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Original paper

Identification of Lactic Acid Bacteria in the corn stalk Fermentation by Fluorescence Quantitative PCR

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Abstract

Lactic acid bacteria play an important role in the fermentation of biological feed. In this experiment, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Lactobacillus casei* and *Lactobacillus rhamnosus* were used to ferment corn stalk by single and mixed bacteria. The changes of *Lactobacillus* flora during fermentation were analyzed by fluorescence quantitative PCR. The results showed that the maximum number of lactic acid bacteria appeared on the 7th day of mixed fermentation, and the total bacteria content could be significantly increased by adding five kinds of lactic acid bacteria at the same time, and the number of each bacteria in mixed fermentation was close to that measured by single fermentation, which indicated that these five kinds of lactic acid bacteria could cooperate during fermentation and help to improve the quality of stalk fermented feed.

Keywords

Fluorescence Quantitative PCR; Lactic acid bacteria; Flora change

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Introduction

Microbial strain is the key to ferment biological feed by adding microbial feed additives (Liu et al. 2019b), because the growth and metabolism of different strains will be different. Most of the reported fermentation methods of bio-feed also adopt the method of co-fermentation of compound strains (Yan et al. 2019). The metabolites produced by probiotic fermentation of feed can effectively prevent the colonization of Gram-negative bacteria in intestine (Lee et al. 2019). Using multi-strain mixed fermentation for bio-feed production is much better than single strain fermentation, because after mixed fermentation of different strains, the differences between them can be used to compensate for each other, and mutually beneficial symbiosis can be achieved (Liu et al. 2019a). For example, Yao et al. (Yao et al. 2018) invented a kind of bio-feed and its production method, which utilized the co-solid-state fermentation tank of *Bacillus subtilis* and yeast to make it a nutritious animal feed; Lan et al. (Lan and Kim 2019) fermented feed by *Bacillus subtilis* and *Bacillus licheniformis*, which improved animal growth performance and animal digestibility of nutrients.

Lactic acid bacteria play an important role in the fermentation of corn stalk bio-feed because anaerobic fermentation is carried out after encapsulation. Bacteria and metabolites of lactic acid bacteria can resist the invasion of pathogenic bacteria to intestinal wall. In addition, both living and dead lactic acid bacteria contain beneficial components of fermented biological feed, which can improve the quality of fermented feed (Ou et al. 2011). At the same time, *Lactobacillus* as the dominant bacteria in animal intestinal tract, fermentation can produce a large amount of lactic acid (Giraffa et al. 2010), acetic acid and some volatile fatty acids, which can reduce the pH of animal digestive tract, thus inhibiting the growth of harmful bacteria and reducing the incidence of animal intestinal diseases (Markkinen et al. 2019); *Lactobacillus* can not only inhibit the growth of pathogenic bacteria, but also adhere to the intestinal cells of animals and play a space-occupying protective role (Mousavi and Mousavi 2019). Among them, *Lactobacillus acidophilus* has the functions of regulating intestinal flora, improving immunity, producing more acid and reducing the activity of toxic microorganisms (Sharma et al. 2018); *Lactobacillus plantarum* is a lactic acid bacteria existing in fermented products such as bread. Adding *Lactobacillus plantarum* to fermented feed can produce amylase, which can make up for the shortcomings of other lactic acid bacteria that generally do not produce amylase (Heo et al. 2018); *Lactobacillus casei* can tolerate enzymes in the mouth, low pH gastric juice environment (Jangra et al. 2019), inhibit the growth of spoilage bacteria and pathogenic bacteria in some foods, but it does not affect the appearance of food, and even optimize food characteristics (Fontana et al. 2018). Therefore, adding *Lactobacillus casei* to feed as a starter can help feed quality production, it also plays an active role in preservation and preservation of feed during

storage (Guo et al. 2018); *Lactobacillus rhamnosus* can enhance the immune function of the body, reduce and eliminate food toxins, and treat intestinal dysbacteriosis diarrhea (Yadav et al. 2018); *Bifidobacterium longum* is a specific anaerobic bacterium, it can inhibit the growth of pathogenic bacteria through its own or secreted metabolites (Elsanhoty et al. 2017). In the fermentation process, it maintains its own advantage of bacteria, interacts with other bacteria and adjusts the relationship between the whole bacteria, so as to achieve the goal of maintaining the balance of bacteria (Slacanac et al. 2005).

This paper mainly studies the evolution of microflora during the fermentation of corn stalk by *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Lactobacillus casei* and *Lactobacillus rhamnosus*, so as to provide reference for the high quality production of corn stalk biological feed and other raw materials.

Materials and Methods

Experimental strains and raw materials

Strains: *Bacillus subtilis* CGMCC 1.1086, *Saccharomyces cerevisiae* CGMCC 2.1527, *Lactobacillus plantarum* CGMCC 1.557, *Lactobacillus acidophilus* CGMCC 1.2467, *Bifidobacterium longum* CGMCC 1.2186, *Lactobacillus casei* CGMCC 1.8727, *Lactobacillus rhamnosus* CGMCC 1.577, purchased from China General Microbial Species Preservation and Management Center (freeze-dried powder, stored at 4°C).

Raw Material: Crushed corn stalk, dried at 45°C and sifted at 20 meshes, stored in dry environment.

Bacillus can tolerate high temperature, high pressure and acidic environment, at the same time, it has good activity of protease and cellulase, etc., which can break the cell wall of agricultural waste and promote the release of nutrients in straw. *Yeast* can produce abundant thallus protein and a small amount of alcohol to induce animal feeding. In addition, the addition of *Bacillus* and *Yeast* in the fermentation can continuously consume oxygen to create a good anaerobic fermentation environment for lactic acid bacteria.

DNA extraction

The standard strain freeze-dried powder was mixed with proper amount of sterile saline, inoculated in PDA solid medium, incubated in constant temperature incubator at 37°C for 24-48 hours, then single colonies were selected by sterile inoculation ring and inoculated into LB medium, MRS medium and malt juice medium respectively, aerobic or anaerobic incubation at 37°C to activate the strains, until the OD₆₀₀ of the strain is 0.8. Fresh strains of standard strains were collected, and bacterial DNA was extracted by bacterial genome extraction kit (Shanghai Biotechnicians); yeast DNA was extracted by fungal genome extraction kit (Takara). The experimental steps were in accordance with the instructions. The PCR products were analyzed by 3% agarose gel electrophoresis and the quality and concentration of DNA were analyzed by ultraviolet spectrophotometer.

Primer Design

The primer sequence (de Almeida et al. 2015; Gueimonde et al. 2004; Makino et al. 2010;

Borshchevskaya et al. 2013), amplified fragment size and annealing temperature used in this experiment are shown in Table 1.

Table 1 Primer Sequence and Annealing Temperature

Name of Strain	Primer Sequence (5'-3')	Amplified fragment size (bp)	Annealing temperature (°C)
<i>Bacillus subtilis</i>	F: AAAATCCGCGCGTATCGTTG	520	53.2
	R: CTCGGCCTGATTCGTATGCT		
<i>Saccharomyces cerevisiae</i>	F: GCGATAACGAACGAGACCCTAA	225	53.2
	R: CCAGCACGACGGAGTTTCACAAGAT		
<i>Lactobacillus plantarum</i>	F: CAGCACTAGATAACGCCCTG	211	53.2
	R : ATGTAGTGCCACGGTCGTTT		
<i>Lactobacillus acidophilus</i>	F: AGACACGGCCCAA ACTCC	231	52.6
	R: GACAACGCTTGCCACCTA		
<i>Bifidobacterium longum</i>	F: GATTCTGGCTCAGGATGAACGC	231	52.6
	R: CTGATAGGACGCGACCCCAT		
<i>Lactobacillus casei</i>	F: CTATAAGTAAGCTTTGATCCGGAGATTT	133	52.6
	R: CTCCTGCGGGTACTGAGATGT		
<i>Lactobacillus rhamnosus</i>	F: TGCTTGCATCTTGATTTAATTTTG	317	52.6
	R: GTCCATTGTGGAAGATTCCC		

Primer Specificity Verification

The conventional PCR reaction was performed by crossing the genomic DNA of each standard strain with

primers (the reaction system is shown in Table 2), and the amplified products of PCR were analyzed by 3% agarose gel electrophoresis.

Table 2 Conventional PCR Reaction System

System Component	Volume (µL)
10×PCR buffer	5
dNTP Mixture	4
Upstream primer	2
Downstream primer	2
DNA template	1.5
ddH ₂ O	35.25
Taq enzyme	0.25

Establishment of Standard Curve

The obtained PCR products was subjected to gelatinization and recovery of the DNA fragment as a standard for Fluorescence quantitative PCR, and the OD_{260/280} value and concentration of DNA fragments were measured. The calculation formula was as follows: copy number = DNA concentration (ng/μL) × 10⁻⁹ × 6.023 × 10²³ / (660 × base number). The standard samples satisfying the purity requirement were diluted by 10 times series to 1 × 10⁻⁸ ~ 1 × 10⁻² copies/μL. The standard curve could be drawn by

taking the logarithm of positive template as abscissa and the initial cycle number (Ct) of fluorescent signals in the process of PCR reaction as ordinate.

Detection of bacterial flora in biological feed by fluorescence quantitative PCR

The diluted DNA samples were used as positive templates and the corresponding primers of each strain were used for quantitative fluorescence PCR. The reaction system of 20 mL is as follows:

Table 3 Fluorescence Quantitative PCR Reaction System

System Component	Volume (μL)
SYBR ^(R) Green Realtime PCR Master	10
ddH ₂ O	6
template	2
Upstream primer	0.8
Downstream primer	0.8
ROX	0.4

The reaction procedure was as follows: first, it was pre-denatured at 95°C for 30 seconds, then amplified at 95°C for 5 seconds and at 60°C for 31 seconds for 40 cycles. After the reaction, the fluorescence signal was recorded by heating to 95°C 15 s, then to 60°C 1 min, and then to 95°C 1 s.

Experimental Design

The fermentation method adopted was a step-by-step fermentation, that is, adding *Bacillus subtilis* and *Saccharomyces cerevisiae* to aerobic fermentation for 12 hours, then adding lactic acid bacteria, immediately encapsulating and anaerobic fermentation. Six samples

were set up, three replicates for each sample. The samples of 0 d, 1 d, 2 d, 3 d, 5 d, 7 d, 10 d, 15 d, 20 d, 25 d and 30 d after encapsulation were respectively taken for quantitative fluorescence PCR. The specific experimental design is shown in Table 4. The above experimental samples were designed to be repeated in three groups at different time periods.

The change of lactic acid bacteria flora in the process of stepwise fermentation without sterilization of raw materials was studied.

Table 4 Experimental materials and fermentation strains

Samples	Raw Material	Water content	Fermentation Strain
A	Corn stalk 80% 、 Bran 20%	35%	<i>Bacillus subtilis</i> , <i>Saccharomyces cerevisiae</i> , <i>Lactobacillus plantarum</i>
B	Corn stalk 80% 、 Bran 20%	35%	<i>Bacillus subtilis</i> , <i>Saccharomyces cerevisiae</i> , <i>Lactobacillus acidophilus</i>
C	Corn stalk 80% 、 Bran 20%	35%	<i>Bacillus subtilis</i> , <i>Saccharomyces cerevisiae</i> , <i>Bifidobacterium longum</i>
D	Corn stalk 80% 、 Bran 20%	35%	<i>Bacillus subtilis</i> , <i>Saccharomyces cerevisiae</i> , <i>Lactobacillus casei</i>
E	Corn stalk 80% 、 Bran 20%	35%	<i>Bacillus subtilis</i> , <i>Saccharomyces cerevisiae</i> , <i>Lactobacillus rhamnosus</i>
F	Corn stalk 80% 、 Bran 20%	35%	<i>Bacillus subtilis</i> , <i>Saccharomyces cerevisiae</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus acidophilus</i> , <i>Bifidobacterium longum</i> , <i>Lactobacillus casei</i> 、 <i>Lactobacillus rhamnosus</i>

Results and Discussion

Primer Specificity Verification

The strains added in this study included *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Lactobacillus casei* and *Lactobacillus rhamnosus*, and the primer specificity was verified after the primer crossing of these five strains. The results were shown in

Figure 1. *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Lactobacillus casei* and *Lactobacillus rhamnosus* had specific amplification at 211 bp, 231 bp, 231 bp, 133 bp and 317 bp, respectively, while other bacteria had no specific amplification in this region. The above results indicate that the primers used in this experiment can specifically identify the fermentation flora in this experiment.

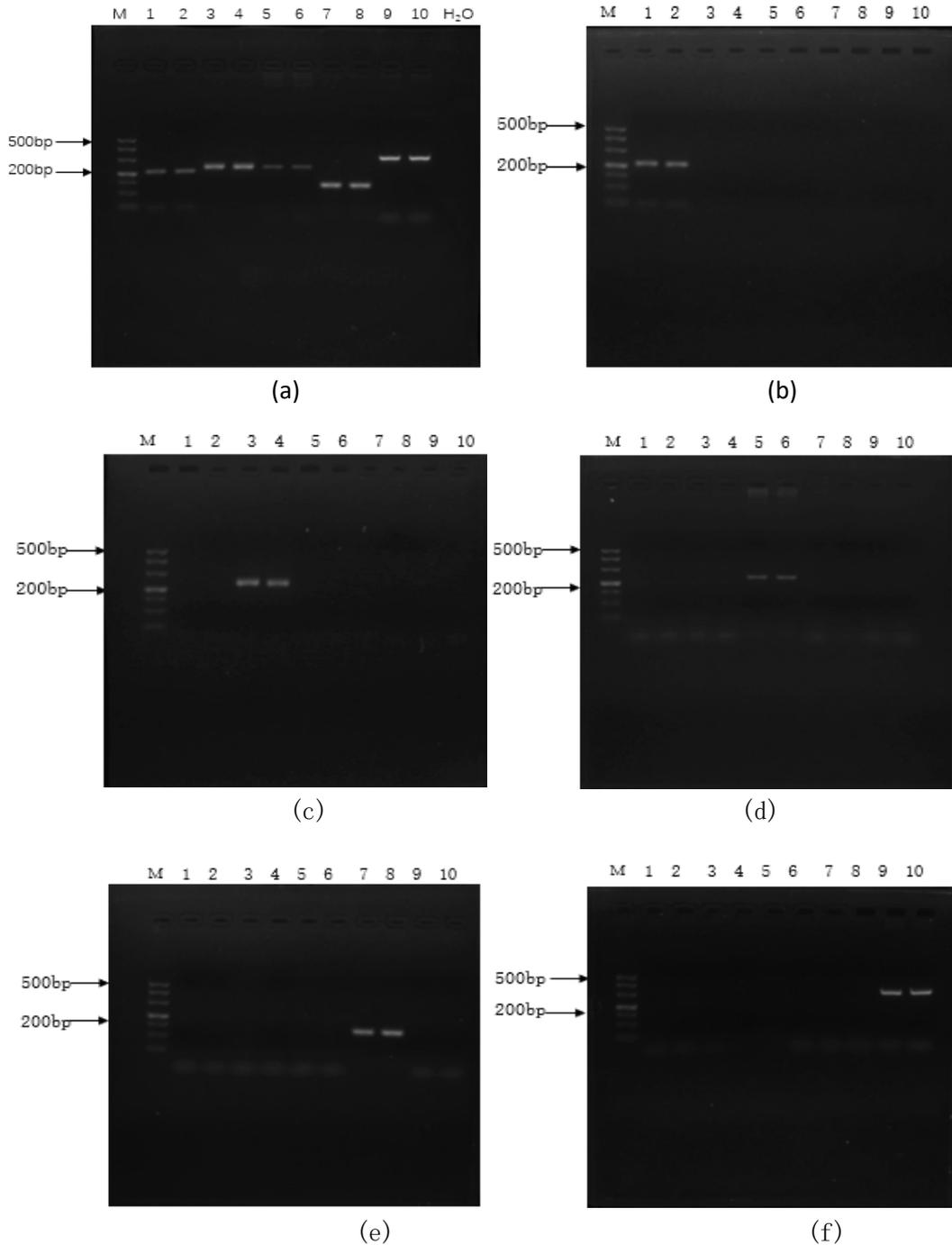


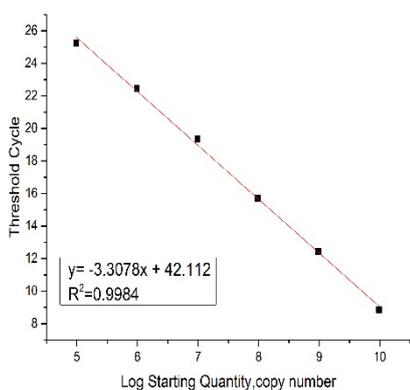
Figure 1. Primer-specific validation map of anaerobic probiotics

(a) M is DNA Marker, 500 bp, 400 bp, 300 bp, 200 bp, 150 bp, 100 bp, 50 bp from top to bottom, lanes 1 and 2 are *Lactobacillus plantarum*, lanes 3 and 4 are *Lactobacillus acidophilus*, 5 Lane 6 are *Bifidobacterium longum*, lanes 7 and 8 are *Lactobacillus casei*, lanes 9 and 10 are *Lactobacillus rhamnosus* (b) *Lactobacillus plantarum* primer-specific validation (c) *Saccharomyces cerevisiae* primer-specific validation (d)

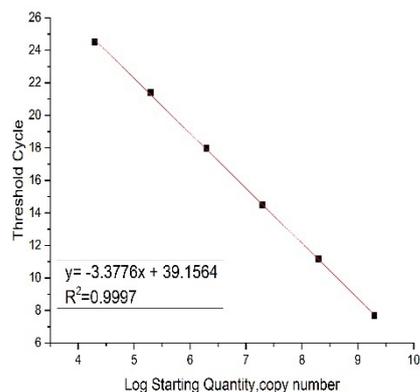
Bifidobacterium longum primer-specific validation (e) *Lactobacillus casei* primer-specific validation (f) *Lactobacillus rhamnosus* primer-specific validation

Establishment of Standard Curve

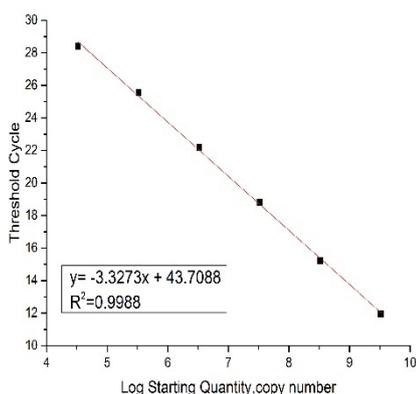
As for the standard curves of the five kinds of lactic acid bacteria, the correlation coefficient R^2 is all greater than 0.99, which can be used as the standard for the detection of the test samples. See Figure 2 for specific standard curves.



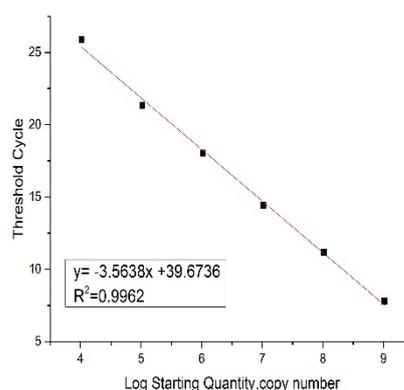
(a)



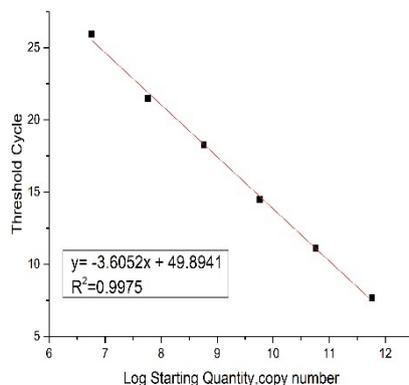
(b)



(c)



(d)



(e)

Figure 2 Standard Curve

(a) *Lactobacillus plantarum* standard curve (b) *Lactobacillus acidophilus* standard curve (c) *Bifidobacterium longum* standard curve (d) *Lactobacillus casei* standard curve (e) *Lactobacillus rhamnosus* standard curve

Melting Curve

The specificity of the primers was further verified by the analysis of the melting curve. The melting curves of the

five strains were all unimodal, indicating that the amplification products were single without the generation of non-target bands and dimers. This was also confirmed by agarose gel electrophoresis, as shown in Figure 3.

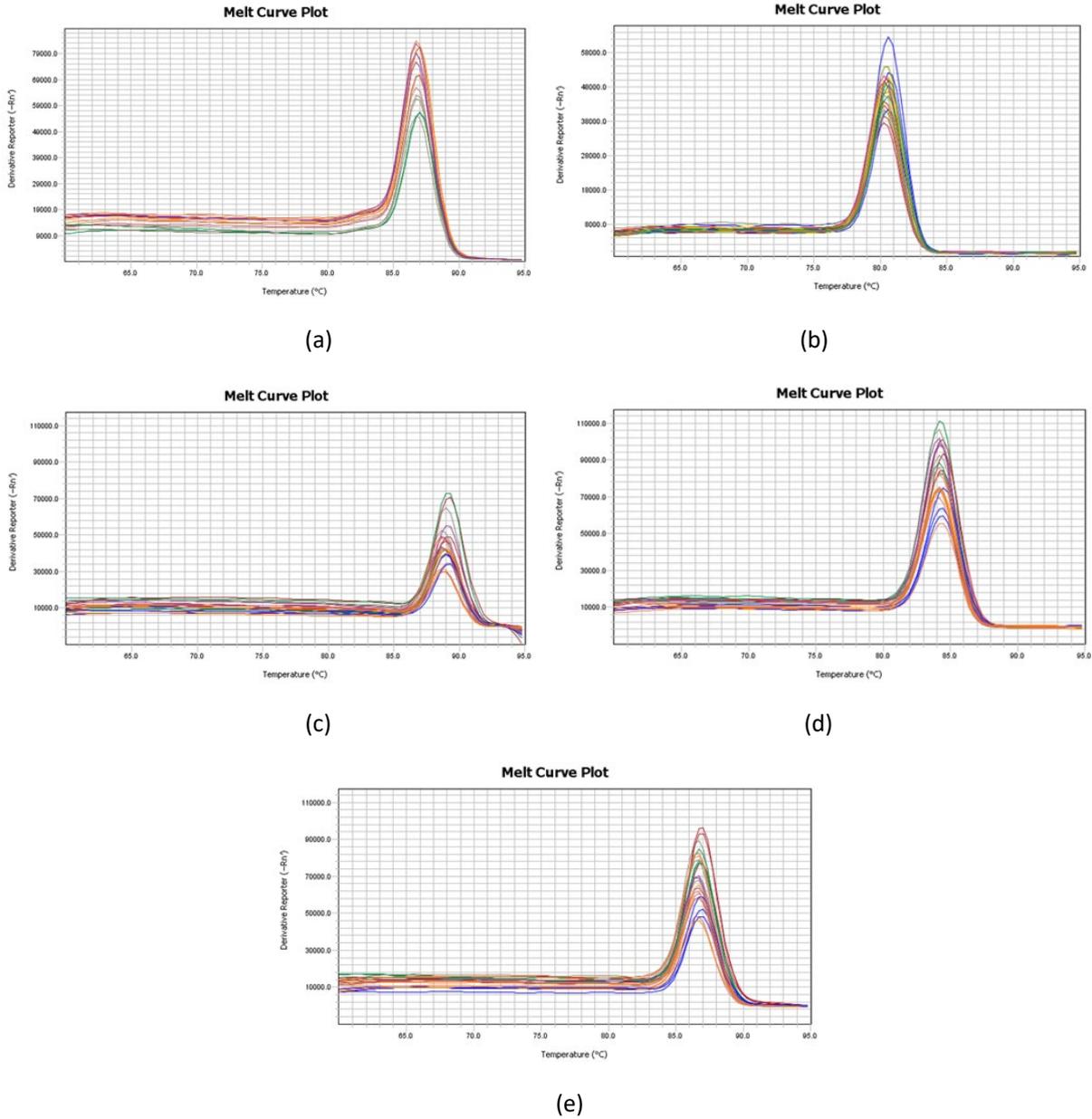


Figure 3 Standard Curve

(a) *Lactobacillus plantarum* standard curve (b) *Lactobacillus acidophilus* standard curve (c) *Bifidobacterium longum* standard curve (d) *Lactobacillus casei* standard curve (e) *Lactobacillus rhamnosus* standard curve

Changes of Lactic Acid Bacteria during Stepwise Fermentation

As can be seen from Figure 4, in general, the number of *Lactobacillus plantarum* in sample F is higher than that in sample A, which peaks on the 7th day of mixed fermentation. The number of

Lactobacillus plantarum in sample A (3.09×10^7 CFU/g) is slightly smaller than that in sample F (4.89×10^7 CFU/g) after fermenting one day, and the number of *Lactobacillus plantarum* in sample F was slightly higher in the middle and late stages of fermentation.

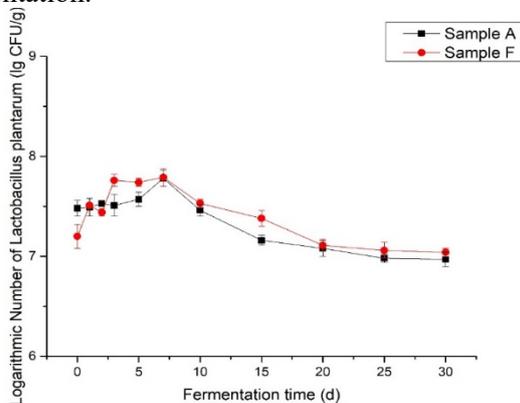


Figure 4 Number changes of *Lactobacillus plantarum* during step fermentation of biological feed
 Sample A: Fermentation with *Lactobacillus plantarum* alone; Sample F: Five lactic acid bacteria were added simultaneously for fermentation

As can be seen from figure 5, the number of *Lactobacillus acidophilus* in sample B is larger than that in sample F; the maximum number of *Lactobacillus acidophilus* in sample B appears on the third day (1.17×10^7 CFU/g), and the peak number of *Lactobacillus acidophilus* in sample F appears on the seventh day (6.46×10^6 CFU/g). According to the figure, the two groups of bacteria tend to be the consistent after 25-30 days, and even the sample F is slightly higher.

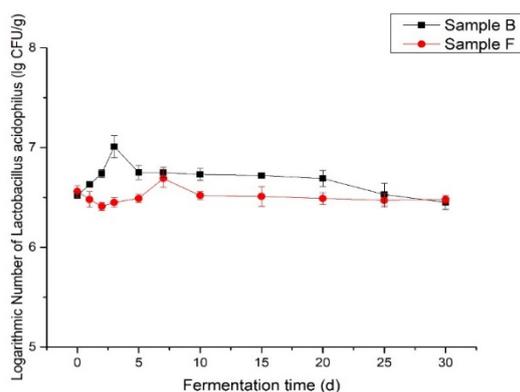


Figure 5 Number changes of *Lactobacillus acidophilus* during step fermentation of biological feed
 Sample B: Fermentation with *Lactobacillus acidophilus* alone; Sample F: Five lactic acid bacteria were added simultaneously for fermentation

As can be seen from figure 6, in the early stage of fermentation, the number in sample C is generally larger than the number of *Bifidobacterium longum* in sample F.

After 15 days of fermentation, the number of *Bifidobacterium longum* in sample F is larger, and the maximum value appears on the 7th day (1.69×10^8 CFU/g). This indicates that five kinds of lactic acid bacteria are fermented at the same time, and the number of *Bifidobacterium longum* did not decrease significantly. The results showed that the simultaneous addition of five lactic acid bacteria promoted the growth of *Bifidobacterium longum*, the peak value of sample C appeared on the third day (5.49×10^8 CFU/g), then decreased slowly, and the number of *Bifidobacterium longum* was maintained at 10^7 orders of magnitude.

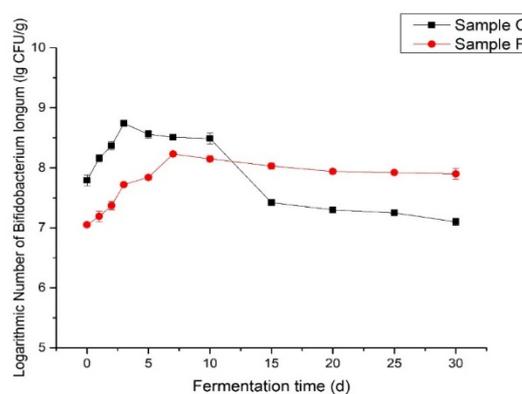


Figure 6 Number changes of *Bifidobacterium longum* during step fermentation of biological feed
 Sample C: Fermentation with *Bifidobacterium longum* alone; Sample F: Five lactic acid bacteria were added simultaneously for fermentation

As shown in Figure 7, the number of *Lactobacillus casei* in sample D is generally higher than that of *Lactobacillus casei* in sample F, indicating that other bacteria begin to compete, and substances produced in the middle and late stages have synergistic or promoting effects on *Lactobacillus casei*. The maximum value of sample D appeared earlier and reached its maximum value (4.68×10^7 CFU/g) on the second day of fermentation, and the number of *Lactobacillus casei* in sample F reached the maximum on the 7th day of mixed fermentation (3.98×10^7 CFU/g).

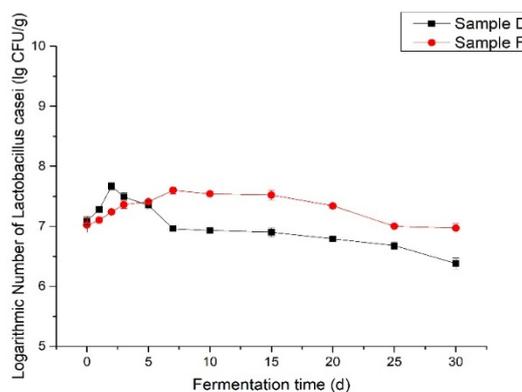


Figure 7 Number changes of *Lactobacillus casei* during step fermentation of biological feed

Sample D: Fermentation with *Lactobacillus casei* alone;
Sample F: Five lactic acid bacteria were added
simultaneously for fermentation

As can be seen from Figure 8, in the initial stage of fermentation, the amount of *Lactobacillus rhamnosus* in sample E is larger than the number of *Lactobacillus rhamnosus* in sample F, and sample E reaches its maximum value (5.62×10^8 CFU/g) on the fifth day of fermentation. Sample F reached a maximum on the 7th day of mixed fermentation (3.16×10^8 CFU/g). In general, the evolution trend of the amount of bacteria was similar, and the fermentation was almost the same on the 30th day.

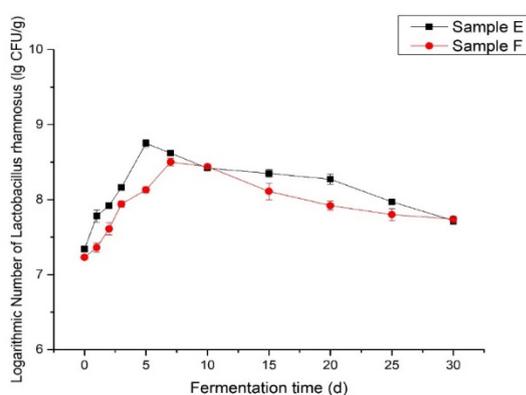


Figure 8 Number changes of *Lactobacillus rhamnosus* during step fermentation of biological feed
Sample E: Fermentation with *Lactobacillus rhamnosus* alone; Sample F: Five lactic acid bacteria were added simultaneously for fermentation

Conclusion

In this chapter, the quantitative analysis of five kinds of lactic acid bacteria, *Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Bifidobacterium longum*, *Lactobacillus casei* and *Lactobacillus rhamnosus*, in the fermentation process of corn stalk bio-feed was carried out by fluorescence quantitative PCR. The results showed that the maximum value of lactic acid bacteria appeared in the 7th day of mixed fermentation. Adding five kinds of lactic acid bacteria at the same time, After seven days of fermentation, it can significantly increase the total bacterial content, and the number of bacterium in mixed fermentation is generally close to that measured by single fermentation. There are slightly more bacteria in the middle and later stages of mixed fermentation, which indicates that the five kinds of lactic acid bacteria have more application value in co-fermentation.

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Compliance with Ethical Standards

Conflict of interest

The authors declared no conflict of interest.

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