

Bovine β -Lactoglobulin Covalent Modification by Flavonoids: Effect on the Allergenicity and Human Intestinal Microbiota

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ABSTRACT: The present study aims to investigate the structure of covalent conjugates of bovine β -lactoglobulin (BLG) and flavonoids (luteolin, myricetin, and hyperoside), and their effect on the allergenicity and human intestinal microbiota. Covalent modification of amino acids in BLG by flavonoids was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and *o*-phthalaldehyde assay. The secondary and conformational structures of BLG were changed by the covalent modification, which were determined by the circular dichroism, Fourier transform infrared spectroscopy, fluorescence spectroscopy, and UV spectroscopy. The enzyme-linked immunosorbent assay (ELISA) and cell experiments indicated that BLG covalent conjugates could reduce IgE/IgG binding capacities and suppress the allergy reactivity of RBL-2H3 cells, suggesting that the covalent modification modulated the balance of T cells. Meanwhile, covalent modification of BLG with these flavonoids can alter the diversity of human intestinal microbiota and the community abundance at phylum, family, and genus levels. The results revealed that covalent modification of BLG with flavonoids alters human intestinal microbiota, might result in the reduction of allergenicity, which could provide information for confirming the relationship between food allergy and the intestinal microbial ecosystem.

KEYWORDS: bovine β -lactoglobulin, flavonoids, allergenicity, covalent modification, human intestinal microbiota

INTRODUCTION

Bovine β -lactoglobulin (BLG) is the major whey protein in bovine milk and has a molecular weight of 18.3 kDa.¹ BLG has many food functional characteristics, such as excellent heat-set gelation characteristics, foamability, and high solubility.^{2–4} However, BLG is one of the nutrition allergens in infant formulas,⁵ which has resulted in the limited use of BLG. Therefore, it is important to reduce the allergenicity of BLG.

At present, there is growing interest in different methods for reducing the allergenicity of BLG, such as enzymatic hydrolysis, nonthermal processing technology,⁶ covalent interaction,^{7,8} and combination therapies.^{9,10} Among the mentioned approaches, covalent interaction presents a very good application for reducing the allergenicity of BLG. For example, glycation can change conformational structures of BLG to reduce allergenicity.¹¹ The allergen–IgE antibody recognition of BLG was reduced by covalent conjugation with bioactive compounds.⁷ Bioactive compounds include flavonoids, phenolic acid, terpenoids, and so on. Luteolin (Lut), myricetin (Myr), and hyperoside (Hyp) are typical flavonoids with a range of diverse bioactivities. Lut has been shown to exert a large number of beneficial functions, for example, anti-inflammatory, antioxidative, and antitumor effects.^{12,13} Myr is a naturally occurring flavonoid that possesses multiple biological functions.^{14,15} Hyp, an important flavonoid glycoside compound, is beneficial for regulating inflammatory reactions.¹⁶ Although various literature reported that covalent interactions with some reducing sugar and polyphenols can affect the allergic reaction of the allergen, the effect of covalent interactions with Lut, Myr, and Hyp on the allergenic capacity of BLG is unknown.

It was reported that flavonoids are considered functional dietary ingredients,¹⁷ and milk protein can serve as vehicles for flavonoids.¹⁸ Van et al. found that the tea catechin–milk protein complex improves the bioavailability of the catechins.¹⁹ Bohin et al. reported that off-tastes of flavonoids could be counteracted by the use of milk proteins.²⁰ Previous studies indicated that the flavonoid–microbiota interaction can modulate the intestinal microbiota composition and flavonoids play a role in the issue using models of cytokine-induced intestinal inflammation to address inflammation. We suspected that the addition of flavonoids to milk protein could be used as an effective method to develop functional foods by targeted delivery of flavonoids to the gut. Furthermore, as an important modulator of metabolism, the intestinal microbiota plays an important role in human health and disease. Some researchers have found that food allergy is related to the composition of intestinal microbiota.²¹ Corzo-Martínez et al.²² reported that the glycosylated casein can induce a significant increase in *Bifidobacteria* compared with healthy infants. Ling et al. reported the fecal microbiota of infants with cow's milk allergy in early life changed and the microbiota diversity in their bodies decreased.²³ However, the effect of covalent interactions between flavonoids (Lut, Myr, and Hyp) and BLG on

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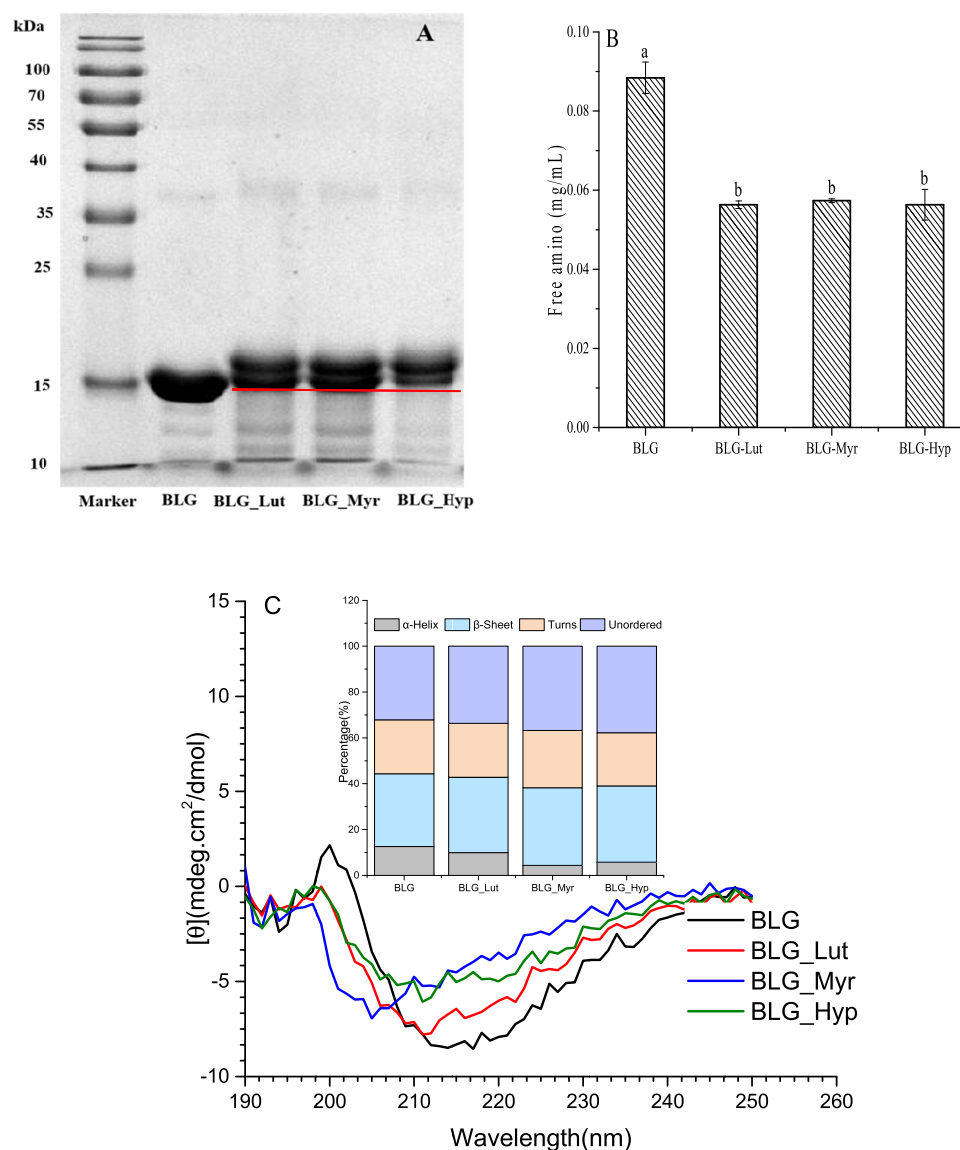


Figure 1. SDS-PAGE (A), free amino groups (B), and secondary structure (C) of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp. Related secondary structural contents analyzed by DichroWeb. Letters (a, b) in the bars mean significantly different ($p < 0.05$).

the human intestinal microbiota of BLG have not been elucidated.

Here, we prepared BLG–flavonoid conjugates (BLG_Lut, BLG_Myr, and BLG_Hyp) using a radical method to investigate whether or not the structural changes of BLG_Lut, BLG_Myr, and BLG_Hyp could reduce allergenicity and alter the human intestinal microbiota. Changes in the structure of BLG–flavonoid conjugates were detected using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), *o*-phthaldialdehyde assay, Fourier transform infrared spectroscopy, fluorescence spectroscopy, and UV spectroscopy. The IgG/IgE binding activity was analyzed by the enzyme-linked immunosorbent assay (ELISA) with rabbit polyclonal antibodies and sera from patients with cow's milk allergy. The influence on the release of cell biological active mediators, such as β -hexosaminidase and histamine, was investigated using the RBL-2H3 cell model. Finally, the influence of BLG–flavonoid conjugates on human intestinal microflora represented by feces was studied *in vitro*.

MATERIALS AND METHODS

Materials. β -Lactoglobulin (BLG) from bovine milk (L3908, $\geq 90\%$) were obtained from Sigma-Aldrich (St. Louis, MO). Pepsin, trypsin, luteolin, myricetin, hyperoside, and other reagents were obtained from Beijing Solarbio Technology Co., Ltd. (Beijing, China).

Sample Preparation. Luteolin (Lut), myricetin (Myr), and hyperoside (Hyp) were dissolved in 95% ethanol, respectively, and stored in a refrigerator at 4 °C for future use. First, 0.5 g of BLG was fully dissolved by adding 50 mL of deionized water, then 1 mL of 30% hydrogen peroxide and 0.25 g of ascorbic acid were added, and the solution was mixed and reacted at room temperature for 2 h. After the reaction finished, the protein solution was added to the Lut solution to achieve a concentration of 0.35 mM Lut. Myr solution and Hyp solution were treated under the same conditions. BLG–Lut mixed solution, BLG–Myr, and BLG–Hyp mixed solution were reacted at room temperature for 24 h. After the reaction stopped, the solution was dialyzed at 4 °C for 48 h in a dialysis bag to remove Lut, Myr, and Hyp that are not bound to BLG. After dialysis, the solution was freeze-dried for future use. Native BLG was named BLG; native BLG covalently modified by Lut, Myr, and Hyp were named BLG_Lut, BLG_Myr, and BLG_Hyp, respectively.

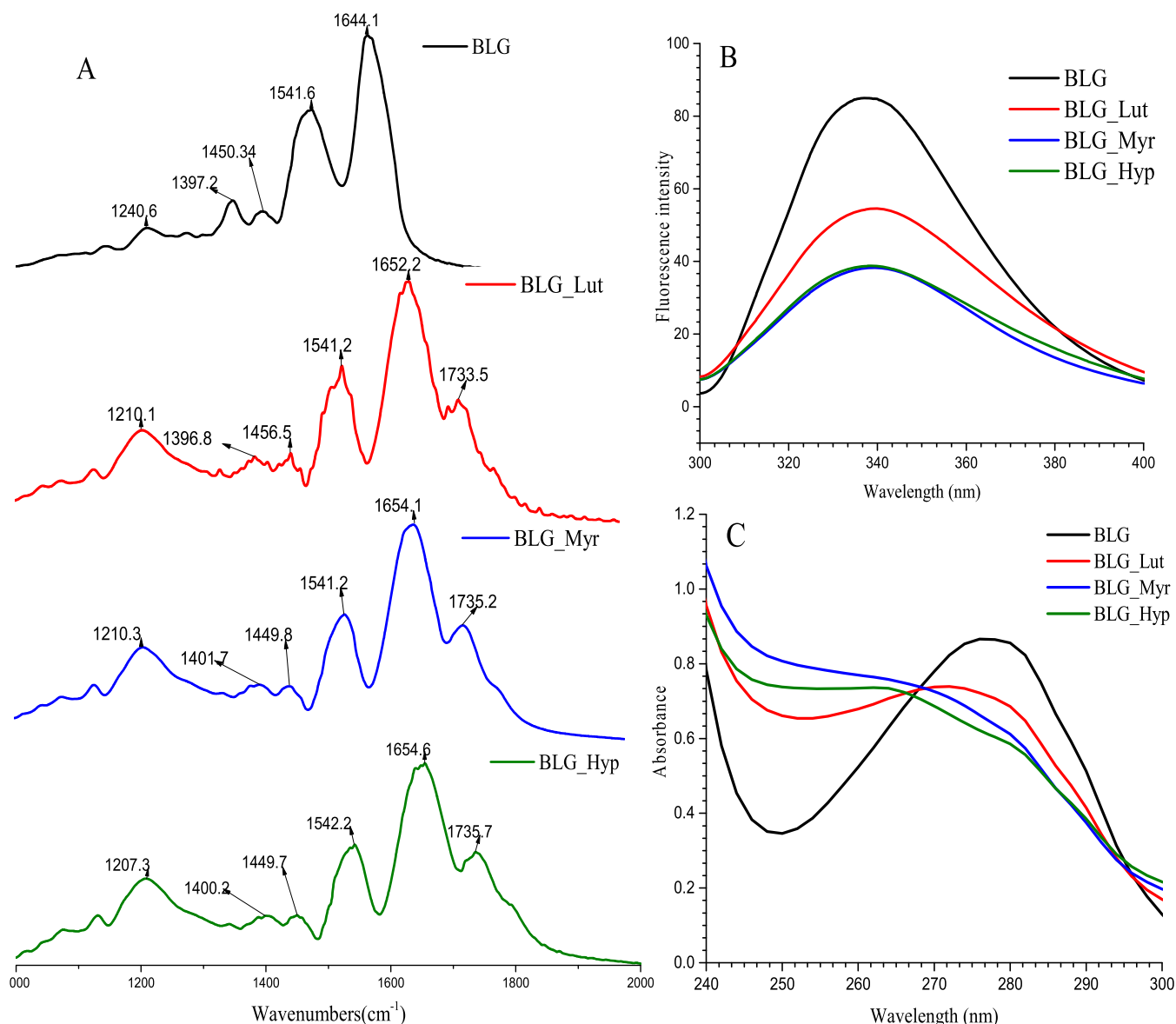


Figure 2. Fourier transform infrared spectroscopy spectra (A), intrinsic fluorescence intensity (B), and UV spectrum (C) for BLG, BLG_Lut, BLG_Myr, and BLG_Hyp.

SDS-PAGE. The SDS-PAGE analysis of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp was performed according to a previous description.⁷ After the BLG, BLG_Lut, BLG_Myr, and BLG_Hyp (1 mg/mL) degeneration finished, buffer was added. SDS-PAGE was performed on a Bio-Rad microprotein electrophoresis system (Bio-Rad Laboratories, Inc., Hercules) using 12% separation gel and 5% stacking gel.

Free Amino Groups. Free amino groups of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp were determined by the *o*-phthaldialdehyde assay.

Determination of the Secondary Structure. Secondary structures of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp were analyzed by Far-UV circular dichroism spectroscopy.

Intrinsic Fluorescence Intensity and UV Spectrum Measurement. Intrinsic fluorescence emission spectra and UV absorption spectra of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp were measured using a Hitachi F-7000 fluorescence spectrophotometer and a UV-2910 spectrophotometer, respectively. The detailed process of the method was performed by our previously reported assay.¹¹

Fourier Transform Infrared (FTIR) Spectroscopy. The infrared spectra of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp study involved use of an FTIR spectrometer with KBr pellets.⁷

IgE/IgG Binding Activity Measurement. Inhibition ELISA assays were used to estimate the IgE and IgG binding activities of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp by the method of Liu et al., which involved mixing the same amounts of sera of five patients to yield a serum pool and rabbit antisera.²⁴ Milk protein-specific IgE levels of five human serum were 6.71, 9.977, 17.1, 43.6, and ≥ 100 kUA/L.

RBL-2H3 Cell Assay. The rat mast cell lines RBL-2H3 were cultured, then human antisera (prepared by mixing the sera of five patients at the same volume) was immediately added, followed by test samples stimulating the cells. The release of histamine and interleukin-6 was measured by the reported methods.²⁴

In Vitro Fermentation of Digested Juice in Human Feces. Fecal samples were obtained from three healthy donors (one male and two females; 18–22 years old) without any type of food allergy, any known metabolic or gastrointestinal disorders, and had avoided probiotics, prebiotics, and antibiotics for 3 months prior to the study. Samples were collected and kept in an anaerobic incubator, then diluted (1:5, w/w) with phosphate buffer (0.1 M, pH 7.4), and homogenized in a stomacher for 1 min at normal speed before analysis. Small-scale *in vitro* fermentation systems were used for the

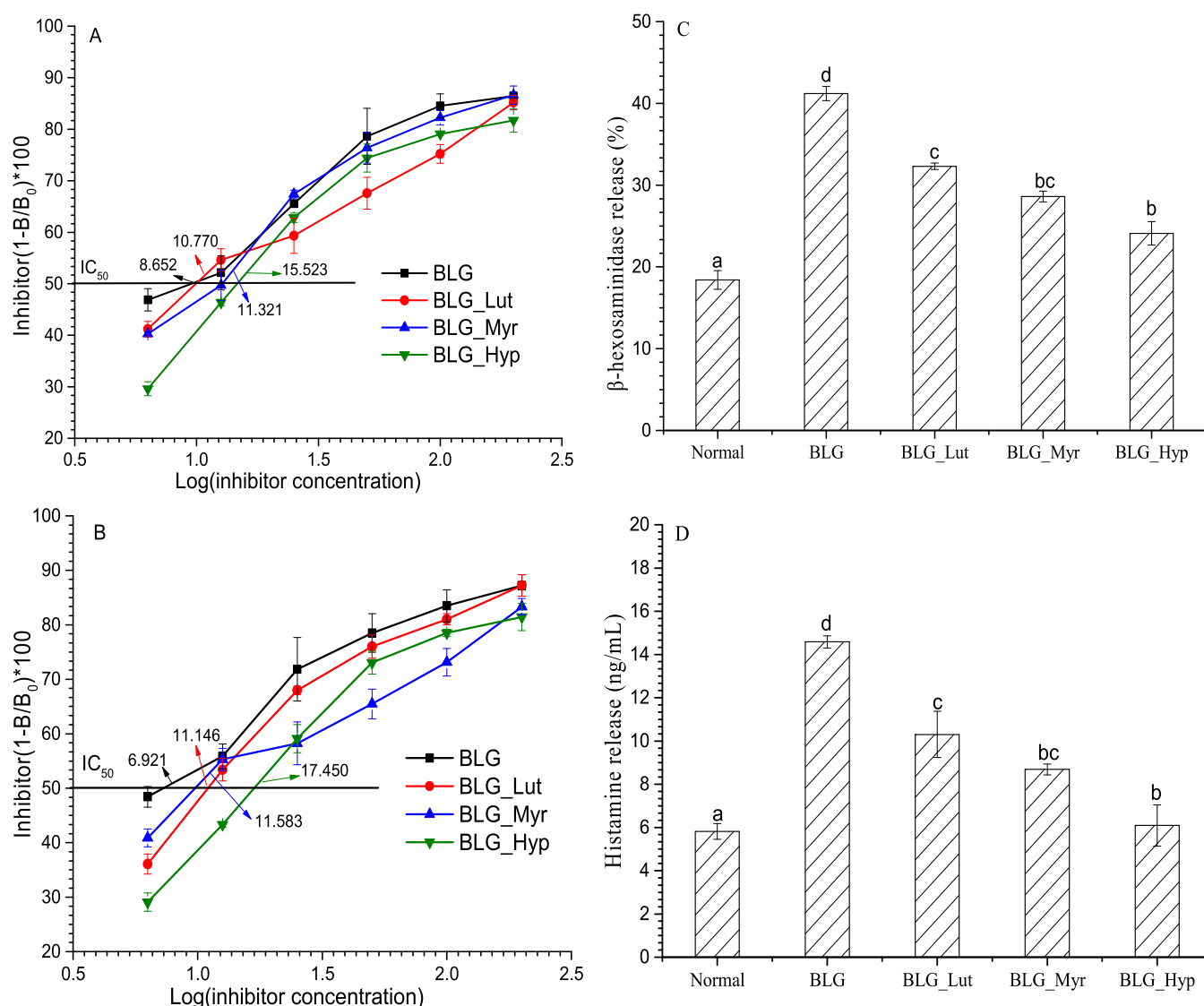


Figure 3. IgE (A)/IgG (B) binding activity and histamine (C) and interleukin-6 release (D) of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp. Letters (a–d) in the bars mean significantly different ($p < 0.05$).

incubation of 10 mg of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp. All additions, inoculations, and incubations were carried out inside the anaerobic incubator.

16S rRNA High-Throughput Sequencing. After the anaerobic culture finished, the feces were collected by centrifugation and stored in a refrigerator at -80°C for future use. DNA extraction and sequencing of samples were processed by Shanghai Majorbio Bio-pharm Technology Co., Ltd. The V4 region of the bacteria 16S ribosomal RNA gene was amplified by PCR using primers 515F5'-GTGCCAGCMGCCGCGTAA-3' and 806R 5'-GCAC-TACHVGGGTWCTAAT-3'. Data analysis was performed using the website (<http://www.isanger.com/index.html>).

Statistical Analysis. Experiment data were statistically analyzed by SPSS (SPSS Inc., IL).

RESULTS AND DISCUSSION

Structural Analysis. The change in molecular weight of BLG was shown through SDS-PAGE after covalent interaction with Lut, Myr, and Hyp. The bands of BLG_Lut, BLG_Myr, and BLG_Hyp showed a slight upward migration compared to the band of native BLG (Figure 1A), which suggests molecular weight increased with the conjugation of BLG and Lut, Myr,

and Hyp. Similar phenomena have been observed in BLG conjugated with polyphenol compounds.^{7,25} This result showed the covalent modification of BLG with Lut, Myr, and Hyp.

The levels of free amino groups of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp were studied. As shown in Figure 1B, the free amino groups of BLG_Lut, BLG_Myr, and BLG_Hyp were markedly lower than that of BLG ($p < 0.05$). The amount of free amino groups decreased in BLG_Lut, BLG_Myr, and BLG_Hyp, suggesting that Lut, Myr, and Hyp may form covalent bonds with the amino acids in BLG.

Circular dichroism spectra were used to measure the secondary structures of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp. In Figure 1C, data indicate that BLG_Lut presents a lower α -helix content and a higher unordered structural content compared to those of BLG. The result suggested that Lut conjugation caused the BLG structure to become more unfolded. In the cases of BLG_Myr and BLG_Hyp, the decrease in the α -helix content and increase in the unordered structural content were also observed, which suggests that part of the α -helix of BLG may be extended to unordered structure,

and the changes in secondary structures of BLG undergo covalent conjugation with flavonoids. The changes in the secondary structure of BLG were consistent with other research results.^{7,26} The changes in the structure of BLG conjugated with Lut, Myr, and Hyp were also explored with FTIR spectroscopy. As shown in Figure 2A, compared with BLG, for BLG_Lut, BLG_Myr, and BLG_Hyp, the amide I bands (C=O stretch) moved from 1644.1 to 1652.2, 1654.1, 1654.6 cm^{-1} , respectively. The spectra of BLG_Lut, BLG_Myr, and BLG_Hyp exhibited other bands at 1733.5, 1735.2, and 1735.7 cm^{-1} (carbonyl group), respectively. This result implied BLG covalently binds to Lut, Myr, and Hyp, leading to the change in the secondary structure of BLG after covalent conjugation with Lut, Myr, and Hyp.

Conformational changes of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp were analyzed by fluorescence and UV spectroscopies. Intrinsic fluorescence spectroscopy can be used to determine the conformational changes around Trp residues. Figure 2B displays that fluorescence intensities of BLG_Lut, BLG_Myr, and BLG_Hyp were lower than that of BLG, which suggests Lut, Myr, and Hyp could shield the area around the Trp residues on the BLG.²⁷ The result was related to conformational changes of BLG after covalent conjugation with Lut, Myr, and Hyp. The changes in the UV spectra of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp are shown in Figure 2C. Compared to BLG, BLG_Lut, BLG_Myr, and BLG_Hyp had a lower maximum UV absorbance at 280 nm. It implied that covalent modification with Lut, Myr, and Hyp could destroy the rigid tertiary structure of BLG.¹¹ Fluorescence and UV spectrum results showed that the BLG covalent modification by Lut, Myr, and Hyp led to change the conformational structure.

Allergenicity Analysis. To determine whether covalent conjugation causes the changes in the allergenicity of BLG, the IgG/IgE binding activities of BLG_Lut, BLG_Myr, and BLG_Hyp were evaluated by ELISA (Figure 3). In Figure 3A, IC_{50} values of BLG_Lut, BLG_Myr, and BLG_Hyp, respectively, shifted to 10.770, 11.321, and 15.523 $\mu\text{g/mL}$, much higher than that of BLG (8.652 $\mu\text{g/mL}$). A similar phenomenon is shown in Figure 3B, IC_{50} values of BLG_Lut, BLG_Myr, and BLG_Hyp were 1.67-, 1.61-, and 2.52-times that of BLG (6.921 $\mu\text{g/mL}$), respectively. Therefore, the IgG/IgE binding to BLG was decreased after covalent modification by Lut, Myr, and Hyp. It was also found that the covalent conjugation with dietary polyphenols reduced the IgG/IgE binding to BLG.⁷ Cell experiments were also used to confirm that covalent conjugation induced the changes in allergenicity of BLG in subsequent experiments.

The release of mediators is related to the occurrence of type I allergic reactions.²⁸ To confirm whether or not covalent conjugation reduces the allergenicity of BLG, a cell experiment was used to evaluate the mediator release from RBL-2H3 cells. In Figure 3C, the release of β -hexosaminidase decreased more in RBL-2H3 cells exposed to BLG_Lut, BLG_Myr, and BLG_Hyp compared with BLG alone. The release of histamine also followed a similar trend (Figure 3D). It can be inferred that the response of RBL-2H3 cells to BLG_Lut, BLG_Myr, and BLG_Hyp alters IgE epitopes on the surface of BLG, hindering the basophil degranulation.²⁴ In summary, ELISA and cell experiment results showed that covalently linking BLG with Lut, Myr, and Hyp leads to the decrease in the IgG/IgE binding activity and suppresses the allergy reactivity of RBL-2H3 cells; this result also implies that the

covalent interaction with flavonoids can reduce the allergenicity of BLG.

Human Intestinal Microbiota Analysis. To study the detailed effects of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp on intestinal microbiota in fermentation samples, high-throughput sequencing of the 16S ribosomal DNA variable region was used to analyze the composition and diversity of the microbial community in the samples after 48 h of fermentation. Figure S1 in the Supporting Information shows 97% similarity of dilution curves in the 16S rDNA sequences from BLG, BLG_Lut, BLG_Myr, and BLG_Hyp samples. The Shannon index decreased in BLG, BLG_Lut, BLG_Myr, and BLG_Hyp, whereas the Simpson index and ACE index increased (Table 1), suggesting that BLG can affect the structure and diversity of

Table 1. Microbial Diversity Indices of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp

sample	Shannon	Simpson	ACE	Chao	coverage
control	2.66404	0.12925	187.18	186.647	0.99952
BLG	2.16587	0.22838	182.242	188.077	0.99947
BLG_Lut	2.29127	0.24398	188.109	187.000	0.99952
BLG_Myr	2.63939	0.17786	200.916	222.600	0.99939
BLG_Hyp	2.37935	0.21249	186.581	192.077	0.99947

intestinal microbiota. Moreover, the Chao index from BLG_Lut, BLG_Myr, and BLG_Hyp was higher than that from the control group, thereby indicating that the covalent interaction with flavonoids can increase the diversity of the intestinal microbiota community and the relative abundance of microbiota.

The microbial community was mainly composed of *Bacteroidota*, *Firmicutes*, *Actinobacteriota*, and *Proteobacteria* (Figure 4A). From Figure 4A we find clearly that BLG, BLG_Lut, BLG_Myr, and BLG_Hyp can alter the relative abundance of *Bacteroidota*, *Firmicutes*, *Actinobacteriota*, and *Proteobacteria*. The relative abundance of *Bacteroidota* increased, and the relative abundance of *Fusobacteriota* decreased in the BLG_Lut, BLG_Myr, and BLG_Hyp compared with BLG. Therefore, the changes in the relative abundance of *Bacteroidota* and *Fusobacteriota* may be associated with food allergy.²³ Therefore, covalently linking BLG with Lut, Myr, and Hyp can greatly affect the stability of microbial community structure.

As shown in Figure 4B, the dominant families included the *Prevotellaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Peptostreptococcaceae*, *Selenomonadaceae*, and *Veillonellaceae* in the fermented juice, and their relative abundance was affected by the tested BLG samples. The relative abundance of *Prevotellaceae*, *Lachnospiraceae*, and *Ruminococcaceae* increased in the BLG_Lut, BLG_Myr, and BLG_Hyp compared to the BLG group. The opposite trend was observed in *Peptostreptococcaceae* and *Selenomonadaceae*. The dominant families may be associated with food allergy.²¹ This result showed that covalently linking BLG with Lut, Myr, and Hyp can greatly affect the microbial community at the family level.

As shown in Figure 4C, the dominant genus composed of *Prevotella*, *Romboutsia*, *Faecalibacterium*, *Megasphaera*, *Agathobacter*, *Dialister*, and *Alloprevotalla* and their relative abundance were affected by the tested BLG samples. The relative abundance of *Prevotella*, *Faecalibacterium*, *Agathobacter* increased and that of *Romboutsia*, *Megasphaera* decreased in the BLG_Lut, BLG_Myr, and BLG_Hyp compared to that of

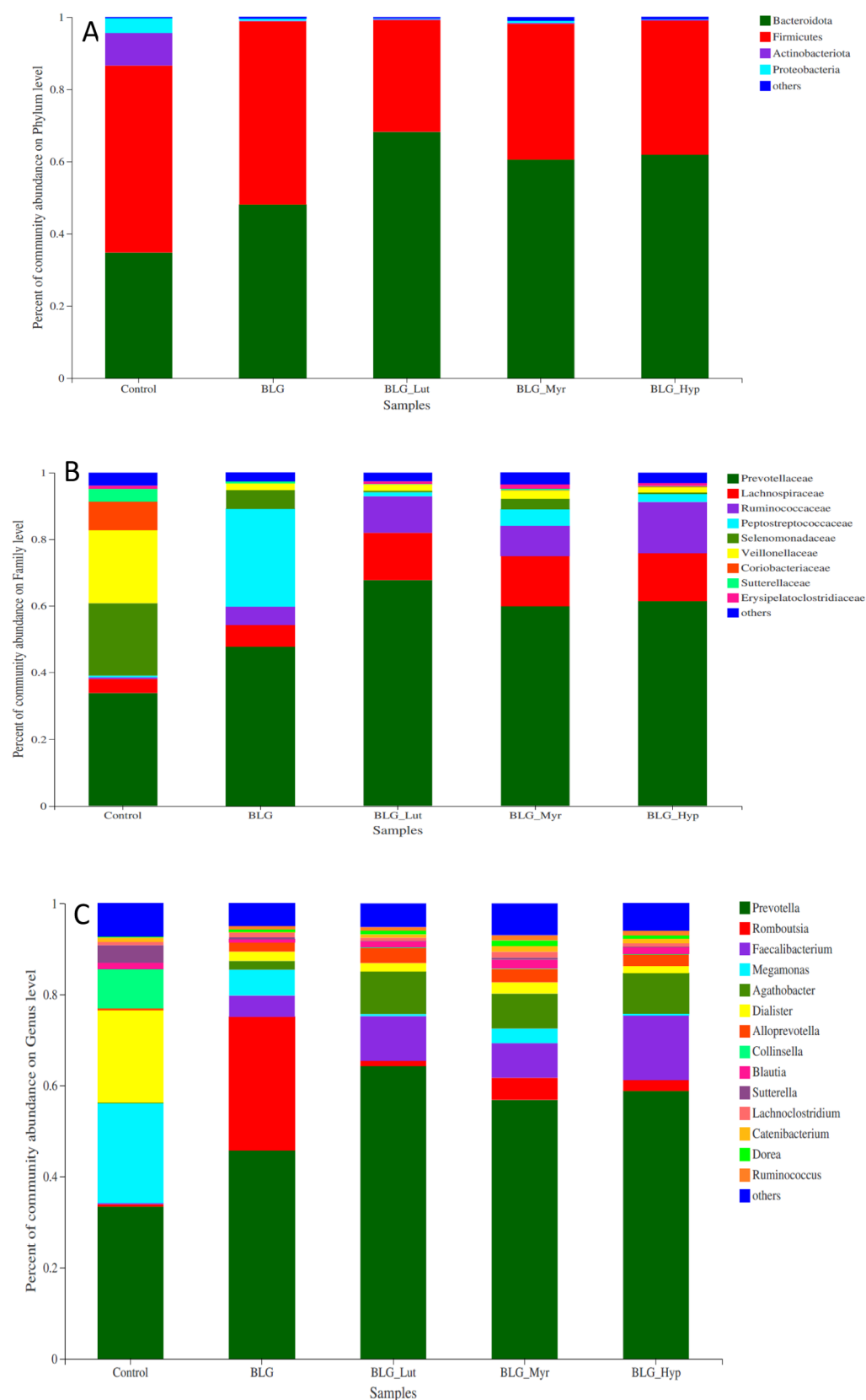


Figure 4. Effect of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp on the community abundance at the phylum level (A), family level (B), and genus level (C).

BLG. This result showed that covalently linking BLG with Lut, Myr, and Hyp can greatly affect the microbial community at the genus level.

The community heatmap at the genus level is shown in Figure 5, and it was found that the community existed according to the evolutionary relationships. Here, red

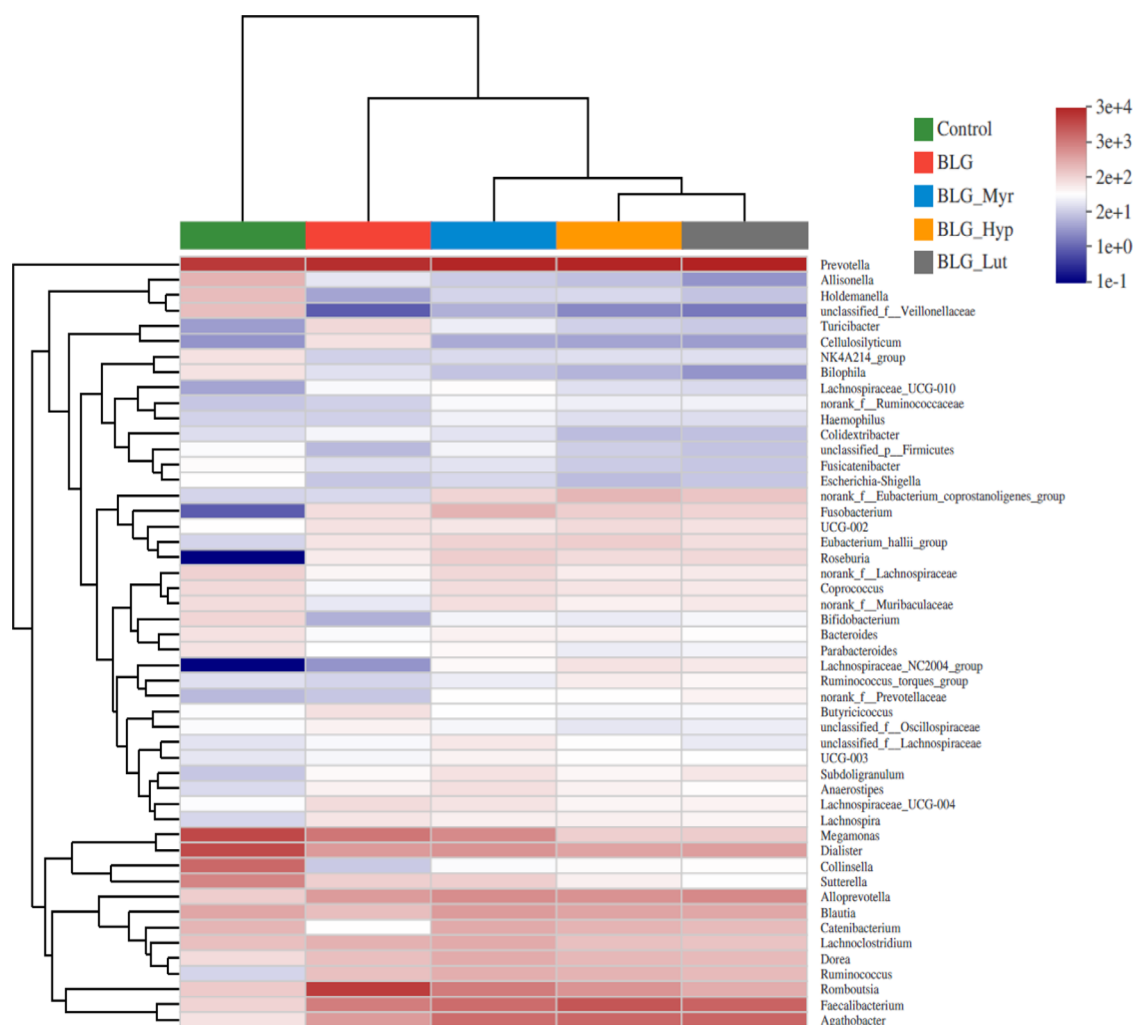


Figure 5. Effect of BLG, BLG_Lut, BLG_Myr, and BLG_Hyp on the community heatmap analysis at the genus level.

represents a core category in BLG, BLG_Lut, BLG_Myr, and BLG_Hyp and mainly includes *Agathobacter*, *Faecalibacterium*, *Romboutsia*, and *Alloprevotalla*. Moreover, data indicated that microbial species were not different between the control group and BLG, BLG_Lut, BLG_Myr, and BLG_Hyp. However, the microbial numbers of the control group and the tested groups were different. The change in microbial numbers exhibited a similar trend to the relative abundance (Figure 4C), impairing the potential allergenicity.²⁹

Effects of the BLG Covalent Interaction with Flavonoids on the Allergenicity and Human Intestinal Microbiota. Bioactive compounds are considered functional dietary ingredients, and milk protein can serve as vehicles for the bioactive compounds. In the current research, we gained insight into BLG covalent modification by flavonoids (Lut, Myr, and Hyp). This study has shown that BLG covalent modification by flavonoids (Lut, Myr, and Hyp) can increase the molecular weight, decrease the amount of free amino groups, and change the secondary structure and conformational structure (Figures 1 and 2). The covalent modification can also decrease allergenicity of BLG, and the reduction is reflected by the decrease of IgG and IgE binding capacities and suppression of the allergy reactivity of RBL-2H3 cells (Figure 3). In addition, the covalent modification also affects the human intestinal microbial ecosystem (Figure 4).

The correlation between the reduction in the allergenicity and altered human intestinal microbiota was discussed in the next step. The conformational and linear epitopes of BLG are related to the potential allergenicity.³⁰ It is reported that the mechanism of the covalent modification by polyphenol is the *o*-quinones mechanism. Many flavonoids are able to be oxidized to their corresponding quinones and semiquinones, which can further undergo covalent reactions with an enormous number of nucleophiles such as cysteine or lysine on the BLG.³¹ The BLG was modified by Lut, Myr, and Hyp, the increase in molecular weight (Figure 1A) and decrease in contents of free amino groups (Figure 1B) indicated they are covalently conjugated each other. This result showed flavonoids could form covalent bonds with residues, which are located in the IgE/IgG epitopes. Thus, the covalent modification sites in BLG can mask the linear IgE/IgG epitope, preventing IgE/IgG from interacting with proteins.⁷ The result can interpret the reduction in the IgE/IgG binding activity (Figure 3A) and the mediators release (Figure 3B) of BLG–flavonoid conjugates. Similar results were achieved by Liu et al., who demonstrated that the covalent modification can mask the linear epitope of allergic proteins.³² On the other hand, conformational epitopes of the protein are linked to its secondary or tertiary structure. The secondary structure of BLG changed after covalent modification by Lut, Myr, and

Hyp (Figures 1C and 2A), affecting conformational epitopes, and led to decrease in the potential allergenicity of proteins. Fluorescence intensity (Figure 2B) and UV spectrum (Figure 2C) of BLG after covalent modification by Lut, Myr, and Hyp considerably changed, suggesting that covalent modification can change the conformational structure of BLG, destroy conformational epitopes, and ultimately reduce the allergenicity of BLG. These results can explain the decrease of the IgE/IgG binding activity (Figure 3A) and the mediator release (Figure 3B) of BLG that covalent modified by Lut, Myr, and Hyp.

Bioactive compounds play an important role in the intestinal microbial ecosystem.³³ Intestinal microbiota can inhibit the cytokines produced by Th2 cells and reduce the release of inflammatory mediators, resulting in alleviating the food allergy symptoms.³⁴ In this article, we focus on the effects of covalently linking BLG with Lut, Myr, and Hyp on the allergenicity and human intestinal microbiota. We found that the human intestinal microbiota changed by BLG treatment (Figures 4 and 5). This situation showed that covalent modification with flavonoids can change the BLG structure and affect the amount of intestinal microbiota in the intestinal tract of infants. The relative abundance of *Bacteroidota* increased and that of *Fusobacteriota* decreased in the BLG–flavonoid conjugates compared with BLG (Figure 4A). Increased *Bacteroidota* count may be related to the balance of T cells, leading to the reduction of the occurrence of allergic reactions.²³ This situation can be used to explain the reduction in the mediator release (Figure 3B). In addition, the relative abundance of intestinal microbiota at the family level (Figure 4B) and genus level (Figure 4C) of BLG modified by flavonoid treatment has similar consequences. After covalent modification, the amount of *Prevotellaceae*, *Lachnospiraceae*, *Ruminococcaceae*, *Prevotella*, *Faecalibacterium*, and *Agathobacter* increased, and these changes also contributed to the reduction in potential allergenicity. Although the human intestinal microbial species were not changed by covalent modification between BLG and flavonoids (Figure 5), the human intestinal microbial numbers were different. Therefore, BLG interacting with Lut, Myr, and Hyp occurred by covalent modification altered the structure and human intestinal microbiota, leading to the reduction in allergenicity of BLG.

In conclusion, this study showed that covalent modification of BLG with luteolin, myricetin, and hyperoside notably decreased IgE/IgG binding capacities and the allergy reactivity of RBL-2H3 cells and altered human intestinal microbiota. This finding may be attributed to structural changes in BLG, which are reflected by changes in the molecular weight, free amino groups, and secondary and tertiary structures. However, the evaluation of protein allergenicity and intestinal microbiota is not comprehensive, and *in vivo* research works in animals or the clinic are needed to confirm that intestinal dysbiosis causes food allergy in the next step.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.1c02482>.

PCR amplification of the 16S rDNA gene of the rarefaction curve from human feces (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

BLG, bovine β -lactoglobulin; Lut, luteolin; Myr, myricetin; Hyp, hyperoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; BLG_Lut, covalent modification of BLG with luteolin; BLG_Myr, covalent modification of BLG with myricetin; BLG_Hyp, covalent modification of BLG with hyperoside

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