**Title:**

Immunostimulatory activity of *Pichia kluyveri*, *Hanseniaspora uvarum*, *Candida intermedia*, *Saccharomyces boulardii* and their derivatives on RAW 264.7 macrophages

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**Abstract**

Yeasts are a group of microorganisms with structural and metabolic characteristics that influence their recognition by immune cells resulting in a species-specific response. Although *Saccharomyces boulardii* is a widely studied probiotic yeast, immunostimulation by non-*Saccharomyces* yeasts still underexplored. Therefore, the aim of this study was to characterize the response induced in macrophages stimulated by yeasts *Pichia kluyveri*, *Hanseniaspora uvarum*, *Candida intermedia* and their derivatives: heat-killed cells, supernatant and DNA. RAW 264.7 murine macrophages were stimulated *in vitro* for 24 h and the response generated was evaluated by analyzing mRNA transcription of cytokines (*IL2*, *IL4*, *IL10*, *IL13*, *IL23*, *TNF-α*), transcription factors (*Bcl6, NFκβ, STAT3*), Toll-like receptor 2 (TLR2) and YM1 protein. Viable and heat-killed cells of *P. kluyveri* and *H. uvarum* were responsible for high levels of relative mRNA transcription of transcription factors and *TLR2* (between 2 – 8-fold increase), however were able to induce only low transcription levels for analyzed cytokines (≤ 2-fold increase). Viable cells of *C. intermedia*

were able to stimulate a significant transcription of *IL4* (7.6-fold increase) and *Bcl6* (4-fold increase), while heat-killed cells stimulated the highest level of *TNF-α* (2.4-fold increase) among yeasts and their derivatives. Furthermore, supernatant from *C. intermedia* culture induced significant ($p < 0.05$) levels of *TLR2* (4.4-fold increase), being the only one among supernatants to present high levels of relative mRNA transcription of *TLR2*. Data found in this work arouse interest in further studies on interaction between non-*Saccharomyces* yeasts and immune system cells, mainly referring to immunomodulatory capacity.

**Keywords:** Non-*Saccharomyces* yeasts, *C. intermedia*, *H. uvarum*, *P. kluyveri*, macrophage, immunostimulation

**Declarations**

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**Conflicts of interest**

The authors declare no competing interests

**Availability of data and material**

All data generated or analyzed during this study are included in this published article

**Code availability**

Not applicable

**Ethics approval**

Not applicable

**Consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Author contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Renan E. A. Piraine, Vitória S. Gonçalves and Neida L. Conrad. The first draft of the manuscript was written by Renan E. A. Piraine and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

## Introduction

Microorganisms in the gastrointestinal (GI) tract impact on metabolism, endocrinological system, GI tract development and immune system regulation [1]. Microbiome present in GI tract is essential for development and function of intestinal mucosa, representing a central barrier in the line of resistance to invasion by pathogens [2]. The microbial community composition is able to prevent and treat bowel disorders (e.g. inflammatory bowel disease), aid in systematic disorders (e.g. allergies) and even enhance the immune response to vaccines [1, 3–5]. Changes in microbial community can be achieved by the administration of probiotic bacteria and yeasts, resulting for example in an immunomodulation in cytokines secretion by immune cells [6].

Gastrointestinal tract is constantly exposed to exogenous substances (bacteria, fungi, peptides, food particles, among others), so 60-70% of the body's immune cells are present in this environment [7]. Macrophages are responsible for initiating response against microorganisms, phagocytizing and identifying them using specific receptors, presenting antigens to other immune cells and participating in the coordination of immune response through the expression of cytokines [8]. Yeasts (microbe-) associated molecular patterns (MAMPs) are recognized by pattern recognition receptors (e.g. TLR2, Dectin-1, among others) on immune cells and by doing so activating or suppressing immune responses [9]. Yeast cell wall components, such as β-glicans and mannans, are recognized by these receptors, inducing specific responses through cytokines and chemokines, which stimulate T cell differentiation [10, 11].

Even though yeasts are part of a same group of microorganisms, they have structural and metabolic differences that influence the stimulation of immune system cells during their recognition, resulting in a variable species-specific response [10]. Probiotic potential of non-*Saccharomyces* yeasts has been widely studied by several groups [12–17], however data regarding the immunomodulation induced by these microorganisms are still scarce on literature. Among non-*Saccharomyces* yeasts, *Hanseniaspora uvarum* (PIT001), *Pichia kluyveri* (LAR001) and *Candida intermedia* (ORQ001) are isolated from our previous studies, which had probiotic characteristics studied *in vitro* (data not shown) and were considered promising yeasts. However, more data are needed regarding their *in vivo* activity and stimulation of effector molecules (cytokines and chemokines), cell receptors and signaling pathways in biological models and cell cultures.

Live yeast cells maintain a microorganism-host interaction through molecules actively produced and secreted, but the simple interaction between non-viable cells (and their cell wall components) with surface receptors expressed by host can be also sufficient to stimulate immune system [6, 18]. Pericolini et al. [19] demonstrate that administration of live or inactivated yeast cells can result in differences in organism's immunomodulation, culminating in different efficacy in controlling pathogens by the host. Yeast secretome is also involved in responses orchestrated by macrophages, as some extracellular proteins present immunogenicity and are related to virulence factors [20]. Proteins secreted by yeasts may also result in changes in cell adhesion and cytokines expression to cell cultures *in vitro* [21]. Nucleic acids from yeasts can be recognized by TLRs (e.g. TLR9) that can signaling for production of interferon (IFN) and other pro-inflammatory genes expression via Toll/Interleukin-1 receptor (TIR) domain or TIR-domain-containing adaptor inducing IFN-β (TRIF) [22].

Yeast cells, their structures and secreted molecules, are recognized as potential stimulators of immune system, so identify responses induced in immune cells is important to characterize this activity. Thus, in this work we investigated response to stimuli using yeast cells (live and heat-killed), culture supernatant and DNA on RAW 264.7 macrophages, evaluating mRNA transcription of cytokines (*IL2, IL4, IL10, IL13, IL23, TNF-α*), transcription factors (*Bcl6, NFκβ, STAT3*), Toll-like receptor 2 (TLR2) and YM1 protein.

## Material and methods

### Strains and culture conditions

Wild isolates *Pichia kluyveri* LAR001, *Hanseniaspora uvarum* PIT001 and *Candida intermedia* ORQ001 were obtained from the microorganism bank of Microbiology Laboratory in the Federal University of Pelotas, as well as the commercial yeast *Saccharomyces boulardii* (a reference probiotic strain), which were cryopreserved in glycerol at -80 °C. Yeasts were grown overnight in YM (Yeast and Malt Extract) medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone and 1% glucose) at 30 °C under constant agitation of 150 rpm.

Murine macrophage-like cell line RAW 264.7 (ATCC® TIB-71™) was grown as monolayers according to Santos et al. [23]. Briefly, cells were incubated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) at 37 °C in a 90% humidity atmosphere with 5% $CO_2$, until approximately 80% confluence in the culture plate.

### Stimuli preparation

Live and inactivated (heat-killed) cells, culture supernatants, and DNA extracted from each yeast were used to stimulate RAW 264.7 cell culture. Yeast cells were washed twice with Phosphate Buffered Saline buffer (PBS) and then $10^8$ CFU/mL of viable cells were stored to be used subsequently during macrophages stimulus. Cell-free supernatant from yeast culture media was obtained by centrifugation at 2.000 × g for 10 min and then separate in aliquots to further stimulate macrophages. Yeast cells at same concentration ($10^8$ CFU/mL) were inactivated by heat and pressure (heat-killed cells), autoclaving at 120 °C with a pressure of 1 atm for 20 min. After inactivation, samples were seeded onto YM agar medium and incubated for 48 h at 28 °C, a control step to assure correct yeast inactivation.

Total DNA from yeasts was extracted following an adaptation of the protocol described by Preiss et al. [24]. A volume of 3 mL from yeast cultures was centrifuged in a DAIKI DTR-16000 centrifuge, at 12.000 × g for 1 min, then it was suspended using 200 uL of breaking buffer solution (2% Triton-X 100 w/v, 1% SDS w/v, 100 mM NaCl, 100 mM Tris pH 8.0, EDTA 1 mM pH 8.0). An amount corresponding to 100 uL of glass microbeads (0.5 uM, Sigma-Aldrich) and 200 uL of phenol/chloroform/isoamyl alcohol (25:24:1) were added to previous solution. Tubes were then vortexed for 2 min, adding TE buffer (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0) after that. Centrifugation was performed at 13.000 × g for 5 min, then aqueous phase (~350 uL) was transferred to a new tube, where 1 mL of ethanol 96% was added,

homogenized and incubated at room temperature for 10 min (with no shaking). Tubes were centrifuged at 13.000 × g for 2 min, supernatants were discarded, and pellets were dried at room temperature for 20 min. DNA was eluted with 50 uL of DNAse free water, then DNA quality was evaluated by electrophoresis on 1% agarose gel (100V, 500 mA, 1 h) and its concentration quantified by Nanovue™ (Biochrom).

**RAW 264.7 cells stimulation**

Stimulation occurred in a yeast:RAW cells ratio of 10:1, following adaptation of the protocol developed by Smith et al. [25]. RAW 264.7 cells were kept under stimulation for 24 h, with incubation in DMEM supplemented with 10% (v/v) FBS at 37 °C in an atmosphere of 90% humidity with 5% $CO_2$. As negative control, cells were stimulated with DMEM medium only. As positive controls, Concanavalin A – ConcA (10 µg) and Zymosan (100 µg) (Sigma-Aldrich) were used. For the assay, 100 µL of live cells or 100 µL of heat-killed cells per well were used as stimuli, at a final concentration of $10^7$ CFU/mL. The same volume of supernatant (100 µL) was used, while for DNA of each yeast the concentration of 850 ng/well was targeted. Total volume for stimulus + culture medium was 1 mL per well, being carried out in triplicates.

**RNA extraction, cDNA synthesis and qPCR**

RNA was extracted using the TRIzol method, according to the manufacturer's instructions. Reverse transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) using approximately 400 ng of RNA. The qPCR reactions were performed using 1 µL of cDNA (synthetized from 400 ng RNA), 5 µL of SYBR Green qPCR Master Mix (Promega, USA), 0.25 µL of each primer (from 10 µg/µL solution), and 3.5 µL of RNAse Free Water (Sigma-Aldrich, USA), for a total reaction volume of 10 µL. Conditions of temperature and time for qPCR reaction were performed according de Avila *et al.* (2016) [26], as follows: denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min.

Relative mRNA transcription levels for *IL2, IL4, IL10, IL13, IL23, TNF-α, Bcl6, NFκB, STAT3, YM1 and TLR2* were determined using qPCR with *β-actin* used as endogenous reference gene. qPCR reactions were performed on a StepOne™ Plus qPCR System (Applied Biosystems). The primers used were described in Table 1. Reaction efficiency for each primer pair was calculated and previously described by [3, 23, 26], and primer specificity was checked from melting curves. All samples were analyzed in triplicate using the comparative threshold cycle (ΔΔCt) method to determine the relative mRNA expression compared to *β-actin* as the reference gene, following a previously described method [27].

*Table 1 here*

**Statistical analysis**

Data were analyzed using GraphPad Prism version 7 Software. Differences in mRNA transcription levels were subjected to analysis of variance by two-way ANOVA with multiple comparison, followed by Tukey's test. Differences between groups were statistically significant when $p < 0.05$. Results of the mRNA levels were expressed graphically using fold increase means and standard deviations.

**Results**

***Saccharomyces boulardii.*** Viable *S. boulardii* cells were able to induce significant ($p < 0.05$) cytokines transcription in RAW cells with 8.6, 8.7, 7.4 and 3-fold increase for *IL2, IL13, IL23, and IL4* respectively (Fig. 1a, d, e and b). Meanwhile for *IL10, TNF-α, YM1, TLR2, Bcl6, NFκβ, STAT3* were detected in low or absent levels (≤ 1.2-fold increase). It was not seen the same response when heat-killed cells were used as stimulus, since relative mRNA transcription for all cytokines remained between 0.5 – 1.2-fold increase (Fig.1). Worth note that transcription factors were induced in higher levels than detected for viable cells, *Bcl6* presenting a 3.3-fold increase transcription, *Nfκβ* and *STAT3* above 1.5-fold increase (1.6 and 1.7-fold, respectively) (Fig. 2a, b and c). Relative mRNA transcription of *TLR2* was low for *S. boulardii* viable cells (0.5-fold increase), however an increase in mRNA levels was observed when its derivatives were used, reaching 1.8-fold increase in heat-killed cells. Supernatant and DNA from *S. boulardii* induced only low levels of mRNA transcription for all genes evaluated, however a remarkable response was observed for *YM1* (2.3-fold increase) in supernatant stimulus (Fig. 3a) and for cytokines *IL2* and *IL10* (1.7-fold increase both) when DNA was used. It was noted that *YM1* mRNA transcription was induced with low (~2-fold increase) levels only in *S. boulardii* supernatant and DNA stimuli, what was not observed for other yeasts and their derivatives (Fig. 3a).

***Pichia kluyveri.*** Live and heat-killed cells of *P. kluyveri* induced a cytokine response close to basal levels, being responsible for low levels in relative mRNA transcription ranging between 0.5 – 1.8-fold increase (Fig. 1). However, these stimuli were able to promote intermediate to high levels of relative mRNA transcription for *Bcl6, Nfκβ* and *STAT3* (Fig.2). Live cells were able to induce a fold increase ranging between 1.7 – 3.7-fold, while heat-killed cells were able to promote high levels of *Bcl6* (7.3-fold increase) and *STAT3* (3.3-fold increase), with a less extent for *Nfκβ* (2.0-fold increase). A high relative mRNA transcription of *TLR2* (Fig. 3b) was observed for live and heat-killed *P. kluyveri* cells (6.3 and 4.3-fold increase, respectively), suggesting that during yeast recognition there was a potent activation of this receptor. Moreover, *P. kluyveri* cells were able to elicit *YM1* transcription (Fig. 3a), even though at low levels (0.8-fold increase). Supernatant from *P. kluyveri* culture was able to induce low levels of *IL-23* and *TNF-α* (1.3 and 1.4-fold increase), being other cytokines below 1.0-fold in relative mRNA transcription (Fig 1). Similar levels were detected for transcription factors *STAT3* (1.3-fold increase) and *Nfκβ* (1.2-fold increase), however a more prominent response was observed in *Bcl6* induction (2.2-fold increase). Stimulate RAW macrophages with supernatant was also able to induce significant levels of *TLR2*, resulting in 2.2-fold increase in relative mRNA transcription. DNA stimulus promoted only basal levels of mRNA transcription for most genes evaluated, nevertheless it was observed for cytokines *IL4* and *IL10* an increase of 1.6 and 1.8-fold.

***Hanseniaspora uvarum***. As observed for *P. kluyveri* stimulus, viable cells of *H. uvarum* also resulted in a cytokine response close to basal levels, in which relative mRNA transcription was inferior to 0.7-fold increase for all (Fig. 1). Live cells of *H. uvarum* resulted in significant levels of *Bcl6* transcription, with 3.4-fold increase, the only transcription factor stimulated with significant levels (Fig. 2a). Although live cells were not able to induce significant levels of cytokines transcription, heat-killed cells of *H. uvarum* were responsible for *IL23* transcription with significant levels ($p < 0.05$) when compared to other stimuli derived from this same yeast, resulting in a 2-fold increase (Fig. 1e). Moreover, inactivated cells provoked low to intermediate transcription levels of *IL2* (1.7-fold increase), *IL10* (1.9-fold increase) and *TNF-* $\alpha$ (1.5-fold increase). Observing all transcription factors evaluated (Fig. 2), relative mRNA transcription was higher when heat-killed cells were used ($p < 0.05$), reaching 5.8-fold increase for *Bcl6*, 1.9-fold increase for *Nfκβ* and 3-fold increase for *STAT3*. Transcription levels of *Bcl6* and *STAT3* mRNA after *P. kluyveri* and *H. uvarum* live and heat-killed cells stimuli were superior to those detected for both Concanavalin A and Zymosan ($p < 0.05$). Stimuli with *H. uvarum* were also able to elicit receptor's transcription, with values around 2.3-fold for live and heat-killed cells (Fig. 3b). These values were higher ($p < 0.05$) than those observed for Zymosan (1.4-fold), suggesting these stimuli were also potent TLR2 activators. Supernatant from *H. uvarum* culture was not able to elicit a consistent cytokine transcription (fold increased ranged between 0.29 – 1.0 for most cytokines), being *TNF-* $\alpha$ transcription the most stimulated (1.5-fold increase) and the only one with relative mRNA transcription above 1-fold. The same was observed for other genes tested, in which low or absent levels of mRNA transcription were detected. DNA from *H. uvarum* was responsible for a 2.2-fold increase in relative mRNA transcription of *IL10*, however it was not observed any other significant values for cytokines, transcription factors, *YM1* or *TLR2*.

***Candida intermedia.*** Live cells of *C. intermedia* were shown to be potent activators of *IL4* transcription (7.7-fold increase), with a significant difference from other stimuli also for *IL13* production (2.3-fold increase) ($p < 0.05$) (Fig. 1b and d). Transcription factors, *YM1* and *TLR2* transcription levels were observed at very low levels after stimulus using viable cells (Fig. 3). Macrophage stimulation using inactivated *C. intermedia* cells revealed that higher levels of *TNF-α* were produced (2.4-fold), when compared to other stimuli from yeasts and their derivatives, which were less than 1.5-fold (Fig. 1f). *IL10* transcription was also observed, however at low levels (1.4-fold increase). Although *Nfκβ* and *STAT3* were detected at low levels (1.5 and 1.6-fold increase, respectively), *Bcl6* relative mRNA transcription was observed at high levels (4.0-fold increase), indicating *C. intermedia* inactivated cells were also *Bcl6* transcription inducers, as detected for *P. kluyveri*, *H. uvarum* and *S. boulardii* (Fig. 2). As observed for previous yeast culture supernatants, it was noted very low levels for most cytokine transcription (≤1.0-fold), except for *IL4* and *TNF-α* which were detected at low levels (1.2 and 1.3-fold increase, respectively). *C. intermedia* culture supernatant also induced a remarkable transcription of *TLR2* (4.4-fold increase) by RAW macrophages (Fig. 3b), evidencing the presence of molecules with ability to stimulate recognition by this receptor. DNA stimulus resulted in a low transcription of *IL4* (1.5-fold increase) and *IL10* (1.7-fold increase), with no significant values for other genes evaluated.

**Controls.** Zymosan, a polysaccharide isolated from *S. cerevisiae* cell wall and distributed commercially, was used to stimulate macrophages, resulting in consistent levels of transcription of *IL10* and *IL23* (5.5-fold increase for both cytokines) and significant levels of *IL4* and *TNF*-α (3.9-fold increase

both). Stimulation by Concanavalin A resulted in a low transcription (1.5 to 2-fold) for all cytokines, except *IL13*, which was observed in concentration practically similar to that of housekeeping gene (basal transcription, 0.2-fold increase only). Zymosan and Concanavalin A stimuli were responsible for low levels of mRNA relevant transcription (ranging 0.5 to 1.5-fold increase) of transcription factors and *TLR2* receptor, however it was seen an intermediate macrophage response in *YM1* for both stimuli (~3-fold increase) (Fig. 4).

*Fig. 1 here*

*Fig. 2 here*

*Fig. 3 here*

*Fig. 4 here*

## Discussion

### Yeasts interact with RAW macrophages in different ways

Yeasts, especially those belonging to *Saccharomyces* spp., are able to participate in macrophages activation through stimuli generated when subjected to interaction with these cells [6, 25, 28]. Macrophages when activated can give rise to distinct populations, being M1 and M2 the main populations described. M1 macrophages (or *classically activated*) are polarized *in vitro* by Th1 cytokines such as GM-CSF (Granulocyte-Macrophage Colony-Stimulating Factor), TNF-α and IFN-γ, alone or together with bacterial LPS (lipopolysaccharide). M1 macrophages express pro-inflammatory cytokines such as IL1, IL6, IL12, IL23 and TNF-α. In contrast, M2 macrophages (or *alternatively activated)* are polarized by Th2 cytokines such as IL4 and IL13, producing anti-inflammatory cytokines such as IL10 and TGF-β [8, 29].

Immune response induced by stimulation with yeasts still needs to be explored, as researchers have characterized different response patterns mediated by probiotics, pathogenic yeasts and their components [30, 31]. Analysis of cytokine expression by human DCs (dendritic cells) in Bazan et al. [10] study have verified a response based on IL12, IL23 and IL27 cytokines when stimulated with different yeast genera, including *Saccharomyces* spp. and *Candida* spp. In our study, *Saccharomyces boulardii* was able to stimulate macrophages to produce high transcription levels of *IL2, IL4, IL13* and *IL23*, with low or only basal expression of other cytokines. IL2 promotes growth and development of peripheral immune cells, initiating a defensive immune response through survival and division of regulatory T cells (Treg) and proliferation of cytotoxic T cells [32]. Santos et al. [33] also reported high levels of *IL2* mRNA transcription by PBMCs (Human Peripheral Blood Mononuclear Cells) from animals supplemented with *S. boulardii*. Cytokines IL4 and IL13 play a fundamental role in immunosuppressive and anti-inflammatory activity, inhibiting or decreasing pro-inflammatory cytokines expression such as TNF-α and IL1β [34, 35]. IL23 has an immunoregulatory and pro-inflammatory role, stimulating IFN-γ and mainly sustaining cell-mediated responses focusing in intracellular infections elimination [32, 34]. As noted by Stier and Bischoff (2016)

[28] *S. boulardii* leads to a general unspecific immune system activation, and variations in patterns of cytokines stimulated can be observed according to cell lines (dendritic cells, macrophages, lymphocytes…) analyzed, stimulus, test conditions (*in vitro* X *in vivo*), *in vivo* challenge with pathogens, among others. Although Smith et al. [6] did not find differences in cytokine-inducing properties among live, UV-irradiated and heat-killed cells, in our study it was detected a considerable variation ($p < 0.05$) in relative mRNA transcription between stimuli with viable and non-viable cells, resulting in a 4 to 6-fold less transcription of *IL2*, *IL13* and *IL23* when evaluating heat-killed cells (non-viable cells) of *S. boulardii*.

*Hanseniaspora uvarum* and *P. kluyveri* are two yeasts which remains underexplored regarding their ability to interact with immune system, so our work is one of the first studies aiming the characterization of immune cells-yeasts interaction. Stimuli using live and inactivated cells from these yeasts maintained a low or basal expression of cytokines evaluated, without a predominance of any of them during cytokine response, as observed for *C. intermedia* and *S. boulardii*. Even so, relative mRNA transcription levels detected were similar to those observed for stimulation with lectin Concanavalin A, a mitogen with well-described activity in macrophage activation [36]. Macrophage response based on *IL4* and *IL13* transcription was observed for live *C. intermedia* cells, with transcription levels for *IL4* 2.5-fold higher than that observed for *S. boulardii*. Variations in metabolic activity and cell wall composition of *Candida* spp. lead to differences in phagocytosis and levels of cytokines being produced by immune cell lines stimulated *in vitro* (such as greater or lesser production of TNF-α) [37] . There are extensive variations in yeast cell wall when comparing different fungal species and strains, such as α-glucans in addition to β-glucans, differences in concentration of chitosan, galactomannans and melanin [11]. Although cell wall composition among *H. uvarum*, *P. kluyveri*, *C. intermedia* and *S. boulardii* is comparable, it was demonstrated by Mateja et al. [38] significant differences in their genera regarding patterns of GPI anchored and non-covalently attached proteins, as well as cell wall thickness, permeability, amounts of mannan and glucans. Thus, different responses induced in immune cells may be related to cell wall complexity and its components [39].

Paraprobiotics are non-viable microorganism cells with capacity to stimulate immune system and modulate its response, amplifying the response through the exposure of immunogenic molecules after an inactivation treatment [18]. After a heat-treatment associated with high pressure, cell inactivation occurs through membranes damage, loss of nutrients and ions, protein denaturation and essential enzymes inactivation, what can lead to modifications in cell coarseness and roughness [18, 40]. These structural and molecular changes influence immune-modulating properties of paraprobiotics, it being reported in some cases even more cytokines production (e.g. IL12) by macrophages stimulated with heat-killed probiotics than viable cells, as observed by Miyazawa [41]. In our study cytokines *IL10*, *IL23* and especially *TNF*-α mRNA transcription levels are potentiated when macrophages are stimulated by heat-killed cells of *C. intermedia* and *H. uvarum*, a behavior also observed for other *Candida* species by Navarro-Arias et al. [37] that stimulated PBMCs with viable and heat-killed *C. albicans*, *C. tropicalis*, *C. guilliermondii*, *C. krusei* and *C. auris*. Based on responses mediated by heat-killed cells, it was suggested also a possible paraprobiotic effect on these yeasts.

In our study, fungal DNA was able to induce a similar pattern of stimuli among all cytokines, however *IL10* was induced at more prominent levels, being the most induced cytokine by these stimuli. Ramirez-Ortiz et al. [42] and Patin et al. [43] related unmethylated fungal CpG DNA recognition with induction of high levels of TNF-α and other pro-inflammatory cytokines expression, however same relationship was not observed in our experiments. Yeast cells have generated significant transcription levels for cytokines (*IL2*, *IL4*, *IL13* and *IL23* for example), however in some cases DNA stimulus resulted in the highest level of cytokine detected among stimuli, for example in *IL10* transcription, in which all DNAs used generated ≥ 1.5-fold increase in relative mRNA transcription. The conditioning of DCs to the predominance of a response based on IL10 expression promotes Foxp3+ regulatory T cells (Treg) secretion, what contributes to intestinal tolerance [25]. In this context, microorganisms or their derivatives with ability to target this cytokine profile promote tolerance to the intestinal microbiota and are able to reduce conditions characterized by excessive inflammation [7, 25]. It is noteworthy that there is also evidence of IL10 destabilizing cytokines mRNA, including IL10 mRNA itself, so a low cytokine detection in the experiment may be due to this fact [34].

We enforce that our results were obtained from a 24 h period of macrophage stimulation and possibly different responses could be observed if different periods were tested, however it was out of scope in our study. Yeast:immune cells ratio, also known as MOI (*multiplicity of infection)*, is also a parameter that differs among studies, what may influence in patterns of cells interaction and cytokine response [44]. Our methods were in line with Smith et al. [6] and Smith et al. [25], which are important and comprehensive reports of immune cells stimulation by yeasts.

**Transcriptional factors role in macrophage stimuli**

Analysis of *Bcl6*, *STAT3* and *NFκβ* mRNA expression revealed that heat-killed cells were the stimuli responsible for highest levels of transcription in all tested yeasts. In these cases, *P. kluyveri* and *H. uvarum*, both in viable and non-viable cells, were the yeasts which induced highest levels in expression of transcription factors. Since *Bcl6* plays a fundamental role in regulation of Th2-type inflammation and it is constantly expressed in monocytes [45], we sought to observe in our study its transcriptional levels in stimulated macrophages. *Bcl6* also regulates macrophage function by repressing IL6 pro-inflammatory cytokine production and differentiation of Th17 cells [46]. Observing transcription values for *Bcl6*, a tendency towards M2 polarization of macrophages stimulated with non-*Saccharomyces* yeast cells is suggested, however more studies must to be conducted to confirm this hypothesis.

During active inflammation occasioned by infections, microorganisms can bind to PRRs (Pattern Recognition Receptors) and activate signaling pathways via MAP kinases and *NFκβ*, resulting in stimulation of cytokines production with inflammatory effects, including those with pro-inflammatory activity (e.g. IL1β) which induce polarization to M1 macrophages [47]. The increase in *NFκβ* transcription is involved with TLR2 receptor activity, which has its expression level increased by mannose and β-glicans recognition [48]. Zymosan is a good stimulator of *NFκβ* activation [49], and as observed in our work,

stimuli with all yeasts cells (viable or non-viable) were able to induce similar or superior expression of *NFκβ* than observed in Zymosan stimulus.

Toll-like receptors are innate immune response infection sensors that participate in the activation or inhibition of macrophage activity via Jak-STAT pathway; signaling via *STAT3* is activated by several cytokines and their receptors, such as IL2, IL6, IL10, IL23 and IL27 [50]. The STAT proteins are transcription factors frequently involved in downstream cytokine signal transduction mediated by TLR2, TLR4 e TLR9 [51]. *STAT3* main role in macrophages is to mediate anti-inflammatory effects of IL10, restricting gene transcription of pro-inflammatory cytokines [51] and repressively impacting *NFκβ* signaling pathways [52]. *STAT3* transcription was observed at higher levels when heat-killed cells were used as a stimulus, with emphasis on *P. kluyveri* and *H. uvarum*, responsible for inducing significant levels of its expression. These results surpassed induction levels of *Bcl6* and *STAT3* by Concanavalin A and Zymosan by 2 to 6-fold. Therefore, there were indicatives of M2 polarization in macrophages by these stimuli.

**Recognition of yeasts and their derivatives by TLR2**

Macrophages are usually in an inactive state, but can be activated through a variety of stimuli during immune response, which are recognized in different ways, for example by PRRs such as TLR2 [8, 9, 29, 30, 53]. Yeast cells and their cell wall components (e.g. Zymosan) are important stimulators of TLR2 and Dectin-1 [49, 54, 55], and in our study it was observed that live and inactivated cells of *P. kluyveri* were responsible for high levels of its transcription, up to 4-fold higher than that detected for macrophage stimulation by Zymosan. Stimulus using *H. uvarum* cells also resulted in TLR2 transcription at significant levels, demonstrating this yeast is also responsible for stimulating the receptor expression. Composition of cell walls may vary among yeast species and strains, what modifies the way that immune system recognizes and process their components [54], culminating in higher or lower levels of receptor expression according to yeast surface structure.

Low TLR2 expression from other stimuli does not necessarily impact cytokine expression, as noted by Smith et al. [25], since recognition of cell wall components may also be dependent on other receptors, such as Dectin-1 and mannose receptors. It could also be observed in our data, as although stimuli from *S. boulardii* and its derivatives resulted in low levels of TLR2 expression, cytokines like IL13 were highly expressed when *S. boulardii* live cells were used to stimulate macrophages response. Variations in TLR2 and cytokine expression levels occasioned by live and inactivated cells stimuli may be explained by β-glicans exposure on the entire yeast cell surface occurring after heat treatment, while intact cells usually expose β-glicans only through budding scars [30].

Molecules present on *P. kluyveri* and *C. intermedia* culture supernatants were also responsible for stimulating significant levels of TLR2, higher than observed for Zymosan stimulus, demonstrating metabolites secreted by yeasts are also important in stimulating receptors in cells of the immune system. Secreted proteins by *Candida* spp. yeasts are linked with TLR2/TLR4 recognition as demonstrated by Wang et al. [56], promoting inflammatory response in DCs and macrophages stimulated *in vitro*. The most

common ligands related to TLR2 are PAMPs (Pathogen-Associated Molecular Patterns) originated from glycolipids, lipopeptides or GPI-anchored structures [57], thus it is suggested that higher receptor mRNA transcription in these cases is related to secretome products or proteins detached from yeast cell wall that have these structures in their conformations [21, 56].

**YM1 marker**

YM1 protein expression is considered a marker for polarization to M2-activated macrophages in mice [58], being initially described in inflammation induced by parasitic infections [59] and observed by our group (data not shown) with relevant expression in M2 macrophages from intestinal mucosa of mice experimentally infected with *Toxocara canis*. Marker expression was not detected for most stimuli, however *P. kluyveri* cells and *S. boulardii* derivatives generated low levels of its transcription. Welch et al. [60] related YM1 expression with IL4 and IL13 production, however this association is questioned by Goren et al. [61], and in our work it was not possible to confirm the same correlation, since live cells of *C. intermedia* and *S. boulardii* (potent activators of these cytokines) had low or absent levels of YM1 transcription.

The highest relative mRNA transcription levels of *YM1* were observed in Concanavalin A and Zymosan stimuli. Concanavalin A showed consistent low levels of mRNA transcription for cytokines, transcription factors and TLR2 on RAW 264.7 macrophages stimulated, however a remarkable response for *YM1* marker could be detected. Zymosan was confirmed as potent *IL10* inducer in immune cells *in vitro* culture, also responsible for a high stimulus of other cytokines, such as *IL4*, *IL23* and *TNF-α*, as well as *YM1* marker.

**Conclusion**

*Saccharomyces boulardii* is a yeast that has different probiotic mechanisms already described, mainly immunomodulation in *in vitro* and *in vivo* models. In the present work, the high cytokines transcription levels observed in RAW cells stimulated with *S. boulardii* corroborated with the hypothesis that its probiotic immune modulation mechanism is mediated by cytokines activation. We observed that non-*Saccharomyces* yeasts, *Pichia kluyveri* LAR001, *Hanseniaspora uvarum* PIT001, *Candida intermedia* ORQ001, and their heat-killed cells, culture supernatants and DNAs are able to stimulate RAW macrophages with distinct responses. Viable and heat-killed cells of *P. kluyveri* and *H. uvarum* were responsible for high transcription levels of transcription factors and *TLR2*, but only low levels of relative mRNA transcription for the studied cytokines. Viable cells of *C. intermedia* were able to stimulate significant levels of *IL4* and *Bcl6*, while heat-killed cells stimulated the highest levels of *TNF-α* among yeasts and their derivatives. Furthermore, supernatant from *C. intermedia* culture stimulated high levels of *TLR2*, being the only one among yeast culture supernatants to present high levels of mRNA relevant transcription.

The data found in this work provoke interest in further studies on immunomodulatory activity present in these yeasts. As next steps, we intend to use them in *in vivo* assays to confirm their possible probiotic effects.

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**Table 1. Sequences of primers used in qPCR reactions.**

| Gene | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| *β-actin* | AGAGGGAAATCGTGCGTGAC | CAATAGTGATGACCTGGCCGT |
| *IL2* | TTGTGCTCCTTGTCAACAGC | CTGGGGAGTTTCAGGTTCCT |
| *IL4* | CCAAGGTGCTTCGCATATTT | ATCGAAAAGCCCGAAAGAGT |
| *IL10* | TTTGAATTCCCTGGGTGAGAA | ACAGGGGAGAAATCGATGACA |
| *IL13* | TCTTGCTTGCCTTGGTGGTC | GGTCTTGTGTGATGTTGCTCAGC |
| *IL23* | CCTTCTCCGTTCCAAGATCCT | ACTAAGGGCTCAGTCAGAGTTGCT |
| *TNF-α* | CTGAGTTCTGCAAAGGGAGAG | CCTCAGGGAAGAATCTGGAAAG |
| *Bcl6* | GCCGGCTCAATAATCTCGTGAACAGGTCC | CCAGCAGTATGGAGGCACATCTCTGTATGC |
| *NFκβ* | AGTGCAAAGGAAACGCCCAGAAG | GCCAGGGCTTCCGGTACTC |
| *STAT3* | CCGATGCCTGTGGGAAGAGTC | TGTCACTACGGCGGCTGTTG |
| *TLR2* | ATGTCGTTCAAGGAGGTGCG | CTGACCGGTGATGCAATTCG |
| *YM1* | GGGCATACCTTTATCCTGAG | CCACTGAAGTCATCCATGTC |

**Figure captions**

**Figure 1: Gene transcription of cytokines during RAW 264.7 stimuli with yeast cells and their derivatives.** Viable yeast cells ($10^7$ UFC/mL), heat-killed inactivated cells ($10^7$ UFC/mL), culture supernatant (100µl) and fungal DNA (850 ng) from *P. kluyveri* (LAR001), *H. uvarum* (PIT001), *C. intermedia* (ORQ001) and *S. boulardii* (reference probiotic strain) were used to stimulate cytokine secretion by RAW macrophages. Relative mRNA transcription of *IL2* (a), *IL4* (b), *IL10* (c), *IL13* (d), *IL23* (e) and *TNF-α* (f) was normalized using *β-Actin* level as reference. Data are shown as mean ±SD (Standard Deviation). Dotted line indicates one fold of relative mRNA expression.

**Figure 2: Gene transcription of transcription factors during RAW 264.7 stimuli with yeast cells and their derivatives.** Viable yeast cells ($10^7$ UFC/mL), heat-killed inactivated cells ($10^7$ UFC/mL), culture supernatant 100µl) and fungal DNA (850 ng) from *P. kluyveri* (LAR001), *H. uvarum* (PIT001), *C. intermedia* (ORQ001) and *S. boulardii* (reference probiotic strain) were used to stimulate transcription factors expression by RAW macrophages *in vitro*. Relative mRNA transcription of *Bcl6* (a), *NFκβ* (b) and *STAT3* (c) was normalized using *β-Actin* level as reference. Data are shown as mean ±SD (Standard Deviation). Dotted line indicates one fold of relative mRNA expression.

**Figure 3: Relative mRNA transcription of YM1 protein and TLR2 receptor during RAW 264.7 stimuli with yeast cells and their derivatives.** Viable yeast cells ($10^7$ UFC/mL), heat-killed inactivated cells ($10^7$ UFC/mL), culture supernatant 100µl) and fungal DNA (850 ng) from *P. kluyveri* (LAR001), *H. uvarum* (PIT001), *C. intermedia* (ORQ001) and *S. boulardii* (reference probiotic strain) were used to stimulate *YM1* protein and *TLR2* expression by RAW macrophages *in vitro*. Relative mRNA transcription of *YM1* marker (a) and *TLR2* (b) was normalized using β-Actin level as reference. Data are shown as mean ±SD (Standard Deviation). Dotted line indicates one fold of relative mRNA expression.

**Figure 4: Concanavalin A and Zymosan stimuli in RAW 264.7 macrophage.** Data represent relative mRNA transcription of cytokines (*IL2, IL4, IL10, IL13, IL23 and TNF-α*), transcription factors (*Bcl6, NFκβ and STAT3*), *YM1* marker and *TLR2* receptor in RAW macrophages stimulated with Concanavalin A and Zymosan, being determined based on mRNA levels of *β-Actin* reference gene. Error bars indicate ±SD (standard deviation). Dotted line indicates one fold of relative mRNA expression.

**Fig. 1**

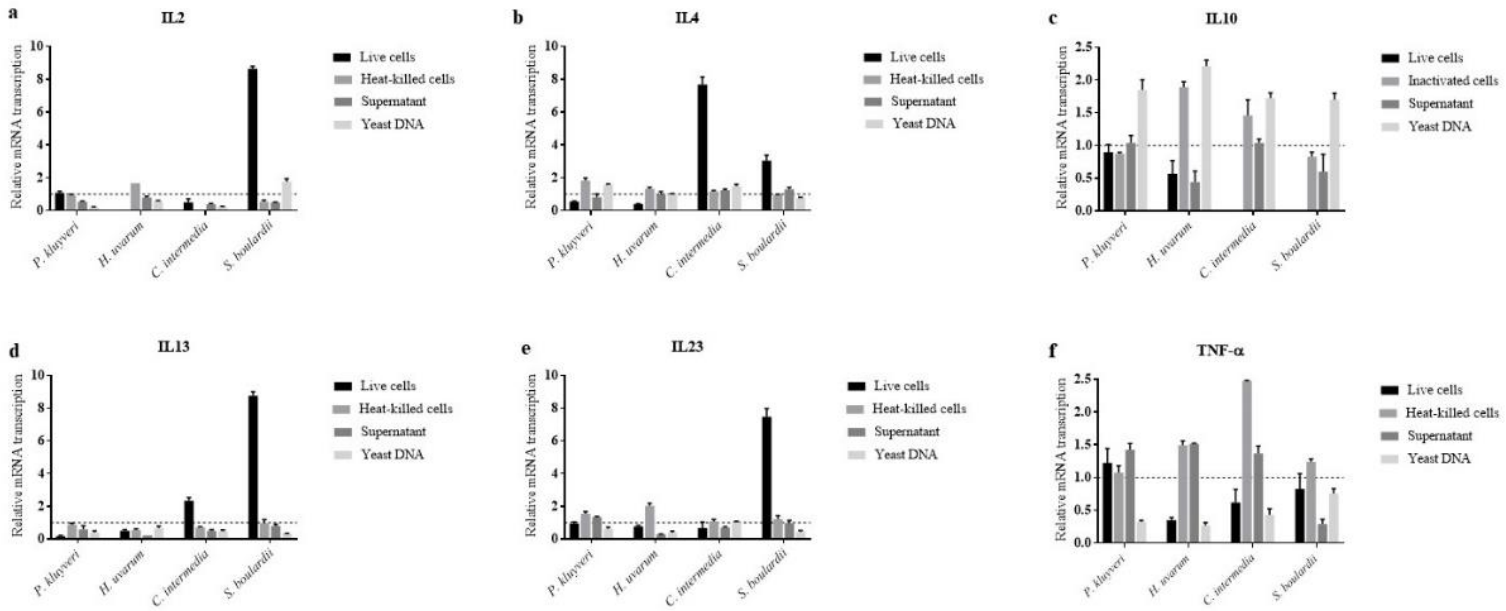



**Fig. 2**

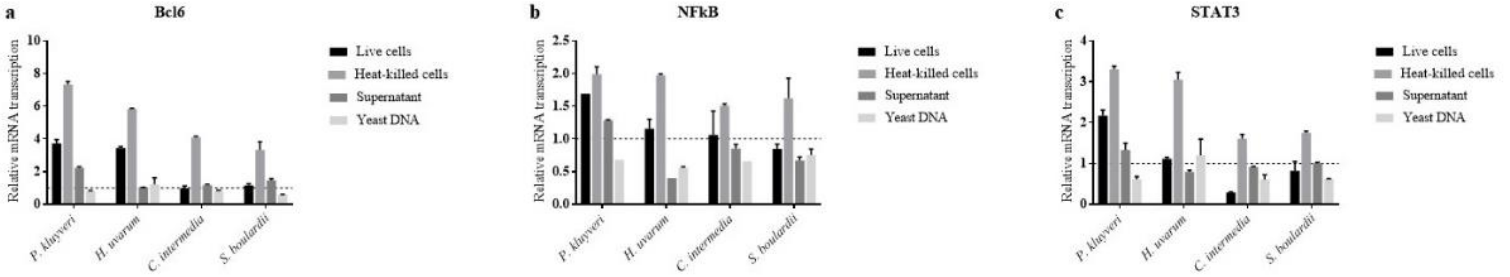



**Fig. 3**

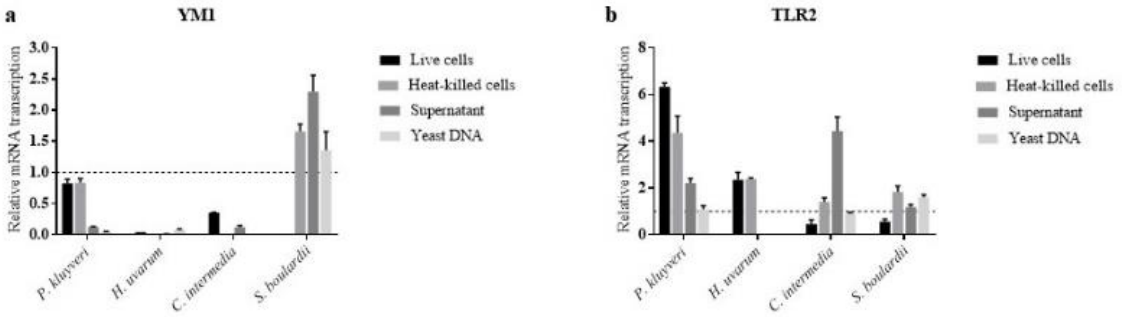
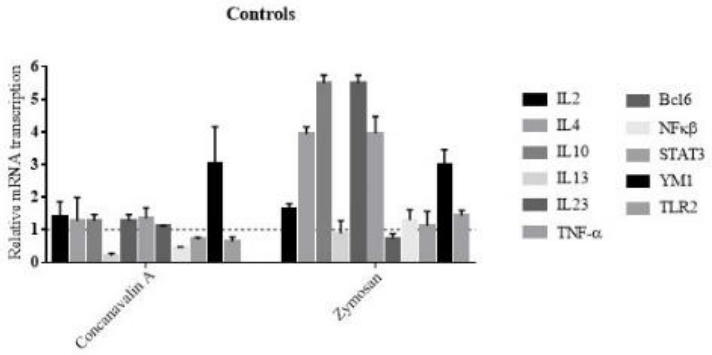
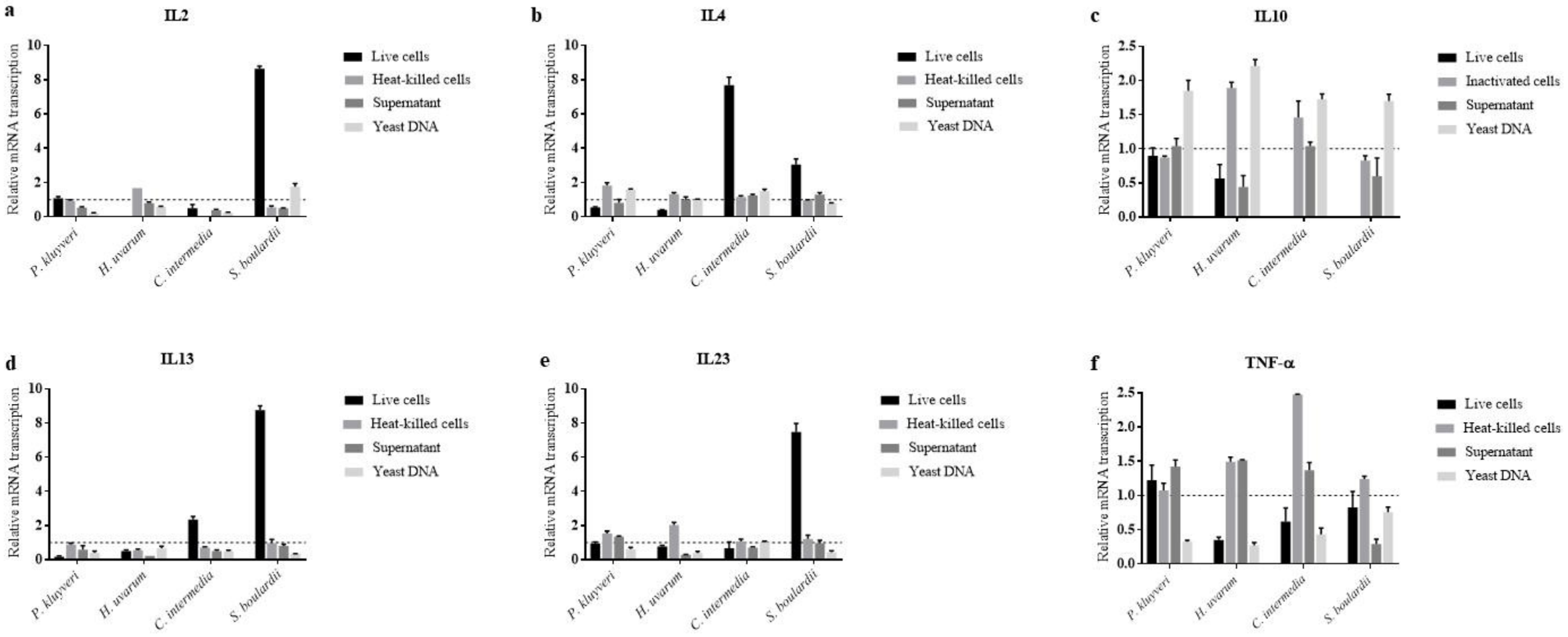
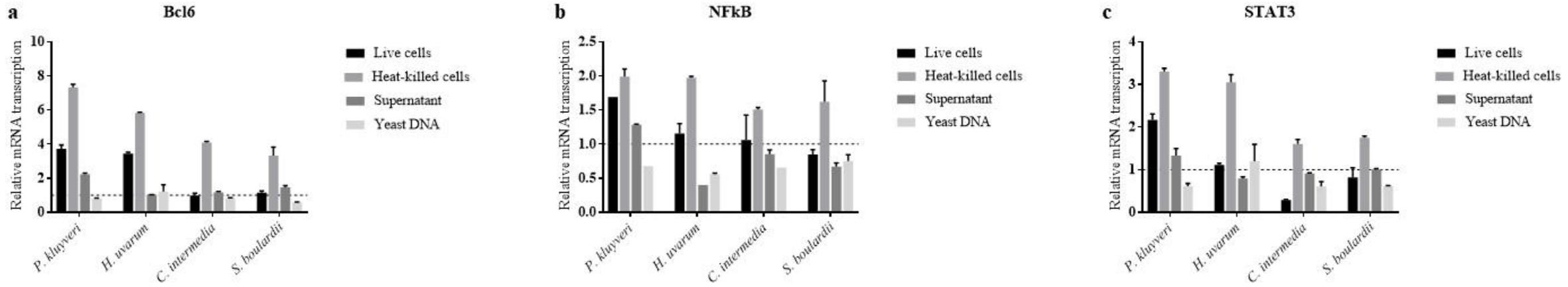
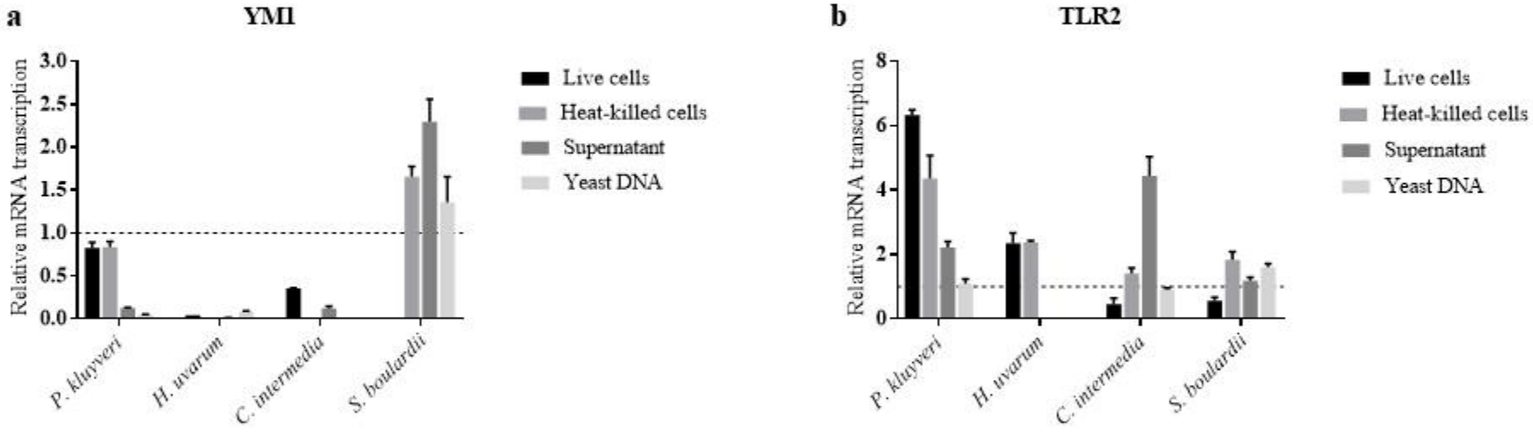


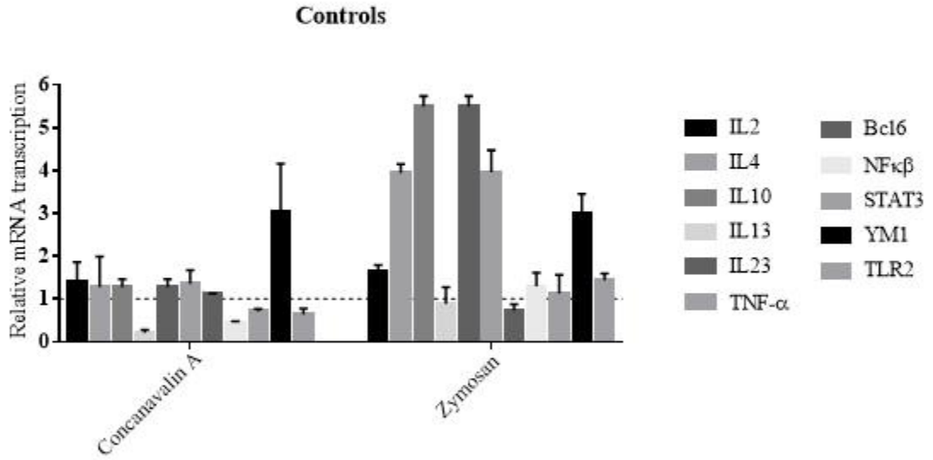
**Fig. 4**


Controls
Relative mRNA transcription
0
1
2
3
4
5
6
Concanavalin A
Zymosan
IL2
IL4
IL10
IL13
IL23
TNF-α
Bcl6
NFκβ
STAT3
YM1
TLR2


a
IL2
Relative mRNA transcription
Live cells
Heat-killed cells
Supernatant
Yeast DNA
P. kluyveri
H. uvarum
C. intermedia
S. boulardii
b
IL4
Relative mRNA transcription
Live cells
Heat-killed cells
Supernatant
Yeast DNA
P. kluyveri
H. uvarum
C. intermedia
S. boulardii
c
IL10
Relative mRNA transcription
Live cells
Inactivated cells
Supernatant
Yeast DNA
P. kluyveri
H. uvarum
C. intermedia
S. boulardii
d
IL13
Relative mRNA transcription
Live cells
Heat-killed cells
Supernatant
Yeast DNA
P. kluyveri
H. uvarum
C. intermedia
S. boulardii
e
IL23
Relative mRNA transcription
Live cells
Heat-killed cells
Supernatant
Yeast DNA
P. kluyveri
H. uvarum
C. intermedia
S. boulardii
f
TNF-α
Relative mRNA transcription
Live cells
Heat-killed cells
Supernatant
Yeast DNA
P. kluyveri
H. uvarum
C. intermedia
S. boulardii


a
Bcl6
Relative mRNA transcription
10
8
6
4
2
0
P. kluyveri
H. uvarum
C. intermedia
S. boulardii
Live cells
Heat-killed cells
Supernatant
Yeast DNA
b
NFkB
Relative mRNA transcription
2.5
2.0
1.5
1.0
0.5
0.0
P. kluyveri
H. uvarum
C. intermedia
S. boulardii
Live cells
Heat-killed cells
Supernatant
Yeast DNA
c
STAT3
Relative mRNA transcription
4
3
2
1
0
P. kluyveri
H. uvarum
C. intermedia
S. boulardii
Live cells
Heat-killed cells
Supernatant
Yeast DNA


a
YM1
Relative mRNA transcription
3.0
2.5
2.0
1.5
1.0
0.5
0.0
P. kluyveri
H. uvarum
C. intermedia
S. boulardii
Live cells
Heat-killed cells
Supernatant
Yeast DNA
b
TLR2
Relative mRNA transcription
8
6
4
2
0
P. kluyveri
H. uvarum
C. intermedia
S. boulardii
Live cells
Heat-killed cells
Supernatant
Yeast DNA


Controls
Relative mRNA transcription
0
1
2
3
4
5
6
Concanavalin A
Zymosan
IL2
IL4
IL10
IL13
IL23
TNF-α
Bcl6
NFκβ
STAT3
YM1
TLR2