PROBIOTICS IN POULTRY FEED: NEW PROMISING PROBIOTIC STRAINS IN THE DIET

Iryna Matiukha[1]*, Olena Krasovska[1,2], Oleksandr Politylo[1], Oleg Vishchur[2], Daria Mudrak[2]

PrJSC „Enzym Group", Luchakivska str., 232, Lviv, Ukraine

Institute of Animal Biology NAAS, Stusa str., 38, Lviv, Ukraine

Abstract

The development and research of feed compounds to enrich diets, ensure health and stimulate productivity is an urgent issue in poultry farming. Probiotics are important in this context, and the key to their successful use is the correct selection of a strain with high probiotic potential, its rational dosage and justification of the expediency and method of introduction into the diet. The aim of the experiment was to research the productivity, blood biochemical parameters and growth performance of broilers using a probiotic feed supplement. It was found that the probiotics application in the feeding of broiler chickens increased live weight, the average daily gain was higher in birds that received all tested probiotic strains compared with control. Additional consumption of probiotic feed supplement by broiler chickens increases normalized biochemical indexes in the blood and improved beneficial intestinal microbiota. The best results were obtained when new promising probiotic strains (produced by Enzym Group) were added to the diet.

Keywords: *yeast, probiotic, strains, antioxidant, enzymes, gastro intestinal, growth performance*

1. INTRODUCTION

The poultry industry has become an important economic activity in many countries. In large-scale rearing facilities, where poultry are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses. Prevention and control of diseases have led during recent decades to a substantial increase in the use of veterinary medicines.

However, the utility of antimicrobial agents as a preventive measure has been questioned, given extensive documentation of the evolution of antimicrobial resistance among pathogenic bacteria. So, the possibility of antibiotics ceasing to be used as growth stimulants for poultry and the concern about the side-effects of their use as therapeutic agents has produced a climate in which both consumer and manufacturer are looking for alternatives. Probiotics are being considered to fill this gap and already some farmers are using them in preference to antibiotics [1, 2, 3].

Salmonella from ingested poultry meat has caused a rise in foodborne illnesses. Annually, 10% of consumers become ill from *Salmonella*, and 25% of all global diarrhea diseases are caused by *Salmonella* cases. Resistant serotypes of *Salmonella* are a growing concern to the public and an issue for food safety (WHO, 2006). Live beneficial bacteria and yeasts such as probiotics might be able to alleviate and overcome this challenge. Probiotics do not only show promising results for a healthier consumer, but they also show increased performance and an increased immunity for the broiler [4, 5]. Specifically, in broilers, research has concluded probiotics both live yeast and bacteria increase resistance to *Salmonella*, *E. coli* or *C. perfringens* infections.

The impact of biotechnology in animal and poultry nutrition is significant. Biotechnology plays a vital role in the poultry feed industry. Dietary changes as well as lack of a healthy diet can influence the balance of the microflora in the gut thus predisposing to digestion upsets and decrease in productivity. A well-balanced ration sufficient in energy and nutrients is also of great importance in maintaining a healthy gut. A great deal of attention has been received from nutritionists and veterinary experts for proper utilization of nutrients and the use of probiotics for growth promotion of poultry.

Many studies have analyzed the effects of probiotics supplementation on animal performance, intestinal ecosystem and morphology, obtaining controversial results [6, 7]. The beneficial effects of probiotics in animal production depend on several factors, including the chosen strain, the level of supplementation, the duration and the frequency of exposure to the probiotic and the physiological condition of the host. *Saccharomyces cerevisiae* is a well-known yeast used worldwide and since ancient times for different purposes, from baking to balance human gut microflora. This yeast species exerts probiotic influence in broilers by promoting the metabolic processes of digestion and nutrient utilization [8].

Saccharomyces yeast, particularly Saccharomyces cerevisiae, has gained recognition as an effective probiotic alternative to antibiotics in broiler diets. This yeast strain contributes to improved gut health by enhancing the balance of the intestinal microbiota, thereby reducing the prevalence of pathogenic bacteria. Studies have shown that Saccharomyces cerevisiae supplementation can lead to increased nutrient absorption, better feed conversion ratios, and overall improved growth performance in broilers. Additionally, S. cerevisiae has been noted for its role in modulating the immune system, leading to enhanced resistance against diseases. The ability of Saccharomyces cerevisiae to improve gut integrity and function makes it a valuable component in antibiotic-free poultry production systems [9, 10].

Besides Saccharomyces cerevisiae, other yeast strains such as *Kluyveromyces marxianus* and *Candida pintolopesii* have also shown promise as probiotic alternatives in broiler diets. These yeasts contribute to enhanced gut health and performance by producing beneficial metabolites like short-chain fatty acids and vitamins that support intestinal health and growth. *Kluyveromyces marxianus*, for example, has been documented to improve digestive enzyme activity and nutrient absorption, thereby promoting better growth rates and feed efficiency in broilers [11]. Additionally, the use of these yeast strains can lead to a reduction in the occurrence of gastrointestinal diseases, further supporting the move towards antibiotic-free poultry production. The growing body of research underscores the potential of various yeast probiotics in enhancing poultry health and productivity in sustainable and antibiotic-free systems [11, 12].

Enzym Group is an expert in fermentation, biotechnology company – a leader in the domestic market of yeast products. Along with baker's yeast and food solutions, Enzym develops modern biotechnological products for animal husbandry. In particular, already a successful product with proven effectiveness probiotic feed additive EnzActive – a probiotic yeast strain of Saccharomyces cerevisiae. The company's scientists are constantly in search of new products and technologies to obtain new generation products for animals – safe, effective, and sustainable. So, at present, a new yeast strain, *Kluyveromyces marxianus*, has attracted attention. The results of the study of the probiotic properties of this strain are the subject of this work.

Kluyveromyces marxianus is a yeast species that has been explored for various industrial applications, including its potential as a probiotic in animal feed. *Kluyveromyces marxianus* (formerly *Kluyveromyces fragilis*) is a lactic yeast isolated from different dairy products, mainly kefir [13, 14]. While the importance of this species in food development and fermentation is well documented, characterizations of its putative probiotic activities have been very limited [15].

The hypothesis behind this study is that dietary supplementation with *Kluyveromyces marxianus* could positively effects on the performances and some vital blood and tissues parameters in broilers rations completely free from antibiotics.

2. MATERIALS AND METHODS

2.1. Animal Experimental Design.

A total of 14-day old Ross 308 chicks were divided (by similar weight) into 5 replicates (experimental units) of 10 birds each and assigned to one of the 5 experimental diets. Animals were raised for 49 days (d) according to the Experimental design and probiotic usage recommendation.

The experiment was carried out under the supervision of certified veterinarians. Chickens were vaccinated before dividing into groups at hatchery against Newcastle disease, Marek disease, infectious

bronchitis, and coccidiosis, then placed in floor pens with wood shavings as litter, supplemental heat in the first period, plastic waterer, and feed *ad libitum* according to the experimental group. The temperature was monitored to ensure the same environmental conditions for all the replicates.

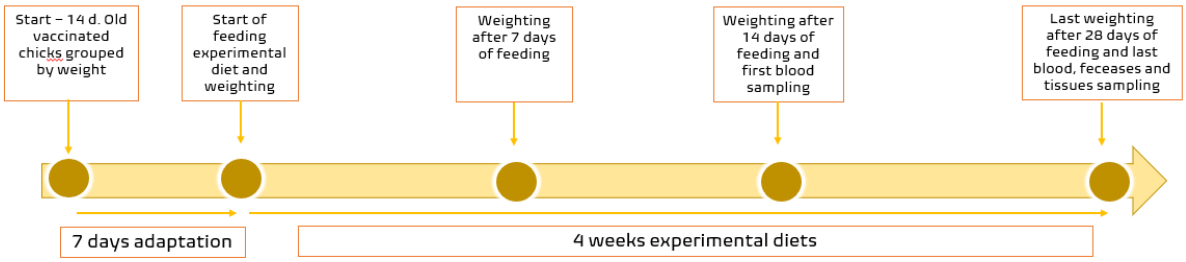

Figure 1. The study scheme of the impact of probiotics in the diet of broilers on the dynamics of bird growth.

Animals were fed the starter formula from d1 to d14 and grower from d21 to d28 and finisher formula from d28 to d49. The dietary treatments after the beginning of the experimental period reported in Table 1, as well as rations formulations.

Table 1. The feeding scheme of experimental diets.

Groups	**Diets NAE (no antibiotic ever)**	
	14 days old – 27 days old	**28 days old – 49 days old**
CON	Basic balanced diet– Grower (GBd)	Basic balanced diet – Finisher (FBd)
Experimental 1,2,3,4	Basic balanced diet – Grower (GBd) + 0,1% of probiotic	Basic balanced diet – Finisher (FBd) + 0,1% of probiotic
Experimental 1 (EI)	GBd / FBd + 0.1% *S. Cerevisiae (EnzActive – produced by Enzym Group)*	
Experimental 2 (EII)	GBd / FBd + 0.1% – *K. Marxianus S1 (produced by Enzym Group - pilot sample R&D)*	
Experimental 3 (EIII)	GBd / FBd + 0.1% - *K. Marxianus S2 (produced by Enzym Group - pilot sample R&D)*	
Experimental 4 (EIV)	GBd / FBd + 0.1% - *K. Marxianus CS (Commercial strain)*	

The health and vitality status of the subjects were evaluated twice daily and at the end of the trial, a post-mortem exam was performed on all animals. On days 15, 21, 28 and 40, all the birds were weighed, and average daily gain (ADG) was calculated. On days 28 and 49, five broilers from each replicate were sacrificed and samples were collected for further analyses.

2.2. Yeast strains origin

Yeasts *Saccharomyces cerevisiae* was grown at the facilities of Enzym group, on a molasses medium in accordance with the developed regulations to produce this strain. Were grown at the facilities of Enzym groups, on a molasses medium in accordance with the developed technological regulations for the

production of this strain. After receiving the yeast cream, it was dried in a spray dryer. As a result, we received product EnzActive – live *Saccharomyces cerevisiae* with a yeast cell count ≥ $1.5*10^{10}$CFU/g.

Yeasts *Kluyveromyces marxianus* (KMS1 and KMS2), had been selected and grown in R&D center of Enzym Company – isolated from dairy products, showed itself as a high-tech and heat-resistant strains compared to other strain candidate strains.

Kluyveromyces marxianus (B0399) is a commercial strain from the yeast market, were used to compare with Enzym's strains.

2.3. Blood sampling.

At days 14th and 21st of trials, 5 chicks from each replicate were randomly selected. Blood samples were collected in tubes with EDTA anticoagulant tubes. Portions of each blood sample were immediately used for determining whole list of biochemical and immunological parameters. The remaining was centrifuged, and their plasma was collected and stored at −80ºC for further enzymatic and chemical analyses. For superoxide dismutase (SOD) determination erythrocytes were washed with ice acetic acid according to methodology.

All procedures on animals were carried out in compliance with European Union regulations (EU Council. Directive 86/609/EEC on the Protection of Animals Used for Experimental and Other Scientific Purposes, 1986. EU Commission).

2.4. Biochemical Parameters.

Malondialdehyde (MDA): The blood was centrifuged at 1,500 × g for 5 min; plasma was collected in labeled tubes and stored at −80°C until analysis. After thawing, 500 µL of plasma was placed in

a labeled glass tube and mixed with the reagents of a commercial kit for the measurement of thiobarbituric acid reactive substances (TBARS). Each tube was covered with a glass marble and

incubated at 95°C for 45 min. The tubes were removed from incubation and allowed to cool in an ice bath for 10 min. Once cooled, the tubes were centrifuged at 3000 × g for 10 min and the supernatant carefully removed from the tubes for analysis. The absorbance of the supernatants was measured at 532 nm using a UV/VIS spectrophotometer [16].

Lipid hydroperoxides (LHP): Determination of the content of lipid hydroperoxides in biological material is achieved by precipitation of proteins with a solution of trichloroacetic acid and extraction of lipids with ethanol followed by the interaction of the studied extracts with ammonium thiocyanate [17]. Tissue collection and preparation of samples for extraction are carried out at t<4 °C. 0.2 ml of blood plasma, which contains 0.5 mg/ml of sodium oxalate in a buffer solution of Ph 7.4, placed in a centrifuge tube with well-fixed cork, add 2.8 ml of ethanol and 0.05 ml of 50% THOK solution. The test tube is closed and shake for 5–6 min. A protein precipitate is formed isolated by centrifugation for 10 min at 3000 rpm. The obtained supernatant, which is an ethanol extract of lipids, is used as an object for determination of lipid hydroperoxides and total lipids, which contain polyunsaturated fatty acids.

Superoxide dismutase (SOD) activity can be assessed using spectrophotometric assays that measure the enzyme's ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide radicals. This method, originally developed by Beauchamp and Fridovich in 1971, relies on monitoring the decrease in absorbance at 560 nm. The reaction involves generating superoxide radicals via a xanthine-xanthine oxidase system, which then reduce NBT to a formazan dye. SOD inhibits this reduction, and the extent of inhibition correlates with the enzyme's activity [18]. The assay for **glutathione (GSH)** content typically involves the use of a spectrophotometric method based on the reaction of GSH with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), also known as Ellman's reagent. In this assay, GSH reduces DTNB to produce a yellow-colored 5-thio-2-nitrobenzoic acid (TNB), which can be measured at 412 nm. The absorbance at this wavelength is proportional to the GSH concentration in the sample [19]. **Transferases:** serum was separated by centrifugation at 6000 rpm for 1.30 minutes and serum analysis was carried out immediately. The serum Alanine Transaminase (ALT) and Aspartate Aminotransferase (AST) were measured using commercial kits (AST (AS101) and ALT (AL100) – Randox, United

Kingdom) and a spectrophotometer. AST and ALT were measured at 546 nm while ALP measured at 405 nm.

The **total protein** in serum was measured by Biuret test and absorbance was measured at 550 nm and serum **albumin** was measured at 630 nm.

2.5. Body Weight Measurement

Body weight (BW) was recorded on a weekly basis for comparative evaluation and interaction effects of all treatment groups. Mortality was observed and recorded on daily basis.

2.6. Statistical analysis.

All data were analyzed by t-Test, using analysis when the overall P value of the experiment was below the value of significance ($P < 0.05$), t test was applied in order to assess the significance of results of single pairs of data.

3. RESULTS AND DISCUSSION

Studying ALT and AST activity in broilers' blood when feeding them probiotics is crucial for evaluating liver health and overall metabolic status. ALT (Alanine Aminotransferase) and AST (Aspartate Aminotransferase) are enzymes that indicate liver function, and their activity levels can reveal potential liver damage or stress.

In our studies, ALT activity on the 14th day of the experiment was within the normal range. However, on the 28th day, the ALT activity in the control group and the group fed a commercial probiotic from the market exceeded the upper allowable limit. In contrast, in broilers that received experimental probiotics in their diet, including the product EnzActive, the ALT level remained within the normal range (Figure 2).

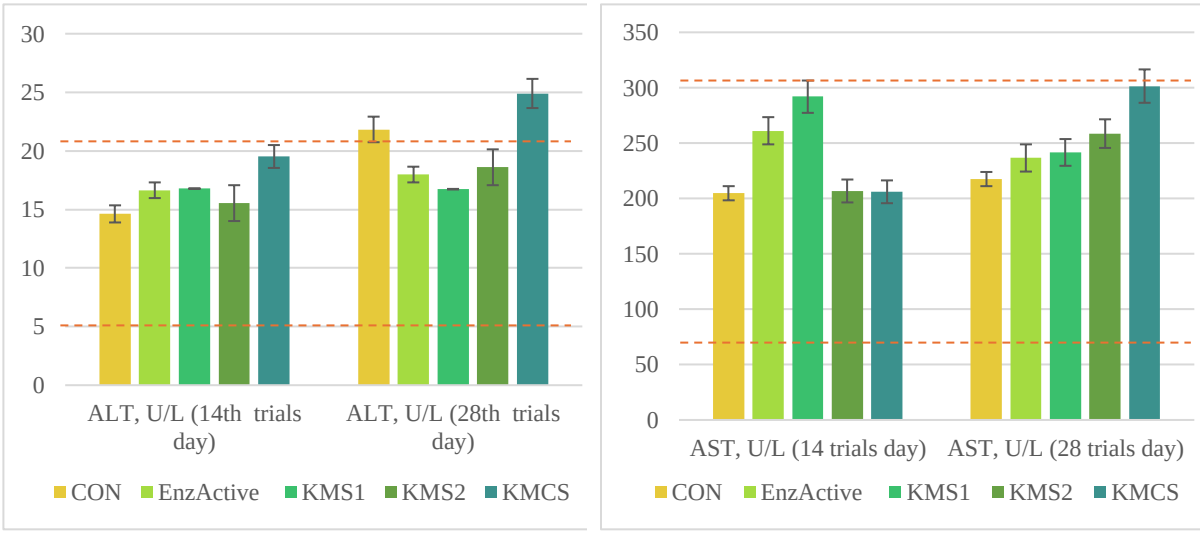

Figure 2. The activity of transferase enzymes in the serum of broilers during the experiment.

As for AST activity, on the 28th day of the experiment, the enzyme activity in the blood of the birds fed EnzActive and KM S1 normalized. The activity slightly intensified in the blood of the broilers that received KM S2 probiotic, but the changes remained within physiological norms. But again, the last group showed excessive enzyme activity growth, which may indicate metabolic disturbances**.**

Probiotics are known to influence gut health and immune response, which can, in turn, impact liver function. Monitoring these enzymes helps in understanding the safety and efficacy of probiotics in

poultry diets, ensuring they promote overall health without causing adverse effects. Research has shown that probiotics can modulate enzyme activity, highlighting their role in maintaining liver health in broilers [20, 21].

There is enough evidence that broilers show inflammatory changes in the liver through their life. Some authors showed that the increased ALT and AST rate in the serum of the COBB 500 broiler could be the cause for general liver lesions. The increased level of AST might be lowered due to lessened growth rate caused by reduced food intake with increased temperature.

The increased AST/ALT ratio indicates that increased heat may cause chronic liver damage in fast growing broilers [22] and this might relate with the occurrence of sudden death syndrome (SDS) when compared with the enzyme levels of dead broilers from sudden death syndrome in other studies [23].

ALT enzyme is found in highest amount in liver and is used to identify acute liver failures [24] as the enzyme is released into the serum immediately after a hepatocellular damage.

The changes in the activity of both enzymes recorded in the blood of birds in the experimental groups that received probiotics produced by the Enzyme company are within physiological norms and do not indicate any protein metabolism disorders or liver and myocardium dysfunctions. In contrast, the enzyme response upon the addition of the commercial product obtained for comparative evaluation prompts consideration of the underlying cause of these changes, as the alterations in activity were significant.

The impact of yeast probiotics on ALT and AST activity in broilers is significant in assessing liver health and overall metabolic function. Studies have shown that incorporating yeast-based probiotics into broiler diets can positively influence liver enzyme levels, potentially reducing liver stress and enhancing metabolic efficiency. For instance, research by Zhang et al. (2020) [25] demonstrated that broilers fed with yeast probiotics exhibited lower ALT and AST levels compared to control groups, indicating improved liver function and reduced tissue damage. Another study by Gao et al. (2017) [26] found similar results, suggesting that yeast probiotics can help maintain enzyme activity within physiological norms, thus supporting better health and growth performance in broilers. These findings highlight the beneficial role of yeast probiotics in poultry nutrition and their potential to promote liver health and metabolic stability. Our results are fully consistent with the findings of these authors.

Probiotics in feed have a positive effect because it can increase the content of lysine analog and aminoethyl cysteine in the digestive tract, most of them are hydrolyzed into amino acids lysine and cysteine and can increase protein retention which plays a role in meat formation [27]. The addition of probiotics increases the activity of the protease enzyme that needed in digestion to break peptide bonds in feed protein to free amino acids needed to the body [28]. Amino acids play a role in composing body tissues and in growth so the increased availability of amino acids will increase growth and body weight [29]. The accumulation of amino acids that can be absorbed by the small intestine, can increase the activity of the protease enzyme [30]. The increased secretion of protease enzymes associated with protein metabolism causes the rate of protein metabolism in the liver increases, so total plasma protein levels increase, with increasing of total plasma albumin. Most of the blood protein comes from ration proteins that undergo digestion and absorption in the intestine in the form of free amino acids that are carried by the portal blood to the liver. Free amino acids are precursors for the synthesis of blood proteins, which are synthesized in the liver. Nearly 90% of blood protein is released into the blood circulation. The main blood proteins are albumin, globulin, and fibrinogen. Blood proteins play a role as blood clotting, enzymes, hormones, immune defense, a role in the inflammatory response, and transport or binding proteins. Blood protein functions are to maintain osmotic pressure, as a source of amino acids for tissues, transport nutrients to cells and waste products to secretory organs, the body's acid-base balance (buffer). Albumin can bind various ligands and is responsible for 80% of the osmotic pressure [31, 32, 33, 34, 35]. Normal levels of total blood protein in chickens are in the range of 32.7−44.0 g / l [36], albumin levels are between 10.9−15.6 g / l.

Based on the results of the study, the effect of giving yeast probiotics on the total protein content of broiler chicken blood is presented in Figure 4.

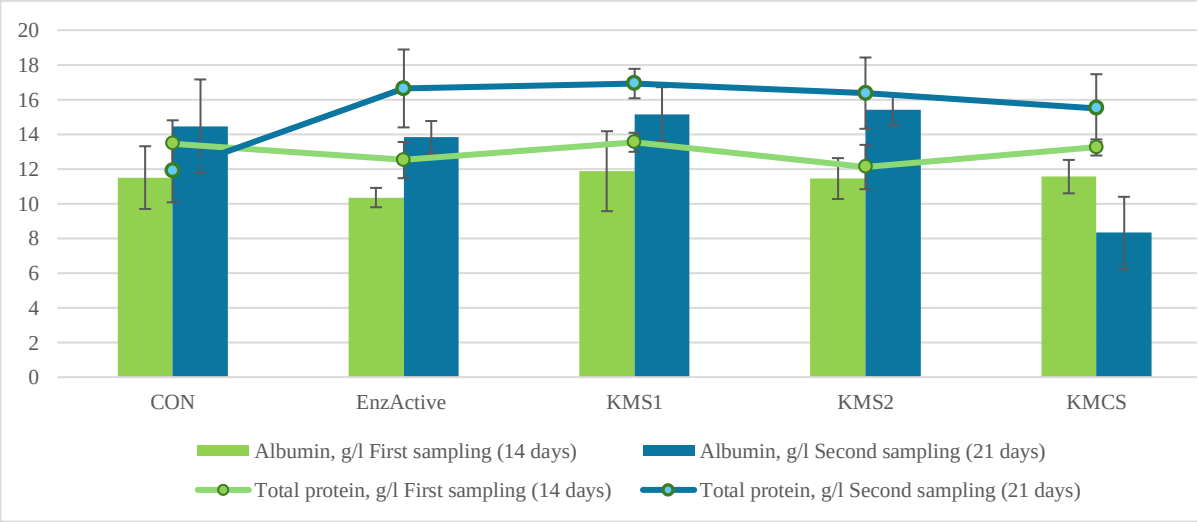

Figure 3. Total Protein and Blood albumin levels.

The treatments with probiotics (EnzActive, KM S1 and KM S2) showed higher total protein and albumin levels compared to the control group. This increase probably attributed to the elevated activity of the protease enzyme in the intestines of the probiotic-treated groups. Probiotics enhance the activity of protease enzymes, which are crucial for breaking down peptide bonds in feed proteins, thereby releasing amino acids needed by the body. Additionally, Wang and Qing (2010) reported that broiler chickens fed with probiotics exhibited a significant increase in protease enzyme activity, leading to improved feed protein utilization during digestion [37].

The activity of probiotics produces short-chain fatty acids that function to repair the microvilli, namely increasing the villi height, width of the villi, and goblet cells in the small intestine, so that the area of absorption of food substances increases. Increasing the number of microbial populations that are beneficial for livestock prevents the development of pathogenic microbes in the digestive tract which leads to increased digestion of food. Increased digestibility and absorption of food substances will have an impact on increasing total blood protein.

Integrating yeast probiotics into broiler diets has demonstrated beneficial effects on protein metabolism, as evidenced by changes in serum proteins, albumin, and globulin levels. Research shows that broilers consuming yeast probiotics display elevated total protein and albumin concentrations, signaling improved liver function and protein synthesis. Moreover, the equilibrium between albumin and globulin, crucial for sustaining immune responses and overall health, is enhanced. For instance, Gao et al. (2017) reported that yeast probiotics increased serum protein levels and optimized the albumin-to-globulin ratio, resulting in better growth performance and health status in broilers. Additionally, Zhang et al. (2020) corroborated these results, showing that dietary yeast probiotics significantly boost protein and albumin levels, thereby supporting the immune system and overall metabolic health of broilers [38, 39].

It is crucial to investigate changes in oxidative stress and antioxidant defense in the blood of broilers when studying the addition of probiotic yeast to their diets. Oxidative changes, provoked by the influence of various stress factors (including thermal), can cause significant cellular damage, leading to impaired growth, health issues, and reduced overall performance in broilers. Probiotic yeast has been shown to enhance antioxidant defense mechanisms, helping to neutralize harmful free radicals and protect cellular integrity. By understanding the impact of probiotic yeast on these parameters, we can optimize broiler diets to improve health outcomes, enhance growth performance, and ensure the sustainable and efficient production of poultry [40, 41]. The oxidative status can be assessed based on primary oxidation [i.e., through the measurement of lipid hydroperoxide (LHP) value] or secondary oxidation [i.e., through the measurement of malondialdehyde (MDA).

Our research showed that by the end of the study, the level of lipid peroxidation products in the blood of broilers in the experimental groups was significantly lower than in the control group (Figure 3).

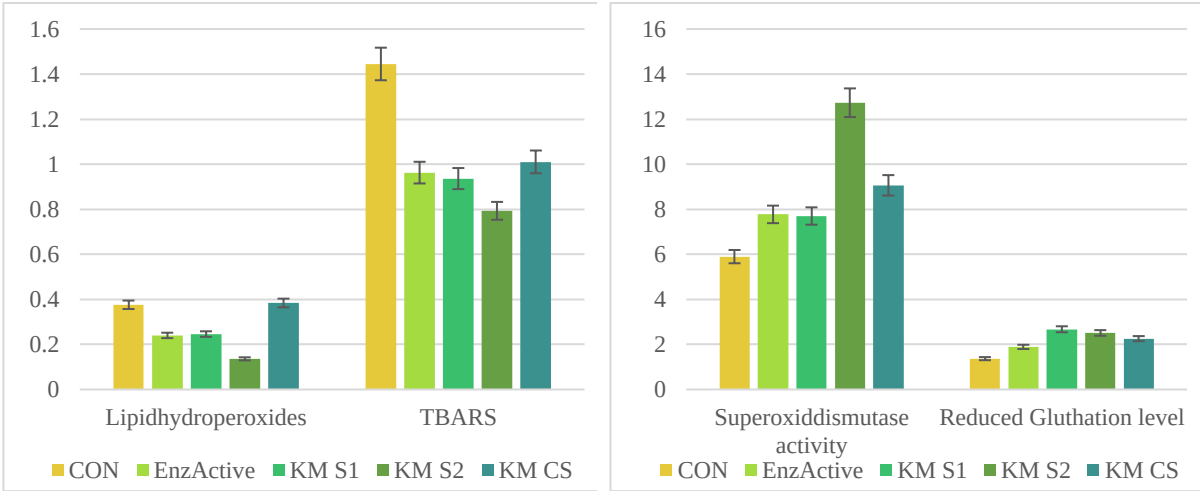

Figure 4. The level of lipid peroxidation products, the activity of superoxide dismutase, and the content of reduced glutathione in the blood of broilers.

This effect was especially pronounced with the addition of the probiotic KM SC2 to the diet, which was our target strain in this research. Consequently, the activity of superoxide dismutase (SOD) was higher in the blood of broilers with probiotics in their diet, as was the content of reduced glutathione compared to the control. The highest SOD activity was recorded in the group that consumed the target probiotic in their diet.

Oxidative metabolism is a normal process in all tissues. During the normal oxidative metabolic process, various reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced. During this normal metabolism, 1 to 2% of oxygen is converted to ROS. ROS are implicated in different disorders, including thermal injury, inflammation, sepsis, mutagenesis, carcinoma, autoimmune diseases and ischemia reperfusion injury. The role of ROS in the injury induced by ischemia reperfusion has been convincingly shown in different organs, including the brain, liver, skin, muscle, lung, intestine, kidneys and heart. However, under some circumstances, increased ROS/RNS production or decreased antioxidant defenses may lead to oxidative stress, in which case the generated reactive species can alter the properties of lipids, proteins and nucleic acids, leading to cellular dysfunction [42].

Probiotic yeasts in broiler diets can significantly influence oxidative stress parameters, including lipid peroxidation and superoxide dismutase (SOD) activity. By enhancing antioxidant defenses, probiotic yeasts help mitigate oxidative damage and support overall health. Research has shown that dietary supplementation with probiotic yeasts reduces lipid peroxidation products and increases SOD activity in broilers. This effect is attributed to the probiotic's ability to improve the antioxidant status of the birds, thus promoting better growth performance and health outcomes [43, 44, 45, 46]. Aluwong, T with his team proved that administering yeast probiotic supplement increased body weight and enhanced serum antioxidant enzyme activities of broiler chickens. Antioxidant enzymes are most effective when acting synergistically with one another or with other components of the antioxidant barrier of the organism when their activity remains balanced. It has been shown that nutrition plays a vital role in maintaining the pro-oxidant-antioxidant balance [47]. In the present study, there was increased in both GSH content and SOD activity. The most importantly growth processes in early life are characterized with the generation of ROS through cellular division and apoptosis [48]. A similar study in turkey reported that mannanoligosaccharides a component of S. cerevisiae used as dietary additive stimulate the mechanisms of oxidative defense and improve the growth performance of the birds [49].

Body weight (BW) was recorded on a weekly basis for comparative evaluation and interaction effects of all treatment groups. Mortality was observed and recorded on daily basis (Figure 5).

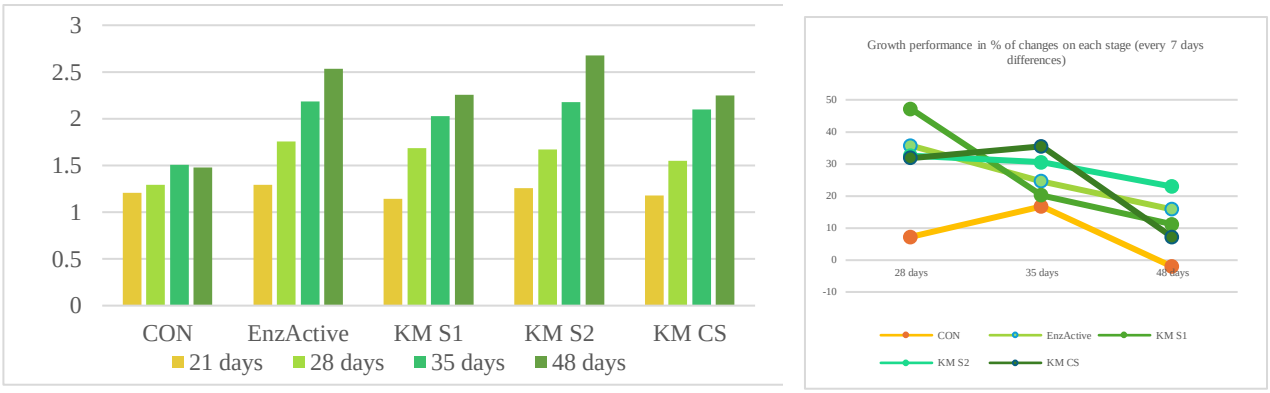

Figure 5. Broilers Body weight (BW) dynamic during trials

The present study revealed that probiotic supplementation had an impressive impact on the body weight of broiler chickens from the second week of life. This may be explained as one of the yeast probiotic beneficial effects of promoting a healthy gastrointestinal tract environment by nourishing the enterocytes, improving ileal mucosal development and reinforcing mucosal barrier function through maintaining epithelial integrity. This finding is similar with the result obtained by Zhang et al. [50], who reported improve growth rate of ma le broiler chickens supplemented with S. cerevisiae. In addition, this finding agrees with the results obtained by several authors [51] in studies with broiler chickens. Moreover, we have demonstrated that new probiotics based on a promising *Kluyveromyces* strain isolated from dairy products can exhibit powerful probiotic effects in poultry diets, with the complete exclusion of antibiotics at all stages of growth.

CONCLUSIONS

The results of the conducted studies indicate that probiotic yeast improves metabolic processes, reduces the development of stress-related effects, and enhances the growth rate of poultry. Considering that antibiotics and other growth stimulants were completely excluded from the diets throughout the study, probiotic yeast, particularly targeted strains of *Kluyveromyces marxianus*, can be successfully recommended for use in diets that are entirely free from antibiotics.

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