

The effect of probiotic nutrition of *Saccharomyces boulardii* and *Saccharomyces cerevisiae* on yield, antioxidant activity and expression of interleukin-6 gene in Holstein calves

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Received August 06, 2020; Accepted October 20, 2020

ABSTRAK

Penelitian dilakukan untuk mengetahui pengaruh probiotik *Saccharomyces boulardii* dan *Saccharomyces cerevisiae* terhadap hasil, aktivitas antioksidan, dan ekspresi gen interleukin-6 pada pedet Holstein. Enam belas pedet dibagi menjadi 4 perlakuan ransum dengan 4 pedet/perlakuan. Pakan perlakuan eksperimental meliputi 1) kontrol (tanpa penggunaan probiotik), 2) probiotik yang larut dalam susu berdasarkan *S. boulardii* (1 g/kg), 3) probiotik yang larut dalam susu berdasarkan *S. cerevisiae* (1 g/kg) dan 4) A campuran probiotik ragi *S. boulardii* dan *S. cerevisiae* sebagai larutan dalam susu sapi (masing-masing 1 g/kg). Rancangan acak lengkap digunakan dalam penelitian ini. Hasil penelitian menunjukkan bahwa pemberian pakan *S. boulardii* dan *S. cerevisiae* tidak berpengaruh nyata dibandingkan dengan kontrol. Tidak ada perbedaan yang signifikan antara perlakuan yang berbeda untuk jumlah enzim antioksidan dibandingkan dengan kontrol. Superoksida dismutase (SOD) pada perlakuan menggunakan probiotik lebih tinggi dibandingkan dengan kontrol. Ekspresi ekspresi gen interleukin-6 meningkat secara signifikan ($P<0,05$) untuk perlakuan 3 dan 4 pada hari ke 10, 30 dan 60 dibandingkan dengan kontrol. Kesimpulannya, penambahan probiotik tidak mengubah sifat kinerja aktivitas antioksidan, tetapi perlakuan *S. cerevisiae* meningkatkan ekspresi gen interleukin-6 yang menunjukkan efek pada sistem kekebalan.

Kata kunci : probiotics, *Saccharomyces*, *Boulard's Saccharomyces*, *Holstein*, *calf*, *performance*

ABSTRACT

An experiment was performed to investigate the probiotic effect of *Saccharomyces boulardii* and *Saccharomyces cerevisiae* on yield, antioxidant activity, and expression of interleukin-6 gene in Holstein calves. Sixteen calves were divided into 4 dietary treatments with 4 calves/treatment. Experimental treatment diets include 1) control (without probiotic use), 2) milk soluble probiotics based on *S. boulardii* (1 g/kg), 3) milk soluble probiotics based on *S. cerevisiae* (1 g/kg) and 4) A mixture of yeast probiotics *S. boulardii* and *S. cerevisiae* as a solution in dairy milk (1 g/kg each). The completely randomized design was used in this experiment. The results showed that feeding *S. boulardii* and *S. cerevisiae* did not have a significant effect on performance traits compared with control. There was no significant difference between the different treatments for the number of antioxidant enzymes compared to the controls. Superoxide dismutase (SOD) in the treatments used probiotics were higher than the control. The expression of interleukin-6 gene expression was increased significantly ($P<0.05$) for treatments 3 and 4 on Days 10, 30 and 60 compared to the control. In conclusion, addition of probiotics did not alter performance traits of antioxidant activity, but the *S. cerevisiae* treatment did increase

interleukin-6 gene expression which suggests an effect on the immune system.

Keywords: probiotik, Saccharomyces, Boulard's Saccharomyces, Holstein, calf, performance

INTRODUCTION

In recent years, the feed composition of infant dairy calves has been considered important for growth and production (Mollenhorst *et al.*, 2016). During the this period, the calves are affected by various types of stresses due to the management system which affects their growth efficiency. Some stresses can reduce the function of growth and the immune system by altering ruminal and intestinal microorganisms, resulting in increased mortality (Seifzadeh *et al.*, 2016). Another harmful effect of these stresses is the production of free radicals. Free radicals are atoms or molecules with single and unsaturated electrons that have a very high chemical reaction (reactivity) to achieve stability, and therefore in the body of living organisms cause damage to various macromolecules such as proteins, lipids, carbohydrates, and nucleic acids and have certain physiological and pathophysiological effects (Halliwell and Gutteridge, 2015).

The most important free radicals are hydrogen peroxide (H₂O₂), anion superoxide (O₂⁻), and radical hydroxyl (OH·) (Poorghasemi *et al.*, 2013).

The most destructive effect of these free radicals is on the cell membrane and the membrane of intracellular organs such as the mitochondrial membrane. These reactive oxygen species (ROS) cause peroxidation of lipids which can damage phospholipid membrane of cells and the cell wall to harden, thus affecting many of the cell's activities (Martarelli *et al.*, 2011).

Production of free radicals and ROS in the body's cells is inevitable, but the body also uses a complex and skilled defense system called antioxidants, which includes enzymatic and non-enzymatic reactions (Martarelli *et al.*, 2011).

The most important enzymatic antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT). Also, non-enzymatic antioxidants include some vitamins, a number of trace elements, and probiotics (Shwartz *et al.*, 2009).

Some researchers believe that enzymatic antioxidants are not enough to prevent oxidative damage, and oral additives can be effective. In recent decades, probiotics have been shown to be one of the most important dietary supplements,

stimulating growth, increasing antioxidant capacity, and boosting the immune system (Moslemipur *et al.*, 2014).

Probiotics are living microorganisms that, if consumed in moderation, can have beneficial effects on host health. These microorganisms are widely used to treat infections, allergies, and inflammatory diseases, and act by balancing the population of intestinal microbial flora. They have been shown to have a positive effect on improving animal performance and function of their immune system (Salarzadeh *et al.*, 2019).

As can be seen, immune responses fall into two categories, humoral (antibody dependent) and cellular (T cell dependent). Removal of soluble antigens and destruction of extracellular microorganisms is the main task of humoral immunity (due to antibodies produced by B cells) and elimination of intracellular microorganisms (due to T cells). Antibodies are a group of proteins in globulin that are involved in immunity and are called immunoglobulins. One of the signals for activating the humoral immune response is cytokines such as interleukin-6, which activate helper T lymphocytes, proliferate and differentiate B cells. In fact, interleukin-6 is effective on plasma cells and lymphocytes, and the main function of this cytokine is to differentiate plasma cells faster and produce more antibodies (Downing *et al.*, 2010).

Probiotics inhibit oxidative stress by reducing inflammatory lesions and increasing the levels of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase. Probiotics increase the ratio of T lymphocytes to suppressive T lymphocytes. Probiotics have been shown to increase interleukin-6 levels and boost the host's immune system (Fantuzzi, 2005).

Saccharomyces are the most important industrial yeast for the production of fermented products, recombinant proteins, and monounsaturated proteins. The genus Saccharomyces includes two well-known species, *Saccharomyces cerevisiae*, and *Saccharomyces bullardii*. *Saccharomyces cerevisiae* is the most famous yeast and fermenter of carbohydrates, which can be effective in the immune system by applying various mechanisms (Martins *et al.*, 2007).

Saccharomyces bollard is also one of the

most widely used probiotics and is commonly used to fight intestinal pathogenic microorganisms and therefore can have special benefits in improving host performance (Rajkowska *et al.*, 2014). In their experiment, Lascano *et al.* (2009) concluded that the probiotic yeast *Saccharomyces boulardii* had the potential to improve intestinal health by reducing the duration and severity of diarrhea.

A number of studies have demonstrated the effects of yeast probiotics on improving performance parameters in livestock (Ayad *et al.*, 2013). Wang *et al.* (2009) concluded in an experiment that adding probiotics at the end of the pig breeding period increased the antioxidant capacity of the animal serum so that the GPX and serum SOD were increased. Hardy *et al.* (2013) also reported in their experiment that the use of probiotics increases the function and antioxidant activity.

Given the importance and sensitivity of dairy calf production and the positive effect of probiotics as an additive on growth, health, and immune system function, the present study aimed to investigate the use of two type of probiotic yeast separately and in combination on performance, antioxidant activity and expression of the interleukin-6 gene in dairy calve.

MATERIALS AND METHODS

The present study used 16 newly born Holstein calves with an average birth weight of 2.5 4 2.5 (unclear what is intended) kg after feeding with colostrum. When 3 days old, the calves were randomly allocated to 4 treatments (sample size = 4 calves/treatment). The experimental treatment diets were 1) control (without probiotic use), 2) daily soluble probiotic based on *Saccharomyces boulardii* (1 g/kg), 3) daily soluble probiotic based on *Saccharomyces cerevisiae* (1 g/kg) and 4) A mixture of *Saccharomyces boulardii* and *Saccharomyces cerevisiae* yeast probiotics as a solution in dairy milk (1 g/kg each).

Saccharomyces cerevisiae CNCM-1077 yeast probiotics and *Saccharomyces cerevisiae* var. *Boulardii* CNCM I-1079 (*Saccharomyces cerevisiae* var. *Boulardii* CNCM I-1079) were used in this study, respectively 2×10^{10} g/CFU was a living yeast cell.

The calves were fed colostrum (10% of body weight) from day one to day three. Since the fourth day, 4 liters of milk per day was fed to the

calves. In addition to milk, the calves had free access to a fully mixed diet. The calves' diets were adjusted using the NRC table (2011). The nutrients used in the diet and its chemical composition are presented in Table 1. 10% of alfalfa (substituted with wheat bran) in sizes of 2 to 3 cm at 35 days of age of calves was added to the initial feed.

Performance

From the fourth day, the calves' food was weighed daily by a digital scale and given to them *ad libidum*. By deducting the remaining amount of food from the initial amount the next morning, the amount of food eaten by each calf was recorded, and the feed consumed for the whole period was calculated. The calves' weight was measured every ten days before the morning meal. To obtain the average weight gain of the whole period, by dividing the difference in the amount of weight at the end of each calf period, the birth weight was used based on the number of days spent in the relevant period. Finally, the feed conversion ratio for each calf was obtained by dividing the feed consumed throughout the period by the weight gain over the entire period.

To measure antioxidant activity, blood sampling was performed before feeding in the morning on the 10th, 30th, and 60th days at the beginning of each treatment. Blood was drawn from the tail vein and allowed to clot. To prepare the serum, the test tubes containing blood samples were centrifuged at 3000 rpm for 20 minutes. Serum samples were stored at -20. C to determine antioxidant parameters.

The activity of antioxidant indicators was measured using Pars Azmoun commercial kits by Eliza Reader device (ELX800 model, Bio-Tek) at a wavelength of 412 nm for glutathione peroxidase activity. The concentration of superoxide dismutase was calculated by nitroblotrazole (NBT) at 560 nm. The activity of the enzyme catalase was also measured by the Claiborne method at 240 nm

Interleukine-6 gene expression (IL)

Blood samples were collected to be examined for IL-6 gene expression at the end of the treatment period. It was transferred to a liquid nitrogen tank at the Molecular Genetics and Biotechnology Laboratory and stored at -80 C until RNA extraction. Total RNA was isolated

Table 1. Nutritional Values and Chemical Compounds of Starter Diet

Dietary compounds	Percentage in diet	Chemical compounds	Percentage in diet
Barley	5	DM	88.851
Corn grain	35	TDN	83.476
Soybean meal	33.2	CP	18.83
Wheat barn	20	CF	4.338
Ground salt	1	EE	8.712
Calcium carbonate	1.2	NDF	10.93
Magnesium oxide	0.3	ADF	5.652
Di-calcium phosphate	0.3	Ca	1.076
Sodium bicarbonate	1	P	0.443
Vitamin Supplement ¹	1.5		
Mineral supplement ²	1.5		
Total	100		

DM: dry matter; TDN: total digestible nutrients; CP: crude protein; CF: crude fibre; EE: ether extracts; NDF: neutral detergent fiber and ADF: acid detergent fiber

¹ Vitamin premix (mg/kg diet): vitamin A: 2000000 IU; vitamin D: 25000 IU; vitamin E: 3000 IU and Antioxidant: 600 mg.

² Mineral premix (mg/kg diet): Cu: 12500 mg; Co: 10 mg; I: 100 mg; Fe: 400 mg; Mn: 10 mg; Zn: 6500 mg and Se: 10 mg.

from blood samples using a one-way test company according to the manufacturer's instructions. To make cDNA from Qiagen's QuantiFast Reverse Transcriptase kit (QIAGEN, 205311), and according to the manufacturer's instructions, the cDNA of each sample was prepared from its extracted RNA. The sequence of primers used to study the IL-6 gene included primers for real-time PCR and primers for the GAPDH gene as the internal control for normalization (Table 2). Using Real-time PCR primers and using Sybergreen, gene expression was evaluated in cDNA samples prepared from the experimental sample (Poorghasemi *et al.*, 2017).

Statistical Analysis

The data collected in a completely randomized design were statistically analyzed using the General Linear Model (GLM) and statistical software SAS (2004). The averages was compared using Duncan's test at 5% probability level. The statistical model of the design was $Y_{ij} = \mu + A_i + e_{ij}$. In this model, Y_{ij} : The value of each observation for the studied attribute, μ : Average

observations, A_i : The effect of experimental treatments and e_{ij} : The effect of experimental error.

RESULTS

Table 3 shows the effect of experimental treatments on performance traits in calves. The difference in weight and mean body weight of calves in experimental treatments did not differ significantly from control ($P > 0.05$). Also, the mean of dry matter intake and conversion factor of any of the treatments were not significantly different from the control ($P > 0.05$). Table 4 presents the results of the effect of experimental treatments on the activity of serum blood antioxidant enzymes. Accordingly, the amount of antioxidant enzymes GPX, CAT, and SOD in the blood serum of calves at the ages of 10, 30, and 60 days was not significantly different between the treatments ($P > 0.05$). Table 5 shows the results of the experimental diets on the expression of the interleukin-6 gene. On the

Table 2. The Sequence of Primers Designed to Real Time PCR

Gene Symbol	Direction	Sequence of the Primers	Product size (pb)
IL-6	Forward	5'-TGATGGATGCTTCCAAACTG-3'	20
	Reverse	5'-GAGCATTGGAAGTTGGGGTA-3'	20
GAPDH	Forward	5'-GTATTGGGCGCCTGGTCACC-3'	20
	Reverse	5'-CGCTCCTGGAAGATGGTGATGG-3'	22

Table 3. The Effect of Experimental Treatments on Calf Performance

Performance Traits	T1	T2	T3	T4	SEM	P-value
Weight (kg)	52.022 ^a	54.447 ^a	56.982 ^a	58.882 ^a	2.708	0.345
Feed Consumption (kg/cow per 60 d)	28.941 ^a	34.906 ^a	34.908 ^a	39.007 ^a	4.259	0.357
Body weight gain (kg/cow per 60 d)	20.750 ^a	25.500 ^a	26.000 ^a	29.329 ^a	3.036	0.395
Feed conversion ratio	1.394 ^a	1.368 ^a	1.342 ^a	1.329 ^a	0.099	0.998

T1: Control; T2: *S. boulardii* (1 g/kg of diet); T3: *S. cerevisiae* (1 g/kg of diet) and T4: *S. boulardii* (1 g/kg of diet) + *S. cerevisiae* (1 g/kg of diet).

The means within the same row with at least one common letter, do not have significant difference (P<0.05). SEM: standard error of the means.

tenth day of the experiment, the expression of interleukin-6 gene expression was significant for treatments 3 and 4 with control (P < 0.05). On days 30 and 60 of the experiment, the difference in expression of the interleukin-6 gene in calves using probiotics increased significantly (P < 0.05).

DISCUSSION

Also, the average dry matter intake of probiotic-containing treatments increased more than in the control group, and the conversion factor of these treatments improved. Researchers have reported that feeding probiotics improved the daily weight gain of dairy calves, possibly due to improved microbial flora and maximum livestock use of dietary nutrients (Riddell *et al.*, 2010). In newborn calves or under environmental stress, the microbial population can change dramatically. It seems probiotics can accelerate their daily weight gain by accelerating the animal's habituation to solid foods and reducing

the number of bacteria that cause infectious diarrhea (Lorenzoni *et al.*, 2012).

According to the results of the present experiment, the treatments did not affect growth characteristics. Neither weight, feed consumption nor feed conversion of the probiotic-treated calves differed significantly from the control treated group.

According to the findings of the present study, the dry matter intake of calves fed probiotics was not affected compared to the control group (Mohamadi Roodposhti and Dabiri, 2012). Although this increase was not significant in the present experiment, its effect on the weight gain of probiotic consuming calves is quite evident. According to the researchers, the increase in dry matter intake in calves depends on the development of ruminal epithelium and its capacity, as well as the rapid adaptation of calves to solid feed (Hossain *et al.*, 2012).

Adding probiotics to the feed consumed

Table 4. The Effect of Experimental Treatments on the Activity of Serum Calf Antioxidant Enzymes at Different Ages

Antioxidant enzymes	T1	T2	T3	T4	SEM	P-value
Glutathione peroxidase (GPX)						
day 10	3.888 ^a	9.615 ^a	10.059 ^a	11.631 ^a	3.298	0.421
Day 30	4.425 ^a	8.802 ^a	10.394 ^a	15.11 ^a	6.430	0.433
Day 60	6.916 ^a	8.822 ^a	12.598 ^a	21.376 ^a	5.887	0.618
Catalase (CAT)						
Day 10	0.270 ^a	0.270 ^a	0.270 ^a	0.270 ^a	0.000	0.802
Day 30	0.270 ^a	0.270 ^a	0.270 ^a	0.270 ^a	0.000	0.916
Day 60	0.271 ^a	0.271 ^a	0.271 ^a	0.271 ^a	0.001	0.693
Superoxide dismutase (SOD)						
Day 10	200 ^a	431.2 ^a	495.6 ^a	518 ^a	97.443	0.162
Day 30	216 ^a	416.7 ^a	522.1 ^a	631.7 ^a	207.663	0.564
Day 60	326.3 ^a	432.2 ^a	543.1 ^a	785.5 ^a	178.637	0.365

T1: Control; T2: *S. boulardii* (1 g/kg of diet); T3: *S. cerevisiae* (1 g/kg of diet) and T4: *S. boulardii* (1 g/kg of diet) + *S. cerevisiae* (1 g/kg of diet).

The means within the same row with at least one common letter, do not have significant difference (P<0.05). SEM: standard error of the means.

by calves at the beginning of their birth has affected the function of the rumen so that these calves will be more inclined to eat than the control. This suggests that probiotics may have increased ruminal development. As a result, as the rumen expands, the number of papillae and the level of absorption increase, which increases the dry matter intake (Nakanishi *et al.*, 1993).

In the present experiment, calves fed probiotics had a better conversion factor than control. Using a mixture of *S. boulardii* and *S. cerevisiae* had the best conversion factor, although no significant difference was observed with control and other treatments. It seems that one of the reasons for the improvement in conversion ratio in probiotic treatments has been the improvement in animal health. Given that probiotics can improve the supply of microbial protein by developing rumen and improving the process

of ruminal fermentation, they will improve the conversion factor. By stimulating cellulose bacteria and bacteria producing ruminal lactate, probiotics increase digestibility and increase the passage of microbial protein through the rumen and increase the supply of microbial protein in the large intestine, which allows livestock to access more nutrients and improve conversion ratio (Hillman *et al.*, 2017).

The results of this study showed that the use of yeast probiotics did not affect the CAT activity of calves' blood serum compared to the control group. Also, according to the results, the amount of GPX and SOD enzymes in calves that had consumed probiotics, although not significantly different from the control was higher in all three measured times. In an experiment conducted on Swiss Brown infant calves, Tunc and Yoruk (2012) concluded that adding 0.15%

Table 5. The Effect of Experimental Treatments on the Expression of Interleukin-6 Gene in Calves at Different Ages

Interleukine-6	T1	T2	T3	T4	SEM	P-value
Day 10	1.000 ^b	0.596 ^b	1.765 ^a	1.768 ^a	0.171	0.003
Day 30	1.000 ^b	2.23 ^a	2.263 ^a	2.862 ^a	0.222	0.002
Day 60	1.000 ^c	5.964 ^b	10.781 ^a	14.381 ^a	1.160	0.000

T1: Control; T2: *S. boulardii* (1 g/kg of diet); T3: *S. cerevisiae* (1 g/kg of diet) and T4: *S. boulardii* (1 g/kg of diet) + *S. cerevisiae* (1 g/kg of diet).

The means within the same row with at least one common letter, do not have significant difference (P<0.05).

SEM: standard error of the means.

probiotics to the basal fluid during the first 12 weeks of infancy had a significant effect on GPX, CAT and SOD had no antioxidant enzymes.

However, various studies have shown that the use of probiotics affects the antioxidant status of the animal so that animals use the ability of probiotics to increase the level of antioxidants and neutralize the effects of various ROS (Truusalu *et al.*, 2004). In fact, probiotics play a stimulating role in the production of antioxidants and the breakdown of free radicals through the production of butyric and hydrogen acids (Martarelli *et al.*, 2011).

Wang *et al.* (2016) proposed that the use of a diet containing probiotics leads to an increase in the body's antioxidant activity. They said that probiotics, as an antigen, stimulated the secretion of antioxidant enzymes and increased antioxidant secretion, leading to the elimination of free and excess radicals produced by high metabolism and adverse environmental stress. It protects the body's tissues and organs by regulating the balance of free radical.

As can be seen from the results, the amount of antioxidant enzymes was higher in 60 days than in 10 and 30 days, which could be due to the increase in high metabolism and the production of more free radicals to increase growth (Persichetti *et al.*, 2014).

The present results for the expression of interleukin-6 gene indicate that both *boulardii*

and *cerevisiae* have been effective on interleukin-6 levels, but at the end of the period, the application of a mixture of both species has been able to increase interleukin-6 levels more than at other times.

Probiotics cause beneficial bacteria in the gut, which have many immunological and metabolic functions. Beneficial bacteria in the gut affect the function of the immune system by interacting with intestinal epithelial cells and lymphatic tissues around the gastrointestinal tract. In fact, probiotics increase the ability of these bacteria to strengthen the body's immune system and mucosal defenses (Dowarah *et al.*, 2018).

Stimulation of the immune system with probiotics may be due to increased T-type lymphocytes, phagocytes, and serum protein levels, which are associated with the secretion of cytokines by cells of the immune system stimulated by probiotics (Bai *et al.*, 2011). Interleukin-6 is secreted by T cells and macrophages to stimulate the immune response (Poorghasemi *et al.*, 2015).

Since interleukin-6 is related to Th2-related lymphocytes, it can be concluded that yeast probiotics *S. cerevisiae*, *S. boulardii* increase the number of Th2 lymphocytes (Fooks and Gibson, 2002). The effects of probiotics on the immune system have been reported in several studies (Scharek *et al.*, 2005; Benyacoub *et al.*, 2003).

However, the exact mechanism of action of probiotics on the immune system and their

differentiating effects on different lymphocytes and cytokines is not yet well understood. One possible mechanism of action is that the cell wall of probiotics has immune-stimulating compounds, such as peptidoglycans, that increase the activity of immune-related cells in the intestinal wall. Antigen-expressing dendritic cells are one of the most important cells in the intestinal wall that respond to the presence of probiotics, and by increasing the expression of cytokines, it ultimately increases the number of T cells in the body (Smits *et al.*, 2005). Studies have also shown that probiotics can delay apoptosis in lymphocytes, thereby increasing the level of lymphocytes in the blood (Scharek *et al.*, 2005).

CONCLUSION

The results of this experiment showed that the use of probiotics as an additive in the diet of calves, although it improved the growth performance and antioxidant activity of calves, but had no significant effect. It seems that the lack of significant performance is due to the amount and type of probiotics consumed. Probably because probiotics in milk reduce palatability, they affect food intake and make performance insignificant. On the other hand, because the experiment was conducted in the warm season, heat stress factors may affect the efficiency of probiotic use. Also, the use of probiotics in the early days of calves' life significantly increased the relative expression of the interleukin-6 gene, which will increase the efficiency of the immune system. In general, the present study showed that although the separate application of *Saccharomyces* species can affect performance, antioxidant activity, and the immune system, the use of a mixture of the two species together can have more favorable effects.

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