UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

RAFAELA ALVES DA SILVA

Antifungal activity of Punicalagin isolated from *Punica granatum* and synergism with Nystatin against *Candida albicans*: cellular metabolism, detection of virulence genes and proteomic analysis

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Orientador: **Profª. Drª. Vanessa Soares Lara**

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ABSTRACT

Antifungal activity of Punicalagin isolated from *Punica granatum* and synergism with Nystatin against *Candida albicans*: cellular metabolism, detection of virulence genes and proteomic analysis

Despite therapeutic advances, opportunistic fungal infectious diseases have increased in prevalence and have become a universal public health problem. *Candida albicans* (CA) is a commensal fungus that under certain environmental conditions can act as an opportunistic pathogen and colonize mucous membranes and tissues causing local and systemic infections. The emergence of drug resistant strains, as well as the increase of immunosuppressed patients, has limited therapeutic options. Therefore the aim of this study was to evaluate 1) The antifungal activity of Punicalagin (P), alone or in combination with Nystatin (N), against two CA strains. 2) The cytotoxicity effect of **P** and **N**, as well as the combination of both, on human primary cells. In the first step, yeasts of CA ATCC 90028 and SC5314 were exposed to **P** and **N** for 24 hours. The Minimal Inhibitory Concentration (MIC) of **P** (50 µg/mL) and **N** (3.9 µg/mL) were determined by the broth microdilution assay. The Checkerboard Assay was performed to verify the synergism between the combinations (8: PN, P8 N/4, P/8 N/2, P/8 N, P/4 N/2, P/4 N, P/2 N/4 and P/2 N/2), which were selected from the 56 combinations initially tested. The fungal metabolism was assessed by the metabolic reduction assay XTT (24h) and Minimum Microbicidal Concentration (MMC) was determined by Colony-forming unit (CFU/mL, 24h). The evaluation of virulence factors gene expression was amplified and quantified by PCR real time (Polymerase Chain Reaction-RT-qPCR, only for P/8 N/4 in 24h). The analysis of proteins related to essential biological processes of the fungus was performed by LC-MS/MS (24h). MIC and MMC of **P** were significantly reduced in the presence of **N**, indicating synergism between both. Once the antifungal potential of **P** was verified, we proposed to evaluate whether this drug acts through the same mechanism of action of **N**, altering the permeability of the cell membrane of the fungus (binding ergosterol). The evaluation of gene expression has demonstrated upregulation of some genes that may be related to a defense mechanism associated with stress or cell death. The proteomics analysis revealed alterations in the expression of several proteins in the fungi exposed to P/8 N/4 in relation to negative control, correlated with important biological processes, such as, energy metabolism,

stress response, drug metabolism, among others. In the second step, cytotoxicity assays involving **P** and **N**, as well as the combination of both, were undertaken on human palate epithelial cells (HPEC) and human gingival fibroblasts cells (HGF) by Alamarblue dye. Similarly, **P** cytotoxicity was reduced when used in combination with **N**. Based on these *in vitro* results, the synergistic antifungal activity produced between **P** and **N** suggested that the combination of drugs, at the concentrations tested, may be a topical therapeutic or preventive alternative to be used in cases of superficial candidiasis, such as denture stomatitis.

Keywords: *Candida albicans,* Oral candidiasis, *Punica granatum,* Antifungals agents.

RESUMO

Atividade antifúngica de Punicalagina isolada de *Punica granatum* e sinergismo com Nistatina sobre *Candida albicans*: metabolismo celular, detecção de genes de virulência e análise proteômica

Apesar dos avanços terapêuticos, doenças infecciosas por fungos oportunistas têm aumentado em prevalência e tornaram-se um problema universal de saúde pública. *Candida albicans* (CA) é um fungo comensal que, sob certas condições ambientais, pode atuar como um patógeno oportunista e colonizar mucosas e tecidos causando infecções locais e sistêmicas. O surgimento de cepas resistentes às drogas convencionalmente utilizadas, assim como aumento de pacientes imunodeprimidos, tem limitado as opções terapêuticas. Portanto o objetivo deste estudo foi avaliar: 1) a atividade antifúngica de Punicalagina (P), com ou sem a associação de Nistatina (N), contra duas cepas de CA. 2) O efeito citotóxico de **P** e **N**, assim como suas combinações, em culturas primárias humanas de células epiteliais de palato (CEPH) e de fibroblastos gengivais (FGH). Na primeira etapa, leveduras de CA ATCC 90028 e SC5314 foram expostas a **P** e **N**, por 24 horas. As concentrações inibitórias mínimas (CIMs) de **P** (50 µg/mL) e **N** (3,9 µg/mL) foram determinadas pelo método de diluição em caldo. O Ensaio Checkerboard foi realizado para verificar o sinergismo entre as combinações (8: PN, P8 N/4, P/8 N/2, P/8 N, P/4 N/2, P/4 N, P/2 N/4 e P/2 N/2), as quais foram selecionadas a partir de 56 combinações inicialmente testadas. O metabolismo fúngico foi avaliado pelo método do XTT (24h) e a Concentração Mínima Microbicida (CMM) foi realizada através de Unidades formadoras de colônias (UFC/mL, 24h). A avaliação da expressão gênica de fatores de virulência foi amplificada e quantificada por PCR em tempo real (Reação em Cadeia da Polimerase-RT-qPCR, apenas para P/8 N/4 em 24h). A análise proteômica para identificação de proteínas alteradas foi realizada por LC-MS/MS (24h). A MIC e MMC de **P** foram significativamente reduzidas na presença de **N**, indicando sinergismo entre ambas. Confirmado o potencial antifúngico, o Ensaio de mecanismo de ação (teste do ergosterol) foi realizado e não foi confirmada a ação de **P** através da ligação ao ergosterol. A avaliação da expressão gênica demonstrou regulação positiva de alguns genes que pode estar relacionado a um mecanismo de defesa associado ao estress ou a morte celular. A análise proteômica revelou proteínas diferencialmente expressas nos fungos expostos a P/8 N/4 em relação ao

controle negativo (sem tratamento), as quais estão relacionadas ao processo de metabolismo energético, resposta ao estress e metabolismo de drogas, dentre outros. Na segunda etapa, CEPH e FGH foram expostas a **P** e **N** e suas combinações por 24h para realização dos ensaios de viabilidade por Alamarblue. Combinada à **N**, a Punicalagina apresentou-se menos citotóxica do que de forma isolada. Com base nesses resultados *in vitro*, o sinergismo antifúngico produzido entre **P** e **N** sugere que a combinação das drogas pode ser uma alternativa terapêutica tópica para ser utilizada nos casos de candidose localizada, como por exemplo, a estomatite protética.

Palavras-chave: *Candida albicans,* Candidíase oral, *Punica granatum,* Agentes antifúngicos.

TABLE OF CONTENTS

1 Introduction

1 INTRODUCTION

Infectious diseases caused by opportunistic fungi have grown abruptly, becoming a public health problem. The high rates of morbidity and mortality associated with these diseases, the increase in the number of immunocompromised patients and the emergence of conventional antifungal resistant strains along with the adverse effects of current therapies, show the importance of the discovery of new drugs (BROWN et al., 2012; PACHAVA et al., 2013; ORGANIZATION, 2014).

Candida albicans (CA) is a commensal fungus and the most common opportunistic pathogen in humans. This fungus colonizes mucosal and abiotic surfaces and can cause local and systemic infections, from candidiasis to candidemia (MAVOR; THEWES; HUBE, 2005; SALERNO et al., 2011; LEE et al., 2014). Oral candidiasis is the most common fungal infection in humans, especially in immunocompromised patients, as well as in the elderly and users of removable total dentures, in this case known as denture stomatitis (DS), clinically manifested as spots or erythematous areas in the palate region (CHANDRA et al., 2001a; SHERMAN et al., 2002; SALERNO et al., 2011; WILLIAMS et al., 2013).

Currently, conventional treatments for DS include topical application of Nystatin (**N**), Amphotericin B, Chlorhexidine, Miconazole and Clotrimazole, are recommended as first-line treatment for uncomplicated cases of oral candidiasis (LYU et al., 2016). **N** is a polyene antifungal that acts by binding ergosterol and disrupts the major lipidic component of the fungal cell membrane resulting in the formation of porin channels. These pores disrupt the integrity of the fungal plasma membrane allowing the efflux of cations, such as K+, and leads to the leakage of cytosolic components which results in cell death, thus are fungicidal drugs (NIIMI; FIRTH; CANNON, 2010; SPAMPINATO; LEONARDI, 2013). Although **N** has excellent therapeutic effectiveness, some disadvantages related to the use of nystatin include: bad taste, drug interation, gastrointestinal side effects such as nausea, vomit and epigastric pain, especially in childrens or elder people. In addition, a relatively high cost of medication and the administration of doses four times per day contribute to low drug adherence (MARTINSON et al., 2009; BAKHSHI et al., 2012; KOVAC; MITIC; KOVAC, 2012; MANSOURIAN et al., 2014; MUKHERJEE et al., 2017).

Although oral Nystatin is the major antifungal agent used to treat oral Candidiasis in HIV-infected patient in resource-limited settings, studies have been conducted to identify lower-cost alternatives to replace the current treatment. Mukherjee et al. conducted a preclinical study with topical gentian violet which has anti-*Candida* potential and has been recommended by the World Health Organization (ORGANIZATION, 2014). This topical treatment showed no statistical differences with Nystatin, besides presenting a substantially lower acquisition cost than Nystatin (MUKHERJEE et al., 2017) .

Resistance of *Candida* strains to polyenes such as Nystatin and Amphotericin B is rare, having been found a nystatin resistance of 11.3% for non-*Candida albicans* species (MOHAMADI; MOTAGHI, 2014) . A study showed a lower susceptibility to Nystatin and Fluconazole in 9 clinical isolates when compared with the reference strain of *C. albicans* ATCC 90028 (SARDI et al., 2016) .

The resistance mechanism is probably due to loss of function of ERG6 or ERG3 gene through mutation, and involved in ergosterol biosynthesis, leading to the low content of ergosterol membrane detected in some resistant fungi (FICHTENBAUM et al., 2000; KANAFANI; PERFECT, 2008; NIIMI et al., 2010; SPAMPINATO; LEONARDI, 2013; MANSOURIAN et al., 2014). However, azole-resistant *C. albicans* is often found in HIV-infected patients with oropharyngeal candidiasis, resulting in cases of refractory candidiasis or therapeutic failure (SKIEST et al., 2007; SPAMPINATO; LEONARDI, 2013). Another point is that there are few potential targets of action to be explored in fungi that are not shared with human cells, both eukaryotes (ROCHA, 2002; CROSARIOL, 2010; DENNING; HOPE, 2010). For these reason leads us to find new strategies for *Candida*-related infections. Antifungals usually present some toxicity, since there are few targets of action in fungi that are not shared with human cells, both eukaryotes

Medicinal plants, used in traditional medicine, have been considered a valuable source of antimicrobial agents, which conjugated to antifungal formulations traditionally used in the clinic, intensify its antimicrobial activity against resistant strains (NASCIMENTO et al., 2000; ALAVARCE et al., 2015; DA SILVA et al., 2017). This interaction between drugs is known as synergism (ENDO et al., 2010; MERTAS et al., 2015). Therapies that use the combination of drugs can increase the action spectrum, improve the antifungal activity and reduce the associated side effects (SUN et al., 2017).

There are several mechanisms proposed for antifungal synergy through the drugs combination: (1) One type of interaction is inhibition of different stages of the same biochemical pathway. (2) Changes in the cell wall or cell membrane permeability promoted by an antifungal agent resulting in increased penetration of other antifungal agent. (3) Inhibition of carrier proteins. (4) Inhibition of different targets in fungal cells simultaneously, as targets of the cell wall and cell membrane (JOHNSON et al., 2004).

Punica granatum (*P. granatum*) is a fruit belongs to the *Lythraceae* family, popularly called "pomegranate" and a potential medicinal plant. Since ancient times, *P. granatum* has been used for the treatment of various diseases. Recently, the plant has attracted increasing interest of researchers in analyzing its composition and biological properties. As referred their antimicrobial properties, inhibits the growth of methicillin resistant *Staphylococcus aureus* (MRSA), *Listeria monocytogenes, Yersinia enterocolitica, Escherichia coli, Human influenza* H3N2 and *C. albicans* (AJAIKUMAR et al., 2005; SEERAM et al., 2005; ADAMS et al., 2006; LANSKY; NEWMAN, 2007; ALTHUNIBAT et al., 2010; ENDO et al., 2010; GLAZER et al., 2012; MANSOURIAN et al., 2014). Almeida et al. (2018) used the *P. granatum* hydroethanolic extract associated with a denture adhesive that resulted in the significant interference of the development of *C. albicans* biofilms formed on thermopolymerizable acrylic resin specimens. Another study showed the use of a gel containing the extract of *P. granatum* as an antifungal agent and demonstrated promising effects for the clinical treatment of denture stomatitis (CÉSAR DE SOUZA VASCONCELOS et al., 2003).

The ellagitannins are the main polyphenols present in the *P. granatum*. Compounds such as granatins A and B, punicalagin and punicalin were isolated from the pericarp and are the main compounds responsible for the antimicrobial activity. Chemical analysis performed in one study showed that pomegranate phenolic compounds showed high levels of hydrolyzable tannins such as punicalin, punicalagin, pedunculagin and punigluconin (CATÃO et al., 2006; DUDONNÉ et al., 2009).

A therapeutic option to be used in combination with **N** is Punicalagin (P), an ellagitannin isolated from *P. granatum*, and one of the main components responsible for its antifungal activity. *In vitro* studies has previously been demonstrated the synergistic antifungal effect between **P** and commercially available Fluconazole using

C. albicans, suggesting that the use of **P** in combination with other antifungals, such as **N** may be an interesting therapeutic strategy for the treatment of oral candidiasis (CATÃO et al., 2006; ENDO et al., 2010; ANIBAL et al., 2013).

Currently, elucidating the molecular mechanisms related to the ability of the *Candida* species to cause infections may be important for the development of new drugs that have as target virulence factors of the pathogen and less undesirable effects to the patients.

Many factors have been involved in the increase of CA pathogenicity, including production of phospholipases, adhesins (ALS Family), hyphae formation, expression of drug resistance genes and production of secreted aspartyl proteinases (SAP) (RÜCHEL et al., 1992; HUBE, 1996; CHAFFIN et al., 1998; WU et al., 2000). The use of Real-time reverse transcriptase–polymerase chain reaction (RT–qPCR) for the amplification of specific mRNA allows the study of the gene expression of these virulence factors.

Studies have shown that the development of mutant strains, whose genes encode proteins responsible for energy metabolism, stress response and biosynthesis of macromolecules that are essential for the fungus, have virulence and consequently attenuated host damage. Thus, investigating which proteins are exclusively or differentially expressed after antifungal treatments contribute to the understanding of several mechanisms involved in *Candida* species pathogenicity (TSANG; BANDARA; FONG, 2012; AOKI et al., 2013).

Here we aim to determine *in vitro* the antifungal activity of Punicalagin and Nystatin, alone and in combination, against two strains of CA associated with oral infections. The discovery of new antimicrobial components is of great relevance, particularly for Dentistry, since infections of the oral cavity of bacterial and fungal origin are relatively common problems, resulting in chronic inflammatory diseases such as *Candida*-associated denture stomatitis.

2 Articles

2 ARTICLES

To achieve both aims of this thesis, the articles presented were written according to Journal of Natural Products and International Journal of Medical Microbiology guidelines, respectively.

- ARTICLE 1 – *In vitro* Antifungal activity of Punicalagin – Nystatin Combinations against *C. albicans* associated with Oral candidiasis.

- ARTICLE 2 - Antifungal activity of Punicalagin and Nystatin used in combination against *Candida albicans*: detection of virulence genes and proteomic analysis.

2.1 Article 1 – In vitro Antifungal activity of Punicalagin – Nystatin Combinations against *C. albicans* associated with Oral candidiasis

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ABSTRACT: Despite therapeutic advances, opportunistic fungal infectious diseases have increased in prevalence and have become a universal public health problem. *Candida albicans* (*C. albicans*) is a commensal fungus that under certain environmental conditions can act as an opportunistic pathogen and colonize mucous membranes and tissues causing local and systemic infections. The emergence of drug resistant strains, as well as the increase of immunosuppressed patients, has limited therapeutic options. The antifungal activity of ellagitannin isolated from *Punica granatum,* Punicalagin **(1)**, alone or in combination with Nystatin **(2)**, was determined against two strains of *C. albicans*. Cytotoxicity assays involving **1** and **2**, as well as the combination of both, were undertaken on human primary oral keratinocytes and gingival fibroblasts cultures. Minimal Inhibitory Concentration (MIC) and Minimum Microbicidal Concentration (MMC) of **1** were significantly reduced in the presence of **2**, indicating synergism between the two drugs, and this behavior was also observed in cytotoxicity assays. The results of this *in vitro* study suggest that the combination of drugs, at the concentrations tested, increases its therapeutic efficacy against strains of *C. albicans,* decreases cytotoxicity on human cells and may be a viable alternative for clinical use.

Infectious diseases caused by opportunistic fungi have grown abruptly, becoming a public health problem. The high rates of morbidity and mortality associated with these diseases, the increase in the number of immunocompromised patients and the emergence of conventional antifungal resistant strains along with the adverse effects of current therapy, show the importance of the discovery of new drugs.[1-3]

Candida albicans is a commensal fungus and the most common opportunistic pathogen in humans. Colonizes mucosal and abiotic surfaces, this fungus can cause local and systemic infections, from candidiasis to candidemia.[4-6] Oral candidiasis is the most common fungal infection in humans, especially in immunocompromised patients, as well as in the elderly and users of removable total dentures, in this case known as denture stomatitis (DS), clinically manifested as spots or erythematous areas in the palate region.[6-9]

Currently, conventional treatments for DS include topical application of **2**, chlorhexidine or miconazole, prior to the use of systemic antifungal drugs. **2** is a polyene antifungal that acts by binding ergosterol and increasing fungal membrane permeability, thus affecting the integrity of the cell wall, and causes leakage of intracellular contents and cell death.[10-12] Although **2** has excellent therapeutic effectiveness, some disadvantages related to the use of nystatin include: bad taste, gastrointestinal side effects such as nausea, vomit and epigastric pain, especially in childrens or elder people. In addition, a relatively high cost of medication and the administration of doses four times per day contribute to low drug adherence. [13-15]

For this reason, medicinal plants, used in traditional medicine, have been considered a valuable source of antimicrobial agents, added to antifungal formulations traditionally used in the clinic.[16] Studies show that these combinations may increase their antimicrobial activity against resistant strains.[17, 18] This interaction between drugs is known as synergism, in which the combination of drugs can increase the spectrum of action, improve antifungal activity and reduce associated side effects.[19]

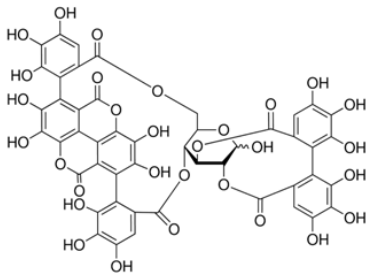
Among these medicinal plants, a therapeutic option to be used in combination with **2** is Punicalagin **(1)**, an ellagitannin isolated from *Punica granatum (Lythraceae)*, and one of the main components responsible for its antifungal activity.[20]

In vitro studies has previously been demonstrated the synergistic antifungal effect between **1** and commercially available Fluconazole using *C. albicans*, suggesting that the use of **1** in combination with other antifungals, such as **2** may be an interesting therapeutic strategy for the treatment of oral candidiasis.[17, 20, 21]

Here we aim to determine in vitro the antifungal activity of Punicalagin and Nystatin, alone and in combination, against two strains of *C. albicans* associated with oral infections.

Chart 1

1



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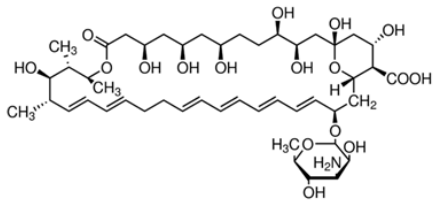

RESULTS AND DISCUSSION

The MIC values of **1** and **2** against *C. albicans* ATCC 90028 and SC 5314 are shown in Table 1. The MIC of the compounds tested alone was 50 µg/mL **(1)** and 3.9 µg/mL **(2)**. When combined, **1** and **2** demonstrated increased antifungal efficacy, with reduction of their MICs. Fungal metabolism varied from 2.02 to 31.5% for the ATCC strain (6.25:3.9; 6.25:1.95) (Figure 1) and 3.46 to 17.2% for strain SC (50:3.9; 6.25:1.95) (Figure 2). Inhibitory concentration indexes suggest that synergism was produced in one of the combinations (FICI $\leq$ 0.5) and partial synergism was produced in three combinations (FICI> 0.5-1). The indifferent effect was produced in 4 combinations (FICI ≥ 1 and <4) and no antagonism was detected (FICI ≥ 4) for both strains tested (Table 1). Combinations of drugs that produce synergy or partial synergy may potentially reduce toxicity and improve outcome for patients with difficult-to-treat infections.[22]

Table 1: MIC of Punicalagin, Nystatin and in Combination, and their combined FIC index values (FICIs), for the two strains tested (using the Checkerboard method).

Punicalagin [A] (µg/mL) *C. albicans* **ATCC 90028 /SC 5314**			**Nystatin [B] (µg/mL)** *C. albicans* **ATCC 90028 /SC 5314**			**FICIs[b]**	**Combinations (1+2)**
MIC[a] single	$MIC_{combination}$ **(1+2)**	FIC[A]	MIC^{a}_{single}	$MIC_{combination}$ **(2+1)**	FIC[B]		
50 (1)	50	1.0	3.9 **(2)**	3.9	1.0	2.0 I[e]	50:3.9
	6.25	0.125		0.975	0.25	0.375 S[c]	6.25:0.975
	6.25	0.125		1.95	0.5	0.625 PS[d]	6.25:1.95
	6.25	0.125		3.9	1.0	1.125 I[e]	6.25:3.9
	12.5	0.25		1.95	0.5	0.75 PS[d]	12.5:1.95
	12.5	0.25		3.9	1.0	1.25 I[e]	12.5:3.9
	25	0.5		0.975	0.25	0.75 PS[d]	25:0.975
	25	0.5		1.95	0.5	1.0 I[e]	25:1.95

[a] The MIC was defined as the lowest concentration causing prominent growth reduction (≥50% reduction in the metabolic activity). [b] FICI = FIC[A] +FIC[B] = (MIC[A] combination/MIC[A] single) + (MIC[B] combination/MIC[B] single). [c] S, synergistic interaction when FICI ≤ 0.5. [d] PS, partial synergism when FICI values between 0,5 - 1,0, [e] I, indifference when ≥ 1 to <4.

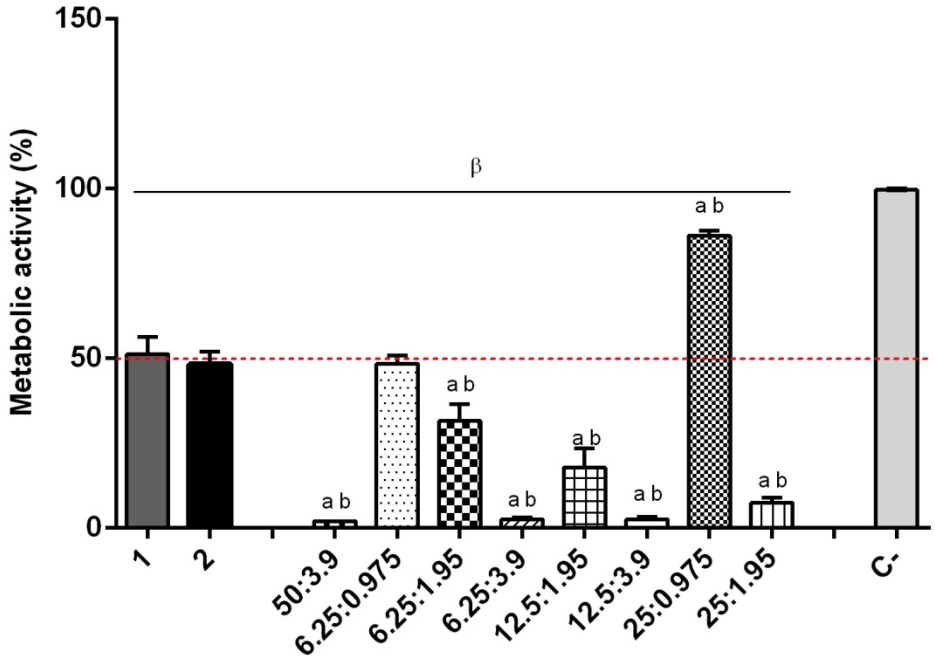

Figure 1: Cell metabolism activity (%) of *C. albicans* ATCC 90028, after 24 h in contact with Punicalagin **(1)**, Nystatin **(2)** and in Combination. The percentage of metabolism was calculated from the respective negative control (culture medium). Statistical difference: β *vs.* C-, a *vs.* **1**, b *vs.* **2**. Dotted line represents 50% of fungal metabolism. Representative results of three experiments in six-fold; *Anova One-Way and Tukey Test.*

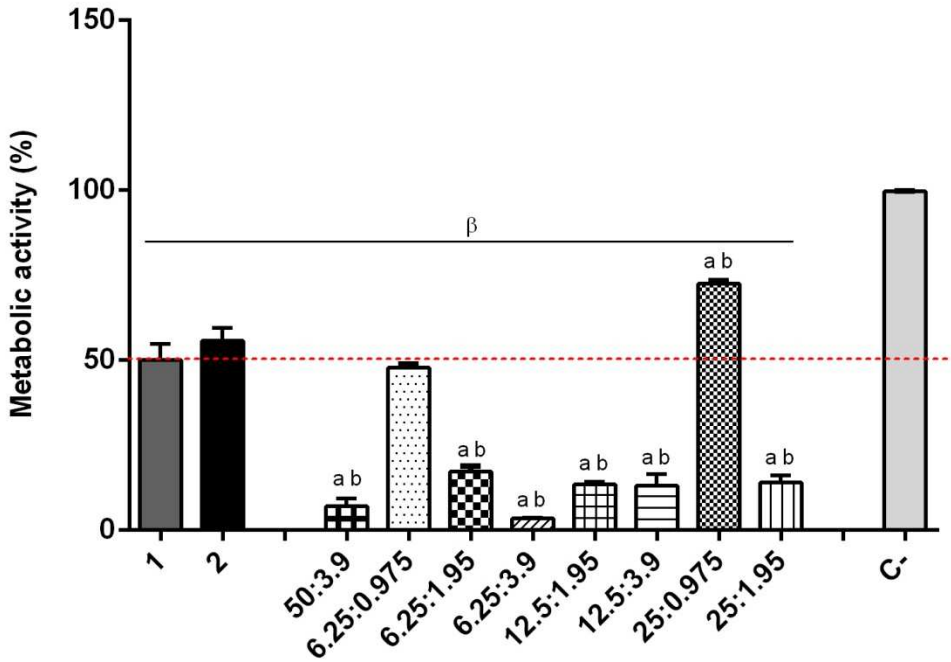

Figure 2: Cell metabolism activity (%) of *C. albicans* SC 5314, after 24 h in contact with Punicalagin **(1)**, Nystatin **(2)** and in Combination. The percentage of metabolism was calculated from the respective negative control (culture medium). Statistical difference: β *vs.* C-, a *vs.* **1**, b *vs.* **2**. Dotted line represents 50% of fungal metabolism. Representative results of three experiments in six-fold; *Anova One-Way and Tukey Test.*

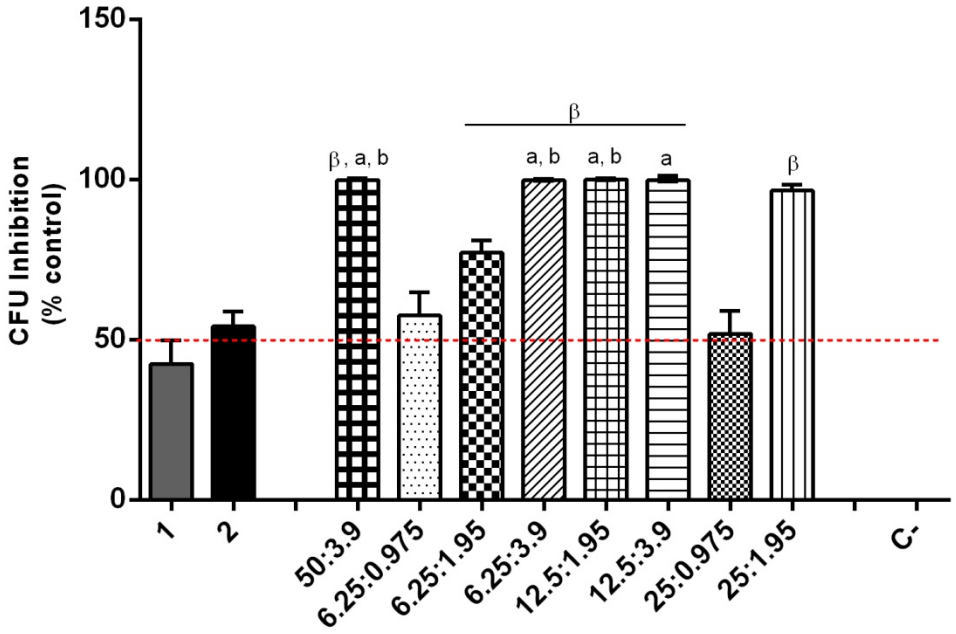

Figure 3. Analysis of the effect of Punicalagin **(1)**, Nystatin **(2)** and in Combination, after 24h, on colony-forming unit (CFU/mL) inhibition (%) of *C. albicans* ATCC 90028. The percent inhibition was calculated from the respective control (culture medium). Statistical difference: β *vs.* C-, a *vs.* **1**, b *vs.* **2**. Dotted line represents 50% of fungal inhibition. Representative results of three experiments in triplicate; *Kruskal-Wallis, Dunn's Test.*

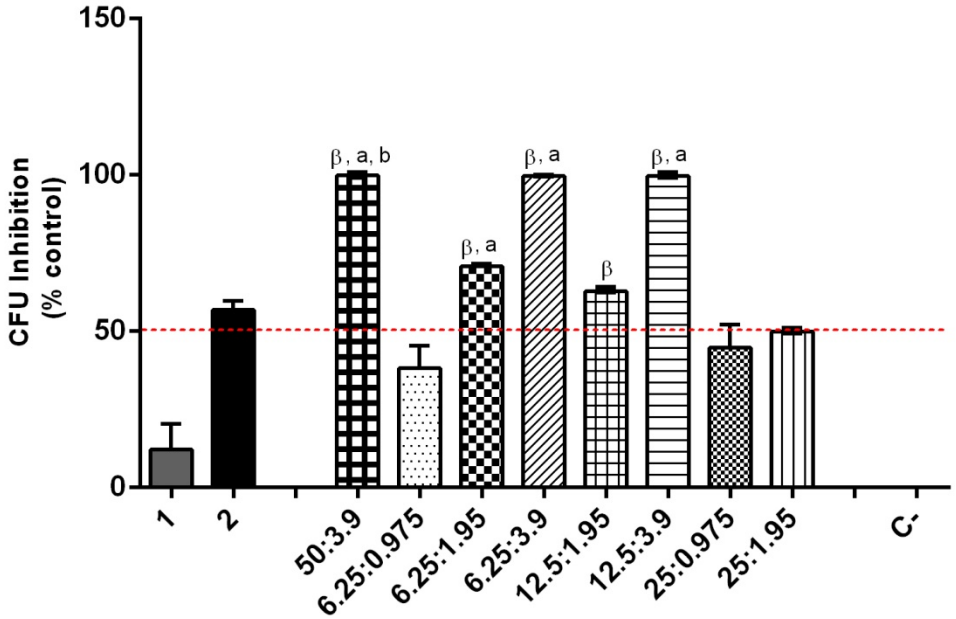

Figure 4. Analysis of the effect of Punicalagin **(1)**, Nystatin **(2)** and in Combination, after 24h, on colony-forming unit (CFU/mL) inhibition (%) of *C. albicans* SC 5314. The percent inhibition was calculated from the respective control (culture medium). Statistical difference: β *vs.* C-, a *vs.* **1**, b *vs.* **2**. Dotted line represents 50% of fungal inhibition. Representative results of three experiments in triplicate; *Kruskal-Wallis, Dunn's Test.*

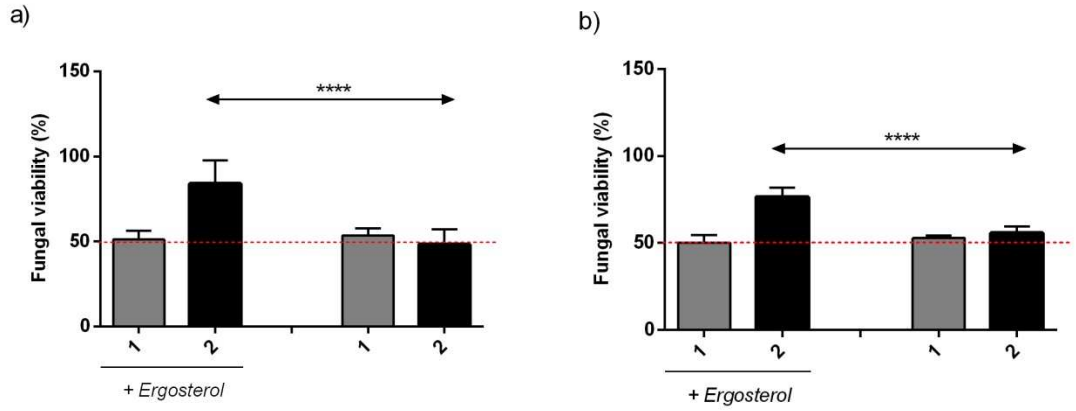

Figure 5. Analysis of the effect of exogenous ergosterol on the MIC of Punicalagin **(1)** and Nystatin **(2)** on *C. albicans* ATCC 90028 (A) or *C. albicans* SC 5314 (B). Arrows represent statistical difference between groups. Representative results of three independent experiments in triplicate. *Anova One-Way, Tukey Test.*

A significant increase in the fungicidal efficacy of **1** and **2** was observed in the *C. albicans* ATCC 90028 colonies when used in combination, ranging from 96.60 to 100.03%, compared to drugs used alone, which were ineffective as fungicidal agents (**1** = 42.5% and **2** = 54.18%). The 6.25:0.975 and 6.25:1.95 combinations also resulted in significantly higher inhibition values (CFU/mL) in relation to drugs alone (57.5 and 77. 15%, respectively) (Figure 3). The same behavior was observed in the *C. albicans* strain SC 5314, where the association of the drugs also significantly increased the inhibition of colony growth, presenting values of 99.9% inhibition (50:3.9) compared to drugs used alone, which also did not show efficacy as fungicidal agents (**1** = 12.2 and **2** = 56.8%). For this strain, the combinations (6.25:1.95; 6.25:3.9; 12.5:3.9) presented inhibition values higher than that obtained with the drugs alone, ranging from 70.9 to 99.8%, although they did not present statistical difference in relation to **2** (Figure 4).

These results demonstrate that the combination of the drugs has fungicidal efficacy, since it was effective in reducing the CFU/mL in the two strains tested. The concentrations that inhibited approximately 100% of fungal growth and which could be recommended for further testing are 6.25: 3.9 and 12.5: 3.9 for both strains tested. These results are related to fungal growth in the planktonic form and not in the biofilm form. Susceptibility studies have revealed that biofilms formed by *C. albicans* can be up to 2000 times more resistant to antifungal drugs than their planktonic counterparts [23-26]. Several factors, such as the presence of extracellular

matrix, expression of resistance genes, presence of persistent cells and altered metabolic rate of biofilm cells, contribute directly to drug resistance. The extracellular matrix plays a vital role in reducing drug penetration into cells and induces drug efflux activities. Secondarily, cell density also contributes to drugs resistance by biofilms. [27-29] For studies involving *C. albicans biofilms*, further trials using a range of drug concentrations should be performed.

Synergism was observed only in one combination (6.25:0.975) while partial synergism was observed in 3 combinations (6.25:1.95; 12.5:1.95; 25:0.975). These results were evaluated through the arithmetic calculus and the interpretation of the FIC index, according to studies previously published in the literature.[30-32] However, there is still great controversy in the literature regarding the interpretation of these data, since several criteria can be used to evaluate the interaction between drugs. Some authors classify interactions only as synergism, indifferent effect and antagonism,[33, 34] while others include the additive effect and do not use the indifferent effect.[35, 36] Other authors also use the weak synergistic effect and the partial synergistic effect with different scales between them.[37] In this study we considered the classification that contemplates the partially synergistic behavior,[22, 30-32] since we observed, in the 6.25:1.95 and 12.5:1.95 combinations, an inhibitory effect greater than the sum of the effects of the individual drugs tested and not an activity equal to the effect predicted by drugs used alone, a behavior that is called additive effect.[38] In our study, we confirmed this premise, where the combination classified as synergism was the one that presented the greatest decrease in MIC, from 8 times to **1** and 4 times to **2**. The combinations 6.25:1.95, 12.5:1.95 and 25:0.975, classified as partial synergism, presented reductions in MIC, but were not the highest reductions observed in the combination, in this case, the lowest MIC of Punicalagin (6.25) combined with MIC of Nystatin 2 × reduced, thus presenting higher values of FICI. The smaller MIC decreases presented higher values of FICI and, therefore, they received an indifferent classification, although 6.25:3.9 and 50:3.9 resulted in greater antifungal effects than drugs alone (**1** and **2**).

Although FICI is the parameter most used to define the interaction between drugs, there are some disadvantages when studying drugs that act against filamentous fungi, such as *C. albicans*, since this index was stipulated for analysis of the interaction of antibiotics. One of the disadvantages is related to the MIC endpoint determination, since the antifungal drugs may have different endpoints for reading

the MIC. This was observed in the study by Te Dorsthorst et al. (2002) and NCCLS document M38-A2,[36, 39] which describe the score 0 for the antifungal Amphotericin B and score 2 for azole, such as Fluconazole and Ketoconazole, which has less defined endpoints than Amphotericin B, for example. However, the advantage of using the Checkerboard method is that it is the most commonly used method, and the data produced can easily be compared with previously published studies.[40]

Endo et al. (2010) demonstrated by means of electromicrography the antifungal activity and the synergism of the combination of **1** with Fluconazole, having observed changes in fungus structure, such as thickening of the cell wall, alterations in the space between the wall and the cell membrane, as well as appearance of vacuoles and reduction of cytoplasmic content.[17] Similarly, we suggest that through the action of **2**, already well known to act by binding ergosterol of the fungi membrane, there was an increase in the permeability of the fungal cells, facilitating the penetration of **1** in the cells, allowing a significant improvement of the efficacy antifungal compared to the drugs alone or in lower concentrations.

Our results demonstrated that the antifungal property of **1**, unlike **2**, is not related to a direct ergosterol binding in the cell membrane of the fungus. In Figure 5, we can see that there was an increase in fungal viability in the presence of exogenous ergosterol in the **2** group, that is, exogenous ergosterol promoted an increase in MIC, demonstrating that the drug interacts with the exogenous ergosterol, keeping the fungal cells viable, leaving to interact with endogenous ergosterol. For **1**, the same phenomenon was not evidenced, since there was no change in cellular viability in the presence or absence of exogenous ergosterol.

The mechanism of action of **1** on cells of different microorganisms was also analyzed by other authors. Xu et al. (2017), evaluated this mechanism on cultures of *Staphylococcus aureus* where a remarkable inhibitory effect on biofilm, an increase in K + ions efflux and, based on morphological analysis, a significant shrinkage of the cytoplasm with membrane damage, however, without cell lysis.[41] Another author proposed different mechanisms for tannins, which include **1**, such as: (i) inhibition of extracellular microbial enzymes; (ii) deprivation of substrates and metal ions necessary for microbial growth and (iii) direct action on microbial metabolism through the inhibition of oxidative phosphorylation.[42] Furthermore, Haslam (1996) proposed that tannins are capable of complexing with other molecules, including macromolecules, such as proteins and polysaccharides leading to cell death.[21, 43]

The studies cited above found that morphological changes for the most part related to fungal cell wall, in this way, we suggest that the Punicalagin mechanism of action may be related to the fungal cell wall, similar to what occurs with the echinocandins. [44, 45] In order to confirm this premise, assays such as the sorbitol test and others that evaluate the morphological characteristics, such as scanning electron microscopy are required.

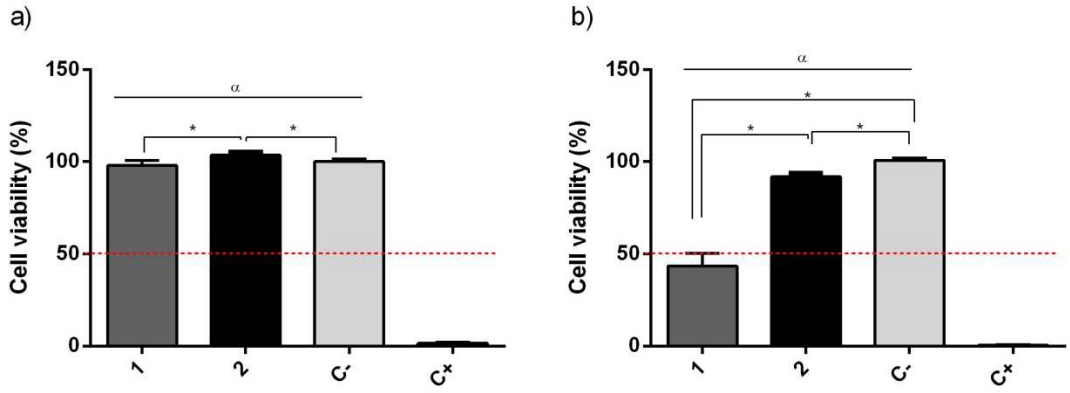

Figure 6: Cell viability (%) of selected concentrations of Punicalagin (**1**) and Nystatin (**2**). (A) Human gingival fibroblasts (HGF). (B) Human palate epithelial cells (HPEC). Statistical difference: (α) *vs.* C + and (*) between groups. Dotted line represents 50% viability. Three independent experiments in triplicate. *Anova one-way, Tukey*.

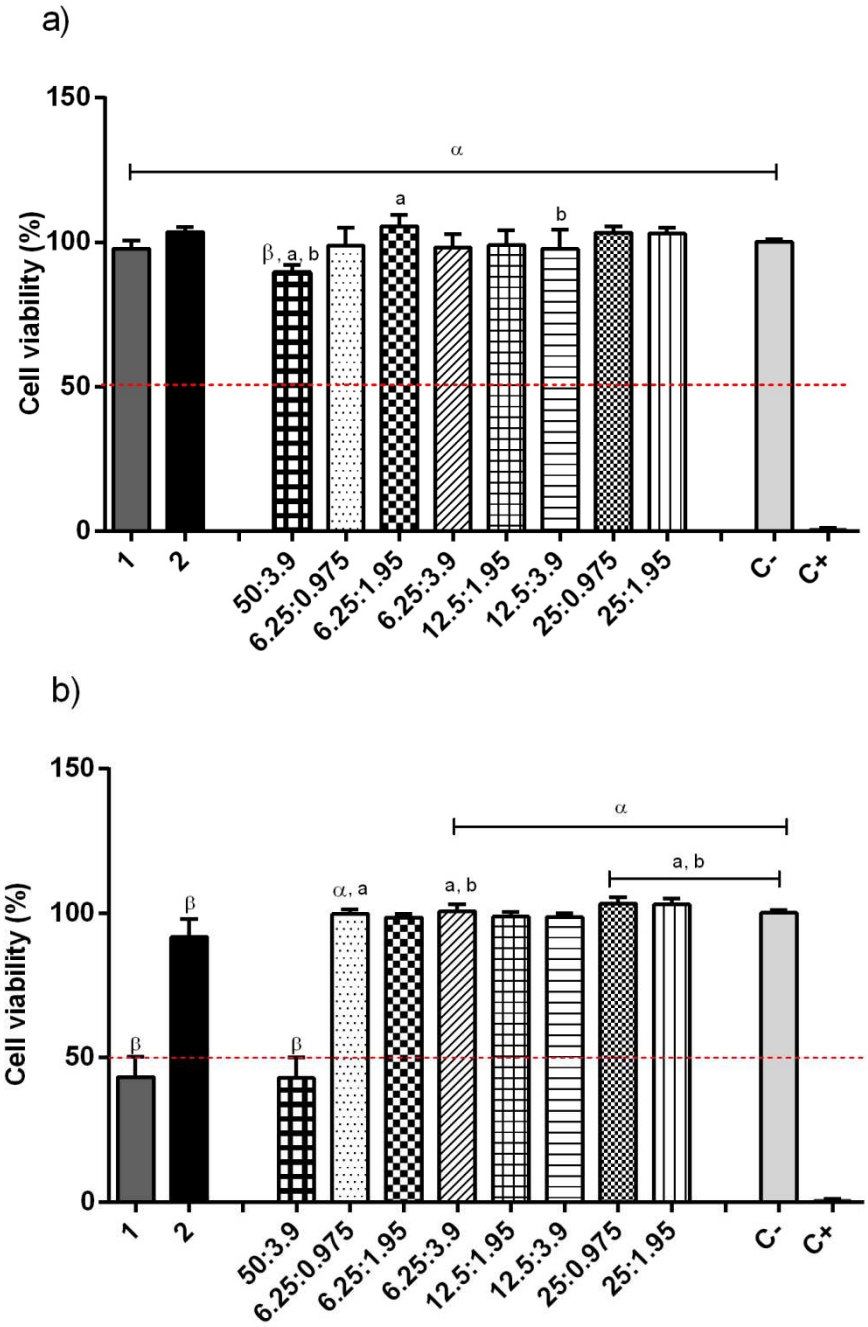

Figure 7. Cell viability (%) of the drugs used alone, Punicalagin (**1**) and Nystatin (**2**), and in Combination. A) Human gingival fibroblasts (HGF). B) Human palate epithelial cells (HPEC). Statistical difference: β *vs.* C-, α *vs.* C +, a *vs.* P, b *vs.* N. Dotted line represents 50% viability. Three independent experiments in triplicate. *Anova one-way, tukey test and Kruskal-wallis, Dunn's test*, respectively.

With respect to the cytotoxicity of the drugs when used alone, **2** presented very low cytotoxicity (8.14% of viability reduction) on HPEC cultures, whereas **1** showed to be very cytotoxic (56.6% viability reduction) (Figure 6B). For HGF cultures, both drugs alone had good viability levels with no viability reduction in **2** and 2.1% reduction in **1** (Figure 6A). In our cytotoxicity assays, HPECs have always shown greater sensitivity

to drugs than HGF, which has also been shown in the literature.[46] However, **1** cytotoxicity was significantly reduced when used in combination with **2**, allowing an increase in HPEC cell viability of 43.4% **(1)** (Figure 6B) for values ranging from 98.6 to 103.1% (Figure 7B). Although the cytotoxicity in combination is reduced in keratinocytes, it is increased in comparison to fungal cells, which is an essential prerequisite for its use in topical therapies.

In conclusion, the antimicrobial synergism produced between **1** and **2** suggests that the combination of the two compounds at the concentrations tested may be a good topical alternative for the treatment of superficial oral candidiasis, such as denture stomatitis. Combined treatment of natural products and conventional drugs is one of the effective treatments against *Candida* species. This therapeutic strategy has been shown to improve drug efficacy, decrease toxicity, side effects and microbial resistance problems.[47-49] Although, with the results of this study, we cannot accurately prove the mechanism of action by which **1** acts, we know that it acts by altering the morphology and intracellular microbial content, affecting the metabolism and the rate of fungal growth. The combination with drugs such as Nystatin might facilitate the entry of **1** through cell membrane and thereby potentiate its antifungal activity.[17] Therefore, the combination of drugs encourages the development of new antifungal therapies, due to the multiple targets that can be achieved by the use of two or more drugs, especially when involving compounds extracted from natural products, and clinical studies are necessary to prove the effectiveness of this association.

EXPERIMENTAL SECTION

Fungal strains and Media. In all experiments, we used the standard strain of the fungus *C. albicans* ATCC 90028, purchased from American Type Culture Collection (ATCC), University Boulevard Manassas (VA, USA), and a clinical strain SC5314, provided by Dr. Arnaldo Lopes Colombo (Special Laboratory of Mycology, Federal University of São Paulo, Brazil). The strains were cultured in YEPD culture medium (1 liter: 10g yeast extract, 10g peptone, 20g dextrose, Acumedia®) and incubated at 30° C for 36 h in incubator (Fanem®, Guarulhos, Brazil). For agar plates, 2.5% (w/v) Bacto agar (BD, Sparks, MD, USA) was added to the medium. Both strains were stored in 20% glycerol at -80° C. Prior to each experiment, the cells were reactivated

on YEPD agar plates.

Reagents. Chemicals such as Nystatin, Punicalagin (≥98% purity (HPLC), from pomegranate, cat. no. P0023-10 mg), XTT, Menadione, Ergosterol and reagents for human oral keratinocyte culture were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All routine reagents were obtained from Thermofisher Scientific (Grand Island, NY, USA).

In vitro antifungal assays. Minimum inhibitory concentrations of **1** and **2** were determined, alone and in combination, against strains of *C. albicans* ATCC 90028 and SC5314. The concentrations of **1** and **2** used were selected based on MIC values previously determined by the broth microdilution assay.[39] Separate 96-well plates were inoculated with 100 µL of Saboraud dextrose culture medium (Difco®, Detroit, Michigan, USA) containing 2.5×10^3 colony forming units/mL (CFU/mL) [50] and 100 µL of the concentrations of **1** (1000, 500, 250, 125 and 50 µg/mL) and **2** (15.6, 7.8, 3.9, 1.95 µg/mL). MIC values were visually determined as the lowest concentration of agents causing ≥50% inhibition of growth over the control group (Culture Medium) (Numerical Scale 2) (data not shown).[50] The Checkerboard Assay, which compares in vitro the efficacy of the combination of two or more antimicrobials by determining the fractional inhibitory concentration index, was performed according to Castro et al. (2015).[51] The fraction inhibitory concentration index (FICindex) was calculated and interpreted as follows: FIC^A = MIC of compound A combined / MIC of compound A alone, while FIC^B = MIC of compound B combined / MIC of compound B alone and FICIs = $FIC^A + FIC^B$. Finally, the values of FICIs were considered: ≤0.5 - synergism; 0.5 to 1 - partial synergism; ≥1 to <4 - indifferent (no interaction) and ≥ 4 - antagonism.[22, 30-32]

Initially, 56 combinations of **1** and **2** were tested. Of these, 8 combinations were selected (Table 1) which resulted in reduction of fungal metabolism without altering the cellular viability of keratinocytes (through the Cytotoxicity Assay). Later, the susceptibility of *C. albicans* to drugs at 8 concentrations was assessed by the metabolic reduction assay XTT (2,3-Bis (2-Methoxy-4-Nitro-5-Sulfophenyl) -5-[(Phenyl-Amino) Carbonyl] -2H-Tetrazolium Hydroxide).[52-54] After inoculation and 24-hour fungal growth, 200 µL of the drug-mediated media (Sabouraud Broth - Difco®,

Detroit, Michigan, USA) were added to the wells of the 96-well culture plates where they remained incubated at 37°C for 24 h. After this time, the wells were washed with PBS 1× and then incubated for a further 4h with the XTT solution (0.25 mg/mL) and menadione (25 mM). Conversion of XTT was measured using a spectrophotometer (Synergy Mx Monochromator-Based Biotek®) at 550 nm. MIC for growth was defined as the lowest concentration of drug that caused ≥ 50% reduction in relative metabolic activity as compared to control (Culture medium).[55] The inhibitory effect of the drugs on metabolism was calculated using the formula: % reduction = (A*well* – A*background*) / (A*drug free well* – A*background*) × 100, in which the background was measured from the blank and (A) refers to absorbance.[11, 50, 56, 57]

The Minimum Microbicidal Concentrations (MMC) of drugs used alone and in combination was determined relative to the two strains tested. After 24 h of fungal growth, the media conditioned with the drugs at the 8 concentrations were added to the 96-well culture plates. After 24 h of exposure, the samples were serially diluted (10^{-1} and 10^{-2}) and plated on Sabouraud Dextrose agar plates containing 1% chloramphenicol. The inoculated plates were incubated at 37 ° C for 48 h before counting the colonies (CFU/mL).

Inhibition percentages were calculated by the formula CFU/mL = number of colonies $\times 10^{n}/q$, where n equals the absolute value of the dilution and q is the quantity of plated suspension in mL for each dilution. The data were ploted using the GraphPad Prisma 6 software.

Once the antifungal potential of **1** was verified, we proposed to evaluate whether this drug acts through the same mechanism of action of **2**, altering the permeability of the cell membrane of the fungus (binding ergosterol). The assay was performed using the minimal inhibitory concentration (MIC) established for both drugs alone, **1** and **2**, using the XTT protocol, in triplicate, in the presence and absence of exogenous ergosterol.

The media conditioned separately with the drugs were added to each well of 96-well culture plates and inoculated with the two *C. albicans* strains at the concentration 2.5 × 10^{3}/well supplemented or not with ergosterol at the concentration of 400 µg/mL. Wells containing only Sabouraud culture media, supplemented or not with ergosterol (negative control – C-) and wells containing ethyl alcohol PA (ergosterol solvent) (Internal Negative Control) were also used. The plates were incubated at 35 ° C for 48 h.[51]

Human cell toxicity test. The cytotoxicity assay was performed using the Alamarblue fluorimetric reagent (Thermofisher, Grand Island, NY, USA), following the manufacturer's guidelines. Human palate epithelial cells/ keratinocytes (HPEC) and human gingival fibroblasts (HGF) were established from biopsies obtained from healthy volunteers during routine surgical procedures (Bauru Dental School - University of São Paulo). This protocol experimental study was previously approved by the ethics committee of the Bauru School of Dentistry - USP (CAAE, 44951715.6.0000.5417). HPEC were cultured as previously described by Klingbeil et al. (2012).[58] HGF were cultured in Dulbecco's Modified Eagles Medium (DMEM) containing 10% FBS and penicillin/streptomycin (100 IU/mL / 100 µg/mL). Both cells were plated at 1×10^4 cells/well concentration in 96-well culture plates and after 24 h of adherence, the corresponding culture media were removed and replaced by the drug-conditioned media and incubated in an humidified incubator at 37 ° C (5% CO2/95% air – Shel Lab®, Cornelius, OR, USA) for 24 h. The concentrations used were based on pilot studies (broth microdilution) that demonstrated the antifungal action from the concentration of 50 µg / mL **(1)**. For Nystatin **(2)**, the established concentration was 3.9 µg/mL (MIC - data not shown).[59]
Subsequently, cell viability was assessed with the combination of drugs (Table 1). Control wells received water (C +) or culture medium (C-) to evaluate the effectiveness of the assay. After 24 h, the contents of the wells were replaced with the Alamarblue reagent and incubated again at 37°C for 4 h. After the period, resofurin was detected in Synergy Mx fluorimeter (Biotek Instruments, Winooski, Vermont, USA) with emission/excitation of 488/600 nm. The viability percentage was calculated and plotted using the GraphPad Prisma 6 software.

Statistical Analyzes. The parametric quantitative results were presented as mean ± standard deviation (SD) and submitted to *ANOVA One-Way Analysis of Variance*, followed by comparative analysis by the *Tukey HSD Test and Two-Way ANOVA Variance Analysis*, followed by the comparative analysis by the *Test of Tukey HSD*. The non-parametric quantitative results were presented as median ± standard deviation (SD) and were submitted to *the Kruskal-wallis* test, followed by the comparative analysis by the *Dunn's Test*. All experiments were performed in triplicate and a minimum of 3 independent experiments. A significance level of 5% was used for all tests.

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Notes

The authors declare no competing financial interest.

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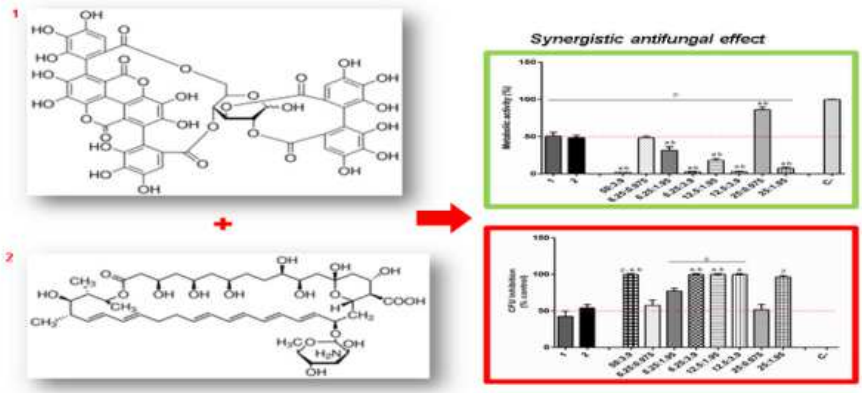
TOC graphic



2.2 Article 2 - Antifungal activity of Punicalagin and Nystatin used in combination against *Candida albicans*: detection of virulence genes and proteomic analysis

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Abstract

Candida albicans (CA) is a commensal fungus and the most common opportunistic pathogen in humans. Colonizing mucosal and abiotic surfaces, this fungus can cause local and systemic infections, from candidiasis to invasive candidemia. The high rates of morbidity and mortality associated with these diseases, the increase in immunocompromised patients, and appearance of resistant strains to the conventional antifungal agents, together with the adverse effects of current therapy, lead to the discovery of new drugs. The combination of drugs such as Nystatin (N) and Punicalagin (P) corresponds to a therapeutic strategy that improves antifungal activity. The understanding of molecular aspects affected by the combination of these drugs is important to clarify the mechanisms involved in the antifungal action and if fungal mechanisms of resistance are affected.

For all experiments, yeasts of CA ATCC 90028 and SC5314 were exposed to P/8 N/4, a drug combination that provided at least 50 % of viable fungal cells, for 24 hours. Genes related to virulence were assessed by Polymerase Chain Reaction (RT-qPCR) and the analysis of proteins related to essential biological processes of the fungus was performed by LC-MS/MS. 24 hours after drug treatments, the fungal material was processed for total RNA and total proteins extraction. The RT-qPCR analysis demonstrated upregulation of some genes, such as ALS-1, ALS-4 and ALS-5 in the ATCC strain and ALS-4 in the SC strain compared to the untreated cells (negative control), that may be related to a defense mechanism associated with stress or cell death. Proteomic analysis revealed changes in the abundance of various proteins correlated with important biological processes, such as, energy metabolism, stress response, drug metabolism, among others. Heat shock proteins as HSP90p, HSP12p and HSP70p were downregulated in P/8 N/4 in relation to negative control and are proteins that play an important role in antifungals drugs resistance. GAPDH was upregulated in P/8 N/4 in relation to negative control and seems to be related to stress response. In conclusion, the results from this study suggest that Punicalagin used in combination with Nystatin represents a possible viable therapeutic strategy for oral candidiasis, by altering important biological

processes involved in the viability and energetic metabolism of the fungus *Candida albicans*.

Keywords: *Candida albicans*, Oral candidiasis, *Punica granatum*, Antifungals agents, Gene expression, Proteomics.

Introduction

C. albicans (CA) is a commensal fungus that can become pathogenic in certain circumstances, depending on the host's immune conditions, besides the expression of virulence factors by these microorganisms, which has led to resistance to commercial antifungal agents (KHAN et al., 2010).

Although *Candida* spp. lives as a commensal in the oral cavity of healthy individuals, these yeasts are able to cause infections in the presence of predisposing conditions of the individual. Oral candidiasis is the most common fungal infection in humans, especially in immunocompromised individuals, as well as in the elderly and users of removable total dentures. In this case, the disease is also known as Denture stomatitis (DS), commonly observed as spots or erythematous areas on the palate (CHANDRA et al., 2001a; SHERMAN et al., 2002; SALERNO et al., 2011; WILLIAMS et al., 2013).

Many factors have been involved in the increase of CA pathogenicity, including production of phospholipases, adhesins (ALS Family), hyphae formation, expression of drug resistance genes and production of secreted aspartyl proteinases (SAP) (RÜCHEL et al., 1992; HUBE, 1996; CHAFFIN et al., 1998; WU et al., 2000).

Studies have shown that the development of mutant strains, whose genes encode proteins responsible for energy metabolism, stress response and biosynthesis of macromolecules that are essential for the fungus, have virulence and consequently attenuated host damage. Thus, investigating which proteins are exclusively or differentially expressed after antifungal treatments contribute to the understanding of several mechanisms involved in *Candida* species pathogenicity (TSANG; BANDARA; FONG, 2012; AOKI et al., 2013).

Currently, elucidating the molecular mechanisms related to the ability of cause infections in species such as *Candida* might be important for the elaboration of new drugs that have as target virulence factors of the pathogen and less undesirable effects to the patients.

Conventional treatments for DS include topical application of Nystatin, chlorhexidine or miconazole prior to the use of systemic antifungal drugs. Despite Nystatin has an excellent therapeutic efficacy, some disadvantages related to the use of nystatin include: bad taste, drug interation, gastrointestinal side effects such as nausea, vomit and epigastric pain, especially in childrens or elder people. In addition,

a relatively high cost of medication and the administration of doses four times per day contribute to low drug adherence (MARTINSON et al., 2009; BAKHSHI et al., 2012; KOVAC; MITIC; KOVAC, 2012; MANSOURIAN et al., 2014; MUKHERJEE et al., 2017). These factors encourage the search for new therapeutic sources, such as medicinal plants, that have been extensively studied and considered a valuable source of antimicrobial agents, which conjugated to antifungal formulations traditionally used in the clinic, intensify its antimicrobial activity against resistant strains (MUKHERJEE et al., 2005; ENDO et al., 2010; SUN et al., 2017). This interaction between drugs is known as synergism (ENDO et al., 2010; MERTAS et al., 2015). Therapies that use the combination of drugs can increase the action spectrum, improve the antifungal activity and reduce the associated side effects (SUN et al., 2017).

The use of Punicalagin (P), the major ellagitannins present in *Punica granatum*, and one of the main responsible for the antifungal activity, in combination with Nystatin (N) represents a possible viable therapeutic strategy for oral candidiasis. Therefore, the present study we aimed to investigate whether this drug combination could interact with the virulence factors expression and interfere in biological processes essential for fungus survival.

Material and methods

Organisms and growth conditions

The present study used strains of *C. albicans* fungus (ATCC 90028, purchased from American Type Culture Collection (ATCC), University Boulevard Manassas, (VA, USA), and the clinical strain SC5314, provided by Dr. Arnaldo Lopes Colombo (Special Laboratory of Micology, Federal University of São Paulo, Brazil). The strains were cultured in YEPD medium (per liter: 10g yeast extract, 10g peptone, 20g dextrose, Acumedia®, Lansing, Michigan, USA) and incubated at 30 °C for 36 hours in incubator (Fanem®, Guarulhos, Brazil). Both strains were stored in 20% glycerol at -80 °C. Prior to each experiment the cells were reactivated on YEPD agar plates. A single CA colony was removed, resuspended in YEPD culture medium and maintained in the incubator (Fanem®, Guarulhos, Brazil) at 30 °C, 180 rpm overnight. Then the cells were collected by centrifugation at 4000 rpm for 5 min and

washed twice with 5 mL of phosphate buffer solution (PBS 1X). The pellet was resuspended in 1 mL of PBS and the cell density adjusted for 2.5 × 10^3 cell.mL^{-1}, using Neubauer chamber (NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS., 2002).

Drug preparation

Stock solutions of Punicalagin and Nystatin were prepared in Methanol at a concentration of 10 mg/mL and 1 mg/mL, respectively. After, the drugs were diluted in Sabouraud Broth medium (BD Difco®, Detroit, Michigan, EUA) to achieve following concentrations: **P** (50 µg.mL^{-1} of Punicalagin); **N** (3.9 µg.mL^{-1} of Nystatin) and the combination P/8 N/4 (6.25 µg.mL^{-1} of Punicalagin + 0.975 µg.mL^{-1} of Nystatin). P/8 N/4 was a drug combination that provided at least 50 % of viable cells (data not shown) and therefore was selected for the experiments.

RT-qPCR analysis

Fungal cells were treated with culture medium containing the drugs for 24 hours and the total RNA was isolated using Ribo Pure - Yeast Kit (Ambion®, Austin, TX, USA), according to the guideline of the manufacturer. The RNA quantification (ng. μL^{-1}) and qualification (1.9 – 2.1) were verified by Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Subsequently, 1 µg of RNA was transcripted to cDNA using QuantiTect Reverse Transcription kit (1 µL of reverse transcriptase enzyme, 1 µL of primers and oligo dT mixture, and 4 µL of buffer solution at 42 °C for 30 min) (Qiagen®, Hilden, Germany). The reaction was stopped at 95 °C for 3 min.

The quantitative expression of target genes was analyzed by RT-qPCR reactions using SYBR Green Master Mix kit (Applied Biosystems®, Warrington, UK), and were read in Viia 7 real-time PCR equipment (Applied Biosystems®, Warrington, UK).

The assays were performed in duplicates at 384 well plates through the following cycling conditions: initial temperature of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The results were normalized with the endogenous gene EFB1 (translation elongation factor EF-1 beta) and represented as

$2^{-(\Delta Ct)}$. The primers and probes sequence (Table 1) were used according to Nailis et al. (2006), Naglik et al. (2008), Samaranayake et al. (2013) and Ibrahim et al. (2015), respectively:

Table 1. Primers sequences used in RT-qPCR.

Gene symbol	Primer/probe	Gene sequence (5'-3')
ALS1	Forward	CAACAGGCACCTCAGCATCTAC
	Reverse	CTCCACCAGTAACAGATCCACTAGTAA
ALS2	Forward	CCAACAACCACCATCACAAC
	Reverse	GGATTTGGCAGTGGAACTTG
ALS3	Forward	CAACTTGGGTTATTGAAACAAAAACA
	Reverse	AGAAACAGAAACCCAAGAACAACCT
ALS4	Forward	TCT GCA ACA CGA GTC AGC TCA
	Reverse	CCG CAC CAA CAC AAG CAT ATA
ALS5	Forward	CTGCCGGTTATCGTCCATTTA
	Reverse	ATTGATACTGGTTATTATCTGAGGGAGAAA
HWP1	Forward	TGGTCCAGGTGCTTCTTCTT
	Reverse	GGTTGCATGAGTGGAACTGA
EAP1	Forward	TGT GAT GGC GGT TCT TGT TC
	Reverse	GGTAGTGACGGTGATGATAGTGACA
ECE1	Forward	GTCGTCAGATTGCCAGAAATTG
	Reverse	CTTGGCATTTTCGATGGATTGT
SAP1	Forward	TTTCATCGCTCTTGCTATTGCTT
	Reverse	TGACATCAAAGTCTAAAGTGACAAAACC
SAP2	Forward	TCCTGATGTTAATGTTGATTGTCAAG
	Reverse	TGGATCATATGTCCCCTTTTGTT
SAP3	Forward	GGACCAGTAACATTTTTATGAGTTTTGAT
	Reverse	TGCTACTCCAACAACTTTCAACAAT
SAP4	Forward	CAATTTAACTGCAACAGGTCCTCTT
	Reverse	AGATATTGAGCCCACAGAAATTCC
SAP5	Forward	CATTGTGCAAAGTAACTGCAACAG
	Reverse	CAGAATTTCCCGTCGATGAGA
SAP6	Forward	CCTTTATGAGCACTAGTAGACCAAACG
	Reverse	TTACGCAAAAGGTAACTTGTATCAAGA
SAP9	Forward	ATTTACTCCACAGTTTATATCACTGAAGGT
	Reverse	CCACCAGAACCACCCTCAGTT
SAP10	Forward	CCCGGTATCCAATAGAATCGAA
	Reverse	TCAGTGAATGTGACGAATTTGAAGA
PLB1	Forward	GGT GGA GAA GAT GGC CAA AA
	Reverse	AGCACTTACGTTACGATGCAACA
PLB2	Forward	TGAACCTTTGGGCGACAACT
	Reverse	GCCGCGCTCGTTGTTAA
PLC	Forward	AGCCACCAATTGGCAAACTTA
	Reverse	ACTGCTTGATTTTAAAGTTGGTTTCC
PLD	Forward	TGTTTACGGTGAAGGGTTGGA
	Reverse	CACTGCTAACCCTTGCTCTCTTG

Proteomic analysis

24 hours after drug treatments, the fungal cells were washed twice with PBS and centrifuged (2000 rpm for 2 min at 24 °C), then were removed from the wells using lysis buffer (6M Urea, 2M Thiureia, 10 mM DTT, 50mM AMBIC e 0.1% SDS (m/v)). For total protein extraction, the solution was transferred to microtubes containing zirconia beads (Ambion®, Austin, TX, USA) and vortexed at 4º C for 30 min (AOKI et al., 2013). Samples were centrifuged again (15000rpm for 5 min at 4 ºC), the protein quantification was performed acording to Bradford method (Bio-Rad, Hercules, USA) (BRADFORD, 1976) and 1µg.µL^{-1} of proteins were transferred to a microtube. Subsequently the procedures were performed as described by Dionizio et al. (2018), In brief, 25 µl of 0.2% RapiGest SF (Waters Division, Milliford, USA, cat#186001861), was added, shaken and 10 µl of 50 mM AMBIC was added for 30 min at 37 °C (DIONIZIO et al., 2018). Samples were then reduced (2.5 µL of 100 mM DTT; Bio-Rad, cat# 161–0611) and alkylated (2.5 µL 300 mM IAA; GE, cat# RPN 6302 V) in the dark for 30 min at room temperature. The digestion was performed with 100 ng of trypsin during overnight at 37 ° C (Promega, cat #V5280). After this, 10 µl of 5% was added, incubated for 90 min at 37 °C and then centrifuged (14000 rpm for 30 min at 6 °C). The supernatant was purified using Spin C18 columns (Pierce, cat #89870) and resuspended in 200 µl of 3% acetonitrile.

LC-MS/MS and bioinformatics analyses

The bioinformatics analysis was performed to compare the groups of interest (Tables A2-A14), as previously reported (ORCHARD, 2012; BAUER-MEHREN, 2013; MILLAN, 2013; LEITE et al., 2014; DIONIZIO et al., 2018). The peptides identification was done on the nanoAcquity UPLC-Xevo QTof MS system (Waters Corporation, Manchester, UK), using Protein Lynx Global Server (PLGS) software, as described by Leite, et al. (2014), searched in the database of *C. albicans* proteins (Swiss Prot, Swiss Institute of Bioinformatics, Geneva, Switzerland). The difference in protein expression among the comparison groups was acquired using Monte-Carlo algorithm in the PLGS software and expressed as $p<0.05$ for down-regulated and and $1-p>0.95$ for up-regregulated proteins. The identified proteins were classified, and attributed to biological functions and origin through CYTOSCAPE® 3.6.1

software (Java® technology) with the aid of ClueGo and ClusterMarker applications.

Statistical analysis

Analysis of the gene expression of CA virulence factors (qRT-PCR) were presented as mean ± standard deviation (SD) and were submitted to Two-way ANOVA analysis, followed by Tukey test for comparative analysis. In the proteomic analysis, the differences in protein expression between the comparison groups were obtained through the PLGS software, according to the peak intensity of the peptide ions, where $p<0.05$ for downregulated proteins and $p>0.95$ for upregulated proteins.

Results

Gene expression of virulence factors of *C. albicans* (RT-qPCR)

The mRNA expression of CA virulence genes against drugs was assessed after 24 hours. The selected concentration of drug combination was at P/8 N/4, a concentration that did not significantly affected the cell viability (data not shown), but generated some effect at the molecular level.

Our results highlighted the maintenance of gene expression levels similar to the Nystatin drug, which has excellent antimicrobial action, as demonstrated in ALS-1 for SC strain (Figure 1A), and ALS-3 for ATCC and SC strains (Figure 1C). Other situations observed were a higher gene expression after exposure to P/8 N/4 (ALS-1 ATCC, ALS-4 SC, ALS-5 ATCC and EAP-1). ALS-2 gene (ATCC and SC), ALS-5 and EAP-1 (SC), showed no statistical difference between C-, P/8 N/4, **N** and **P** (Figures 1B, 1E, 1F). In relation to HWP1 gene (Hyphal wall protein 1), the expression after exposure to P/8 N/4 significantly decreased compared to the negative control (C-) and **P**, and was similar to Nystatin for the ATCC strain. Regarding to SC strain, the values were still lower than negative control (C-) and **P**, equivalent to Nystatin values, but with no statistical difference (Figure 2A). Another specific hypha gene, ECE, had significantly decreased expression in negative control (C-), ATCC strain, and similar to expression levels after exposure to Nystatin. For the SC strain, the same pattern was observed, but without statistical difference between C-, P/8 N/4, **N** and **P** (Figure 2B).

Regarding to the phospholipase family genes (PLC, PLD, PLB1 e PLB2) (Figures 3A-D), was observed a inhibition in PLC gene expression after exposure to P/8 N/4 compared to negative control (C-) and **P**, mainly in the SC strain, nevertheless no statistical differences were observed. Besides, the same expression patterns in the PLD gene was observed after exposure to P/8 N/4 for SC strain. In relation to PLB2 (Figure 3D), ATCC strain, the expression decreased in P/8 N/4 compared to negative control (C-), with no statistical difference; while in SC strain there was a decrease of the expression in relation to C-, **N** and **P**, also without significant difference.

In PLD ATCC and PLB1 ATCC, there was an increase in expression levels of P/8 N/4 in relation to C-, **N** and **P** (Figures 3B and C). In SC strain treated with P/8 N/4, **N**, **P** and in negative control (C-), PLB1 gene expression levels were lower and similar between them (Figure 3C).

The genes of the aspartyl proteinases family (secreted aspartyl proteinases - SAPs) are shown in Figure 4 (A-H). After exposure to P/8 N/4, both strains showed a decrease in expression levels of the SAP-4, SAP-5 and SAP-6 genes compared to negative control (C-), except in SAP-4 SC, which the expression levels were higher than C-, however smaller than **N** (Figure 4D).

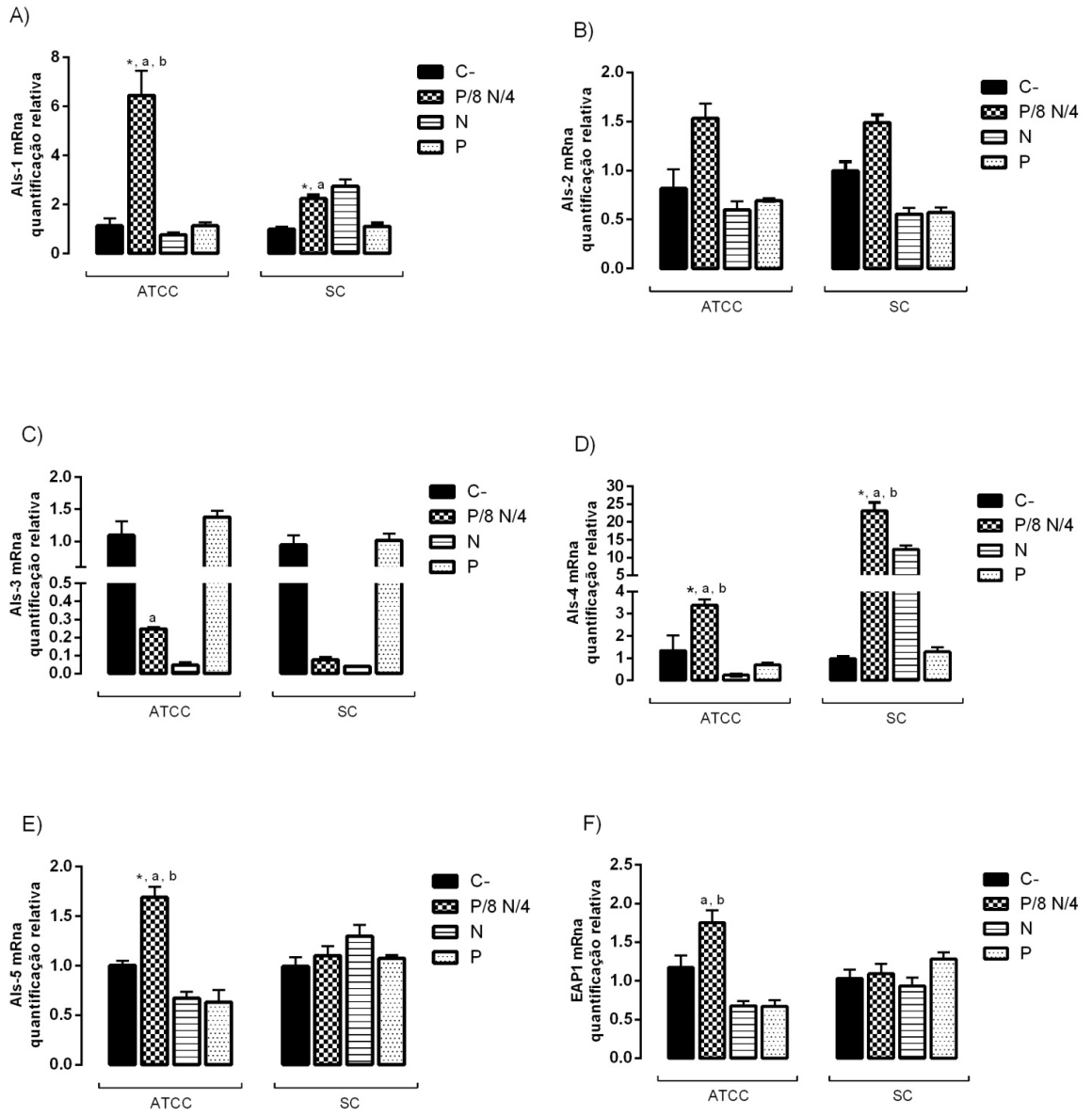

Figure 1. Relative quantification of mRNA expression of Agglutinin-like sequence (ALS) family gene (A-E) and EAP-1 (F) gene. *, a, b represent statistical difference compared to C-, **P** and **N**, respectively. Three independent experiments. Anova Two-Way, Tukey Test.

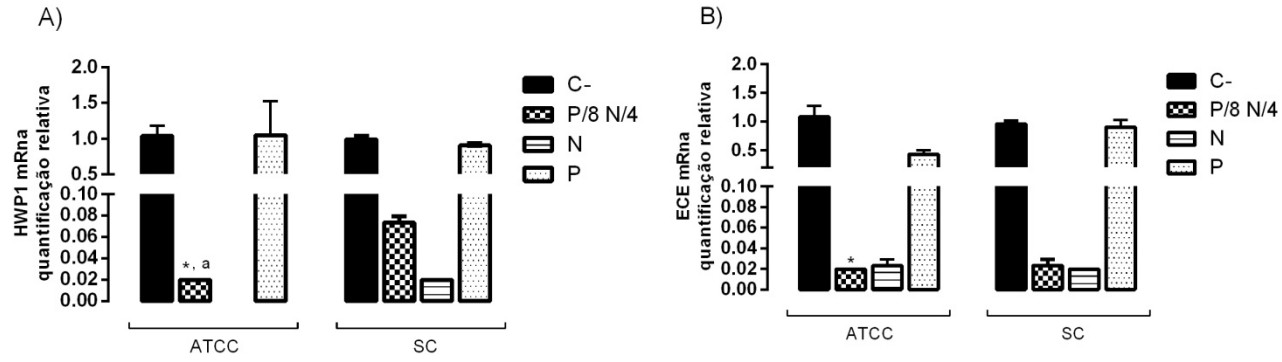

Figure 2. Relative quantification of mRNA expression of HWP1(A) and ECE (B). *, a, b represent statistical difference compared to C-, **P** and **N**, respectively. Three independent experiments. Anova Two-Way, Tukey Test.

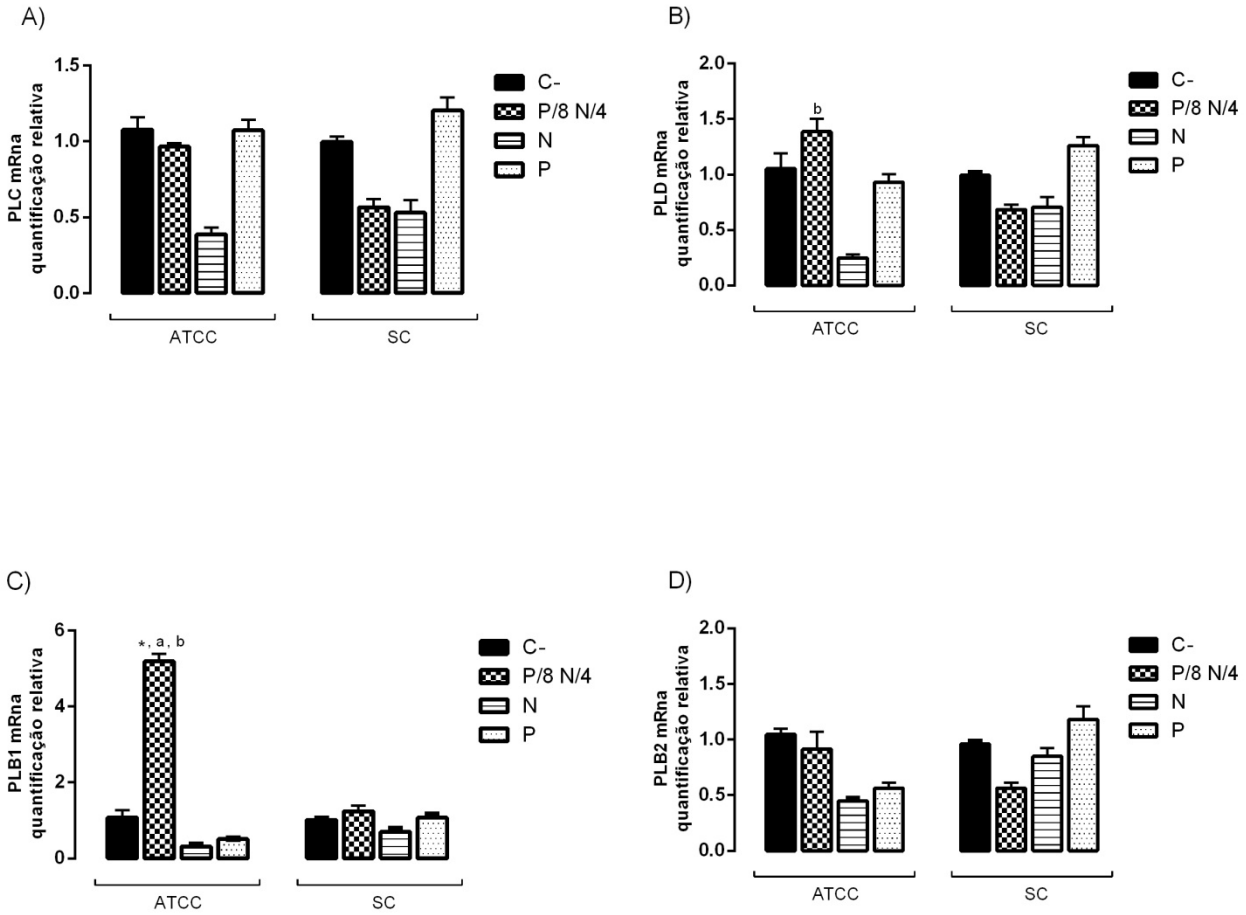

Figure 3. Relative quantification of mRNA expression of phospholipase PLC family genes: PLC (A), PLD (B), PLB1 (C) and PLB2 (D). *, a, b represent statistical difference compared to C-, **P** and **N**, respectively. Three independent experiments. Anova Two-Way, Tukey Test.

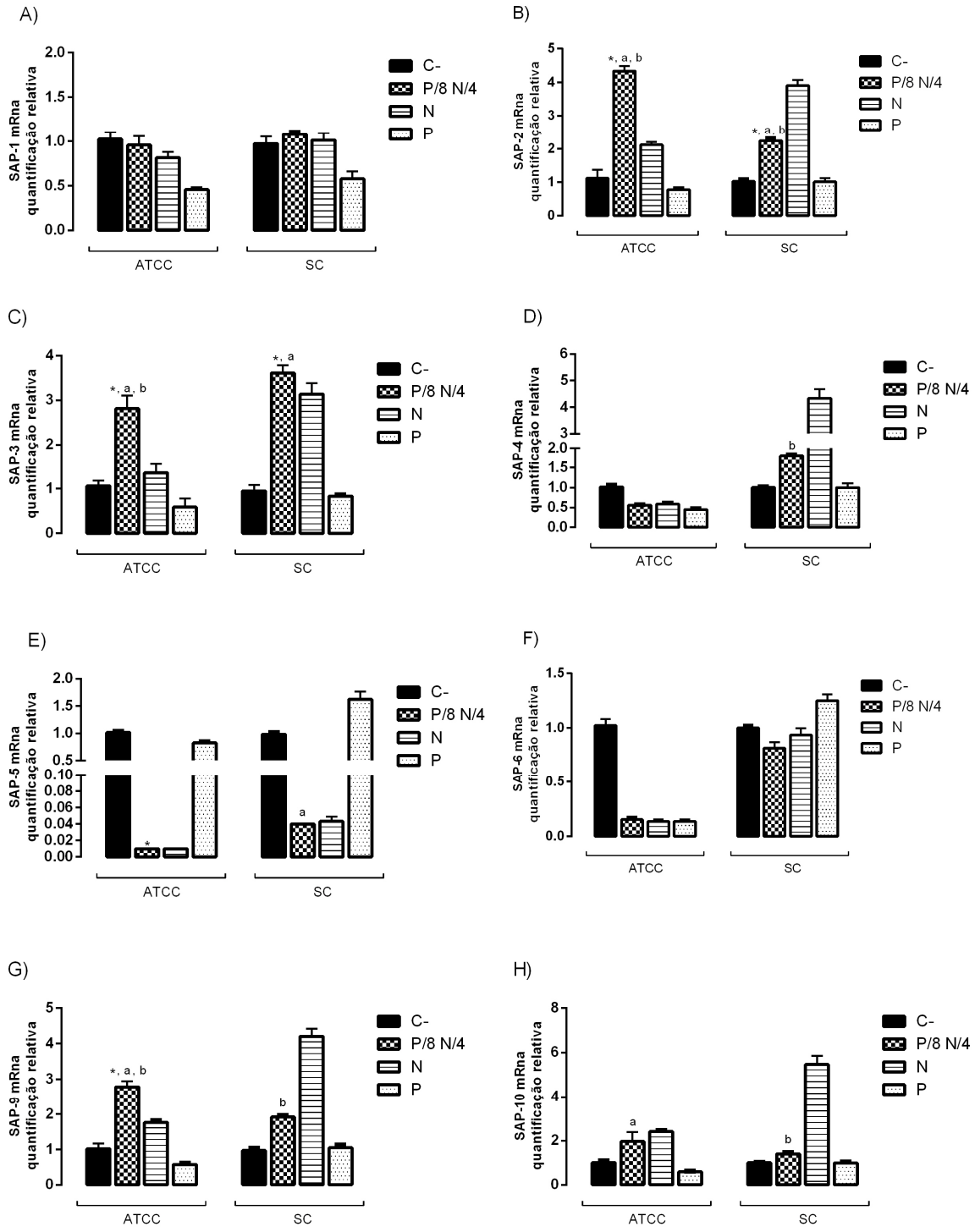

Figure 4. Relative quantification of mRNA expression of aspartyl proteinases (SAPs) family gene. SAP-1 (A), SAP-2 (B), SAP-3 (C), SAP-4 (D), SAP-5 (E), SAP-6 (F), SAP-9 (G) and SAP-10 (H). *, a, b represent statistical difference compared to C-, **P** and **N**, respectively. Three independent experiments. Anova Two-Way, Tukey Test.

Proteomic analysis of fungal cells

The total number of proteins uniquely identified in the P/8 N/4, **P**, **N** treatments and negative control (C-), for ATCC 90028 strain were 45, 6, 14 and 141, respectively (Supplementary Table A2-A5). In the quantitative analysis of the P/8 N/4 *vs*. C-, 75 proteins with change in expression were detected (Supplementary Table A9). As the comparison **P** *vs.* C-, 1 protein with change in expression was found (Supplementary Table A10). About **N** *vs*. C-, 55 proteins with change in expression were identified (Supplementary Table A11).

In the SC 5314 strain, the total number of proteins identified exclusively (unique) in P/8 N/4, **N** and negative control (C-) were 38, 53 and 87, respectively (Supplementary Table A6-A8) and there were no proteins unique in the **P** treatment. The difference among P/8 N/4 and negative control (C-) showed alteration in 63 proteins expression (Supplementary Table A12). In **P** *vs*. C-, 3 proteins with change in expression were found (Supplementary Table A13), and in **N** *vs*. C-, 243 proteins with change in expression were detected (Supplementary Table A14).

With regard to ATCC 90028 strain, Figures 5 and A1 (Supplementary Figure A1) show the functional classification according to the biological processes and cellular components altered, respectively, with the most significant term for the comparison P/8 N/4 *vs*. C-. The same analyzes were performed comparing **P** *vs*. C- (Figures 6 and Supplementary Figure A2) and **N** *vs*. C- (Figures 7 and Supplementary Figure A3).

The group exposed to the P/8 N/4 treatment had the largest alteration, with change in 11 functional categories (Figure 5). Among them, the categories with the highest percentage of associated genes were: Drug metabolism process (23.9%), %), Generation of metabolic and energy precursors (19.7%), Biosynthetic process of carboxylic acid (16.9%), Translation (15.5%), Cellular response to heat (5.6%), Cellular response to antibiotic (4.2%), Biosynthetic process of amino acids of serine family (2.8%), Chlamydospore formation (2.8%), Pentosan phosphate pathway (2.8%), Translational elongation (2.8%) and Protein ubiquitination process (2.8%). The cellular component analysis showed that the amount of categories was similar between the comparisons, ranging 3 to 5 categories with altered processes (Supplementary Figure A1). With regard to SC 5314 strain, Figures 8 and A4 (Supplementary Figure A4) showed the functional classification according to the

biological processes and changes in cellular components, respectively, with the most significant term for the P/8 N/4 *vs*. C-. The same analyzes were performed between **P** *vs*. C- (Figures 9 e Supplementary Figure A5) and in **N** *vs*. C- (Figures 10 and Supplementary Figure A6).

The group exposed to **P** treatment influenced 11 functional categories (Figure 9). Among them, the categories with the highest percentage of associated genes were: Biosynthesis process of organic compounds containing nitrogen (30%), Process of drug metabolism (21%), Metabolic process of carbohydrates (13%), Phosphorylation of nucleoside diphosphate (10%), Cell homeostasis (7%), Metabolic process of antibiotic (6%), Transport of transmembrane protons (5%). Cellular response to heat (3%), Protein ubiquitinization process (2%), Biosynthetic amino acid process of the aspartate family (2%) and Isomerization of peptidyl-prolyl proteins (2%). The analysis of cellular component, revealed that the amount of categories was similar between comparisons, ranging 3 to 7 categories with processes affected (Supplementary Figure A4).

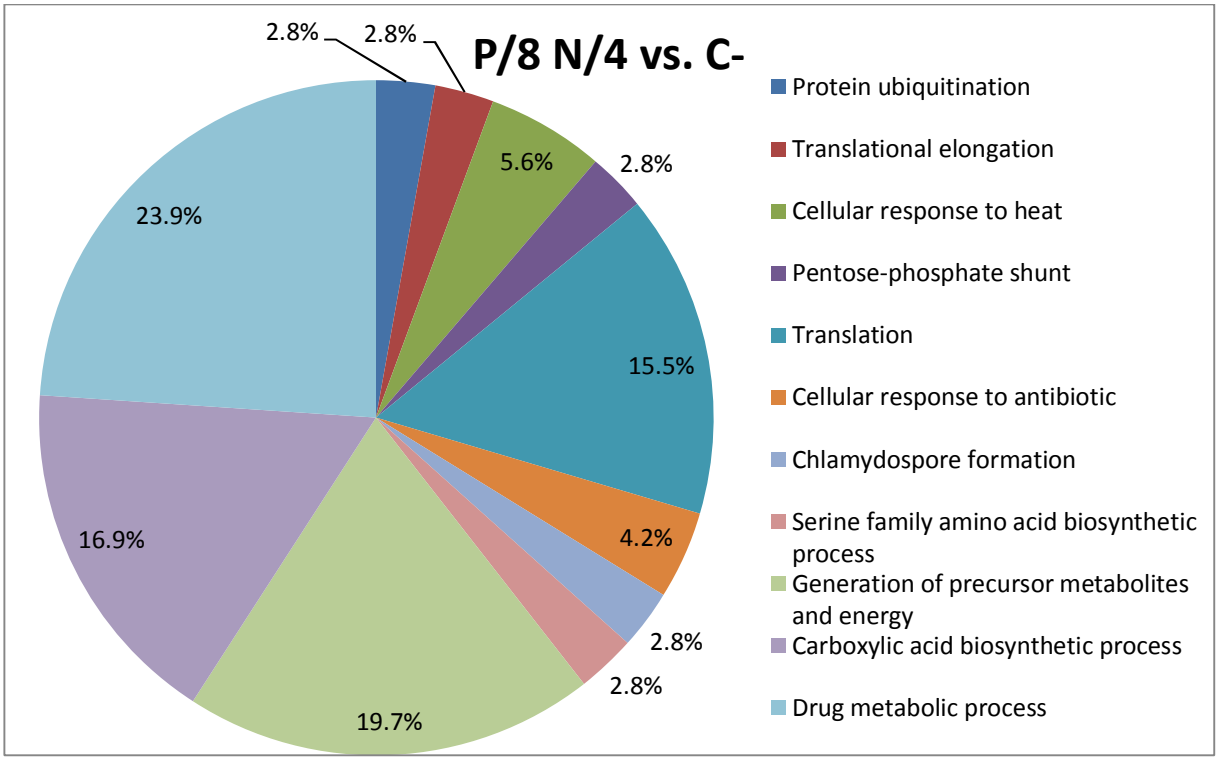

Figure 5. Functional distribution of proteins identified with differential expression in the *C. albicans* ATCC90028 cells exposed to P/8 N/4 *vs.* C-. Categories of proteins based on GO annotation Biological Process. Terms significant (Kappa = 0.04) and distribution according to percentage of number of genes association. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® pluggins of Cytoscape® 3.6.1.

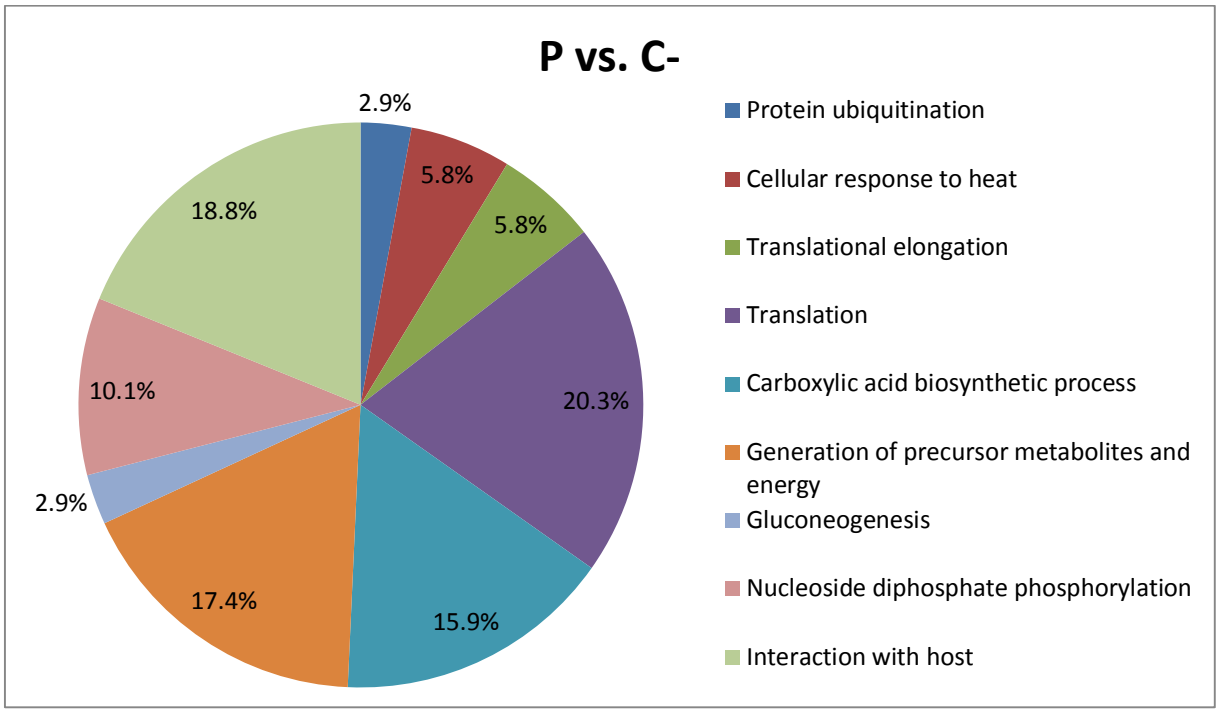

Figure 6. Functional distribution of proteins identified with differential expression in the *C. albicans* ATCC90028 cells exposed to P vs.C-. Categories of proteins based on GO annotation Biological Process. Terms significant (Kappa = 0.04) and distribution according to percentage of number of genes association. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® pluggins of Cytoscape® 3.6.1.

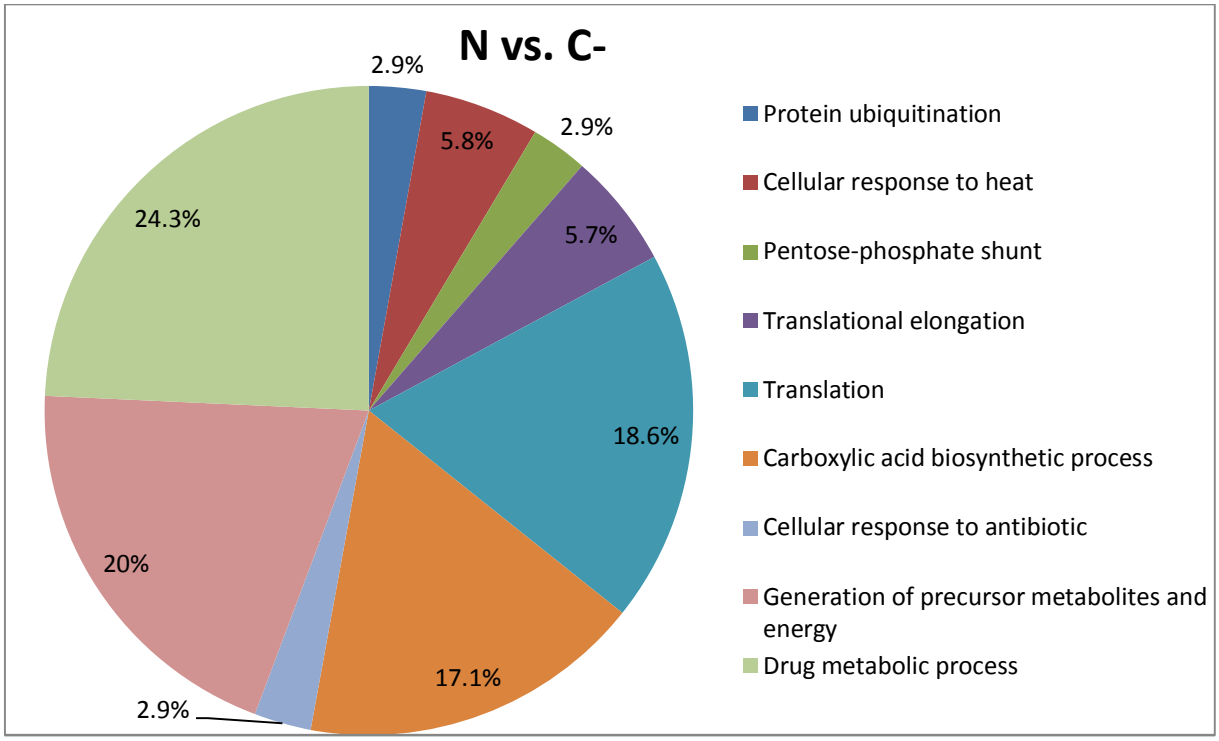

Figure 7. Functional distribution of proteins identified with differential expression in the *C. albicans* ATCC90028 cells exposed to N *vs.*C-. Categories of proteins based on GO annotation Biological Process. Terms significant (Kappa = 0.04) and distribution according to percentage of number of genes association. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® pluggins of Cytoscape® 3.6.1.

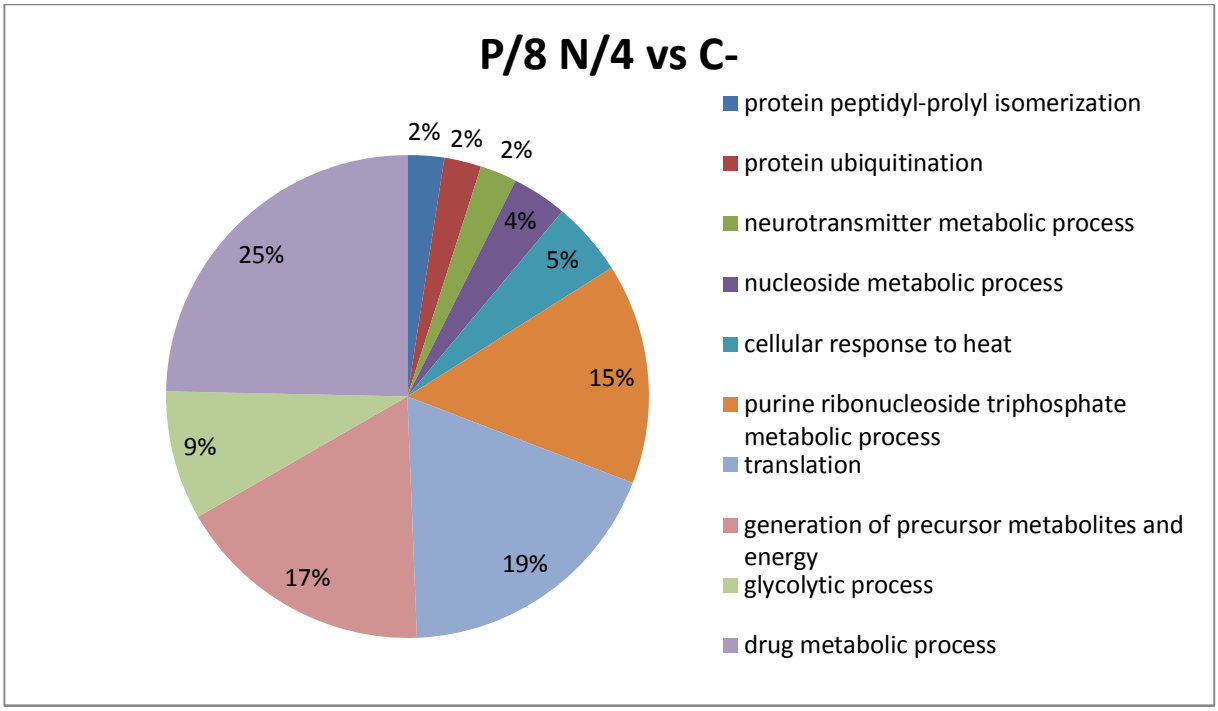

Figure 8. Functional distribution of proteins identified with differential expression in the *C. albicans* SC cells 5314 exposed to P/8 N/4 *vs.* C-. Categories of proteins based on GO annotation Biological Process. Terms significant (Kappa = 0.04) and distribution according to percentage of number of genes association. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® pluggins of Cytoscape® 3.6.1.

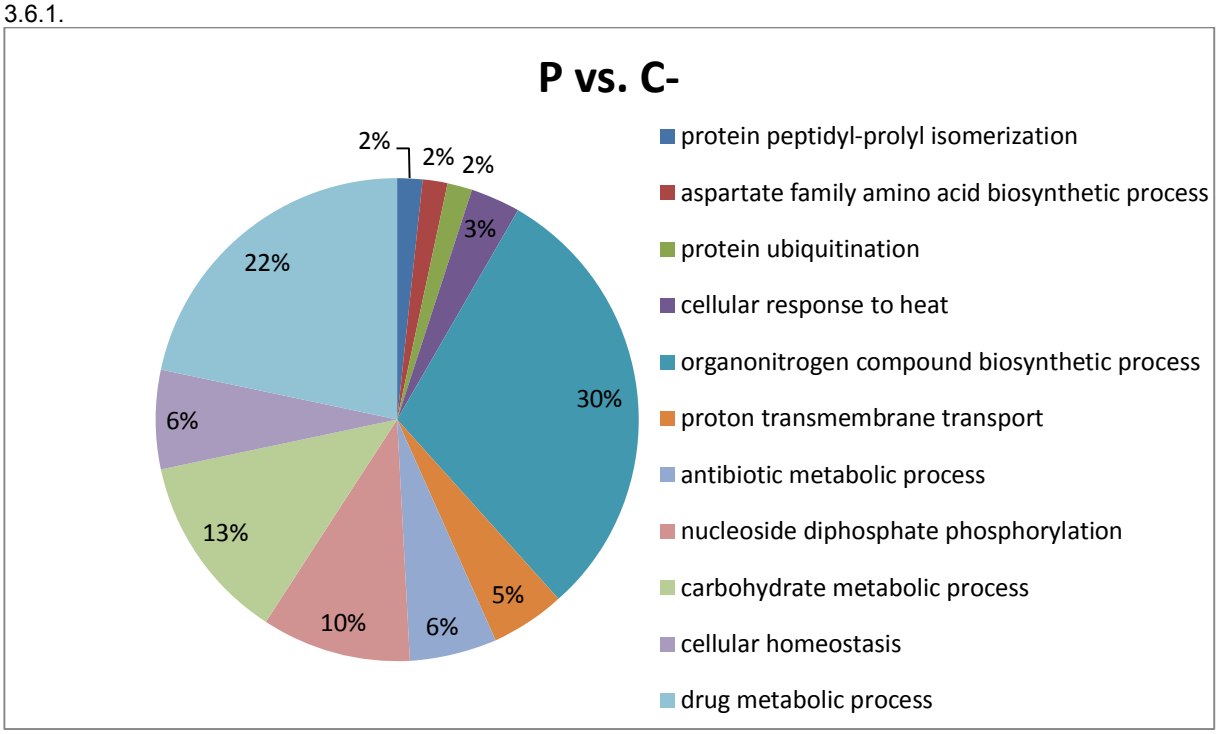

Figure 9. Functional distribution of proteins identified with differential expression in the *C. albicans* SC 5314 cells exposed to P *vs.* C-. Categories of proteins based on GO annotation Biological Process. Terms significant (Kappa = 0.04) and distribution according to percentage of number of genes association. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® pluggins of Cytoscape® 3.6.1.

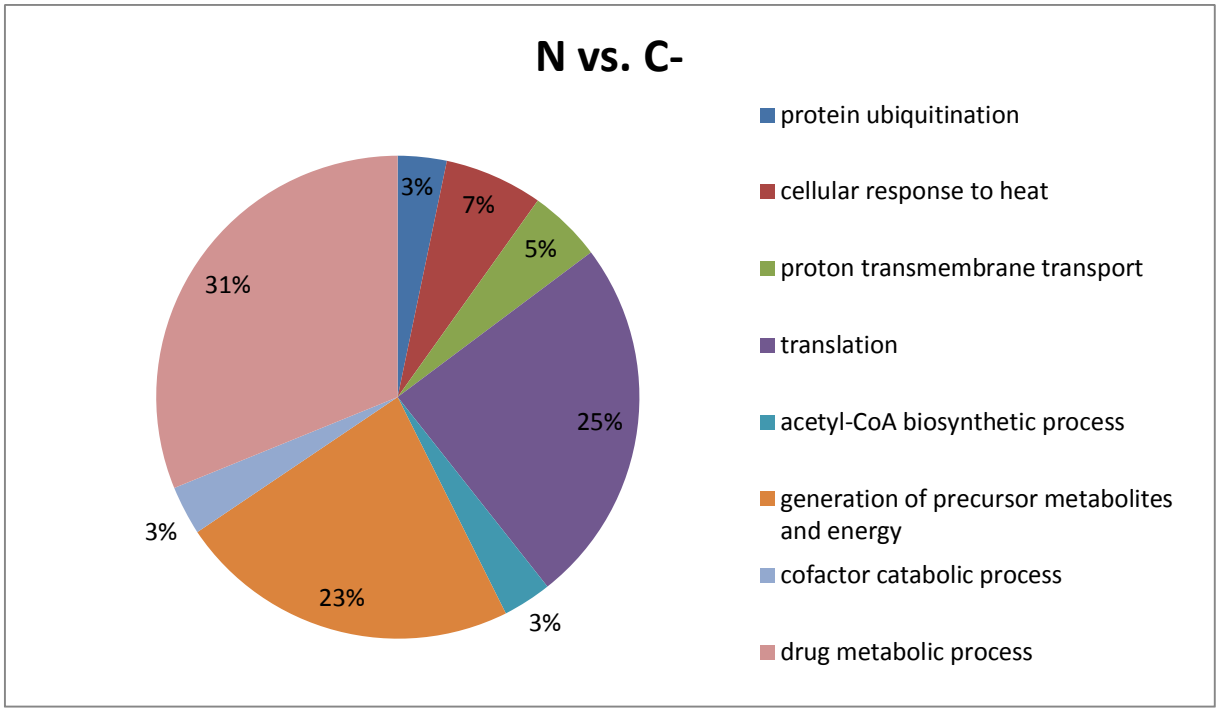

Figure 10. Functional distribution of proteins identified with differential expression in the *C. albicans* SC 5314 cells exposed to N *vs.* C-. Categories of proteins based on GO annotation Biological Process. Terms significant (Kappa = 0.04) and distribution according to percentage of number of genes association. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® pluggins of Cytoscape® 3.6.1.

Discussion

The therapeutic search for the control of candidoses is being widely discussed in the scientific literature. The present study demonstrated that the virulence factors of *C. albicans* (CA), ATCC and SC strains, against Punicalagin (P) and Nystatin (N) treatments undergo complex modulations in gene expression. It has been demonstrated that **P** has fungicidal activities once stimulate a decrease fungus cell wall thickness and in the cytoplasmic content. These actions are most evident when **P** is administered together with fluconazole (ENDO et al., 2010). The **N** exhibits antifungal action due to a direct binding to ergosterol and the increases in fungus membrane permeability, thus affecting the integrity of the cell wall, causing extravasation of intracellular constituents (NIIMI; FIRTH; CANNON, 2010; SPAMPINATO; LEONARDI, 2013) and also blocking fungus adhesion (ELLEPOLA; SAMARANAYAKE, 1998).

Regarding to the CA virulence factors, no significant difference in the gene expression of ALS 1-5 was identified in both CA strains after the treatment with **N** and **P** compared to negative control. In contrast, the treatment with drug combination (P/8 N/4) in sub-inhibitory concentration caused a significant increase in the gene

expression of ALS-1, ALS-4 and ALS-5 in the ATCC strain, and ALS-4 in the SC strain compared to the negative control. Similar results were found in SAPs gene expression, exhibiting increased level in SAP-2, SAP-3 and SAP-9 in ATCC strain, and SAP-2 in SC strain, after the treatment with P/8 N/4 compared to negative control (C-).

A hypothesis for the increase in virulence factors gene would be a response of fungi to antifungals administered in subinhibitory concentrations. Some studies have also demonstrated an increase in gene expression of ALS and SAP in strains treated with subtoxic doses of fluconazole (WU et al., 2000; BARELLE et al., 2007). Barelle et al. (2007) showed that the gene induction was involved to a defense mechanism of CA stress-related. The authors also demonstrated that the up-regulation of different SAPs is under different controls, such as the yeast-hyphae transition, nevertheless these effects appear to be transient *in vivo*. Wu et al. (2000) identified an increase in the expression of SAPs and did not correlate this increase to cell death or non-specific release of SAP, since it did not detect a reduction in the number of CFUs and no significant release of enolase, an enzyme constitutive of the glycolytic pathway. Thus, they correlated that the exposure to sub-inhibitory doses of fluconazole may result in an increased extracellular production of SAP by strains capable of overexpressing genes related to a multidrug resistance efflux pump (MDR1), associated to an increase in CA virulence *in vivo* (WU et al., 2000). However, in our results, we can correlate the up-regulation of some genes with the cell death process, because in CFU studies, P/8 N/4 demonstrated a reduction in fungal growth compared to the negative control (data not shown). Additionally, although enolase had its expression decreased relative to the control, identified by proteomic analysis, this could be connected to the drug exposure time, which was 24 hours, differently of the short exposures reported in those studies. A similar result was observed in strains resistant to some fungicides such as fluconazole and itraconazole (COPPING et al., 2005; COSTA et al., 2010).

In agreement with these results, the therapy with another fungicide, caspofungin, promotes an increase of SAP-5 gene expression and does not modulate the expression of the others SAPs and PLB1 (RIPEAU et al., 2002). Dimethylamino dodecyl methacrylate (DMADDM) has antimicrobial activity and has been incorporated in several dental materials. This strategy has been shown to be effective against fungi, because interfere in adhesion, which may be occasioned by

the decreased expression of some virulence factors such as ALS-3 and HWP1 (ZHANG, K. et al., 2016). In the present study, ALS-3 and HWP expression was reduced in relation to the negative control in the two strains treated with P/8 N/4. The intervention in fungus adhesion processes may be crucial in oral candidiasis treatment, preventing the fungus attachment to the mucosal surfaces and devices such as total removable prostheses (ANTLEY; HAZEN, 1988; ELLEPOLA; SAMARANAYAKE, 1998).

The microorganism's virulence also depends on their hemolytic abilities. Regarding to fungi specie, phospholipases are correlated with this ability, the PLB1, PLB2, PLC and PLD are the most described in the literature (LEONOV et al., 2017). The present study did not detected differences between the phospholipases evaluated, except of the PLB1 gene expression which was increased in the ATCC strain after P/8 N/4 treatment. However, the treatment with fluconazole inhibited the PLC expression (WILLIS et al., 2001) and caspofungin treatment did not affected PLB1 gene expression (RIPEAU et al., 2002), as well as DMADDM did not modified PLD gene expression (ZHANG, K. et al., 2016).

These results demonstrate the complexity of fungus behavior against to antifungals. The mechanism of action, time of treatment, and drug concentration are determinant in the success of the fungal infections control. To understand the mechanisms involved in fungal resistance, the use of antifungals at sub-inhibitory doses may be interesting for *in vitro* studies, however, it is difficult to extrapolate to what occurs *in vivo*. Thus, these results encourage the development of clinical trials to evaluate the efficacy of this combination (Punicalagin and Nystatin) and other combinations by changing the concentrations of each drug.

According to the gene ontology analysis, the main functional categories were related to energy metabolism, translation, drug metabolic process and stress response. Several proteins had their expression inhibited after P/8 N/4 treatment compared to negative control (C-) and the main biological process associated with these proteins were: Energy metabolism (Glucose-6-phosphate isomerase, Triose phosphate isomerase, Phosphoglycerate kinase, Phosphoglycerate mutase, Enolase, Pyruvate kinase, Alcohol dehydrogenase, Aconitate hydratase (Aconitase), Malate dehydrogenase, Acetyl-coenzyme-A hydrolase, Methionine synthase, Bifunctional phosphoribosylaminoimidazole carboxamide formyltransferase/IMP cyclohydrolase, ATP synthase subunit alpha, ATP synthase subunit beta and

Inorganic pyrophosphatase); Protein synthesis (Translation elongation factor 2 and various ribosomal proteins, eg. 60S ribosomal protein L13); Stress response (70-kDa heat shock protein Ssa1, 70-kDa heat shock protein Ssb1, 70-kDa heat shock protein Ssc1 and 70-kDa heat shock protein Sse1), Drug metabolic process (Succinate dehydrogenase [ubiquinone], Peroxiredoxin TSA1, Pyruvate kinase, Enolase, Phosphoglycerate mutase and Aconitate hydratase).

The biological processes affected through the reduction of these proteins may be associated with damaged and fragile cells formation, reduced metabolic activity and growth (SILVA-ROCHA et al., 2017).

Heat shock protein 90 (HSP90p) is one of the most abundant proteins in the cytosol of eukaryotes, corresponding to 1 to 2% of the total proteins. Studies demonstrated that HSP90 plays an important role in antifungal drugs resistance, where the inhibition of HSP90p reduced the CA strains resistance to antifungal fluconazole (COWEN; LINDQUIST, 2005; BLUM et al., 2013). In our results, HSP90p expression was lower in relation to C- after P/8 N/4 and N treatment, for SC5314 strain. With regard to ATCC 90028, strain, it was detected only in the C- group.

HSP12p, in *C. glabrata*, for example, is an overexpressed gene in fluconazole-resistant mutant strains. Its overexpression seems to related to a protective role of the cell wall and cell membrane proteins, besides to increase the initial phase of hypha formation and reduce the effect of farnesol on the filamentation inhibition, consequently, being able to play a facilitating role in the hyphae formation (FU; DE SORDI; MÜHLSCHLEGEL, 2012).

The increase of heat shocks proteins is generally related to higher tolerance of fungi to stress, allowing the cells to survive under unfavorable conditions (BENTLEY; FITCH; TUITE, 1992; BURNIE et al., 2006). In our study all heat shocks proteins had their expression decreased after P/8 N/4 treatment, for the two strains evaluated.

Glycolytic enzymes are relevant during the CA pathogenesis, behaving as main inducers of host immune response and are the main allergens during candidiasis (STROCKBINE et al., 1984; SHEN et al., 1991; ISHIGURO et al., 1992; SWOBODA et al., 1993; ITO et al., 1995; GIL-NAVARRO et al., 1997; GOZALBO et al., 1998). Enolase (ENO1) is one of the most abundant glycolytic enzymes in CA cytosol. It binds to human plasminogen and this interaction promotes an increase in the fibrinolytic capacity of the fungus, providing the invasion and dissemination (CHAFFIN et al., 1998; CROWE et al., 2003). Both Enolase and Heat shock protein

70 (HSP70p) are proteins that have been reported as important antigens in many infectious diseases (BIANCO et al., 1986). In our study, these two proteins had decreased expression in relation to the negative control after P/8 N/4 and **N** treatments, in ATCC and SC strains. In addition, three glycolytic enzymes were identified: phosphoglycerate kinase (PGK), glyceraldehyde phosphate dehydrogenase (GAPDH), which demonstrated laminin and fibronectin binding properties, and alcohol dehydrogenase (ADH) (PENDRAK; KLOTZ, 1995; CHAFFIN et al., 1998; GOZALBO et al., 1998).

Phosphoglycerate kinase (PGK) catalyzes the hydrolysis of 1,3-bisphosphoglycerate to 3-phosphoglycerate with the production of ATP molecule. PGK was overexpressed in resistant strains of *Staphylococcus aureus* compared to susceptible strains (MACKENZIE et al., 2002). In CA, it has been suggested that overexpression of this protein is correlated with fluconazole resistance (YAN et al., 2007). In our results, the PGK expression was decreased in the **P**, **N** and P/8 N/4 treatments in relation to the negative control, thus suggesting interference in one of the resistance mechanisms of the fungus.

Glyceride-3-phosphate dehydrogenases (GAPDH) are a family of proteins that have several activities in different locations within the cell, besides to have a well characterized role in the glycolysis process (SIROVER, 1999). GAPDH catalyzes the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate and the concomitant reduction of NAD+. Studies have identified GAPDH on bacteria the cell surface (CHAFFIN et al., 1998), playing different roles, such as interactions with host cells. Another study has shown that secretion of GAPDH protein within the cell wall is enhanced by some stress conditions, as starvation, in *S. cerevisiae* yeasts (GIL; DELGADO; GOZALBO, 2001). In our results there was an increase in GAPDH expression in the P/8 N/4 or **N** treatments, in both strains tested. This suggests that the expression is could be associated to the stress response provided by the use of antifungal drugs.

Alcohol dehydrogenase (ADH1p), on cell surface and cytoplasm, catalyzes the reduction of acetaldehyde to ethanol generating NAD+ and participating in multiple biological processes such as biofilm formation, fermentation and interaction with host (WANG et al., 2012). A possible relationship between ADH of CA and receptors for vitronectin and fibronectin has also been suggested (PENDRAK; KLOTZ, 1995). Previous studies have found that ADH1p overexpression refers to fluconazole

resistance in CA (ZHU; LU, 2005) and that fluconazole induces ADH1 gene expression (WANG et al., 2012). In our results, we obtained an increased expression of ADH1p after **P** treatment in ATCC strain, and **N** treatment in SC strain. However, in the drug combination (P/8 N/4), the expression was decreased compared to the negative control. Another study similarly identified ADH1p overexpressed after separately fluconazole and tetrandrine treatment; nevertheless, when combined, there was a decrease in the expression, suggesting that ADH1p expression is involved in the synergism mechanism against CA (ZHANG et al., 2013).

Malate dehydrogenase (MDH1p) is a considerable enzyme for the fungi bioenergetic metabolism. MDH1p participates in the glyoxalate cycle, which allows fungal cells to use fatty acids as a substrate for gluconeogenesis (TYLICKI et al., 2008). The glyoxalate cycle is required for fungal virulence. In CA infections, Lorenz and Fink (2001) have shown high rates of genes induction that encode enzymes involved in the glyoxalate cycle, such as isocitrate lyase, malate synthase, citrate synthase, and also malate dehydrogenase in CA during internalization by mammalian cells. CA exhibits a metabolic program by which the glyoxalate cycle and gluconeogenesis are activated during the early stages of infection. Subsequent progression of systemic disease is dependent of glycolysis (LORENZ; FINK, 2001). Our results showed a reduction of malate dehydrogenase expression in the **P**, **N** and P/8 N/4 treatments in relation to the negative control, in both strains tested. Thus, not only the decrease of MDH1p, but also other proteins involved with the fermentative or oxidative metabolism, can be effective as antifungals (TYLICKI et al., 2008).

Another interesting result was the identification of Acetyl-CoA-acetyltransferase, encoded by ERG10 gene, only in the negative control, in both ATCC and SC strains. This data allows to assume that there was an interference in the ergosterol production, as such a protein is necessary in the first step of this biosynthesis through its condensation in acetoacetyl-CoA (BUURMAN et al., 2004).

In healthy individuals, phagocytic cells, such as macrophages (EVRON, 1980), monocytes and neutrophils (SCHUIT, 1979; MARÓDI; KORCHAK; JOHNSTON, 1991), act against *Candida* infections, producing various growth inhibitors and cytotoxic compounds, including microbicidal enzymes and reactive oxygen and nitrogen species (PETERSON; CALDERONE, 1978; VAZQUEZ-TORRES; BALISH, 1997). A potentially effective artifice against CA is nitric oxide (NO) (ULLMANN et al., 2004).

One way of protecting microorganisms from the toxicity of nitric oxide is through enzymes that convert NO into less toxic molecules. Flavohemoglobin is a NO dioxygenase, encoded by YHB1 gene. In CA, it is induced by NO and converts it to nitrate (GARDNER et al., 1998; ULLMANN et al., 2004). Amphotericin B promote oxidative damage to fungal cells and several oxidative stress response genes are overexpressed in response to the drug (LIU et al., 2005), such as YHB1, which in our results was also overexpressed compared to the negative control after treatment with P/8 N/4 and **N** in both strains evaluated.

CA produces and secretes various hydrolytic enzymes, including secreted aspartyl proteinases and phospholipases. The proteolytic activity of secreted aspartyl proteinases (Saps), encoded by SAP gene, has been considered essential for CA virulence in several studies, being composed of 10 proteins, SAP1 to SAP10 (ODDS, 1988; SANGLARD et al., 1997). Aspartyl proteinases are secreted by *Candida* pathogenic species *in vivo* during infection (MACDONALD; ODDS, 1980; BERNARDIS et al., 1990; DE BERNARDIS et al., 1995). The enzymes are secreted *in vitro* when the microorganism is cultured in the presence of exogenous protein (usually bovine serum albumin) as a source of nitrogen. Besides, not only the exogenous protein is essential for enzymatic induction, but the pH of the medium seems to act directly on aspartyl proteinases synthesis (CHAFFIN et al., 1998).

In CA, the ALS family (Agglutinin-like sequence) consists of at least four genes that can encode cell wall proteins (HOYER et al., 1995). Although ALS genes were originally isolated from expression in hyphae cultured with RPMI 1640 and not in yeast forms cultured with YEPD medium, the genes do not appear to be regulated by the yeast-hyphae transition, but dependent of the components in the culture medium (CHAFFIN et al., 1998).

Phospholipases secreted by CA generate tissue damage in host cells, rupturing epithelial cells membranes and allowing that the hyphae extremities infiltrate in cytoplasm cytoplasm of host cells, it means, consist in a tissue invasion mechanism. In several studies we observed that for the induction of these enzymes *in vitro*, medium modified by vitamins are used, in addition the fetal bovine serum in its composition. Besides of the differentiated nutritional factor, fungal growth is performed for 7 days before the supernatants are collected (MACDONALD; ODDS, 1980; IBRAHIM et al., 1995; D'EÇA JÚNIOR et al., 2011; ELLS et al., 2014).

Due to the above conditions, we hypothesize that ALS, SAP and

phospholipase family proteins were not identified in our samples, since the composition, culture medium pH and fungal growth time were not adequate for the synthesis and secretion of these proteins at detectable levels by the mass spectrometer.

Differences in protein expression among the both strains evaluated were not analyzed at this first moment, because initially it was not the aim of our study. Indeed, these differences are expected, since one of the strains is clinical (SC 5314), and therefore exhibit resistance and virulence factors different from the standard strain (ATCC 90028). This evaluation will be carried out later.

In summary, the advent of new therapeutic alternatives for oral candidosis treatment is very relevant and the use of natural products has been increasing (NASCIMENTO et al., 2000). Our results suggest that P/8 N/4 could act against *C. albicans*, directly interacting with proteins implicated in structural organization of fungus morphology and in essential energy metabolic processes. This may reflect in damage capacity to its structure and filamentation reduction, resulting in imperfect and non-viable cells.

Declaration of interest

The authors declare no conflicts of interest.

Acknowledgements

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Supplementary material

Appendix A

Suppl Table A2. Proteins identified exclusively in the P/8 N/4 group, *C. albicans* ATCC 90028.

[a]Acess number	Protein name	*PLGS score*
C4YIE3	60S ribosomal protein L17	107.35
Q5AGX8	Acetyltransferase component of pyruvate dehydrogenase complex	38.93
A0A1D8PIB2	Asparagine synthase (Glutamine-hydrolyzing) 2	34.1
Q5A6R2	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	34.69
O42696	CaNIK1 protein	24.5
B9WKZ5	Cell division control protein 4 homologue_ putative	21.69
C4YJC1	Cytochrome c oxidase polypeptide IV_ mitochondrial	192.73
Q5ALV5	Cytochrome c oxidase subunit IV	192.73
Q5AJZ3	Ditrans_polycis-polyprenyl diphosphate synthase ((2E_6E)-farnesyl diphosphate specific)	35.74
Q5AAF4	Formin BNR1	33.53
A0A1D8PHP4	Golgi transport complex subunit	53.58
B9WHD5	Heat shock protein_ putative	255.84
O74713	High-affinity glucose transporter	118.67
Q9URL9	Histidine kinase	24.5
Q5AHA0	Histidine protein kinase 1	17.46
Q5A599	Histidine protein kinase NIK1	24.5
Q5AIA2	Homoserine dehydrogenase	68.24
A0A1D8PNQ9	Lap4p	45.34
Q59TU5	Mrf1p	63.69
A0A1D8PI00	NAD-specific glutamate dehydrogenase	41.66
O93851	Nik1p	24.5
B9WAX2	Nuclear transport factor_ putative	18.87
A0A1D8PM92	Nucleocytoplasmic transporter	25.79
B9WF95	O-acetylhomoserine o-acetylserine sulphydrylase_ putative	24.84
O13289	Peroxisomal catalase	92.03
O74711	Peroxisomal targeting signal receptor	61.08
A0A1D8PRM7	Phosphoenolpyruvate carboxykinase	70.59
C4YI55	Protein MET17	24.84
Q59TE0	Ribosomal 60S subunit protein L17B	107.35
A0A1D8PGP2	rRNA-processing protein	36.83
Q5A8X6	Succinate--CoA ligase [ADP-forming] subunit alpha_ mitochondrial	176.74
A0A1D8PSD4	Sys3p	15.05
B9WGV8	Threonyl-tRNA synthetase_ cytoplasmic_ putative	26.54
B9WD11	U3 small nucleolar RNA-associated protein_ putative	31.06
A0A1D8PN55	Uncharacterized protein	34.56
A0A1D8PDR3	Uncharacterized protein	44.89
A0A1D8PNM4	Uncharacterized protein	48.84
A0A1D8PNW3	Uncharacterized protein	59.63
A0A1D8PQR8	Uncharacterized protein	44.12
A0A1D8PTW2	Uncharacterized protein	35.36
B9WC14	Uncharacterized protein	36.97
Q5ABF8	Uncharacterized protein	26.76
G1U9Z8	Uncharacterized protein CaJ7.0123	40.85
B9WBH7	White colony protein_ putative	1750.47
B9WHX1	Xenobiotic compound monooxygenase_ DszA family_ putative	57.27

Identified proteins are organized according to the alphabetical order of proteins. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Suppl Table A3. Proteins identified exclusively in the P group, *C. albicans* ATCC 90028.

[a]Acess number	Protein name	*PLGS score*
A0A1D8PRK3	Uncharacterized protein	84.67
C4YT59	Uncharacterized protein	84.67
Q5ADM2	Uncharacterized protein	46.95
G1UAQ5	Uncharacterized protein CaJ7.0502	84.67
B9W7T1	Spliceosomal factor U2AF small subunit_ putative	82.11
B9WIZ1	Subunit of RNase MRP and of nuclear RNase P_ putative	83.68

Identified proteins are organized according to the alphabetical order of proteins. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Suppl Table A4. Proteins identified exclusively in the N group, *C. albicans* ATCC 90028.

[a]Acess number	Protein name	*PLGS score*
B9WMM0	Cystathionine gamma-lyase_ putative	122.27
Q9B8D8	Cytochrome c oxidase subunit 2	109.06
Q5APG7	Protein EFR3	33.19
A0A1D8PNG9	Threonine synthase	47.85
B9WMV2	Transporter of the Major Facilitator Superfamily (MFS)_ putative	60.91
C4YQH1	Uncharacterized protein	264.1
C4YDA3	Uncharacterized protein	23.02
C4YGH8	Uncharacterized protein	35.17
C4YI87	Uncharacterized protein	113.59
C4YP88	Uncharacterized protein	171.85
C4YT24	Uncharacterized protein	474.2
Q5A0W1	Uncharacterized protein	35.17
B9WGH4	Vacuolar inheritance protein_ putative	67.9
Q59MN0	Vacuolar protein 8	67.9

Identified proteins are organized according to the alphabetical order of proteins. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Suppl Table A5. Proteins identified exclusively in the control group, *C. albicans* ATCC 90028.

[a]Acess number	Protein name	*PLGS score*
C4YFR9	10 kDa heat shock protein_ mitochondrial	1075.73
P40910	40S ribosomal protein S1	170.00
Q5ADQ6	40S ribosomal protein S12	448.32
Q96W53	40S ribosomal protein S14	213.33
C4YQM1	40S ribosomal protein S15	561.71
C4YNQ0	40S ribosomal protein S19-B	188.04
C4YTD8	40S ribosomal protein S28-A	449.06
C4YMQ1	40S ribosomal protein S29-A	431.42
P47837	40S ribosomal protein S4	85.72
A0A1D8PL99	40S ribosomal protein S6	297.22
Q5AJ93	40S ribosomal protein S7	1184.77
Q5PS51	60 kDa chaperonin (Fragment)	457.00
A0A1D8PQS0	60S acidic ribosomal protein P0	269.64
Q9HFQ4	60S acidic ribosomal protein P2-B	255.07
B9WE59	60s acidic ribosomal protein_ putative	255.07
B9WBX0	60s ribosomal protein [l11-b]_ putative	249.23
Q9UVJ4	60S ribosomal protein L10a	177.82
C4YJY8	60S ribosomal protein L11	249.23
C4YPY4	60S ribosomal protein L12	1760.28
A0A1D8PLC9	60S ribosomal protein L20	328.21
C4YSV1	60S ribosomal protein L25	88.40
C4YF39	60S ribosomal protein L26-B	276.10
Q9P843	60S ribosomal protein L27	319.23
P47831	60S ribosomal protein L28	693.43
C4YKL4	60S ribosomal protein L3	215.59
A0A1D8PCX8	60S ribosomal protein L6	100.73

C4YCU7	60S ribosomal protein L8-B	166.29
B9WLA5	60s ribosomal protein_ putative	693.43
Q5AFA8	Abp1p	94.63
A0A1D8PH52	Acetyl-CoA C-acetyltransferase	197.04
Q9P837	Actin binding protein (Fragment)	84.71
A0A1D8PQ26	Adenosine kinase	201.43
Q5A4Q1	Adenylate kinase	391.68
Q5A516	ADP/ATP carrier protein	187.91
Q59R28	Alpha-1_2-mannosyltransferase MNN26	63.48
Q5AL11	Amino acid transporter	36.53
P46586	ATP phosphoribosyltransferase	51.75
B9WD23	ATP synthase subunit_ mitochondrial_ putative	287.49
Q5A5R4	ATP-dependent DNA helicase	34.00
C4YTQ8	ATP-dependent molecular chaperone HSP82	117.05
A0A1D8PFB8	Cell division control protein	30.70
P43069	Cell division control protein 25	28.07
C4YP07	Chromatin remodelling complex ATPase chain ISW1	61.44
A0A1D8PSH3	Citrate synthase	1116.64
P53698	Cytochrome c	75.04
Q5APK5	Cytochrome c oxidase subunit Va	146.26
Q59RQ6	Dihydrolipoyl dehydrogenase	184.29
Q5AND3	DNA helicase	47.64
Q5A5N2	DNA-dependent ATPase	42.74
A0A1D8PG90	ESCRT-III subunit protein	83.48
Q5A7P7	F1F0 ATP synthase subunit 5	287.49
A0A1D8PHL7	F1F0 ATP synthase subunit h	916.91
Q5A6L1	Fumarase	72.44
C4YLR9	Fumarate hydratase_ mitochondrial	68.91
Q9P841	Galactose/glucose transporter (Fragment)	240.20
Q9P4E9	GTP-binding nuclear protein GSP1/Ran	103.24
P83774	Guanine nucleotide-binding protein subunit beta-like protein	423.27
B9WK86	Heat shock protein 82 homolog_ putative	110.88
P46598	Heat shock protein 90 homolog	124.98
Q96VB9	Heat shock protein homolog SSE1	141.58
C4YE44	Heat shock protein Hsp88	141.58
C4YR68	Heat shock protein STI1	151.53
Q5AD47	Hexose transporter	267.73
A0A1D8PG81	Hgt7p	267.73
A0A1D8PG82	Hgt8p	267.73
P48989	Histone H2B.1	139.07
Q59VP1	Histone H2B.2	131.35
Q5A9D9	Homoisocitrate dehydrogenase	134.15
A0A1D8PN90	Hsp90 cochaperone	145.40
P83777	Inorganic pyrophosphatase	1674.23
B9WBW2	Integral membrane protein_ eisosome component_ putative	771.77
A0A1D8PS79	Isocitrate dehydrogenase [NADP]	156.46
Q5A310	ISWI chromatin-remodeling complex ATPase ISW2	61.44
Q59NN4	Long-chain fatty acid transporter	30.86
G1UA43	Lysine/glutamic acid-rich protein	18.49
B9WH01	Major ADP/ATP carrier protein of the mitochondrial inner membrane_ putative	69.25
A0A1D8PCV3	mRNA splicing protein	83.71
Q5A940	Multiprotein-bridging factor 1	199.69
C4YRH4	NAD(P)H-dependent D-xylose reductase I_II	192.74
Q5ADT1	Negative regulator of sporulation MDS3	15.60
Q5A784	Ofr1p	77.51
B9WK13	Part of 40S ribosomal subunit_ putative	423.27
P22011	Peptidyl-prolyl cis-trans isomerase	284.51
Q5ACI8	Peptidyl-prolyl cis-trans isomerase D	314.67
B9WEV5	Plasma membrane protein_ putative	67.53
Q5AI15	Polyadenylate-binding protein_ cytoplasmic and nuclear	58.05
Q5ADL0	Pre-mRNA-processing ATP-dependent RNA helicase PRP5	64.51
B9WKX9	Prolyl-tRNA synthetase_ cytoplasmic_ putative	68.40
A0A1D8PR99	Protein disulfide isomerase	109.01

A0A1D8PKD1	Rct1p	294.48
Q59KG2	Respiratory growth induced protein 1	322.22
A0A1D8PDT3	Ribosomal 40S subunit protein S14B	213.33
A0A1D8PK22	Ribosomal 40S subunit protein S15	561.71
A0A1D8PK61	Ribosomal 40S subunit protein S19A	188.04
A0A1D8PQN0	Ribosomal 40S subunit protein S28B	449.06
A0A1D8PTR4	Ribosomal 40S subunit protein S29A	504.40
A0A1D8PHW1	Ribosomal 60S subunit protein L11B	249.23
Q5AJF7	Ribosomal 60S subunit protein L12A	1760.28
A0A1D8PPT5	Ribosomal 60S subunit protein L23B	1025.85
A0A1D8PPS1	Ribosomal 60S subunit protein L25	96.39
A0A1D8PCQ5	Ribosomal 60S subunit protein L26B	276.10
A0A1D8PSC5	Ribosomal 60S subunit protein L28	693.43
Q59LS1	Ribosomal 60S subunit protein L3	215.59
A0A1D8PHF5	Ribosomal 60S subunit protein L31B	2780.77
Q5ANA1	Ribosomal 60S subunit protein L8B	174.50
C4YSS4	Ribosomal protein	177.82
B9WDI1	Ribosomal protein of the large subunit_ putative	1760.28
B9WE58	Ribosomal protein of the small subunit_ putative	561.71
Q5ANH5	Ribosomal protein P2B	255.07
B9WBJ8	Ribosomal protein_ large subunit_ putative	2780.77
A0A1D8PF11	Rpl82p	166.29
A0A1D8PU61	S-(hydroxymethyl)glutathione dehydrogenase	383.55
O13426	Serine hydroxymethyltransferase_ cytosolic	111.22
Q5ADT0	Slk19p	27.90
G1UAE2	SOM1 protein	120.26
A7ISD6	Sti1	182.92
Q9P8T3	Telomerase reverse transcriptase 1	78.20
Q9P8T2	Telomerase reverse transcriptase 2	78.20
A0A1D8PEA0	Tertp	78.20
Q5A017	Transaldolase	92.94
P0CY34	Transcriptional repressor TUP1	77.18
Q5ADR6	Translation initiation factor eIF2B subunit delta	68.12
A0A1D8PNK3	Trifunctional aldehyde reductase/xylose reductase/glucose 1-dehydrogenase (NADP(+))	192.74
Q5A399	Tyrosine protein phosphatase	96.02
A0A1D8PP59	Ubiquinol--cytochrome-c reductase subunit	174.25
B9WIP9	Ubiquinone biosynthesis O-methyltransferase_ mitochondrial	47.96
B9WGD1	Ubiquitin carboxyl-terminal hydrolase_ putative	58.22
A0A1D8PFU8	Uncharacterized protein	1075.73
A0A1D8PGN6	Uncharacterized protein	42.40
A0A1D8PIA8	Uncharacterized protein	85.49
A0A1D8PJP3	Uncharacterized protein	76.50
A0A1D8PK71	Uncharacterized protein	89.83
A0A1D8PMG9	Uncharacterized protein	30.53
A0A1D8PPV6	Uncharacterized protein	263.68
A0A1D8PQF2	Uncharacterized protein	87.65
A0A1D8PRS9	Uncharacterized protein	104.88
Q59PD4	Uncharacterized protein	175.29
O94028	Uncharacterized protein Ca49C10.14	83.71
G1UAF7	Uncharacterized protein CaJ7.0374	109.01
B9WAE7	Vacuolar protein sorting protein 24_ putative	83.48

Identified proteins are organized according to the alphabetical order of proteins. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Suppl Table A6. Proteins identified exclusively in the P/8 N/4 group, *C. albicans* SC 5314.

[a]Acess number	Protein name	*PLGS score*
C4YFR9	10 kDa heat shock protein_ mitochondrial	536.33
C4YJX2	40S ribosomal protein S9-B	82.58
Q9HFQ7	60S acidic ribosomal protein P1-A	472.02
C4YD35	Altered inheritance of mitochondria protein 21	60.93
A0A1D8PP00	Arginase	308.85

A0A1D8PIB2	Asparagine synthase (Glutamine-hydrolyzing) 2	144.53
A0A1D8PKB9	Branched-chain-amino-acid aminotransferase	125.26
B9WN03	BTB domain and ankyrin repeat protein_ putative	46.00
O42696	CaNIK1 protein	51.25
Q9P8X1	Crm1p	48.60
C4YJU4	Cytochrome c oxidase polypeptide VI_ mitochondrial	154.13
C4YGB8	D-3-phosphoglycerate dehydrogenase 1	149.89
B9WEB0	Dynein heavy chain_ cytosolic_ putative	14.76
A0A1D8PRR9	Exportin	52.59
C4YQN7	Fatty acid synthase alpha subunit reductase	39.69
Q59P43	GTP-binding nuclear protein	318.24
C4YMN0	Heat shock protein 104	111.86
G9BX82	Heat shock protein 70	4114.71
A0A1D8PCL1	Hgt1p	74.07
O74713	High-affinity glucose transporter	76.53
O74271	Histidine kinase	51.25
B9W8C8	Kinetochore subunit_ putative	417.3
H8WVN7	Late-stage biofilm-induced gene in C. albicans	72.81
G1UA43	Lysine/glutamic acid-rich protein	134.57
C4YRH4	NAD(P)H-dependent D-xylose reductase I_II	225.99
Q5ADT1	Negative regulator of sporulation MDS3	186.32
O93851	Nik1p	51.25
B9WEV5	Plasma membrane protein_ putative	27.39
B9W8V8	Pre-mRNA splicing complex subunit_ putative	72.1
C4YKB0	Protein YOP1	205.64
B9WLS6	Ribosomal protein_ small subunit_ putative	287.17
Q59WE2	SCF ubiquitin ligase subunit	417.3
C4YDT0	Suppressor of kinetochore protein 1	417.3
C4YP91	Uncharacterized protein	134.57
C4YP92	Uncharacterized protein	179.5
C4YS31	Uncharacterized protein	135.41
C4YTW8	Uncharacterized protein	51.25
G1UAV9	Uncharacterized protein CaJ7.0309	631.86

Identified proteins are organized according to the alphabetical order of proteins. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Suppl Table A7. Proteins identified exclusively in the N group, *C. albicans* SC 5314.

[a]Acess number	Protein name	*PLGS score*
B9W7A3	14-3-3 protein_ minor isoform_ putative	115.04
C4YI06	2-(3-amino-3-carboxypropyl)histidine synthase subunit 2	111.94
Q5ALV6	40S ribosomal protein S26	97.01
C4YIP1	60S ribosomal protein L33-A	343.19
C4YTM0	Acetyltransferase component of pyruvate dehydrogenase complex	67.30
Q5AEF9	Allantoate permease	82.47
B9W7H8	Aspartyl-tRNA synthetase_ mitochondrial	38.57
Q5AK53	ATP-dependent 6-phosphofructokinase	60.98
C4YI53	ATP-dependent RNA helicase SUB2	186.51
Q59US5	Bifunctional cysteine synthase/O-acetylhomoserine aminocarboxypropyltransferase	160.54
B9W7P8	Butanediol dehydrogenase_ putative	79.56
C4YEV5	Cell division control protein 12	75.21
B9WMM0	Cystathionine gamma-lyase_ putative	210.45
Q5APK5	Cytochrome c oxidase subunit Va	512.42
Q59SR3	Dal9p	82.47
Q59SJ9	Diphthamide biosynthesis protein 2-1	111.94
A0A1D8PL26	Diphthamide biosynthesis protein 2-2	132.03
B2B9R2	ECE1	49.68
Q07730	Extent of cell elongation protein 1	113
A0A1D8PPY8	Fimbrin	82.49
B9WBX6	Flavodoxin-like reductase_ putative	175.51
Q5AD47	Hexose transporter	102.88
B9WBI5	Importin beta-2 subunit_ putative	56.9

A0A1D8PCV7	L-aminoadipate-semialdehyde dehydrogenase	38.5
B9WAE0	Leucine-rich repeat-containing protein_ conserved	82.49
Q59TC4	Lys22p	81.37
B9W8Y7	Malate synthase	385.1
A0A1D8PL61	Midasin	50.16
A0A1D8PGY4	Mitogen-activated protein kinase	44.43
B9W6I9	NATB N-terminal acetyltransferase non-catalytic subunit_ putative	70.71
Q9UR58	O-acetylhomoserine O-acetylserine sulphydrylase	160.54
Q59T35	Osm1p	53.51
A0A1D8PHZ6	Pds5p	34.22
B9WCA6	Phosphatidylinositol 4_5-bisphosphate 5-phosphatase_ putative	75.75
C4YI55	Protein MET17	110.56
Q5A0Z9	Pyruvate dehydrogenase E1 component subunit alpha	75.61
B9WI96	Regulator of Ty1 transposition_ putative	125.69
A0A1D8PHH4	Ribosomal 60S subunit protein L33A	343.19
B9WDL4	Ribosomal protein of the large subunit_ putative	185.66
A0A1D8PKJ4	Saccharopine dehydrogenase (NADP+_ L-glutamate-forming)	177.78
A0A1D8PCY5	Septin	75.21
Q9HGT6	Serine--tRNA ligase_ cytoplasmic	91.24
C4YM97	Succinate--CoA ligase [ADP-forming] subunit beta_ mitochondrial	566.95
B9WH30	Topoisomerase 1-associated factor_ putative	112.3
Q5A017	Transaldolase	458.89
Q5A6A4	tRNA N6-adenosine threonylcarbamoyltransferase	106.52
C4YJ68	Uncharacterized protein	86.13
C4YHX9	Uncharacterized protein	40.96
C4YIP0	Uncharacterized protein	53.51
C4YJ67	Uncharacterized protein	45.07
C4YJW7	Uncharacterized protein	34.81
Q5A398	Uncharacterized protein	137.19
B9W744	Vaculoar membrame protein_ putative	57.72

Identified proteins are organized according to the alphabetical order of proteins. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Suppl Table A8. Proteins identified exclusively in the control group, *C. albicans* SC 5314.

[a]Acess number	Protein name	*PLGS score*
B9WD61	2-oxoglutarate dehydrogenase E1 component_ mitochondrial_ putative	77.40
B9WJJ9	40S ribosomal protein (S18)_ putative	613.23
C4YR43	40S ribosomal protein S11	151.84
B9WEU1	40S ribosomal protein S12	685.51
B9W994	40S ribosomal protein S17 subunit_ putative	962.16
C4YCW2	40S ribosomal protein S17-B	962.16
Q5A7K0	40S ribosomal protein S24	744.04
C4YRM5	40S ribosomal protein S25-B	278.41
C4YTD8	40S ribosomal protein S28-A	3087.89
C4YRI8	40S ribosomal protein S5	144.85
B9W733	60S ribosomal protein L10_ putative	334.68
B9W9T2	60s ribosomal protein l14_ putative	443.73
C4YFM9	60S ribosomal protein L16-A	402.09
C4YTN5	60S ribosomal protein L5	417.66
C4YNQ6	60S ribosomal protein L8-B	488.4
Q5AFA8	Abp1p	176.93
A0A1D8PH52	Acetyl-CoA C-acetyltransferase	153.29
C4YTE2	Acyl-CoA-binding protein 2	242.33
P0CH96	Adenylosuccinate synthetase	64.26
B9WBI3	Aspartate aminotransferase_ cytoplasmic_ putative	93.04
A0A1D8PRY3	ATP synthase subunit gamma	436.3
B9WBK5	ATP synthase subunit_ mitochondrial_ putative	280.88
A0A1D8PRM6	ATP-dependent DNA helicase RRM3	55.37
P0CT51	Blood-induced peptide 1	440.22
A0A1D8PNN8	Cam1-1p	154.87
A0A1D8PG90	ESCRT-III subunit protein	168.27
Q59ZE0	F1F0 ATP synthase subunit 4	300.41
A0A1D8PKV4	Fum12p	164.35

B9WHD5	Heat shock protein_ putative	12095.79
A0A1D8PIM0	Ifr1p	66.32
A0A1D8PSE7	Ifr2p	80.04
A0A1D8PS79	Isocitrate dehydrogenase [NADP]	771.08
B9WK63	Large subunit ribosomal protein_ putative	417.66
A0A1D8PQN3	Long-chain fatty acid transporter	254.39
A0A1D8PU56	Long-chain fatty acid-CoA ligase	83.17
A0A1D8PRG4	Mam33p	399.28
Q5AHH0	Mediator of RNA polymerase II transcription subunit 1	41.51
B9WJV2	Mitochondrial matrix acidic protein_ putative	151.22
C4YLN8	Mitochondrial protein import protein MAS5	151.07
Q59M70	NADH-cytochrome b5 reductase 2	244.3
Q9UVL1	Non-histone chromosomal protein 6	347.92
P53696	Profilin	372.77
Q5AP65	Protein FMP52_ mitochondrial	115.14
B9WE72	Ray38p homologue_ putative	159.13
A0A1D8PKD1	Rct1p	1457.11
Q5AND4	Rdi1p	217.92
B9WGE2	Reticulon-like protein	270.77
B9WE89	Rho GDP-dissociation inhibitor_ putative	114.76
Q5A2L2	Ribose phosphate diphosphokinase subunit	63.54
A0A1D8PI15	Ribosomal 40S subunit protein S10A	590.78
A0A1D8PN83	Ribosomal 40S subunit protein S11A	500.08
A0A1D8PEY9	Ribosomal 40S subunit protein S17B	965.84
A0A1D8PQQ5	Ribosomal 40S subunit protein S18B	613.23
A0A1D8PNQ6	Ribosomal 40S subunit protein S25B	278.41
A0A1D8PQN0	Ribosomal 40S subunit protein S28B	3087.89
Q5AG43	Ribosomal 40S subunit protein S5	169.68
Q5AIB8	Ribosomal 60S subunit protein L10	351.01
A0A1D8PFL9	Ribosomal 60S subunit protein L14B	748.92
Q5AB87	Ribosomal 60S subunit protein L16A	402.09
Q5AGZ7	Ribosomal 60S subunit protein L5	417.66
Q5ANA1	Ribosomal 60S subunit protein L8B	504.01
Q5A6R1	Ribosomal protein L15	1658.58
A0A1D8PK40	Ribosomal protein L19	604.52
B9WMR3	RNA Pol II transcript 3' end formation complex subunit_ putative	90.37
A0A1D8PF11	Rpl82p	441.62
Q5AGZ9	RuvB-like helicase 2	111.02
A0A1D8PU61	S-(hydroxymethyl)glutathione dehydrogenase	67.72
B9W743	SNO-family glutamine amidotransferase_ putative	182.55
A0A1D8PTR7	Tropomyosin	164.6
P10875	Tubulin beta chain	212.77
A0A1D8PSQ3	Type I HSP40 co-chaperone	151.07
P52495	Ubiquitin-activating enzyme E1 1	42.61
A0A1D8PNW3	Uncharacterized protein	67.31
B9W7D1	Uncharacterized protein	88.38
B9WF63	Uncharacterized protein	29.64
C4YG27	Uncharacterized protein	443.73
C4YHS4	Uncharacterized protein	347.92
C4YJ60	Uncharacterized protein	168.27
C4YKT1	Uncharacterized protein	66.32
C4YLE5	Uncharacterized protein	80.04
C4YMP9	Uncharacterized protein	143.4
C4YQN5	Uncharacterized protein	430.83
C4YRK4	Uncharacterized protein	149.25
C4YRT4	Uncharacterized protein	67.31
B9WAV1	Uroporphyrin-III c-methyltransferase_ putative	61.25
B9WAE7	Vacuolar protein sorting protein 24_ putative	168.27
B9WHX1	Xenobiotic compound monooxygenase_ DszA family_ putative	67.31

Identified proteins are organized according to the alphabetical order of proteins. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Suppl Table A9. Proteins identified with significantly altered expression in the P/8 N/4 in comparison to control, *C. albicans* ATCC 90028.

[a]Acess number	Protein name	*Gene*	*Processo Biológico*	*PLGS score*	Ratio P/8 Nis/4 *vs.* C-
Q59MV9	Flavohemoprotein	YHB1	response to toxic substance, response to defense-related host nitric oxide production	119.86	6.42
C4YMJ2	Uncharacterized protein	CAWG_02074	oxidoreductase activity	119.86	6.23
B9WGB7	Translation elongation factor 1-beta (EF-1-beta)_ putative	EFB1	Protein biosynthesis	229.26	2.12
Q92211	Glyceraldehyde-3-phosphate dehydrogenase	TDH1	cell wall organization, glucose metabolic process	5063.29	1.6
Q9P8Q7	Isocitrate lyase	ICL1	tricarboxylic acid cycle, pathogenesis	112.88	1.57
Q59T44	40S ribosomal protein S8	RPS8A	translation	950.45	1.39
P83779	Pyruvate decarboxylase	PDC11	magnesium ion binding	784.68	1.22
Q0ZID4	GTPase cytoplasmic elongation factor 1 alpha (Fragment)	tef1	translation elongation factor activity	3080.42	0.84
M4Q0Q2	Translation elongation factor 1-alpha (Fragment)	N/A	Protein biosynthesis	3080.42	0.84
P46614	Pyruvate kinase	CDC19	cellular response to starvation, filamentous growth.	472.56	0.81
C4YDJ3	Elongation factor 1-alpha	CAWG_00588	translation elongation factor activity, interaction of plasminogen	3842.91	0.78
Q59QD6	Elongation factor 1-alpha 2	TEF2	translational elongation	3846.06	0.78
P0CY35	Elongation factor 1-alpha 1	TEF1	interaction with host, translational elongation	3842.91	0.77
B9WGY2	Cofilin_ putative	----	actin filament depolymerization, endocytosis	687.25	0.72
C4YI58	Ornithine aminotransferase	CAWG_03767	ornithine metabolic process	225.74	0.71
C4YMM3	40S ribosomal protein S20	RPS20	translation	3145.47	0.68
Q5A389	Ribosomal 40S subunit protein S20	RPS20	translation	3186.83	0.68
B9WMK5	Ribosomal protein_ small subunit_ putative	Cd36_33780	translation	3145.47	0.68
P82611	Aconitate hydratase_ mitochondrial	ACO1	induction by symbiont of host defense response	231.25	0.66

P82610	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (Methionine synthase)	MET6	fungal growth	404.4	0.65
P14235	Actin	ACT1	ATP binding. DNA repair, cellular response to oxidative stress	246.26	0.61
P28877	Plasma membrane ATPase 1	PMA1	Hydrogen ion transport, Ion transport. Transport	649.46	0.55
B9WC24	Heat shock protein	Cd36_21650	Stress response	395.63	0.53
B9WMK0	Cytoplasmic ATPase that is a ribosome-associated molecular chaperone_ putative	Cd36_33730	cellular response to glucose starvation	1128.27	0.49
Q5A1E8	Succinate dehydrogenase [ubiquinone] flavoprotein subunit_ mitochondrial	SDH12	Electron transport	117.22	0.49
P87222	Ribosome-associated molecular chaperone SSB1	SSB1	cellular response to glucose starvation	1128.27	0.48
P0CT51	Blood-induced peptide 1	BLP1	pleiotropic stress-tolerance phenotype	839.84	0.47
P83784	Heat shock protein SSC1_ mitochondrial	SSC1	Stress response	409.53	0.47
P83781	Mitochondrial outer membrane protein porin	POR1	porin activity	302.34	0.46
P82612	Phosphoglycerate mutase	GPM1	interaction with host	3738.51	0.45
A0A1D8PHF8	Wh11p	WH11	Pathogenesis, phenotypic switching, response to stress, biofilm formation.	4953.01	0.44
P43074	White colony protein WHS11	WHS11	response to stress	4953.01	0.44
Q5ANP2	Nascent polypeptide-associated complex subunit alpha	EGD2	protein transport	1388.01	0.43
A0A1D8PFV1	Ribosomal 60S subunit protein L4B	RPL4B	translation	581.25	0.43
P83773	Acetyl-CoA hydrolase	ACH1	cellular response to alkaline pH, acetate metabolic process	242.61	0.41
C4YFR7	60S ribosomal protein L4-B	CAWG_00043	translation	581.25	0.4
Q6YK78	29 kDa IgE-binging protein	N/A	IgE-reacting componentes. human allergic disorders	776.48	0.39
Q5AF03	Glyoxalase 3	GLX3	Stress response	1160.38	0.39
O74261	Heat shock protein 60_ mitochondrial	HSP60	cellular response to heat, protein refolding	1289.54	0.39
C4YQ30	Protein SNO4	CAWG_02584	----	776.48	0.39

P0CU34	Peroxiredoxin TSA1-B	TSA1B	cell redox homeostasis, antioxidant activity, Maintenance of genomic stability	5671.38	0.38
P46273	Phosphoglycerate kinase	PGK1	cell wall organization, glycolytic process.	1329.22	0.38
Q9Y7F0	Peroxiredoxin TSA1-A	TSA1	cellular response to oxidative stress, filamentous growth, Maintenance of genomic stability	5671.38	0.37
Q5AG68	Nucleoside diphosphate kinase	YNK1	nucleoside diphosphate phosphorylation	1742.78	0.35
A0A1D8PDC4	ATP synthase subunit alpha	ATP1	ATP biosynthetic process	1536.64	0.33
C4YEG9	Sphingolipid long chain base-responsive protein LSP1	CAWG_00926	Endocytosis, negative regulation of protein kinase activity, response to heat	1573.41	0.33
A0A1D8PDD1	Lipid-binding protein	PIL1	---	1591.58	0.32
O59931	60S ribosomal protein L13	RPL13	translation	800.81	0.31
C4YCU4	60S ribosomal protein L2	CAWG_00328	translation	868.72	0.3
A0A1D8PP43	Adh1p	ADH1	interaction with host, biofilm formation, , interaction of plasminogen	4220.94	0.3
P43067	Alcohol dehydrogenase 1	ADH1	interaction with host, biofilm formation, interaction of plasminogen	4220.94	0.3
Q9URB4	Fructose-bisphosphate aldolase	FBA1	glycolytic process, interaction with host. Adherence, interaction of plasminogen.	1665.66	0.3
A0A1D8PF08	Ribosomal 60S subunit protein L2A	RPL2	translation	868.72	0.3
B9W7P2	Uncharacterized protein	Cd36_04430	Endocytosis, negative regulation of protein kinase activity, response to heat	1573.41	0.3
A0A1D8PKZ9	ATP synthase subunit beta	ATP2	ATP synthesis coupled proton transport	5140.73	0.29
G9BX82	Heat shock protein 70	hsp70	Stress response	3706.66	0.28
P41797	Heat shock protein SSA1	SSA1	antimicrobial humoral response, cellular response to heat, interaction with host .	3706.66	0.28
P46587	Heat shock protein SSA2	SSA2	antimicrobial humoral response, interaction with host, response to toxic substance	3486.87	0.28
C4YFZ1	Heat shock protein SSA4	CAWG_00103	Stress response	3706.66	0.28
A0A1D8PTI7	40S ribosomal protein S27	RPS27	translation	612.73	0.26
Q5AMP4	Malate dehydrogenase	MDH1-1	carbohydrate metabolic process	5031.61	0.26

A0A1D8PGT5	Aldehyde dehydrogenase (NAD(P)(+))	ALD5	Oxidoreductase	1617.67	0.25
P30575	Enolase 1	ENO1	entry into host, fibrinolysis, filamentous growth, glycolytic process.	2283.16	0.25
Q59QN6	Formate dehydrogenase	FDH1	glycine catabolic process	756.51	0.23
B9WDM7	Probable chaperone protein Hsp31 homologue_ putative	Cd36_82570	metabolic process	516.93	0.23
B9WCY3	ATP-dependent helicase_ putative	Cd36_24760	DNA replication. DNA repair.	34.99	0.2
Q9P940	Triosephosphate isomerase	TPI1	filamentous growth, glycolytic process.	670.01	0.19
C4YR96	Uncharacterized protein	CAWG_04595	response to stress	8265.21	0.19
C4YR94	Uncharacterized protein	CAWG_04593	response to stress	8416.93	0.16
A0A1D8PNC7	Hsp12p	HSP12	response to stress	8430.04	0.15
Q5AHH4	Small heat shock protein 21	HSP21	cellular response to oxidative stress, pathogenesis, filamentous growth.	2871.02	0.14

Identified proteins are organized according to the ratio score. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Suppl Table A10. Proteins identified with significantly altered expression in the P in comparison to control, *C. albicans* ATCC90028.

[a]Acess number	Protein name	*Gene*	*Processo Biológico*	*PLGS score*	Ratio P *vs.* C-
P43067	Alcohol dehydrogenase 1	ADH1	interaction with host, biofilm formation, interaction of plasminogen	640.6	1.08

Identified proteins are organized according to the ratio score. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Suppl Table A11. Proteins identified with significantly altered expression in the N in comparison to control, *C. albicans* ATCC 90028.

[a]Acess number	Protein name	*Gene*	*Processo Biológico*	*PLGS score*	Ratio N *vs.* C-
Q59MV9	Flavohemoprotein	YHB1	filamentous growth, pathogenesis, response to toxic substance, response to defense-related host nitric oxide production	119.86	5.81
C4YMJ2	Uncharacterized protein	CAWG_02074	oxidoreductase activity	119.86	5.75
Q92211	Glyceraldehyde-3-phosphate dehydrogenase	TDH1	cell wall organization, glucose metabolic process, Binding to fibronectin and laminin, antigenic properties	5063.29	1.51
A0A1D8PM35	Translation elongation factor 1 subunit beta	EFB1	Protein biosynthesis	1429.45	0.8
P78590	Elongation factor 1-beta	EFB1	Protein biosynthesis	1429.45	0.76

Q5A0M4	Elongation factor 2	EFT2	cellular response to drug, drug binding	183.07	0.68
A0A1D8PGY0	Ribosomal 60S subunit protein L21A	RPL21A	translation	2244.78	0.64
C4YJX1	60S ribosomal protein L21-A	CAWG_04142	translation	2244.78	0.63
O94039	Transketolase 1	TKT1	metabolic process	275.02	0.59
Q5ABC3	Elongation factor Tu	TUF1	translation elongation factor activity	2961.42	0.58
P82611	Aconitate hydratase_ mitochondrial	ACO1	induction by symbiont of host defense response	231.25	0.57
P28877	Plasma membrane ATPase 1	PMA1	Hydrogen ion transport, Ion transport, Transport	649.46	0.54
P14235	Actin	ACT1	ATP binding. DNA repair, cellular response to oxidative stress	246.26	0.53
P82610	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (Methionine synthase)	MET6	cellular response to heat, induction of host defense response	404.4	0.51
P46614	Pyruvate kinase	CDC19	cellular response to starvation, filamentous growth.	472.56	0.51
P83784	Heat shock protein SSC1_ mitochondrial	SSC1	Stress response	409.53	0.5
C4YDJ3	Elongation factor 1-alpha	CAWG_00588	translation elongation factor activity	3842.91	0.47
Q59QD6	Elongation factor 1-alpha 2	TEF2	translational elongation	3846.06	0.47
P0CY35	Elongation factor 1-alpha 1	TEF1	interaction with host, translational elongation	3842.91	0.46
C4YMM3	40S ribosomal protein S20	CAWG_02106	translation	3145.47	0.45
B9WMK5	Ribosomal protein_ small subunit_ putative	Cd36_33780	translation	3145.47	0.45
Q0ZID4	GTPase cytoplasmic elongation factor 1 alpha (Fragment)	tef1	translation elongation factor activity	3080.42	0.44
Q5A389	Ribosomal 40S subunit protein S20	RPS20	translation	3186.83	0.44
M4Q0Q2	Translation elongation factor 1-alpha (Fragment)	N/A	Protein biosynthesis	3080.42	0.44
P83773	Acetyl-CoA hydrolase	ACH1	cellular response to alkaline pH, acetate metabolic process	242.61	0.39
Q5AF03	Glyoxalase 3	GLX3	Stress response	1160.38	0.38
A0A1D8PMW6	Cofilin	COF1	actin filament depolymerization	919.67	0.34
C4YQ30	Protein SNO4	CAWG_02584	----	776.48	0.34
Q6YK78	29 kDa IgE-binging protein	N/A	---	776.48	0.33
B9WMK0	Cytoplasmic ATPase that is a ribosome-associated molecular chaperone_ putative	Cd36_33730	cellular response to glucose starvation	1128.27	0.33
P87222	Ribosome-associated molecular chaperone SSB1	SSB1	cellular response to glucose starvation	1128.27	0.33
P46273	Phosphoglycerate kinase	PGK1	cell wall organization, glycolytic process, interaction with host	1329.22	0.32
Q5ANP2	Nascent polypeptide-associated complex subunit alpha	EGD2	protein transport	1388.01	0.31
P82612	Phosphoglycerate mutase	GPM1	interaction with host	3738.51	0.31
Q9Y7F0	Peroxiredoxin TSA1-A	TSA1	cellular response to oxidative stress, filamentous growth. fungal-type cell wall organization, antioxidant activity, maintenance of genomic stability	5671.38	0.29
C4YJ74	Heat shock 70 kDa protein C	CAWG_03887	ATP binding	484.81	0.28
A0A1D8PHF8	Wh11p	WH11	Pathogenesis, phenotypic switching, response to stress.	4953.01	0.28
P43074	White colony protein WHS11	WHS11	response to stress	4953.01	0.28
Q9URB4	Fructose-bisphosphate aldolase	FBA1	glycolytic process, interaction with host. Adherence, interactions with plasminogen	1665.66	0.27
P0CU34	Peroxiredoxin TSA1-B	TSA1B	cell redox homeostasis, antioxidant activity, maintenance of	5671.38	0.26

			genomic stability		
Q59QN6	Formate dehydrogenase	FDH1	glycine catabolic process	756.51	0.24
G9BX82	Heat shock protein 70	hsp70	Stress response	3706.66	0.24
B9W9W8	Heat-shock protein (HSP) 70_ putative	Cd36_12490	cellular response to heat	3638.44	0.24
O74261	Heat shock protein 60_ mitochondrial	HSP60	cellular response to heat, protein refolding	1289.54	0.23
P41797	Heat shock protein SSA1	SSA1	antimicrobial humoral response, cellular response to heat, interaction with host	3706.66	0.23
C4YFZ1	Heat shock protein SSA4	CAWG_00103	Stress response	3706.66	0.23
P0CG73	Polyubiquitin	UBI1	activation of protein kinases	1050.81	0.23
P43067	Alcohol dehydrogenase 1	ADH1	interaction with host, interactions with plasminogen	4220.94	0.22
P46587	Heat shock protein SSA2	SSA2	antimicrobial humoral response, interaction with host, response to toxic substance	3486.87	0.22
C4YI48	ATP synthase subunit beta	CAWG_03756	ATP synthesis	4550.58	0.21
O60036	Ubiquitin fusion protein	UBI3	translation	1050.81	0.21
A0A1D8PDC4	ATP synthase subunit alpha	ATP1	ATP biosynthetic process	1536.64	0.2
Q5ADS0	Ubiquitin	UBI4	cell morphogenesis, filamentous growth, phenotypic switching, virulence	1050.81	0.2
Q5AG68	Nucleoside diphosphate kinase	YNK1	nucleoside diphosphate phosphorylation	1742.78	0.18
A0A1D8PDD1	Lipid-binding protein	PIL1	----	1591.58	0.15
B9WDM7	Probable chaperone protein Hsp31 homologue_ putative	Cd36_82570	metabolic process	516.93	0.15
C4YEG9	Sphingolipid long chain base-responsive protein LSP1	CAWG_00926	Endocytosis, negative regulation of protein kinase activity, response to heat	1573.41	0.13
P30575	Enolase 1	ENO1	Fibrinolysis, filamentous growth, gluconeogenesis, glycolytic process	2283.16	0.12
Q5AMP4	Malate dehydrogenase	MDH1-1	carbohydrate metabolic process	5031.61	0.12
C4YJP6	Aldehyde dehydrogenase_ mitochondrial	CAWG_04064	Oxidoreductase	1617.67	0.11
A0A1D8PGT5	Aldehyde dehydrogenase (NAD(P)(+))	ALD5	oxidoreductase activity	1617.67	0.1
Q5AHH4	Small heat shock protein 21	HSP21	cellular response to oxidative stress, pathogenesis, filamentous growth	2871.02	0.09

Identified proteins are organized according to the ratio score. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Suppl Table A12. Proteins identified with significantly altered expression in the P/8 N/4 in comparison to control, *C. albicans* SC 5314.

[a]Acess number	Protein name	*Gene*	*Processo Biológico*	*PLGS score*	Ratio P/8 N/4 *vs.* C-.
Q59MV9	Flavohemoprotein	YHB1	filamentous growth, pathogenesis, response to toxic substance, response to defense-related host nitric oxide production	84.33	10.697
Q5AJB1	V-type proton ATPase catalytic subunit A	TFP1	cellular response to oxidative stress, virulence	65.28	2.484
Q9B8D8	Cytochrome c oxidase subunit 2	COX2	mitochondrial electron transport, cytochrome c to	328.31	1.584

			oxygen		
O13426	Serine hydroxymethyltransferase_cytosolic	SHM2	glycine biosynthetic process from serine, tetrahydrofolate interconversion	161.44	1.522
A0A1D8PRG5	Ribosomal protein P1B	RPP1B	translation	618.61	1.507
Q59TE0	Ribosomal 60S subunit protein L17B	RPL17B	cellular response to drug	1124.64	1.448
B9W8L0	Transketolase	Cd36_07840	metabolic process	249.34	1.336
A0A1D8PL14	Ornithine-oxo-acid transaminase	CAR2	cellular response to drug	609.33	1.31
Q9URB4	Fructose-bisphosphate aldolase	FBA1	glycolytic process, interaction with host, adherence, interactions with plasminogen	4255.64	1.209
P0CY35	Elongation factor 1-alpha 1	TEF1	interaction with host, translational elongation	12193.48	1.083
P82612	Phosphoglycerate mutase	GPM1	glycolytic process, interaction with host	8358.03	1.062
Q92211	Glyceraldehyde-3-phosphate dehydrogenase	TDH1	cell wall organization, , Binding to fibronectin and laminin, antigenic properties	19392.47	1.051
C4YJQ8	Elongation factor 2	EFT2	Protein biosynthesis	2265.29	0.923
C4YFR7	60S ribosomal protein L4-B	CAWG_00043	translation	1675.21	0.878
P28877	Plasma membrane ATPase 1	PMA1	ATP biosynthetic process	926.48	0.869
P82611	Aconitate hydratase_ mitochondrial	ACO1	induction by symbiont of host defense response	456.91	0.844
P46614	Pyruvate kinase	CDC19	cellular response to starvation, filamentous growth	3454.37	0.827
O59931	60S ribosomal protein L13	RPL13	translation	3283.63	0.819
Q59T44	40S ribosomal protein S8	RPS8A	translation	1656.95	0.803
P46273	Phosphoglycerate kinase	PGK1	cell wall organization, glycolytic process, interaction with host.	6128.65	0.803
P83782	Cytochrome b-c1 complex subunit 2_ mitochondrial	QCR2	oxidation-reduction process	504.59	0.787
O74261	Heat shock protein 60_ mitochondrial	HSP60	cellular response to heat. protein refolding	3246.94	0.787
P83778	Malate dehydrogenase_ cytoplasmic	MDH1	carbohydrate metabolic process	1949.7	0.779
P83781	Mitochondrial outer membrane protein porin	POR1	porin activity	1095.88	0.771
Q5A6R2	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	ADE17	purine nucleotide biosynthetic process	192.81	0.756
C4YPY4	60S ribosomal protein L12	CAWG_02538	translation	1511.49	0.756
P14235	Actin	ACT1	ATP binding. DNA repair, cellular response to oxidative stress	2965.15	0.733
P83775	Putative NADPH-dependent methylglyoxal reductase GRP2	GRP2	pyruvate metabolic process	845.55	0.726
Q96VB9	Heat shock protein homolog SSE1	MSI3	cellular response to drug, virulence	176.49	0.719
Q5AJ93	40S ribosomal protein S7	RPS7A	translation	1033.64	0.719
O13434	Phosphoenolpyruvate carboxykinase (ATP)	PCK1	gluconeogenesis	872.73	0.677
Q59N40	Aspartate aminotransferase	AAT21	biosynthetic process, cellular amino acid metabolic process	354.37	0.644
P46587	Heat shock protein SSA2	SSA2	antimicrobial humoral response, interaction with host, response to toxic substance	5767.98	0.638
P41797	Heat shock protein SSA1	SSA1	antimicrobial humoral response, cellular response to heat, interaction with host	6734.11	0.631

A0A1D8PNC7	Hsp12p	HSP12	response to stress	13212.9	0.595
P30575	Enolase 1	ENO1	Fibrinolysis, filamentous growth, glycolytic process .	8652.08	0.583
P43067	Alcohol dehydrogenase 1	ADH1	interaction with host, biofilm formation, interactions with plasminogen	10194.2	0.56
Q96W68	Heat shock protein Hsp104	HSP104	protein metabolic process	409.23	0.543
Q9P843	60S ribosomal protein L27	RPL27	translation	865.55	0.522
P25997	Elongation factor 3	CEF3	translational elongation	449.9	0.463
O13289	Peroxisomal catalase	CAT1	filamentous growth. virulence	1482.22	0.463
B9W877	Catalase	Cd36_06380	hydrogen peroxide catabolic process	1475.49	0.445
P22011	Peptidyl-prolyl cis-trans isomerase	CYP1	protein folding	2274.62	0.44
Q59KG2	Respiratory growth induced protein 1	RGI1	energy reserve metabolic process	584.92	0.44
P87066	Tubulin alpha chain	TUB1	cellular response to drug, microtubule-based process	147.94	0.427
C4YTQ8	ATP-dependent molecular chaperone HSP82	CAWG_05553	response to stress	2363.47	0.427
B9WKI6	Protein disulfide-isomerase_ putative	Cd36_72900	cell redox homeostasis	214.92	0.423
A0A1D8PFS4	6-phosphogluconate dehydrogenase_ decarboxylating	GND1	D-gluconate metabolic process	1059.75	0.423
P83774	Guanine nucleotide-binding protein subunit beta-like protein	ASC1	cell adhesion, filamentous growth. virulence	1492.09	0.423
A0A1D8PTP9	Chaperone ATPase	HSP104	Pathogenesis, biofilm formation	455.54	0.419
P87222	Ribosome-associated molecular chaperone SSB1	SSB1	cellular response to glucose starvation	2615.29	0.415
Q9P940	Triosephosphate isomerase	TPI1	filamentous growth, gluconeogenesis, glycolytic process.	1252.96	0.415
P46598	Heat shock protein 90 homolog	HSP90	regulation of apoptotic process, pathogenesis, filamentous growth, cellular response to drug	2363.47	0.403
Q5A516	ADP/ATP carrier protein	PET9	transmembrane transporter activity	1606.12	0.353
P47837	40S ribosomal protein S4	RPS4	translation	506.79	0.336
P83777	Inorganic pyrophosphatase	IPP1	phosphate-containing compound metabolic process	1702.37	0.323
B9WEC5	40S ribosomal protein S0	RPS0	translation	925.63	0.196
Q5AHH4	Small heat shock protein 21	HSP21	cellular response to oxidative stress, pathogenesis, filamentous growth	5822.92	0.115

Identified proteins are organized according to the ratio score. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Suppl Table A13. Proteins identified with significantly altered expression in the P in comparison to control, *C. albicans* SC 5314.

[a]Acess number	Protein name	*Gene*	*Processo Biológico*	*PLGS score*	Ratio P *vs.* C-
O94038	Alcohol dehydrogenase 2	ADH2	alcohol dehydrogenase (NAD) activity, zinc ion binding	1103.56	1.840
P46273	Phosphoglycerate kinase	PGK1	cell wall organization, glycolytic process, interaction with host	6128.65	0.427

Identified proteins are organized according to the ratio score. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

Suppl Table A14. Proteins identified with significantly altered expression in the N in comparison to control, *C. albicans* SC 5314.

[a]Acess number	Protein name	*Gene*	*Processo Biológico*	*PLGS score*	Ratio N *vs.* C-
Q59MV9	Flavohemoprotein	YHB1	filamentous growth, response to toxic substance, response to defense-related host nitric oxide production	84.33	27.113
C4YMJ2	Uncharacterized protein	CAWG_02074	oxidoreductase activity	82.73	27.113
Q5A5V6	Pyruvate dehydrogenase E1 component subunit beta	PDB1	acetyl-CoA biosynthetic process from pyruvate, glycolytic process.	209.57	6.11
Q5AJB1	V-type proton ATPase catalytic subunit A	TFP1	cellular response to oxidative stress	65.28	4.055
C4YPU2	Vacuolar ATP synthase catalytic subunit A	CAWG_02495	cellular protein metabolic process	48.32	3.896
B9WIG8	Ketol-acid reductoisomerase_ mitochondrial	Cd36_60880	isoleucine biosynthetic process, mitochondrial genome maintenance, valine biosynthetic process	335.26	2.387
Q92211	Glyceraldehyde-3-phosphate dehydrogenase	TDH1	cell wall organization, glucose metabolic process, , interactions with plasminogen, antigenic properties	19392.47	2.117
Q6YK78	29 kDa IgE-binging protein	N/A	---	2733.01	2.054
C4YQ30	Protein SNO4	CAWG_02584	---	2733.01	2.054
P0CG73	Polyubiquitin	UBI1	activation of protein kinases	488.11	1.954
A0A1D8PM35	Translation elongation factor 1 subunit beta	EFB1	negative regulation of actin filament bundle assembly, Protein biosynthesis	3485.73	1.916
P78590	Elongation factor 1-beta	EFB1	Protein biosynthesis	3485.73	1.896
Q5AF03	Glyoxalase 3	GLX3	Stress response	3407.41	1.896
Q5A109	Ubiquitin fusion protein	UBI3	protein ubiquitination, ribosome biogenesis, translation	488.11	1.878
Q5ADS0	Ubiquitin	UBI4	cell morphogenesis, filamentous growth	488.11	1.859
B9WDM7	Probable chaperone protein Hsp31 homologue_ putative	Cd36_82570	metabolic process	1391.24	1.84
P83780	Glucose-6-phosphate isomerase	PGI1	glycolytic process	362.49	1.804
O74254	Glucoamylase 1	GAM1	cell wall organization, biofilm formation	84.1	1.751
A0A1D8PF79	Glutamate decarboxylase	GAD1	glutamate metabolic process	468.65	1.751
O94083	Eukaryotic translation initiation factor 5A	ANB1	positive regulation of translational elongation, positive regulation of translational termination	1156.99	1.682
A0A1D8PEW1	Gca2p	GCA2	biofilm formation	80.84	1.665
A0A1D8PF68	S-adenosylmethionine synthase	SAM2	One-carbon metabolism	67.84	1.665
P0C8K9	Cytochrome c oxidase subunit 1	COX1	mitochondrial electron transport, cytochrome c to oxygen	70.59	1.649
Q5A900	Ribosomal 40S subunit protein S2	RPS21	translation	640.44	1.649
Q5ABC3	Elongation factor Tu	TUF1	translation elongation factor activity	2585.57	1.616
Q9P926	Nuclear transport factor 2	NTF2	protein transport	718.81	1.537
P43074	White colony protein WHS11	WHS11	response to stress	1159.72	1.537
A0A1D8PES3	Ran GTPase-binding protein	NTF2	----	718.81	1.522
A0A1D8PHF8	Wh11p	WH11	phenotypic switching, response to stress, biofilm formation	1159.72	1.522
P82612	Phosphoglycerate mutase	GPM1	interaction with host	8358.03	1.507
P41797	Heat shock protein SSA1	SSA1	antimicrobial humoral response, cellular response to heat,	6734.11	1.492

			interaction with host.		
B9W9W8	Heat-shock protein (HSP) 70_ putative	Cd36_12490	cellular response to heat	6725.27	1.492
C4YF14	Pyridoxine biosynthesis protein PDX1	CAWG_01124	pyridoxal phosphate biosynthetic proces	179.75	1.492
P46587	Heat shock protein SSA2	SSA2	antimicrobial humoral response, interaction with host, response to toxic substance	5767.98	1.477
D6NHM0	Cytochrome c oxidase subunit 2	cox2	copper ion binding, cytochrome-c oxidase activity	274.76	1.462
G1UAT9	Uncharacterized protein CaJ7.0321	CaJ7.0321	cell redox homeostasis	435.29	1.462
C4YTW9	Mitochondrial peroxiredoxin PRX1	CAWG_05615	cell redox homeostasis, antioxidante activity, maintenance of genomic stability	435.29	1.448
B9WLR5	UTP-glucose-1-phosphate uridylyltransferase_ putative	Cd36_30070	intein-mediated protein splicing, UDP-glucose metabolic process	73.73	1.448
A0A1D8PCX8	60S ribosomal protein L6	RPL6	cellular response to starvation, filamentous growth, translation	1702.62	1.433
B9W7K3	Acetyl-coenzyme A synthetase	Cd36_04040	acetyl-CoA biosynthetic process from acetate, histone acetylation, replicative cell aging	145.19	1.419
O94014	Rehydrin-like protein	Ca35A5.08	cell redox homeostasis	435.29	1.419
P82610	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase (Methionine synthase)	MET6	cellular response to heat, induction by symbiont of host defense response	443.12	1.323
P46614	Pyruvate kinase	CDC19	cellular response to starvation, filamentous growth.	3454.37	1.323
O93827	Mannose-1-phosphate guanyltransferase	MPG1	cell cycle, cell wall mannoprotein biosynthetic process	823.35	1.31
C4YQT4	Cofilin (Fragment)	CAWG_04431	actin filament depolymerization	1245.59	1.297
Q9URB4	Fructose-bisphosphate aldolase	FBA1	glycolytic process, interaction with host, Aherence, interactions with plasminogen	4255.64	1.284
B9W7B8	Polyadenylate-binding protein	Cd36_03170	regulation of translational initiation	52.02	1.284
A0A1D8PP43	Adh1p	ADH1	Activation of host defense response, interaction with host and biofilm formation, interactions with plasminogen	10285.05	1.271
P43067	Alcohol dehydrogenase 1	ADH1	induction by symbiont of host defense response, interaction with host, biofilm formation, interactions with plasminogen	10194.2	1.271
P87206	Eukaryotic initiation factor 4A	TIF1	translational initiation	668.79	1.271
A0A1D8PRG5	Ribosomal protein P1B	RPP1B	translation	618.61	1.221
C4YFZ1	Heat shock protein SSA4	CAWG_00103	Stress response	6725.27	1.185
P82611	Aconitate hydratase_ mitochondrial	ACO1	induction by symbiont of host defense response	456.91	1.174
B9W8L5	Elongation factor 1-alpha	Cd36_07890	Protein biosynthesis, interactions with plasminogen	8821.45	1.174
Q59QD6	Elongation factor 1-alpha 2	TEF2	translational elongation	12193.48	1.174
O94039	Transketolase 1	TKT1	metabolic process	781.98	1.174
P0CY35	Elongation factor 1-alpha 1	TEF1	interaction with host, translational elongation	12193.48	1.162
Q5A0M4	Elongation factor 2	EFT2	cellular response to drug, drug binding	2254.25	1.094
Q9Y7F0	Peroxiredoxin TSA1-A	TSA1	cellular response to oxidative stress, filamentous growth, fungal-type cell wall organization, antioxidant activity , maintenance of genomic stability	4839.09	1.083
P83781	Mitochondrial outer membrane protein porin	POR1	porin activity	1095.88	0.914

P14235	Actin	ACT1	ATP binding. DNA repair, cellular response to oxidative stress	2965.15	0.896
O74261	Heat shock protein 60_ mitochondrial	HSP60	cellular response to heat, protein refolding	3246.94	0.887
A0A1D8PTI7	40S ribosomal protein S27	RPS27	translation	1237.24	0.878
C4YHT1	60S ribosomal protein L20	CAWG_03632	translation	2296.91	0.878
O13289	Peroxisomal catalase	CAT1	cellular response to starvation. filamentous growth, pathogenesis	1482.22	0.861
Q59QN6	Formate dehydrogenase	FDH1	glycine catabolic process	6207.56	0.852
A0A1D8PNC7	Hsp12p	HSP12	response to stress	13212.9	0.844
B9WIF6	Phosphoglycerate kinase	PGK1	cell wall organization, glycolytic process, activation of host defense response and interaction with host	4773.61	0.827
C4YPY4	60S ribosomal protein L12	CAWG_02538	translation	1511.49	0.819
Q5AJF7	Ribosomal 60S subunit protein L12A	RPL12	translation	1511.49	0.819
A0A1D8PDD1	Lipid-binding protein	PIL1	---	2375.08	0.811
C4YEG9	Sphingolipid long chain base-responsive protein LSP1	CAWG_00926	Endocytosis, negative regulation of protein kinase activity, response to heat	2375.08	0.803
B9W7P2	Uncharacterized protein	Cd36_04430	Endocytosis, negative regulation of protein kinase activity, response to heat	2375.08	0.803
Q5AJ93	40S ribosomal protein S7	RPS7A	translation	1033.64	0.795
C4YCU4	60S ribosomal protein L2	CAWG_00328	translation	1955.9	0.795
A0A1D8PF08	Ribosomal 60S subunit protein L2A	RPL2	translation	1955.9	0.795
A0A1D8PDC4	ATP synthase subunit alpha	ATP1	ATP biosynthetic process	2736.19	0.787
Q5A6R2	Bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	ADE17	purine nucleotide biosynthetic process	192.81	0.787
Q59T44	40S ribosomal protein S8	RPS8A	translation	1656.95	0.779
Q59RR7	Phosphotransferase	GLK4	cellular glucose homeostasis, glycolytic proces	411.51	0.779
P83778	Malate dehydrogenase_ cytoplasmic	MDH1	carbohydrate metabolic process	1949.7	0.771
C4YNQ0	40S ribosomal protein S19-B	CAWG_02832	translation	1945.81	0.763
A0A1D8PTP9	Chaperone ATPase	HSP104	Pathogenesis, biofilm formation	455.54	0.763
P46598	Heat shock protein 90 homolog	HSP90	regulation of apoptotic process, pathogenesis, filamentous growth, cellular response to drug	2363.47	0.763
C4YTQ8	ATP-dependent molecular chaperone HSP82	CAWG_05553	response to stress	2363.47	0.756
B9WK86	Heat shock protein 82 homolog_ putative	Cd36_71850	response to stress	2327.88	0.756
A0A1D8PK61	Ribosomal 40S subunit protein S19A	RPS19A	translation	1945.81	0.756
B9WEA9	Ribosomal protein of the small subunit_ putative	Cd36_85180	translation	1945.81	0.756
A0A1D8PKZ9	ATP synthase subunit beta	ATP2	ATP synthesis coupled proton transport	7123.18	0.748
A0A1D8PP59	Ubiquinol--cytochrome-c reductase subunit	orf19.4016	catalytic activity, metal ion binding	419.41	0.741
O59931	60S ribosomal protein L13	RPL13	translation	3283.63	0.733
A0A1D8PPS1	Ribosomal 60S subunit protein L25	RPL25	translation	2802.5	0.733
C4YJY0	Sphingolipid long chain base-responsive protein PIL1	CAWG_05772	---	1089.88	0.733
C4YGJ3	Pyruvate decarboxylase	CAWG_03171	carboxy-lyase activity	9731.4	0.719

C4YFR7	60S ribosomal protein L4-B	CAWG_00043	translation	1675.21	0.712
B9WI65	Core subunit of the ubiquinol-cytochrome-c reductase complex_ mitochondrial_ putative	Cd36_54830	catalytic activity, metal ion binding	374.71	0.712
A0A1D8PFV1	Ribosomal 60S subunit protein L4B	RPL4B	translation	1713.28	0.712
Q5AHH4	Small heat shock protein 21	HSP21	cellular response to oxidative stress, pathogenesis, filamentous growth	5822.92	0.712
Q8TGC7	Catalase (Fragment)	cta	response to oxidative stress	1143.4	0.691
C4YQ17	60S ribosomal protein L9-B	CAWG_02571	translation	1564.24	0.684
Q59LS1	Ribosomal 60S subunit protein L3	RPL3	translation	288.03	0.684
O94038	Alcohol dehydrogenase 2	ADH2	alcohol dehydrogenase (NAD) activity, zinc ion binding	1103.56	0.677
Q5AEN2	Ribosomal 60S subunit protein L9B	RPL9B	translation	1588.52	0.677
P25997	Elongation factor 3	CEF3	translational elongation	449.9	0.664
O42766	14-3-3 protein homolog	BMH1	filamentous growth, pathogenesis	184.21	0.657
Q5AG68	Nucleoside diphosphate kinase	YNK1	nucleoside diphosphate phosphorylation	2920.23	0.644
A0A1D8PPT5	Ribosomal 60S subunit protein L23B	RPL23A	translation	847.75	0.644
Q0ZID4	GTPase cytoplasmic elongation factor 1 alpha (Fragment)	tef1	translation elongation factor activity	10142.36	0.625
A0A1D8PQS0	60S acidic ribosomal protein P0	RPP0	ribosome biogenesis	491.35	0.619
Q9P8Q7	Isocitrate lyase	ICL1	tricarboxylic acid cycle, pathogenesis	571.53	0.619
B9WAK8	Cytochrome c oxidase polypeptide IV_ mitochondrial_ putative	Cd36_16480	cytochrome-c oxidase activity	751.91	0.607
A0A1D8PIW1	Etr1p	ETR1	oxidoreductase activity	98.15	0.607
O13434	Phosphoenolpyruvate carboxykinase (ATP)	PCK1	gluconeogenesis	872.73	0.607
P22011	Peptidyl-prolyl cis-trans isomerase	CYP1	protein folding	2274.62	0.6
Q9P940	Triosephosphate isomerase	TPI1	cellular response to starvation, filamentous growth	1252.96	0.6
C4YFU0	6-phosphogluconate dehydrogenase	CAWG_00066	D-gluconate catabolic process	792.83	0.595
P87222	Ribosome-associated molecular chaperone SSB1	SSB1	cellular response to glucose starvation, filamentous growth	2615.29	0.589
B9WMK0	Cytoplasmic ATPase that is a ribosome-associated molecular chaperone_ putative	Cd36_33730	cellular response to glucose starvation	2615.29	0.583
A0A1D8PG82	Hgt8p	HGT8	transmembrane transporter activity	391.62	0.583
Q5A2A1	Succinate dehydrogenase [ubiquinone] flavoprotein subunit_ mitochondrial	SDH1	Electron transport	24.1	0.571
P47831	60S ribosomal protein L28 (Fragment)	RPL28	translation	2779.57	0.566
A0A1D8PGT5	Aldehyde dehydrogenase (NAD(P)(+))	ALD5	oxidoreductase activity	3259.08	0.566
P47837	40S ribosomal protein S4	RPS4	translation	506.79	0.543
Q9P844	40S ribosomal protein S21	RPS21	translation	990.7	0.527
Q5PS51	60 kDa chaperonin (Fragment)	cpn60	ATP binding	1484.41	0.517
P83774	Guanine nucleotide-binding protein subunit beta-like protein	ASC1	cell adhesion, filamentous growth, virulence	1492.09	0.517
Q5AF44	Thioredoxin peroxidase	AHP1	cell redox homeostasis	675.84	0.517
C4YHK7	Uncharacterized protein	CAWG_03554	cell redox homeostasis	675.84	0.517
Q9HFQ4	60S acidic ribosomal protein P2-B	RPP2B	translational elongation	848.07	0.512

B9WK13	Part of 40S ribosomal subunit_ putative	Cd36_71100	invasive growth in response to glucose limitation	1445.71	0.512
C4YTI4	Uncharacterized protein	CAWG_05477	invasive growth in response to glucose limitation.	1445.71	0.512
A0A1D8PL99	40S ribosomal protein S6	RPS6A	translation	1245.37	0.507
A0A1D8PFV7	Mdg1p	MDG1	----	349.24	0.477
A0A1D8PHF5	Ribosomal 60S subunit protein L31B	orf19.3572.3	translation	3863.16	0.472
Q96W54	40S ribosomal protein S22-A	RPS22A	translation	1081.63	0.463
B9WBJ8	Ribosomal protein_ large subunit_ putative	Cd36_19890	translation	3863.16	0.463
C4YNX7	Elongation factor 1-gamma 1	CAWG_02910	translation elongation factor activity	149.25	0.454
Q5A4Q1	Adenylate kinase	ADK1	ADP biosynthetic process	228.92	0.445
Q9P843	60S ribosomal protein L27	RPL27	translation	865.55	0.432
A0A1D8PKC3	Translation elongation factor EF1B gamma	CAM1	Protein biosynthesis	149.25	0.419
B9WBD0	Large ribosomal subunit protein_ putative	Cd36_19200	translation	499.86	0.415
A0A1D8PK22	Ribosomal 40S subunit protein S15	RPS15	translation	758.12	0.415
C4YR94	Uncharacterized protein	CAWG_04593	response to stress	13212.9	0.415
P83777	Inorganic pyrophosphatase	IPP1	phosphate-containing compound metabolic process	1702.37	0.399
P0CU35	40S ribosomal protein S22-B	RPS22B	translation	1081.63	0.395
P30575	Enolase 1	ENO1	entry into host. Fibrinolysis, filamentous growth, glycolytic process.	8652.08	0.391
Q5A516	ADP/ATP carrier protein	PET9	transmembrane transporter activity	1606.12	0.361
P40910	40S ribosomal protein S1	RPS1	translation	603.47	0.333
A0A1D8PSH3	Citrate synthase	CIT1	tricarboxylic acid cycle	3050.01	0.31
B9WH01	Major ADP/ATP carrier protein of the mitochondrial inner membrane_ putative	Cd36_50590	Transport	1360.54	0.287
P83783	Adenosylhomocysteinase	SAH1	one-carbon metabolic process, S-adenosylhomocysteine catabolic process	279.44	0.273

Identified proteins are organized according to the ratio score. The ID is based on protein ID from the UniProt protein database (http://www.uniprot.org/).

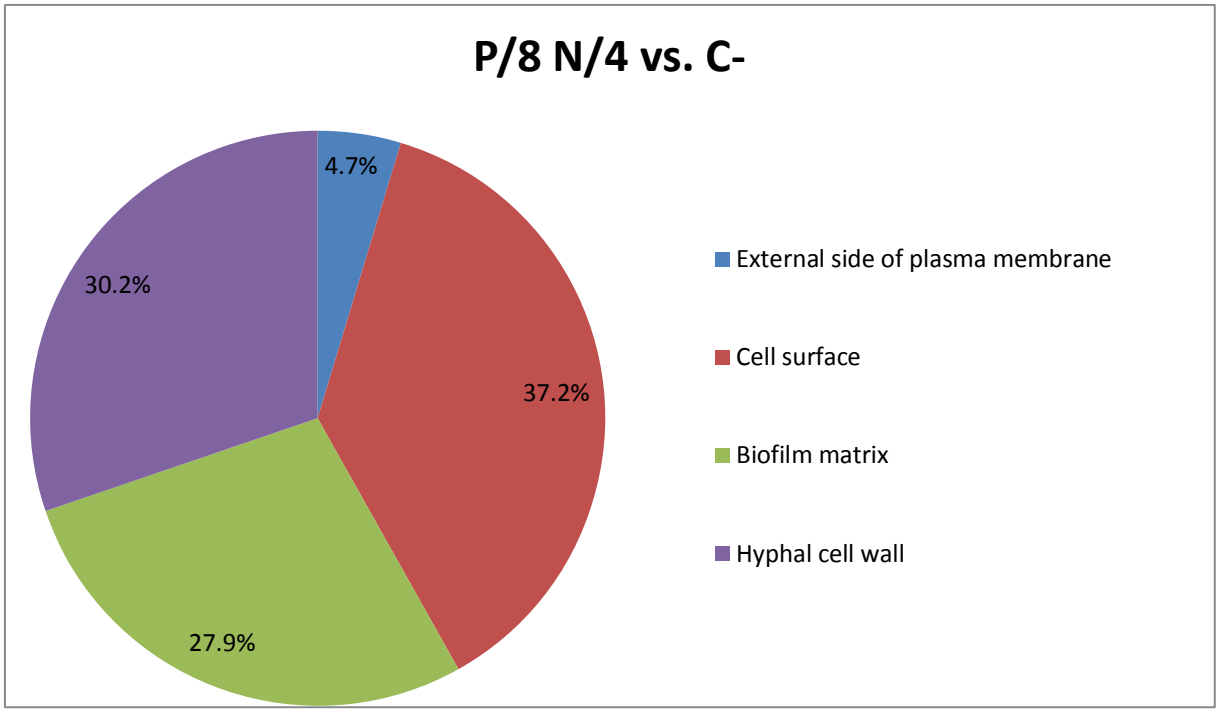

Figura A1. Functional distribution of proteins identified with differential expression in the *C. albicans* ATCC90028 cells exposed to P/8 N/4 *vs.* C-. Categories of proteins based on *GO annotation Cellular Component.* Terms significant (Kappa = 0.04) and distribution according to percentage of number of genes association. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® pluggins of Cytoscape® 3.6.1.

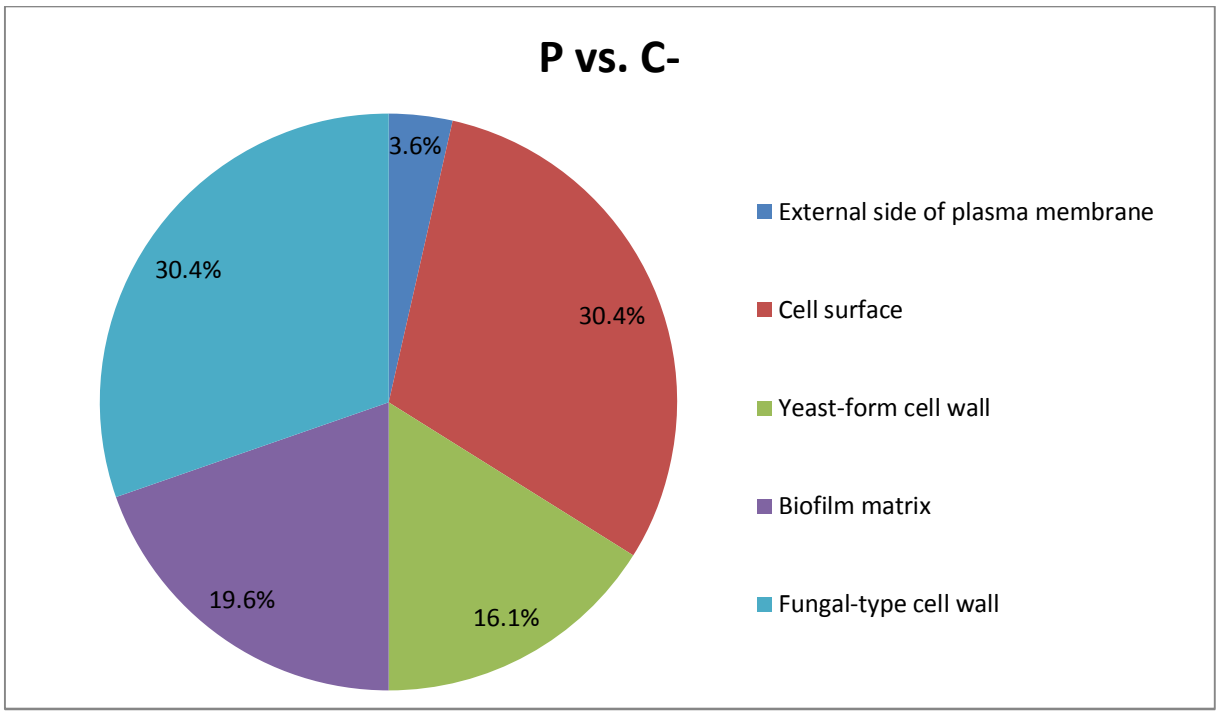

Figura A2. A1. Functional distribution of proteins identified with differential expression in the *C. albicans* ATCC90028 cells exposed to P *vs.* C-. Categories of proteins based on *GO annotation Cellular Component.* Terms significant (Kappa = 0.04) and distribution according to percentage of number of genes association. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® pluggins of Cytoscape® 3.6.1.

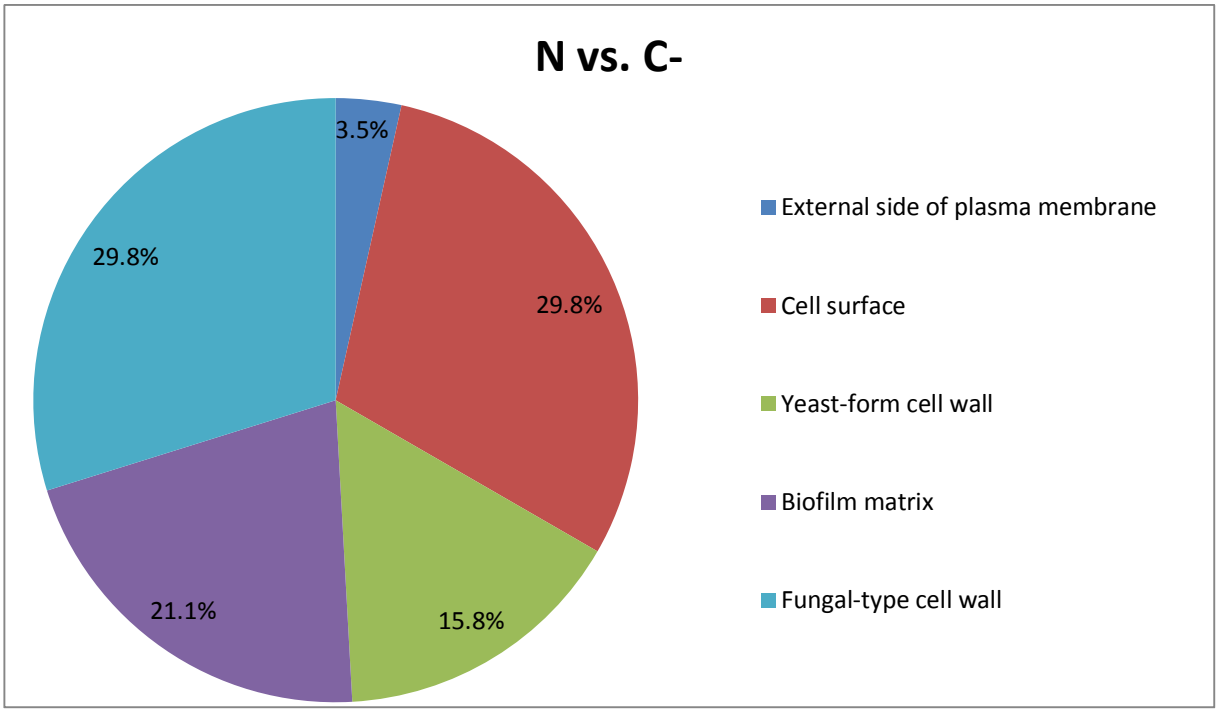

Figura A3. Functional distribution of proteins identified with differential expression in the *C. albicans* ATCC90028 cells exposed to N *vs.* C-. Categories of proteins based on *GO annotation Cellular Component.* Terms significant (Kappa = 0.04) and distribution according to percentage of number of genes association. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® pluggins of Cytoscape® 3.6.1.

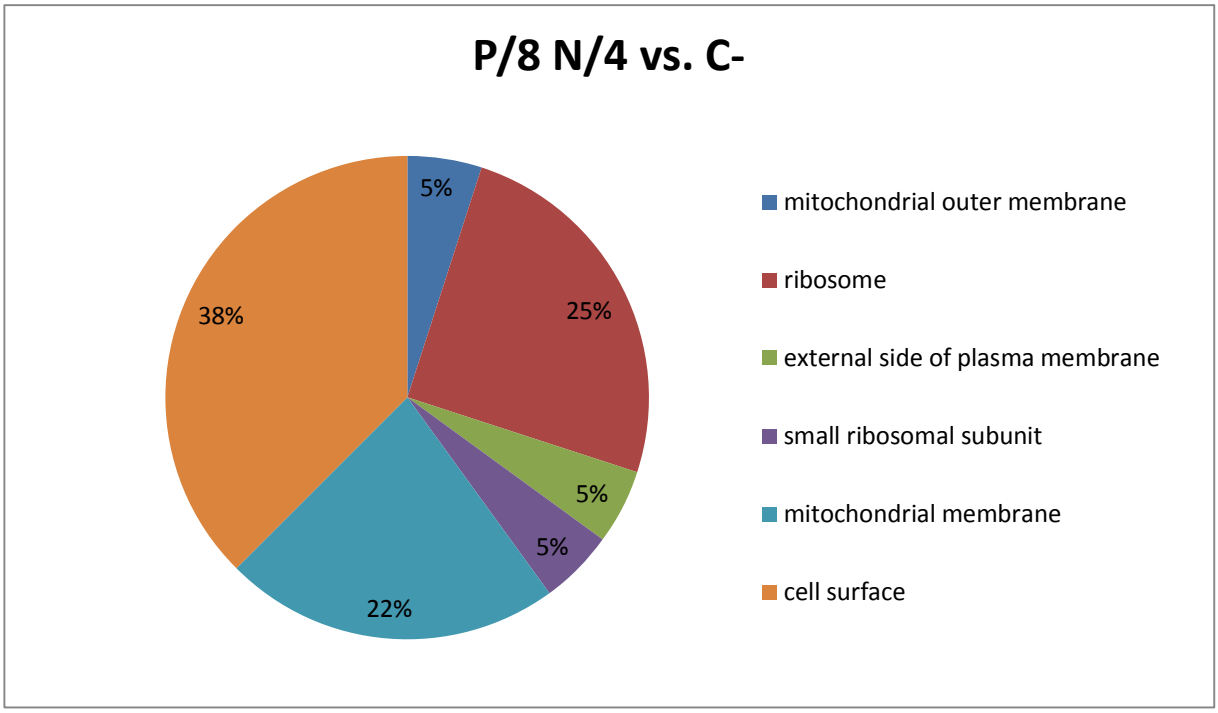

Figura A4. Functional distribution of proteins identified with differential expression in the *C. albicans* SC 5314 cells exposed to P/8 N/4 *vs.* C-. Categories of proteins based on *GO annotation Cellular Component.* Terms significant (Kappa = 0.04) and distribution according to percentage of number of genes association. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® pluggins of Cytoscape® 3.6.1.

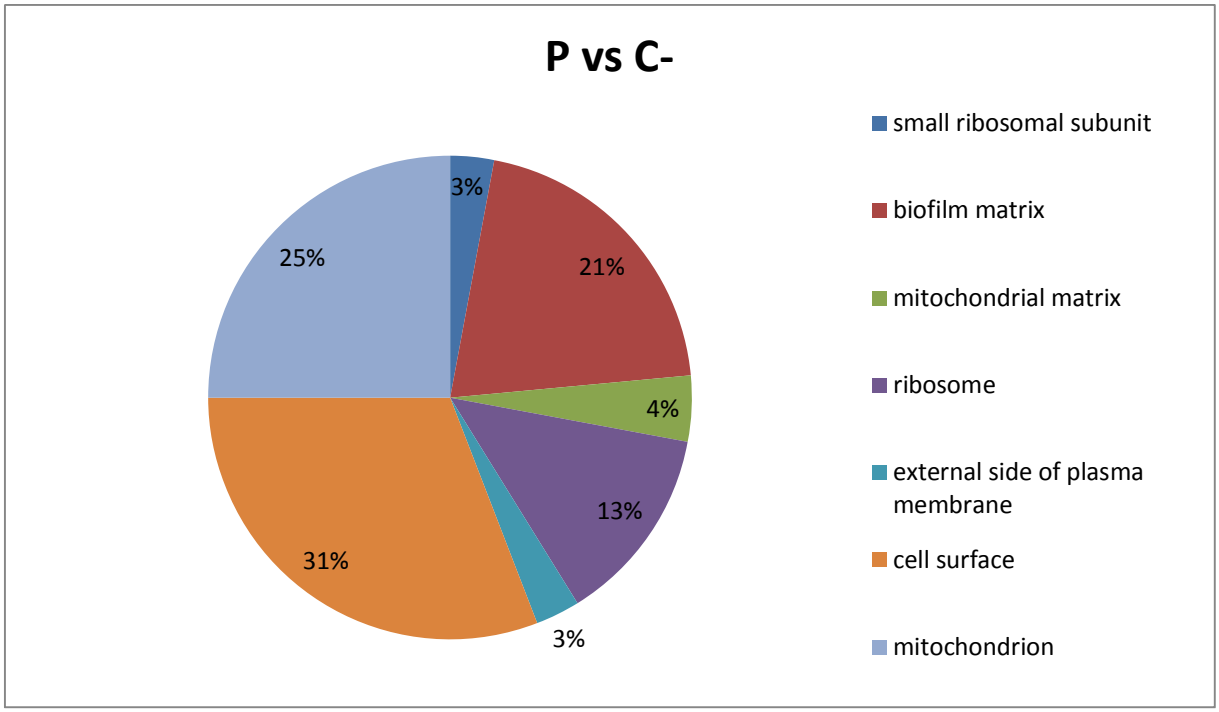

Figura A5. Functional distribution of proteins identified with differential expression in the *C. albicans* SC 5314 cells exposed to P *vs.* C-. Categories of proteins based on *GO annotation Cellular Component.* Terms significant (Kappa = 0.04) and distribution according to percentage of number of genes association. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® pluggins of Cytoscape® 3.6.1.

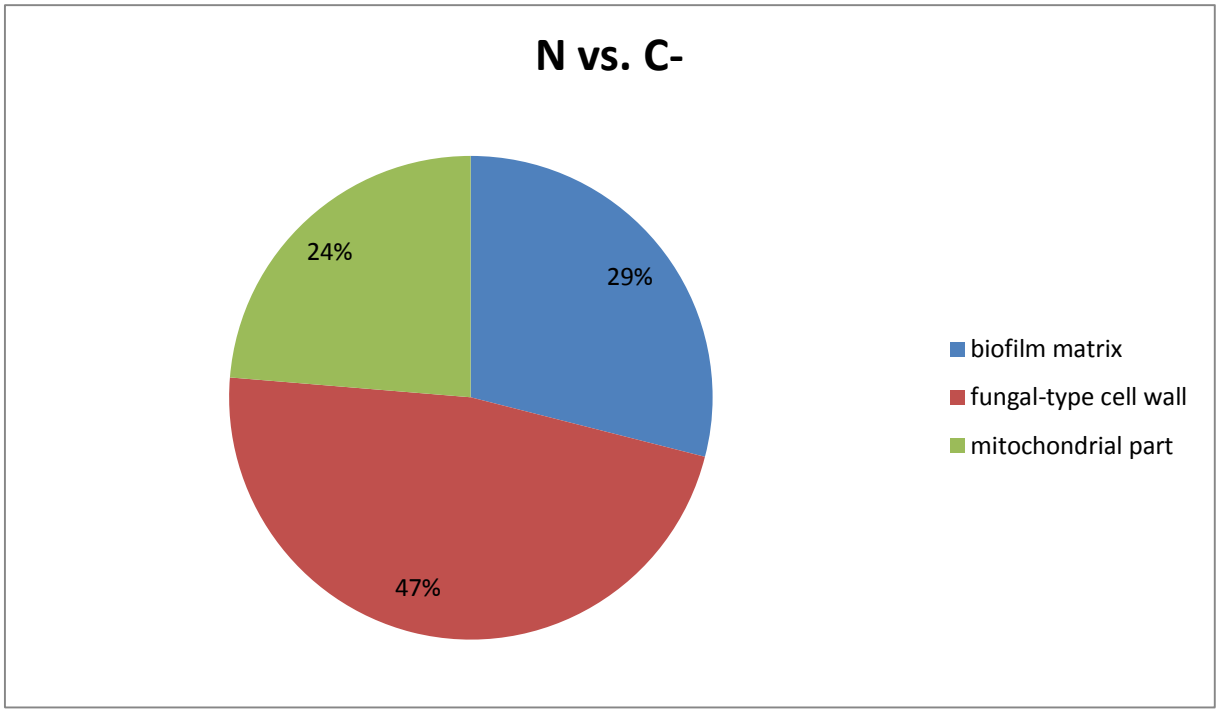

Figura A6. Functional distribution of proteins identified with differential expression in the *C. albicans* SC 5314 cells exposed to N *vs.* C-. Categories of proteins based on *GO annotation Cellular Component.* Terms significant (Kappa = 0.04) and distribution according to percentage of number of genes association. Proteins access number was provided by UNIPROT. The gene ontology was evaluated according to ClueGo® pluggins of Cytoscape® 3.6.1.

3 Discussion

3 DISCUSSION

Due to the increase of resistant strains to the conventional antifungal agents, the search for new therapeutic alternatives has been the focus of several studies (ENDO et al., 2010; MERTAS et al., 2015; SUN et al., 2017).

The antifungal drug combinations is a potential strategy to control the evolution of drug resistance and can be an effective solution for *Candida* infections, improving the action spectrum, increasing therapeutic efficacy and reducing toxicity and side effects (SUN et al., 2017).

Antifungals usually present some toxicity, since there are few targets of action in fungi that are not shared with human cells, both eukaryotes (ROCHA, 2002; CROSARIOL, 2010). Medicinal plants may be considered as new sources of active compounds for producing antimicrobial agents, which added to the antifungal formulations traditionally used in the clinic increase their antimicrobial activity against resistant strains (ENDO et al., 2010; MERTAS et al., 2015).

Thus, the main purpose of this study was to evaluate the effect against *C. albicans* of Punicalagin (P), the major ellagitannins present in *P. granatum*, and one of the main responsible for the antifungal activity, in combination with the conventional antifungal agent, Nystatin (N). Besides, we evaluated cytotoxic effect of drugs on human cells.

The first paper of this thesis initially tested the Minimal Microbicidal Concentrations (MIC) of the drugs Punicalagin (P) and Nystatin (N) alone that were established in 50 and 3.9 µg/mL, respectively. Subsequently the Checkerboard assay, which is widely used to compare *in vitro* the efficacy of the combination of two or more antimicrobials, was performed. When used in combination, **P** and **N** demonstrated good antifungal results with reduction of their previously established MICs, with a significant increase of the antifungal activity in comparison to the drugs alone; besides identifying the synergism in 4 combinations (P/8 N/4, P/8 N/2, P/4 N/2 and P/2 N/4). In addition, fungal metabolism was dramatically reduced in some combinations (2%) compared to drugs alone, as well as the inhibition of fungal growth was very high (100%) as opposed to the results of drugs alone that were ineffective as fungicides.

Endo et al. (2010) also identified a potently synergistic action of **P** in combination with fluconazole with the reduction of fluconazole MIC, in addition to detecting changes in fungus morphology, such as thickening of the cell wall and presence of vacuoles. Other studies proved the antifungal action of *P. granatum* and correlated with tannins, among them, Punicalagin, one of the main compounds responsible for this activity (AJAIKUMAR et al., 2005; SEERAM et al., 2005; ADAMS et al., 2006; ; LANSKY; NEWMAN, 2007 ALTHUNIBAT et al., 2010; ANIBAL et al., 2013). In the present study, **P** demonstrated high cytotoxicity in human palate epithelial cells, however, in combination with **N** showed values close to those obtained with untreated cells (negative control), an essential prerequisite for topical therapy, since it was possible to obtain lower concentrations of the drugs, with lower or no cytotoxic potential for human cells. In contrast, cytotoxicity to fungal cells was increased.

The synergistic antifungal effect produced between **P** and **N** suggests that the combination of the two compounds may be a good topical alternative for the treatment of superficial oral candidiasis, such as denture stomatitis (DS). Combined treatment of natural products and conventional drugs is one of the effective treatments against *Candida* species. This therapeutic strategy has been shown to improve drug efficacy, decrease toxicity, side effects and antimicrobial resistance problems (CUI et al., 2015; OLFA et al., 2015; MERTAS et al., 2015).

Although we cannot accurately prove by which **P** pathway acts, studies have shown that it causes morphological changes usually related to fungal cell wall and in the intracellular microbial content, which might affect metabolism and fungal growth rate (ENDO et al., 2010) . In this way we suggest that the **P** mechanism of action may be related to the fungal cell wall, similar to what occurs with the echinocandins (DUNYACH et al., 2011; SPAMPINATO; LEONARDI, 2013). In order to confirm this premise, assays such as the sorbitol test and others that evaluate the morphological characteristics, such as scanning electron microscopy are required.

In addition, the combination with drugs such as Nystatin might facilitate the entry of **P** through cell membrane and thereby potentiate its antifungal activity (ENDO et al., 2010).

Based on the results of the 1st paper, the focus of the 2nd paper was to study more deeply fungal mechanisms altered by the action of drugs alone and in combination. For the 2nd paper, we decided to study only one combination classified

as synergy by the checkerboard method (P/8 N/4 and drugs alone) in order to investigate the molecular mechanisms altered, but in a drug combination that provided at least 50 % of viable cells. The other combinations could give us a better result on the mechanisms involved in the antifungal action, however, did not provide enough material for adequate analysis. We also followed the 24-hour exposure to drugs according to the 1st paper.

The second paper demonstrated that the virulence factors of CA, ATCC and SC strains, against **P** and **N** treatment undergo complex modulations in gene expression. Regarding the virulence factors of CA, in the present study, no significant difference in the gene expression of ALS 1-5 was identified in both CA strains after treatment with **P** and **N** compared to the control. In contrast, the treatment with drug combination (P/8 N/4) in sub-inhibitory concentration, caused a significant increase in the gene expression of ALS-1, ALS-4 and ALS-5 in the ATCC strain, and ALS-4 in the SC strain compared to the control. Similar results were found in SAPs gene expression, exhibiting increased level in SAP-2, SAP-3 and SAP-9 in ATCC strain, and SAP-2 in SC strain, after the treatment with P/8 N/4 compared to the negative control (C-).

A hypothesis for the increase in virulence factors gene would be a response of fungi to antifungals administered in sub-inhibitory concentrations. Some studies have also demonstrated increased gene expression of ALS and SAP in strains treated with fluconazole subdoses (WU et al., 2000; BARELLE et al., 2007). Barelle et al. (2007) showed that the gene induction was involved to a defense mechanism of CA stress-related. The authors also demonstrated that the up-regulation of different SAPs is under different controls, such as the yeast-hyphae transition, nevertheless these effects appear to be transient *in vivo*. Wu et al. (2000) identified an increase in the expression of SAPs and did not correlate this increase to cell death or non-specific release of SAP, since it did not detect a reduction in the number of CFUs and no significant release of enolase, an enzyme constitutive of the glycolytic pathway. Thus, they correlated that exposure to sub-inhibitory doses of fluconazole may result in increased extracellular production of SAP by strains capable of overexpressing genes related to a multidrug resistance efflux pump (MDR1), associated to an increase in CA virulence *in vivo* (WU et al., 2000). However, in our results, we can correlate the up-regulation of some genes with the cell death process, because in CFU studies P/8 N/4 demonstrated a reduction in fungal growth compared to the

negative control (data not shown). Additionally, although enolase had its expression decreased relative to the control, identified by proteomic analysis, this could be connected to the drug exposure time, which was 24 hours, differently of the short exposures reported in those studies. A similar result was observed in strains resistant to some fungicides such as fluconazole and itraconazole (COPPING et al., 2005; COSTA et al., 2010).

In agreement with these results, the therapy with another fungicide, caspofungin, promotes an increase of SAP-5 gene expression and does not modulate the expression of the others SAPs and PLB1 (RIPEAU et al., 2002).

Dimethylamino dodecyl methacrylate (DMADDM) has antimicrobial activity and has been incorporated in several dental materials. This strategy has been shown to be effective against fungi, because interfere in adhesion, which may be occasioned by the decreased expression of some virulence factors such as ALS-3 and HWP1 (ZHANG et al., 2016). In the present study, ALS-3 and HWP expression was reduced in relation to the control in the two strains treated with P/8 N/4. The intervention in fungus adhesion processes may be crucial in oral candidiasis treatment, preventing the fungus attachment to the mucosal surfaces and devices such as total removable dentures (ANTLEY; HAZEN, 1988; ELLEPOLA; SAMARANAYAKE, 1998).

The virulence of microorganisms also depends on their hemolytic abilities. Regarding to fungi species, phospholipases are correlated with this ability and PLB1, PLB2, PLC and PLD are the most described in the literature (LEONOV et al., 2017). The present study did not detect statistical significance of gene expression, except of the PLB1 gene expression which was increased in the ATCC strain after P/8 N/4 treatment. However, the treatment with fluconazole inhibited the PLC expression (WILLIAMS et al., 2013) and caspofungin treatment did not affected PLB1 gene expression (RIPEAU et al., 2002), as well as DMADDM did not modified PLD gene expression (ZHANG et al., 2016).

These results demonstrate the complexity of fungus behavior towards antifungals agents. The mechanism of action, time of treatment, and drug concentration are determinant in the success of the fungal infections control. To understand the mechanisms involved in fungal resistance, the use of antifungals at sub-inhibitory doses may be interesting for *in vitro* studies; however, it is difficult to

extrapolate to what occurs *in vivo*. Thus, these results encourage the development of clinical trials to evaluate the efficacy of this combination (**P** and **N**) and other combinations by changing the concentrations of each drug.

In agreement with the results of the gene expression, through the proteomic analysis it was possible to detect a large amount of altered proteins in relation to the control without treatment, proteins associated to biological processes important for fungus viability related to energy metabolism, translation, metabolic processes and stress response. The biological processes affected through the reduction of these proteins may be associated with damaged and fragile cells formation, reduced metabolic activity and growth.

The increase of heat shocks proteins is generally related to the greater tolerance of fungi to stress, allowing the cells to survive in unfavorable conditions (BENTLEY et al., 1992; BURNIE et al., 2006). In our study, all heat shocks proteins had their expression decreased after treatment with P/8 N/4, for the two strains evaluated.

Glycolytic enzymes are relevant during the CA pathogenesis, behaving as main inducers of host immune response and are the main allergens during candidiasis (STROCKBINE et al., 1984; SHEN et al., 1991; ISHIGURO et al., 1992; SWOBODA et al., 1993; GIL-NAVARRO et al., 1997).

Enolase (ENO1) is one of the most abundant glycolytic enzymes in the *C. albicans* cytosol. It binds to human plasminogen and this interaction promotes an increase in the fibrinolytic capacity of the fungus, facilitating invasion and dissemination. Both Enolase and Heat shock protein 70 (HSP70p) are proteins that have been reported as important antigens in various infectious diseases (BIANCO et al., 1986). In our study, these two proteins had decreased expression in relation to the control after treatment with P/8 N/4 and **N**, in ATCC and SC strains. In addition, three glycolytic enzymes were identified: phosphoglycerate kinase (PGK), glyceraldehyde phosphate dehydrogenase (GAPDH), which demonstrated laminin and fibronectin binding properties, and alcohol dehydrogenase (ADH) (PENDRAK; KLOTZ, 1995; CHAFFIN et al., 1998; GOZALBO et al., 1998).

In our results, the PGK expression was decreased in the treated strains in relation to the negative control, thus suggesting interference in one of the resistance mechanisms of the fungus.

Glyceride-3-phosphate dehydrogenases (GAPDH) are a family of proteins that

have several activities in different locations within the cell, besides to have a well characterized role in the glycolysis process (SIROVER, 1999). A study showed that in yeast of *S. cerevisiae* secretion of GAPDH protein within the cell wall is enhanced by some stress conditions, such as starvation (GIL et al., 2001). In our results there was an increase in GAPDH expression in the P/8 N/4 or **N**-treated strains. This suggests that the expression is could be associated to the stress response provided by the use of antifungal drugs.

Alcohol dehydrogenase (ADH1p), on cell surface and cytoplasm, catalyzes the reduction of acetaldehyde to ethanol generating NAD+ and participates in multiple biological processes such as biofilm formation, fermentation and interaction with host (WANG et al., 2012). Previous studies have found that overexpression of ADH1p is related to fluconazole resistance in *C. albicans* (ZHU; LU, 2005) and that fluconazole induces ADH1 gene expression (WANG et al., 2012). In our results, we obtained an increased expression of ADH1p after **P** treatment in ATCC strain, and **N** treatment in SC strain. However, in the drug combination (P/8 N/4), the expression was decreased compared to the control. Another study similarly identified ADH1p overexpressed after separately fluconazole and tetrandrine treatment; nevertheless, when combined, there was a decrease in the expression, suggesting that ADH1p expression is involved in the synergism mechanism against *C. albicans* (ZHANG et al., 2013).

Malate dehydrogenase (MDH1p) is a considerable enzyme for the fungi bioenergetic metabolism. MDH1p participates in the glyoxalate cycle, which allows fungal cells to use fatty acids as a substrate for gluconeogenesis (TYLICKI et al., 2008). The glyoxalate cycle is required for fungal virulence. *C. albicans* exhibits a metabolic program by which the glyoxalate and gluconeogenesis cycle are activated during the early stages of infection. Our results showed a reduction of malate dehydrogenase expression in both treated strains relative to the negative control. Thus, not only the decrease of MDH1p but also of other proteins involved with the fermentative or oxidative metabolism can be effective as antifungals (TYLICKI et al., 2008).

Another interesting result was the identification of Acetyl-CoA-acetyltransferase, encoded by ERG10 gene, only in the negative controls, in both ATCC and SC strains. This data allows to assume that there was interference in the ergosterol production, as such a protein is necessary in the first step of this

biosynthesis through its condensation in acetoacetyl-CoA (BUURMAN et al., 2004).

In healthy individuals, phagocytic cells, such as macrophages (EVRON, 1980), monocytes and neutrophils (SCHUIT, 1979; MARÓDI et al., 1991), act on *Candida* infections, producing various growth inhibitors and cytotoxic compounds, including microbicidal enzymes and reactive oxygen and nitrogen species (PETERSON; CALDERONE, 1978; VAZQUEZ-TORRES; BALISH, 1997). A potentially effective artifice against CA is nitric oxide (NO) (ULLMANN et al., 2004).

One way of protecting microorganisms from the toxicity of nitric oxide is through enzymes that convert NO into less toxic molecules. Flavohemoglobin is an NO-dioxygenase encoded by the YHB1 gene. In *C. albicans,* it is induced by NO and converts it to nitrate (GARDNER et al., 1998; ULLMANN et al., 2004). Amphotericin B promote oxidative damage to fungal cells and several oxidative stress response genes are overexpressed in response to the drug (LIU et al., 2005), such as YHB1, which in our results was also overexpressed compared to control after treatment with P/8 N/4 and **N** in both strains evaluated.

Hydrolytic enzymes, such as aspartyl proteinases and phospholipases, produced and secreted by *C. albicans*, as well as proteins encoded by ALS (agglutinin-like sequence) genes were not detected in the proteomic analysis, although they were expressed in the PCR assay. We hypothesize that it may have occurred because the composition, culture medium pH and fungal growth time were not adequate for the synthesis and secretion of these proteins at detectable levels by the mass spectrometer (MACDONALD; ODDS, 1980; IBRAHIM et al., 1995; CHAFFIN et al., 1998; D'EÇA JÚNIOR et al., 2011; ELLS et al., 2014).

Differences in protein expression among the both strains evaluated were not analyzed at this first moment, because initially it was not the aim of our study. Indeed, these differences are expected, since one of the strains is clinical (SC 5314), and therefore exhibit resistance and virulence factors different from the standard strain (ATCC 90028). This evaluation will be carried out later.

In this context, the results of the present study and other studies in the search for new therapeutic alternatives through the drug combination encourage the development of clinical trials to evaluate the efficacy of this combination for the treatment of oral candidosis.

4 Conclusions

4 CONCLUSIONS

1) Synergistic antifungal activity produced between **P** and **N** suggested that the combination of drugs, at the concentrations tested, may be a viable alternative to be applied as preventive treatment or topical therapy for superficial candidiasis, such as denture stomatitis.

2) P/8 N/4 appears to exert an antifungal effect on *C. albicans* by direct interaction with important proteins related to structural organization of fungus morphology and in essential metabolic energy processes. This might be related to the capacity of the drugs used in combination to cause harm to fungi structure and reduction of filamentation, resulting in defective and non-viable cells.

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Appendix

APPENDIX A – DECLARATION OF EXCLUSIVE USE OF ARTICLE IN THESIS

We hereby declare that we are aware of the article "***In vitro* Antifungal activity of Punicalagin – Nystatin Combinations against *C. albicans* associated with Oral candidiasis"**, which will be included in Ph.D. thesis of the student Rafaela Alves da Silva. This article was exclusively used in this thesis and may not be used in other works of the Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

Bauru, May 17th, 2018

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APPENDIX B – DECLARATION OF EXCLUSIVE USE OF THE ARTICLE IN THE THESIS

We hereby declare that we are aware of the article **"Antifungal activity of Punicalagin and Nystatin used in combination against *Candida albicans*: detection of virulence genes and proteomic analysis**", which will be included in Ph.D. thesis of the student Rafaela Alves da Silva. This article was exclusively used in this thesis and may not be used in other works of the Graduate Programs at the Bauru School of Dentistry, University of São Paulo.

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Annexes

ANNEXES

ANNEX A – Ethics committee approval

FACULDADE DE ODONTOLOGIA DE BAURU-USP

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: "INFLUÊNCIA DA INCORPORAÇÃO DOS FITOTERÁPICOS E. GIGANTEUM E P. GRANATUM SOBRE MATERIAIS RESILIENTES UTILIZADOS NA CONFECÇÃO DE PRÓTESES REMOVÍVEIS BUCAIS: TOPOGRAFIA, CARACTERIZAÇÃO QUÍMICA E AÇÃO CONTRA CANDIDA ALBICANS".

Pesquisador: Rafaela Alves da Silva Alavarce

Área Temática:

Versão: 2

CAAE: 44951715.6.0000.5417

Instituição Proponente: Universidade de Sao Paulo

Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

DADOS DO PARECER

Número do Parecer: 1.114.009

Data da Relatoria: 17/06/2015

Apresentação do Projeto:

Este trabalho será desenvolvido com uma aluna de pós graduação na área de patologia e visa a avaliar se materiais utilizados para o revestimento temporário de próteses removíveis bucais, previamente incorporados especificamente com os fitoterápicos Equisetum giganteum e Punica granatum, apresentamatividade antimicrobiana e antiaderente; atividade anti-inflamatória sobre células epiteliais de palato humano (CEPH); citotoxicidade sobre células humanas e alterações de superfície. Para tanto, envolverá aparticipação de um único participante que deverá autorizar mediante o TCLE complementar, a doação de fragmento do palato duro já obtido e congelado em trabalho prévio aprovado pelo CEP.

Objetivo da Pesquisa:

O objetivo apresentado é de avaliar se materiais utilizados para o revestimento temporário de próteses removíveis bucais, previamente incorporados com os fitoterápicos Equisetum giganteum e Punica granatum, apresentam atividade antimicrobiana e antiaderente; atividade anti-inflamatória sobre células epiteliais de palato humano (CEPH); citotoxicidade sobre células humanas e alterações de superfície.

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FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.114.009

Avaliação dos Riscos e Benefícios:

Foram apresentados adequadamente:

Riscos: Os riscos envolvidos serão provenientes da cirurgia que será realizada nos pacientes durante a pesquisa "Avaliação do recobrimento radicular pela técnica de enxerto conjuntivo subepitelial associado ao condicionamento radicular com ácido cítrico ou terapia fotodinâmica - estudo clínico randomizado", já aprovada por este CEP e que irá ceder o material residual armazenado para a pesquisa em questão, e são aqueles comuns a qualquer tratamento odontológico (pequeno sangramento durante a limpeza, sensibilidade, pequeno desconforto), que ocorreriam, mesmo que não estivesse participando da pesquisa. A presente pesquisa, como irá trabalhar com material biológico armazenado não apresenta riscos. O número do protocolo de aprovação por este CEP foi solicitado e incluído no item justificativas da dispensa do TCLE do Plataforma Brasil.

Benefícios: A descoberta de novos componentes antimicrobianos é de grande relevância, particularmente para a Odontologia, já que infecções da cavidade bucal, de origem bacteriana e fúngica, são problemas relativamente comuns, resultando em doenças inflamatórias crônicas como, por exemplo, a estomatite protética associada a Candida. Levando-se em conta o aumento percentual da população idosa e que a EP acomete frequentemente idosos usuários de prótese total superior, torna-se fundamental a realização de novos estudos sobre alternativas terapêuticas para a EP, que sejam simultaneamente antimicrobiana, antiaderente, anti-inflamatória e não tóxica para os tecidos bucais, visando a melhora da qualidade de vida desta população idosa.

Comentários e Considerações sobre a Pesquisa:

Este estudo tem como objetivo avaliar in vitro se um reembasador resiliente e um condicionador tecidual, modificados por meio da incorporação prévia com os fitoterápicos E. giganteum e P. granatum, apresentam atividades antimicrobiana, antiaderente e anti-inflamatória, sem alteração de suas propriedades mecânicas (topografia) e composição química. Neste contexto, estas plantas medicinais poderiam desempenhar um papel importante no tratamento da EP.

Considerações sobre os Termos de apresentação obrigatória:

- O apoio financeiro foi descrito adequadamente e está consistente com apresentação do termo de outorga.
- A utilização desse material em pesquisas vinculadas à linha de pesquisa acima citada, será somente após a aprovação de um Comitê de Ética em Pesquisa em Seres Humanos e mediante a

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assinatura de um documento, por parte do responsável pela guarda do material, cedendo essas células. Para utilização do material biológico cedido em pesquisas futuras, conforme item 5 da Resolução CNS 441/2011. Dessa forma, o termo de cessão de responsabilidade da Profa. Dra. Carla A. Damante foi adequadamente apresentado, bem como a justificativa de que apenas pacientes que apresentaram a cessão para futuros trabalhos da mesma linhas na assinatura do TCLE do momento da coleta cirúrgica seriam elegíveis.

- Foi informado adequadamente que o tecido todo será descartado após este trabalho, não havendo sobras.

Recomendações:

Não se aplica

Conclusões ou Pendências e Lista de Inadequações:

Aprovado.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

Esse projeto foi considerado APROVADO na reunião ordinária do CEP de 17.06.2015, com base nas normas éticas da Resolução CNS 466/12. Ao término da pesquisa o CEP-FOB/USP exige a apresentação de relatório final. Os relatórios parciais deverão estar de acordo com o cronograma e/ou parecer emitido pelo CEP. Alterações na metodologia, título, inclusão ou exclusão de autores, cronograma e quaisquer outras mudanças que sejam significativas deverão ser previamente comunicadas a este CEP sob risco de não aprovação do relatório final. Quando da apresentação deste, deverão ser incluídos todos os TCLEs e/ou termos de doação assinados e rubricados, se pertinentes.

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FACULDADE DE ODONTOLOGIA DE BAURU-USP

Continuação do Parecer: 1.114.009

BAURU, 18 de Junho de 2015

Assinado por:
Izabel Regina Fischer Rubira Bullen
(Coordenador)

Endereço: DOUTOR OCTAVIO PINHEIRO BRISOLLA 75 QUADRA 9
Bairro: VILA NOVA CIDADE UNIVERSITARIA **CEP:** 17.012-901
UF: SP **Município:** BAURU
Telefone: (14)3235-8356 **Fax:** (14)3235-8356 **E-mail:** cep@fob.usp.br

DETALHAR NOTIFICAÇÃO

– DADOS DA VERSÃO DO PROJETO DE PESQUISA

Título da Pesquisa: "INFLUÊNCIA DA INCORPORAÇÃO DOS FITOTERÁPICOS E. GIGANTEUM E P. GRANATUM SOBRE MATERIAIS RESILIENTES UTILIZADOS NA CONFECÇÃO DE PRÓTESES REMOVÍVEIS BUCAIS: TOPOGRAFIA, CARACTERIZAÇÃO QUÍMICA E AÇÃO CONTRA CANDIDA ALBICANS".
Pesquisador Responsável: Rafaela Alves da Silva
Área Temática:
Versão: 2
CAAE: 44951715.6.0000.5417
Submetido em: 10/06/2015
Instituição Proponente: Universidade de Sao Paulo
Situação da Versão do Projeto: Aprovado
Localização atual da Versão do Projeto: Pesquisador Responsável
Patrocinador Principal: FUNDACAO DE AMPARO A PESQUISA DO ESTADO DE SAO PAULO

Comprovante de Recepção: PB_COMPROVANTE_RECEPCAO_490

– DADOS DA NOTIFICAÇÃO

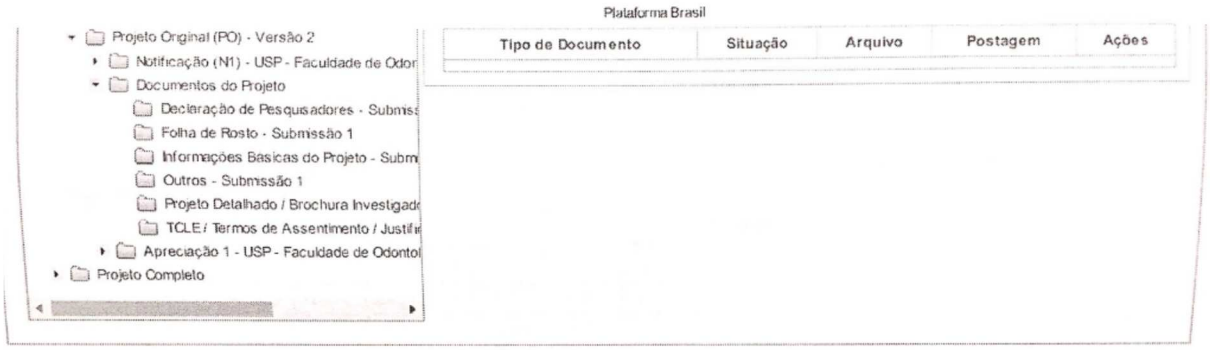
Tipo de Notificação: Envio de Relatório Final
Detalhe:
Justificativa:
Data do Envio: 26/04/2018
Situação da Notificação: Aguardando confirmação de indicação de relatoria

– DOCUMENTOS DO PROJETO DE PESQUISA

Versão Atual Aprovada (PO) - Versão 2	Tipo de Documento	Situação	Arquivo	Postagem	Ações

brasil.saude.gov.br/visao/pesquisador/gerirPesquisa/gerirPesquisaAgrupador.jsf

Maristela Petenuci Ferrari
Secretária- SRTE 43052
Setor de Apoio às Comissões
e Convênios-FOB-USP



HISTÓRICO DE TRÂMITES

Apreciação	Data/Hora	Tipo Trâmite	Versão	Perfil	Origem	Destino	Informações
N1	26/04/2018 18:15:34	Indicação de Relatoria	2	Secretária	USP - Faculdade de Odontologia de Bauru da USP	USP - Faculdade de Odontologia de Bauru da USP	
N1	26/04/2018 18:15:16	Aceitação do PP	2	Secretária	USP - Faculdade de Odontologia de Bauru da USP	USP - Faculdade de Odontologia de Bauru da USP	
N1	26/04/2018 16:23:53	Notificação enviada	2	Pesquisador	PESQUISADOR	USP - Faculdade de Odontologia de Bauru da USP	

asil.saude.gov.br/visao/administrador/4x4Novo/detalharProjetoAgrupadorApreciacao.jsf

DOCUMENTOS DO PROJETO DE PESQUISA

ersão Atual Aprovada (PO) - Versão 2
Projeto Original (PO) - Versão 2
Notificação (N1) - USP - Faculdade de Odor
Documentos do Projeto
Declaração de Pesquisadores - Submiss
Folha de Rosto - Submissão 1
Informações Básicas do Projeto - Submi
Outros - Submissão 1
Projeto Detalhado / Brochura Investigad
TCLE / Termos de Assentimento / Justific
Apreciação 1 - USP - Faculdade de Odontol
rojeto Completo

Tipo de Documento	Situação	Arquivo	Postagem	Ações
Parecer Consubstanciado do CEP	Aceito	PB_PARECER_CONSUBSTANCIADO_CEP_2669089.pdf	22/05/2018 17:30:40	

LISTA DE APRECIAÇÕES DO PROJETO

Apreciação	Pesquisador Responsável	Versão	Submissão	Modificação	Situação	Exclusiva do Centro Coord.	Ações
N1	Rafaela Alves da Silva	2	26/04/2018	22/05/2018	Aprovado	Sim	
PO	Rafaela Alves da Silva	2	10/06/2015	18/06/2015	Aprovado	Não	

ANNEX B – Manuscript submission letter confirmation from Journal of Natural Products

ScholarOne Manuscripts

Journal of Natural Products

Home

Submission Confirmation

Print

Thank you for your submission

Submitted to	Journal of Natural Products
Manuscript ID	np-2018-003913
Title	In vitro Antifungal activity of Punicalagin – Nystatin Combinations against *C. albicans* associated with Oral candidiasis
Authors	da Silva, Rafaela Lopes, Marcelo de Castro, Ricardo Ishikiriama, Bella Luna Ferrari, Tatiane Porto, Vinicius Neppelenbroek, Karin Garcia, Cindy Lara, Vanessa

manuscriptcentral.com/acs-np