Modulation of mice fecal microbiota by administration of casein glycomacropeptide

Qingsen Chen, Jinyi Cao, Yuchen Jia, Xueji Liu, Yali Yan, Guangchang Pang

The Tianjin Key Laboratory of Food Biotechnology College of Biotechnology and Food Science, Tianjin University of Commerce, Tianjin, China

Abstract

Casein glycomacropeptide (GMP) is known to promote the *in vitro* growth of *Bifidobacteria* and *Lactobacilli*. In this paper, we used conventional culture techniques and fluorescent *in situ* hybridization (FISH) techniques to investigate the effect of casein GMP on mice fecal microbiota. The population structure of the intestinal microbiota, including *Lactobacillus*, *Bifidobacteria*, *Enterococcus*, coliforms and *Enterobacteriaceae*, was tested and compared. After consecutive administration of casein GMP for 15 days, numbers of *Lactobacillus* and *Bifidobacteria* increased significantly ($P<0.01$), numbers of *Enterobacteriaceae* and Coliforms decreased significantly ($P<0.05$) while no significant changes were observed for *Enterococcus*. The detection limits of FISH technique were significantly lower ($P<0.01$) than the traditional culture method. These results suggested that consumption of casein GMP had a prebiotic effect on male BALB/c mice. Casein GMP helped establish a healthier intestinal microbiota. Additionally, FISH was proved to be a rapid and relatively low-cost detection method that can be used to further our understanding of human intestinal microbiota.

Introduction

All animal intestinal tracts, as well as those of humans, harbor complex microorganism communities. These bacterial communities are comprised of up to 400 to 500 bacterial species forming a complex ecosystem. Records indicate that there is an estimated 1012-1014 microorganisms per gram of fecal material.[1-3] The intestinal microbiota play both positive and negative roles. A healthy intestinal microbiota can help digest food, and metabolize endogenous and exogenous compounds, and can potentiate the immune system and prevent pathogen colonization in the gastrointestinal tract (GIT).[2,4] However, an unbalanced gut microbiota can cause diseases such as diarrhea and inflammatory bowel disease (IBD). Therefore, sustaining a well-balanced gut microbiota by promoting the colonization of beneficial bacteria is crucial to human health.

It has been observed that some *Lactobacillus* and *Bifidobacterium* strains have a beneficial effect on hosts by adhering to the surface of intestinal cells and colonizing the gastrointestinal tract.[5,6] Thus, analyzing intestinal microbiota in the research for functional food components that promote beneficial gut bacteria had been one of the objectives in this field.

Casein glycomacropeptide (GMP) is a casein-derived peptide, cleaved from κ-casein by chymosin at 105Phe-106Met during cheese making. The concentration of casein GMP ranges from 1.2 to 1.5 g/L in sweet whey, comprising 15-20% of the total protein.[7] Casein GMP is a peptide with many known biological functions, including binding of cholera and *Escherichia coli* enterotoxins,[8] inhibition of bacterial and viral adhesion,[9] suppression of gastric secretions,[10] modulation of immune system responses,[11] and promotion of bifidobacterial growth whose prebiotic activity was attributed to its glycosylated N-acetylneuraminic acid component.[12,13]

Because of its unique carbohydrate composition and versatile biological activities, casein GMP is considered a potential ingredient for functional foods and nutraceuticals. Idota *et al.*[14] found that casein GMP had promoted *in vitro* growth in *Bifidobacteria* and *Lactobacilli*, but the effect of casein GMP on the intestinal microflora has not been tested.

Identification and enumeration of gastrointestinal microbiota commonly use traditional culture techniques. Culture-based studies provided us with the first insights into the diversity and complexity of the gastrointestinal tract ecosystem. However, the conventional culture method has a number of disadvantages, for example, it is laborious and time-consuming. It is also limited by the possibility of culturing the studied microorganisms, and a certain expertise and specialized equipment are required to isolate strict anaerobes.[15] Such drawbacks have often hindered related research.

Recent years have seen the development of new molecular techniques, especially those based on 16S rRNA sequences. These molecular techniques are essential for the study of microbial diversity, including understanding the distribution, function and adaptation of such microorganisms in different environments. The techniques are also important in understanding the relationship between microbial diversity and specific metabolic activities.

Fluorescence *in situ* hybridization (FISH) was first used to detect the bacterial ecosystem in 1988.[16] In recent years, this method has been developed and applied to the study of intestinal microbiota.[17-19] In this study, to investigate the modulation of mice fecal microbiota upon administration of casein GMP, we applied both a culture-dependent method and FISH to evaluate changes in gut microflora.

Depending on the probes chosen, FISH can be used to detect bacteria from different phylogenetic term. Specific oligonucleotide probes and primers have been designed for many bacterial species that are known to be present in the GIT. But because the complex microflora of the human gut is difficult to study with probes at a species level due to the vast diversity of this ecosystem, in this study, we chose probes that are designed to recognize major genera or groups of microbes.[17]

Correspondence: Qingsen Chen, East of Jinba Road, Beichen District, College of Biotechnology and Food Science, Tianjin University of Commerce, PR China.
Tel. +86.22.2666.7631 - Fax: +86.22.27725382.
E-mail: chqsen@tjcu.edu.cn

Key words: casein glycomacropeptide (GMP), fecal microbiota, fluorescent *in situ* hybridization (FISH) techniques, traditional culture method.

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Contributions: QC was study project leader; JC is responsible for carrying out the main study, research data collection and writing the report; YJ specific probes and wrote the report; XL FISH techniques; YY helped with conventional culture techniques and involved in guidance of research; GP helped in data analysis.

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Materials and Methods

Animals and diets

Male 6-week old BALB/c mice (n=20; provided by The Academy of Military Medical Sciences of China, China) were fed normal fodder for one week and divided equally into two groups thereafter. The control group

(group A) received the basal diet and an equal volume of normal saline per day. The casein GMP group (group B) was administered the basal diet and 0.5 mg/mL casein GMP (Tatua Dairy Co-operative Co., Ltd, New Zealand) at the dose of 0.2 mL per day; casein GMP purity (of protein) 71%; sialic acid on protein 5.6%.

Strains and probes

Lactobacillus helveticus TS206 strain was optimized and preserved in our laboratory. The 16S rRNA-targeted oligonucleotide probes (Invitrogen, USA) used in this study are listed in Table 1. The EUB338 probe was linked with 6-carboxy-x-rhodamine (ROX) at the 5′ end. The group-specific probes were linked with fluoresceine isothiocyanate (FITC) at their 5′ ends.

Fluorescent *in situ* hybridization analysis

Before performing FISH hybridization, the specificity of probes was tested using *L. helveticus* TS206. Briefly, *L. helveticus* TS206 was cultured in MRS broth at 37°C for 12 h. The bacterial cells were harvested and pelleted by centrifugation (3500×g for 10 min at 4°C), washed once with phosphate-buffered saline (PBS, pH 7.2), and adjusted to a final concentration of 10^{-7} CFU/mL. The suspension was hybridized with Lacb722 probe as described below. As a negative control, the specificity of Bif164 probe was also tested by *L. helveticus* TS206 in a similar manner.

FISH hybridization was performed as described by Franks *et al.* with slight modifications.[20] After administration of casein GMP for 15 days, fresh stool were collected from the Group A (control group) and Group B (casein GMP group) mice in sterile plastic centrifugal tubes (5 mL; RNase-free), weighed and kept for no longer than 12 h at 4°C before processing. Portions (0.3 g) of each stool were suspended in 2.7 ml of filtered (0.2-um-pore-size filter, Millipore Cor-poration, USA) PBS (130 mM NaCl, 3 mM $NaH_2PO_4 \cdot 2H_2O$, 7 mM $Na_2HPO_4 \cdot 12H_2O$, pH 7.2) and vortexed to homogenize the samples. The suspension was centrifuged at 700 g for 2 min to remove debris. The supernatant was diluted 1:3 with PFA (4% (w/v) paraformaldehyde in PBS), fixed at 4°C for 16 h, and stored in 50% (v/v) ethanol/PBS at -70°C for use.[21]

The suspension fixed with PFA as described above was incubated with 10 ul of 10 mg/ml lysozyme dissolved in 100 mM Tris-HCl (pH 7.2) for 20 min at 37°C before hybridization. The fixed cell suspension (10 μl) was applied to a pre-cleaned glass slide (RNase-free) and dried at room temperature. The cells were then dehydrated with a series of solutions containing 50%, 80%, and 99.5% ethanol (3 min for each concentration). The cells fixed on the glass slide were hybridized with 25 uL hybridization buffer overnight at 46°C in a humid chamber. The hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.1% SDS, 30% (v/v) formamide, 10% (w/v) dextran sulfate) contained 20 ng/uL of fluorescently labeled probe. The fixed cell suspension can be diluted with PBS (pH 7.2) based on the quantity of the target organism. After hybridization, the cells were subsequently transferred into washing buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), filtered through a 0.2-um-pore-size filter), incubated at 48°C for 30 min to remove non-specific binding of the probe, rinsed briefly with distilled water, and air-dried.

Table 1. 16S rRNA-targeted oligonucleotide probes for fluorescent *in situ* hybridization analysis.

Probe	OPD-code	Probe sequence (5' to 3)	Target organism
EUB 338 [21]	S-D-Bact-0338-a-A-18	GCTGCCTCCCGTAGGAGT	All Bacteria
Enter1432 [22]	S-G-Enter-1432-a-A-15	GTTTTGCAACCCACT	Enterobacteriaceae
Bif164 [23]	S-G-Bif-0164-b-A-18	CATCCGGYATTACCACCC	Bifidobacterium
Lacb722 [24]	S-G-Lacto-722-a-A-25	YCACCGCTACACATGRAGTTCCACT	Lactobacillus group
ENC221 [25]	not applicable	CACCGCGGGTCCATCCATCA	Enterococcus spp

R=G/A, Y=T/C, M=A/C, K= G/T, S= G/C, W= A/T, H=A/C/T, B=G/T/C, V=G/C/A, D=G/A/T, N=G/A/T/C

Enumeration of bacteria in the fecal samples by fluorescent *in situ* hybridization

Fluorescent cells were counted in triplicate for appropriately diluted samples. Digital images of the slides were taken using a Nikon Eclipse Ti-U fluorescent microscope (Nikon, Japan). These images were analyzed and fluorescent cells were counted using NIS-Element BR image analysis software (Nikon, Japan). Depending on the amount of fluorescent cells, 15 randomly chosen microscopic fields were imaged.

Analysis by conventional culture techniques

Fresh stool were collected in sterile plastic centrifugal tubes, weighed (approx. 0.1 g) and processed immediately. After thorough mixing, 10-fold dilutions were prepared in sterile saline. Aliquots of adequate dilutions were plated in triplicate onto different selective media: *Lactobacillus* were enumerated on MRS broth; *Enterobacteriaceae* on Violet Red Bile Dextrose Agar (VRBDA);[22] *Bifidobacteria* on Tryptone-Peptone-Yeast Extract (TPY) agar with 2 ug/L dicloxacillin;[23] *Enterococcus* on Bile Esculin Azide Agar; and coliforms on MacConkey Agar. The plates described above were incubated anaerobically at 37°C for 48 h except for the coliforms that were incubated aerobically at 37°C for 24 h (24). All bacterial counts (colony-forming units [CFU] per g of wet feces) were transformed to logarithms (log_{10} CFU) for statistical analysis. Media (Hopebio) used in this study were obtained from Qingdao Hope Bio-Technology Co. Ltd, China.

Data analyses

Data are expressed as means±SD. The differences in bacterial counts between different samples or between different techniques were determined by t-test using SPSS for Windows, version 11.5 (IBM SPSS Statistics, USA). P>0.05 was considered significant.

Results

Analysis of the fecal microbiota by conventional culture techniques

The differences in target microbiota counts of fecal microbiota at different time points are shown in Table 2. *Lactobacillus* and *Bifidobacteria* in the fecal sample increased after supplementing with GMP. Their counts reached 109 and 1010 CFU/g (wet weight). After 15 days of treatment, the differences compared to their basal level were significant (P<0.01). *Enterobacteriaceae* and coliforms in the fecal sample decreased significantly after 15 days of casein GMP treatment (P<0.05). During the whole trial, there was no significant change in *Enterococcus* in the control group or in the experimental group.

Specificity of 16S rRNA-targeted oligonucleotide probes

Using the above-mentioned methods, the specificity of 16S rRNA-targeted oligonucleotide probes was analyzed. It was confirmed that the probes only hybridize with their target strains. Specifically, more than 90% of the Lacb722 probes were specifically hybridized with their corresponding target strain *L. helveticus* TS206 (Figure 1A), whereas no fluorescent signal was detected using Bif164 probes (Figure 1B).

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Analysis of the fecal microbiota by fluorescent *in situ* hybridization techniques

Five probes (Table 1) were used to test mice fecal microbiota before and after administration of casein GMP. The results obtained with FISH are shown in Table 3. The numbers of fecal microorganism obtained by culture techniques were significantly lower than those obtained by FISH. The *Lactobacillus* group (probe Lacb722) accounted for 9.27±2.60% of the total population of fecal microbiota after being supplemented with casein GMP for 15 days. Compared with group B (casein GMP group), group A (control group) had a significantly lower number of targets for the *Lactobacillus* group (2.05±1.06%). The *Bifidobacteria* group (probe Bif164) accounted for 6.84% (±1.36%) of the total population. This was significantly higher than the control group (1.67±0.95%). The *Enterococcus* group and the *Enterobacteriaceae* group make up 0.92% (±0.28%) and 0.96% (±0.25%) of the total population, respectively. These proportions were significantly lower than those in the control group (2.66±1.23% and 2.63±1.01%, respectively).

Comparison of conventional cultivation method and fluorescent *in situ* hybridization

The trend was consistently detected by both methods, but the level of bacteria detected by FISH was much higher than that detected by conventional cultivation techniques. The accuracy of the culture-dependent approach is limited by the selectivity of the media. FISH is limited by the specificity of the probe. However, the advantage of FISH technique is obvious in that it can enumerate culturable and unculturable bacteria. More importantly, the accuracy of FISH is much higher than the culture-dependent approach, reaching above 90%, and this technology is also fast, practical, and better reflects the main gastrointestinal microbiota profile.

Discussion

Early works have found that casein GMP promotes the *in vitro* growth of *Bifidobacteria* and *Lactobacilli*, but the related research *in vivo* is still lacking. Our study has demonstrated that use of casein GMP can significantly increase the intestinal concentration of *Lactobacillus* and *Bifidobacteria*. These intestinal microbiota are essential to the health of the host and play a role in regulating immune responses, inhibiting exogeneous or harmful bacteria, and facilitating digestion and absorption of food ingredients.[2] The accumulation of probiotics (*Lactobacillus* and *Bifidobacteria*) with administration of casein GMP to the male BALB/c mice facilitates competitive exclusion of pathogens, thereby promoting non-specific host resistance to microbial pathogens. Related studies have also demonstrated that *Bifidobacteria* can potentiate the secretion of secretory IgA (SIgA)[25] that prevents the spreading of intestinal bacteria across the gut mucosa by coating intestinal bacteria and blocking their interaction with the epithelium.[26]

The consumption of casein GMP can signifi-

Table 2. Enumeration (log CFU/g of feces) of *Lactobacillus*, *Bifidobacteria*, *Enterococcus*, *Enterobacteriaceae*, Coliforms in fecal samples before and after 5, 10, 15 days of assumption of casein glycomacropeptide by conventional cultivation techniques.

Target microbiota	Time points	Plate counts (log CFU/g wet feces, n=10) means±SD	
		group A	group B
Lactobacillus	D_0	8.21±1.45	8.12±1.21
	D_5	8.36±1.28	8.61±1.08*
	D_{10}	8.06±0.98	9.02±1.25°
	D_{15}	8.29±1.36	9.38±0.96°
Bifidobacteria	D_0	9.45±0.98	9.05±2.06
	D_5	9.66±1.22	9.76±1.53*
	D_{10}	9.61±1.56	10.01±1.01°
	D_{15}	9.58±0.86	10.08±1.44°
Enterococcus	D_0	7.38±1.32	8.01±0.65
	D_5	7.83±0.77	8.12±0.92
	D_{10}	7.63±1.01	7.96±1.22
	D_{15}	7.86±1.32	7.65±1.11
Enterobacteriaceae	D_0	8.38±2.01	8.56±1.32
	D_5	8.81±1.08	8.84±0.96
	D_{10}	8.19±1.21	8.46±1.63
	D_{15}	8.63±0.95	7.65±1.41*
Coliforms	D_0	8.08±1.33	8.36±0.68
	D_5	8.49±0.86	8.69±1.54
	D_{10}	7.95±2.01	8.56±1.63
	D_{15}	8.36±1.06	7.84±1.98*

Significant differences among target microbiota counts of fecal microbiota in different period (*P<0.05, °P<0.01). D, administration period (days).

Table 3. Enumeration (log CFU/g of feces) and percentage of *Lactobacillus*, *Bifidobacteria*, *Enterococcus* spp, *Enterobacteriaceae* in faecal samples before and after 5, 10, 15 days of assumption of casein glycomacropeptide by for fluorescent *in situ* hybridization.

Target microbiota	Time points	Fuorescent *in situ* hybridization counts (log CFU/g of feces, n=10) Means±SD		Percentage of target microbiota in fecal total bacteria Means±SD	
		group A	group B	group A	group B
Lactobacillus	D_0	10.43±1.22	10.26±0.99	1.92±0.62	1.83±0.38
	D_5	10.15±1.35	10.26±1.26	2.08±0.77	2.02±0.53
	D_{10}	10.77±1.46	11.18±0.98	2.00±0.96	8.36±2.21°
	D_{15}	10.32±1.89	11.63±0.78°	2.05±1.06	9.27±2.60°
Bifidobacteria	D_0	10.34±0.87	10.23±1.25	2.06±0.63	1.88±0.72
	D_5	10.26±1.36	10.32±1.48	1.92±0.58	3.94±1.02*
	D_{10}	10.63±2.39	11.11±1.02°	1.88±0.45	5.37±2.21°
	D_{15}	10.41±1.05	11.36±1.16°	1.67±0.95	6.84±1.36°
Enterococcus spp	D_0	10.47±2.03	10.26±1.54	2.04±0.63	2.17±0.58
	D_5	9.81±1.66	10.56±2.01	1.87±1.01	1.82±0.63
	D_{10}	10.02±1.32	10.29±1.55	2.72±0.97	1.93±0.32
	D_{15}	10.24±0.98	10.00±0.59	2.66±1.23	1.92±0.28
Enterobacteriaceae	D_0	10.41±1.27	10.60±1.65	2.32±1.03	2.45±1.01
	D_5	10.29±1.85	10.15±0.64	2.86±0.96	1.75±0.51
	D_{10}	10.97±0.72	9.98±0.88*	2.27±1.13	1.35±0.36*
	D_{15}	10.56±1.46	9.82±1.21*	2.63±1.01	0.96±0.25*

Significant differences among target microbiota counts of fecal microbiota in different periods (*P<0.05, °P<0.01). D, administration period (days).

cantly decrease the intestinal concentration of potential pathogens such as *Enterobacteriaceae* and coliforms, which might be associated with the sialic acid component (N-acetylneuraminic acid) in casein GMP. The sialic acid unit of casein GMP has been found to inhibit a variety of toxins, viruses and bacteria that caused infection through receptor association[27] since sialic acid receptors found on the surface of most bacteria are required for their initial infection.[27] Casein GMP, with an exogenous source of sialic acid might, therefore, inhibit bacterial adhesion by blocking the cell surface receptors preventing the bacteria from targeting the epithelial cell lining. Thus, the consumption of casein GMP has a prebiotic effect on the establishment of healthy intestinal microbiota, as well as prevention of infection.

The intestinal tract is a complex ecosystem of microorganisms. Therefore, it is important to establish an accurate, rapid and stable method of analysis to understand the human gut microbial community. In our study, we applied both culture method and FISH techniques in the analysis of mice feces. Both are able to monitor the difference in the numbers of major representatives of the intestinal flora. Cultural-based studies were able to provide a basic understanding of the diversity and complexity of the GIT ecosystem; however, they only allow the detection of culturable bacteria, are time-consuming and results are frequently biased due to the selectivity of the culture medium. So estimations of conventional bacteria by cultivation methods are inaccurate. On the other hand, it is well known that anaerobic bacteria are predominant in the GIT, 40-80% of the total microscopic counts can not be recovered by culture.[3] So it is impossible to accurately examine the intestinal microflora using traditional culture techniques. Molecular approaches based on nucleic acid such as FISH and quantitative real time polymerase chain reaction (qPCR) can overcome the shortcomings of the culture method. FISH is limited by the specificity of the probe and the efficacy of the probe binding to the bacterial target. The probes (EUB338, Enter1432, Bif164, Lacb722, ENC221) in our study have been widely used in related research and have been shown to have a reliable specificity. FISH can enumerate culturable and unculturable bacteria and detect 90% of the total intestinal microorganism. Therefore, for accurate and fast identification, FISH is probably more appropriate than conventional culture techniques because FISH can provide information about the presence, number, morphology and spatial distribution of microorganisms. Moreover, FISH gives a detailed picture of the microenvironments without any selective purification or amplification steps.

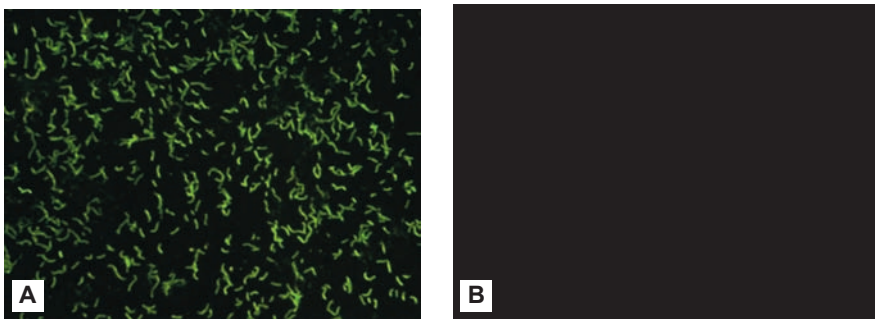

Figure 1. A) The positive hybridizing signal of *Lactobacillus helveticus* TS206 detected by Lacb722 probe. *Lactobacillus helveticus* TS206 was used to confirm the specificity of Lacb722 probe. *L. helveticus* TS206 was incubated in MRS broth at 37°C for 12 h. The bacterial cells were pelleted by centrifugation(3500×g, 10 min, 4°C) and washed once with phosphate-buffered saline (PBS, pH 7.2). The result of the FISH experiment with the *L. helveticus* TS206 showed that the specificity of the Lacb722 probe was high with the labeling efficiency above 90%. B) The positive hybridizing signal of *Lactobacillus helveticus* TS206 detected by Bif164 probe. *Lactobacillus helveticus* TS206 was used to verify the specificity of Bif164 probe. L *helveticus* TS206 was incubated in MRS broth at 37°C for 12 h. The bacterial cells were pelleted by centrifugation (3500×g, 10 min, 4°C), and washed once with phosphate-buffered saline (PBS, pH 7.2). The result of the FISH experiment with the *L. helveticus* TS206 revealed that the labeling efficiency was 0%.

Conclusions

Casein GMP is one of the biologically active components of milk. In this paper, we investigated the effect of casein GMP on modulating the intestinal microbiota *in vivo* using both a conventional culture method and FISH. The results showed that casein GMP had obvious biological capability to modulate the intestinal flora; more specifically, numbers of *Lactobacillus* and *Bifidobacteria* increased significantly ($P < 0.01$), whereas numbers of *Enterobacteriaceae* and coliforms decreased significantly ($P < 0.05$). Casein GMP showed a prebiotic effect as it helped to modulate the intestinal microbiota and establish intestinal microbiota that is considered to be healthier. The study provides theoretical support for casein GMP to be developed into an excellent source of functional food.

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