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ORIGINAL PAPER

In vitro Effects of Prebiotics and Synbiotics on *Apis cerana* Gut Microbiota

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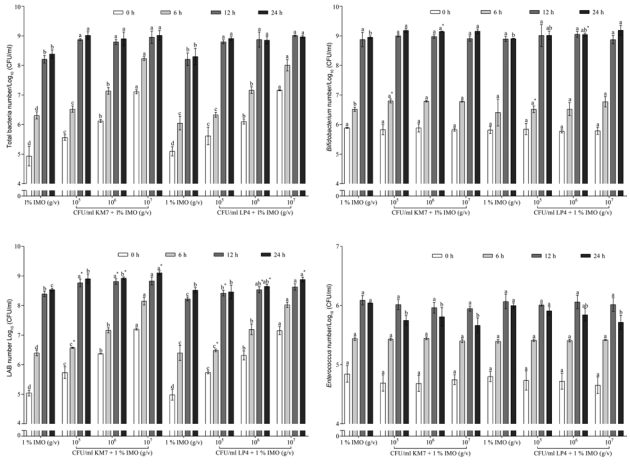
Abstract

This study aimed to investigate *in vitro* effects of the selected prebiotics alone, and in combination with two potential probiotic *Lactobacillus* strains on the microbial composition of *Apis cerana* gut microbiota and acid production. Four prebiotics, inulin, fructo-oligosaccharides, xylo-oligosaccharides, and isomalto-oligosaccharides were chosen, and glucose served as the carbon source. Supplementation of this four prebiotics increased numbers of *Bifidobacterium* and lactic acid bacteria while decreasing the pH value of *in vitro* fermentation broth inoculated with *A. cerana* gut microbiota compared to glucose. Then, two potential probiotics derived from *A. cerana* gut at different dosages, *Lactobacillus helveticus* KM7 and *Limosilactobacillus reuteri* LP4 were added with isomalto-oligosaccharides in fermentation broth inoculated with *A. cerana* gut microbiota, respectively. The most pronounced impact was observed with isomalto-oligosaccharides. Compared to isomalto-oligosaccharides alone, the combination of isomalto-oligosaccharides with both lactobacilli strains induced the growth of *Bifidobacterium*, LAB, and total bacteria and reduced the proliferation of *Enterococcus* and fungi. Consistent with these results, the altered metabolic activity was observed as lowered pH in *in vitro* culture of gut microbiota supplemented with isomalto-oligosaccharides and lactobacilli strains. The symbiotic impact varied with the types and concentration of *Lactobacillus* strains and fermentation time. The more effective ability was observed with IMO combined with *L. helveticus* KM7. These results suggested that isomalto-oligosaccharides could be a potential prebiotic and symbiotic with certain lactobacilli strains on *A. cerana* gut microbiota.



K e y w o r d s: *Apis cerana*, isomalto-oligosaccharides, *Bifidobacterium*, lactic acid bacteria, metabolic activity

Introduction

Honeybees can provide humans with various bee products, such as pollen, honey, royal jelly, and propolis (Nainu et al. 2021). Honeybees are also important pollinators worldwide (Khalifa et al. 2021), increasing crop yields and maintaining the stability of ecosystems and the diversity of plant communities (Dai et al. 2018). A previous study has shown that 85% of the main crops directly related to food rely on pollination by insects such as honeybees (Klein et al. 2007). The gut microbial composition of honeybees is simpler than that of humans (Kwong and Moran 2016). The honeybee gut harbors a diverse microbial community, among which lactic acid bacteria (LAB) and bifidobacteria are beneficial in the intestine (Ge et al. 2021). On the contrast,

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some pathogenic bacteria such as *Paenibacillus larvae* and *Melissococcus pluton* have been found to cause diseases in honeybees (Fünfhaus et al. 2018). Both probiotics and prebiotics benefit the intestinal health of animals and humans by improving the gut microbiota balance, epithelial barrier, and immune function of hosts (Sanders et al. 2019). Thus, maintaining gut homeostasis is of great significance in honeybees.

Apis cerana, a native honeybee species in China, has a beekeeping history of more than 1700 years (Wang et al. 2021). In 2017, the number of *A. cerana* was estimated to be two million in China and play a critical role in crop production (Chen et al. 2017). Antibiotics have been commonly used in bee colonies to treat and control diseases of honeybees caused by bacterial pathogens such as *P. larvae* (Raymann et al. 2017). However, emerging evidence has reported that antibiotic treatment could reduce gut microbial diversity, weaken nutrient metabolism and immune function, affect physiological and behavioral development, and even increase mortality in honeybees (Raymann et al. 2017; Ortiz-Alvarado et al. 2020; Duan et al. 2021). Therefore, the need for alternatives to antibiotics to maintain honeybee fitness has emerged.

A prebiotic is defined as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" by the International Scientific Association for Probiotics and Prebiotics (ISAPP) (Gibson et al. 2017). Commonly used prebiotics include inulin (INU), fructo-oligosaccharides (FOS), xylo-oligosaccharides (XOS), and isomalto-oligosaccharides (IMO) (Carlson et al. 2017; Poeker et al. 2018; Sorndech et al. 2018; Tandon et al. 2019). Prebiotics can alter the gut microbiota composition and promote host health by accumulating organic acids (Rastall and Gibson 2015). Prebiotics, which mainly favor bifidobacteria and lactobacilli, can be fermented to generate short-chain fatty acids (SCFAs) with colonic bacteria to fight chronic diseases (Tornero-Martínez et al. 2019). A synbiotic is a mixture comprising live microorganisms and substrate(s), selectively utilized by host microorganisms that confer a health benefit on the host (Swanson et al. 2020). Lactic acid bacteria, the most widely used probiotics, antagonize the pathogenic bacteria and influence the gut microbiota of the honeybees (Williams 2010; Audisio et al. 2015; Ramos et al. 2020). These studies highlight the potential of prebiotics and synbiotics modifying the gut microbiota of honeybees and consequently improving host health. However, little is known about the effects of prebiotics and synbiotics on the intestinal microbiota of *A. cerana*. This study aimed to determine the prebiotic properties of selected commonly used prebiotics using *in vitro* fermentation of gut microbiota of *A. cerana*. The most prebiotic potential was observed in IMO. Furthermore, IMO's *in vitro* symbiotic effect with lactobacilli, which has shown probiotic properties in our previous study, was determined. The obtained results may provide insight into the potential outcomes of IMO to improve the health of honey bees.

Experimental

Materials and Methods

LAB strains and incubation condition. Strains *Limosilactobacillus helveticus* KM7 (CGMCC No. 16042) and *Lactobacillus reuteri* LP4 (CGMCC No. 16043) deposited in the China General Microbiological Culture Collection Center, were isolated from the intestines of adult worker honeybees from *A. cerana* in our previous study and showed probiotic properties (Wang 2018; Lei et al. 2020). Each strain was incubated with Man Rogosa and Sharpe (MRS) broth at 37°C and grown to stationary phase for 24 h in an anaerobic jar (Anaero-Jar TM 2.5l, Oxoid Ltd., Basingstoke, UK), including a gas-generating package (AnaeroPack, Mitsubishi Gas Chemical Co., Tokyo, Japan) prior to experiments. After centrifuging at 3,944 × *g* for 10 min at 4°C, the pellets were resuspended in MRS broth.

In vitro fermentation of *A. cerana* gut microbiota in the presence of prebiotics

Gut homogenate. Thirty-six naturally foraging adult worker bees of *A. cerana,* aged 18 days, were collected in summer (July) from a single colony, with robust population and without identified diseases, maintained in the apiary of the Yunnan Agricultural University in Kunming, China (longitude 102°45′30.5″ E, latitude 25°8′5.8″ N). Collected bees were placed in 50 ml sterilized centrifuge tubes and then pumped in CO_2 until the honeybees were unconscious. The whole bees were washed in 75% (v/v) ethanol. The guts were dissected aseptically using forceps and 1 g of gut samples were diluted with 10 ml of phosphate buffer saline (PBS), followed by cutting into pieces in an anaerobic workstation. The collection of gut samples were processed within 15 min. The experiment was performed in triplicates.

Gut fermentations. FOS (catalog No. S11133), INU (catalog No. S11143), IMO (catalog No. S11134), and XOS (catalog No. S11137) (Shanghai Yuanye Biological Technology Co., Ltd, Shanghai, China) were chosen, and glucose (GLU) was used as a nonselective control. *In vitro*, static batch culture fermentations were performed with five FOS, INU, IMO, XOS, and GLU treatments in three replicates using the serum bottle, as described by Rycroft et al. (2001) the simple modification. Briefly, the basal medium (peptone 2 g/l, yeast

extract 2 g/l, NaCl 0.1 g/l, K_2HPO_4 0.04 g/l, KH_2PO_4 0.04 g/l, $MgSO_4 \cdot 7H_2O$ 0.01 g/l, $CaCl_2 \cdot 6H_2O$ 0.01 g/l, $NaHCO_3$ 2 g/l, L-cysteine 0.5 g/l, bile salt 0.5 g/l, Tween80 2 ml/l, vitamin K_1 10 µl, and hemin chloride 0.05 g/l) (100 ml) supplemented with 1% GLU, 1% FOS, 1% INU, 1% IMO, or 1%XOS was placed into the serum bottle and sterilized. According to the literature (Likotrafiti et al. 2014; Henrique-Bana et al. 2020) and our preliminary study, selected prebiotics were added to a final concentration of 1% (m/v). Afterward, the bottles were refluxed with O_2-free N_2 and covered. Each bottle filled with 100 ml sterilized medium was then inoculated 1 ml of the gut homogenate and maintained at 37°C in an anaerobic jar (AnaeroJar TM 2.5l, Oxoid Ltd., Basingstoke, UK) including a gas-generating package (AnaeroPack, Mitsubishi Gas Chemical Co., Tokyo, Japan). Samples were collected after 0, 6, 12, and 24 h of fermentation to analyze viable microbial cells and pH. In the preliminary study, no total cultivable bacteria, *Bifidobacterium*, LAB, *Enterococcus,* and total fungi were detected in the basal medium in *in vitro* culture of gut microbiota of *A. cerana.* The explanation may be that the basal medium without supplemented carbohydrates could not support the *in vitro* growth of honeybees gut microbe (Long et al. 2015). Therefore, in the present study, the effect of the basal medium in the absence of prebiotics or glucose was not investigated.

Enumeration of Bifidobacterium and LAB. The number of *Bifidobacterium* and LAB was determined using the plate count method described by Abdel-Moneim et al. (2020). Briefly, 1 ml fermentation broth was 10-fold serially diluted in PBS. The viable count was enumerated by plating onto MRS agar (Catalog No.: HB0384-5, Qingdao Haibo Biotechnology Co., Ltd, Qingdao, China) or *Bifidobacterium* selective medium agar containing 10 g/l of peptone, 5 g/l of liver extract, 3 g/l of beef extract, 5 g/l of yeast extract, 8 g/l of peptone from casein, 0.5 g/l dissolved starch, 1 g/l of NaCl, 1 g/l of K_2HPO_4, 1 g/l of KH_2PO4, 10 g/l of GLU, 0.01 g/l of $FeSO_4 \cdot 7H_2O$, 0.005 g/l of $MnSO_4$, 0.5 g/l of L-cysteine, 1 ml of Tween80, and 20.0 g/l of agar (Catalog No.: HB0394, Qingdao Haibo Biotechnology Co., Ltd, Qingdao, China) within 15 min and incubated at 37°C in an anaerobic jar (AnaeroJar TM 2.5L, Oxoid Ltd., Basingstoke, UK) including an anaeroPack (Mitsubishi Gas Chemical Co., Tokyo, Japan) for 48 h. The colonies grown on *Bifidobacterium* selective medium for further microscopic examination were identified. Enumeration was repeated three times, and the number of microbial colonies (CFU) was recorded.

Measurement of pH. After fermentation at 0, 6, 12, and 24 h, 10 ml of fermentation broth from each culture bottle was taken and transferred to 50 ml sterile centrifuge tube. The pH meter (Shanghai INESA Instrument Co., Ltd, Shanghai, China) was used to measure pH.

In vitro fermentation of *A. cerana* gut microbiota in the presence of prebiotic IMO combined with *L. helveticus* KM7 or *L. reuteri* LP4

Gut fermentations. Gut samples and basal medium with 1% (g/v) IMO were prepared as described above. One milliliter of gut homogenate and 1 ml of freshly cultured *L. helveticus* KM7 and *L. reuteri* LP4 (10^5, 10^6, or 10^7 CFU/ml) was seeded into 100 ml of basal medium supplemented with IMO and maintained at 37°C in an anaerobic jar (AnaeroJar TM 2.5L, Oxoid Ltd., Basingstoke, UK) including an anaeroPack (Mitsubishi Gas Chemical Co., Tokyo, Japan). IMO without the presence of *Lactobacillus* strains was served as control. Samples were collected after 0, 6, 12, and 24 h of fermentation to measure total bacteria, bacteria, *Bifidobacterium*, fungi, and *Enterococcus*, respectively.

Enumeration of bacteria and fungi and analysis of pH. After fermentation of 0, 6, 12, and 24 h, 1 ml of fermentation broth was collected and mixed with 9 ml of PBS. Enumeration of *A. cerana* gut microbiota was performed using plate count methods. Serial dilutions were performed as described above and placed onto different selective media to determine the microbial amounts. Agars of MRS and *Bifidobacterium* selective medium were used as described above to count LAB and *Bifidobacterium*, respectively. The diluted broth was placed onto Luria Bertani nutrient agar (Catalog No.: HB0129, Qingdao Haibo Biotechnology Co., Ltd, Qingdao, China) and incubated at 30°C for 48 h to count total aerobic bacteria (Abdel-Moneim et al. 2020). The dilution was placed onto *Enterococcus* selective agar (Bile Aesculin Azide Agar, catalog No.: HB0133, Qingdao Haibo Biotechnology Co., Ltd, Qingdao, China) and incubated at 37°C for 48 h to count *Enterococcus* sp., which can be recognized by producing colonies with a black halo around (hydrolysis of esculin) (Śliżewska et al. 2019). To enumerate total fungi, the dilutions were placed onto potato dextrose agar (Catalog No.: HB0233, Qingdao Haibo Biotechnology Co., Ltd, Qingdao, China) with 0.01% (w/v) chloramphenicol and incubated at 28°C for 72 h to count total fungi number with the addition of microscopic examination, based on China National Standard Microbiological Examination of Foods (GB4789.15-2016). For pH measurement, fermentation broth samples were collected and subjected to analysis as described above.

Statistical analysis. Experimental data analysis was performed using SPSS18.0. One-way ANOVA was used to compare the differences between the data of multiple different treatment groups. The independent student *t*-test was performed to compare the data differences between the two treatment groups. $p < 0.05$ Indicated a significant difference. The results were expressed as mean ± standard deviation (SD).

Results

In vitro fermentation of *A. cerana* gut microbiota in the presence of prebiotics

Bididobacterium and LAB enumeration. As shown in Table I, *Bifidobacterium* multiplied over time in each group. Compared to the GLU group, the *Bifidobacterium* number in the IMO group significantly rose from fermentation for 6 h until 24 h ($p < 0.05$). At the fermentation of 12 h, the number of *Bifidobacterium* in INU, IMO, and XOS group was significantly higher than that in the GLU group ($p < 0.05$), and in the IMO group the number was significantly higher than in INU and XOS groups ($p < 0.05$). At 24 h, *Bifidobacterium* amount in the IMO group was significantly more than those in GLU, INU, FOS, and XOS groups ($p < 0.05$). *Bifidobacterium* multiplication benefited from four kinds of prebiotics: IMO, INU, FOS and XOS. IMO provided the best promotion effect in the intestinal of *A. cerana*.

The number of LAB in each group of the fermentation broth supplemented with oligosaccharides or glucose increased in a time-dependent manner (Table I). Compared to the GLU group, IMO showed a significant increasing effect on LAB growth at all time points. The LAB number in both INU and XOS groups was higher than those in the GLU group after 12 and 24 h of fermentation. There was no significant difference in the LAB number between IMO and INU groups ($p > 0.05$). *In vitro* fermentation of IMO and INU can significantly promote the LAB multiplication in the gut of *A. cerana*.

pH value. The pH value in all groups was significantly reduced, and the extent of pH reduction varied with prebiotics type (Table II). The pH value in each group did not differ after 6 h of fermentation ($p > 0.05$). After 12 h of fermentation, compared to the GLU group, the pH value in INU, IMO and XOS groups was

Table I
Number of *Bifidobacterium* and LAB in the prebiotic fermentation broth of *A. cerana* gut microbiota.

Number of gut microbiota / Log_{10} (CFU/ml)	Time (h)	Treatment				
		GLU	FOS	INU	IMO	XOS
Bifidobacterium	0	5.85 ± 0.06^{Ca}	5.82 ± 0.05^{Ca}	5.83 ± 0.02^{Ca}	5.82 ± 0.01^{Ca}	5.80 ± 0.02^{Ca}
	6	5.63 ± 0.03^{Db}	5.66 ± 0.16^{Cab}	5.83 ± 0.13^{Cab}	5.94 ± 0.19^{Ca}	5.81 ± 0.15^{Cab}
	12	6.99 ± 0.08^{Bc}	6.46 ± 0.25^{Bd}	7.79 ± 0.08^{Bb}	8.31 ± 0.36^{Ba}	7.43 ± 0.24^{Bb}
	24	8.15 ± 0.05^{Ad}	8.41 ± 0.08^{Ac}	8.85 ± 0.02^{Ab}	9.10 ± 0.12^{Aa}	8.52 ± 0.18^{Ac}
LAB	0	5.01 ± 0.02^{Da}	4.99 ± 0.03^{Ba}	5.00 ± 0.02^{Da}	5.01 ± 0.05^{Ca}	4.98 ± 0.02^{Ca}
	6	6.03 ± 0.03^{Cb}	5.06 ± 0.16^{Bc}	6.10 ± 0.09^{Cab}	6.34 ± 0.19^{Ba}	5.21 ± 0.15^{Cc}
	12	7.29 ± 0.08^{Bc}	5.76 ± 0.25^{Ad}	8.09 ± 0.08^{Bab}	8.28 ± 0.33^{Aa}	7.73 ± 0.24^{Bb}
	24	7.76 ± 0.14^{Ac}	5.67 ± 0.35^{Ad}	8.36 ± 0.11^{Aab}	8.61 ± 0.02^{Aa}	8.19 ± 0.16^{Ab}

GLU – glucose; FOS – fructo-oligosaccharides; INU – inulin; IMO – isomalto-oligosaccharides; XOS – xylo-oligosaccharides
A-D – different uppercase letters between treatments using the same prebiotic at the different fermentation timing denote significance ($p < 0.05$)
a-d – different lowercase letters between different treatments at the same time fermentation point denote significance ($p < 0.05$)

Table II
The pH value in the prebiotic fermentation broth of *A. cerana* gut microbiota.

Time (h)	pH value				
	GLU	FOS	INU	IMO	XOS
0	6.98 ± 0.03^{Aa}	7.00 ± 0.04^{Aa}	6.98 ± 0.06^{Aa}	7.01 ± 0.02^{Aa}	6.99 ± 0.03^{Aa}
6	6.89 ± 0.04^{Ba}	6.91 ± 0.05^{Ba}	6.89 ± 0.07^{Aa}	6.89 ± 0.03^{Ba}	6.90 ± 0.06^{Ba}
12	6.13 ± 0.07^{Cb}	6.31 ± 0.03^{Ca}	5.71 ± 0.02^{Bd}	5.57 ± 0.07^{Ce}	5.97 ± 0.05^{Cc}
24	5.78 ± 0.07^{Db}	6.22 ± 0.04^{Da}	5.59 ± 0.04^{Cc}	5.48 ± 0.03^{Dd}	5.65 ± 0.03^{Dc}

GLU – glucose; FOS – fructo-oligosaccharides; INU – inulin; IMO – isomalto-oligosaccharides; XOS – xylo-oligosaccharides
A-D – different uppercase letters between treatments using the same prebiotic at the different fermentation timing denote significance ($p < 0.05$)
a-d – different lowercase letters between different treatments at the same fermentation point time denote significance ($p < 0.05$)

reduced significantly ($p < 0.05$), while the value of pH in the FOS group was significantly higher ($p < 0.05$). After 24 h of fermentation, compared to the GLU group, the pH value in INU, IMO and FOS groups was reduced significantly ($p < 0.05$), while the value of pH in the FOS group significantly increased ($p < 0.05$). Among all treatment groups, the lowest pH value was found in the IMO group after both 12 and 24 h of fermentation. These data indicate that *in vitro* fermentation of IMO could decrease pH value of intestinal microbial in adult worker *A. cerana*.

In vitro fermentation of *A. cerana* gut microbiota in the presence of prebiotic IMO combined with *L. helveticus* KM7 or *L. reuteri* LP4

Bacteria enumeration. During 24 h *in vitro* fermentation, the combination of probiotic and IMO significantly increased the total bacteria number in the fermentation broth (Fig. 1A). Compared to IMO treatment, after 6, 12, and 24 h of fermentation, the number of *L. helveticus* KM7 adding groups and *L. helveticus* KM7 combined with IMO groups increased significantly ($p < 0.05$). Similarly, relative to the IMO group, significantly higher bacteria counts in the *L. reuteri* LP4 combined with IMO were observed after 6, 12, and 24 h of fermentation ($p < 0.05$). Inducing the IMO effect on bacteria combined with *L. helveticus* KM7 or *L. reuteri* LP4 was similar. These data indicated that the combination of IMO with *L. helveticus* KM7 or *L. reuteri* LP4 improved bacteria *in vitro* fermentation of intestinal microflora of adult worker honeybee of *A. cerana*.

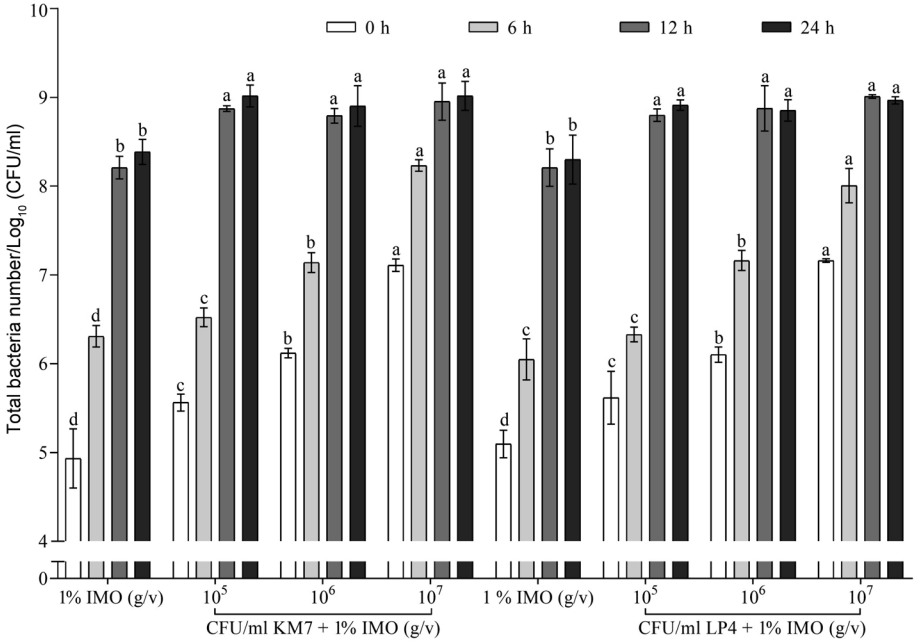
After 6 or 24 h of fermentation, the use of *L. helveticus* KM7 and IMO caused a significant increase in *Bifidobacterium* number in the fermentation broth relative to that of IMO alone ($p < 0.05$) (Fig. 1B). However, no significant difference was seen between IMO supplemented with *L. helveticus* KM7 inoculated at concentrations of 10^5–10^7 CFU/ml ($p > 0.05$). A similar effect was found in IMO supplemented with *L. reuteri* LP4. These results indicated that LAB and IMO synergistically facilitated *Bifidobacterium* proliferation in the fermentation broth of intestinal microflora of *A. cerana*.

The combination of IMO and *L. helveticus* KM7 significantly induced LAB growth *in vitro* in the fermentation broth of intestinal microflora of *A. cerana* ($p < 0.05$) (Fig. 1C). This inductive impact depended on the inoculation concentration of KM7 strain. After 24 h of fermentation, concurrent addition of 10^7 CFU/ml of KM7 and IMO significantly increased LAB number in fermentation broth from that in IMO only ($p < 0.05$). Consistent with findings, the LAB number was also significantly higher in the fermentation broth supplemented with IMO compared to that in IMO only ($p < 0.05$). The more significant inductive effect on LAB growth was observed with IMO combined with KM7 strain. These results revealed that *L. helveticus* KM7 and *L. reuteri* LP4 improved the LAB number in the fermentation broth of intestinal microbiota of *A. cerana* supplemented with IMO, and this effect was specific for LAB species.

Compared to the IMO group, after 6 and 12 h, IMO supplemented with *L. helveticus* KM7 did not significantly alter the number of *Enterococcus* while significantly reducing *Enterococcus* growth only after 24 h of *in vitro* fermentation ($p < 0.05$) (Fig. 1D). The inoculation concentration of *L. helveticus* KM7 did not reduce the *Enterococcus* number. Combination of IMO and *L. reuteri* LP4 of 107 CFU/ml inoculation concentration decreased the *Enterococcus* proliferation after 24 h of fermentation ($p < 0.05$), suggesting that only administration of an adequate number of *L. reuteri* LP4 combined with IMO could modulate *Enterococcus* growth. Concurrent addition of IMO and *L. helveticus* KM7 or *L. reuteri* LP4 significantly decreased number of *Enterococcus* in the *in vitro* fermentation broth of intestinal microbiota of *A. cerana,* respectively, and *L. helveticus* KM7 showed more remarkable ability than *L. reuteri* LP4.

Fungi enumeration. After 6, 12, and 24 h of fermentation, more numerous fungi in the *in vitro* fermentation groups of *L. helveticus* KM7 combined with IMO were detected than the IMO group ($p < 0.05$) (Fig. 2). No significant differences among *L. helveticus* KM7 groups added at different doses were seen ($p > 0.05$). Interestingly, a combination of IMO with *L. reuteri* LP4 did not significantly change fungi growth at all indicated timing of *in vitro* fermentation ($p > 0.05$), suggesting that reducing the impact on the fungi growth of LAB when concurrently was dependent on LAB species. The strain of *L. helveticus* KM7 rather than *L. reuteri* LP4 combined with IMO could reduce the number of fungi *in vitro* fermentation broth of intestinal microbiota of adult worker bees of *A. cerana*.

pH value. Starting with 6 h during *in vitro* fermentation, a significant reduction in pH was found in groups of IMO with either *L. helveticus* KM7 or *L. reuteri* LP4 ($p < 0.05$) (Fig. 3). After 6 h, *L. helveticus* KM7 at medium (10^6 CFU/ml) and high (10^7 CFU/ml) concentrations, pH value in the fermentation broth of *L. helveticus* KM7 and IMO was lower than *L. reuteri* LP4 and IMO group significantly ($p < 0.05$). After 12 and 24 h, the pH value of groups with IMO and *L. helveticus* KM7 at the same concentration was significantly lower than that of those with IMO and *L. reuteti* LP4 ($p < 0.05$). Strain *L. helveticus* KM7 had a stronger impact than *L. reuteri* LP4. Decreasing pH values were dependent on the inoculation concentration. The combination of IMO with either *L. helveticus* KM7 or *L. reuteri* LP4 could lower the pH of *in vitro* fermentation broth of intestinal microbiota of *A. cerana*.


0 h
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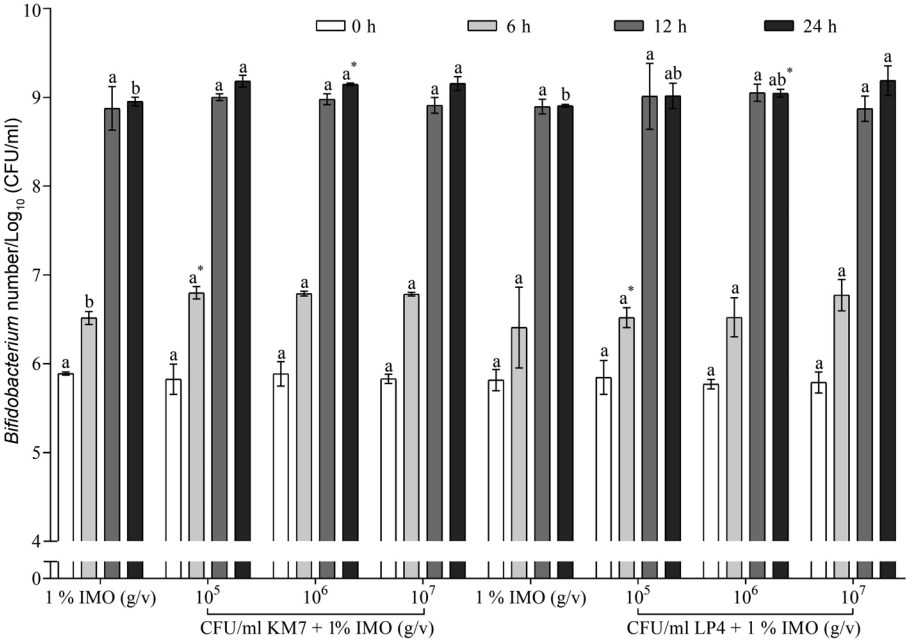
Total bacteria number/Log10 (CFU/ml)
1% IMO (g/v)
10^5
10^6
10^7
1 % IMO (g/v)
CFU/ml KM7 + 1% IMO (g/v)
CFU/ml LP4 + 1% IMO (g/v)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10^6
10^7
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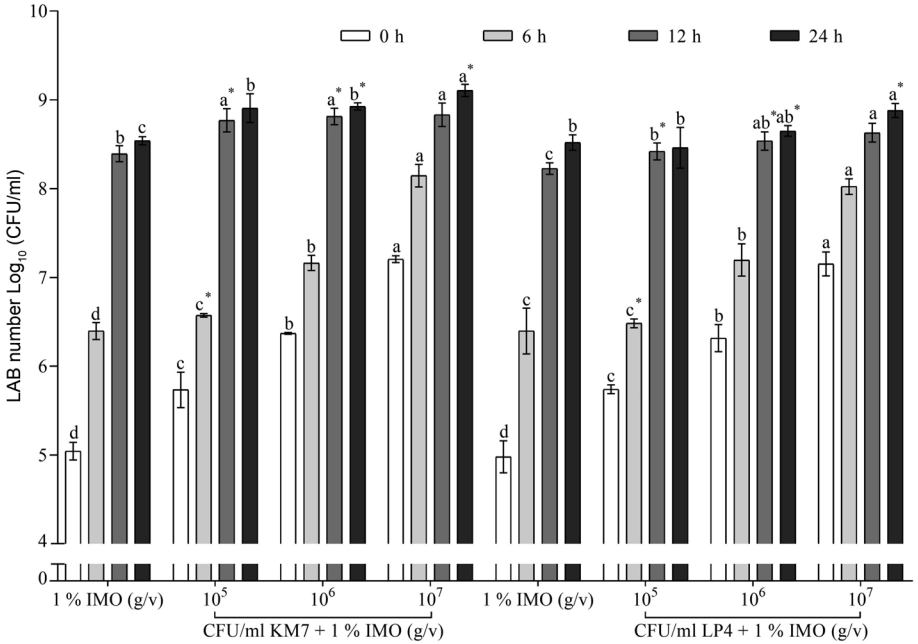
CFU/ml LP4 + 1 % IMO (g/v)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LAB number Log10 (CFU/ml)
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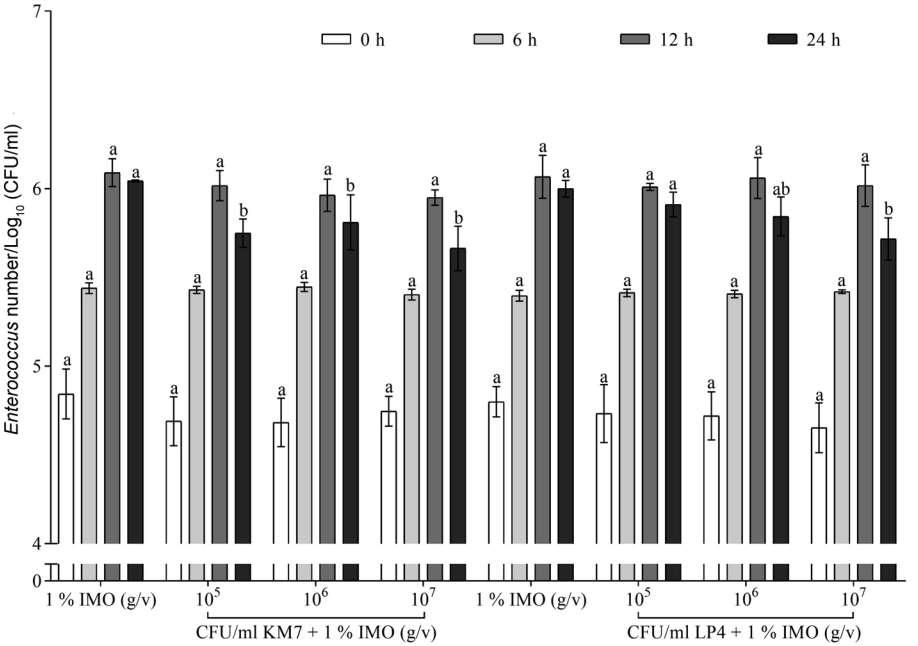
10^6
10^7
CFU/ml KM7 + 1 % IMO (g/v)
CFU/ml LP4 + 1 % IMO (g/v)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24 h
Enterococcus number/Log10 (CFU/ml)
1 % IMO (g/v)
10^5
10^6
10^7
CFU/ml KM7 + 1 % IMO (g/v)
CFU/ml LP4 + 1 % IMO (g/v)

Fig. 1. Effect of IMO in combination with *L. helveticus* KM7 or *L. reuteri* LP4 on bacteria during the *in vitro* fermentation of *A. cerana* gut microbiota; A) the number of total bacteria, B) *Bifidobacterium*, C) LAB and D) *Enterococcus* were determined. IMO is the abbreviation of isomalto-oligosaccharides. A graphic was created with different treatment grouping on the x-axis and microbes counts on the y-axis.

* – significance between treatments using the different probiotic strains added at the same concentration for the same fermentation period ($p < 0.05$)

a-d – different lowercase letters between different treatments at the same time fermentation point denote significance ($p < 0.05$); results were expressed as mean ± SD (n = 3)

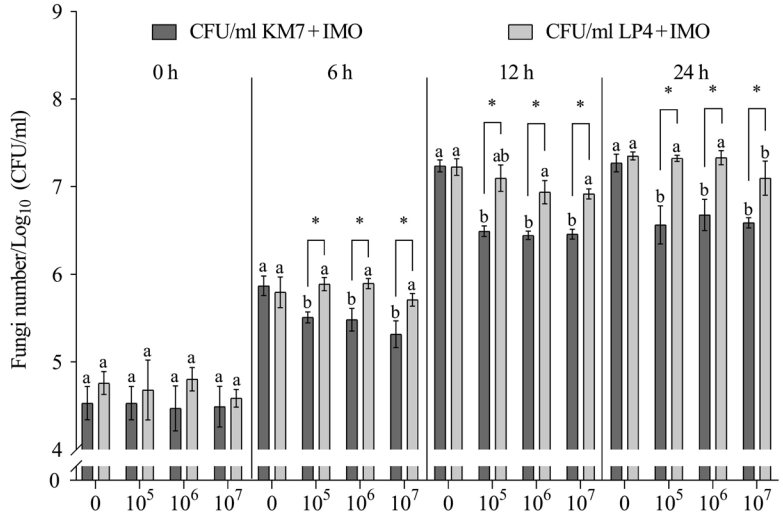

Fig. 2. Effect of IMO in combination with *L. helveticus* KM7 and *L. reuteri* LP4 on fungi during *in vitro* fermentation of *A. cerana* gut microbiota. IMO is the abbreviation of isomalto-oligosaccharides.

* – significance between treatments using the different probiotic strains added at the same concentration for the same fermentation period ($p < 0.05$)

a-d – different lowercase letters between different treatments at the same time fermentation point denote significance ($p < 0.05$); results were expressed as mean ± SD (n = 3)

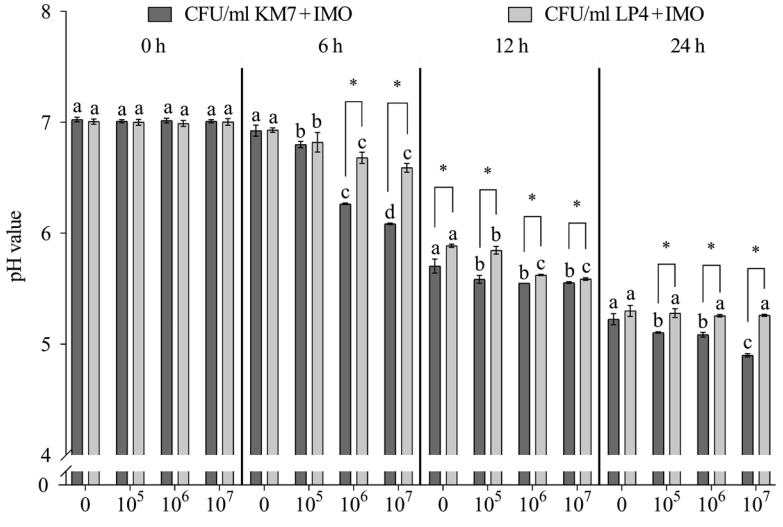

Fig. 3. Effect of IMO in combination with *L. helveticus* KM7 and *L. reuteri* LP4 on pH during *in vitro* fermentation of *A. cerana* gut microbiota. IMO is the abbreviation of isomalto-oligosaccharides.

* – significance between treatments using the different probiotic strains added at the same concentration for the same fermentation period ($p < 0.05$)

a-d – Different lowercase letters between different treatments at the same fermentation time point denote significance ($p < 0.05$); results were expressed as mean ± SD (n = 3)

Discussion

In this study, the experiment design evaluated *in vitro* effects of prebiotics alone, and in combination with *Lactobacillus* on microbial composition and acid production of *A. cerana* microflora. Our results have shown that selected prebiotics including FOS, INU, IMO, and XOS stimulate the growth of LAB and *Bifidobacterium* compared to GLU, and this effect varied with the type of prebiotics. Lowered pH was also found with these selected prebiotics, reflecting bacterial metabolic activity induced with them. LAB and *Bifidobacterium* are commensals in *A. cerana* gut, providing beneficial effects and effects on growth of these strains could be used to evaluate prebiotics (Watson et al. 2013). LAB are recognized to be lactic acid producers in honeybees' gut (Ellegaard et al. 2015) and increased numbers of these bacteria contribute to enhanced lactic acid levels with prebiotics treatment. Several *in vitro* and *in vivo* studies using these prebiotics have reported similar findings to our results. For instance, Fehlbaum et al. (2018) observed that FOS, inulin, GOS, and XOS caused the *Bifidobacerium* growth and XOS and GOS, but not FOS and inulin, resulted in the *Lactobacillus* growth after 24 h fermentation broth inoculated with adult fecal microbiota. An animal study reported that

the intragastrical administration of FOS, GOS, or IMO to BALB/c mice for 17 days increased the fecal levels of *Bifidobacterium* and *Lactobacillus*, and beneficial effect on these bacteria was dependent on the type and the used dosages (Wang et al. 2017). In our study, prebiotic IMO showed greater inductive effects on LAB and *Bifidobacterium* and metabolism compared to other prebiotics. IMO has been a partly digestible carbohydrate and a well-established functional food in Asia for decades (Goffin et al. 2011). The ability of IMO to benefit intestinal microbiota has been reported in rats, pigs, and humans, and varied prebiotic potential compared to other types of prebiotics has been observed in several studies (Koleva et al. 2014; Okazaki and Katayama 2019; Logtenberg et al. 2021). For example, in a recent study comparing IMO and GOS *in vitro* fermentation using infant fecal inoculum of 2- and 8-week-old infants IMO showed more inductive effects on *Bifidobacterium* and acetate and lactate production than GOS (Logtenberg et al. 2021). By contrast, the results in rats fed a high-fat diet observed by Okazaki and Katayama (2019) suggested that both FOS increased fecal *Bifidobacterium* and *Lactobacillus* and IMO had no impact on these beneficial bacteria. Overall, this evidence suggests the impact of different prebiotics on intestinal microbiota composition may depend on prebiotics type, host species, and physiological conditions, which may contribute to IMO's most favorable prebiotic properties on *A. cerana* in this study.

Increasing evidence in both *in vitro* and *in vivo* studies indicates that synbiotics, a combination of probiotics and prebiotics, have beneficial effects on modulating host microbiota composition and metabolism (Pandey et al. 2015). In our previous study, *L. helveticus* KM7 or *L. reuteri* LP4 derived from *A. cerana* gut have shown the potential probiotic properties and were chosen to evaluate the symbiotic effect combined with IMO. The data revealed that IMO combined with *L. helveticus* KM7 or *L. reuteri* LP4 exhibited symbiotic potential through improving proliferation of *Bifidobacterium*, LAB, and total bacteria and decreasing growth of total fungi and *Enterococcus* in *A. cerana* gut microbiota compared to IMO alone. In addition, lowered pH was observed in the combination of IMO with *L. helveticus* KM7 or *L. reuteri* LP4 relative to IMO alone, confirming the bacteria fermentation in *Apis cerana* gut microbiota. However, the increase in total LAB *in vitro* fermentation of *A. cerana* gut microbiota with IMO in the presence of lactobacilli might be due to the exogenously applied *Lactobacillus,* and this need to be elucidated in further study using molecular techniques in order to specify the inoculated or endogenous LAB. Bacteria present in the honeybee gut play a beneficial role in maintaining host health through helping utilize and absorb nutrients, protecting from xenobiotics, and acidifying the gut environment to reduce pathogens colonization (Raymann et al. 2018; Pachla et al. 2021). However, yeasts have been recognized as dominant fungi in the gut of the *Apis* genus, but the increased abundance of yeast has been related to diseases and malnutrition or response to antibiotics and insecticides (Gilliam and Prest 1972; Ptaszyńska et al. 2016). The infection with intestinal fungal pathogens such as *Nosema apis* and *N. ceranae* has been recognized to cause shortened lifespan and reduction in colony size of honeybees (Houdelet et al. 2021). Our results suggest that IMO combined with selected *Lactobacillus* strains maintain gut microbiota homeostasis of *A. cerana*. Stimulation of *Bifidobacterium* growth in *A. cerana* gut microbiota supplemented with IMO and *Lactobacillus* strains agreed with the report of Gmeiner et al. (2000), supported by the evidence of the symbiosis between certain *Bifidobacterium* and lactobacilli strains (Driessen and Deboer 1989; Molly et al. 1996). Consistent with these findings, our study indicates IMO's *in vitro* symbiotic effect with lactobacilli in honeybees of *A. cerana*. Further research is needed to confirm the symbiotic impact of IMO using honeybees.

Conclusion

In the current study, *in vitro* effect of prebiotics alone and combined with *Lactobacillus* on the microbial populations *in vitro* fermentation of *A. cerana* gut microbiota. IMO was the most favorable prebiotic to increase *Bifidobacterium* and LAB growth and microbial metabolic activity. Furthermore, IMO in combination with *L. helveticus* KM7 or *L. reuteri* LP4 led to a tremendous increase in *Bifidobacterium* and LAB and a more significant decrease in *Enterococcus*, fungi, and pH compared to IMO alone. These results highlight the potential of IMO as the prebiotic and symbiotic with lactobacilli for honeybee production. However, *in vitro* study cannot provide all the information needed to understand the effect of IMO and its mechanism fully, and further research supported by *in vivo* studies is warranted before ascribing prebiotic and synbiotic properties to IMO in the honey bees.

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Ethical statement

All procedures performed in the present study were accepted by the Local Animal Care and Ethics Committee for Animal Experiment in Yunnan Agricultural University (Kunming, China); permission no. YNAU2019uwyh013.

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Author contributions

Qiuye Lin and Zhenhui Cao conceived and designed the experiments; Mingkui Lv, Qingzhi Lei and Huanjuan Yin performed the experiments; Mingkui Lv, Qingzhi Lei, Tiannian Hu and Sifan Wang analyzed the data; Mingkui Lv wrote the paper; Kun Dong, Hongbin Pan and Yiqiu Liu gave important suggestions; Qiuye Lin and Zhenhui Cao revised the manuscript.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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