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Original paper

Feasibility study of fluorescence quantitative PCR for the detection of microecological dynamics in fermented maize stover feeds

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Abstract

During the fermentation of corn stalk bio-feed, the quantity of bacteria in corn stalk bio-feed was counted by fluorescence quantitative PCR and plate colony counting respectively. The comparative analysis of these studies was used to explore the feasibility of fluorescence quantification methods and changes in microbiota during fermentation. The results showed that the standard deviation of fluorescence quantitative method was smaller than that of plate method, but the trend was similar. The biomass of *Bacillus subtilis*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* reached their maximum on the third, fifth and fifth day respectively, and then decreased gradually and maintained at a certain level. The experiment showed that the fluorescence quantitative PCR method can accurately quantify the number of bacteria in corn stalk bio-feed, and it is a better method to quantitatively detect the dynamic changes of different kinds of bacteria in corn stalk bio-feed.

Keywords

Fluorescence Quantitative PCR; Fermented corn stalk; Flora change

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Introduction

Corn stalk is an important agricultural by-product with high abundance, high energy content and low cost (Kaya et al., 2018). In recent years, a large amount of corn stalks have been burned in the field, resulting in biomass loss and environmental pollution (Zhang et al., 2019). Microorganisms can convert corn stalk into animal protein feed products (Gado et al., 2017). Fermented feed, as a new type of green and environment friendly feed, has become a hot spot in animal nutrition research (Wang et al., 2018).

Lactobacillus is the earliest and most widely used probiotic bacteria (Singhal et al., 2019). It is a kind of anaerobic bacteria. It can regulate the normal flora of gastrointestinal tract, maintain microecological balance (Vieco-Saiz et al., 2019), improve food digestibility (Gao et al., 2019), reduce serum cholesterol, control endotoxin, and inhibit the growth and reproduction of intestinal spoilage bacteria and the production of spoilage products (Zhou et al., 2019b). It can improve the fermentation quality of corn stalk and preserve more nutrients (Liu et al., 2019a). *Bacillus subtilis* is a non-lethal spore with strong tolerance to external high temperature, high pressure and acid and alkali (Sahraei et al., 2019). Moreover, various enzymes synthesized by *Bacillus subtilis* itself (lipase, protease, cellulase, etc.) (Wu et al., 2019) play a role in the digestive tract with the digestive enzymes in animals and improve the digestive level of animals (Li et al., 2019). *Bacillus subtilis* can consume a large amount of oxygen to maintain the intestinal anaerobic environment (Blavi et al., 2019), thus promoting the growth of anaerobic probiotics such as lactobacillus, inhibiting the growth of aerobic pathogenic bacteria, and maintaining the balance of intestinal flora in animals (Zhou et al., 2019a). Yeast can synthesize protein by using sugar and inorganic salts from corn stalk (Liu et al., 2019b), and also can synthesize a small amount of vitamins and growth factors (Camara et al., 2019). The nutritional value of protein synthesized by yeast is close to animal protein. So yeast is often used as a fermentation bacteria for protein feed (Alvarez-Ramirez et al., 2019).

The detection methods of bacterial flora mainly include traditional culture methods and molecular biology methods (Chon et al., 2019). The traditional culture method is the basic method for microbial identification, but there are many problems, such as time-consuming, high cultivation requirements, and many influencing factors (Xiong et al., 2019). The emergence of molecular biology technology makes up for many shortcomings of traditional culture methods. It has the advantages of short detection time, high sensitivity and accurate results (Wang et al., 2019). Fluorescence quantitative polymerase chain reaction can use DNA extracted from samples for quantitative and qualitative analysis (Zhang et al., 2018). Although there have been many reports about the establishment and application of quantitative PCR detection methods, there are few studies on the establishment of quantitative PCR detection methods for the fermentation system of biological

feed, among which the establishment of quantitative PCR detection methods for fermented biological feed flora of corn stalk has not been reported.

At present, the research on fermented feed mostly focuses on the changes of nutrient composition and application effects of feed (Pan et al., 2019), while there are few reports on the changes of microflora in fermented feed. In order to quickly and quantitatively monitor the dynamic changes of *Bacillus subtilis*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* in the fermentation process of corn stalk, a method of rapid detection of bacterial biomass by fluorescence quantitative PCR was established in this experiment, in order to study the production process parameters of fermented feed from the aspect of bacterial flora variation, and to provide guidance for further optimization of production process.

Quantitative real-time fluorescent PCR (qPCR) is a new nucleic acid quantification technique based on conventional PCR. It is based on the addition of fluorescent chemicals to the reaction system. As the amplification products accumulate during the PCR reaction, the intensity of the fluorescence signal increases in equal proportion. At this stage of the reaction, the reaction components are not restricted and the Ct value is highly reproducible, making it a more reliable method of measuring the starting copy number than measuring the cumulative PCR product amount at the endpoint (Giulietti et al., 2001). Absolute quantification is a method for the quantitative analysis of unknown samples using a known standard curve. The standard for absolute quantification is usually a clone of a plasmid, cDNA or PCR product containing the same amplification fragment as the sample to be tested, and the Ct value of each dilution is measured by multiplying the standard and plotting the standard curve using the logarithm of the copy number of the standard as the horizontal coordinate and the measured Ct value as the vertical coordinate (Dhanasekaran et al., 2010). The initial amount of the unknown sample was deduced from the standard curve.

Due to the complexity of fermented feed ingredients, there is no comprehensive quality assessment system for fermented feed in China. Currently, it is generally accepted that the content of live probiotic bacteria is an important indicator for assessing the quality of fermented feeds. The traditional standard method for testing the probiotic content of fermented feeds is the plate count method. Since some lactic acid bacteria can also grow aerobically, the plate method can significantly overestimate the total aerobic bacteria in fermentation processes involving lactic acid bacteria.

Materials and Methods

Bacillus subtilis (CGMCC 1.1086), *Saccharomyces cerevisiae* (CGMCC 2.1527) and *Lactobacillus plantarum* (CGMCC 1.557) were purchased from the China General Microbiological Culture Collection Center (freeze-dried powder, stored at 4°C). The laboratory-preserved *Bacillus subtilis*, *Lactobacillus plantarum* and *Saccharomyces*

cerevisiae were inoculated into fresh liquid LB medium, and then expanded at 30°C. After the bacteria recovered (OD₆₀₀ = 0.8), they were added to corn stalk at 5% inoculation volume for fermentation.

Corn stalks were crushed and passed through a 30 mesh sieve. Take 1 g of the fermented corn stalk and 9 mL of

water, followed by 10-20min of mixture, then take the supernatant, centrifuge for 2 min at high speed, then discard the supernatant and reserve the sediment. The total DNA of each fungus was extracted by Fungal/Bacterial DNA Kit, and the DNA samples were stored at -20°C for reserve.

Table 1. Primer sequence

Strain name	Sequence (5'-3')	Amplification size (bp)
<i>Bacillus subtilis</i>	F: AAAATCCGCGCGTATCGTTG R: CTCGGCCTGATTCGTATGCT	520 bp (<i>gyrA</i> , L. N. Borshchetskaya et al., 2013)
<i>Saccharomyces cerevisiae</i>	F: GCGATAACGAACGAGACCCTAA R: CCAGCACGACGGAGTTTCACAAGAT	225 bp (18s rRNA, Imma Andorra et al., 2011; Hiroshi Makino et al., 2010; Adriana Araújo de Almeida et al., 2014)
<i>Lactobacillus plantarum</i>	F: CAGCACTAGATACCGCCCTG R: ATGTAGTGCCACGGTCGTTT	211 bp (<i>recA</i> gene, Eunice C. Ilha et al., 2015; Xiong, T et al., 2018)

The primer sequences used in this experiment and the size of the amplified fragments can be seen in Table 1 (Borshchetskaya et al., 2013; Makino et al., 2010; Pontonio et al., 2019).

To detect the primer specificity, the genomic DNA of the standard strain was obtained for routine PCR reaction. The reaction system is 50 µL as follows: ddH₂O 35.25 µL, 10 × PCR buffer 5 µL, dNTP Mixture 4 µL, Upstream primer 2 µL, Downstream primer 2 µL, DNA template 1.5 µL, Taq enzyme 0.25 µL. The amplification was carried out by long Gene's NMG96G PCR. The reaction procedure was as follows: pre-denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min, a total of 30 cycles were completed, and elongation at 72°C for 10 min. The conventional PCR reaction was performed by crossing the genomic DNA of *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Lactobacillus plantarum* with primers, the amplified products of conventional PCR were analyzed by 3% agarose gel electrophoresis.

Fluorescence quantitative polymerase chain reaction system was as follows: SYBR^(R) Green Realtime PCR Master 10 µL, ddH₂O 6 µL, template 2 µL, forward primer 0.8 µL, reverse primer 0.8 µL, ROX 0.4 µL. The amplification and analysis were carried out by 7300 fluorescence quantitative PCR. Using the corresponding primers and templates for quantitative fluorescence PCR, the relationship between the number of template cycles

and the fluorescence intensity of different copies can be obtained.

The DNA fragments recovered from the gel-cut products were used as the standard for quantitative fluorescence PCR, and the OD_{260/280} value and concentration of the fragments were measured and converted to the copy number of 1µL of each standard strain for making standard curves. The standard products were diluted 10 times to 1 × 10⁻⁷–1 × 10⁻² copies/µL, and SYBR Green was used as fluorescent dye for real-time quantitative PCR. The standard is a single-copy cloning plasmid containing the target fragment, and a series of standard products are used as templates for the fluorescence quantitative PCR reaction. Converting the concentration of the series of standards into the copy number of plasmid DNA copies, and the calculation formula is shown as follows:

$$\text{Copy number} = \text{DNA concentration (ng/}\mu\text{L)} \times 10^{-9} \times 6.023 \times 10^{23} / (660 \times \text{base number})$$

After the reaction is completed, the Ct value is automatically analyzed by the system software, and the standard curve is generated.

The DNA of the sample to be tested was quantified by fluorescence quantitative polymerase chain reaction according to the same reaction system and reaction conditions of the preparation standard curve. Standard calibration and ddH₂O instead of DNA template were used as negative control in each experiment. Three parallel samples were made for each sample to ensure the

validity and repeatability of the test data. The copy number of the three bacteria in the fermentation sample was calculated according to the cycle number and standard curve, and the results were expressed by the logarithm of the number of bacterial copies per gram of sample (base 10).

Results

Primer specificity verification

The conventional PCR products of the standard strains were analyzed by 1% agarose gel electrophoresis.

The results showed that *Bacillus subtilis* had a specific band at 520 bp, While *Saccharomyces cerevisiae* and *Lactobacillus plantarum* had no specific bands. *Saccharomyces cerevisiae* had a specific band at 224 bp, but *Bacillus subtilis* and *Lactobacillus plantarum* had no such band. *Lactobacillus plantarum* also has its own specific band at 211 bp (Fig. 1). The above results indicate that the primers designed in this experiment can correctly and specifically identify the three kinds of bacteria, which provides a basis for quantitative determination of three kinds of bacteria by using these primers.

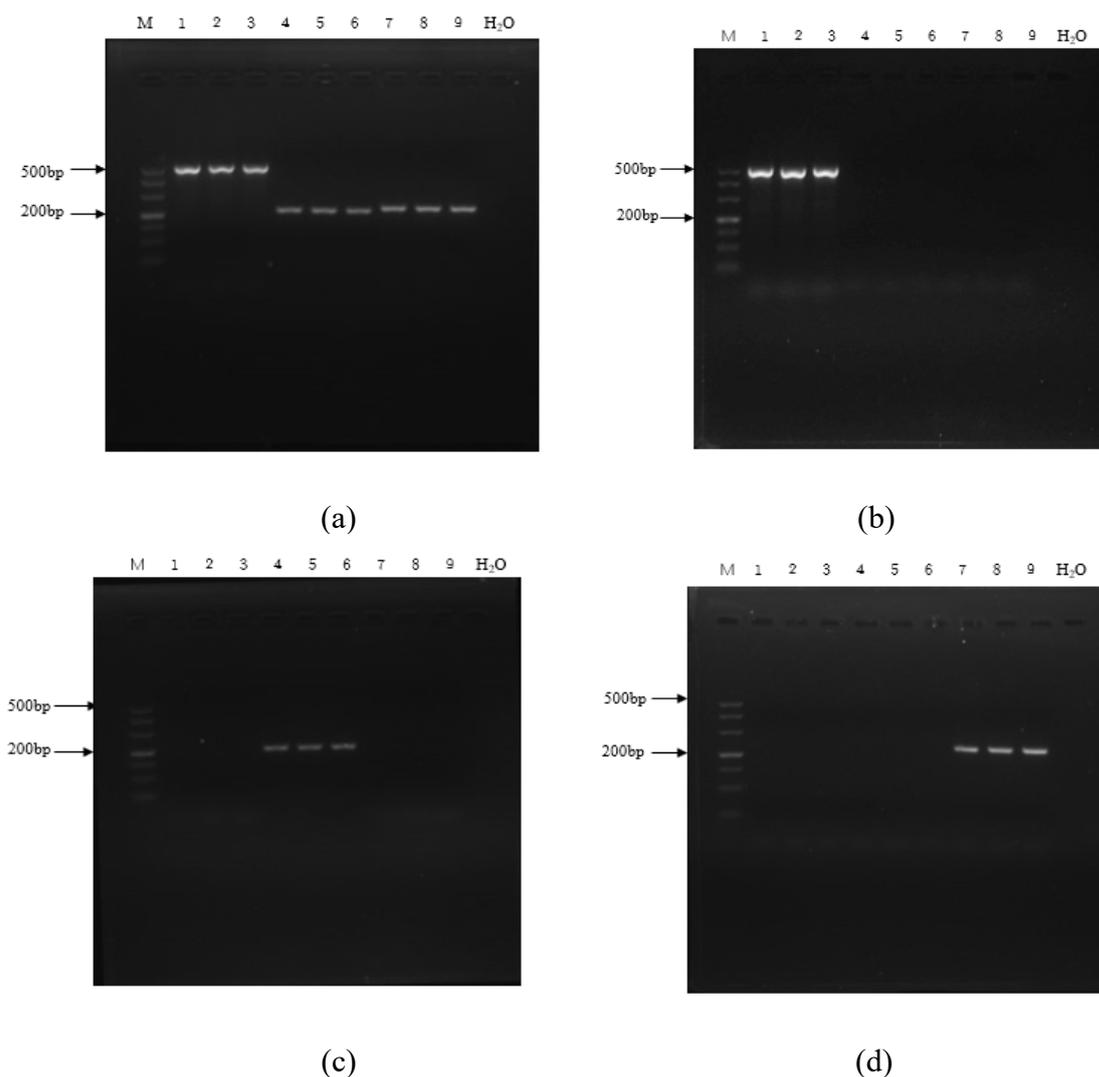


Figure 1. Validation of primer specificity by PCR. *M*: DL500 marker; (a) Lane1-3: *Bacillus subtilis*(520 bp); Lane4-6:*Lactobacillus plantarum*(211 bp); Lane7-9: *Saccharomyces cerevisiae*(225 bp); (b) Specificity Verification of *Bacillus subtilis* Primers(520 bp); (c) Specificity Verification of *Lactobacillus plantarum* Primers(211 bp); (d) Specificity Verification of *Saccharomyces cerevisiae* Primers(225 bp).

Standard curve establishment

The logarithms of the standard samples with different copies were taken as abscissa coordinates, and the initial cycle number (Ct) which reached the fluorescence threshold in the process of quantitative PCR was taken as ordinate to obtain the standard curves of the three bacteria,

which provides a reference standard for the quantification of the sample to be tested (Fig. 2). As shown in Fig. 2, the correlation coefficient of standard curve R^2 is above 0.99, and the amplification efficiency is $90\% \leq E \leq 110\%$, Which is in line with the basic requirements of qPCR.

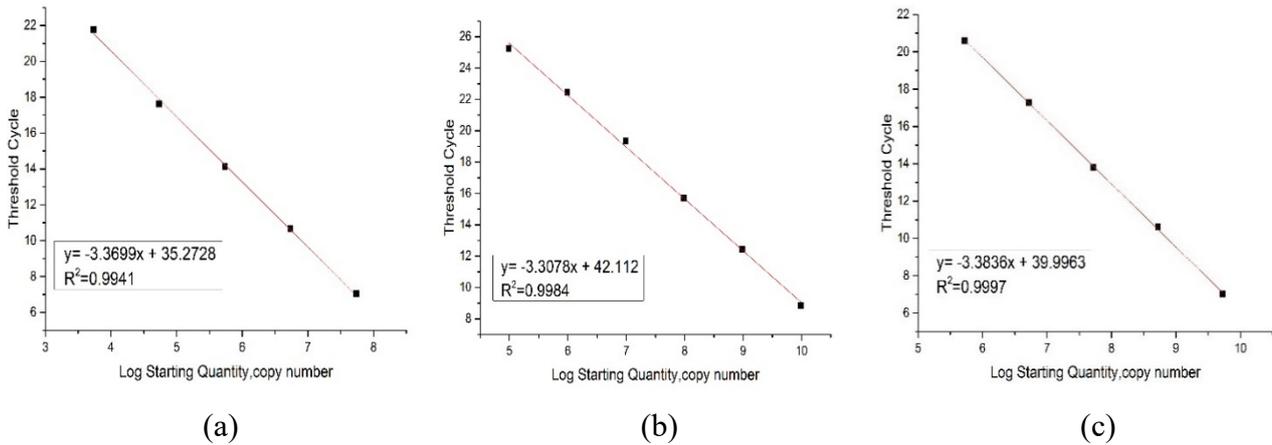


Figure 2. Standard curve. (a) *Bacillus subtilis* standard curve; (b) *Lactobacillus plantarum* standard curve; (c) *Saccharomyces cerevisiae* standard curve

Melting curve

After the reaction was completed, the melting curves of the three bacteria were analysed. The results showed that the amplification products were single and that the DNA

fragments bound by the SYBR Green II fluorescent dye were targeted (Fig. 3).

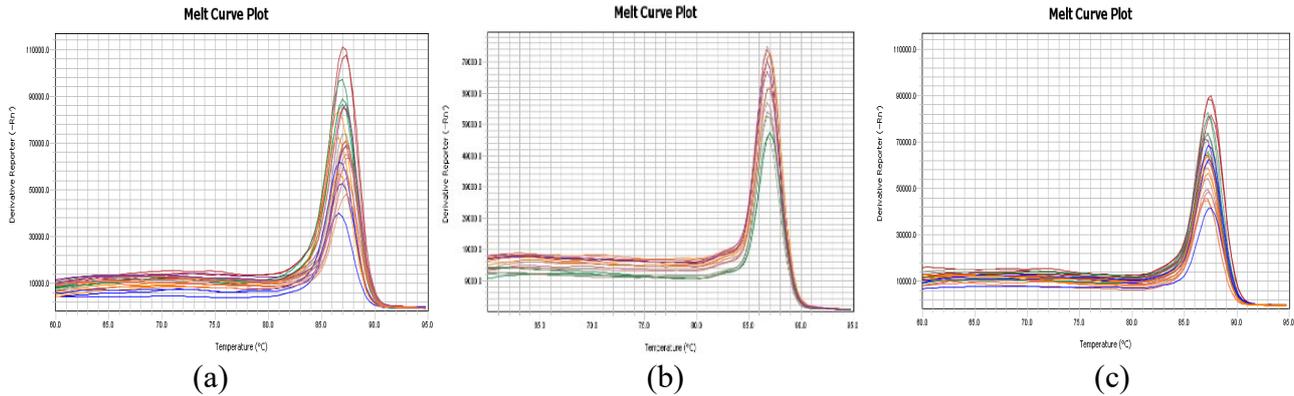


Figure 3. Melting curve. (a) *Bacillus subtilis* melting curve; (b) *Lactobacillus plantarum* melting curve; (c) *Saccharomyces cerevisiae* melting curve.

Comparison between plate method and fluorescence quantitative PCR

DNA samples were extracted at day 0, 1, 2, 3, 5, 7, 10, 15, 20, 25 and 30 d for quantitative fluorescence PCR. As shown in Fig. 4, 5 and 6, the results of fluorescence quantitative PCR and traditional plate colony counting are different. The standard deviation of the results of fluorescence quantitative PCR is more smaller than that of the plate counting method, but the trend of bacterial quantity is approximately the same.

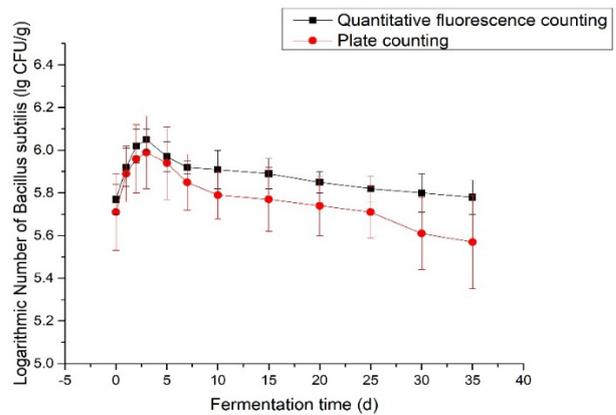


Figure 4. Counting *Bacillus subtilis* by Fluorescence Quantitative PCR and Plate method

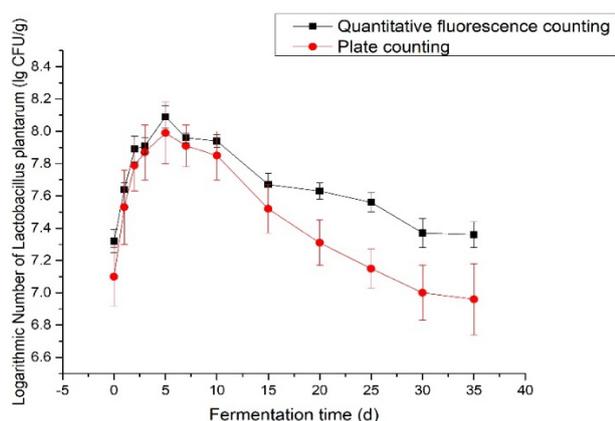


Figure 5. Counting *Lactobacillus plantarum* by Fluorescence Quantitative PCR and Plate method

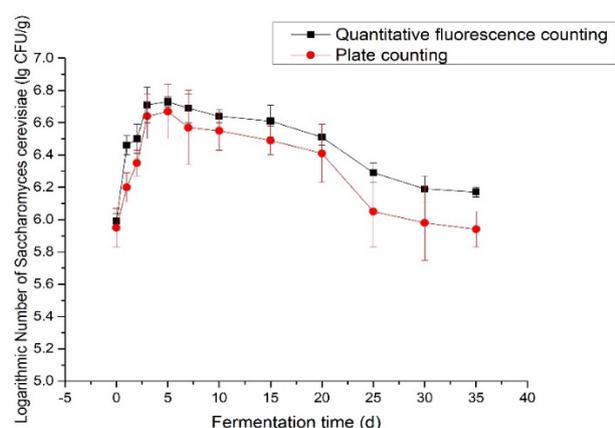


Figure 6. Counting *Saccharomyces cerevisiae* by Fluorescence Quantitative PCR and Plate method

Discussion

The traditional bacterial quantitative methods can only detect culturable bacteria quantitatively, but can not detect many unculturable and unknown bacteria, including the dead bacteria. Moreover, their sensitivity is very low, time-consuming and laborious, and they are susceptible to the influence of operation methods (Cardoso et al., 2019). In addition, due to the different growth conditions and growth rates of various bacteria in fermented feed flora, the culture identification method is not accurate enough (Tabanelli et al., 2019).

In this study, a rapid quantitative detection method for *Bacillus subtilis*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae* in the fermentation process of corn stalk bio-feed was established. The results of the plate counting method in the experiment were higher than those measured by the fluorescence quantitative PCR method, this may be because the traditional plate counting method only counts live bacteria, while the fluorescence quantitative PCR method is based on the concentration and purity of DNA in the samples of corn stalk bio-feed, the bacterial DNA of all states is amplified together, so that the results

can accurately reflect all the colonies of the target bacteria, including live bacteria and dead bacteria, making the results higher (Ilha et al., 2016). As a result, the experimental results are different from those of traditional methods. Despite the inactivity of the dead bacteria, many metabolites were still reserved, for example, the cell walls of the dead bacteria still contain dextran and mannan, which can adsorb toxins. Lactic acid bacteria can inhibit the adhesion and invasion of pathogenic bacteria to intestinal wall as well as cell debris and its metabolites. As long as the intake is large, it also has good complex immune function, which can promote the proliferation of bifidobacteria and regulate the balance of bacterial flora. The dead cells of bifidobacteria, like live bacteria, can inhibit the growth of tumors and reduce the formation of carcinogens. Dead cells are not affected by gastric acid and bile, and are not afraid of heating; therefore, both living probiotics and dead probiotics contain beneficial components of fermented bio-feed, which can improve the quality of fermented feed (Adams, 2010), so we believe that the fluorescence quantitative PCR can more accurately identify the amount of bacteria in corn stalk bio-feed.

The results of fluorescence quantitative PCR counting can well reflect the change rule of bacterial quantity in the fermentation process of corn stalk bio-feed. The bacterial quantity in the initial stage of bio-feed fermentation is higher, which may be due to the synergistic effect of three bacteria. *Bacillus subtilis* proliferates in the early stage of fermentation and consumes a lot of oxygen, thus promoting the growth of lactic acid bacteria; yeast can also synthesize proteins from sugars and inorganic salts in corn stalks, and can also synthesize small amounts of vitamins and growth factors.

The highest bacterial biomass occurs on the fifth day when corn stalk is fermented as bio-feed. With the prolongation of fermentation time, the quantity of *Bacillus subtilis* and *Saccharomyces cerevisiae* declined continuously, when the pH drops to a certain level, the growth of *Lactobacillus plantarum* is also inhibited, showing a slow downward trend and gradually becoming stable.

Conclusions

The numbers of *Bacillus subtilis*, *Lactobacillus plantarum* and *Saccharomyces cerevisiae*, as determined by fluorescent quantitative PCR, reached their maximum on days 3, 5 and 5 respectively, showing a rapid increase, followed by a slow decrease and finally a plateau. Fluorescence Quantitative PCR technology can quantify the number of different kinds of bacteria in the fermentation process of corn stalk bio-feed quickly and accurately, and the experiment has good repeatability, which lays the experimental foundation for the future detection of the same genus and different species of bacteria. It provides an effective detection method for the research of corn stalk bio-feed, and also provides guidance for optimizing the fermentation process of other bio-feed.

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Compliance with ethical standards

The authors declare that they have no conflict of interest. The authors declared no conflict of interest. This article does not contain any studies with human participants performed by any of the authors.

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