



IMMUNOBIOLOGICAL EFFECTS OF MARINE *Debaryomyces hansenii*-DERIVED LYSATES ON GOAT PERIPHERAL BLOOD LEUKOCYTES †

[EFECTOS INMUNOBIOLOGICOS DE LISADOS DERIVADOS DE LA LEVADURA MARINA *Debaryomyces hansenii* EN LEUCOCITOS DE SANGRE PERIFÉRICA DE CABRA]

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SUMMARY

Background. Successful immunomodulatory effects of yeast structural and secretory bioactive compounds have been demonstrated on several animal species. Likewise, studies have shown the ability of *Debaryomyces hansenii* to stimulate both immune and antioxidant responses on fish, crustaceans, mollusks, and recently, goats. **Objective.** To analyze the immunostimulant activity of lysates derived from five strains of the marine yeast *D. hansenii* in goat peripheral blood leukocytes using *in vitro* assays. **Methodology.** For this purpose, leukocytes from caprine peripheral blood were isolated and incubated (24 h) with lysate-derived *D. hansenii* strains and Zymosan (positive control). **Results.** The results revealed that these strains significantly increased cell immune parameters, such as phagocytic capacity, reactive oxygen species (respiratory burst) and nitric oxide production, and myeloperoxidase activity. Antioxidant enzyme assays in leukocytes stimulated with lysate-derived *D. hansenii* demonstrated significant increases in superoxide dismutase and catalase activities. **Implications.** This *in vitro* study using peripheral blood leukocytes suggest that lysates derived from marine yeast *D. hansenii* strains could be considered immunostimulants for goats through their ability to stimulate the non-specific immune parameters and the antioxidant immune mechanism, without affecting leukocyte viability. **Conclusion.** In particular, the results indicated that *D. hansenii* BCS001 was able to modulate the immune parameters in goat immune cells. Therefore, additional *in vivo* immunostimulatory assays deserve further research, including other species of economic interest in food production.

Keywords: Immune response; immunostimulant; ruminants; bioactive compounds; antioxidant activity.

RESUMEN

Antecedentes. Se han demostrado efectos inmunomoduladores exitosos de compuestos bioactivos secretorios y estructurales de levaduras en varias especies animales. Así mismo, diversos estudios han demostrado la capacidad de *Debaryomyces hansenii* para estimular la respuesta inmune y antioxidante en peces, crustáceos, moluscos, y recientemente en caprinos. **Objetivo.** Analizar la actividad inmunoestimulante de lisados derivados de cinco cepas de la levadura marina *D. hansenii* en leucocitos aislados de sangre periférica de cabra, a través de ensayos *in vitro*. **Metodología.** Para ello, fueron aislados leucocitos de sangre periférica de cabra, e incubados durante 24 h en presencia de lisados de *D. hansenii* y zimósán (control positivo). **Resultados.** Los resultados revelaron que estas cepas aumentaron significativamente los parámetros inmunes evaluados en las células, como la capacidad fagocítica, producción de especies reactivas de oxígeno (estallido respiratorio), producción de óxido nítrico y la actividad de mieloperoxidasa. Los ensayos de enzimas antioxidantes demostraron aumentos significativos en las actividades de superóxido dismutasa y catalasa. **Implicaciones.** Este estudio *in vitro* utilizando leucocitos de sangre periférica, sugiere que los lisados derivados de las cepas de la levadura marina *D. hansenii* pudieran ser considerados inmunoestimulantes para caprinos, debido a su habilidad para estimular parámetros inmunes no específicos y el mecanismo inmune antioxidante, sin afectar la viabilidad de los leucocitos. **Conclusión.** En particular, los resultados indicaron que la cepa *D. hansenii* BCS001 fue sobresaliente al modular los parámetros inmunes en los leucocitos de cabra. Por lo tanto,

† Submitted January 20, 2021 – Accepted September 10, 2021. This work is licensed under a CC-BY 4.0 International License. ISSN: 1870-0462.

merece más investigación a través de ensayos adicionales *in vivo*, incluyendo también otras especies de interés económico en la producción de alimentos.

Palabras clave: Respuesta inmune; inmunoestimulante; rumiantes; compuestos bioactivos; actividad antioxidante.

INTRODUCTION

Currently, yeasts have represented an attractive source of valuable compounds because their cell is a rich source of bioactive compounds (proteins, cytoplasmic enzymes, polysaccharides, etc.) valuable in biotechnology, pharmacology, and food industry (Liu *et al.*, 2016). Particularly, their β -glucans (Wilson *et al.*, 2015), mannoproteins (Pietrella *et al.*, 2001), chitin (Brodaczewska *et al.*, 2015), nucleotides (Waititu *et al.*, 2017), polyamines (Reyes-Becerril *et al.*, 2011a), and other bioactive compounds are molecules capable of stimulating the immune system by inducing activation or increasing the activity of any of their functional components.

Some molecules that are not synthesized by mammals are identified as non-self by our immune cells, inducing immune responses. This response can occur through innate (fast initial response) or adaptive mechanisms (prolonged effective immunity) (Tizard, 2009). Infectious agents are detected by proteins referred to as toll-like receptors (TLR), capable of binding to specific structural molecular models present in mainly bacterial microorganisms (Aderem and Ulevitch, 2000). The inflammatory response is part of innate immunity, and it is triggered when tissues are injured by bacterial, toxins, heat or any cause, in this process, components that promote the accumulation of phagocytic cells are released (neutrophils and macrophages), which are responsible for destroying most of the invading microorganisms through pathogen-killing mechanisms (reactive oxygen (ROS) and nitrogen (RNS) species), involving specialized enzymes such as NADPH oxidase, nitric oxide synthase and myeloperoxidase (MPO) (Tizard, 2009).

Yeasts have gained interest in ruminant production systems mainly because of their effects on improving productive performance, digestibility, and ruminal microbiome balance (Pienaar *et al.*, 2015; Ma *et al.*, 2015; Hansen *et al.*, 2017; He *et al.*, 2017; Xu *et al.*, 2017). In this regard, some studies have been conducted and focused on productive performance, blood metabolite balance, and microbiome responses in yeast supplemented animals (Hadjipanayiotou *et al.*, 1997; Giger-Reverdin *et al.*, 2004; El-Ghani, 2004; Stella *et al.*, 2007; Pal *et al.*, 2010; Zicarelli *et al.*, 2016). To our knowledge, few reports have been related to the effect of yeast on the immune system in goats, which were mainly conducted in cows and calves (Magalhães *et al.*, 2008; Ma *et al.*, 2015; Vohra *et al.*, 2016; He *et al.*, 2017).

Marine yeasts have emerged as an effective immunostimulant, among them the marine yeast *Debaryomyces hansenii* (Reyes-Becerril *et al.*, 2008). *Debaryomyces* spp. is a non-pathogenic and extremophilic yeast (osmotolerant, halotolerant, xerotolerant), initially isolated from marine environments although it has been isolated from many habitats (Breuer and Harms, 2006). Studies have shown *D. hansenii* ability to stimulate both immune and antioxidant responses in fish species *in vivo* (Reyes-Becerril *et al.*, 2012) and *in vitro* (Angulo *et al.*, 2017). Moreover, yeast-stimulated leukocytes and then exposed to *Vibrio parahaemolyticus* infection resulted in significantly lower apoptosis induced by the pathogen, improved phagocytic capacity, increased nitric oxide production and reactive oxygen species, as well as peroxidase and superoxide dismutase activities (Angulo *et al.*, 2017). Recently, it has been found that marine *D. hansenii* strains could be considered as potential immunostimulants for goat production systems. The authors showed that live yeast and β -glucans were able to modulate the immune system by promoting cell viability, phagocytic activity, antioxidant immune response, immune-related gene expression, and induction of trained immunity in goat peripheral blood leukocytes and newborn goats (Medina-Córdova *et al.*, 2018; Angulo *et al.*, 2018; Angulo *et al.*, 2019; Angulo *et al.*, 2020). Remarkably, Vrzał *et al.* (2015) reported that administering a patented commercial immunostimulant product based on *Candida albicans*, *C. krusei*, *C. glabrata*, and *Propionibacterium acnes* lysates prolonged the relapse period of vulvovaginal affections and had a positive effect on cellular immunity qualities in women. Therefore, this study was designed to evaluate the immunostimulant activity of lysates derived from five marine yeast *D. hansenii* strains in goat peripheral blood leukocytes using *in vitro* assays.

MATERIALS AND METHODS

Yeast Strains

Debaryomyces hansenii strains used in this study belong to the yeast collection isolated from marine environments (Table 1) from Centro de Investigaciones Biológicas del Noroeste, S.C. (CIBNOR) (Hernández-Saavedra, 1990). The marine yeast strains were isolated from the sea surface (0 m), and 100 m (MBSL; meters below sea level) and cultured by cross-streaking in YPD agar prepared with distilled water and supplemented with 0.05 % chloramphenicol at 30 °C.

Table 1. *Debaryomyces hansenii* strains from the Centro de Investigaciones Biológicas del Noroeste, S.C. (CIBNOR) collection of yeasts isolated from marine environments.

Yeast	Code	Depth of collection (m)
<i>Debaryomyces hansenii</i> var. <i>hansenii</i>	DhhBCS001	0
<i>Debaryomyces hansenii</i> var. <i>hansenii</i>	DhhBCS002	100
<i>Debaryomyces hansenii</i> var. <i>hansenii</i>	DhhBCS003	100
<i>Debaryomyces hansenii</i> var. <i>hansenii</i>	DhhBCS004	0
<i>Debaryomyces hansenii</i> var. <i>hansenii</i>	DhhBCS005	0

Immunological assays

Lysate preparation

Yeast biomass was obtained by inoculating a loopful of pure isolated yeast in 2000 mL of Yeast Peptone Dextrose broth (YPD; Sigma, St. Louis, MO, U.S.A.) supplemented with antibiotic (chloramphenicol 1 μ L/mL) and incubated on a rotary shaker at 30 °C, 150 rpm from 48-72 h with constant aeration. Subsequently, yeast suspensions were adjusted at 1×10^8 cells/mL and then sonicated at 90 % of amplitude twice for 3 min. Finally, they were centrifuged at 720 g, 4 °C, and supernatants were used on assays (Liu *et al.*, 2016).

Isolation of leukocytes from caprine peripheral blood

For the *in vitro* study, peripheral blood samples from a herd of Saanen \times Nubian crossbred goat, clinically healthy, homogeneous in number of calving and physiological state, maintained under semi-intensive conditions were collected in sodium heparin-containing BD Vacutainer® (Franklin Lakes, NJ, U.S.A.) tubes to isolate leukocytes under sterile conditions following Azmi *et al.* (2006) with slight modifications. Blood was diluted with phosphate-buffered saline (PBS, pH 7.4) (1:1 v/v), then placed in falcon tubes containing Histopaque®-1077 (Sigma, St. Louis, MO, U.S.A.), and centrifuged at 1500 rpm, 10 °C for 20 min. The buffy coat containing peripheral blood white cells was collected and washed with PBS. Then, the cells were suspended in RPMI 1640 supplemented with fetal bovine serum (3 %). Finally, peripheral blood leukocytes were observed and counted with an Automated Cell Counter - TC20 (BioRad, Hercules, CA, U.S.A.) and adjusted to 1×10^6 cells/mL of sRPMI. A drop of leukocyte suspension

was stained with trypan blue (Sigma, Cat. T-8154, St. Louis, MO, U.S.A.) to calculate viability, and leukocyte suspension with a viability of N95 % was used for *in vitro* experiment (Reyes-Becerril *et al.*, 2016).

Stimulation test of peripheral blood leukocytes

One milliliter of the peripheral blood leukocyte suspension was placed into 24-well flat-bottomed microtiter plates (Nunc, Waltham, MA, U.S.A.). Subsequently, 125 μ L of lysate suspension were added and incubated at 37 °C with 85 % relative humidity and 5 % CO₂ atmosphere for 24 h. As positive control, leukocytes were incubated with 125 μ L of Zymosan A from *S. cerevisiae* (Sigma, Z4250, 200 μ g/mL, St. Louis, MO, U.S.A.) regularly used for its activating effect on the immune response (Pietretti *et al.*, 2013; Vallejos-Vidal *et al.*, 2016). Leukocytes incubated with PBS were used as negative control. Finally, for immunological assays peripheral blood leukocytes stimulated with lysates were dispensed into 96-well microtiter plates (Nunc) by triplicate.

Cell viability

The resazurin reduction assay was used to determine the treatment effect on goat peripheral blood leukocyte viability according to Riss *et al.* (2016) with slight modifications. Briefly, 100 μ L/well of leukocytes (1×10^6 cells/mL of sRPMI) plus yeast lysates were placed on 96-well microtiter plates (Nunc, Waltham, MA, U.S.A.) with 20 μ L of resazurin solution (Resazurin sodium salt, Sigma, Cat: R7017, St. Louis MO, U.S.A.) and incubated at 37 °C for 4 h. Fluorescence was recorded in Varioskan™ (Flash 2.4.5, Thermo Scientific, Waltham, MA, U.S.A.) with excitation at 530 nm and emission at 580 nm. Prior to these calculations, fluorescence readings for wells without cells were subtracted from those for the experimental wells to account for background. Peripheral blood leukocytes without lysates and those incubated with dimethyl sulfoxide (DMSO) at 10 % final concentration were used as negative and positive viability controls, respectively. The assay was performed in triplicate, and viability (%) was calculated using the following formula: % viability = $A_{\text{sample}}/A_{\text{negative control}} \times 100$.

Cell viability greater than 75 % is considered non-cytotoxic according to ISO 10993-5 and generally accepted in other reports in this field.

Phagocytic activity

The phagocytosis of *S. cerevisiae* (strain S288C) by goat peripheral blood leukocytes stimulated with lysates was studied by flow cytometry analysis (S3e Cell Sorter, Bio-Rad, Hercules, CA, U.S.A.)

(Rodríguez *et al.*, 2003). To label *S. cerevisiae* cells with fluorescein isothiocyanate (FITC; Sigma, St. Louis, MO, U.S.A.), they were stirred in a shaker (IUL) at 40 cycles/min and incubated with 5 mg/mL FITC at 22 °C in dark conditions for 15 min. After the yeast cells were labelled, free FITC was removed by washing the cells twice in PBS and adjusting to 5×10^7 cells/mL of sRPMI to be used for flow cytometric study. After lysate stimulation, leukocytes were washed, and 60 µL of labelled *S. cerevisiae* cells were added. Cell suspensions were mixed, then centrifuged (400 g, 37 °C, 5 min), resuspended in sRPMI, and finally incubated at 37 °C for 30 min. At the end of incubation time, samples were placed on ice to stop phagocytosis, and 400 µL ice-cold PBS was added to each sample. Fluorescence of the extracellular yeast cells was quenched by adding 40 µL ice-cold trypan blue (0.4 % in PBS). Standard samples of FITC-labeled *S. cerevisiae* or leukocytes alone were included in each phagocytosis assay. All samples were performed in a flow cytometer set to analyze the phagocytic cells showing the highest forward scatter (FSC) and moderate sideward scatter (SSC). Phagocytic capacity was defined as the mean fluorescence intensity.

Oxidative and nitrosative stress responses and antioxidant enzymatic activities

Respiratory burst

The respiratory burst activity of the leukocytes isolated from caprine peripheral blood and stimulated with yeast lysates was measured as the production of reactive oxygen intermediates using the nitro blue tetrazolium (NBT) reduction assay according to Kemenade *et al.* (1994). Leukocytes (100 µL) were incubated with NBT solution (1 mg/mL; Sigma, St. Louis, MO, U.S.A.) in darkness for 2 h, washed, incubated for 10 min with methanol (70 % v/v), washed again and resuspended in 2 M KOH-DMSO. Optical density (OD) was measured at 655 nm in a microplate reader (BIORAD, Model 3550-UV, Hercules, CA, U.S.A.).

Nitric oxide (NO) production

Nitric oxide production in the stimulated leukocytes was determined according to Neumann *et al.* (1995). Since NO is an unstable molecule and degrades to nitrite and nitrate, leukocytes were used for the extracellular NO production determination based on Griess reagent that quantifies supernatant leukocyte nitrite content. Briefly, 100 µL of leukocytes (1×10^6 cells/mL) were incubated with an equal Griess reagent (Sigma, St. Louis MO U.S.A.) volume in a 96-well plate and mixed properly. The cells were incubated at room temperature under dark conditions for 15 min. Reduced nitrite was measured by taking the OD at 490

nm in a microplate reader (3550-UV microplate reader Bio-Rad, Hercules, CA, U.S.A.). Nitrite production in cell supernatants was represented as nitrite concentration in µM using a standard curve.

Superoxide dismutase and catalase activities

SOD activity was measured in goat peripheral blood leukocytes by the percentage reaction inhibition rate of enzyme with water-soluble tetrazolium dye (WST-1) substrate and xanthine oxidase using a SOD Assay Kit (Sigma, St. Louis, MO, U.S.A.) according to the manufacturer's instructions. Each endpoint assay was monitored at 450 nm, which is the absorbance wavelength for the colored product of the WST-1 reaction with superoxide. After 20 min of reaction at 37 °C, the OD was recorded; % inhibition was normalized by mg protein and shown as SOD activity units.

Catalase (CAT) activity was assayed by Clairborne (1985) method, following the decrease in absorbance of H₂O₂ at 655 nm. One unit of enzyme activity is defined as the amount of enzyme required to degrade 1 mmol of H₂O₂ in 1 min and expressed as U/mg protein.

Myeloperoxidase activity

Total myeloperoxidase (MPO) activity in stimulated leukocytes was measured according to Quade and Roth (1997) with slight modifications. Briefly, 20 µL of leukocytes stimulated with yeast lysates were incubated with 100 µL of a solution containing 20 mM 3, 3',5, 5'-tetramethylbenzidine hydrochloride (TMB, Sigma, St. Louis, MO, U.S.A.) and 5 mM H₂O₂. Both reagents, substrates of MPO, were prepared on the same day. The colorimetric reaction was stopped by adding 50 µL of 2 M sulfuric acid (H₂SO₄) after 2 min. The OD was recorded at 450 nm in a microplate reader (Bio-Rad 3550-UV, Hercules, CA, U.S.A.).

Statistical analysis

All statistical analyses for this experiment were performed using the statistical package SigmaPlot (Version 12.0). Data were analyzed using one-way analysis of variance and student t-test to determine the differences among the treatments. Mean separations were performed using Tukey's test. Differences at $p < 0.05$ were considered significant.

RESULTS

Cell viability

Viability of the goat peripheral blood leukocytes exposed to lysates from *D. hansenii* strains was determined by the resazurin reduction assay after 24 h

of incubation are shown in Fig. 1. No significant differences in leukocyte viability were observed when compared among the lysate treatment groups.

Moreover, the results obtained in leukocytes treated with DMSO showed a significant ($p < 0.05$) viability decrease (29.9 %).

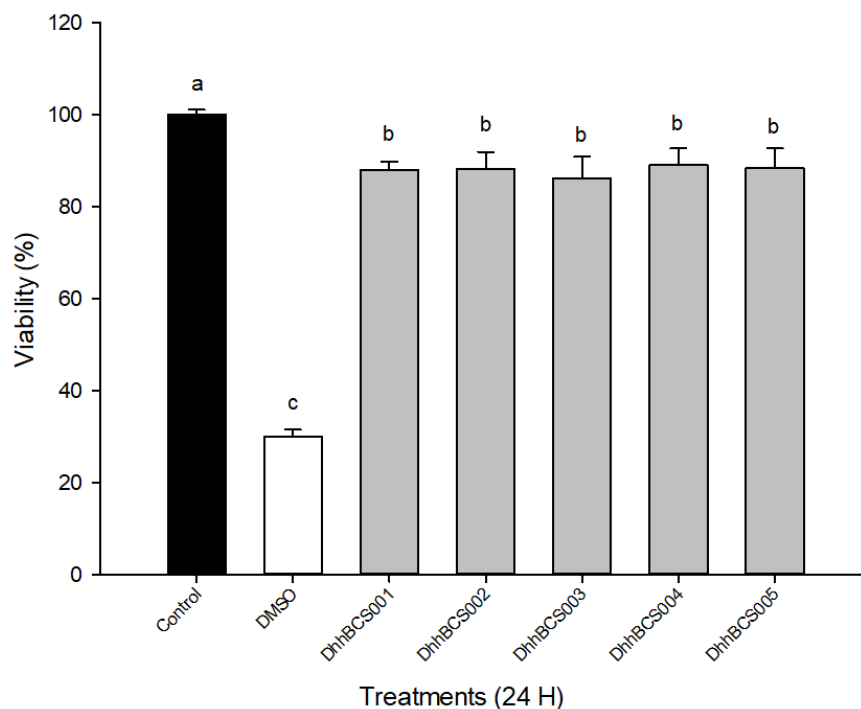


Figure 1. Effect of lysates from *Debaryomyces hansenii* strains on viability (%) in goat peripheral blood leukocytes determined by the resazurin reduction assay. Results are expressed as cell viability (%) (mean \pm standard error (SE)). All experiments were performed by triplicate. Different letters denote significant difference ($p < 0.05$) among groups.

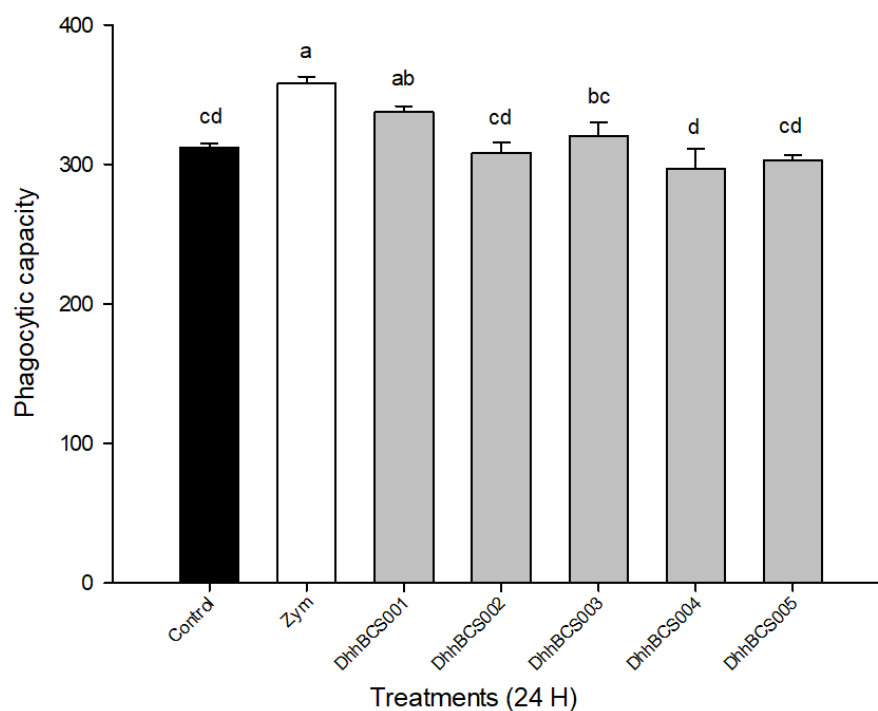


Figure 2. Phagocytic capacity of goat peripheral blood leukocytes immunostimulated with lysates of *Debaryomyces hansenii* strains after 24 h of incubation at 37 °C and 5 % of CO₂. Data are shown as mean \pm standard error (SE), according to variance analysis. Different letters denote significant difference ($p < 0.05$) among groups.

Phagocytic capacity

Regarding phagocytic capacity, goat peripheral blood leukocytes stimulated with lysate from DhhBCS001 strain showed similar phagocytic capacity (relative number of ingested yeasts per cell) than those in Zymosan positive control treatment and were significantly ($p < 0.05$) higher compared to the control group (PBS, non-stimulated leukocytes). No significant differences were observed in the remaining treatments when compared to the control group (Fig. 2).

Oxidative and nitrosative stress responses and antioxidant enzymatic activities

Respiratory burst

As shown in Fig. 3, all lysate treatments from *D. hansenii* strains clearly induced the highest reactive oxygen species (ROS) production in the respiratory burst assay after 24 h of incubation compared to the control group (PBS).

Nitric oxide (NO) production

Nitric oxide production significantly increased ($p < 0.05$) in goat peripheral blood leukocytes treated with Zymosan and lysate from the marine strain DhhBCS001 when compared to the control treatment (PBS, Fig. 4). In contrast, nitric oxide production in the remaining groups was statistically ($p < 0.05$) similar to control.

Superoxide dismutase and catalase activity

The highest SOD activity ($p < 0.05$) was observed in leukocytes stimulated with lysates from the marine yeast strains DhhBCS002, DhhBCS003, DhhBCS004 and DhhBCS005 compared to the control treatments (PBS and Zymosan) (Fig. 5). The significant increases ($p < 0.05$) of catalase activity were observed in all the groups stimulated with lysates from *D. hansenii* strains, as well as those in the Zymosan group compared to the control treatment (PBS, Fig. 6).

Myeloperoxidase activity

Myeloperoxidase activity increased ($p < 0.05$) in goat peripheral blood leukocytes stimulated with lysates obtained from the marine yeast strain DhhBCS002 compared with the control groups (PBS and Zymosan, Fig. 7).

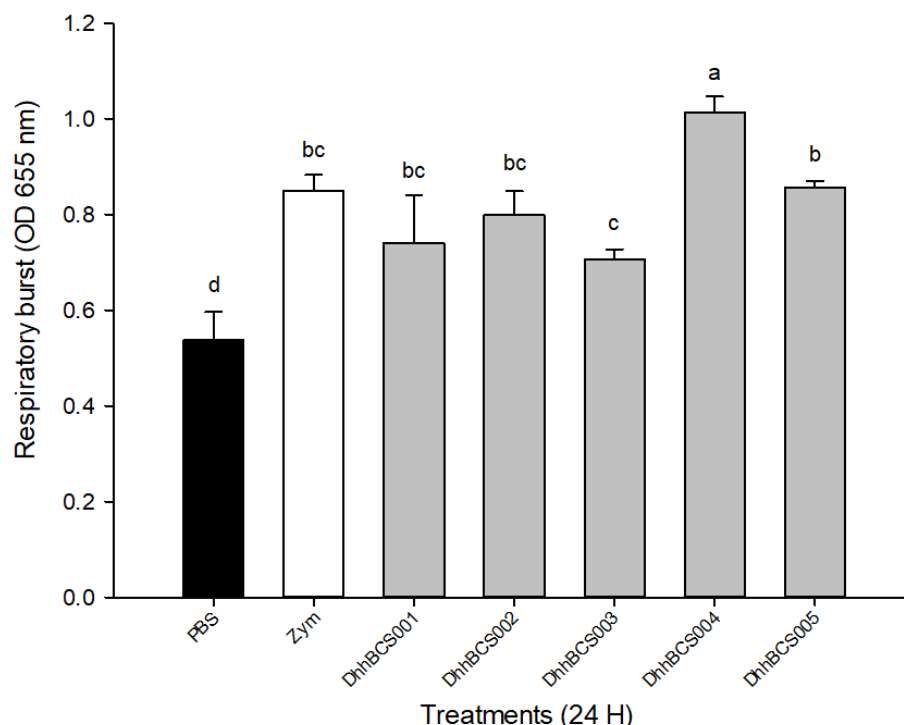


Figure 3. Reactive oxygen species (ROS) production of peripheral blood leukocytes immunostimulated by lysates of *Debaryomyces hansenii* strains, after 24 h of incubation at 37 °C and 5 % of CO₂. Each bar represent the mean \pm standard error (SE) of three replicates. Different letters denote significant difference ($p < 0.05$) among groups.

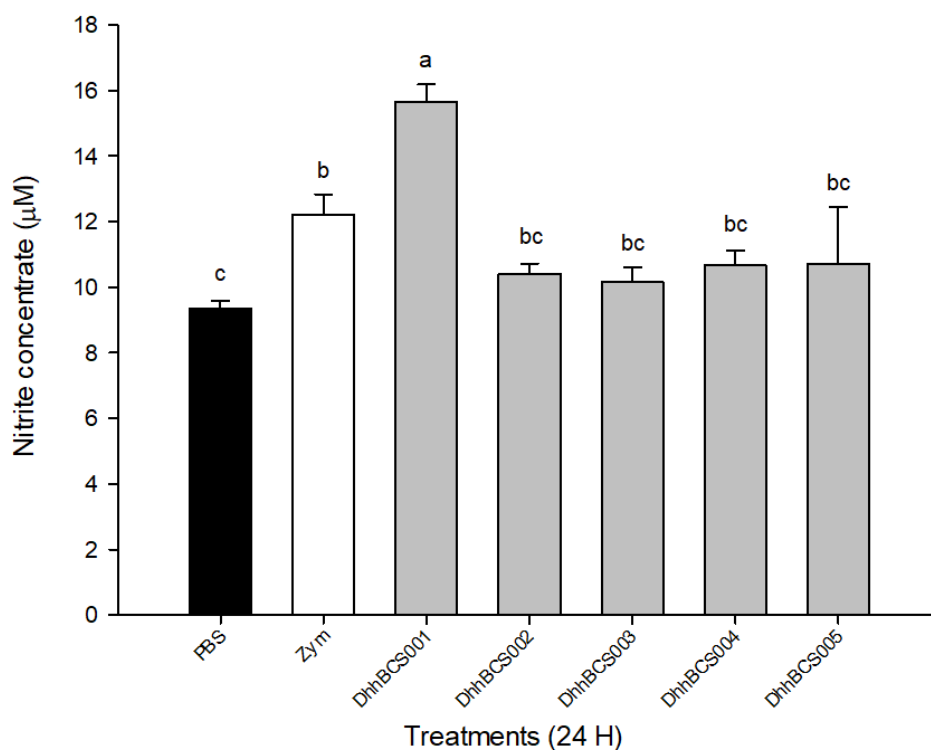


Figure 4. Nitric oxide (NO) production by leukocytes stimulated with lysates from *Debaryomyces hansenii* strains, after 24 h of incubation at 37 °C and 5 % of CO₂. Data are expressed as the percentage of control. Each bar represent the mean \pm SE of three replicates. Different letters denote significant difference ($p < 0.05$) among groups.

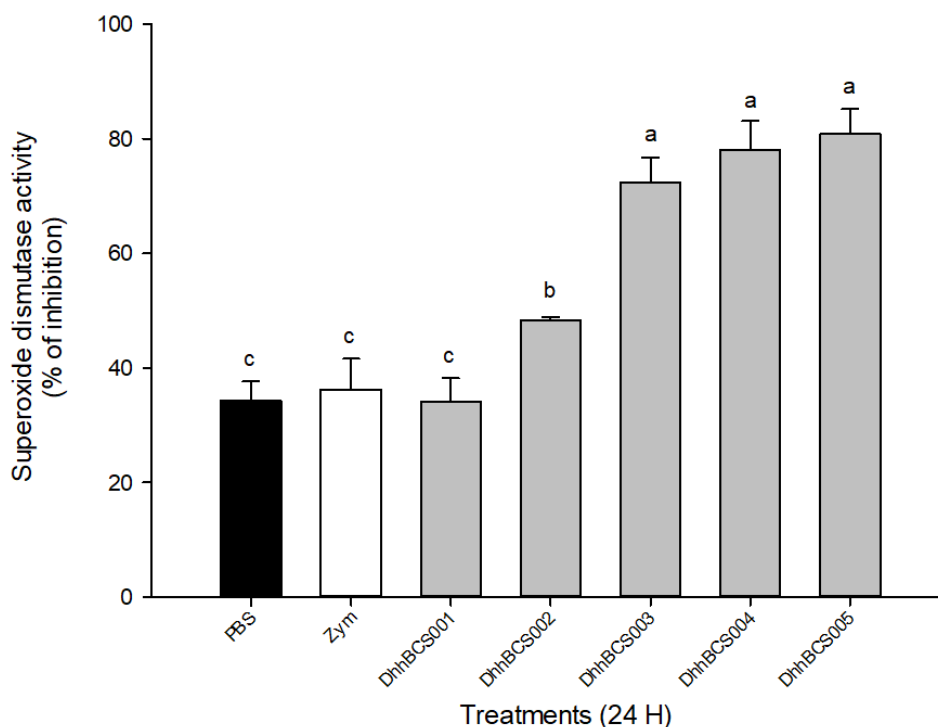


Figure 5. Superoxide dismutase activity in leukocytes stimulated with lysates of *Debaryomyces hansenii* strains after 24 h of incubation at 37 °C and 5 % CO₂. Data are expressed as U/mg protein. Each bar represent the mean \pm standard error (SE) of three replicates. Different letters denote significant difference ($p < 0.05$) among groups.

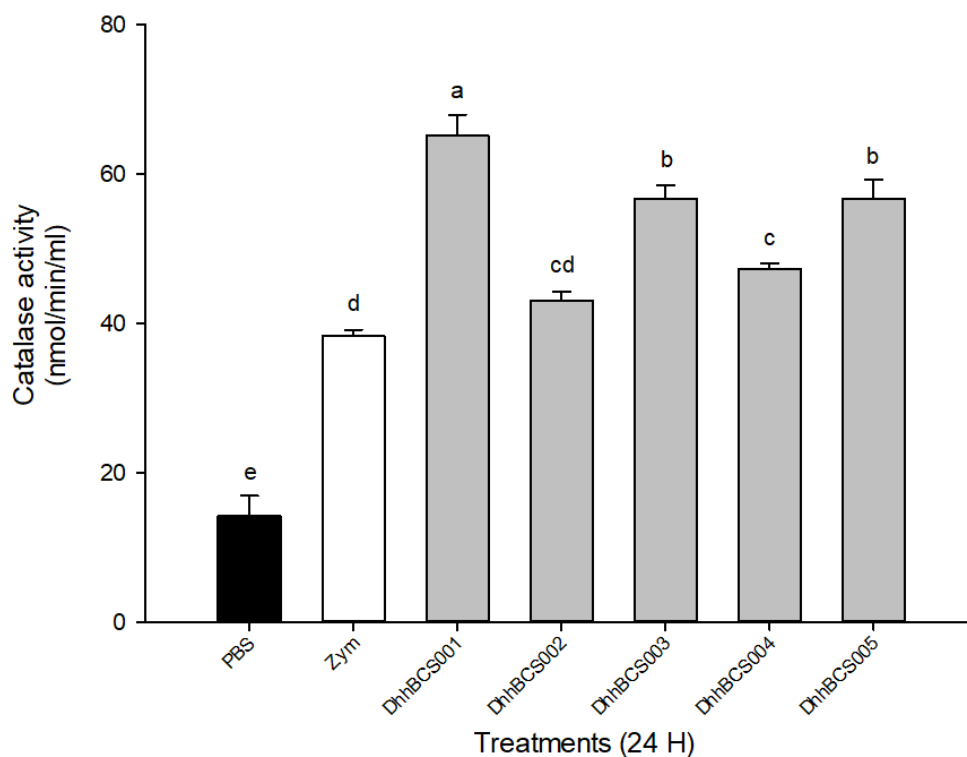


Figure 6. Catalase activity in leukocytes stimulated with lysates of *Debaryomyces hansenii* strains after 24 h of incubation at 37 °C and 5 % CO₂. Each bar represent the mean \pm standard error (SE) of three replicates. Different letters denote significant difference ($p < 0.05$) among groups.

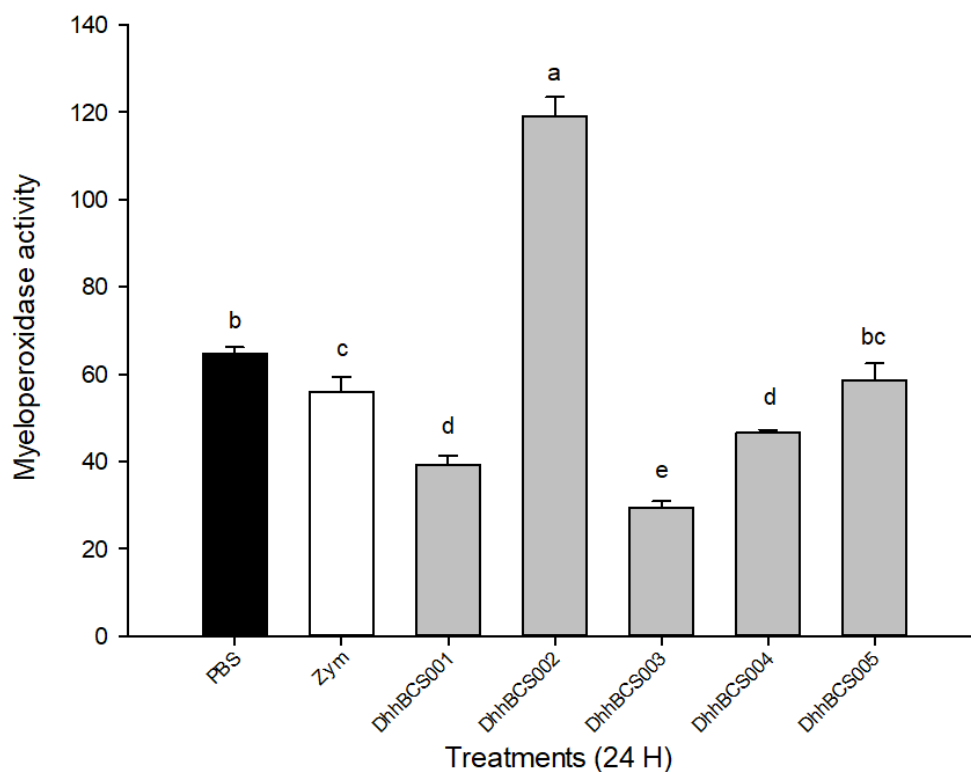


Figure 7. Myeloperoxidase activity (MPO) on leukocytes stimulated with lysates from *Debaryomyces hansenii* strains, after 24 h of incubation at 37 °C and 5 % of CO₂. Data are expressed as U/mL. Each bar represent the average \pm SE of three replicates. Different letters denote significant difference ($p < 0.05$) among groups.

DISCUSSION

Successful immunomodulatory effects of yeast structural and secretory bioactive compounds, such as β -glucan, mannoproteins, chitin, and nucleic acids have been demonstrated on several animal species (Ortuño *et al.*, 2002). On this regard, *D. hansenii* has shown probiotic effects on fish (Tovar-Ramírez *et al.*, 2010; Reyes-Becerril *et al.*, 2011b), crustaceans (Pacheco *et al.*, 2012), mollusks (Macey and Coyne, 2006), and recently, live and β -glucans derived from this yeast species had immunostimulatory effects on goats (Angulo *et al.*, 2018; Medina-Córdova *et al.*, 2018; Angulo *et al.*, 2019). In the same way, it should be noted that yeast lysate offers a lower cost advantage over β -glucan purification, while free immunostimulant compounds in yeast lysate are better available compared to whole cells. Therefore, these advantages bring interesting questions on yeast lysate immunostimulatory effects, which could help to control bacterial infections.

Cell viability

This study evaluated lysates derived from several marine yeast *D. hansenii* strains to determine their immunostimulatory activity on goat peripheral blood leukocytes (PBL) through *in vitro* immunological assays. A probiotic or immunostimulant should stimulate the immune response without causing cytotoxicity to the host cells (Kim and Austin 2006). Here, *D. hansenii*-derived lysates had no cytotoxic effects in PBL at the concentrations tested; thus, they can be considered safe for goats. Additionally, according to the European Food Safety Authority (EFSA), *D. hansenii* has been reported as safe for use in animals and humans (BIOHAZ, 2012). Phagocytic activity, radical production (NBT), nitric oxide, myeloperoxidase and two antioxidant enzymes (SOD and CAT) in PBL were selected because they may be related with immunostimulation.

Phagocytic capacity

Phagocytosis is the mechanism of innate immunity by which macrophages, monocytes and neutrophils engulf and digest foreign particles into endocytic vesicles called phagosomes. This process, in turn, triggers several antimicrobial activities that use a wide variety of associated mechanisms, which include cellular activation, the production of oxygen-free (ROS: superoxide anion (O_2^-), hydroxyl radicals (OH^\cdot) and hydrogen peroxide (H_2O_2)) and nitrogen-free radicals (RNS: nitric oxide), and cytokines driving the inflammatory response (Bekkering *et al.*, 2016; Vallejos-Vidal *et al.*, 2016). The results in this study showed that the phagocytic capacity increased in leukocytes incubated with *D. hansenii* BCS001 lysates, as well as those with Zymosan (positive

control) compared to the non-stimulated group. Interestingly, Magalhães *et al.* (2008) observed an increase in the mean number of phagocytized bacteria and proportion of phagocytized bacteria killed by *S. cerevisiae* incubation in Holstein calf leukocytes. In another study, β -glucan from *S. cerevisiae* stimulated phagocytic activity in pig peripheral blood monocytes and neutrophils (Vetvicka and Oliveira, 2014).

Respiratory burst

Likewise, we also observed that respiratory burst activity (ROS production) was higher in leukocytes stimulated with lysates derived from all marine yeast *D. hansenii* strains compared to the non-stimulated group. Remarkably, phagocytic and respiratory burst activities have been also observed in Pacific red snapper (*Lutjanus peru*) leukocytes stimulated with *D. hansenii* and exposed to live *V. parahaemolyticus* (Angulo *et al.*, 2017). Similar to the results in this study, *D. hansenii*-derived β -glucan were able to stimulate phagocytic and respiratory burst activities in goat PBL with and without bacterial challenge (Angulo *et al.*, 2018; Medina-Córdova *et al.*, 2018). Interestingly, Angulo *et al.* (2019) also observed that respiratory burst activity (ROS) increased in leukocytes from newborn goats supplemented with *D. hansenii* CBS8339.

Nitric oxide (NO) production

Moreover, phagocytosis stimulation can lead to nitric oxide (NO) generation as an immune mediated mechanism. The NO is a small free radical modulator of multiple physiological functions that contribute to killing viral infected cells, tumor cells and pathogenic bacteria (MacMicking *et al.*, 1997; Du *et al.*, 2015). In this study, NO production increased in leukocytes stimulated with lysates from *D. hansenii* BCS001 strain. Recently, Angulo *et al.* (2017) reported that fish leukocytes stimulated with *D. hansenii* BCS004 strain and challenged with *V. parahaemolyticus* increased NO production. Similarly, goat PBL enhanced NO production upon stimulation with live and β -glucans from marine *D. hansenii* (Angulo *et al.*, 2018; Medina-Córdova *et al.*, 2018; Angulo *et al.*, 2019).

Superoxide dismutase and catalase activity

Although ROS production is a defense mechanism, it may also induce toxic effects in the host cells, named oxidative stress, when generated in excess. Thus, the multiple intracellular signaling pathways that control oxidative stress can be activated to keep cell homeostasis, including antioxidant enzyme activity (Case, 2017; Mahaseth and Kuzminov, 2017). Superoxide dismutase (SOD) and catalase (CAT) are key enzymes that convert ROS into oxygen species less dangerous to the organism. Here, nearly all tested

strains significantly increased SOD and CAT activities in goat PBL. On this regard, Reyes-Becerril *et al.* (2011) reported an increase in hepatic SOD and CAT activities by oral administration of *D. hansenii* CBS8339 in the fish *Mycteroperca rosacea* upon *Aeromonas hydrophila* infection. In line with these findings, SOD activity increased in Pacific red snapper (*L. peru*) leukocytes stimulated with *D. hansenii* BCS004 and exposed to *V. parahaemolyticus* (Angulo *et al.*, 2017). Similarly, SOD and CAT activities increased in leukocytes isolated from newborn goats orally supplemented with live *D. hansenii* CBS 8339 and challenged with *E. coli* (Angulo *et al.*, 2019). Likewise, SOD and CAT activities increased in goat PBL stimulated with β -glucans-derived from *D. hansenii* (Medina-Cordova *et al.*, 2018). The observed effects could be associated with the cellular enzymatic antioxidant mechanism to a better redox balance because SOD converts O_2^- into H_2O_2 , which is then converted to water and oxygen by CAT.

Myeloperoxidase activity

Furthermore, myeloperoxidase (MPO) is a hemoprotein secreted during neutrophil and macrophage activation that plays an important role as a defense mechanism. This enzyme uses H_2O_2 as substrates to generate hypochlorous acid, a toxic compound for invasive pathogens. Intriguingly, leukocytes stimulated with *D. hansenii* BCS002 increased the MPO activity compared with the Zymosan and non-stimulated groups. This effect was observed in leukocytes from fish and goats orally supplemented with *D. hansenii* BCS004 and *D. hansenii* CBS8339, respectively (Angulo *et al.*, 2017; Angulo *et al.*, 2019). Additionally, previous studies have shown that yeast glucans can increase MPO activity (Meena *et al.*, 2013). In this sense, Medina-Córdova *et al.* (2018) found an increase of MPO activity in goat PBL with β -glucans derived from *D. hansenii* BCS005. Overall, this and previous studies have demonstrated that yeasts have different structural and secretory immunomodulatory compounds with the ability to promote leukocyte activity triggering innate immune responses in different animals (Brattgjerd *et al.*, 1994; Cheng *et al.*, 2004; Batbayar *et al.*, 2012).

CONCLUSIONS

The results of this study *in vitro* using peripheral blood leukocytes suggest that lysates derived from marine yeast *D. hansenii* strains could be considered immunostimulants for goats through an increase in the non-specific immune parameters and the antioxidant immune mechanism, without affecting leukocyte viability. Particularly lysate from *D. hansenii* BCS001 have the potential to prepare goat leukocytes to respond more efficiently to bacterial infections, through improved phagocytic capacity, nitric oxide

production and catalase activity. However, additional *in vivo* immunostimulatory assays are required, evaluating various administration routes and including other species of economic interest in food production.

Acknowledgments

The authors thank Erika Alamillo and Crystal Guluarte for technical assistance and Diana Fischer for editorial services in English.

Funding. This research was financially supported by grants from CONACYT (INFR-2014-01/225924 and PDCPN2014-01/ 248033).

Conflict of interest. The authors declare no competing or financial interests.

Compliance with ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Data availability. Data are available with Dr. Carlos Angulo, eangulo@cibnor.mx upon request.

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