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Comparative Effects of Fermentation ff Sugar Kefir Grains (*Tibicos*) and Tetracyclin on Three Major Ileum Bacteries in Broiler Chickens

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Abstrak

Antibiotik seperti tetrasiklin dalam industri peternakan menghadapi pembatasan yang ketat akibat meningkatnya resistensi antimikroba. Fermentasi tibicos sebagai probiotik alami, memiliki potensi sebagai alternatif yang memadai dan aman untuk mengatur keseimbangan mikrobiota di usus. Penelitian ini bertujuan untuk membandingkan efektivitas fermentasi tibicos dan tetrasiklin dalam mengatur populasi tiga bakteri utama (*Escherichia coli*, *Lactobacillus*, dan *Salmonella* spp.) di usus halus ayam broiler. Sembilan puluh ayam broiler strain Indian River dibagi menjadi tiga perlakuan dengan tiga ulangan (n=10 per ulangan): kontrol dengan air murni (KN), 45 ppm tetrasiklin (KP), dan 5% fermentasi tibicos (FT). Perlakuan diberikan melalui air minum *ad libitum* dari usia 8 hingga 35 hari. Populasi bakteri dianalisis menggunakan metode *Total Plate Count* (TPC) pada sampel ileum berusia 35 hari. Analisis data menggunakan pendekatan multivariat termasuk Bray-Curtis dissimilarity, PERMANOVA, *Principal Coordinates Analysis* (PCoA), dan *Similarity of Percentage Analysis* (SIMPER). Analisis dissimilaritas Bray-Curtis menunjukkan perbedaan moderat dalam komposisi mikrobiota antara perlakuan dengan nilai 0,064854 (FT vs KN), 0,069687 (FT vs KP), dan 0,07451 (KN vs KP). Analisis SIMPER mengidentifikasi *Escherichia coli* sebagai kontributor utama perbedaan antara perlakuan, dengan kontribusi sebesar 39,50% (KP vs KN, p=0,005), 65,70% (KP vs FT, p=0,001), dan 41,10% (KN vs FT, p=0,001). Analisis Koordinat Utama menunjukkan pengelompokan yang jelas antara perlakuan dengan fermentasi tibicos menghasilkan profil mikrobiota yang berbeda dari kontrol dan tetrasiklin. Fermentasi tibicos menunjukkan efektivitas yang sebanding dengan tetrasiklin dalam memodulasi komposisi bakteri ileum terutama dalam mengendalikan populasi patogen sambil mempertahankan stabilitas *Lactobacillus*.

1. INTRODUCTION

The global poultry industry is experiencing significant transformation due to stringent restrictions on antibiotic growth promoters (AGPs) stemming from the emergence of antimicrobial resistance. The widely utilized antibiotic growth promoter tetracycline (AGP) in the broiler chicken industry has enhanced production by managing intestinal diseases through gut microbiota (Castanon, 2007). Prolonged tetracycline usage has been linked to the emergence of antibiotic-resistant bacteria, potentially jeopardizing public health and the stability of ecological systems. The crisis has reached a pivotal moment as the World Health Organisation identifies antimicrobial resistance as one of this century's most pressing global health challenges. Numerous avian pathogens, such as Salmonella species and Escherichia coli, have demonstrated resistance to tetracyclines, with prevalence rates in certain countries consistently increasing (Huyghebaert *et al.*, 2011).

The poultry industry must seek biologically efficient, cost-effective, and environmentally sustainable alternatives. The gut microbiota influences overall health and the quality of grilled chicken. This role can be fulfilled through competitive exclusion, immune modulation, nutrient metabolism, and barrier function (Oakley *et al.*, 2014). The dynamic equilibrium between beneficial bacteria such as Lactobacillus and the regulation of pathogenic bacteria like Escherichia coli and Salmonella spp. Dictates optimal digestive efficiency, nutrient absorption, and infection resistance (Rinttilä & Apajalahti, 2013). The ileum is part of the small intestine. It is a vital locus for microbiota research due to its function in nutrient absorption and as a barrier against pathogen translocation to the colon (Sergeant *et al.*, 2014). The primary challenge in substituting AGP is identifying options with significant antimicrobial selectivity, which will aid in managing pathogens without adversely affecting beneficial commensal bacteria. Regarding the maintenance of optimal gut flora equilibrium, most commercially available probiotics and feed additives have failed to replicate the efficacy and array of advantages provided by AGP (Patterson & Burkholder, 2003).

Tibetan kefir or tibicos is a natural probiotic consortium comprising a diverse collection of lactic acid bacteria and yeast in mutualistic symbiosis. It offers an innovative method for altering gut microbiota. Tibicos fermentation yields diverse bioactive metabolites, such as organic acids, bacteriocins, enzymes, and naturally occurring antimicrobial substances, which function synergistically, in contrast to monoculture probiotics (Marsh *et al.*, 2014). Tibicos demonstrates advantages over synthetic probiotics regarding high microbial diversity, enhanced adaptability, and cost-effectiveness in mass production. The tibicos fermentation mechanism is believed to interact in various ways to affect bacteria. Organic acids produced during fermentation establish an acidic environment that inhibits the proliferation of gram-negative pathogenic bacteria while maintaining native lactic acid bacteria (Ricke, 2003). The synthesized bacteriocins and antimicrobial peptides are targeted explicitly at certain infections. Competitive exclusion arises from competition for adhesion sites and nutrients, while immune modulation through interaction with gut-associated lymphoid tissue enhances host resistance (Bermudez-Brito *et al.*, 2012).

Despite the notable possibility of tibicos for various applications, comparative efficacy studies against conventional antibiotics are limited, particularly concerning grilled chickens. Comprehensive and direct comparisons between tibicos fermentation and tetracycline are essential. These tests must produce significant scientific data for potential commercial applications of these compounds through innovative analytical techniques. A comprehensive evaluation of the efficacy of these treatments must incorporate several essential elements: the ability to manage specific pathogens, the maintenance of beneficial bacteria, and the overall impact on microbiota composition and stability. The ratio of Lactobacillus to Escherichia coli is a reliable indicator of gut health in poultry and a predictor of production performance (Kabir, 2009). An optimal ratio indicates harmonious flora, efficient nutrient absorption, and improved disease resistance.

Comparative studies must incorporate assessments of these criteria as clinically significant endpoints. This study aims to evaluate the efficacy of 45 ppm tetracycline and 5% tibicos fermentation in regulating the populations of three significant bacteria (*Escherichia coli*, *Lactobacillus*, and *Salmonella* spp.) in the ileum of broiler chickens. The research hypothesis posits that tibicos fermentation offers greater antimicrobial selectivity in protecting beneficial bacteria than tetracycline, thereby regulating pathogenic bacteria. The evaluation utilizes a complete multivariate analysis technique, facilitating an in-depth understanding of microbiota modulation patterns and the marker of bacterial species that serve as significant differentiators between treatments.

2. MATERIALS AND METHODS

The study was conducted in November 2024 in a confined poultry facility and the Tropical Animal Research Center laboratory at the Faculty of Animal Science at Gadjah Mada University. The tibicos fermentation process used 10% (w/v) palm sugar, which was sterilized by heating to 100°C for three hours. The solution was cooled to 25°C and then maintained in a refrigerator at 4°C for 24 hours to stabilize it. Under aseptic conditions, the cooled palm sugar solution was inoculated with tibicos at a concentration of 5% (v/v). Fermentation occurred for 48 hours at an ambient temperature of 25 to 28 °C. Sterile gauze filtered the fermenting product, separating the tibicos biomass from the filtrate. The filtered fermented product must be stored in a refrigerator at 4°C and utilized within 48 hours to maintain microbial viability.

Cages measuring 50x40x40 cm and equipped with a battery cage system house grill chicken. It ensures precise control over treatment consumption and eliminates cross-contamination between treatments. The relative humidity is maintained at 60 - 65% in a regulated environment with temperatures of 28 - 32°C during weeks 1 - 2 and 26 - 28°C thereafter. Lighting is supplied continuously for 24 hours. Commercial starting feed containing 22% crude protein and 3,000 kcal/kg metabolizable energy was provided ad libitum and was free of antibiotics, growth promoters, and coccidiostats. Fresh water was substituted every 24 hours to ensure microbial viability and freshness in the tibicos fermentation process. Daily water intake is monitored, and no significant differences between treatments are observed. The institutional research ethics committee has approved all maintenance operations adhering to animal welfare guidelines. After the chickens fasted for 12 hours, day 35 was dedicated to sampling under aseptic conditions to ensure consistency and stability of the sampling environment.

By animal welfare standards, the chickens were euthanized via cervical dislocation, and a 5 cm segment of the ileum was excised from the ileocecal junction. Samples were promptly placed in sterile containers cooled with ice following aseptic collection using sterilized instruments. The ileum samples were analyzed within 24 hours of collection to maintain bacterial viability. Using a stomacher for two minutes, one gram of ileum sample was homogenized in nine milliliters of sterile phosphate-buffered saline (PBS). A decimal dilution series from 10^{-10} to 10^{-6} was produced in sterile PBS for each sample.

Selective media tailored to each target bacterium were employed in the microbiological analysis using the total plate count (TPC) method. E. coli analysis was conducted on eosin methylene blue (EMB) agar following a 24-hour incubation at 37 °C. The IMViC (indole, methyl red, Voges-Proskauer, citrate) test was performed on the incubated samples. Lactobacillus colonies were enumerated on Man-Rogosa-Sharpe (MRS) agar at a pH of approximately 6.2, and the samples were incubated anaerobically at 37 °C for 48 hours. Gram staining and a negative catalase test were used for confirmation. Salmonella spp calculations were analyzed using Salmonella-Shigella (SS) agar media and incubated at 37 °C for 24 hours (Bobany *et al.*, 2010). Serological testing and the Triple Sugar Iron (TSI) biochemical assay were used for confirmation. Microbial colony counts were performed on plates containing 25 - 250 colonies by microbiological criteria. The results indicate log CFU/g of the ileal sample. Each sample was replicated three times to ensure the accuracy and precision of the results.

The Bray-Curtis index was used to evaluate variations in microbiota composition across treatments through a population dissimilarity analysis of bacteria. Before the investigation, the bacterial population data (log CFU/g) were transformed into relative abundance. The Bray-Curtis index was calculated using the formula:

Bray-Curtis Dissimilarity Index = 1 - (2W/A + B).
Where:
W is the minimum abundance of identical species in both communities.
A represents the aggregate abundance of all species within the initial community.
B represents the total abundance of all species in the second community.

The index value ranges from 0 to 1. A value of 0 signifies identical composition, while values exceeding 0 indicate differences (Anderson, 2017). The Bray-Curtis index values were statistically tested using R Studio version 4.5.0 and the "vegan" package to examine the permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2017; Ricotta & Pavoine, 2022).

Principal coordinates analysis (PCoA) illustrated spatial variations in microbiota composition via the cmdscale function using the Bray-Curtis dissimilarity matrix. Conversely, the similarity of percentage analysis (SIMPER) determined species contributions to treatment variations using the simper function with a contribution threshold of 90%. PERMANOVA was used to evaluate variations in microbiota composition and structure across treatments using the adonis2 function with 999 permutations (Shi *et al.*, 2020). The significance level employed in statistical testing was α = 0.05.

3. RESULTS AND DISCUSSION

Figure 1 shows how the Bray-Curtis values are spread out between treatments and how the composition of the microbiota changes. The fact that the values in the 0.06 - 0.08 range are pretty evenly spread out shows that the microbiota composition of the tested treatments was only slightly different. A value of 0 means that the microbial composition is the same, and a value of 1 means entirely different (Anderson, 2017). The plot shows that the Bray-Curtis dissimilarity values are spread differently between treatments, meaning the microbial composition differs. Most of the dissimilarity values are close to zero, which means that the treatments being compared have microbial compositions that are very similar or almost the same.

On the other hand, a distribution with higher values shows that the microbial composition is different between treatments, with some treatments having very different compositions. A wide range of Bray-Curtis dissimilarity values means that there is more variation in dissimilarity between samples or treatments. On the other hand, a narrow range means that the microbial composition between groups is more similar.

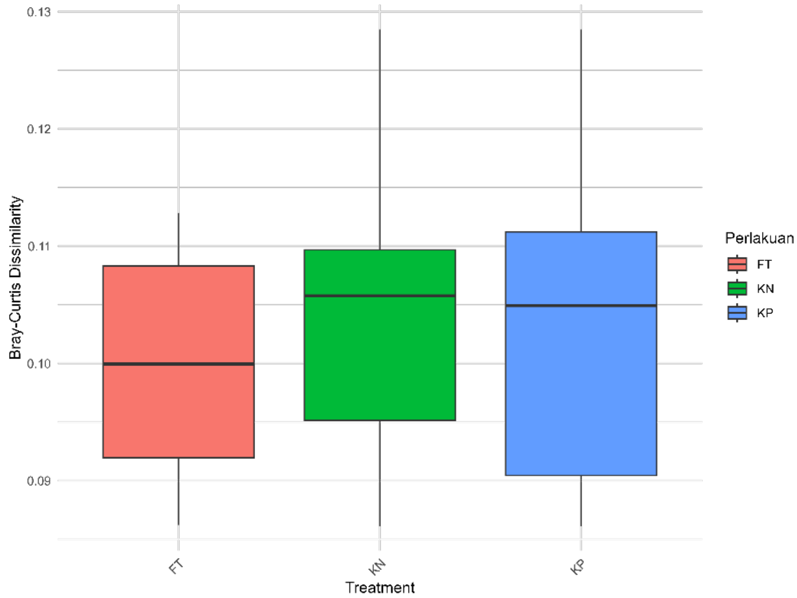

Figure 1. Plot histogram Bray-Curtis dissimilarity.

Table 1 shows how the treatments that were looked at differ based on Bray-Curtis dissimilarity values. The data show that the treatments being compared have different types of microbes. The difference in value between FT and KN is 0,064854, but the difference between FT and KP is bigger at 0,069687. The results show that the microbial compositions are slightly different between the two treatments, but these differences are insignificant. On the other hand, the dissimilarity value between KN and KP is 0,07451, which means that the two treatments are more different. The dissimilarity matrix shows that FT, KN, FT, and KP have microbial compositions that are more similar. On the other hand, KN and KP are more different. The Bray-Curtis dissimilarity values in this study (0,064854 – 0,07451) are comparable to those seen by Zhao *et al.* (2013), which shows that natural probiotics change the microbiota less than synthetic antibiotics. This slight difference shows that using tibicos to ferment sugar palms can change the microbiota in a controlled way without causing dysbiosis, which can be bad for gut health (Oakley *et al.*, 2014). Lozupone and Knight (2005) said that if the Bray-Curtis dissimilarity value is less than 0.1, changes in composition are biologically acceptable and will not upset the balance of gut microbiota.

Table 1. Dissimilarity matrix between treatments

	FT	KN	KP
FT		0,064854	0,069687
KN	0,064854		0,07451
KP	0,069687	0,07451	

Note: FT = Tibicos fermentation 5%, KN = Control, KP = Tetracycline 45 ppm

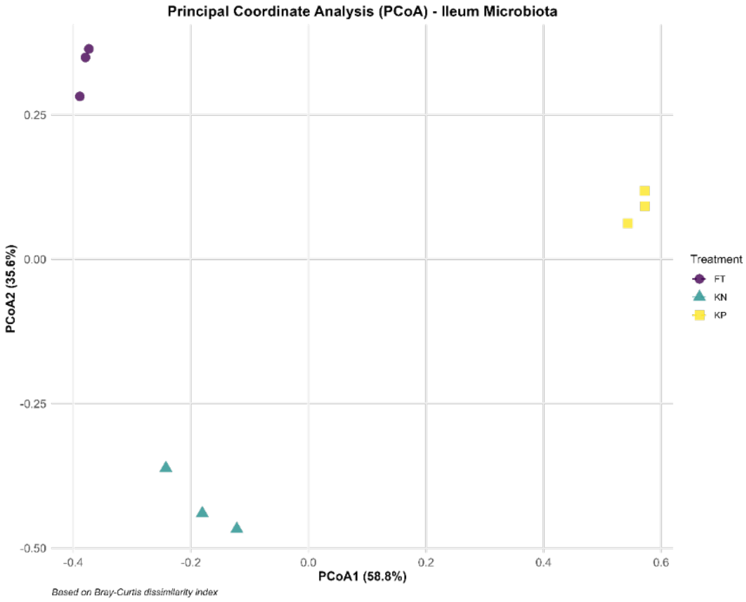

Figure 2. Principal Coordinate Analysis (PCoA)

The PCoA results demonstrated the variations in microbiota among the treatments across two dimensions.The analysis demonstrated that the treatments were grouped, displaying distinct spatial attributes. The KP (tetracycline) treatment established a distinct cluster in the upper right quadrant, signifying a divergent microbiota profile relative to the other treatments. The control (KN) treatment tended to cluster in the upper left quadrant. The tibicos fermentation (FT) treatment exhibited a more concentrated distribution, positioned between the control and tetracycline (KP) treatments. The structure of these groups corresponds with Anderson and Willis (2003) regulations of ecological ordination, which posit that the Euclidean distances between points reflect the extent of variation in community composition. The tibicos fermentation treatment is situated between the control and tetracycline treatments, indicating that tibicos fermentation has a less significant impact than tetracycline yet still produces changes from the baseline condition. Legendre and Legendre (2012) assert that the compact clustering of the FT treatment signifies uniform effects across all replicates. It is a crucial element in guaranteeing the dependability of commercial applications.

The SIMPER analysis results indicate that Escherichia coli is the principal factor influencing the variations noted across all treatment comparisons. In comparing KP versus KN, Escherichia coli comprised 39,50% of the total, a statistically significant proportion (p=0,005). In the comparison of KP and FT, there was a notable increase in the contribution of Escherichia coli (65,70%, p=0,001), suggesting potential disparities in the Escherichia coli population between tetracycline and tibicos fermentation processes. The dominance of Escherichia coli as the principal differentiator in all treatment comparisons (39,5 – 65,7% contribution) corresponds with the conclusions of Rinttilä and Apajalahti (2013), who posited that Escherichia coli is a reliable model for evaluating the effectiveness of antimicrobial agents in poultry. Consistent differences ($p < 0,05$) for Escherichia coli across all comparisons suggest that both tetracycline and tibicos fermentation have specific targets against this opportunistic pathogen (Huyghebaert *et al.*, 2011). The significant percentage of Escherichia coli in the KP versus FT comparison (65,70%) highlights the effectiveness of both treatments in managing Escherichia coli, albeit via different mechanisms and to differing degrees. This discovery corroborates the hypothesis that broad-spectrum antibiotics and natural probiotics experience distinct selective pressures (Gaggìa *et al.*, 2010).

Table 2. Similarity of Percentage Analysis between treatments.

Comparative test	Microorganism	Ration	Contributions (%)	P-value
KP vs KN	Escherichia coli	2,65	39,50	0,005
	Lactobacillus	7,89	35,00	0,192
	Salmonella spp	4,79	25,50	0,228

KP vs FT	Escherichia coli	16,62	65,70	0,001
	Lactobacillus	4,26	21,50	0,835
	Salmonella spp	0,93	12,80	0,999
KN vs FT	Escherichia coli	11,48	41,10	0,001
	Lactobacillus	9,04	32,10	0,116
	Salmonella spp	6,36	22,80	0,984

Note: FT = Tibicos fermentation 5%, KN = Control, KP = Tetracycline 45 ppm

A comparative analysis of KP and FT revealed that Escherichia coli was predominantly responsible for the observed variations, constituting 65,70% of the total variability and yielding a statistically significant p-value of 0,001. This finding underscores the significance of this species in distinguishing between the two treatments. Conversely, Lactobacillus accounted for a mere 21,50% of the total, exhibiting a p-value of 0,835, indicating its contribution as non-significant Salmonella spp. The lowest significant effect was exhibited, with a percentage of 12,80% and a statistically significant p-value (0.999). This finding indicates that this species did not play a substantial role in differentiating between KP and FT. A subsequent comparison of KN and FT revealed that Escherichia coli exhibited a 41,10% contribution and a p-value of 0,001, substantiating the findings' statistical significance. Lactobacillus constituted 32,10% of the total, yet its p-value (0,116) indicated that the outcomes were not statistically significant for Salmonella spp. Constituted 22,80% of the total, yet its elevated p-value (0,984) indicated that the discrepancy in Salmonella spp.'s contribution was not statistically substantial.

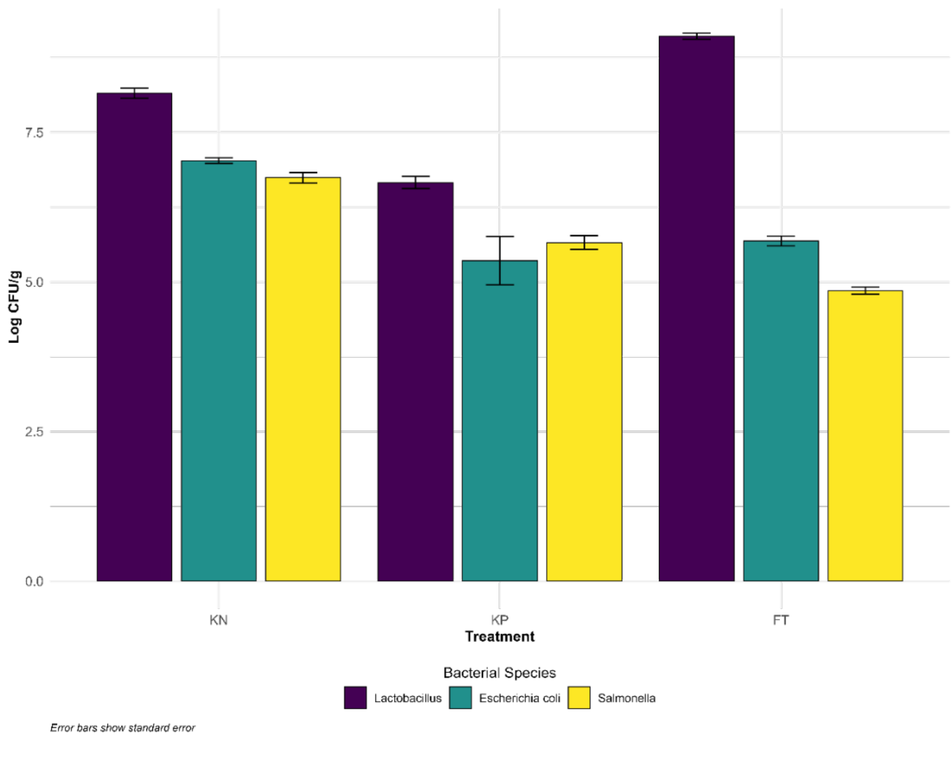

Figure 3. Comparison of treatment on microorganism abundance.

The study examined the quantity of three distinct bacterial types, Escherichia coli, Lactobacillus, and Salmonella, in each of the three treatments. As illustrated in Figure 3, KN, KP, and FT are the primary factors to be considered. The experimental design comprises three bars for each treatment, each representing a distinct type of microbe. The coloration of the bars serves as a taxonomic indicator, denoting the species of fish inhabiting each distinct region. The most prevalent strain of bacteria, identified as Escherichia coli and depicted in purple, was observed to occur most frequently in the KN treatment group. The KP and FT treatment groups exhibited a lower prevalence of this strain, followed by the KN treatment group. Consequently, the KN treatment resulted in a greater abundance or presence of Escherichia coli than the other two treatments. The green Lactobacillus demonstrates a uniform distribution across all three treatments, with the KN treatment exhibiting a marginal increase in abundance compared to the KP and FT treatments. Salmonella (yellow) was observed less frequently than the other two species, with minimal variation among the treatments. However, the KN treatment slightly increased compared to KP and FT.

The effectiveness of tibicos fermentation in regulating Escherichia coli is believed to result from a complex interplay of mechanisms working together.   Fermentation creates an acidic environment (low pH) by producing organic acids, including lactic acid and acetic acid. This property poses a considerable obstacle to the proliferation of gram-negative pathogenic bacteria, including Escherichia coli (Hilmi *et al.*, 2024). Secondly, the lactic acid bacteria in tibicos produce bacteriocins and other natural antimicrobial substances that can eliminate pathogenic bacteria (Cotter *et al.*, 2005). Thirdly, studies have shown that competitive interactions between probiotic microorganisms and pathogenic bacteria, including competition for nutrients and adhesion sites, confer a competitive advantage to beneficial bacteria (Collado *et al.*, 2007). Fourthly, evidence suggests that Peyer's patches can increase the local intestinal immune system, thereby occluding the establishment of pathogens (Bermudez-Brito *et al.*, 2012).

4. CONCLUSION

Tibicos fermentation 5% exhibited antimicrobial selectivity towards Salmonella spp. and Escherichia coli while concurrently enhancing Lactobacillus proliferation. Although effective in pathogen control, Tetracycline demonstrated an 18.3% reduction in Lactobacillus levels.

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