



Title	Functional evaluation of bean husk as a new feed ingredient for monogastric animals
Author(s)	Htun Myint
Citation	北海道大学. 博士(農学) 甲第13149号
Issue Date	2018-03-22
DOI	10.14943/doctoral.k13149
Doc URL	http://hdl.handle.net/2115/73223
Type	theses (doctoral)
File Information	Htun_Myint.pdf



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Functional evaluation of bean husk as a new feed ingredient for
monogastric animals

(単胃動物のための新規飼料素材としてのマメ外皮の機能評価)

Doctor of Philosophy

in Division of Agrobiology, Graduate School of Agriculture
at Hokkaido University, Japan

Htun Myint

Abstract

Bean husk is an agricultural by-product that is generated from the processing of bean for human consumption. Bean husk is rich in dietary fiber and phytochemicals such as polyphenols and considered a new feed source candidate for various animals, but still underutilized. Bean husk fiber is reported to selectively stimulate the growth of fiber-degrading bacteria in the gastrointestinal tract of herbivorous livestock animals such as ruminants. Therefore, bean husk has gained attention as a new possible source of functional fiber for other animals, in particular monogastrics. The objective of the present study was to evaluate effect of bean husk on blood parameters, cecal fermentation and microbial community in rats and dogs; the results are expected to provide insights into the future development of functional foods for monogastric animals.

For evaluation of chickpea (*Cicer arietinum*) husk, feeding study using fifteen male SD rats (5 weeks of age) was conducted. Rats were divided into three groups; they were fed one of the following diets for 3 weeks: purified diet (AIN 93G) containing 5% cellulose (CEL), an identical diet in which cellulose was replaced by corn starch (STA) or by chickpea husk (CPH). Chickpea husk contained high polyphenolic content and significant superoxide dismutase and 2,2-diphenyl-picrylhydrazyl scavenging activities. In a feeding experiment, CPH showed lowered cholesterol levels and improved antioxidant activity represented by reduced thiobarbituric acid reactive substances in blood. CPH showed increased cecal levels of total short-chain fatty acids and butyrate. CPH presented with lowered cecal indole and skatole concentrations, as did CEL. Cecal bacterial changes were notable in CPH, evidenced by differences in denaturing gradient gel electrophoresis banding patterns. These results indicate that chickpea husk feeding can improve the antioxidative status of rats through its polyphenolic components and modulate the hindgut environment by its fibrous components.

For evaluation of lablab bean (*Dolichos lablab*) husk and soybean (*Glycine max*) husk, a feeding study using twenty male SD rats (5 weeks of age) was conducted. Rats were divided into 4 groups and fed one of the following diets for 3 weeks: purified diet (AIN 93G) containing 5% cellulose (CEL), or the same diet in which cellulose was replaced by corn starch (STA), lablab bean husk (LBH), or soybean

husk (SBH). Feed intake, body weight, anatomical parameters, and cecal ammonia level did not differ significantly among diets. Rats on LBH and SBH showed higher concentrations of cecal short-chain fatty acid and lactate than those on CEL. Rats on CEL, SBH, and LBH exhibited decreased cecal indole and skatole levels. LBH yielded increased cecal abundance of beneficial bacteria such as *Akkermansia muciniphila*, bifidobacteria, and *Oscillibacter* relatives, as demonstrated by either quantitative real-time PCR (qPCR), MiSeq, or clone library analysis. SBH favoured the growth of lactobacilli as assessed by both qPCR and MiSeq, and favoured the growth of bifidobacteria as assessed by MiSeq. In comparison with STA, LBH and SBH yielded decreased cecal abundance of bacteria related to *Dorea massiliensis*, as demonstrated by qPCR, MiSeq, and clone library analysis. Both types of bean husk were found to contain oligosaccharides that might selectively stimulate the growth of beneficial bacteria. Based on these results, the two species of bean husk tested are considered functional for promoting the gut health of monogastric animals.

For the confirmation of the functionality of soybean husk, a feeding study using four Shiba dogs (7–48 months in age and 7.5 ± 1.7 kg in body weight) was conducted. Dogs fed a commercial diet supplemented with 5.6% soybean husk after feeding control cellulose diet, showed an increase in short-chain fatty acids, such as acetate and butyrate, and lactate, and a decrease in indole and skatole in the feces compared to those fed a 5.6% cellulose diet. qPCR assay showed that soybean husk supplementation stimulated the growth of lactobacilli, *Clostridium* cluster IV including *Fecalibacterium prausnitzii*, *Clostridium* cluster XIVa, *Bacteroides-Prevotella-Porphyromonas* group but inhibited the growth of *Clostridium* cluster XI. Therefore, soybean husk is suggested to be applicable as a functional fiber in the formulation of canine diets.

Acknowledgements

At first, thanks go to my supervisor, Professor Yasuo Kobayashi. He supervised my academic path in both master's and PhD study. His observations and comments helped me to establish the overall direction of my research and to move forward with it. He encouraged me to grow as a scientist. His support, advice and guidance throughout my study has been crucial to complete it and his patience and understanding to me, has been much appreciated.

Thanks also go to my co-supervisors, Professor Masashi Takahashi (Laboratory of Animal Genetics and Reproduction), Associate Professor Satoshi Koike especially for his knowledge and advice available to me and Assistant Professor Yutaka Suzuki. Their critical reviewing on this thesis is much appreciated.

Thanks also go to Professor Tin Ngwe (Rector (Retired), University of Veterinary Science, Yezin, Myanmar). His introduction with my supervisor, is highly appreciated. I have been fortunate to receive vital support and encouragement from Professor Aung Aung and my teachers from the Department of Physiology and Biochemistry, University of Veterinary Science, Yezin, Myanmar. I am grateful for that.

Thanks also go to Japanese Government, Monbukagakusho (MEXT) Scholarship Program and Myanmar Government, Ministry of Agriculture, Livestock and Irrigation. Their financial support and official arrangement on my study are much appreciated.

Thanks also go to my tutor, Dr. Naoki Fukuma (Assistant Professor, Obihiro University of Agriculture and Veterinary Medicine), my colleagues, Yu Iwahashi and Hiroyuki Kishi, and present and past lab members. Their support and contribution are much appreciated.

Finally, to my parents and my wife Yi Yi Mon, thank you so much for your love that helped keep me focussed on my study in Japan.

Table of contents

	Page
Abstract	1
Acknowledgements	3
Table of Contents	4
Chapter 1 Introduction and literature review	6
1.1 Health benefits of dietary fiber and polyphenols	6
1.2 Functional components of bean husk	8
1.3 Application of bean husk as feed ingredients	10
Chapter 2 Function of chickpea husk in rats	13
2.1 Introduction	13
2.2 Materials and methods	14
2.2.1 Animals and diets	14
2.2.2 Analysis of chemical compositions	14
2.2.3 Analysis of antioxidant activities	15
2.2.4 Analysis of blood and cecal parameters	16
2.2.5 Analysis of microbiota	16
2.2.6 Statistical analysis	18
2.3 Results	18
2.3.1 Components of chickpea husk and growth performance	18
2.3.2 Antioxidant activities of cecal digesta and blood	18
2.3.3 Cecal fermentation and blood parameters	19
2.3.4 Cecal microbiota	19
2.4 Discussion	19
Chapter 3 Function of lablab bean husk and soybean husk in rats	22
3.1 Introduction	22
3.2 Materials and methods	23
3.2.1 Animals and diets	23
3.2.2 Chemical analyses of bean husk	24
3.2.3 Measurement of blood parameters and cecal fermentation products	25
3.2.4 Microbiota analysis by qPCR	26
3.2.5 Microbiota analysis by 16S rDNA clone library	26
3.2.6 Microbiota analysis by MiSeq	27
3.2.7 Statistical analysis	28
3.3 Results	28
3.3.1 Nutritional composition of bean husk	28
3.3.2 Growth and cecal characteristics of rats	29
3.3.3 Microbiota	29
3.4 Discussion	31
Chapter 4 Function of soybean husk in dogs	36
4.1 Introduction	36
4.2 Materials and methods	37
4.2.1 <i>In vitro</i> fermentation experiment	37
4.2.2 <i>In vivo</i> feeding experiment	38
4.2.3 Chemical analysis	39
4.2.4 Microbial analysis	39
4.2.5 Statistical analysis	40
4.3 Results	40

	4.3.1 <i>In vitro</i> fermentation experiment	40
	4.3.2 <i>In vivo</i> feeding experiment	41
4.4	Discussion	42
Chapter 5	General discussion	45
	Conclusion	47
	Tables and figures	48
	References	65

Chapter 1

Introduction and literature review

Agricultural by-products are considered as a source of functional food that are known for prevention and treatment of various chronic diseases, such as constipation, heart diseases, diabetes, obesity and some forms of cancer (Marlett *et al.* 2002). Due to the rising awareness of health and nutrition, functional food such as prebiotics has been gained attention to innovate its production. Development of new functional food from less expensive resources such as agricultural by-products have been the focus of my research.

There are various types of agricultural by-products such as crop waste and residues, by-products from fruit- and vegetable-processing industry, by-products from sugar, starch and confectionary industry, by-products from grain- and legume-milling industry and oil industry, and by-products from distilleries and breweries (Ajila *et al.* 2012). Those by-products contain functional components having health benefits and are therefore valuable supply of nutrients to animals.

1.1 Health benefits of dietary fiber and polyphenols

Among functional components, dietary fiber has been known for its role playing in many physiological functions and in the prevention of diseases such as cardiovascular disease, diabetes, obesity and cancer (Slavin 2013). There are many plant sources rich in dietary fiber such as cereals, legumes, vegetables, fruits and nuts. Dietary fiber is carbohydrate polymers with ten or more monomeric units, which are neither digested nor absorbed in the human small intestine (Codex Alimentarius 2010). Dietary fiber is generally classified into two main types including insoluble and soluble dietary fiber depending on its solubility in water. Structural or nonviscous fiber such as cellulose, some hemicelluloses and lignins is insoluble dietary fiber, whereas viscous fiber such as pectins, arabinoxylan, glucans and algal polysaccharides is usually soluble dietary fiber (Williams *et al.* 2017). The insoluble fiber is related to both water absorption and intestinal regulation, whereas the soluble fraction is associated with microbial regulation, reduction of cholesterol in blood and decrease

of glucose absorption by small intestine. Insoluble and soluble dietary fiber has been reported to be able to bind bile acids while passing through small intestine without digestion. Bile acid is synthesized in the liver and secreted in small intestine from the destruction of endogenous cholesterol. Soluble dietary fiber due to its viscosity, creates thickening of the unstirred layer lining the intestinal lumen, thereby reducing the flow of bile acids towards the epithelial cells for uptake. Insoluble fiber directly binds to or entraps bile acids, thereby preventing re-absorption in small intestine and increasing their excretion. Therefore, bile acids are end products of cholesterol catabolism which is the most important route to eliminate surplus cholesterol from the blood circulation. Fermentation of particular dietary fiber in large intestine produces short-chain fatty acid (SCFA). Those acids, in particular propionate and acetate, can suppress endogenous cholesterol synthesis. Moreover, bacterial de-conjugation of bile acids causes mass excretion of bile acids in the feces (Fechner *et al.* 2014, Gunness *et al.* 2016, Andersson *et al.* 2013). Therefore, fiber, SCFAs and bacterial de-conjugation of bile acids will concertedly induce new synthesis of bile acids from endogenous cholesterol in the liver, contributing to the reduced blood cholesterol.

Dietary fiber selectively stimulate the growth and/or activity of intestinal bacteria, particularly *Bifidobacterium* and *Lactobacillus* which plays an important role in protection against pathogens and reduces the concentration of harmful fermentation products in the gastrointestinal tract (Gibson & Roberfroid 1995). These prebiotic effect is due to the fermentation of dietary fiber, leading to the production of SCFAs, principally acetate, propionate and butyrate. Acetate has been shown to stimulate proliferation of normal crypt cell, enhance ileal motility, increase colonic blood flow and involve in adipogenesis and host immune system (Ono *et al.* 2004, Hong *et al.* 2005, Brown *et al.* 2003). Propionate has been shown to regulate hypocholesterolemic and anti-carcinogenic effects (Hosseini *et al.* 2011, Martinez-Villaluenga *et al.* 2005, Xiong *et al.* 2004, Hinnebusch *et al.* 2002, Jan *et al.* 2002). Butyrate is an energy source for enterocytes and has been found to act as a protectives against experimental tumorigenesis cells and exhibit anti-inflammatory properties by the inhibition of TNF- α production in colonic epithelial cells (Bailon *et al.* 2010, Luhrs *et al.* 2002).

Apart from dietary fiber, polyphenols are another component of agricultural by-products having potential health benefits. Low molecular weight polyphenols such as monomeric and dimeric structures may be readily absorbed in the small intestine and distribute into body through systemic circulation. Some polyphenols (oligomeric and polymeric polyphenols such as condensed or hydrolysable tannins) associated with dietary fiber are not hydrolysable by digestive enzymes in the upper intestinal tract. Once they reach to the large intestine, some of them get fermented along with dietary fiber and are extensively metabolised by microbiota, leading to the formation of absorbable metabolites such as phenylacetic, phenylpropionic, and phenylbutyric acids (Rechner *et al.* 2004). These aromatic acids are well absorbed through the intestinal barrier and may actually be responsible for systemic health effects. Bacteria belonging to *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Eubacterium*, *Enterococcus* and *Blautia* have been reported to be responsible for the breakdown and biotransformation of polyphenols into low molecular weight metabolites in gastrointestinal tract (Espin *et al.* 2017). On the other hand, non-fermentable and non-absorbable polyphenols present in the large intestine may contribute to a healthy antioxidant environment by scavenging free radicals and counteracting effects of dietary pro-oxidants. Therefore, dietary fiber associated with polyphenols may exert beneficial effects by creating improved fermentation and antioxidant environment in large intestine. Agricultural by-products rich in functional components such as dietary fiber and polyphenols, could be added to different foods for animals as well as human, to yield beneficial effects. Bean husk is one of such agro-byproduct and may have a potential to utilize in animal nutrition.

1.2 Functional component of bean husk

Among the agricultural by-products available, bean husk forms major feed resources. It is available in plenty to the extent of over 2.0 million tonnes per annum in the world from over 26 million tonnes of dry bean production (FAOSTATS 2014), since husk corresponds to 8-11% of whole seed (Kanatt *et al.* 2011). Bean husk is rich in dietary fiber, ranging from dry weight contents of 75% in chickpea husk to 87% in lentils husk and 89% in pea husk (Dalgetty & Baik 2003). Husk of pea, lupin

and soybean are reported to contain soluble and insoluble dietary fiber (Guillon & Champ 2002). Indeed, the total dietary fiber accounts for 89%, 80% and 75% in the husk, respectively. Soluble dietary fiber are contained at 7%, 8% and 10 %, respectively. Pea husk was characterized as rich in cellulosic glucose, pectins and hemicellulose and identified to contain heteroxylans, glucans, and arabinans (Renard *et al.* 1997). Le Goff *et al.* (2001) confirmed the presence of xylogalacturonans in the pectins from pea husk. Both of bean and its husk (eg. soybean) contain raffinose family oligosaccharides, also known as important group of α -galactooligosaccharides (Fan *et al.* 2015). The oligosaccharides in the raffinose family include raffinose, stachyose, and verbascose. Raffinose is a trisaccharide, composed of one galactose monomer attached to a sucrose molecule via α -(1-6) glycosidic linkage. The stachyose, tetrasaccharide and verbascose, pentasaccharide are synthesized from raffinose by the addition of α -D-linked galactosyl units. However, little information is accumulated for the detailed characterization of dietary fiber in bean husk. Further characterization of saccharides in husk may elucidate their functional properties in detail.

Carbohydrate fractions of bean husk including oligosaccharides, polysaccharides and other fermentable non-starch polysaccharides are considered to modulate gastrointestinal fermentation and microbial community in animals. In particular, raffinose family oligosaccharides have been reported to be utilized by selected beneficial bacteria in *in vitro* and to influence growth of *Bifidobacterium* in the colon of rats (Saito *et al.* 1992, Gulewicz *et al.* 2002). Thus, bean husk has a great potential to be used as a new functional food due to its high content of both insoluble and soluble dietary fiber. However, they are either underutilized or discarded as an agricultural waste.

Another functional component of bean husk is polyphenols which are one of the most important groups of secondary metabolites in plants having antinutrient properties such as low digestibility and inhibitory effect on absorption of protein and other nutrients in animals (Sreerangaraju *et al.* 2000). On the other hand, polyphenols have to be found to possess beneficial activities such as anti-diabetic, anticancer, anti-inflammatory, cardioprotective, osteoprotective, neuroprotective, antiasthmatic, antihypertensive, antiageing, antiseptic, cerebrovascular protection,

cholesterol lowering, hepatoprotective, antifungal, antibacterial and antiviral properties (Ganesan & Xu 2017, Daglia 2012). Polysaccharides and polyphenols were detected in the husk of chickpea, horse gram, mung bean, black gram, green gram, pigeon pea, lentils and faba bean (Lai *et al.* 2010, Sreerama *et al.* 2010a, Kanatt *et al.* 2011, Girish *et al.* 2012, Basha *et al.* 2017, Mirali *et al.* 2017, Karatas *et al.* 2017). The previous reports confirmed that all husk had good antioxidant potential protecting animal body cells such as λ -DNA and red blood cell, against oxidative damage, and scavenging superoxide and free radicals and also had good antibacterial activities against food borne pathogens such as *Bacillus cereus*. Furthermore, phenolic substances including condensed tannin-, and lignin-concentrated bean husk can bind considerable amount of bile acid (Karatas *et al.* 2017). Bean husk could therefore have application in food of various animals including human in terms of health promotion. Therefore, it is very important to consider about functional activities of a diet containing bean husk in gastrointestinal tract of monogastric animals and to access its benefit. Bean husk can be exploited as a new source of novel natural feed ingredient or additive to utilize their potential nutritional value.

1.3 Application of bean husk as feed ingredient

Bean husk has been basically used as ruminant feed. Faba bean husk was evaluated for potential application in dairy cow and found that 30% inclusion of husk improved yield and fat content of milk (Al-Saiady 1998). Chickpea husk and lablab bean husk were proposed to use as easily digestible supplemental fiber that caused a positive associative effect on fiber digestion because they selectively stimulated rumen microbiota (Ngwe *et al.* 2012, Fuma *et al.* 2012). Soybean husk has been widely used in dairy cows as a replacement of grain in diet (Ipharraguerre & Clark 2003).

However, study on nutritional significance of bean husk in monogastric animal is limited although it is evaluated to use as the ingredients in the formulation of various food products for human health. For instance, mung bean husk incorporated in biscuits, was recommended for the people suffering from diabetes and constipation due to its low glycemic index (Bora & Kulshrestha 2015).

Dietary fiber from soybean husk was shown for potential utilization in snacks with enrichment of fiber for human health (Yang *et al.* 2014).

Bean husk application in monogastrics is related to animal health. For instance, van der Meulen *et al.* (2010) demonstrated the effects of pea husk and faba bean husk as preventives of enterotoxigenic *Escherichia coli* colonization in ileum which is an important etiological agent responsible for post-weaning diarrhea in piglets. Both of bean husk diminish adhesion of *E. coli* to epithelial cells by increasing intestinal water-holding capacity. Therefore, the reduction of intestinal fluid loss by both of bean husk may contribute to prevent gastrointestinal disorders. In another study, pea husk has been found to disturb the enterotoxigenic *E. coli* K88 cell adhesion, while faba bean husk interfere with the enterotoxin, by binding to the intestinal receptor, leading to the reduction of the numbers of *E. coli* attaching to the intestinal mucosa (Becker *et al.* 2012). Thus, bean husk has functional properties to prevent colonization by potential pathogens and thereby reduces the risk of infections in the gastrointestinal tract of monogastric animals.

Other biological properties of bean husk were also reported. When fermentation characteristics of whole pea, pea husk and pea inner fiber were compared with wheat bran, sugar beet pulp, and other feed ingredients in *in vitro* system using colonic bacteria from pigs, pea and pea husk showed a higher fermentation rate and SCFA production than other substrates (Jha *et al.* 2011). Dietary inclusion of pea husk enhanced bacterial fermentation and yielded highest SCFA production especially butyrate in ileum and colon of growing pigs (Jha & Leterme 2012). Bean husk has the ability in gut fermentation as its whole seed has. The supplementation of pea husk fiber in the diet of pigs reduced the abundance of pathogens involved in irritable bowel syndrome such as *Clostridium* cluster I and *Enterobacteriaceae* family and increased those of beneficial bacteria belonging to *Lactobacillus* group and *Clostridium* cluster IV (Luo *et al.* 2017). Therefore, main health-promoting effects are indicated to exist in bean husk.

However, it remains unclear whether other bean husks such as chickpea husk, lablab bean husk and soybean husk possess functional activity in monogastric animal, while some of them have

been utilizing in ruminant animals. The objective of this study was to evaluate nutritional properties of chickpea husk, lablab bean husk, and soybean husk as a new functional feed ingredient for monogastric animals. Determination of changes in blood parameters, gastrointestinal fermentation and microbial community by three different types of bean husk in rats as a model of monogastric animals, is expected to understand the nutritional and health effects of each supplemented husk. Dogs were also used as experimental animals to further evaluate functions of soybean husk that is most abundantly available among husks tested. This study might give insights into understanding of functionality and practical use of the husk in monogastric animals.

Chapter 2

Function of chickpea husk in rats

2.1 Introduction

Chickpea (*Cicer arietinum*), also called Bengal gram bean or garbanzo bean, is a member of the *Fabaceae* family of legumes and its global production was over 14 million tons in 2014 (FAOSTATS 2014). Large quantities (over 907,000 tonnes) of chickpea husk accumulate annually as an agro-byproduct after dehulling for human consumption. Chickpea husk is a new feed source for animals and could be a potential source of dietary fiber, antioxidants and microbial modulators.

It has been reported that chickpea husk can be used as a source of dietary fiber (Dalgetty & Baik 2003), in which pectins (Urias-Orona *et al.* 2010) and oligosaccharides such as raffinose, stachyose and verbascose (Sreerama *et al.* 2010a) are constituents. Additionally, the presence of tannin (Sreerangaraju *et al.* 2000), flavonoids, phenolic acids and anthocyanins (Sreerama *et al.* 2010b) in chickpea husk were also reported. Chickpea husk possesses water-holding capacity (Dalgetty & Baik 2003), which plays an important physiological role in gut function and control of blood parameters. In light of these components and physical function, chickpea husk is recommended for use as a fiber supplement for ruminant animals (Sreerangaraju *et al.* 2000, Ngwe *et al.* 2012) and as a health inducer for hypercholesterolemic rats (Mittal *et al.* 2009).

Most plants contain fiber and polyphenols, which are resistant to digestive enzymes of the upper gastrointestinal tract. Therefore, they are transported to the large intestine, where they undergo fermentation. Chickpea husk, rich in both dietary fiber and polyphenols, is expected to favorably regulate metabolic activities, and may improve the health status of monogastric animals through its physiological and functional properties. Therefore, this study was carried out to functionally evaluate chickpea husk in association with antioxidant properties, blood parameters, gut fermentation and microbiota of rats as a model of monogastric animals.

2.2 Materials and methods

2.2.1 Animals and diets

Fifteen male Sprague-Dawley rats (five weeks of age) were purchased from Japan SLC, Inc., Hamamatsu, Japan. They were individually housed in plastic cages in an environmentally controlled room (temperature 23°C, light-dark cycle 12 h (12-24 hours for lighting), relative humidity 55-60%) and grouped into three treatments (five rats/group) with an average body weight of 142.5±7.2 g. All rats had free access to water and diet. One group was given a purified diet (AIN-93G; CLEA Japan Inc., Tokyo, Japan) containing 5% cellulose powder as a fiber source, which was used as the control diet (CEL). The other two groups were given the same purified diet in which 5% cellulose was replaced with either 5% corn starch (STA) or 5% chickpea husk (CPH). The starch diet did not contain any fiber source and was regarded as a negative control. In preliminary experiment, 5% inclusion of chickpea husk did not show any detrimental effect on health and growth performance. Therefore, 5% supplementation was taken in the present evaluation. Corn starch was purchased from Wako, Osaka, Japan. Desi type chickpea husk was imported from a pulse processing plant (Yezin, Nay Pyi Taw, Myanmar) via Prof. Tin Ngwe, University of Veterinary Science, Myanmar. At the end of the 21-day feeding period, rats were anaesthetized by xylazine-HCl injection. After laparotomy, blood samples were taken from the heart, and the prepared blood plasma was kept at -30°C until analysis. Cecal digesta were sampled and stored at -80°C until analysis.

The animal protocol used in this study was in accordance with the Guidelines for Animal Experiments, Hokkaido University (2007) and the Act on Welfare and Management of Animal (2005).

2.2.2 Analysis of chemical composition

The proximate composition of chickpea husk was analyzed according to the methods of the Association of Official Analytic Chemists (AOAC 1990). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed by the method of Goering and Van Soest (1975). Total phenolic content, total flavonoids and condensed tannin were analyzed as follows. About

200 mg of chickpea husk was extracted with 10 mL of acetone/water (70:30, v/v). The mixture was shaken at 130 rpm in a 30°C water bath for 3 h, and then kept overnight at room temperature. The mixture was centrifuged (4°C, 3,000×g, 10 min) and the supernatant was used in the analysis. The total phenolic content was determined using Folin-Ciocalteu's reagent as described by Makkar *et al.* (1993). The results were reported as tannic acid equivalent (0.1 mg/mL) on a dry matter basis. Non-tannin phenolic compounds were evaluated after removing phenolic compounds from the sample by precipitation with polyvinyl polypyrrolidone. Total tannin was calculated by subtracting non-tannin phenolic compounds from total phenolic compounds. Total flavonoids were assayed by the aluminum chloride colorimetric method (Dewanto *et al.* 2002). The condensed tannin was determined using the vanillin-HCl assay (Price *et al.* 1978). Total flavonoids and condensed tannin were reported as catechin equivalent (0.3 mg/mL) on a dry matter basis.

2.2.3 Analysis of antioxidant activity

Antioxidant activity was determined by the following procedure. For chickpea husk, a ground sample (200 mg) was soaked in 10 mL of acetone/water (70:30, v/v) for 15 min in a 70°C water bath. During incubation, sample tubes were vortexed at 5-min intervals and then centrifuged (4°C, 3,000 × g, 20 min). The obtained supernatant was used for analysis of superoxide dismutase (SOD) enzyme activity by a SOD Test Wako (Wako, Osaka, Japan), according to the manufacturer's instructions. The 2,2-diphenyl-picrylhydrazyl (DPPH) free radical scavenging activity was determined using the method of Brand-Williams *et al.* (1995). The extracted sample (0.2 mL) was added to 500 µmol/L DPPH in ethanol (1.8 mL) and thoroughly mixed. After 30-min incubation in the dark, the absorbance was measured at 517 nm. The absorbance of the control was obtained by replacing the sample with cellulose. Percent DPPH scavenging activity was calculated as: $((\text{Control absorbance} - \text{Sample absorbance}) / (\text{Control absorbance})) \times 100$. For cecal digesta, samples thawed on ice (1 g) were suspended in 5 mL of 0.85% saline, vortexed, and centrifuged (4°C, 7,800 × g, 5 min). The supernatant was used for analysis of SOD and DPPH radical scavenging activities. Water was used as the blank in

the DPPH assay. Lipid peroxidation products were assayed by quantifying the malondialdehyde formed in terms of thiobarbituric acid-reactive substances (TBARS) by a TBARS Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). Plasma SOD, DPPH and TBARS were also determined after blood plasma was diluted four-fold with water.

2.2.4 Analysis of blood and cecal parameters

Blood plasma thawed on ice was employed for the analysis of total cholesterol (T-Cho CII kit, Wako) and glucose (Glu-E kit, Wako) according to the manufacturers' instructions. The cecal digesta supernatant was used for pH measurement, by an electrode (F-51; HORIBA, Kyoto, Japan), and also for the analysis of SCFA, ammonia, indole and skatole. SCFA was determined by gas chromatography (GC-14B; Shimadzu, Kyoto, Japan) equipped with a 0.53 mm × 30 m capillary column (ULBON HR-20 M; Shimadzu) based on the procedure of Suto (1973). Ammonia nitrogen (NH₃-N) was measured by the phenol-hypochlorite reaction method (Weatherburn 1967) using a microplate reader at 660 nm (ARVO MX, Perkin Elmer, Japan). Assays for indole and skatole were carried out by colorimetric methods (Walstra *et al.* 1999).

2.2.5 Analysis of microbiota

DNA from cecal digesta was extracted and purified using the repeated bead beating plus column (RBB + C) method (Yu & Morrison 2004) with a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The concentration of purified DNA was quantified by a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were diluted to 10 ng/μL before being subjected to PCR amplification.

Bacterial communities were analyzed by PCR-DGGE (denaturing gradient gel electrophoresis) as described by Muyzer *et al.* (1993). The PCR reaction mixture (25 μL) contained 2.5 μL of 10× PCR buffer, 2.0 μL of deoxynucleotide triphosphate, 0.5 μL of each primer (341f-GC and 534r, 20 pmol/μL), 18.375 μL of water, 0.125 μL of bovine serum albumin (20 mg/mL), 0.125 μL of TaKaRa Ex Taq®

polymerase (Takara Bio, Tokyo, Japan) and 1.0 µL of template DNA. Electrophoresis of PCR products was performed using a DCode System (Bio-Rad, Hercules, CA, USA). PCR fragments were separated by an 8% polyacrylamide gel using 1.0× TAE buffer (20 mmol/L Tris-acetate pH 7.4, 10 mmol/L sodium acetate, 0.5 mmol/L Na₂ ethylenediaminetetraacetic acid) with a linear 35-60% gradient of denaturant (100% denaturant corresponds to 7 mol/L urea and 40% deionized formamide) at 60°C for 13 h at a constant voltage of 90 V. DNA bands were visualized by silver staining (Muyzer *et al.* 1998) and scanned (ES 2200; EPSON, Nagano, Japan). Cluster analysis was performed by the unweighted pair group method with arithmetic mean algorithm (UPGMA) based on the Dice correlation coefficient (BioNumerics software version 6.0; Applied Maths, Sint-Martens-Latem, Belgium). Banding pattern similarities were analyzed by examination of clusters formed after creation of the algorithm-based dendrogram.

Bacterial communities were also quantitatively characterized by real-time PCR (qPCR) assays with species/group specific primers for the representative groups, such as total bacteria (Muyzer *et al.* 1993), total lactobacilli (Walter *et al.* 2001; Heilig *et al.* 2002), four different *Lactobacillus* species including *Lactobacillus acidophilus* (Haarman and Knol 2006), *Lactobacillus gasseri* (Song *et al.* 2000), *Lactobacillus johnsonii* (Furet *et al.* 2004) and *Lactobacillus reuteri* (Dommels *et al.* 2009), total bifidobacteria (Kok *et al.* 1996), *Fecalibacterium prausnitzii*, *Eubacterium hallii* (Ramirez *et al.* 2009), *Clostridium coccoides*-*Eubacterium rectale* group (Rinttila *et al.* 2004), *Escherichia coli* (Malinen *et al.* 2003) and *Clostridium perfringens* (Wang *et al.* 1994). The qPCR was carried out on a Light Cycler 480 real-time PCR system (Roche Applied Science, Mannheim, Germany). The PCR reaction mixture (20 µL) contained 10.0 µL of KAPA SYBR (Kapa Biosystems, Woburn, MA, USA), 0.2 µL of each primer, 8.6 µL of water and 1.0 µL of template DNA. Results from samples in each group were averaged and reported as a relative proportion of each species (group) in total bacteria.

2.2.6 Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA). Tukey's multiple comparison test was used to resolve differences among treatment means using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). Statistical differences were considered to be significant at $P < 0.05$. Differences between treatments at $P < 0.10$ were considered as a trend toward significance.

2.3 Results

2.3.1 Components of chickpea husk and growth performance

Chemical composition and antioxidant status of chickpea husk are detailed in Table 1. Non-structural carbohydrates or non-fibrous carbohydrates are soluble components that include starch, soluble sugars and soluble fibers. Its calculated amount was 28.8%. Meanwhile, structural carbohydrates are represented by cellulose (55.6%), lignin (3%) and hemicellulose (3%). Total phenolic content reported as tannic acid equivalent, total flavonoids reported as catechin equivalent, calculated total tannin and condensed tannin reported as catechin equivalent were 2.7%, 2.5%, 2.1% and 1.0%, respectively. Chickpea husk possessed SOD enzyme activity and scavenged DPPH radicals with the values of 32.0% and 31.0%, respectively. Rats fed the three different diets showed equal food intake and growth without notable differences in feed conversion ratios. Weights of representative organs, including digesta content, did not differ among groups (data not shown).

2.3.2 Antioxidant activities of cecal digesta and blood

SOD, DPPH and TBARS of cecal digesta and blood are shown in Table 2. SOD activity of cecal digesta in CPH was lower ($P < 0.05$) than those in CEL and STA. CEL showed the highest SOD activity among the groups. Diets did not affect DPPH scavenging activity in the cecal digesta. Higher TBARS ($P < 0.05$) was found in the cecal digesta of CPH than those of CEL and STA. However, there was no difference in TBARS of cecal digesta between CEL and STA. Although blood SOD and DPPH did not

differ among the groups, a significant reduction of plasma TBARS ($P < 0.05$) was found in CPH compared to CEL and STA. No difference of plasma TBARS was found between CEL and STA.

2.3.3 Cecal fermentation and blood parameters

Cecal pH, cecal fermentation products and blood glucose and cholesterol are shown in Table 3. A lower cecal pH ($P < 0.05$) was observed in CPH compared to CEL and STA. In comparison to CEL, CPH showed increased ($P < 0.05$) total SCFA and butyrate concentrations. The ammonia concentration did not differ among the diets. STA showed higher ($P < 0.05$) indole and skatole levels in comparison with the other two diets containing fiber sources. The diets did not influence blood glucose concentrations. CPH showed a lower level of total cholesterol ($P < 0.05$) in comparison with CEL and STA.

2.3.4 Cecal microbiota

Cluster analysis of PCR-DGGE banding profiles from the cecal digesta showed that CPH showed altered cecal total microbiota, which was clustered in a single group (Figure. 1). However, none of the diets affected bacteria assayed by quantitative PCR, including total bacteria, total lactobacilli, four *Lactobacillus* species (*L. acidophilus*, *L. gasseri*, *L. johnsonii* and *L. reuteri*), total bifidobacteria, *F. prausnitzii*, *E. hallii*, *Clostridium coccoides-Eubacterium rectale* and *E.coli* in the cecal digesta (Table 4).

2.4 Discussion

Plant phenolic compounds are considered to be powerful antioxidants because of their ability to donate hydrogen or electrons to stable free radicals, leading to the formation of non-toxic species and therefore inhibiting the propagation phase of lipid oxidation. Chickpea husk used in the present study contained polyphenols and possessed antioxidant potential according to the *in vitro* evaluation of SOD and DPPH free radical-scavenging activities (Table 1). Therefore, chickpea husk might be an important feed source to improve the antioxidative status of animals. Although the cecal digesta did not show any improvement of antioxidant activity, plasma TBARS was significantly reduced with

chickpea husk intake (Table 2). One possible mechanism for the reduced plasma TBARS may involve the inhibitory action of polyphenols and their metabolites against oxidation reactions. Conjugated forms of phenolic acids, flavonoids and bacterial metabolites of flavonoids can be absorbed by intestinal cells into the circulation (Marin *et al.* 2015), where they might inhibit lipid oxidation caused by oxidative stress. Thus, the phenolic compounds of chickpea husk or their metabolites may enhance protection against blood lipid oxidation, which is a risk factor in cardiovascular disease induced by oxidative stress.

Chickpea husk produced more SCFA in the cecum, especially butyrate, an important substrate for the proliferation of intestinal epithelial cells and improvement of gut health, accompanied by a lower pH compared to cellulose (Table 3). This may be due to the fermentation of insoluble and soluble dietary fibers in chickpea husk that promote butyrate-producing bacteria. The most abundant component in chickpea husk is insoluble fiber such as cellulose, lignin and hemicellulose. In particular, soluble fiber in chickpea husk, represented by pectin, might become readily available for microbial fermentation. On the other hand, polyphenols are potential modulators of cecal fermentation because they or their metabolites affect cecal bacteria. The tannin in chickpea husk might reduce indole and skatole formation by inhibiting either microbial activity directly or by reducing the availability of proteins for bacterial metabolism. Lowering of indole and skatole, tryptophan metabolites associated with odor, is a good index in the application of chickpea husk to feed for companion animals, especially those kept indoors. This improved fermentation is related to alterations in the cecal microbial community by chickpea husk feeding, evidenced by the different DGGE banding patterns (Fig. 1) but not the qPCR assay results. It is possible that these bacterial changes are mainly due to the soluble fiber fraction of chickpea husk. In our unpublished data, pure culture study revealed that chickpea husk has lactogenic and bifidogenic effects, possibly caused by the presence of oligosaccharides. Similarly, Madhukumar and Muralikrishna (2012) reported that xylo-oligosaccharides derived from chickpea husk could be utilized by bifidobacteria, lactobacilli and *Pediococci* spp in pure culture. Beside dietary fiber, it is also assumed that modification of the

microbiota might be due to polyphenols present in chickpea husk through inhibitory (Kanatt *et al.* 2011) or stimulatory (Pozuelo *et al.* 2012) effects. Such changes in the bacterial community in the presence of both fibers and polyphenols in chickpea husk might contribute to the increase of SCFA and the decrease of indole and skatole. Cecal samples were taken to evaluate the function of chickpea husk in rat hindgut in the present study, because most of the previous studies on prebiotics have dealt with cecal fermentation. We found some favorable actions of chickpea husk on cecal fermentation (Table 3) and would expect similar ones in lower segment of the large intestine, based on the fact that cecal, colonic and fecal samples of mice share essentially common bacterial operational taxonomic units (Gu *et al.* 2013).

Notably, the lowest total cholesterol concentration was observed in rats fed chickpea husk (Table 3). It can be speculated that such a beneficial effect is probably due to the tannin present in chickpea husk. Tannin inhibits pancreatic lipase and increases fecal excretion of dietary lipids including cholesterol, which indirectly lowers blood lipid and cholesterol levels. In fact, Zou *et al.* (2014) reported hypolipidemic effect of persimmon tannin in rats. Another factor to lower blood cholesterol is dietary fiber. Mallillin *et al.* (2008) point out that all fibers, both soluble and insoluble, can entrap bile acid and prevent its re-absorption. Chickpea husk is rich in such fiber that accelerates excretion of bile acids into feces. Consequently, endogenous cholesterol is utilized for bile acid synthesis in the liver, possibly resulting in lowered blood cholesterol levels. Furthermore, SCFA, mainly propionate and butyrate may suppress cholesterol synthesis in the liver (Beyer-Sehlmeyer *et al.* 2003). Therefore, the above factors as a whole, that is the tannin, fiber and SCFA originating from chickpea husk, might be responsible for the decrease of blood cholesterol in the present study.

The present study confirmed that chickpea husk is a potential source of dietary fiber, antioxidants and cecal bacterial inhibitors or stimulants. Chickpea husk contains functional polyphenols and fibers that could serve as an antioxidant and hindgut modulator in monogastric animals. It is anticipated that more detailed analyses will be performed in future to confirm the mechanisms proposed here.

Chapter 3

Function of lablab bean husk and soybean husk in rats

3.1 Introduction

Lablab bean (*Dolichos lablab*), commonly referred to as hyacinth bean or field bean, is a major pulse crop in South and South East Asia. Feeding with lablab bean husk has been shown to lead to the alteration of rumen microbiota and activity by selective stimulation of specific fibrolytic bacteria (Fuma *et al.* 2012), and to facilitate increased fiber digestibility in sheep (Ngwe *et al.* 2012). These results suggest that this material can be used as an easily digestible supplemental fiber for ruminant animals. Lablab bean husk contains cellulose and hemicellulose (Fuma *et al.* 2012, Htay *et al.* 2014), as well as pectic polysaccharides that incorporate arabinogalactan as a major sugar, including arabinose and rhamnose (Muralikrishna & Tharanathan 1994). Therefore, lablab bean husk is worthy of evaluation for its possible use as a feed ingredient not only for ruminants, but also for monogastric animals.

Soybean (*Glycine max*) husk is widely used as a ruminant feed and has been proposed for use as a feed for lactating dairy cows (Ipharraguerre & Clark 2003). Soybean husk contains complex carbohydrates including pectin, hemicellulose, and cellulose; and 77-88% of the pectin content of soybean husk is contained as galacturonic acid (Mullin & Xu 2001, Monsoor 2005, Karr-Lilienthal *et al.* 2005, Gnanasambandam & Proctor 1999). Specifically, the major cell wall polysaccharide in soybean husk is galactomannan, which is composed of galactose, mannose, arabinose, and glucose (Hussein *et al.* 1998). Soybean husk also has been proposed for use as an energy feed for ruminants (Ipharraguerre & Clark 2003) based on the observation that this material modifies rumen fermentation by permitting increases in the cellulolytic microbial population in dairy cows (Belanche *et al.* 2012), a property also reported for lablab bean husk (Fuma *et al.* 2012, Ngwe *et al.* 2012). Soybean husk was suggested for use as an ingredient in dog food that would not negatively affect nutrient digestion (Cole *et al.* 1999); previous work has shown that consumption of soybean husk yields a decrease in the level of harmful fecal fermentation products, including sulphide and indole, along with an increase in SCFAs with a

concomitant reduction of fecal pH (Simpson *et al.* 2002). Soybean husk feeding in pigs decreases fecal pH and ammonia emission without negatively affecting growth performance (Wang *et al.* 2009); the non-starch polysaccharide in soybean husk is believed to promote the activity of lactobacilli and bifidobacteria that utilize ammonia. Recently, soybean husk fed to piglets has been reported to improve intestinal anatomical morphology and microbial community due to decreased proliferation of *E. coli* (Pascoal *et al.* 2015).

Bean husk is considered an efficient source of soluble and insoluble dietary fiber that could be utilized as a feed supplement for the improvement of gastrointestinal health of monogastric animals. Therefore, supplementation of animal diets with bean husk that contains such beneficial fiber is expected to stimulate the generation of favourable fermentation products in the gastrointestinal tract through microbial modulation. Such intestinal microbiota-mediated functionality of bean husk in monogastric animals is still under investigation.

The objective of the present study was to evaluate the utility of lablab bean husk and soybean husk in cecal fermentation and microbial community regulation in rats; these results are expected to provide insights into the future development of functional foods for monogastric animals.

3.2 Materials and methods

3.2.1 Animals and diets

This feeding experiment was conducted using twenty male Sprague Dawley rats (Japan SLC, Inc., Hamamatsu, Japan), aged 5 weeks old. Rats were housed individually in plastic cages in an environmentally controlled room maintained at 23°C with a 12-h/12-h light-dark cycle (lights on from 12:00-24:00) and 55-60% relative humidity. Rats were divided into four groups (n=5 per group) with an average body weight of 159.1±5.1 g. Diet and water were provided *ad libitum*. One group was given a purified diet (AIN 93G; CLEA Japan Inc., Tokyo, Japan) that contains 5% cellulose powder as a fiber source. This feed (designated CEL) was used as the control diet. The other three groups were given the same purified diet in which the 5% cellulose was replaced by either corn starch (STA), lablab bean

husk (LBH), or soybean husk (SBH). STA did not contain any fiber source and was regarded as a negative control. Corn starch was purchased from Wako (Osaka, Japan). Lablab bean husk was kindly provided by Professor Tin Ngwe (University of Veterinary Science, Myanmar), and was obtained from a local pulse processing plant (Yezin, Nay Pyi Taw, Myanmar) where the beans had been soaked in water and de-hulled by hand. Soybean husk (Japanese product) was obtained through ZEN-NOH (Tokyo, Japan). All of the rats were measured for body weight weekly and for feed intake daily; the feed conversion ratios were calculated from these data. After 3 weeks, rats were euthanized with xylazine HCl. At necropsy, gastrointestinal segments (oesophagus, stomach, small intestine, caecum, and colon) and their contents were harvested and weighed. Cecal digesta was sampled and stored at -80°C for the analysis of fermentation products and microbiota. Blood was sampled from the heart by syringe, centrifuged to obtain plasma, and frozen as above. The animal experiment was carried out in compliance with the Guidelines for Animal Experiments, Hokkaido University (2007) and the Act on Welfare and Management of Animal (2005).

3.2.2 Chemical analyses of bean husk

The chemical composition of both types of bean husk was analysed in triplicate, including testing for dry matter, crude protein, crude fat, and crude ash using the Association of Official Analytic Chemists methods (AOAC 1990). NDF and ADF were determined using a fiber analyser (A200, ANKOM, Fairport, NY). Heat stable amylase was used in the NDF determination; the resulting values are inclusive of residual ash. Analysis of ADL was also performed (Goering & Van Soest 1975). For sugar analysis, the soluble fraction of the bean husk was obtained by water extraction (100 g husk per litre water for 24 h). The resulting extract was freeze-dried; a 500-mg aliquot was dissolved in 1.2 ml of water and the sugar composition was analysed by thin-layer chromatography (TLC) using a silica gel 60 F₂₅₄ plate (Merck, Darmstadt, Germany). The TLC developing solvent consisted of isopropanol/1-butanol/water (12:3:4, v/v/v). Maltooligosaccharide mixture (2% w/v) was used as a standard. Sugars were visualized by spraying the TLC plate with anisaldehyde-sulfuric acid reagent (1% anisaldehyde

and 2% sulfuric acid in acetic acid) followed by heating of the plate. To purify the oligosaccharides included in the water extract, the extract was separated by gel-filtration column chromatography using a Bio-gel P-2 Extra Fine column (Bio-Rad, Hercules, CA); 1.5 cm i.d. \times 100 cm). Water was used as mobile phase with a flow rate of 3 ml/h. One hundred fractions (4 ml per tube) were collected, and sugar concentrations were measured by the phenol-sulfuric acid method (Dubois *et al.* 1956) using glucose as a standard. Fractions containing oligosaccharides, including fractions S1, S2, S3, L1, L2, and L3 (see Results), were used for further analyses. Molecular masses of the purified oligosaccharides were analysed by electrospray ionization mass spectrometry (ESI-MS) using an Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA). Structural characterization of fractions L1 and L2 was performed by dissolving the samples in D₂O and obtaining ¹³C-NMR spectra using a Bruker AMX500 NMR spectrometer (Bruker, Germany).

3.2.3 Measurement of blood parameters and cecal fermentation products

Blood plasma was analysed for glucose and total cholesterol by using commercially available kits (Wako). Cecal digesta (1 g) was suspended in saline (5 ml) and vortexed, and the mixture was then centrifuged (4°C, 7,800 $\times g$, 5 min). The resulting supernatant was used to measure pH by electrode (F-51, HORIBA, Kyoto, Japan), and also subjected to the analysis of SCFA, lactate, succinate, ammonia, indole, and skatole concentrations. SCFA was determined by gas chromatography (GC-14B, Shimadzu, Kyoto, Japan) equipped with 0.53 mm \times 30 m capillary column (ULBON HR-20M, Shimadzu) as previously reported (Suto 1973). D-lactate, L-lactate, and succinate were measured by D/L Lactic Acid Assay Kits and Succinic Acid Assay Kits (Megazyme, Bray, Ireland) according to manufacturer's instructions for the respective kits. Ammonia nitrogen (NH₃-N) was measured by the phenol-hypochlorite reaction method (Weatherburn 1969) using a microplate reader (ARVO MX; Perkin Elmer, Waltham, MA) at 660 nm. Assays for indole and skatole were carried out by colorimetric methods (Walstra *et al.* 1999).

3.2.4 Microbiota analysis by qPCR

Bacterial DNA from each cecal sample was extracted using the repeated bead beating plus column method (Yu & Morrison, 2004) with a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Purified DNA was diluted appropriately before further analysis (for qPCR and 16S rDNA clone library). Bacterial communities were quantitatively characterized by qPCR assays with species/group-specific primers for the representative groups, including total bacteria, total lactobacilli, different *Lactobacillus* species (*L. acidophilus*, *L. gasseri*, *L. johnsonii*, *L. reuteri*), total bifidobacteria, *F. prausnitzii*, *E. hallii*, *Clostridium coccoides*-*Eubacterium rectale* group, *Bacteroides*-*Prevotella*-*Porphyromonas* group (Rinttila *et al.* 2004), *E. coli*, and *C. perfringens*, using a LightCycler 480 real-time PCR system (Roche Applied Science, Mannheim, Germany). Primers for OTU 4, OTU 5, OTU 6, and OTU 10 (detected in the 16S rDNA library; see Results) were newly designed. The sequences of the primers (5'-3') for these assays were F: ACGCGAAGAACCTTACCTGA and R: GCACGTGTGTAGCCCTGG for OTU 4 (*Dorea massiliensis*), F: ACGTGACGGTACCTGGA and R: GGGACACAGTTTTGAGAG for OTU 5 (*Oscillibacter* relatives), F: GGACTCGCGTCCGATTAGT and R: ACTGGTGTTCCTCCCCATC for OTU 6 (*Ruminococcus bromii*), and F: CAGCACGTGAAGGTGGGGAC and R: CCTTGCGGTTGGCTTCAGAT for OTU 10 (*Akkermansia muciniphila*). Primer specificity, quantification range, and other validation reactions were performed as described by Koike *et al.* (2007). Results from samples were averaged among the animals in each dietary group and reported as a log value (gene copy number per g digesta) for total bacteria and also as a relative abundance (%) of each species or group in total bacteria.

3.2.5 Microbiota analysis by 16S rDNA clone library

Bacterial DNA from each cecal digesta was amplified by PCR with the primers 27F (AGAGTTTGATCATGGCTCAGA) and 1525R (AAGGAGGTGWTCCARCC). The reaction mixture for library construction consisted of 2.5 µl of 10 × Ex-*Taq* buffer, 2.0 µl of 2 mM dNTP, 0.5 µl of each primer, 18.375 µl of water, 0.125 µl of Takara Ex-*Taq* polymerase, and 1.0 µl of template DNA. PCR conditions (22 cycles) were as follows: initial denaturation at 94°C for 0.5 min, annealing at 58°C for 0.5 min, and

extension at 72°C for 1.5 min. Final extension was performed at 72°C for 7 min. The PCR product was purified by a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) after performing agarose gel electrophoresis, then ligated with pGEM-T Easy Vector (Promega, Madison, WI) and transformed into *E. coli* JM109 (Nippon Gene, Tokyo, Japan). White colonies that developed on ampicillin- and Xgal-containing Luria-Bertani plates were employed for plasmid isolation using a QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany); the purified plasmid DNA was subjected to sequence analysis (TAKARA, Shiga, Japan). Sequence classification was conducted using Mothur ver. 1.29 software (Schloss *et al.* 2009). After removal of the chimeric sequences, sequences sharing $\geq 97\%$ identity were grouped into operational taxonomic units (OTUs). The nucleotide sequences were compared with known sequences in the GenBank database using BLASTn (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to obtain similarity values. The Clustal X program (<http://www.clustal.org/>) for multiple sequence alignment and phylogenetic analysis was used. A neighbour-joining tree with a Kimura-2 correction was created (Collins *et al.* 1994). The sequences obtained were deposited in the DDBJ nucleotide sequence database under the accession numbers AB969309 through AB969665.

3.2.6 Microbiota analysis by MiSeq

For the comprehensive analysis of microbial communities, MiSeq analysis was performed. Hypervariable V3-V4 regions of 16S rDNA were amplified by using the universal bacterial primer set (Herlemann *et al.*, 2011), consisting of S-D-Bact-0341-b-S-17 (CCTACGGGNGGCWGCAG) and S-D-Bact-0785-a-A-21 (GACTACHVGGGTATCTAATCC), where N stands for A, C, G or T, W for A or T, H for A, T or C, and V for A, C or G. The PCR reaction mixture (50 μ l) contained 10 μ l of 5 \times PrimeSTAR buffer, 4.0 μ l of dNTP, 1.0 μ l of each primer, 32.5 μ l of water, 0.5 μ l of PrimeSTAR polymerase, and 1.0 μ l of template DNA. PCR reaction conditions (30 cycles) were as follows: denaturation at 98°C for 10 sec, annealing at 55°C for 15 sec, and extension at 72°C for 30 sec. Following amplification, DNA was purified using a High Pure PCR Product Purification Kit (Roche) and sequenced (Illumina Miseq 2000, San Diego, CA).

Raw Illumina fastq files were demultiplexed, qualitatively checked, and analysed by Quantitative Insights into Microbial Ecology (QIIME v1.9.0) as described previously (Caporaso *et al.*, 2010).

3.2.7 Statistical analysis

All data were analysed by a one-way analysis of variance (ANOVA). Tukey's procedure for multiple comparison was used to resolve differences among treatment means by using SPSS 16.0 software (SPSS, Chicago, IL, USA). Differences were considered to be significant if $P < 0.05$ and trend if $P < 0.10$.

3.3 Results

3.3.1 Nutritional composition of bean husk

Chemical composition of the two different types of bean husk tested is shown in Table 5. Insoluble dietary fiber, represented by cellulose and hemicellulose, predominated in both types of bean husk. Non-fibrous carbohydrates, consisting of soluble components such as starch, soluble sugars, and soluble fibers, accounted for 23.6% in lablab bean husk and 24.0% in soybean husk. TLC analysis indicated the presence of three different oligosaccharides in each type of husk (denoted as L1, L2, and L3 for lablab bean husk, and S1, S2 and S3 for soybean husk, with respective degrees of polymerization as shown in Figure. 2). After purification by gel-filtration, the molecular mass of the individual oligosaccharides in sodiated form, determined by ESI-MS, were estimated as follows: L1 and S1 had the same mass of 504 Da, while L2 and S2 shared the same mass of 666 Da. L3 and S3, whose molecular mass was estimated as 794 Da, could not be further analysed, presumably due to complexity of their structure. The ^{13}C -NMR spectra of L1 and L2 coincided exactly with those of authentic raffinose and stachyose, respectively. Therefore, L1 and L2 were identified as raffinose and stachyose, respectively. Lablab bean husk was found to contain 0.24% oligosaccharides, in which raffinose (0.04%), stachyose (0.12%), and unknown oligosaccharides (0.1%) were constituents. In the

same manner, soybean husk was found to contain 0.26% oligosaccharides, in which raffinose (0.03%), stachyose (0.2%), and unknown oligosaccharides (0.03%) were constituents.

3.3.2 Growth and cecal characteristics of rats

Growth performance and cecal fermentation profiles are summarized in Table 6. For animals maintained for 3 weeks on the diets, no significant difference was found among the groups for feed intake, body weight gain, feed conversion ratio (all in Table 6), or weights of GI segments, contents, and internal organs (data not shown). Rats fed LBH and SBH showed lower ($P < 0.05$) cecal pH than those fed CEL and STA. The total SCFA concentration in the cecum ($P < 0.05$) was elevated in both the LBH and SBH groups compared to those in the CEL and STA groups. Similarly, the cecal acetate concentration was higher ($P < 0.05$) in LBH and SBH than in CEL and STA. The butyrate concentration in SBH was higher ($P < 0.05$) than that in CEL and STA, but did not differ from that in LBH; butyrate in LBH was higher ($P < 0.05$) than that in STA but did not differ from that in CEL. Total lactate concentration was higher in SBH and STA than in CEL, an effect that was largely due to the higher concentration ($P < 0.05$) of L-lactate. Succinate concentration in SBH was the highest among the groups and significantly higher ($P < 0.05$) than those in CEL and STA, but did not differ from that in LBH. Ammonia concentration did not differ among the groups. Rats fed STA showed the highest concentration of indole and skatole among the groups. For indole, LBH-fed rats exhibited the lowest levels, followed by CEL and then SBH. However the SBH effect on indole did not achieve significance. Skatole concentrations were lowest in rats fed LBH followed by CEL and SBH, and all of these were lower ($P < 0.05$) than STA.

3.3.3 Microbiota

A phylogenetic tree of clones derived from the bacterial 16S rDNA clone library analysis is shown in Figure 3. A total of 503 clones having nearly full-length 16S rDNA sequences were obtained and formed 39 OTUs. *Lactobacillales*, *Clostridium* cluster IV, and *Clostridium* cluster XIVa were the

major clusters, to which 434 of 503 clones and 29 of 39 OTUs belonged. Bacteria belonging to OTU 4 (*Dorea massiliensis*) were frequently detected in STA (85% in total clones) but not in LBH or SBH. Bacteria belonging to OTU 5 (*Oscillibacter* relatives) were frequently seen in LBH (58%) and CEL (42%) but not in SBH or STA. OTU 6 (*Ruminococcus bromii*) was detected in CEL (53%), LBH (16%), and SBH (31%), but not in STA. OTU 10 (*Akkermansia muciniphila*) was detected only in LBH and SBH, in proportions of 67 and 33%, respectively.

New qPCR assays for OTU 4 (*Dorea massiliensis*), OTU 5 (*Oscillibacter* relatives), OTU 6 (*R. bromii*), and OTU 10 (*A. muciniphila*) were successfully established with amplification efficiencies of 1.866-1.982 within ranges of 10^2 - 10^9 copies of the respective target DNA. Each primer set had specificity sufficient to identify the target bacteria, as validated by mini-clone library sequencing (24-72 clones for each exhibited 100% identity in sequence). The qPCR assay values for representative cecal bacteria are shown in Table 7. The *Lactobacillus* group was the dominant bacterial taxon in the cecal digesta. In comparison with CEL and STA, SBH showed a higher ($P < 0.05$) proportion of total lactobacilli, *L. acidophilus*, and *L. reuteri*, but proportions in SBH did not differ significantly from those in LBH. Rats fed LBH had higher ($P < 0.05$) abundance of *A. muciniphila* than those fed CEL and STA. However, the abundance of *A. muciniphila* did not differ significantly between rats fed LBH or SBH. LBH also showed a higher ($P < 0.05$) proportion of bacteria belonging to OTU 5 (*Oscillibacter* relatives) in comparison with STA and SBH, but this proportion did not differ from that with CEL. Rats fed on STA showed a higher ($P < 0.05$) proportion of *Dorea massiliensis* compared to the rats fed LBH or SBH. However this proportion did not differ significantly from that in rats fed CEL. The abundance of total bacteria, *L. gasseri*, *L. johnsonii*, *F. prausnitzii*, *E. hallii*, total bifidobacteria, *R. bromii*, *E. coli*, *Bacteroides-Prevotella-Porphyromonas* group, and *Clostridium coccoides-Eubacterium rectale* group were not affected by diets.

After quality filtering of the MiSeq data, a total of 1,011,982 sequence reads (CEL: 234987, STA: 290834, LBH: 251263, SBH: 234898) remained from 20 samples. Alpha diversity indices including Chao 1, observed species, Shannon, and phylogenetic diversity, and relative abundance of microbial

taxa at phylum and genus level ($> 0.01\%$) are shown in Table 8. No difference was found among the groups for alpha diversity indices. The number of observed species ranged from 441-603. At the phylum level, rats fed SBH showed a higher ($P < 0.05$) abundance of Actinobacteria than those fed CEL or STA, but the value in SBH did not differ significantly from that in LBH. Within the Actinobacteria phylum, members of the genus *Bifidobacterium* showed different abundances, with LBH and SBH showing higher ($P < 0.05$) abundance than CEL and STA. The relative abundance of Bacteroidetes did not differ among the diet groups. However, rats fed LBH or SBH showed higher ($P < 0.05$) abundances of genus *Prevotella* than did those fed CEL or STA. STA showed higher ($P < 0.05$) relative abundance of unclassified Rikenellaceae than did LBH and SBH, but the value did not differ significantly from that of CEL. Firmicutes was the most abundant bacterial phylum detected in the cecum of the rats. In comparison with CEL, LBH showed lower ($P < 0.05$) abundance of Firmicutes, but the value did not differ significantly from that of STA and SBH. Within this phylum, *Lactobacillus* was highest ($P < 0.05$) in SBH in terms of relative abundance. STA showed higher ($P < 0.05$) relative abundance of genus *Dorea* than did LBH and SBH, but the STA value did not differ significantly from that of CEL. The relative abundance of unclassified Ruminococcaceae was found to be higher ($P < 0.05$) in CEL than in SBH, but the value was not significantly different from that in LBH or STA. Rats fed LBH had a higher ($P < 0.05$) abundance of phylum Verrucomicrobia than did rats fed CEL and STA, but LBH and SBH did not differ significantly for this parameter. Almost all phylum Verrucomicrobia were constituted by genus *Akkermansia*, the proportion of which was higher ($P < 0.05$) in LBH than in CEL and STA.

3.4 Discussion

The addition of 5% bean husk to the diet of rats had no significant effect on feed intake, body weight, feed conversion ratio, or anatomical data, including the weights of GI tract segments and contents. Blood parameters such as glucose and cholesterol were within normal ranges and not different among the groups maintained on distinct diets (data not shown). Therefore, either of two different types of bean husk is proposed for use as a feed ingredient for monogastric animals;

supplementation of rat feed with 5% bean husk did not exhibit detrimental effects on growth performance or general health status.

Since the husk of different beans contains different amount of dietary fiber (Table 5), their functions may be inherently different in the cecum of rats. Also, separation of soluble sugars revealed the presence of oligosaccharides, including raffinose, stachyose, and others, in the two tested species of bean husk. Soybean husk contained higher level of stachyose, which is a non-reducing tetrasaccharide composed of two galactoses, one glucose, and one fructose. Lablab bean husk feeding yielded distinct effects in the cecum such as increases of SCFAs, especially acetate; soybean husk feeding yielded similar effects but with a distinguishing feature of increased lactate (Table 6). The increases of these beneficial organic acids with both types of bean husk were probably the main reason for the decrease of pH, reflecting fermentation of bean husk fiber in the cecum. The increase in the concentration of acetate by lablab bean husk feeding might correspond to the increase in the proportion of acetate-producing bacteria, particularly *Akkermansia*, that was detected by the MiSeq, clone library, and qPCR analyses; a similar effect on bifidobacteria was supported only by the MiSeq data (Tables 7 and 8, Figure. 3). Previous studies in obese mice have suggested that dietary fibers such as oligofructose favour the growth of *Akkermansia* in the cecum (Everard *et al.* 2011, Everard *et al.* 2013). The abundance of *Akkermansia* inversely correlates with body weight and type 1 diabetes in mice and humans (Everard *et al.* 2013, Santacruz *et al.* 2010). Therefore, increased abundance of *Akkermansia* by lablab bean husk feeding is expected to yield a beneficial health impact in animals. On the other hand, soybean husk feeding yielded increases in acetate and lactate concentrations, possibly as a result of the enrichment of total lactobacilli, including *L. acidophilus* and *L. reuteri* that was detected by qPCR and MiSeq analyses. These lactobacilli, particularly *L. reuteri*, have been found to initially utilize raffinose-family oligosaccharides, which are converted to α -galactooligosaccharide (Teixeira *et al.* 2012). α -galactooligosaccharide is presumably further utilized by other acetate and lactate producers such as bifidobacteria, as detected by the MiSeq analyses in the present study. Lactobacilli and bifidobacteria are well known for their probiotic activities, increasing in abundance in

rats upon supplementation of feed with raffinose oligosaccharides (Dinoto *et al.* 2006) and pectic arabinogalactan (Daguet *et al.* 2006). Lablab bean husk feeding was also distinguished from soybean husk feeding by its stimulatory effect on bacteria belonging to OTU 5, a member of *Clostridium* cluster IV and the closest relative to *Oscillibacter valerigienes*. Relatives of *Oscillibacter* were found to increase in animals fed diets rich in fermentable polysaccharides such as resistant starch (Walker *et al.* 2011). Another distinct phenomenon in soybean husk feeding was the increased cecal level of succinate, a precursor of propionate; this succinate may have been produced by *Prevotella*, a genus whose abundance increased from 0 to 0.01% on feeding with husk-supplemented diets. Therefore, in the present study, the raffinose family oligosaccharides and/or possibly pectin contained in both types of bean husk are hypothesized to be a main cause for the observed lactogenic and bifidogenic effects, and for the increased abundance of other beneficial bacteria.

Interestingly, bacteria related to *Dorea* spp. (a constituent of *Clostridium* cluster XIVa), regarded as a potentially harmful group, were not found (as assessed by the clone library) in rats fed either type of bean husk. Lower values of this group for both LBH and SBH than for STA in the qPCR assay and in the MiSeq analysis, may suggest functional improvement of the gastrointestinal health of the host. Hooda *et al.* (2012) reported that the fecal population of *Dorea* is decreased in healthy men consuming soluble polysaccharides. If the same situation applies in the present study, the presence of pectic polysaccharide in both of bean husk appears to have indirectly inhibited the growth of *Dorea* spp. in the caecum of rats. Increased abundance of the members of *Clostridium* cluster XIVa, including *Dorea* spp., has been observed in patients with irritable bowel syndrome (Rajilic-Stojanovic *et al.* 2011). Thus, present findings indicated that having a diet without any fiber (for example starch) can create health problems and may be a risk factor for intestinal health. Bacterial coverage in our clone library was not sufficiently wide in the present study, given the small number of sequence reads (503 in total). However, the sequences that were obtained showed changes in the clone libraries associated with the various feeding regimens; these differences were useful for focusing attention on specific groups of bacteria (*Akkermansia*, *Dorea*, etc.), thereby suggesting the development of primers for

quantification of those groups. In fact, qPCR assay values for these groups corresponded well with the detection frequency of those groups in the MiSeq data.

The present study raised the possibility that there was a reduced population of opportunistic and pathogenic bacteria in the low-pH condition of the cecum, such that production of harmful fermentation products remained low as a result of the enhanced growth of beneficial bacteria such as lactobacilli and bifidobacteria. In particular, the lowered indole and skatole in the cecum of rats fed either type of bean husk might be due to fibers that regulate bacterial nitrogen metabolism (Xu *et al.* 2002). Additionally, the insoluble fibers are expected to adsorb these bacterial putrefactive compounds, facilitating the excretion of those products. Similar findings were obtained in a previous report (An *et al.* 2013), indicating that fermentable fibers (including oligosaccharides and polysaccharides) suppress the accumulation of the bacterial putrefactive compounds that are responsible for the malodour of flatus and feces. Therefore, we hypothesize that the formation of indole and skatole in the cecum were suppressed primarily by the presence of dietary fibers in the bean husks, which provide physical features such as increased adsorption ability, acceleration of excretion, and other functional activities.

In conclusion, supplementation with bean husk promoted cecal fermentation in rats, as indicated by higher concentrations of SCFAs and lactate, and decreased accumulation of bacterial putrefactive compounds. Two types of husk were tested; both exhibited remarkable selectivity against gut bacteria. Similar bifidogenic effects and suppressive effects on *Dorea* spp. were found with both lablab and soybean husks.

With respect to specific effects on rat cecal microbiota, lablab bean husk stimulated the growth of *A. muciniphila* and *Oscillibacter*-related bacteria, while soybean husk stimulated the growth of members of the *Lactobacillus* group, including *L. acidophilus* and *L. reuteri*. Both types of bean husk were shown to contain oligosaccharides that might enrich for these beneficial bacteria, thereby inhibiting the colonization of the intestinal tract by potential pathogens. Therefore, either of the

tested bean husk types is expected to serve as a promoter of hindgut health and could find use as a functional feed ingredient for monogastric animals.

Chapter 4

Function of soybean husk in dogs

4.1 Introduction

Soybean husk represents a large agro-byproduct and contains 63-81% total dietary fiber, of which 4-12% is soluble fiber (Cole *et al.* 1999). Because of these dietary fibers and polyphenols (Zhang *et al.* 2011), soybean husk can be considered as an alternative functional food supplement for monogastric animals. Both soybean and its husk contain oligosaccharides such as raffinose (0.3-1%), stachyose (0.8-4%) and verbascose (0.1-0.2%) (Fan *et al.* 2015). Recent study indicated that dietary supplementation with soybean husk decreased *E. coli* numbers and increased acetate production in the gastrointestinal (ileal) tract of piglets (Pascoal *et al.* 2015). More recently, in a rat study, soybean husk was proposed as a functional food ingredient in light of its solubility, viscosity, water-holding ability, cholesterol-binding and bile acid-binding capacities and hypocholesterolemic activity (Liu *et al.* 2016).

Accordingly, soybean husk is a potential health promoting additive not only for domestic animals, but also for long-living monogastrics such as companion animals. In fact, dietary supplementation with 16% soybean husk reduced the body weight of obese dogs by restricting energy absorption without any changes in behavior or feed intake (Sabchuk *et al.* 2014). To the best of our knowledge, little study on the effect of soybean husk supplementation of pet foods on intestinal microbes has been conducted since Cole *et al.* (1999) reported that soybean husk (as an effective dietary fiber source) did not negatively affect nutrient digestibility in dogs. A study employing dietary supplementation with a combination of 5% each of soybean husk and beet pulp in dogs showed improved fermentation; however, no specific changes in the microbiota as analyzed by denaturing gradient gel electrophoresis were observed (Simpson *et al.* 2002). With its potential nutritive and physiological effects, soybean husk should be investigated for its functional capacity relating to the gastrointestinal microbiota and health of dogs.

Thus, the objective of this study was to obtain preliminary information regarding this functional fiber source in improving the gastrointestinal health of dogs by focusing on fecal fermentation characteristics and microbes.

4.2 Material and methods

4.2.1 *In vitro* fermentation experiment

Cellulose (Sigma-Aldrich, Tokyo, Japan), soybean husk (ZEN-NOH, Tokyo, Japan) (Table 9) and enzyme-treated soybean husk were used as substrates for the *in vitro* fermentation experiments. In comparison with pure cellulose as a control fiber, we expected to assess physicochemical functions specific to soybean husk. Soybean husk was ground using a 1 mm sieve and employed in the fermentation study. Enzyme-treated soybean husk (to simulate stomach and small intestinal digestion) was prepared according to the method of Panasevich *et al.* (2013). Briefly, 1 g of soybean husk for triplicate tubes was weighed and incubated for 6 h in 25 mL of 0.1 M phosphate buffer, 10 mL of 0.2 M hydrochloric acid and 1 mL of 1% pepsin solution (Wako, Osaka, Japan) at 39°C in a shaking water bath to simulate gastric digestion. Then, the pH was adjusted to 6.8 by 6N NaOH (6M NaOH in 1 L water) and 1 mL of 5% pancreatin solution (Sigma-Aldrich) was added to each tube. Incubation continued at 39°C for 18 h to simulate small intestinal digestion. Digesta samples were precipitated by the addition of four volumes of 95% ethanol, allowed to stand for 1 h and filtered by suction. Residues were dried to a constant weight and applied to the *in vitro* fermentation experiment as enzyme-treated soybean husk. Cellulose was also treated in the same manner and used as a control substrate.

Feces as the inoculum source were obtained from three Toy Poodle dogs (6.5 ± 3.5 months in age, 2.9 ± 0.4 kg in body weight) in a pet shop (Petland, Sapporo, Japan). The animals were housed individually and fed a commercially available dry diet (120 g/d, Royal Canin Mini Junior; ROYAL CANIN JAPON, Tokyo, Japan, Table 9) twice daily (08.00 and 17.00 hours) with free access to drinking water. Freshly voided feces (within 15 min of defecation) were collected. Equal amount of feces from three

dogs were mixed and diluted (1:10 w/v) with McDougall's buffer (McDougall 1948), homogenized, filtered through four layers of surgical gauze and sealed in a 125 mL serum bottle into which CO₂ was flushed. Two series of *in vitro* fermentation experiments, one for intact soybean husk and the other for enzyme-treated soybean husk, were performed. A 0.1 g portion of substrate in triplicate Hungate tubes was added to 10 mL of fecal inoculum under CO₂ flushing and incubated at 39°C for 24 h. Cellulose (Table 1) was used as a control substrate. After incubation, samples were analyzed for pH, SCFA, lactate, ammonia, indole and skatole. The remaining samples were frozen at -80°C for microbial analyses.

4.2.2 *In vivo* feeding experiment

Four healthy Shiba dogs (7–48 months in age and 7.5 ± 1.7 kg in body weight) reared by a dog breeder (Shiroi, Chiba, Japan) were used. All dogs were housed individually and fed twice daily (08.00 and 17.00 hours) with commercial diet (170 g/day, three dogs fed Aikengenki; Unicharm, Tokyo, Japan, or one dog fed Science Diet; Hill's Colgate, Tokyo, Japan, Table 9) to which 10 g cellulose powder or soybean husk powder were supplemented (5.6% of total diet). The fiber sources were the same as used for the *in vitro* experiment. The dogs were fed the cellulose-supplemented diet for the first 7 days, and then were switched to the soybean husk-supplemented diet for another 7 days. On the 6th and 7th day of each dietary period, feces were collected a total of four times (morning and evening for both days) and mixed. Mixed feces from individual dogs in each period (1 g) was suspended in 10 mL of distilled water, homogenized, and centrifuged (4°C, 12,000 × *g*, 2 min), and the supernatants were used for the analysis of fermentation metabolites. Fecal samples were also employed for DNA extraction to monitor fecal microbes. The study protocol conformed to the Guidelines for Animal Experiments, Hokkaido University (2007) and the Act on the Welfare and Management of Animals (2005). Written informed consent was obtained from the owners of all animals used.

4.2.3 Chemical analysis

The proximate analysis of soybean husk was performed according to the methods of the Association of Official Analytic Chemists (AOAC 1990). Fiber fractions including NDF, ADF and ADL were determined according to the methods described by Goering and Van Soest (1975), where NDF was assayed using a heat-stable amylase. The values were expressed inclusive of residual ash. Cellulose was calculated as the difference between ADF and ADL. Hemicellulose was calculated as the difference between NDF and ADF. Non-structural carbohydrate (NSC) or non-fibrous carbohydrate (NFC) were calculated by subtracting the sum of NDF, ash, protein and fat from 100. Soluble dietary fiber was determined using total dietary fiber assay kits K-TDFR (Megazyme International, Wicklow, Ireland) according to the manufacturer's instructions. All analyses were performed in triplicate and the results are reported on a dry matter basis. Determinations of total polyphenols, total flavonoids and total tannin were performed.

Fecal pH was measured using a pH electrode (F-51; HORIBA, Kyoto, Japan), while ammonia was determined by the phenol-hypochlorite reaction method, and indole and skatole by colorimetric methods using a spectrophotometer (ARVO MX; Perkin Elmer, Yokohama, Japan). For SCFA and lactate, 0.2 g of feces were mixed with 600 μ L of distilled water and 200 μ L of 25% meta-phosphoric acid, vortexed, kept at 4°C overnight, and centrifuged (4°C, 13,000 $\times g$, 20 min). The supernatant obtained was subjected to gas chromatographic analysis of SCFA (GC-14B; Shimadzu, Kyoto, Japan) using a 0.53 mm \times 30 m capillary column (ULBON HR-20M; Shimadzu) and D/L-lactic acid assay of lactate using a kit (Megazyme, Bray, Ireland) according to the manufacturer's instructions.

4.2.4 Microbial analysis

DNA was extracted from *in vitro* cultures and *in vivo* fecal samples and purified using the repeated bead beating plus column (RBB+C) method (Yu & Morrison 2004) with QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The DNA concentration was quantified by a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Extracted fecal DNA was appropriately diluted and

subjected to qPCR assay to characterize the following representative bacterial species/groups: total bacteria, total lactobacilli, four species of *Lactobacillus* (*L. acidophilus*, *L. johnsonii*, *L. gasseri* and *L. reuteri*), total bifidobacteria, *Clostridium* cluster IV (*Clostridium leptum* subgroup) (Matsuki *et al.* 2004), *F. prausnitzii*, *Clostridium* cluster XIVa (*Clostridium coccoides* group) (Song *et al.* 2004), *E. hallii*, *Clostridium* cluster XI (Song *et al.* 2004), *C. perfringens*, *Clostridium* cluster I (Song *et al.* 2004), *Bacteroides-Prevotella-Porphyromonas* group, *Prevotella* (Matsuki *et al.* 2002), *Akkermansia muciniphila* (Png *et al.* 2010) and *E. coli* using specific primers. The qPCR assay was performed using a Light Cycler 480 real-time PCR system (Roche Applied Science, Mannheim, Germany). Results are reported as a log value of the gene copy number per gram feces for total bacteria and as a relative proportion of each species/group in total bacteria for specific bacteria.

4.2.5 Statistical analysis

Student's *t*-test (SPSS 16.0 software; SPSS Inc., Chicago, IL, USA) was performed to determine the significance of changes in the fermentation metabolites and bacterial species/groups in samples from both *in vitro* and *in vivo* experiments. *P*-values of less than 0.05 and less than 0.1 were considered as statistically significant and trend, respectively.

4.3 Results

4.3.1 *In vitro* fermentation experiment

Fermentation metabolites resulting from the *in vitro* fermentation with intact or enzyme-treated soybean husk are presented in Table 10. Incubation with either intact soybean husk or enzyme-treated soybean husk produced greater ($P < 0.01$) amounts of total SCFAs (including acetate, propionate and butyrate) and lactate, with a significant reduction ($P < 0.05$) in pH compared with cellulose. Only acetate was increased ($P < 0.01$) in enzyme-treated soybean husk compared with cellulose in terms of the molar proportion. Lower ($P < 0.01$) amounts of indole and skatole were detected for intact soybean husk than cellulose, whereas enzyme-treated soybean husk showed a

decreasing trend ($P < 0.1$) of indole production compared with cellulose. Neither intact nor enzyme-treated soybean husk affected ammonia production.

The qPCR analysis of microbes in the *in vitro* fermentation with intact or enzyme-treated soybean husk is presented in Table 11. Fermentation with either intact or enzyme-treated soybean husk resulted in increased ($P < 0.05$) numbers of bifidobacteria as compared with cellulose. Levels of total bacteria, total lactobacilli and *E. coli* were unaffected by supplementation with intact and enzyme-treated soybean husk.

4.3.2 *In vivo* feeding experiment

Fecal fermentation metabolites of dogs fed diets supplemented with either soybean husk or cellulose are presented in Table 12. Soybean husk supplementation significantly decreased ($P < 0.01$) fecal pH compared with cellulose. Feces of dogs fed the soybean husk diet had higher ($P < 0.05$) levels of total SCFAs, acetate, butyrate and lactate than those fed the cellulose diet. Propionate was unaffected by soybean husk supplementation. Soybean husk had no effect on the molar proportion of SCFA. The fecal concentrations of indole and skatole in dogs fed the soybean husk diet were lower ($P < 0.05$) than those fed the cellulose diet. Fecal ammonia concentration was unaffected by soybean husk supplementation.

Fecal microbiota of dogs fed a diet supplemented with either soybean husk or cellulose is presented in Table 13. Soybean husk supplementation led to a greater ($P < 0.05$) relative proportion of total lactobacilli than observed for cellulose supplementation. The levels of various *Lactobacillus* species, including *L. acidophilus*, *L. johnsonii*, *L. gasseri* and *L. reuteri* were unaffected by soybean husk supplementation. The levels of *Clostridium* cluster IV and its member *F. prausnitzii* were higher ($P < 0.05$) in dogs fed the soybean husk diet than in those fed the cellulose diet. Dogs fed the soybean husk diet showed a higher ($P < 0.05$) *Clostridium* cluster XIVa level than those fed the cellulose diet. In contrast, there was a reduction ($P < 0.01$) in the proportion of *Clostridium* cluster XI in dogs fed soybean husk diet compared with those fed cellulose diet. The proportion of *Bacteroides-Prevotella*-

Porphyromonas group was higher ($P < 0.05$) in dogs fed soybean husk diet than in those fed cellulose diet. The levels of total bacteria, total bifidobacteria, *E. hallii*, *C. perfringens*, *Clostridium* cluster I, *Prevotella*, *A. muciniphila* and *E. coli* were not affected by soybean husk supplementation.

4.4 Discussion

In vitro study revealed that soybean husk supplementation significantly increased fermentation products such as SCFAs and lactate, indicating the fermentability of soybean husk, in comparison with cellulose (Table 10). This was also confirmed by supplementation with enzyme-treated soybean husk, although a smaller increase compared to intact soybean husk was observed. This difference in fermentability between intact and treated soybean husk is due to the disappearance of rapidly fermentable components during enzymatic digestion, leaving behind the more slowly fermenting components such as cellulose. Because intact soybean husk is rich in both soluble and insoluble dietary fiber, the microbiota in the culture could be modulated, leading to a decrease in putrefactive compounds, such as indole and skatole, produced from the remaining fecal protein. The pH-lowering and bifidogenic effects of soybean husk (Tables 10 and 11) are proposed to be key factors for reducing the production of these bacterial putrefactive compounds. This has been suggested to benefit gastrointestinal health (Jha and Berrocso, 2016). However, this effect was not obvious in enzyme-treated soybean husk, with the exception of the tendency toward decreased indole levels. Overall, the *in vitro* fermentation study suggested that soybean husk can be favorably fermented in the hindgut of dogs, possibly contributing to gut health.

Dietary supplementation of cellulose or soybean husk did not affect feed intake and body weight, and showed no detrimental effects such as diarrhea and general illness in dogs used. Although we did not collect nutritional data, adverse effect on growth performance of dogs might not be expected by supplementing 5% soybean husk in the diet as suggested by Cole *et al.* (1999). The feces of dogs on soybean husk diet had higher SCFAs including acetate and butyrate, and also lactate, leading to a low pH (Table 12). These beneficial organic acids are indicative of increased microbial

activity, reflecting changes in specific groups of bacteria in dogs fed the soybean husk diet. Soybean husk appears to be butyrogenic, since both the concentration and molar proportion of butyrate increased. This might be attributable to the increased proportion of butyrate producers in *Clostridium* clusters IV and XIVa (Table 13). The increased proportion of *Clostridium* cluster IV following soybean husk supplementation is likely due to increased numbers of *F. prausnitzii*. *Fecalibacterium* spp. are prominent in the feces of healthy dogs (Garcia-Mazcorro *et al.* 2012) and these species have been shown to possess anti-inflammatory properties, evidenced by the protective effect of butyrate production on inflammatory bowel disease in dogs (Suchodolski *et al.* 2012). The increase in such butyrate producers could be explained by the increased abundance of total lactobacilli, as there is cross-feeding mechanism between lactate producers and butyrate producers. In fact, the metabolic mechanism of lactate to butyrate has been previously reported (Duncan *et al.* 2004).

It is possible that other specific *Lactobacillus* species may be stimulated by soybean husk besides those assessed in the present study, in reference to the large difference in qPCR assay values between total lactobacilli (14.2%) and the sum of the four *Lactobacillus* spp. determined (<0.60%) (Table 13). Lactobacilli unquantified could be *L. fermentum*, *L. rhamnosus*, *L. salivarius*, *L. murinus*, *L. animalis*, *L. sanfranciscensis* and *L. paraplantarum* detected in dogs (Grzeskowiak *et al.* 2015) and also uncultivated ones. The bifidogenic effects of soybean husk supplementation observed in the *in vitro* study (Table 11) were not confirmed by the *in vivo* feeding study. This can be partly explained by differences in the microbiota between dog breeds and between the main diet compositions. Members of *Bacteroides-Prevotella-Porphyromonas* are known as important fiber degraders, and pectin has been reported to increase the numbers of this group (Ivarsson *et al.* 2014). Therefore, soybean husk containing such components might contribute to the increase in *Bacteroides-Prevotella-Porphyromonas* numbers.

In soybean husk supplementation, the fecal levels of indole and skatole as microbial metabolites of tryptophan were decreased in the present feeding study (Table 12). This can be partly explained by the decrease in *Clostridium* cluster XI numbers (Table 13), the growth of which was

negatively influenced by the low pH from organic acid production from soybean husk. Consequently, this may have decreased the metabolism of undigested or endogenous proteins, since its members are protein utilizers, although *C. perfringens* numbers were unchanged by soybean husk feeding. Moreover, the polyphenol content and swelling property of soybean husk (Kosmala *et al.* 2014) might be other reasons for the reduction in these putrefactive compounds responsible for fecal odor. Sensory evaluation for fecal odor was not conducted in the present study. However, less unpleasant odor of feces from dogs fed soybean husk could be noticed while handling and analyzing the samples. The inhibitory effect of soybean husk on indole and skatole production is thought to be beneficial, since these compounds are regarded as a risk factor for colon cancer (Windey *et al.* 2012). In this regard, soybean husk supplementation could be useful for companion animals that are long-lived and/or kept indoors.

The present studies are not considered to be of sufficient duration (7 days) to assess adaptation of the hindgut microbiota. However, the observed beneficial shifts in lactobacilli, *Clostridium* cluster IV including *F. prausnitzii*, *Clostridium* cluster XIVa, *Bacteroides-Prevotella*-*Porphyromonas* and organic acid production suggest that soybean husk supplementation improves gastrointestinal health in dogs. Further *in vivo* studies are needed to confirm this observation, using long-term study with different levels of soybean husk in the feed formulation. Detailed evaluation of soybean husk for nutrition, health and animal performance is possible in relation to microbial modulation once optimal supplementation level is established.

Chapter 5

General discussion

In the present study, chickpea husk, lablab bean husk and soybean husk were found to contain both of soluble (represented by non-structural carbohydrate) and insoluble (NDF) dietary fiber. However, each bean husk contains different amounts of polyphenols and fiber. In particular, chickpea husk was rich in tannin, resulting in high antioxidative effects. Meanwhile, soluble sugar from lablab bean husk and soybean husk appeared as two main spots on thin layer chromatography, and were identified as raffinose and stachyose. When these different types of bean husk were used in three different feeding studies using rat and dogs, beneficial health effects were found. Main findings are summarized in Table 14.

Functional property of chickpea husk was evaluated by using rats. The lower concentration of total cholesterol was observed in rats fed chickpea husk diet in comparison with starch. Lower blood cholesterol level is probably due to tannins and dietary fiber present in chickpea husk. Tannins of chickpea husk can bind to bile acid in rat intestine and inhibit its reabsorption, facilitating bile acid synthesis in the liver for which plasma cholesterol is used. This can promote the reduction of blood cholesterol level. Beside tannins, both of soluble and insoluble fiber in chickpea husk might in part inhibit absorption of cholesterol source from dietary lipids by acceleration of fecal lipid excretion through lipid binding ability of tannins. Chickpea husk dietary supplementation showed the reduction of TBARS in comparison with cellulose and starch supplementation. Tannins in chickpea husk might be responsible for the prevention of superoxide formation and lipid peroxidation. In fact, TBARS lowering effect of tannin has been demonstrated in serum of rabbits (Jurgonski *et al.* 2014). Tannin-rich fiber found in tea was reported as a lipid binding agent that reduced blood cholesterol level and improved antioxidant activity in mice, because tannins interfere with fatty acid metabolism and it enhance endogenous antioxidant defense system or participate in the regeneration of other antioxidant compounds (Guo *et al.* 2016). Cholesterol-lowering effect of chickpea husk and protective

effect of chickpea husk against lipid oxidation suggest the functional importance of this by-product in prevention and care for cardiovascular diseases and overall health in animals.

Lablab bean husk feeding increased cecal level of total SCFA including acetate and butyrate and lowered cecal pH, indicating improvement of hindgut fermentation. In particular, lablab bean husk feeding showed higher acetate level that was related to increased abundance of *Akkermansia* confirmed by both of qPCR and Miseq. This mucin degrading species is known to convert mucins to acetate (Dao *et al.* 2016). Such prebiotics-like activity might be due to pectin or oligosaccharides possibly contained in lablab bean husk. Consumption of pectin or guar gum was found to increase *Akkermansia* in rats fed high-fat diets (Jakobsdottir *et al.* 2013) and its abundance has been negatively associated with metabolic diseases such as obesity and diabetes (Everard *et al.* 2011, Santacruz *et al.* 2010). Therefore, increased abundance of *Akkermansia* by lablab bean husk feeding has a beneficial health impact with respect to prevention and care for obesity, diabetes and their associated metabolic disorders in monogastric animals, especially long-living animals including companion animals and even humans.

Soybean husk feeding in rats increased total SCFA, acetate, butyrate in comparison with cellulose and starch feeding. Major polysaccharides and oligosaccharides in soybean husk seem to be utilized by cecal microbes and improved the cecal environment through organic acid production, leading to low pH. The most favorable aspect of soybean husk feeding in rats was the increase of cecal lactobacilli which were predominant acetate and lactate producers confirmed by both qPCR and MiSeq. The presence of pectin or raffinose family oligosaccharides in soybean husk might be the main factor to increase lactobacilli, because lactobacilli have the ability to break down raffinose and stachyose by their α -galactosidase activity (Pan *et al.* 2017), leading greater SCFA production. Soybean husk could possess a beneficial health impact in another aspect, since importance of lactobacilli have been known for the immune development in the gastrointestinal tract (Van Baarlen *et al.* 2013).

Soybean husk was supplemented in the diet of dogs to evaluate its impact. Feces of dogs fed on soybean husk diet had higher levels of total SCFA and lactate than those on cellulose diet. These

increases of beneficial organic acids with concomitant reduction of pH are indicative of increased microbial growth and activities. Lactogenic effect of soybean husk found in rat study was confirmed in dog. This caused increase of lactate that might be consumed by *Clostridium* cluster IV including *F. prausnitzii* and *Clostridium* cluster XIVa to produce butyrate as demonstrated by Duncan *et al.* (2004). Increased abundance of those butyrate producers are considered favorable, because these species have been shown to possess anti-inflammatory properties due to their potential protective effect on inflammatory bowel disease in dog by producing butyrate (Suchodolski *et al.* 2012). Therefore, supplementation of soybean husk in the diet of dog has a beneficial health impact with respect to its lactogenic effect and potential anti-inflammatory properties.

Conclusion

The results in first experiment indicate that chickpea husk dietary supplementation (5%) improves cardiovascular health through lowering of blood cholesterol and lipid peroxidation product (TBARS) by its polyphenols and dietary fibers in chickpea husk. The results in second experiment indicate that 5 % inclusion of lablab bean husk and soybean husk in the diet promotes cecal fermentation of rats, as represented by higher SCFA. Lablab bean husks exhibits a remarkable selectivity against *Akkermansia*, while soybean husk particularly favors the growth of lactobacilli in the cecum of rats. Improvement of hindgut fermentation and lactogenic effect by soybean husk were confirmed in third experiment using dogs. The improvement of hindgut fermentation and increased proportion of beneficial bacteria by supplementation of bean husk may be useful for promoting gastrointestinal health of monogastric animals. However, further studies are needed to fully understand the association of bean husk feeding with health of animals including humans. In this stage, the characterization and purification of individual functional components from bean husk needs further studies to confirm which one is responsible for the prebiotic effect and other health benefits in animals as well as humans. Bean husk could be valuable in the development of nutritional and drug therapies to combat different health ailments.

Tables and figures

Table 1 Chemical composition and antioxidant status of chickpea husk

	Chickpea husk
Dry matter (%)	92.0
----- % in dry matter -----	
Cellulose ^a	55.6
Hemicellulose ^b	3.0
Acid detergent lignin	3.0
Ash	4.0
Crude protein	4.5
Fat	1.0
Non-fibrous carbohydrate ^c	28.8
 Total phenolic compound	 2.7
Total flavonoid	2.5
Total tannin	2.1
Condensed tannin	1.0
 Superoxide dismutase	 32.0
2,2-diphenyl-picrylhydrazyl (DPPH)	31.0

^a = Acid detergent fiber – Acid detergent lignin.

^b = Neutral detergent fiber – Acid detergent fiber.

^c = 100 - (Neutral detergent fiber + Ash + Protein + Fat).

Table 2 Antioxidant status of cecal digesta and blood of rats fed experimental diets (Mean \pm SD)

Parameters	Cecal digesta						Blood					
	CEL		STA		CPH		CEL		STA		CPH	
Superoxide dismutase (%)	24.2	\pm 3.2 ^a	16.7	\pm 3.4 ^b	9.0	\pm 4.3 ^c	18.3	\pm 3.0	34.9	\pm 17.6	25.9	\pm 8.1
2,2-diphenyl-picrylhydrazyl (%)	18.4	\pm 6.5	20.9	\pm 2.4	13.0	\pm 2.5	5.1	\pm 1.8	3.0	\pm 1.5	3.4	\pm 2.0
Thiobarbituric acid-reactive substances (μ mol malondialdehyde)	4.2	\pm 1.4 ^b	5.5	\pm 1.0 ^b	6.9	\pm 1.5 ^a	56.0	\pm 27.2 ^a	55.1	\pm 18.0 ^a	19.8	\pm 7.1 ^b

CEL (control): AIN 93G purified diet containing 5% cellulose, STA: negative control diet in which cellulose is replaced by corn starch, CPH: treatment diet in which cellulose is replaced by chickpea husk.

^{a b c} = Mean values with different superscript letters within a row are significantly different ($P < 0.05$).

Table 3 Cecal fermentation and blood parameters of rats fed experimental diets (Mean \pm SD)

Parameters	CEL			STA			CPH		
Cecal digesta									
pH	8.3	±	0.1 ^a	8.3	±	0.2 ^a	7.9	±	0.1 ^b
Total SCFA (μmol/g digesta)	37.6	±	6.4 ^b	46.8	±	5.9 ^a	46.1	±	3.4 ^a
Acetate (μmol/g digesta)	23.2	±	2.3	27.1	±	4.5	27.5	±	1.9
Propionate (μmol/g digesta)	7.9	±	2.9	10.4	±	1.7	8.7	±	1.7
Butyrate (μmol/g digesta)	3.1	±	0.9 ^b	5.4	±	1.5 ^a	6.5	±	1.0 ^a
Acetate (molar %)	62.4	±	6.2	57.7	±	5.4	59.8	±	2.2
Propionate (molar %)	20.6	±	4.9	21.9	±	1.4	18.9	±	2.7
Butyrate (molar %)	8.2	±	1.3	11.8	±	4.1	14.1	±	1.9
Ammonia (μgN/g digesta)	131	±	35.8	156.7	±	26.6	137.4	±	32.3
Indole (μg/g digesta)	14.6	±	2.4 ^b	32.2	±	6.9 ^a	17.5	±	5.4 ^b
Skatole (μg/g digesta)	113	±	35.0 ^b	201.5	±	94.6 ^a	124.5	±	36.2 ^b
Blood									
Total glucose (mg/dl of plasma)	158.2	±	69.2	191.4	±	46.2	102.3	±	20.4
Total cholesterol (mg/dl of plasma)	103.9	±	5.8 ^a	121.8	±	11.3 ^a	83.6	±	3.3 ^b

CEL (control): AIN 93G purified diet containing 5% cellulose; STA: negative control diet in which cellulose is replaced by corn starch; CPH: treatment diet in which cellulose is replaced by chickpea husk.

^{a b} = Mean values with different superscript letters within a row are significantly different ($P < 0.05$).

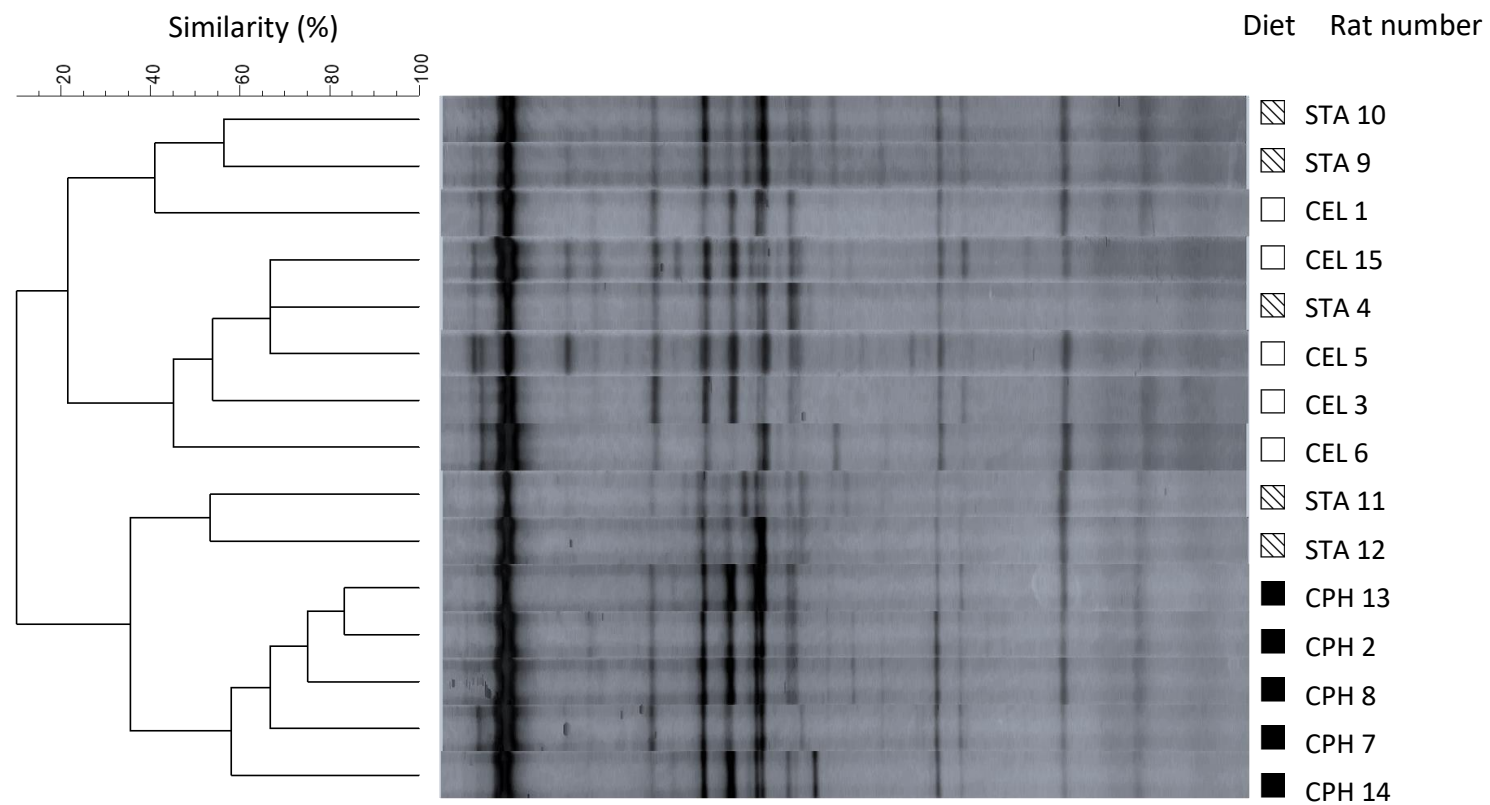


Figure 1 Similarity of DGGE banding profiles generated from DNA of cecal bacteria of rats fed experimental diets. CEL (Control) (□): AIN 93G purified diet containing 5% cellulose, STA (▨): negative control diet in which cellulose is replaced by corn starch, CPH (■): treatment diet in which cellulose is replaced by chickpea husk.

Table 4 Cecal bacteria of rats fed experimental diets (Mean \pm SD)

Bacteria	CEL			STA			CPH		
Total bacteria (Log copy/g digesta)	11.2	\pm	0.1	11.2	\pm	0.1	11.3	\pm	0.1
Proportion (Relative % in total bacteria)									
Total lactobacilli	45.0	\pm	10.6	46.0	\pm	17.1	53.0	\pm	4.6
<i>Lactobacillus acidophilus</i>	0.2	\pm	0.04	0.2	\pm	0.06	0.4	\pm	0.08
<i>Lactobacillus gasseri</i>	0.001	\pm	8E-04	0.0005	\pm	1E-04	0.0003	\pm	0.0001
<i>Lactobacillus johnsonii</i>	20.7	\pm	5.5	16.3	\pm	3.9	20.9	\pm	4.7
<i>Lactobacillus reuteri</i>	2.4	\pm	0.3	1.7	\pm	0.4	2.8	\pm	0.4
Total bifidobacteria	2.1	\pm	1.4	0.1	\pm	0.07	1.7	\pm	1.2
<i>Faecalibacterium prausnitzii</i>	0.003	\pm	7E-04	0.001	\pm	4E-04	0.003	\pm	0.0005
<i>Eubacterium halli</i>	0.001	\pm	0.001	0.0005	\pm	2E-04	0.0003	\pm	0.0001
<i>Clostridium perfringens</i>	ND			ND			ND		
<i>Escherichia coli</i>	0.1	\pm	0.07	0.2	\pm	0.09	0.02	\pm	0.01
<i>Clostridium coccoides-Eubacterium rectale</i> group	6.8	\pm	1.5	4.6	\pm	1.4	6	\pm	1.5

CEL (control): AIN 93G purified diet containing 5% cellulose, STA: negative control diet in which cellulose is replaced by corn starch, CPH: treatment diet in which cellulose is replaced by chickpea husk.

ND = not detected (not within quantifiable range).

Table 5 Chemical composition of bean husk used

	Lablab bean husk	Soybean husk
Dry matter (%)	92.0	91.0
----- % in dry matter -----		
Crude ash	3.3	4.5
Crude protein	5.9	9.2
Ether extract	1.2	1.4
Carbohydrate		
Cellulose ^a	52.0	46.8
Hemicellulose ^b	13.8	14.0
Lignin	0.2	0.2
Non fibrous carbohydrate ^c	23.6	24.0
Raffinose	0.1	0.1
Stachyose	0.1	0.2

^a: calculated as acid detergent fiber – acid detergent lignin

^b: calculated as neutral detergent fiber – acid detergent fiber

^c: calculated as 100 – (neutral detergent fiber + ash + protein + fat)

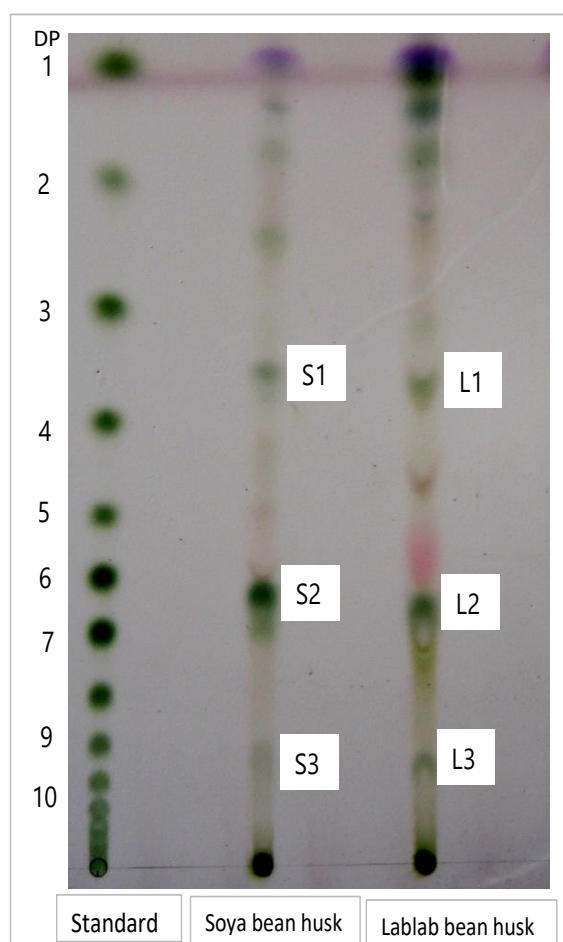


Figure 2 Thin-layer chromatography of soluble sugars from soybean husk and lablab bean husk. L1, L2, L3, S1, S2, and S3 are prominent oligosaccharide fractions.

DP: degree of polymerization

Table 6 Growth performance and cecal fermentation products of rats fed experimental diets. (Mean \pm SD)

Parameters	CEL	STA	LBH	SBH
Feed intake (g)	426.8 \pm 16.5	420.8 \pm 8.4	459 \pm 19.8	442.1 \pm 27.4
Initial body weight (g)	159.3 \pm 5.3	158.7 \pm 6.4	159.3 \pm 5.5	159.3 \pm 5.1
Body weight gain (g)	153 \pm 7.2	157.8 \pm 24.3	170 \pm 12.7	173.1 \pm 11.6
Food conversion ratio (g feed/g gain)	2.7 \pm 1.0	2.7 \pm 1.0	2.7 \pm 1.0	2.6 \pm 0.9
Cecal fermentation profile				
pH	7.7 \pm 0.4 ^a	7.8 \pm 0.4 ^a	7.3 \pm 0.2 ^b	7.3 \pm 0.2 ^b
Short chain fatty acid (SCFA)				
Acetate (μ mol/g digesta)	26.3 \pm 4.4 ^b	27.5 \pm 3.1 ^b	34.4 \pm 2.8 ^a	33.8 \pm 2.8 ^a
Propionate (μ mol/g digesta)	8.4 \pm 1.3	8.1 \pm 5.5	9.3 \pm 2.2	8.5 \pm 0.4
Butyrate (μ mol/g digesta)	5.9 \pm 1.3 ^{bc}	4.2 \pm 2.7 ^c	7.8 \pm 0.9 ^{ab}	9.7 \pm 0.6 ^a
Total SCFA (μ mol/g digesta)	42.9 \pm 6.6 ^b	40.5 \pm 8.6 ^b	57.4 \pm 10.2 ^a	54.4 \pm 3.4 ^a
D-lactate (μ mol/g digesta)	1.7 \pm 2.1	3.4 \pm 1.6	3.7 \pm 1.1	4.0 \pm 1.5
L-lactate (μ mol/g digesta)	2.7 \pm 1.3 ^b	5.4 \pm 1.3 ^a	4.6 \pm 1.1 ^{ab}	5.2 \pm 1.7 ^a
Total lactate (μ mol/g digesta)	4.4 \pm 2.6 ^b	8.9 \pm 2.5 ^a	8.4 \pm 2.0 ^{ab}	9.2 \pm 2.6 ^a
Succinate (μ mol/g digesta)	0.1 \pm 0.2 ^b	0.3 \pm 0.5 ^b	1.1 \pm 1.0 ^{ab}	2.1 \pm 1.7 ^a
Ammonia (μ gN/g digesta)	82.1 \pm 27	98.7 \pm 25.4	90.1 \pm 27.6	61.1 \pm 39.2
Indole (μ g/g digesta)	12.8 \pm 3.1 ^{bc}	21.7 \pm 2.5 ^a	11.3 \pm 1.6 ^c	16.6 \pm 2.4 ^{ab}
Skatole (μ g/g digesta)	71.2 \pm 24.5 ^b	174.3 \pm 31.8 ^a	64.5 \pm 12.3 ^b	94.1 \pm 29.3 ^b

CEL (Control): AIN 93G purified diet containing 5 % cellulose, STA: negative control diet in which cellulose was replaced by corn starch, LBH: treatment diet in which cellulose was replaced by lablab bean husk, SBH: treatment diet in which cellulose was replaced by soya bean husk. ^{a b c}: Mean values within a row with superscript letters are significantly different ($P < 0.05$).

Table 7 Cecal bacterial species and groups in rats fed experimental diets analyzed by real-time PCR (Mean \pm SD)

Species/group	CEL			STA			LBH			SBH		
Total bacteria (log copy/g digesta)	10.9	\pm	0.2	11.1	\pm	0.1	10.5	\pm	0.1	10.5	\pm	0.2
Proportion (Relative % in total bacteria)												
Total lactobacilli	24.6	\pm	10.7 ^b	27.5	\pm	8.4 ^b	32.5	\pm	7.1 ^{ab}	56.6	\pm	4.3 ^a
<i>Lactobacillus acidophilus</i>	0.2	\pm	0.1 ^b	0.2	\pm	0.1 ^b	0.5	\pm	0.1 ^{ab}	1.3	\pm	0.4 ^a
<i>Lactobacillus gasseri</i>	9E-06	\pm	5E-06	0.00001	\pm	5E-06	0.00001	\pm	1E-06	0.00001	\pm	4E-06
<i>Lactobacillus johnsonii</i>	32.4	\pm	11.1	28.5	\pm	9.9	30.5	\pm	7.2	41.5	\pm	8.8
<i>Lactobacillus reuteri</i>	0.6	\pm	0.3 ^b	0.5	\pm	0.1 ^b	1.4	\pm	0.7 ^{ab}	2.2	\pm	0.8 ^a
<i>Faecalibacterium prausnitzii</i>	0.1	\pm	0.0	0.0	\pm	0.0	0.0	\pm	0.0	0.0	\pm	0.0
<i>Eubacterium hallii</i>	0.0009	\pm	0.0	0.0006	\pm	0.0	0.0004	\pm	0.0	0.02	\pm	0.01
Total bifidobacteria	0.06	\pm	0.02	0.03	\pm	0.01	0.03	\pm	0.01	0.01	\pm	0.002
OTU 10 (<i>Akkermansia muciniphila</i>)	3.5	\pm	1.6 ^b	4.1	\pm	1.4 ^b	42.3	\pm	9.4 ^a	15.9	\pm	10.1 ^{ab}
OTU 4 (<i>Dorea massiliensis</i>)	3.2	\pm	1.0 ^{ab}	6.9	\pm	1.5 ^a	2.2	\pm	0.2 ^b	1.8	\pm	0.2 ^b
OTU 5 (<i>Oscillibacter</i> relatives)	12.0	\pm	3.5 ^{ab}	0.0	\pm	0.0 ^b	27.5	\pm	5.0 ^a	2.0	\pm	0.5 ^b
OTU 6 (<i>Ruminococcus bromii</i>)	4.2	\pm	1.1	3.0	\pm	0.7	4.5	\pm	1.0	3.1	\pm	0.8
<i>Escherichia coli</i>	0.6	\pm	0.5	0.7	\pm	0.5	0.2	\pm	0.1	0.03	\pm	0.01
<i>Bacteroidetes-Prevotella-Porphylomonas</i> group	0.5	\pm	0.4	1.3	\pm	0.7	0.2	\pm	0.1	0.02	\pm	0.01
<i>Clostridium coccoide-Eubacterium rectale</i> group	10.2	\pm	3.2	12.6	\pm	2.8	5.0	\pm	0.8	2.9	\pm	1.7

CEL (Control): AIN 93G purified diet containing 5% cellulose, STA: negative control diet in which cellulose was replaced by corn starch, LBH: treatment diet in which cellulose was replaced by lablab bean husk, SBH: treatment diet in which cellulose was replaced by soybean husk. ^{a b c}: Mean values within a row with different superscript letters are significantly different ($P < 0.05$). OTU 4, 5, 6 and 10 refer to Fig. 3.

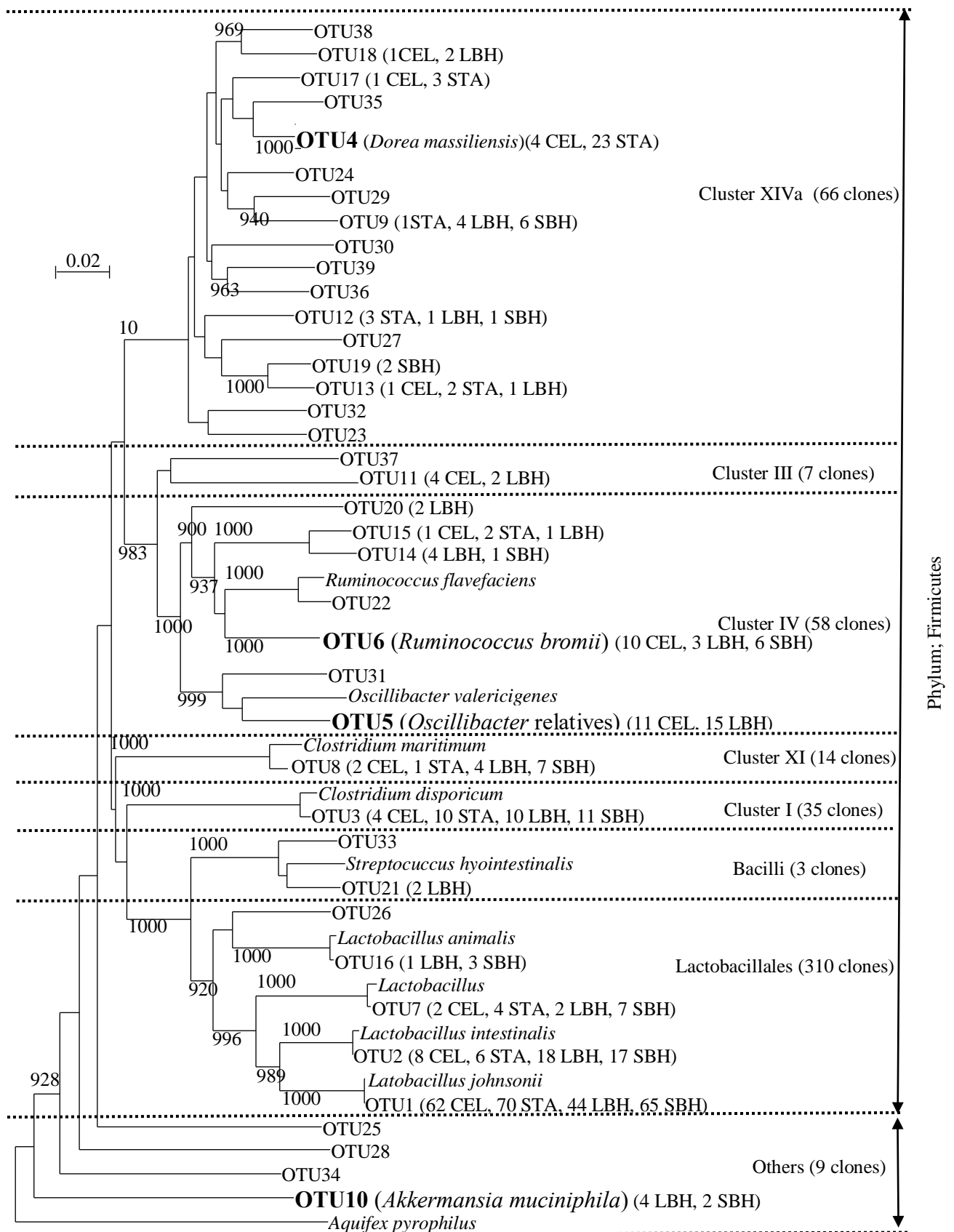


Figure 3 Phylogenetic clustering of cecal bacterial 16S rDNA clone sequences of rats fed experimental diets: CEL: AIN 93G purified diet containing 5% cellulose, STA: negative control diet in which cellulose was replaced by corn starch, LBH: treatment diet in which cellulose was replaced by lablab bean husk, SBH: treatment diet in which cellulose was replaced by soybean husk. Clones having >97% sequence identity were considered to belong to a distinct OTU; the number of clones in each OTU is indicated in brackets. OTUs employed for qPCR results are shown in bold. The horizontal bar represents nucleotide substitutions per sequence position.

Table 8 Biodiversity of cecal bacterial communities and relative abundance (%) of bacterial taxa at phylum and genus level in rats fed experimental diets (analyzed by MiSeq)

		CEL	STA	LBH	SBH
Alpha diversity					
Chao1		857.64	1112.65	782.52	862.56
Observed species		441	603	464	508
Shannon		4.32	4.72	3.91	4.13
Phylogenetic diversity		31.18	39.44	34.03	35.16
Phylum/family/Genus					
Actinobacteria		4.17 ^b	4.58 ^b	4.89 ^{ab}	7.98 ^a
Micrococcaceae	<i>Rothia</i>	0.45	0.58	0.9	0.66
Bifidobacteriaceae	<i>Bifidobacterium</i>	0.15 ^b	0.12 ^b	1.65 ^a	2.64 ^a
Coriobacteriaceae	Unclassified	2.27	1.5	1.12	2.7
	<i>Adlercreutzia</i>	1.26	2.3	1.16	1.85
Bacteroidetes		1.48	7.48	0.30	1.1
Bacteroidaceae	<i>Bacteroides</i>	0.62	3.77	0.05	0.37
Porphyromonadaceae	<i>Parabacteroides</i>	0.07	0.38	0.02	0.04
	<i>Prevotella</i>	0 ^b	0 ^b	0.01 ^a	0.01 ^a
Rikenellaceae	Unclassified	0.19 ^{ab}	0.85 ^a	0.04 ^b	0.28 ^b
S24-7	Unclassified	0.48	2.19	0.13	0.3
[Odoribacteraceae]	<i>Odoribacter</i>	0.09	0.15	0.03	0.05
Firmicutes		88.02 ^a	81.69 ^{ab}	62.96 ^b	67.91 ^{ab}
Lactobacillaceae	<i>Lactobacillus</i>	3.93 ^b	6.94 ^b	3.67 ^b	13.49 ^a
Streptococcaceae	<i>Lactococcus</i>	0.89	1.01	0.71	0.78
	<i>Streptococcus</i>	0.23	0.4	0.33	0.35
	<i>Turicibacter</i>	2.52	4.69	1.21	2.88
Unclassified	Unclassified	12.62	9.22	8.81	8.01
Christensenellaceae	Unclassified	0.18	0.17	0.06	0.06
Clostridiaceae	Other	2.44	3.49	5.44	8.62
	Unclassified	7.86	14.15	10.67	10.42
	<i>Lachnospiraceae</i>	5.8	2.53	1.32	1.44
	<i>Blautia</i>	5.87	4.06	0.95	0.71
	<i>Coprococcus</i>	0.37	0.35	0.65	0.33
	<i>Dorea</i>	6.71 ^{ab}	15.04 ^a	0.91 ^b	3.41 ^b
	[<i>Ruminococcus</i>]	0.23	0.36	0.42	0.54
Ruminococcaceae	Unclassified	20.81 ^a	7.34 ^{ab}	14.62 ^{ab}	4.2 ^b
	<i>Oscillospira</i>	0.9	2.43	1.79	1.9
	<i>Ruminococcus</i>	10.45	4.71	9	7.73
Erysipelotrichaceae	Unclassified	2.24	0.49	0.37	1.36
	<i>Allobaculum</i>	3.23	2.54	1.61	0.91
	<i>Coprobacillus</i>	0.21	0.27	0.01	0.32
	[<i>Eubacterium</i>]	0.27	0.27	0	0.05
Verrucomicrobia		4.63 ^b	2.45 ^b	30.19 ^a	21.9 ^{ab}
Verrucomicrobiaceae	<i>Akkermansia</i>	4.63 ^b	2.45 ^b	30.19 ^a	21.9 ^{ab}

CEL (Control): AIN 93G purified diet containing 5% cellulose, STA: negative control diet in which cellulose was replaced by corn starch, LBH: treatment diet in which cellulose was replaced by lablab bean husk, SBH: treatment diet in which cellulose was replaced by soybean husk. ^{a b c}: Mean values within a row with superscript letters were significantly different ($P < 0.05$).

Table 9 Chemical composition of basal diets and dietary fiber supplements

items	Basal commercial diet			Supplemented fiber	
	Royal canin [†]	Aiken- genki [†]	Science diet [†]	Soybean husk	Cellulose [#]
Moisture %	10.5	10.0	10.0	9.0	7.8
	----- Dry matter basis -----				
Crude protein %	29.0	23.0	25.0	9.2	nil
Ether extract %	18.0	10.0	17.0	1.4	nil
Crude ash %	-	8.5	8.5	4.5	nil
Crude fiber %	6.4	4.5	3.0	47.0	100
Cellulose %	-	-	-	46.8	100
Hemicellulose %	-	-	-	14.0	nil
Lignin %	-	-	-	0.2	nil
Non fibrous carbohydrate % [‡]	-	-	-	24.0	nil
Soluble fiber % [§]	-	-	-	6.1	nil
Total polyphenols (mg/g)	-	-	-	2.0	nil
Total flavonoids (mg/g)	-	-	-	0.9	nil
Tannin (mg/g)	-	-	-	0.5	nil

[†]: Nutritional facts available in products (for products, see text) are shown.

[‡]: Calculated as 100-(neutral detergent fiber+ash+protein+fat).

[§]: Analyzed by dietary fiber assay kits.

[#]: Sigma-Aldrich, Tokyo, Japan

-: Not available.

Table 10 Fermentation metabolites after in vitro fermentation of substrates using fecal inocula from dogs[§] (Mean±SD)

Parameters	Substrate		P-value	Substrate treated by enzymes*		P-value
	Cellulose	Soybean husk		Cellulose	Soybean husk	
pH	7.1 ± 0.0	6.9 ± 0.0	0.026	7.0 ± 0.0	6.8 ± 0.0	0.036
Total SCFA (mmol/g substrate)	2.6 ± 0.5	4.1 ± 0.6	0.003	1.2 ± 0.1	1.7 ± 0.2	0.004
Acetate (mmol/g substrate)	1.7 ± 0.4	3.0 ± 0.4	0.001	0.8 ± 0.1	1.2 ± 0.1	0.003
Propionate (mmol/g substrate)	0.4 ± 0.1	0.6 ± 0.1	0.011	0.3 ± 0.0	0.4 ± 0.0	0.007
Butyrate (mmol/g substrate)	0.3 ± 0.0	0.4 ± 0.1	0.035	0.1 ± 0.0	0.2 ± 0.0	0.021
Acetate (molar %)	71.0 ± 3.2	75.6 ± 0.3	0.112	68.5 ± 0.3	71.2 ± 0.3	<0.001
Propionate (molar %)	16.3 ± 1.4	14.7 ± 0.3	0.241	21.3 ± 0.3	21.7 ± 0.4	0.230
Butyrate (molar %)	10.9 ± 1.7	9.7 ± 0.0	0.306	7.3 ± 0.8	7.0 ± 0.1	0.431
Lactate (mmol/g substrate)	0.02 ± 0.0	0.03 ± 0.0	0.025	0.01 ± 0.0	0.02 ± 0.0	0.001
Ammonia (mg N/g substrate)	3.8 ± 0.1	3.4 ± 0.5	0.171	4.0 ± 0.2	3.9 ± 0.0	0.341
Indole (µg/g substrate)	23.6 ± 0.4	17.1 ± 0.2	0.003	37.4 ± 10.3	27.9 ± 2.2	0.095
Skatole (µg/g substrate)	324.1 ± 4.3	269.4 ± 3.6	0.001	325.2 ± 19.3	332.8 ± 28.7	0.670

[§]:Fecal inoculum was prepared by suspending fresh feces in McDougall's buffer in 1:10 ratio (w/v).

*:Substrate was digested by pepsin followed by pancreatin for 18 h.

Table 11 Microbiota changes after in vitro fermentation of substrates using fecal inocula from dogs[§] (Mean ± SD)

Bacteria	Substrate		P-value	Substrate treated by enzymes*		P-value
	Cellulose	Soybean husk		Cellulose	Soybean husk	
Total bacteria (Log copy/g substrate)	12.1 ± 0.0	12.0 ± 0.0	0.136	11.4 ± 0.1	11.5 ± 0.1	0.398
Proportion (Relative % in total bacteria)						
Total lactobacilli	0.9 ± 0.1	0.8 ± 0.1	0.171	5.1E-05 ± 0.0	4.8E-05 ± 0.0	0.874
Total bifidobacteria	2.5 ± 0.2	4.4 ± 0.2	0.011	0.3 ± 0.0	0.6 ± 0.1	0.001
<i>Escherichia coli</i>	1.5 ± 0.3	1.9 ± 0.6	0.431	3.3 ± 0.4	3.3 ± 0.2	0.860

[§]:Fecal inoculum was prepared by suspending fresh feces in McDougall's buffer in 1:10 ratio (w/v).

*:Substrate was digested by pepsin followed by pancreatin for 18 h.

Table 12 Fecal fermentation metabolites of dogs fed experimental diet (Mean±SD)

Parameters	Diet supplemented with		<i>P</i> -value
	Cellulose ¹	Soybean husk ²	
pH	6.4 ± 0.6	5.9 ± 0.3	0.004
Total short chain fatty acid (μmol/g feces)	139.1 ± 28.4	158.3 ± 25.3	0.035
Acetate (μmol/g feces)	78.7 ± 14.5	91.2 ± 15.9	0.031
Propionate (μmol/g feces)	50.5 ± 14.8	54.5 ± 12.5	0.320
Butyrate (μmol/g feces)	6.8 ± 2.4	10.6 ± 2.9	<0.001
Acetate (molar %)	55.8 ± 4.9	57.6 ± 4.6	0.169
Propionate (molar %)	36.5 ± 5.7	34.7 ± 5.7	0.222
Butyrate (molar %)	5.5 ± 2.2	6.3 ± 1.7	0.063
Lactate (μmol/g feces)	13.9 ± 4.5	21.0 ± 7.4	0.007
Ammonia (μg N/g feces)	0.1 ± 0.1	0.1 ± 0.0	0.251
Indole (μg/g feces)	4.7 ± 0.7	4.1 ± 1.0	0.018
Skatole (μg/g feces)	5.2 ± 0.9	3.8 ± 1.1	0.001

¹Cellulose: 170 g basal feed supplemented with 10 g cellulose was fed daily.

²Soybean husk: 170 g basal feed supplemented with 10 g soybean husk was fed daily.

Table 13 Fecal microbiota of dogs fed experimental diet (Mean \pm SD)

Bacteria	Diet supplemented with		<i>P</i> -value
	Cellulose ¹	Soybean husk ²	
Total bacteria (Log copy/g feces)	10.3 \pm 0.1	10.1 \pm 0.3	0.587
Proportion (Relative % in total bacteria)			
Total lactobacilli	5.6 \pm 2.7	14.2 \pm 4.5	0.044
<i>Lactobacillus acidophilus</i>	2.0E-04 \pm 0.0	3.0E-04 \pm 0.0	0.699
<i>Lactobacillus johnsonii</i>	3.0E-04 \pm 0.0	4.0E-03 \pm 0.0	0.527
<i>Lactobacillus gasseri</i>	4.0E-05 \pm 0.0	4.0E-05 \pm 0.0	0.817
<i>Lactobacillus reuteri</i>	0.4 \pm 0.2	0.6 \pm 0.4	0.479
Total bifidobacteria	0.1 \pm 0.1	0.1 \pm 0.1	0.728
Clostridial cluster IV (<i>Clostridium leptum</i> subgroup)	1.2 \pm 0.9	2.8 \pm 0.6	0.034
<i>Faecalibacterium prausnitzii</i>	0.4 \pm 0.3	2.3 \pm 1.1	0.044
Clostridial cluster XIVa (<i>Clostridium coccoides</i> group)	18.4 \pm 5.8	33.3 \pm 9.7	0.043
<i>Eubacterium hallii</i>	0.001 \pm 0.0	0.002 \pm 0.0	0.143
Clostridial cluster XI	30.5 \pm 4.8	15.4 \pm 6.2	0.006
<i>Clostridium perfringens</i>	0.2 \pm 0.1	0.2 \pm 0.2	0.986
Clostridial cluster I	1.3 \pm 1.2	0.7 \pm 0.5	0.613
<i>Bacteroides-Prevotella-Porphyromonas</i> group	3.1 \pm 1.7	6.8 \pm 1.5	0.029
<i>Prevotella</i>	0.2 \pm 0.1	0.7 \pm 0.5	0.101
<i>Akkermansia muciniphila</i>	6.2E-05 \pm 0.0	1.0E-04 \pm 0.0	0.264
<i>Escherichia coli</i>	0.2 \pm 0.1	0.3 \pm 0.2	0.167

¹Cellulose: 170 g basal feed supplemented with 10 g cellulose was fed daily.

²Soybean husk: 170 g of basal feed supplemented with 10 g soybean husk was fed daily.

Table 14 Summarized data of the functional activities of each supplemented bean husk used

Parameters	Chickpea husk		Lablab bean husk		Soybean husk		
	in rats		in rats		in rats		in dogs
	vs Cellulose	vs Starch	vs Cellulose	vs Starch	vs Cellulose	vs Starch	vs Cellulose
Blood							
Cholesterol	↓	↓					
TBARS	↓	↓					
Cecal/fecal digesta							
pH	↓	↓	↓	↓	↓	↓	↓
Acetate			↑	↑	↑	↑	↑
Butyrate	↑			↑	↑	↑	↑
Total SCFA	↑		↑	↑	↑	↑	↑
Lactate					↑		↑
Indole		↓		↓			↓
Skatole		↓		↓		↓	↓
Total lactobacilli					↑	↑	↑
<i>Lactobacillus acidophilus</i>					↑	↑	
<i>Lactobacillus reuteri</i>					↑	↑	
Total bifidobacteria			↑	↑	↑	↑	
<i>Akkermansia muciniphila</i>			↑	↑		↑	
<i>Dorea massiliensis</i>				↓		↓	
Clostridial cluster IV							↑
<i>Faecalibacterium prausnitzii</i>							↑
Clostridial cluster XIVa							↑
Clostridial cluster XI							↓
<i>Bacteroides-Prevotella-Porphyromonas</i>							↑

↑ Significantly increased

↓ Significantly decreased

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