

Investigation on the changes of *Bifidobacterium* and lactic acid bacteria in soybean meal fermented feed

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Abstract

The main objective of this research was to explore the dynamic changes of *Bifidobacterium* and lactic acid bacteria (LAB) in the process of feed fermentation under anaerobic condition, so as to increase the number of fermented bacteria of *Bifidobacterium* from the aspect of strain combination. The results showed that when *Bifidobacterium lactis* (*B. lactis*, i.e. *Bifidobacterium animalis subsp. lactis*) fermented with *Bacillus coagulans* or *Lactobacillus paracasei*, the maximum number of *B. lactis* in those samples was 9.42 times and 4.64 times of that of fermented sample with *B. lactis* only. The soybean meal was fermented by *B. lactis*, *L. paracasei* and *B. coagulans*, and the number of *B. lactis* reached the maximum after fermented 10 days, which was 6.13 times of that in unfermented sample. The reducing sugar content and highest activity of α -galactosidase were higher than the control. These results suggest that *B. coagulans* and *L. paracasei* can promote the growth of *B. lactis*. It is inferred that *B. coagulans* can metabolize normally in aerobic,

micro-aerobic and anaerobic environments, consume oxygen, produce digestive enzymes, and cooperate with *L. paracasei* to produce metabolic products benefit for the growth of *B. lactis*.

Keywords: qPCR, Microflora changes, Soybean meal, *Bifidobacterium*, lactic acid bacteria

1 Introduction

Fermented feed is rich in small peptides , organic acids and other functional factors. The macromolecule and anti-nutritional factors in raw material are degraded through microbial fermentation, and the quality of fermented feed is directly affected by the evolution of fermentation probiotics. Mixed culture fermentation has become the main fermentation method for the preparation of biological feed. Compare with monoxenic fermentation, mixed culture fermentation can better reflect the synergism and complementarity among microorganisms. Ding et al.(Ding et al., 2020) optimized the fermentation conditions of tea residue, in which fermentation strains were *Bacillus subtilis*, *Aspergillus niger* and *Saccharomyces cerevisiae*, and the crude protein, reducing sugar, and cellulose activity of the fermentation products were significantly improved compared with those before fermentation. Previous studies have indicated that mixed culture fermentation can improve the nutritional quality of feed by reducing antinutritional factors in soybean meal, corn and other raw materials and by increasing nutrient bioavailability(Li et al., 2019; Shi, Zhang, Lu, & Wang, 2017). Mixed culture fermentation system is very complicated with more relationships

among fermented probiotics. Chen et al.(Chen, Shih, Chiou, & Yu, 2010) revealed that *Aspergillus oryzae* and *Lactobacillus casei* could degrade the antigenic protein in soybean meal. Nowadays, there are many studies on technology optimization of fermented feed with mixed strains, but there are few reports on the interaction mechanism between fermentation strains. There are abundant metabolites in the several strains coculture environment, and the interaction and metabolic pathways of different strains are different from those of single culture. Therefore, screening the strains with synergistic relationship will be the core content of fermented feed study.

Aerobic fermentation, which is easy to be polluted by pathogenic bacteria in the environment, is characterized with more equipment invest and obvious material loss. Anaerobic fermentation process avoids the waste of raw materials and the possibility of contamination during fermentation. Moreover, anaerobic fermentation process doesn't need oxygen, so it consumes less energy than aerobic fermentation. Lactic acid bacteria (LAB) are the main fermentation microorganism in anaerobic fermentation process. The growth characteristics and fermentation effect of LAB are quite different with each other. *L. fermentum* and *L. plantarum* are dominating fermentation microorganism(Camu et al., 2007). *L. paracasei* and *L. rhamnosus* have strong ability of acid producing performance and good health function(Demers-Mathieu, St-Gelais, Audy, Laurin, & Fliss, 2016). *B. coagulans* produces both lactic acid and spores, which is favorable to long term storage of feed(Zhou et al., 2020). Compared with *Bacillus subtilis*, *B. coagulans* can metabolize

normally in both micro-aerobic and anaerobic environments, consume oxygen and produce lactic acid. Compared with LAB, *B. coagulans* has more abundant enzyme included protease, amylase and xylanase. *Bifidobacterium* is anerobic and can produce a variety of digestive enzymes and produce B vitamins such as folic acid, directly providing nutrients to the animal(Bujna, Farkas, Tran, Dam, & Nguyen, 2018). Meanwhile, *Bifidobacterium* can make good use of stachyose in soybean meal to degrade anti-nutritional factors in raw materials(Grmanová, Rada, Sirotek, & Vlková, 2010), which has been applied in broiler breeding(Li, Lu, Wu, & Lien, 2014). Research suggested that *Bifidobacterium* tend to exhibit weak growth even in milk, and always need long fermentation time and strict condition of anaerobiosis(Abdulmir, Yoke, Nordin, & Abu, 2010). It may be that *Bifidobacterium* are more likely to be affected by factors such as oxygen, pH, storage temperature than other bacteria(Kurmann & Rasic, 1991). *Bifidobacterium* are mostly used as microbial agents added to the diet directly, but studies on *Bifidobacterium* as fermented probiotics for feed are rarely reported.

In this study, soybean meal was used as the main fermentation raw material, and *B. lactis* was mixed with different LAB for fermentation. The microflora changes of different fermentation stages were measured by quantitative PCR (qPCR), and the quality of fermented feed products was detected.

2 Materials and methods

2.1 Strains and culture methods

Bacillus subtilis CGMCC 1.1086, *Saccharomyces cerevisiae* CGMCC 2.1527 and *Lactobacillus rhamnosus* CGMCC 1.577 used in this study were obtained from China General Microbiological Culture Collection Center (CGMCC). *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Lactobacillus paracasei*, *Bacillus coagulans* and *Bifidobacterium animalis subsp. lactis* were preserved strains in our laboratory.

B. subtilis was grown in LB medium at 30 °C with a shaking of 180 rpm. *S. cerevisiae* was grown in YPD medium at 28 °C with a shaking of 200 rpm. *Bifidobacterium* and LAB were grown anaerobically in MRS medium at 37°C. When the optical density at 600 nm for the growing cultures was between 0.8 and 1.0, the strains were used in solid-state fermentation.

2.2 pH and total acid

Soybean meal 45 g, soybean residue 45 g, and inferior wheatmeal 10 g were used as fermentation medium, and water content was adjusted to 55% after inoculation with a 2% ratio of experimental strains. The mixture was then packaged in polythene fermented bags and sealed by heat. All the bags were incubated at 37°C under anaerobic condition. Samples of different fermentation days were taken to test the total acid content and pH value. Total acid content (g of lactic acid*g⁻¹) of feed was estimated according to the method Jemaa et al(Ben Jemaa et al., 2017).

2.3 Solid-state fermentation process

Soybean meal 45 g, soybean residue 45 g, inferior wheatmeal 10 g were used as fermentation medium. Inoculum amount of *B. subtilis* and *S. cerevisiae* was totally

2%, inoculum rate of *L. fermentum*, *L. plantarum*, *L. paracasei*, *L. rhamnosus* and *B. coagulans* was 2% individually, and water content was adjusted to 55%. The mixture was then packaged in polythene fermented bags and sealed by heat. All the bags were incubated at 37°C under anaerobic condition. Feed samples of different fermentation time were stored at -20°C for further analysis.

Under the above fermentation conditions, *B. lactis* was mixed with different LAB for fermentation. The concrete operation process was shown in Table 1.

Table 1 Combination of fermentative strains

Sample	Fermented strains
A	<i>B. subtilis</i> , <i>S. cerevisiae</i> , <i>B. lactis</i> , <i>L. fermentum</i>
B	<i>B. subtilis</i> , <i>S. cerevisiae</i> , <i>B. lactis</i> , <i>L. plantarum</i>
C	<i>B. subtilis</i> , <i>S. cerevisiae</i> , <i>B. lactis</i> , <i>L. paracasei</i>
D	<i>B. subtilis</i> , <i>S. cerevisiae</i> , <i>B. lactis</i> , <i>L. rhamnosus</i>
E	<i>B. subtilis</i> , <i>S. cerevisiae</i> , <i>B. lactis</i> , <i>B. coagulans</i>
F	<i>B. subtilis</i> , <i>S. cerevisiae</i> , <i>B. lactis</i> , <i>L. paracasei</i> , <i>B. coagulans</i>

2.4 Primer sets

The BLAST search tool was used to check the specificity of each primer set(Hidaka, Horie, Akao, & Tsuno, 2010; Schwendimann, Kauf, Fieseler, Gantenbein-Demarchi, & Miescher Schwenninger, 2015; Sheu et al., 2010). The primer sequences and amplicon size were shown in Table 2. Primers were synthesized by GENERAL BIOL (China).

Table 2 Primer sequence

Species	Primer sequence (5'-3')	Primer length (bp)
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<i>L. fermentum</i>	F: GCACCTGATTGATTTTGGTCG R: GGTATTAGCATCTGTTTCCAAATG	103 bp
<i>L. plantarum</i>	F: GGAGCCGCTATTAGTATTTTCAT R: AATACAAGCAAGTCTTGGACCAG	107 bp
<i>L. paracasei</i>	F: TTCACCAGATGGAAGGACTC R: TCAATGTTCGCTGCCTGT	274 bp
<i>L. rhamnosus</i>	F: GACGCAGCCGGTTGACCCAA R: GGCGGCAGTTGCCCCAGAAT	376 bp
<i>B. coagulans</i>	F: GCATGGAGGAAAAAGGAA R: CCCGGCAACAGAGTTTTA	262 bp
<i>B. lactis</i>	F: TCACGACAAGTGGGTTGCCA R: GTTGATCGGCAGCTTGCCG	178 bp

121 2.5 PCR reaction

122 The total volume of PCR reaction mixture was 20 μ L, with 10 μ L Taq Master
 123 Mix (Vazyme, China), 2 μ L DNA template, 1 μ L of each primer and 6 μ L ddH₂O.
 124 The PCR programs consisted of the following steps: Denaturation at 95°C for 3 min,
 125 followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and
 126 extension at 72°C for 1 min. After completion, extension at 72°C for 10 min. The
 127 amplified products were subjected to gel electrophoresis in 3% agarose gels and
 128 visualized by ethidium bromide staining.

129 2.6 Preparation of samples

130 1 g feed sample was put into a 50 mL centrifuge tube with 10 mL PBS buffer
 131 and then shaken for 20 min to disperse the microbial cells. After filtration with gauze,
 132 1 mL of the filtrate was centrifuged at 12000 r/min for 2 min, the precipitates were

washed with PBS buffer and stored at -20°C. The total DNA of each sample was extracted by FastPure Bacteria DNA Isolation Mini Kit (Vazyme, China).

2.7 Quantitative PCR assay

2.7.1 Construction of standard curve

The genomic DNA extracted from standard strains were performed PCR amplification with specific primers. The size of the PCR products was detected by gel electrophoresis in 3% agarose gels, and the bands were accurately cut to obtain the specific fragments of standard strains. The target fragments were purified by Gel Extraction Kit (Sangon Biotech, China). Ten-fold serial dilutions of the purified target fragments, in which concentration was determined, was detected by qPCR. Standard curves were obtained by plotting the Ct values against the target gene copy number. The standard curves of experimental strains were as follows: (1) *L. fermentum*: $y = -3.1891x + 35.108$. (2) *L. plantarum*: $y = -3.14x + 34.444$. (3) *L. paracasei*: $y = -3.6016x + 37.823$. (4) *L. rhamnosus*: $y = -3.4245x + 35.303$. (5) *B. coagulans*: $y = -3.4285x + 34.501$. (6) *B. lactis*: $y = -3.6457x + 36.8455$.

2.7.2 qPCR amplification conditions

qPCR reactions were performed in the QuantStudio 3 Real-Time PCR System (Applied Biosystems, USA). The total volume of qPCR amplification was 20 μ L, containing 10 μ L of TB Green Premix Ex Taq (Takara, Japan), 6 μ L of ddH₂O, 2 μ L of template DNA, 0.8 μ L of each primer and 0.4 μ L of ROX. Thermal cycling conditions consisted of 1 cycle at 95°C for 30 s, and 40 cycles at 95°C for 5 s followed

by 60°C for 30 s. A melting-curve analysis was performed at the end of each qPCR assay

2.8 Small peptide

Using Kjeldahl method, refer to GB/T 22492-2008 “Soy peptides power”. The measured small peptide content refers to the proportion of small peptide in the crude protein content of the measured sample. The small peptides consist of oligopeptides, a small number of polypeptides and free amino acids.

2.9 α -galactosidase activity

The standard α -galactosidase activity was assayed by the release of p-nitrophenol from pNPG. α -galactosidase activity (U/g) of feed was detected according to the method Huang et al (Huang et al., 2018). One unit of enzyme activity (U) was defined as the amount of enzyme releasing 1 μ mol of p-nitrophenol from 10 mmol/L of pNPG per minute at 37°C with pH 5.5.

2.10 Reducing sugar

3,5-Dinitrosalicylic acid (DNS) colorimetric determination of the reducing sugar content in fermented feed was adopted. The details of the analytical process referred to the method proposed by Zhao et al (Zhao, Li, Zhang, Zhang, & Kong, 2013).

2.11 Analysis of secondary structure of protein

FTIR absorption spectra from 4000 to 400 cm^{-1} were acquired by Nicolet iS50 (Nicolet, USA). The amide I band performed in the region of 1700-1600 cm^{-1} (Table 3) was analyzed by “PeakFit 4.12” procedure with second-derivative analysis and

Fourier self-deconvolution, and “Origin 8.5” procedure was applied to quantify individual component bands according to curve fit. The details of the analytical methods have been reported in previous work(Wang et al., 2014).

Table 3 The standard assignment of amide I band

Secondary structure	Amide I bands/cm ⁻¹
β-sheet	1640~1610
Random coil	1650~1640
α-helix	1658~1650
β-turn	1700~1660

2.12 Statistical analysis

All results are expressed as mean±standard deviation.

3. Results and discussion

3.1 Changes of acidity during feed fermentation

As shown in Fig.1(a), the total acid content in feed fermentation showed a gradual growth trend. From 0 to 10 days, the total acid content increased rapidly. As time went on, the total acid content tended to be stable. Among them, *L. rhamnosus* had the strongest acid-forming capacity, and the total acid content up to 5.34%.

From Fig.1(b), we can easily see that due to the blooming of LAB, and organic acid produced constantly, the pH value of fermentation products has decreased since the start of fermentation. Over time, the pH value of 6 group all decreased, but the reduced degree was different. The feed inoculated with *L. rhamnosus* had a stable pH ranged from 4.0 to 4.2 after fermentation for 6 days. The pH value of other groups was ranged from 4.3 to 4.5 after fermentation for 30 days, which was basically

consistent with the change of total acid in fermentation process.

3.2 Changes of *B. lactis* and LAB during feed fermentation

As shown in Fig.2(a), the biomass of *L. fermentum* and *L. plantarum* both reached their maximum on the third day, for 1.84×10^7 copies/g and 8.88×10^7 copies/g, respectively. Along with the accumulation of organic acid, pH in the feed declined, and the growth of the bacteria itself was inhibited, but the quantity of bacteria decreased slowly in the fermentation anaphase. The biomass of *L. paracasei* and *L. rhamnosus* reached their maximum on the first day, for 1.84×10^7 copies/g and 8.88×10^7 copies/g, respectively. *B. coagulans* also reached its maximum value (1.27×10^7 copies/g) on the first day, and was 3.74 times as much as those before fermentation. In the early time of fermentation, *B. coagulans* mostly existed in the form of vegetative cell, but the spores were gradually generated as the time went on, leading to the quantity decrease of *B. coagulans*. Although *B. coagulans* could form spores with strong stress resistance, its growth was still affected to some extent. *B. lactis* grew slowly in the early time of fermentation, and reached the maximum value (5.54×10^6 copies/g) on the tenth day, which was 3.15 times as much as that before fermentation, and then decreased gradually and maintained at a certain level.

Changes of microflora in fermented feed with *B. lactis* and each LAB individually were shown in Fig.2(b) and Fig.2(c). We can easily find that *B. lactis* in sample A reached the maximum value (7.26×10^6 copies/g) on the fifteenth day of fermentation, which was 4.94 times of that before fermentation, indicating that *L.*

fermentum had no inhibitory effect on *B. lactis*. *B. lactis* reached the maximum on the tenth day of fermentation (9.35×10^6 copies/g), which was 2.21 times of the number of bacteria before fermentation. *L. plantarum* played a dominant role in fermentation, and grew rapidly as the early stage, and used the nutrients in raw materials preferentially. Interspecific competition may lead to the growth restriction of *B. lactis*. In sample D, *B. lactis* grew slowly and the peak value of bacteria quantity was 1.16 times the number of that before fermentation. *L. rhamnosus* is rich in acid, which resulted in lower pH of fermentation medium. Environmental pH was an important factor affecting the growth of *Bifidobacterium*, the decomposition rate of carbohydrate depends on 6-phosphofructokinase under anaerobic conditions, and the enzyme activity influences glycolytic and lactic acid flux (Li et al., 2016). When *Bifidobacterium* grows in meta-acidic environment, 6-phosphofructokinase is feedback inhibited or repressed (Desjardins, Roy, & Goulet, 1990), so the activity of *Bifidobacterium* is affected, which is not conducive to its growth. Both samples C and E reached their peak values on the first day of fermentation, and their bacterial biomass was 10.98 and 14.30 times of that before fermentation, respectively, which accelerated the fermentation process. *L. paracasei* and *B. coagulans* could promote the growth of *B. lactis* to some extent.

From Fig.2(c), we see that, under anaerobic fermentation conditions, *L. fermentum*, *L. plantarum*, *L. paracasei*, *L. rhamnosus* and *B. coagulans* were inoculated with *B. lactis* individually. The number of *L. fermentum* was 1.66×10^7

copies/g before fermentation (0 d), and reached its peak value (8.37×10^9 copies/g) on the first day. The number of *L. plantarum* before fermentation was 5.84×10^7 copies/g, and reached the maximum on the tenth day (4.06×10^8 copies/g). *L. paracasei* and *L. rhamnosus* both reached the maximum value on the first day with 2.98×10^7 copies/g and 7.71×10^7 copies/g, respectively. *B. coagulans* reached the peak value (1.23×10^7 copies/g) on the third day of fermentation. After 15 days of fermentation, the number of *B. coagulans* decreased significantly due to the decrease of vegetative cells and spore formation.

In the mixed-culture fermentation process of *B. lactis*, *L. paracasei* and *B. coagulans*, the changes of bacterial biomass are shown in Fig.2(d). *B. lactis* reached the maximum value on the tenth day of fermentation with 2.31×10^7 copies/g, which was 6.13 times of that before fermentation, and increased compared with that of single fermentation. *L. paracasei* also reached maximum (7.58×10^7 copies/g) on tenth day and then maintained at a certain level. *B. coagulans* reached the maximum value (2.59×10^7 copies/g) on the first day of fermentation, and then the bacteria quantity gradually decreased. Oxygen and acidity are the key factors affecting the survival of *Bifidobacterium*. *B. coagulans* not only has the characteristics of *Bacillus* but also produces lactic acid. *B. coagulans* consumed part of oxygen and yielded protease during the early time of fermentation, which provided a good growth environment for *Bifidobacterium*. The growth of *L. paracasei* was stable, and the acid production was suitable. It seemed that *L. paracasei* had no obvious competitive inhibition effect on

B. lactis when pH of feed environment was reduced. The results showed that the synergistic fermentation of *B. lactis*, *L. paracasei* and *B. coagulans* had application value.

3.3 Changes of small peptide, α -galactosidase activity and reducing sugar content during feed fermentation

The changes of small peptide contents in mixed fermentation process of *B. lactis*, *L. paracasei* and *B. coagulans* was shown in Fig.3. The content of small peptides increased rapidly after 3 days of fermentation. It is possible that strains were in exponential phase at initial fermentation, with strong activity and rapid growth. At the same time, protease was produced to hydrolyze the protein in the feed. The content of small peptides increased slowly after 15 days of fermentation and reached 22.65% after 30 days of fermentation.

Bifidobacterium and *Lactobacillus* species can produce different levels of α -galactosidase and hydrolyze various glycosidic anti-nutritional factors. Studying on the activity of α -galactosidase in the fermentation process can make the correct planning and design of fermentation process. As shown in Fig.4, strains were in exponential phase at initial fermentation, and the enzyme activity gradually increased. The highest enzyme activity of control and sample F were 4.608 U/g and 7.378 U/g respectively after fermentation. In addition, the control maintained relatively stable enzyme activity during stationary phase, and α -galactosidase activity of sample F decreased and was lower than that of control during fermentation anaphase. It is

possible that α -galactosidase in various sources had different properties such as protease resistance and galactose tolerance. With the accumulation of metabolites, α -galactosidase inhibitors in the fermentation substrate might influenced the enzyme activity(Huang et al., 2018).

The changes of reducing sugar content during fermentation were shown in Fig.5. Trend of reducing sugar in sample F was consistent with the control, and fermentation microorganisms transformed the saccharides in feed raw materials into alcohols, organic acids and other substances, and the reducing sugar content gradually decreased as fermentation process went on. From 0 to 6 days, the microorganism grew and multiplied rapidly, and consumed a lot of reducing sugar. After fermentation for 15 days, the reducing sugar content of control and sample F increased to 6.56 mg/g and 8.44 mg/g respectively, and held steady gradually. After fermentation for 30 days, the reducing sugar contents of control and sample F were 7.14 mg/g and 8.00 mg/g, respectively. Microorganism yielding glycosidase and amylase in the fermentation process to hydrolyze non-reducing sugar in feed into glucose and other little molecular carbohydrates to provide energy for the growth of microorganisms. The reducing sugar content of sample F was generally higher than that of control. Sample F had high α -galactosidase activity, which degraded the non-reducing sugar such as raffinose and stachyose into reducing sugars. Meanwhile, as the number of microorganisms gradually stabilized, enzyme activity decreased and reducing sugar consumption also decreased.

3.4 Changes of distinct secondary structure elements of protein in fermented feed

The decomposition of the amide I band was performed in the region of 1700-1600cm⁻¹, and the amide I vibration arises mainly from the C=O stretching vibration of protein backbone(Barth, 2007). Amide I vibration is sensitive to secondary structure. A second-derivative analysis and Fourier self-deconvolution were used to resolve and quantify secondary structural components of feed protein. As shown in Table 4, The proportion of secondary structure of protein in the microbial fermented feed (Sample F) was different from that of the unfermented feed. The β -sheet content of the unfermented soybean meal was the highest, which was 34.53%, followed by β -turn, which was 30.08%, and the content of α -helix and random coil was lower, which were 16.30% and 19.10%, respectively, α -helix / β -fold was 47.21%. The ratio of α -helix in fermented feed decreased by 5.07%, and the ratio of β -turn increased by 5.15%. There was little difference in β -sheet and random coil, and the ratio of α -helix / β -folding decreased. It was reported that the decrease of α -helix component may be related to the increase of protein digestibility(Wang et al., 2014). New peak appeared after fermentation (Fig.6), which was speculated to be due to the increase of small peptide content during feed fermentation, leading to protein recombination(Yasar, Tosun, & Sonmez, 2020).

Table 4 Changes of protein secondary structure relative content

Secondary structure (%)	Before fermentation	After fermentation
α -helix	16.30±1.31	11.23±1.18
β -sheet	34.53±1.43	35.60±0.92

β -turn	30.08±1.15	35.23±1.78
random coil	19.10±0.87	17.94±1.04
α - helix/ β - sheet	47.21±0.02	31.54±0.04

4 Conclusion

In this study, *L. rhamnosus* had the strongest acid producing ability. When *B. lactis*, *L. paracasei* and *B. coagulans* were used to ferment soybean meal feed together, the peak biomass of *B. lactis* was 6.13 times of that before fermentation, which was higher than sample fermented with *B. lactis* only (3.15 times). The peak value of α -galactosidase activity was 1.6 times higher than the control. On the thirtieth day of fermentation, the content of reducing sugar was 8.00 mg/g, and the content of small peptides was 22.65%, which was 2.7 times of that before fermentation, the proportion of secondary structure of protein in feed also changed. These results suggested that *L. paracasei* and *B. coagulans* could promote the growth of *B. lactis*, and fully ferment soybean meal feed with a synergistic effect.

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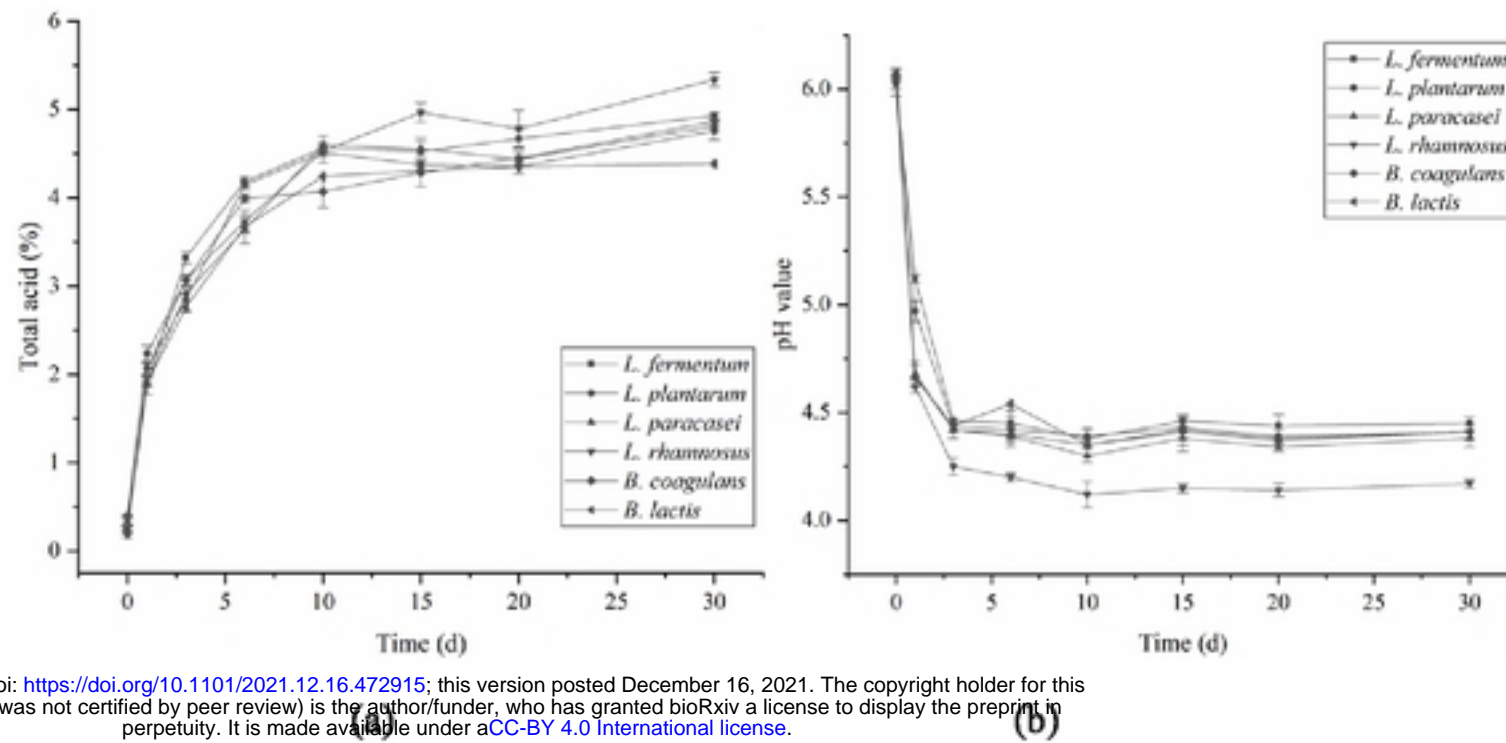
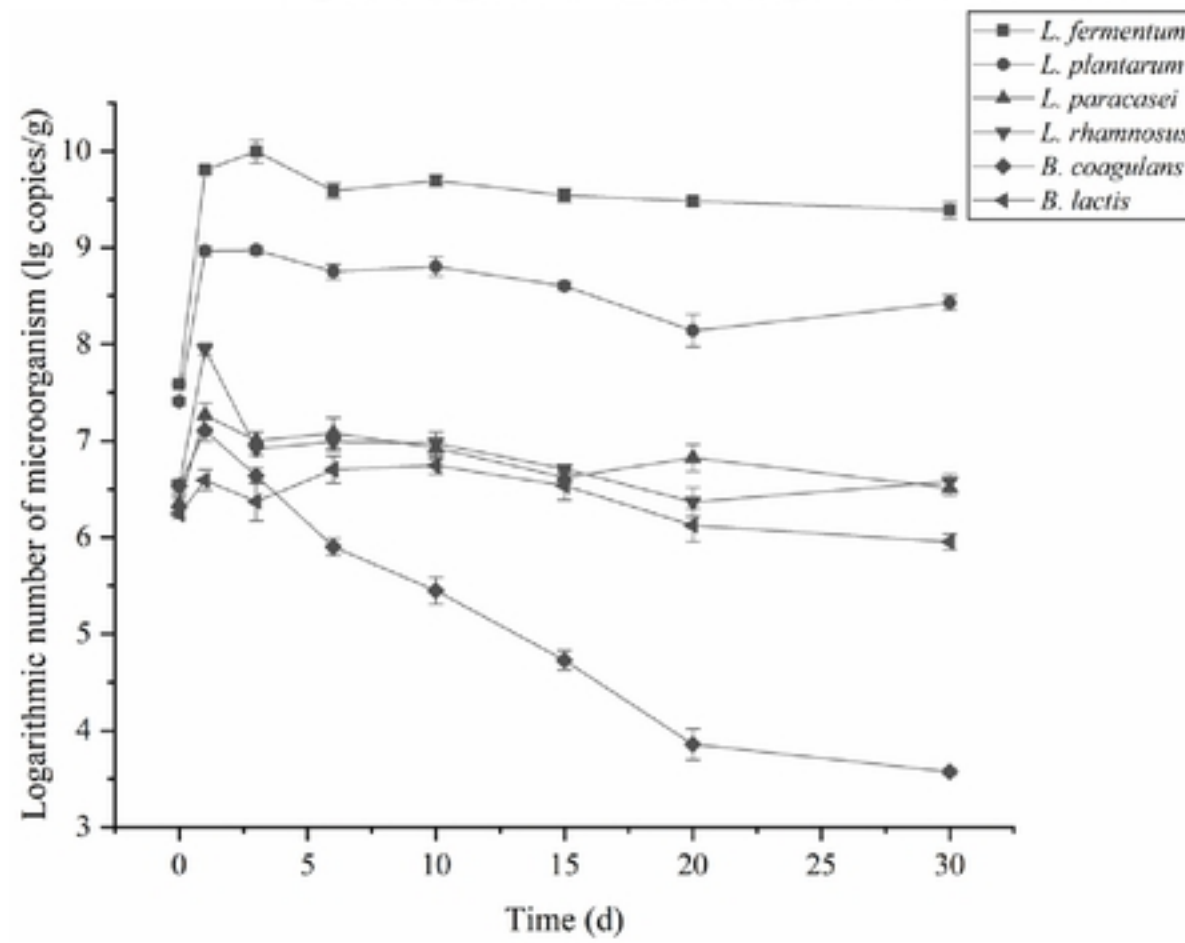
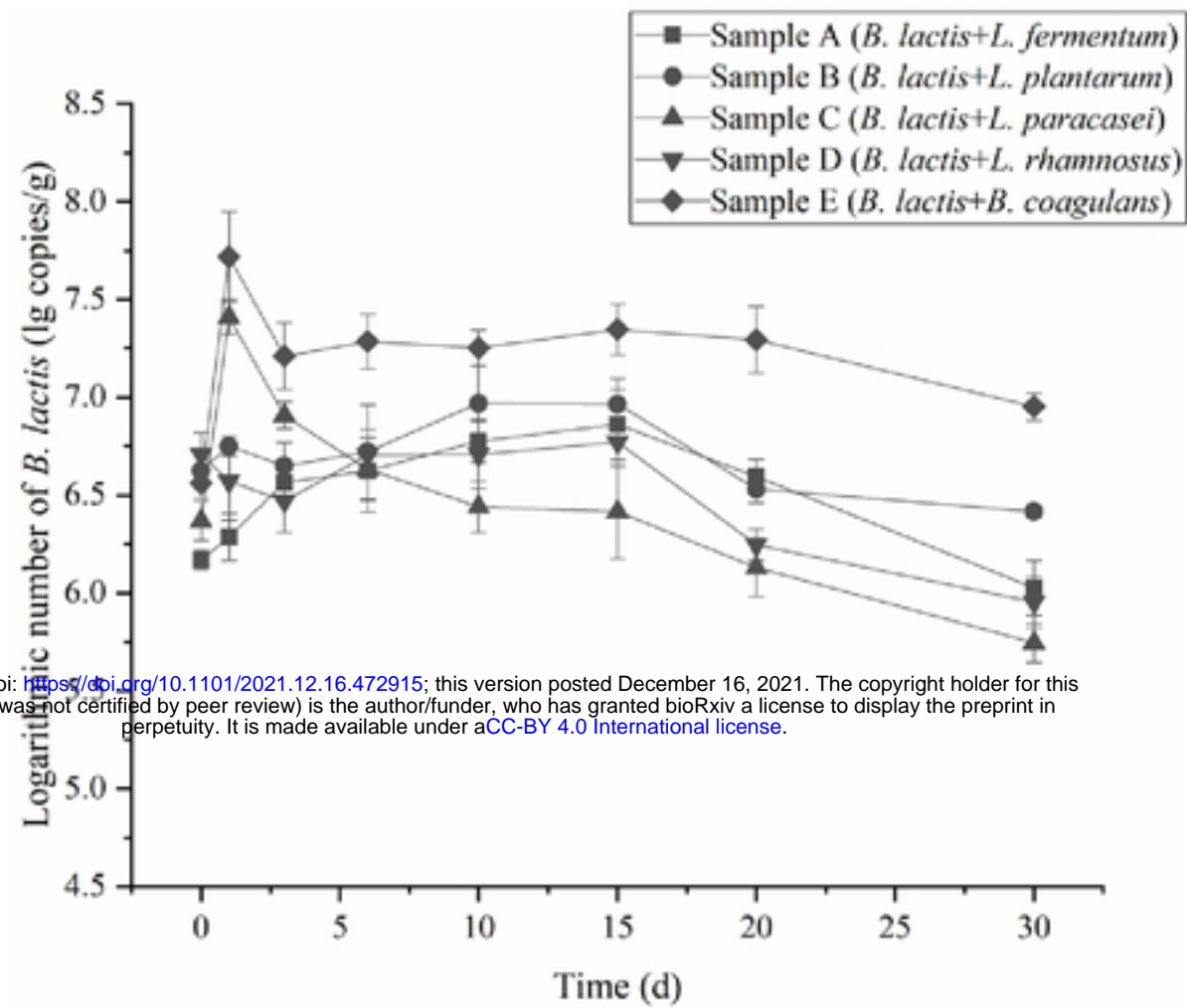


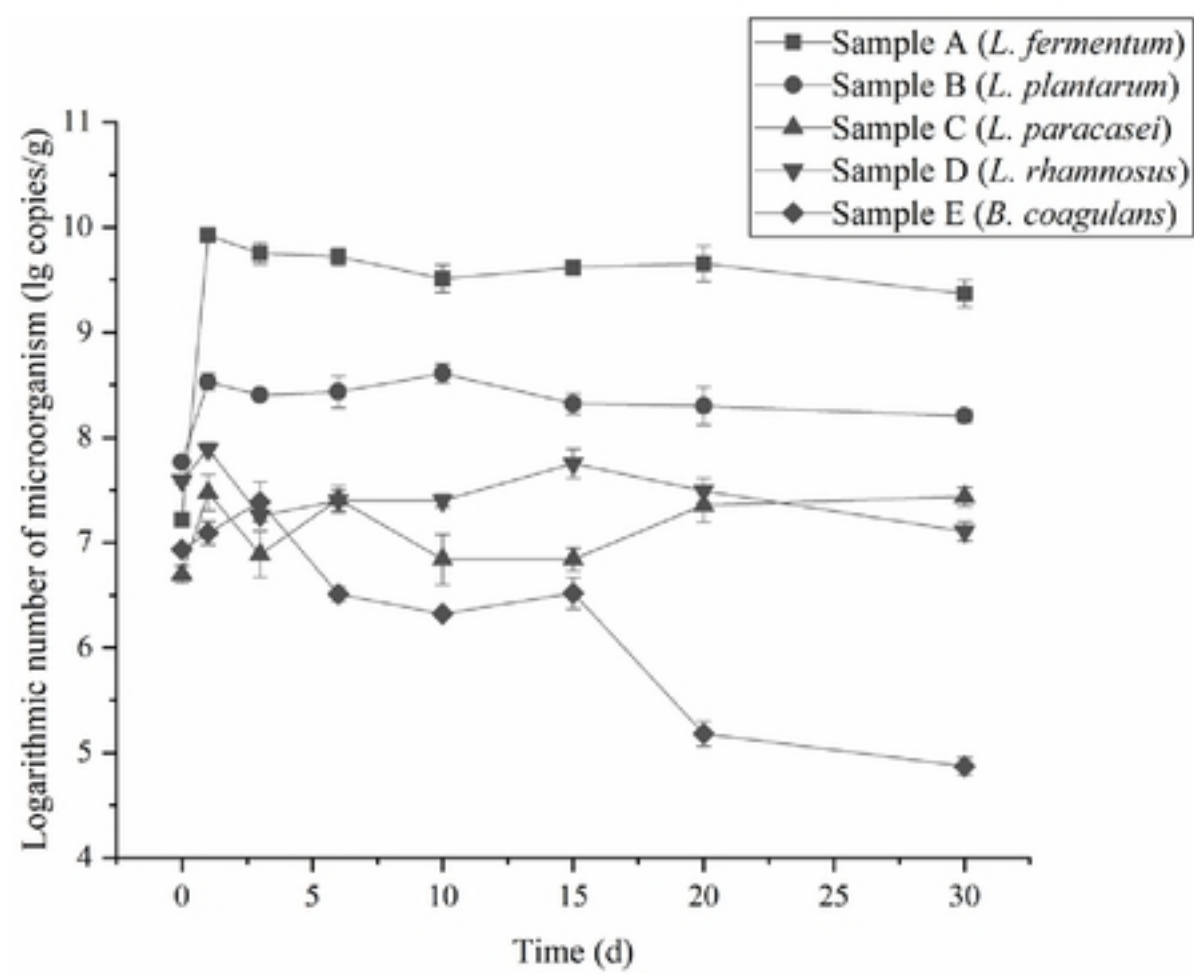
Fig.1 Changes of total acid and pH value in fermentation process



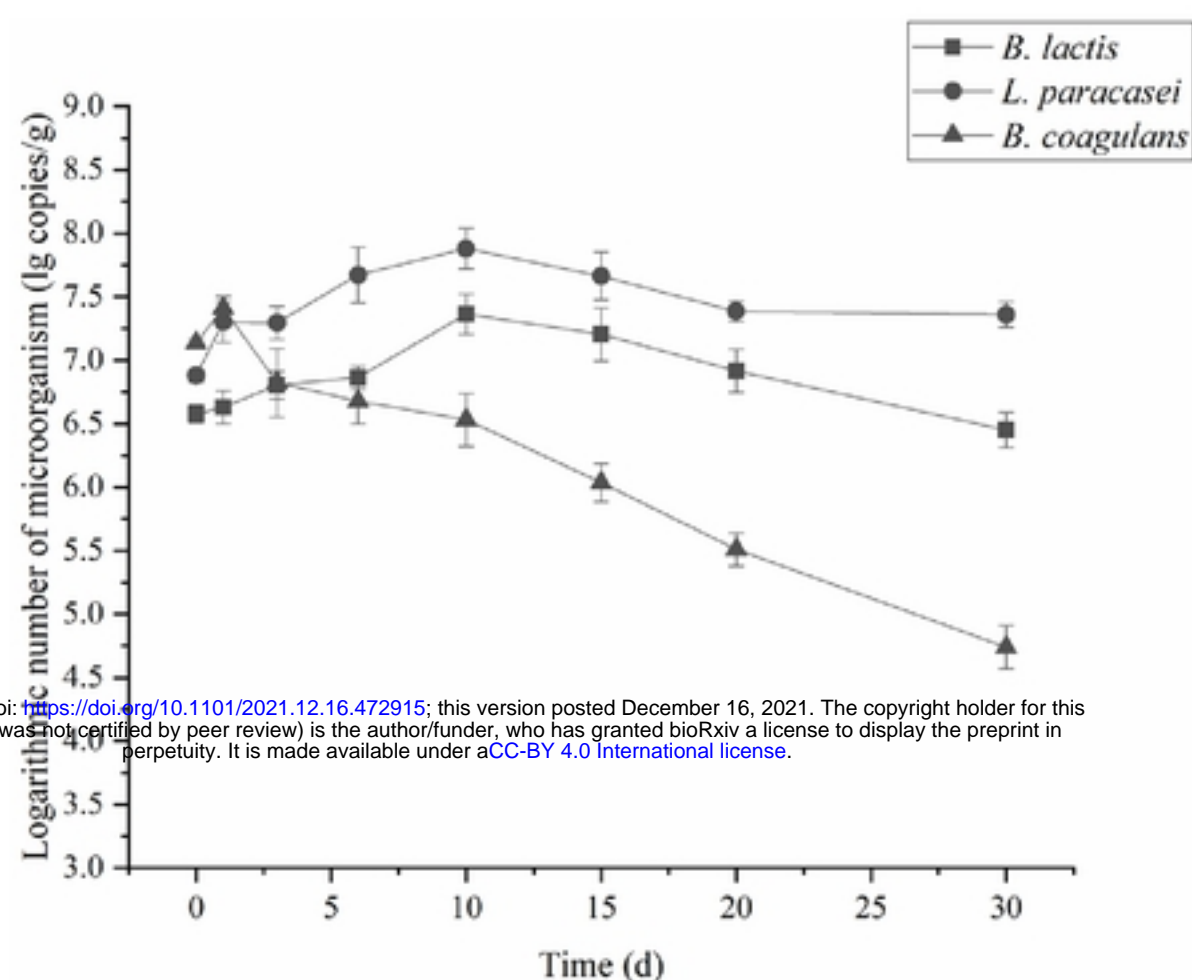
(a)



(b)



(c)



(d)

Fig.2 Changes of microflora in fermentation process:(a) fermentation with 6 strain in this study respectively based on *B. subtilis* and *S. cerevisiae*; (b) and (c): fermentation with *B. lactis* and different LAB based on *B. subtilis* and *S. cerevisiae*; (d) sample F: fermentation with *B. lactis*, *L. paracasei* and *B. coagulans* based on *B. subtilis* and *S. cerevisiae*.

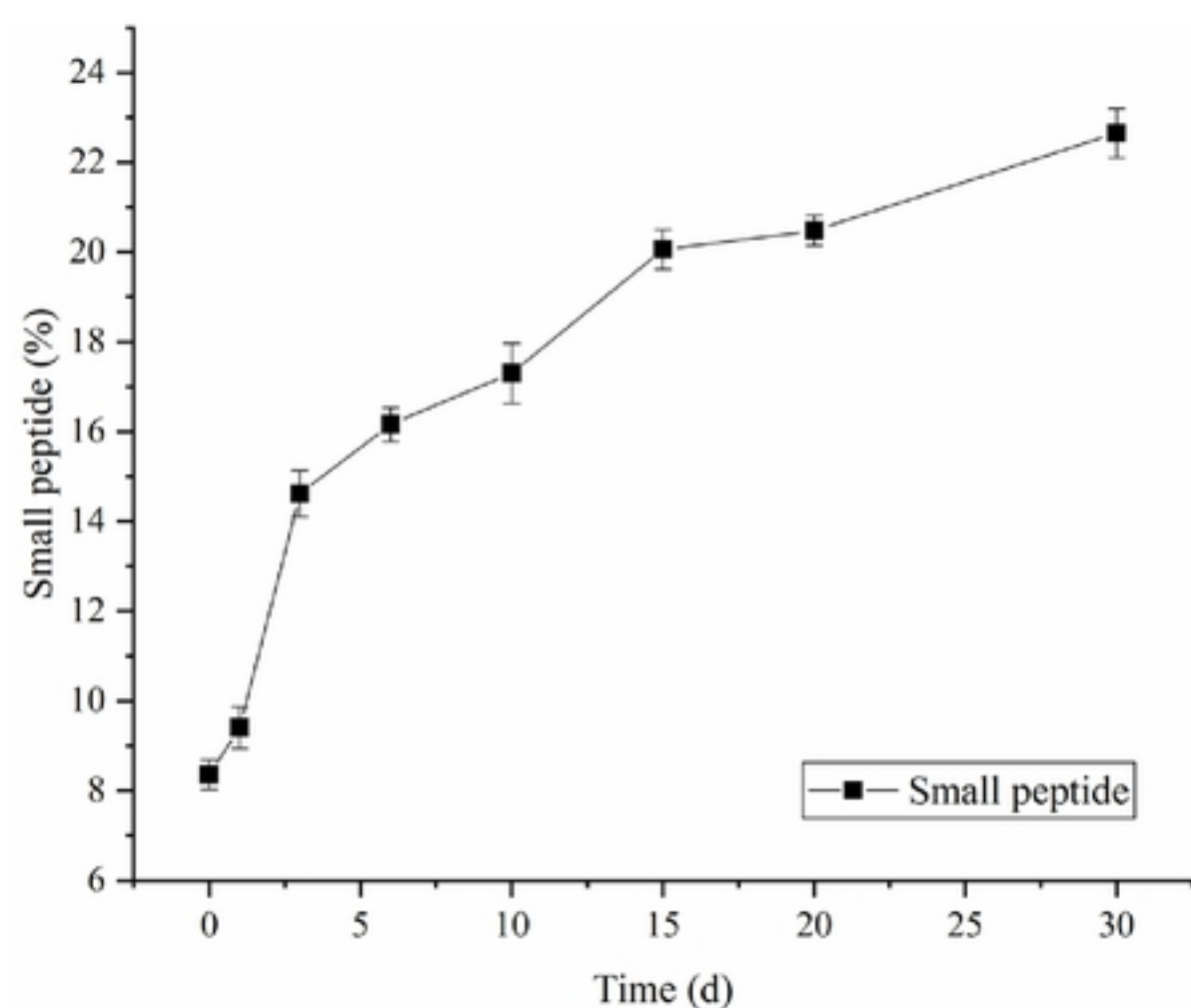


Fig.3 Changes of small peptides during fermentation

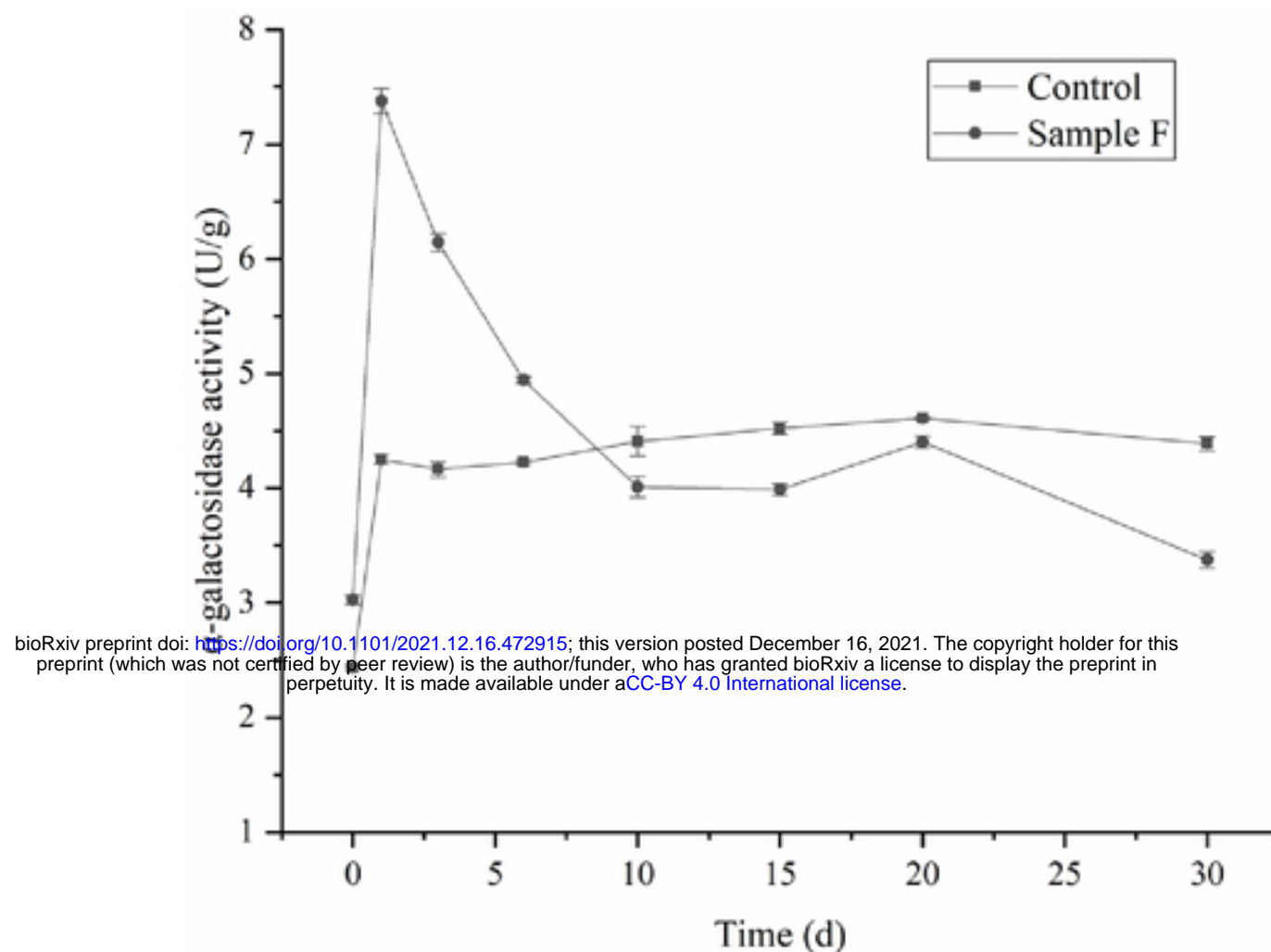


Fig.4 Changes of α -galactosidase activity during fermentation: **Control** fermentation with *B. subtilis*, *S. cerevisiae* and *B. lactis*; **Sample F** fermentation with *B. subtilis*, *S. cerevisiae*, *B. lactis*, *L. paracasei* and *B. coagulans*

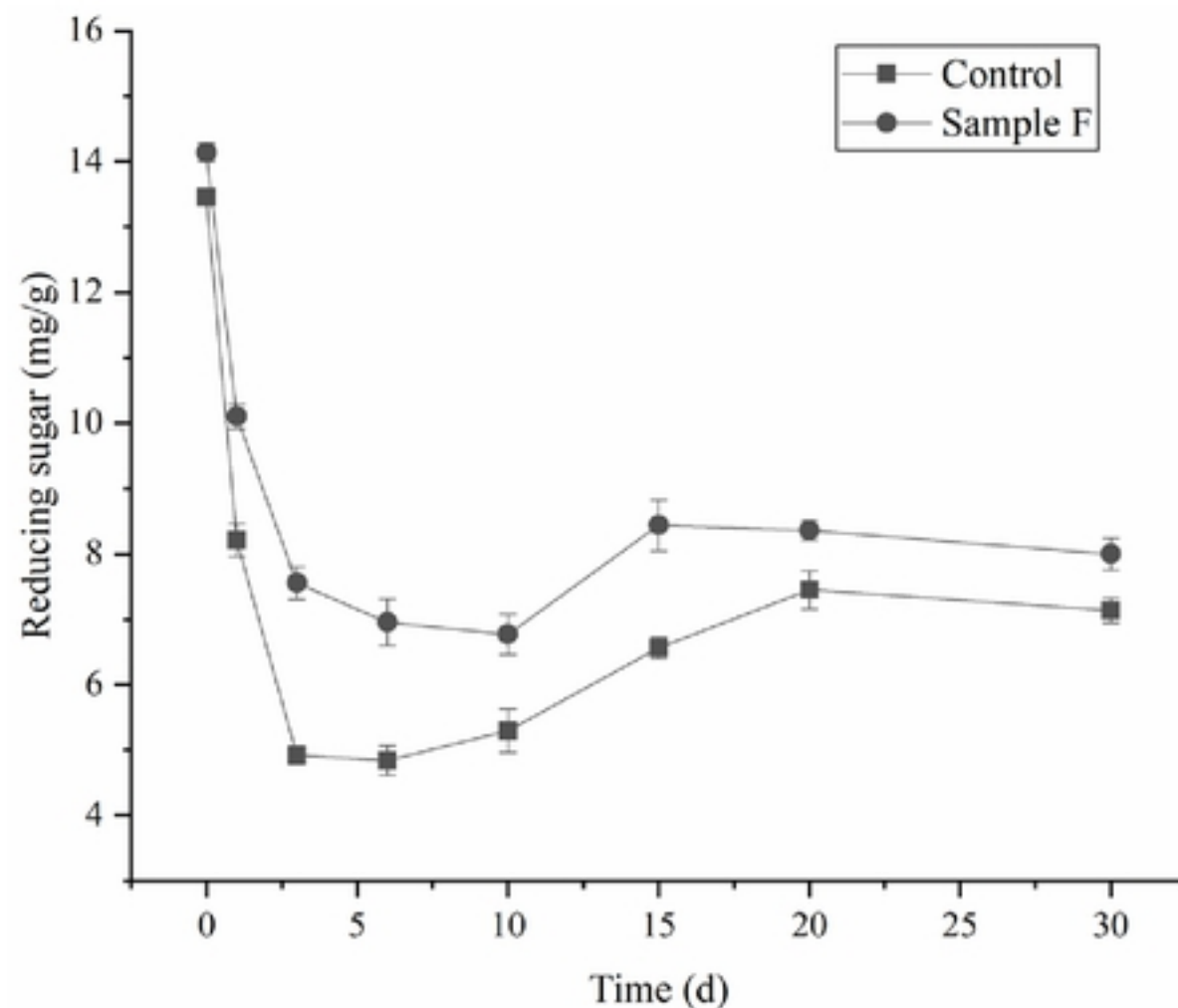
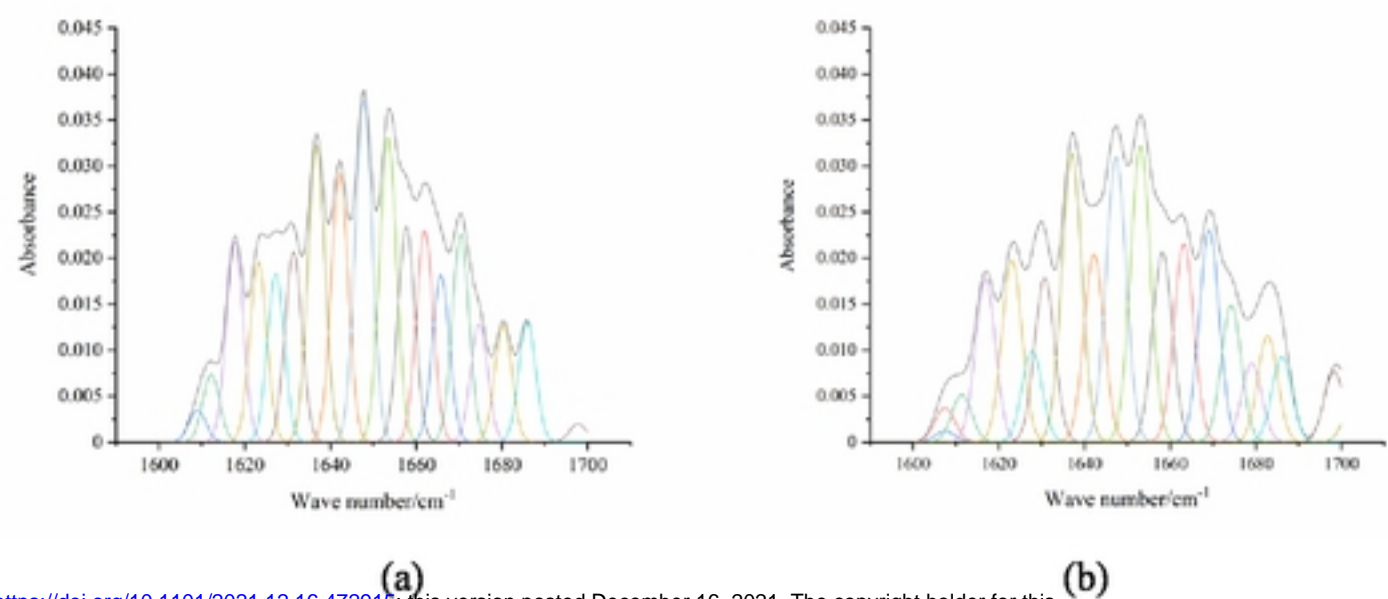


Fig.5 Changes of reducing sugar content during fermentation: **Control** fermentation with *B. subtilis*, *S. cerevisiae* and *B. lactis*; **Sample F** fermentation with *B. subtilis*, *S. cerevisiae*, *B. lactis*, *L. paracasei* and *B. coagulans*

Figure 6 (color print)



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Fig.6 The amino I band curve fitting results of proteins.(a) fermentation raw material; (b) fermented feed