African Journal of Biotechnology Vol. 10(19), pp. 3704-3708, 9 May, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2677
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Effects of different dietary protein sources on cecal microflora in rats

Hongwei Qi, Zhentian Xiang, Guoquan Han, Bing Yu, Zhiqing Huang and Daiwen Chen*

Institute of Animal Nutrition, Key Laboratory for Animal Disease-Resistance Nutrition of China Ministry of Education, Sichuan Agricultural University, Ya'an, Sichuan 625014, People's Republic of China.

Accepted 6 April, 2011

The aim of this study was to test the effects of balanced diets respectively containing soya protein isolate, zein and casein as the sole protein source on the amount of total bacteria*, Lactobacillus, Bifidobacterium* and *Escherichia* in cecal digesta of growing rats. The casein increased the amount of *Lactobacillus* and *Bifidobacterium* than the soya protein and zein. Soy protein was better than the zein in the role of increasing the numbers of *Lactobacilli* and *Bifidobacterium* in the cecal of rats.

Key words: Protein, *Lactobacillus, Bifidobacterium*, *Escherichia coli,* rats, quantitative real-time PCR.

INTRODUCTION

Billions of bacteria are harbored in the intestinal tract of humans and animals (Backhed et al., 2005). The human and rat gastrointestinal tract (GIT) represents a dynamic ecosystem composing of a highly complex and diverse community of aerophilic and anaerobic microbes, which are involved in the fermentation of ingested feed and the components secreted by the host into the GIT (Pieper et al., 2009). The diet, microbiota and GIT interactions of mammals are extremely complex (De Angelis et al., 2006). Diet has a direct influence on the intestinal microbes of both composition and vitality of this community (Bauer et al., 2006). Regulating the composition and metabolic activity of the intestinal microbiota through the diets to improve gut metabolism and health is an increasing focus of nutritionists in the post-antibiotic era (Pieper et al., 2009).

It has been proposed that the protein level in animal feed could influence the intestinal microbiota. The diet with high level protein causes animal intestinal microbial disorders and diarrhea (Bikker et al., 2006). Animal protein and plant protein have different effects on infant intestinal microflora (Hoey et al., 2004). Both soya protein and zein are always included in the animal diet formulations in swine and poultry production (Zhao et al., 2007). Although abundant in natural products, soy protein and zein are known to have lower protein utilization for growth as compared with the animal proteins. This study chose three protein sources (soya protein isolate (SPI), zein and casein) to assess the effects of different protein sources on the cecal microflora of growing rats.

*Corresponding author. E-mail: daiwenc@yahoo.com. Tel: +86-835-2882088. Fax: +86-835-2885106.

MATERIALS AND METHODS

The experimental procedures followed the actual law of animal protection which was approved by the Animal Care Advisory Committee of Sichuan Agricultural University. A total of 30 male Sprague-Dawley rats (62.55 g) were divided into homogeneous groups of ten based on the initial average body weight in a completely randomized design for 14 days. Rats were housed individually in stainless-steel metabolism cages (25 × 15 × 15 cm) in a temperature- and humidity-controlled room, maintained at 22 ± 1°C on a 12 h light-dark cycle started at 07.00 h. The animals were given free access to water and the restricted-fed food.

Diets and feeding

The experimental proteins tested included SPI, zein and casein. Casein (Sigma, St Louis, MO, USA), SPI (ADM International, Inc., Chicago, IL, USA) and zein (Wako, Osaka, Japan) were purchased commercially. Isonitrogenous (168 g/kg DM) and isoenergetic (19 510 kJ) diets were formulated with sole protein sources following the recommendations of the American Institute of Nutrition (AIN) to meet the nutritional requirements for growing rats. They had free access to water and diet and the feed intake of rats were similar. The composition of the diets is shown in Table 1.

Table 1. Composition of the balanced diets.

Ingredient (%)	Casein	Soya protein isolate	Zein
Casein	21.40		
SPI		20.44	
Zein			24.50
Cornstarch	39.90	13.50	36.80
Sucrose	10.00	10.00	10.00
Dextrinized cornstarch	13.50	13.50	13.50
Soybean oil	7.00	7.00	7.00
Fiber	5.00	5.00	5.00
Mineral mix*	2.94	2.93	2.94
Vitamin mix¥	0.06	0.06	0.06
Choline bitartracte	0.20	0.20	0.20
Total	100.00	100.00	100.00
Analysed content			
Crude protein (%)	16.82	16.80	16.80
Total energy (MJ/kg)	19.51	19.52	19.51
Lysine (g/kg)	12.39	9.33	1.76
Methionine (g/kg)	4.62	1.62	3.74
Threonine (g/kg)	6.13	5.22	4.22
Phenylalanine (g/kg)	8.16	8.07	9.93
Valine (g/kg)	8.46	5.47	4.94
Leucine (g/kg)	14.22	11.16	24.84
Isoleucine (g/kg)	7.82	6.38	6.28

*Mineral mixture (per kg diet): 2.9 g $CaCO_3$; 13.1 g $CaHPO_4$; 1.3 g NaCl; 8.4 g K_2SO_4; 3.5 g $MgSO_4.H_2O$; 122.8 mg $FeSO_4.H_2O$; 21.1 mg $CuSO_4{\cdot}5H_2O$; 33.1 mg $MnSO_4.H_2O$; 36.6 mg $ZnSO_4.H_2O$; 3.9 mg KI; 15 mg Na_2SeO_3. ¥Vitamin mixture (per kg diet): 8100 mg vitamin A; 150 mg vitamin D_3; 48 mg DL-α-tocopheryl acetate; 6 mg vitamin K_3; 6 mg vitamin B_1; 9 mg vitamin B_2; 12 mg vitamin B_6; 45 mg vitamin B_{12}; 255 mg D-biotin; 3 mg folic acid; 69 mg nicotinamide; 30 mg D-pantothenic acid.

Digesta sample collection

After 14 days of treatment, all of the rats were anesthetized by ether and sacrificed after 1 h feeding. The abdominal cavity was opened to expose the gastrointestinal tract. The digesta of cecum were removed immediately and were stored at -80℃ until further analyses. All the experimental procedures followed the actual law of animal protection which was approved by the Animal Care Advisory Committee of Sichuan Agricultural University.

Extraction of DNA from digesta

Bacterial DNA was isolated from the digesta samples using an E.Z.N.A.TM stool DNA isolation kit (Omega Bio-Tek, Doraville, CA), according to the manufacturer's protocol. The pellet was suspended in 200 µl TE buffer [10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA], and the mixture was briefly mixed on a vortex mixer. The suspension was placed in a boiling water bath for 1 min, subjected to 3 freeze-thaw cycles alternating between -70 for 3 min and 100 for 2 min, and then centrifuged at 10 000 *g* for 5 min. 100 µl aliquot of the supernatant was transferred to a sterile tube and was stored at -20℃ until PCR testing (Yang et al., 2008). The concentration was determined by a spectrophotometer (Beckman Coulter DU 800, Fullerton, CA).

Designing and validation of primers

Primers and probe (Table 2) were designed with Primer Express 3.0. 16S rRNA sequences of maximum species of each genus encountered were downloaded from the GenBank database as well as EMBL and DDBJ, in which the aim was to avoid any non-specific amplification; the sequences of all the genera fetched from the database were submitted to DNAStar (MegAlign) programme (DNASTAR, Inc., Madison, WI). These sequences were then submitted to alignment where the maximum number of species belonging to one genus was aligned and the regions showing conservations were selected as genus-specific primers and probe. To further ensure that the oligonucleotide sequences were complementary pairing with the target genus only, they were checked with GenBank program BLAST (NCBI BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) and RDP program Check-Probe (Details about RDP data and analytical functions can be found at http://rdp.cme.msu.edu/). Primers (Table 2) for all the bacteria were obtained from the published work of Fierer et al. (2005). All the primers and probe were commercially synthesized from invitrogen.

Quantitative PCR conditions

Quantitative real-time PCR was carried out with IQ5 real-time PCR detection System (Bio-Rad, CA, USA) and optical grade 96-well

Table 2. Sequences of oligonucleotide primers and probe.

Gene	Primer (5'-3') and probe sequence	Product length (bp)
Lactobacillus		
FQ-RSF	GAGGCAGCAGTAGGGAATCTTC	126
FQ-RSR	CAACAGTTACTCTGACACCCGTTCTTC	
FQ-RS-P	AAGAAGGGTTTCGGCTCGTAAAACTCTGTT	
Escherichia coli		
FQ-DCF	CATGCCGCGTGTATGAAGAA	96
FQ-DCR	CGGGTAACGTCAATGAGCAAA	
FQ-DC-P	AGGTATTAACTTTACTCCCTTCCTC	
Bifidobacterium		
FQ-SQF	CGCGTCCGGTGT GAAAG	121
FQ-SQR	CTTCCCGATATCTACACATTCCA	
FQ-SQ-P	ATTCCACCGTTACACCGGGAA	
Total bacteria (Fierer et al., 2005)		200
Eub338F	ACTCCTACGGGAGGCAGCAG	
Eub518R	ATTACCGCGGCTGCTGG	

plates were used. The reaction system was composed of 12.5 µl SYBR Premix Ex Taq (2×), 1 µl of forward and 1 µl of reverse primers (100 nM), 9.5 µl ddH_2O and 1 µl DNA in each reaction for detecting the total bacteria. The total reaction volume was 25 µl. The PCR conditions were as follows: 1 cycle of predenaturation at 95°C for 20s; 40 cycles of denaturation at 95°C for 5 s; annealing at 60°C for 30 s and extension at 72°C for 50 s. The melting curve conditions were 95°C for 30 s, 55°C for 1 min and 95°C for 1 min (temperature change velocity: 0.5°C/s). PrimerScriptTM PCR kit (Perfect Real Time) (TaKaRa, Dalian, China) was used for *Lactobacillus*, *Escherichia coli* and *Bifidobacterium*. The reaction protocol was composed of 1 cycle of predenaturation at 95°C for 2 min; 50 cycles of denaturation at 95°C for 15 s; annealing at 60°C for 30 s and extension at 72°C for 50 s. The total reaction volume was 20 µl. The reaction system was composed of 8 µl realMasterMix (2.5×), 1 µl probe enhancer solution (20×), 1 µl of forward and 1 µl of reverse primers (100 nM), 0.3 µl probe (100 nM), 7.7 µl ddH_2O and 1 µl DNA in each reaction for detecting *Lactobacillus*, *E. coli* and *Bifidobacterium*.

Standard curve

For the quantization of total bacteria in the test samples, two target genes were amplified, cloned and used to generate the standard curves. The amplified products were eluted from the agarose gel using TIANquick mini purification kit (TIANGEN, Beijing, China) and were cloned into the pMD19-T vector (TaKaRa, Dalian, China). Plasmids DNA was purified using the E.Z. N.A TM plasmid miniprep kit (OMEGA Bio-Tek, USA). Clones were screened for the inserts using the appropriate restriction enzymes, and positive clones were sequenced. The standard strains of *Lactobacillus*, *E. coli* and *Bifidobacterium* were cultured anaerobically or aerobically in LB broth supplemented at 37°C for 12 to 48 h. The total genomic DNA from the different reference strains was extracted and purified by using the method described in the related kit manual (E.Z.N.A. TM Bacterial DNA Kit, OMEGA Bio-Tek, USA). DNA concentration of the plasmids preparation was determined by spectrophotometer (Coulter DU 800, Beckman, USA) and the copy number was calculated using the following formula: (DNA concentration in µg/µl × 6.0233 × 10^{23} copies/mol) / (DNA size (bp) × 660 ×10^{6}). A 10-fold dilution series of the plasmid DNA was prepared and used to generate the standard curve. Target copy numbers for each reaction were calculated from the standard curves.

Statistical analysis

The results were analyzed using SPSS17.0. A one-way ANOVA procedure was carried out for all the data. All results were expressed as means ± SD. The results were statistically analyzed by using least significant difference test. $P < 0.05$ was considered significant.

RESULTS

Quantities of total bacteria, *Lactobacillus, Bifidobacterium* and *Escherichia* in the cecal digeata

Real-time PCR analysis was performed to determine the amount of total bacteria, *Lactobacillus, Bifidobacterium* and *Escherichia* in the cecal digesta of the rats. The amount of total bacteria in cecal digesta fed casein and SPI were significantly higher than that in zein group ($P < 0.05$); there was no significantly difference between the casein and SPI treatments. The amount of *Lactobacillus* was higher in casein treatment than in the other two

Table 3. The amount of the total bacteria, *Lactobacillus, Bifidobacterium* and *Escherichia* in the cecal digesta of rats among different dietary protein sources treatments.

Treatment	Casein	SPI	Zein
Total bacteria (Log copies/g)	10.80±0.14[a]	10.69±0.10[a]	10.29±0.22[b]
Lactobacillus (Log copies/g)	6.57±0.16[a]	6.33±0.08[b]	5.35±0.18[c]
Bifidobacterium (Log copies/g)	7.72±0.42[a]	7.06±0.53[b]	6.55±0.19[b]
Escherichia (Log copies/g)	5.77±0.32[c]	7.68±0.14[a]	6.21±1.10[b]

The results are expressed as mean values ± the standard deviation for all the samples.
[a, b, c]Mean values without a common superscript letter among the groups were significantly different ($P < 0.05$).

treatments ($P < 0.05$). The amount of *Bifidobacterium* was higher in casein treatment than in the other two treatments, and that of the SPI treatment was higher than that of zein. The amount of *Escherichia* was higher in SPI treatment than in the other two treatments and the amount of *Escherichia* was higher in zein treatment than in casein treatment (Table 3).

The ratio of *Lactobacillus*, *Bifidobacterium* and *Escherichia* based on total bacteria in the cecal digesta of rats

The ratio of *Lactobacillus* based on the total bacteria was higher in the treatment fed casein than in the other two treatments, and that of the SPI treatment was higher than the Zein treatment ($P < 0.05$). The ratio of *Bifidobacterium* based on the total bacteria was higher in the casein treatment than in the other two treatments, and that of the zein treatment was lower than the other two treatments ($P < 0.05$). The ratio of *Escherichia* based on the total bacteria was higher in SPI treatment than in the other two treatments ($P < 0.05$) (Table 4).

DISCUSSION

In this study, casein, SPI and zein with a high concentration of true protein (about 90% of total protein) were used as a sole protein source in order to avoid additional effects of others compositions on the metabolic responses. The crystal amino acid supplementations were not included in the diets. The profile of amino acids in each protein meal played a key role in the regulation of the intestinal microflora of rats. All the nutrients in the experiment diets used in this study were similar except the profile of amino acids (Table 1).

The amount of the total bacteria in the cecal digesta of casein and SPI treatments were significantly higher than that in zein treatment ($P < 0.05$), which showed that the diet containing casein or SPI was not only the condition for the host growth, but also the microbial fermentation substrate for intestinal bacteria.

Both *Lactobacilli* and *Bifidobacterium* are believed to play an important role on animal and human health and immune function (Orrhage and Nord, 2000; Zhang et al., 2010). It is considered to be the target organism due to their potential to inhibit the growth of pathogenic bacteria that may prevent intestinal disorders (Mikkelsen et al., 2004). Yazawa et al. explained that *Bifidobacteria* are able to suppress pathogenic bacteria (*E. coli*) because they utilize oligo- and polysaccharides which other intestinal bacteria cannot use (Yazawa et al., 1978). Stimulation of the *Lactobacillus* within the GIT of piglets is extremely important, not only due to the potential effect of the bacteria on gut function and health (Stewart, 1997), but also because of their possible antagonistic activities against pathogenic bacteria (Simpson et al., 2000). It has been assumed that significant bifidogenic activity may also be associated with casein (Bezkorovainy and Topouzian, 1981; Petschow and Talbott, 1990). In this study, the treatment of fed casein not only increased the quantity of *Lactobacillus* and *Bifidobacterium*, but also enhanced the ratio of *Lactobacillus* and *Bifidobacterium* based on the total bacteria. Our data is consistent with that of previous report (Bezkorovainy and Topouzian, 1981).

E. coli are the predominant facultative anaerobe of the human colonic flora (Tyler et al., 1991). There are many species of *E. coli*, which are pathogens, such as, enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and enteroaggregative *E. coli* (EAEC) (Vial et al., 1990). The amount of *Escherichia* were higher in SPI treatment than in the other two treatments (Table 3). The possible reason was the presence of the soybean antigen protein in soya protein. The soybean antigen is known to result in intestinal mucosal injury, which increases the propagation and adhesion of *E. coli* on the intestinal wall as well as the susceptibility to disease (Urban et al., 2001).

The data in this work showed that soya protein was better than zein in the role of increasing beneficial bacteria such as *Lactobacilli* and *Bifidobacterium*, though, both isolated soy protein and zein were plant proteins. This may be because zein was extremely imbalance in the amino acid composition, especially the short of lysine (Geiger et al., 1952). The lysine was only 0.18% in the diet of zein treatment (Table 1). The imbalance amino acid pattern could have led to impaired host and intestinal

Table 4. The ratio of *Lactobacillus*, *Bifidobacterium* and *Escherichia* based on total bacteria in cecal digesta of rats among three different dietary protein sources treatments.

Treatment	Casein	SPI	Zein
Lactobacillus/total bacteria	$(5.98\pm0.48^{a})\times10^{-5}$	$(4.35\pm0.46^{b})\times10^{-5}$	$(1.27\pm0.62^{c})\times10^{-5}$
Bifidobacterium/total bacteria	$(9.89\pm0.58^{a})\times10^{-4}$	$(3.58\pm0.40^{ab})\times10^{-4}$	$(2.38\pm0.22^{b})\times10^{-4}$
Escherichia/total bacteria	$(9.92\pm3.91^{b})\times10^{-6}$	$(9.65\pm0.83^{a})\times10^{-4}$	$(8.58\pm2.33^{b})\times10^{-5}$

The results are expressed as mean values ± the standard deviation for all samples.
[a, b, c]Mean values without a common superscript letter among groups were significantly different ($P < 0.05$).

bacteria growth.

In summary, we determined the amount of total bacteria, *Lactobacillus, Bifidobacterium* and *Escherichia* in the digesta of rat cecums by quantitative real-time PCR with probe. The different protein sources affected the quantity of the total bacteria, *Lactobacillus, Bifidobacterium* and *Escherichia* of the rats. The casein increased the amount of *Lactobacillus* and *Bifidobacterium* than soya protein and zein. Soy protein was better than zein in the role of increasing the numbers of *Lactobacilli* and *Bifidobacterium* in the cecal of rats.

Acknowledgements

This work was supported by the Program for Changjiang Scholars and Innovative Research Team in University of China (IRT0555-5) and the earmarked fund for Modern Agro-industry Technology Research System of China (CARS-36).

REFERENCES

Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI (2005). Host-bacterial mutualism in the human intestine. Science, 307: 1915-1920.

Bauer E, Williams BA, Smidt H, Mosenthin R, Verstegen MW (2006). Influence of dietary components on development of the microbiota in single-stomached species. Nutr. Res. Rev. 19: 63-78.

Bezkorovainy A, Topouzian N (1981), *Bifidobacterium bifidus* var. Pennsylvanicus growth promoting activity of human milk casein and its derivatives. Int. J. Biochem. 13: 585-590.

Bikker P, Dirkzwager A, Fledderus J, Trevisi P, le Huerou-Luron I, Lalles JP, Awati A (2006). The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets. J. Anim. Sci. 84: 3337-3345.

De Angelis M, Siragusa S, Berloco M, Caputo L, Settanni L, Alfonsi G, Amerio M, Grandi A, Ragni A, Gobbetti M (2006). Selection of potential probiotic *lactobacilli* from pig feces to be used as additives in pelleted feeding. Res. Microbiol. 157: 792-801.

Fierer N, Jackson JA, Vilgalys R, Jackson RB (2005). Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl. Environ. Microbiol. 71: 4117-4120.

Geiger E, Courtney GW, Geiger LE (1952). The physiological availability of some essential amino acids present in zein, in the acid and in the enzymatic hydrolyzate of zein. Arch. Biochem. Biophys. 41: 74-82.

Hoey L, Rowland IR, Lloyd AS, Clarke DB, Wiseman H (2004). Influence of soya-based infant formula consumption on isoflavone and gut microflora metabolite concentrations in urine and on faecal microflora composition and metabolic activity in infants and children. Br. J. Nutr. 91: 607-616.

Mikkelsen LL, Naughton PJ, Hedemann MS, Jensen BB (2004). Effects of physical properties of feed on microbial ecology and survival of Salmonella enterica serovar *Typhimurium* in the pig gastrointestinal tract. Appl. Environ. Microbiol. 70: 3485-3492.

Orrhage K, Nord CE (2000). Bifidobacteria lactobacilli in human health. Drugs Exp. Clin. Res. 26: 95-111.

Petschow BW, Talbott RD (1990). Growth promotion of Bifidobacterium species by whey and casein fractions from human and bovine milk. J. Clin. Microbiol. 28: 287-292.

Pieper R, Bindelle J, Rossnagel B, Van Kessel A, Leterme P (2009). Effect of carbohydrate composition in barley and oat cultivars on microbial ecophysiology and proliferation of *Salmonella enterica* in an *in vitro* model of the porcine gastrointestinal tract.: Appl. Environ. Microbiol. 75: 7006-7016.

Simpson JM, McCracken VJ, Gaskins HR, Mackie RI (2000). Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. Appl. Environ. Microbiol. 66: 4705-4714.

Stewart CS (1997). Microorganisms in hindgut fermentors: Gastrointestinal microbial. 2: 142-186.

Tyler SD, Johnson WM, Lior H, Wang G, Rozee KR (1991). Identification of verotoxin type 2 variant B subunit genes in *Escherichia coli* by the polymerase chain reaction and restriction fragment length polymorphism analysis. J. Clin. Microbiol. 29: 1339-1343.

Urban D, Irwin W, Kirk M, Markiewicz MA, Myers R, Smith M, Weiss H, Grizzle WE, Barnes S (2001). The effect of isolated soy protein on plasma biomarkers in elderly men with elevated serum prostate specific antigen. J. Urol. 165: 294-300.

Vial PA, Mathewson JJ, DuPont HL, Guers L, Levine MM (1990). Comparison of two assay methods for patterns of adherence to HEp-2 cells of Escherichia coli from patients with diarrhea.: J. Clin. Microbiol. 28: 882-885.

Yang JL, Wang MS, Cheng AC, Pan KC, Li CF, Deng SX (2008). A simple and rapid method for extracting bacterial DNA from intestinal microflora for ERIC-PCR detection. World J. Gastroenterol. 14: 2872-2876.

Yazawa K, Imai K, Tamura Z (1978). Oligosaccharides and polysaccharides specifically utilizable by bifidobacteria. Chem. Pharm. Bull. (Tokyo). 26: 3306-3311.

Zhang M, Wang XQ, Zhou YK, Ma YL, Shen TY, Chen HQ, Chu ZX, Qin HL (2010). Effects of oral *Lactobacillus plantarum* on hepatocyte tight junction structure and function in rats with obstructive jaundice. Mol. Biol. Rep. 37: 2989-2999.

Zhao J, Harper AF, Estienne MJ, Webb KJ, McElroy AP, Denbow DM (2007). Growth performance and intestinal morphology responses in early weaned pigs to supplementation of antibiotic-free diets with an organic copper complex and spray-dried plasma protein in sanitary and nonsanitary environments. J. Anim. Sci. 85: 1302-1310.