Tolerance of Clostridium butyricum to Feed Processing and Digestive Tract Environment in Broiler Chickens (Postprint)

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Abstract

This study investigated the tolerance of Clostridium butyricum to adverse conditions during feed pelleting and within the digestive tract of broiler chickens by simulating feed pelleting conditions and the gastrointestinal environment. The tolerance of C. butyricum to temperature, pressure, artificial gastric juice, and intestinal digestive enzymes was evaluated. Temperature tolerance test: experimental diets containing C. butyricum were treated at 85°C for 2.5, 5.0, and 7.5 min, with the control group left untreated. Pressure tolerance test: experimental diets containing C. butyricum were treated at pressures of 0.20, 0.30, and 0.40 MPa for 5 min, with the control group left untreated. Artificial gastric juice tolerance test: experimental diets containing C. butyricum were treated in artificial gastric juice with pH values of 2.00, 3.00, and 4.00 for 48 min, with the control group using pH 7.00 phosphate buffer solution (PBS) instead of artificial gastric juice. Intestinal digestive enzyme tolerance test: experimental diets containing C. butyricum were first treated with pH 3.00 artificial gastric juice for 48 min, then treated in an intestinal digestive enzyme mixture for 198 min, with the control group using pH 7.00 PBS instead of intestinal digestive enzymes. Each treatment group consisted of three replicates. Following the experimental treatments, the viable count of C. butyricum in the experimental diets was determined by plate counting method, and the survival rate was calculated. The results showed that after treatment at 85°C for 2.5, 5.0, and 7.5 min, the survival rates of C. butyricum were 70.43%, 52.69%, and 46.35%, respectively; after treatment at pressures of 0.20, 0.30, and 0.40 MPa for 5 min, the survival rates were 64.38%, 87.14%, and 101.74%, respectively; after treatment in artificial gastric juice with pH values of 2.00, 3.00, and 4.00 for 48 min, the survival rates were 113.27%, 123.07%, and 78.52%, respectively; after treatment in the intestinal digestive enzyme mixture for 198 min, the survival rate was 47.71%. These results indicate that C. butyricum can tolerate the high-temperature and high-pressure environments during broiler chicken feed processing, exhibits good

tolerance to artificial gastric juice, but shows relatively low tolerance to intestinal digestive enzymes.

Full Text

Tolerance of *Clostridium butyricum* to Feed Processing Conditions and the Digestive Tract Environment of Broilers

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Abstract: This study investigated the tolerance of *Clostridium butyricum* to adverse environments during feed pelleting and within the broiler digestive tract through in vitro simulations. The tolerance of *C. butyricum* to high temperature, pressure, artificial gastric juice, and intestinal digestive enzymes was systematically evaluated. For the temperature tolerance test, experimental diets containing *C. butyricum* were treated at 85°C for 2.5, 5.0, and 7.5 min, with an untreated control group. For the pressure tolerance test, the diets were treated at 0.20, 0.30, and 0.40 MPa for 5 min, with an untreated control. For the artificial gastric juice test, diets were treated for 48 min in gastric juice at pH 2.00, 3.00, and 4.00, while the control group received phosphate-buffered saline (PBS) at pH 7.00. For the intestinal digestive enzyme test, diets were first treated for 48 min in artificial gastric juice (pH 3.00), then subsequently treated for 198 min in an intestinal digestive enzyme mixture, with the control receiving pH 7.00 PBS. Each group included three replicates. Following treatment, viable counts of *C. butyricum* were determined by plate counting to calculate survival rates.

The results demonstrated that after exposure to 85°C for 2.5, 5.0, and 7.5 min, the survival rates of *C. butyricum* were 70.43%, 52.69%, and 46.35%, respectively. Following pressure treatments of 0.20, 0.30, and 0.40 MPa for 5 min, survival rates were 64.38%, 87.14%, and 101.74%, respectively. After 48 min treatment in artificial gastric juice at pH 2.00, 3.00, and 4.00, survival rates were 113.27%, 123.07%, and 78.52%, respectively. Following intestinal digestive enzyme treatment for 198 min, the survival rate was only 47.71%. These findings indicate that *C. butyricum* can tolerate the high temperature and pressure conditions of feed processing and exhibits good tolerance to artificial gastric juice, but shows relatively low tolerance to intestinal digestive enzymes.

Keywords: *Clostridium butyricum*; tolerance; survival rate

Clostridium butyricum is an anaerobic probiotic bacterium that colonizes the intestinal tract and effectively promotes intestinal development and improves livestock performance, making it a promising green feed additive for animal pro-

duction. For viable probiotic additives, tolerance to extreme conditions during pelleting and within the digestive tract determines both their commercial feasibility and practical efficacy in livestock applications. During its late growth and metabolic phases, *C. butyricum* forms spores characterized by slow metabolism, low water content, and strong resistance to adverse conditions [1]. Previous studies have reported that *C. butyricum* possesses certain tolerance to high temperature, gastric acid, and bile salts [2-6]. This study evaluated the tolerance of *C. butyricum* to temperature, pressure, artificial gastric juice, and intestinal digestive enzymes by simulating feed pelleting conditions and the broiler digestive environment, aiming to assess the feasibility and influencing factors for its application in livestock feed.

1.1 Materials and Reagents

The *C. butyricum* powder used in this study contained 2$×10^{11}$ CFU/g and was purchased from Beijing Bio-Strong Science & Technology Co., Ltd. Key reagents included: TSN agar (Qingdao Hope Bio-Technology Co., Ltd.); pepsin, trypsin, chymotrypsin, amylase, lipase, and phosphate-buffered saline (Beijing Aoboxing Biotechnology Co., Ltd.); and poultry bile salts (Kaima Biochemical Co., Ltd.).

Equipment included: a clean bench (Shanghai Boxun Industry & Commerce Co., Ltd.), a precision pH meter (Testo, Germany), a constant temperature water bath oscillator (SHA-B, Hunan Lichen Instrument Technology Co., Ltd.), a constant temperature incubator (MCO-15AC, Sanyo, Japan), an anaerobic culture jar (C-32, Mitsubishi, Japan), anaerobic gas packs (C-35, Mitsubishi, Japan), and vacuum pressure bottles (Xibei Experiment).

A corn-soybean meal basal diet for broilers was formulated according to the Chinese agricultural industry standard NY/T 33-2004 and practical broiler production conditions. The composition and nutrient levels are shown in . The basal diet was sterilized by dry heat at 160°C for 150 min, cooled, and adjusted to 17-18% moisture content. *C. butyricum* powder was then mixed with the basal diet to achieve a final concentration of approximately 10^9 CFU/kg.

1.2 Experimental Methods

1.2.1 High Temperature Tolerance Test High temperature tolerance was evaluated using a water bath to simulate pelleting temperatures. Experimental diet samples were treated at 85°C [7] for 2.5, 5.0, and 7.5 min, with an untreated control group. Each treatment included three replicates. Following treatment, samples were serially diluted and plated to determine viable counts and calculate survival rates.

1.2.2 High Pressure Tolerance Test Pressure tolerance was evaluated to simulate pelleting conditions. Experimental diet samples were placed in vacuum pressure bottles and pressurized with nitrogen gas (N_2) to 0.20, 0.30, and 0.40

MPa [8] for 5 min, with an untreated control group. Each treatment included three replicates.

1.2.3 Artificial Gastric Juice Tolerance Test Artificial gastric juice was prepared by adjusting PBS (pH 7.00) to pH 2.00, 3.00, and 4.00 using 37% hydrochloric acid, then adding pepsin at 0.89 U/mL. The solution was sterilized by filtration through a 0.22 μm membrane filter. To simulate the gastric environment of broilers, 1 g of experimental diet was placed in a 250 mL sterile Erlenmeyer flask with 9 mL of artificial gastric juice at each pH level. The control group received 9 mL of PBS (pH 7.00). Each treatment included three replicates. Samples were incubated in a 41°C water bath oscillator at 120 r/min, with timing beginning after 5 min and samples removed after 48 min [9]. Treated samples were centrifuged at 4,000 r/min, the supernatant was discarded, and the pellet was washed three times with sterile PBS before resuspension in 9 mL PBS for serial dilution and plate counting to determine viable counts and calculate survival rates.

1.2.4 Intestinal Digestive Enzyme Tolerance Test The intestinal digestive enzyme mixture was prepared by adjusting PBS to pH 6.00 and adding enzymes to achieve final concentrations of 780.76 U/mL amylase, 1.09 U/mL trypsin, 0.04 U/mL chymotrypsin, and 80.36 U/mL lipase [10]. The solution was sterilized by filtration through a 0.22 μm membrane filter and prepared fresh before use. To simulate intestinal digestion, 1 mL of sample previously treated with artificial gastric juice (pH 3.00) for 48 min was centrifuged, the supernatant discarded, and the pellet washed three times with sterile PBS before resuspension in 9 mL of intestinal digestive enzyme mixture. The control group received 9 mL of PBS (pH 7.00). Each treatment included three replicates. Samples were incubated in a 41°C water bath oscillator at 120 r/min, with timing beginning after 5 min and samples removed after 198 min [10]. Following treatment, samples were centrifuged at 4,000 r/min, washed three times with sterile PBS, and resuspended in 9 mL PBS for serial dilution and plate counting to determine viable counts and calculate survival rates.

1.3 Statistical Analysis

All experimental data were initially processed and survival rates calculated using Excel 2003. One-way ANOVA in SPSS 19.0 was used to analyze significant differences in viable counts. Duncan' s multiple comparison test was applied to detect inter-group differences when significant effects were identified. $P<0.05$ was considered statistically significant.

2 Results

2.1 High Temperature Tolerance of *C. butyricum*

As shown in , *C. butyricum* exhibited certain tolerance to high temperature, though its resistance decreased with prolonged exposure. Viable counts after treatment at 85°C for 2.5, 5.0, and 7.5 min were all significantly lower than the control group ($P<0.05$). Survival rates gradually decreased with increasing treatment time, though the rate of decline diminished over time. While all treatment groups differed significantly from the control in viable counts ($P<0.05$), it should be noted that the initial viable count of *C. butyricum* was high, and the high temperature treatment did not cause a change in the order of magnitude.

2.2 High Pressure Tolerance of *C. butyricum*

As shown in , viable counts in the 0.20 and 0.30 MPa groups were significantly lower than the control ($P<0.05$), while the 0.30 MPa group did not differ significantly from the control ($P<0.05$). The 0.20 MPa group showed the lowest survival rate at 64.38%, while survival rates in the 0.30 and 0.40 MPa groups increased progressively to 87.14% and 101.74%, respectively.

2.3 Artificial Gastric Juice Tolerance of *C. butyricum*

As shown in , viable counts in all treatment groups did not differ significantly from the control ($P>0.05$). However, survival rates in the pH 2.00 and 3.00 groups were slightly higher than the control.

2.4 Intestinal Digestive Enzyme Tolerance of *C. butyricum*

As shown in , viable counts after intestinal digestive enzyme treatment were significantly lower than the control ($P<0.05$), with a survival rate of only 47.71%.

3 Discussion

3.1 High Temperature Tolerance

Research methodologies for evaluating high temperature tolerance of *C. butyricum* vary among studies. Wang [2] reported that when *C. butyricum* suspension was water-bathed for 15 min, survival rates remained above 84.23% at temperatures below 75°C, but dropped to only 27.41% at 80°C. Xie [3] found that survival rate was 6.48% after treatment at 80°C for 30 min. However, these studies evaluated temperature tolerance in liquid environments, whereas the present study examined tolerance under actual feed pelleting conditions, specifically when *C. butyricum* powder was mixed with broiler basal diet. The results showed a survival rate of 70.43% after 2.5 min at 85°C, with gradual decline as treatment time increased. Therefore, while high temperatures during feed conditioning and pelleting affect *C. butyricum* activity, extended exposure causes substantial inactivation and reduces efficacy.

3.2 High Pressure Tolerance

No previous studies have reported on the pressure tolerance of *C. butyricum*, though some studies indicate that certain bacterial spores exhibit resistance to high pressure [11]. The present results show that viable counts were significantly lower than the control after treatment at 0.20 and 0.30 MPa for 5 min, but did not differ significantly from the control at 0.40 MPa. This demonstrates that *C. butyricum* can tolerate pressure conditions within a certain range during feed pelleting.

3.3 Artificial Gastric Juice Tolerance

Probiotics exhibit stress responses to acidic conditions in the digestive tract to ensure survival [12]. Wang [2] and Liao [4] reported that *C. butyricum* survival rates decreased to varying degrees after acid treatment, though differences were not significant. In the present study, viable counts did not differ significantly from the control after 48 min treatment in artificial gastric juice at pH 2.00, 3.00, and 4.00, indicating that *C. butyricum* possesses adequate tolerance to gastric acid.

3.4 Intestinal Digestive Enzyme Tolerance

The significant reduction in viable counts after intestinal digestive enzyme treatment differs from results reported by Wang [2] and Ouyang et al. [5]. This discrepancy may be attributed to the prior treatment of experimental diets with artificial gastric juice in the present study, which likely activated some spores into vegetative cells with reduced stress resistance. Subsequent exposure to intestinal digestive enzymes would then cause substantial destruction of these vegetative cells. Pre-treatment with artificial gastric juice maximally simulates the actual conditions in the broiler digestive tract, suggesting that *C. butyricum* has relatively low tolerance to intestinal digestive enzymes in vivo.

4 Conclusions

1. Under simulated feed pelleting conditions, the survival rate of *C. butyricum* decreases with extended high temperature treatment.
2. Survival rates under different pressure conditions show a declining trend with increasing pressure.
3. *C. butyricum* exhibits adequate tolerance to artificial gastric juice.
4. *C. butyricum* shows relatively low tolerance to intestinal digestive enzymes.

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Note: Figure translations are in progress. See original paper for figures.