Master's Thesis of Science in Agriculture

Development of Broccoli By-product Silage by MHA (Methionine Hydroxy Analogue) Treatment and Its Evaluation on Substitutional Effect for Hanwoo Feed

메티오닌 수산화 유도체(MHA) 처리 브로콜리 부산물사일리지 개발 및 한우 사료 대체 효과 평가

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Wang Xin

Department of International Agricultural Technology

Graduate School of International Agricultural Technology

Seoul National University

Development of Broccoli By-product Silage by MHA (Methionine Hydroxy Analogue) Treatment and Its Evaluation on Substitutional Effect for Hanwoo Feed

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By

Wang Xin

Supervised by
Prof. Sang Kee Kang

Major of International Agricultural Development
Department of International Agricultural Technology
Graduate School of International Agricultural Technology
Seoul National University

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Approved as a qualified thesis
for the Degree of Master of Science in Agriculture
by the committee members

Chairman **Kyoung Hoon Kim, Ph.D.** _____

Member **Sang Kee Kang, Ph.D.** _____

Member **Jong Geun Kim, Ph.D.** _____

Abstract

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Wang Xin
Major of International Agricultural Development
Department of International Agricultural Technology
The Graduate School
Seoul National University

Nowadays, the comprehensive utilization and recycling of agricultural by-products are important, which could reduce environmental pollution and promote the sustainable development of agriculture.

Every year in Pyeong - chang area, South Korea, a large amount of broccoli by-products are abandoned. In this study, broccoli by-product silage with 0.4 % MHA (Methionine hydroxy analogue) to replace the fermented process was used to evaluate the substitutional effect to the conventional feed for Hanwoo. MHA pH is lower than 2. When used as a feed additive, it can be used as methionine nutrition supplement to promote the growth and

development of animals. In this experiment, MHA treated groups and MHA untreated groups were compared to test (chemical composition, organic acids, pH, microbial, sulforaphane, beta-carotene and antioxidants). Then select the most appropriate formula for mass production, the mass broccoli by-product silage was fed to pregnant Hanwoo cattle, and their substitutability for conventional feed was evaluated by the health states (body weight, body shape measurements, metabolic profile test, complete blood count) of Hanwoo.

The results showed that there was no significant difference in TDN and RFV between MHA broccoli by-product silage and control silage (inoculated with *L.plantarum* 5×10^7 cfu / kg of the silage), that means MHA has no adverse effect to the feed value. It can be seen from the microbial growth and organic acid content that the addition of MHA (the highest concentration of 0.8 % in this study) can not completely replace the fermentation process, but MHA treated group content more lactic acid and less acetic acid, and there was no or very little butyric acid, it can be calculated MHA was beneficial to the fermentation process for environmental lactic acid bacteria and inhibited the activity of undesirable bacteria. For the beta-carotene, the content of beta-carotene increased nearly 60 % - 80 % after fermentation, and high concentrate MHA group (0.4 % and 0.8 % treated group) losses less then 20 % beta-carotene after 10 days fermented. The ability of antioxidation also

increased after fermentation. Through the *in vivo* test, the health status of the two groups (BB-TMR group and Con-TMR group) was compared to evaluate the desirability of the feed. After comparison, there was no difference between the two groups in terms of weight, MPT, CBC, and various health assessment indicators. But as for fatty acid composition, BB-TMR group contains more oleic acid and less stearic acid, which is very valuable for health. In terms of the content of functional substances, there was also no difference in the content of vitamin A, and antioxidants levels in plasma. Finally, it can be concluded the MHA broccoli by-product silage can substantiate the conventional feed as the Hanwoo feed.

Key word: Broccoli by-product, Broccoli by-product silage, MHA (Methionine hydroxy analogue), Substantiation, conventional TMR, Hanwoo feed

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List of Abbreviations and Formula

ACN: Acetonitrile

ADF: Acid detergent fiber

ALB: Albumin

BB: Broccoli by-product

BBS: Broccoli by-product silage

BP: Beet pulp

BT: Base TMR

BF: Barley straw forage

BUN: Blood urea nitrogen

CFU: Colony forming unit

CP: Crude protein

CS: Corn silage

DDM: Digestible dry matter

DIM: Days in milk

DM: Dry matter

DMI: Dry matter intake

DW: Distilled water

DPPH: 2,2-diphenyl-1-picrylhydrazyl

EE: Ether extract

GAE: Gallic acid equivalent

GC: Gas chromatography

Glb: globulin

HGB: Hemoglobin

IACUC: Institutional Animal Care and Use Committee

LAB: Lactic acid bacteria

LC: liquid chromatography

LDL: Low density lipoprotein

MCV: Mean corpuscular volume

NDF: Neutral detergent fiber

NFC: Non-fiber carbohydrate

NEFA: Non-esterified fatty acid

RB: Rice bran

RBC: Red blood cells

RFV: Relative feed value

ROS: Reactive oxygen specie

RT: Room temperature

SEM: Standard error of the mean

TDN: Total digestible nutrients

TAG: Triglycerides

TMR: Total mixed ration

TPC: Total phenolics content

WB: Wheat bran

WBC: White blood cells

1. Introduction

With the development of society, the green-biotechnology including agricultural has become an important way to solve the problem of resource reuse and environmental pollution at home and abroad. Following the principle of sustainable development, green food production in a specific way and actively developing green agriculture has become a strategic measure to meet international challenges (Steer, 2008). There are many ways of comprehensive utilization and recycling of agricultural and sideline products. In this study, broccoli by-product silage which with 0.4 % MHA (Methionine hydroxy analogue) to replace the fermented process were used to evaluate the substitutional effect to the commercial feed for Hanwoo feed.

In this experiment, we choose broccoli by-product (majorly leaves and stem part) as a potentially functional bio-resource additive to evaluate the substitution for commercial feed. In the part of *in vitro* experiment, different concentrations of MHA were added to make silage. The main evaluation items were feed quality and retention of some functional substances, including beta-carotene, total phenolic acid content, sulforaphane, etc. The purpose of adding MHA is to directly reduce the pH

value and inhibit the activity of bacteria to replace the process of feed fermentation for long - term preservation of the feed.

Through the comprehensive evaluation of the results, the most appropriate formula is selected to mass production. For animal experiments, 16 pregnant Hanwoo cattle were divided into two groups. The substitution of the feed was evaluated by comparing the broccoli by-product silage and commercial silage. At the same time, the substitutability of feed was evaluated by the health status of Hanwoo. Through these tests, it can be concluded that the TMR containing MHA broccoli by-product silage can substitute commercial feed as the daily feed of Hanwoo cattle.

2. Literature Review

2.1. Purpose and Necessity of Using Agricultural By-products

Agricultural by-products refer to non-economic products in agricultural production, including crop straw, livestock manure, waste after agricultural products processing (such as animal viscera, bone, hair, distiller's grains) and other renewable resources (Xu & Geelen, 2018). Through the comprehensive development and utilization of agricultural and sideline products, it can not only turn waste into treasure, increase income, but also reduce environmental pollution, increase fuel, feed, fertilizer, industrial raw materials, etc., and promote the sustainable development of agriculture (Alic, Branscomb, & Brooks, 1992).

With the development of society, green agriculture has become an important way to solve the problem of resource reuse and environmental pollution at home and abroad. Following the principle of sustainable development, green food production in a specific way and actively developing green agriculture has become a strategic measure to meet international challenges (Steer, 2008).

There are many ways of comprehensive development and utilization

of agricultural and sideline products. According to their uses, there are mainly five kinds: making fertilizer, making feed, producing edible fungus, producing industrial products and making energy. Therefore, one of the goals of this study is to select one agricultural by-product, conduct a valuation as a feed and establish a feeding technique for its use (Aguilar‐Rivera, Llarena‐Hernández, Michel‐Cuello, Gámez‐Pastrana, & de Jesús Debernardi‐Vazquez, 2017).

2.2. Broccoli By-Product

2.2.1. Generation of broccoli by-product in Korea

In South Korea, the planting area of broccoli is 2,014 ha nationwide, with a total planting weight of 25,101 tons, of which the total planting amount of Jeju Island is 75.51 % of the total output, and the output of Gang - won Province is 10% of the total output. As the edible part of the broccoli is only the flower, about 7821 tons of by-products of broccoli are wasted in Gang - won Province every year.

2.2.2. Characteristics and utilization of broccoli and broccoli by-product

Broccoli, also known as cauliflower, broccoli and green cauliflower, is a variety of broccoli in ems that are drawn out. The top of the plant is a group of closely integrated flower buds. The flower buds are green, so it is also called broccoli. The leaves are blue-green, gradually turning to dark blue-green, and the wax powder is increasing. Petioles long and narrow. There are two types of leaf forms: broadleaf and longleaf (Clarke et al., 2011).

One of the broccoli's biggest advantages is its nutrient content. It's loaded with a wide array of vitamins, minerals, fiber and other bioactive compounds (Ouda & Mahadeen, 2008). Broccoli is also a good source of polyphenolic compounds with high antioxidant activity, and it could play a significant role in the prevention of diseases associated with oxidative stress, such as cardiovascular and neurodegenerative diseases as well as cancer. (Hwang, J.-H., & Lim, S.-B. (2015). Antioxidant and anticancer activities of broccoli by-products from different cultivates and maturity stages at harvest. Preventive Nutrition and Food Science, 20(1), 8–14.) Most people only eat cauliflower, which accounts for about 30% of the vegetable biomass. For this reason, research is usually focused on the

flower part, while information about the nutritional characteristics of other parts of broccoli is usually limited. Only a few authors have described nutrients and antioxidants. Activity of broccoli by-products (DOM í nguez PERLES et al., 2010; Guo, Lee, Chiang, Lin and Chang, 2001; Hwang and Lim, 2015; soengas, Carta, Francisco, sotelo and Velasco, 2012).

At present, there are few studies on agricultural production by-products of broccoli. Broccoli, from which only~10–15% of the total aerial biomass of the plant is consumed (Liu, Zhang, Ser, Cumming, & Ku, 2018). A previous study showed that broccoli root contains high amounts of glucosinolate and quinone reductase detoxifying enzyme-inducing activity (Lee, Ku, Becker, & Juvik, 2017). However, utilizing broccoli root tissue is not easy because of the difficulty in harvesting and processing the lignified root tissues. Although broccoli and collard greens belong to the same species (*Brassica oleracea*), and collard leaf is utilized as the edible part, broccoli leaf is seldom utilized for food. Some people consume broccoli stems, but this is not the norm. Commercial broccoli florets usually have ~10 cm of the attached stem. Although the stems nearest to florets are tender and edible the bottom stem is lignified and not acceptable for food consumption. The potential consumption of stems and leaves would increase productivity and sustainability of the World's broccoli crop

by increasing yield from 15% up to as much as 83 % the bottom stem is lignified and not acceptable for food consumption. So using by-products as feed will have a good prospect.

This study mainly uses the by-product of broccoli as an additive to replace the common commercial feed. Previous studies in our research group have shown that the content of functional substances such as sulforaphane in the silage with the by-products of broccoli and broccoli as additives will decrease significantly after fermentation (Kwon, 2018), so this time, MHA（Methionine hydroxy analogue）is used as an acidifier to replace the original fermentation process of the silage.

2. 3. Production of Silage

2.3.1. Fermentation versus acid treatment for the silage production

Silage is pasture grass that has been 'pickled'. It is a method used to preserve the pasture for ruminant to eat later when natural pasture isn't good, like in the dry season (McDonald, Henderson, & Heron, 1991). The grasses are cut and then fermented to keep as much of the nutrients (such as sugars and proteins) as possible. The fermentation is carried out by microscopic organisms living in the grass. The process must be carried out under acidic conditions (around pH 4-5) in order to keep nutrients and provide a form of food that ruminant will like to eat. Fermentation at higher pH results in silage that has a bad taste, and lower amounts of sugars and proteins.

The advantages of silage are that it can be stored for a long time. When the fresh straw or forage is processed into silage, it will become more resistant to the storage, and will not deteriorate in the sealed environment for 1 - 2 years. The nutritional value of silage is higher. Silage can keep the nutritional components of fresh straw or forage to the greatest extent, especially microelements and vitamins. The palatability of silage is better. After sealed fermentation, the silage will become tender and sour,

and its palatability is better. The digestibility of silage is higher, silage is more tender than green dry straw, and contains some probiotics such as lactic acid bacteria, so the digestibility of silage is higher. However, silage also has disadvantages. For example, silage has a lot of acidity. Silage will produce some acidic substances in the fermentation process. Therefore, the acidity of silage is higher. If silage is fed in large quantities for a long time without adding other forage, it may cause too much gastric acid or even gastric acid poisoning, which will affect the digestion and health of beef cattle. Silage can make cows abort. In the same way, because of the acidity of silage, pregnant cows may have abortion, premature delivery and weak calving when they eat a lot of silage(Woolford, 1984).

With the development of agricultural technology, acidifier will be added in the process of making feed, which has the advantages of reducing feed pH value and acid binding force, promoting the activation of zymogen in stomach, promoting intestinal microecology balance, preventing animal intestinal pathogenic microbial diseases, promoting nutrient digestion, etc. However, the addition of acid will destroy the absorption of vitamins and mineral elements in the feed and the absorption speed in the stomach is too fast, and inhibit the normal development of gastric acid secretion and gastric function (Henderson, 1993).

2.3.2. MHA as an acidifier

Methionine hydroxy analogue (MHA) is a chemical compound with dark brown mucus appearance. pH is below 2. When used as feed additive, it can be used as a substituent for an essential amino acid, methionine to promote growth of domestic animals, especially for poultry. (Rostagno & Barbosa, 1995).

Because the fermentation process will affect the content of functional substances in the feed materials, MHA could be used to directly replace the fermentation process. The ideal state of this experiment is that because the pH value of MHA is very low, during or before fermentation, the pH value of feed can directly reach the final stable pH value, so that the feed can be preserved under the condition of low pH value, and the functional substances in feed can also be retained.

2. 3. 3. Lactic acid bacteria as an inoculant

For the silage production, most frequently used microorganism is lactic acid bacteria as an inoculant for fermentation, which increases the efficiency of fermentation and it enhances the storage of the silage. There are many strains, and the most common ones are *Lactobacillus plantarum*, *Streptococcus faecium*, *Pediococcus* strains and so on (Drouin, Mari, &

Schmidt, 2019). There are about 10~10^3 cfu/g of lactate bacteria present in the forage, which are cut and increased to 10^3~10^7 cfu/g when it makes in silos, but most are hetero-type and do not have high fermentation efficiency (Kim et al., 1999). The treatment effect of inoculant generally improves the fermentation of the silage, especially by reducing protein breakdown, reducing the ammonia nitrogen and increasing the digestion rate of the silage (Haigh et al., 1996; Smith et al., 1993). The *Lactobacillus* inoculation group in this paper is a control group with MHA as additive.

2.4. Functional Components in Broccoli By-product

2.4.1. Sulforaphane

Sulforaphane, is a kind of isothiocyanate, which is obtained by hydrolysis of glucosinolates (Glu) by myrosinase enzyme in plants. It is rich in broccoli, kale, North turnip and other cruciferous plants (Ishida, Hara, Fukino, Kakizaki, & Morimitsu, 2014). It is a common antioxidant and the best plant active substance found in vegetables. Futhion is a kind of sulfur-containing compound, which is hydrolyzed by black mustard enzyme. It is a yellow or colorless liquid at room temperature, insoluble in water, but easily soluble in methanol, dichloromethane, acetonitrile and other organic solvents, and easy to decompose under high temperature and

alkaline conditions. Glucosinolates and myrosinase are stable in plant tissue cells and vacuoles, and only when plant tissue is destroyed can they hydrolyze to sulforaphane (Angelino et al., 2015). As the hydrolysis process is affected by pH, temperature, water and other factors, the yield of sulforaphane is often reduced (Barba et al., 2016).

Sulforaphane not only has a strong anti-cancer activity but also has a strong anti-oxidation ability (Su et al., 2018). It is recognized as one of the natural products with anti-cancer, anti-cancer and beauty effects. As early as the beginning of the 20th century, researchers have studied the anti-cancer and anti-cancer effects of sulforaphane (Bose et al., 2018). It is found that sulforaphane can inhibit liver cancer, colon cancer, breast cancer, prostate cancer, induce phase II enzyme, activate the production of anti-cancer substances, kill white blood cells. In addition, sulforaphane can adjust the outermost immune system skin, enhance human immunity (Soundararajan & Kim, 2018). Tests in humans and mice by US researchers have shown that sulforaphane helps the lungs clear harmful bacteria

2.4.2. Beta-carotene and vitamin A

Bate-carotene is one of the carotenoids. It is an orange fat-soluble

compound. It is the most common and stable natural pigment in nature. Reddish purple to dark red crystalline powder, slightly peculiar odor (Mortensen, 2006). Bate-carotene dilute solution is orange yellow, easily soluble in dichloromethane, chloroform, carbon disulfide and other organic solvents. When the concentration of the solution increases, it is orange, because the polarity of the solvent can be slightly red. Unstable in the presence of oxygen, heat and light. It is stable in a weak base (Khoo, Azlan, Tang, & Lim, 2017).

The antioxidation of beta-carotene mainly lies in its ability of scavenging free radicals. beta-carotene molecules contain many double bonds, which are easily oxidized in the presence of light, heat, oxygen and free radical ions with strong activity, so as to protect the body from damage (Lobo, Patil, Phatak, & Chandra, 2010). There are a lot of lipid peroxidation and free radical reaction in organism, which lead to the decline of cell function, the aging of organism and the occurrence of diseases. The existence of beta-carotene can reduce lipid peroxidation (Gottlieb, Zarling, Mobarhan, Bowen, & Sugerman, 1993). Therefore, the activity of carotenoids which can scavenge free radicals and quench singlet oxygen has been widely concerned. Beta-carotene can form gap connection between cells to make the cytoplasm communicate with each other, and

regulate the metabolic response by exchanging small molecules. Recently, it has also been proved that beta-carotene added to animal food as feed additive has certain specific functions for animals. For example, when feeding cows without beta-carotene, we often observe "asymptomatic" fever, as well as delayed ovulation, follicular cyst, delay and reduce the formation of corpus luteum. In serious cases, it can lead to reproductive disorders and placenta stagnation. All of these symptoms can be corrected by adding bate - carotene to the feed (Zielińska, Wesołowska, Pawlus, & Hamułka, 2017).

Beta-carotene, which can be converted into vitamin A after ingestion of human digestive organs, is currently a safer product to supplement vitamin A (only supplement chemical synthetic vitamin A, when excessive, it will cause poisoning). It can maintain the health of eyes and skin, improve the condition of night blindness and rough skin, and help protect the body from free radicals (Brown, Bron, Harding, & Dewar, 1998).

2.4.3. Phenolic acids and antioxidant

Phenolic acids are a group of secondary plant metabolites, widely spread throughout the plant kingdom and in foods of plant origin (Herrmann & Nagel, 1989). Research on phenolic acids has been carried

out because of their biological and pharmacological properties, especially antioxidant activity (Shahidi, Janitha, & Wanasundara, 1992). It has been determined that the antioxidant effect of these plants products is mainly attributed to phenolic compounds such as flavonoids and phenolic acids, ascorbic acid, vitamin E and different carotenoids (Sawadogo et al., 2006). These natural antioxidants are very effective to prevent the destructive processes caused by oxidative stress induced by free radicals (Gruz, Ayaz, Torun, & Strnad, 2011). Free radicals or reactive oxygen species (ROS) have been implicated in the pathology of many diseases, including cancer, coronary artery diseases, hypertension, diabetes and neurodegenerative disorders, in addition to aging (Ferguson, 2010). Phenolic compounds are the secondary metabolic derivatives of pentose phosphate, shikimate and phenylpropanoid metabolic pathway with aromatic rings bearing one or more hydroxyl groups (Randhir, Lin, Shetty, & Lin, 2004). The structural diversity within different phenolic compounds due to the hydroxyl group substitutions in the aromatic ring makes these compounds biologically more activity and potential (Khorasani Esmaeili, Mat Taha, Mohajer, & Banisalam, 2015) .The scavenging effect of BBS on DPPH (1,1-diphenyl-2-pyridyl hydrazine) was also examined.

Chapter1. Production of Broccoli By-product Silage by MHA Treatment

1. Introduction

With the improvement of people's living standards, people's demand for meat food is growing, especially for high-grade meat, which effectively promotes the development of animal husbandry. The development of animal husbandry needs a lot of food and feed as support, so it is an important issue to utilize and recycle of agricultural by products. It has been payed attention as new feed resources recently.

A large amount of wastes, such as straw, fruit shell and other plant fiber materials, are obtained after harvesting grains or vegetables in agricultural production every year. These materials are renewable resources. If they are only discarded on the ground, they will waste resources and pollute the environment. Therefore, the use of these agricultural by-products to make new feed will improve the ecological environment, form a virtuous cycle of agricultural production, and promote sustainable development of agriculture and animal husbandry.

The major task is to develop a functional cattle feed using broccoli

by-products as feed. Some functional substances can be found in broccoli, such as sulforaphane, Beta-carotene, etc. If the development of functional beef is realized through the by-products feed of broccoli that gets a double advantage.

2. Materials and Methods

2.1. Silage Preparation

2.1.1. Experimental production BBS preparation

Broccoli by-product (*Brassica oleracea var.Italica*), which indicates stems and leaves, were obtained from Pyeong-chang area in Korea. In order to meet the nutritional needs of feed for animals, other agriculture products must be as additives to supplying essential nutrition. Also because broccoli by-product has a high moisture content, beet pulp (BP) used as a moisture absorbent and carbohydrate source. At the same time, wheat bran was also used as energy source. The purpose was the evaluation of MHA effect instead of fermentation, so a group of inoculated with Lactic acid bacteria (*Lactobacillus plantarum*, LAB, 5×10^7 cfu/kg of the silage) inoculation was used as a control.

Production of broccoli by-product silage was proceeded in mid-July

and BB were cut into 5-10cm long segments by cutting machine before silage making process. The *in vitro* section was set up into 5 treatment groups (about 36kg scale in each treatment group and triplicated) which treated by different amounts of BP, WB and MHA. For BP and WB, 3.6kg was added to each group because the results of the previous part of the experiment showed that the feed with 3.6kg was the most palatable. The addition amount of MHA is 0, 0.2%, 0.4%, 0.8% respectively. For the LAB group, the concentration of inoculated *lactobacillus* is 200mg. Samples were taken for analysis on 0, 10, 20, 30 and 60 days respectively. The control group silage only used broccoli by-product, BP, WB with DW (distilled water). Spray the MHA and DW evenly on the feed, mix equality, put into the bucket after compaction and store in a shaded place. The manufacturing process of the broccoli by-product silage is shown in Figure 1.

Figure 1. The manufacturing process of the broccoli by -product silage. (A)-(B) Steps for cutting down the broccoli by-product. (C)-(D) Steps for mixing broccoli by-product and additives. (E)-(F) Steps for suppression and storage the silage.

2.1.2. Feed value analysis of BBS

The BBS samples were collected about 500g and placed in convection dry oven at 65℃ up to 72 hours for the determination of dry matter (DM) content. After drying, DM was analyzed and all the dried samples were grounded by a Willey Mill with 1 mm screen (Thomas Scientific, Inc., New Jersey, USA). Crude protein (CP) and ash were detected in the same way explained in AOAC (Method 990.03 and 942.05, respectively (International, 2006). The neutral fiber (NDF) detected method was explained in Van Soest (P. v. Van Soest, Robertson, & Lewis, 1991). The acid detergent fiber (ADF) contents were determined by Van (P. Van Soest & McQueen, 1973). The ether extract content was analyzed in the same way as the Barbosa (Barbosa et al., 2017). The Ammonia-N was analyzed in the same method in AOAC (Ebeling, 1968). Total digestible nutrient (TDN) and relative feed value (RFV) were calculated by the known formula described by (Powell & Holland, 1990). TDN was calculated from ADF value (TDN% = 88.9 - (0.79 × ADF%). And RFV was estimated through digestible dry matter (DDM% = 88.9 - 0.779 × ADF%) and dry matter intake (DMI% = 120 / NDF%) as RFV = (DMI% × DDM%) / 1.29.

2.1.3. Analysis of organic acid contents in BBS

After each sampling, it shall be stored in a - 20 ℃ freezer until the next procedure. 10g frozen silage sample was thawed and mixed with 90ml distilled water in the conical flask and placed in the refrigerator at 4℃ for 24 hours. Meantime, the conical flask were shaken by hand every 2 hours. After that, the silage samples filtered through 4 layers of gauze and measured by pH meter (model AG 8603; Seven Easy pH, Mettler-Toledo, Schwerzenbach, Switzerland).

To get the extract samples for organic acid measurement, 10 g silage sample mixed with 90ml distilled water (DW) in conical flask and incubated in the fridge at 4℃ for 24 hours. After incubation, the silage samples filtered by 4 layers of gauze and filter paper (Whatman No. 6, AVANTEO). In organic acids that include acetic acid, butyric acid and propionic acid were measured by using gas chromatography (GC-iacuc, Agilent 7890B, USA). A sample volume of 1 μL was injected with capillary column Nukol with a length of 30m × 0.25mm (Sigma-Aldrich, Germany). The injector was set at 220℃ and the split rate was 1:40. The flame ionization detector (FID) was set to 220℃ and the column oven was set to 200℃. The ramp temperature was increased to 10℃ at 20℃/min and hold for 19min. Lactic acids were measured by D-LACTIC ACID and

L-LACTIC ACID ASSAY Kit (Megazyme). The protocol was followed by the manufacturer's instruction. In brief, add blank and diluted samples 1.6 mL in the 5mL tube respectively, and mixed with 0.5mL lactic acid buffer, 0.1mL NAD^+ and 0.02mL D-GPT. After full mixing and 3mins incubating at room temperature, 200μL blank and samples were taken out in 96 well plates that were read in a spectrophotometer at the absorbance of 340nm. Then, start the reactions by addition of 0.02mL D-LDH and L-LDH, after full mixing and 10mins incubating read again by the spectrophotometer. The lactic acid contents were calculated followed by this formula:

$$C = V \times MW / \varepsilon \times d \times v \times \Delta A_{lactic\ acid}$$

Where, V = final voluem (mL), MW = molecular weight of lactic acid (g/mol), Ɛ = extinction coefficient of NADH at 340nm (6300, $1 \times mol^{-1} \times cm^{-1}$), d = light path (cm), v = sample volume (mL), $\Delta A_{lactic\ acid}$ = determine the absorbance difference for both blank and samples.

2.1.4. Analysis of microbial growth in BBS

De Man, Rogosa and Sharp agar (MRS) medium, potato dextrose agar medium and nutrition agar medium were used for lactic acid bacteria, fungi and yeast, total microorganisms, respectively, for silage (only fungi and yeast were counting) by spread-plate method (Madigan, Martinko, &

Parker, 1997). 10 g silage sample was dissolved in 100 ml physiological saline (0.9 % NaCl solution). And shaken for 20 minutes on incubator on room temperature. After standing the sample, the extract was serially diluted by 0.9 % NaCl solution 10^{-2} - 10^{-4} times, then 100 µl of each was inoculated on agar surface and spread evenly by one - off plastic spreader. The agar plates with samples were incubated at 30℃ for 24 - 48 hours. After incubating, the colony forming units per gram (CFU / g) microorganisms were counted on agar plates on agar plates and calculated according to the dilution factor.

2.2. Analyses of Functional Substances in BBS

2.2.1. Sulforaphane

BBS samples were collected from the silage made for feeding trial and storage in the freezer at - 20 ℃. Sulforaphane content was analyzed to compare the change during the manufacturing process. The samples were weighed and put into the 50 ml glass tube (1 g fresh sample or 150 mg lyophilized sample). Then add 4 ml acid water (pH 6), and the mixture was incubated at 30 ℃ in water bath 2.5h. At this step, the evaporation of water was observed to have decreased the extract by about 70 % from the initial weight of the mixture. Sulforaphane was extracted with 20 ml of

dichloromethane by vortexing for 1 min and incubating 1h at room temperature. Then the sample was filtered through Whatman no. 41 paper. The purification method was designed by (Bertelli, Plessi, Braghiroli, & Monzani, 1998), using SPE silica cartridge (SiOH, ThermoFisher Scientific) 3 ml disposable columns. Before using, the silica gel cartridge was washed by 3 ml dichoromethane. Sulforaphane was extracted by passing 20 ml of organic extract through the cartridge with 3 ml of ethyl acetate and eluting the Sulforphane with 3 ml of methanol. The methanol extract was evaporated to dryness in a vacuum oven at 45 ℃ until evaporating all liquid and redissolved with 2 ml acetonitrile. The final solution was vortexed for 1 min and filtered with a membrane of 0.45 μm. 20 μl of solution will be used for analysis by UPLC. All samples are triplicated. The condition of the instrument was shown in Table 1.

Table 1. Instrumental conditions of UPLC for determination of Sulforphane in BBS.

Column	ACQUITY BEH C18, 2.1mm × 50mm (Waters,USA)
Mobile phase	A: 100% Distilled water, B: 100% Acetonitrile
Gradient	Isocratic
Flow rate	0.1 mL/min
Injection	20μL
Temperature	4°C
Absorbance	202nm

2.2.2. Beta-carotene

For our BBS samples, 1 g of lyophilized samples were used. 1 g BBS sample dissolved in 10 ml 6 % pyrogallol ethanol and sonicate for 10 minutes. After sonication, 7 ml of 60 % KOH was added in and mix by vortexing. After mixing evenly, the mixture was incubated at 80 ℃ water bath and vortexed every 20 minutes. Then the mixture sonicated in ice for 10 minutes. 10 ml 2 % NaCl and 15 ml extract solution (hexane : acetate 85 : 15) was added. After vortexing, centrifuge at 3600 rpm for 5 minutes. Add the extract solution and centrifugate three times. When all the bate - carotene are washed and get the top layer to evaporator tube. Evaporate the liquid completely and dissolve it in 1 ml $CHCl_3$ that measured by the UPLC system. The condition of the instrument was shown in Table 2.

Table 2. Instrumental conditions of UPLC for determination of β-carotene.

Column	ACQUITY BEH C18, 2.1mm × 50mm (Waters,USA)
Mobile phase	A：70 % acetonitrile : methanol (85:15) B: 30 % dichloromethanol
Flow rate	1000 μL / min
Injection	3.5 μL
Temperature	35 ℃
Pressure	4.9 MPa
Detector	UV 254 nm

2.2.3. DPPH radical scavenging activity

1 g of lyophilized sample was extracted by 10 ml of 70% ethanol at room temperature for 24 hours. After extracting, centrifuged for 15 minutes at 6400 rpm and filtered by 0.2 µL PTFE filter (ThermoFisher Scientific). The extracted samples were stored in firefighter for other experiments. First of all, prepare 100 µM DPPH (Sigma-Aldrich, Germany) solution dissolved in methanol. 50 µL extracted samples reacted with 200 µL DPPH solution and shaking for 1 minute. Control was made by 70 % ethanol mixed with 200 µL of 100 µM DPPH solution. The absorbance measured at 517 nm is used to express the binding efficiency of free radicals by the spectrophotometer (SpectraMax M3, Molecular Devices, USA).

DPPH radical scavenging activity capacity follows the formula:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{(Absorbance\ of\ control - Asorbance\ of\ test\ sample)}{Absorbance\ of\ control} \times 100$$

2.2.4. Total phenolic contents

The extraction solution of the sample is the same as the DPPH assay. The total phenolic content determined by Follin-ciocalteu. (Deng et al., 2013) with gallic acid (Sigma-Aldrich, Germany) as the standard. 1 mL extracted samples were reacted with 1 mL 5 folded Follin- ciocalteu

reagent and incubation in dark condition for 3 minutes. After incubation, add 1 mL 10 % sodium carbonate and reacted in a dark condition for 1 hour. The total phenolic contents were determined at 750 nm by spectrophotometer and displayed as gallic acid equivalent (GAE).

3. Result and discussion

3.1. Evaluation of BBS Quality

3.1.1. Evaluation of chemical composition and feed value

With the increase of MHA content, of which the T 0.8 % group DM was the highest. The result is given in table 3. The DM content of some treatment fluctuated in the early stage of preservation. After that, the DM content of each treatment decreased gradually with the prolongation of storage time. The content of crude protein in the con 1 group was higher than other treatment groups, but the difference was not significant. As for the EE content, the con 1 and con 2 groups showed an obvious upward trend compared with other MHA treated groups, and the content of the MHA treated group was relatively stable. For CF, ADF and ADF has no significant difference. In the case of TDN, there was no significant difference in all of the groups, but the lowest TDN value was shown in the

con1 group on 60 days. RFV was the lowest value at 148.38 at T 0.4 group on 10 days, the highest value at 197.61 at T 0.2 % group on 30 days. For RFV, con 1, con 2 and T 0.2 % groups, the decrease was positive, while the other two groups showed an upward trend because high content NDF has reduced RFV. RFV decreased significantly in con 1 group after 30 days storage, which was due to the increase of bacterial activity again. So MHA treatment prevent bacterial growth, the feed value has been preserved in MHA groups. High content of MHA groups inhibited the growth rate of NH_3-N / TN ratio, the result was shown in Figure 2. During the storage period, RFV and TND values has no difference between the MHA treated groups and the untreated groups, that means MHA has no effect to the feed value.

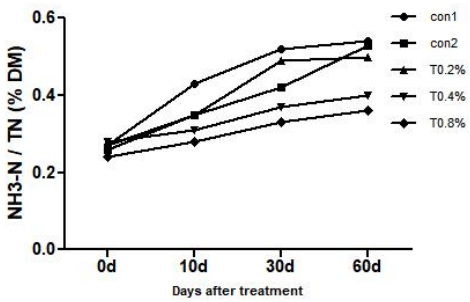


Figure 2. Changes of NH3-N / TN ratio

Table 3. Evaluation of Chemical composition and Feed value

Sample	Date	DM	CP	EE	CF	Ash	ADF	NDF	TDN	RFV
			% DM							
con1	0d	24.70	18.65	2.94	13.37	8.51	18.82	37.06	74.03	186.35
	10d	24.20	18.67	2.81	17.83	9.89	21.89	39.13	71.61	170.80
	30d	22.07	20.59	4.02	17.22	11.57	21.04	34.69	72.28	194.44
	60d	20.53	20.30	3.84	19.26	11.74	24.08	43.96	69.88	148.43
	Mean	22.88	19.55	3.40	16.92	10.43	21.46	38.71	71.95	175.00
con2	0d	24.00	18.26	2.91	14.08	8.35	21.60	41.57	71.84	161.28
	10d	27.40	18.04	3.30	17.20	8.14	22.49	43.15	71.13	153.88
	30d	26.53	17.75	6.23	16.33	8.27	20.45	36.73	72.74	184.80
	60d	25.00	19.59	3.03	18.26	10.18	23.47	43.02	70.36	152.70
	Mean	25.73	18.41	3.87	16.47	8.74	22.00	41.12	71.52	163.17
T0.2%	0d	26.63	19.14	3.55	14.84	9.17	19.36	38.58	73.61	177.99
	10d	26.53	17.20	2.91	18.99	8.43	23.68	42.21	70.19	155.27
	30d	23.67	20.41	3.95	17.19	11.24	21.13	34.10	72.21	197.61
	60d	23.33	18.67	3.05	16.98	10.26	22.16	40.18	71.39	165.85
	Mean	25.04	18.86	3.37	17.00	9.78	21.58	38.77	71.85	174.18
T0.4%	0d	27.33	17.76	3.03	17.83	8.25	23.48	43.15	70.35	152.22
	10d	26.33	20.15	2.34	17.31	9.91	23.29	44.36	70.50	148.38
	30d	24.20	18.32	3.47	16.94	8.92	21.79	36.59	71.69	182.86
	60d	26.07	19.30	3.83	15.82	9.88	19.39	39.02	73.58	175.93
	Mean	25.98	18.88	3.17	16.98	9.24	21.99	40.78	71.53	164.85
T0.8%	0d	24.00	19.05	3.36	15.84	9.12	20.67	40.42	72.57	167.54
	10d	27.67	18.43	2.92	15.39	8.97	21.17	40.16	72.18	167.72
	30d	27.60	18.37	4.58	17.12	8.69	23.66	37.25	70.21	175.98
	60d	25.40	19.56	3.55	16.02	9.92	20.96	38.97	72.34	173.23
	Mean	26.17	18.85	3.60	16.09	9.18	21.62	39.20	71.82	171.12
rbb		89.00	27.00	1.93	17.21	17.67	22.60	40.18	71.05	165.06
BP			7.47	1.05	16.47	3.79	21.28	36.10	72.09	186.36
WB			15.34	3.53	19.76	4.02	24.96	39.77	69.18	162.46

DM, dry matter; CP, crude protein; EE, ether extract; CF, crude fiber; ADF, acidic detergent fiber; NDF, neutral detergent fiber; TDN, total digestible nutrients; RFV, relative feed value;con 1, blank control; con 2, lactic acid bacteria inoculation group; T0.2%, 0.2% MHA treatment group; T0.4%, 0.4% MHA treatment group; T0.8%, 0.8% MHA treatment group.

3.1.2. Determination of organic acid contents in BBS

Originally, it is hypothesized that MHA treatment to broccoli by-product would directly decrease the pH, then the silage product could be preserved for a long time without fermentation process, because of prevention effect of low pH on bacterial growth. But from the results, both pH and organic acids show varying degrees of change. It is concluded that there is still a fermentation process after adding MHA.

In comparison with the different content of MHA, the pH of 0 day decreased with the increase of MHA content, the con1 group pH level was the highest (5.2), the T0.8% group pH level was the lowest (4.69). Taken samples every ten days, and the pH was 4±0.3 in the final 60 days.

Through the comparison of the five sampling results, the pH of the con 2 group inoculated with lactic acid bacteria increased again after 30 days (3.97 to 4.25), but the other groups did not. This is because when the pH was reduced to a certain extent, the lactic acid bacteria itself also has no longer active, some spoilage yeasts use lactic acid as an energy source to continue to grow, so as to make the pH rise again. In other text groups which added MHA, the pH still decreased or stabilized, so it is speculated that MHA plays an important role in maintaining the pH stability. From the

T0.8% group, the content of lactic acid and acetic acid were not high (avg.11.54% and 1.07%, respectively), but the pH was only 3.86, which proved that MHA can inhibit the activity of bacteria, reduce energy loss, and play a direct role in the reduction of pH. The content of acetic acid decreased with the increase of MHA, and the content of con 2 (avg. 3.03%) was lower than that of the control group (avg. 4.76%), but higher than that of MHA groups (avg. 1.93%, 1.42%, 1.07%, respectively). Acetic acid or propionic acid as the spoilage yeast inhibitor, although acetic acid and propionic acid content was not high, the silage has no corrupt, which means MHA can inhibit the spoilage yeast activity. The results were shown in Table 4 and Figure 3.

Overall, although MHA can not completely inhibit the fermentation process, in some aspects, it was beneficial to the fermentation process for environmental lactic acid bacteria and played a great role in inhibiting the activity of undesirable bacteria and the stability of the silage.

Table 4. Determination of organic acid contents in BBS

Sample	Data	pH	Organic acid (% DM)			
			lactic acid	Acetic acid	Propionic acid	Butyic acid
con1	0d	5.50^{a}	1.82^{c}	0.57^{d}	0.08^{a}	0.11^{b}
	10d	4.11^{b}	15.95^{b}	2.14^{c}	0.13^{a}	0.14^{b}
	20d	4.11^{b}	20.53^{a}	2.66^{bc}	0.52^{a}	0.50^{ab}
	30d	4.48^{b}	21.34^{a}	3.83^{ab}	0.29^{a}	0.84^{a}
	60d	4.3^{b}	25.30^{a}	4.76^{a}	0.49^{a}	0.37^{ab}
	Mean	4.50^{A}	16.99^{A}	2.79^{A}	0.30^{A}	0.39^{A}
con2	0d	5.22^{a}	5.75^{b}	0.82^{b}	0.00^{b}	0.00^{b}
	10d	4.10^{bc}	8.30^{b}	2.95^{a}	0.00^{b}	0.00^{b}
	20d	3.96^{c}	20.11^{a}	2.58^{a}	0.03^{b}	0.03^{b}
	30d	3.97^{c}	21.91^{a}	2.30^{a}	0.04^{b}	0.12^{b}
	60d	4.25^{b}	21.26^{a}	3.03^{a}	1.99^{a}	1.03^{a}
	Mean	4.30^{A}	15.47^{A}	2.34^{AB}	0.41^{A}	0.24^{AB}
T0.2%	0d	5.28^{a}	1.24^{d}	0.48^{b}	0.00^{a}	0.00^{a}
	10d	4.18^{b}	9.85^{c}	2.28^{a}	0.03^{a}	0.00^{a}
	20d	3.80^{b}	17.81^{b}	1.66^{b}	0.00^{a}	0.00^{a}
	30d	4.11^{b}	21.93^{a}	2.66^{a}	0.05^{a}	0.05^{a}
	60d	4.04^{b}	19.89^{ab}	2.59^{a}	0.00^{a}	0.06^{a}
	Mean	4.28^{A}	14.14^{A}	1.93^{AB}	0.02^{A}	0.02^{B}
T0.4%	0d	5.19^{a}	0.98^{c}	0.42^{d}	0.00^{a}	0.00^{a}
	10d	3.95^{bc}	10.62^{b}	1.45^{bc}	0.00^{a}	0.00^{a}
	20d	3.98^{b}	18.52^{a}	2.03^{a}	0.00^{a}	0.00^{a}
	30d	3.85^{bc}	21.10^{a}	1.90^{ab}	0.00^{a}	0.00^{a}
	60d	3.76^{c}	21.49^{a}	1.32^{c}	0.00^{a}	0.00^{a}
	Mean	4.15^{A}	14.54^{A}	1.42^{AB}	0.00^{A}	0.00^{B}
T0.8%	0d	4.69^{a}	0.64^{c}	0.43^{b}	0.00^{a}	0.00^{a}
	10d	4.02^{b}	7.24^{b}	1.09^{a}	0.00^{a}	0.00^{a}
	20d	3.82^{c}	16.07^{a}	1.21^{a}	0.00^{a}	0.00^{a}
	30d	3.82^{c}	18.04^{a}	1.21^{a}	0.00^{a}	0.00^{a}
	60d	3.86^{c}	15.70^{a}	1.40^{a}	0.00^{a}	0.03^{a}
	Mean	4.04^{A}	11.54^{A}	1.07^{B}	0.00^{A}	0.01^{B}

con 1, blank control; con 2, lactic acid bacteria inoculation group; T 0.2 %, 0.2 % MHA treatment group; T 0.4 %, 0.4 % MHA treatment group; T 0.8 %, 0.8 % MHA treatment group.

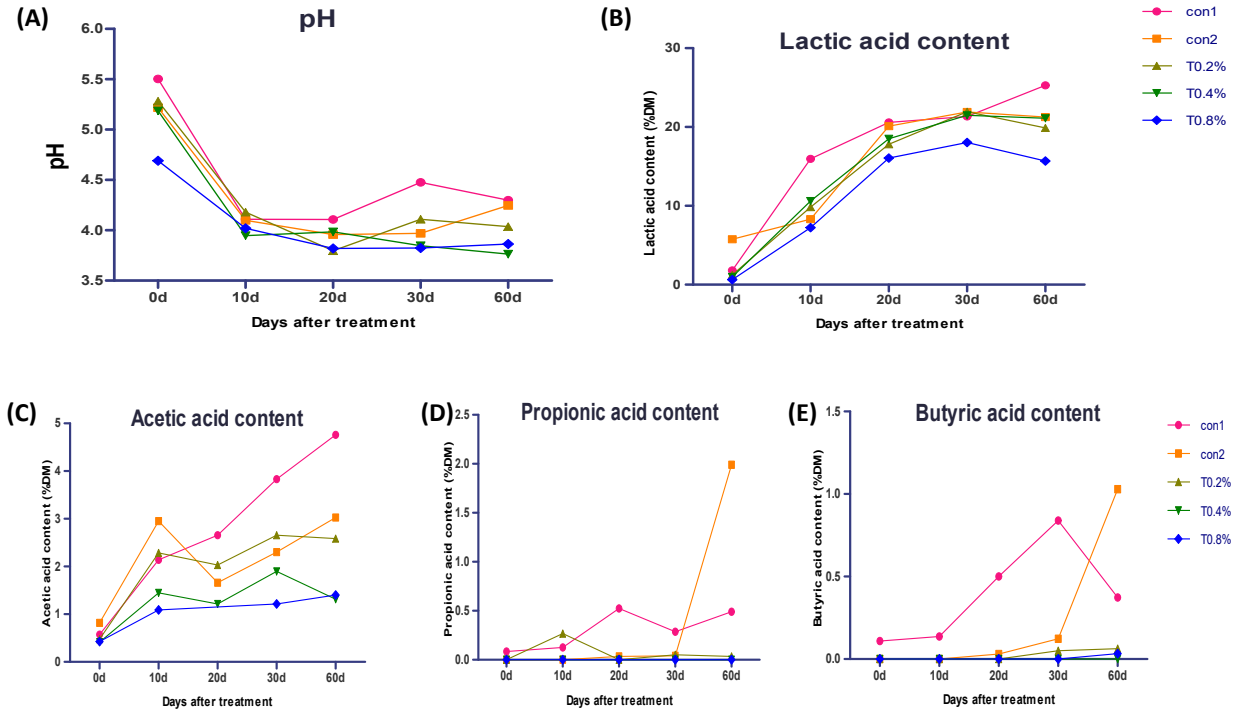


Figure 3. Trends of organic acids content (%DM) and pH

3.1.3. Microbial growth in BBS

The microbes results in this experiment were shown in Table 5 and Figure 4. The original purpose of the experiment was to replace the fermentation process with MHA additive, so the count of microorganisms was used to evaluate

whether MHA replaced the fermentation process or not. Some strains of lactic acid bacteria (LAB) produce bacteriocins which can inhibit the growth of other microbes (Gollop, Zakin, & Weinberg, 2005). Con1 that has no treatment group only 4.82 log CFU / g of LAB developed. con2 group (inoculate LAB) and other MHA treated groups shows a similar count of LAB. According to the above situation that the content of lactic acid and acetic acid was increasing continuously, lactic acid bacteria still had activity, so it can be seen that MHA did not completely inhibit the fermentation process. The content of lactic acid bacteria in the con 2 group was on the rise, while that in MHA groups was on the rise first and then on the decline, and the content of lactic acid bacteria continued to rise. It can be inferred MHA as an additive that can directly reduce the pH value, thus inhibiting the activity of some pernicious bacteria, but the activity of lactic acid bacteria still existed and entered the stable period ahead of time. Fungi

and yeast are aerobic microorganisms present in silage that can lead to spoilage during fermentation (Muck, 2010). Con 1 groups had a distinct putrid smell after 10 days and produced butyric acid (see above for details).

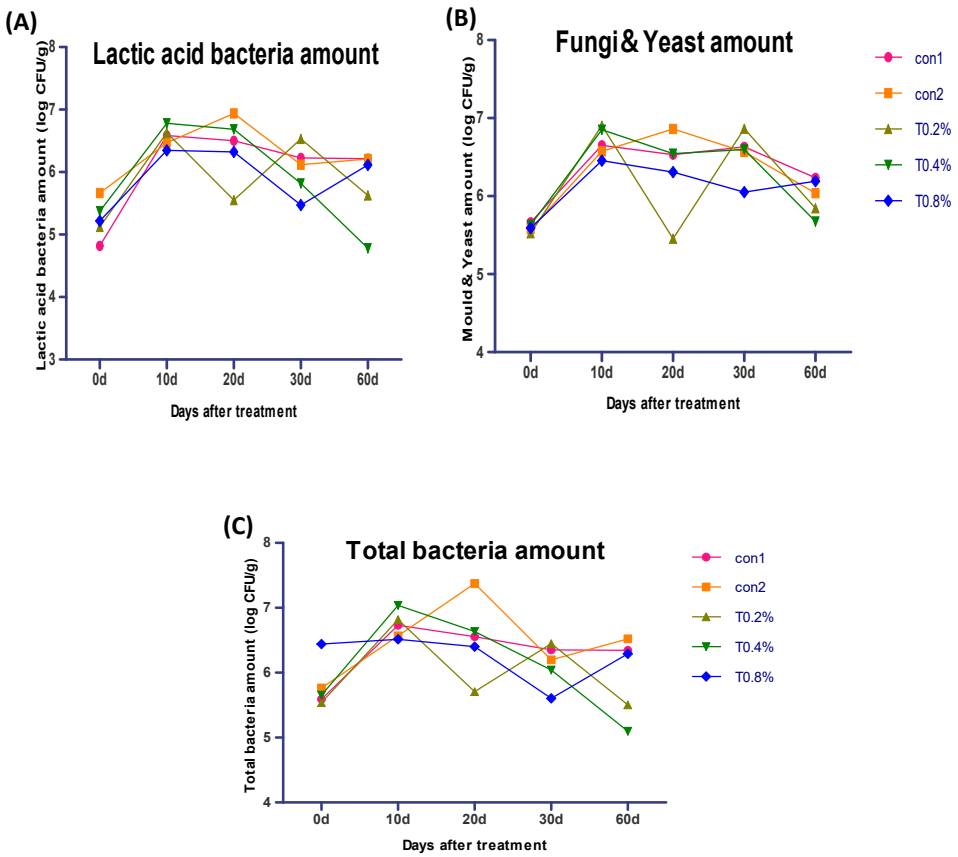


Figure 4. Microbes growth in each group

Table 5. Effect of dates and different treatments on microbes activities of BBS

Treatment	Date	LAB	F&Y	TB
		log CFU/g		
con1	0d	4.82^{c}	5.66^{c}	5.59^{b}
	10d	6.58^{ab}	6.65^{a}	6.73^{a}
	20d	6.71^{a}	6.71^{a}	6.78^{a}
	30d	6.23^{b}	6.63^{ab}	6.35^{a}
	60d	6.21^{b}	6.23^{ab}	6.35^{a}
	Mean	6.11^{A}	6.37^{A}	6.36^{A}
con2	0d	5.67^{c}	5.58^{c}	5.77^{c}
	10d	6.68^{ab}	6.74^{ab}	6.74^{b}
	20d	6.94^{a}	6.86^{a}	7.37^{a}
	30d	6.12^{bc}	6.57^{ab}	6.20^{bc}
	60d	6.21^{bc}	6.04^{bc}	6.52^{b}
	Mean	6.32^{A}	6.36^{A}	6.52^{A}
T0.2%	0d	5.12^{c}	5.53^{b}	5.54^{b}
	10d	6.63^{a}	6.91^{a}	6.82^{a}
	20d	5.55^{b}	5.46^{b}	5.71^{b}
	30d	6.53^{a}	6.87^{a}	6.45^{a}
	60d	5.77^{b}	5.97^{b}	5.72^{a}
	Mean	5.92^{A}	6.15^{A}	6.05^{A}
T0.4%	0d	5.37^{bc}	6.63^{ab}	5.65^{bc}
	10d	6.78^{a}	6.57^{ab}	7.04^{a}
	20d	6.27^{ab}	6.87^{a}	6.26^{ab}
	30d	5.82^{ab}	6.6^{ab}	6.04^{b}
	60d	4.78^{c}	6.05^{b}	5.10^{c}
	Mean	5.80^{A}	6.54^{A}	6.02^{A}
T0.8%	0d	5.19^{b}	5.97^{a}	6.41^{a}
	10d	6.35^{a}	6.45^{a}	6.51^{a}
	20d	6.32^{a}	6.31^{a}	6.40^{a}
	30d	5.47^{b}	6.05^{a}	5.61^{b}
	60d	6.11^{a}	6.19^{a}	6.29^{a}
	Mean	5.89^{A}	6.19^{A}	6.24^{A}

LAB, lactic acid bacteria; F & M, fungi and yeast; TB, total bacteria; con 1, blank control: con 2, inoculate lactic acid bacteria group: T0.2%, content 0.2% MHA treatment group; T0.4%, content0.4% MHA treatment group; T0.8%, content 0.8% MHA treatment group.

3.2. Evaluation of Functional Substances in BBS

3.2.1. Sulforaphane

Sulforaphane is a kind of anticancer substance existing in broccoli, and the silage was made from the by-product of broccoli. The content of sulforaphane in the broccoli by-product was 10.48 mg/kg. Because of the sulforaphane is abundant in flowers, the content of steams and leaf is not much. The content and change trend of sulforaphane on different days and additive content are shown in Table 6. In some broccoli cultivars, glucoraphanin can hydrolysis by the action of myrosinase yields sulforaphane (Matusheski, Juvik, & Jeffery, 2004). However, sulforaphane is not the only hydrolysis product of glucoraphanin, since the reaction strongly depends on the chemical conditions (pH, temperature, presence of cations and ascorbic acid) (Ludikhuyze, Rodrigo, & Hendrickx, 2000). Sulforaphane synthesis is favored by high temperature (up to 70 ℃) and neutral pH (Mahn & Reyes, 2012), whereas nitrile formation occurs at low temperatures (below 50 ℃) and acid pH (Howard, Jeffery, Wallig, & Klein, 1997).

At 0 day, all the five groups contained sulforaphane, the highest content of which was T 0.4 % (16.08 mg / kg), but there was no significant difference ($P > 0.05$). From the 10th day of sampling, each group showed a

significant downward trend ($P < 0.05$). The deceased tend showed in Figure 5. As mentioned above, sulforaphane synthesis is related to pH and temperature, after 10 days the silage pH was 4 ± 0.2, although there is no specific temperature, according to the silage water content (more than 70 %), the fermentation temperature can not be higher than 40 ℃. Therefore, these external conditions may affect the myrosinase activity and affect the synthesis of sulforaphane. Because there are too many factors affecting the synthesis of sulforaphane, it is difficult to preserve it in the process of feed production.

Table 6. Sulforphane content in BBS in different days and treatment.

Treatment	Sulforphane content (mg/kg)					SEM	P-value
	0d	10d	20d	30d	60d		
con 1	14.85[a]	0.98[b]	0.27[b]	0.03[b]	1.13[b]	3.13	
con 2	5.91[a]	2.81[ab]	0.58[b]	0.00[b]	0.88[b]	1.51	
T0.2%	4.29[a]	3.27[ab]	0.43[b]	0.00[b]	0.65[b]	1.5	<0.05
T0.4%	16.08[a]	3.62[ab]	0.50[b]	0.00[b]	0.52[b]	6.41	
T0.8%	9.50[a]	2.41[b]	1.05[c]	0.00[c]	0.48[c]	0.56	

con 1, blank control: con 2, lactic acid bacteria inoculation group: T0.2%, 0.2% MHA treatment group; T0.4%, 0.4% M HA treatment group; T0.8%, 0.8% MHA treatment group. SEM, standard error of the mean

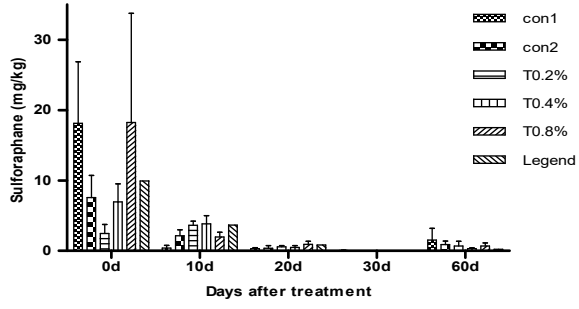


Figure 5. Sulforphane content in BBS in different days and treatment.

3.2.2. Beta-carotene

Effect of different days and treatment of maturity on bate-carotene concentration was shown in Figure 6. On the whole, the sample with the most content on the 10th day. The purpose of this experiment is to replace the fermentation process with MHA and maintain some functional ingredients. In terms of bate-carotene content, the BBS which added MHA is 10 times more than fermented BBS. The bate-carotene content of fermented BBS was shown in this paper (Kwon, 2018). The differences between groups are shown in Table 7. The group without MHA and with less MHA content changed obviously with the increase of days. In the T0.4% and T0.8% groups, the production of bate-carotene began to stabilize after increasing to the tenth day, so MHA had an effect on the stability of bate-carotene.

The experimental results showed that the bate-carotene content increased and then decreased. Some studies showed well-fermented silage usually had beta-carotene losses of less than 20% (Moreno, Carvajal, López-Berenguer, & García-Viguera, 2006), it also means high content MHA group (T0.4% and T0.8%）has good fermentation quality.

Table 7. Bate-cartene content in BBS in different days and treatment.

Treatment	Bate-carotene content (mg/100g)					SEM	P-value
	0d	10d	20d	30d	60d		
con 1	8.50[c]	73.50[a]	29.43[c]	40.07[ab]	17.45[c]	12.03	
con 2	11.20[b]	61.35[a]	51.10[a]	40.73[ab]	39.55[a]	6.68	
T0.2%	11.35[a]	55.53[a]	31.05[b]	42.37[a]	22.03[c]	7.19	<0.05
T0.4%	8.30[c]	47.10[a]	36.10[ab]	39.85[a]	32.43[b]	10.58	
T0.8%	8.63[c]	37.33[a]	30.63[b]	29.80[b]	32.95[b]	6.72	

con 1, blank control: con 2, lactic acid bacteria inoculation group: T0.2%, 0.2% MHA treatment group; T0.4%, 0.4% M HA treatment group; T0.8%, 0.8% MHA treatment group. SEM, standard error of the mean

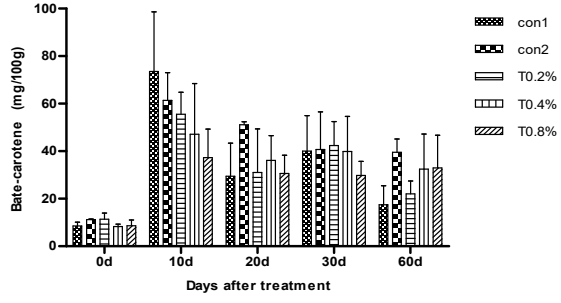


Figure 6. Bate-carotene content in BBS in different days and treatmen

3.2.3. Antioxidants

It can be seen from the results on Figure 7 of the above discussion that the BBS fermentation process after MHA addition has not disappeared. During fermentation, LAB produces substances such as aroma compounds, ascorbic acid, glutathione, phenolic compounds, which enhance the safety of antioxidant activities (Fessard et al., 2017). Phenolic acids are covalently bound to polysaccharides in plant cell walls. Many kinds of bacteria including L. Plantarum display cinnamoyl esterase activities which have been shown to release phenolic acids from the plant cell walls (Chew, Wong, & Michal, 1993). This action of LAB causes the phenolic acid content to increase, which is, the TPC content in the fermented BBS. In addition, the increase in TPC content, which is antioxidant, resulted in increased DPPH radical scavenging activity. Total phenolics and antioxidant activity are highly correlated (Piluzza & Bullitta, 2011). So the more TPC in the sample, the higher DPPH radical scavenging activity. A lot of substances such as carotenoids, tocopherols, vitamin, phenolic acids, flavonoids, etc. have antioxidant activity. In this experiment, DPPH's free radical scavenging ability does increase with the increase of TPC content. In the test results after the 10th day of sampling, TPC content tends to be

stable, but DPPH's free radical scavenging ability has increased significantly, the specific reasons need further study.

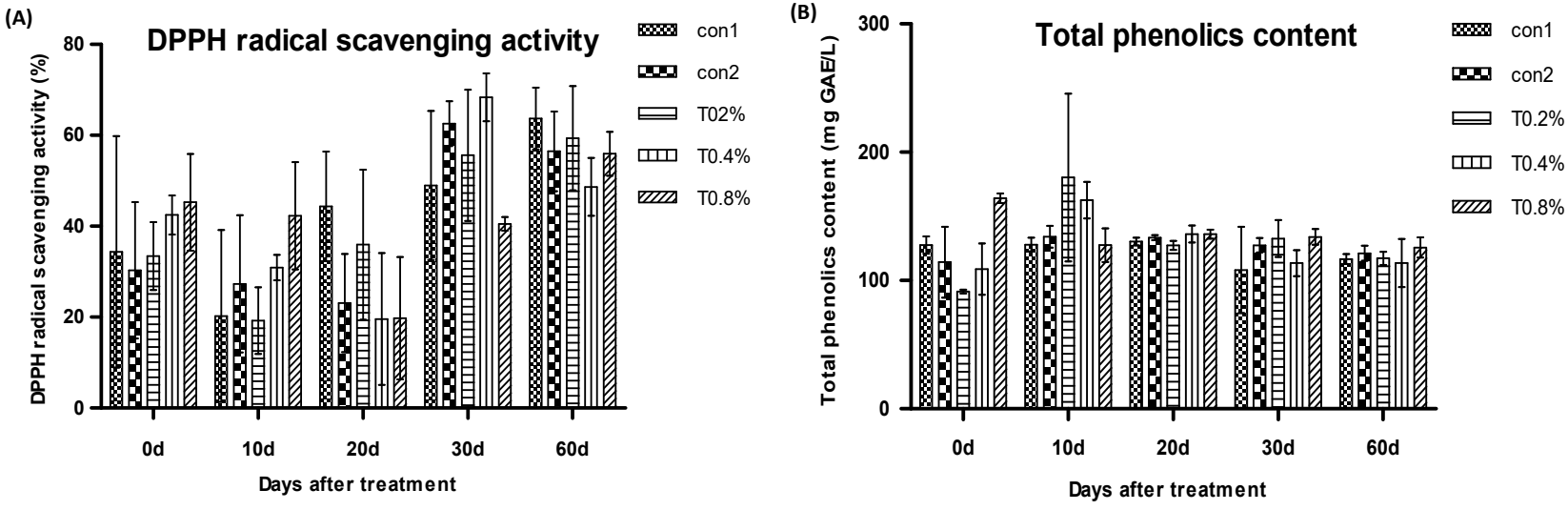


Figure 7. DPPH radical scavenging activity (%) (A) and total phenolic content (mg GAE / L) (B) in each treatment group

4. Mass Production of MHA BBS

4.1. Production Process of BBS for Animal Trials

4.1.1. Production condition of BBS

For the animal feeding trials, around 6,500 kg of MHA-BBS feed production was planed with following condition. For the MHA treatment, 0.4 % of MHA treatment was applied because it showed good result in feed value and character istics in previous *in vitro* experiments, also conditioning economic aspect.

Broccoli (*Brassica oleracea var. Italica*) by-product that included stems, leaves and a little bit low quality flowers obtained from Pyeong-chang area in Korea. In order to meet the nutritional needs of experimental animals, the best formula of BBS production was obtained that have the same total digestible nutrients as the CS (corn silage) of the farm. Except for the by-products of broccoli, other agricultural by-products were used from the farm.

4.1.2. Production of BBS

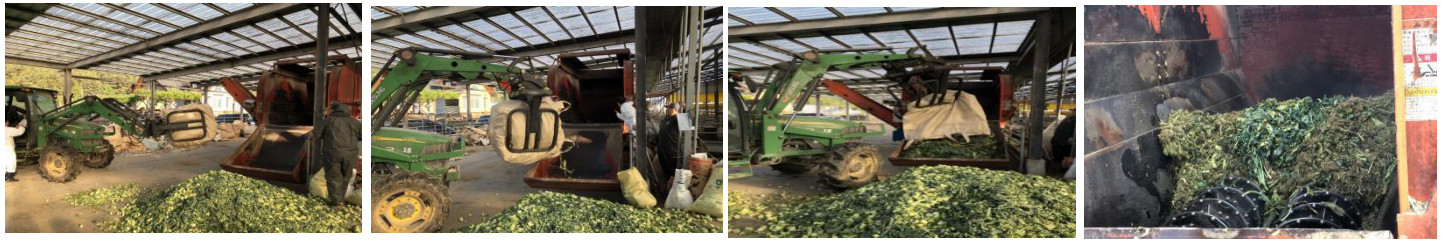
The BBS (the silage made by MHA treated BB) was produced at a

commercial Hanwoo farm (Ye-Darm Farm) in Pyeong-chang area at the end of October. All of the Agriculture by-products were the same materials used in control treatment. The BBS and CS content were shown in Table 8. For the mass production, the TMR mixer was used that located in the Ye-Darm farm to produce large-scale BBS. First, put the prepared by-products of broccoli into the machine, cut into small pieces with a length of 3 - 5cm, and then add other agricultural by-products and Supplements in turn. Finally, after full mixing, sprinkle 0.4 % MHA evenly, and then put them into the silage plastic bag for sealing and preservation after uniform mixing. Samples were taken at 0d, 24d, 45d and before opening. The BBS making process was shown in Figure 8.

Table 8. The composition of BB TMR and Con TMR

Broccoli by-product TMR	%	Control TMR	%
1. Broccoli by-product	57.86	1. Corn silage	70.9
2. Perilla meal	6.49	2. Perilla meal	9.36
3. Limestone	1.05	3. Limestone	0.87
4. Salt	0.98	4. Salt	0.71
5. Rice bran	6.76	5. Rice bran	12.43
6. Wheat straw	52.21	6. Wheat straw	68.11
7. Buckwheat husk	10.72	7. Barley brewers grains	16.42
8. Beet pulp	9.39	8. Bean dregs	10.96
9. Maltose rice	17.6	9. Maltose rice	10.23
10. Barley brewers grains	6.42		
11. Bean dregs	5.97		
12. Corn silage	24.55		

(A) The preparation process of broccoli by-product



(B) Addition and mixing of other agriculture by-products

(C) MHA addition and silage preservation

Figure 8. A large amount of BBS manufacturing process

4.2. Evaluation of Mass BBS Quality

4.2.1.Evaluation of chemical composition and feed value

In the case of the mass production of broccoli by-product silage, the chemical composition has no significant differences in the BBS. In the feed value assessment of BBS, the chemical composition can be evaluated by TDN and RFV. The chemical composition results are shown in Table 9. Except for the BF, all of the BBS and CS group were more the 70 % of TDN and RFV was estimated to be more than 200. DM in the CS was significantly higher than that in the BBS, because the moisture content of the by-product of broccoli was about 90%, which affected the total dry matter content. In the case of CP and EE, the content of CS was higher than the BBS. The Ash content in BBS was 1.5 times more than CS. And the CF, ADF and NDF content has no significant difference between the two groups.

Table 9 . Chemical composition of mass production BBS and CS

Treatment	DM	CP	EE	CF	Ash	ADF	NDF	Ammonia	TDN	RFV
	%DM									
BBS-0d	39. 81	21.76	4.17	13.08	9.75	19.07	30.9	0.4	73.83	222.91
BBS-45d	39. 2	23.09	5.33	11.87	10.05	18.2	29.65	0.46	74.52	234.43
BBS-102d	37.81	21.98	3.66	13.38	10.13	17.58	31.69	0.45	75.01	220.76
CS	46.31	33.85	9.4	11.56	6.36	15.52	32.09	0.73	76.64	222.66
BF	29.77	5.77	1.95	31.01	10.28	36.39	59.71	0.11	60.15	94.34

BBS-0d, broccoli by-product sampling on 0 day; BBS-24d, broccoli by-product sampling on 24 day; BBS-45d, broccoli by-product sampling on 45 days; BBS-102d, broccoli by-product sampling on 102 days; CS, con silage; BF, barley straw forage

4.2.2. Determination of organic acid contents in BBS

Organic acid composition of mass product BBS was shown in Table 10. With the preservation time of BBS, the lactic acid content from 0 day to the final day was increasing. The comprehensive lactic acid content and the above bacterial activity also mean that the BBS also has a fermentation process. Lactic acid is the main acid product during the silage fermentation. As the time passed, the content of lactic acid in BBS increased obviously, and the pH decreased, this shows that the fermentation process is well underway. Compared with BBS, the content of lactic acid in the control group was lower, but the content of acetic acid was higher, and the pH was also higher. It was consistent with the conclusion that in the silage stored, higher acetic acid was produced, which is consistent with their final higher pH values by Coskuntuna (Coskuntuna, Koc, Ozduven, & Coskuntuna, 2010). BBS showed higher content in lactic acid but lower content in acetic acid compared with CS. The highest lactic acid content was on BBS-102d, even with butyric acid (1.26 % DM), but depended on the Wieringa's conclusion that mentions grass silage contained less than 50 - 100 % DM butyric acid can be considered as good-medium quality silage (Wieringa, 1966). However, the reason for the low lactic acid content and high pH of

TMR in the control group maybe was farm factors (low production, more cattle), which led to the insufficient time for fermentation of silage.

Table 10. Organic acid composition of BBS and CS

Treatment	pH	Organic acid (% DM)			
		Lactic acid	Acetic acid	Propionic acid	Butyic acid
BBS-0d	5.75	1.55^{f}	2.78^{d}	0.25^{c}	0.95^{ab}
BBS-24d	3.8	19.61^{c}	6.02^{c}	0.41^{bc}	0.59^{b}
BBS-45d	3.84	23.03^{b}	6.67^{c}	0.24^{c}	0.85^{ab}
BBS-102d	3.82	28.58^{a}	9.43^{b}	0.68^{a}	1.26^{a}
CS	5.07	15.23^{d}	15.16^{a}	0.51^{ab}	0.07^{c}
BF	4.66	8.87^{e}	10.55^{b}	0.57^{ab}	1.14^{a}

BBS-0d, broccoli by-product sampling on 0 day; BBS-24d, broccoli by-product sampling on 24 day; BBS-45d, broccoli by-product sampling on 45 days; BBS-102d, broccoli by-product sampling on 102 days; CS, con silage; BF, barley straw forage

4.2.3. Microbial growth in BSS

The viable count of microbes in this experiment in this experiment was shown in Table 11. It can be seen from the above that the addition of MHA to BBS did not completely inhibit the fermentation process, It can be seen from the Table 11 that MHA has little effect on the activity of lactic acid bacteria, from 0 day to the last day of sampling, there was no difference in the activity of lactic acid bacteria. Similarly, there was no significant difference in the number of total bacteria, fungi and yeast. In CS, the number of lactic acid bacteria, total bacteria, fungi and yeast is the highest. That because some probiotics were added to the CS in the farm, especially yeast and bacillus. But BBS has no added any probiotics and content more lactic acid and little acetic acid, so BBS has the better fermentation quality.

Table 11. Microbes activities of BBS and CS

Treatment	LAB	F&Y	TB
		log CFU/g	
BBS-0d	4.68^{b}	4.65^{bc}	5.17^{a}
BBS-24d	4.59^{b}	4.84^{ab}	5.31^{a}
BBS-45d	4.78^{b}	5.01^{ab}	5.40^{a}
BBS-102d	4.79^{b}	4.98^{ab}	5.23^{a}
CS	5.81^{a}	5.69^{a}	5.71^{a}
BF	4.01^{c}	4.15^{c}	4.26^{b}

LAB, lactic acid bacteria; F & M, fungi and yeast; TB, total bacteria; BBS-0d, broccoli by-product sampling on 0 day; BBS-24d, broccoli by-product sampling on 24 day; BBS-45d, broccoli by-product sampling on 45 days, BBS-102, broccoli by-product sampling on 102 day; CS, corn silage; BF, barley straw forage

4.3. Evaluation of Functional substances in BBS

4.3.1. Beta-carotene

In mass production, the content of beta-carotene is significantly lower than that of the laboratory test group. It is speculated that there are two reasons: the first one is that there are relatively more other additives in mass production, so the content of by-products of broccoli is relatively small; the other one is that the quality of broccoli by-products is different, and there are many yellow leaves in large-scale production of broccoli, so the quality is also affected One of the reasons for carotene. Because the addition of MHA did not completely replace the fermentation process, after fermentation, the content of beta-carotene in BBS increased significantly, about 80%. This finding is also reflected in other studies. However, the content of CS was very low, which was similar to BBS-0d. Because there was no fermentation cycle in CS, the content of beta-carotene after full fermentation of con could not be obtained. The result was shown in Table 12 and Figure 9.

Table 12. Beta-carotene content of BBS and CS

Treatment	Beta-carotene (mg / kg)	SEM	P-value
BBS-0d	0.5	0.12	
BBS-24d	3.27	0.16	
BBS-45d	3.74	0.02	＜0.0001
BBS-102d	2.7	0.09	
CS	0.33	0.01	

BBS-0d, broccoli by-product sampling on 0 day; BBS-24d, broccoli by-product sampling on 24 day; BBS-45d, broccoli by-product sampling on 45 days; BBS-102d, broccoli by-product sampling on 102 days; CS, con silage.

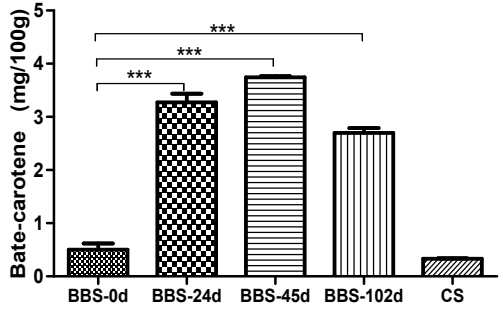


Figure 9. Bate - carotene content in BBS and CS

4.3.2. Antioxidants

In BBS, the results of mass products and laboratory test groups are similar, the antioxidant activity of mass production in BB TMR and CS are shown in Table 13. DPPH radical scavenging activity also drops first and then ascend, TPC is on the rise with the number of days. The difference between BBS and CS was shown in Figure 10. For DPPH radical scavenging activity, there was no difference between BBS-102d and CS. For total phenolics content, BBS-102d had 24.61mg GAE / kg more than CS and showed significant difference ($P < 0.05$). However, for antioxidant activities that including DPPH radical scavenging activity and TPC, the activity of forage was the highest, 38.33 % and 250.78 mg GAE / kg, respectively.

Table 13. Antioxidant activity of mass production in BBS and CS

Treatment	DPPH radical scavening activity (%)	SEM	P-value	Total phenolics content (mg GAE / kg)	SEM	P-value
BBS-0d	52.99	0.95	0.0248	88.72	1.03	< 0.0001
BBS-24d	45.59	3.35		107.59	3.48	
BBS-45d	31.73	2.22		107.50	1.8	
BBS-102d	42.35	5.37		159.52	9.98	
CS	19.42	3.16		134.91	3.16	
BF	38.33	2.56		250.78	4.47	

BBS-0d, broccoli by-product sampling on 0 day; BBS-24d, broccoli by-product sampling on 24 day; BBS-45d, broccoli by-product sampling on 45 days; BBS-102d, broccoli by-product sampling on 102 days; CS, con silage; BF, balery straw forage

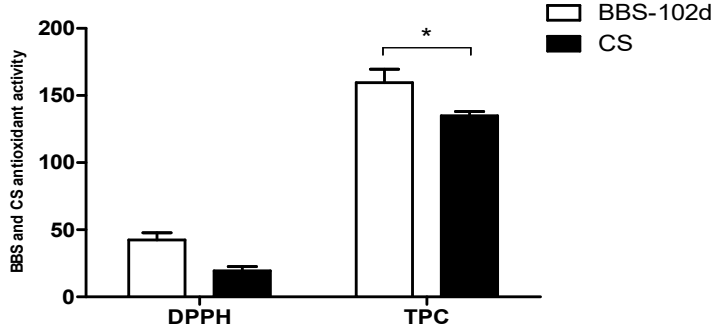


Figure 10. DPPH radical scavenging activity (%) and TPC (mg GAE / kg) in BBS and CS

Chapter2. Evaluation of MHA BBS Substantiational Effect for Hanwoo Feed

1. Introduction

Nowadays, with the development of society and the progress of the economy, people pay more and more attention to the quality of meat and the pursuit of human health. The purpose of this experiment is not only to use the agricultural by-products of broccoli to produce feed instead of commercial feed but also to use the nutrients in the by-products of broccoli to improve and enrich the nutrients in meat. So as to protect the environment and establish a positive relationship between health food and by-product resources.

Hanwoo is the most preferred in the diet culture of South Korea. After feeding BBS, the health status of 16 pregnant Hanwoo (every groupconsis of 8 heads, respectively) was compared and evaluated the substitution efficiency of BBS for commercial feed.

2. Materials and Methods

2.1. Animals and Experimental Desgin

The Hanwoo were used for the feeding trial following the policy and regulations for the care and use of experimental animals, and all animals were used in this experiment that in accordance with the approval of the Institutional Animals Care and Use Committee (SNU-200505-1).

16 pregnant Hanwoo cattle was used and separated into 2 groups according to the body weight. The two groups were fed with BB TMR (Bro-group) and conventional TMR (Con-group) respectively. The whole period of the experiment was 87 days and 5 times of sampling has been proceed. The items of sampling were body weight, body shape measurement, blood sampling and body condition score. After blood sampling, complete blood count (CBC), MPT, vitamin A and fatty acid were detected. Through the above experimental data to evaluate the health of pregnant Hanwoo. The sampling time and items are shown in Figure 11.

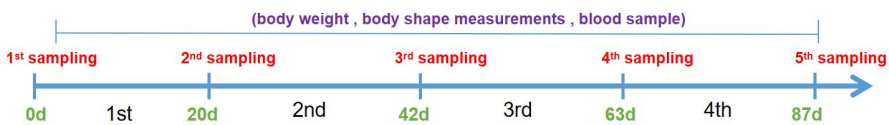


Figure 11. Sampling time and items in the feeding trial

2.2. Experimental Diet and Feeding

During the experimental trial, all Hanwoo cattle could move freely cowshed (except for feeding time). The feeding time was at 7:00 a.m. and 6:30 p.m. respectively. BBS and Con-TMR were fed first, and forage was fed after all of the silage were finished. The feeding method was shown in Table 14. In the whole process of animal experiments, three adjustments were made to the feeding mode according to the changed of body weight.

Table 14. The feeding method during feeding period

Group	Type	1st (0-20d)	2nd(20-42d)	3rd(42-63d)	4nd(63-87d)
		kg			
Con-group	CS	6	6	6	6
	BF	6	6	6	6
	BT	1	1	3	3
Bro-group	BBS	6	8	8	8
	BF	6	6	6	6
	BT	1	1	3	3

CS, corn silage; BF, barley straw forage; BT, base TMR; BBS, broccoli by-product silage.

2.3. Hanwoo Body Shape Measurement and Blood Sampling

For the method of body measurement, measure Hanwoo' s body length, heart-length, withers height and hip height by the tape measure. Keep the animal's head next to the posts or shelves to make sure the cattle are fixed. First, find the jugular vein, then shave all the hair around it. Use iodine and alcohol to disinfect the skin of the sampling department. Press one side of the jugular vein near the heart with the left hand, and touch the vein with the right index finger to expand it. Then the needle was inserted into the jugular vein at a 45 - degree angle to the vein. Take a sample after seeing the blood return.

2.4. Metabolic profile test

For the metabolic profile test (MPT), the plasma was used. The plasma recovered from the blood samples after centrifuging at 4 ℃ at 3500 rpm for 15 minutes. After centrifuge, the plasma was stored at -80 ℃ until required.

MPT was performed with a Toshiba Acute Biochemical Analyzer - TBA - 40FR Toshiba Medical Instruments, Otawara-shi, Tochigi-ken, Japan) according to this method that was described (Deluyker, Gay, Weaver, & Azari, 1991). The protein metabolism indicators consist of TP (total

protein), Alb (albumin), and UREA (urea). Energy metabolism includes Glu (glucose), T-Cho (total cholesterol), and LCFA (long-chain fatty acid). For liver and kidney function, AST (aspartate aminotransferase) and GGT (gamma-glutamyl transpeptidase) and Crea (creatinine). For mineral metabolism, Ca (calcium), IP (inorganic phosphorus, and Mg (magnesium)(Piao et al., 2015).

2.5. Complete blood count

Complete blood count（CBC) can be an important and powerful diagnostic tool as a component of minimum databases. It can be used to monitor the response to therapy, to gauge the severity of an illness, or as a starting point for formulating a list of differential diagnosis (Barger, 2003).

The CBC measurement method following the same method (Alam, Kim, Kim, Na, & Kim, 2012). The CBC was analyzed using an automatic hematology analyzer (Scil Vet abc, Scil Animals Care Company , USA). First, the blood samples (containing EDTA) were put into the roller mixer, and for the proper rolled at 16 mm amplitude and 33 rpm. All samples enter the analyzer in the same way, and after the start button was pressed, the blood samples were taken by the inject needle automatically. The result will be shown after 90 s and was received in printed form as well.

2.6. Fatty Acids

Long Chain Fatty Acids (LCFAs) are fatty acids with aliphatic tails longer than 12 carbons (Labatut & Pronto, 2018) and physiologically important because they combine to form triacylglycerol and provide energy storage form in adipose tissue of animals (Belal et al., 2017). Supplemental fats differing in origin are used to improve the energy density of diets in ruminant production. Fats are an effective energy supplement, being very digestible with a high metabolic use for animals (Suksombat, Meeprom, & Mirattanaphrai, 2016).

Briefly, after getting the blood samples, centrifuged at 3000 rpm for 15 minutes to obtain the plasma, and the plasma samples were sorted at - 80 ℃ for analyses fatty acid composition. 1 ml plasma samples (milk 1.5 ml, pork meat 1 g, processed meat 1 g, oli 40 µl) were added in the glass tube with cover. After that, 1 ml C11 (the concentration is 0.5 mg / ml, solvent in MeOH) was added and vortexing. Then 700 µl of 10 N KOH and 5.3 ml MeOH were added respectively and mixed evenly. Put the mixed sample into a 55 ℃ water bath for 1.5 hours, and vortexing every 20 minutes. After cooling, add 580 µl of 24 N H_2SO_4 and continue to put it in a 55 ℃ water bath for 1.5 hours, vortex every 20 minutes. After cooling again, add 3 ml of hexane and continue to vortex for 5 minutes.

After mixing evenly, centrifuge for 5 minutes at 3000rpm at 20 ℃, take out the upper liquid and put it into GC sample bottle for testing. The situation of the GC for determination of fatty acid is shown in Table 15.

Table 15. The situation of GC for determination of fatty acid

Column	SP-2560 (100 m ×0.25 mm × 0.2 um)
Injection Volume	1 µl
Split ratio	1 / 30
Flow rate	1.1757 mL / min
Oven temperature	100 ℃ for 5 min Ramp to 24 ℃ at 4 ℃ / min and hold for 14 min
Injector Temperature	250 ℃
Detector Temperature	260 ℃

2.7. Vitamin A

Plasma concentration of retinol is an accepted indicator to assess the vitamin A (retinol) status in cattle (Raila et al., 2017). The determination method of vitamin A in this experiment is the same as this method (Karpińska, Mikołuć, Motkowski, & Piotrowska-Jastrzębska, 2006). After getting the blood samples, centrifuged at 3000 rpm for 15 minutes to obtain the plasma, and the plasma samples were sorted at - 80 ℃ and waiting for testing. After mixing 500 µl of plasma, 10 µl of 1000 ppm of retinyl acetate and 1 ml of methanol and vortexing, the vortices continued after adding 1.5

ml of hexane. Centrifuge at 2000 rpm for 15 minutes, took out the upper liquid, evaporated with nitrogen at 15 psi for 5 minutes, after it was completely dried, added 1 ml of methanol for dissolution, filter with 0.2 μm syringe filter, and then put the samples into LC special bottle for testing. The situation of UPLC for the determination of vitamin A is shown in Table 16.

Table 16. The situation of HPLC for determination of vitamin A

Column	PDA
Mobile phase	Acetonitrile : MeOH (1 : 1)
Gradient	Isocratic (3 min run cycle)
Flow rate	0.4 mL/min
Injection Volume	3 μL
Column Temperature	30 °C
Sample Temperature	20 °C

2.8. Antioxidants

Up to now, the Folin-Ciocalteu method is the basic and most used method to determine the concentration of total phenolic content in biological samples such as plasma, urine, and animal organs. Even though not so widely used for biological samples, the DPPH test can be a choice for the assessment of the antioxidant activity (Chedea & Pop, 2019).

1 mM of DPPH soluble in 200 ml of 80 % methanol, the plasma samples were diluted with 80 % methanol and centrifuged for 5 minutes at 10000 rpm, after that 5 µl plasma supernatant was taken and mixed with 245 µl DPPH solution. After 30 minutes reaction in dark condition, the absorbance measured at 517 nm is used to express the binding efficiency of free radicals by the spectrophotometer (SpectraMax M3, Molecular Devices, USA).

DPPH radical scavenging activity capacity also follows the formula:

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{(Absorbance\ of\ control - Asorbance\ of\ test\ sample)}{Absorbance\ of\ control} \times 100$$

Phenolic content in plasma samples determined by Folin - Ciocalteu reagent. The plasma samples were centrifuged at 10000 rpm for 10 minutes at room temperature. After centrifuged, 1 ml DW, 1ml Folin reagent (folded by 5 times) and 50 µl plasma sample supernatant well-mixed and reaction in dark condition for 3-6 minutes, and then added 1 ml 10 % Na_2CO_3. After 70 - 75 minutes incubation at room temperature in the dark condition, the total phenolic contents were determined at 750 nm by spectrophotometer and displayed as gallic acid equivalent (GAE).

3. Result and Discussion

3.1. Body Weight and Body Shape Measurements

During this experiment, 16 pregnant Hanwoo were divided into test group and control group. The body weight comparison between the control group and test group was compared by the slope of the body weight data for the test period. Since the weight of the two groups of Hanwoo was not the same before the test and the pregnancy period was also not the same, it is not meaningful to compare the average body weight changes between the two groups. The standardization of the body weight of a cow is a pregnant or non-pregnant conditions is the first step to meet their nutrient requirements (Paulino, Fonseca, Henriques, Valadares Filho, & Detmann, 2010). One of the purposes of this feeding test is whether BB TMR can replace Con TMR to meet the nutritional needs of pregnant cows. The trend of body weight change during the test trial was shown in Table 17, according to the analysis, there was no significant difference in body weight ($P > 0.05$) between the two groups after the end of the whole feeding period. The slope of body weight change of the two groups was shown as Figure 12, and the slope was calculated at the beginning of the second sampling, because at the beginning of the experiment, we gave the

same amount to the two groups, but due to the high moisture content of BBS, it resulted in weight loss. After adjustment, the weight of BB-TMR group increased again. By comparing the slope of body weight change between the two groups, we can find that there was no significant difference between the two groups, therefore, it is concluded that BB TMR can meet the basic nutritional needs of pregnant cows.

Table 17. The trend of body weight change during the test trial

Treatment	0d	20d	42d	63d	87d	SEM	P-value
BB-TMR group	509.38	501.88	520.14	529.29	545.57	7.66	$p > 0.05$
Con-TMR group	505.38	514.00	528.63	543.38	564.00	10.47	

BB-TMR group, the test group fed by broccoli by-product TMR during feeding trail; Con-TMR group, the test group fed by control (corn) TMR during feeding trail.

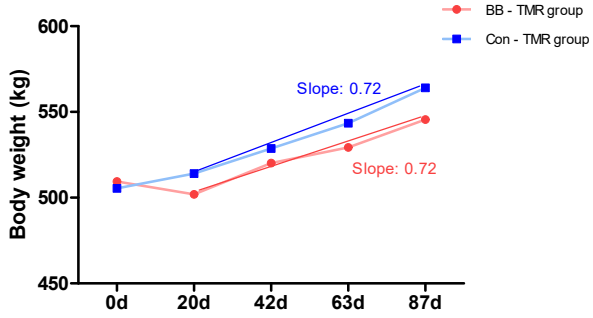


Figure 12. Comparison of body weight increasing slope between control and test group during test period

Physical condition test indicators include heart girth, body length, withers height and hip height. These four terms also calculate the slope from the second sampling, because the body weight fluctuation maybe can affect the accuracy of these data. Although the slope of each index was different, there was no significant difference (P ＞ 0.05) after analysis.

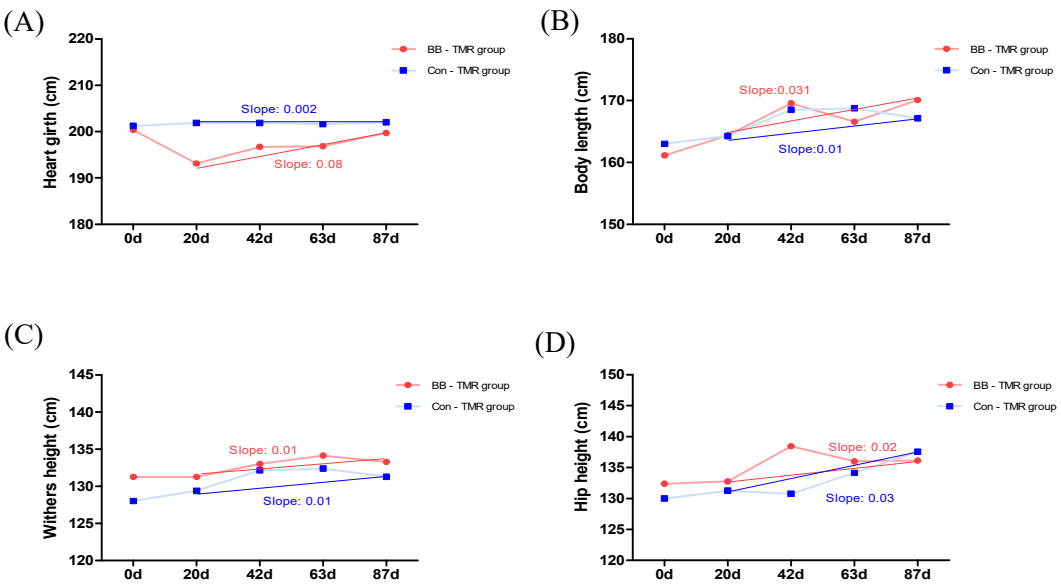


Figure 13. Comparison of body measurements slope between control and test group during test period

3.2. Vitamin A Contents in the Plasma

Vitamin A content in the plasma samples analyzed in BB-TMR group and Con-TMR group during the experiment period. The Vitamin A content result was shown in Table 18 and Figure 14. Since the experimental animals were pregnant cows, the two groups were supplemented with vitamin A 30 g every day as the base during the experiment. During the whole experiment, the content of vitamin A showed an overall upward trend. The group with the highest content was the BB-TMR group (1.71 mg / 100 ml) with the last sampling, while the group with the lowest content was Con-TMR group (1.30 mg / 100 ml) with the first sampling. It can be seen from the table 18 that the vitamin A content of BB-TMR group is slightly higher than Con-TMR group, but there was no difference during the test period between the two groups after analysis ($P > 0.05$). The results noted that there was no significant difference in bate-carotene content between the mass-produced BB-TMR group and the Con-TMR group. Because vitamin A is very important for pregnant cows, the farm supplemented vitamin A 22.5 g every day. Because the content of beta-carotene in mass BBS production is not high, the vitamin A content in plasma has no significant difference between the two groups.

Table 18. Vitamin A content in plasma in BB-TMR group and Con-TMR group

Treatment	Vitamin A content in plasma (mg / 100ml)					SEM	P-value
	0d	20d	42d	63d	87d		
BB-TMR group	1.36	1.63	1.66	1.59	1.71	0.063	P ＞0.05
Con-TRM group	1.30	1.63	1.52	1.37	1.56	0.061	

BB-TMR group, the test group fed by broccoli by-product TMR during feeding trail; Con-TMR group, the test group fed by control (corn) TMR during feeding trail.

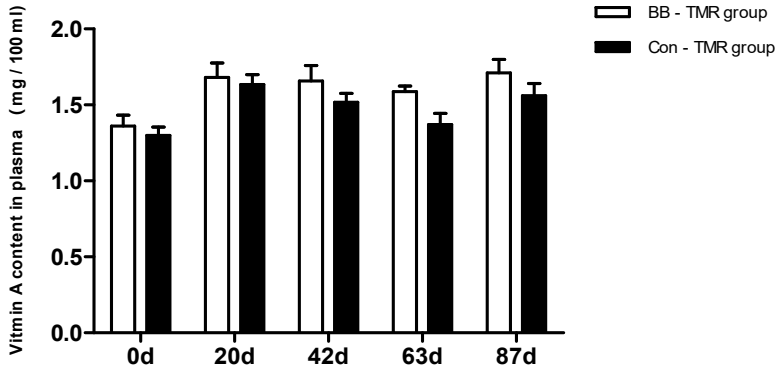


Figure 14. Vitamin A content in plasma during test period

3.3. Fatty Acid Composition in Plasma

The composition of fatty acid was shown in Table 19 and Figure 15. There was no difference in fatty acid composition in plasma between BB-TMR group and Con-TMR group. It is worth mentioning that the content of stearic acid and oleic acid is slightly different between the two groups. Stearic acid is a saturated fatty acid that, in contrast to other long-chain saturated fatty acids, does not raise plasma LDL (low-density lipoprotein) cholesterol (Baer, Judd, Kris-Etherton, Zhao, & Emken, 2003). Although there was no significant difference between the two groups, the content of Con-TMR group was slightly higher. This may be due to the high fat content of corn in CS. It is widely accepted that oleic acid, and oleic acid may have many beneficial health effects. Among such effects are improved insulin sensitivity, and endothelium-dependent flow-mediated vasodilatation, lowering of LDL cholesterol and an increase in HDL (high-density lipoprotein) cholesterol (Estévez-González et al., 2010). In BB-TMR group, the content of oleic acid in bovine plasma was higher, which may be related to the intake of by-products of broccoli. The specific reasons need further study.

Table 19. Fatty acid content in plasma in BB-TMR group and Con-TMR group

	Fatty acid (%)	0d	20d	42d	63d	87d	SEM	P - value
BB-TMR group	Palmitic acid (C16 : 0)	9.37	8.80	9.56	10.07	10.18	0.25	> 0.05
	Stearic acid (C18:0)	15.62	13.76	16.56	21.00	15.71	1.31	> 0.05
	Oleic acid (C18:1n9c)	10.05	10.67	12.42	11.46	11.24	0.40	> 0.05
	Linoleic acid (C18:2n6c)	33.40	27.49	29.78	33.00	33.75	1.22	> 0.05
	Gamma-linolenic acid (C18:3n6)	—	1.02	0.79	—	1.09	0.05	> 0.05
	α-linolenic acid (C18:3n3)	4.23	3.25	4.16	4.38	4.61	0.23	> 0.05
	Dihomogamma-linolenic acid (C20:3n6)	2.41	1.59	2.22	2.25	2.21	0.14	0.012
	Arachidonic acid (C20:4n6)	3.23	3.21	2.86	3.04	2.90	0.08	> 0.05
Con-TMR group	Palmitic acid (C16 : 0)	8.46	9.17	12.50	10.38	9.58	0.25	> 0.05
	Stearic acid (C18:0)	16.02	15.56	16.88	17.29	15.33	1.31	> 0.05
	Oleic acid (C18:1n9c)	11.13	9.39	9.27	10.01	9.13	0.40	> 0.05
	Linoleic acid (C18:2n6c)	31.66	29.82	34.00	34.72	36.36	1.22	> 0.05
	Gamma-linolenic acid (C18:3n6)	—	1.20	0.97	—	0.87	0.05	> 0.05
	α-linolenic acid (C18:3n3)	4.06	3.06	4.33	3.82	3.97	0.23	> 0.05
	Dihomogamma-linolenic acid (C20:3n6)	2.51	2.01	2.24	2.30	2.40	0.14	> 0.05
	Arachidonic acid (C20:4n6)	3.07	2.67	2.38	2.31	2.31	0.08	> 0.05

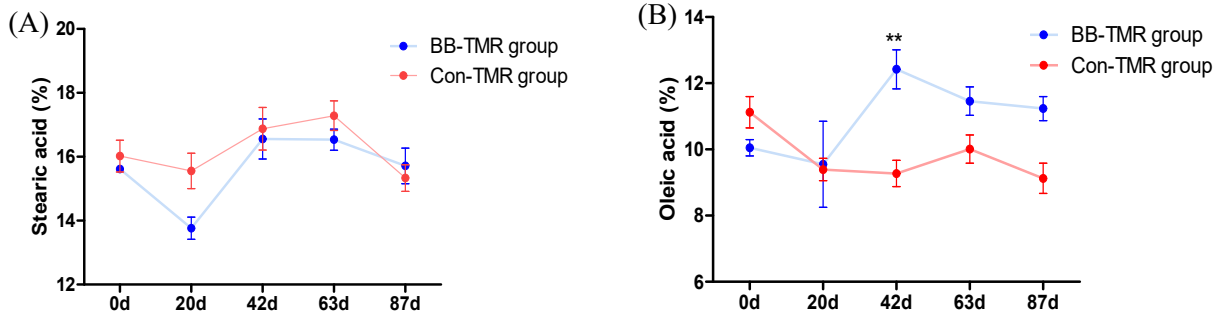


Figure 15. Stearic acid (%) and oleic acid (%) in BB-TMR and Con-TMR group

3.4. Antioxidant activity in plasma

The Folin-Ciocalteu method, measuring the total phenolic content (TPC), is the reference assay to measure phenolics in foods as well as their presence in plasma, urine, and even different organs. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay (DPPH assay) is one of the various analytical methods that have been developed to measure the antioxidant capacity mainly in plant and food extracts (Chedea & Pop, 2019). Because the results of the DPPH free radical scavenging rate used in this experiment are not stable, the partial antioxidant capacity of plasma is analyzed and discussed by TPC content. The content of TPC in both groups increased first and then decreased, but there was no significant difference between the two groups. In the second sampling, the difference between the two groups was large. It was inferred that the second sampling was due to the insufficient feed intake in the Bro - group. The result was shown in Table 20 and Figure 16.

Table 20. Total phenolics content in plasma in BB-TMR group and Con-TMR group

Treatment		Total phenolics content (mg GAE / kg)	SEM	P-value	Treatment		Total phenolics content (mg GAE / kg)	SEM	P-value
BB-TMR group	0d	1148.96	18.01	>0.05	Con-TMR group	0d	1116.15	26.3	>0.05
	20d	1308.75	43.77			20d	1629.17	146.3	
	42d	1020.19	23.31			42d	971.83	34.89	
	63d	1068.27	26.88			63d	1001.54	33.83	
	87d	1238.96	18.81			87d	1161.46	19.63	

BB-TMR group, the test group fed by broccoli by-product TMR during feeding trail; Con-TMR group, the test group fed by control (corn) TMR during feeding trail.

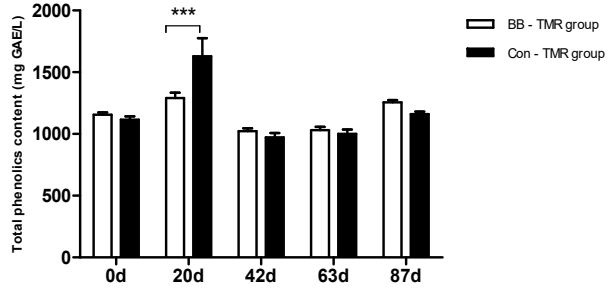


Figure 16. TPC (mg GAE / kg) in BB-TMR group and Con-TMR group

3.5. Metabolic Profile Test

Blood metabolic profile tests (MPT) are simple cost-effective biochemical tests that are mostly used to identify nutritional and/or management challenges in dairy cattle herds, but they also can be simply used to find animals that are clinically healthy (Madreseh-Ghahfarokhi & Dehghani-Samani, 2020). Blood samples are mainly used to assess energy profile, protein profile, enzyme and mineral profile.

MPT test items include blood glucose, ketone body, blood urea nitrogen, blood volume ratio, total protein, albumin, globulin, total cholesterol, aspartate transaminase, gamma glutamine transpeptidase, calcium, phosphorus and magnesium, etc. Main energy profile are: glucose (GLU), cholesterol (COL-T), can be used to evaluate nutritional programs; triglycerides (TAG), that can produce adenosine triphosphate; β - hydroxybutyrate (β - HBA), most abundant and important ketone body; non-esterified fatty acid, related to lipomobilisation and negative energy balance. Main protein profile: blood urea nitrogen (BUN): indicator of the energy intake and indication between fermentable carbohydrates and rumen degradable protein(Van, 2016); albumin (ALB), which related to the hepatic insufficiency; globulin (GLOB), that can increase the response to

an inflammatory process (Whitaker, 2000); total protein (PROT-T), which effect the kidney damage, liver damage and nutritional health. The enzyme γ glutamyl transpeptidase (γ - GT) is an essential indicator of hepatic lesions and function (Stojević, Piršljin, Milinković-Tur, Zdelar-Tuk, & Ljubić, 2005). Main mineral profile are calcium (Ca) and inorganic phosphorus (iP) due to their importance in the rapidity of metabolic reactions and their role in the transmembrane transport systems (Houillier, 2014). The results are shown in Figure 17 to Figure 19.

In all the test items, except for BUN, there was no difference between the BB-TMR group and the Con-TMR group. Because of the direct relationship between BUN and energy intake, there was a significant difference between the two groups at the second sampling, urea nitrogen is the end product of protein metabolism, combined with the changes in body weight, that can be known the protein intake was not enough in the second sampling. After adjusting the feeding mode, there was no difference between the two groups. Therefore, it can be inferred that BB TMR will not have adverse effects on animal health.

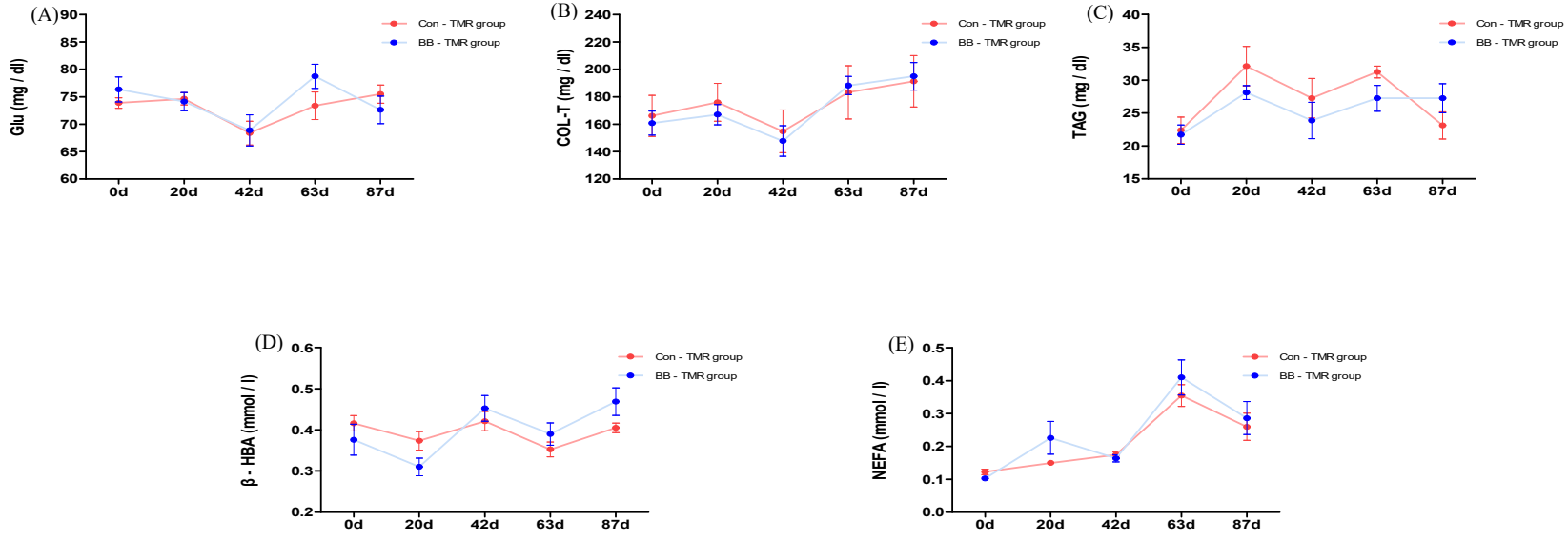


Figure17. The result of energy profile in BB-TMR group and Con-TMR group

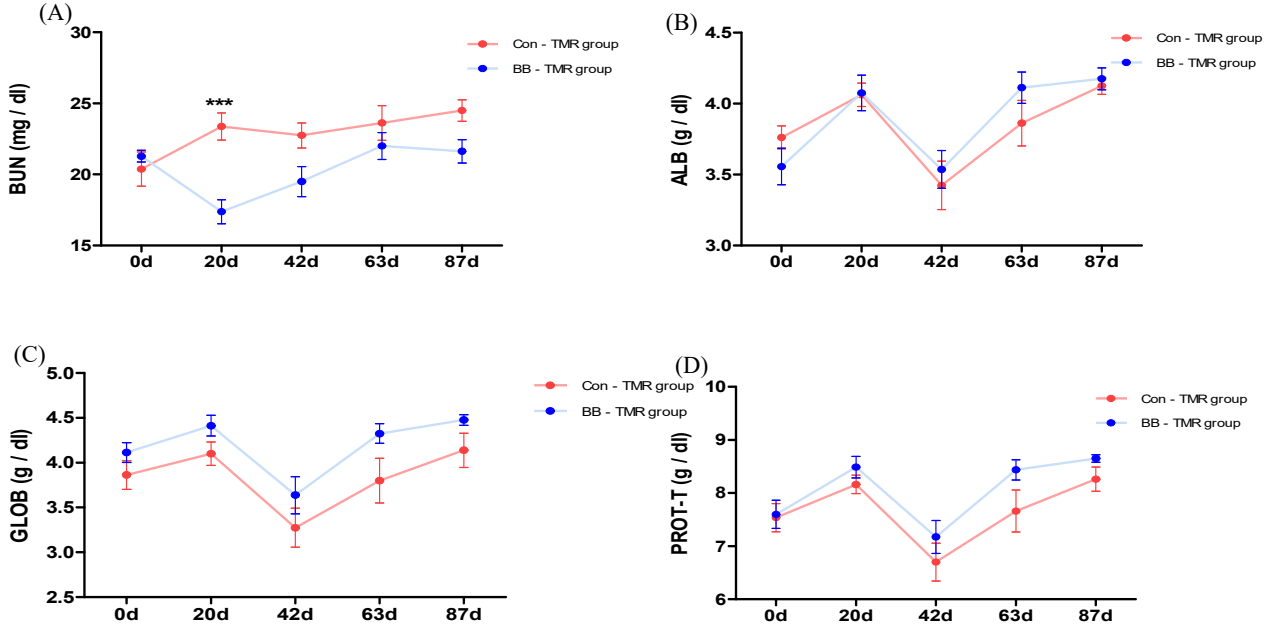


Figure 18. The result of protein profile in BB-TMR group and Con-TMR group

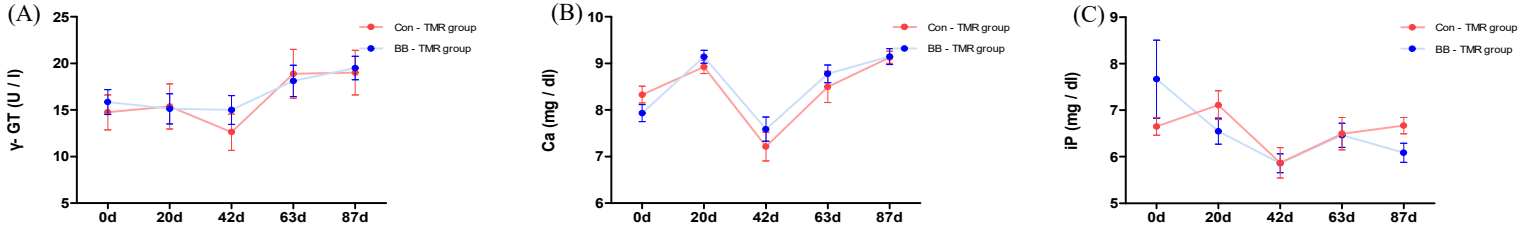


Figure 19. The result of enzyme and mineral in BB-TMR group and Con-TMR group

3.6. Complete Blood Count

A complete blood count can be an important extension of the physical examination in ruminants and may be used to suggest certain disease processes when exam findings are vague and are useful for establishing prognosis in many cases (Jones, 2011).

White blood cells (WBCs), can help your body fight bacteria. If you have too much, it could be a sign of inflammation, infection, medical reaction, or other health conditions. If the infection rate is low, you have a higher risk of infection. Drugs, viral infections, or bone marrow diseases can also cause low counts. Red blood cell (RBC), they carry oxygen all over the body. They also help carry carbon dioxide. If red blood cell count is too low, you may have anemia or other diseases. Hemoglobin (HGB), is a protein that contains oxygen in your blood. Hematocrit (HCT), this test tells you how much blood is made up of red blood cells. A low score may be a sign that you don't have enough iron, a mineral that helps your body produce red blood cells. A high score may mean you are dehydrated or otherwise. Mean corpuscular volume (MCV), is the average size of your red blood cells. If they are larger than usual, your maximum confidence interval will be higher. This can happen if you have low levels of vitamin

B12 or folate. If your red blood cells are small, you may have anemia. Platelet (PLT), these help blood clot. Lymphocytes (Lym), this is a kind of white blood cell, and it is the smallest white blood cell. It is produced by lymphoid organs and mainly exists in the circulating lymph in the lymphatic vessels. It is an important cellular component of the immune response function of the body. Neutrophile, a type of white blood cell that helps heal damaged tissues and resolve infections. Neutrophil blood levels increase naturally in response to infections, injuries, and other types of stress. They may decrease in response to severe or chronic infections, drug treatments, and genetic conditions. (George-Gay & Parker, 2003)

From the results of this experiment, there was no significant difference in the Con-TMR group and BB-TMR group except for the platelet content in the second result. The second sampling time was the weight loss period of the BB-TMR group, after adjustment, there was no significant difference with the control group. It can be seen that BB TMR has no effect on the health of cattle and can be used as a substitute feed. CBC results are shown in Figure 20.

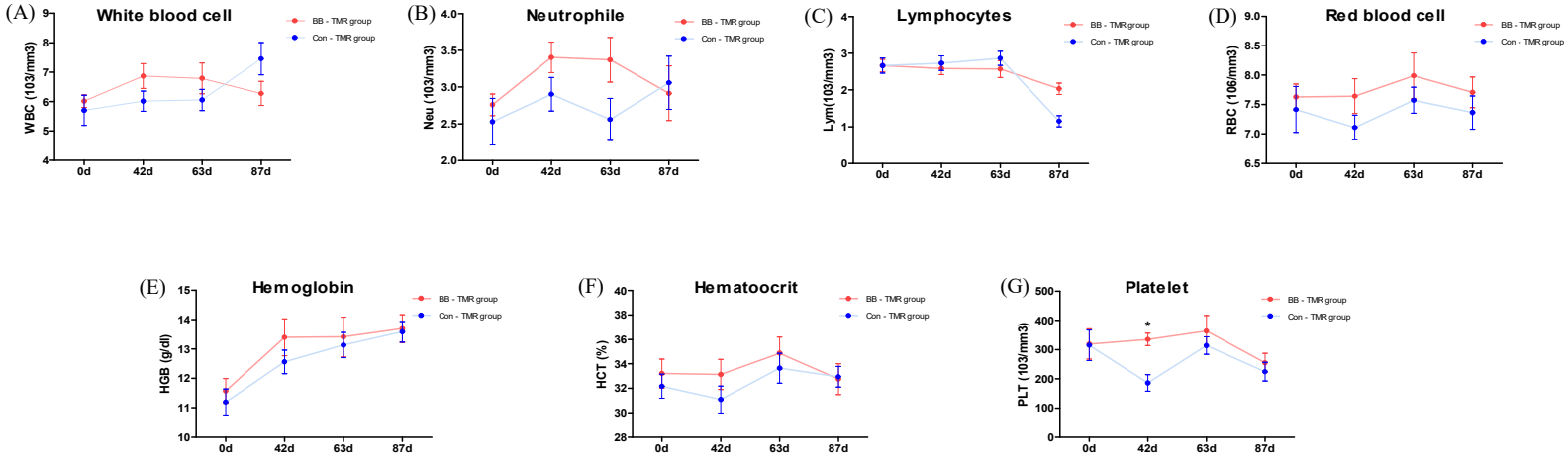


Figure 20. The result of Complete blood count in BB-TMR group and Con-TMR group

3.Conclusion

We conducted a study on the development and evaluation of MHA broccoli by-product silage on substitutional effect for Hanwoo feed. In this study, comprehensive economic budget and experimental results, the best formula is that 80 % (weight) broccoli by-products are mixed with 10 % (weight) BP and WB respectively which mixed with 0.4 % MHA was shown in the *in vitro* test. The results showed that there was no significant difference in TDN and RFV between MHA broccoli by-product silage and control silage (inoculated with *L.plantarum* 5×10^7 cfu / kg of the silage), that means MHA has no adverse effect to the feed value. It can be seen from the microbial growth and organic acid content that the addition of MHA (the highest concentration of 0.8 %) can not completely replace the fermentation process, but MHA treated group content more lactic acid and less acetic acid, and there was no or very little butyric acid, it can be concluded MHA was beneficial to the fermentation process for environmental lactic acid bacteria and played a great role in inhibiting the activity of undesirable bacteria. Specifically, pH remained stable after the decline, and there was no secondary fermentation. The addition of MHA could quickly inhibit the activity of bacteria and reduce the trend of

corruption. For the beta-carotene, the content of beta-carotene increased nearly 60 % - 80 % after fermentation, and high concentrate MHA group (0.4 % and 0.8 % treated group) losses less then 20 % beta-carotene after 10days fermented. The ability of antioxidation also increased. With regard to the mass production of feed products, after considering economic and nutritional aspect, the final determination of 0.4 % MHA addition for mass production.

In the part of animal experiment, the health status of the two groups (BB-TMR group and Con-TMR group) was compared to evaluate the desirability of the feed. After comparison, there was no difference between the two groups in terms of weight, MPT, CBC, and various health assessment indicators. In terms of the content of functional substances, there was also no difference in the content of carotene and antioxidants level in plasma. The results showed that 0.4% MHA had no adverse effect on the health of cattle.

Originally, it is hypothesized that after treated MHA to broccoli by-product silage, the fermentation process could be totally replace, because of prevention effect of low pH on bacteria growth. According to the *in vitro* test result, fermentation process has not be replaced but MHA provide a good environment for the fermentation. So this study was very

valuable.

In conclusion, the broccoli by-product is a valuable feed resource, and after evaluation, it can be substitute the Hanwoo feed. We also believe that the comprehensive development and utilization of broccoli by-products are important that can reduce environmental pollution and promote the sustainable development of agriculture.

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Abstract in Korean

현대사회에 있어 환경오염을 줄이고 지속 가능한 농업 발전의 증진을 위한 농업부산물의 종합적 이용 및 재활용은 매우 중요하다. 브로콜리는 화뢰(꽃)부분을 채취해 식용하는 십자화과의 채소로 우리나라에서는 주로 제주도(전체 생산량의 약 70%)와 강원도(전체 생산량의 약 15%)가 주산지로 연간 25,000 여톤을 생산하고 있는데, 이 때 잎, 줄기 등 화뢰 생산량의 약 2.5 배(연간 약 60,000 톤)의 비식용 부위가 부산물로써 그대로 노지에 폐기되는 것으로 추정되고 있다.

본 연구에서는 기존의 발효 공정 대신 메티오닌 수산화 유도체(Methionin Hydroxy Analogue)를 처리한 브로콜리 부산물 사일리지에 대해 적합한 배합비를 선정한 후 대량생산하여 한우 번식우에 대해 급여시험을 진행하였으며, 한우시험축의 건강지표(체중, 체형 측정, 대사 판정 시험, 총 혈구 분석결과) 및 혈장 내 기능성 성분 분석 결과를 토대로 상용 사료 대비 한우 번식우의 유지사료로써의 가치를 평가하였다.

우선 MHA 를 0.2, 0.4, 0.8% 수준별로 처리한 사일리지를 제조하여 MHA 처리구와 MHA 미처리구의 일반 성분, 유기산 함량, pH, 미생물 성장 정도와 함께 설포라판(sulforaphan)과 베타카로틴(beta-carotene) 등의 기능성 물질의 함량 변화와 항산화능을 비교하였다.

그 결과 MHA 처리 브로콜리 부산물 사일리지와 락토바실러스 플란타룸(Lactobacillus plantatum)을 5×107cfu/kg 수준으로 접종 한 대조구에서 TDN 과 RFV 는 유의적인 차이가 없는 것으로 보아 MHA 처리가 사료 가치에 유해한 영향를 미치지 않는 것으로 판단할 수 있었다. 미생물 분석 결과와 유기산 함량으로 보아, MHA (최고 농도 0.8%)를 첨가하여 발효 과정을 완전히 대체 할 수는 없으나 MHA 처리구에서 젖산 함량이 높고 초산(acetic acid) 함량이 적으며, 낙산(butyric acid) 함량은 매우 적거나 없는 것

으로 나타났다. 이로 미루어 볼 때 MHA는 사일리지 재료 (브로콜리 부산물)의 pH를 직접적으로 낮추어 유산균의 발효 과정에 있어 유리한 환경을 조성하며, 유해균의 활성을 억제하는 것으로 추정할 수 있었다. 설포라판의 경우 모든 실험구에서 시간이 지남에 따라 급격히 감소하는 경향을 나타내었고, 베타카로틴에 대해서는 발효 후 베타카로틴 함량이 60 ~ 80% 증가하며, 발효 10일 이후 MHA 농도가 높은(0.4%, 0.8%) 처리구에서 베타카로틴이 20% 이내의 함량이 손실되는 결과를 보였다. 항산화 활성도 역시 발효 후 향상되는 경향을 보였다.

한우 번식우를 대상으로 한 사양시험을 통하여 MHA 처리 브로콜리 사일리지 기반의 TMR(BB-TMR)을 급여한 실험구와 농가에서 자가배합하여 사용하는 기존 TMR(Con-TMR)을 급여한 대조구의 건강 상태 및 혈장 내 기능성 성분을 비교하여 MHA 처리 브로콜리 부산물의 사료대체 효과를 검정하였다. 그 결과 실험구과 대조구는 체중, MPT, CBC등 각종 건강 평가 지표에서 차이를 보이지 않았다. 그러나 혈장 내 지방산 조성의 경우, 단일 불포화지방산인 올레인산(oleic acid)의 경우 실험구가 대조구에 비해 유의적으로 높은 결과를 보였으나, 포화지방산인 스테아르산(stearic acid)의 경우에는 실험구에서 대조구보다 낮은 결과를 나타냈다. 혈장 내 비타민A와 항산화 물질의 함량은 실험구와 대조구에서 유의적 차이를 보이지 않았다.

이러한 결과를 종합적으로 고려해 볼 때, MHA 처리 브로콜리 부산물 사일리지는 한우 번식우의 유지를 위한 농가 자가배합 TMR을 제조함에 있어서 경제적인 대체자원으로 활용할 수 있을 것으로 판단되었다.

주요어: 농림 부산물, 자원 재활용, 브로콜리 부산물 사일리지, MHA(메티오닌 수산화 유도체), 한우 번식우

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