maintained conditions of approximately 80% relative humidity for dry-aged beef with *Mucor flavus* or not. For dry-aged beef with the mold, the mold strain was cultured on PDA plates (Merck Ltd.) for a week and then allowed to contact each piece of meat.

7-2-3 Quantitative analysis of amino acids

Samples from the surface of the edible part of the aging beef were collected after being trimmed. Three samples were obtained in one meat block. These were cut and homogenised. Analysis of amino acids is the same as 5-2-4. The values were adjusted for the concentration of moisture content after aging.

7-2-4 Volatile compound analysis

1.0 g of dry-aged beef samples were placed in a 20 mL of glass vials sealed with a poly tetra fluoro ethylene/silicone septum (Gl sciences inc., Eindhoven, Netherlands). The volatile compounds were prepared according to the modified method (Yu et al., 2008) of the headspace solid-phase micro extraction (HS-SPME) using a divinylbenzene/carboxen/ polydimethylsiloxane (50/30 μm thickness) SPME fiber (Supelco Co., Pennsylvania, USA). Volatile components were injected with split less mode onto a InertCap Pure WAX ProG 2 M column (0.25 mm × 60 m, df = 0.25 μm, Gl sciences inc.) at a flow rate of 1.23 mL/min helium at 40°C (2 min) − (5.0°C/min) – 230°C (5 min) in the GCMS-QP2010 Ultra (Shimadzu corp., Kyoto, Japan) system. As

MS conditions, ion source and interface temperature were 230 °C and 240 °C, and the MS detector was operated in scan mode (20–600 m/z) using electron impact ionisation (70 eV). Based on the data obtained from GCMS, the NIST11 Mass Spectrum Library (National institute of standards and technology, USA) and FFNSC 2 Flavour and Fragrance Natural & Synthetic Compounds GCMS Library (Chromaleont srl, Italy) were used to identify the volatile compound in each peak with the highest similarity score of mass spectra. Identified compounds containing Si and Ni were removed for Figure 7-4 and Table 7-1 because they were not originally from beef. In addition, identified compounds below 85% of similarity were removed for Table 7-1.

7-2-5 Statistical analysis

Tukey's test was used for identifying differences ($P < 0.05$) in each amino acid group between before and after aging using Rstudio Version 1.4.1717.

7-3 Results and Discussion

7-3-1 Amino acids content

After dry-aging, *Mucor flavus* covered beef sirloin with white hyphae (Figure 7-1). Previously, the amino acid content of the interior meat samples did not increase stably during dry-aging with the mold (Chapter 6). Regarding amino acids contained in beef meat, there are several steps of proteinogenic degradation with enzyme reaction. The way to produce free proteinogenic amino acids assumes to be from short peptides and not directly from protein. In fact, the sum of amino acids increased more as the temperature for aging, meaning an acceleration of enzyme reaction depending on high temperature (Savell, 2008). Actually, the amino acid content of each beef meat varies according to which step of meat to be in, depending on how many days elapsed after slaughter before delivery. Therefore, molecular biological techniques make it difficult to elucidate the difference in the underlying mechanism of amino acid production among different aging processes. This time, the surface after trimming was chosen as the sample to analyse amino acids. The surface was not where the hyphae of the mold extended. Actually, amino acid contents depend on choosing the sampling point. No difference of amino acid increase in the interior meat samples of aged beef conducted under 80% and 100% relative humidity has been reported (Chapter 5). These sample points were over 1 cm depth from the surface after trimming, and the moisture contents were almost the same (about 74%). In this result, both amino acid contents in dry- or wet-aged beef were similar. However, moisture contents were lower in the immediate sample inner of the trimming part (about 71%) than the interior meat. It was hypothesised that there is a gradient of moisture content within about 1 cm from the surface of the edible part to the inner side (Figure 7-2), and there would also be a gradient of amino acids content. Also, that would lead to a more proteinogenic degradation process. In fact, total amino acids significantly increased in the meat surface after dry-aging with the mold ($P < 0.01$) even though the

moisture content was adjusted (Figure 7-3). Additionally, sweet-tasting amino acids and savory-tasting amino acids significantly increased after dry-aging with the mold ($P < 0.05$). That means each amino acid increased due to the proteinogenic degradation process. Although the trimming part becomes the cause of the burnt deposit turning out black, the surface of the edible part must be most concentrated and most tasty; releasing good odors after it is grilled because amino acids or other components are concentrated in part. In fact, amino acids react with sugars by heating, resulting in a savory flavor (Okumura, 1993), which also contributed to various smells on dry-aged beef.

7-3-2 GCMS chromatogram

In Okinawa, using mold is becoming common for producing dry-aged beef. However, the effect of dry-aging with mold is not obvious, such as *Mucor flavus* on aroma volatiles in beef. Accordingly, odor analysis should be conducted to discover volatile compounds that *Mucor flavus* can add to beef, useful information about added value for dry-aged beef we produce in Okinawa. The mold strain must produce a savory flavor that adds value to dry-aged beef companies in Okinawa produce. From the result of GCMS chromatogram, distribution of total ion chromatogram of volatile compounds from dry-aged beef with the mold was considerably different from that of dry-aged beef without mold despite some peaks being identified as the same compounds in both types of dry-aged beef (Figure 7-4). And the number of peaks was higher in dry-aged beef with the mold (75 peaks) than that of dry-aged beef without mold (67 peaks). Besides, peaks detected in dry-aged beef with the mold were scattered horizontally within 30 min of retention time. In contrast, relatively many peaks in dry-aged beef without mold were plotted within 20 min of retention time. Accordingly, higher polar compounds existed in dry-aged beef with the mold because the Wax column has a highly polar polyethene glycol stationary phase. As a result, there is a high possibility that *Mucor flavus* can produce higher polar compounds

on beef meat.

7-3-3 Aroma volatile compounds

Table 7-1 shows the volatile compounds detected in dry-aged beef with *Mucor flavus*. Among acids detected in dry-aged beef with the mold, two compounds were also detected in dry-aged beef without mold, such as acetic acid and 2-methyl-propanoic acid. However, three compounds, Heptanoic acid, 2-Oxooctanoic acid and Undecanoic acid, were detected only in dry-aged beef with the mold. As for alcohols, six compounds, 1-Hexanol, 1-Heptanol, 1-Octanol, trans-2-Octen-1-ol, trans-2-Nonen-1-ol and Phenylethyl Alcohol were also detected in dry-aged beef without mold. In contrast, two compounds, cis-Hept-4-enol and 3-(Methylthio)-1-propanol, were detected only in dry-aged beef with the mold. As for aldehydes, one compound, Octanal, was also detected in dry-aged beef without mold. Six compounds, trans-2-Octenal, trans-2-Nonenal, 2,4-trans,trans-Nonadienal, Decanal and 2-Undecenal, were detected in dry-aged beef with the mold. As for esters, two compounds, n-Caproic acid vinyl ester and Benzoic acid, 2-formyl-4,6-dimethoxy-, 8,8-dimethoxyoct-2-yl ester were also detected in dry-aged beef without mold. Four ester compounds, 1-Norbornanemethanol, acetate, cis-3-Octen-1-ol, acetate, Decanoic acid, ethyl ester and Ethyl 9-hexadecenoate were detected only in dry-aged beef with the mold. All three heterocyclic compounds were detected only in dry-aged beef with the mold, such as 2,3,4,5-Tetrahydropyridine, 5-Ethyldihydro-2(3H)-furanone and 2-Acetyl-2-thiazoline. Three acid compounds detected in mold-aging are aliphatic higher acids and could be produced by the mold during aging. It was reported that acids were almost not detected in dry-aged beef produced in the aging room with air flow (Mikami *et al*., 2021). That means acids could be volatilised by air flow. On the contrary, there was no air flow in the dry box where dry-aging was conducted in the present study, and accordingly, acids could remain easily on the meat. As for alcohols, six compounds were also detected in

dry-aged beef without mold. Among of them, 1-Hexanol, 1-Heptanol and 1-Octanol were also detected in wet-aged beef (Mikami *et al*., 2021). They are described as malty, popcorn-like, corn-like and citrus smells (Migita *et al*., 2017; Feng *et al*., 2019). As a result, they are supposed to be generated during the aging process of meat, not related to the mold. On the contrary, three compounds were detected only in dry-aged beef with the mold. Unfortunately, it is not known what they smell, but there is a possibility that the mold can produce those compounds. As for aldehydes, one compound was also detected in dry-aged beef without mold. In fact, heptanal, trans-2-octenal, and trans-2-nonenal were detected in dry- and wet-aged beef (Mikami *et al*., 2021); however, in this study, trans-2-octenal smelled nutty only when found in dry-aged beef with mold. In addition, 2,4-trans,trans-Nonadienal and 2-Undecenal, aliphatic higher aldehydes were detected in dry-aged beef with the mold. As a result, they are unique compounds in dry-aged beef with the mold, *Mucor flavus*. Additionally, these smells are described as nutty and boiled meat, which can be a savory flavor you smell. As for esters, two compounds were also detected in dry-aged beef without mold. Four ester compounds were detected only in dry-aged beef with the mold, but the smell they imparted was not investigated in details. However, it is known that esters have a fruity aroma and they could be involved in contributing to the aroma and flavor of mold-aging beef. Interestingly, all heterocyclic compounds were detected only in dry-aged beef with mold. Heterocyclic compounds could have a unique aroma. In fact, heterocyclic compounds are of interest because of their varied occurrence in food flavors and their valuable organoleptic characteristics. Even though heterocyclic aroma chemicals are found only in minute amounts in foods, their powerful odors and low odor thresholds, as expressed by high values, make them key in boosting flavors and fragrances (Zviely, 2008). Accordingly, they also could play an important role in savory flavor (perhaps ‘mellow and intense’) in dry-aged beef with *Mucor flavus*. As a result, the number of volatile compounds by GCMS in dry-aged beef

with the mold was higher, and several peaks were related to the savory flavor possessed only by dry-aged beef with the mold.

7-3-4 Next step

Lee *et al*. (2021) reported that 37 volatile compounds, including aldehydes, furans, ketones, etc. were detected in aged beef. The method of sample gas collection was conducted by headspace without SPME and incubation for 10 mins at 80 ℃. Among detected compounds, only one compound, hexanoic acid, was detected in the present study. This time, the gas collection method was conducted using SPME and incubation for 10 mins at 60 ℃. The temperature difference must be why detected compounds are totally different between them. Of course, higher temperature makes it easier to detect more compounds because the higher temperature can volatilise more compounds on meat, and it is closer to actual beef restaurants serve. The next step is to analyse volatile compounds using GCMS with incubation for higher temperatures and research the composition of fatty acids and others during mold-aging.

Figure 7-1. Appearance of *Mucor flavus* covering dry-aged beef.

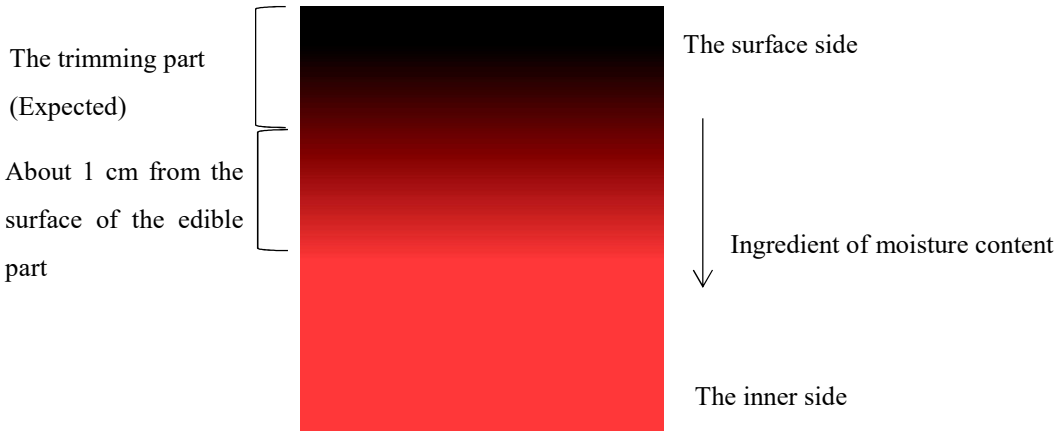

Figure 7-2. A cross-section from the surface to the inner side in dry aged beef where an ingredient of moisture content presumably occurred.

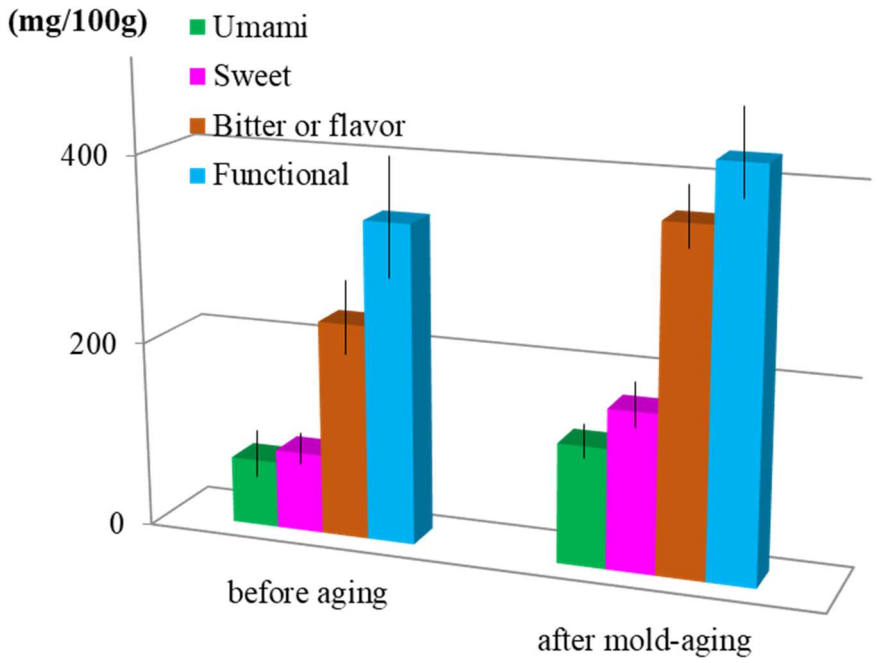

Figure 7-3. The levels of each amino acid group in the surface meat before and after dry-aging with the mold.
Amino acid groups are classified as functional, bitter or flavor-, sweet- or umami-tasting. Significant differences were detected in total ($P < 0.01$), sweet ($P < 0.05$) and bitter or flavor ($P < 0.05$).

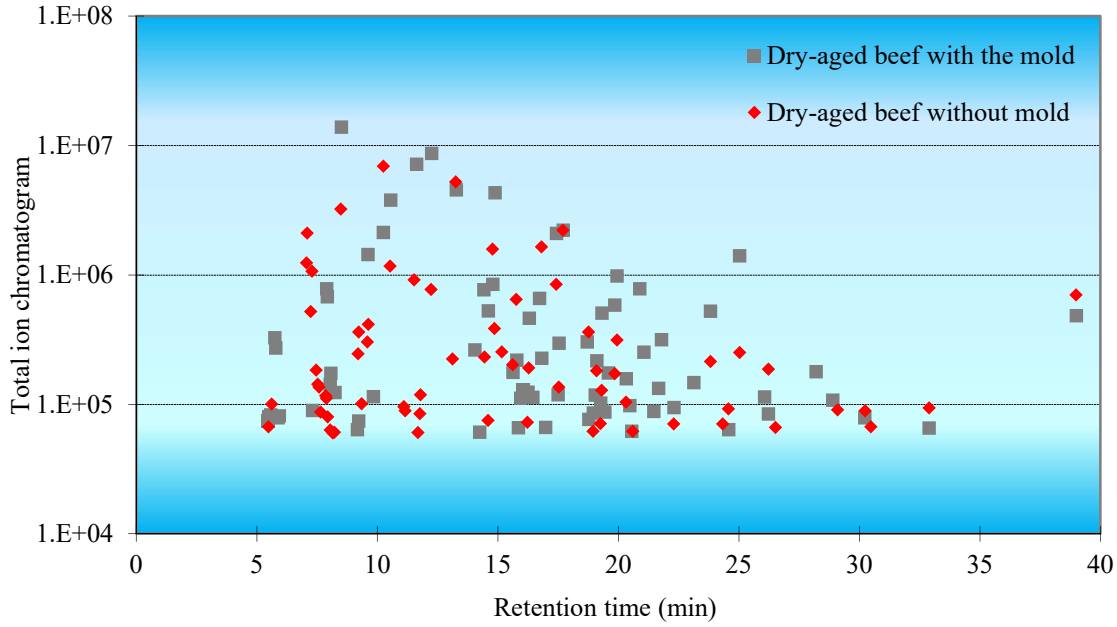

Figure 7-4. Total ion chromatogram of volatile compounds vs retention time by GCMS with dry-aged beef with the mold or not.

Table 7-1. Identified volatile compounds from dry-aged beef with *Mucor flavus*.

Category		Volatile compounds	Aroma	Retention Index	Peak area
Acids		Acetic acid	sour, pungent, vinegar [a, b, c]	576	5.E+05
		2-methyl-propanoic acid		711	3.E+05
		Heptanoic acid		1136	1.E+05
		2-Oxooctanoic acid		1309	6.E+04
		Undecanoic acid		1471	2.E+05
Alcohols		1-Hexanol	popcorn-like [d]	860	9.E+06
		1-Heptanol	woody, oily, corn-like [d]	960	4.E+06
	Alkene	**cis-Hept-4-enol**		968	1.E+05
		1-Octanol	green, citrus, roasted, meat-like [d, e]	1059	2.E+06
	Alkene	trans-2-Octen-1-ol	grassy, plant-like [d]	1067	3.E+05
	Thio	**3-(Methylthio)-1-propanol**		912	8.E+05
	Alkene	trans-2-Nonen-1-ol		1167	3.E+05
	Benzen	Phenylethyl Alcohol	rosy, floral [b, e]	1136	1.E+06
Ketones	Pyrroline	**2-Acetyl-1-pyrroline**	**popcorn [f]**	945	7.E+06
Aldehydes		**Heptanal**	**fatty, green, floral [g, f]**	905	8.E+05
		Octanal	fatty, soapy, peel [h, e]	1005	4.E+06
	Alkene	**trans-2-Octenal**	**fatty, green, citrus, nutty, cooked flour [i, e, f]**	1013	3.E+05
	Alkene	**trans-2-Nonenal**	**green, cucumber, fatty [e]**	1112	7.E+05
	Alkadien	**2,4-trans,trans-Nonadienal**	**nutty, fatty [f]**	1120	6.E+04
		Decanal	**rubber tubing, smokey [j]**	1204	1.E+05
	Alkene	**2-Undecenal**	**fatty, boiled meat [j]**	1311	3.E+05
Esters	Vinyl	n-Caproic acid vinyl ester		974	2.E+05
		1-Norbornanemethanol, acetate		1181	1.E+05
	Alkene	**cis-3-Octen-1-ol, acetate**		1191	1.E+05

		Decanoic acid, ethyl ester	1381	9.E+04
		Ethyl 9-hexadecenoate	1986	1.E+05
	Benzen	Benzoic acid, 2-formyl-4,6-dimethoxy-, 8,8-dimethoxyoct-2-yl ester	2658	1.E+05
Ether		**1-Ethoxy-cis-2-heptene,**	1000	4.E+04
Heterocycle	Pyridine	**2,3,4,5-Tetrahydropyridine**	740	8.E+05
	Furanone	**5-Ethyldihydro-2(3H)-furanone**	986	7.E+04
	Thiazoline	**2-Acetyl-2-thiazoline**	1104	1.E+05
Others	Alkene	3,5,5-Trimethyl-1-hexene	757	2.E+05
	Cyclohexene	**3-Methylcyclohexene**	763	7.E+04
	Cyclopenten	**5-Hexyl-3,3-dimethyl-1-cyclopentene**	1274	1.E+05

Bold letter indicates volatile compounds detected only in dry-aged beef with *Mucor flavus*, not in that without mold. Aroma descriptions were obtained from the following publications: a Frauendorfer and Schieberle (2008); b Majcher *et al*. (2020); c Yu *et al*. (2019); d Migita *et al*. (2017); e Feng *et al*. (2019); f Yang *et al*. (2008); g Liu *et al*. (2019); h Vera *et al*. (2020); i Bi *et al*. (2020); j Song *et al*. (2010).

7-4 Abstract

Okinawa Industrial Technology Center succeeded in isolating the mold strain, *Mucor flavus*, creating savory odors during beef aging. Several companies already produce dry-aged beef products using the isolated mold strain in Okinawa. It will be a new food product as the Okinawan brand for tourists from all over the world. After trimming, each amino acid in the meat surface was analysed by LCMS and aroma volatile compounds from the headspace were analysed by solid-phase micro extraction followed by GCMS to reveal the effect of dry-aging with *Mucor flavus* on beef taste and aroma. Each amino acid in the surface of dry-aged beef with the mold increased. It was hypothesised that there is a gradient of moisture content from the surface of the edible part to the inner side of the dry-aged beef, where amino acids easily generated from the protein degradation. Amino acids react with sugars by heating, resulting in savory flavor. Besides, the peak number of volatile compounds using GCMS was more detected in dry-aged beef with the mold than that of dry-aged beef without mold. There is a high possibility that *Mucor flavus* can produce higher polar compounds on beef meat. Several aldehydes detected only in dry-aged beef with the mold were related to savory flavors, such as nutty and boiled meat.

Chapter 8

General discussion

Okinawa has a subtropical climate, and tropical grasses have been growing there for a long time. In Okinawa, area of grass pasture covers more than 95% of the area with 4 major grass varieties recommended, i.e. *Ch. gayana*, *D. eriantha*, *M. maximus* and *C. nlemfuensis* in the past 15 years. Transvala has become the major grass sown in Okinawa and represented 30.1% of total area sown to grass in Okinawa in 2021 because of its perceived excellent characteristics and its suitability for local conditions (Hanagasaki 2022). This grass variety has contributed tremendously to the beef industry. However, other types of grass are also required because Okinawa offers a variety of climatic and geographical conditions. Regarding this, it is observed that Basilisk and MG5 has several characteristics that make it suitable for Okinawa conditions. Transvala is a creeping type plant and can only propagate vegetatively, whereas Basilisk and MG5 are erect type of plants that mainly propagated via seeds. Accordingly, growing Basilisk and MG5 can complement Transvala. The forage production study has provided valuable information on the relative performance of a range of tropical grasses including Basilisk and MG5 over a number of years in Okinawa. Basilisk was obviously superior to Transvala in terms of yields of DM, digestible DM and CP, suggesting that it could be an acceptable substitute. In addition, Basilisk and MG5 performed as well as all other cultivars evaluated in most years and outperformed some cultivars in some years. Basilisk maintained a high level of production and had higher DM yield than other recommended grass varieties of Okinawa Prefecture, supporting claims that this cultivar has a good level of tolerance of drier conditions. In particular, Basilisk and MG5 are drought tolerant in some islands experiencing drought. In fact, Basilisk maintained a higher level of production than other recommended grass varieties of Okinawa Prefecture while rainefall in 2003 was considerably lower than the long-term mean of the area. Further, total DM

yield and total digestible DM yield of both Basilisk and MG5 during 2006–2008 were significantly higher than that of *Urochloa mutica* (syn. *Brachiaria mutica*), one of recommended grass varieties of Okinawa Prefecture, whereas the corresponding total CP yields (Basilisk at 10.4 t/ha and MG5 at 10.9 t/ha) were similar to those of other *Urochloa* cultivars ($P > 0.05$).

Considering the situation in Okinawa, these two grass varieties should be planted there. Based on results of the current study, both varieties were approved to be added to the list of grasses recommended for use in Okinawa Prefecture for improving beef production in 2016. Consequently, if Basilisk and MG5 are introduced and planted widely in Okinawa, they will induce the production of high-quality forage, which could reduce the annual cost of breeding cows (Kouki and Ebina 2009). Conversely, high-quality forages, such as Basilisk and MG5, are required to establish beef brands. In a trial of growing Japanese black calves using Transvala hay showed results comparable to those shown by timothy hay (Nagatoshi et al. 2007). Basilisk and MG5 maintained a high production level and had a higher DM yield, digestible DM yield and CP yield than that of Transvala, which can be useful in fattening beef toward the beef brand because of the type of feed associated with the beef brand (Chapter 2). However, Basilisk and MG5 rarely produce their seeds under the circumstances in Okinawa. Accordingly, strategies for increased usage of these grass varieties should be developed. The standard method for vegetative propagation of grasses in Okinawa is to cut stems (culms) to retain 2 joints (nodes), the cuttings then being inserted into a mixture of soil and potting mix. Actually, Transvala can root and grow easily with this method (Mochizuki et al. 2005). As for MG5, this method normally results in a success rate of about 10% of plantlets being produced. Results have been similar when a commercial rooting accelerator (TGG010S or TGG020S, both from the auxin group; Tokai Global Greening Co. Ltd.) was used, indicating that treatment with that plant hormone had no effect on rooting of MG5 and Basilisk cuttings. In the current

study, the new two propagating methods were developed. Both methods that produce rooted cuttings resulted in a sufficient percentage of plantlets. With approximately 77% of plantlets produced, direct inserting the lower cuttings of stems into the soil is a successful and practical method for the vegetative propagation of MG5 (Chapter 3). Nevertheless, a suitable supply of seeds is required for the wider production of cultivars. Thus, the next step is to ensure an import route for the quantity of Basilisk and MG5 seeds. In fields, tropical grasses have been used not only for direct feeding and pasture but also as silage in Okinawa. Transvala can be made into high-quality silage and acts as useful and convenient forage if produced via appropriate processing. The current study revealed that good-quality Transvala silage is related to a very wide range of LAB. *Lactobacillus* was the obvious predominant flora in Transvala silage, sorghum silage and corn silage, all of which were of acceptable quality. To increase the possibility of stimulating proliferation of these beneficial LAB as early as possible, wilting grass sufficiently to below 70% moisture seems critical. In addition, compacting fresh forage effectively and wrapping silage properly to minimize the amount of air in the silage should enhance the chances that the environment in the silage would be suitable for rapid proliferation of LAB (Chapter 4). It is obvious that choosing excellent LAB needs to produce tropical grass silages superior in quality. Indeed, Basilisk and MG5 also can become good quality silages if they are produced properly because they have abundant nutrients. Identifying the LAB most suitable for producing Basilisk and MG5 silages is needed in further studies. It is hoped that these findings on forage production and quality, vegetative propagation and silage research will contribute to the basis of a feed intake study in beef cattle, which is necessary for commercializing Okinawan beef brand.

After establishing the beef brand, we need to boost Okinawa's unique food brand using local beef or imported beef to promote Okinawa's economy. The current study demonstrated the effects of both dry- and wet-aging processes on Okinawan delivered

cow beef and Australian beef. The positive effects, such as an increase in free amino acids and a decrease in hardness, in dry-aged beef were almost the same as those found in wet-aged beef. In fact, no significant differences were found between the two beef aging methods with regard to all studied variables, except drip, cooking and productive losses. On one hand, drip loss significantly decreased and cooking loss showed a decreasing trend during the dry-aging process; on the other hand, cooking loss significantly increased during the wet-aging process. In other words, dry-aged beef could become juicy during the dry-aging process. Besides, the tenderness observed in biting them indicates the clear difference between dry- and wet-aged beef. Upon chewing wet-aged beef, it was observed that its tenderness remained for longer than that of dry-aged beef, whereas dry-aged beef was much easier to cut with the teeth. In the study, I cannot derive definitive conclusions regarding the difference in amino acid increase in the interior meat samples during both dry- and wet- aging processes. Nonetheless, the results showed that glutamic acid increased stably, which is essential for the umami taste of meat, for beef during both types of aging. It was reported that meat from Japanese brown cattle have high levels of glutamic acid and leucine, which increase stably during the aging process (Sugioka et al. 2015). However, alanine did not increase but fluctuated sharply and non-proteinogenic amino acids also did not increase during the aging process. The results in the study were very similar to these results. In particular, the results in Okinawan delivered cow beef were exactly the same as these. In conclusion, I discovered there are several advantages to dry-age Okinawan delivered cow beef and Australian beef (Chapter 5).

The mold strain we discovered was found to belong to the *Mucor* genus, which is used for preparing various food products, including sausage, salami, cheese, tofu and vegetables, worldwide (Stephanie et al. 2017). Therefore, *Mucor* is a relatively safe mold genus, and its fermentation effects are expected to improve the quality of meat in terms of certain aspects. In the study, during mold-aging process, the mold mycelium was

observed to sparsely spread on the meat during the first week and to completely cover it after 2 weeks, producing light gray-colored meat and the mold grew stably even after that. While it grew, productive loss did not increase between the weeks 3 and 4 of aging and did not exceed 30%, a significantly lower level than that in dry-aged beef without mold. This was an interesting finding, and the mold strain possibly prevented moisture evaporation from the meat and trim once it had completely covered the meat surface. Substantial yield losses and increased processing times that are associated with the dry aging process require to increase prices for the sale and distribution of dry-aged product (Laster 2007). Decreasing productive loss of dry-aged beef is very important because it directly influence the retail segment of the beef industry. Under the circumstances, there is a possibility that covering the meat by the mold increases retail yield and it may be the key of dry-aging process. The hardness of mold-aged beef gradually decreased during the aging process and had significantly decreased after 4 weeks of aging. Proteases and collagenolytic enzymes produced by certain molds have previously been shown to break down the muscle and connective tissues of meat (Dashmaa et al. 2016). In contrast, the hardness of dry-aged beef without the mold did not significantly decrease. However, a definitive conclusion that only mold-aged beef became more tender cannot be drawn from the study findings. No significant decrease was observed during the dry-aging process without the mold due to the large error margins in the data. It is possible that dry-aged beef without the mold becomes softer as the natural enzymes in beef have been reported to break down proteins and connective tissue in the muscle, leading to more tender beef (Baird 2008; Campbell et al. 2016). Notably, the breaking stress values were similar at the endpoints of the two aging methods in the study. By the way, the amino acid content of the interior meat samples did not stably increase during mold-aging process. However, it is particularly interesting that the amino acids increased on the meat surface where the mold grew during the mold-aging process but not during the dry-aging process without

the mold. The presence of GABA, one of the functional amino acids famous for its sedative properties, and other amino acids significantly increased after 2 weeks of mold-aging, when the mold completely covered the surface meat. As a result, trimmings of the mold-aged beef caused an increase in amino acids, even the type of amino acids such as, proline and aspartic acid, that beef does not usually contain. Accordingly, it is possible to use the trimmings of mold-aged beef to prepare jerky as a functional food item (Chapter 6). There are many steps to produce amino acids after beef slaughtering. It is hypothesized that the change in amino acid composition in the interior meat stems from normal change even in dry- and mold-aged meats because there was no difference in the amino acid composition between the dry-, wet- and mold-aging processes. Indeed, it is ideal that the trials using beef are approved via the same terms that are used after slaughter; however, it is difficult to obtain these beef meats from foreign countries. In fact, local produced beef meats were not enough for trials in Okinawa. Nevertheless, it is of great significance that I can detect the increase in amino acid concentration on the surface of dry-aged beef with the mold after trimming, which is one of the advantages of mold-aged products. In general, beef is served in the form of steak in restaurants after grilling at least the outer surface of the meat. The reaction between the amino acids and sugars results in the grilled savory flavor of the steak. Accordingly, a higher amino acid content can promotes this reaction, resulting in a more savory flavor. Further, it adds the unique flavor of mold-aged beef as some compounds release nutty and boiled meat flavor. It would be one of contributions to creating food brands to attract many tourists (Chapter 7). These results related to increased free amino acid content and decreased hardness during dry-aging and savory flavor produced by the mold during dry-aging is expected to contribute to the elucidation of key information on the aging mechanism of beef meat, which is necessary for commercializing Okinawan beef or food brands.

In this context, the two prefectural recommended grasses play an important role in

establish of Okinawan brand beef, such as special grass-fed beef, owing to the valuable nutritive content discovered in the current study. Actually, MG5, with high digestible crude protein and total digestible nutrients, resulted in higher body-weight gain in cattle than Transvala and *Chloris gayana* cv. Katambora (Nakanishi et al. 2006). In general, cattle raised on grass tend to have leaner meat, which is more suitable for dry aging in terms of increased free amino acid content and decreased hardness. Therefore, as a grass-fed brand of beef unique to Okinawa is going to be established, the meat should be improved using the dry-aging technique developed in this study for the Okinawan beef food brand. Besides, the technique of mold-aging method using *Mucor flavus* would establish Okinawan food brand because of the advantage I discovered in the study. I hope Okinawan brand beef and its dry- and mold-aging beef products, Okinawan food brand, are established as world's brand and become popular all over the world.

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