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Milmed *Saccharomyces Cerevisiae* Activity on Central Nervous System Cells

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Abstract

Objective: The aim of the present study was to evaluate the effects of incubation of CNS cells, namely microglia and neuronal-like cells, with Milmed *Saccharomyces cerevisiae* yeast [Milmed]. Cytotoxicity tests and dose-response curves were performed; the phenotype assumed by the cells after incubation with yeast, in the presence or in the absence of LPS, was also evaluated

Materials and Methods: Milmed, a treated yeast preparation grown in medium enriched with glucose or glycerol and with different nitrogen availability, was added at different concentrations to microglial BV2 cells, U-937 monocytic cell line, SK-N-SH, IMR-32 neuroblastoma cell line. Cells were incubated with Milmed at different concentrations for 24 and 48 hrs. Cell viability was determined by MTS and Trypan Blue exclusion.

Results: Morphological analysis revealed that microglial and monocyte-like cells were able to engulf and phagocytize the yeast. Milmed did not display any toxicity until the concentration of 10^7/well. Indeed, even at this concentration it is likely that depletion of nutrient rather than toxicity may account for the observation. It exhibited a trophic activity upon neuroblastoma cells, as confirmed by the induction of NGF and BDNF mRNAs. When added to LPS treated BV2 cells, Milmed induced the recovery of resting phenotype, that is a spindle cell body with several cytoplasmic elongations, differing from the LPS stimulated phenotype: round, enlarged and with minor and shorter ramifications, suggesting an anti-inflammatory activity of Milmed yeast.

Conclusion: Milmed yeast does not produce cytotoxic effects neither upon microglia cells nor upon cells of neuronal origin, where it plays a trophic activity. It seems to play an anti-inflammatory activity which suggests its possible use as a nutraceutical.

Keywords: Yeast; Toxicity; Microglia; Neuroblastoma cells; Inflammation; Neurotrophins

Abbreviations: BDNF: Brain-Derived Neurotrophic Factor; CNS: Central Nervous System; LPS: Lipopolysaccharide; NGF: Nerve Growth Factor

Introduction

The intestinal microbiota is essential for the absorption of nutrients, induction of fermentation processes, and for the regulation of intestinal permeability. Yeasts are part of the intestinal microbiota and participate in the maintenance of metabolic homeostasis. There is enormous variability in the composition of the microbiota, not only based on individual characteristics but also on dietary changes and exposure to antibiotics, prebiotics and probiotics. Probiotics have been defined as live microorganisms, which when administered in an adequate amount, confer a beneficial health effect to the host [1]. *Saccharomyces cerevisiae* [*S. cerevisiae*] have been available for the last decades as dietary supplements because of their high contents of vitamin B, proteins, peptides, amino-acids and trace minerals [2]. Different yeast species, such as *Saccharomyces cerevisiae* var. *boulardii,* have been included into probiotics, since they have shown beneficial activities to promote health and cooperate in the treatment of some diseases. For example, *Saccharomyces* was shown to modulate favorably the microbiota, helping the development of those bacterial species that promote health: *S. boulardii* supplementation in mice alters the balance of major intestinal phyla inducing a significant increase in Bacteroides and a marked decrease in Firmicutes, Proteobacteria and Tenericutes [3]; induce the release of several enzymes from the brush border, driving the adsorption or degradation of nutrients accumulating in the lumen, and play immunomodulatory effects, such as regulation of cytokine production [4]. Besides, as outlined in a recent paper by Staniszewski and Kordowska-Wiater [5] yeasts owe potential for new probiotic products with novel properties, which are not offered by bacteria-based probiotics and may be used also for the production of functional foods [6].

One extremely interesting activity displayed by probiotics is the ability to modulate the immune system functioning, to participate in the development and maintenance of innate and adaptive immunity, acting in particular upon the production of cytokines therewith suppressing inflammation by inhibiting proinflammatory cytokine production [7,8] or by increasing the production of anti-inflammatory cytokines [9] as well as the enhancement of innate immunity [10]. In addition, it has been shown that *Saccharomyces boulardii* administration modulates short-chain fatty acids that in turn exert immunomodulatory properties [11]. As a matter of fact, *S. boulardii*, administered daily by oral gavage to leptin-resistant obese and type 2 diabetic mice (db/db) for 4 weeks, stimulated the reduction of pro-inflammatory cytokines such as IL-6 and IL-1beta [3]. A study conducted upon obese subjects showed that the intake of *Saccharomyces cerevisiae* var. *boulardii* for 60 days at mealtimes determined a significant weight loss and a decrease of BMI, fat mass, insulin, HOMA Index and uric acid [12]. *Saccharomyces cerevisiae* was very useful also for the prevention and treatment of various immunological conditions by suppressing production of IgE causing type I allergy symptoms and for the prevention of food allergy [13] as assessed from patients' self-reports.

The ability to modulate the activity of the innate immune system is of great potential as most chronic-degenerative diseases are accompanied by low-grade chronic inflammation which is also one of the main risk factors for the development of cerebrovascular diseases. Alzheimer's disease [AD] is a chronic-degenerative disease that affects several areas of the brain, in particular the hippocampus and the prefrontal cortex, which are responsible for spatial orientation and memory processing. Morphological analyses of the affected areas revealed accumulations of amyloid substance to form senile plaques around which microglial cells, polarized in a pro-inflammatory manner, were detected [14]. AD is in constant increase in the elderly and to date no effective therapies are available. The possibility to modulate the innate immune system and to decrease the inflammation that characterizes the development of the disease could at least allow to slow it down. The aim of the present study was to evaluate the effects of incubation of CNS cells, namely

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microglia and neuronal-like cells, with Milmed yeast. Cytotoxicity tests and dose-response curves were performed; the phenotype assumed by the cells after incubation with yeast, in the presence or in the absence of LPS, was also evaluated in order to assess eventual morphological alterations related to a pro- or anti-inflammatory phenotype.

Materials and Methods

Yeast strains and growth

The treatment and preparation of *S. cerevisiae* with electromagnetic waves in the Extreme High Frequency (EHF) range of 30–300 GHz produces a treated yeast extract, given the name Milmed. This treatment was developed through the pioneering work of Golant [15-17] on *S. cerevisiae*, a strain that is obtained from the International Research Center ‘‘Beer and Beverage XXI Century’’, Moscow, Russia. The yeast was cultured in wort which was produced through meaded malt extract. The treatment of the yeast in an electromagnetic field of super-high frequencies with electromagnetic waves in the EHF range of 30–300 GHz produced the treated yeast extract [16], after which the yeast was re-cultured at 25–28 °C for 48 h [18].

Un-treated and treated strains from Milmed AB (www.milmed.de) were grown under shaking overnight at 28°C in YP (1% yeast extract, 2% bactopeptone; DIFCO), supplemented with 2% glucose or 3% glycerol, SD (yeast nitrogen base w/o amino acids 0,67%) glucose 2% or glycerol 3% media. Cells were harvested at exponential phase and the number of cells quantified by spectrophotometry at 600nm.

1 OD = 13.33 x 10^6 cells/ml.

For each experiment, 10^7 cells were collected and washed in PBS. To verify temperature inactivation, yeast cells incubated at 60°C for 15 min, were centrifuged for 5 min at 3200 rpm to collect cells, plated on YP plus agar 2% for 24 h and examined for the eventual colony formation.

Cell lines

Human neuroblastoma cell lines IMR 32 and SK-N-SH were a kind gift of Prof. Giannini, U937 were a kind gift of prof Nervi. BV2 murine microglia cell line was a kind gift of Dr. Mangino.

Murine BV2 microglia cell line were cultured in growth medium containing Dulbecco’s modified Eagle’s medium (DMEM, Euroclone, Italy) supplemented with 10% fetal bovine serum (FBS),2mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified incubator under 5% CO_2.

Human IMR 32 and SK-N-SH neuroblastoma cell lines were cultured in growth medium containing Minimum Essential Medium (MEM) (Gibco), with Earle’s salts and Sodium bicarbonate, without L-Glutamine, liquid sterile-filtered supplemented with 10% Fetal bovine serum (FBS), 2mM glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified incubator under 5% CO_2.

For all experiments, cells were washed twice with warm PBS.

Treatments

BV-2 murine microglia cells, U-937 human monocytic cells, SK-N-SH, SH-SY-5Y and IMR 32 human neuroblastoma cells were seeded in 48 well plates (cell density of $3x10^4$ cells/well) in duplicate and incubated at 37°C with different concentrations of treated and untreated yeast grown in different media (YP 2% Glucose, or YP 3% Glycerol, SD 2% glucose) for 24, 48, 72 hrs and compared to control cells. The yeast concentrations used were 10^7, 10^5, 10^3, $5x10^2$,10^2, $0.5x10^2$/ well.

Determination of Cell Viability and Cytotoxicity

Trypan Blue exclusion assay

Cell viability was determined by Trypan-Blue exclusion assay. Trypan-blue (Euroclone, Italy) exclusion assay is a simple and rapid method measuring cell viability that determines the number of viable cells and dead cells. It is based on the principle that live cells with an intact membrane are able to exclude the dye whereas dead cells without an intact membrane take up the dye. For the Trypan blue exclusion test, BV-2 were seeded onto 24-well plates at a density of 3×10^4/well. After treatments, cells were detached with 1× Tripsin-EDTA, and 100 µL of cell suspension were mixed with 100 µL of Trypan blue solution, and cell counts were performed using a Neubauer chamber. Blue stained cells were considered nonviable.

Real-time PCR

Total RNA was extracted from control and treated cells using the miRNeasy Micro kit (Qiagen, Hilden, Germany) and quantified using NanoDrop One/One C (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was generated using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystem, Foster City, CA, USA). Quantitative real-time PCR (qPCR) was performed for each sample in triplicate on an Applied Biosystems 7900HT Fast real-time PCR System (Applied Biosystem, Cheshire, UK) through the program SDS2.1.1 (Applied Biosystem, Foster City, CA, USA) using the Power SYBR® Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA). The primers for real-time PCR amplification were designed with UCSC GENOME BROWSER (http://genome,cse.ucsc.edu/;university of California, Santa Cruz) (Table 1). The primer pair sequences were matched by BLASTn to the genome sequence to identify the primer locations with respect to the exons. A comparative threshold cycle (CT) method was used to analyze the real-time PCR data. The amount of target, normalized to the endogenous reference of GAPDH *primers* (ΔCT) and relative to the calibrator of untreated control (ΔΔCT), was calculated by the equation $2^{-\Delta\Delta CT}$ as previously described [19].

Immunofluorescence microscopy

Cell cultures were stimulated with lipopolysaccharide (LPS), a prototypical microbial antigen in the presence or absence of Milmed in different dilutions. Briefly, BV2 cells grown in chamber-slides, were incubated with Milmed grown in YP 2% Glucose, or YP 3% Glycerol, or SD 2% glucose or SD 3% glycerol, in the presence or in the absence of LPS (strain 0111:B4, Sigma.Aldrich,10 ng /ml) for 24 h. Cells were subsequently fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at 25°C, followed by treatment with 0.1 M glycine in PBS for 20 min at 25°C and with 0.1% Triton X-100 in PBS for additional 5 min at 25°C to allow permeabilization. To analyze cytoskeletal actin reorganization, microglial cells were incubated with Phalloidin-Tetramethylrhodamine B isothiocyanate (Phalloidin-TRITC), 1:50 for 45 min at 25°C. Nuclei were highlighted with the 40,6-diamine-20-phenylindole dihydrochloride (DAPI) obtained from Sigma-Aldrich (Saint Louis, MO, USA). Coverslips were finally mounted with mowiol for observation. Fluorescence signal was analyzed by recording stained images using an AxioObserver inverted microscope, equipped with the ApoTome System (Carl Zeiss Inc., Oberkochen, Germany).

Table 1: The primers for real-time PCR amplification.

	Foward	Reverse
hBDNF	AAA CAT CCG AGG ACA ACG TG	AGA AGA GGA GGC TCC AAA GG
hNGF	TAA AAA GCG GCG ACT CCG TT	ATT CGC CCC TGT GGA AGA TG
hGAPDH	ACA GTC AGC CGC ATC TTC	GCC CAA TAC GAC CAA ATC C

Statistical analyses

Data are expressed as mean ± SD. Statistical analysis, including Paired and Unpaired T-test, were performed using GraphPad Prism 8 (GraphPad Software, La Jolla, USA). Statistically significant results are indicated in figures as follows: * , P<0.05; ** , P<0.01; *** P<0.001, **** P<0.0001.

Results

Yeast preparation and growth capability

To study the ability of yeast to modify cell lines growth we added yeasts alive or not to the cell cultures. Yeast cells were grown at 28°C under shaking and harvested at exponential phase. 10^7 cells were collected and the same amount was inactivated at 60°C for 15 min. Yeast cells viability was verified by incubating both alive and inactivated yeasts at 28°C for 24h on YP solid medium. Figure 1 shows that temperature treatment was able to kill yeasts compared to untreated cells, as shown by the quantitative values reported in the graph.

We carried out several experiments in order to obtain a dose-response curve to ascertain whether or not Milmed may produce any toxicity to the cultures, dependent upon concentration. As shown in Figure 2 no toxicity was observed by Trypan blue staining on BV2 microglia cell cultures incubated with a concentration to 10^5. The 10^7 concentration was discarded since it showed to be toxic probably because yeast cells were completely consuming the nutritional elements necessary for the trophism of microglial cells.

The addition of yeast at a concentration of 10^5 to the cultures did not alter functional properties of these phagocytic cells: as shown in Figure 3 cells took up the yeast and after 48hr the yeast was completely phagocytized and cells looked birefringent and viable.

Overlapping results were obtained repeating these treatments with the human monocytic cell line U937 (Figure 4).

Yeast added to different human neuroblastoma cell lines produced no toxicity, stimulating on the contrary cell proliferation (Figure 5)

We then added the same yeast concentrations to human

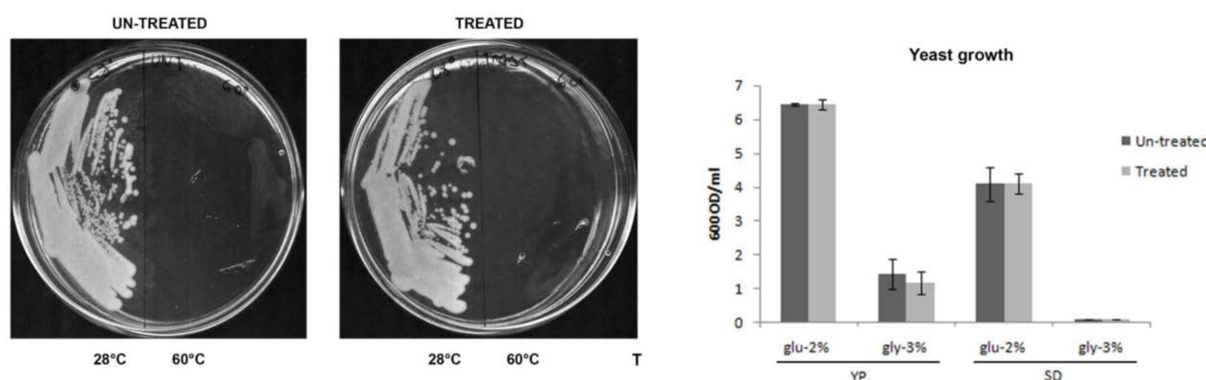

Figure 1: Growth of untreated and treated yeast at different temperature and in different culture conditions: on the first panel plates containing YP agar seeded with yeast grown at 28°C (left) or 60 °C (right) are depicted. Both untreated and treated yeast grow at 28 °C but are killed at 60°C showing no different resistance to high temperature for both samples. Right panel: growth of untreated and treated yeast in YPD enriched with 2% glucose or 3% glycerol is shown: no difference was measured between the two yeast samples. Data are expressed as mean ± SD.

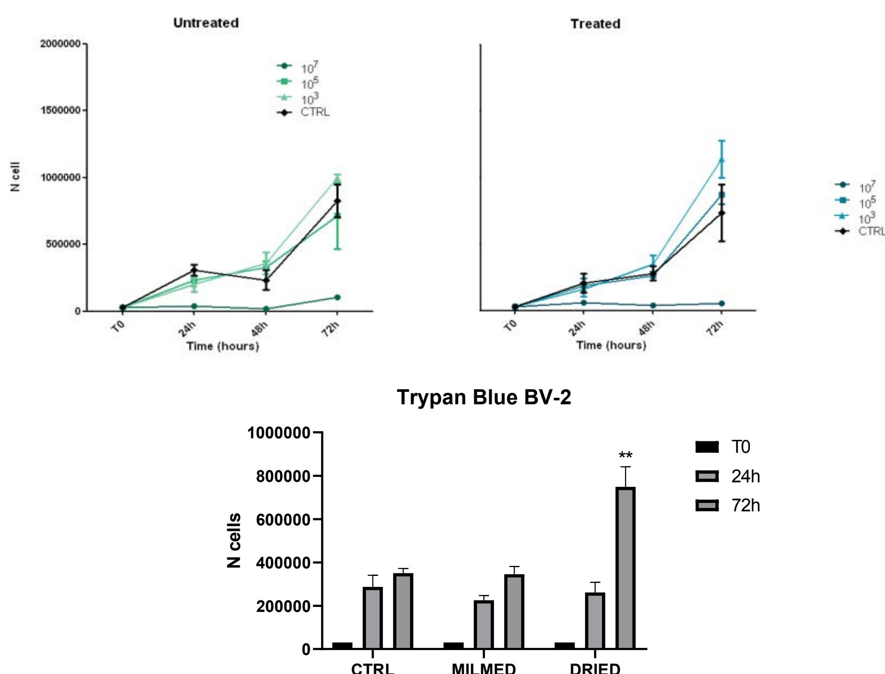

Figure 2: BV2 microglia cells incubated with different Milmed yeast concentrations were analyzed by Trypan Blue dye exclusion at different times (0, 24,48,72 h). No toxicity was observed except with the concentration of 10^7 yeast cells, probably because the yeast consumed all the nutrients present in the medium. (paired T test Vs CTRL : ** P<0.01).

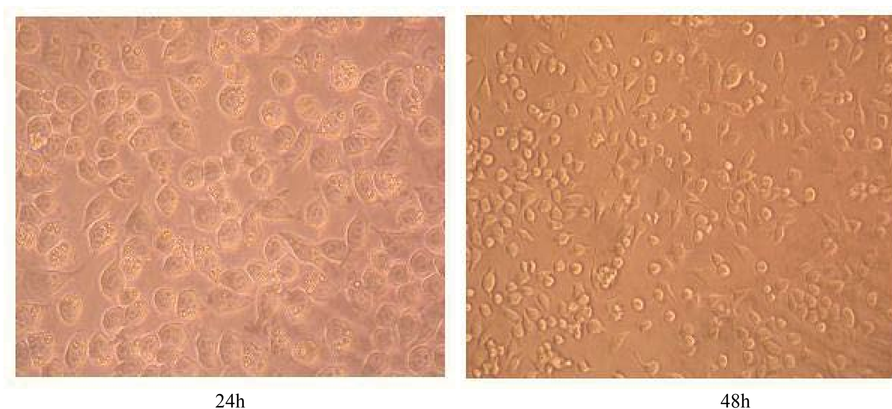

Figure 3: Milmed yeast added to BV2 microglia cells was phagocytized, as shown in the picture on the left, where it appears inside the cells, while at 48 h it is no longer visible; the cells are alive, retain their original shape and show no signs of toxicity.

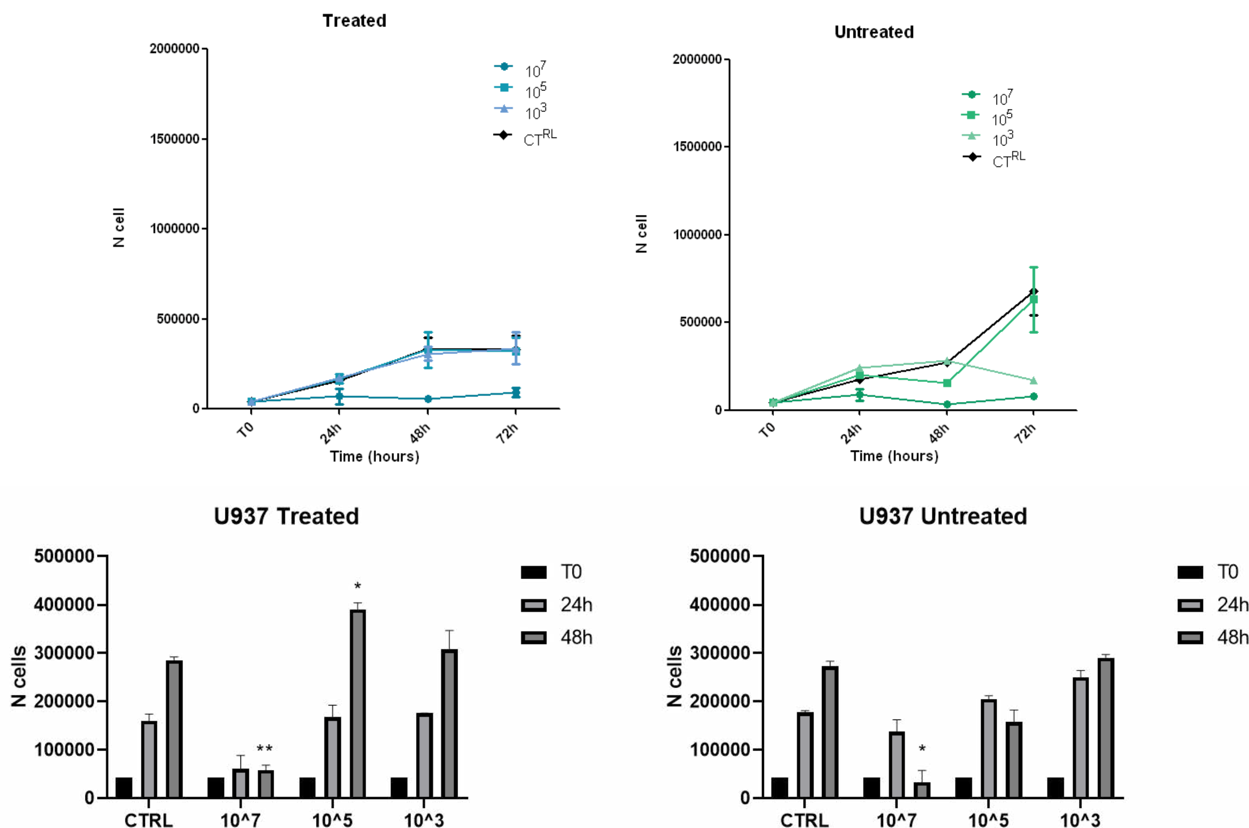

Figure 4: Milmed yeast grown at 28°C or 60°C was added to the human monocytic U937 cell line. The results obtained are comparable to those depicted in Fig. 3: only the yeast concentration of 10^7 cells is toxic. (paired T test Vs CTRL : * P<0.05; ** P<0.01).

neuroblastoma cell line SK-N-SH. The results obtained seemed to indicate a proliferative effect on these cells with concentrations equal to or less than 10^5. In the light of this scenario, we repeated the treatment using lower yeast concentrations. A trophic activity was observed with both liquid and dried Milmed batches (Figure 6).

Figure 7 shows the BDNF and NGF mRNAs expression following Milmed yeast addition to human SK-N-SH neuroblastoma cells. Yeast addition promoted the expression of both neurotrophins.

Results show an increased production of neurotrophin mRNAs following Milmed treatment. Same results are obtained by adding dried Milmed.

In Figure 8 the effects of yeast added at different concentrations and grown in different conditions are shown. We studied the metabolic characteristic of Milmed yeasts with the perspective to analyse a possible effect of differential metabolic yields on cell line growth. Yeast was grown in rich medium YP (Yeast, Peptone) containing all metabolite needed for yeast viability and in SD medium (Synthetic Defined without amino acids) containing only ammonium sulphate as nitrogen base, in which yeast is forced to activate anabolic pathways for amino acids synthesis. Moreover, we supplemented YP with glucose 2%, which allows only fermentation, or glycerol 3%, a respiratory carbon source, to analyse yeast catabolic properties. In YP glycerol 3% the growth was slower compared to YP glucose 2%, as expected for brewer's yeast. Different concentrations of yeast grown in the above described conditions were added to BV2 microglial cells. As shown in. Figure 8 the effects on BV2 cells growth were different depending on yeast metabolism modulated by different cultural conditions; no cytotoxicity was observed with any Milmed sample.

In order to ascertain yeast-driven functional effects on microglial

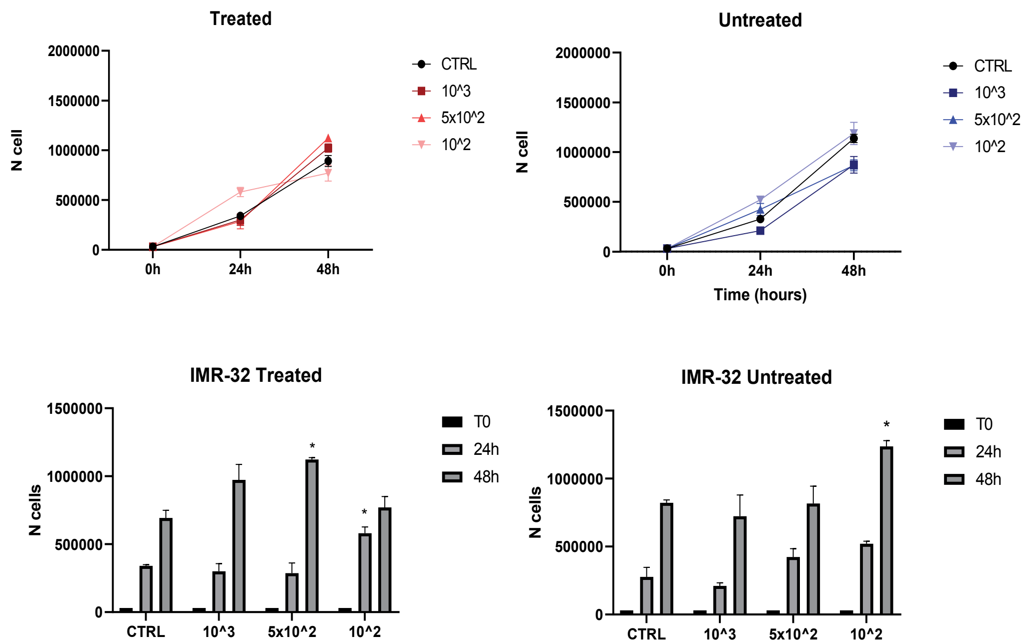

Figure 5: Milmed yeast added to IMR-32 human neuroblastoma cells. No toxicity but a positive trend towards proliferation was observed. (paired T test Vs CTRL : * P<0.05).

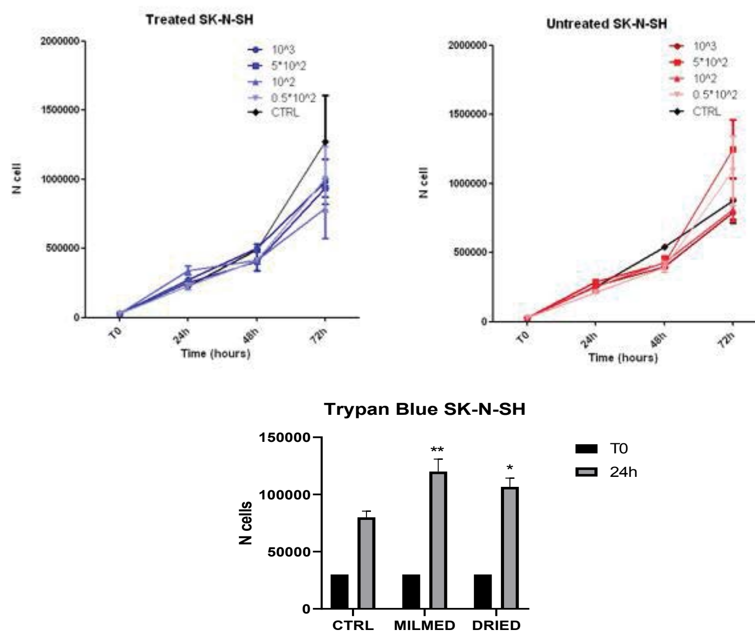

Figure 6: Milmed yeast added to SK-N-SH human neuroblastoma cell line. A proliferative effect with concentrations equal to or less than 10^5 was observed, both with liquid and dried Milmed batches. (paired T test Vs CTRL : * P<0.05; ** P<0.01).

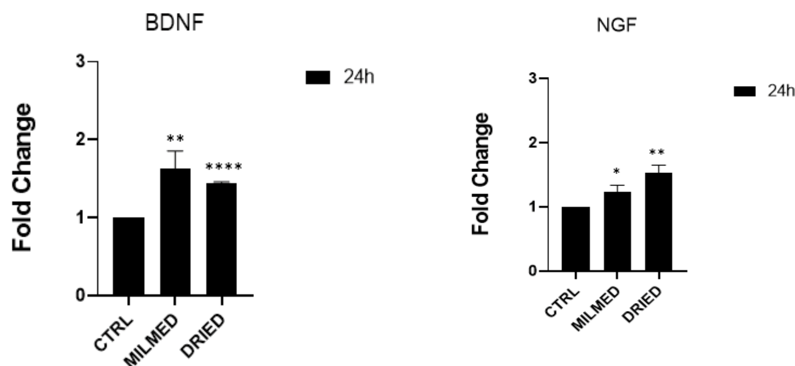

Figure 7: The expression of BDNF and NGF mRNAs following Milmed treatment. Human SK-N-SH neuroblastoma cells grown with 5 x 10^2 Milmed to 24 hr. (Unpaired T test *, P<0.05; ** P<0.01; **** P<0.0001).

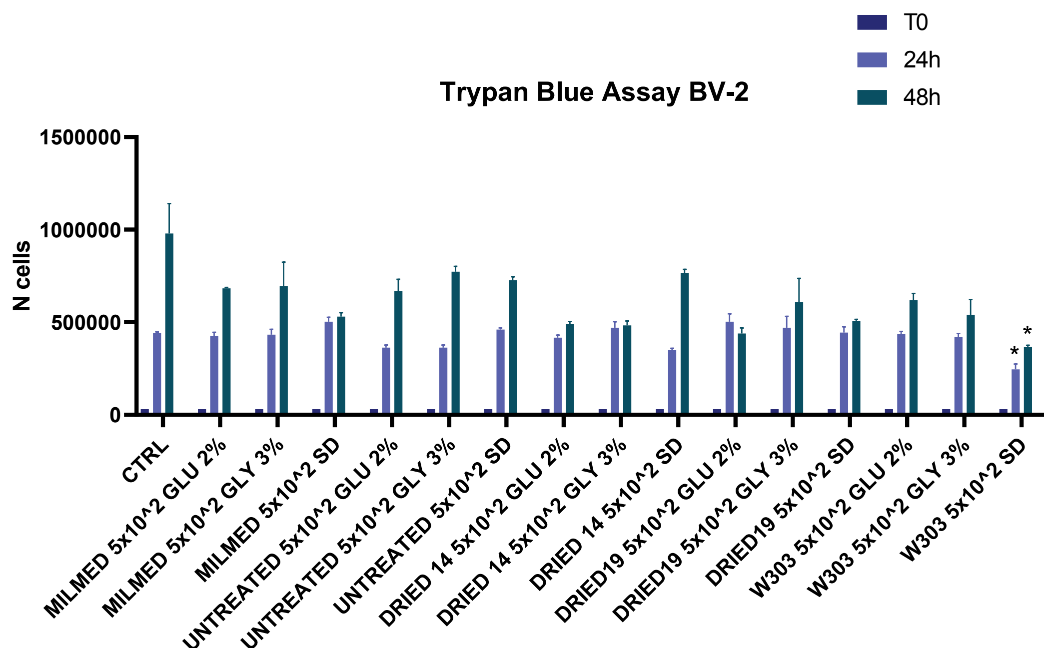

Figure 8: Determination of cell viability and cytotoxicity in the presence of different amounts of Milmed yeast grown in different conditions. The effects on BV2 cells growth were different depending on yeast metabolism determined by different cultural conditions. No significant difference were detected with Milmed yeast; on the contrary W303, a laboratory strain, included as a control, decreased cell viability. (paired T test * P<0.05)

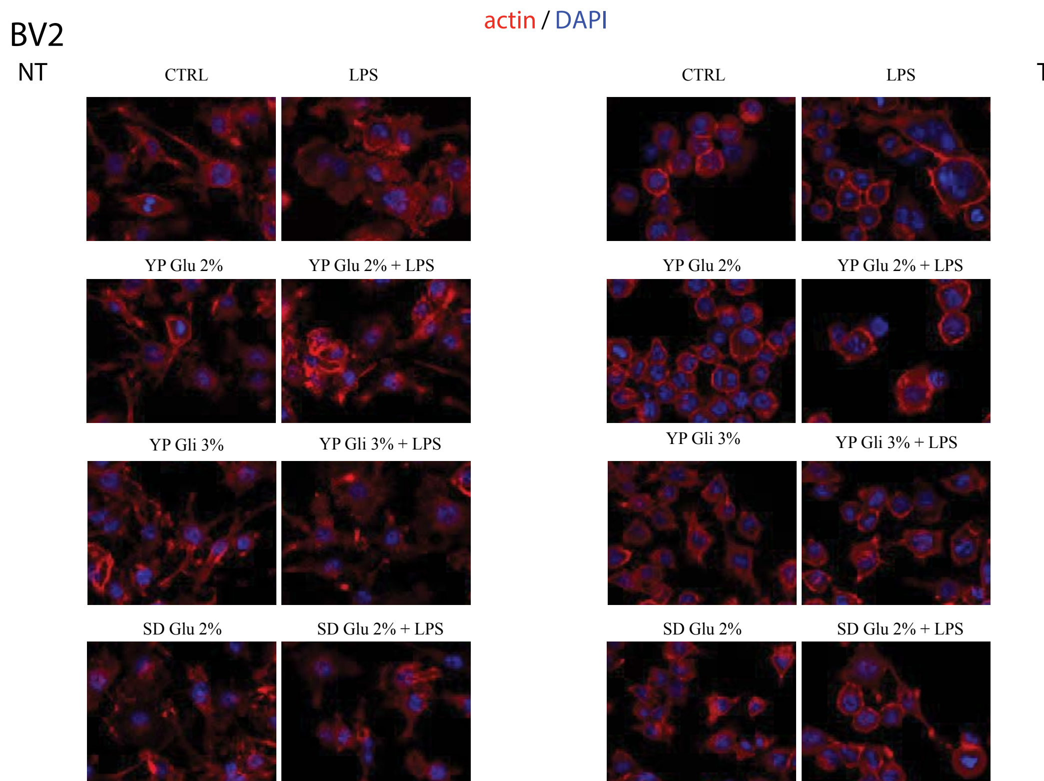

Figure 9: BV-2 cells treated by LPS in the presence or in the absence of Milmed yeast grown in different conditions. Cell shape was analyzed by staining actin cytoskeleton. LPS stimulation induced dramatic changes in cell morphology. Milmed yeast addition was able to restore the anti-inflammatory phenotype of BV2 cells.

cells, we performed some immunofluorescence experiments aimed at evaluating the presence of any effects on cytoskeleton, since the cytoskeletal rearrangement corresponds to phenotypic and functional modifications associated with migration and other functional processes (Figure 9).

In the case of untreated Milmed yeast (left panel) the cell shape is conserved after the addition of the yeast, and many cells bearing cytoplasmic elongations are observed. If LPS, a powerful pro-inflammatory stimulus, is added to the cultures, cells become enlarged and round. It is well known that quiescent cells have a ramified spindle-like phenotype, whereas pro-inflammatory cells become round and acquire an ameboid phenotype. Moreover, in the absence of LPS actin cytoskeleton is mainly organized in filopodia, whereas LPS seems to induce actin depolymerization. Thus, the presence of yeast seems to

maintain the resting phenotype. The addition of treated yeast (right panel) induces a drastic change in cell morphology. In fact, already in the control sample, cells appear round, attached to each other as a chain and the actin does not present typical structures in which it is usually reorganized. Similar results were obtained when the cells were incubated with treated yeast and grown in YP glucose 2%. Cells are round also in the case of treated yeast grown in YP glycerol 3% or SD glucose 2%, in addition, actin is organized in filopodia. In all the cases observed, stimulation with LPS leads to an enlargement of the cell and causes actin to organize in filopodia.

Discussion

The present results imply that Milmed milli-wave length frequency, electro-magnetical treated yeast does not produce cytotoxic effects neither upon microglia cells nor upon cells of neuronal origin, as well as the untreated yeast showing an absence of cytotoxicity. Microglia phagocytize the yeast in the first 24 hrs, being not affected apparently by this process. Much interest ought to be focused also upon the results obtained with Milmed yeast grown in different metabolic media, added to human neuroblastoma cell lines: the yeast induces trophic activity with optimal results at a concentration of 5 x 10^2 yeast cells/well and striking results were obtained with yeast grown under nutrient deprivation conditions. Several hypotheses may be entertained in order to explain these effects. Under such conditions [20], yeast accumulates trehalose, a disaccharide acting as a molecular chaperone, involved in refolding of partially denatured proteins [21]. Trehalose seems to play a key role in neuroprotection mechanisms, perhaps through the regulation of autophagy as suggested in a recent review [22]. Previous studies have shown that dietary polysaccharides derived from yeast may improve cognition and well-being in middle-aged adults [23]; extend lifespan in *Caenorhabditis elegans* [24]; partial recovery in mouse models with neurologic defects [25] and yeast together with physical exercise ameliorated recovery in a MPTP mouse model of Parkinson's disease [18], where trehalose counteracted dopamine level decrease in the corpus striatum [26]. The bulk of studies on neurodegenerative diseases animal models showed that after trehalose supplements animals lived longer with reduced neuropathology [22].

Moreover, trehalose was shown to down-regulate the production of pro-inflammatory cytokines in LPS-stimulated microglial cells [27].

Our results show that treated Milmed yeast added in the presence of pro-inflammatory agent, i.e. LPS, seems to extinguish the effect of the pro-inflammatory trigger, at least partially. Microglia cells partly reacquire an elongated form, losing some phylopodia. Upon activation, microglia also undergo dramatic morphologic changes, from resting ramified shape into activated amoeboid morphology [28]. We demonstrated the ability of Milmed yeast to induce remodeling of LPS-stimulated microglia to acquire the branched spindle-like morphology characteristic of quiescent unstimulated cells. As a matter of fact, immunofluorescence experiments show that Milmed yeast added to microglia cells induce morphological changes linked to actin cytoskeleton remodeling. It is known that actin cytoskeleton dynamics shape microglia effector functions [29] since it has a fundamental role in various cellular processes such as migration, morphogenesis, cytokinesis, endocytosis and phagocytosis [30]. Furthermore, actin polymerization, depolymerization and branching drive dendritic spine morphogenesis on neuronal cells maintaining long-term memory [31]. Several studies reported the positive impact of *Saccharomyces boulardii* on disease outcome in clinical studies of inflammatory bowel diseases [32,33] and its impact on inflammatory cytokine production by human dendritic cells [4]. These effects were attributable to the binding of beta glucan extracted from wall to Dectin-1 receptor [34] modulating innate immunity cell behavior.

Conclusion and Perspectives

The conclusions of the present study may be summarized as follows: (i) Even at the highest doses of Milmed yeast there appears not to exist toxicity since cellular growth was induced to such an extent that nutrient depletion caused cell loss, (ii) The marked effects of Milmed yeast upon cell proliferation for both microglia and neuroblastomas confirms the significance of the observed trophic effects, and (iii) in the presence of pro-inflammatory agent, i.e. LPS, Milmed yeast seems to extinguish the effect of the pro-inflammatory trigger.

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