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Isolation and Characterization of Wild Yeast Strains Producing Beta Glucans; Probiotic Systemic Effect in Mice under Conditions of Environmental Stress

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Abstract

Probiotics are living microorganism intended to confer health benefits to the host organism. Some of them produce and retain group of metabolites helping the good conditions of organism, some of the effects of probiotics are mediated by metabolites that promote good health by improving the host´s immune response and antioxidant capacity. β-glucans are glucose-polymer present in yeast, fungi, and plants that can stimulate cellular immunological response and reduce oxidative stress damages in both, vertebrate and invertebrate species. In this study, we isolated and characterized wild yeast strains from different regions of Mexico. About 250 different isolates were obtained from fermented fruit extracts, of which 44 were selected and further characterized microbiologically, biochemically and molecularly. Restriction fragment length polymorphism of PCR-amplified internal transcribed spacer (ITS) region revealed a wide diversity among selected isolates, and this was confirmed by DNA sequencing. Biochemically, diversity was supported by a wide range of β-glucans levels produced under laboratory conditions. Two identified species, *Saccharomyces cerevisiae* and *Debaryomyces hansenii* show the most promising β-glucans profiles and were subjected to a kinetic study over 24 hours. The highest β-glucans levels were obtained in stationary phase of growth. Both strains were evaluated as a probiotic. Evaluation as probiotics in mice under environmental stress conditions showed that animals receiving yeast strain as a dietary supplement regained weight more rapidly and had better liver, renal and immunological functions compared to a control group fed with a normal diet. β-glucans also proved to be effective in reducing oxidative stress damage and protecting renal, hepatic and immunological functions in support of systemic positive effect.

INTRODUCTION

Yeasts are eukaryotic microorganisms commonly found in the environment, and often isolated from sugar-rich substrates undergoing fermentation [1,2]. Examples include naturally occurring strains on the skin of fruits and berries such as grapes, apples and peaches, and plant exudates such as plant saps or cacti. Some yeasts are found in association with soil and insects [1,2]. As chemo organotrophic organisms, yeasts use organic compounds as a source of energy and do not require sunlight to grow. Carbon is obtained mostly from hexose sugars, such as glucose and fructose, or disaccharides such as sucrose and maltose. Some species can metabolize pentose sugars such as ribose alcohols, and organic acids [3]. Yeast species either require oxygen for aerobic cellular respiration (obligate aerobes) or are anaerobic, with some anaerobes capable of using aerobic methods of energy production (facultative anaerobes) [3]. Yeasts grow better in a neutral or slightly acidic pH environment. The ecological function and biodiversity of yeasts are relatively unknown compared to those of other microorganisms [4].

Probiotics are live microorganisms that confer health benefits to the host when administrated in adequate amounts [1, 2]. The use of probiotics is based on the role of these microorganisms as possible modulators of the intestinal microbiota and the immune system [5]. Probiotics have a wide spectrum of immunomodulatory effects as they are capable of acting on innate and acquired immunity, protecting the host against infections and promoting more optimal organic response to environmental conditions that induce oxidative stress processes [6,7].

Yeasts are particularly interesting microorganisms due to their diversity, versatility and wide practical uses. Yeasts are

widely accepted as an important component of human food and animal feed. Yeasts are a subject of permanent research, and they display important properties that are used in the production of certain compounds, enzymes and toxins [7]. Among these compounds are β-glucans, glucose homopolysaccharides with variable degrees of branching that are linked through β-(1 → 3) and β-(1 → 4) bonds. Evidence from in vitro and in vivo studies suggests that β-glucans can be used as prebiotics thanks to their ability to promote the growth of beneficial microorganisms such as *Lacto bacillus* ssp. and *Bifido bacterium* ssp. in the gut microbiota of mammals, including humans [8]. Another property, mainly attributed to β-glucans, is the modulation of the immune system [9].

In the present work, we isolated native yeast strains from different fruit extracts collected from different regions of Mexico. A total of 250 isolates were obtained of which, 44 were selected for further microbiological, biochemical and molecular characterization. One of selected yeasts was tested in mice under environmental stress conditions. The results of this study support positive systemic effect of dietary supplementation with selected strain of *Saccharomyces cerevisiae* and the positive use of this strain as probiotics in animals and humans. Probiotic supplementation could have a dramatic impact on livestock productivity by preventing weight loss, improving gastrointestinal health, boosting the immunological response to common pathogens and promoting more optimal organic response to environmental conditions.

MATERIALS AND METHODS

Culture media

The Martins Rose Bengal Agar medium (glucose 10 g/L, peptone 5 g/L, K_2HPO_4 0.5 g/L, KH_2PO_4 0.5 g/L, $MgSO_4.7H_2O$ 0.5 g/L, Rose Bengal 30 mg/L, agar 15 g /L, yeast extract 0.5 g/L and streptomycin sulfate 30 mg/l), was used for the isolation of native yeast strains and supplemented with either glucose (MRAG) or xylose (MRAX) as carbon source. YPG (yeast extract 10 g/L, peptone 10 g/L, glucose 20 g/L and YPX (yeast extract 10 g/L, peptone 10 g/L, xylose 20 g/L) media were testing β-glucans production in the 44 selected strains. For solid media microbiological agar was added at 15g/L.

Strain isolation

Native yeast strains were isolated from guava, orange, mango, papaya, apple, lemon, cucumber, banana and pineapple ordered from different Mexican regions with disparate climatic and soil conditions. An integral crude extract was obtained from 10g of each fruit sample by maceration followed by addition of 25 mL of sterile distilled water and. The extract was transferred to a 250mL Erlenmeyer flask and incubated for 5 days at 30°C with moderate agitation (100 rpm / min). At the end of this period, the pH of fermented fruit cultures was measured. One milliliter of fermented extract from each culture were serially diluted up to 10^5 and were spread on selective medium MRA or MRA-X medium supplemented with streptomycin at 30 mg/ L at 30°C for 48 hours.

Colony morphology

Individual yeast colonies were streaked from fresh MRAG or MRAX plates and grown at 30°C for 48 hours to study colony morphology according to the following criteria: 1) size at the time of incubation diameter in mm from a fractions of mm up to 10 mm; 2) form (punctate, irregular, circular, rhizoid, fusiform); 3) elevation (flat, monticular, elevated, umbelliform, convex, umbilicated); 4) border or contour (whole, wavy, lobate, serrated, filamentous or curly); 5) color (white, yellow, black, brown, orange, etc.); 6) surface (smooth or rough); 7) appearance (moist, dry, cottony, powdery, velvety, hairy, grainy); 8) Consistency (soft or butyrous, viscose, membranous or hard); 9) reflected light (bright or matte); and 10) transmitted light (translucent or transparent).

Total DNA extraction

Total DNA was extracted from each strain using the method of Hoffman and Winston [10]. Briefly, 5 mL of MRAG or MRAX liquid medium in 15 mL Falcon tubes were inoculated and grown at 30ºC and 110 rpm/mL for 48 hours. Cells were collected by centrifugation (5minutes at 3500 rpm), washed one time with 500 µL of sterile water, and resuspended with the mixture (200 µL of Winston solution, 200 µL of Tris-saturated phenol/chloroform and glass), this mixture were vortexes at full speed for 5 minutes, then 200 µL TE (10mM Tris, 1mM EDTA) was added and centrifuged for 1 minute at 10000 rpm. DNA was precipitated by adding 1 mL of absolute ethanol to the supernatants, followed by inverting 6 to 10 times and centrifuge at full speed for 5 minutes. DNA pellets were washed with 500 µL of ethanol 70%, allowed to air dry for 15 minutes and dissolved in 50 µL of sterile water.

PC ramplification of the ITS regions

The 5.8S rDNA internal transcribed spacer (ITS) regions were amplified by polymerase chain reaction (PCR) using the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). PCR reactions were prepared in a total volume of 25 µL containing 1 µL of total DNA, 12.5 µL of Master Mix (Thermo Fisher Scientific, Massachusetts, USA), 9.5 µl of water, 1 µL (Cf:10 µM) oligonucleotide ITS1 and 1 µL (Cf: 10 µM) of ITS4oligonucleotide. Amplification was performed in a thermocycler (Labnet, New Jersey, USA) under the following conditions: denaturation 94ºC for 5 min, 35 cycles of amplification (94ºC for 1 min, 52ºC for 2 min, 72ºC for 2 min), and termination stage: 72ºC for 10 min. The PCR products were visualized on a 1% agarose gel, using E-gel Imager (Thermo Fisher Scientific, Massachusetts, USA) with blue light base [11]. The length of ITS sequences ranged from 600 to 750 bp.

RFLP of amplified ITS regions

Restriction fragment length polymorphism (RFLP) analysis of the PCR-amplified ITS regions was performed using the method of Granchi., [12]. Briefly, 20 µL of the PCR products were drop onto 0.025 µm VSWP membranes (Millipore, Sigma, USA) left to be dialyzed onto sterile water for 30 minutes. Dialyzed PCR products were recovered [9], and digested with Hha I, Hae III and Hinf I [12], according to supplier´s instructions (Thermo Fisher Scientific, USA). The digestions were visualized on 2% TAE agarose by electrophoresis. PCR products were also sequenced and selected yeast strains were identified by BLAST [11-13].

β-glucan determination

Levels of 1,3:1,6-β-glucan were measured using β-glucan Assay Kit (K-YBGL β-glucan Assay Kit, Megazyme™, Ireland) according to the manufacturer´s instruction as previously reported elsewhere [14]. The β-glucan levels were determined in preliminary massive test and then in kinetic test to establish the optimal growth phase for β-glucan production in the selected high producing yeasts.

Cell adhesion assay

Mouse intestinal epithelial cells were obtained from our institutional cell & culture biobank and maintained in Eagle's minimal essential medium (MEM) supplemented with glutamine (0.584 g/L),sodium bicarbonate (3.7 g/L), antibiotics (penicillin 100 µg/ml, streptomycin 100 µg/mL, spectinomycin100 µg/mL) and 5% bovine fetal serum (FBS) at pH 7.2. T75 Culture flasks were maintained in atmosphere of 5% CO_2 at 37°C [15]. For the assay, cell monolayers of β-glucans high producer strains were washed three times with prewarmed MEM medium without FBS serum, fixed with cold methanol, stained with Giemsa stain. Cells were analyzed under the microscope and adherence score calculated. Strains with the highest score selected for further tests.

Animal care, husbandry and probiotic testing

Mouse experiments were performed in accordance with the Guidelines for Ethical Conduct in the Care and Use of Nonhuman Animals in Research and the Institutional Committee of Ethics, Animal Care and Welfare [16]. Six-week-old BALB/c mice were housed in temperature controlled (22-25ºC) on a 12h light/dark cycle (12h/12h) with access to water and food *ad libitium*. The normal diet consists in NUTRICUBOS, provided from Lab Chows (https://www.nutrimentospurina.com). A total of 20 mice were divided into 4 groups of 5 mice each, two groups were fed with normal control diet, and the other two groups were fed with NUTRICUBOS enriched with 20% w/w of one of the selected yeast strains. For each experimental diet, one of the two groups, were subjected to oxidative stress. Oxidative stress was induced by perturbing living conditions in a number of ways: change of room, food deprivation, diminishing of water supply and alteration of the acoustics for three days (at days 11, 12, 13 of diet exposure). At the end of the test the animals were transferred to another facility and the normal living conditions were restored. The animals were weighed daily until day 30th.

Blood biochemical analysis

Murine blood samples were collected through the ophthalmic plexus using a glass Pasteur pipette at the day 0 (prior to day exposure) and at the day 10th, 13th and 30th of experiment. Samples were centrifuged at 1831 x g for plasma collection. Liver and renal functions were evaluated by measuring of 11 biochemical markers (glucose, total protein, serum albumin, urea, uric acid, creatinine, alanine transaminase, aspartate transaminase, alkaline phosphatase, and serum glutathione peroxidase and serum total antioxidant capacity). Measurements were done using commercial kits according to the manufacturers': a)glucose (Mouse Glucose Assay Kit, Crystal Chem., Elk Grove Village, IL, USA); b) total protein (Total Protein Assay Kit, Bio Scientific Corporation, Austin, TX, USA); c) Serum Albumin (Quanti Chrom™ BCG Albumin Assay Kit, Bio Assay Systems, Hayward, CA,USA); d) Urea BUN (Mouse Blood Urea Nitrogen ELISA Kit, Creative Diagnostics, Shirley, NY, USA); e) Uric Acid (Quanti Chrom™ Uric Acid Assay Kit, Bio Assay Systems, Hayward, CA, USA); f) Creatinine (Mouse Creatinine CREA ELISA, Kamiya Biomedical Co., Seattle, WA, USA); g) Alanine Transaminase (EnzyChrom™ Alanine Transaminase Assay Kit, Bio Assay Systems, Hayward, CA, USA); h) Aspartate Transaminase (EnzyChrom™ Aspartate Transaminase Assay Kit, Bio Assay Systems, Hayward, CA, USA);i)Alkaline phosphatase (Mouse Alkaline Phosphatase (ALP) ELISA, Kamiya Biomedical Co., Seattle, WA, US); j) Serum GSH-Activity (Mouse Glutathione (GSH) Colorimetric Cuvette Detection Kit (Innovative Research, MI, USA) and k) Plasma Total Antioxidant Capacity (Total Antioxidant Capacity Assay Kit, ABCAM, Cambridge, MA, USA).

Mouse immunological status

The immunological status of animals were evaluated by the determining of lymphocytes, neutrophils, monocytes, eosinophils and basophils during diet-exposure experiment using hemocytometer [17].

Statistical analysis

Data were analyzed using one-way ANOVA followed by Dunnett'sor Fisher's protected least significant difference multiple comparison testing in SPSS13.0 (SPSS, Chicago, IL, USA). When necessary, data were transformed for normalization and to reduce heterogeneity of variance, p-values less than 0.05 were statistically considered significant.

RESULTS AND DISCUSSION

Isolation and characterization of wild yeast strains

Approximately 1500 yeast species described so far about a dozen are used at industrial scale and some 70-80 species have been shown to possess potential value for biotechnology [18]. It has been suggested that known yeast species represent roughly 5% of the total number that may exist in the nature [18]. The current applications of yeasts have been greatly expanded beyond classical applications over the past decades. Yeasts are also recognized as probiotics. Yeast cell wall contains β-glucans that can function as antioxidant by preventing damages caused by oxidative stress. Oxidative stress results in several altered metabolic and inflammatory conditions and has been center of extensive research in both human and veterinary medicine [19-21].

In this study we isolated native yeast strains from fruit extracts collected across Mexico as a part of biorepository project. A total of 256 strains were isolated, 187 in MRA containing glucose as carbon source and 69 in MRA containing xylose. The fruit type and region of origin, the final pH generated during fermentation and the carbon source used during isolation were important factors contributing to the diversity of the yeast collection. In addition to sugars, many fruits also contain acids such as citric acid and other metabolites. Thus yeasts have evolved to survive in a slightly acid condition and consequently fermentation performs best in the pH ranging 4.5 to 5.5. We analyzed fruit extracts with acid (pH 3

to 5), neutral (pH 6 to 7) and alkaline (pH 8 to 9) pH values. Acid and alkaline media are stressing conditions and yeast isolated from those conditions can be used in similar environments, that are the case of *Debaryomyces hansenii* that have been used as a probiotic on the immune and antioxidant systems of juvenile leopard grouper *Mycteroperca rosacea* exposed to stress [22]. Yeast culture on media containing xylose allowed us to isolate wild xylose-metabolizing strains, suitable for the biotransformation of hemicellulose to produce alcohol [23] or xylitol [24].

After analysis of colony morphology in isolated strains we selected 44for further microbiological and molecular analysis. Initially, we performed RFLP analysis of ITS regions by the method of Granchi et al., [12] but we were unable to identified individual isolates. Because of limitations of conventional and ITS-RFLP methods alone we decided to use DNA sequencing and sequence comparison of the 5.8S rRNA gene and ITS1-ITS4 regions [13], for accurate identification of the yeast strain. We used a combination of Heras-Vázquez et al. [25], and Doniger et al. [26], methodologies. A high degree of diversity among selected strains was evidenced by RFLP analysis of PCR-amplified ITS *Zygosaccharomyces* ssp., *Yarrowia* ssp., *Rodotorula* ssp. and *Piscia* ssp. Native yeast population has been studied by other authors, demonstrating the existing great diversity of yeasts in different environments and substrates [27-34].The Mexican regions where fruit were collected from have different climatic and soil conditions increased our chances to obtain a greater biodiversity in obtained yeast collection (Table 1). Biodiversity of yeast, as it occurs with other microorganisms, is evolutionary result of adaptation processes to a variety of climatic and soils conditions in close ecological interrelation among all living communities in every ecosystem [35-41].

β-glucans production and selection of promising yeast strains

β-glucan production was quantified for each of 44 selected yeast strains (Table 2, Figure 1). Six strains showed the highest β-glucans levels and were selected for further studies: 1. *Zygosaccharomyces* ssp.YMG4 (from Mango extract, final pH4.0, glucose as carbon source) with β-glucans production of 29.61 µg/mL/per 25mg wet weight; 2. *Debaryomyces hansenii* YAX22 (from apple extract, final pH 3.0, xylose as carbon source) with a production of 32.87 µg/mL/25 mg wet weight; 3. *Debaryomyces hansenii* YPX28 (from papaya extract, final pH 8.0, Xylose as a carbon source) with a production of 41.14 µg/mL/25 mg wet weight; 4. *Saccharomyces cerevisiae* YLG36 (From Lemon extract, final pH 7.0, glucose as a carbon source) with a raised production of 41.77 µg/mL/25 mg wet weight; 5. *Saccharomyces cerevisiae* YPG43 (from Pineapple extract, final pH3.0, glucose as a carbon source) produced 40.26 µg/mL/25 mg wet weight and 6. *Debaryomyces hansenii* YPX44 (from Pineapple extract, final pH 3.0, xylose as a carbon source) produced 49.78 µg/mL/25 mg wet weight.

Successful probiotic microorganisms must be able to colonize digestive tract of the host animal by adhering to the intestinal mucosa at least temporarily. In a preliminary experiment, the adhesion capacity of selected high β-glucan producers was evaluated *in vitro* using mouse intestinal epithelial cells. *Saccharomyces cerevisiae* LX36 and *Debaryomyces hansenii* PG43 showed good adherence capacity.

Yeast growth dynamic and β-glucan production

Saccharomyces cerevisiae LX36 and *Debaryomyces hansenii* PG43 strains were used to optimize the culture conditions for β-glucan production. The highest β-glucan levels were obtained at 28ºC, 130 rpm agitation for 24 hours, at early stationary phase (Figure 2,3). *Debaryomyces hansenii* was selected because his ecological, physiological and genetic characteristics making this organism metabolically versatile [42,43]. *D. hansenii* an osmo-, halo- and xero-tolerant, yeast, can be found in many food products but also in hyper saline waters, such as the coastal marshes and salt lakes [44]. Some strains can be cultivated in media with up to 25% NaCl [44]. This species is generally nonpathogenic, halophile, osmotolerant and oleaginous microorganism, which make it very attractive platform for basic and applied biotechnological research, including a potential use as probiotics. *Debaryomyces hansenii* PG43 strain is a high β-glucan producer that encourages its use in aquiculture, particularly in shrimp, mussels and oysters farming [42-44], and experiment in such invertebrate species are in progress because the difficulties in establishing experimental facilities at this moment.

Saccharomyces cerevisiae LX36 was selected to test its probiotic function in mice under induced environmental stress. *Saccharomyces cerevisiae* is probably the most common microorganism widely exploited in food industry. It is well known that this yeast specie promotes both human and animal health [45-46], and enhances the bioavailability of minerals through hydrolysis of different compounds as phytates, folate biofortification, mycotoxins and xenobiotics detoxification [47-49]. Many studies promote *Saccharomyces cerevisiae* strains as probiotics restoring gastrointestinal microbial population balance [49]. The different adhesion capacity observed among different strains is seems to be related with the expression of *Flo* (Flocculim) adhesion-family genes encoding for protein responsible for cell-cell and cell-surface adherence [50]. Rapid evolution and divergence of *Flo* genes resulted in great diversity of *Flo* genes, that are usually transcriptionally silent in laboratory yeast strains, a consequence of nonsense mutation in the transcriptional activator *FLO8* [50-53]. Each yeast cell family cells harboring small reservoir of different adhesion-encoding genes. Furthermore, it has been reported that *S. cerevisiae* is able to enhance the survival and therapeutic potential of the probiotic *Lactobacillus rhamnosus* [54], which has been used to prevent and treat vaginal infection [55].

Effect of diet supplementation with dry Saccharomyces cerevisiae YLG36 in mice

Dry yeast powder from *Saccharomyces cerevisiae* LX36 was administrated to mice as a dietary probiotic 10 days before three days period of induced environmental stress. Animals subjected to stressing conditions fed with yeast supplement regained weight more rapidly compared to those animals fed with the control non-supplemented diet (Figure 4). Blood biochemical markers related to liver, renal (Table 3, Figures 5-7) and immunological functions were also evaluated (lymphocytes, neutrophils, monocytes, eosinophils and basophils) (Table 4,

Table 1: Characterization of yeast strains selected based on colony morphological analysis. Restriction analysis of the 600-750 pb ITS amplicon with Hha I, Hae III and Hinf I resulted in 36 polymorphic groups. Double bands are indicated with a 2 in parenthesis. Classification of yeast isolates based on ITS sequencing and BLAST search is also shown.

Strain Nº	Fruit of Origin	Final pH	Carbon Source	Restriction Length Polymorphism Fragment (bp)				Classification by ITS sequencing and blast reaching
				Hha I	Hae III	Hinf I	RFLP Group	
1	Mango	4	Xylose	300 (2)	480 (2)	320 (2)	1	*Debaryomyces hansenii.*
2	Mango	4	Xylose	100 (2), 200 (2)	550	320, 350	2	*Debaryomyces hansenii.*
3	Mango	4	Glucose	300 (2)	750	450, 300	3	*Saccharomyces cerevisiae*
4	Mango	4	Glucose	300 (2)	350 (2)	340, 250 (2)	4	*Zygosaccharomyces ssp.*
5	Mango	4	Glucose	350 (2)	500	250, 300	5	*Saccharomyces cerevisiae*
6	Mango	4	Glucose	700	500	250, 350	6	*Zygosaccharomyces ssp.*
7	Guava	5	Glucose	700	500, 100 (2)	350, 380	7	*Saccharomyces cerevisiae*
8	Guava	5	Glucose	300, 230 (2)	800	400 (2)	8	*Saccharomyces cerevisiae*
9	Guava	5	Xylose	300, 250 (2)	800	400, 350	9	*Candida intermedia*
10	Guava	5	Xylose	150 (2), 200 (2)	500, 100 (2)	450, 380	10	*Candida intermedia*
11	Guava	5	Xylose	150 (2), 200	550, 100 (2)	400, 350	11	*Pichia stipitis*
12	Orange	3	Glucose	300,170 (2)	700	350, 400	12	*Zygosaccharomyces ssp.*
13	Orange	3	Glucose	200 (2), 100 (2)	500, 100	250, 350	13	*Saccharomyces cerevisiae*
14	Orange	3	Glucose	200 (2), 100 (2)	500, 100	250, 300	13	*Saccharomyces cerevisiae*
15	Orange	3	Glucose	300, 200 (2)	380 (2)	180, 280 (2)	14	*Rhodotorula ssp.*
16	Apple	3	Xylose	300, 200 (2)	700	400, 350	15	*Debaryomyces hansenii.*
17	Apple	3	Xylose	300, 200	650, 100	400, 350	16	*Candida intermedia*
18	Apple	3	Xylose	300, 250 (2)	540	350 (2)	17	*Pichia stipitis*
19	Apple	3	Xylose	300, 200 (2)	700	300, 380	18	*Pichia stipitis*
20	Apple	3	Glucose	300, 250 (2)	700	300, 380	18	*Pichia kudriavzevii*
21	Apple	3	Glucose	300, 250 (2)	750	350, 400	18	*Saccharomyces cerevisiae*
22	Apple	3	Xylose	300, 200 (2)	700, 100	350, 380	19	*Debaryomyces hansenii*
23	Banana	5	Xylose	300, 200 (2)	500, 100 (2)	300, 350	20	*Candida intermedia*
24	Banana	5	Xylose	300, 200 (2)	500, 100 (2)	300, 350	20	*Debaryomyces hansenii*
25	Banana	5	Glucose	350 (2)	500, 100 (2)	300, 350	20	*Saccharomyces cerevisiae*
26	Banana	5	Glucose	350 (2)	450, 100 (2)	300, 350	21	*Saccharomyces cerevisiae*
27	Papaya	8	Xylose	350, 230 (2)	500	300, 350	22	*Debaryomyces hansenii*
28	Papaya	8	Xylose	350 (2)	600	380 (2)	23	*Debaryomyces hansenii*
29	Cucumber	9	Glucose	750	430 (2)	750	24	*Pichia kudriavzevii*
30	Cucumber	9	Glucose	350, 300	350 (2)	700	25	*Yarrowia ssp.*
31	Cucumber	9	Glucose	350, 200 (2)	500	320, 400	26	*Pichia kudriavzevii*
32	Cucumber	9	Glucose	350, 300	350, 450	320,400	27	*Yarrowia lipolytica*
33	Lemon	7	Glucose	350 (2)	500, 350	250, 380	28	*Saccharomyces cerevisiae*
34	Lemon	7	Glucose	350 (2)	430, 250	350, 450	29	*Saccharomyces cerevisiae*
35	Lemon	7	Glucose	350 (2)	350, 500	200 (2), 280	30	*Saccharomyces cerevisiae*
36	Lemon	7	Glucose	380 (2)	480	300, 350	31	*Saccharomyces cerevisiae*
37	Lemon	7	Glucose	350 (2)	380 (2)	390 (2)	32	*Candida utilis*
38	Lemon	7	Xylose	350 (2)	580, 200	450, 300	33	*Yarrowia lipolytica*
39	Lemon	7	Xylose	350 (2)	390 (2)	400 (2)	33	*Yarrowia lipolytica*
40	Pineapple	3	Xylose	370 (2)	480 (2)	400 (2)	33	*Debaryomyces hansenii.*
41	Pineapple	3	Xylose	380 (2)	580	200(2) , 300	34	*Pichia kudriavzevii*
42	Pineapple	3	Xylose	350 (2)	450, 350	350 (2)	35	*Pichia kudriavzevii*
43	Pineapple	3	Glucose	350, 400	500 (2)	350 (2)	36	*Saccharomyces cerevisiae*
44	Pineapple	3	Xylose	350 (2)	300, 350	350 (2)	37	*Debaryomyces hansenii*

(2) Number of observed DNA fragments with approximately the same length

Table 2: β-glucan production of selected yeast strains. Shaded lanes correspond to strains with the highest production levels.

No	Strain	Cod	Fruit of Origen	Final pH	Carbon Source	β-glucans (µg/mL) per 25mg wet weight)
1	*Debaryomyceshansenii.*	YMX1	Mango	4	Xylose	16,68
2	*Debaryomyceshansenii.*	YMX2	Mango	4	Xylose	16,46
3	*Saccharomyces cerevisiae*	YMG3	Mango	4	Glucose	15,68
4	*Zygosaccharomyces ssp.*	YMG4	Mango	4	Glucose	29,61
5	*Saccharomyces cerevisiae*	YMG5	Mango	4	Glucose	17,08
6	*Zygosaccharomyces ssp.*	YMG6	Mango	4	Glucose	17,09
7	*Saccharomyces cerevisiae*	YGG7	Guava	5	Glucose	17,94
8	*Saccharomyces cerevisiae*	YGG8	Guava	5	Glucose	16,01
9	*Candida intermedia*	YGX9	Guava	5	Xylose	16,38
10	*Candida intermedia*	YGX10	Guava	5	Xylose	17,60
11	*Pichia stipitis*	YGX11	Guava	5	Xylose	16,63
12	*Zygosaccharomyces ssp.*	YOG12	Orange	3	Glucose	17,29
13	*Saccharomyces cerevisiae*	YOG13	Orange	3	Glucose	15,61
14	*Saccharomyces cerevisiae*	YOG14	Orange	3	Glucose	15,94
15	*Rhodotorula ssp.*	YOG15	Orange	3	Glucose	16,60
16	*Debaryomyces hansenii.*	YAX16	Apple	3	Xylose	16,834
17	*Candida intermedia*	YAX17	Apple	3	Xylose	18,09
18	*Pichia stipitis*	YAX18	Apple	3	Xylose	16,72
19	*Pichia stipitis*	YAX19	Apple	3	Xylose	16,35
20	*Pichia kudriavzevii*	YAG20	Apple	3	Glucose	16,61
21	*Saccharomyces cerevisiae*	YAG21	Apple	3	Glucose	16,59
22	*Debaryomyces hansenii*	YAX22	Apple	3	Xylose	32,87
23	*Candida intermedia*	YBX23	Banana	5	Xylose	16,60
24	*Debaryomyceshansenii*	YBX24	Banana	5	Xylose	17,61
25	*Saccharomyces cerevisiae*	YBG25	Banana	5	Glucose	17,63
26	*Saccharomyces cerevisiae*	YBG26	Banana	5	Glucose	12,77
27	*Debaryomyces hansenii*	YPX27	Papaya	8	Xylose	40,82
28	*Debaryomyces hansenii*	YPX28	Papaya	8	Xylose	41,14
29	*Pichia kudriavzevii*	YCG29	Cucumber	9	Glucose	13,86
30	*Yarrowia ssp.*	YCG30	Cucumber	9	Glucose	13,56
31	*Pichia kudriavzevii*	YCG31	Cucumber	9	Glucose	13,99
32	*Yarrowialipolytica*	YCG32	Cucumber	9	Glucose	13,89
33	*Saccharomyces cerevisiae*	YLG33	Lemon	7	Glucose	13,47
34	*Saccharomyces cerevisiae*	YLG34	Lemon	7	Glucose	13,12
35	*Saccharomyces cerevisiae*	YLG35	Lemon	7	Glucose	14,46
36	*Saccharomyces cerevisiae*	YLG36	Lemon	7	Glucose	41,77
37	*Candida utilis*	YLG37	Lemon	7	Glucose	16,82
38	*Yarrowialipolytica*	YLX38	Lemon	7	Xylose	14,97
39	*Yarrowialipolytica*	YLX39	Lemon	7	Xylose	13,85
40	*Debaryomyces hansenii.*	YPX40	Pineapple	3	Xylose	14,35
41	*Pichia kudriavzevii*	YPX41	Pineapple	3	Xylose	14,18
42	*Pichia kudriavzevii*	YPX42	Pineapple	3	Xylose	14,10
43	*Saccharomyces cerevisiae*	YPG43	Pineapple	3	Glucose	40,26
44	*Debaryomyce shansenii*	YPX44	Pineapple	3	Xylose	49,78

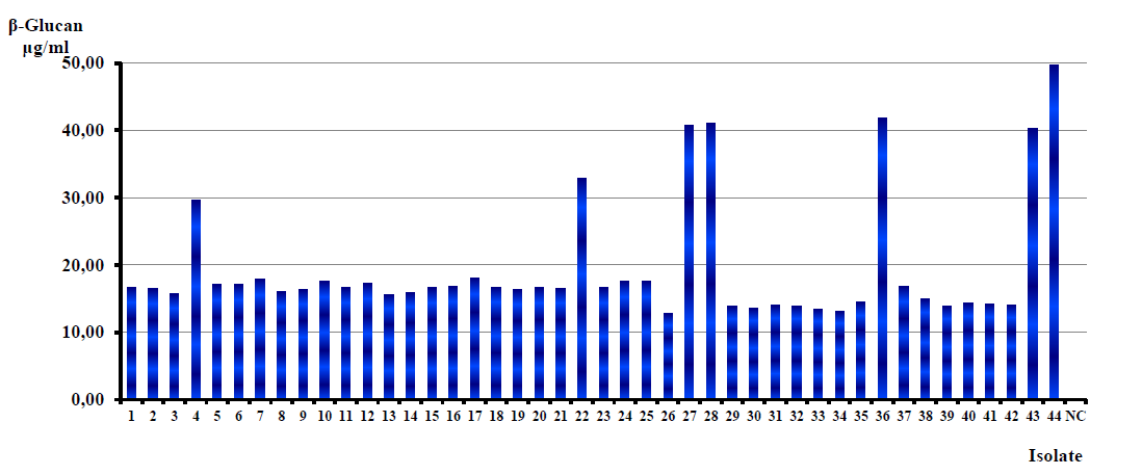

Figure 1 Determination of total β-glucan levels expressed as µg/ml/25 mg of wet weight. NC: Negative control (no cells). The strains selected to further studies were: *Zygosaccharomyces* ssp. MMG4, *Debaryomyces hansenii* YMX22, *Debaryomyces hansenii* YPX28, *Saccharomyces cerevisiae* YLG36, *Saccharomyces cerevisiae* YPG43and *Debaryomyces hansenii* YPX44.

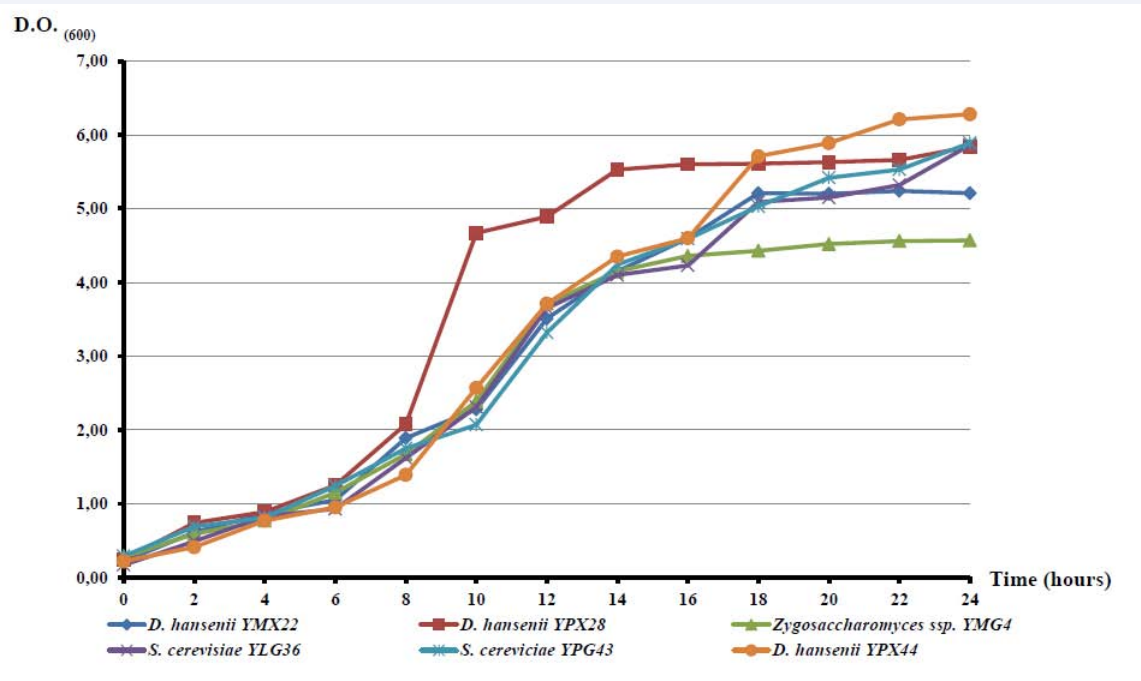

Figure 2 Growth β-glucan production by selected strain in YPG or YPX media at 28°C and 130 rpm for 24 hours.

Figures 8,9). Compared to the control not stressed groups, an alteration from normal values was observed particularly at the end of induced stress period (day 13) in two groups subjected to induced stress, but the group received normal diet without yeast supplement during the previous 10 days is the most affected when the induced stress was suspended. The oxidative stress induced by malnutrition and inadequate environmental living conditions as the change resulted from livestock handling. These changes resulted in metabolic dysfunction, decreased availability of essential nutrients, loss of electrolytes and amino acids, increased metabolic requirements, homeostatic and energy imbalance, physiological dysregulation and overall a poor health condition [56]. The beneficial effect of diet supplementation with yeast rich in β-glucans is obvious when compare the evolution of weight, liver, renal, immunological and antioxidant blood markers. In general metabolic alterations are less severe and animals recover completely and more rapidly when normal environmental conditions were restored. Kanagasabapathy [57], reported positive effects of β-glucans rich extract from *Plerotus sajor caju* to prevent obesity and oxidative stress in mice fed on high fat diet.

Below is a description of biochemical and immunological tests results including a description of observed evolutionary

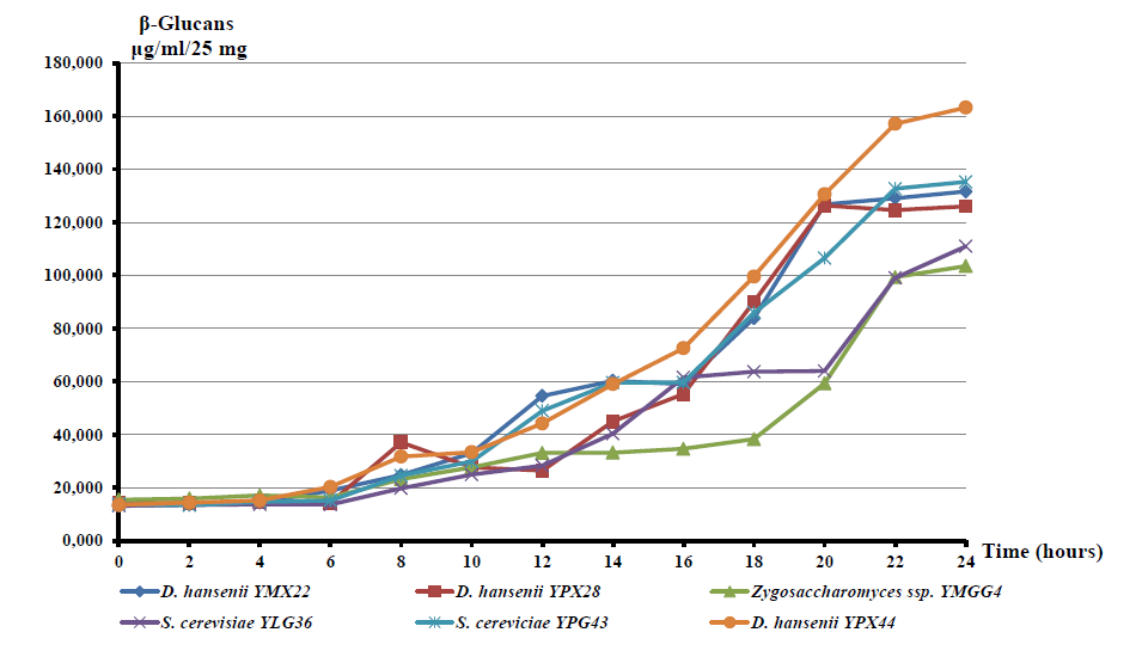

Figure 3 Dynamics of β-glucan production by selected strain in YPG or YPX media at 28°C and 130 rpm for 24 hours.

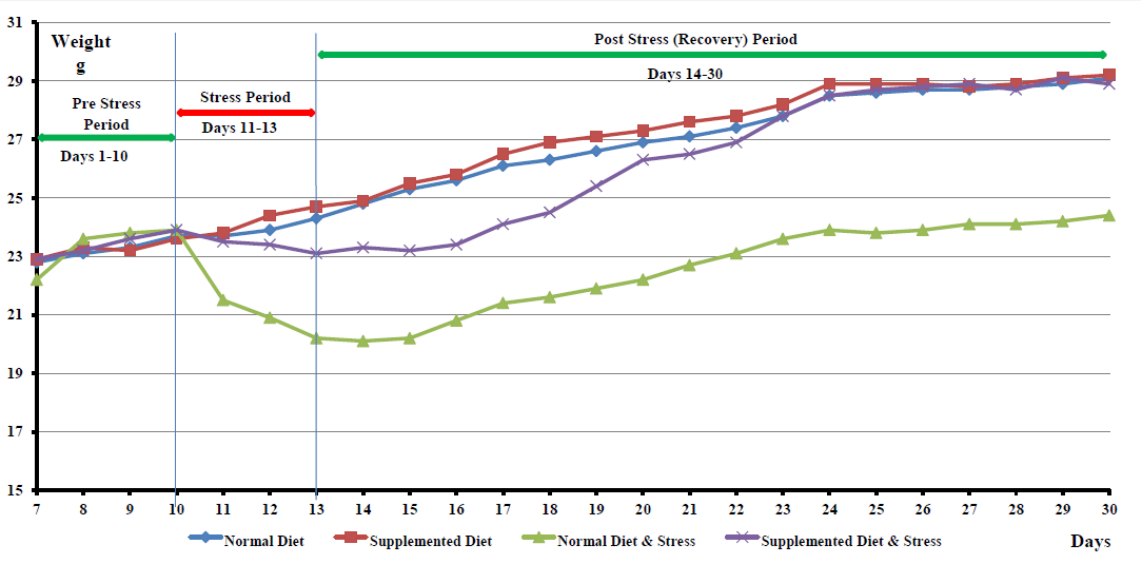

Figure 4 Effect diet supplementation with 25% dry S. cerevisiae YPG43 (rich in β-glucans) in 6week-old BALB/c mice. Treatments correspond to: ND-control diet (not stressed), SD-supplemented diet (not stressed), NDS-control diet subjected to stress, and SDS-supplemented diet subjected to stress. The stress period during days 11, 12, 13 consisted in changes in living conditions, food deprivation, change of housing location, and reduced water supply. The best response to induced stress and best weight recovery were observed in the animal group fed the supplemented diet. The blood tests establishing parameters of renal, hepatic and immunological functions were done at days 1, 10, 13 and 30. n= 5 in each experimental group.

tendency for each measured markers (Table 3,4). Our results are consistent with those obtained by others authors, although some variation in specific values can be observed due the experimental conditions, the characteristics of induced stress and the fact that we analyzed only mice periphery blood [58,59]. The same tendency in analyzed serum indicators has been found in rats and dogs [60,61].

Glucose: Glucose is a key diagnostic marker of energy metabolism and overall metabolic health associated with diabetes mellitus, but also indicative of hyperactivity of thyroid, pituitary or adrenal glands. Hypoglycemia can be associated with insulin secreting tumors, myxedema, hypopituitarism orhypoadrenalism. The balance between oxidative and antioxidant processes appears to be sensitive to glucose levels with moderate elevations of glucose affecting the oxidative

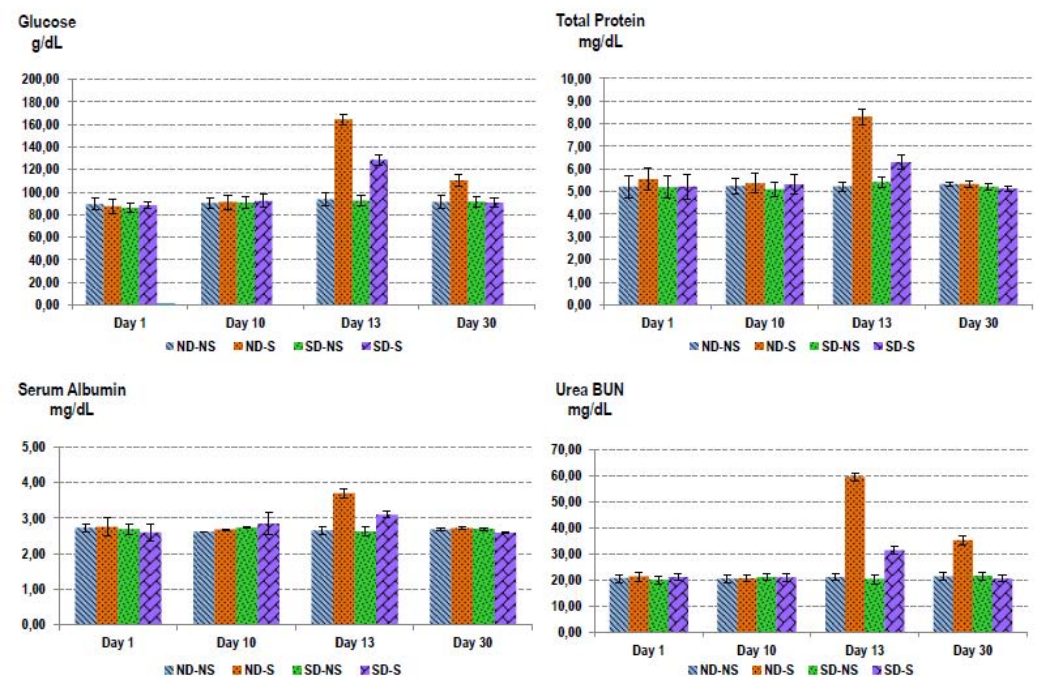

Figure 5 Glucose, total protein, serum albumin and urea BUN test results for BALB/c mice. Deviation from normal levels was observed in stressed groups, but values should return to normal after stress conditions disappear. Only animals in the stressed group fed probiotic supplemented diet recovered completely at the end of the experiment. ND: Control diet, not stressed; NDS: Control diet, stressed; SD: Supplemented diet, not stressed; SDS: Supplemented diet, stressed. N= 5 in each experimental group.

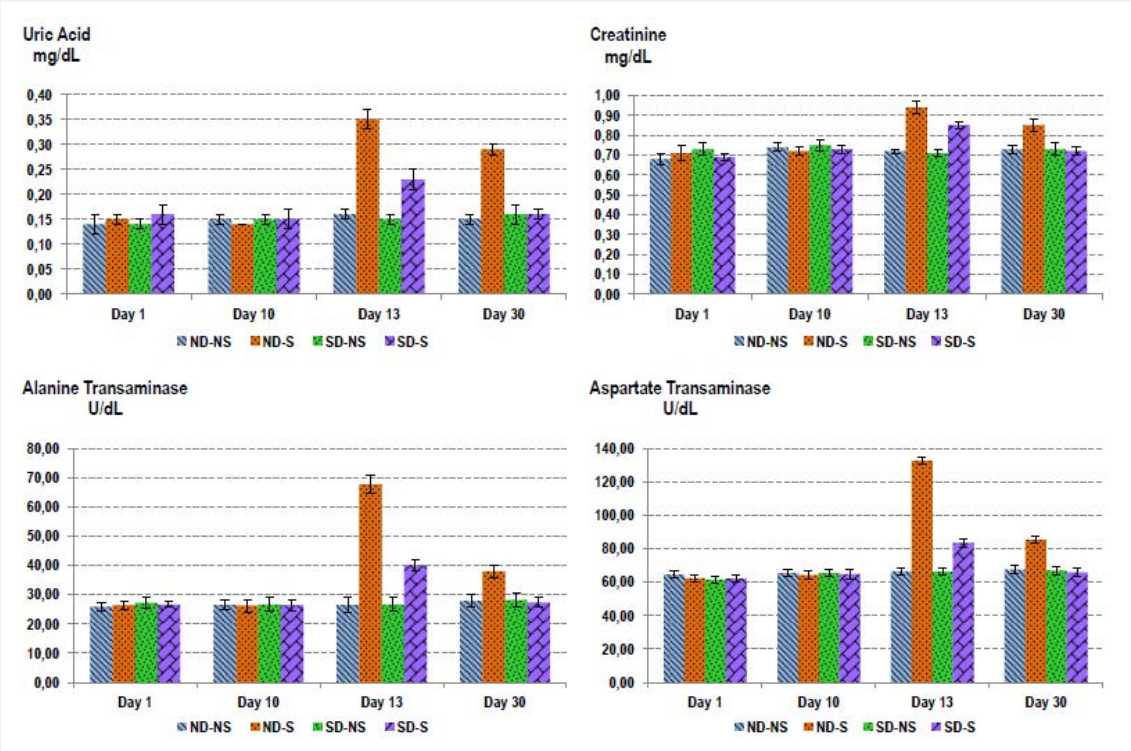

Figure 6 Uric acid, creatinine, alanine transaminase and aspartate transaminase test results for BALB/c mice. Deviation from normal levels was observed in stressed groups, but values should return to normal after stress conditions disappear. Only animals in the stressed group fed probiotic supplemented diet recovered completely at the end of the experiment. ND: Control diet, not stressed; NDS: Control diet, stressed; SD: Supplemented diet, not stressed; SDS: Supplemented diet, stressed. N= 5 in each experimental group.

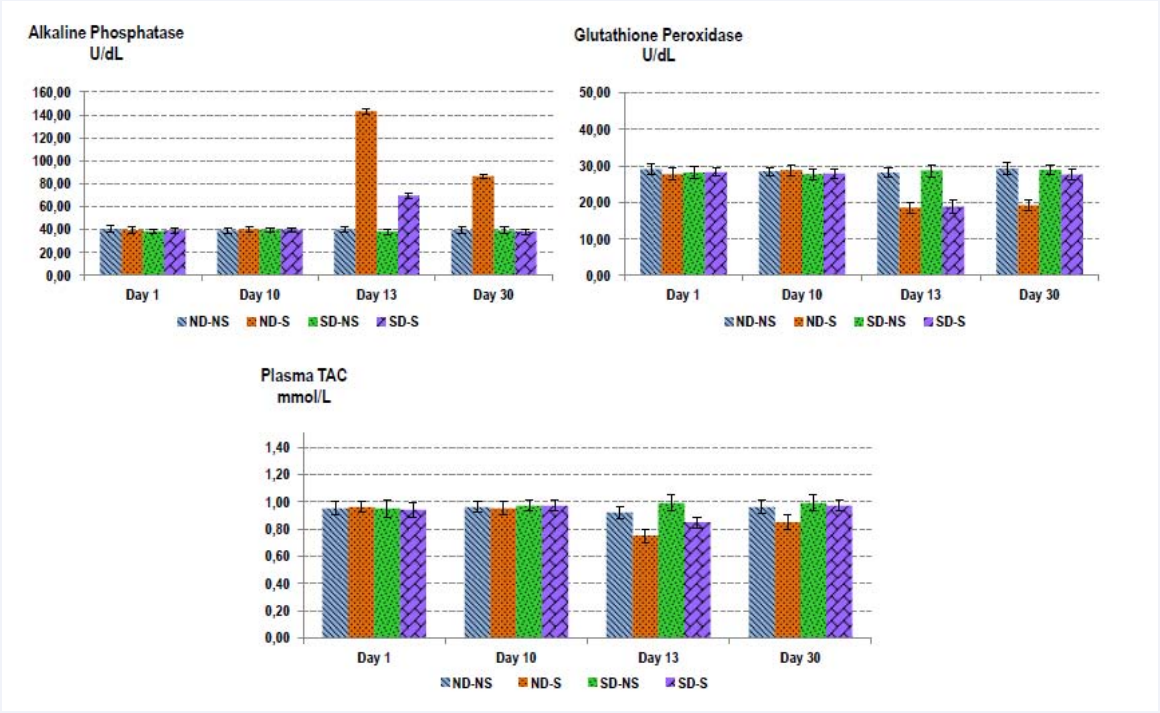

Figure 7 Alkaline phosphatase, glutathione peroxidase and plasma total antioxidant capacity (TAC) test results for BALB/c mice. Deviation from normal levels was observed in stressed groups, but values should return to normal after stress conditions disappear. Only animals in the stressed group fed probiotic supplemented diet recovered completely at the end of the experiment. ND: Control diet, not stressed; NDS: Control diet, stressed; SD: Supplemented diet, not stressed; SDS: Supplemented diet, stressed. N= 5 in each experimental group.

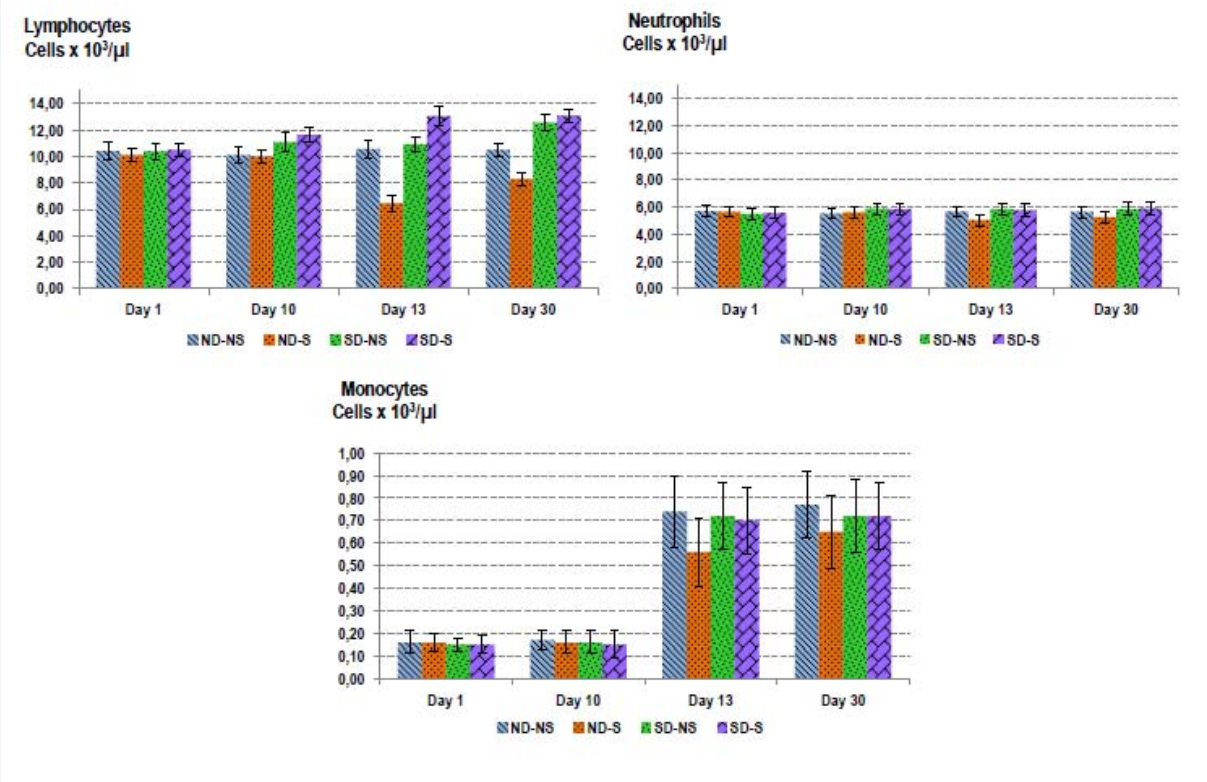

Figure 8 Lymphocyte, neutrophil and monocytes coin YPG or YPX media at 28°C and 130 rpm for 24 hours.in BALB/c mice. ND: Control diet, not stressed; NDS: Control diet, stressed; SD: Supplemented diet, not stressed; SDS: Supplemented diet, stressed. N= 5 in each experimental group.

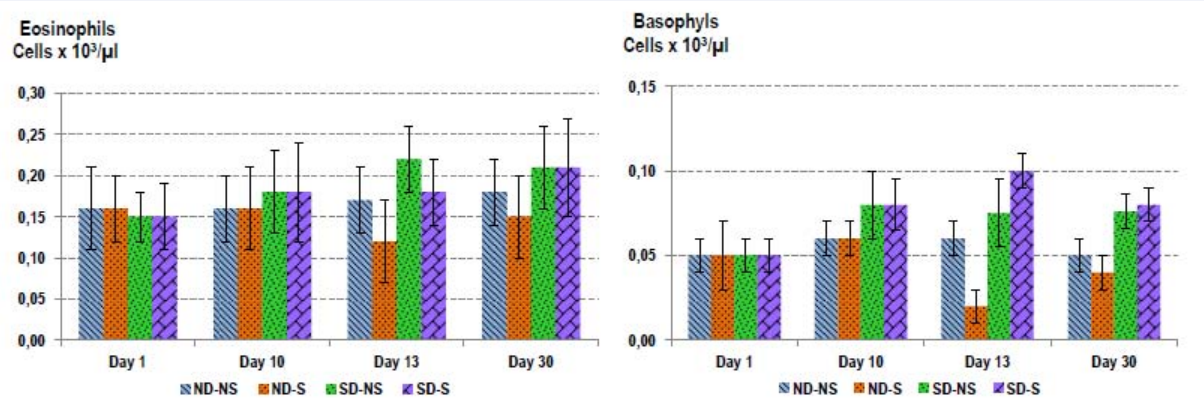

Figure 9 Eosinophil and basophil counts in BALB/c mice. ND: Control diet, not stressed; NDS: Control diet, stressed; SD: Supplemented diet, not stressed; SDS: Supplemented diet, stressed. N= 5 in each experimental group.

Table 3: Blood biochemical tests performed in peripheral blood of BALB/c mice. ND: Control diet, not stressed; NDS: Control diet, stressed; SD: Supplemented diet, not stressed; SDS: Supplemented diet, stressed. N= 5 in each experimental group.

No	Test	Group	1st day	s	10th day	s	13th day	s	30th day	s	Remarks
1	Glucose g/dL	ND	89.11	5.22	90.21	4.52	93.45	5.72	91.08	5.91	Stable normal glucose values
		NDS	87.23	6.31	91.03	6.57	164.26	4.74	110.35	4.72	Glucose increases at day 13th, incomplete recovery of normal values at day 30th.
		SD	85.90	4.17	90.42	5.34	92.12	4.52	91.37	4.77	Stable normal glucose values
		SDS	88.35	3.25	92.03	5.62	128.45	4.51	90.42	4.09	Glucose increases at day 13th, complete recovery of normal values at day 30th.
2	Total Protein mg/dL	ND	5.21	0.51	5.24	0.35	5.22	0.21	5.32	0.09	Stable normal total protein values
		NDS	5.54	0.47	5.36	0.42	5.31	0.34	5.32	0.12	Total Protein increases at day 13th, incomplete recovery of normal values at day 30th.
		SD	5.19	0.49	5.09	0.32	5.41	0.24	5.21	0.15	Stable total protein values
		SDS	5.21	0.56	5.31	0.43	5.29	0.31	5.13	0.13	Total Protein increases at day 13th, complete recovery of normal values at day 30th.
3	Serum Albumin mg/dl	ND	2.72	0.11	2.61	0.01	2,65	0.12	2.67	0.03	Stable normal Serum Albumin values
		NDS	2.75	0.26	2.67	0.02	3,69	0.13	2.71	0.04	Serum Albumin increases at day 13th, incomplete recovery of normal values at day 30th.
		SD	2.68	0.16	2.73	0.01	2,62	0.12	2.69	0.03	Stable normal glucose values
		SDS	2.59	0.25	2.84	0.31	3,09	0.09	2.58	0.01	Serum Albumin increases at day 13th, complete recovery of normal values at day 30th.
4	Urea BUN mg/dL	ND	20.62	1.51	20.41	1.45	21.01	1.32	21.32	1.43	Stable normal Urea values
		NDS	21.32	1.75	20.58	1.41	59.47	1.29	35.21	1.58	Urea increases at day 13th, incomplete recovery of normal values at day 30th.
		SD	20.04	1.43	21.04	1.25	20.31	1.67	21.05	1.39	Stable normal Urea values
		SDS	21.03	1.37	20.91	1.36	31.32	1.54	20.51	1.32	Urea increases at day 13th, complete recovery of normal values at day 30th.

5	**Uric Acid mg/dL**	**ND**	0.14	0.02	0.15	0.01	0.16	0.01	0.15	0.01	Stable normal Uric Acid values
		NDS	0.15	0.01	0.14	0.01	0.35	0.02	0.29	0.01	Uric Acid increases at day 13th, incomplete recovery of normal values at day 30th.
		SD	0.14	0.01	0.15	0.01	0.15	0.01	0.16	0.02	Stable normal Uric values
		SDS	0.16	0.02	0.15	0.02	0.23	0.02	0.16	0.01	Uric Acid increases at day 13th, complete recovery of normal values at day 30th.
6	**Creatinine mg/dL**	**ND**	0.68	0.03	0.74	0.02	0.72	0.01	0.73	0.02	Stable normal Creatinine values
		NDS	0,71	0.04	0.72	0.02	0.94	0.03	0.85	0.03	Creatinine increases at day 13th, incomplete recovery of normal values at day 30th.
		SD	0.73	0.03	0.75	0.03	0.71	0.02	0.73	0.03	Stable normal Creatinine values
		SDS	0.69	0.02	0.73	0.02	0.85	0.02	0.72	0.02	Creatinine increases at day 13th, complete recovery of normal values at day 30th.
7	**Alanine Transaminase U/dL**	**ND**	25.82	1.43	26.41	1.57	26.45	2.74	27.93	2.15	Stable normal ALTP values
		NDS	26.34	1.34	26.18	2.01	67.72	2.92	37.94	2.26	Activity increase at day 13th, incomplete recovery of normal values at day 30th.
		SD	27.21	1.72	26.63	2.37	26.64	2.31	28.09	2.45	Stable normal ALT values
		SDS	26.62	1.05	26.34	1.96	39.96	2.08	27.32	1.76	Activity increases at day 13th, complete recovery of normal values at day 30th.
8	**Aspartate Transaminase U/L**	**ND**	64.6	2.05	65.38	1.95	66.41	2.05	67.42	2.58	Stable normal ALP values
		NDS	62.31	2.28	64.07	2.38	132.54	1.75	85.25	1.79	Activity increase at day 13th, incomplete recovery of normal values at day 30th.
		SD	61.45	2.34	65.43	2.09	66..21	1.98	66.73	2.58	Stable normal ALP values
		SDS	62.05	1.94	64.78	2.81	83.42	2.27	65.72	2.36	Activity increases at day 13th, complete recovery of normal values at day 30th.
9	**Alkaline Phosphatase U/L**	**ND**	40.52	2.74	39.32	2.21	40.15	2.05	39.54	2.75	Stable normal ALP values
		NDS	39.67	2.45	40.07	2.32	142.95	2.15	86.56	1.59	Activity increase at day 13th, incomplete recovery of normal values at day 30th.
		SD	38.45	2.03	39.86	1.97	38.25	2.35	39.15	2.81	Stable normal ALP values
		SDS	39.37	2.51	39.78	2.17	69.62	2.43	38.23	2.39	Activity increases at day 13th, complete recovery of normal values at day 30th.
10	**Serum Glutathione Peroxidase *(GSH-Px) nmol/ml**	**ND**	29.12	1.45	28.5	1.09	28.18	1.34	29.31	1.81	Stable normal GSH-Px values
		NDS	27.76	1.62	28.8	1.45	18.54	1.55	19.21	1.48	Activity decrease at day 13th, incomplete recovery of normal values at day 30th.
		SD	28.21	1.59	27.7	1.38	28.73	1.68	28.89	1.21	Stable normal GSH-Px values
		SDS	28.32	1.05	27.9	1.32	18.82	1.72	27.67	1.43	Activity decrease at day 13th, complete recovery of normal values at day 30th.
11	**Serum Total Antioxidant Capacity nmol/L**	**ND**	0.95	0.05	0.96	0.04	0.92	0.04	0.96	0.05	Stable normal TAC values
		NDS	0.96	0.04	0.95	0.05	0.75	0.05	0.85	0.05	Activity decrease at day 13th (after stress), incomplete recovery of normal values at day 30th.
		SD	0.95	0.06	0.97	0.04	0.99	0.06	0.99	0.06	Stable normal TAC values
		SDS	0.94	0.05	0.97	0.04	0.85	0.04	0.97	0.04	Activity decrease at day 13th, complete recovery of normal values at day 30th.

*GSH nmol of reduced glutathione per ml

Table 4: White blood cells counts in BALB/c mice. ND: Control diet, not stressed; NDS: Control diet, stressed; SD: Supplemented diet, not stressed; SDS: Supplemented diet, stressed. N= 5 in each experimental group.

No	Test	Group	1st day		10th day		13th day		30th day		Description of tendency
			Cells x 10^3/µl	s	Cells x 10^3/µl	s	Cells x 10^3/µl	s	Cells x 10^3/µl	s	
1	Lymphocytes	ND	10.42	0.7	10.09	0.6	10.55	0.7	10.49	0.5	Without significant changes
		NDS	10.1	0.5	9.97	0.5	6.45	0.6	8.29	0.5	Decrement at 13th day, Slow recovery at 30th day
		SD	10.37	0.6	11.08	0.7	10.92	0.6	12.55	0.6	Increment
		SDS	10.51	0.5	11.64	0.6	13.05	0.7	13.08	0.5	Increment at 10th day
2	Neutrophils	ND	5.70	0.42	5.51	0.39	5.67	0.39	5.63	0.41	Without significant changes
		NDS	5.65	0.41	5.62	0.42	5.04	0.41	5.26	0-44	Decrease at 13th day, slow recovery at 30th day
		SD	5.48	0.40.	5.88	0.42	5.84	0.42	5.86	0.47	Increment at 10th day
		SDS	5.55	0.41	5.84	0.41	5.77	0.43	5.88	0.47	Increase is observed at 30th day
3	Monocytes	ND	0.71	0.12	0.74	0.17	0.74	0.16	0.77	0.15	Without significant changes
		NDS	0.70	0.13	0.71	0.16	0.56	0.15	0.65	0.16	Decrease level at 13th day, Slow recovery at 30th day
		SD	0.71	0.13		0.16	0.72	0.15	0.72	0.16	Without significant changes
		SDS	0.69	0.15	0.71	0.15	0.70	0.15	0.72	0.15	Without significant changes
4	Eosinophils	ND	0.16	0.05	0.16	0.04	0.17	0.04	0.18	0.04	Without significant changes
		NDS	0.16	0.04	0.16	0.05	0.12	0.05	0.15	0.05	Decrement at day 13th, Slow recovery at ay 30th.
		SD	0.15	0.03	0.18	0.05	0.22	0.04	0.21	0.05	Increment at 10th day
		SDS	0.15	0.04	0.18	0.06	0.18	0.04	0.21	0.06	Increment and fast recovery
5	Basophils	ND	0,05	0.01	0.06	0.01	0.06	0.01	0.05	0.01	Without significant changes
		NDS	0.05	0.02	0.06	0.01	0.01	0.02	0.04	0.01	Decrement at 13th day, Slow recovery at day 30th.
		SD	0.05	0.01	0.08	0.02	0.075	0.02	0.076	0.01	Increment at 10th day
		SDS	0.05	0.01	0.08	0.01	0.10	0.01	0.08	0.1	Increment at 10th day

status [62]. Chronic hyperglycemia can induce oxidative stress in organism, by the generation of free radicals, which may increase lipid peroxidation, depletes antioxidants, and enhances oxidative stress in individuals with type 2 diabetes [63]. Drastic Animals change in glucose levels were not observed but feed yeast supplemented diet showed better values compared with the animals fed control diet which showed increased glucose levels at day 13th (post stress) with couldn't completely return to normal values at day 30th (Table 3). The relationship between glucose fluctuations and oxidative stress markers has a multifactorial character [64].

Total protein: Total protein concentration market showed increased levels at day 13th in stressed animals fed with control diet (post stress) but at day 30th the return to normal values was almost completely, we considered that since all animal were health the organism can assimilate induced stress and values of total protein could return to normality. In animals fed supplemented diet the variations in total protein levels were not significant.

BSA: Albumin plays an important role in the regulation and maintenance of homeostasis due its anti-oxidant and anticoagulant actions. Albumin also functions as a carrier for nutritional factors and drugs, and is an effective plasma pH buffer. Abnormal serum albumin is a good indicator for morbidity and mortality, and may reveal liver disease, nephritic syndrome, malnutrition and protein-losing enteropathies in the context of

other plasma markers. High albumin levels can also be associated with dehydration and starvation [65]. Mice exposed to stress-inducing conditions showed increased serum albumin level at day 13th. The group fed the probiotic-supplemented diet returned to normal levels at day 30th but animals fed control diet normal BSA levels. But the BSA level were not well correlated with total protein level as we expected, probably increased on BSA level in mice fed control diet and stressed was joined with decreases in other serum proteins.

Urea BUN: Urea is the major end product of protein catabolism in animals. It is the primary vehicle for removal of toxic ammonia from the body. Urea determination is very useful in assess kidney function. In general, increased urea levels are associated with nephritis, renal ischemia, urinary tract obstruction, and certain extra renal diseases, congestive heart failure, liver diseases and diabetes. Decreased levels indicate acute hepatic insufficiency or may result from over-vigorous parenteral fluid therapy. Urea level in animals fed with probiotic supplemented slightly increased at day 13th and return to normal level at day 30th compared with control group fed normal diet and stressed, where urea level increased dramatically and the complete return to normal levels was not completely reached at the 30th day.

Uric acid: Uric Acid is the waste product of purine degradation. Uric acid is an important indicator in diagnose and evaluation of kidney function. The metabolism of purines to uric acid requires the action of xanthine oxido reductase (XOR), a dual-function enzyme that has xanthine oxidase (XO) or xanthine dehydrogenase (XDH) activities. XOR exist as XDH, a NAD^+-dependent dehydrogenase that catalyzes the hydroxylation of hypoxanthine to xanthine and then the hydroxylation of xanthine to uric acid with the production of NADH. Uric acid has a protective antioxidant action at physiological concentrations, whereas in excess it has been associated with conditions such as hypertension and nephrolithiasis. Additionally, uric acid may induce an inflammatory response and oxidative stress when released from injured cells in damaged tissue [66]. In mammals, XDH can be converted into XO under a variety of pathophysiological conditions, such as inflammation, ischemia/reperfusion injury, or liver damage by viral infection or toxic substances [67]. Both XO and XDH may generate the reactive oxygen species (ROS) superoxide anion (O_2•-) and hydrogen peroxide (H_2O_2) during the oxidation of substrates. This reaction is inversely dependent on pH and O_2 tension and is thus favored under hypoxic conditions. In the reverse reaction, urate has the ability to scavenge oxygen radicals. Therefore hyperuricemia may be responsible for increase in serum antioxidant capacity. Both uric acid and ascorbic acid are strong reducing agents (electron donors) and potent antioxidants. In humans, over half the antioxidant capacity of blood plasma comes from hydrogen urate ion [66]. Current evidences regarding the antioxidant role of uric acid and suggests that it has an important role as an oxidative stress marker and a potential therapeutic role as an antioxidant. Further well designed clinical studies are needed to clarify the potential use of uric acid (or uric acid precursors) in diseases associated with oxidative stress. It has been proposed that UA may represent one of the most important low-molecular-mass antioxidants in the human biological fluids. Current evidence suggests uric acid can be an important oxidative stress marker and a potential therapeutic antioxidant Apart from its action as radical scavenger, UA uriate can also chelate metal ions, like such as iron and copper, converting them to poorly reactive forms unable to catalyze free-radical reactions [66-71]. UA level in animals fed with probiotic supplemented slightly increased at day 13th and return to normal level at day 30th compared with control group fed normal diet and stressed, where increased in UA levels to high compared with normal values and the complete return to normal levels was not completely reached at the 30th day.

Creatinine: Creatinine is synthesized in the body at a fairly constant rate. Creatine is generated from creatine-phosphate as a result of muscle contractions. Creatinine is excreted into in the urine. In healthy individuals, creatinine is independent of diet and is fairly constant. The creatinine clearance test is very convenient for measuring and evaluating glomerular filtration rate. In kidney disease plasma creatinine levels become elevated due to reduced creatinine filtration and excretion rate. Our mice feed the control diet had increased creatinine levels at the 13th day (post-stress induction) and had an incomplete recovery at day 30. These results are consistent with previous reports showing impaired kidney function, at least temporary, under oxidative stress conditions [72-73]. The animals fed probiotic supplemented diet showed less increase in creatinine level and completely return to normal level when experiment concluded.

Alanine transaminase: Alanine Transaminase (ALT) allows the conversion of alanine and α-ketoglutarate to pyruvate and glutamate. This enzyme plays a central in process such as gluconeogenesis and amino acid metabolism. ALT is located mainly in the liver, and, and in less quantity in other organ as kidney, heart, muscle, and pancreas tissues. Usually le serum levels of ALT are low, and increased serum ALT activity is widely used as a marker for liver damage. Mice exposed to stress-inducing conditions showed increased serum ALT levels immediately after stress induction at 13th day, animals fed with the probiotic-supplemented diet returned to normal levels at 30th day. Our observations are well correlated with the results obtained by Cichoż-Lach and Michalak [74].

Aspartate transaminase: Aspartate Transaminase (AST), also known as serum glutamic oxaloacetic transaminase (GOT), facilitates the conversion of aspartate and β-ketoglutarate to oxaloacetate and glutamate. AST is a pyridoxal phosphate-dependent enzyme that exists in cytoplasmic and mitochondrial forms, GOT1 and GOT2, respectively. AST plays a role in gluconeogenesis, amino acid metabolism and the urea and tricarboxylic acid cycles AST is usually part of test panels for the evaluation of liver function and the diagnosis of conditions such as, myocardial infarction, acute pancreatitis, acute hemolytic anemia, severe burns, acute renal disease and trauma. In our study, similar to ALT results, mice exposed to stress-inducing conditions showed increased serum AST levels immediately after stress induction, but only animals fed the probiotic-supplemented diet recovered completely.

Alkaline phosphatase: Alkaline phosphatase (ALP) drives the hydrolysis of phosphate esters in an alkaline environment. This reaction produces an organic radical and inorganic phosphate. In mammals, this enzyme is found mainly in the liver

and bones. Increased serum ALP levels are a good an indicator of impaired liver function and have been associated with conditions such as malignant biliary obstruction, primary biliary cirrhosis, primary sclerosing cholangitis, hepatic lymphoma and sarcoidosis [75,76]. Mice exposed to stress-inducing conditions showed increased serum ALP levels immediately after stress induction at 13th day, but animals fed the probiotic-supplemented diet showed less increase and the normal ALP levels were recovered completely at the 30th day.

Glutathione peroxidase: Glutathione peroxidase (GSH-Px) is a common antioxidant enzyme that functions to scavenge ROS similar to superoxide dismutase and catalase. GSH-Px plays an important role in hemoglobin protection from oxidation. The increasing concentration of reduced glutathione oxidation can affect erythrocyte's membrane and enzymatic systems, particularly during the glycolysis [76,77]. In our study, GSH-Px activity decreased at day 13th post-stress induction, but returned to normal levels only in mice feed the probiotic-supplemented diet by day 30.

Plasma total antioxidant capacity (TAC): Oxidative stress can be assessed by measuring the concentration and dynamics of endogenous antioxidant compounds. However, these measurements can be somewhat inconsistent due to variations in the nature of ROS produced. Measurement of the total antioxidant capacity (TAC) of bodily fluids is believed to be a more useful indicator of the ability of antioxidants to protect against oxidative stress damage. TAC can determine spectrophotometrically in plasma [61]. Glutathione peroxidase (GSH-Px) is a common antioxidant enzyme that functions to scavenge ROS similar to superoxide dismutase and catalase. GSH-Px plays an important role in hemoglobin protection from oxidation. The increasing concentration of reduced glutathione oxidation can affect erythrocyte's membrane and enzymatic systems, particularly during the glycolysis [76,80]. In our study, GSH-Px activity decreased at day 13th post-stress induction, but returned to normal levels only in mice feed the probiotic-supplemented diet by day 30. Our results showed that mice feed control diet had a lower TAC at day 13 post-stress induction and did not return completely to normal levels by day 30. But the alteration in this parameter is much lower than we have expected. TAC assay is mostly focused on low molecular weight, chain breaking excluding the contribution made by antioxidant enzymes and metal-binding proteins [81].

Immunological status: The immunological status of animals was evaluated by the determining lymphocyte, neutrophil, monocyte, eosinophil, and basophil counts (Table 4, Figures 7-9). The total leukocyte count decreased in the animal group fed control diet, but not in the same proportion among the different types of leukocyte. Lymphocytes were the most affected cell type, falling 35.3% from 9.97x10^3 to 6.45 x10^3 cells/µL between days 10 and 13 post-stress induction. In the groups fed the probiotic-supplemented diet, there was an increase in lymphocyte count prior to stress induction (day 10), but the group fed with normal control diet the number of lymphocytes fell and complete recovery of normal values were not observed. A decrease in cell count was also observed for the other cell populations: -10.32% (neutrophils), -21.12% (monocytes), -25% (eosinophils) and -6.6% (basophils) in mice fed control diet. These results suggest that the probiotic-supplemented diet had a stimulatory effect on the proliferation of different leukocyte populations, thus providing better protection against infections [80,81]. Malnutrition always results in an immune deficiency in addition other alteration in living and environmental conditions leading to oxidative stress increase the level of physiological, metabolic and immunological affectations [59]. We hypothesize that β-glucans in the yeast cell wall, which are absent from mammalian cells, play an important role in the overall stimulation of innate and acquired immunity [80- 82].

These results support the idea of systemic positive effect of dietary supplementation with booth selected yeast strains and the possibility to apply selected yeasts as a probiotic in humans and animal. In animal production during handling and manipulation of animals, occur changes in their living condition (change of feeding, transfers to another location, transport to a new region, etc) as well as changes in animal live cycle (birth, breastfeeding or breeding, weaning, calving, etc). These drastic changes in living conditions and stages of the life cycle can have a dramatic impact on the productivity of the livestock by weight loss, slow weight gain, organ physiological malfunction and diminish of immunological defenses [82-85]. We seek to minimize its consequences with dietary supplements as preventive treatment. The protection is probably due to multiple mechanisms involving free radical scavenger properties and increasing the antioxidant status.

CONCLUSION

Oxidative stress induced by dietary restriction and other adverse environmental conditions can lead to adverse health effects such as body weight loss, immunosuppression and altered metabolism. In this study, we demonstrate that diet supplementation with high β-glucan producing yeast as a probiotic can have a positive health impact by reducing or preventing the noxious effects of oxidative stress. Our results support the possible use of one specific yeast strains (*Saccharomyces cerevisiae* LX36) as a probiotic in humans and animal. During animal production, changes in living conditions due to handling and manipulation (e.g., changes in feeding and housing location, and life cycle changes such as forced mating, delivery, breastfeeding, weaning, calving, etc) could have a dramatic impact on livestock productivity due to weight loss, slow weight recovery, metabolic dysfunction and reduced immunological competence. We seek to minimize the adverse effects of oxidative stress by supplementing animal feed with high β-glucan-producing probiotics as prophylactic treatment.

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