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Study on the Antibacterial Rule in Fermented Feed with Different Amounts of $CaCO_3$ by Quantitative Real-Time Polymerase Chain Reaction

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Abstract: Fermented feed is needed to maintain the vitality of probiotics and cannot be sterilized. Fermented feeds, especially those with added $CaCO_3$, have a high risk of contamination with pathogens. *Escherichia coli*, *Staphylococcus aureus*, and *Shigella flexneri* are the main pathogenic bacteria threatening animal breeding. This study developed a new microbial quantitative real-time PCR analysis method to investigate the antibacterial rule in fermented feed with different amounts of $CaCO_3$. Moreover, using the qPCR method, we found that the feed pH decreased slowly with the increase of $CaCO_3$ addition. In the early stage of fermentation, $CaCO_3$ addition promoted three pathogenic bacteria growth. In the middle and late fermentation, $CaCO_3$ addition inhibited the growth of *Escherichia coli* and *Shigella flexneri*, and the greater the $CaCO_3$ addition, the stronger the inhibitory effect. The $CaCO_3$ addition reduced the growth inhibition of *Staphylococcus aureus*, and the inhibition effect was weaker with the increase of $CaCO_3$ addition. From the inhibitory effect on intestinal pathogenic bacteria such as *Escherichia coli* and *Shigella flexneri*, the optimal addition amount of $CaCO_3$ was 12%. At this level of addition, the number of *Lactiplantibacillus plantarum* subsp. *plantarum*, *Lacticaseibacillus rhamnosus*, and *Bacillus subtilis* were also the highest, and the content of organic acids with antibacterial effects was also the highest. The addition of $CaCO_3$ had an inhibitory effect on the growth of pathogenic bacteria, mainly attributed to the promotion of the growth of *Lactiplantibacillus plantarum* subsp. *plantarum* and *Lacticaseibacillus rhamnosus*, and the organic acid of its fermentation product had an inhibitory effect on pathogenic bacteria. This study provides theoretical guidance for the antibacterial rule of high-pH fermented feed with different amounts of $CaCO_3$.

Keywords: animal nutrition; organic acid; solid-state fermentation; *Escherichia coli*; *Lacticaseibacillus rhamnosus*

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1. Introduction

Pathogenic bacteria (PB) are a huge threat to animal breeding. Main PB are *Escherichia coli*, *Staphylococcus aureus* and *Shigella flexneri* [1]. If an animal is infected with these PB, it will cause serious bodily disease and even death. Studies have reported that *Escherichia coli* infection can cause diarrhea, meningitis, sepsis, atherosclerosis, etc. [2], and *Staphylococcus aureus* infection can cause vomiting, diarrhea, and abdominal distension [3]. Animal feed is one of the main sources of PB. Traditional feed can be sterilized by high-temperature cooking and other methods. However, fermented feed is rich in a large number of probiotics, and sterilization before use will cause serious damage to the probiotics and greatly reduce the probiotic effect of fermented feed. Moreover, the water content of fermented feed is generally higher than that of traditional feed, and the possibility of spoilage leading to carrying PB is higher.

Soybean meal (SBM) and bean dregs (BDs) are high-quality raw materials for the production of fermented feed. SBM is known for its balanced amino acid composition and high protein content compared to other plant protein sources [4]. The protein content of SBM is generally 45–55%, of which more than 80% is water-soluble [5]. BDs are the by-products in the processing of soybean products such as soy milk and tofu, which are nutritious but inexpensive. BDs are high in fat, protein, isoflavones, and other nutrients [6]. The solid-state fermentation process is widely used in the production of fermented feed due to its simple equipment and low production cost [7]. Because some nutrients in BDs and SBM are destroyed in the process of high-temperature sterilization, their nutrient and energy utilization rates are very low, and their potential utilization value is not fully realized. Therefore, solid-state fermentation materials such as BDs and SBM often do not undergo sterilization treatment, and the fermentation process cannot achieve an absolutely sterile environment. This greatly increases the risk of carrying PB in fermented feed. The probiotics used in animal nutrition are mainly strains of different genera, such as *Lactobacillus*, *Bacillus*, *Pediococcus*, and *Enterococcus* [8]. Other probiotics are microscopic fungi, such as *Saccharomyces cerevisiae* [9]. These probiotics are also added to fermented feed as fermentation strains and produce some beneficial products for animal health. Studies have reported that many fermentation products, such as organic acids, antibacterial peptides, and bacteriocins, have certain antibacterial effects [10,11]. *Lactobacillus rhamnosus* and *Lactobacillus plantarum* are probiotics that can be added to animal feed [12], and they can produce organic acids [13]. Organic acids can lower the pH of feed and inhibit the growth of PB. Because of its antibacterial and immune regulatory activities, it has been applied in animal production [14]. Nithya and Halami [15] found that the antibacterial peptide secreted by *Bacillus subtilis* exhibited promising antibacterial properties and can be considered a potential candidate as a biopreservative agent. *Saccharomyces cerevisiae* and its cellular components (mannaoligosaccharides, glucooligosaccharides, and enzymes) can improve the growth, immunity, and intestinal health of cultured animals [16]. The proliferation of microorganisms forms nutrient competition for PB and has an inhibitory effect on their growth. However, the antibacterial rule of fermented feed on specific PB remains to be studied.

Calcium (Ca) is crucial for egg formation, eggshell strength, and thickness. Adequate Ca intake by laying hens is key to ensuring the quality of eggshells [17]. Research has shown that when laying hens continue to consume Ca, they can fully utilize their egg production performance [18]. Therefore, in the process of laying hens breeding, Ca supplements such as $CaCO_3$ are often added to the feed. In addition, during the fermentation process, $CaCO_3$ is often added to co-ferment with microorganisms to form organic acid calcium, small peptide calcium, etc. [19,20], which can effectively improve the absorption and utilization of Ca and increase the nutritional value of fermented feed. The added $CaCO_3$ can neutralize the organic acids secreted by the microorganisms so that the fermentation environment maintains a high pH value and a high Ca^{2+} osmotic pressure. This may cause the fermentation strains to not grow and metabolize normally or may even cause them to die. The fermentation process maintains a constant high pH, which is easily contaminated by PB such as *Escherichia coli*, *Staphylococcus aureus*, and *Shigella flexneri*, resulting in fermentation failure.

The traditional microbial quantitative analysis method is mainly to determine the species and quantity of microorganisms by enrichment culture on plates and evaluation of the microorganism morphology and culture characteristics [21]. Traditional quantitative analysis methods are time-consuming and laborious, and different strains have a large influence on each other [22]. In addition, there are a large number of microorganisms that cannot be cultured in the sample, which leads to lower test results [23]. This is also one of the reasons why some feed production enterprises have poor accuracy in microbial detection in feed products. Quantitative real-time PCR (qPCR) is increasingly applied to microbial quantitative analysis. The microbial quantitative analysis by qPCR is efficient, simple, and accurate [24]. However, the accurate analysis of qPCR depends on the specificity of the

primers used for the particular strain. The specificity of the strain primer is especially important when determining the various strains in a mixed multi-strain sample.

This study developed a qPCR method for the determination of fermenting microorganisms and PB. *Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*, and different amounts of $CaCO_3$ were added simultaneously at the beginning of fermentation. The growth of fermenting microorganisms, PB, and changes in organic acid content in fermented feed were analysed to investigate the antibacterial rule of fermented feed with different amounts of $CaCO_3$. Moreover, the results of this study are expected to be applied to the microbial quantitative detection process of feed products in feed production enterprises in the future.

2. Materials and Methods

2.1. Materials and Reagents

BDs and SBM were purchased from a bean processing factory in Zhenjiang City (Jiangsu, China). All solvents and chemicals are analytical reagent grade or higher and are purchased from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China). Acid protease and papain were purchased from Ningxia Xia Sheng Enzyme BioEngineering Co., Ltd. (Ningxia, China), and the activity of these enzymes is 60,000 U/g.

2.2. Microorganisms

Lactiplantibacillus plantarum subsp. *plantarum* (CGMCC 1.557), *Lacticaseibacillus rhamnosus* (CGMCC 1.2467), *Bacillus subtilis* (CGMCC 1.1086), and *Saccharomyces cerevisiae* (2.1527) were purchased from the China General Microbiological Culture Collection Center. *Shigella flexneri* (CMCC 51572) was purchased from the National Center for Medical Culture Collections. *Escherichia coli* (ATCC 43888) and *Staphylococcus aureus* (ATCC 6538) were preserved by our laboratory.

2.3. Expanding the Culture of Strains and Configuration of Culture Media

Luria–Bertani (LB) liquid medium (1 L): 10 g tryptone, 10 g sodium chloride, and 5 g yeast extract were accurately weighed in a 1 L beaker. A total of 950 mL of distilled water was then added to the beaker and blended on a magnetic stirrer. The liquid was placed in a 1 L volumetric bottle and filled to 1000 mL. The medium was then evenly divided into four 500 mL conical glass bottles. These cone-shaped glass bottles containing the medium were then autoclaved at 121 °C for 20 min. Malt extract medium (1 L): 130.1 g wort powder was accurately weighed and placed in a 1 L beaker filled with 700 mL water. After the powder was heated and dissolved, the liquid was placed in a 1 L volume bottle, and the volume was fixed to 1000 mL. The liquid was divided into four 500 mL conical glass bottles. It was then autoclaved at 121 °C for 15 min. *Bacillus subtilis*, *Lactiplantibacillus plantarum* subsp. *plantarum*, *Lacticaseibacillus rhamnosus*, *Escherichia coli*, *Staphylococcus aureus*, and *Shigella flexneri* were cultured with LB medium at 37 °C for 16 h, respectively. *Saccharomyces cerevisiae* was cultured with malt extract medium at 28 °C for 16 h.

2.4. Primer Specificity Verification

The strains were cultured to OD_{600} = 1.00, and the DNA of each bacterial was extracted with the FastPure Bacteria DNA Isolation Mini Kit (Vazyme, Nanjing, China); moreover, yeast DNA was extracted by fungal genome extraction kit (Takara, Kusatsu, Japan). The specificity of each primer was verified by routine PCR with the DNA of other strains. The reaction system was as follows: 2× Rapid Tag Master Mix 10 μL (Vazyme, Nanjing, China), Upstream primer 0.8 μL, Downstream primer 0.8 μL, DNA template 2 μL, ddH_2O 6.4 μL. 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 was at 72 °C for 10 min. The PCR-amplified products were analyzed by 3% agarose gel electrophoresis. The primer sequences used in this study can be seen in Table 1 [25–27].

Table 1. Specific primers of strains.

Strains	Primers (5′-3′)	Amplified Fragment
Bacillus subtilis	F: CGTAGAGCCACTTGAGCG R: CTGCCGTTACAGTTCCTT	257
Saccharomyces cerevisiae	F: GCGATAACGAACGAGACCCTAA R: CCAGCACGACGGAGTTTCACAAGAT	225
Lactiplantibacillus plantarum subsp.	F: GTGGTGCGGTCGATATTTTAGTT R: TCAGCCGCGCTTGTAACC	108
Lacticaseibacillus rhamnosus	F: GACGCAGCCGGTTGACCCAA R: GGCGGCAGTTGCCCCAGAAT	376
Escherichia coli	F: GCACTAAAAGCTTGGAGCAGTTC R: AACAATGGGTCAGCGGTAAGGCTA	178
Staphylococcus aureus	F: GCGATTGATGGTGATACGGTT R: AGCCAAGCCTTGACGAACTAAAGC	279
Shigella flexneri	F: GAGATTCCTGCTCCGCTAA R: TGCGAGGTAGTTGACATTGG	323

2.5. Establishment of Strain Standard Curves for qPCR Determination

The establishment of strain standard curves refers to the method of Yu, Dong, and Lu [28] with slight modification. The DNA fragments amplified by specific primers were recovered as the standard for qPCR. The concentration of the fragments was measured and converted to the copy number of each standard strain for making standard curves. The calculation formula was copy number = DNA concentration (ng μL^{-1}) $\times$ 10^{-9} $\times$ 6.023 $\times$ 10^{23}/(660$\times$ base number). The standards were diluted 10 times to 10^{-7}~10^{-2} copies μL^{-1}, and SYBR Green was used as fluorescent dye for qPCR. qPCR reaction system was SYBR Qpcr Master Mix (Vazyme, Nanjing, China) 10 µL, Upstream primer 0.8 µL, Downstream primer 0.8 µL, DNA template 2 µL, ddH_2O 6.4 µL. The reaction procedure was as follows: predenaturation at 95 °C for 30 s, amplification at 95 °C for 5 s, 60 °C for 31 s, a total of 40 cycles. After the reaction, it was warmed to 95 °C 15 s, then to 60 °C 1 min, and then to 95 °C 1 s. The amplification and analysis were carried out by Thermofisher QuantStudio 3 (Thermo Fisher Scientific, Singapore). 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.

2.6. Solid-State Fermentation Process

The solid-state fermentation process referred to the research of Heng et al. [29] and made slight modifications based on our experimental results (Figure 1). Firstly, 120 g BDs and 120 g SBM are mixed in a plastic bowl with a bare mouth. The mixture was stirred evenly and fermented under the aerobic condition at 30 °C for 14 h. Then, *Saccharomyces cerevisiae*, *Lactiplantibacillus plantarum* subsp. *plantarum*, *Lacticaseibacillus rhamnosus*, and 0.65% acid protease + 0.65% papain (*w*/*w*) were added. The mixture was stirred evenly and was put into a fermentation bag containing a one-way vent. Finally, the fermentation bag was sealed, and the fermentation was conducted under an anaerobic condition at 30 °C in a constant temperature incubator. Three PB (*Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*) and different amounts of $CaCO_3$ (0, 2, 4, 8, and 12%, *w*/*w*) were added before sealing the fermentation bag for anaerobic fermentation. The inoculum size of each strain was 1.25% (*v*/*w*), and the strains were cultured to OD_{600} = 1.00.

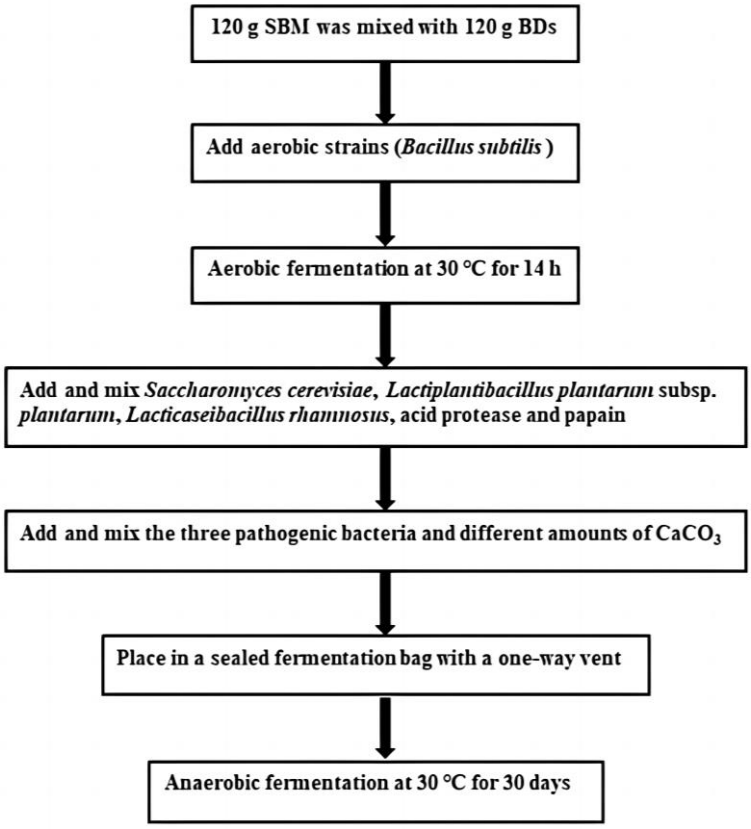

Figure 1. Initial fermentation process.

2.7. Changes of Fermented Feed pH and Organic Acid Content with Different Amounts of $CaCO_3$

Samples were taken on days 0, 1, 4, 7, 11, 15, 20, and 30 of fermentation to determine the feed pH. Samples were taken on 0, 7, 15, and 30 days of fermentation to determine the organic acid content.

2.8. Microorganism Detection with qPCR

Samples were taken at 12 h, 1 day, 4 days, 7 days, 11 days, 15 days, 20 days, and 30 days of fermentation to determine three PB by qPCR. Samples were taken on days 0, 7, 15, and 30 of fermentation to determine *Bacillus subtilis* by qPCR. Samples were taken at 12 h, 7 days, 15 days, and 30 days of fermentation to determine *Saccharomyces cerevisiae*, *Lactiplantibacillus plantarum* subsp. *Plantarum*, and *Lacticaseibacillus rhamnosus* by qPCR. The extraction of microbial DNA in fermented feed also refers to the method of Yu, Dong, and Lu [28] with slight modification. Sample (1.00 g) was mixed with 10 mL deionized water, shaken for 20 min, and filtered through a 200 mesh sieve. Filtrate (2 mL) was used to extract DNA for qPCR, and bacterial DNA was extracted by the FastPure Bacteria DNA Isolation Mini Kit (Vazyme, Nanjing, China); yeast DNA was extracted by fungal genome extraction kit (Takara). The remaining qPCR steps are as described above.

2.9. Determination of Fermented Feed pH and Organic Acids

Feed pH was determined according to the method of Elfalleh et al. [30]. The sample (2.00 g) was mixed with 20 mL of deionized water, soaked for 2 h, and measured with a pH meter (Sartorius PB-10, Sartorius, Beijing, China). Organic acids were determined by the method of Moghaddam, Zhang, and Du [31] with slight modification. Sample (1.00 g) was mixed with 20 mL pH 2.7 HCl solution, sonicated for 30 min, shaken and soaked for 3.5 h, and then centrifuged at 6000× *g* for 35 min. The supernatant was diluted with HCl solution (pH 2.0) at 1:1 and filtered through a 0.45 μm filter membrane before HPLC analysis (SHIMADZU, Kyoto, Japan). Chromatographic conditions: mobile phase,

0.01 mol L^{-1} KH_2PO_4 (pH 2.7):methanol = 97:3; chromatographic column: Welch AQ-C18; detection wavelength: 210 nm; flow rate: 0.6 mL min^{-1}. The total organic acid content was the sum of each acid content.

2.10. Statistical Analysis

All experiments were performed in triplicate. All data are expressed as the mean ± SD and were analyzed using one-way analysis of variance (ANOVA) in SPSS 25.0 and plotted using Origin Pro8. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Strain Primer Specificity Verification

In this study, except for the nucA gene used in *Saccharomyces cerevisiae* and *Staphylococcus aureus*, the other five bacterial strains all used the recA gene, and these two genes have been confirmed to have been optimized for amplification methods and used for quantitative analysis of micrograms numbers [25–27]. Each strain primer used in the experiment can only bind to its own DNA, not to other strains of DNA (Figure 2), so the specificity of each strain primer is good.

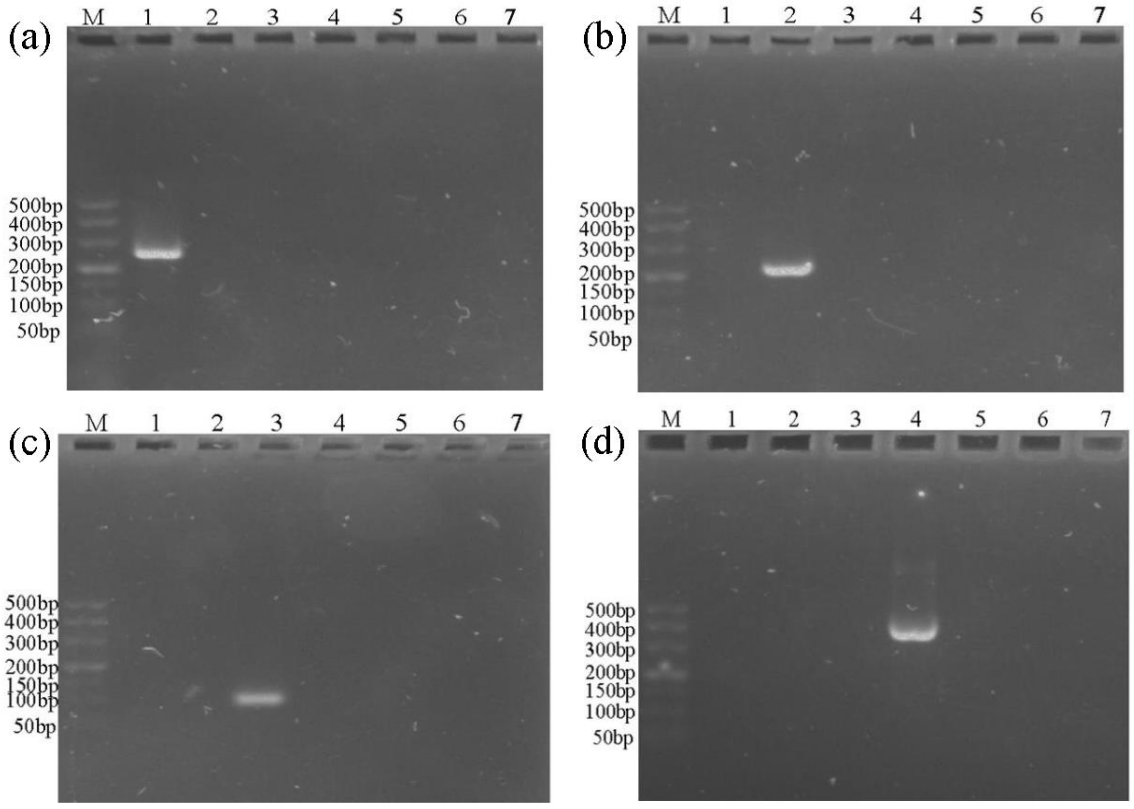

Figure 2. *Cont.*

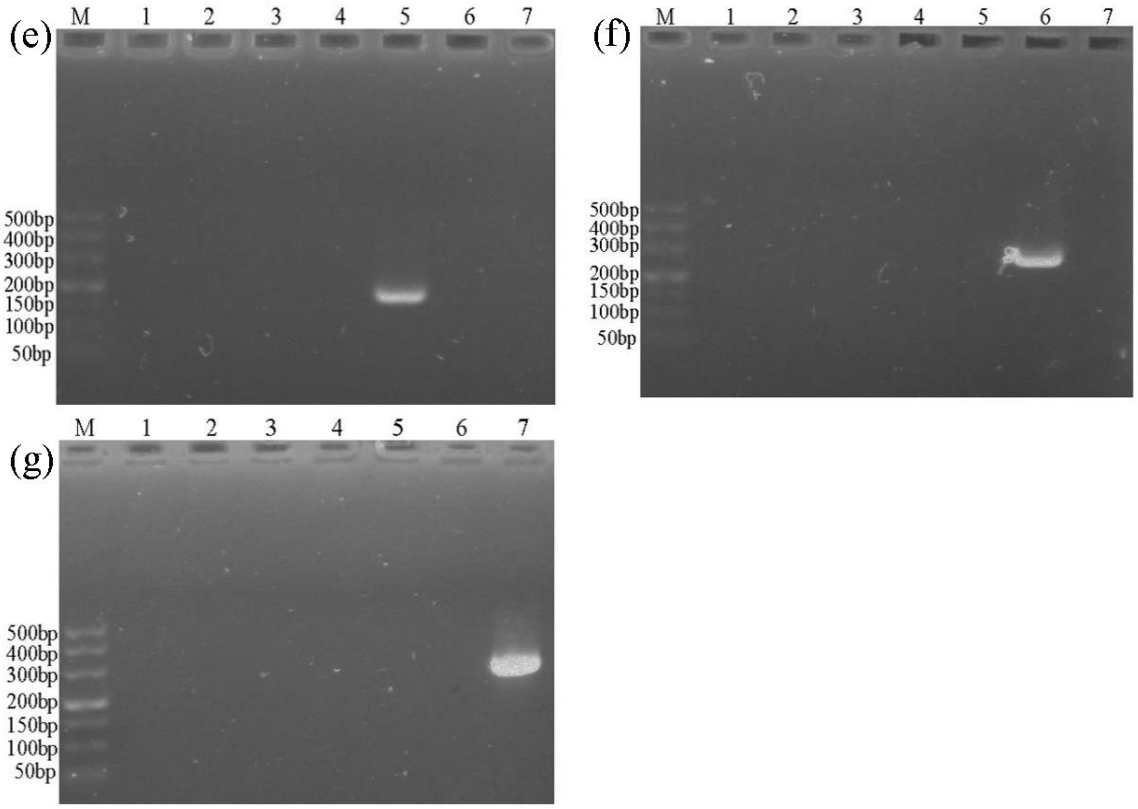

Figure 2. Specificity verification of strain primers. (**a**) *Bacillus subtilis*; (**b**) *Saccharomyces cerevisiae*; (**c**) *Lactiplantibacillus plantarum* subsp. *plantarum*; (**d**) *Lacticaseibacillus rhamnosus*; (**e**) *Escherichia coli*; (**f**) *Staphylococcus aureus*; (**g**) *Shigella flexneri*. M: DNA Marker (50~500 bp); 1: *Bacillus subtilis*; 2: *Saccharomyces cerevisiae*; 3: *Lactiplantibacillus plantarum* subsp. *plantarum*; 4: *Lacticaseibacillus rhamnosus*; 5: *Escherichia coli*; 6: *Staphylococcus aureus*; 7: *Shigella flexneri*.

3.2. Establishment of qPCR Strain Standard Curves

During the PCR process, the fluorescence signal of the sample amplification products shows an s-shaped curve with exponential cycle, linear cycle, and plateau phase [32]. In the exponential phase, there is a linear relationship between the initial concentration of the sample and the number of amplification cycles (Ct value) corresponding to the sample. The standard curves of strains are shown in Table 2.

Table 2. Standard curves of strains.

Strains	Standard Curves	Related Coefficient	Efficiency Coefficient
Bacillus subtilis	Y = −3.3493X + 35.973	R^2 = 0.9996	98.87%
Saccharomyces cerevisiae	Y = −3.4505X + 38.2299	R^2 = 0.9977	94.90%
Lactiplantibacillus plantarum subsp. *plantarum*	Y = −3.4474X + 35.226	R^2 = 0.9999	95.01%
Lacticaseibacillus rhamnosus	Y = −3.4732X + 36.781	R^2 = 0.9951	94.05%
Escherichia coli	Y = −3.3464X + 39.203	R^2 = 0.9990	98.98%
Staphylococcus aureus	Y = −3.4632X + 38.2299	R^2 = 0.9991	94.42%
Shigella flexneri	Y = −3.2115X + 35.226	R^2 = 0.9985	104.48%

3.3. Changes of Fermented Feed pH with Different Amounts of $CaCO_3$

As shown in Figure 3, with the increase of $CaCO_3$ addition, the pH drop of fermented feed slows down. Without $CaCO_3$ addition, the pH of fermented feed was lower than 4.5 in 4 days of fermentation and dropped to about 4.1 in 30 days of fermentation. The pH of fermented feed with 2% $CaCO_3$ dropped to below 4.5 in 15 days and was around 4.3 in 30 days. The pH of fermented feed with 4% $CaCO_3$ dropped to about 4.5 in 30 days. The

pH of fermented feeds with 8% and 12% $CaCO_3$ was always above 5.0 during one month of fermentation.

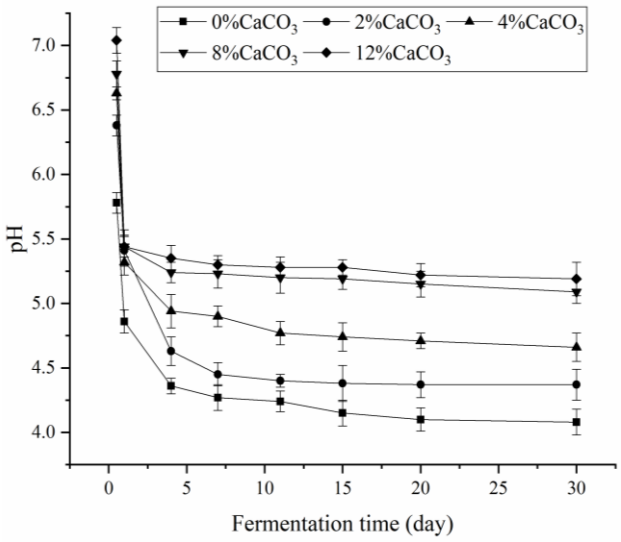

Figure 3. Changes of fermented feed pH with different amounts of $CaCO_3$.

3.4. Growth Changes of Escherichia coli, Staphylococcus aureus, and Shigella flexneri with Different Amounts of $CaCO_3$

As shown in Figure 4a, in the early stage of fermentation, the addition of $CaCO_3$ promoted the growth of *Escherichia coli*, of which 4% was the most obvious, followed by 8%. The addition of $CaCO_3$ made the maximum growth of *Escherichia coli* (2.63×10^7 copies g^{-1}) appear in advance to the 4th day, while the maximum growth of *Escherichia coli* (2.14×10^7 copies g^{-1}) appeared on the 7th day without the addition of $CaCO_3$, and the maximum growth of *Escherichia coli* in the feed with the addition of $CaCO_3$ was higher than that in the feed without the addition of $CaCO_3$. In the middle and later stages of fermentation, the number of *Escherichia coli* decreased with the increase of $CaCO_3$ addition.

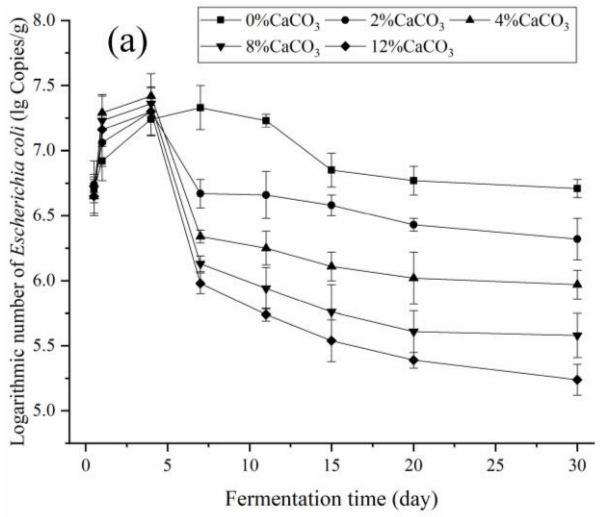

Figure 4. *Cont.*

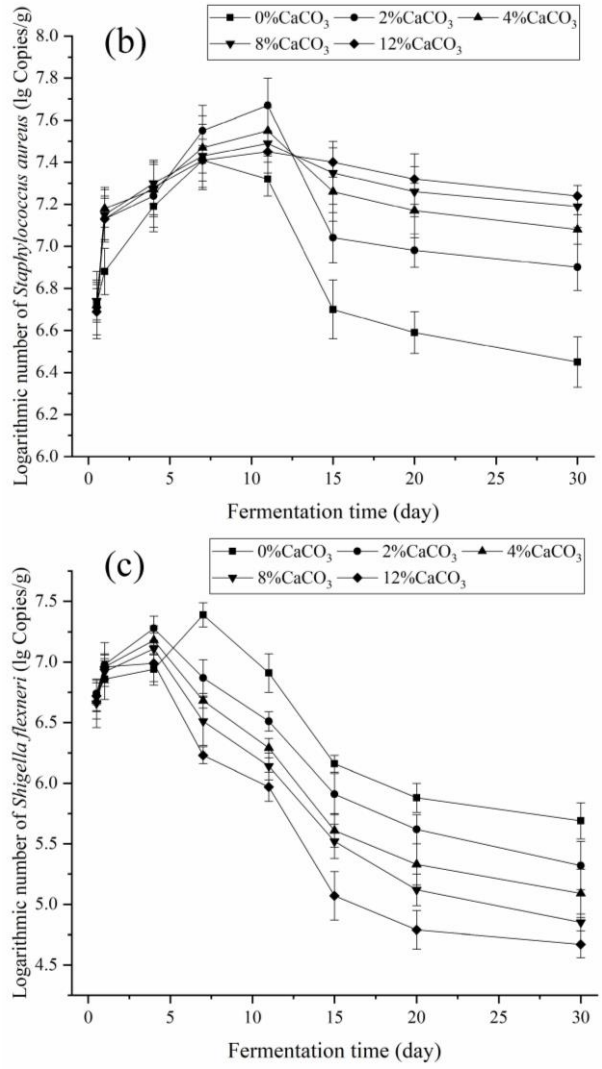

Figure 4. Growth changes of pathogenic bacteria with different amounts of $CaCO_3$. (**a**) *Escherichia coli*; (**b**) *Staphylococcus aureus*; (**c**) *Shigella flexneri.*

As shown in Figure 4b, in the early stage of fermentation, $CaCO_3$ addition promoted the growth of *Staphylococcus aureus*, and with the increase of $CaCO_3$ addition, the promotion effect was weakened; the greatest promotion effect was 2% $CaCO_3$. In the middle and late stages of fermentation, the number of *Staphylococcus aureus* gradually decreased, and with the increase of $CaCO_3$ addition, the number of *Staphylococcus aureus* increased. The growth trend of *Shigella flexneri* in all groups of experiments increased first and then decreased (Figure 4c). In the early stage of fermentation, the growth rate of *Shigella flexneri* in the feed with $CaCO_3$ addition was faster than that in the feed without $CaCO_3$ addition. In the middle and late stages of fermentation, the number of *Shigella flexneri* continued to decrease in all groups of experiments.

3.5. Changes of Organic Acid Content and Growth Changes of Fermenting Strains with Different Amounts of $CaCO_3$

As shown in Figure 5a, at 7 days of fermentation, the $CaCO_3$ addition reduced the number of *Bacillus subtilis*. The addition of 2% $CaCO_3$ slowed down the decline of *Bacillus subtilis* in the late stage of fermentation. As shown in Figure 5b, the addition of 2% and 4% $CaCO_3$ promoted the growth of *Saccharomyces cerevisiae* in the early stage of fermentation. In the late stage of fermentation, the addition of $CaCO_3$ slowed down the decay of *Saccharomyces cerevisiae*. In the fermentation process containing anaerobic fermentation,

lactic acid bacteria grow rapidly and are the dominant fermenting strains [29]. As shown in Figure 5c,d, the addition of $CaCO_3$ promoted the growth of *Lactiplantibacillus plantarum* subsp. *plantarum* and *Lacticaseibacillus rhamnosus* throughout the fermentation process. The number of both lactic acid bacteria increased with the increase of $CaCO_3$ addition. In addition, as shown in Figure 6, the content of organic acids in fermented feed increased with the addition of $CaCO_3$. In the early stage of fermentation (0–7 days), the organic acid content increases rapidly. After 7 days of fermentation, the organic acid content showed a slow increasing trend.

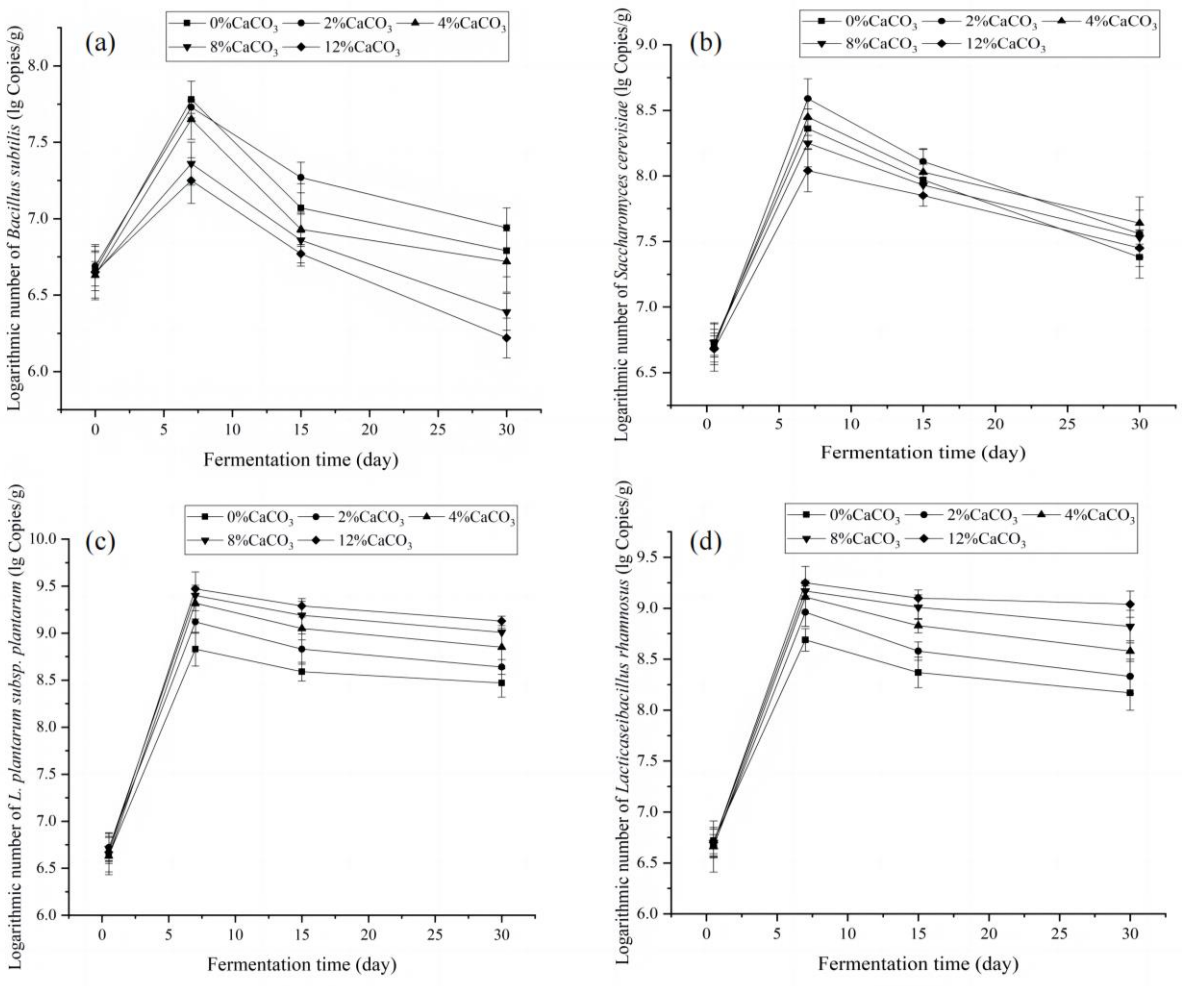

Figure 5. Growth changes of fermenting strains with different amounts of $CaCO_3$. (**a**) *Bacillus subtilis*; (**b**) *Saccharomyces cerevisiae*; (**c**) *Lactiplantibacillus plantarum* subsp. *plantarum*; (**d**) *Lacticaseibacillus rhamnosus*.

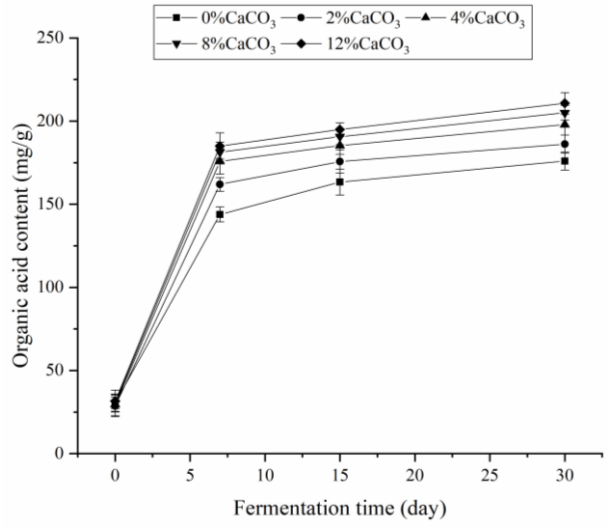

Figure 6. Changes of organic acid content with different amounts of $CaCO_3$.

4. Discussion

It is reported that fermented feed with a pH lower than 4.5 has a better antibacterial effect, and long-term high pH is likely to contaminate PB [33,34]. During the experiment, none of the fermented feeds with $CaCO_3$ showed obvious white spots, odor, etc. We speculate that it is possible that the fermented feeds still have a good antibacterial effect under the high pH caused by the addition of $CaCO_3$.

The growth trend of *Escherichia coli*, *Staphylococcus aureus*, and *Shigella flexneri* increased first and then decreased in all groups of experiments (Figure 4a–c). In the early stage of fermentation, the addition of $CaCO_3$ made the feed pH high, which was favorable to the growth of *Escherichia coli*, and the growth competition ability of *Escherichia coli* was stronger than other strains. In the middle and later stages of fermentation, the number of *Escherichia coli* decreased with the increase of $CaCO_3$ addition. This indicated that the addition of $CaCO_3$ promoted the inhibition of *Escherichia coli* by fermented feed, and the inhibition effect was stronger with the increase of $CaCO_3$ addition. Different from *Escherichia coli*, the addition of $CaCO_3$ weakened the growth inhibition of fermented feeds to *Staphylococcus aureus*, and the inhibition effect became weaker with the increase of $CaCO_3$ addition. This may be because *Staphylococcus aureus* was a gram-positive bacterium with thicker cell walls [35] that was more resistant to antimicrobial substances in fermented feed. The addition of $CaCO_3$ had a significant inhibition effect on the growth of *Shigella flexneri*. The higher the $CaCO_3$ addition, the faster the number of *Shigella flexneri* decreased, indicating that the inhibitory effect of fermented feed on *Shigella flexneri* was stronger.

At present, it is not clear which amount of $CaCO_3$ is the most appropriate. Because the quantities of *Bacillus subtilis*, *Lactiplantibacillus plantarum* subsp. *plantarum* and *Lacticaseibacillus rhamnosus* are the highest with a $CaCO_3$ addition under 12%, the production of organic acids is also the highest, and this additional amount has the best inhibition effect on the *Escherichia coli* and *Shigella flexneri*. But, the inhibition against *Staphylococcus aureus* was the worst. The addition of 4% $CaCO_3$ is more suitable for the growth of *Saccharomyces cerevisiae*. The exploration of the most suitable amount of $CaCO_3$ addition is the content of our next research.

The growth of microorganisms plays an important role in the antimicrobial effect of fermented feed. Adding an appropriate amount of $CaCO_3$ is beneficial for the reproduction of fermentation strains such as *Bacillus subtilis* and *Saccharomyces cerevisiae* in this study. The addition of $CaCO_3$ promotes the growth of *Lactiplantibacillus plantarum* subsp. *plantarum* and *Lacticaseibacillus rhamnosus* to varying degrees. The proliferation of microorganisms such as *Lactiplantibacillus plantarum* subsp. *plantarum* and *Lacticaseibacillus rhamnosus* had a competitive inhibitory effect on the growth of *Escherichia coli*, *Staphylococcus aureus*,

and *Shigella flexneri*. *Lactiplantibacillus plantarum* subsp. *plantarum* and *Lacticaseibacillus rhamnosus* can also secrete lactic acid bacteriocins, organic acids, and other antibacterial substances, which have a good inhibitory effect on PB. Peng et al. [36] found bacteriocin LP 21-2 produced by *Lactiplantibacillus plantarum* SHY 21-2 had a broad antimicrobial spectrum against gram-positive bacteria and gram-negative bacteria, including *Staphylococcus aureus* ATCC25923, *Salmonella typhi* CMCC50071.

$CaCO_3$ is one of the frequently added raw materials in fermented feed, and its co-fermentation with microorganisms can form organic acid calcium, small peptide calcium, etc. [19,20], which can effectively improve the absorption and utilization rate of calcium and enhance the nutritional value of fermented feed. In addition, the addition of $CaCO_3$ significantly increased the content of organic acids in the fermented feed (Figure 6). The antimicrobial effect of lactic acid is believed to be exerted through the ability of undissociated acids to enter cells, disrupt pH homeostasis, and, thus, cause damage to nucleic acids and proteins [33]. The main components of organic acids in this fermented feed were lactic acid and acetic acid. Costa et al. [37] found that decreases in counts of *L. monocytogenes* or *S. Enteritidis* in "Minas Frescal" cheese and ground chicken breast, respectively, were related to increases in lactic and acetic acid contents and decreases in pH values. Bacteriocins can effectively inhibit the growth and reproduction of pathogenic microorganisms and have great feeding value [38]. Vázquez, González, and Murado [39] found that compared with bacteriocin, lactic acid and acetic acid are more effective at inhibiting PB. However, most of the organic acids were in the form of organic acid radicals due to the presence of $CaCO_3$. This may be due to organic acid ions also having good antibacterial effects. Although the addition of a large amount of $CaCO_3$ resulted in the high pH of the fermented feed, it promoted the production of large amounts of organic acid radicals, which had a good growth inhibition effect on PB. The quality of the feed remains unaffected, and the increased organic acid radicals also improve the quality of the fermented feed.

This study established a rapid detection method for the number of microorganisms in the fermented feed fermentation process. This is more accurate and rapid than the traditional method of using plate enrichment culture and evaluating microbial morphology and culture characteristics to determine the type and quantity of microorganisms in products [18]. Although this method cannot distinguish dead cell DNA, resulting in a judgment of excessive quantity, this study provides a rapid and quantitative analysis of microorganisms during the fermentation process of fermented feed, providing a theoretical basis for the prevention of PB pollution, safe production, and preservation of fermented feed. Propidine monoazide (PMA) is a common live bacterial dye, which combines with qPCR technology (PMA-qPCR) to not only specifically quantify the number of strains but also distinguish between live and dead cells [40]. It has been applied to the quantitative detection of microorganisms in beverages [41]. This method may be applied to the rapid and accurate quantitative detection of microorganisms in fermented feed, which is also our next research direction.

5. Conclusions

In order to improve the quality and stability of fermented feed and to more accurately detect changes in microbial biomass during the fermentation process of fermented feed, we have established a rapid qPCR method for detecting microbial changes in fermented feed. The relationship between the addition of $CaCO_3$ and the changes in the number of probiotics, PB, and the organic acid content was established through this method. The addition of $CaCO_3$ significantly inhibited the growth of *Escherichia coli* and *Shigella flexneri* and reduced the growth inhibition of *Staphylococcus aureus*. It also promoted the growth of fermenting strains and a large amount of organic acid production, which made the fermented feed have a certain antibacterial effect. For the inhibitory effect of intestinal PB, the additional amount of 12% $CaCO_3$ is the most suitable. At this level of addition, the number of *Lactiplantibacillus plantarum* subsp. *plantarum*, *Lacticaseibacillus rhamnosus*, and *Bacillus subtilis* are the highest, and the content of organic acids with antibacterial effects is also the highest. However, this method cannot distinguish between dead cell

DNA, resulting in a judgment of excessive quantity. However, this study provides a new rapid detection method for quickly adjusting feed production processes by detecting the number of microorganisms in the fermentation production process of fermented feed and provides a theoretical basis for preventing PB pollution, safe production, and preservation of fermented feed.

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