# Studies on Effects of Indigestible Sugars on Nutrient Digestibility and Nitrogen Metabolism in Rabbits

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# Chapter 1

# GENERAL INTRODUCTION

## 1.1 Rabbit is important economic animals

The domestic rabbit (*Oryctolagus cuniculus*) is a descendent of wild rabbit and widespread over the world (Moreki 2007). Rabbits are important sources of food, particularly in Europe (Italy, France and Spain) and Asia (China). According to USDA, domesticated rabbits produce an all white meat product that is high in protein and low in fat and cholesterol (Table 1.1). According to Colin and Lebas (1995), the world carcass production of rabbit was up to 1.6 million tons. In addition rabbit fur has been used as fertilizer, glue or for felting in hats and coats. Another significant use of rabbits is in cosmetic, medical and pharmaceutical research laboratories. Therefore rabbit is very important economic animals. In their natural environment, rabbits are gregarious and prolific, and they are completely herbivorous monogastric animals (Moreki 2007). Rabbit capability to utilize type of high fiberous diet, together with the high reproduction ability, rabbits are able to breed year-round, and due to having a relatively short generation interval, they are uniquely poised to provide animal protein for developing countries, where grain can only be justified for human use (Irlbeck 2001). However, to ensure optimum performance provide high metabolic rate, a high daily nutrient and energy is needed. To support optimal growth, the importance of nutritional strategies has increased significantly considered as feed costs, pathological conditions associated with energy and nutrient deficiencies of rabbits (Carabaño & Piquer 1998). However it is not easy to modify the nutrition of rabbits to improve the efficiency of production, because the rabbit is a unique monogastric and herbivorous animal with an unusual digestive physiology.

**Table 1.1** Nutritional values of rabbit and other common meats

| Animal meat | Protein (%) | Fat (%) | Calories (per oz.) | Cholesterol (mg per oz.) |
|---|---|---|---|---|
| Rabbit[1] | 22.8 | 6.3 | 44 | 55 |
| Beef[2] | 29.9 | 10.1 | 61 | 73 |
| Catfish[3] | 22.3 | 5.9 | 40 | 20 |
| Chicken[4] | 28.9 | 7.4 | 54 | 80 |
| Lamb[5] | 28.1 | 9.5 | 58 | 78 |
| Pork[6] | 27.7 | 14.8 | 60 | 72 |
| Turkey[4] | 28.9 | 4.9 | 48 | 65 |

Source: USDA. [1] Wisconsin Meat Facts and Analysis, Fact Sheet ME 87-3, USDA Handbook 8-17, 1989. (whole carcass); [2] USDA Handbook 8-13 Reo., 1989. (composite sample); [3] NutriFacts Seafood. (whole); [4] USDA Handbook 8-5, 1979. (composite sample); [5] USDA Handbook 8-10, 1991. (composite sample); [6] USDA Handbook 8-17, 1989. (composite sample) From Lukefahr at al. (1998)

**1.2 Digestible system of the rabbit**

The rabbit is a monogastric herbivorous animal, and its digestive physiology is well adapted to high intake of plant cell walls (Bellier & Gidenne 1996; Gidenne & Lebas 2002). The digestive system of the rabbit is characterized by the relative importance of the cecum and colon when compared with other species (Portsmouth 1977), and is classified as hindgut fermenters (Cheeke 1987; McNitt et al. 1996). In the digestible system of rabbit, stomach comprises 15% of the gastrointestinal tract (Brewer 2006). The stomach is normally never empty (Davies & Davies 2003; Brewer 2006) and pH of from 1 to 5 (Chamorro et al. 2007), contribution of food intake and degradation. The small intestine is 22% of the gastrointestinal tract, within its duodenum is relatively long in the rabbit (Brewer 2006). The small intestine contains of digestive enzymes and nutrient digestion and absorption occurs in this area.

Large intestine is composed of the cecum and colon (proximal colon and distal colon). The rabbit cecum is very large (see Fig. 1.1), with a capacity of about 10 times that of the stomach and about 40% of the gastrointestinal tract (Brewer 2006). The large intestine is very important area of the rabbit digestive system. Rabbit cecum is the main area of bacterial activity (Bellier *et al.* 1995; Gidenne *et al.* 2002). As a cecum fermenter, the rabbit has a special nutritional system called cecotrophy. This process allows rabbits to re-ingest their microbial product in the cecum, as cecotrophs. Cecotrophy in small herbivorous mammals is considered as an adaptation to the metabolic disadvantage of small body size, especially when feeding on low quality diets.

The rabbit has an important nutritional function with a mechanism for colon separates liquid digesta from solid particles, and operates retrograde transport of liquid digesta containing bacteria in the cecum (Hörnicke 1981; Sakaguchi *et al.* 1992; Sakaguchi 2003). This inherent capability maintains the population of microorganisms in the cecum of rabbits. This separation mechanism is consistent with lower fiber digestibility due to the short fermentation period in rabbits (Sakaguchi 2003, see Fig. 1.2 and Fig. 1.3). In rabbit, liquid digesta is in the cecum have a longer retention than solid digesta. Separation and retrograde flow of digesta and bacteria are made possible

by the function is called colonic separation mechanism (Sperber 1985; Björnhag 1987). The large particle digesta or undegestible materials, which are separated from the fine particle and fluid digesta, are rapidly excreted as hard feces, they are low in minerals and high in fiber content (Hörnicke 1981; Cheeke 1987). The colonic separation mechanism results in a hight density of bacteria in the cecum contents, which is successively consumed as a cecotroph (Sakaguchi 2003). These digestive characteristic are adapted to high intake of dietary fiber and provide sufficient nutrient requirements for rabbits.

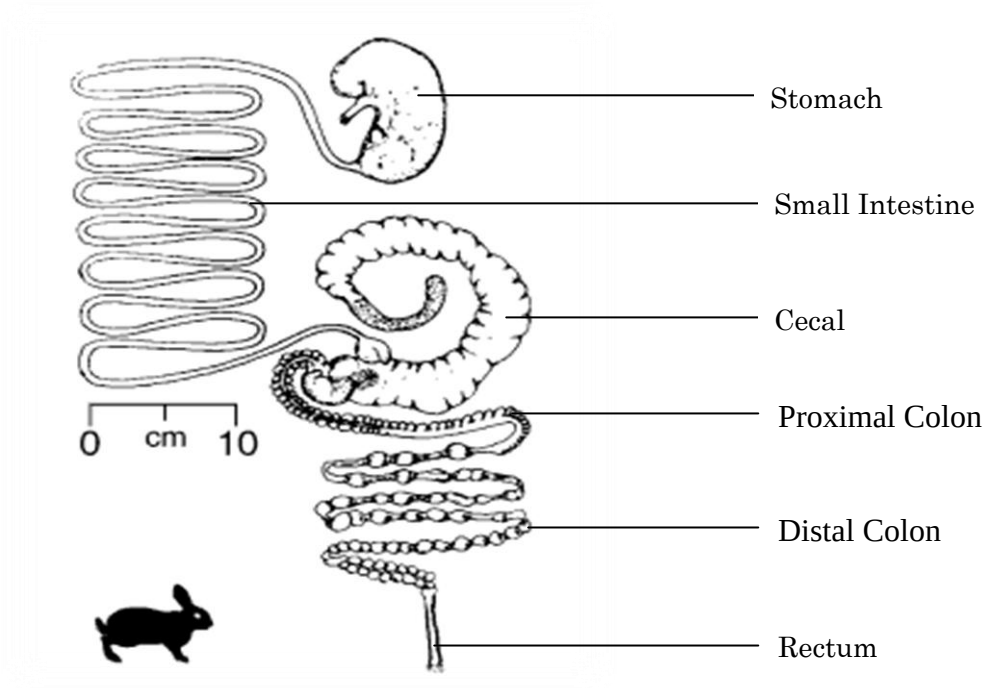


**Fig. 1.1** The rabbit (oryctolagus Cuniculus) digestive system. From Stevens and Hume (1998)

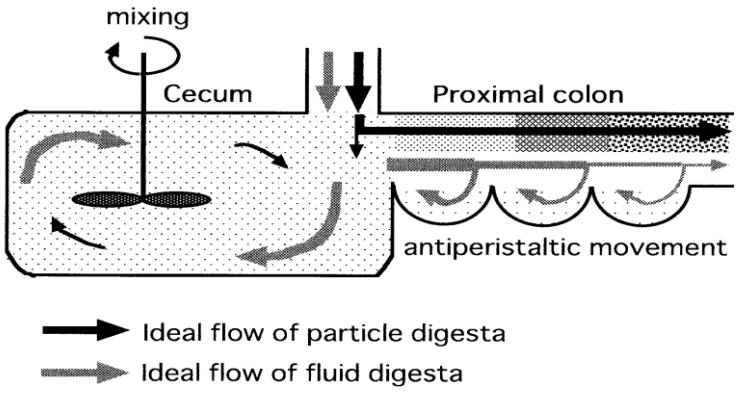

**Fig. 1.2** Possible model of selective retention of fluid digesta in the cexum. From Sakaguchi (2003).

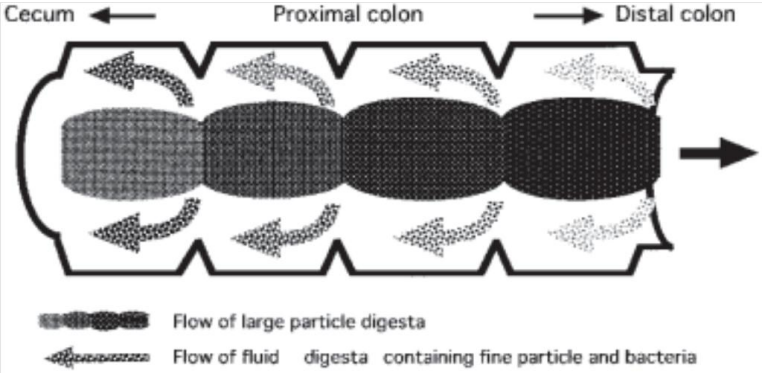


**Fig. 1.3** Schematic model of colonic separation of digesta in the proximal colon. From Sakaguchi (2003).

### 1.3 Cecotrophy in the rabbit

Hindgut fermentable animal was excretion two kinds of feces, hard and soft feces, which are produced as a result of bacterial proliferation in the cecum. The soft feces are directly re-ingested by the rabbit as a source of nutrients. The soft feces, also termed cecotrophes. In the rabbit, cecotrophy was rhythmical patterns of feeding and excretion has been shown to occur. During certain times of the day rabbit produce ingest special feces (cecotrophes or soft feces) with content of high bacterial protein, B-group vitamins, minerals and less fiber (Hörnicke 1981; cheeke 1987; Belenguer *et al.* 2002; Sakaguchi 2003). During other hours rabbits excrete hard feces, which contain content of high fiber and low minerals (Table 1.2). Cecotropy has considerable nutritional significant for nitrogen (N) supplementation in rabbit. This special digestive system compares the ruminant species in term of microbial protein recycling (Belenguer *et al.* 2002). It can provide up to 30% of the daily N intake, the re-ingested protein is characterized by a high digestibility and an elevated content of essential amino acids (Hörnicke 1981), which is mostly derived from cecal microbes (Belenguer *et al.* 2002). Rabbit digestive physiology follows a circadian rhythm characterized mainly by the practice of caecotrophy (Gallouin 1983) and variations in cecal fermentative activity (Gidenne 1986; vernay 1987). In rabbit, low fecal excretion in the light period corresponding to the excretion and ingestion soft feces and to a low feed intake, and peak of fecal excretion in the night period after the feed intake had increased (Hörnicke et al. 1984). This circadian rhythm was not affected by dietary fiber level of diet, but dietary fiber level has an effect on feed intake and excretion fecal level (Grigorov 1989;

Bellier & Gidenne 1996).

In small herbivorous mammals, Cecotrophy is a very important approach to obtain nutrients. Cecotrophy provide animal with necessary nutrients, such as protein, minerals, essential amino acids and vitamins. This special digestive system compares the ruminant species in terms of microbial protein recycling (Belenguer *et al.* 2002).

**Table 1.2** Average chemical compositions of cecal contents, soft and hard feces

| Ingredients | cecum | soft feces | hard feces | reference |
|---|---|---|---|---|
| Dry matter (g/kg) | 200 | 340 | 470 | 3,4,5,6,7 |
| Crude protein (g/kg DM) | 280 | 300 | 170 | 3,4,5,6,7 |
| Crude fiber (g/kg DM) | 170 | 180 | 300 | 3,4,5,6,7 |
| MgO (g/kg DM) | | 12.8 | 8.7 | 2 |
| CaO (g/kg DM) | | 13.5 | 18 | 2 |
| $Fe_2O_3$ (g/kg DM) | | 2.6 | 2.5 | 2 |
| Inorganic P (g/kg DM) | | 10.4 | 6.0 | 2 |
| Organic P (g/kg DM) | | 5.0 | 3.5 | 2 |
| $Cl^-$ (mmol/kg DM) | | 55 | 33 | 2 |
| $Na^+$ (mmol/kg DM) | | 105 | 38 | 2 |
| $K^+$ (mmol/kg DM) | | 260 | 84 | 2 |
| Bacteria ($10^{10}$/g DM) | | 142 | 31 | 2 |
| Nicotinic acid (mg/kg) | | 139 | 40 | 1 |
| Riboflavin (mg/kg) | | 30 | 9 | 1 |
| Panthotenic acid (mg/kg) | | 52 | 8 | 1 |
| Cianocobalamine (mg/kg) | | 3 | 1 | 1 |

1, Kulwich *et al*.(1953); 2, adapted from Hörnicke & Björnhag (1980); 3, Carabaño *et al*. (1988); 4, Carabaño *et al*. (1989); 6, Fraga *et al*. (1991); 6, Motta-Ferreira *et al*. (1996); 7, Carabaño *et al*. (1997). Adapted from Carabaño & Piquer (1998).

### 1.4 Indigestible but fermentable carbohydrates

For the past few years, indigestible but fermentable carbohydrate is industrial produced from natural sources (see Fig. 1.4). Therefore, indigestible carbohydrates are researched or used by wide range (for example: laboratory research, food industry, medical and pharmaceutical research etc.). Some indigestible carbohydrates have functional effects similar to soluble dietary fiber such as enhancement of a healthy gastrointestinal tract, improvement of glucose control, and modulation of the metabolism of triglycerides (Roberfroid & Slavin 2000). Indigestible carbohydrates escape digestion of the stomach or small intestine and reach the large intestine. In the

large intestine, they are modification of colonic microflora (Mussatto & Mancilha 2007), fermented by intestinal bacteria production of organic acids and gases (Cummings 1984; Cummings & Macfarlane 2002; Oku & Nakamura 2002), change excretion nitrogen balance (Younes et al. 1995a; Xiao Li et al. 2011), and stimulate intestinal mineral absorption (Oku 1996; Oku 1997). In addition, indigestible carbohydrates selectively stimulate the growth or metabolic activity of bacteria species benefic for health, proportioning and improvement in the composition of the colonic microflora, and thus improving the host health, therefore it is classified as prebiotics (Voragen 1998; Roberfroid & Slavin 2000).

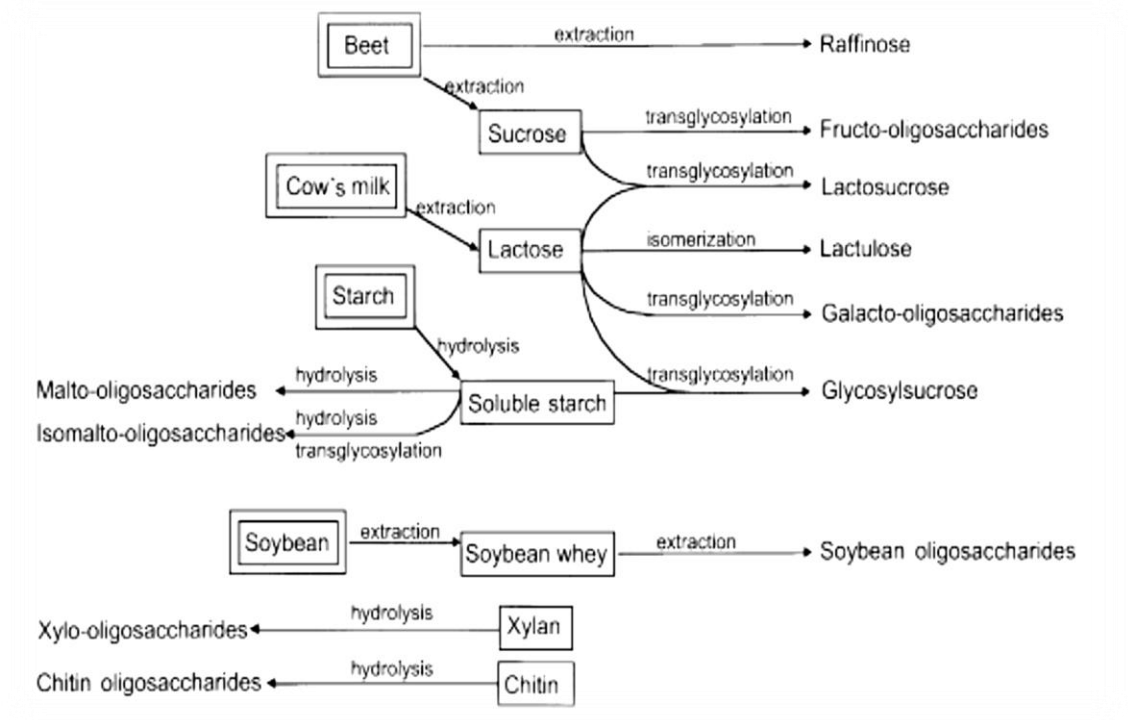


**Fig. 1.4** Schematic representation of production processes of non-digestible oligosaccharides. From Sako et al. 1999.

### 1.5 Effect of indigestible but fermentable carbohydrates on nitrogen balance

In the cecum, bacterial growth required nitrogen and energy sources. They are substances from both dietary and endogenous origin that have escaped digestion in the small bowel, such as resistant starches, plant cell-wall polysaccharides, variety of mucopolysaccharides, and proteins (Younes et al. 1995b). Fermentable carbohydrates escape digestion of the stomach or small intestine and reach the large intestine, where they can be induce an enlargement of the large intestine (Demigné & Rémésy 1982; Levrat et al. 1991), and partly or totally fermented by the microflora into short chain

fatty acids (Nyman 2002), and profoundly increase microbial multiplication in rats (Younes et al. 1995b, 2001). The fermentable carbohydrates stimulate bacterial deaminase and urease activity, leading to a substantial increase in ammonia, which is the major source of N for microbial flora (Varel et al. 1974), utilized by the cecum large microbial population in the synthesis of proteins. The high bacterial content of the cecal content pass through the cecotrophes re-ingested and take part in body protein synthesis of rabbits (Griffiths & Davies 1963).

When the diet contain of rich contents indigestible but fermentable carbohydrate, they enlarge the cecum, and hence larger surface of exchange. This process due to elevated cecal blood flow, promote high flux of urea into the cecum (Younes et al. 1995a). In the cecum, ureolytic activity is increased by fermentable carbohydrate (Rémésy *et al.* 1992), hence it has a high rate of ammonia production and reabsorption (Rémésy & Demigné 1989). Indigestible but fermentable sugar D-mannitol provided a significant contribution to decreasing urinary nitrogen excretion and the improvement of N utilization in adult rabbit (Hanieh & Sakaguchi 2009; Xiao Li *et al.* 2011; Xiao Min *et al.* 2013). In addition, fructooligosaccharide increases the N retention rate and decreases urinary N excretion in rabbit and guinea pigs (Tsuzuki *et al.* 2010; Kawasaki *et al.* 2013).

### 1.6 Effect of indigestible but fermentable carbohydrates on nutrient digestibility

The indigestible sugars, such as maltitaol, lactinol, D-mannitol, and fructooligosaccharide, have been shown to mineral bioavailability in humans and rats (Goda et al. 1995; Ammann et al. 1988; Jin et al. 2013; Ohta et al. 1995). In addition, it has been shown that when the fructooligosaccharide and D-mannitol are added to the diets of rats, the ash absorption is increased (Nishiyama *et al.* 2009). It has been suggested that indigestible sugars increase the rate of transepithelial diffusion of minerals in the small intestine (Kishi *et al.* 1998) and the solubilization of minerals (Demigné *et al.* 1980). The addition of indigestible sugar D-mannitol to the diet was shown to increase the fiber digestibility (Hanieh & Sakaguchi 2009; Xiao Min et al. 2013) and crude ash absorption (Xiao Li *et al.* 2011; Xiao Min *et al.* 2013) in adult rabbits. Additionally, the absorption promoting effect of colonic fermentation on mineral absorption after the ingestion of fructooligosaccharide has been reported in rats (Ohta *et al.* 1994). This information indicated that the nutrient digestibility or absorption was affected by feeding indigestible sugar in host.

### 1.7 Present study

In the previous study, we demonstrated that feeding D-mannitol reduced urinary N excretion and improved dietary nitrogen utilization in adult rabbit (Hanieh & Sakaguchi 2009; Xiao Li *et al.* 2011, 2012). This improved dietary nitrogen utilization was get through increased the transfer of blood urea N to the large intestine for bacterial N synthesis in rabbits (Xiao Li *et al.* 2011, 2012). Additionally, fiber digestibility is significantly lower in rabbits than in other herbivores (Sakaguchi *et al.* 1992). The addition of indigestible sugar D-mannitol to the diet was shown to increase the fiber digestibility in rabbits (Hanieh & Sakaguchi 2009); however the mechanism is not clearly understood. Chiou *et al.* (1998) suggested that fiber digestibility is dependent on retention time in gastrointestinal tracts in rabbits. Therefore, it is conceivable that effect of D-mannitol on N utilization, fiber digestibility, and retention time of digesta were estimated to investigate the effect of D-mannitol on nutrients utilization in rabbits.

A lot of researchers have been reported indigestible but fermentable sugar of fructooligosaccharides (FOS) has an effect on nitrogen balance of the rats and human. Further, addition of FOS to the diet has been shown to significantly improve the utilization of N in rabbits and guinea pigs (Tsuzuki & Sakaguchi 2010; Kawasaki *et al.* 2012). In the present study, we determined the effect of FOS on N transfer from the blood to cecal microbes (uszing of $^{15}$N-urea) and on N utilization and nutrient digestibility in mature rabbits. It was found that FOS can act as an energy source for bacterial proliferation, increasing the transfer of blood urea N to the cecum for bacterial N synthesis. However, the nutrient digestibility was not affected by FOS feeding mature rabbits.

Continuously, effect of FOS on N utilization, transfer of blood urea N to cecal microbial N in young rabbits fed urea compound diet (using of $^{15}$N-urea). The result indicated that FOS to the urea containing diet of rabbits improved N retention, but was failed to reduce N excretion in urine. Furthermore, cecal bacterial growth or proliferation did not efficiency utilize FOS to the diet containing urea. Those results indicated that FOS was not act as an energy source for bacterial proliferation, increasing the transfer of blood urea N to the cecal for bacterial N synthesis in urea containing diet.

In the end, we study the effect of other indigestible but fermentable sugar on N utilization and nutrient digestibility in adult rabbits except of D-mannitol and FOS. The result indicated that the effect of Lactosucrose, Sorbitol and Raffinose were not similar to that of mannitol or FOS.

# Chapter 2

## Effect of D-mannitol on Nitrogen Retention, Fiber Digestibility, and Digesta Transit Time in Adult Rabbits


### 2.1 Abstract

The aim of the current study was to elucidate the effect of gastrointestinal retention time of digesta on fiber digestibility in adult rabbits fed indigestible, but fermentable, sugar D-mannitol. The adult rabbits were fed a commercial diet containing 5% glucose or D-mannitol. Total feces and urine were collected during the experimental period. Nitrogen (N) balance, digestibility of nutrients, and gastrointestinal mean retention time (MRT) were measured. The results indicated that urinary excretion was significantly lowered, whereas N retention and N accumulation rate were significantly increased in the D-mannitol group compared with the glucose group ($P < 0.05$). However, fecal N excretion was unaffected. Absorption of crude ash (CA) and acid detergent fiber (ADF) digestibility were significantly higher in the D-mannitol group compared with the glucose group ($P < 0.05$). The addition of D-mannitol to the diet did not affect the MRT of liquid digesta, but increased the MRT of solid digesta compared with the glucose group ($P < 0.05$). These results suggest that the addition of D-mannitol to the diet stimulates cecal bacterial growth, thereby increasing N utilization and digesta retention time.



**Keywords:** adult rabbits, D-mannitol, fiber digestibility, N retention, retention time.


### 2.2 Introduction

Rabbits are herbivorous monogastric animals that have a digestive tract adapted for a high intake of dietary fiber. Rabbits possess a very large cecum, which is the main area of bacterial activity (Bellier *et al.* 1995). The bacteria in the cecum of rabbits can ferment digestible and fermentable non-fiber components. Rabbits produce two kinds of feces, hard and soft feces, which are produced as a result of bacterial proliferation in the cecum. The soft feces are directly re-ingested by the rabbit as a source of nutrients. The soft feces, also termed cecotrophes, contain high quality bacterial protein, B-group

vitamins, and less fiber (Hörnicke 1981; Belenguer *et al.* 2002; Sakaguchi 2003). These features result in a higher utility of feed protein in rabbits.

The rabbit colon separates liquid digesta from solid particles, and operates retrograde transport of liquid digesta containing bacteria in the cecum (Hörnicke 1981; Sakaguchi *et al.* 1992; Sakaguchi 2003). This inherent capability maintains the population of microorganisms in the cecum of rabbits. Bacterial growth requires a source of energy. The main sources of energy for microbial growth in the cecum are feed residues that contain little fermentable carbohydrates. All indigestible carbohydrates escape the digestion of the stomach or small intestine and reach the large intestine where they can be fermented and used as a source of energy for bacterial growth. Circulating blood urea and nitrogenous residues represent the major sources of nitrogen (N) for bacterial protein synthesis in the cecum. When the availability of indigestible carbohydrates, which stimulates bacterial growth in the cecum, is substantial, a greater flow of urea from blood to the cecum occurs, and results in an increase of bacterial N in feces. This may result in an increase in N consumption through cecotrophy and may improve N utilization (Xiao *et al.* 2011).

Conversely, fiber represents the majority of carbohydrates present in grasses. The majority of dietary fiber will be excreted without a long retention or fermentation in the cecum of rabbits because of the colonic separation mechanism detailed above. Thus, fiber digestibility is significantly lower in rabbits than in other herbivores (Sakaguchi *et al.* 1992). The addition of indigestible sugar D-mannitol to the diet was shown to increase the fiber digestibility in rabbits (Hanieh & Sakaguchi 2009); however the mechanism is not clearly understood. Chiou *et al.* (1998) suggested that fiber digestibility is dependent on retention time in gastrointestinal tracts in rabbits. In rats, supplementation of indigestible sugars fructooligosaccharide and galactooligosaccharide to diets elongated digesta retention time in the gut (Sakaguchi *et al.* 1998). Thus, it is possible that the addition of D-mannitol as an indigestible sugar to the diet prolongs digesta retention time in the large intestine and increases fiber digestibility in rabbits. In the present study, the effect of D-mannitol on N utilization, fiber digestibility, and retention time of digesta were estimated to investigate the effect of D-mannitol on nutrients utilization in rabbits.

## 2.3 Material and methods

### 2.3.1 Animals and diets

Six adult rabbits (1.61 ± 0.16 kg) were used for experiments. Before the experiments, the animals were maintained on a commercial diet (Labo R Stock, Nihon Nosan, Tokyo, Japan), which contained 16.3% crude protein (CP), 25.3% ADF, 9.30% crude ash (CA), 91.3% dry matter (DM), and 3.40% ether extract (EE). The animals were kept in temperature-controlled rooms at 25 ± 1°C and individually housed in metabolic cages (diameter 38 cm, height 30 cm). The rabbits were randomly assigned to one of two treatments to minimize individual differences in the animals regarding the diets. The treatment was designed with its own corresponding control group, and was conducted in two successive phases. The two successive phases was intervals 30 days. The animals were fed an experimental diet for 12 days. The diet was given as 50 g per daily metabolic body size ($kg^{0.75}$). Daily food intake was recorded. After an initial 5 days of acclimatization, feces were collected for 7 days to estimate digesta retention time. The animals were not prevented cecotrophy in this study.

The experimental diets included addition of glucose (Nacalai Tesque Inc., Kyoto, Japan) or D-mannitol (Nacalai Tesque Inc.) to the commercial diet at 50 g/kg. Animals were given free access to water during all experimental periods.

The study was approved by, and performed, in accordance with the experimental animal care committee of Okayama University.

### 2.3.2 Markers

Cr-mordanted Italian ryegrass (*Lolium multiflorum*) cell-wall constituents (Cr-CWC) as a particulate marker, and Co-EDTA (Dojindo Laboratories Inc., Kumamoto, Japan) as a liquid digesta marker were used to estimate retention time. Cr-CWC was prepared according to the method described by Udén *et al.* (1980).

### 2.3.3 Experimental methods

***Retention time***

At the end of the acclimatization period, gut digesta markers (Cr-CWC 0.5 g and Co-EDTA 0.25 g) were administered. The experimental diet was then given. Fecal

samples were collected every hour for the first 12 h (12 × 12 samples), every 2 h for the following 36 h (18 × 12 samples), and every 6 h for a further 120 h (20 × 12 samples) after dosing. The collected feces were dried for 24 h in a circulation dryer (60°C). Urine was collected 5 days later in the sampling period. Urine, which adhered to the cage, was collected using boric acid (Nakarai Tesque Inc., Kyoto, Japan), rinsed and then stored in a refrigerator at 4°C.

***Methods of analysis***

To determine concentration of Cr and Co dried fecal samples ashed in an electric furnace at 550°C for 8 h. The ashed samples were treated according to the method described by Williams *et al*. (1962). Analysis of Cr and Co concentrations were measured using an atomic absorption spectrophotometer (AA-80; Nippon Jarrell-Ash, Kyoto, Japan).

Single exponential regression equations were fitted statistically to the time-course decline of the fecal concentration of markers in rabbit. A turnover time of each marker was estimated from the decline in fecal concentration of marker by the function (Brandt & Thacker 1958): $Y = Ae^{-kt}$ where Y is the concentration of markers in feces at time t. A is constant depending on the level of the markers. K is a regression constant, and t is the time interval (h) after administration of the marker and each sampling time in the middle of the previous sampling period. 1/k is the reciprocal of the regression constant k, which is considered to be mean retention time (MRT) of the marker in the digestive tract segment. The slope of this equation represents the dilution of the marker at the site of the gastrointestinal tract reservoir. The MRT in the gastrointestinal tract was calculated by 1/k + TT.

Feed and feces were collected during the study of metabolism, after grinding. The DM content of diets and feces was determined by drying the samples at 135°C for 2 h. CA content was estimated by ashing at 550°C for 6 h. The N content of the diet, urine, and feces, and the ADF content of fecal samples and diets were determined according to the method of the Association of Official Analytical Chemists (AOAC 1990). Each ingredient's digestibility nitrogen accumulation rate was calculated using the following equation:

Nutrient digestibility (%) = absorption nutrient / (dietary nutrient intake) ×100

Nitrogen accumulation rate (%) = (intake nitrogen - excretion nitrogen) / (dietary

nitrogen intake) ×100

### 2.3.4 Statistical analysis

Data were calculated as mean ± standard deviation. Significant differences among the data were analyzed using paired *t*-tests (Microsoft Office Excel 2007; Microsoft Corporation). Differences were considered statistically significant at $P < 0.05$.

## 2.4 Results

Table 2.1 shows N accumulation rate and N balance during the experimental period. N excretion in urine was significantly decreased in rabbits fed with a diet containing D-mannitol ($P < 0.05$). The N retention and N accumulation rate were significantly higher compared with the glucose group ($P < 0.05$). However, there was no significant difference in N intake and excretion in feces between the two groups.

**Table 2.1** Nitrogen balance and nitrogen retention rate in rabbits fed a glucose or mannitol containing diet (n = 6)

| | Glucose | Mannitol |
|---|---|---|
| Feed intake (g dry matter/day) | 56.5±13.0 | 53.4±9.26 |
| Nitrogen intake (g/day) | 1.46±0.30 | 1.45±0.20 |
| Nitrogen excretion in feces (g/day) | 0.50±0.12 | 0.52±0.09 |
| Nitrogen excretion in urine (g/day) | 0.91±0.16 | 0.72±0.12* |
| Nitrogen retention (g/day) | 0.05±0.19 | 0.21±0.15* |
| Nitrogen accumulation rate† (%) | 1.72±13.9 | 14.0±8.82* |

Values are means ± SD.

* Significantly different from glucose groups ($P < 0.05$)

† Ratio of nitrogen retention to nitrogen intake

Nutrient digestibility or absorption is shown in Table 2.2. Dry matter and CP digestibility were not significantly different between the two groups. However, ADF digestibility and CA absorption were significantly higher in the D-mannitol group compared with the glucose group ($P < 0.01$, $P < 0.05$ respectively).

**Table 2.2** Digestibility (%) or absorption of nutrient in rabbits fed a glucose or mannitol containing diet (n = 6)

| | Glucose | Mannitol |
|---|---|---|
| Dry matter | 53.7±3.28 | 53.4±3.22 |
| Crude protein | 64.7±3.14 | 64.5±2.86 |
| Acid detergent fiber | 13.0±3.52 | 21.5±4.65** |
| Crude ash | 50.6±3.61 | 53.2±2.77* |

Values are mean ± SD.

*, **Significantly different from glucose groups ($P < 0.05$, $P < 0.01$, respectively).

Turnover time (1/k, h), transit time (TT, h), and MRT (h) of the digesta markers are shown in Table 2.3. Retention time of liquid markers was not different between the two groups. The 1/k and MRT of solid markers were significantly higher (approximately double) in the mannitol group compared with the glucose group ($P < 0.05$). 1/k represents the retention time of the digesta in the gastrointestinal tract, and may, therefore, reflect the digestibility of the nutrient (Chiou *et al.* 1998).

**Table 2.3** Turnover time (1/k, h), transit time (TT, h) and mean retention time (MRT, h) of digesta markers in rabbits fed a glucose or mannitol containing diet (n = 6)

| | Glucose | Mannitol |
|---|---|---|
| Co-EDTA | | |
| 1/k | 48.1±28.1 | 59.4±25.1 |
| TT | 4.50±0.00 | 4.83±0.52 |
| MRT | 52.6±28.1 | 64.2±25.2 |
| Cr-CWC | | |
| 1/k | 27.3±9.27‡ | 56.6±23.5* |
| TT | 4.50±0.00 | 4.83±0.52 |
| MRT | 31.8±9.27‡ | 61.4±23.7* |

Values are mean ± SD.

*Significantly different from glucose groups ($P < 0.05$)

‡Significantly different from Co-EDTA ($P < 0.05$)

**2.5 Discussion**

It has been reported that fermentable carbohydrates flow into the cecum, where they are fermented to organic acids, mainly short chain fatty acids (SCFAs), and used for proliferation of cecal bacteria (Levrat *et al.* 1991; Gidenne 1997). In rabbits, fermentable sugars can be used as a source of energy for cecal bacterial growth (Davies & Davies 2003). Some urea-N enters the cecum from the blood (Knutson *et al.* 1977), and is transferred to ammonia by bacterial ureolytic activity (Forsythe & Parker 1985). The ammonia-N transferred from blood urea is used for bacterial N synthesis (Demigné & Rémésy 1979; Younes *et al.* 1995). As a result, fermentable sugar stimulates microbial proliferation and increases microbial protein levels. This process results in a decrease of urinary N excretion (Younes *et al.* 1995). In the present study the intake of D-mannitol significantly decreased urinary N excretion. This resulted in N accumulation, and the N retention rate was significantly higher compared with the glucose group, consistent with our previous investigation (Xiao *et al.* 2011).

Ash absorption was increased in rabbits fed D-mannitol. The addition of fructooligosaccharide and D-mannitol to the diets of rats has been shown to increase ash absorption (Nishiyama *et al.* 2009). It has been suggested that indigestible sugars increase the rate of transepithelial diffusion of minerals in the small intestine (Kishi *et al.* 1998) and the solubilization of minerals (Demigné *et al.* 1980). Additionally, the absorption promoting effect of colonic fermentation on mineral absorption after the ingestion of fructooligosaccharide has been reported in rats (Ohta *et al.* 1994). It is possible that the improved absorbability of ash by D-mannitol in the present experiment may be caused by a similar mechanism.

In rabbits, dietary fiber can have an effect on gastrointestinal retention time (Laplace 1978) and the separation of particles in the digestive tract (Carabaño *et al.* 1988). Dietary fiber maintains a high rate of digesta passage. This avoids accumulation of particle digesta in the cecum, which can reduce feed intake and impair growth (De Blas et al. 1999). Dietary fiber is a substrate for cecal microorganisms. Fermentation produces mainly SCFAs, which is used as energy for the animal (Hörnicke 1981). These

suggest that dietary level of fiber in the diet could influences feed utilization and growth performance in rabbits.

The rabbit colon separates liquid from solid digesta, and operates retrograde transport of liquid containing bacteria to the cecum. This inherent capability maintains the population of microorganism in the cecum of the rabbit. The liquid marker in digesta separated by the colonic separation system is excreted in soft feces. On the other hand, almost of particle digesta flow into large gut is transported to distal region of the colon without entering into the cecum. This means less amount of particle digesta enter into cecum and in soft feces. Therefore, influence of cecotorphy on the retention time and transit time of particle digesta (Cr-CWC in this study) must be negligible under usual feeding condition.

In the present study, D-mannitol extended the retention time of solid-phase contents in rabbits. Owing to the elongated retention time, bacteria may have more time to ferment fiber components in the diet. This likely explains the increase of ADF digestibility in rabbits fed D-mannitol, because it has been reported that longer digesta retention times partially explain higher ADF digestibility (Sakaguchi 2003). The supplementation of the indigestible sugars, fructooligosaccharide and galactooligosaccharide, to diets has been shown to elongate retention time in rats (Sakaguchi *et al.* 1998). It is possible that similar effects of D-mannitol on the gastrointestinal tract were induced in rabbits in the present study.

In animals fed glucose, the digesta retention time was significantly longer for the liquid marker than for the solid marker. This may be achieved by the separation of solid and liquid phase digesta in the colon of rabbits, maintaining the liquid phase digesta longer than the solid phase in the cecum (Sakaguchi *et al.* 1992; Sakaguchi 2003). However, there was no difference in the digesta retention time between solid and liquid phase markers in the D-mannitol group. The results of the current study show that the colonic separation mechanism is disordered after D-mannitol feeding, but the mode of action of D-mannitol on the separation mechanism is still unclear.

It was reported that supplementation of starch for incubated rumen bacteria, as an energy source, promotes the growth of bacteria that require neither amino acids nor branched-chain fatty acids. This growth was followed by the growth of amino acid-dependent bacteria, and finally by nutritional interdependence among ruminal

bacteria and growth of branched-chain fatty acid-dependent cellulolytic bacteria (Miura *et al.* 1983). This may explain, in part, the results of the present study, which showed improvement in the digestibility of ADF in D-mannitol fed rabbits.

In conclusion, the addition of D-mannitol as an indigestible, but fermentable, sugar to the diet of rabbits improved N retention by reducing N excretion in urine. Fiber digestibility (ADF) was also increased by D-mannitol and was likely because of the prolonged retention time of digesta.

# Chapter 3

## Transfer of Blood Urea Nitrogen to Cecal Microbes and Nitrogen Retention in Mature Rabbits are Increased by Dietary Fructooligosaccharides


### 3.1 Abstract

To estimate the effect of fructooligosaccharides (FOS) on N utilization, seven mature rabbits were fed a diet containing 5% glucose or FOS for 8 days. During the last 5 days, total feces and urine were collected to measure N levels (Experiment 1). To examine N transfer from the blood to cecal microbes, eight rabbits were fed the same diets as in Experiment 1. After 9 days of feeding, 2 g of glucose or FOS was given orally. Two hours later 20 mg of $^{15}$N-urea was administered via the ear vein, and 1 h later cecal and blood samples were collected (Experiment 2). Urinary N excretion was lowered by FOS feeding ($P < 0.05$). Total bacterial N and $^{15}$N in the cecum was significantly higher in FOS-fed animals ($P < 0.05$). Urea N in the cecum was lower in FOS-fed rabbits ($P < 0.05$). Similarly, $^{15}$N atom % excess of cecal urea N was also lower in FOS-fed rabbits than in glucose-fed rabbits. These results suggest that FOS in the diet increases the transfer of blood urea N to the cecum for bacterial synthesis, thereby increasing N utilization.



**Key words:** fructooligosaccharides, N retention, blood urea, bacterial N, mature rabbit.


### 3.2 Introduction

Fructooligosaccharides (FOS) are not hydrolyzed by digestive enzymes (Oku *et al.* 1984) but instead are rapidly fermented by hindgut flora (Tokunaga *et al.* 1989). It has been reported that, in rats, fermentation of FOS stimulates the growth and proliferation of cecal bacteria that utilize blood urea N, thus resulting in a decrease in urinary N excretion (Delzenne *et al.* 1995; Mussatto & Mancilha 2007).

It is well-established that when refractory carbohydrates enter the gut they induce an enlargement of the large intestine, which results in greater intestinal surface area and blood flow (Younes *et al.* 1995b) and rapidly increases the flux of urea into the large intestine (Rémésy & Demigné 1989). In addition, cecal total urease activity is

increased by oligosaccharides (Rémésy *et al.* 1992), resulting in a high rate of ammonia production and re-absorption in the large intestine (Rémésy & Demigné 1989). Refractory carbohydrates stimulate bacterial growth and proliferation in the cecum and reduce circulating urea N concentrations. Ammonia produced in the large intestine can be utilized for bacterial protein synthesis in the rat (Rémésy & Demigné 1989). The proliferation of cecal bacteria that is stimulated by the presence of carbohydrates in the large intestine requires a large amount of N, which leads to reduced concentrations of ammonia N within the large intestine (Rémésy & Demigné 1989). This suggests that refractory carbohydrates that enter the large intestine promote bacterial growth and proliferation when there is a sufficient supply of circulating blood urea as a source of N for bacterial protein synthesis. This process induces an increase in fecal excretion of bacterial N (Viallard 1984; Langran *et al.* 1992).

In rabbits, a considerable portion of endogenous urea is transferred to the gastrointestinal tract (Regoeczi *et al.* 1965), and the exchange of N between blood urea and ammonia within the lumen of the cecum has been well-established (Forsythe & Parker 1985a, b). All refractory carbohydrates escape the digestive processes in the stomach and small intestine, and reach the large intestine where they can be completely fermented and used as a source of energy for bacterial growth. Circulating blood urea and nitrogenous residues represent the major sources of N for bacterial protein synthesis in the cecum. When the availability of refractory carbohydrates, which stimulate bacterial growth in the cecum, is substantial a greater flow of urea from the blood to the cecum occurs and results in an increase of bacterial N in feces (Mortensen 1992; Younes *et al.* 1995b). This is particularly essential for cecotrophic animals like rabbits. Rabbits re-ingest a significant amount of cecotrophs (or soft feces), which significantly contributes to meeting their protein requirement (Hörnicke 1981; Belenguer *et al.* 2005). It has been observed that feeding D-mannitol as a refractory, but fermentable, sugar increased protein N in rabbit cecotrophs (Hanieh & Sakaguchi 2009; Xiao *et al.* 2011).

In a previous study, we demonstrated that feeding D-mannitol improved dietary nitrogen utilization and increased the transfer of blood urea N to the large intestine for bacterial N synthesis in rabbits (Xiao *et al.* 2011, 2012). Further, addition of FOS to the diet has been shown to significantly improve the utilization of N in rabbits and guinea pigs (Tsuzuki & Sakaguchi 2010; Kawasaki *et al.* 2012). In this study, we determined

the effect of FOS on N transfer from the blood to cecal microbes and on N utilization in mature rabbits.

## 3.3 Material and methods

### 3.3.1 Diets

Before the experiments the animals were maintained on a commercial diet (Labo R Stock, Nihon Nosan, Tokyo, Japan), which contained 16.3% crude protein (CP), 25.3% acid detergent fiber (ADF), 9.30% crude ash (CA), 91.3% dry matter (DM), and 3.40% ether extract (EE). The animals were kept in temperature-controlled rooms at 25 ± 1°C and individually housed in metabolic cages (diameter 38 cm, height 30 cm).

The experimental diets were prepared by the addition of glucose (Nacalai Tesque Inc., Kyoto, Japan) or FOS (Nacalai Tesque Inc.) to the commercial diet at 50 g/kg. Animals were allowed free access to water during all experimental periods. The animals were fed 50 g of the diet per daily metabolic body size ($kg^{0.75}$), and daily food intake was recorded. The animals were not prevented from performing cecotrophy in this study.

The study was performed with the approval of, and in accordance with, the guidelines of the experimental animal care committee of Okayama University.

### 3.3.2 Animals and sampling

***Experiment 1***

Seven mature rabbits (4 months old, 1.88 ± 0.22 kg) were fed either the FOS-supplemented diet or a glucose-supplemented diet during each of two 8 days experimental periods. During the first period, four of the seven animals were fed the glucose diet and the other three animals were fed the FOS diet. This was followed by a 7 days rest period, and then the animals were fed the other diet for the second phase of the experiment (Table 3.1). Total excreted urine was collected daily for 5 days into 10 ml 1.5% boric acid solution to prevent evaporation of N, and stored at 4°C until analysis. Feces were collected for 5 days, oven-dried at 60°C for 24 h, ground to a particle size of < 1 mm, and stored at room temperature until further analysis.

**Table 3.1** Experimental design in Experiment 1

| Feeding period | Glucose group (n=7) | FOS group (n=7) |
|---|---|---|
| Phase 1 (8-d feeding trial) | Animal A, B and C | Animal D, E, F and G |
| 7-d intervals | | |
| Phase 2 (8-d feeding trial) | Animal D, E, F and G | Animal A, B and C |

The animals in each group were exchanged between the phase 1 and 2.

***Experiment 2***

Eight mature rabbits (4 months old, 1.94 ± 0.11 kg) were randomly assigned to one of two treatment groups (FOS or control diet), and fed experimental diets for 9 days. After 9 days feeding and 3 h prior to euthanasia 3 ml of a water solution containing 2 g glucose or FOS was given orally and 2 h later 20 mg $^{15}$N-urea (99.2 atom%, SI Science Co., Ltd., Sugito, Saitama, Japan) in 1 ml saline (8.5 g sodium chloride/l) was administered via the ear vein. One hour after the injection of $^{15}$N-urea, blood was taken from an abdominal artery and the portal vein under terminal anesthesia (pentobarbital sodium). All samples were collected between 09.00 hour and 10.00 hour. Serum was collected by centrifugation at 1,800 × *g* for 20 min and stored at −30°C until further analysis. The liver, small intestine, cecum, colon and rectum were removed and weighed, placed on ice, and stored at −30°C for subsequent analysis.

### 3.3.3 Analytical procedures

The DM content of diets and feces was determined by drying the samples at 135°C for 2 h. CA content was estimated by ashing at 550°C for 6 h. The N content of the diet, urine, and feces, and the ADF content of fecal samples and diets were determined according to the method described by the Association of Official Analytical Chemists (AOAC 1990). Nutrient digestibility or absorbability and nitrogen accumulation rate were calculated using the following equations:

(1) Apparent digestibility or absorbability (%) = 100 × (nutrient intake – fecal nutrient output) / nutrient intake;

(2) Nitrogen accumulation rate (%) = 100 × (N intake – fecal N – urinary N) / N intake.

The digesta in each gut segment were homogenized, and the pH was measured

immediately after collection using a digital pH meter (Horiba Ltd., Kyoto, Japan). The moisture content of the digesta was determined by drying a sample at 105°C for 24 h. The concentration of organic acids in the homogenized cecal contents was determined by HPLC (Column: 2 Shim-pack SCR-102H, Detector: Shimadzu CDD-6A; Shimadzu Corporation, Kyoto, Japan). A liver sample was homogenized in physiological saline (8.5 g sodium chloride/l). The homogenized liver sample, and samples of the contents of the small intestine, cecum, colon, and serum were treated with one-fifth of their volume of trichloroacetic acid (TCA) solution (500 g/l), centrifuged (12,000 × *g* for 20 min at 4°C), and washed twice with TCA solution (50 g/l) (Fürst & Jonsson 1971). The TCA precipitates were analyzed for total N and protein N using a CN analyzer (Yanaco CN Corder, MT-700, J-Science, Kyoto, Japan). The deproteinized supernatants of the cecal contents were collected, and urea within the supernatants was converted to ammonia by treatment with urease (*EC* 3.5.1.5; Nacalai Tesque Inc.) according to the procedure described by Obara *et al.* (1994). The urease-treated samples and non-urease-treated samples were steam distilled with the addition of magnesium oxide or sodium hydroxide, and ammonia was trapped in boric acid and titrated with sulfuric acid (0.05 *N*) for urea N and ammonia N determination (Nolan & Leng 1972). Samples of the contents of the cecum and colon were diluted in physiological saline, shaken well and placed in a refrigerator for 24 h at 4°C to dislodge adherent bacteria (Minato & Suto 1978). Bacteria residue was obtained by centrifugation (20,000 × *g* for 20 min at 4°C) of the supernatant after centrifugation at 500 × *g* for 5 min at 4°C (Belenguer *et al.* 2005), and the N content of the isolated bacteria was determined using a CN analyzer. Serum ammonia N and urea N concentrations were measured using commercially available kits (Ammonia Test-Wako and Urea N B Test-Wako, Wako Pure Chemical Industries Ltd., Osaka, Japan). The concentrations of $^{15}N$ in total N, protein N, bacterial N, urea N, and ammonia N of each sample were analyzed by mass spectrometry (MSI-150-MT-600 NC Model, Quadrupole Mass Spectrometry System, J-Science, Kyoto, Japan).

### 3.3.4 Statistics

Data are presented as means ± standard deviations. In Experiment 1, the significance of between treatment differences was assessed using a nonparametric

method (Wilcoxon Rank Sum Test, Excel statistics, SSRI Co., Tokyo, Japan). In Experiment 2, differences between group means were assessed using a nonparametric method (Mann-Whitney *U*-Test, Excel statistics, SSRI Co., Tokyo, Japan). Differences were considered significant at $P < 0.05$.

**3.4 Results**

Table 3.2 shows the feed intake, apparent digestibility or absorbability of nutrients, and N balance during Experiment 1. The DM intake and weight gain (approximately 10 g/day in both groups) of the rabbits were not significantly influenced by FOS feeding. The apparent digestibility or absorbability of DM, CP, CA and ADF were not significantly different between the two groups.

Urinary N excretion was significantly lower in rabbits fed a diet containing FOS (0.7-fold of the glucose group, $P < 0.05$). The N retention and the N retention rate based on N intake and based on digested N were significantly higher in the FOS group compared with the glucose group (2.3-fold, 2.2-fold, and 2.2-fold, respectively, $P < 0.05$; Experiment 1).

**Table 3.2** Feed intake, apparent digestibility or absorbability of nutrients, and N balance in rabbits fed a glucose- or FOS-containing diet

| | Glucose | FOS |
|---|---|---|
| Feed intake (g dry matter/day) | 73.6±9.88 | 73.6±8.84 |
| Apparent digestibility or absorbability (g/g) | | |
| Dry matter | 0.58±0.02 | 0.58±0.02 |
| Crude protein | 0.67±0.03 | 0.66±0.02 |
| Crude ash | 0.54±0.03 | 0.54±0.02 |
| Acid detergent fiber | 0.25±0.03 | 0.25±0.03 |
| N balance | | |
| N intake (g/day) | 1.96±0.26 | 1.95±0.23 |
| Fecal N (g/day) | 0.64±0.10 | 0.65±0.08 |
| Urinary N (g/day) | 1.09±0.23 | 0.78±0.15* |
| N retained (g/day) | 0.23±0.10 | 0.52±0.25* |
| N retention rate based on N intake | 0.12±0.05 | 0.26±0.10* |
| N retention rate based on N digested | 0.18±0.07 | 0.39±0.15* |

Values are means ± SD (n=7).

* Significantly different from the glucose group ($P < 0.05$).

Table 3.3 shows the liver weight and gut weight. There was no significant difference in the fresh weight of organs between groups. However, the ratio of total cecal weight and the weight of cecal contents to body mass were both significantly higher in the FOS group compared with the glucose group (1.3-fold and 1.4-fold, respectively, $P < 0.05$). Furthermore, the DM weight of the cecal contents was greater in the FOS group compared with the glucose group (16.6 ± 3.25 and 11.3 ± 2.53 g). In the gut, with the exception of the cecum, there were no other between-group differences.

**Table 3.3** Weight of the liver and gut segments in rabbits fed a glucose- or FOS-containing diet

| | Fresh weight (g) | | % of body weight ratio | |
|---|---|---|---|---|
| | Glucose | FOS | Glucose | FOS |
| Liver | 43.0±3.82 | 40.2±1.43 | 2.19±0.24 | 2.07±0.08 |
| Small Intestine | | | | |
| Total weight | 26.9±3.26 | 28.2±2.50 | 1.37±0.12 | 1.45±0.11 |
| Tissue weight | 14.0±2.86 | 12.4±0.57 | 0.71±0.13 | 0.64±0.02 |
| Content weight | 13.0±2.41 | 15.7±2.29 | 0.66±0.11 | 0.81±0.11 |
| Cecum | | | | |
| Total weight | 60.9±11.9 | 79.0±13.5 | 3.10±0.54 | 4.04±0.51* |
| Tissue weight | 16.2±0.46 | 16.2±0.91 | 0.83±0.05 | 0.83±0.08 |
| Content weight | 44.6±11.8 | 62.8±13.1 | 2.27±0.55 | 3.21±0.51* |
| Colon and Rectum | | | | |
| Total weight | 29.4±2.79 | 28.7±6.41 | 1.50±0.10 | 1.49±0.41 |
| Tissue weight | 14.3±2.12 | 12.6±0.70 | 0.73±0.08 | 0.65±0.06 |
| Content weight | 15.1±1.01 | 16.1±6.33 | 0.77±0.05 | 0.84±0.37 |
| Body weight | 1961±81.4 | 1948±139 | — | — |

Values are mean ± SD (n=4). Content weight = Total weight – Tissue weight.

* Significantly different from the glucose group ($P < 0.05$)

The concentration and total amount of organic acids and the pH of the cecal contents are presented in Table 3.4. The pH was significantly lower ($P < 0.05$) in FOS-fed rabbits. The total amount (μmol) of organic acids had a tendency to be higher in FOS-fed rabbits (1.6-fold of the glucose group). However, there was no significant difference in concentrations of individual organic acids and in total short chain fatty acid (SCFA) between the two groups.

**Table 3.4** Luminal pH, concentration (μmol/g contents) and total amount (μmol) of organic acids in the cecal content of rabbits

| | Glucose | FOS |
|---|---|---|
| pH | 6.6±0.1 | 6.4±0.1* |
| Organic acid concentration | | |
| Succunic acid | 1.37±0.38 | 1.30±0.47 |
| Lactic acid | 2.93±1.02 | 3.13±0.47 |
| Formic acid | 0.34±0.10 | 0.31±0.03 |
| Acetic acid | 14.8±3.65 | 18.2±2.33 |
| Propionic acid | 6.16±5.00 | 6.04±3.59 |
| Butyric acid | 3.37±1.69 | 5.48±1.65 |
| Total SCFA | 24.3±6.68 | 29.7±2.28 |
| SCFA molar ratio (Ac/Pr/Bu)[2] | 61/25/14 | 61/20/19 |
| Total amount of organic acids | 1354±602 | 2198±575 |

Values are means ± SD (n=4).

* Significantly different from the glucose group ($P < 0.05$).

Table 3.5 shows total N and $^{15}N$ concentrations in the gut contents on a DM basis. The N concentration and $^{15}N$ atom % excess did not differ between the two groups. However, total N in the small intestine and cecum was significantly higher in FOS-fed rabbits. The cecal $^{15}N$ atom % excess was not increased by the FOS diet; however, the cecal contents of the rabbits fed FOS weighed significantly more than

those of the rabbits fed the glucose-containing diet, resulting in a significantly greater $^{15}N$ excess.

**Table 3.5** Total N and $^{15}N$ concentrations in the gut contents (DM basis)

| | N concentration (% of DM) | Total N (mg) | $^{15}N$ atom % excess | Amount of $^{15}N$ excess (mg) | % of $^{15}N$ injected |
|---|---|---|---|---|---|
| Small intestine | | | | | |
| Glucose | 8.43±1.13 | 184±27.4 | 0.15±0.04 | 0.28±0.10 | 2.96±1.00 |
| FOS | 7.83±1.44 | 237±32.6* | 0.18±0.06 | 0.43±0.12 | 4.55±1.28 |
| Cecum | | | | | |
| Glucose | 5.00±0.43 | 560±99.9 | 0.20±0.09 | 1.13±0.62 | 11.7±6.48 |
| FOS | 4.64±0.17 | 769±137* | 0.26±0.09 | 1.89±0.46* | 19.7±4.74* |
| Colon and Rectum | | | | | |
| Glucose | 4.88±0.48 | 198±36.0 | 0.22±0.04 | 0.44±0.12 | 4.41±1.18 |
| FOS | 4.92±0.23 | 216±87.2 | 0.21±0.06 | 0.43±0.10 | 4.34±0.97 |

Values are means ± SD (n=4).

*, Significantly different from the glucose group ($P < 0.05$)

$^{15}N$ atom % excess = 100 / [2 ($^{14}N^{14}N$ / $^{14}N^{15}N$) + 1] (atom %) - $^{15}N$ natural abundance ratio (0.366%)

Amount of $^{15}N$ excess = Total N × $^{15}N$ atom % excess

Table 3.6 shows the concentration and amount of bacterial N and $^{15}N$ in the contents of the cecum and colon. There was no difference in the concentration of microbial N between the two groups; however, the cecal $^{15}N$ atom % excess had a tendency to be higher in the FOS group compared with the glucose group ($P = 0.083$). Total N and amount of $^{15}N$ excess in cecal bacteria were significantly greater in FOS-fed rabbits ($P < 0.05$, respectively). The addition of FOS to the diet did not affect colonic bacterial N and $^{15}N$ concentrations.

**Table 3.6** Bacterial N and $^{15}N$ concentration in the large intestine (DM basis)

| | N concentration (% of DM) | Total N (mg) | $^{15}N$ atom % excess | Amount of $^{15}N$ excess (mg) | % of $^{15}N$ injected |
|---|---|---|---|---|---|
| Cecum | | | | | |
| Glucose | 3.92±0.72 | 437±92.7 | 0.06±0.05 | 0.28±0.23 | 2.95±2.43 |
| FOS | 3.49±0.24 | 625±25.7* | 0.17±0.10 | 0.91±0.32* | 9.52±3.28* |
| Colon | | | | | |
| Glucose | 4.19±0.48 | 122±29.4 | 0.17±0.05 | 0.20±0.07 | 2.04±0.73 |
| FOS | 4.10±0.44 | 140±66.6 | 0.23±0.06 | 0.30±0.08 | 2.98±0.76 |

Values are means ± SD (n=4).

*, Significantly different from the glucose group ($P < 0.05$)

$^{15}N$ atom % excess = 100 / [2 ($^{14}N^{14}N$ / $^{14}N^{15}N$) + 1] (atom %) - $^{15}N$ natural abundance ratio (0.366%)

Amount of $^{15}N$ excess = Total N × $^{15}N$ atom % excess

Table 3.7 shows the protein N and $^{15}N$ concentrations in the liver and gut contents on a DM basis. FOS did not affect protein N concentrations and $^{15}N$ atom % excess in the liver and gut contents. Consequently, total protein N and amount of $^{15}N$ excess did not differ between the two groups.

**Table 3.7 Protein N and $^{15}N$ concentrations in the liver and gut contents (DM basis)**

| | N concentration (% of DM) | Total N (mg) | $^{15}N$ atom % excess | Amount of $^{15}N$ excess (mg) | % of $^{15}N$ injected |
|---|---|---|---|---|---|
| Liver | | | | | |
| Glucose | 9.74±0.11 | 1479±179 | 0.18±0.03 | 2.71±0.58 | 28.2±6.00 |
| FOS | 9.48±1.26 | 1218±168 | 0.20±0.03 | 2.36±0.30 | 24.6±3.14 |
| Small intestine | | | | | |
| Glucose | 8.27±1.06 | 181±30.0 | 0.12±0.03 | 0.22±0.08 | 2.25±0.86 |
| FOS | 7.25±1.00 | 221±37.6 | 0.11±0.06 | 0.24±0.10 | 2.53±0.99 |
| Cecum | | | | | |
| Glucose | 4.92±0.44 | 551±103 | 0.17±0.09 | 0.92±0.61 | 9.56±6.39 |
| FOS | 4.48±0.27 | 744±143 | 0.14±0.10 | 1.11±0.88 | 11.5±9.12 |
| Colon | | | | | |
| Glucose | 4.57±0.66 | 131±26.6 | 0.18±0.05 | 0.24±0.09 | 2.37±0.92 |
| FOS | 4.73±0.53 | 164±87.8 | 0.21±0.03 | 0.37±0.26 | 3.71±2.57 |

Values are means ± SD (n=4).

$^{15}N$ atom % excess = 100 / [2 ($^{14}N^{14}N$ / $^{14}N^{15}N$) + 1] (atom %) - $^{15}N$ natural abundance ratio (0.366%)

Amount of $^{15}N$ excess = Total N × $^{15}N$ atom % excess

Table 3.8 shows the concentrations of ammonia N and urea N, and $^{15}N$ atom % excess in wet cecal contents and in serum from arterial or portal vein blood. The concentration of cecal urea N was lower in the FOS group compared with the glucose group ($P < 0.05$). Similarly, the $^{15}N$ atom % excess was lower in rabbits fed a diet containing FOS ($P < 0.05$). Concentrations of serum ammonia N and urea N in arterial blood did not differ between the two groups. The serum urea N concentration in blood from the portal vein had a tendency to be lower in the FOS group compared with the glucose group; however, the $^{15}N$ atom % excess of urea N, and ammonia N in serum from the portal vein did not differ between the two groups.

**Table 3.8 Concentrations of ammonia N, urea N, and $^{15}N$ in the cecal contents, and arterial and portal blood**

| | Glucose | FOS |
|---|---|---|
| Cecum | | |
| Ammonia N | | |
| N concentration (μg/g wet content) | 198±37.2 | 156±14.5 |
| $^{15}N$ atom % excess | 1.56±0.41 | 0.90±0.36 |
| Urea N | | |
| N concentration (μg/g wet content) | 108±21.3 | 80.3±7.23* |
| $^{15}N$ atom % excess | 2.07±0.43 | 1.19±0.30* |
| Arterial blood serum | | |
| Ammonia N | | |
| N concentration (μg/dl) | 106±51.3 | 99.7±35.0 |
| $^{15}N$ atom % excess | 1.26±1.10 | 1.15±0.72 |
| Urea N | | |
| N concentration (mg/dl) | 22.7±2.41 | 21.7±1.66 |
| $^{15}N$ atom % excess | 1.74±1.13 | 1.27±1.09 |
| Portal blood serum | | |
| Ammonia N | | |
| N concentration (μg/dl) | 601±323 | 550±263 |
| $^{15}N$ atom % excess | 1.65±0.61 | 1.36±0.58 |
| Urea N | | |
| N concentration (mg/dl) | 21.0±2.57 | 17.9±1.65 |
| $^{15}N$ atom % excess | 1.88±0.61 | 1.49±0.54 |

Values are mean ± SD (n=4).

*, Significantly different from the glucose group ($P < 0.05$)

$^{15}N$ atom % excess = 100 / [2 ($^{14}N^{14}N$ / $^{14}N^{15}N$) + 1] (atom %) - $^{15}N$ natural abundance ratio (0.366%)

### 3.5 Discussion

**Nitrogen utilization and nutrient digestibility**

It has been reported that fermentable carbohydrates undergo fermentation to organic acids in the cecum. These organic acids, mainly SCFA, serve as an energy source for cecal bacteria (Levrat *et al.* 1991; Gidenne 1997). In rabbits, fermentable sugars can be used as a source of energy for cecal bacterial growth or proliferation (Davies & Davies 2003). Some urea N enters the cecum from the blood (Knutson *et al.* 1977), and is converted to ammonia by bacterial ureolytic activity (Forsythe & Parker 1985a). This ammonia N transferred from blood urea is then used for bacterial N synthesis (Demigné & Rémésy 1979; Younes *et al.* 1995b). As a result, fermentable sugar stimulates microbial proliferation and increases microbial protein levels. This process results in a decrease in urinary N excretion (Younes *et al.* 1995a, b, 1996). In this study, the addition of FOS to the diet resulted in significantly lower urinary N excretion and a higher retention rate of N compared with the glucose group. Similarly, we have previously observed that the feeding of D-mannitol improved N retention and decreased urinary N excretion in rabbits (Xiao *et al.* 2011; Xiao *et al.* 2013).

In this study, nutrient digestibility and absorption were not affected by the addition of FOS to the diet. We previously reported that the addition of FOS to guinea pig feed increased CA absorption and ADF digestibility (Kawasaki *et al.* 2013). In addition, it has been reported that the addition of FOS to feed increased the absorbability of CA in rats (Ohta *et al.* 1994; Nishiyama *et al.* 2009). These reports indicate that FOS promotes CA absorption in the rat and guinea pig; however, this did not occur in rabbits fed a FOS diet in this experiment.

In this experiment, we did not prevent loss of fecal component by heating at 60°C for the sample preparation. This might induce somewhat underestimate of the concentration of dry matter and N in the feces, resulting in a little overestimate of the digestibility of dry matter and N retention. However, this overestimate may not be a serious impact factor on the comparison between groups.

**Cecal organic acids**

In rats, FOS takes 3 to 4 h to reach the large intestine after oral ingestion, and is readily fermented by cecal bacteria to produce SCFA (Oku & Nakamura 2002), which

are utilized as a source of energy by epithelial cells of the large intestine (Henning & Hird 1972; Roediger 1980) and also as a source of synthesis of fatty acids and glucose in internal metabolism. In addition, both the amount and concentration of organic acids in the cecum were much higher in rats fed a FOS-containing diet (Sakaguchi *et al.* 1998), and in rats fed FOS the SCFA composition was considerably different from that of control rats (Younes *et al.* 1995b). However, in our rabbits, we found the SCFA composition in the FOS group did not greatly differ from that of the glucose group. Our results are consistent with a previous study that reported SCFA concentrations to be lowest in the cecotrophy period (09.00 hour to 13.00 hour) than at other times of the day (Bellier & Gidenne 1996).

**Bacterial N and $^{15}$N accumulation**

In this study, FOS entering the large intestine considerably enlarged the cecal content. This might induce a longer retention time of the cecal contents (Sakaguchi *et al.* 1998). In addition, the presence of carbohydrates in the large intestine stimulates bacterial proliferation, and this process requires a source of N (Levrat *et al.* 1993; Younes *et al.* 1995b). As a consequence, we found greater total N and bacterial N in the cecal of FOS-fed rabbits than in those in the glucose-fed group. These results indicate that FOS escapes enzymatic digestion in the small intestine and reaches the large intestine, where it can be fermented and used as a source of energy for bacterial growth.

The main sources of energy for microbial growth in the cecum are feed residues that contain small amounts of fermentable sugars. The results of this study indicate that FOS could be used as a source of energy for bacterial growth. Circulating blood urea and nitrogenous residues represent the major sources of N for bacterial protein synthesis in the cecum. When the availability of refractory but fermentable carbohydrates is substantial enough to stimulate bacterial growth in the cecum, then a greater flow of urea from the blood to the cecal lumen must be induced. This results in an increase of bacterial N. In our study, cecal bacterial $^{15}$N atom % excess was higher and cecal urea $^{15}$N atom % excess was significantly lower in rabbits fed the FOS diet compared with those in the glucose group. This result indicates that FOS stimulates bacterial growth and proliferation by utilizing urea N from the blood. A similar result was previously obtained in rabbits fed D-mannitol (Xiao *et al.* 2012).

**Blood urea N and urinary N**

When FOS enters the large intestine, the cecal wall becomes enlarged, resulting in increased blood flow to the cecum (Younes *et al.* 1995b). Thus, blood urea may be a substantial source of N in the large intestine (Mason 1984; Viallard 1984; Rémésy & Demigné 1989; Langran *et al.* 1992). The difference in concentrations of urea between arterial and portal blood can be thought of as an extraction of urea by the gastrointestinal tract. Our results indicate that the extraction of urea by the gastrointestinal tract was 2.6-fold greater in the FOS-fed group compared with the glucose group. Cecal total urease activity is increased by oligosaccharides (Rémésy *et al.* 1992; Younes *et al.* 1995b), resulting in a high rate of ammonia production and re-absorption in the large intestine (Rémésy & Demigné 1989), and could provide a favorable trans-mucosal gradient for passive diffusion of urea. FOS may increase the permeability of urea from arterial blood into the gastrointestinal tract. In the gastrointestinal tract, urea is degraded by urease to form ammonia, which is used by cecal bacteria to synthesize bacterial protein. This microbial protein is rapidly re-ingested by rabbits as cecotrophs (soft feces), which contain a larger amount of microbial protein than hard feces (Griffiths & Davies 1963). An increase in the diffusion of blood urea into the cecum and subsequent cecotrophy induces a decrease in blood urea N and a reduction in the amount of ammonia absorbed from the cecum into the blood, thus reducing urinary N excretion (Zervas & Zijlstra 2002). Our results likely indicate an increase in this process. Cecal urea N concentrations and $^{15}N$ atom % excess in urea N considerably decreased by feeding FOS, and cecal bacterial N and the amount of $^{15}N$ excess increased. On the other hand, the urea N concentration in serum from the portal vein significantly decreased.

In conclusion, the results of this study suggest a possible mechanism for the increase in N utilization caused by FOS in mature rabbits. FOS can act as an energy source for bacterial proliferation, increasing the transfer of blood urea N to the cecum for bacterial N synthesis. The reduction of urinary N excretion by FOS feeding signifies not only an improvement of dietary protein quality, but also a reduction of environmental eutrophication.

## Chapter 4

# Influence of Fructooligosaccharide on Nitrogen Utilization, Transfer of Blood Urea Nitrogen to Cecal Microbial Nitrogen in Young Rabbits Fed Urea Containing Diet


### 4.1 Abstract

To estimate the effect of fructooligosaccharide (FOS) on nitrogen (N) utilization in young rabbits fed a urea containing diet, ten rabbits were fed a commercial diet with urea 10 g/kg with added glucose or FOS 50 g/kg each for 8 days. During the last 5 days, total feces and urine were collected to measure N balances (Experiment 1). To demonstrate N transfer from the blood to cecal microbes, twelve rabbits were fed the same diets as in Experiment 1. After 9 days of feeding, 2 g of glucose or FOS and 40 mg $^{15}$N-urea was given orally. Two hours later cecal and blood samples were collected (Experiment 2). N retention rate were significantly higher in the FOS feeding group ($P < 0.05$). The $^{15}$N atom % excess of portal blood serum urea was lower in urea diet contain FOS feeding group. However, cecal total N, bacterial N and amount of excess $^{15}$N was not between-group different. The proximal colon amount of excess $^{15}$N was significantly lower in the FOS feeding group ($P < 0.05$). These results suggest that FOS to the urea containing diet was not affect transfer of blood urea N to the cecum for bacterial synthesis, thereby increasing N utilization.



**Keywords:** blood urea, microbial N, N utilization, urea-fed, young rabbit.


### 4.2 Introduction

In rabbits cecum contains $10^{11}$/g bacterial (Forsythe *et al.* 1985), those bacterial growth requires nitrogen (N) and energy source. In the rabbit about 40% of endogenous urea enters the digestive tract and is degraded by the cecal bacteria (Regoeczi *et al.* 1965). Endogenous urea was regarded main source of intestinal ammonia (Sabbaj et al. 1970; Summerskill & Wolpert 1970), and utilized by cecal bacterial growth or proliferation.

Fermentable fructooligosaccharide (FOS) is not split by intestinal disaccharidases. It across into the larger intestine and is rapidly fermented by intestinal

flora. It has been reported that, in rats, fermentation of FOS stimulates the growth and proliferation of cecal bacteria that utilize blood urea N, thus resulting in a decrease in urinary N excretion (Delzenne et al. 1995; Mussatto & Mancilha 2007). In a previous study, we found that FOS feeding improves dietary N utilization and increase the transfer of blood urea N to the large intestine for bacterial N synthesis in the rabbit (Xiao Min *et al.* 2013). These reports suggested that FOS seems to fermentable D-mannitol (Xiao Li *et al.* 2012), which inter to the larger intestine after promoting bacterial growth and proliferation when blood urea requires considerable supply source of N for bacterial protein synthesis and this process induce the increase of fecal bacteria N excretion (Viallard 1984, Langran *et al.* 1992).

Urea is important N source for ruminants, and has been examined extensively to describe its metabolism in the ruminants. The urea is highly soluble and diffusion into the intestine and hydrolyzed to ammonia in the rats (Chao & Tarver 1953). In the rabbits cecum contains high urease activity, so can efficiently utilizing of urea N source (Marounek et al. 1995). In addition, adult rabbits can utilize injection or orally administered urea then quantities highly significant in nitrogen metabolism of low-protein diet (Houpt 1963). However, in low-protein diet addition of urea did not enhanced growth of rabbits (Olcese & Pearson 1948). The approximately 290 mg/kg of Urea N was given intravenously of the rabbits, 24 h after found that Urea N was not retention in body fluids (Nolan & Heisinger 1971). Moreover, bacterial dry weight in cecal content has a tendency to be lower, and the cecal concentration of ammonia N was significantly increase in urea supplementation group than the control group. However, bacterial dry weight in cecal content was significantly increase, and cecal concentration of ammonia N was significantly decreased in rabbits fed a diet supplemented with urea-bentonite group (Abdl-Rahman et al. 2011). Those reports suggest that, dietary urea did not efficiently utilize by cecal bacterial in the rabbits. Nevertheless, urea-bentonite combination points to a synchronous supply of energy and ammonia N in the cecum for continuous growth of cecal microbes (Abdl-Rahman et al. 2011). When cecal bacterial growth stimulated by indigestible but fermentable FOS, the bacterial growth required of N was supply for Non-protein nitrogen of urea. The urea feed efficiently utilized by FOS feeding rabbits supply for N sources. In this study, we determined the effect of FOS on N transfer from the blood to cecal microbes and on N

utilization in feeding urea containing diet of rabbits.

## 4.3 Material and methods

### 4.3.1 Diets

Before the experiments the animals were maintained on a commercial diet (Labo R Stock, Nihon Nosan, Tokyo, Japan), which contained 16.3% crude protein (CP), 25.3% acid detergent fiber (ADF), 9.30% crude ash (CA), 91.3% dry matter (DM), and 3.40% ether extract (EE). The animals were kept in temperature-controlled rooms at 25 ± 1°C and individually housed in metabolic cages (diameter 38 cm, height 30 cm).

The experimental diets included addition of glucose (Nacalai Tesque Inc., Kyoto, Japan) or FOS (Nacalai Tesque Inc.) 50 g/kg and respectively added urea (Nacalai Tesque Inc.) 10 g/kg to the commercial diet. Animals were allowed free access to water during all experimental periods. The animals were daily food intake was recorded. The animals were not prevented from performing cecotrophy in this study.

The study was performed with the approval of, and in accordance with, the guidelines of the experimental animal care committee of Okayama University.

### 4.3.2 Animals and sampling

***Experiment 1***

Ten young rabbits (1.47 ± 0.11 kg, 3 months old) were used for experiments. The rabbits were randomly assigned to one of two treatments to minimize individual differences in the animals regarding the diets. The animals were fed an experimental diet for 8 days, comprising 3 days for adaptation and 5 days for collection of samples. The experiment diet was given as 50 g per daily metabolic body size ($kg^{0.75}$). Total excreted urine was collected daily for 5 days into 10 ml 1.5% boric acid solution to prevent evaporation of the N, and stored at 4°C until analysis. Feces were collected for 5 days, oven-dried at 60°C for 24 h, ground to a particle size of <1 mm, and stored at room temperature until further analysis.

***Experiment 2***

Twelve young rabbits (1.50 ± 0.12 kg, 3 months old) were randomly assigned to

one of two treatments groups, and fed an experimental diet for 9 days. After 8 days of experiment diet was given as 50 g per daily metabolic body size ($kg^{0.75}$), one day before experiment day, experiment diet was given as 50 g per daily metabolic body size ($kg^{0.75}$) further addition of 60 g. After 9 day feeding and 2 h prior to euthanasia 3mL of a water solution containing 2 g glucose or FOS and 40 mg $^{15}$N-urea (99.2 atom%, SI Science Co. Ltd, Sugito, Saitama, Japan) was respectively given orally. Two hour after the orally administration of $^{15}$N-urea, blood was taken from an abdominal artery and the portal vein under terminal anesthesia (pentobarbital sodium). All samples were collected between 09.00 hour and 10.00 hour. Serum was collected by centrifugation at 1,800 × *g* for 20 min and stored at -30°C until further analysis. The liver, small intestine, cecum, proximal colon, distal colon and rectum were removed and weighed, placed on ice, and stored at -30°C for subsequent analysis.

### 4.3.3 Chemical analyses and analytical procedures

The DM content of diets and feces was determined by drying the samples at 135°C for 2 h. CA content was estimated by ashing at 550°C for 6 h. The N content of the diet, urine, and feces, and the ADF content of fecal samples and diets were determined according to the method described by the Association of Official Analytical Chemists (AOAC 1990). Nutrient digestibility or absorbability and nitrogen accumulation rate were calculated using the following equations:

Apparent digestibility or absorbability (%) = 100 × (nutrient intake – fecal nutrient output) / nutrient intake;

Nitrogen accumulation rate (%) = 100 × (N intake – fecal N – urinary N) / N intake.

The digesta in each gut segment were homogenized, and the pH was measured immediately after collection using a digital pH meter (Horiba Ltd, Kyoto, Japan). The moisture content of the digesta was determined by drying a sample at 105°C for 24 h. The concentration of organic acids in the homogenized cecal contents was determined by HPLC (Column: 2 Shim-pack SCR-102H, Detector: Shimadzu CDD-6A; Shimadzu Corporation, Kyoto, Japan). A liver sample was homogenized in physiological saline (8.5 g sodium chloride/L). The homogenized liver sample, and samples of the contents of the small intestine, cecum, proximal and distal colon, and serum were treated with one-fifth of their volume of trichloroacetic acid (TCA) solution (500 g/L), centrifuged

(12,000 × *g* for 20 min at 4°C), and washed twice with TCA solution (50 g/L) (Fürst and Jonsson 1971). The TCA precipitates were analyzed for total N and protein N using a CN analyzer (Yanaco CN Corder, MT-700, J-Science, Kyoto, Japan). The deproteinized supernatants of the cecal contents were collected, and the urea within the supernatants was converted to ammonia by treatment with urease (*EC* 3.5.1.5; Nacalai Tesque Inc.) according to the procedure described by Obara et al. (1994). The urease-treated samples and non-urease-treated samples were steam distilled with the addition of magnesium oxide or sodium hydroxide, and ammonia was trapped in boric acid and titrated with sulfuric acid (0.05 *N*) for urea N and ammonia N determination (Nolan and Leng 1972). Samples of the contents of the cecum, proximal and distal colon were diluted in physiological saline, shaken well and placed in a refrigerator for 24 h at 4°C to dislodge adherent bacteria (Minato and Suto 1978). Bacteria residue was obtained by centrifugation (20,000 × *g* for 20 min at 4°C) of the supernatant after centrifugation at 500 × *g* for 5 min at 4°C (Belenguer et al. 2005), and the N content of the isolated bacteria was determined using a CN analyzer. Serum ammonia N and urea N concentrations were measured using commercially available kits (Ammonia Test-Wako and Urea N B Test-Wako; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The concentrations of $^{15}N$ in total N, protein N, bacterial N, urea N, and ammonia N of each sample were analyzed by mass spectrometer (MSI-150-MT-600 NC Model, Quadrupole Mass Spectrometry System; J-Science, Kyoto, Japan).

### 4.3.4 Statistical analysis

Data were calculated as mean ± standard deviation. The significant of between treatment differences was analyzed by Student`s *t*-tests (Microsoft Office Excel 2007; Microsoft Corporation). Differences were considered statistically significant at $P < 0.05$.

## 4.4 Results

Table 4.1 shows the feed intake, apparent digestibility or absorbability of nutrients, and N balance during the Experiment 1. During the experiment period, feed intake, weight gain and feed efficiency was not significantly influenced by addition of FOS to the diet containing urea. Apparent digestibility or absorption rate was not

distinctly different between the two groups.

N intake, N excretion in feces and urine were not significantly affected by in urea fed with a diet containing FOS. However, N retention, N retention rate based on intake and based on apparently digestibility were significantly higher compared with the control group ($P < 0.05$).

**Table 4.1** Feed intake, apparent digestibility or absorbability of nutrients and N balance in diets containing urea

| | GU | FU |
|---|---|---|
| Feed intake (g dry matter/day) | 64.9±4.36 | 64.7±3.43 |
| Apparent digestibility or absorbability (g/g) | | |
| Dry matter | 0.53±0.01 | 0.53±0.02 |
| Crude protein | 0.70±0.03 | 0.69±0.04 |
| Crude ash | 0.54±0.01 | 0.53±0.01 |
| Acid detergent fiber | 0.17±0.02 | 0.15±0.03 |
| N balance | | |
| N intake (g/day) | 1.85±0.12 | 1.86±0.09 |
| N feces (g/day) | 0.51±0.55 | 0.58±0.06 |
| N urine (g/day) | 1.12±0.19 | 0.91±0.17 |
| N retained (g/day) | 0.18±0.12 | 0.37±0.17* |
| N retention rate based on intake | 0.10±0.07 | 0.20±0.08* |
| N retention rate based on apparently digested | 0.22±0.16 | 0.38±0.10* |

Values are means ± SD (n=5). GU, commercial diet containing 5% glucose and 1% urea; FU, commercial diet containing 5% fructooligosaccharides and 1% urea.

* Significantly different from glucose groups ($P < 0.05$).

**Table 4.2** Liver weight and development of the gut

| | Fresh weight (g) | | Body weight ratio (%) | |
|---|---|---|---|---|
| | GU | FU | GU | FU |
| Liver | 49.5±3.51 | 44.8±6.44 | 3.18±0.22 | 2.91±0.33 |
| Stomach | | | | |
| Total weight | 66.0±16.2 | 65.7±10.2 | 4.23±1.04 | 4.25±0.37 |
| Wall weight | 11.3±1.71 | 12.0±1.38 | 0.72±0.10 | 0.78±0.04 |
| Content weight | 54.7±14.8 | 53.8±8.96 | 3.51±0.96 | 3.48±0.35 |
| Small Intestine | | | | |
| Total weight | 26.6±4.58 | 28.6±1.54 | 1.71±0.29 | 1.86±0.15 |
| Wall weight | 10.6±1.99 | 10.5±1.28 | 0.68±0.12 | 0.68±0.06 |
| Content weight | 15.9±3.31 | 18.1±1.13 | 1.02±0.21 | 1.19±0.14 |
| Cecum | | | | |
| Total weight | 65.3±14.1 | 76.1±5.20 | 4.18±0.88 | 4.95±0.37* |
| Wall weight | 14.1±2.68 | 13.9±1.02 | 0.90±0.17 | 0.90±0.08 |
| Content weight | 51.2±11.8 | 62.2±4.54* | 3.28±0.74 | 4.05±0.31* |
| Proximal Colon | | | | |
| Total weight | 15.0±2.08 | 13.0±3.98 | 0.96±0.13 | 0.83±0.17 |
| Wall weight | 6.64±0.51 | 6.41±0.76 | 0.43±0.03 | 0.42±0.03 |
| Content weight | 8.33±2.07 | 6.62±3.24 | 0.53±0.13 | 0.42±0.16 |
| Distal Colon | | | | |
| Total weight | 9.59±5.27 | 10.1±4.36 | 0.61±0.33 | 0.64±0.22 |
| Wall weight | 3.63±0.51 | 3.13±0.57 | 0.23±0.03 | 0.20±0.02* |
| Content weight | 5.96±4.84 | 6.92±3.88 | 0.38±0.30 | 0.44±0.21 |
| Rectum | | | | |
| Total weight | 5.25±2.12 | 5.73±1.57 | 0.34±0.13 | 0.37±0.07 |
| Wall weight | 1.91±0.44 | 1.75±0.26 | 0.12±0.03 | 0.11±0.02 |
| Content weight | 3.34±2.22 | 3.98±1.66 | 0.21±0.14 | 0.25±0.08 |
| Body weight | 1559±77.3 | 1542±147 | — | — |

Values are means ± SD (n=6). GU, commercial diet containing 5% glucose and 1% urea; FU, commercial diet containing 5% fructooligosaccharides and 1% urea.

* Significantly different from glucose groups ($P < 0.05$).

Table 4.2 shows liver weight and development of the gut after urea feed intake during the experimental period. The cecal content weight was significantly higher in FOS feeding group compared with the glucose group. Consequently, the cecal total weight of body weight ratio was significantly higher in urea fed with a diet containing FOS ($P < 0.05$). The distal colon wall weight of body weight ratio was significantly lower in intake FOS feeding group compared with the glucose group ($P < 0.05$). In the gut, with the exception of the cecum, there were no other between-group differences. The liver weight had a tendency to be lower in the FOS feeding group compared with the glucose group ($P = 0.076$). However, body weight ratio of liver weight was not different between the two groups.

**Table 4.3** Luminal pH, concentration (μmol/g contents) and total amount (μmol) of organic acids in the cecal content of rabbits

| | GU | FU |
|---|---|---|
| pH | 6.35±0.16 | 6.30±0.18 |
| Organic acid concentration | | |
| Succinic acid | 2.29±2.54 | 1.41±1.24 |
| Lactic acid | 3.54±1.58 | 2.62±0.57 |
| Formic acid | 0.20±0.03 | 0.29±0.20 |
| Acetic acid | 24.9±5.03 | 27.7±3.88 |
| Propionic acid | 5.20±1.37 | 9.15±3.90* |
| Butyric acid | 8.44±1.74 | 7.95±2.57 |
| Valeric acid | 0.39±0.30 | 0.19±0.24 |
| Total SCFA | 38.6±5.53 | 44.8±9.71 |
| SCFA molar ratio (Ac/Pr/Bu)[2] | 65/13/22 | 62/20/18 |
| Total amount of organic acids | 2294±572 | 3054±536* |

Values are means ± SD (n=6). GU, commercial diet containing 5% glucose and 1% urea; FU, commercial diet containing 5% fructooligosaccharides and 1% urea.

$^{15}N$ atom % excess = 100 / [2 ($^{14}N^{14}N$ / $^{14}N^{15}N$) + 1] (atom %) - $^{15}N$ natural abundance

ratio (0.366%).

Amount of $^{15}N$ excess = Total N × $^{15}N$ atom % excess.

* Significantly different from glucose groups ($P < 0.05$).

The concentration and total amount of organic acids and the pH in the cecal content are presented in Table 4.3. The total amount (μmol) of organic acids was significantly higher in urea diet addition of FOS feeding rabbits ($P < 0.05$). Though, the concentration of the individual SCFAs (acetic, propionic, butyric) and total SCFA were not significantly different between two groups. In addition, the SCFAs fermentation pattern was not greatly different between the two groups.

**Table 4.4 Total N and $^{15}N$ concentration in gut contents (DM basis)**

| | Proportion of N (% of DM) | Total N (mg) | $^{15}N$ atom % excess | Amount of excess $^{15}N$ (mg) | % of $^{15}N$ administration |
|---|---|---|---|---|---|
| Small intestine | | | | | |
| GU | 9.89±3.62 | 325±117 | 0.22±0.08 | 0.76±0.51 | 3.98±2.66 |
| FU | 10.4±3.85 | 339±77.1 | 0.24±0.07 | 0.85±0.42 | 4.45±2.18 |
| Cecum | | | | | |
| GU | 5.50±1.84 | 687±148 | 0.29±0.16 | 1.88±0.95 | 9.77±4.95 |
| FU | 4.75±1.21 | 707±149 | 0.20±0.08 | 1.42±0.62 | 7.41±3.23 |
| Proximal colon | | | | | |
| GU | 4.87±0.30 | 104±26.2 | 0.26±0.09 | 0.28±0.13 | 1.46±0.70 |
| FU | 4.60±0.69 | 78.2±27.5 | 0.19±0.05 | 0.14±0.04* | 0.74±0.22* |
| Distal colon | | | | | |
| GU | 4.99±0.48 | 85.0±69.3 | 0.22±0.09 | 0.16±0.05 | 0.81±0.28 |
| FU | 4.63±0.70 | 89.5±38.0 | 0.17±0.08 | 0.14±0.06 | 0.71±0.33 |
| Rectum | | | | | |
| GU | 6.28±1.70 | 62.3±38.8 | 0.20±0.09 | 0.11±0.09 | 0.59±0.47 |
| FU | 6.12±1.65 | 70.8±19.6 | 0.19±0.08 | 0.13±0.06 | 0.69±0.33 |

Values are means ± SD (n=6). GU, commercial diet containing 5% glucose and 1% urea; FU, commercial diet containing 5% fructooligosaccharides and 1% urea.

$^{15}N$ atom % excess = 100 / [2 ($^{14}N^{14}N$ / $^{14}N^{15}N$) + 1] (atom %) - $^{15}N$ natural abundance ratio (0.366%).

Amount of $^{15}N$ excess = Total N × $^{15}N$ atom % excess.

* Significantly different from glucose groups ($P < 0.05$).

Table 4.4 shows the total N and $^{15}N$ concentration in the gut contents on a DM basis of the experimental period. In experimental period, cecal total N and $^{15}N$ concentration was no difference between the two groups. The amount of $^{15}N$ excess in proximal colon was significantly decreased in urea diet addition to FOS feeding group compared with the glucose feeding group.

**Table 4.5 Protein N and $^{15}N$ concentration in liver and larger intestine (DM basis)**

| | Proportion of N (% of DM) | Total N (mg) | $^{15}N$ atom % excess | Amount of excess $^{15}N$ (mg) | % of $^{15}N$ administration |
|---|---|---|---|---|---|
| Liver | | | | | |
| GU | 9.70±1.06 | 1634±124 | 0.28±0.11 | 4.68±1.89 | 24.4±9.85 |
| FU | 9.29±1.13 | 1385±123* | 0.24±0.19 | 3.14±2.32 | 16.4±12.1 |
| Small intestine | | | | | |
| GU | 7.23±1.54 | 236±31.5 | 0.13±0.03 | 0.30±0.08 | 1.57±0.43 |
| FU | 7.25±0.64 | 247±38.6 | 0.16±0.08 | 0.37±0.18 | 1.93±0.92 |
| Cecum | | | | | |
| GU | 3.67±1.23 | 456±104 | 0.12±0.13 | 0.69±0.71 | 3.58±3.68 |
| FU | 4.34±1.32 | 640±152* | 0.16±0.10 | 1.03±0.64 | 5.35±3.34 |
| Proximal colon | | | | | |
| GU | 4.50±0.19 | 96.9±26.1 | 0.16±0.09 | 0.17±0.11 | 0.87±0.55 |
| FU | 4.48±0.30 | 78.7±35.7 | 0.16±0.08 | 0.12±0.07 | 0.64±0.37 |
| Distal colon | | | | | |
| GU | 4.53±0.50 | 81.7±76.5 | 0.11±0.09 | 0.08±0.06 | 0.40±0.32 |
| FU | 4.51±0.44 | 89.9±44.9 | 0.12±0.05 | 0.10±0.04 | 0.50±0.19 |

Values are means ± SD (n=6). GU, commercial diet containing 5% glucose and 1% urea; FU, commercial diet containing 5% fructooligosaccharides and 1% urea.

$^{15}N$ atom % excess = 100 / [2 ($^{14}N^{14}N$ / $^{14}N^{15}N$) + 1] (atom %) - $^{15}N$ natural abundance ratio (0.366%).

Amount of $^{15}N$ excess = Total N × $^{15}N$ atom % excess.

* Significantly different from glucose groups ($P < 0.05$).

Table 4.5 shows the protein N and $^{15}N$ concentrations in the liver and gut contents on a DM basis. The total protein N in liver was significantly lower in the urea diet contain of FOS feeding group than that of in the urea diet contain of glucose feeding group ($P < 0.05$). This must be relation with tendency to decrease in liver weight. The cecal total protein N was significantly higher in the urea diet contain of FOS feeding group compared with glucose feeding group ($P < 0.05$).

**Table 4.6 Bacterial N and $^{15}N$ concentration in lager intestine (DM basis)**

| | Proportion of N (% of DM) | Total N (mg) | $^{15}N$ atom % excess | Amount of $^{15}N$ excess (mg) | % of $^{15}N$ administration |
|---|---|---|---|---|---|
| Cecum | | | | | |
| GU | 3.20±0.31 | 412±62.0 | 0.09±0.06 | 0.36±0.24 | 1.86±1.24 |
| FU | 3.01±0.21 | 455±81.9 | 0.14±0.11 | 0.67±0.58 | 3.48±3.04 |
| Proximal colon | | | | | |
| GU | 4.22±0.30 | 90.4±23.2 | 0.15±0.06 | 0.14±0.07 | 0.73±0.35 |
| FU | 3.83±0.51 | 65.5±22.4* | 0.13±0.02 | 0.08±0.03* | 0.43±0.17* |
| Distal colon | | | | | |
| GU | 3.75±0.31 | 68.3±64.6 | 0.13±0.10 | 0.08±0.06 | 0.40±0.29 |
| FU | 3.46±0.40 | 68.6±33.1 | 0.13±0.08 | 0.07±0.04 | 0.36±0.19 |

Values are means ± SD (n=6). GU, commercial diet containing 5% glucose and 1% urea; FU, commercial diet containing 5% fructooligosaccharides and 1% urea.

$^{15}N$ atom % excess = 100 / [2 ($^{14}N^{14}N$ / $^{14}N^{15}N$) + 1] (atom %) - $^{15}N$ natural abundance ratio (0.366%).

Amount of $^{15}N$ excess = Total N × $^{15}N$ atom % excess.

* Significantly different from glucose groups ($P < 0.05$).

Table 4.6 shows the concentration and amount of bacterial N and $^{15}N$ in the contents of the cecum and colon. The cecal and distal colon bacterial N and $^{15}N$ concentration was no difference between the two groups. Total N and amount of $^{15}N$ excess in there proximal colon bacteria were significantly decrease in the urea diet contain of FOS feeding group ($P < 0.05$, respectively).

**Table 4.7 Concentration of ammonia N, Urea N and $^{15}N$ in cecal content, arterial blood and portal blood serum**

| | GU | FU |
|---|---|---|
| Cecum Ammonia N | | |
| N concentration (μg/g content) | 201±34.4 | 193±23.8 |
| $^{15}N$ atom % excess | 1.04±0.58 | 1.40±0.71 |
| Cecum Urea N | | |
| N concentration (μg/g content) | 117±32.0 | 109±24.3 |
| $^{15}N$ atom % excess | 1.80±1.34 | 1.15±0.66 |
| Arterial blood serum Ammonia N | | |
| N concentration (μg/dl) | 285±188 | 265±84.4 |
| $^{15}N$ atom % excess | 0.93±0.82 | 0.51±0.46 |
| Arterial blood serum Urea N | | |
| N concentration (mg/dl) | 25.2±5.13 | 26.9±3.63 |
| $^{15}N$ atom % excess | 1.30±1.18 | 1.32±0.97 |
| Portal blood serum Ammonia N | | |
| N concentration (μg/dl) | 464±80.1 | 487±185 |
| $^{15}N$ atom % excess | 1.04±0.45 | 0.62±0.34 |
| Portal blood serum Urea N | | |
| N concentration (mg/dl) | 15.9±6.77 | 19.4±5.54 |
| $^{15}N$ atom % excess | 1.69±0.61 | 1.22±0.16* |

Values are means ± SD (n=6). GU, commercial diet containing 5% glucose and 1% urea; FU, commercial diet containing 5% fructooligosaccharides and 1% urea.

$^{15}N$ atom % excess = 100 / [2 ($^{14}N^{14}N$ / $^{14}N^{15}N$) + 1] (atom %) - $^{15}N$ natural abundance ratio (0.366%).

* Significantly different from glucose groups ($P < 0.05$).

Table 4.7 shows the concentration of ammonia N and urea N, and $^{15}N$ atom % excess in wet cecal contents and in serum from arterial or portal vein blood. The concentration of cecal urea N did not differ between the two groups. However, $^{15}N$ atom % excess was tendency to decrease in urea diet addition to FOS feeding group than that of in the glucose feeding group. Concentration of serum ammonia N and urea N in arterial blood did not differ between the two groups. The serum ammonia N concentration in the blood from the portal vein has a tendency to be lower in urea diet contain FOS feeding group than in the glucose feeding group ($P = 0.051$). The serum urea N concentration in blood from the portal vein did not differ between the two groups. However, $^{15}N$ atom % excess has a significantly decrease in urea diet contain FOS feeding group compared with the glucose feeding group ($P < 0.05$).

**4.5 Discussion**

In the cecum contain a lot of urease, and urease activity was higher in this area compared with other intestinal segments of the rabbits (Forsythe *et al.* 1985). Usually, endogenous urea was degraded by cecal bacterial, and supply major source of ammonia for the cecal bacterial growth or proliferation. For rabbit, the fermentable carbohydrate entered the large intenstine after the intake and stimulated bacterial growth, which result in the insufficient of N source for endogenous urea. At that time, blood urea was degraded by urease supply ammonia for bacterial growth necessary N in the larger intestine. The result, urinary N was greatly decreased in the feeding fermentable carbohydrate rabbits. In previous study, we found that feeding of D-mannitol or FOS by resulted higher N retention in cecum caused by greater urea conversion from blood, which was coupled with lowering of urinary N excretion in rabbits (Xiao Li *et al.* 2011, 2012; Xiao Min *et al.* 2013). These findings indicate that FOS stimulated bacterial proliferation in cecum, which promoted ammonia utilization for bacterial protein synthesis by reducing ammonia absorption and increasing the transfer of blood urea N to the cecum. However, in this study supplementation of FOS to the urea containing diet did not affect cecal total N contents, bacterial N contents and portal blood serum urea N

concentration. This result suggested that FOS did not improve N utilization of urea containing diet.

In this study, the small intestine concentration of total N was higher than previous study (0.64-fold, Xiao Min *et al.* 2013). In addition, the $^{15}N$ atom % excess of cecal ammonia N, Urea N and bacterial N was no deference in previous study (Xiao Min *et al.* 2013). These results may indicate that large proportion of oral administration of urea were concentrated in small intestine, then gradually degradation in this area, and diffusion into the portal system and used, mainly in the liver, for nonessential amino acids synthesis (Chao *et al.* 1953).

Our experiment result indicated that cecal content weight and total amount of organic acids was greatly increased in FOS containing diet than in glucose containing diet. Those results are consistent with previous study (Xiao *at al.* 2013). This higher cacal contenr weight could be associated with the FOS feeding. This result could be in association with that FOS effect of cecal contents and mean retention time of the rats (Sakaguchi et al. 1998).

In present study, supplementation of FOS to the urea containing diet was significantly increased of N retention rate based on intake. However, the urinary N excretion was not affected by FOS feeding rabbits. In our previous study, the experiment diet as a commercial pellet diet (Lab Diet Hi-Fiber Rabbit, PMI Nutrition international, LLC, Brentwood, UA, USA) supplemented D-mannitol to the urea containing diet significantly decrease urinary N excretion and significantly increase N retention rate based on intake in growing rabbits (Xiao Li *et al.* 2012). This is consistent with recommendations of protein requirement (CP, 16%) for growing rabbits (NRC 1977). However, in present study addition of 1% urea to the commercial diet (CP, 16.3%) was higher to protein requirements of growing rabbits. This result indicated that in the rabbits cannot efficiently utilization of higher than protein requirement (CP, 16%). Nolan *et al.*（1971）suggested that dietary factors influence urea-N utilization in ruminant, probably have similar effects occur in the rabbits.

In conclusion, the addition of FOS as an indigestible, but fermentable, sugar to the urea containing diet of rabbits improved N retention, but was failed to reduce N excretion in urine. In addition, cecal bacterial growth or proliferation did not efficiency

utilize of NPN source of FOS to the diet containing urea. Those results indicated that FOS was not act as an energy source for bacterial proliferation, increasing the transfer of blood urea N to the cecal for bacterial N synthesis in urea containing diet.

# Chapter 5

## Effects of Lactosucroe, Sorbitol and Raffinose on Nutrient Digestibility and Nitrogen Utilization in Adult Rabbits


### 5.1 Abstract

The effect of indigestible carbohydrate on apparent digestibility of nutrients and nitrogen (N) utilization, have been investigated in adult rabbits fed a commercial diet. In experiment 1, eighteen adult rabbits were fed a commercial diet with glucose or lactosucrose and sorbitol 50 g/kg each. In experiment 2, fourteen adult rabbits were fed a commercial diet with glucose or raffinose 30 g/kg each. Total feces and urine were collected during the experimental period. The results indicated that urinary N excretion has a tendency to decrease in lactosucrose and sorbitol groups than in the glucose group. Consequently, the N retention, and the N retention rate based on N intake and based on digested N tend to increase in the lactosucrose and sorbitol groups compared with the glucose group (Experiment 1). Urinary N excretion, N retention, and the N retention rate based on N intake and based on digested N was not different between the two groups (Experiment 2). These results suggest that the all indigestible carbohydrate did not act as an energy source for bacterial proliferation, increasing the transfer of blood urea N to the cecum for bacterial N synthesis in rabbits.



**Key words:** indigestible carbohydrate, nutrient digestibility, nitrogen utilization, adult rabbit


### 5.2 Introduction

Rabbits possess a very large cecum, which is the main area of bacterial activity (Bellier *et al.* 1995; Gidenne *et al.* 2002). As a cecum fermenter, the rabbit has a special nutritional system called cecotrophy. This process allows rabbits to re-ingest their microbial product in the cecum, as cecotrophs. During certain times of the day rabbit produce ingest special feces (cecotrophes or soft feces) with content of high bacterial protein, B-group vitamins, minerals and less fiber (Hörnicke 1981; cheeke 1987; Belenguer *et al.* 2002; Sakaguchi 2003). During other hours rabbits excrete hard feces, which contain content of high fiber and low minerals. Cecotropy has considerable nutritional significant for nitrogen (N) supplementation in rabbit. This special digestive system compares the ruminant species in term of microbial protein recycling (Belenguer *et al.* 2002). It can provide up to 30% of the daily N intake, The re-ingested protein is

characterized by a high digestibility and an elevated content of essential amino acids (Hörnicke 1981), which is mostly derived from cecal microbes (Belenguer *et al.* 2002).

Indigestible carbohydrates escape digestion of the stomach or small intestine and reach the large intestine, where they can be partly or totally fermented (Nyman 2002) and profoundly increase microbial multiplication in rats (Younes et al. 1995, 2001). The indigestible carbohydrates stimulate bacterial deaminase and urease activity, leading to a substantial increase in ammonia, which is the major source of N for microbial flora (Varel et al. 1974), utilized by the cecum large microbial population in the synthesis of proteins. The high bacterial content of the cecal content pass through the cecotrophes re-ingested and take part in body protein synthesis of rabbits (Griffiths & Davies 1963). Indigestible but fermentable sugar D-mannitol provided a significant contribution to the improvement of N utilization in adult rabbit (Hanieh & Sakaguchi 2009; Xiao Li *et al.* 2011; Xiao Min *et al.* 2013). In addition, fructooligosaccharide increases the N retention rate and decreases urinary N excretion in rabbit (Tsuzuki *et al.* 2010; Xiao Min *et al.* 2014).

The indigestible sugars, such as maltitaol, lactinol, D-mannitol, and fructooligosaccharide, have been shown to mineral bioavailability in humans and rats (Goda et al. 1995; Ammann et al. 1988; Jin et al. 2013; Ohta et al. 1995). In addition, it has been shown that when the fructooligosaccharide and D-mannitol are added to the diets of rats, the ash absorption is increased (Nishiyama *et al.* 2009). The addition of indigestible sugar D-mannitol to the diet was shown to increase the fiber digestibility (Hanieh & Sakaguchi 2009; Xiao Min et al. 2013) and crude ash absorption (Xiao Li *et al.* 2011; Xiao Min *et al.* 2013) in adult rabbits. This information indicated that the nutrient digestibility or absorption was affected by feeding indigestible sugar in host.

In the previous study it has been reported that indigestible but fermentable sugar of D-mannitol and fructooligosaccharides have great effect on N utilization and nutrient digestibility or absorption in rabbits. Davies & Davies (2003) suggested that fermentable sugars can be used as a source of energy for cecal bacterial growth in rabbits. Consequently, all indigestible but fermentable carbohydrate influence nitrogen utilization and nutrition digestibility or absorption in rabbits. In the present study, we estimated the effects of lactosucrose ($4^{G}$-β-D-galactosylsucrose), sorbitol and raffinose on N utilization and nutrient digestibility in adult rabbits.

## 5.3 Material and methods

### 5.3.1 Animal and diets

Before the experiments the animals were maintained on a commercial diet (Labo R Stock, Nihon Nosan, Tokyo, Japan), which contained 16.3% crude protein (CP), 25.3% acid detergent fiber (ADF), 9.30% crude ash (CA), 91.3% dry matter (DM), and 3.40% ether extract (EE). The animals were individually housed in metabolism cages with wire-mesh bottoms (diameter 38 cm, height 30cm) for feces and urine collection, and maintained in an air-conditioned room at an ambient temperature of 23-25°C and a relative humidity of 50-60% with the lights on from 05.30 to 17.30 hours.

*Experiment 1*

Eighteen adult rabbits (1.17 ± 0.08 kg) were used for experiments. The rabbits were randomly assigned to one of three treatments to minimize individual differences in the animals regarding the diets. The experimental diets included addition glucose (Nacalai Tesque Inc., Kyoto, Japan), lactosucrose (Ensuiko Sugar Refining Co. Ltd., Tokyo, Japan) and sorbitol (Nacalai Tesque Inc.) to the commercial diet at 50 g/kg (Table 5.1). The diet was given as 50 g per daily metabolic body size ($kg^{0.75}$). Daily food intake was recorded. The animals were given free access to water during all experimental periods. The animals were not prevented cecotrophy in this study.

Each feeding trial lasted 8 days, comprising 3 days for adaptation and 5 days for collection of samples for each phase. The animals were weighed at the beginning and termination of the experiment. Excreted urine was collected daily in 1.5% boric acid solution to prevent evaporation of N. total urine was collected for 5 days, diluted to 1000 ml with distilled water and stored at -4°C until analysis. Feces were collected for 5 days, oven-dried at 60°C for 24 h, ground to sizes of <1 mm and stored at room temperature until further analysis.

The study was performed with the approval of, and in accordance with, the guidelines of the experimental animal care committee of Okayama University.

*Experiment 2*

Fourteen adult rabbits (1.94 ± 0.12 kg) were used for experiments. The rabbits were randomly assigned to one of two treatments to minimize individual differences in the animals regarding the diets. The experimental diets included addition glucose (Nacalai Tesque Inc.), raffinose (Nacalai Tesque Inc.) to the commercial diet at 30 g/kg (Table 5.1). The other procedures were the same as those for experiment 1.

**Table 5.1** Chemical compositions of experimental diets (g/kg dry matter)

| | Experiment 1 | | | Experiment 2 | |
|---|---|---|---|---|---|
| | Glucose 1 | Lactosucrose | Sorbitol | Glucose 2 | Raffinose |
| Dry matter | 964 | 965 | 961 | 907 | 903 |
| Organic matter | 908 | 909 | 910 | 912 | 916 |
| Crude protein | 164 | 169 | 159 | 155 | 149 |
| Acid-detergent fiber | 224 | 221 | 230 | 226 | 222 |
| Crude ash | 92 | 91 | 90 | 88 | 84 |

Glucose 1, commercial diet containing 5% glucose; lactosucrose, commercial diet containing 5% lactosucrose; sorbitol, commercial diet containing 5% sorbitol; glucose 2, commercial diet containing 3% glucose; raffinose, commercial diet containing 5% raffinose.

### 5.3.2 Chemical analyses

The dry matter (DM) content of diets and feces was determined by drying the samples at 135°C for 2 h. The crude ash (CA) content was estimated by ashing at 550°C for 6 h. The N content of the diet, urine, and feces, and the acid-detergent fiber (ADF) content of fecal samples and diets were determined according to the method of the Association of Official Analytical Chemists (AOAC 1990). Nutrient digestibility and nitrogen accumulation rate were calculated using the following equations:

Apparent digestibility or absorbability (%) = 100 × (nutrient intake – fecal nutrient output) / nutrient intake

Nitrogen accumulation rate (%) =100 × (N intake – fecal N – urinary N) / (N intake)

### 5.3.3 Statistical analyses

Data are calculated as mean ± standard deviations. In Experiment 1, the significance of between treatment differences was assessed using nonparametric one-way analysis of variance (ANOVA; Tukey method; Excel statistics, SSRI Co., Tokyo, Japan). In Experiment 2, differences between group means were assessed using student's *t*-tests (Microsoft Office Excel 2007; Microsoft Corporation). Differences were considered significant at $p < 0.05$.

## 5.4 Results

Table 5.2 shows the feed intake, apparent digestibility or absorbability of nutrients, and N balance during Experiment period. In Exp 1 and Exp 2, the daily feed intake (DM), N intake and fecal N excretion in indigestible sugar group were not

different from that of control group. Therefore, feed conversion or growth performance was not affected by oligosaccharide groups compared with glucose groups. In Exp 1 the urinary N excretion has a tendency to decrease in lactosucrose and sorbitol groups than in the glucose group. Consequently, the N retention, and the N retention rate based on N intake and based on digested N tend to increase in the lactosucrose and sorbitol groups compared with the glucose group. However, in Exp 2 the urinary N excretion, N retention, and the N retention rate based on N intake and based on digested N was not different between the two groups. In the two experiments, apparent digestibility or absorbability of DM, CP, CA and ADF were not significantly different between the glucose group and indigestible sugar groups.

**Table 5.2** Feed intake, apparent digestibility or absorbability of nutrients, and N balance in experiment periods.

| | Experiment 1 (n = 6) | | | Experiment 2 (n = 7) | |
|---|---|---|---|---|---|
| | Glucose | Lactosucrose | Sorbitol | Glucose | Raffinose |
| Feed intake (g dry matter/day) | 58.8±3.90 | 57.2±1.63 | 56.8±3.83 | 70.2±15.3 | 69.8±12.2 |
| Apparent digestibility or absorption rate (%) | | | | | |
| Dry matter | 60.4±2.22 | 60.1±6.06 | 58.5±1.02 | 56.5±1.32 | 54.5±2.39 |
| Crude protein | 74.5±2.98 | 74.3±4.74 | 73.0±2.76 | 67.0±2.60 | 68.8±2.77 |
| Crude ash | 57.7±1.77 | 58.2±6.86 | 54.2±1.55 | 56.0±2.39 | 55.0±2.61 |
| Acid detergent fiber | 25.3±4.56 | 24.8±11.1 | 23.9±2.15 | 25.7±4.15 | 24.8±4.64 |
| N balance | | | | | |
| N intake (g/day) | 1.58±0.10 | 1.58±0.04 | 1.51±0.11 | 1.89±0.44 | 1.82±0.34 |
| Fecal N (g/day) | 0.40±0.05 | 0.40±0.08 | 0.40±0.07 | 0.59±0.16 | 0.60±0.12 |
| Urinary N (g/day) | 0.72±0.11 | 0.57±0.23 | 0.52±0.26 | 1.23±0.22 | 1.11±0.25 |
| N retained (g/day) | 0.46±0.17 | 0.61±0.30 | 0.58±0.22 | 0.07±0.10 | 0.10±0.16 |
| N retention rate based on N intake (%) | 29.1±9.51 | 38.6±19.0 | 38.7±14.9 | 2.98±4.62 | 5.33±8.62 |
| N retention rate based on N digested (%) | 38.6±11.2 | 50.6±24.3 | 53.3±21.5 | 4.41±6.66 | 8.21±13.7 |

Glucose 1, commercial diet containing 5% glucose; lactosucrose, commercial diet containing 5% lactosucrose; sorbitol, commercial diet containing 5% sorbitol; glucose 2, commercial diet containing 3% glucose; raffinose, commercial diet containing 5% raffinose.

**5.5 Discussion**

In the previous study, we demonstrate that indigestible but fermentable oligosaccharides D-mannital and fructo-oligosaccharide effectively depress urinary N and enhance N retention of the adult rabbits (Hanieh & Sakaguchi 2009; Xiao Li et al. 2011, 2012; Xiao Min et al. 2013). These studies suggested that fermentable carbohydrates escaped digestion in the small intestine, rich flow into the large intestine, where they are fermented to organic acids, mainly short chain fatty acids (SCFAs), and used as a source of energy for cecal bacterial growth or proliferation in host (Levrat *et al.* 1991; Gidenne 1997; Davies & Davies 2003). When adding indigestible but fermentable carbohydrates in the feed, the bacterial multiplication of optimal N that is provided by the small intestine undigested protein, endogenous protein diffuse into digestive contents of blood urea (Mason et al. 1984; Moran et al. 1990; Cummings & Macfarlane 1991). Circulating blood urea and nitrogenous residues were major source of N for bacterial protein synthesis in rabbit cecum.

In the present study, the addition of lactosucrose, sorbitol and raffinose to the diet did not affect N utilization. However, in the previous study, the supplementation of D-mannital (Hanieh & Sakaguchi 2009; Xiao Li *et al.* 2011; Xiao Min *et al.* 2013) and fructooligosaccharide (Tsuzuki *et al.* 2010; Xiao Min *et al.* 2014) to the diet significantly increased N utilization in rabbits. These results indicate that whole of the indigestible but fermentable carbohydrates did not stimulate bacterial proliferation in the cecum, which utilization of blood urea N to the cecal bacterial N synthesis in the rabbits. Dietary supplementation with lactosucrose decreased the pH value of the large intestine in rats (Kishino et al. 2006). In addition, the release of hydrogen gas will culminate after taking lactosucrose over 30 min in healthy adult (Fujita et al. 1991). These result suggested that lactosucrose was rapidly and effectively fermented by cecal bacteria. In the present study may be caused by a similar mechanism, that the lactosucrose was not efficiently utilized by cecal bacterial proliferation in rabbit. Sorbitol was detected in the urine of human and rats subjects after oral doses of maltitol (Kearsley & Lian-Loh 1982; Lian-Loh et al. 1982). This information could suggested sorbitol was not fermented by cecal microbial. Sorbitol had been absorbed only partially in the small intestine, and undergoes fermentation in the lower part of the digestive tract (Schell Dompert & Siebert 1980).

In conclusion, lactosucrose, sorbitol and raffinose were not similar to mannitol or fructo-oligosaccharide effect on N utilization in rabbits. All indigestible but

fermentable carbohydrate did not act as an energy source for bacterial proliferation, increasing the transfer of blood urea N to the cecum for bacterial N synthesis in rabbits.

# Chapter 6

## GENERAL DISCUSSION

In this study, it was demonstrated that indigestible oligosaccharide could increase nutrient digestibility and nitrogen utilization in rabbits. And provide a possible mechanism for the increment of acid detergent fiber (ADF) digestibility and nitrogen (N) retention by the consumption of indigestible oligosaccharides.

Rabbit can provide the important source of food for human. Especially in European countries, the rabbit is an important farm animal. The rabbit meat is high in protein, low in fat and cholesterol. Therefore physicians have recommended rabbit meat to patients with coronary condition. In recent years, in Asian countries people also begin to pay their attention to dietary health. So we speculated that the rabbit will gradually replace the poultry in Asia. Recently, in European countries the people begin to pay an attention to the relationship between animal forage additives and human health. However, indigestible oligosaccharides are commonly contained in natural products, and widely used in functional foods, because the indigestible oligosaccharides have important physicochemical and physiological properties beneficial to health of human (Mussatto & Mancilha 2007).

The rabbit is a monogastric herbivore, and its digestive system is very adaptative to high fiber diet. Rabbits possess a very large cecum, and which is the main area of bacterial activity (Bellier *et al.* 1995; Irlbeck 2001; Gidenne *et al.* 2002). As a cecal fermenter, the rabbit has a special nutritional system called cecotrophy. This process allows rabbits to re-ingest their microbial product in the cecum, as cecotrophs. Cecotropy has considerable nutritional significant for N supplementation in rabbit. It can provide up to 30% of the daily N intake, The re-ingested protein is characterized by a high digestibility and an elevated content of essential amino acids (Hörnicke 1981), which is mostly derived from cecal microbes (Belenguer *et al.* 2002). The rabbit large intestine has an important nutritional function with a mechanism for selective retention of fine particles and solutes (Sakaguchi 2003). This separation mechanism is consistent with lower fiber digestibility due to the short fermentation period in rabbits (Sakaguchi 2003). These digestive characteristic are adapted to high intake of dietary fiber and provide sufficient nutrient requirements for small body size of herbivores animal as rabbits.

A lot of papers have been reported indigestible oligosaccharide escape digestion of the stomach or small intestine and reach the large intestine, where they can be induce

enlargement of the large intestine, and partly or totally fermented by the microflora into short chain fatty acids (SCFAs) and profoundly increase microbial multiplication. The multiplicatied bacteria re-ingested through the cecotrophes and take part in body protein synthesis of rabbits (Griffiths & Davies 1963). At the same time, an indigestible oligosaccharide effects nutrient digestibility or absorbability in the host. In previous studies, an indigestible sugar D-mannitol provided a significant contribution to decrease of urinary nitrogen excretion and the improvement of N utilization in adult rabbit (Hanieh & Sakaguchi 2009; Xiao Li *et al.* 2011). Additionally, the addition of indigestible sugar D-mannitol to the diet was shown to increase the fiber digestibility in rabbits (Hanieh & Sakaguchi 2009). Chiou *et al.* (1998) suggested that fiber digestibility is dependent on retention time in gastrointestinal tracts and separation of particles in the digestive tract (Carabaño *et al.* 1988) in rabbits. Dietary fiber is a substrate for cecal bacterial fermentation produces mainly SCFAs, used as energy for cecal bacterial growth. In chapter 2, D-mannitol extended the retention time of solid-phase contents in rabbits. Owing to the elongated retention time, bacteria may have more time to ferment fiber components in the diet. This likely explains the increase of ADF digestibility in rabbits fed D-mannitol, because it has been reported that longer digesta retention times partially explain higher ADF digestibility (Sakaguchi 2003). However, the indigestible sugars, such as fructo-oligosaccharide (chapter 3), lactosucrose, sorbitol, and raffinose (chapter 5) had no effect on ADF digestibility for rabbit in diet except D-mannitol (chapter 2).

In recent years, a lot of researchers have been reported indigestible sugar, FOS comparatively effects on nitrogen balance of the rats (Younes et al. 1995b). In our laboratory, addition of FOS to the diet has been shown to significantly improve the utilization of N in rabbits and guinea pigs (Tsuzuki & Sakaguchi 2010; Kawasaki *et al.* 2012). Many of the studies indicated that indigestible sugar, enter into the cecum stimulate bacterial proliferation and urease activity. In addition, Younes at al. (1995b) has been shows that indigestible carbohydrates enter into the gut they induce an enlargement of the cecum, which results in greater intestinal surface area and blood flow. At the same time, indigestible sugar can be fermented and used as a source of energy for bacterial proliferation. In this study, our examined that possible mechanism of FOS on N transfer from the blood to cecal microbes (using of $^{15}$N-urea) and on N utilization and nutrient digestibility in mature rabbits (chapter 2). The cecal content of DM, bacterial N, and total N was significantly improved in FOS feeding rabbits compared with glucose feeding rabbits. Furthermore, total amount of $^{15}$N excess in cecal bacteria was significantly greater and $^{15}$N atom % excess of cecal ammonia was lower in rabbits fed

a diet containing FOS (chapter 3). These results showed that FOS like mannitol enter into the large intestine stimulated bacterial growth, and blood urea was provided as N source for the growth of bacterial.

Based on this study (chapter 3), we try to give N source for the growth of cecal bacterial provided by non-protein source such as urea added to FOS containing diet (chapter 4). In chapter 4, we estimated that dietary urea could be used by microbial growth and make use of the mechanisms in addition of dietary urea to the FOS containing diet. The result cecal content of bacterial N was not increased in FOS containing diet of urea feed. Further, there was no different between two groups in the cecal urea content and $^{15}N$ atom % excess. These results indicated that when FOS stimulated cecal bacterial proliferation required N source cannot be provided by the feeding of urea. The arterial blood serum ammonia N concentration was fairly higher by the addition of urea (chapter 4) than that in the study without the addition of urea (approximately 2.7-fold, chapter 3). This can indicate that the dietary urea is degraded in stomach or small intestine and is directly absorbed into the blood. This study shows that FOS cannot improve the utilization of urea N in rabbits.

## Chapter 7

## SUMMARY

The rabbit is a hindgut fermenter animal. The cecal proliferation of bacterial is ingested by coprophagy, and this bacterial protein significantly contributes to the protein nutrition of the rabbit is known. In addition, rabbit colon separates liquid digesta from solid particles, and operates retrograde transport of liquid digesta containing bacteria in the cecum. This inherent capability maintains the population of microorganisms in the cecum of rabbits. Bacterial growth requires a source of energy. The major energy sources are undigested endogenous N and feed residue in the upper digestive tract.

Indigestible but fermentable carbohydrates escape digestion of the stomach or small intestine and reach the large intestine. They can be partly or totally fermented by the microflora into short chain fatty acids (SCFAs), and profoundly increase microbial multiplication. It has been reported that adding fermentable carbohydrate D-mannitol to the feed stimulates microbial proliferation and increases microbial protein levels. This process results in a decrease of urinary N excretion and N accumulation, and N retention rate becomes significantly higher in feeding mannitol diet rabbit. Moreover, fermentable sugars influence fiber digestibility and mineral absorption in the rabbit.

Therefore, it was demonstrated that indigestible oligosaccharide could affect increase in nutrient digestibility and nitrogen utilization in rabbits. It also provides a possible mechanism for the increment of acid detergent fiber (ADF) digestibility and nitrogen (N) retention by the consumption of indigestible oligosaccharide. The research content is as follows:

### 1. Effect of D-mannitol on Nitrogen Retention, Fiber Digestibility, and Digesta Transit Time in Adult Rabbits

The aim of the current study was to elucidate the effect of gastrointestinal retention of digesta on fiber digestibility in adult rabbits fed indigestible, but fermentable, sugar D-mannitol. The adult rabbits were fed a commercial diet containing 5% glucose or D-mannitol. Total feces and urine were collected during the experimental period. Nitrogen (N) balance, digestibility of nutrients, and gastrointestinal mean retention time (MRT) were measured. The results indicated that urinary excretion was significantly lowered, whereas N retention and N accumulation rate were significantly increased in the D-mannitol group compared with the glucose group ($P < 0.05$). However, fecal N excretion was unaffected. Absorption of crude ash (CA) and acid

detergent fiber (ADF) digestibility were significantly higher in the D-mannitol group compared with the glucose group ($P < 0.05$). The addition of D-mannitol to the diet did not affect the MRT of liquid digesta, but the MRT of solid digesta was longer compared with the glucose group ($P < 0.05$). These results suggest that the addition of D-mannitol to the diet stimulates cecal bacterial growth, thereby increasing N utilization and digesta retention time.

**2. Transfer of Blood Urea Nitrogen to Cecal Microbes and Nitrogen Retention in Mature Rabbits are Increased by Dietary Fructooligosaccharides**

To estimate the effect of fructooligosaccharides (FOS) on N utilization, seven mature rabbits were fed a diet containing 5% glucose or FOS for 8 days. During the last 5 days, total feces and urine were collected to measure N levels (Experiment 1). To examine N transfer from the blood to cecal microbes, eight rabbits were fed the same diets as in Experiment 1. After 9 days of feeding, 2 g of glucose or FOS was given orally. Two hours later 20 mg of $^{15}$N-urea was administered via the ear vein, and 1 h later cecal and blood samples were collected (Experiment 2). Urinary N excretion was lowered by FOS feeding ($P < 0.05$). Total bacterial N and $^{15}$N in the cecum was significantly higher in FOS-fed animals ($P < 0.05$). Urea N in the cecum was lower in FOS-fed rabbits ($P < 0.05$). Similarly, $^{15}$N atom % excess of cecal urea N was also lower in FOS-fed rabbits than in glucose-fed rabbits. These results suggest that FOS in the diet increases the transfer of blood urea N to the cecum for bacterial synthesis, thereby increasing N utilization.

**3. Influence of fructooligosaccharide on nitrogen utilization, transfer of blood urea nitrogen to cecal microbial nitrogen in young rabbits fed urea containing diet**

To estimate the effect of fructooligosaccharide (FOS) on nitrogen (N) utilization in young rabbits fed a urea containing diet, ten rabbits were fed a commercial diet with urea 10 g/kg with added glucose or FOS 50 g/kg each for 8 days. During the last 5 days, total feces and urine were collected to measure N balances (Experiment 1). To demonstrate N transfer from the blood to cecal microbes, twelve rabbits were fed the same diets as in Experiment 1. After 9 days of feeding, 2 g of glucose or FOS and 40 mg $^{15}$N-urea was given orally. Two hours later cecal and blood samples were collected (Experiment 2). N retention rate were significantly higher in the FOS feeding group ($P < 0.05$). The $^{15}$N atom % excess of portal blood serum urea was lower in urea diet contain FOS feeding group. However, cecal total N, bacterial N and amount of excess $^{15}$N was not between-group different. The proximal colon amount of excess $^{15}$N was

significantly lower in the FOS feeding group ($P < 0.05$). These results suggest that FOS to the urea containing diet did not affect transfer of blood urea N to the cecum for bacterial synthesis, thereby increasing N utilization.

**4. Effect of indigestible carbohydrate on nutrient digestibility and nitrogen utilization in adult rabbits**

The effect of indigestible carbohydrate on apparent digestibility of nutrients and nitrogen (N) utilization has been investigated in adult rabbits fed a commercial diet. In experiment 1, 18 adult rabbits were fed a commercial diet with glucose or lactosucrose and sorbitol 50 g/kg each. In experiment 2, 14 adult rabbits were fed a commercial diet with glucose or raffinose 30 g/kg each. Total feces and urine were collected during the experimental period. The results indicated that urinary N excretion has a tendency to decrease in lactosucrose and sorbitol groups than in the glucose group. Consequently, the N retention and the N retention rate based on N intake and digested N tend to increase in the lactosucrose and sorbitol groups compared with the glucose group (Experiment 1). Urinary N excretion, N retention, and the N retention rate based on N intake and based on digested N was not different between the two groups (Experiment 2). These results suggest that the all indigestible carbohydrate did not act as an energy source for bacterial proliferation, increasing the transfer of blood urea N to the cecum for bacterial N synthesis in rabbits.

The research shows that FOS and mannitol was increased fiber digestibility, mineral absorption and availability of protein, result promoting efficiency in utilization of the grass components. It is considered which fermentable sugars in the cecum provide energy source for bacterial growth, and prolonged transition time of digestive tract contents. However, it is not possible for FOS to be converted non-protein N source of urea into the body protein N. Moreover, other fermentable sugars did not affect N utilization and nutrient digestibility in adult rabbits except for D-mannitol and FOS. These results show that improvement of fiber digestibility or mineral absorption and increase in protein utilization are affected by the kind of carbohydrates and feed conditions in the rabbits. In the future, expression condition of the effect of indigestible sugars on feed ingredients utilization should be considered when fermentable sugars are applied to diet of the rabbit feed.

## 学位論文の概要

草食性の単胃動物ウサギは典型的な後腸発酵動物で、盲腸内で増殖した微生物を食糞によって摂取し、この微生物態タンパク質がウサギのタンパク質栄養に大きく貢献していることが知られている。また、ウサギの結腸には消化管内容物を選択的に保留する機能があり、繊維質を速やか排泄する一方、消化や発酵し易い非繊維質を盲腸内に逆送して、長時間滞留させる働きが備われている。この結腸機能が盲腸内微生物の増殖に重要な役割を果たしている。盲腸内微生物の増殖はエネルギーや窒素（N）源が必要で、上部消化管で消化されなかった飼料残渣や内因性 N がそれらの主要なものになると考えられる。

難消化発酵性糖質（発酵性糖質）は胃や小腸で発酵されずに盲腸まで届き、そこで発酵されて微生物増殖のエネルギー源になる。これまでに発酵性糖質であるマンニトールをウサギの飼料へ添加することによって、微生物増殖が刺激され、N 源として血液から移行した尿素-N の微生物態タンパク質への移行量が増加することが明らかにされている。その結果尿中に排泄される N 量が低下し、更に盲腸内で増殖した微生物は食糞により摂取され有効に利用されるので、総 N 排泄量が低下し N 利用性は向上することになる。このほか、発酵性糖質は他の栄養素と微生物発酵基質として競合する可能性や、発酵産物である有機酸の作用などによって、ウサギにおいても飼料中繊維やミネラルの消化吸収に影響することが考えられる。

そこで、本研究では発酵性糖質がウサギの消化管機能や栄養素代謝に及ぼす効果を総合的に把握することをめざして、栄養素消化率や体内 N 動態に及ぼす難消化発酵性糖質の影響やその作用機構について以下の点を検討した。

### １．成熟ウサギの N 蓄積、繊維消化率、消化管内容物滞留時間に及ぼすマンニトールの影響

発酵性糖質であるマンニトールがウサギの N 蓄積に及ぼす効果の再現性を確認し、マンニトールが繊維消化率と、微生物による繊維消化と密接に関係すると考えられる消化管内容物の移行に及ぼす影響を調べた。市販固形飼料にグルコースあるいはマンニトールを 5%添加した飼料を、成熟ウサギ（n=6）に代謝体重当たり 50 g/日与えて 12 日間飼育し、飼育開始 5 日経過後から消化管内容物滞留時間測定のために経時的に糞を採取し、飼育開始 7 日経過後から飼料消化率や N 出納試験を実施した。その結果、マンニトール添加群ではグルコース添加群と比較して、尿中排泄 N 量が有意（$P<0.05$）に少なかったが、糞中排泄 N 量には影響はなく、N 蓄積率が有意（$P<0.05$）に高かった。また、マンニトール添加群の酸性デタージェント繊維と粗灰分消化率はグルコース添加群と比較して

有意（P<0.05）に高かった。消化管内容物滞留時間は、液相マーカーではマンニトール摂取による影響はみられなかったが、固相マーカーではグルコース添加群と比較して有意（P<0.05）に長くなった。以上のことから、マンニトールをウサギの飼料へ添加することにより、盲腸内微生物増殖が刺激され、血中尿素の微生物への転換量と食糞によって摂取される微生物量の増大がもたらされた可能性を追認できた。またマンニトール摂取は繊維消化率の向上をもたらし、この効果は、固相内容物の滞留時間がマンニトール摂取によって増加したことから、繊維成分が微生物による消化過程を比較的長時間受けることができた結果と判断された。

**２．ウサギ血中尿素の盲腸内微生物への移行と体内 N 代謝に及ぼすフラクトオリゴ糖の影響**

フラクトオリゴ糖（FOS）の摂取がウサギの N 利用に及ぼす影響とその作用メカニズムを調べた。まず FOS の N 出納に及ぼす影響を調べるために、成熟ウサギを試験飼料で 8 日間飼育し、後半の 5 日間で代謝試験を行った。試験飼料は市販飼料に、グルコースあるいは FOS を 5%添加した飼料を代謝体重当たり 50 g/日与えた（実験 1）。次に血中尿素の動態に及ぼす FOS の影響を調べるために、実験 1 と同様の飼料で 9 日間飼育し、飼育最終日の 6:00～7:00 にグルコースあるいは FOS を 2 g 経口投与し、2 時間経過後 $^{15}$N-尿素を 20 mg 耳静脈から投与した。その 1 時間後に腹大動脈および門脈血、消化管内容物を採取し、窒素成分ならびにその中の $^{15}$N 量を測定した（実験 2）。その結果、グルコース添加群と比較して FOS 添加群の尿中排泄 N 量が有意（$P < 0.05$）に少なく、N 蓄積量は有意（$P < 0.05$）に多かった。盲腸内容物の微生物態 N と $^{15}$N 量は FOS 添加で有意（$P < 0.05$）に増加した。一方盲腸内容物中尿素態 N 濃度及びその $^{15}$N atom % excess は FOS 添加で有意（$P < 0.05$）に低下した。以上の結果から、ウサギでは FOS がマンニトールと同様に盲腸内微生物の増殖を促し、微生物増殖に利用される血中尿素 N 量を増大させ、増殖した微生物体は食糞により利用されることから、飼料 N の利用効率を向上させたことが推察される。

**３．尿素配合飼料摂取ウサギの N 利用と血中尿素動態に及ぼす FOS の影響**

ウサギの飼料中尿素-N の利用性と、飼料中尿素-N 利用に及ぼす FOS の影響を調べた。まずウサギを尿素配合飼料で 8 日間飼育し、後半の 5 日間で代謝試験を行い、N 出納を測定した。試験飼料は市販飼料に尿素を 1%配合し、グルコースあるいは FOS を 5%添加した飼料を代謝体重当たり 50 g/日与えた（実験 1）。次に血中尿素-N の体内動態に及ぼす FOS の影響を調べるために、実験 1 と同様の飼料で 9 日間飼育した。飼育最終日の 7:00～8:00 に $^{15}$ N-尿素 40 mg とグルコ

ースあるいはFOS 2 gを同時に経口投与し、1 時間後に腹大動脈および門脈血、消化管内容物を採取し、N 成分量やそれらの $^{15}N$ 量を測定した（実験 2)。その結果、グルコース添加群と比較してFOS 添加群の体内 N 蓄積量ならびに N 蓄積率は有意（$P < 0.05$）に上昇したが、尿中 N 排泄量の減少効果は、尿素無配合時の FOS 添加の効果に比べて小さかった。盲腸内容物中の微生物態 N 量と $^{15}N$ 量は FOS 添加でやや高い値を示したが有意な差ではなかった。以上の結果からFOS の添加は盲腸微生物増殖を刺激することで N の利用性を向上させるが、摂取した尿素の盲腸内微生物態 N への移行量は限定的であることが推察される。すなわち飼料に添加された尿素はウサギにとって有効な N 源になりにくいものと考えられる。

**４．成熟ウサギの N 蓄積、栄養素消化率に及ぼす難消化発酵性糖質の影響**

マンニトールとFOS で認められた栄養素消化や N 代謝に及ぼす効果が、他の難消化発酵性糖質にも備わっているかどうかを確認するために、ラクトスクロース、ソルビトール、ラフィノースを用いて以下の試験を行った。成熟ウサギを試験飼料で 8 日間飼育し、後半の 5 日間で代謝試験を行った。試験飼料は市販飼料に、グルコースあるいはラクトスクロース、ソルビトールをそれぞれ 5%添加した飼料を代謝体重当たり 50 g/日与えた（実験 1)。また、試験飼料は市販飼料に、グルコースあるいはラフィノースを 3%添加した飼料を代謝体重当たり 50 g/日与えた（実験 2)。その結果、グルコース添加群と比較してラクトスクロースあるいはソルビトール添加群では尿中排泄 N 量が低下する傾向があり、体内 N 蓄積量もやや増加したが有意な差ではなかった。またラフィノース添加群とグルコース添加群の間に尿中排泄 N 量や体内 N 蓄積の差異はほとんど見られなかった。また、二つの実験における栄養素消化率について有意な差が認められなかった。以上の結果からラクトスクロース、ソルビトール、ラフィノースは、ウサギの N 出納や栄養素消化率に対して、FOS やマンニトールのような影響を与えないと考えられる。

以上より、FOS やマンニトールには、飼料中の繊維成分やミネラルの消化吸収や摂取したタンパク質の利用性を向上させる効果があり、結果として草類成分の利用効率を促進するということができる。これらの効果は盲腸に到達して微生物のためのエネルギー源になり微生物増殖を増加させること、消化管内容物の移行を遅延させること等によって引き起こされると考えられる。しかし、飼料に添加した尿素-N の利用性はFOS によって向上させることはできなかった。また、供試した発酵性糖質のうちFOS やマンニトール以外の糖質にはFOS やマンニトールと同様の効果を見出すことはできなかった。これらのことは、繊維

成分やミネラルの消化吸収率の改善、またタンパク質の利用性を向上させる効果の発現は、糖質の種類や飼料条件によって影響を受けることを示している。今後、ウサギの飼育飼料の成分配合設計等への応用の前に、発酵性糖質の飼料成分利用性改善効果発現条件の詳細な解明が待たれる。

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