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Candida albicans Release Intracellular Bacteria When Treated With Amphotericin B

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Abstract

Background: Coexistence of bacteria and yeast in a myriad of microbial communities is indicative of their intimate relationship, which could be the intracellular existence of bacteria inside yeast. In this study, the intracellular existence of bacteria inside yeast and bacterial release from amphotericin B-treated yeasts was examined using polymerase chain reaction (PCR) and microscopy.

Methods: Released bacteria, B1 from Y1 (a gastric yeast) and B2 from Y2 (an oral yeast) were identified as *Staphylococcus hominis* and *Staphylococcus haemolyticus* by biochemical tests as well as amplification of *Staph*-specific *tuf and 16S rRNA* genes. PCR products were sequenced and matched with *Staphylococcus* published sequences in GenBank. PCR was also used for amplification of *Staph-tuf* and *Helicobacter pylori-16S rRNA* genes from DNAs of 50 yeasts (20 oral, 20 gastric and 10 fecal). Microscopy was used for observing bacterium-like bodies (BLBs) inside the yeasts vacuole.

Results: Thirty-two yeasts (64%) carried *Staph-tuf* gene, 20 yeasts (40%) carried *H. pylori 16S rRNA* gene, 14 yeasts (28%) carried both genes, 12 yeasts (24%) carried neither, 6 yeasts (12%) carried only *H. pylori 16S rRNA* gene, and 18 yeasts (36%) carried only *tuf* gene. Amplified products of *tuf* (370 bp) and *16S rRNA* (756 bp) genes from B1 and Y1, and B2 and Y2 showed high similarity to *S. hominis* and *S. hemolyticus,* respectively. Microscopic observations showed BLBs inside the yeasts vacuoles, which could be related to the released bacteria. These BLBs were alive and could be observed in successive generations of yeasts.

Conclusion: Amplification of *Staphylococcus*- and *H. pylori*- specific genes from yeasts showed that the intracellular BLBs could belong to *Staphylococcus* species and *H. pylori*. Release of culturable staphylococci from 2/50 (4%) yeasts showed that not all yeasts release bacteria, and bacterial release takes place under unknown conditions. However, it could be triggered by amphotericin B or hydrolytic enzymes. Coexistence of staphylococci and *H. pylori* genes could represent a mixed endosymbiotic bacterial population in Fungi such as yeast. By selecting certain bacterial associates, the diversity of microbial communities could be determined. These selected bacteria could have an intracellular origin, being released under certain conditions.

Keywords: Amphotericin B, *Candida albicans*, *Helicobacter pylori,* Intracellular bacteria, Staphylococci

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Introduction

Bacteria and fungi, during more than 1 billion years of coexistence in different niches, have learned to exchange unknown diverse signals to share antagonistic or synergistic interactions with each other.[1–3] Being equipped with fungal lytic enzymes, is a hallmark of bacteria that control fungal communities.[4,5] However, fungi with different antibacterial activities are powerful predators of bacteria.[5] It seems that it is the long-term intimate and synergistic relationship between bacteria and fungi that has enabled them to persistently conquer the world and occupy a wide range of habitats, including those impossible for other microorganisms to live in.[6–8] This success could not have been achieved by either fungi or bacteria alone. The intimate extracellular association between bacteria and yeast in a myriad of distinct niches could be indicative of the likelihood of intracellular existence of bacteria inside fungi. It has been proposed that the *N*-glycosylation of the protein components of the rigid cell walls of ancestral eukaryotic cells led to the development of flexible extracellular matrices and the emergence of a phagotrophic lifestyle, with cells engulfing other cells. Accordingly, free-living unicellular eukaryotes acted as predators of bacteria for food and essential nutrients.[9] This phenomenon was the basis for the establishment of an endosymbiotic relationship as a milestone in the evolution of eukaryotes.[10,11] Accordingly, it is reasonable to assume that engulfed bacteria inside the phagosome of eukaryotic cells evolved in a way to change their destiny from being destroyed or serving as slaves, providing nutrients for their host, to become endosymbiont partners, being mutually beneficial.[11]

Several reports describe the endosymbiotic relationship between bacteria and eukaryotes including animals,[12] insects,[13] sponges[14] and plants.[15,16] It is believed that intracellular bacteria have evolved to reside inside the membrane-bound vacuoles of eukaryotic cells,[17] where they adapted to survive,[18] maintain their cell structure,[19]

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establish themselves as endosymbiotic partners for entire life span of the host, and be inherited by the next generations.[20] One of the best studied examples of the intracellular association between bacteria and eukaryotes is related to arbuscular mycorrhizal fungi,[21–24] first discovered by electron microscopic observations in 1970.[25] In recent studies on different fungi, fluorescent staining kits for bacterial detection and viability studies and also amplification of the bacterial *16S rRNA* gene have been used to demonstrate the existence of non-culturable endosymbiotic bacteria in the fungal vacuole.[26–29]

In our previous studies, light microscopic observations of wet mounts of *Candida* yeasts that were co-isolated with *Helicobacter pylori* from gastric biopsies, showed the existence of fast-moving bacterium-like bodies (BLBs) inside the vacuoles of yeasts. Attempts to culture BLBs for recovering *H. pylori* or other bacteria from disrupted yeasts were unsuccessful. However, *H. pylori*-specific genes were detected in *Candida* yeast isolates from stomach,[30] oral cavity,[31,32] food[33] and vagina.[34] Moreover, immunodetection of *H. pylori*-specific proteins in the protein pool of *Candida* yeasts demonstrated that *H. pylori* is well equipped with protective proteins to survive and establish itself in the vacuole of the yeast cell.[35] It was concluded that *Candida* yeast could serve as a sophisticated niche for *H. pylori*, while providing essential nutrients for its growth and multiplication, it protects the bacterium against environmental stresses and facilitates its spread within human hosts.[36]

This study was designed to examine the likelihood of bacterial release from yeast cells upon treatment with antifungal amphotericin B (amph B), which is known to disrupt fungal plasma and vacuole membranes. We proceeded our previous study in which 50 *Candida* yeasts isolates from oral cavity, stomach and feces, were recruited to compare antifungal activity of 3 non-antifungal drugs with that of 2 antifungals: ketoconazole and amph B.[37] Macrodilution method was used, according to the Clinical and Laboratory Standards Institute (CLSI) standard (M27-P), for determination of minimum fungicidal concentration (MFC) of the above mentioned drugs. Bacterial cells were released into the medium from two *Candida albicans* out of 50 amph B-treated *Candida* yeasts, at sub-lethal concentrations of the antifungals (gastric yeast, Y1, at 0.25 and 0.5 μg/mL and oral yeast, Y2, at 0.5 μg/mL). It is noteworthy that to eliminate any exogenous bacterial contamination before starting the assay, yeasts were sub-cultured on yeast extract glucose chloramphenicol (YGC) agar (Merck, Darmstadt, Germany) for more than 10 times. Furthermore, serial dilutions of amph B for all yeasts were prepared from the same stock, and growth medium showed no bacterial growth before use. For each yeast isolate, growth medium tubes containing different concentrations of amph B were inoculated from the same fungal suspension. Microscopic examination of wet mounts and gram-stained smears from the 2 broths showed the existence of gram-positive cocci. Culturing the contaminated broths on brain heart infusion (BHI) agar (Merck, Darmstadt, Germany) yielded pure cultures of gram-positive cocci. The present study was performed to answer the following questions: Where did the gram-positive cocci come from and did amph B play a key role in the release of bacteria from yeast cells? The released bacteria, B1 from Y1 and B2 from Y2, were identified by biochemical tests, and the likelihood of their intracellular existence inside the yeast vacuole was examined using polymerase chain reaction (PCR) and microscopic methods. To assess the coexistence of *H. pylori* with B1 and B2 inside the yeast vacuole, PCR was used for detection of *H. pylori*-specific *16S rRNA* gene.

Materials and Methods

Identification of Released Bacteria (B1 and B2) by Biochemical Tests

The purpose of this part of the study was to identify the released bacteria, B1 from Y1 (a gastric yeast) and B2 from Y2 (an oral yeast), according to macroscopic, microscopic and biochemical characteristics. The B1 isolate produced 0.5–2 mm pale yellow, smooth, opaque, raised and butyrous colonies. The B2 isolate produced 2–5 mm white-cream, smooth, opaque, raised and butyrous colonies. Microscopic observations on gram-stained smears showed that both isolates were gram-positive cocci arranged in pairs and tetrads. The results of biochemical tests showed that both isolates were catalase positive, oxidase negative, produced acid from glucose aerobically, lipase negative, ornithine decarboxylase negative, reduced nitrate to nitrite, produced acetoin, novobiocin sensitive, and showed good growth on mannitol salt agar, nutrient agar containing 10% and 15% NaCl and also aesculin agar containing 40% bile salt. B1 was urease positive, but hemolysin and arginine dihydrolase negative. B2 was urease negative, but showed weak hemolysis and positive arginine dihydrolase activity. Comparison of the results with information in Bergey's Manual of Systematic Bacteriology[38] showed that the biochemical characteristics of the B1 isolate corresponded to *Staphylococcus hominis*, and those of B2 corresponded to *Staphylococcus haemolyticus*.

Confirmation of B1 and B2 Identities by Detection of *Staphylococcus*-Specific *tuf* and *16S rRNA* genes

For confirming the identity of the bacterial isolates, B1 and B2, PCR was performed with primers designed for detection of *Staphylococcus*-specific *tuf*[39] and *16S rRNA* genes.[40,41] A clinical isolate of *S. aureus*, whose

identity was confirmed by biochemical tests, was used as the positive control. DNA was extracted from the bacterial cells according to the method of Sambrook and Russell.[42] PCR was carried out in a total volume of 25 μL, containing 10× PCR buffer (Sinacolon, Tehran, Iran), 3mM $MgCl_2$ (Sinacolon, Iran), 0.2 mM dNTPs mix, 10 pmole of each primer, 1 U of *Taq* DNA polymerase (Cinagen, Tehran, Iran) and 100 ng of bacterial DNA. The amplification steps of the *tuf* gene from bacteria included: initial denaturation at 94°C for 3 minutes; followed by 40 amplification cycles of 1 second at 95°C, 30 seconds at 55°C and 30 seconds at 72°C; with a final step of extension at 72°C for 3 minutes. Amplification steps of the *16S rRNA* gene from bacteria included: initial denaturation at 94°C for 10 minutes; followed by 30 amplification cycles of 45 seconds at 94°C, 45 seconds at 55°C and 75 seconds at 72°C; with a final step of extension at 72°C for 10 minutes. PCR products were electrophoresed using 1.5% agarose gel and their size was determined using a molecular ladder. Furthermore, PCR products were sequenced and matched with *Staphylococcus* published sequences in GenBank by the BLAST program (https://www.ncbi.nlm.nih.gov).

Detection of *Staphylococcus*-Specific *tuf* and *16S rRNA* Genes in Yeasts

The purpose of this study was to examine the likelihood of intracellular existence of B1 and B2 inside the yeasts Y1 and Y2, as well as in the remaining 48 yeasts. Total DNA was extracted from 50 yeasts (20 oral, 20 gastric and 10 fecal) according to the method of Sambrook and Russell.[42] The specifically designed primers and the control *S. aureus* described in the previous section were also used for detection of the *Staphylococcus tuf* gene in total DNA extracted from 50 yeasts. For confirming the molecular similarity between bacteria in yeasts Y1 and Y2 and the released ones, detection of the *Staphylococcus 16S rRNA* gene was also performed. The amplification steps of each gene from yeast were as described in the previous section, except for the amount of template DNA (300–500 ng) and the annealing temperature, which was optimized to 53.7–54°C for the *Staphylococcus tuf* gene and 54–55°C for the *Staphylococcus 16S rRNA* gene. PCR products were electrophoresed and their size was determined according to a molecular ladder. Furthermore, PCR products amplified from Y1 and Y2 were sequenced and matched with *Staphylococcus* published sequences in GenBank by the BLAST program (https://www.ncbi.nlm.nih.gov).

Detection of the *Helicobacter pylori 16S rRNA* Gene in Yeasts

The DNA of 50 yeasts was examined for the presence of the *H. pylori 16S rRNA* gene, using specifically designed primers.[43] A clinical isolate of *H. pylori*, whose identity was previously confirmed by PCR, was used as a positive control. The total volume of the reaction mixture and the amount of template DNA were as described in the previous section. The amplification steps for the *H. pylori 16S rRNA* gene from yeasts and control *H. pylori* included: initial denaturation at 94°C for 3 minutes; followed by 33 amplification cycles of 45 seconds at 94°C, 60 seconds at 56°C and 60 seconds at 72°C; with a final step of extension at 72°C for 5 minutes. PCR products were electrophoresed and their size was determined, using a molecular ladder.

Microscopic Observation of BLBs Inside the Yeast Vacuole

Yeast cells were examined by phase-contrast and fluorescent microscopes for observation of BLBs inside their vacuoles. Before the microscopic studies, all 50 yeasts were sub-cultured on YGC agar more than 10 times to eliminate the chance of contamination by extracellular bacteria. Then, wet mounts were prepared from the 48 hours cultures of 50 yeasts on YGC agar and were examined by light microscope for observation of BLBs inside the vacuoles of the yeast cells. Photographs were taken of the fast-moving BLBs inside the vacuoles of Y1 and Y2, at 4 time intervals (0, 2, 4 and 6 seconds). Furthermore, Y1 and Y2 yeast cells were stained by a Live/Dead BacLight Bacterial Viability Kit (Invitrogen, USA), which specifically interacts with bacterial DNA, revealing the bacterial nature of BLBs and their viability. Yeast cells from the 48 hours cultures on YGC agar were suspended in distilled water and their turbidity was adjusted to a 0.5 McFarland's standard. A 0.5 mL volume of each yeast suspension was mixed with 1.5 μL of fluorescent stain containing equal volumes of SYTO 9 and propidium iodide. After a quick vortex and incubation in the dark for 15 minutes, a 10 μL volume of each yeast suspension was placed on a glass slide and examined by the 100× lens of a fluorescent microscope. Movies of the live and fast-moving BLBs inside the vacuoles of the yeasts were recorded. Photographs were prepared and showed the movements of BLBs at 4 time intervals (0, 2, 4 and 6 seconds).

Co-culture of B1 and B2 With Their Respective Yeasts, Y1 and Y2

Co-culture was performed to examine the probability of cell wall lytic enzymes production by B1 and B2. Suspensions of B1 (*S. hominis)*, B2 (*S. haemolyticus*), Y1 and Y2 in BHI broth (Merck, Darmstadt, Germany) with the turbidity of 0.5 McFarland units were prepared. Equal volumes of each bacterial suspension and their respective yeasts were used to prepare co-cultures. After incubation

at 37°C, wet mounts and gram-stained preparations were examined for yeast cell lysis every 24 hours

Results

Identification of Released Bacteria (B1 and B2) by Biochemical Tests

The results of biochemical tests showed the identity of bacterial isolates B1 and B2 as *S. hominis* and *S. haemolyticus*, respectively. Both bacterial isolates were facultative anaerobes, produced acid from glucose, and showed positive results for catalase, nitrate reduction and acetoin production. The most prominent feature of B1 was urease activity and of B2 was hemolysin activity.

Confirmation of B1 and B2 Identities by Detection of *Staphylococcus*-Specific *tuf* and *16S rRNA* Genes

Electrophoresis of the amplified products of *Staphylococcus*-specific genes from bacterial isolates B1 and B2 showed bands with the size of 370 bp for *Staphylococcus tuf* gene and 756 bp for *Staphylococcus 16S rRNA* gene, which were similar to those of the control *S. aureus* (Figure 1). Analysis of the sequenced product of the *Staphylococcus tuf* gene detected in B1 showed 99% similarity to several strains of *S. hominis* such as DSM20328, ShmMCP1038 and ShmNN414 with accession numbers of HM352924, HM071884 and GU968751, respectively. Similar analysis of the *tuf* gene detected in B2 revealed 100% similarity to several strains of *S. haemolyticus* such as ShlMCP953, ShlMCP764-2 and ShlMCV28 with accession numbers of HM071886, HM071868 and HM032771, respectively. The PCR product of the *Staphylococcus 16S rRNA* gene amplified from B1 showed 99% similarity to *Staphylococcus* sp. strain ABYHD3-3 with accession number of KX645700, several strains of *S. hominis* such as 169 and CAU5267 with accession numbers of MF399394 and MF429571, respectively. Similar analysis of the *16S rRNA* gene detected in B2 showed 100% similarity to several strains of *S. haemolyticus* such as CAU7880 and CAU2265 with accession numbers of MF429096 and MF428974, respectively as well as to several strains of *Staphylococcus* sp. such as CAU1314 with accession number of MF428945.

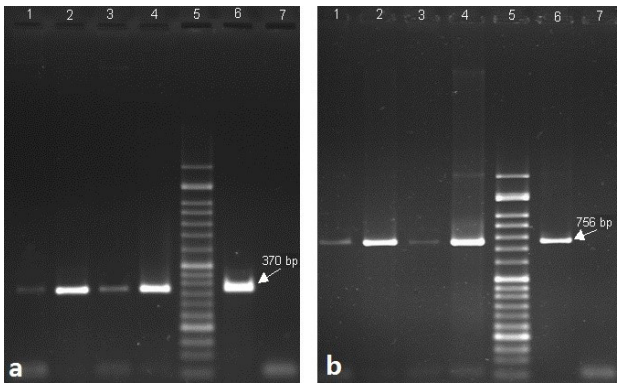

Figure 1. Electrophoresis of Amplified Products of the **a)** *Staphylococcus*-specific *tuf* Gene and **b)** *Staphylococcus*-Specific *16S rRNA* Gene, From Yeast Y1 (Lane 1), Bacterium B1 (Lane 2), Yeast Y2 (Lane 3), Bacterium B2 (Lane 4) And Control *S. aureus* (Lane 6). Lane 5 is a 50 bp ladder and lane 7 is a control without the template (negative control).

Detection of *Staphylococcus*-Specific *tuf* and *16S rRNA* Genes in Yeasts

Electrophoresis of the amplified products of the *Staphylococcus tuf* gene from Y1, Y2 and the remaining 48 yeasts showed bands with the size of 370 bp, which were similar to the size of amplified products from control, B1 and B2. Out of 50 yeasts, 32 (64%) contained the *Staphylococcus tuf* gene. The size of the amplified products of the *Staphylococcus 16S rRNA* gene from Y1 and Y2 was 756 bp, similar to those amplified from control, B1 and B2 (Figure 1). Analysis of the sequenced products of the *Staphylococcus tuf* gene amplified from Y1 showed 99% similarity with *S. hominis*, with accession number of AF298802, and several strains of *S. hominis* such as ShmMCV1 and ShmMCV22 with accession numbers of HM032754 and HM032769, respectively. However, the *Staphylococcus tuf* gene amplified from Y2, with the best alignment, showed 98% sequence similarity to several strains of *S. haemolyticus* such as ShlMCV14, ShlNN878 and ShlNN708 with accession number of HM032764, GU997237 and GU997229, respectively. Sequence analysis of the amplified product of the *Staphylococcus 16S rRNA* gene from Y1 showed 100% similarity to *Staphylococcus* sp. strain ABYHD3-3, *Staphylococcus* sp. strain ABYHD2-4 and several strains of *S. hominis* such as 169 with accession numbers of KX645700, KX645697 and MF399394, respectively. Analysis of the sequenced product of the *Staphylococcus 16S rRNA* gene amplified from Y2 revealed 100% similarity to uncultured bacterium clone ncd1507b08c1, several strains of *S. haemolyticus* such as CAU7870 and CAU2265 with accession numbers of JF127752, MF429096 and MF428947, respectively.

Detection of the *Helicobacter pylori 16S rRNA* Gene in Yeasts

The *H. pylori 16S rRNA* gene was detected in 20/50 (40%) yeasts, including Y1 and Y2, with the size of 521 bp. The size of the PCR products was the same as control *H. pylori*.

Frequency of *Staphylococcus tuf* and *Helicobacter pylori 16S rRNA* Genes in 50 Yeasts

Among 50 yeasts, 32 (64%) carried the *Staphylococcus tuf* gene and 20 (40%) contained the *H. pylori 16S rRNA* gene. A total of 14 yeasts (28%) carried both genes, 12 (24%) contained none of the genes, 6 (12%) carried only the *H. pylori 16S rRNA* gene and 18 (36%) carried only

the *Staphylococcus tuf* gene.

Microscopic Observation of BLBs Inside the Yeast Vacuole

Light microscopic observations on all 50 yeasts showed the presence of fast-moving BLBs inside the yeasts vacuoles. Phase-contrast microscopy photographs of Y1 showed fast-moving BLBs inside the vacuoles, which appeared like a cavity (Figure 2). Fluorescent microscopy of the stained Y1 showed green, live and fast-moving BLBs inside the yeasts vacuoles (Figure 3). Both light and fluorescent microscopic observations on wet mount preparations of yeasts showed the presence of BLBs inside the yeasts vacuoles even after more than 10 times sub-culture on YGC. This indicates the persistence of BLBs in successive generations of yeasts.

Co-culture of B1 and B2 With Their Respective Yeasts, Y1 and Y2

Microscopic observations on wet mounts and gram-stained preparations of co-cultures did not show lysis of yeast cells up to 2 weeks. Yeast cells appeared intact with their oval morphology under the light microscope. Culture of co-cultures was negative for yeasts, but positive for gram-positive cocci.

Discussion

In this study**,** out of 50 amph B-treated *Candida* yeasts, 2 *C. albicans*, Y1 and Y2, released culturable gram-positive cocci: *S. hominis* and *S. haemolyticus,* respectively. Microscopic observations of BLBs inside yeasts vacuoles after several sub-cultures showed that BLBs were alive and transmitted to the next generations of yeasts.

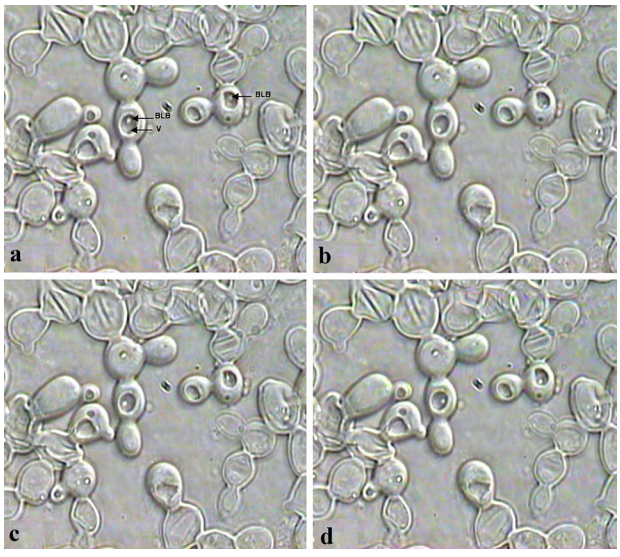

Figure 2. Phase-Contrast Microscopy of Y1 Yeast. Photographs taken at 0, 2, 4 and 6 s (**a, b, c** and **d,** respectively) show fast-moving BLBs inside vacuoles (V) (× 1250).

Amplification of *Staphylococcus*-specific genes *tuf* and *16S rRNA* from B1 and B2, as well as Y1 and Y2, showed that live and fast-moving BLBs could belong to *Staphylococcus* species. Furthermore, amplification of the *H. pylori 16S rRNA* gene from 2 yeasts showed that non-culturable *H. pylori* could be found among BLBs observed inside yeasts vacuoles. Yeasts that showed the existence of BLBs, but were negative for the *Staphylococcus tuf* or *H. pylori 16S rRNA* gene, might contain other bacterial endosymbionts whose identification needs further study. Some reports indicate that endosymbiotic bacteria exist in low numbers in host cells and are often difficult to detect. Moreover, in most cases, they are non-culturable due to being highly adapted to their own host. However, others have shown that endobacteria possess multicopies of small genomes containing genes needed for survival inside the host, as well as those for providing essential metabolites for the host.[46,47] Considering that several studies have reported that endobacteria are non-culturable,[21,24] the positive culture of B1 and B2 indicates that the culturability of some endobacteria can happen only under certain unknown conditions. The question raised in this study was: Did amph B play a role in bacterial release from *Candida* yeast?

Yeast cell wall is mainly composed of complex polymers of β-1,3- and β-1,6-glucans, mannoproteins, and chitin.[48,49] For cell wall lysis, a synergistic action of β-1,3-glucanases, β-1,6-glucanases, proteases, mannanases and chitinases is necessary.[4] Bacteria[50] and fungi[51] comprise 2 major groups of glucanase-producing microorganisms. A considerable number of studies describe bacteria from natural habitats, such as members of the genus *Bacillus*[52] that are equipped

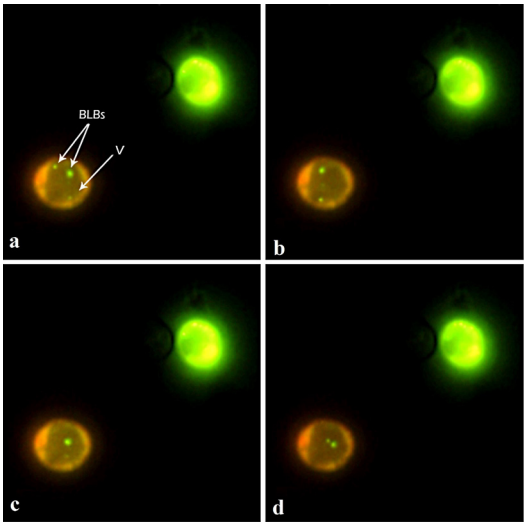

Figure 3. Fluorescent Microscopy of Y1 Yeast. Live and green BLBs are demonstrated inside yeast vacuole. Photographs taken at 0, 2, 4 and 6 seconds (a, b, c and d, respectively) show two fast-moving BLBs inside the yeast vacuole (V). Red yeast cells had started dying due to UV radiation, while BLBs inside vacuole were still alive and green (×1000).

with cell-wall degrading enzymes able to lyse viable fungal cells to reach nutrients for growth.[5,53] However, yeasts are well-known for having multiple glucanases[54] that are mainly implicated in cell growth and budding.[55] It is not clear how biosynthesis and activity of β-glucanases are regulated through the life cycle of yeast.[54,55] It seems that environmental factors such as culture medium composition, pH, temperature, aeration and growth phase may affect their biosynthesis and activity.[56] In the present study, co-culture of *S. hominis* and *S. haemolyticus* with their respective yeasts did not lead to lysis of yeast cells, although yeasts did not form colonies. This suggests that glucanases and other hydrolytic enzymes involved in disruption of the yeast vacuole and cell envelope could originate from the contents of the yeast vacuole or other endobacteria present in the vacuole. Moreover, release of culturable gram-positive bacteria from disrupted yeasts might have happened under specific conditions, triggered by amph B which disrupts vacuolar and cell membranes, and causes cation leakage and cell death.[57,58]

Bacteria and fungi are close and indivisible partners in degrading biological waste materials and nutrient cycling in nature,[59,60] producing fermented beverages,[61] pharmaceuticals[62] and fuel ethanol,[19] and formation of persistent polymicrobial biofilms on human body surfaces.[63] Although the details and importance of complex interactions between these 2 major groups of microorganisms are poorly understood,[5,7,64] their concurrence in a diverse range of niches is not accidental. Thus, it should follow the principles of evolution of symbiosis within living organisms.

Released bacteria from *Candida* yeasts Y1 and Y2 were identified as *S. hominis* and *S. haemolyticus,* respectively. Recent reports have shown frequent isolation of multidrug-resistant *S. hominis*[65] and *S. haemolyticus*[66] from patients with severe infections such as bacteremia or meningitis. Furthermore, a considerable number of reports have described the frequent coexistence of *Candida* yeast and staphylococci in chronic biofilms and systemic infections that exhibited antibiotic resistance, which were major causes of morbidity and mortality.[67] In an interesting study that compared the coexistence of staphylococci and fungi in cheese[68] with the coexistence of the same microorganisms in the human respiratory tract[69] and skin,[70] it was concluded that similar to eukaryotic hosts that have evolved to be selectively colonized with specific microbial communities,[71,72] fungi have also evolved to coexist with certain bacteria in free-living microbiomes. Accordingly, fungal selection of certain bacteria might be important in determining the diversity of microbial communities in different niches. In this regard, fungal selection of bacteria in the microbiome of human body surfaces or food materials such as cheese, could be considered important in the development of infectious diseases such as *Staphylococcus*-related urinary tract infections.[68] Extracellular coexistence of *Candida* yeast with *H. pylori* in the human stomach also shows an intimate and selective relationship between 2 microorganisms that could be related to the development of gastric diseases.[73]

In this study, microscopic observations of live BLBs inside the yeast vacuole, along with detection of *Staphylococcus tuf* and *16S rRNA* as well as *H. pylori 16S rRNA* genes in the total DNA of Y1 and Y2 yeasts, showed the intracellular existence of *S. hominis* and *S. haemolyticus* along with *H. pylori* in *C. albicans* yeasts. Establishment inside the vacuole of yeast, like in the vacuoles of other eukaryotic cells, is a genuine way for endobacterium to escape destructions occurring in the host-cell cytoplasm[74] and extracellular milieu,[75] while reaching nutrients for survival and multiplication. In this regard, free-living and ubiquitous yeast could act as a vehicle for the spread of its intracellular bacteria in the environment and within hosts, including humans.[36] Accordingly, yeast might be considered as a mysterious and magic pot that contains a mixed population of endosymbiotic bacteria that are released under certain unknown conditions. This phenomenon could be the missing link in the chain of events that maintain bacteria and yeasts as the principal coordinators of microbial interactions that profoundly affect the balance of power in the microbial world. Yeasts which are more resistant to environmental stresses than bacteria[76] could remain as permanent reservoirs of endosymbiotic bacteria that, by releasing them, restore the bacterial microbiome in soil after a famine crisis, in industrial fermentation facilities after an effective cleaning and in the human body after antibiotic therapy.

Authors' Contribution

AT, FE and FS designed the experiment. AT did the research work and prepared figures. FE did the literature review. FS wrote the paper.

Conflict of Interest Disclosures

The authors have no conflicts of interest.

Ethical Statement

Not applicable.

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