Master's Thesis of Science in Agriculture

Development and Evaluation of Broccoli By-product Silage as a Substitutional Ingredient of TMR for Dairy Cows

브로콜리 부산물 사일리지의 제조와 착유우를 위한
완전배합사료 대체원료로써의 가치 평가

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Development and Evaluation of Broccoli By-product Silage as a Substitutional Ingredient of TMR for Dairy Cows

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Abstract

Development and Evaluation of Broccoli By-product Silage as a Substitutional Ingredient of TMR for Dairy Cows

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One way to achieve a sustainable livestock industry is to increase the by-product utilization from agriculture and feed industry. However, commercial use of agricultural by-product has been limited due to the lack of information on the value of feed, methods of use, and harmful components of natural food resources. In Pyeong-chang, Gang-won province, Korea, over 4,000 t of broccoli flowers are grown annually, but their by-products (stems and leaves) of about 6,000 t per year are discarded. Therefore, a study was designed to establish the optimal broccoli by-products silage formula using a combination of various additives and to test the cow's performance in a dairy cattle

feeding trial to determine whether it would be valuable a substitute to the commercial dairy TMR.

Chopped broccoli by-products (BB) have been mixed with several agricultural by-product additives with or without lactic acids bacteria (LAB) inoculation in a series of silage preparation trial. And feed value, feed fermentation quality and palatability was tested to establish optimal formulation for broccoli by-product silage (BBS). The optimal BBS was obtained when BB combined with beet pulp & wheat bran (20% of the total silage weight) and *L. plantarum* inoculum (5×10^7 cfu/kg). The mixture was put into 20L plastic box as tight as possible to remove air and covered with lid, and incubated in a barn for 45 days. BBS resulted in high levels of CP (19 %), TDN (71.7 %), NE_L (1.67 Mcal/kg) and high quality fermentation with high lactic acid content. For the feeding trial, 9 ton of BBS was prepared using a TMR mixer to chop the BB and mix well the additives. Silage was put into vinyl-lined ton-bag with tight pressure to remove air. BBS was incubated in a barn condition for 45 days. 27 Holstein cows were need to test the feed value of BBS. Control cow feed was 20 kg of commercial TMR (Cargill agri-purina, Korea) mixed with chopped 10 kg of rye silage, total 30 kg of TMR feed was fed daily. Test TMR was prepared mixing 10 kg of chopped rye silage and 16 kg of commercial TMR and 10 kg of BBS which replaces 20 % (4 kg) of commercial TMR. The feeding trial lasted for 8 weeks; the first 4 weeks were control period feeding control TMR feed and the next 4 weeks was test period feeding test TMR. 27 Holstein cattle data was separated to 4 groups according to days in milk (DIM) (group A: 238 ± 90.1, B: 146.8 ± 22.9, C: 73.9 ± 6.9, D: 23.5 ± 12.6). The milk yield was verified by comparing the slope of the test period with the control period using the daily milk yield data from individual cow collected. Milk sampling was conducted once a week to analyze the components and functional substances. As a result, it was determined that there was no significant difference in the milk yield compared to the control period. It was also determined that there was no significant difference in the milk components including

milk fat, protein, lactose, SnF, MUN compared to the control period. We judged that BBS did not affect the change in the milk production. Also, analysis of functional substances in silage showed that sulforaphane a key functional molecule for anti-cancer decreased by about 99 % and beta-carotene increased by 120 % during the BBS fermentation ($p < 0.001$). In addition, antioxidant activity increased by about 55 % ($p < 0.005$). During the test period, the beta-carotene content in milk significantly increased in most groups (linear, $p < 0.05$), but vitamin A showed no significant change. Total phenolics content (TPC) tended to decrease in most groups, while antioxidant activity tended to increase. Therefore, BBS had a high deed feed value and fermentation quality and concluded that when replacing 20 % of commercial dairy TMR with BBS, it did not adversely affect to the cow's milking performance. We also believe that the use discarded BB as feed are important to improve the sustainability of livestock industry in Korea and there are good potential for BBS as a TMR ingredient at least in dairy cows.

Keywords : Broccoli by-product, by-product silage, cow feed, cow TMR, functional components

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List of Abbreviations and Formula

AcN: Acetonitrile

ADF: Acid detergent fiber

BB: Broccoli by-product

BBS: Broccoli by-product silage

BP: Beet pulp

BWB: Buckwheat bran

CC: Corn cob

CD: Cassava distillers dried grains

CFU: Colony forming unit

CP: Crude protein

cmTMR: Control mixed total mixed ration

cTMR: Commercial total mixed ration

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

ESM1: Epithiospecifier modifier 1

ESP: Epithiospecifier protein

FID: Flame ionization detector

GAE: Gallic acid equivalent

GC: Gas chromatography

IACUC: Institutional Animal Care and Use Committee

LAB: Lactic acid bacteria

LC: liquid chromatography

LDL: Low density lipoprotein

MA: Mixed additive

MMU: Mobile milking unit

MUN: Milk urea nitrogen

NDF: Neutral detergent fiber

NFC: Non-fiber carbohydrate

NRC: National Research Council

RB: Rice bran

RFV: Relative feed value

RIP: Degradable intake protein

ROS: Reactive oxygen species

RT: Room temperature

SEM: Standard error of the mean

SnF: Solid-not-fat

TDN: Total digestible nutrients

TMR: Total mixed ration

TPC: Total phenolics content

tTMR: Test total mixed ration

UIP: Undegradable intake protein

WB: Wheat bran

WSC: Water soluble carbohydrate

1. Introduction

The domestic livestock industry is now on a road of qualitative growth minding the sustainable eco-friendly livestock industry with qualitative growth from the past paradigm of quantitative growth. However, the reality of the feed supply and demand sector in the domestic livestock industry is highly dependent on foreign countries due to limited land and natural resources. To achieve sustainable livestock industry in Korea, feed animals other than the human food, raise regionally appropriate animals, keep animals healthy, adopt smart supplements, track costs and benefits, study best practice etc. are required (Mark C. et al., 2014). One way to satisfy these demands is to use domestic natural resources as much as possible. Therefore, the goal of this study is to utilize agricultural by-products as valuable forms that are generated and discarded in large quantities when growing crops and vegetables. It is also an additional goal to make eco-friendly functional livestock products, considering modern consumers demanding a well-being diet.

We have selected the broccoli by-product (stems and leaves) as the functional agricultural bioresourcse in Pyeong-chang, Korea. Broccoli (*Brassica oleracea L. italica*) is a vegetable crop that has been marketed as a health-promoting food because it naturally has high content of bioactive phytochemicals such as glucosinolates, sulforaphane, phenolic compounds, vitamin C, and mineral nutrients (Ares et al., 2013). But, 70 % of the broccoli crops that have these beneficial components consist of inedible

by-products parts. Yi et at. (2015) studied about effects of replacement of concentrate mixture by broccoli by-products on lactating performance in dairy cows. They found the lactating performance by pelletizing broccoli by-products to replace concentrate in dairy cows. As a result, it was concluded that it did not affect the diary performance and was suitable as a dairy cattle feed.

In Korea, Broccoli is harvested in early summer and late autumn. It is very important that broccoli by-product should be collected and preserved by drying or silage preparation in a very short time. To utilize the broccoli by-products, we chose to prepare silage for a dairy feed, and the optimal formula for broccoli by-product silage was established and assessed the value and quality of the feed. For the feed value test, TMR feed was prepared which replace part of the commercial dairy TMR with broccoli by-product silage and feeding trial was performed to determine the change in cow's milk production and milk quality. In addition, functional substances were analyzed in broccoli by-product silage and milk obtained from the feeding trial. Through these tests, it was showed that broccoli by-product silage is a good ingredient of diary TMR feed.

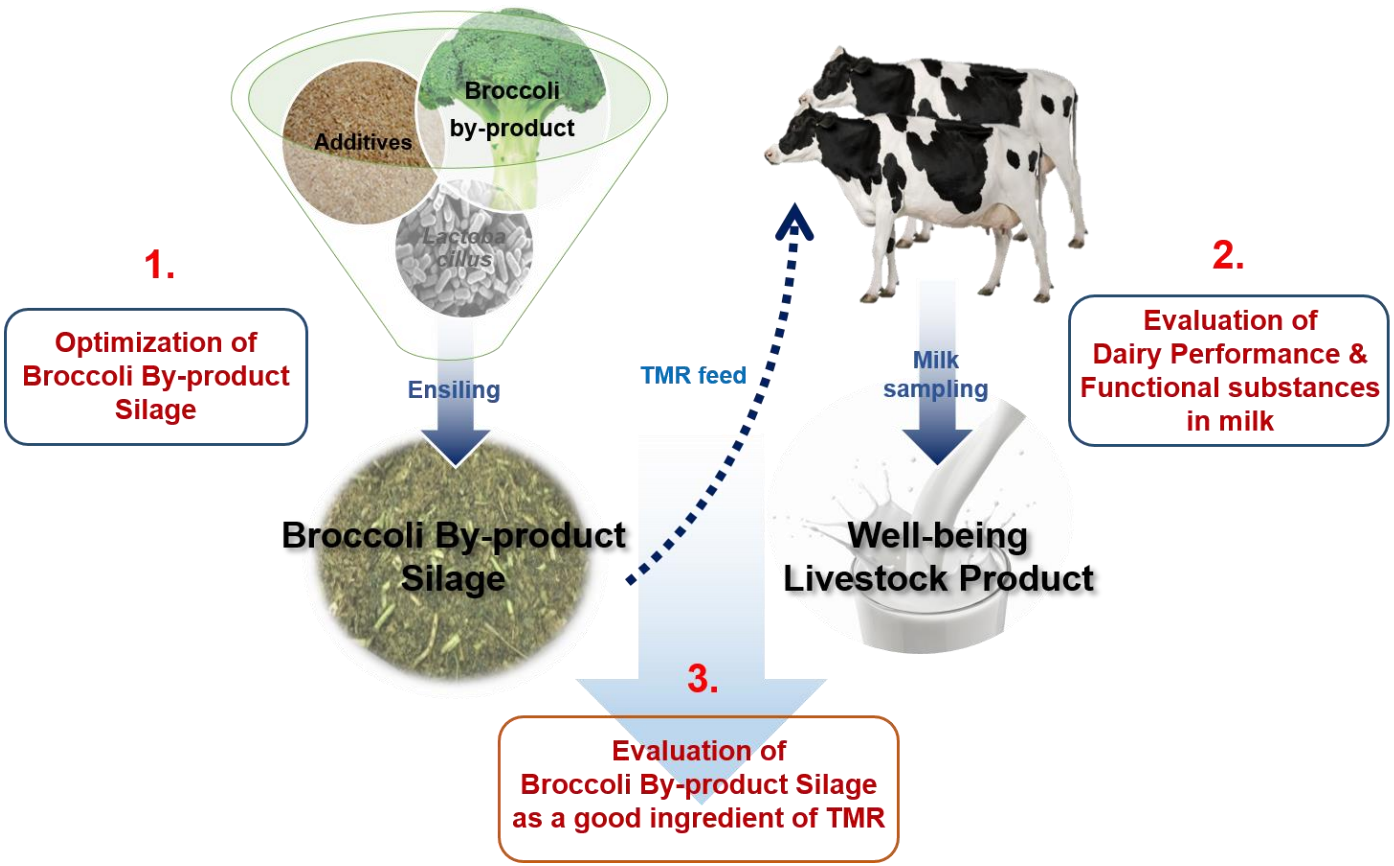

Figure 1. Aim of the study (preparation and evaluation of the broccoli by-product silage)

2. Literature Review

2. 1. Purpose and Necessity of Utilization of Natural Resources

Recently, the supply and demand of feed materials has been deteriorating due to changes in international grain supply and demand which occur by abnormal climate changes or increase in a number of new methods to utilizing crops (developing countries in Asia, producing biofuels and so on). Such conditions could continue for a long time, rather than for a temporary situation, so it is necessary to take proper measures in terms of the domestic livestock industry, which relies mostly on foreign imports. Measures to cope with the imbalance in grain supply and demand can be implemented, such as finding stable importers of crops, expanding imports from a single market to multiple markets and directly participating in the futures market. These methods may be used to address stable supply and demand for crops, but there are still limitations in eliminating the costs of soaring international grain prices.

A recent measure to stabilize feed prices and the imbalance in supply and demand of crops is to utilize the natural resources generated during the production process of agricultural products available. This is expected to have a huge impact in conjunction with the green growth industry. However, natural feed resources are produced in a variety of regions and seasons, and most of the by-products are not easy to transport due to their high moisture or bulk. In addition, there is not much use of agricultural by-

products due to lack of information on the value of feed, methods of use, and harmful components of natural food resources (Oh et al., 2012). 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 techniques for its use.

2. 2. Broccoli By-product

2. 2. 1. Characteristics of broccoli and broccoli by-product

Broccoli (*Brassica oleracea L. italica*) is an edible green plant whose large flowering head is eaten as a vegetable. Broccoli has been marketed as a health-promoting food because it naturally has high content of bioactive phytochemicals such as glucosinolates, sulforaphane, phenolic compounds, vitamin C, and mineral nutrients. Thus, broccoli plays a role in the prevention of chronic diseases, such as cardiovascular and carcinogenic pathologies, and breast and prostate cancers (Ares et at., 2013; Moreno et al., 2006). Broccoli has also been found to exhibit antioxidant activity that prevents oxidative stress related to many diseases (Borowski et al., 2008). Broccoli by-product are stems and leaves. Broccoli parts being used for food are mostly florets, which make up 30% of the whole broccoli. 70% of whole broccoli is by-product like stems and leaves. Many by-products of the agricultural industry including broccoli by-product may be useful as source of nutrients and potentially functional ingredients, giving the opportunity to obtain added value products. So it is worth utilizing the broccoli by-

product as a feed source (Domínguez-Perles et at., 2010).

2. 2. 2. Generation of broccoli by-product in Korea

Broccoli is grown at 1,545 ha nationwide, with Jeju Island accounting for 85 % of the 1,314 ha, with 17,490 t of production. Among them, Gang-won Province accounts for about 10 % of its production. Gang-won Province harvests more than 4,000 t of broccoli annually, and a lot of inedible parts such as leaves and stems, excluding broccoli flowers, are generated (estimated to be around 6,000 t/year) as by-products (MAFRA, 2012). However, the by-products are left in the fields, leading not only to a waste of resources but also to a detrimental effect on the environment.

Figure 2. The occurrence of broccoli by-product in Pyeong-chang, Korea

2. 2. 3. Utilization of broccoli by-product

A number of studies have been done on the potential to utilize many nutritional or functional substances from broccoli by-products. Liu et al. (2018) reported that comprehensive nutrient and bioactive compounds profile represents a useful resource for the evaluation of broccoli by-product utilization in the human diet, and as feedstock for bioactive compounds for industry. Domínguez-Perles et at. (2010) reported that the possibility of adding health-promoting value to this inexpensive and unused broccoli by-product is worth the effort of recycling it to obtain bioactive components that could benefit the food and drug industry.

Few studies have been done using broccoli by-products as a feed. In Megías et al. (2014) study, to evaluate broccoli by-product suitability as a feed, the fermentative, chemical and different pesticide parameters were determined. Yi et at. (2015) studied about effects of replacement of concentrate mixture by broccoli by-products on lactating performance to dairy cows. They utilized the pelletized broccoli by-product as a feed and replaced the commercial concentrate. When the concentrate was replaced by pelletized broccoli by-product at a level of 20%, no adverse effects were found on the gas volume or its rate constant during ruminal fermentation. In dairy cows feeding trial, no significant difference in milk yield was observed and a significant increase was found in milk fat content in the pelletized broccoli by-product group ($p<0.05$). These results indicated that pelletized broccoli by-product could be included in dairy cattle diets at a suitable level to replace concentrate mixture without any adverse effects on dairy

performance.

These results suggest that it is worth to utilize the broccoli by-products as a feed and therefore we selected as agricultural by-product bio-resources.

2. 3. Factors affecting to the silage quality

2. 3. 1. Dry matter and chemical composition

The content of the dry matter (DM) in the silage can have a significant impact on the silage quality. This has to do with the DM of the crops materials that make the silage. Proper amount of DM content is important for crop. The weather at which crops exist, for example, drought, frost, and extreme temperatures affect the growth of the grass and the DM content, thus influencing photosynthesis (Nelson et al., 1994). The recommended optimum DM for ensiling forage ranges from 35% to 45% (Hargreaves et al., 2009).

A high DM in the silage prevents the formation of anaerobic conditions and packaging density in the manufacture of the silage. This condition prevents the high population of lactobacillus because low water content inhibits the growth of the microbes (Rizk et al., 2005). According to one study, a high DM of silage has shown that the population and growth of lactobacillus is hampered, resulting in lower lactic acid concentrations and slower pH rather than optimal DM content silage (Whiter et al., 2001). In cereal silages, water-soluble carbohydrate (WSC) and protein concentrations

within the forage are the two most important substances before ensiling (Hargreaves et al., 2009). Protein and organic acid concentrations in the forage also affect the silage fermentation.

2. 3. 2. Lactic acid bacteria inoculants

The silage inoculants are microbial additives, which is mainly made up of lactic acid bacteria, which increases the efficiency of fermentation and it enhances the storage of the silage. Inoculant uses lactic acid producing bacteria in various plant or silage by selecting the bacteria which have high adaptability to various moisture content or temperature ranges in the silage. There are many strains, and the most common ones are *Lactobacillus plantarum*, *Streptococcus faecium*, *Pediococcus* strains and so on (Richard et al., 1991). There are about $10\sim10^3$ cfu/g of lactate bacteria present in the forage, which are cut and increased to $10^3\sim10^7$ 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). Alfalfa silage inoculated with *L. plantarum* had no effect on dry matter intake (DMI), but combining it with *E. faecium* increased DMI by steers compared to the control (McAllister et al., 1998). However, one study showed that using different strains of inoculant did not affect the feed intake of sheep (McAllister et al., 1995). Another study showed that Steers' DMI increased when only *L. plantarum* was used as an inoculant

(Steen et al., 1989). Weinberg et al. (2003; 2004) were tested to determine the mechanism of the fermentation characteristics and the ability for ruminal fibre digestibility according to silage inoculum. Their results showed that the LAB in silage inoculants could cause favorable shifts in rumen microbial ecology and fermentation patterns and improve DM and NDF digestibility (Weinberg et al., 2007).

2. 3. 3. Ensilage process

The fermentation process occurs when the silages are under anaerobic conditions caused by lactic acid bacteria (LAB) that turn sugars into organic acids. Lactic acid, produced by lactic acid bacteria, improve the protectability for the silage preservation, preventing growth of other spoilage microorganisms. There are two pathways of fermentation occur during ensiling; homolactic fermentation and heterolactic fermentation. Homolactic fermentation is reduced to 2M of lactic acid in the presence of an aldose enzyme and heterolactic fermentation is reduced to equimolar amounts of lactic acids, acetic acid, carbon dioxide and ethanol. Both homo- and hetero-lactic bacteria have flexible metabolic pathways and are also capable of fermenting pentose (xylose and arabinose) sugars into mainly lactic and acetic acids via the pentose-phosphate pathway (Mcdonald et al., 1981; Woolford et al., 1984., Wesseh et al., 2013).

Several factors can affect the fermentation process. For example, during the fermentation process, the fermentation results can be significantly different depending on the type of grass, the timing of harvest, whether or not lactobacillus are treated, and the type of silos (McAllister et al., 1998). Also, the respiration of the plant, the activity

of enzymes, and the clostridial and yeast fermentation show side effects (Muck et al., 1988).

2. 4. Relation between Feed and Milk Production

"Forage quality" encompasses the inherent characteristics which determines its voluntary intake while "forage nutritive value" is a measure of the inherent characteristics of the forage consumed and includes nutrient concentration, digestibility and the nature of the end products of digestion and their impact on animal performance (Moore et al., 1994; Weseh et al., 2013). Even with economics, there is a greater influence in assessing the quality of the forage. Therefore, the nutritional value of the forage or silage is determined by intake, digestibility, and utilization efficiency of animals (Woolford et al., 1984). In addition, inoculant treatment can be manufactured with improved fermentation, thereby improving the palatability of the livestock and improving the DMI (Yan et al., 1996).

In dairy farm, the aim is to maximize profits and reduce the feed cost to the maximum without reducing lactation performance. For this reason, experts or nutritionists not only find an efficient way to reduce feed cost efficiently, but also try to maintain milk production and yield. In addition, milk production has grown from previous years with the selection of outstanding individuals and managements (VandeHarr et al., 2006). In the past 25 years, the milk production has increased by about 2 % per year. Changing

the milk composition by controlling the feed nutrition is currently widely used.

As previously discussed, high quality feed from inoculants can increase the palatability and intake of livestock. Increased intake also affects livestock, increasing the daily weight, increasing the milk yield, and improving the productivity of livestock. In the study of Sutton et al. (1989), They said that some nutritional ingredients changed the concentration of milk fat and milk protein. It was also said that diet change could change the rumen function.

The first thing to consider when feeding dairy cattle is protein. A review by Santos et al. (1998) concluded that diets containing higher concentrations of rumen degradable intake protein (RIP), rather than greater levels of rumen undegradable intake protein (UIP), will have increased milk production with a reduction in milk protein concentrations. Insufficient quantities of RIP reduce the production of ammonia in the rumen, which in turn reduces the milk protein percentages. The second thing to consider when feeding dairy cattle is carbohydrates. Carbohydrates are the primary source of energy for cows and can be divided into two fraction (NRC, 2001); structural carbohydrates and nonstructural carbohydrates. Maintaining balance between these two carbohydrates maintains proper rumen function. There are results that the amount of starch in the feed affects the rumen pH. According to a study conducted in Oba et al. (2003a, 2003b), lower starch content fed to dairy cows increased the rumen pH. There have also been reports that too little amount of neutral detergent fiber (NDF) and acidic detergent fiber (ADF) in feed not only reduce milk fat and milk yield, but also can causes

some metabolic diseases (Ishler et al., 1996). Therefore, appropriate amounts of carbohydrates in the feed are important because they have a large impact on cow production.

Yi et at. (2015) studied about effects of replacement of concentrate mixture by broccoli by-products on lactating performance in dairy cows. They utilized the pelletized broccoli by-product as a feed and replaced the commercial concentrate. When the concentrate was replaced by pelletized broccoli by-product at a level of 20%, no significant difference in milk yield was observed and a significant increase was found in milk fat content in the pelletized broccoli by-product group ($p < 0.05$).

2. 5. Functional Components in Broccoli by-product

2. 5. 1. Sulforaphane

Cruciferous vegetables like broccoli, cabbage contain high concentration of vitamins, minerals and a special group of phytochemicals, sulfur-containing glucosides called glucosinolates (Bellostas et al., 2007). Through the hydrolysis process by endogenous enzyme myrosinase, a variety of bioactive substances are produced. The chemical structure of glucosinolates and other factors such as pH and temperature during enzymatic process form the bioactive substances like thiocyanates, nitriles, sulfate, isothiocyanate, D-glucose and oxazolidine-2-thiones (Bones et al., 1996; Fenwick et al., 1983). Sulforaphane which is a naturally occurring isothiocyanate is a well-known anticancer compound present in broccoli and broccoli by-products, possess potent cancer chemopreventive activity. Glucoraphanin (a kind of glucosinolate) is the precursor of sulforaphane and glucoraphanin can be changed to sulforaphane by myrosinase binding enzymes (epithiospecifier protein, ESP; epithiospecifier modifier 1, ESM1) and other factors (Moreno et at., 2006; Ares et al., 2013). Thus, the sulforaphane conversion can be changed by genetic and environmental factors, and it also presents an opportunity for broccoli to undergo nutritional modification. Sulforaphane restrains phase I enzymes and phase II enzymes. These reaction promotes the apoptosis and cell cycles arrest, and inhibits the metastasis and angiogenesis (Wang et al., 2016). Therefore, the sulforaphane content analysis was conducted on broccoli byproduct silage and milk as a one of the functional substances.

2. 5. 2. Beta-carotene and Vitamin A

Beta-carotene is an organic, strongly colored red-orange pigment abundant in plants and fruits. It is a member of the carotenes, which are terpenoids (isoprenoids), synthesized biochemically from eight isoprene units and thus having 40 carbons. Beta-carotene is the most common form of carotene in plants. In nature, beta-carotene is a precursor (inactive form) to vitamin A via the action of beta-carotene 15,15'-monooxygenase (Susan et al., 1998). Vitamin A is a group of unsaturated nutritional organic compounds that includes retinol, retinal, retinoic acid (Fennema et al., 2008). In this study, we focused to retinoic acid which is an important hormone-like growth factor for epithelial and other cells (Tanumihardjo et al., 2011). Beta-carotene is an antioxidant that enhances immunity with reproductive and mammary (Chew et al., 1993). However, in most grains and fermentation feed, beta-carotene exists in small amounts because of heat damage and decomposition during storage periods. In particular, many studies suggest that the amount of beta-carotene is reduced after fermentation in silage which need fermentation period necessarily. The major reasons for the decreasing beta-carotene is non-oxidative changes, which are destroyed by high heat generation as well as low pH during fermentation or oxidative changes, which are destroyed by exposure to light (Kalac et al., 1981; Kasangi et al., 2010). Beta-carotene is added to the feed to improve the health or productivity of dairy cows. According to the results of Ondarza et al. (2009), beta-carotene supplementation increased percentage of milk fat yield in early-lactation and mature cows. But milk production was not affected.

2. 5. 3. Phenolic acids and Antioxidant activity

Over the past decade, many studies have been interested in edible plants that contain a lot of secondary metabolites (such as phytochemicals). Recent studies showed that cruciferous vegetables (broccoli, cabbage, cauliflower etc.) contain a lot of antioxidants such as carotenoids, tocopherols, vitamin and phenolic acids which are source of natural antioxidants and they protect the human body against damage by reactive oxygen species (ROS) (Cartea et al., 2011). Among the antioxidants possessing antioxidant capacity, phenolic compounds are one of the most important components. Phenolic compounds are widely distributed in the plant kingdom and the structure is shaped by one or more hydroxyl groups attached to a single or more aromatic ring. Phenolics are produced as secondary metabolites through the shikimic acid pathway in plants. Recently, there has been an increasing interest in the bioavailability and biological effects of phenolics in plants. Phenolic compounds play a role in protecting plants from insect, fungi, viruses and bacteria, and controlling hormones in plants. Furthermore, phenolic compounds are worth doing research because they have potential health promoting effects (Crozier et al., 2009; Vallejo et al., 2002). In many studies, phenolic compounds have health related anti-inflammatory, enzyme inhibition, antimicrobial, antiallergic, and anticancer activities, but the most important role of phenolic compounds is antioxidant activity (Plumb et al., 1997; Cushnie et al., 2005) The phenolic compounds absorb and neutralize the reactive oxygen species (ROS) as an antioxidant activity. The ROS, generated by the oxidation process, is a defense mechanism against infection and it's an

important part. However, the occurrence of excessive free oxygen radicals causes tissue damage. If there is an imbalance between the ROS and the antioxidant mechanisms, the ROS will cause oxidative modification of the cellular membrane or intracellular molecules and result in the accumulation of lipid peroxides. These oxidative stress is associated with cancer, aging, atherosclerosis, and inflammation, as well as neurodegenerative disease such as Parkinson's and Alzheimer's disease. Thus, antioxidants, such as phenol compounds, are considered to be possible protective substances that can reduce oxidation damage from ROS in the human body and plays a role in preventing the occurrence of many chronic diseases and low density lipoprotein (LDL) related to arteriosclerosis (Shen et al., 2010).

3. Materials and Methods

3. 1. Silage Preparation

3. 1. 1. 1st Broccoli silage preparation

Broccoli by-product (*Brassica oleracea var. Italica*) which indicate stems and leaves obtained from Pyeong-chang in Korea. Other agricultural by-product such as beet pulp (BP), wheat bran (WB), rice bran (RB) and buckwheat bran (BWB) were used as additives to make the 1st broccoli by-product silage (BBS). All additives were obtained from feed company (Han-you B&F, Korea). Because of high moisture content in broccoli by-product (BB) (up to 90% of moisture), BP was used as a moisture absorbent and carbohydrate source. WB and RB were used as a carbohydrate source to make a condition of high quality fermentation. BWB was selected as a one of local agricultural by-product. Also lactic acid bacteria (*Lactobacillus plantarum*, LAB, 5×10^7 cfu/1kg of the silage) inoculum was used to encourage a high quality lactic acid fermentation. The chemical composition of the materials used in the 1st BBS preparation are listed in Table 1.

Table 1. Ingredients and chemical composition for 1st broccoli by-product test silage

Items (% DM)	BB	BP	RB	WB	BWB
DM (% FW)	7.38	90.12	90.16	91.33	93.99
NDF	30.34	42.78	20.27	32.87	59.19
ADF	25.51	25.81	8.07	10.86	48.4
CP	27.27	8.12	12.47	14.28	10.09
EE	2.97	0.69	15.96	3.56	1.83
Ash	22.07	3.53	7.7	3.4	2.27
NFC	17.35	44.88	43.6	45.89	26.62

DM, dry matter; FW, fresh matter; NDF, neutral detergent fiber; ADF, acidic detergent fiber; CP, crude protein; EE, ether extract; NFC, non-fiber carbohydrate; BB, broccoli by-product; BP, beet pulp; RB, rice bran; WB, wheat bran; BWB, buckwheat bran.

1st BBS preparation proceeded in early July and BB were chopped by agricultural cutting machine (5 ~ 10 cm length) before into silage making process. The 1st BBS experiments were conducted in 10 treatment groups (about 20kg scale in each treatment and 3 replicated) by combinations of BB and various additives (20% or 30%); moisture content 65 ~ 75%, or LAB inoculation. The control treatment silage only used broccoli by-product without LAB inoculum. Fermentation of all treatments had lasted up to 45 days in the shade room.

3. 1. 2. 2nd Broccoli silage preparation

Broccoli by-product were used in common with 1st BBS preparation. Other agricultural by-product such as corn cob (CC), cassava distillers dried grains (CD), mixed additives (MA, combination of cassava distillers dried grains and mushroom grains), beet pulp (BP), wheat bran (WB) and rice bran (RB) were used as additives to

make the 2nd BBS. All additives were obtained from feed company (Han-you B&F) and MA were mixed form of MA and mushroom grains. Also lactic acid bacteria inoculum was used in common with 1st BBS preparation. The chemical composition of the materials used in the 2nd BBS preparation are listed in Table 2. CD and MA were analyzed just crude fiber content, 25.6 and 21.7 respectively.

Table 2. Ingredients and chemical composition for 2nd broccoli by-product test silage

Items (% DM)	BB	CB	CD	MA	BP	RB	WB
DM (% FW)	14.78	92.13	88	88.2	90.12	90.16	91.33
NDF	21.01	80.43	-	-	42.78	20.27	32.87
ADF	17.20	45.79	-	-	25.81	8.07	10.86
CP	18.04	1.72	10.5	14.9	8.12	12.47	14.28
EE	5.15	0.44	2	3.5	0.69	15.96	3.56
Ash	17.44	1.39	19	20.5	3.53	7.7	3.4
NFC	38.36	16.02	-	-	44.88	43.6	45.89

DM, dry matter; FW, fresh matter; NDF, neutral detergent fiber; ADF, acidic detergent fiber; CP, crude protein; EE, ether extract; NFC, non-fiber carbohydrate; BB, broccoli by-product; CC, corn cob; CD, cassava distillers dried grains; MA, mixed additives; BP, beet pulp; RB, rice bran; WB, wheat bran.

2nd BBS preparation proceeded in early December and the experiments were conducted in 13 treatment groups (about 20kg scale in each treatment and 3 replicated) by combinations of BB and various additives (18% or 26%); moisture content 65 ~ 75%. Fermentation of all treatments had lasted up to 45 days in the shade room. The manufacturing process of the 1st and 2nd BBS is shown in Figure 3.

Figure 3. Broccoli by-product silage manufacturing process. (A) - (C) Steps for cutting down the broccoli by-product. (D) - (F) Steps for mixing broccoli by-product and additives. (C) - (I) Steps for suppression and fermentation of manufactured silage.

3. 1. 3. Chemical composition and feed value analyses of BBS

The silage samples were collected about 700g and placed in convection dry oven (model VS -1202D9, Vision Scientific Co., Ltd., Korea) at 65°C up to 72 hours. After drying, Dry matter (DM) was analyzed and grounded to pass through a 1mm sieve (Thomas Scientific Model 4, New Jersey, USA). Crude protein (CP) and ash were analyzed by the same method explained in AOAC (Method 990.03 and 942.05, respectively; AOAC International, 2007). Ammonia-N was analyzed by the same method explained in AOAC (1990). The neutral detergent fiber (NDF) content was estimated using the method of Van Soest (1991) and contents of acid detergent fiber (ADF) was determined according to Van Soest (1973). Total digestible nutrient (TDN) can be calculated by known ADF (Holland et al., 1990); TDN (%) = 88.9 – (0.79 × % ADF). Relative feed value (RFV) can be calculated by digestible dry matter [% DDM = 88.9 – (0.779 × % ADF)] and dry matter intake (% DMI = 120 / % NDF) (Holland et al., 1990).

3. 1. 4. Fermentation quality analyses of BBS

The silage samples were collected about 700g and placed in freezer at -20°C until next procedure. 10g of each silages samples added to 90mL of distilled water (DW) and placed in fridge at 4°C for 24 hrs. And then, the silage samples filtered by 4 layers of gauze and pH measured using a pH meter (model AG 8603; Seven Easy pH, Mettler-Toledo, Schwerzenbach, Switzerland) immediately after filtering.

To analyze the organic acids (volatile fatty acids), 10g of each silages samples added to 90mL of distilled water (DW) and incubated in fridge at 4°C for 24 hrs. After incubation, the silage samples filtered by 4 layers of gauze and filter paper (Whatman No. 6, AVANTEC). The extract was used to following experiments. In organic acids, acetic acids and butyric acids were measured by using gas chromatography (GC-iacuc, Agilent 7890B, USA). A sample volume of 1μL was injected with capillary column Nukol with a length of 30m × 0.25mm (Sigma-Aldrich, Germany). The injector was set at 220°C and split rate was 1:40. The flame ionization detector (FID) was set to 220°C and the column oven was set to 200°C. The ramp temperature was increased to 10°C at 20°C/min and hold for 19min. Lactic acids were measured by using liquid chromatography (Xevo TQ MS ACQUITY UPLC and TQD, Waters, USA). A sample volume of 5μL was injected with column ACQUITY BEH Amide with length 2.1mm × 100mm (Waters, USA). Mobile phase A was 50/50 AcN/H_2O with 10mM of CH_3COONH_4 (pH 9.0) and mobile phase B was 95/5 AcN/H_2O with 10mM of CH_3COONH_4 (pH 9.0). Flow rate was 0.6 mL/min and sample temperature was 5°C. MRM condition was 88.92 > 42.9.

3. 1. 5. Sensory test of BBS by human

After fermentation, the 1st and 2nd BBS were evaluated in visual score (leafiness, odor, color, softness and mold). 5 assessors participated in this test and visual score was estimated by the criterion written by Burns and Gary (1991). The total score is 20 points, and the test was conducted, grading 1 (excellent) to 4 (bad).

Figure 4. Sensory test of BBS by human

3. 1. 6. Palatability test of BBS by cattle

Twenty-six Holstein cattle (10 ~ 14 months old) were assigned to study on the palatability of 1st BBS preparation. Well fermented silages selected through human sensory test were offered and every cattle could access to every silage freely for 1hr. In 2nd BBS test, nine Hanwoo cattle (24 months old) were assigned to study on the palatability. Every 2nd BBS were assigned to this test and it was lasted for 6hrs. During the 2nd BBS preparation test, the cold weather (December) made the silage less accessible, so extended the palatability test time to 6 hrs. Voluntary intake (kg) was determined as the difference in the amount of silage DM offered at the beginning of a time and the DM remaining at end of the test. After the time, preference of a particular silage by cattle was determined by calculating the consumed silage amount and comparing it to the level of consumption of other silages. Silages that were consumed more were considered to be more palatable.

Figure 5. Palatability test of BBS by cattle

3. 1. 7. Selection of optimal formula of BBS preparation

Based on the results of all silage tests which were mentioned in upper part including feed value, fermentation quality, human sensory and cattle palatability test, the optimal formula of broccoli by-product silage preparation was selected. To select the formula, we focused on the results of cattle palatability test if the results of some silages from feed value and fermentation quality tests were similar with other silages. The selected formula of BBS preparation was used to make a large scale BBS for feeding trial.

3. 1. 8. 3rd BBS preparation for feeding trial

A large amount of BBS preparation was done feeding trial using selected formula as a 9,000kg scale. Broccoli by-product including stems and leaves obtained in the month of July at Pyeong-Chang, Korea. Additives and inoculum were also selected as an optimal formula from the evaluation tests. To preparation of this work, large scale of TMR mixer machine was used which is located in Animal Farm of Seoul National University.

3. 2. Feeding Trial using BBS

The *in vivo* experiments and following analyses were conducted at Animal Farm and Laboratory of Animal Metabolism in Seoul National University, Pyeong-chang, Republic of Korea. All animals used in this study were cared in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Seoul National University, Republic of Korea.

3. 2. 1. Animals and experimental design

27 Holstein cattle were used and separated to 4 groups according to days in milk (DIM) (group A: 238 ± 90.1, B: 146.8 ± 22.9, C: 73.9 ± 6.9, D: 23.5 ± 12.6). The feeding sections didn't separate to each groups. The feeding trial lasted for 8 weeks; the first 4 weeks was control period and the next 4 weeks was test period. During experimental periods, all Holstein cattle could move freely in their cowshed. Milk sampling were collected once a week during experimental periods by using mobile milking unit (MMU, DeLavel, Sweden) located in Animal farm.

3. 2. 2. Experimental diets and feeding

Control cow feed was 20 kg of commercial TMR (Cargill Agri purina, Korea) mixed with chopped 10 kg of rye silage, total 30 kg of TMR feed was fed daily. Test TMR was prepared mixing 10 kg of chopped rye silage and 16 kg of commercial TMR and 10 kg of BBS which replaces 20 % (4 kg) of commercial TMR. The feeding trial lasted for 8 weeks; the first 4 weeks were control period feeding control TMR feed and the next 4 weeks was test period feeding test TMR. The mixed ratio adopted through the results of chemical and energy value analyses between commercial TMR and BBS. All Holstein cows fed the test TMR which were also randomly allocated to cows from auto feeding machine. During this time, test TMR were made in every morning (08:00 am) and fed three times a day to experimental cows. Chemical analyses conducted with the same methods which were mentioned at the upper part.

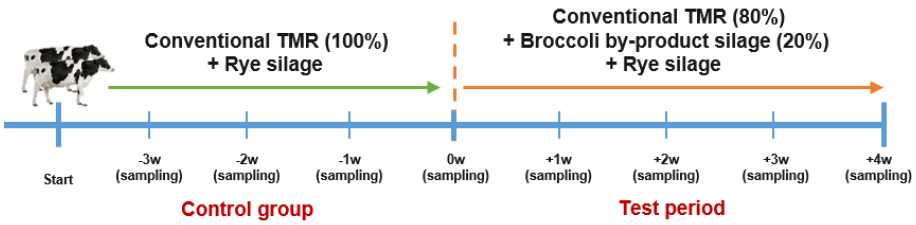

Figure 6. Period and methods of feeding trial in dairy cows

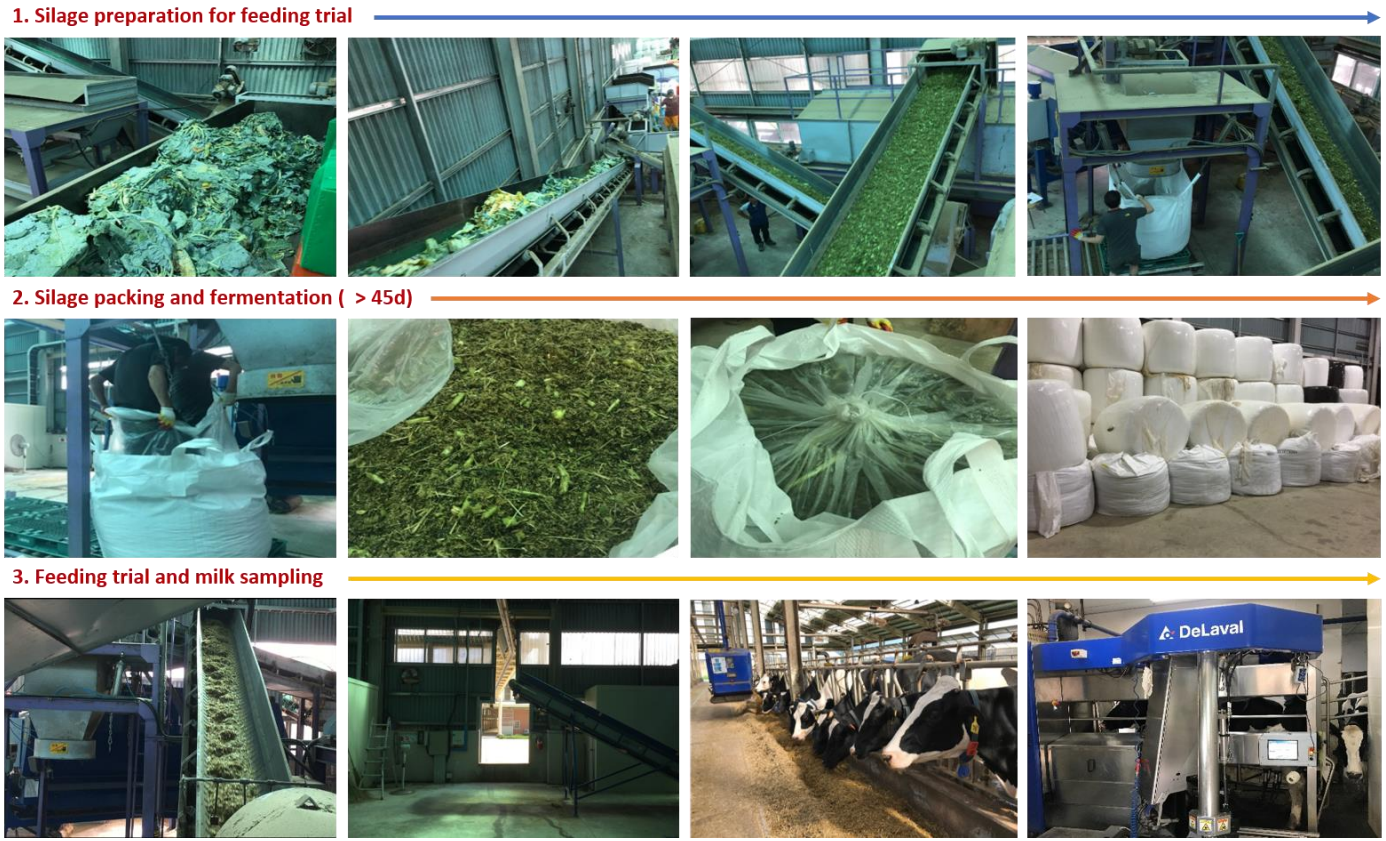

Figure 7. A large amount of BBS manufacturing process and feeding trial in dairy cows

3. 3. Analyses of Milk Production

Milk production (kg/d) were automatically recorded at each milking using the mobile milking unit (MMU) system (DeLaval, Sweden). Because of linear experiment periods (control period and test period), milk yield analyzed by comparing of the slope of daily milk yield between control and test period. Lactation curve provides information about the pattern of milk yield during lactation and it also provides a summary of the pattern of milk yields determined by the biological efficiency of cows (Scott et al., 1996; Jingar et al., 2014). Mln et al. (2005) was identified as a slope change to compare the daily milk yield of goats from the first year to the second year according to the DM intake of concentrate. Therefore, in this experiment, comparison of the difference of slope for each period conducted to evaluate the degree of milk yield changes.

Milk samples for components analyses were collected on once a week during experiment periods. Milk component samples were collected into tubes containing 2-bromo-2-nitropropane-1,3-diol which can prevent infection from some bacteria and analyzed individually by Central Milk Analysis Institute (Korea) for fat, crude protein, lactose, solid-not-fat (SnF), milk urea nitrogen (MUN) content using infrared spectroscopy (Milkoscan 4000, Foss Electric, Hillerod, Denmark)

3. 4. Analyses of Functional Components in BBS and Milk

3. 4. 1. Sulforaphane

BBS samples were collected from the silage which made for feeding trial and storage in freezer at -20°C. Sulforaphane analyses conducted to compare the changes during silage fermentation. The samples were weighed out in a glass tube of 50 mL (1 g fresh sample or 150 mg lyophilized sample), then 4 mL of acidic water (pH 6) was added, and the mixture was incubated at 30°C in water bath for 2.5 h. At this step, the evaporation of the 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 stored at room temperature during 1 h. Then, the resulting solution was filtered through Whatman no. 41 paper. The sulforaphane was purified with the method designed by Bertelli et al. (1998), using SPE silica cartridge (SiOH, ThermoFisher Scientific) 3 mL disposable columns. Prior to use, the silica gel cartridge was conditioned with 3 mL of dichoromethane. Sulforaphane was extracted by passing 20 mL of organic extract through the cartridge, washing the cartridge with 3 mL of ethylacetate (which was then discarded) and eluting the sulforaphane with 3 mL of methanol. The methanol extract was evaporated to dryness in a vacuum oven at 45°C for 2 h, and redissolved with 2 mL of acetonitrile. The resulting solution was vortexed for 30 s and filtered with a membrane of 0.45 μm. A 20 μL sample of this solution was injected onto the column of the UPLC system. All samples were analyzed in triplicate.

Milk samples were collected on every weeks during experimental period and storage

in freezer at -80°C. Sulforaphane analysis in milk was little bit different with silage sample. 10 mL of milk samples were weighed out and add the Hcl to make pH 3.0. This acidic milk centrifuged for 20 min (3,000 rpm) and supernatants were separated in a glass tube of 50 mL. Sulforaphane was extracted with 20 mL of dichloromethane by tube up and down gently for 1 min and stored at room temperature during 1 h. The next steps conducted in the same ways with the silage sample analysis.

Stock standard solution of sulforaphane was prepared by dissolution in acetonitrile and stored at 4 °C in the dark. Sulforaphane was measured by using Liquid chromatography (Xevo TQ MS ACQUITY UPLC and TQD, Waters, USA). A sample volume of 1 μL was injected with column ACQUITY BEH C18 with length 2.1 mm × 50 mm (Waters, USA). Mobile phase A was 100% of distilled water and mobile phase B was 100% of acetonitrile. The analysis were carried out isocratically at a flow rate of 0.1 mL/min, employing as the mobile phase a mixture of 70% of A : 30% of B (v/v). Sample and column temperature were 4 °C and 45 °C, respectively. The sulforaphane was detected at 202 nm. The total time between injections was 6 min.

3. 4. 2. Beta-carotene and Vitamin A

In the case of BBS samples, 2g of dried BBS powder samples were used to analysis. In the case of milk samples, 1g of lyophilized milk samples were used to analysis. The pretreatment methods for beta-carotene and vitamin A were same. BBS sample or milk sample reacted with 10 mL of 6% pyrogallol and sonicate for 10 min. After sonication, 7 mL of 60% KOH add and vortexing 1min. Then, the mixture incubated in water bath

at 80 °C for 1 hr and added 10 mL of 2% NaCl and 15 mL of hexane – acetate mixture (85:15). After vortexing this mixture, centrifuge at 5,000rpm for 15min (repeated 3 times). Next, 1 mL of chloroform added and measured by HPLC system. The HPLC methods used according to the method of Shin et al. (2015).

3. 4. 3. DPPH radical scavenging activity

In the case of BBS samples, 1g of lyophilized BBS sample was extracted with 10mL of 10% ethanol for 24 hrs at RT and then centrifugation for 15 min (6,400 rpm). The liquid supernatant filtered using 0.2 μm PTFE filter (ThermoFisher Scientific) and the extracted samples were storage for further study. Antioxidant activities of BBS were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method (Nguyen et al., 2015). 50 μL of BBS extract samples were reacted with 200 μL of 100 μM DPPH (Sigma-Aldrich, Germany) which was melted in methanol solution and shaking gently for 1 min. Control was made that 50 μL of 70 % ethanol mixed with 200 μL of 100 μM DPPH solution. The level of color reduction in the solution indicated the efficiency of radical binding. Absorbance was measured at wavelength of 517 nm on a spectrophotometer (SpectraMax M3, Molecular Devices, USA) after 30 min incubation at RT.

In the case of milk samples, the samples were defrosted in a water bath at 35 ~ 40 °C. Once defrosted, they were homogenized by vortexing for 1 min. 8 mL of milk were measured and transferred to a 30 mL glass tube. 10mL of 50 % methanol (diluted with deionized water, v/v) were added while vortexing for 1min in order to dissolve the

sample. 500 μL of Carrez 1 solution (zinc acetate) were added and dissolved while vortexing for 1min. 500 μL of Carrez 2 solution (hexacyanoferrate-2-potassium 3-hydrate) were added and dissolved while vortexing for 1min. Then, 5 mL of acetonitrile were added and vortexing for 1min. The solution was complemented to 25 mL with 50% methanol (diluted with deionized water, v/v) in the glass tube. The mixture was then allowed to stand for 25 min until complete clot protein precipitation. The resulting solution was placed in a centrifuge tube and centrifuged at 10,000 rpm and 5 °C for 15 min. Two phases were obtained; a solid (protein and lipid fraction) and a liquid. The liquid extracted samples were storage for further study (Cecilia et al., 2015). Antioxidant activities of milk were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method (Gayathri., 2014). 0.5 mL of milk extract samples were reacted with 1 mL of 100 μM DPPH (Sigma-Aldrich, Germany) which was melted in methanol solution and shaking gently for 1 min. Control was made that 1.5 mL of methanol mixed with 1.5 mL of 100 μM DPPH solution. Absorbance was measured at wavelength of 517 nm after 30 min incubation at RT.

DPPH radical scavenging activity was converted into percentage of antioxidant activity using the following equation:

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

3. 4. 4. Total phenolic contents (TPC)

BBS extract method was same with BBS sample DPPH assay. The total phenolic contents were determined by Folin ciocalteu's method (Masayo and Watanabe et at., 2010) with gallic acid (Sigma-Aldrich, Germany) as the standard. 1 mL of BBS extract samples were reacted with 1mL of diluted Follin ciocalteu's reagent (5 folded by adding deionized water) and incubation for 3 min in dark condition. Then, 1 mL of 10 % Na_2CO_3 (w/v) was added and reacted for 30 min in dark condition. The TPC were determined by spectrophotometer at 750 nm and presented as gallic acid equivalent (GAE).

Milk extract method was same with milk sample DPPH assay. The total phenolic contents were also determined by Folin ciocalteu's method with gallic acid (Sigma-Aldrich, Germany) as the standard. 12 μL of milk extract samples mixed with 50 μL of deionized water and reacted with 15 μL of Follin ciocalteu's reagent and incubation for 3 min in dark condition. Then, 15 μL of 10 % Na_2CO_3 (w/v) was added and reacted for 30 min in dark condition. The TPC were determined by spectrophotometer at 750 nm and presented as gallic acid equivalent (GAE).

3. 6. Statistical Analysis

The data for milk yield, milk components, sulforaphane, beta-carotene, vitamin A, total phenolics acid and DPPH radical scavenging activity were analyzed using SAS PROC MIXED (version 9.4). Milk yield, milk components and sulforaphane were analyzed as repeated measures assuming an AR (1) covariance structure (Littell et al., 1998). Beta-carotene, vitamin A, Total phenolics acid and DPPH radical scavenging activity Pareto analysis of variance (ANOVA) was used to evaluate the observed data and the regression coefficients of linear, quadratic and cubic and their effects were generated. Statistical differences were considered significance at $P < 0.05$ and a trend at $0.05 \leq P < 0.10$.

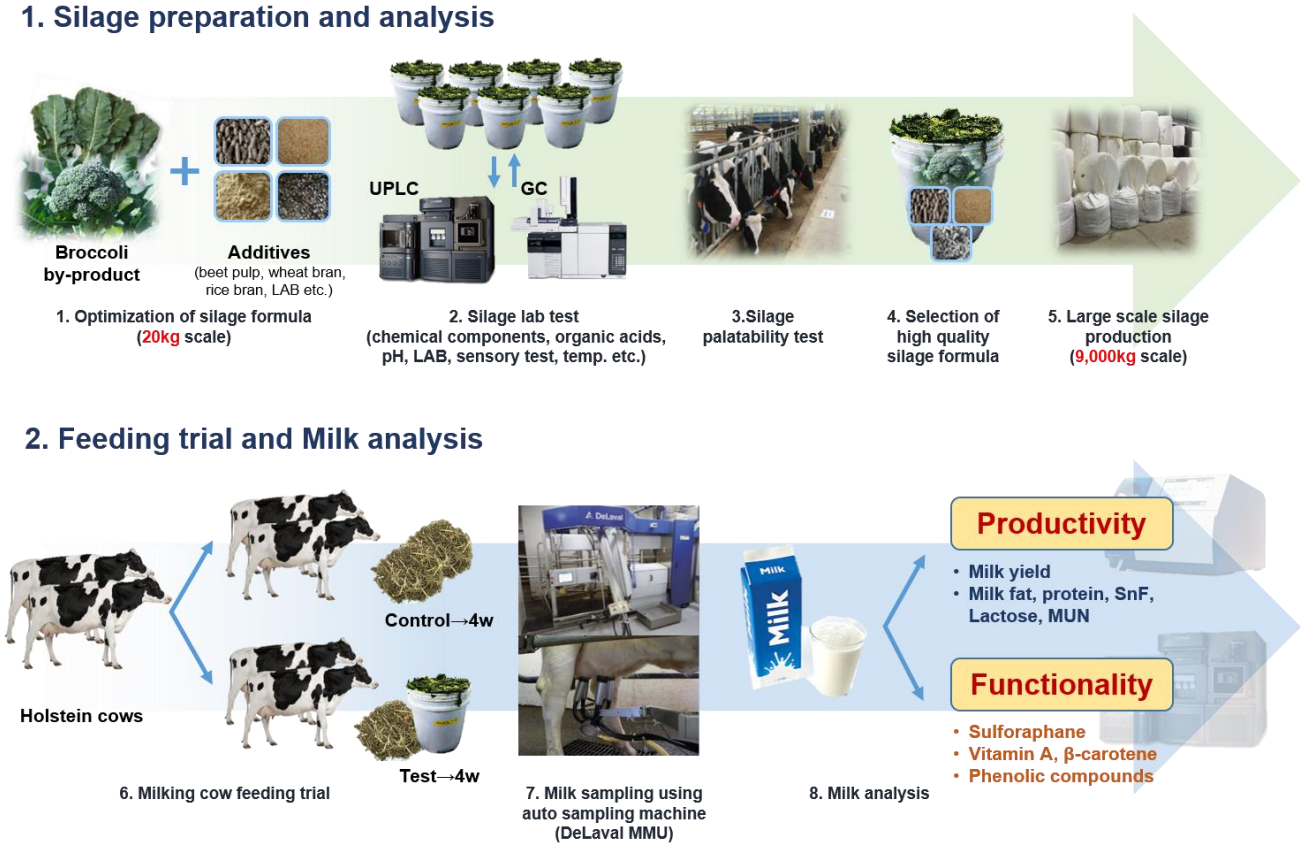

Figure 8. Experimental flow chart

4. Results and Discussions

4. 1. 1st Broccoli By-product Silage Preparation

4. 1. 1. Evaluation of Chemical composition and Feed value

In the feed value assessment of BBS, good and bad were judged based on TDN and RFV. The results are given in table 3. In comparison with LAB inoculum usage to BBS, there were not significant differences in all of the silages except for the silage with BWB additive and control silage. Most of the cases in which LAB inoculum were treated and those that were not treated were more than 70 % of TDN and RFV was estimated to be around 200 except for the silage with BWB additive and control silage. The results showed that tests, which showed an increase in cow intake in feed treated with *Lactobacillus plantarum* (Steen et al., 1989), were not conducted but were predictable. It was also predicted based on Weinberg's results (2007) that silage, which treated LAB inoculum, increased DM and NDF digestibility. CP content also showed no difference in LAB treatment.

In comparison with additives content (20% or 30%), TDN and RFV values were good for most silages and no consistent differences were seen. However, a higher CP content was measured in silages with 20 % of additives. This is because the high protein content of broccoli is believed to contain more in the silage with 20% of additives than 30% of ones.

In comparison with the types of additives (WB, RB, BWB, non), it was found that TDN and RFV are similar in all silages except for the silage with BWB additive. When the WB and RB were compared, TDN values were measured slightly higher in the silages with WB additive, but were not significant. In case of RFV, there were no consistent results, but they had all high value. TDN was the lowest value at 58.22 % in the silage with BWB and 66.58 % in the control silage without the additives, which is higher than in the BWB used silage. TDN is calculated by ADF and indicates a higher value with lower ADF content (Holland et al., 1990). So, the TDN value was lower than other silages due to the high content of ADF (38.84 %) within the BWB. In addition, high content NDF (49.98 %) has also reduced RFV values.

Table 3. Feed value evaluation of the 1st BBS formulas according to chemical components

Silage formulas			DM (% FW)	NDF (% DW)	ADF (% DW)	CP (% DW)	TDN (% DW)	RFV
LAB[1)]	Additive content	Additives						
LAB	20%	WB	23.21	32.87	20.12	18.23	73.01	207
		RB	22.32	32.97	20.78	17.54	72.48	205
	30%	WB	31.23	34.42	18.72	16.6	74.11	201
		RB	32.22	31.3	18.85	15.68	74.01	221
No LAB	20%	WB	26.37	35.34	21.4	18.36	71.99	190
		RB	21.45	33.01	20.71	18.12	72.54	205
	30%	WB	32.84	33.62	19.76	16.89	73.29	203
		RB	28.82	35.11	21.02	15.77	72.29	192
LAB	30%	BWB	33.21	49.98	38.84	15.04	58.22	109
No LAB	- (only BB)		7.38	32.71	28.25	18.94	66.58	190

WB, wheat bran; RB, rice bran; BWB, buckwheat bran; BB, broccoli by-product; DM, dry matter; NDF, neutral detergent fiber; ADF, acidic detergent fiber; CP, crude protein; TDN, total digestible nutrients; RFV, relative feed value.
[1)] *Lactobacillus plantarum,* 5×10^7 cfu/kg silage.

4. 1. 2. Evaluation of fermentation quality

In the fermentation quality assessment of BBS, high or low quality was judged based on pH and organic acids. In silages, which treated LAB inoculum, the pH level was lower (avg. 4.07) than that of the silages that did not treat the same conditions (avg. 4.49). In addition, the lactic acid content was high in most LAB treated silages, and the acetic acid content was lower than that of the silages that did not treat the same conditions.

In comparison with additives content (20 % or 30 %), silages with 20% of additives showed higher pH than those with 30 % of additives and there were no consistent results with the lactic acid content. However, according to Flieg's score which was purposed by Flieg et al. (1938), when compared to organic acids content ratio (acetic acid, lactic acid, butyric acid), the results showed that the silages with 30 % of additives higher than those with 20% of additives, received higher scores.

In comparison with the types of additives, at the same conditions, the silages with RB had a lower pH level than the silage with WB and had a higher lactic acid content. In addition, the acetic acid content was lower in the silage with RB except for the silage with 30% of additives treated with LAB inoculum. In the case of the silage with BWB additive, the previous assessment of the feed value received low ratings, but not bad in the fermentation quality. In the case of the control silage, it was found that lactic acid content was very low (0.06 %) and the acetic acid content was high (10.38 %), and the pH level was also high (6.8). However, due to the low amount of butyric acid, the Flieg's score was not lower (50) in control silage. On the contrary, no LAB treat silage with 20 %

of WB additive had the lowest quality due to high butyric acid content (0.77 %). The results are given in Table 4.

Overall, the results showed that treating LAB inoculum, using 30 % of additives and using RB additive were of good fermentation quality. However, considering the high lactic acid content and low pH levels, the results show that all of LAB treated silages were of good fermentation quality.

Table 4. Quality evaluation of the 1st BBS formulas according to pH and organic acids content

Silage formulas			pH	Organic acid (% DM)			Flieg's score[2]	Grade[3]
LAB[1]	Additives content	Additives		Acetic acid	Lactic acid	Butyric acid		
LAB	20%	WB	4.57	4.72	7.59	0	64	2
		RB	3.93	2.7	12.68	0.04	93	1
	30%	WB	3.92	1.26	9.68	0	98	1
		RB	3.88	1.32	9.8	0	98	1
No LAB	20%	WB	5.12	4.6	2.96	0.77	13	5
		RB	4.47	3.36	9.67	0	77	2
	30%	WB	4.45	2.81	6.59	0	72	2
		RB	3.92	1.62	10.09	0	98	1
LAB	30%	BWB	4.23	3.53	6.69	0	68	2
No LAB	- (only BB)		6.8	10.38	0.06	0.2	50	3

WB, wheat bran; RB, rice bran; BWB, buckwheat bran; BB, broccoli by-product; DM, dry matter.
[1] *Lactobacillus plantarum,* 5×10^7 cfu/kg silage.
[2] Flieg et al. (1938) determined the method how to evaluate the silage quality as a score.
[3] Quality grade according to Flieg's score: 1 (100~81), 2 (80~61), 3 (60~41), 4 (40~21), 5 (< 20).

4. 1. 3. Sensory test by human

As a result of human sensory test, LAB treated silages and the silages with 30 % of additives scored higher in the evaluation score. Among them, LAB treated silage with 30 % of WB additive received the highest score with 20 points, while control silage received the lowest score with 5 points. However, in terms of grade, all silages had excellent ratings of 2 or higher except for control silage.

4. 1. 4. Palatability test by Holstein cows

The palatability test was conducted by feeding the LAB inoculum treated silages to Holstein cows. The results are given in table 5 and figure 9. As a result, the silages with 20 % of additives were intake more than silages with 30 % of additives. When comparing the intake amount of the silages with WB, silage with 20 % of additives showed 18 % higher than that of 30 %. When comparing the intake amount of the silages with RB, silage with 20 % of additives showed 12 % higher than that of 30 %. Control silage with no additives consumed 5.56 % and had the lowest palatability to Holstein cows. The control silage had significantly lower palatability because it was judged that the low lactic acid content and high acetic acid content would have caused decomposition, not lactic acid fermentation. In comparison with the types of additives, among WB, RB, and RWB, the silage with RWB had the lower intake of 27.5 % compared to other silages (excluding control silage) and silages with WB showed higher intake than RB (avg. 13.66 %). In particular, in the silage with 20% additives, the silage with WB consumed 97.33 % and showed the highest palatability among the all silages.

Therefore, in the first BBS test, LAB treated silage with 20 % of WB or RB additive were found to be good feed value and fermentation quality, and the cow's intake was also high. In terms of cow palatability test, the optimal formula for BBS preparation was determined to use 80 % of the BB, 20 % of BP/WB additives and LAB inoculum.

Table 5. Palatability test of intake of 6 BBS by Holstein cows for 1hr

Silage formulas			Initial (kg DM)	Remaining (kg DM)	Intake (%)	Preference
LAB[1)]	Additives content	Additives				
LAB	20%	WB	7.19	7	97.33	1
		RB	8.37	6.75	80.67	2
	30%	WB	10.14	8.04	79.33	3
		RB	9.71	6.67	68.67	4
		BWB	8.54	2.35	27.5	5
No LAB	- (only BB)		1.6	0.09	5.56	6

WB, wheat bran; RB, rice bran; BWB, buckwheat bran; BB, broccoli by-product; DM, dry matter.
[1)] *Lactobacillus plantarum,* 5×10^7 cfu/kg silage.

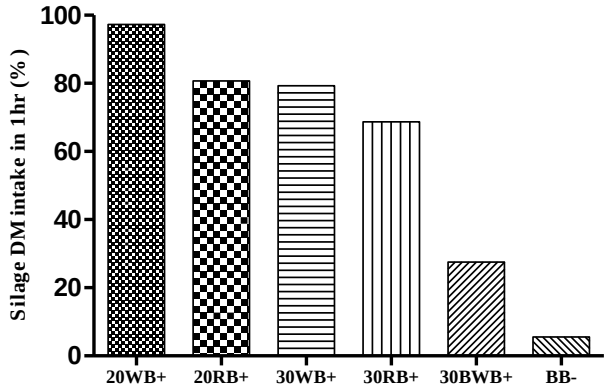

Figure 9. Palatability test of intake of 6 BBS by Holstein cows for 1hr

4. 2. 2nd Broccoli By-product Silage Preparation

4. 2. 1. Evaluation of Chemical composition and Feed value

In the second test, we used lactobacillus in all silages and compared only the content (26 % or 18 %) and types (CC, WB, RB, CD, MA, BP) of additives. The results are given in table 6. In the feed value assessment of BBS, as in the first test, good and bad were judged based on TDN and RFV. There were no significant differences between TDN and RFV values in all silages in comparison with the additive content. Overall, the TDN and RFV values of the silages using CC as a common additive or CC only did not result in higher results than other additives. In particular, for silage using CC only and silage made by mixing CC and CD, TDN was the lowest at about 58 % on average. RFV also had lower results than other silages at 100 or less. On the other hand, the value of TDN and RFV was very high in silages using BP only and silage mixed with RB and WB. In particular, silage mixed with RB and WB, received the highest rating at 77.4 % for TDN and 264 for RFV. According to a study by Yulistiani and Puastuti et al. (2012), corn cob is reported to have a very high fiber content. Therefore, using DD, CD, and MA as a silage additive was less valuable in terms of feed value than using WB, RB, and BP.

Table 6. Feed value evaluation of the 2nd BBS formulas according to chemical components

Silage formulas			DM (% FW)	NDF (% DW)	ADF (% DW)	CP (% DW)	TDN (% DW)	RFV
Additive content	Additive 1	Additive 2						
26%	CC	WB	32.38	47.86	27.61	14.52	67.1	131
		RB	33.15	47.49	27.92	13.73	66.9	132
		CD	30.39	56.74	40.92	13.50	56.6	94
		MA	29.80	53.11	34.29	15.33	61.8	109
	CC	-	29.92	62.76	37.34	9.97	59.4	89
	BP	-	30.28	35.33	24.37	16.35	69.6	184
18%	CC	WB	30.79	45.63	27.21	16.36	67.4	138
		RB	27.09	42.71	26.03	15.42	68.4	150
		CD	25.51	51.95	38.46	14.93	58.5	107
		MA	26.01	49.55	33.06	16.72	62.8	119
	CC	-	26.23	63.15	38.47	10.92	58.5	87
	BP	-	23.88	33.81	23.67	18.72	70.2	194
	RB	WB	25.45	27.32	14.63	21.85	77.4	264

CC, corn cob; WB, wheat bran; RB, rice bran; CD, cassava distillers dried grains; MA, mixed additives; DM, dry matter; NDF, neutral detergent fiber; ADF, acidic detergent fiber; CP, crude protein; TDN, total digestible nutrients; RFV, relative feed value.

4. 2. 2. Evaluation of fermentation quality

In the fermentation quality assessment of BBS, as in the first test, high or low quality was judged based on pH and organic acids. The results are given in Table 7. There were no significant differences on pH and organic acid in all silages in comparison with the additive content (26 % and 18 %). The pH of the whole silages was given as a value of 3.93 ~ 4.39. In organic acids content, all silages had high lactic acid content and low acetic acid and butyric acid content. Therefore, Flieg's score also showed excellent results in the all silages. Unlike a feed value test, fermentation quality assessment showed good results in the silages using CC, CD and MA. The silages using BP, WB, and RB also showed good fermentation quality, as did the feed value test result. The use of WB or RB as an additive in combination with CB increased the lactic acid content, which resulted in excellent score of 100 for Flieg's score. In addition, if RB and WB were used as additives alone, the lactic acid content was the highest at 15.39 %, which appeared to have the best effect of lactic acid fermentation. Thus, LAB can use large amount of carbohydrate sources in the WB and RB as an energy source.

Table 7. Feed value evaluation of the 2nd BBS formulas according to chemical components

Silage formulas			pH	Organic acid (% DM)			Flieg's score[1)]	Grade[2)]
Additive content	Additive 1	Additive 2		Acetic acid	Lactic acid	Butyric acid		
26%	CC	WB	4.03	0.97	11.42	0	100	1
		RB	3.96	0.76	13.10	0	100	1
		CD	4.15	1.65	7.65	0.03	93	1
		MA	4.33	1.61	9.54	0.29	86	1
	CC	-	4.36	1.64	5.77	0.05	84	1
	BP	-	4.08	1.67	7.72	0	93	1
18%	CC	WB	4.03	1.03	10.98	0.06	100	1
		RB	4.01	1.05	13.08	0	100	1
		CD	4.3	2.06	11.00	0.14	96	1
		MA	4.39	1.97	9.60	0.09	96	1
	CC	-	4.62	2.12	5.45	0.12	77	2
	BP	-	4.13	2.42	9.23	0	88	1
	RB	WB	3.93	0.98	15.39	0	100	1

CC, corn cob; WB, wheat bran; RB, rice bran; CD, cassava distillers dried grains; MA, mixed additives, BP, beet pulp.
[1)] Flieg et al. (1938) determined the method how to evaluate the silage quality as a score.
[2)] Quality grade according to Flieg's score: 1 (100~81), 2 (80~61), 3 (60~41), 4 (40~21), 5 (< 20).

4. 2. 3. Sensory test by human

As a result of the sensory test, all silages received a rating of 2 or higher. Among them, the silage with 20 % of CC additive received the highest score of 7.33. Although he received the lowest score (12.5) in the silage using RB and WB, the rating was good. In other words, just as the results of a quality assessment showed good fermentation quality for all silages, the results were excellent for sensory test.

4. 2. 4. Palatability test by Hanwoo

Based on the superior quality of all silages in the previous results, all of the silage was used for the palatability test by Hanwoo. The results are given in Table 8 and Figure 10. A palatability evaluation for 6 hrs conducted on 9 Hanwoo cattle showed that the silage using beet pulp only, it was found to have a 100 % intake rate in less than 2 hours, making it the most palatable. Silage using CC and WB also had excellent results at 99.5 %, followed by silage using RB and WB (90 %). In cases of different additives and silage with the same conditions, there was a higher intake rate in silage, where the additive was 18 %. In particular, CC and silage, which used CD or MA, were low in intake and were judged to have a low palatability.

There was something unusual about the test conducted on the silage using CC. At the silage with 18 % of CC, it was 91 % of intake ratio and at the silage with 26 % of CC, it was 72 % of intake ratio. After checking the remaining volume of these silages, most of the broccoli by-products were consumed and there was a large amount of CC left. According to a study by Yulistiani and Puastuti et al. (2010), corn cob has a very high fiber content, so it is less palatable, but it is reported that if fermented, it will improve. However, contrary to their results, palatability was still bad after fermentation. It is also thought that corn cob was inefficient to use as an additive in feed because it could be easily infected by things like *Aspergillus flavus*, toxic fungi (Yulistiani et al., 2017). Therefore, due to the high NDF and ADF characteristics and the rigid nature of CC, it was judged that the palatability was not good to use as a feed additive.

Table 8. Palatability test of intake of 13 BBS by Hanwoo for 6hrs

Silage formulas			Initial (kg DM)	Remaining (kg DM)	Intake (%)	Preference
Additive content	Additive 1	Additive 2				
26%	CC	WB	3.32	0.80	76	5
		RB	2.98	0.86	71	7
		CD	3.03	2.18	28	11
		MA	2.71	2.19	19	12
	CC	-	2.60	0.73	72	6
	BP	-	2.55	0	100	1
18%	CC	WB	3.24	0.02	99.5	2
		RB	3.04	0.91	70	8
		CD	2.99	2.03	32	10
		MA	3.08	1.11	64	9
	CC	-	2.55	0.23	91	3
	BP	-	2.62	0	100	1
	RB	WB	2.39	0.24	90	4

CC, corn cob; WB, wheat bran; RB, rice bran; CD, cassava distillers dried grains; MA, mixed additives, BP, beet pulp.

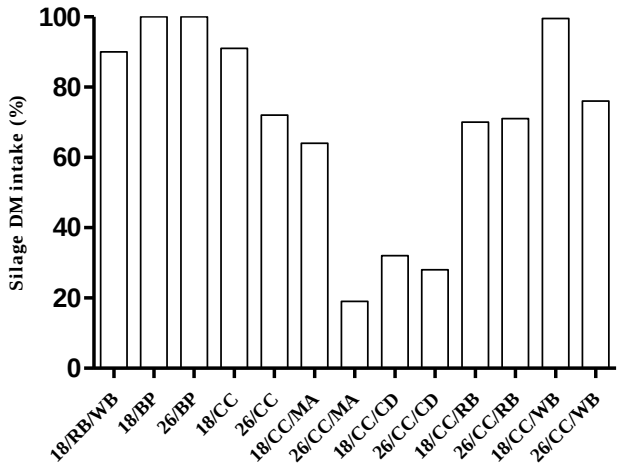

Figure 10. Palatability test of intake of 13 BBS by Hanwoo for 6hrs

4. 2. 5. Check the silage temperature during fermentation

During BBS fermentation, a temperature check test was conducted in the website to determine the characteristics of fermentation using i-button which is a computer chip (Maxim integrated, USA). As a result, the temperature of silage was the highest at about 32℃ within five days after fermentation began at 26 ℃ when it was manufactured. Then, the temperature gradually decreased and about 10 days later it decreased back to the temperature of the day of manufacture (figure 11). It was judged that the temperature gradually drops after the fermentation date because of the changing seasons during the experiment period. The efficacy of inoculants for silage might also be affected by high ensiling temperatures (Weinberg et al., 2000). The temperature seems to have risen to maximum within five days to finish fermentation, but the results show that anaerobic fermentation must be maintained from 21 to 28 days at least to minimize production losses during silage changeover (Kung. 2000-2001)

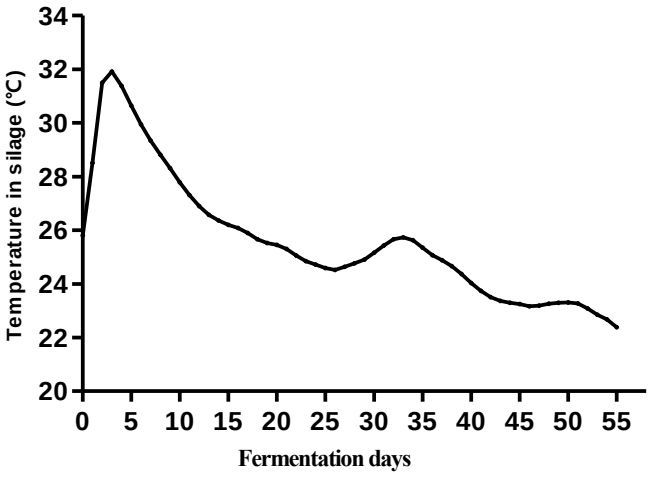

Figure 11. Silage temperature during fermentation

4. 3. Selection of Optimal Formula for BBS

4. 3. 1. Optimal formula and BBS preparation for feeding trial

To summarize the results of the 1st BBS preparation, for the content of additives (20 % or 30 %) or types of additives (WB or RB), there were better results in terms of feed value and in the fermentation quality assessment, the results showed that the silage with 30% of additives were slightly better in case of LAB inoculum used. However, it was found that all silages had a good effect on the lactic acid fermentation and based on this, it was found that the cattle had the best palatability in the LAB treated silage with 20 % WB additive. To summarize the results of the 2nd BBS preparation, the use of CC as a BBS additive produced good fermentation quality, but the results showed that it was inappropriate in terms of feed value and palatability of cattle because it is assumed to be the high NDF and ADF of CC. There were also results that the use of BP as an additive, as studied by O'Kely et al. (1992), was better fermented and nutritious than that of using CC.

Therefore, the optimal formula of broccoli by-product silage was judged to be LAB inoculum treated silage with 20 % of wheat bran additive. The following feeding trial was made with the aforementioned formula, but using rice bran as an additive may be considered an alternative to the wheat bran.

4. 3. 2. Comparison of chemical composition between commercial TMR and BBS

Comparing the commercial dairy TMR (cTMR) with the broccoli by-product (BBS) chemical composition made from selected optimal formula, CP content was 2.5% higher and NDF 7.9% lower on BBS rather than cTMR. EE was 0.5 % lower and CA was 2.2 % lower on BBS. Net energy for lactation (NE_L) also showed a slightly higher value of 0.05 Mcal/kg of DM on BBS. NE_L determined both were appropriate because milk production between 20 and 30 kg/d meets NE_L requirements (MoA, 2004). NFC was also 7.3 % higher and TDN based on ADF was 5.1 % higher on BBS. In other words, the BBS showed higher nutritional value due to a higher CP and TDN content than cTMR. However, the purpose of the feeding trial was to replace some portion of cTMR with BBS, not just BBS. Therefore, when we checked the chemical composition of BBS and cTMR, BBS confirmed that the contents of CP, NFC, and TDN, which are important to the cow's performance, are similar. In addition, based on the slightly higher NEL required energy to produce milk, some portion of the cTMR could be replaced with BBS by 1 : 1 on the basis of DM [1 : 2.73 (v/v) = cTMR : BBS, basis of FW]. In conclusion, it was decided to replace 20 % of cTMR with BBS on FW basis (v/v) and use it for feeding trial as a silage mixed test TMR (tTMR). The results are given in Table 9.

Table 9. Chemical composition and energy value of the commercial dairy TMR and broccoli by-product silage

	Commercial dairy TMR (cTMR)	Broccoli by-product silage (BBS)
Composition (% of DM)		
DM (%)	62.2	22.8
CP	16.5	19
NDF	39.3	31.4
EE	4.4	3.9
CA	11.1	8.9
UIP	5.9	5.7
DIP	10.4	13.3
NFC	29.4	36.7
TDN	66.6	71.7
NE_L (Mcal/kg of DM)	1.62	1.67

TMR, total mixed ration; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acidic detergent fiber; EE, ether extract; CA, crude ash; UIP; undegraded intake protein; DIP, degraded intake protein; TDN, total digestible nutrients; NEL, net energy for lactation

4. 3. 3. Comparison of chemical composition of TMR for feeding trial

In fact, when the Holstein was fed TMR, it was fed by mixing the cTMR and rye silage with a 2:1 ratio (v/v). Thus, the control mixed TMR (cmTMR) was conducted using TMR which was used in the commercial farm during control period. During the test period, only 20 % of cTMR were replaced with BBS in cmTMR of the control period and the amount of rye silage was maintained as a test TMR. Due to the equivalent chemical component content of the existing cTMR and BBS, cmTMR and tTMR also showed the same level of component content. The detailed components contents are listed in Table 10.

Table 10. Chemical composition of TMR for feeding trial

	Control mixed TMR[1)] (cmTMR)	Test TMR[2)] (tTMR)	Difference
Composition (% of DM)			
CP	15.86	15.91	+0.05
NDF	41.12	40.19	-0.93
ADF	30.15	28.77	-1.38
EE	4.1	3.89	-0.21
CA	12.62	12.17	-0.45
NFC	26.8	28.19	+1.39
TDN	65.08	66.17	+1.09

TMR, total mixed ration; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acidic detergent fiber; EE, ether extract; CA, crude ash; TDN, total digestible nutrients.
[1)] Commercial TMR + rye silage
[2)] Commercial TMR + broccoli by-product silage (20% of cTMR) + rye silage

4. 4. Evaluation of Milk Production

4. 4. 1. Milk yield comparison during experimental period

The milk yield comparison between the control period and the test period was compared by the slope of the daily milk yield data for each period. Lactation curve provides information about the pattern of milk yield during lactation and it also provides a summary of the pattern of milk yields determined by the biological efficiency of cows (Scott et al., 1996; Jingar et al., 2014). Considering the typical lactation curve of milking cattle for 305 days, comparing the daily milk yield of the cattle that performed linear feeding trial was low during the test period. So, it was meaningless to compare the average milk yield between the control period and the test period since the milk yield decreases gradually over time. First, the milk yield of the cattle in each group was analyzed by graphic lactation curve using the mean value. In addition, the daily average milk yield data for each group are presented using a trend line through the SAS program (Figure 9). As shown in the figure 12-B, it was shown that the milk yield of each group were stretched over the control period and the test period, respectively. The control period was the period for feeding cmTMR, which indicates the original milk yield curve of the cattle. Therefore, the slope of the milk yield data of each group could be obtained during the control period and the decreased trend could be seen. In this situation, it was expected that the milk yield of the test period would be increased against to the milk yield gradient of the control period if the milk yield increased by feeding the tTMR which was mixed with BBS.

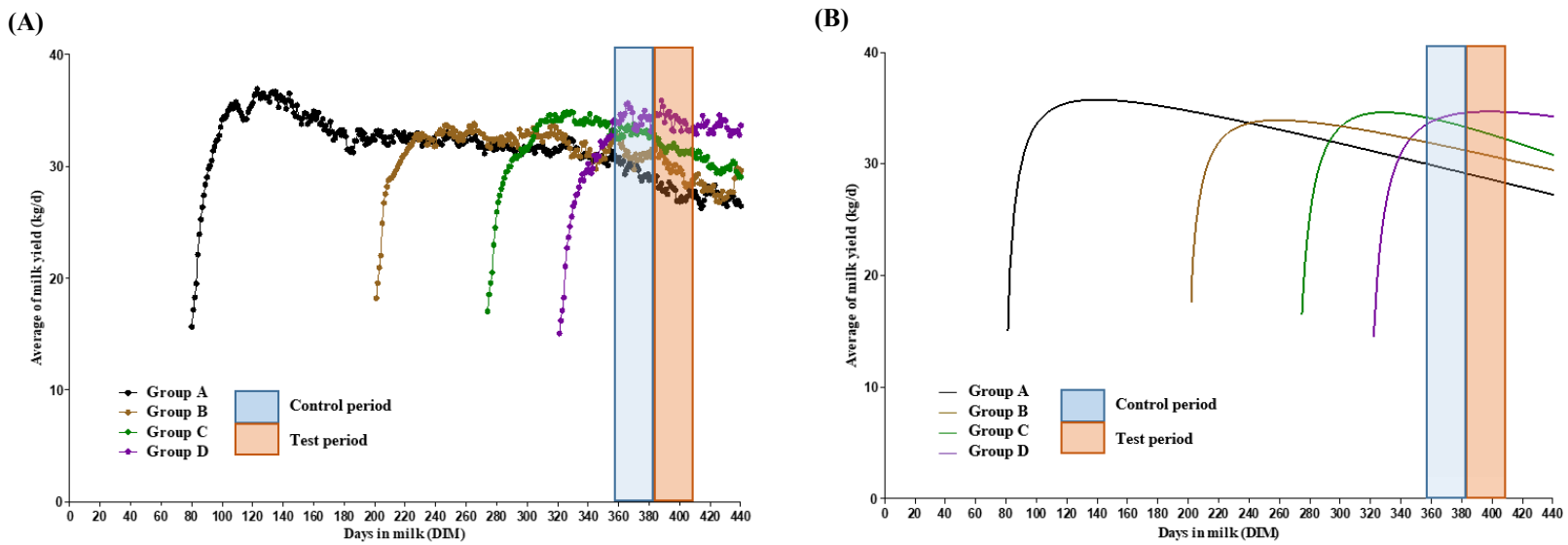

Figure 12. Lactation curves of test Holstein cows. (A) Check the actual milk yield from the daily yield data of each group. (B) Trend lines of lactation curve.

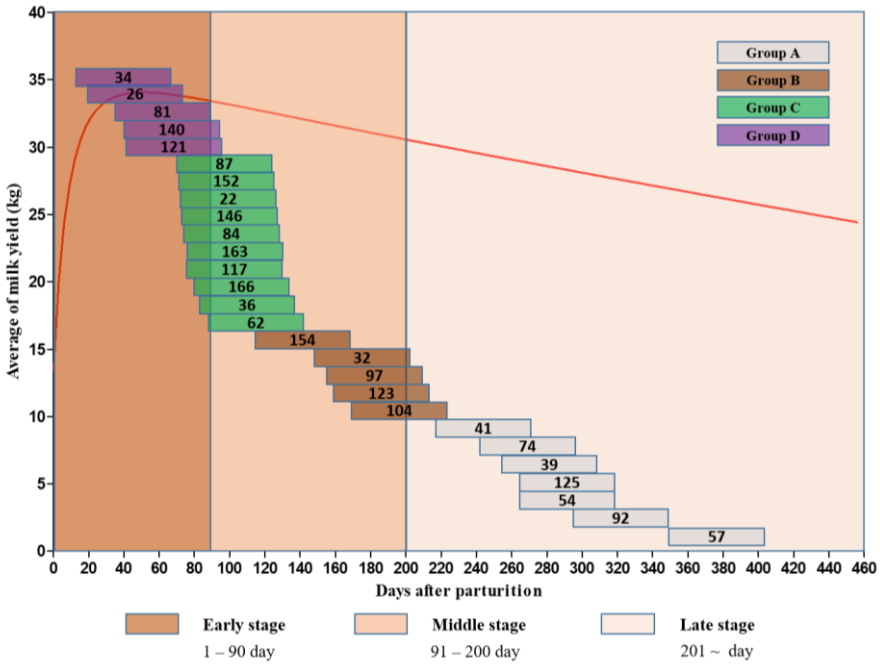

Figure 13. Distribution chart of the lactation stages of each cows during the test period

Table 11. Slope comparison of milk yield in each group

Group	Slope of milk yield		Variation	SEM	P-value
	Control period	Test period			
A	-0.023	-0.020	+	0.060	0.308
B	-0.076	-0.039	+	0.078	0.664
C	-0.043	-0.092	-	0.039	0.243
D	0.160	-0.065	-	0.069	0.031

As shown by the slope in the test period (Figure 12 and 13), group A showed the lowest milk yield. However, group D was the latest parturition cattle group with the milk yield increasing during the control period and the milk yield gradually decreasing during the test period. When comparing the slopes (Table 11), the slope of the control period in group A was - 0.023 and the slope of the test period seemed to increase by - 0.020, but there was no significant difference ($p > 0.05$). Group B, like group A, was - 0.076 in the control period and the slope of the test period seemed to increase by - 0.039, but there was no significant difference ($p > 0.05$). Slope of group C was - 0.043 in the control period and the slope of the test period seemed to decrease by - 0.092, but there was no significant difference ($p > 0.05$). The slope of the control period in Group D was 0.160, a growing trend, but during the test period, the slope significantly decreased to - 0.065. However, Group D, as described earlier, was not adequate to determine the effect of tTMR, as it was expected to decrease in the test period, since the group D was the latest parturition cattle group. In conclusion, as a result of feeding tTMR to dairy cattle during the test period, it was determined that there was no significant difference in the milk yield compared to the control period and we judged that BBS did not affect the change in the milk yield.

4. 4. 2. Milk components comparison during experimental period

In the milk components part, we compared milk fat, protein, lactose, SnF, and MUN content of the 4 groups of Holstein cows with the average of data from the control period and the test period. Excepting for MUN, the milk components were compared to the dairy milk components yield (kg/d) by multiplying the milk yield (kg/d) of the sampling day by the milk components content (%). In milk fat, there was an increase in the all groups except for group A, especially group C showed the highest content at 1.33 kg/d during the test period, but there was no significant difference due to large variation between the populations. There was also no significant difference when comparing the mean values of the data for the entire group. Yi et al. (2015) reported significant increases in milk fat after the pelletizing of the broccoli by-product to the dairy cattle ($p < 0.05$). Ondarza et al. (2009) reported that the extra salary for beta-carotene increased the milk fat content (%), but in this experiment, a small amount of BBS in tTMR was not effective to milk fat. Protein was increased in all groups except group A, especially group D showed the highest increase to 1.11 kg/d, just like milk fat, but there was no significant difference. It was expected that the protein content in milk would increase with a CP content that was slightly higher than tTMR including the BBS, and although there was a tendency to increase, there was no significant difference. Lactose and SnF were also not significantly different. MUN content showed positive results with a decrease of 1.49 mg/dL from 16.1 mg/dL (control period) to 14.61 mg/dL (test period), while other groups showed no significant difference. The normal concentration range of the MUN

in milk is 12 ~ 18 mg/dL (Yoon et al., 2013). High concentration of MUN in milk indicates excess dietary protein or inefficient conversion of protein, which causes higher feeding costs and environmental pollution (Jonker et al., 1998; Rzewuska et al., 2013). MUN depends not only on nutrition but also on other factors, such as year and calendar month when the milk samples were collected, sample type, stage of lactation and so on (Wattiaux et al., 2005; Bastin et al., 2009; Rzewuska et al., 2013). Our results showed that MUN concentration tended to decrease across the entire group and was about 14 mg/dL. However, both the control period and the test period were within the normal MUN concentration range and therefore were considered positive. As a result of feeding tTMR to dairy cattle during the test period, it was determined that there was no significant difference in the milk components including milk fat, protein, lactose, SnF, MUN compared to the control period and we judged that BBS did not affect the change in the milk components. The results are given in Table 12 and Figure 14.

Table 12. Comparison of milk components of Holstein cows

Milk production (kg/d)	Experimental period	Group				
		A	B	C	D	All
Fat	Control	1.32	1.14	1.23	1.26	1.26
	Test	1.27	1.31	1.33	1.32	1.32
	SEM	0.077	0.089	0.117	0.132	0.055
	P-value	0.545	0.124	0.408	0.646	0.225
Protein	Control	1.05	0.91	0.95	0.72	0.93
	Test	1.00	1.02	1.04	1.11	1.04
	SEM	0.068	0.093	0.072	0.091	0.040
	P-value	0.479	0.281	0.471	0.154	0.165
Lactose	Control	1.60	1.36	1.59	1.66	1.74
	Test	1.62	1.51	1.59	1.77	1.80
	SEM	0.108	0.134	0.102	0.175	0.060
	P-value	0.809	0.314	0.954	0.571	0.341
SnF	Control	2.12	2.44	2.78	2.86	2.73
	Test	2.02	2.73	2.85	3.11	2.82
	SEM	0.178	0.240	0.180	0.285	0.105
	P-value	0.526	0.301	0.714	0.427	0.380
MUN (mg/dL)	Control	13.27	14.54	16.10	15.30	15.34
	Test	14.33	14.14	14.61	14.68	14.41
	SEM	0.654	0.438	0.353	0.693	0.261
	P-value	0.316	0.408	0.002	0.422	0.002

SnF, solid-not-fat; MUN, milk urea nitrogen; SEM, standard error of the mean

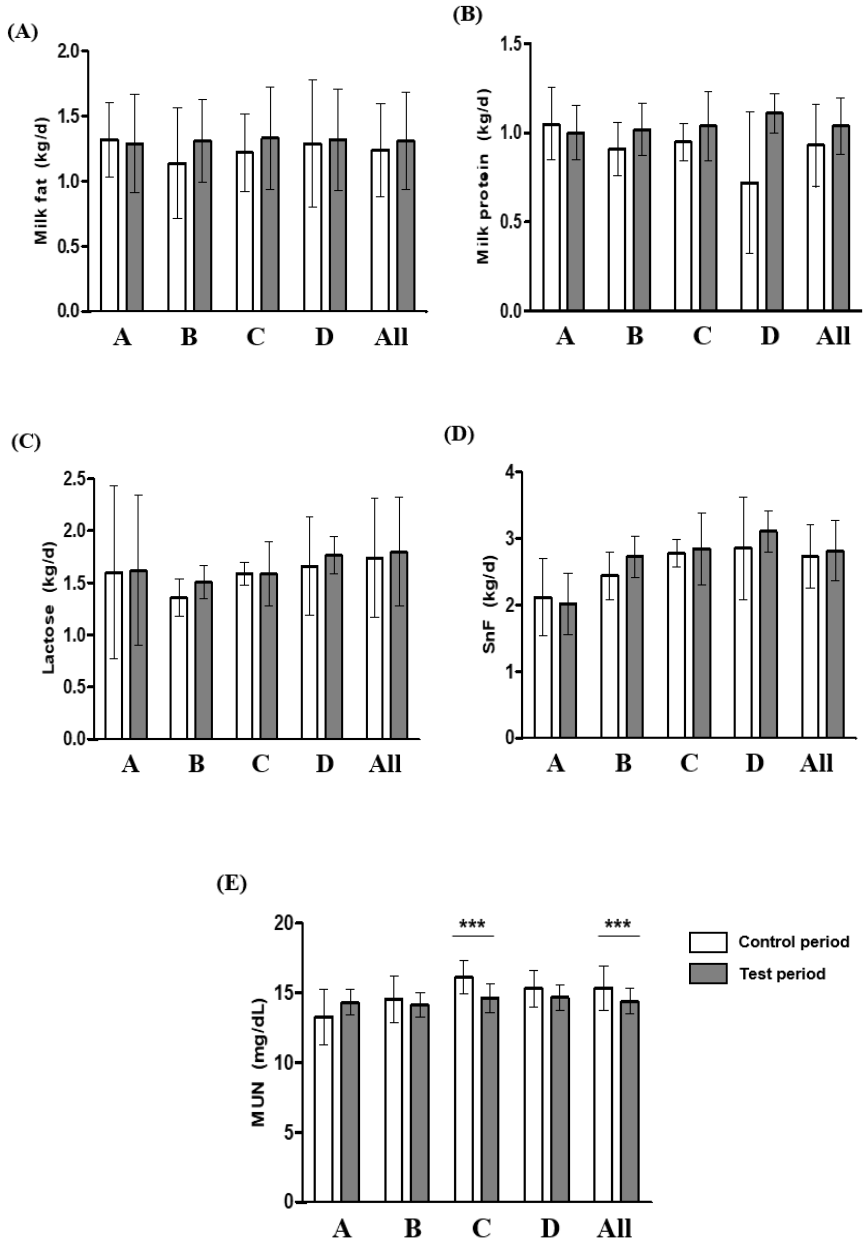

Figure 14. Comparison of milk production of Holstein cows between control and test groups. (A) Milk fat production (kg/d), (B) milk protein production (kg/d), (C) lactose production (kg/d), (D) SnF production (kg/d), (E) MUN content (mg/dL) for each group.

4. 5. Evaluation of Functional Substances in BBS and Milk

4. 5. 1. Functional substances content in BBS and TMR

Sulforaphane

The content of sulforaphane, which is high in broccoli and is known as an anticancer substance, was measured in broccoli, broccoli by-product and broccoli by-product silage. The results are shown in Table 13.

Table 13. Sulforaphane content in ingredients and BBS

	Sulforaphane (mg/kg DM)
Broccoli	1013.6 ± 9.18
Broccoli by-product	338.7 ± 24.1
Broccoli by-product silage	91.64 ± 12.85

Sulforaphane was present at about three times lower levels than broccoli in the broccoli by-product. Using this broccoli by-product, the BBS which had a 20 % of additive content, was found to exist at 91.64 ppm, about 3.78 times lower than the broccoli by-product. During the fermentation period, a test was conducted to verify that sulforaphane in BBS was well preserved (Table 14 and Figure 15). As a result, the 91.64 ppm of sulforaphane in BBS before fermentation was reduced by about 96.5% to 3.2 ppm in the first week after fermentation. The second week of fermentation saw a decrease of about 65.18% from the first week, and until the sixth week it averaged to about 0.916 ppm. Thus it was found that sulforaphane could be metabolized or degraded

by other substances during BBS fermentation. Kawakish and Namiki et al. (1969) said the isothiocyanates compounds, such as sulforaphane, slowly degraded at room temperature and cause water to break down. The results of Jung et al. (2016) also suggested that the rate of degradation of the sulforaphane compound was closely related to temperature as well as water. Their results suggest that when broccoli was kept at room temperature, the sulforaphane content decreased to less than half of its initial content after three days. My experiment also found that sulforaphane was drastically reduced within a week after the fermentation of BBS.

Table 14. Sulforaphane content in the BBS during fermentation

	Sulforaphane content (mg/kg DM) during fermentation							SEM	P-value
	0w	+1w	+2w	+3w	+4w	+5w	+6w		
BBS	91.6^{a}	3.20^{b}	1.114^{c}	0.735^{c}	1.11^{c}	1.017^{c}	0.597^{c}	3.32	<.0001

BBS, broccoli by-product silage; SEM, standard error of the mean
a, b, c Means within the same row with different superscripts differ significantly ($p < 0.05$)

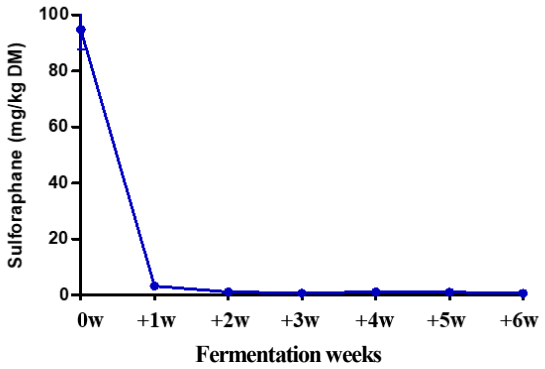

Figure 15. Sulforaphane content in the BBS during fermentation

Beta-carotene and Vitamin A

An analysis was conducted to find out how many beta-carotene and Vitamin A exist in the broccoli manufacturing BBS and what happens after fermentation. An analysis was conducted to determine how much beta-carotene and vitamin A contained in broccoli were present in BBS when manufactured as silage and what changes were made after fermentation. Also, how the content of beta-carotene and vitamin A changed from cmTMR used in the control period and tTMR used in the test period (replacing 20 % of cmTMR with BBS). The results are shown in Table 15 and Figure 16. In BBS, beta-carotene content existed at 12.0 ppm before fermentation and increased to 28.9 ppm after fermentation by about 140 % ($p < 0.001$). Vitamin A content was 0.7 ppm before fermentation, but decreased to 0 after fermentation ($p < 0.05$).

Table 15. Beta-carotene and Vitamin A content in broccoli silage and TMR

	BBS		SEM	P-value	TMR		SEM	P-value
	b/f	a/f			cmTMR	tTMR		
Beta-carotene (mg/kg)	12.0	28.9	1.02	0.0003	3.01	12.6	0.510	0.0002
Vitamin A (mg/kg)	0.702	ND	0.102	0.0085	1.81	0.840	0.195	0.0248

BBS, broccoli by-product silage; TMR, total mixed rations; cmTMR, control mixed TMR; tTMR; test TMR, b/f, before fermentation; a/f, after fermentation; SEM, standard error of the mean.

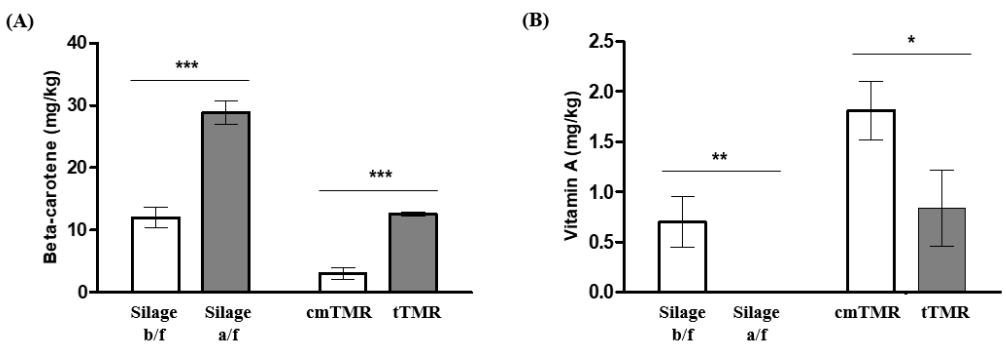

Figure 16. (A) Beta-carotene (mg/kg) and (B) Vitamin A (mg/kg) content in BBS and TMR

Many studies showed that there was a decrease in beta-carotene content in silage after fermentation (Carter et al., 1960; Kasangi et al., 2010;). Also in the results of Nozière et al. (2006), well-fermented silages usually had beta-carotene losses of less than 20%. On the other hand, as our experiment showed, there were also experiments that showed an increase in beta-carotene after fermentation. Angthong et al. (2015) fermented lucaena silage for 111 days, and conducted a beta-carotene analysis during fermentation, resulting in increased beta-carotene during the ensiling period. However, the reason for the increase in beta-carotene was not revealed. Denter et al. (1981) performed tempe fermentation using *Rhizopus* strains and the results showed that beta-carotene increased by four times after fermentation. Based on these results, it was possible that the BBS could have been fermented by fungi or bacteria that would have been synthesized of beta-carotene other than *L. plantarum.* For cmTMR, beta-carotene was present at 3.0 ppm and increased to 12.6 ppm at tTMR by approximately 320 % ($p < 0.001$) but the vitamin A content was reduced by about 55 % at tTMR ($p = 0.05$). This was expected to be increased by mixing 20% of BBS with cmTMR.

Antioxidant activity test

To confirm the anti-oxidant activity, DPPH radical scavenging activity and total phenolic acids (TPC) tests were conducted at BBS and TMR. On BBS, DPPH radical scavenging activity was 39.11 % before fermentation, but increased by about 326.8 % to 166.92 % after fermentation ($p < 0.001$). In addition, the TPC increased about 20.58% from 166.92 mg GAE/kg to 201.28 mg GAE/kg after fermentation ($p < 0.05$). For cmTMR, DPPH radical scavenging activity was 21.25% and for tTMR, it was 60.19%, an increase of about 183.25% ($p < 0.05$). TPC content also increased from tTMR to 203.41 mg GAE/kg, approximately 34.25 %. The increasing antioxidants activity will be due to the increase in beta-carotene and TPC. There are also results that fermentation due to the action of LAB increases antioxidant activity (Martin and Mater et al., 2005). Thus, in view of these results, fermentation improves antioxidant activity. The results are shown in Table 16 and Figure 17.

Table 16. TPC and DPPH radical scavenging activity in broccoli silage and TMR

	BBS		SEM	P-value	TMR		SEM	P-value
	b/f	a/f			cmTMR	tTMR		
Total phenolics content (mg GAE/kg)	166.92	201.28	3.03	0.001	151.52	203.41	0.85	<.0001
DPPH radical scavenging activity (%)	39.11	60.53	2.15	0.002	21.25	60.19	1.87	0.0001

BBS, broccoli by-product silage; TMR, total mixed rations; cmTMR, control mixed TMR; tTMR, test TMR, b/f, before fermentation; a/f, after fermentation; SEM, standard error of the mean; GAE, gallic acid equivalents.

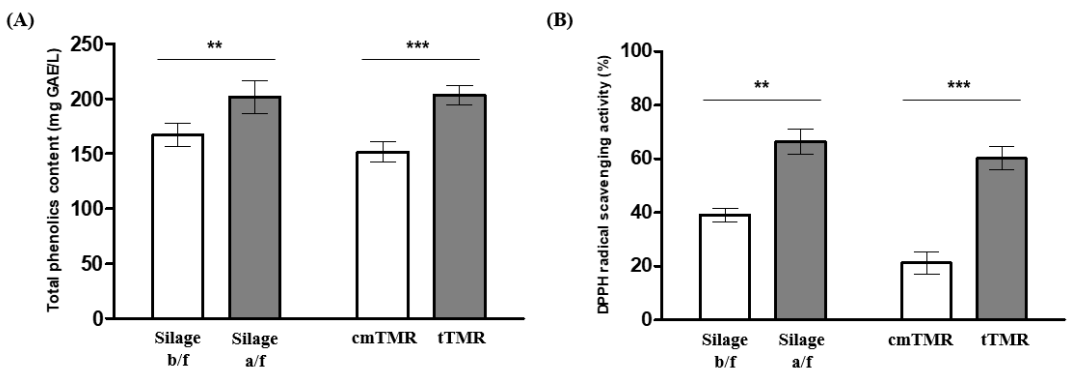

Figure 17. (A) TPC (mg GAE/L) and (B) DPPH radical scavenging activity (%) in BBS and TMR

During fermentation, LAB produce substances such as aroma compounds, ascorbic acid, glutathione, phenolic compounds, which enhance the safety or antioxidant activities (Divya et al., 2012; Cagno et al 2011; Filannino et al., 2013). Phenolic acids are covalently bound to polysaccharides in plant cell walls (Vries R. P. et al., 1960). Many kinds of bacteria including *L. plantarum* display cinnamoyl esterase activities which have been shown to release phenolic acids from plant cell wall (Christov et al., 1993). This action of LAB causes the phenolic acids 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 (Paixao et al., 2007). So the more TPC in the sample, the higher DPPH radical scavenging activity. The results showed that TPC increased 20.58 % and DPPH radical scavenging activity increased 54.77 % after fermentation on BBS. TPC is not the only factor in which DPPH radical scavenging activity increases. A lot of substances such as carotenoids, tocopherols, vitamin, phenolic acids, flavonoids etc. have antioxidant activity (Cartea et al., 2011). In this experiment, increasing DPPH radical scavenging activity is likely to have been caused by the increase in TPC and other antioxidants. The production of these antioxidants can be enhanced by fermentation. Lactate bacteria have been studied for beneficial physiological effects such as resistance to pathogens, immune-potentiating activity, and anti - tumor activity (Havenaar and Velt et al., 1992; Apostolidis, 2008). Lactate bacteria are capable of producing secondary metabolites with anti-oxidation potential, according to a recent study (Kaizu et al., 1993; Ljungh and Wadstrom, 2006). According to

research by Pyo et al. (2005), LAB fermentation of soybean increased the radical scavenging ability for DPPH and ABTS (3-ethylbenzothiazoline-6-sulphnic acid) due to the increase of isoflavone aglycones produced during fermentation. In addition, in tTMR, a mix of BBS, an increase of about 183 % compared to cTMR. Therefore, if dairy cattle take a tTMR, it could have a positive effect on their physiology. In particular, the consumption of feed with enhanced antioxidant functions will affect oxidation-linked diseases (Shetty, 2004; Pyo et al., 2005; Ljungh and Wadstrom, 2006).

4. 5. 2. Functional components in milk during experimental period

Sulforaphane

Sulforaphane was not detected in milk during the test period. In previous BBS tests, we found that the sulforaphane content had decreased by more than 95 % after fermentation and we were expected not to be transferred to the milk if the cattle ate tTMR, which contained 20 % of BBS, and neither were the results.

Beta-carotene and Vitamin A

During the test period (+1w ~ +4w), beta-carotene and vitamin A content in the milk samples analyzed in each group. The results are shown in Table 17 and Figure 18. Although group A's beta-carotene in milk tended to decrease over time by feeding tTMR, there was no significant difference (linear, $p = 0.07$). Group B had a 0.324 mg/100mL of beta-carotene in the first week after receiving a tTMR and significantly increased linearly to 0.413 mg/100mL in the fourth week (linear, $p < 0.001$; quadratic, $p < 0.05$). Group C had a 0.37 mg/100mL of beta-carotene in the first week after receiving a tTMR and tend to increase to 0.479 mg/100mL in the fourth week (linear, $p < 0.05$; cubic, $p = 0.074$). Group D had a higher average beta-carotene content in milk than other groups and increased from 0.47 mg/100mL in the first week to 0.58 mg/100mL in the fourth weeks (linear, $p < 0.05$; quadratic, $p < 0.05$). When comparing all groups of data, there was significantly increased from 0.385 mg/100mL in the first week to 0.449 mg/100mL in the fourth week (linear, $p < 0.05$). The vitamin A content in milk of group A was

significantly decreased from 2.93 mg/100mL in the first week to 1.07 mg/mL in the fourth week (linear, $p = .0001$; quadratic, $p < 0.05$; cubic, $p = 0.002$). The results are shown in Table 18 and Figure 19. During the test period, Group B averaged 2.23 mg/100mL, Group C averaged 2.93 mg/100mL, and Group D averaged 2.75 mg/100mL, with no significant difference ($p > 0.1$). When comparing all groups of data, there was no significant difference during test period ($p > 0.1$). Therefore, when the cows were fed a tTMR mixed with BBS, the beta-carotene content in the milk was significantly increased in the all the groups except group A and the vitamin A content decreased significantly in group A, but there were no significant changes in the rest of the groups. It was noted that the beta-carotene content was increasing after the silage fermenation. Also, the tTMR increased by about four times. Therefore, there was a possibility that beta-carotene can be deposited by taking a tTMR.

Table 17. Beta-carotene content in milk samples during test period

Group	Beta-carotene content (mg/100mL) in milk during test period				SEM	P-values		
	+1w	+2w	+3w	+4w		Linear	Quadratic	Cubic
A	0.375	0.346	0.352	0.322	0.017	0.072	0.954	0.366
B	0.324	0.402	0.417	0.413	0.014	0.002	0.018	0.472
C	0.370	0.420	0.385	0.479	0.023	0.022	0.376	0.074
D	0.471	0.441	0.481	0.580	0.027	0.016	0.044	0.921
All	0.385	0.402	0.409	0.449	0.021	0.038	0.591	0.635

SEM, standard error of the mean.

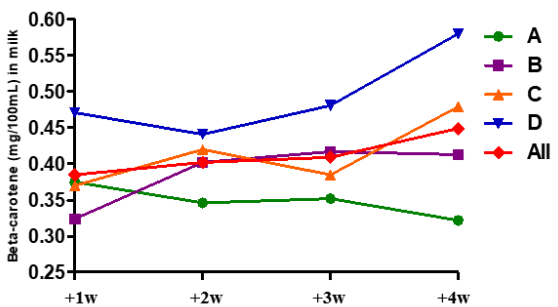

Figure 18. Beta-carotene content in milk samples during test period

Table 18. Vitamin A content in milk samples during test period

Group	Vitamin A content (mg/100mL) in milk during test period				SEM	P-value		
	+1w	+2w	+3w	+4w		Linear	Quadratic	Cubic
A	2.93	2.19	2.67	1.07	0.161	0.0001	0.028	0.002
B	2.00	2.55	1.99	2.39	0.468	0.778	0.882	0.348
C	2.76	2.89	2.55	3.52	0.332	0.222	0.240	0.369
D	2.82	2.37	2.82	3.02	0.250	0.374	0.228	0.344
All	2.63	2.50	2.51	2.50	0.212	0.704	0.776	0.887

SEM, standard error of the mean.

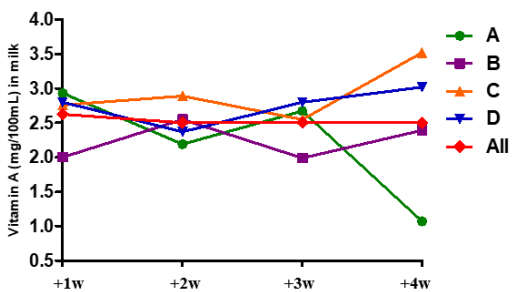

Figure 19. Vitamin A content in milk samples during test period

Antioxidant activity

After feeding the BBS to dairy cattle, total phenolics content (TPC) test and DPPH radical scavenging activity (%) test were conducted to determine the antioxidant activity in milk (Table 19 and Figure 20). In group A, there was no significant difference in TPC for four weeks, and the average was 2.41 mg GAE/L in milk ($p > 0.1$). In group B, also there was no significant difference in TPC for four weeks, and the average was 2.27 mg GAE/L in milk ($p > 0.1$). In group C the average was 2.43 mg GAE/L in the first week and significantly decreased to 2.10 mg GAE/L in 4 weeks (linear, $p < 0.01$). On the other hand, group D increased significantly from 2.12 mg GAE/L in the first week to 2.36 mg GAE/L in the fourth week. In the DPPH radical scavenging activity results (Table 20 and Figure 21), group A significantly increased the scavenging activity from 7.61 % in the first week to 20.84 % in the fourth week (linear, $p = 0.05$). In group B, however, there was no significant difference with an average of 23.47 % during the test period ($p > 0.1$). Group C tended to increase the scavenging activity from 19.09 % in the first week to 24.89 % in the fourth week (linear, $p = 0.072$). Group D, like Group A, significantly increased the scavenging activity from 9.82 % in the first week to 25.31 % in the fourth week (linear, $p < 0.05$). The average value of all group's antioxidant activity data was significantly increased for each week ($p = 0.0001$). TPC content could be an indicator of antioxidant activity. For silage, TPC and DPPH radical scavenging activity increased simultaneously after fermentation. Although TPC decreased, other substances may have acted to increase antioxidant activity (Cartea et al., 2011).

Table 19. Total phenolics content in milk samples during test period

Group	Total phenolic content (mg GAE/L) in milk during test period				SEM	P-values		
	+1w	+2w	+3w	+4w		Linear	Quadratic	Cubic
A	2.58	2.32	2.36	2.38	0.181	0.348	0.271	0.601
B	2.34	2.24	2.28	2.22	0.150	0.507	0.852	0.616
C	2.43	2.28	2.12	2.10	0.121	0.005	0.539	0.521
D	2.12	2.33	2.40	2.36	0.109	0.038	0.125	0.930
All	2.39	2.30	2.26	2.24	0.076	0.043	0.484	0.914

SEM, standard error of the mean.

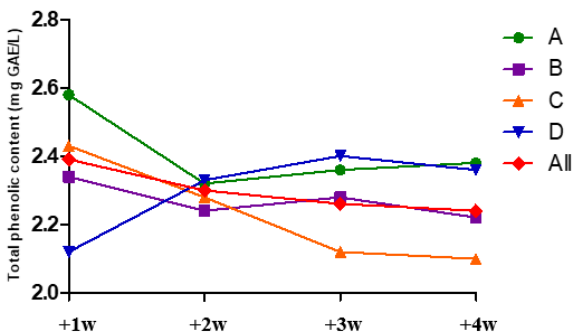

Figure 20. Total phenolics content in milk samples during test period

Table 20. DPPH radical scavenging activity in milk samples during test period

Group	DPPH radical scavenging activity (%) in milk during test period				SEM	P-values		
	+1w	+2w	+3w	+4w		Linear	Quadratic	Cubic
A	7.61	19.3	20.4	20.8	5.13	0.052	0.140	0.555
B	23.5	22.4	23.0	25.0	4.05	0.690	0.602	0.969
C	19.1	19.0	23.6	24.9	3.52	0.072	0.973	0.694
D	9.82	21.2	24.7	25.3	5.13	0.009	0.173	0.745
All	15.2	20.5	22.9	24.0	2.23	0.0001	0.185	0.800

SEM, standard error of the mean.

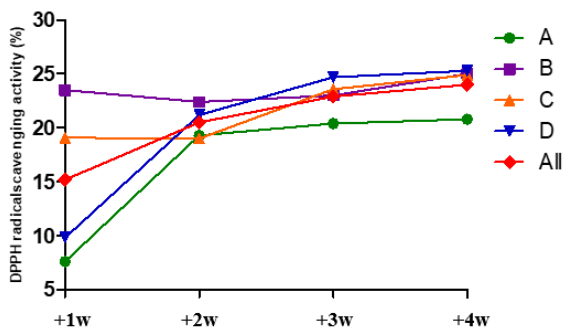

Figure 21. DPPH radical scavenging activity in milk samples during test period

5. Conclusion

We conducted a study on the broccoli by-product silage preparation method and its feed value in the TMR form for dairy cow's performance. The best method was mixing 80 % (weight) of chopped broccoli by-products (BB) with 10 % (weight) beet pulp & 10 % wheat bran inoculated with *L. plantarum* (5×10^7 cfu/kg of the silage). This mixture was put into plastic box with lid or vinyl-lined ton-bag in tight pressure to remove air as much as possible and fermented for 45 days at ambient temperature. This formula gave the best feed value, feed quality and palatability tested on dairy cows. In order to conduct feeding trial for BBS, chemical composition and NE_L of the commercial TMR (cTMR) and BBS were compared. BBS showed slightly higher CP, TDN and NEL values with no significant difference in a DM basis. In the experimental period of feeding trial, cows were fed 30kg of cmTMR (20 kg of cTMR + 10 kg of rye silage) for 4 weeks of the control period followed by 38kg of tTMR [16 kg of cTMR + 10 kg of BBS (equivalent to 4 kg of cTMR in DM) + 10 kg of rye silage] for 4 weeks of the test period. There was no significant difference in the milk yield between to the control and the test period. There was also no significant difference in the milk components including milk fat, protein, lactose, SnF, MUN between the groups. We concluded that BBS did not affect the change in the milk production (milk yield and milk components). In the analysis of functional substances in silage and milk, sulforaphane disappeared by about 99 % and beta-carotene increased by 120 % during the BBS fermentation ($p < 0.001$). In addition, antioxidant activity increased by about 55 % ($p < 0.005$). During the test period, the beta-carotene content in milk significantly

increased in most groups (linear, $p < 0.05$), but vitamin A showed no significant results. TPC tended to decrease in most groups, while antioxidant activity tended to increase.

Therefore, BBS was excellent in feed value and quality and concluded that BBS is a good feed source in a TMR form and by ensiling broccoli by-product could be preserved for a long time. We also believe that the use of unused agricultural by-products as feed are important to improve the sustainability of livestock industry in Korea and there are good potential for BBS as a TMR ingredient at least in dairy cows.

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7. 요약

현대 한국의 축산업은 제한적인 토지와 사료자원으로 인한 사료원료에 대한 해외 의존도가 높으며 최근 해외 농작물 작황 부진으로 인한 사료비 상승이 지속됨에 따라 농가 생산비 대비 사료비 부담이 가중되고 있다. 이에 대한 대책으로는 농작물 재배 당시 발생되는 유용 농산 부산물을 사료화하여 재활용하는 방법이 있다. 강원도 평창군에서는 연간 약 4,000 톤 이상의 식용 가능한 브로콜리가 출하되지만, 부산물인 줄기와 잎은 약 6,000 톤 이상 발생하지만 이는 전량 폐기되어지고 있다. 따라서 본 연구에서는 유용물질을 다량 함유하고 있는 브로콜리 부산물을 사료원료로 재활용 하기위해 계절적으로 단기간에 생산되는 다즙성 부산물의 보존성을 높이기 위한 사일리지를 제조하기로 하고 최적의 사일리지 제조 포뮬라를 확립하며, 착유우 급여 시험을 통해 유 생산성과 기능성 측면을 확인 후 완전배합사료 대체원료로써의 가치 평가를 진행하였다. 최적의 사일리지 제조 포뮬라 확립 시험에서는 브로콜리 부산물을 주 재료로 하여 농산 부산물 기원 부형제들과 유산균 접종원 혼합을 통해 TDN과 RFV를 기반으로 한 사료 가치평가, 유기산 비율에 따른 발효 품질평가, 급여 시험을 통한 기호성 평가 등을 진행하였다. 그 결과 브로콜리 부산물에 비트펄프와 밀기울을 동량으로 (총 사일리지 무게의 20%) 혼합하고 유산균 (*L. plantarum*)을 접종한 사일리지에서 높은 함량의 CP (19 %), TDN (71.7 %) 그리고 NEL (1.67 Mcal/kg) 수치를 보였으며, 높은 함량의 젖산으로 인한 고품질 발효 특성과 우수한 기호성을 나타내었다. 착유우를 대상

으로 한 사양시험에서는 27두의 홀스타인 젖소 (DIM 기준 4그룹)를 대상으로 진행되었으며, 기존 급여되는 완전배합사료의 20%를 브로콜리 부산물 사일리지로 대체 (건물 기준 1:1)하여 시험 사료로 사용하였다. 총 시험기간은 8주로써, 4주의 대조군 기간과 이어서 4주의 실험군 기간으로 연이어 진행하였다. 유량 분석은 개체 별 일일 유량 데이터를 활용하여 만들어진 유량 곡선의 기울기를 이용하여 대조군 기간 대비 실험군 기간의 기울기 증감을 통해 분석하였다. 또한 주차 별 우유 샘플링을 통해 유성분 (fat, protein, lactose, SnF, MUN) 분석과 기능성 물질 (sulforaphane, beta-carotene, vitamin A, pheonolics) 분석을 진행하였다. 그 결과 유량과 유성분에서 브로콜리 부산물 사일리지 급여 후 유의적인 차가 없었으며 젖소의 생산성에는 특이적인 영향을 미치지 않았다고 판단하였다. 기능성 물질 분석에서는 사일리지 발효 후 sulforaphane의 함량이 약 99 % 감소하였으며 beta-carotene 함량은 120 % 증가하였고 항산화 활성도가 55 % 증가하는 결과를 보였다. 우유 내에서는 실험군기간의 대부분의 젖소 그룹에서 beta-carotene 함량이 유의적으로 증가하였지만 vitamin A는 변화가 없었고 항산화 활성도는 증가하는 경향을 보였다.

따라서, 본 연구를 통해 전량 폐기되는 브로콜리 부산물을 비트펄프, 밀기울, 유산균 접종원 혼합을 통한 사일리지 형태로 사료화 하였을 경우 우수한 사료 품질을 보였으며, 착유우를 대상으로 한 사양 시험을 통해 완전배합사료의 20 %를 대체할 수 있어 브로콜리 부산물 사일리지가 완전배합사료 대체원료로써의 가치가 우수한 것으로 판단되어 지속가능하고 축산업의 발전을 위해 TMR 원료로 개발할 필요가 있다고 보여진다.

주요어: 브로콜리 부산물, 부산물 사일리지, 젖소 사료, 젖소 TMR, 기능성 물질

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