Investigator Brochure protocol Part II

TOXICITY, GROWTH, AND ANTI-INFLAMMATORY ACTION UPON CELL CULTURES BY THE TREATED YEAST, MILMED

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INTRODUCTION

The preparation of beer over the centuries has led to the selection of yeasts particularly suited to the fermentation processes necessary to the production of the beverage. Two types of brewing yeasts were originally classified based on flocculation behavior: top fermenting (ale and weiss yeast) (Jentsch, 2007) and bottom fermenting (lager yeast).
The nutritive effects of beer consumption are known: from the pleasure derived from the particularly satisfying taste, to the effects on the cardiovascular system and perhaps also upon the nervous system. Most of the studies in the literature have taken into consideration the beverage, neglecting, in the past, the study of these nutritive effects of beer that may be associated directly with the yeast species used in brewing.
Great interest has been increasingly focused, on the relationship between diet and individual resilience to environmental stimuli, promoting or otherwise inhibiting or slowing the development of diseases, especially the chronic-degenerative ones. The characterization of these mechanisms may allow the development of nutritional protocols, able not only to maintain homeostasis but also to treat chronic degenerative diseases for which, at present, there are no specific, restorative pharmacological interventions. It is also possible to hypothesize the implementation of a diet therapy able to perform synergistic effects with pharmacotherapy.
Neurological diseases, neurodegenerative and cerebrovascular diseases in particular, represent one of the most relevant sectors for public health in Europe due to their great epidemiological and socio-economic impact. With the global increase in lifespan expectancy, the number of individuals presenting chronic-degenerative diseases such as dysmetabolic diseases, atherosclerosis, Parkinson's

disease and Alzheimer's disease continues to grow, (Fotuhi et al., 2009). Concurrently, ageing is associated with immunosenescence and with a chronic low-grade inflammation (inflammaging) that seems to be involved in the etiopathogenesis of several disease profiles. (Maiese, 2018). Several studies aim at setting up nutritional protocols to: i) maintain the homeostasis and performance in healthy individuals, and ii) treat chronic-degenerative diseases.
Research conducted over the past decade has allowed us to identify and analyze some potentially neurotoxic factors, including cytokines, chemokines, biomarkers of oxidative stress. The cytokines present in the brain seem to be involved not only in the induction and amplification of inflammatory processes, but also in the regulation of neurotransmitters such as serotonin and noradrenaline involved in modulating mood. The role of cytokines in the encephalic compartment extends to altering neuroendocrine functions leading to an imbalance in glucocorticoid secretion and resistance, in the release of growth factors such as BDNF which, in turn, influence neurogenesis and synaptic plasticity. Cytokines play an important role in neurological development in neurogenic processes in adult life as they are involved in the signaling mechanisms responsible for the survival, proliferation, differentiation of neural cells and the processes that regulate migration and synaptogenesis. Most of the prevailing studies have been focused upon anti-ageing activity of several nutraceuticals (Cosin-Tomas et al., 2019; Kotha and Luthria, 2019; Lin et al., 2019).

A recent study performed on weaned piglets analyzed the effects of dietary supplementation with Saccharomyces cerevisiae cell wall extract (Liu et al., 2017). The results showed that Saccharomyces cerevisiae cell wall extract supplementation of up to 0.15% produced an improved growth of the animals, increased the serum concentration of some essential and nonessential amino acids, as well as increasing the activities of anti-oxidant enzymes. Moreover, mannan oligosaccharides, polysaccharides that present a linear polymer of mannose sugar, derived from the outer cell wall of the yeast Saccharomyces cerevisiae have shown potential for the reduction of inflammation (Wang et al., 2016; Baurhoo et al., 2012; Hoving et al., 2018).

AIM OF THE STUDY

For all of these effects and for the fact that recently, food-derived bioactive compounds have been shown as a regulator against various chronic diseases due to their low toxicity, as opposed to drugs that induce severe side effects, we carried out several experiments to analyze the effects of Saccharomyces cerevisiae (MILMED), grown in different conditions, on CNS cells, that is microglial or neuroblastoma cell lines.
Microglia belong to the monocyte-macrophage lineage and are responsible for, among several other structural-functional activities, immune surveillance within the brain. Microglial cells are characterized by the extreme plasticity of their polarization: they respond traumatic injury as well as to infections (essentially inflammation) by secreting several cytokines, including IL-1beta, IL-18, IL-6, TNF-alpha; their continuous release leads to the amplification and the chronicity of inflammatory phenomena constituting the basis of chronic-degenerative diseases. Microglial cells are characterized by the extreme plasticity of their polarization: they respond to harmful stimuli by acquiring a pro-inflammatory phenotype whereas they acquire an anti-inflammatory phenotype when they eliminate pathogens and cellular debris by phagocytosis and collaborate to wound healing and restore homeostasis. The cells then assume a specific phenotype according to the

environmental stimuli they receive (Cherry et al., 2014). It is therefore extremely interesting to identify compounds that are able to favor microglia polarization towards M2 phenotype. It is possible to discriminate between the M1 and M2 phenotypes following the expression of the specific markers such as Arginase-1 for M2or alternatively activated macrophage-microglia and iNOS for M1 or classically activated macrophage-microglia. Arginase converts arginine to proline and ornithine and contribute to wound repair; moreover it compete with iNOS since they share the same target, that is arginine, in this way arginase inhibit the synthesis of nitric oxide.
In this scenario we aimed at ascertaining the effect of yeast addition to cell culture looking at their viability, at their cytoskeleton remodeling at their expression of M1 as well as M2 markers
Our first task was to ascertain the eventual toxicity of yeast cells added to the culture; our second task was to evaluate the eventual cytoskeleton modifications, which underly migration processes as well as functional and phenotypic changes.

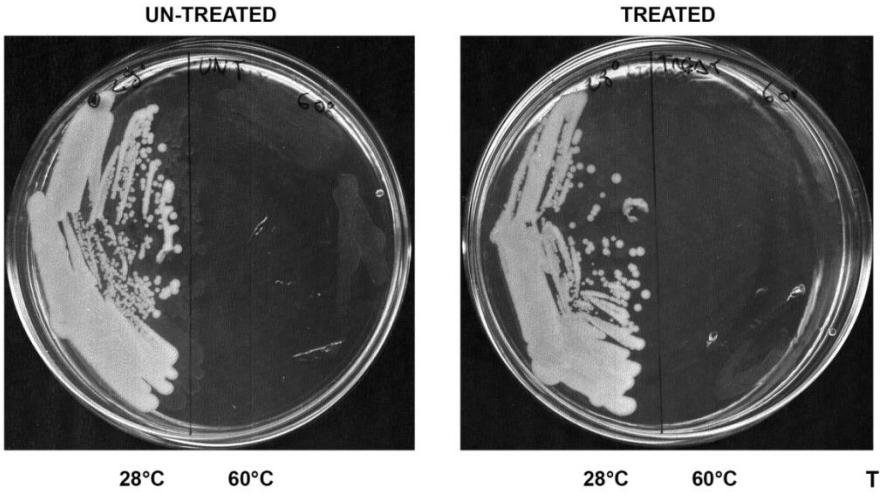
Experimental protocols

The cytotoxic effect was determined by Trypan Blue exclusion and MTS assay after exposure to a series of Milmed concentrations for 24, 48, 72 hrs.

Yeast strains and growth
In the first experiments *S. cerevisiae* Un-treated, Treated and Frozen strains from Milmed AB were grown with shaking at 28 ° C in complete medium YPD (1% yeast extract, 2% bactopeptone, 2% dextrose; DIFCO): inoculated into 10 ml YPD, incubated overnight.
Culture samples were read in a spectrophotometer to determine the number of cells / ml (1 OD = 13.33 x 10^6 cells/ml).
This procedure was repeated for each of the 3 samples received. Each sample was divided into 2 aliquots (3 ml each). One aliquot was not treated further and thus was constituted by live yeast, the second one was inactivated at 60 ° C.

- Untreated sample: Yeast alive were centrifuged 5' at 3200 rpm to collect cells; the supernatant was recovered and filtered (0.2 micron pores)
- Inactivated yeast: were treated at 60°C for 15' and centrifuged 5' at 3200 rpm to collect cells; the supernatant was recovered and filtered (0.2 micron pores)

All the samples were checked with overnight incubation at 28 ° C on YPD solid medium to confirm the proper growth / inactivation. Similarly the supernatant filtrate of each sample was checked too.
S. cerevisiae Un-treated and Treated strains from Milmed AB were grown under shaking overnight at 28°C in YP (yeast extract 1%, peptone 2%) glucose 2% or glycerol 3%, SD (yeast nitrogen base w/o amino acids 0,67%) glucose or glycerol 3% media. Cells were harvested at exponential phase and the number of cells quantified by spectrophotometry at 600nm. For each experiment, 10^7 cells were collected and washed in PBS. To verify temperature inactivation, yeast cells were plated on YP plus agar 2% for 24 h. in the last experiments *S. cerevisiae* Un-treated, Treated and Dried strains from Milmed AB (www.milmed.de) were grown with shaking at 28 ° C in complete medium YPD (1% yeast extract, 2% bactopeptone, 2% dextrose; DIFCO): inoculated into 10 ml YPD, incubated overnight.



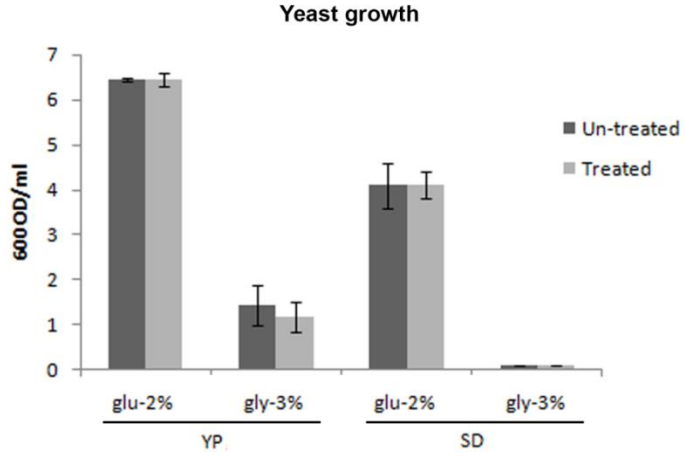


Figure 1. S. Cerevisiae, untreated and treated yeast strains from Milmed Unico AB grown under shaking at 28° or 60° C in YP (see above) glucose (2%) or glycerol (3%), SD (see above) glucose or glycerol media.

Cell cultures

BV-2 murine microglia cells, were cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), 2mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).
Human U-937, macrophage cell line, was cultured in RPMI-1640 medium containing 10% fetal bovine serum penicillin (100 U/ml), and streptomycin (100 μg/ml).
Human SH-SY5Y, neuroblastoma cell line was cultured in DMEM F-12 medium10% Fetal bovine serum (FBS), 2mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml).
Human IMR 32 and SK-N-SH, neuroblastoma cell lines, were cultured in Minimum Essential Medium (MEM) With Earles 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).
For all experiments the cells were washed twice with phosphate –buffered saline (PBS).
All the cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °and culture medium was replaced every 2–3 days.

Cell Treatments

The toxicity of various types of Milmed on different neuroblastoma cell lines was evaluated

- **SK-N-SH**: This cell line was established in cell culture from human metastatic neuroblastoma tissue and has been used as a target cell line in cell-mediated cytotoxicity assays (Biedler et al., 1973, 1978). SK-N-SH cells exhibit a neuronal phenotype and have multiple neurochemical markers. They have been characterized by high dopamine hydroxylase activity, as well as a low activity level of glutamic acid decarboxylase, the enzyme responsible for the conversion of glutamate to GABA.
- **IMR-32**: This cell line was established from an abdominal mass occurring in a 13-mo-old Caucasian male. The human neuroblastoma cell line IMR-32 exhibits both cholinergic and adrenergic properties. The IMR-32 human neuroblastoma cell line expresses both cholinergic and adrenergic neurotransmitter properties (Tumilowicz et al., 1970; Schlesinger et al., 1976; West et al., 1977).
- **SH-SY-5Y:** is a thrice cloned (SK-N-SH -> SH-SY -> SH-SY5 -> SH-SY5Y) subline of the neuroblastoma cell line SK-N-SH (see ATCC HTB-11) which was established in 1970 from a metastatic bone tumor. SH-SY5Y cells have a reported saturation density greater than 1 X 10^6 cells/cm^2. They are reported to exhibit moderate levels of dopamine beta hydroxylase activity. Cells with neuroblast-like morphology are positive for tyrosine hydroxylase (TH) and dopamine-β-hydroxylase characteristic of catecholaminergic neurons, whereas the epithelial-like counterpart cells lacked these enzymatic activities.
- **U937**: This cell line was isolated from the histiocytic lymphoma of a 37-year-old male patient and are used to study the behavior and differentiation of monocytes. U937 cells

mature and differentiate in response to a number of soluble stimuli, adopting the morphology and characteristics of mature macrophages. U937 cells are of the myeloid lineage and so secrete a large number of cytokines and chemokines either constitutively (e.g. IL-1 and GM-CSF) or in response to soluble stimuli. TNFα and recombinant GM-CSF independently promote IL-10 production in U937 cells.

- **BV2:** The BV2 cell line was generated by transduction of neonatal primary microglia with the v-raf/v-myc carrying J2 retrovirus. (Blasi et al., 1990).BV2 cells were involved in the inflammatory response in the CNS, cells belonging to the monocyte-macrophage line and responsible for innate immunity in the CNS. Physiologically present in the quiescent state (resting) it can be activated by different signals such as brain traumas or environmental stimuli. In physiological conditions the "resting" microglia shows a highly branched morphology and guarantees the maintenance of brain homeostasis. In neuropathological conditions, on the other hand, it undergoes rapid activation taking an amoeboid form (Kettenmann et al., 2013). Activated microglia can assume two different functional phenotypes: the classically activated, pro-inflammatory type M1 phenotype, and the alternatively activated, anti-inflammatory type M2 phenotype, associated, respectively, with neurotoxic functions, with production of pro-inflammatory cytokines, and to neuroprotective functions, with production of anti-inflammatory cytokines (Peña-Altamira et al., 2017). When microglia is activated, it morphologically transforms and is responsible for regulating brain function and cell survival. However, microglia can become over-activated with overproduction of compounds that at high concentrations can become toxic and cause extensive damage, inducing neurological and neuronal dysfunctions.

In the first experiments BV-2 murine microglia cells described above ($5x10^3$ cells/well in 96-wells microplate, $3x10^4$ in 48-wells microplate) were stimulated with *S. cerevisiae* Un-treated, Treated and Frozen strains from Milmed AB for 24,48,72 hours with different concentrations of Milmed AB grown at 28°C or Heat inactivated (grown at 60°C) or Filtered supernatants of Milmed culture. The yeast concentrations used were 10^7, 10^5, 10^3, $5x10^2$,10^2, $0.5x10^2$.
In the other experiments BV-2 murine microglia cells, U-937 monocytic human cell, SK-N-SH neuroblastoma cells, SH-SY-5Y neuroblastoma cells and IMR 32 neuroblastoma cells were seeded in 48 well plates (cell density of $3x10^4$ cells/well) and incubated at 37°C with different concentrations of treated and untreated yeast grown in different media (Yp G2%, YP Gly 3%, SD G2%) 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, $0.5x10^2$.
In the last experiments BV-2 murine microglia cells were seeded in 48 well plates (cell density of $1x10^6$ cells/well) and incubated at 37°C with $5x10^2$ treated, untreated and dried yeast grown in Yp G2% media for 72, 96, 120 hrs and compared to control cells.

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. 10 µl cells suspension were mixed with 10 µl trypan blue solution. After 3 min, cells were observed under a light microscope using Burker's chamber: blue stained cells were considered nonviable.

Immunofluorescence microscopy

BV2 cells grown in chamber-slides, were incubated with Milmed grown in YP 2% Glucose, or YP 3% Glycogen, or SD 2% glucose or SD 3% glycogen, in the presence or in the absence of LPS (10 ng /ml)(Sigma Aldrich, Saint Louis, MO, USA) 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 rhodamine-conjugated phalloidin (TRITC-phalloidin), 1:50 for 45 min at 25°C, or, alternatively with the following primary antibodies: anti-arginase-1 and anti-iNOS were from Cell Signalling (Cell Signaling, Danvers, Massachusetts, United States). The primary antibodies were visualized, after appropriate washing in PBS, using FITC-conjugated goat anti-rabbit Alexa Flour 488 secondary antibody (Invitrogen, Carlsbad, CA, USA). Actin cytoskeleton was visualized using Phalloidin-Tetramethylrhodamine B isothiocyanate (Phalloidin-TRITC), 4' ,6-diamidine-20-phenylindole dihydrochloride (DAPI) were obtained from Sigma-Aldrich (Saint Louis, MO, USA) (1:50) for 45 min at 25°C. 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.).

ELISA

BV-2 murine microglia cells were seeded in 6-well plates (cell density of $1x10^6$ cells/ml) and incubated with different treatment of treated, untreated, frozen and dried yeast grown in Yp G2 for 24hrs with and without LPS(20ug/ml, 100ng/ml, 50ng/ml, 20ng/ml and 10ng/ml)(Sigma Aldrich, Saint Louis, MO, USA), and compared to control cells. The yeast concentrations used was $5x10^2$. The cell-free supernatant was collected after 24h stimulation with LPS, and IL-1beta, IL-6, IL-18 and TNF-alpha were measured by ELISA kits (Immunological Sciences, Roma, RM,Italy) according to the manufacturer's instructions. The absorbance at 450nm was determined using a microplate reader. (DATA NOT SHOWN: still repeating the assay)

Statistical analysis

All data are expressed as a mean ± standard deviation (SD). One-way analysis of variance (ANOVAs) was performed using GraphPad Prism version 6 (GraphPad Software Inc., San Diego, CA, USA) to assess between-group differences. P value <0.05 was considered statistically significant.

MTS cell proliferation assay

The cell viability will be evaluated by using Promega's Cell Titer Aqueous assay with MTS tetrazolium, in which viable cells convert MTS tetrazolium into a formazan-coloured product ($OD_{490\ nm}$) 5,000 cells/well were seeded in a 96-well plate and incubated for 24, 48, 72 h in the presence or in the absence of different Milmed concentrations, as specified for each experiment. The cell growth was then measured at each time, in order to determine a dose-response curve. Following the manufacturer's instructions, 20 µl of MTS solution were added to 200 µl of culture media and incubated for 2 h at 37 °C and the optical density measured at 492 nm with a multi-well scanning Elisa reader (Tecan, Switzerland). Five independent experiments were performed in triplicate and values calculated by Magellan™ software.

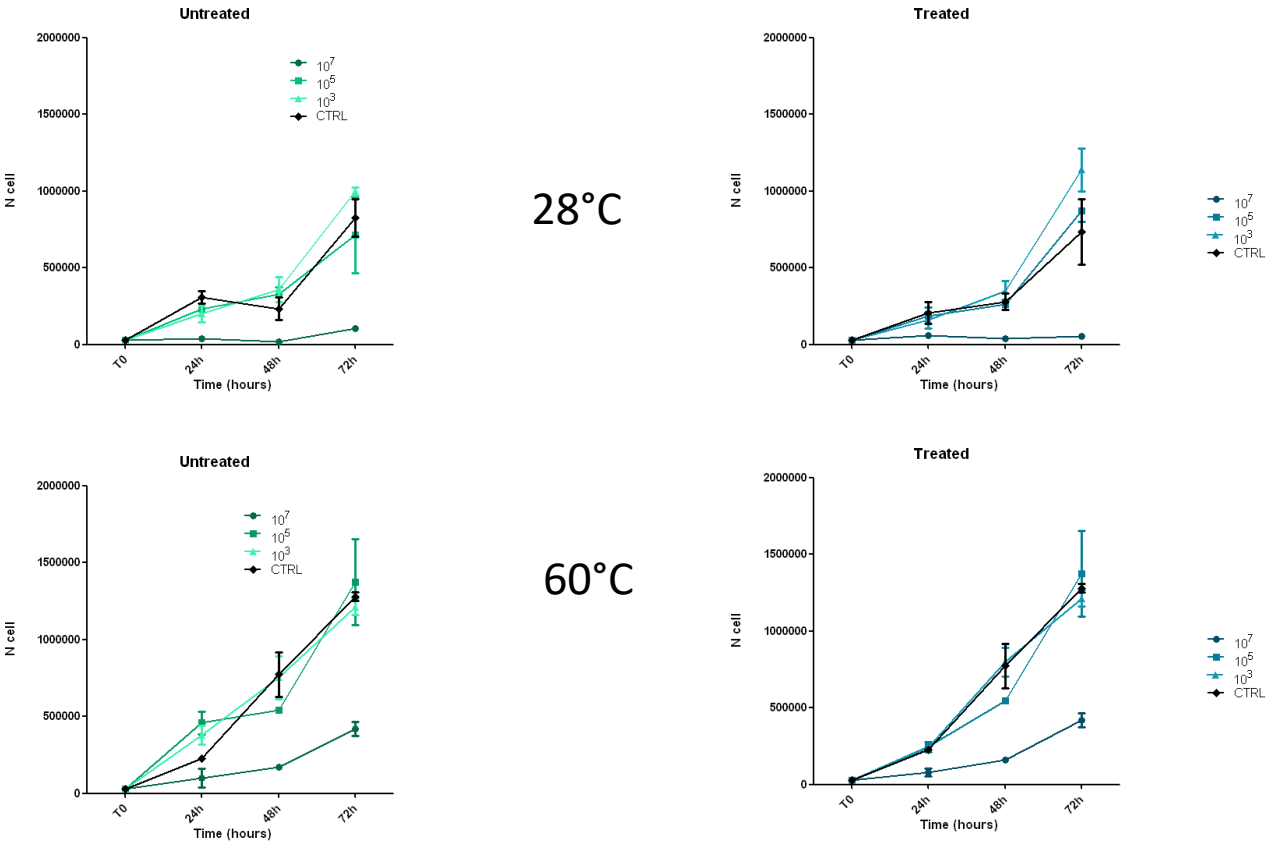
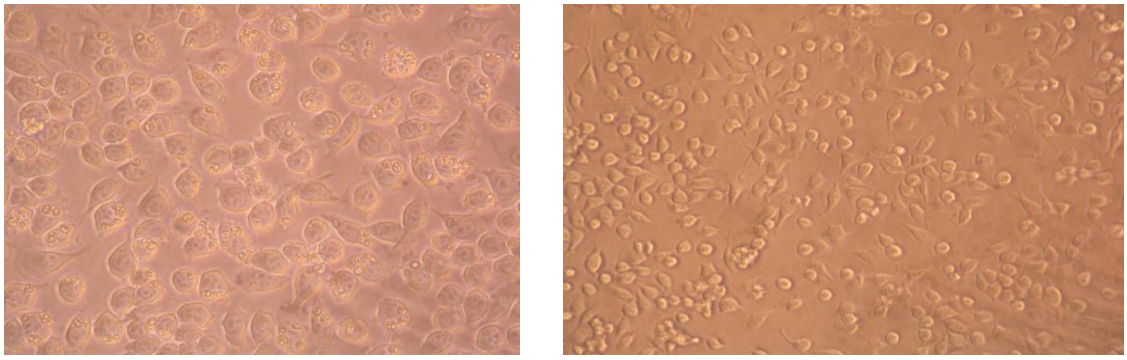


Figure 2. Murine BV2 incubated with treated and untreated Milmed grown at 28°C and 60°C



24h 48h

Figure 3. Yeast was phagocytized by BV2 cells (24 h culture) and disappeared leaving cells very complete (48h culture)

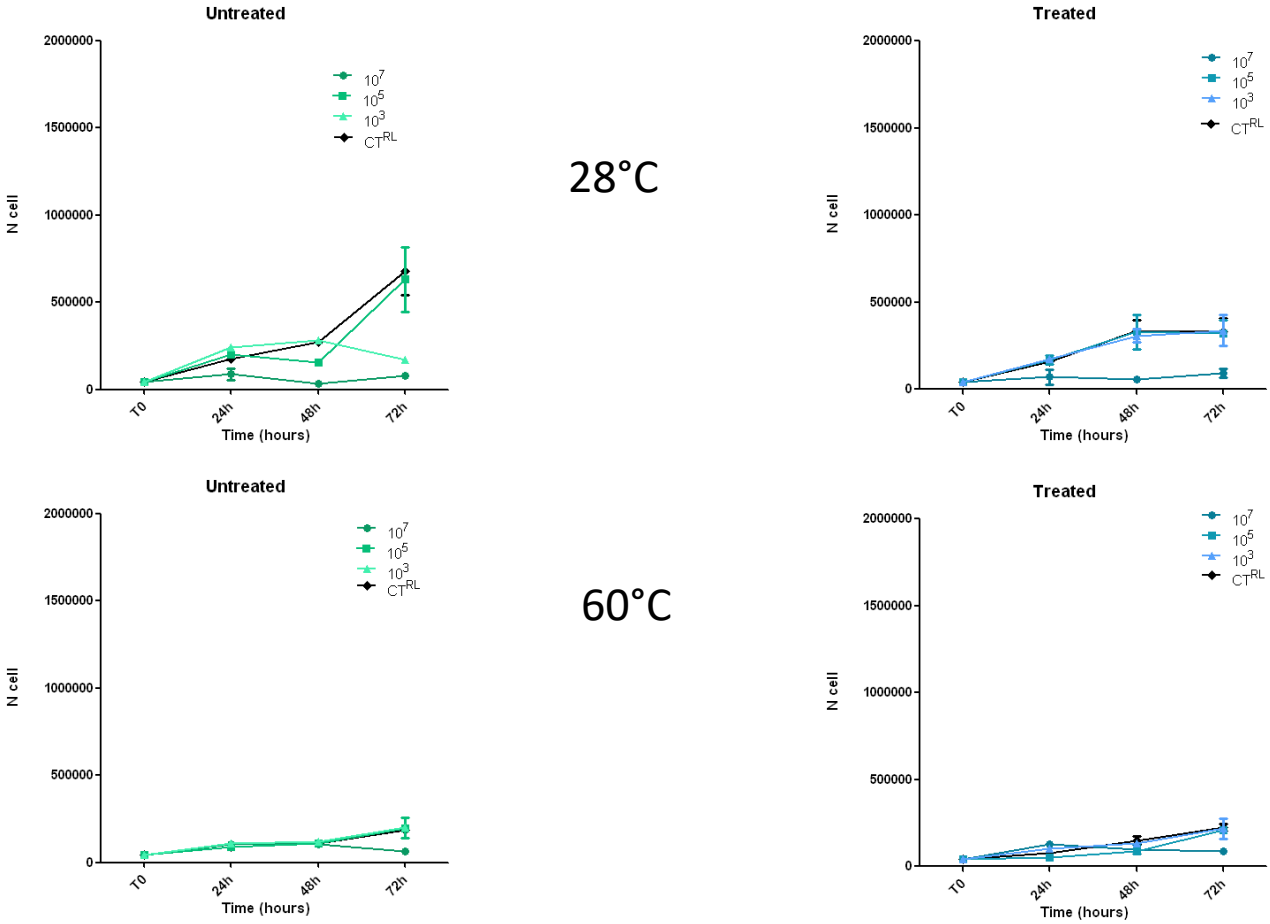


Figure 4. U937 cells incubated with untreated and treated yeast grown at 28°C and 60°C

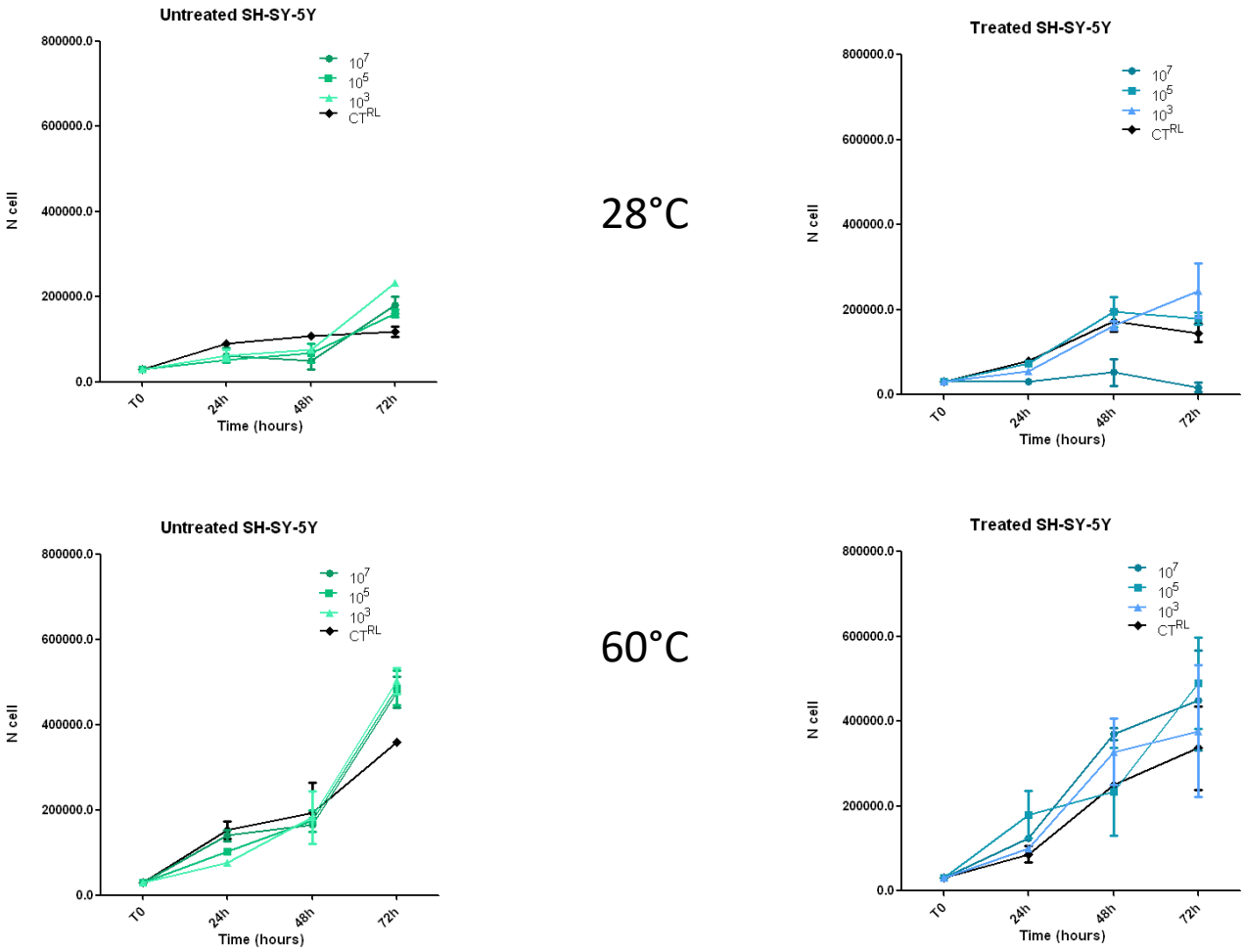


Figure 5. SH-SY-5Y human neuroblastoma cells incubated with untreated and treated yeast grown at 28°C and 60°C

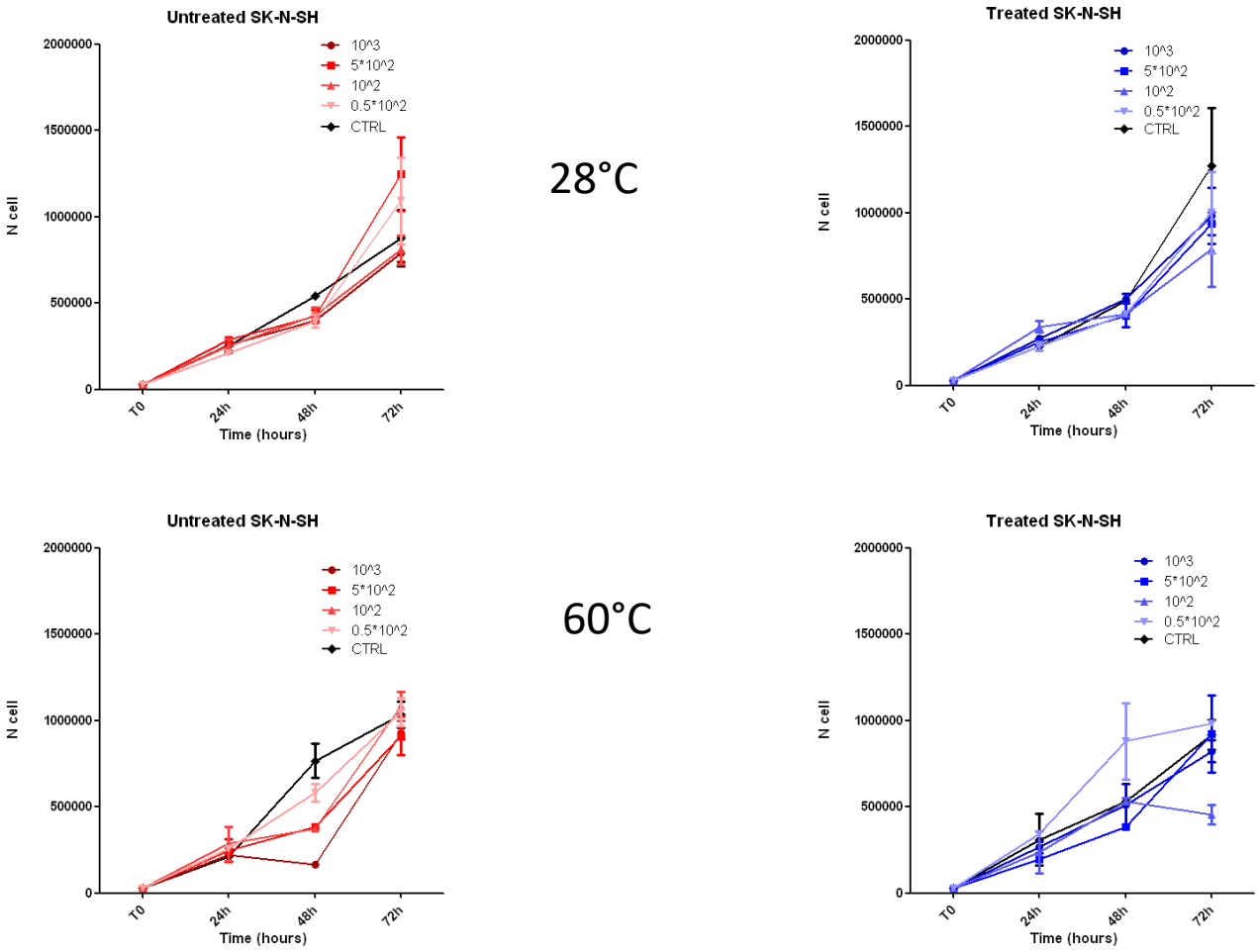


Figure 6. SK-N-SH human neuroblastoma cells incubated with untreated and treated yeast grown at 28°C and 60°C

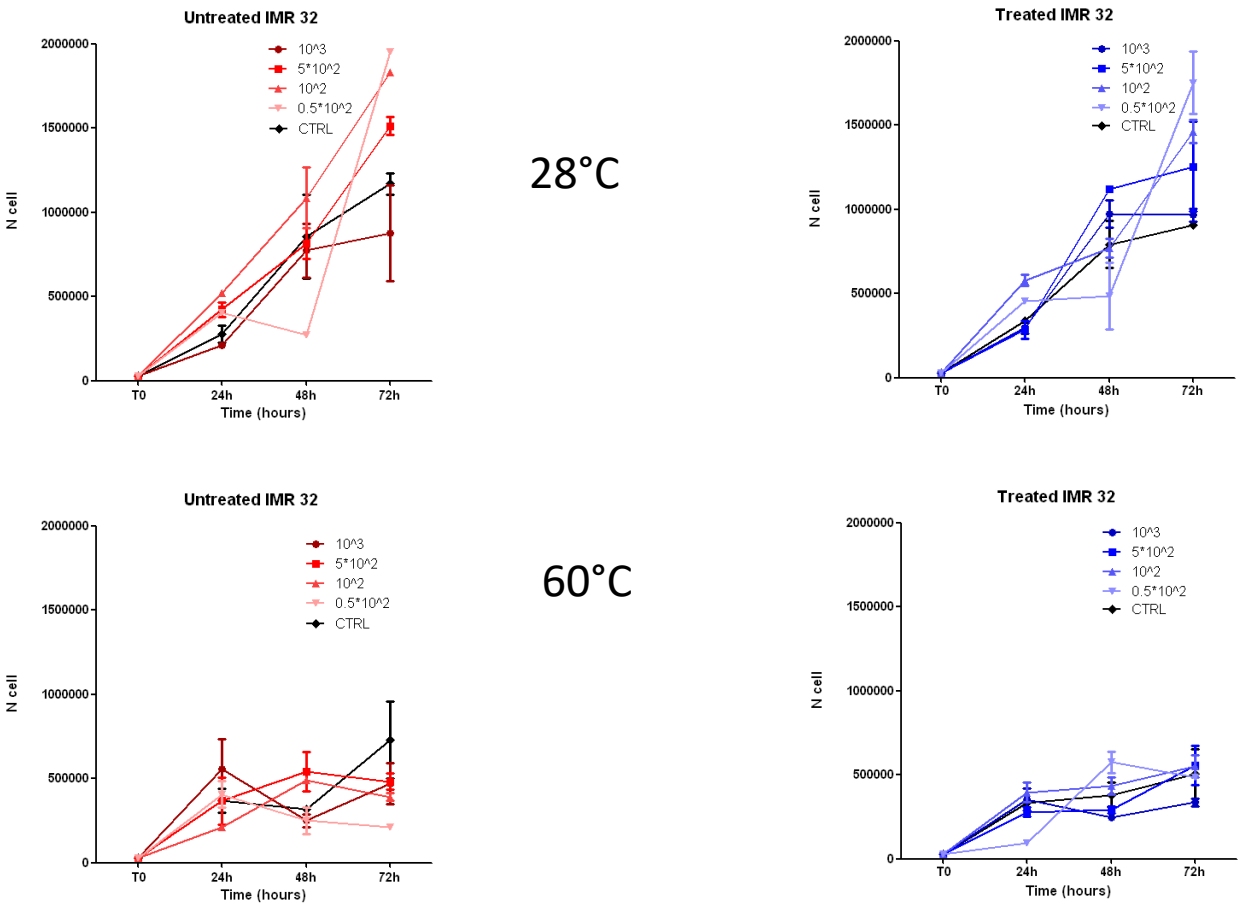
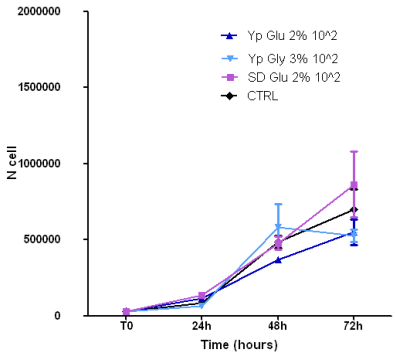
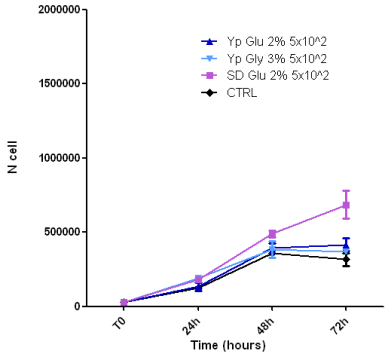


Figure 7. IMR 32 human neuroblastoma cells incubated with untreated and treated yeast grown at 28°C and 60°C

Figure 8. BV2 and SK-N-SK cells incubated with untreated yeast grown in different media at 28°C



BV2 murine microglia cells incubated with untreated Milmed grown at 28°C



SK-N-SH human neuroblastoma cells incubated with untreated Milmed grown at 28°C

RESULTS

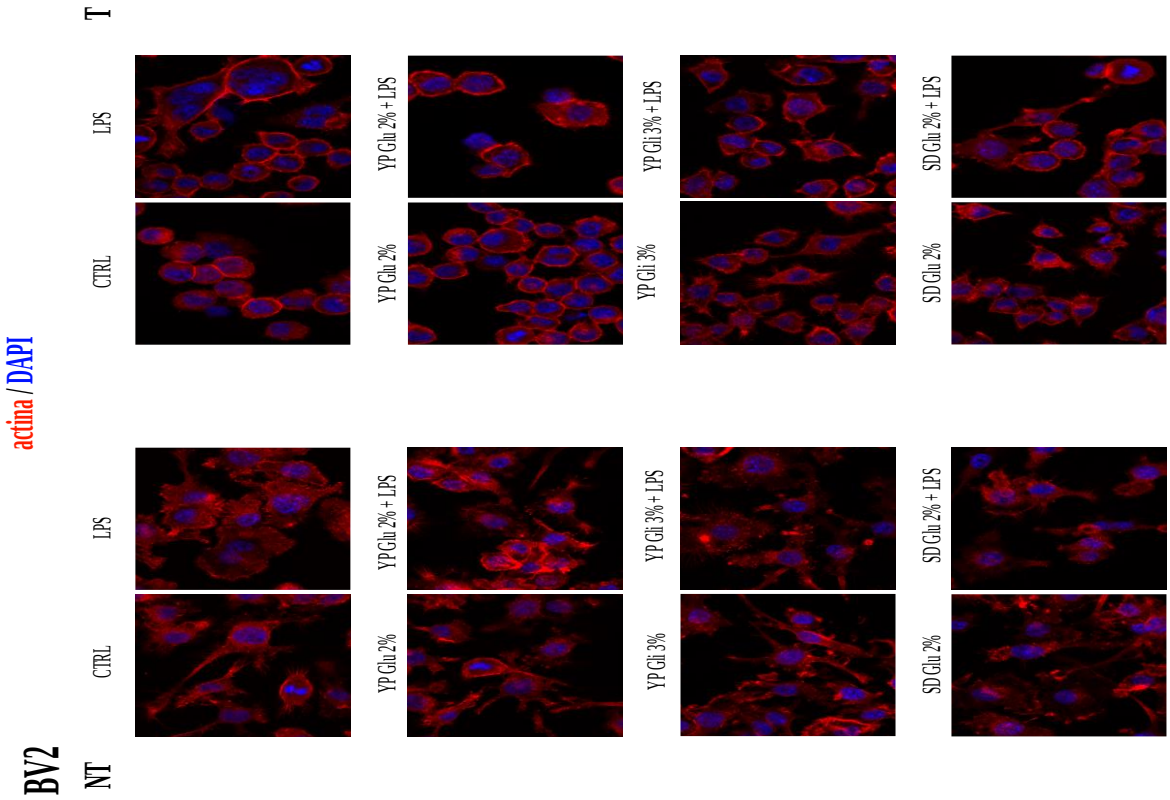
Yeast preparation and growth capability

To study the ability of yeast to modify cell lines behavior we started testing administration of yeasts alive or not. 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. Fig 1 shows that temperature treatment was able to kill yeasts compared to untreated cells.

In Fig. 9, 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 analyze a possible effect of differential metabolic yields on cell lines growth. Yeast growth capability was tested 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 both YP and SD with glucose 2%, which allows only fermentation, or glycerol 3%, a respiratory carbon source, to analyze yeast catabolic properties. We grew cells at 28°C and 37°C and

we found that at 37°C yeast cells were not able to grow, indicating that human body temperature do not allow yeast replication. In YP gly-3% the growth was slower compared to YP glu-2%, as expected for brewer's yeast. In SD glu-2% yeast growth was slow but efficient, while in SD gly-3% yeast was not able to growth. Yeast grown in SD medium was able to increase human neuroblastoma cell growth.

Figure 9. The effects of yeast added at different concentrations and grown in different conditions areshown.



NT= UNTREATED YEAST **T= TREATED YEAST**

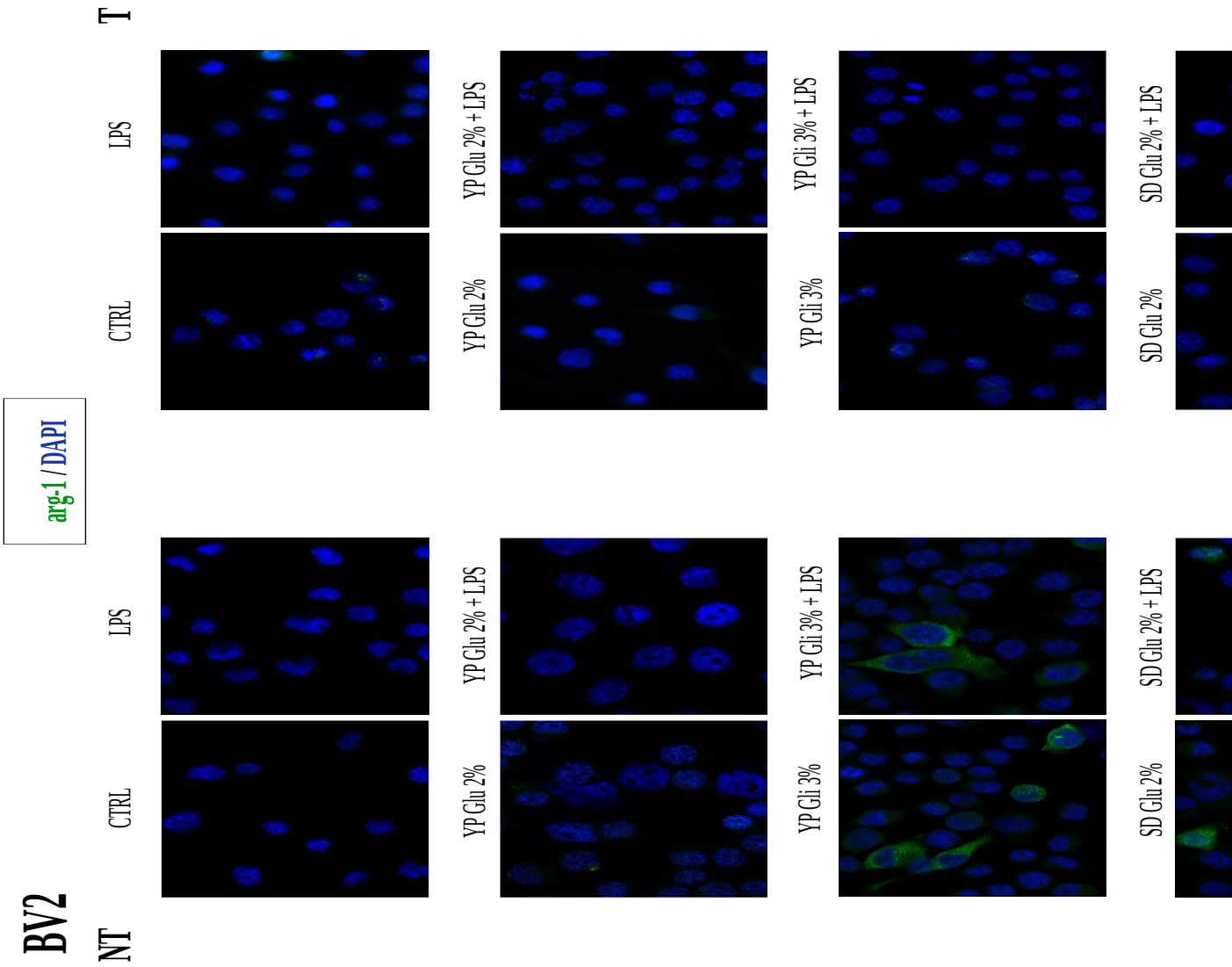


Figure10. BV2 cells incubated with untreated Milmed (NT) or treated Milmed (T) grown in different metabolic media: Expression of arginase-1 (M2 or anti-inflammatory macrophages marker) was analyzed by immunofluorescence . Nuclei were counterstained with DAPI (blue colour).

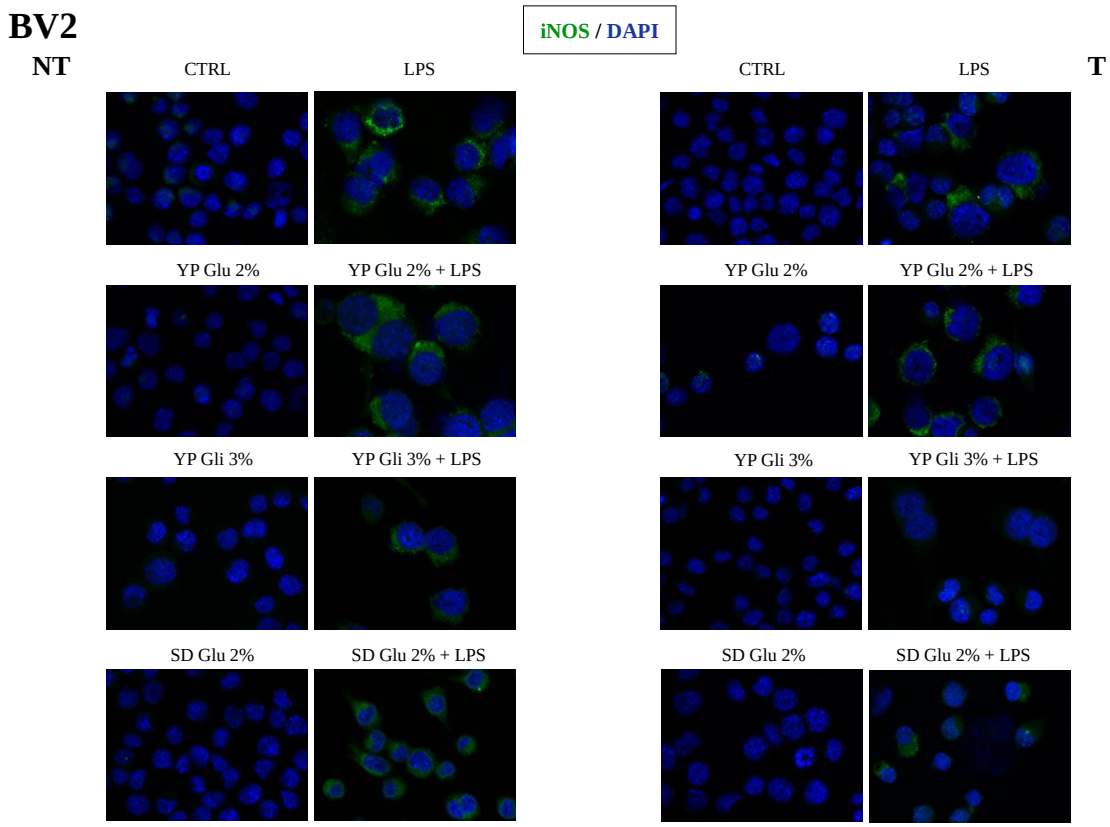


Figure 11. BV2 cells were incubated with untreated Milmed (NT) or treated Milmed (T) grown in different media in order to see if yeast metabolic products may have different effects on microglia cells. iNOS expression (marker of M1 or pro-inflammatory macrophages) was analyzed by immunofluorescence. Nuclei were stained with DAPI.

RESULTS

Yeast preparation and growth capability

To study the ability of yeast to modify cell lines behavior we started testing administration of yeasts alive or not. 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. Fig 1 shows that temperature treatment was able to kill yeasts compared to untreated cells.

In order to ascertain yeast-driven functional effects on microglial cells we performed some immunofluorescence experiments aimed at evaluating any effects on cytoskeleton, since the cytoskeletal rearrangement corresponds to phenotypic and functional modifications associated with migration and other functional processes. In Fig. 9 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 analyze a possible effect of differential metabolic yields on cell lines growth. Yeast growth capability was tested 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 both YP and SD with glucose 2%, which allows only fermentation, or glycerol 3%, a respiratory carbon source, to analyze yeast catabolic properties. We grew cells at 28°C and 37°C and we found that at 37°C yeast cells were not able to grow, indicating that human body temperature do not allow yeast replication. In YP gly-3% the growth was slower compared to YP glu-2%, as expected for brewer's yeast. In SD glu-2% yeast growth was slow but efficient, while in SD gly-3% yeast was not able to growth. Yeast grown in SD medium was able to increase human neuroblastoma cell growth.

In the case of untreated Milmed (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 amoeboid 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. Fig.10 shows the expression of arginase-1 a biomarker of anti-inflammatory macrophage phenotype. Samples incubated with the untreated yeast and those incubated with the treated yeast, under different growth conditions, do not show the arginase labeling. The only differences are appreciated in the samples incubated with untreated yeast grown in YP Gly 3% or SD Glu 2%, as also in the corresponding ones in the presence of LPS (left panel), where some cells are labelled with anti-arginase. In these cells the fluorescence signal appears to be dotted and localized in the cytoplasm, indicating an anti-inflammatory response of the cells in these conditions.

Fig. 11 shows the expression of iNOS, a marker of M1 polarized cells. BV-2 incubated with yeast treated or not under different growth conditions, do not show iNOS labeling. Samples treated in the same way in the presence of LPS, display iNOS, with the exception of the cells incubated with the treated yeast grown in YP 3% GLY with LPS. These observations indicate that in the microglia cells LPS induces an inflammatory response in basically all the conditions examined.

We carried out some experiments in order to obtain a dose-response curve to ascertain if Saccharomyces Cerevisiae yeast may produce any toxicity to the cultures, depending on its concentration. As shown in Fig. 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 Fig. 3 cells took up the yeast and after 48 hrs the yeast was completely phagocytized and cells appeared very happy. The same treatment was repeated with yeast grown at 60°C and with Treated yeast

Overlapping results were obtained repeating these treatments with the human monocytic cell line U937 (Fig. 4). We then added the same concentrations of yeast to human neuroblastoma cell line SH-SY-5Y (Fig. 5). The results obtained seemed to indicate a proliferative effect on these cells with concentrations equal to or less than 10^5. Because of this we repeated the treatment using lower yeast concentrations. (Fig. 6). Yeast added to different human neuroblastoma cell lines produced no toxicity, stimulating on the contrary cell proliferation (Fig.7 and Fig. 8).

Dry Milmed yeast does not show significant differences compared to the Milmed yeast for 24, in fact the counts after 24h show no cytotoxic effects even if the number of cells turns out to be slightly lower than that of the untreated cells (ctrl). All this is confirmed by microscopic photos, but at later times (72h, 96h and 120h) the dry Milmed yeast shows an exponential growth that negatively affects cell viability, that means that yeast is growing so fast that it withdraws all nutrients for its own growth, probably subtracting all nutrients to cells.

Discussion

Our results suggest that Milmed yeast does not produce cytotoxic effects neither on microglia cells nor on cells of neuronal origin. Microglia phagocytize yeast in the first 24 hrs being not apparently affected by this process, even in view of the fact that, as shown by our results, yeasts at 37°C, while retaining their vitality, are not able to replicate. Much interest has to be focused on the results obtained with Milmed yeast grown in different metabolic medium, added to human neuroblastoma cell lines: yeast has a trophic activity with best results at a concentration of 10^3 yeast cells/well and striking results are obtained with yeast grown in nutrient deprivation conditions. In such conditions (Van Dijck et al., 1995) yeast accumulates trehalose, a disaccharide acting as a molecular chaperone, involved in refolding of partially denatured proteins (Singer and Lindquist, 1998). Trehalose seems to play a key role in neuroprotection mechanisms, perhaps through the regulation of autophagy as suggested in a recent review (Lee et al., 2018). Previous studies have shown that dietary polysaccharides derived from yeast may improve cognition and well-being in middle-aged adults (Best et al., 2010); extend lifespan in Caenorhabditis elegance (Seo et al., 2018); partially recover mouse models with neurologic defects (Nelson et al., 2013). In a MPTP mouse model of Parkinson's disease, trehalose counteracted dopamine level decrease in the corpus striatum (Sarkar et al., 2014). The bulk of studies on neurodegenerative diseases animal models showed that after trehalose supplies animals lived longer with reduced neuropathology (Lee et al., 2018).
Moreover, trehalose was shown to down-regulate the production of pro-inflammatory cytokines in LPS-stimulated microglial cells (Bussi et al., 2017).

Our results show that 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 phyllopodia, and decrease the expression of iNOS; at the same time the addition of Milmed seems to stimulate the expression of arginase-1 which, competing for the iNOS substrate, would decrease its activity, polarizing microglia to the non-classical activated M2 (anti-inflammatory) phenotype. Upon activation, microglia also undergoes dramatic morphologic changes, from resting ramified shape into activated amoeboid morphology (Block et al., 2007). We showed the ability of Milmed yeast to remodels LPS-stimulated microglia to acquire the branched spindle-like morphology characteristic of quiescent unstimulated cells.

Much interest is focused on the relations between diet and epigenetic modifications that may have relevant impact on resilience against environmental stimuli, promoting or on the contrary inhibiting the development of diseases, especially the chronic-degenerative ones.

It is now known that the genome accessibility and stability depends on epigenetic mechanisms that do not alter the DNA sequence, but dynamically modulate gene transcription.

All epigenetic mechanisms, as the activity of enzymes and proteins that modify the chromatin structure, are influenced by external signals. Thus, lifestyle, including eating habits and exercise, can influence genome transcription and stability and can be inherited from one generation to the next.

Among the most important molecular mechanisms of epigenetic regulation there is post-translational modification of histones tails which produce several effects on gene expression (for example acetylation, methylation, phosphorylation, ubiquitynation).Several studies dealing with caloric restriction and lifespan lead to the identification in the yeast Saccharomyces Cerevisiae of a protein with deacetylase activity NAD + dependent able to deacetylate histone proteins and different transcription factors; it was called SIR2 (Silent Information Regulator 2) as it is able to silence some genes located near telomeres and behaves as a pro-longevity factor. It is considered the progenitor of the sirtuin family, histone-deacetylases that are involved in the regulation of the replicative lifespan: in fact, its deletion involves a decrease of the replicative lifespan. Moreover, it has been implicated in the mechanisms underlying the lengthening of the lifespan following caloric restriction, not only in yeast, but also in higher eukaryotes (Wierman and Smith, 2014: Chen et al., 2015). As a matter of fact sirtuins have been claimed as putative therapeutic targets for many human diseases, including metabolic and age-dependent neurological disorders (Maxwell et al, 2011).

Immunofluorescence experiments show that Milmed added to microglia cells induce morphological changes linked to actin cytoskeleton remodeling. It is known that actin cytoskeleton dynamics shape microglia effector functions (Uhlemann et al., 2016) since it has a fundamental role in various cellular processes such as migration, morphogenesis, cytokinesis, endocytosis and phagocytosis (Tojkander et al., 2012). Furthermore, actin polymerization, depolymerization and branching drive dendritic spine morphogenesis exert effects upon neuronal cells maintaining long-term memory (Basu and Lamprecht, 2018).

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