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Sugar-Rich Foods Carry Osmotolerant Yeasts with Intracellular *Helicobacter Pylori* and *Staphylococcus* spp

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ABSTRACT

BACKGROUND

Sugar-rich foods are of the main components of daily human meals. These foods with high sugar and low water content kill bacteria. However, osmotolerant yeasts survive and multiply. The aim of this study was to examine the occurrence of intracellular *Helicobacter pylori (H. pylori)* and *Staphylococcus* spp. in yeast isolates from sugar-rich foods.

METHODS

Thirty-two yeast isolates from fresh fruits, dried fruits, commercial foods, and miscellaneous foods were identified by the sequencing of amplified products of 26S rDNA. Fluorescence microscopy and LIVE/DEAD bacterial viability kit were used to examine the occurrence of live bacteria inside the yeast's vacuole. Immunofluorescence assay was used to confirm the identity of intracellular bacteria as *H. pylori* and *Staphylococcus*. Polymerase chain reaction (PCR) was used for the detection of 16S rDNA of *H. pylori* and *Staphylococcus* in the total DNA of yeasts.

RESULTS

Yeasts were identified as members of seven genera; *Candida*, *Saccharomyces*, *Zygosaccharomyces*, *Pichia*, *Meyerozyma*, *Metschnikowia*, and *Wickerhamomyces*. Intravacuolar bacteria were stained green with a bacterial viability kit, revealing that they were alive. Immunofluorescence assay confirmed the identity of intracellular *H. pylori* and *Staphylococcus* spp. PCR results revealed that among the 32 isolated yeasts, 53% were *H. pylori*-positive, 6% were *Staphylococcus*-positive, 18.7% were positive for both, and 21.8% were negative for both.

CONCLUSION

Detection of *H. pylori*- and *Staphylococcus*-16S rDNA in yeast isolates from dried fruits, and commercial foods showed the occurrence of more than one kind of endosymbiotic bacterium in yeasts' vacuoles. While the establishment of *H. pylori* and *Staphylococcus* in yeast is a sophisticated survival strategy, yeast serves as a potent bacterial reservoir.

KEYWORDS:
Sugar-rich foods, Yeast, Intracellular bacteria, *Helicobacter pylori*, *Staphylococcus* spp

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INTRODUCTION

The gastrointestinal tract is the main portal of entry into the human body for food- and water-borne microorganisms. *Helicobacter pylori (H. pylori)* is a bacterium involved in peptic diseases with unknown environmental sources and route of transmission.[1] On the one hand, close contact is believed to be the

main route of transmission of *H. pylori*, which occurs from mother to child and among siblings.[2] On the other hand, *H. pylori* is considered as a gastric colonizer whose entry into the human stomach may occur along with the ingestion of food and water.[3] However, there is no convincing evidence to indicate the survival of *H. pylori* in food [4] and water.[5] It has been suggested that food processing steps exert physical and chemical stresses on *H. pylori*, and thus different foods cannot be considered as vehicles for carrying *H. pylori* to the human gastrointestinal tract.[6] Furthermore, several reports demonstrated that *H. pylori* inside foodborne yeast could be protected from stressful conditions present in different food materials.[7] On the other hand, *Staphylococcus* spp. are typically found in both fermented and non-fermented animal and plant foods.[8] The coexistence of *Staphylococcus* and yeast has been frequently reported, for example, in food and microbial biofilms.[9] A clinically significant yet not fully elucidated fungal-bacterial interaction is the one occurring between Candida albicans and *Staphylococcus* spp.[10]

Yeasts are ubiquitous unicellular fungi that live as saprophytes on plant or animal materials, preferentially using sugars as carbon and energy sources.[11] Yeasts are equipped with different hydrolytic enzymes, such as glycosidases, cellulases, proteinases, and lipases,[11-13] which enable them to use different kinds of substrates and thus thrive in a wide range of environmental niches. Reports indicate that compared with bacteria, yeasts are more tolerant of stressful conditions such as acidic pH and are also able to grow in a wider range of water activity.[14]

Sugar-rich foods with low water activity are considered to be stressful materials that are hostile to microbial life, causing bacterial death due to osmotic shock.[15] However, osmotolerant yeasts not only tolerate the osmotic shock but are capable of growing under such conditions. It has been demonstrated that osmotolerant yeasts accumulate glycerol or other polyols in response to low water activity, maintaining or restoring an inside-directed driving force for water across their cell membrane.[11] Moreover, these polyols, with their hydroxyl groups, retain intracellular polymers in hydrated form, thus preserving enzyme activity.[16-18] Yeasts that tolerate environments with high-sugar and low-water contents are osmotolerant yeasts encompassing most of the ascomycetes.[19] Fresh fruits with high levels of sugars and other nutrients and intermediate (15-50%) water content provide favorable conditions for microbial growth.[20] However, bacteria cannot tolerate the acidic pH of these fruits and are thus eliminated, allowing osmotolerant yeasts to multiply and become established as the normal microflora in the sweet niche of fruits.[21] Dried fruits, preserved fruits, and fruit syrups are also sugar-rich foods with low water activity that carry osmotolerant yeasts as their normal microflora.[14] Different kinds of sugars that are used as additives in sweet foods might also carry osmotolerant yeasts. These yeasts are common contaminants of sugar factories and those that process concentrated solutions of sugars.[22] Sugars are produced from molasses of sugar beet or sugar cane that have high microbial contents, mainly consisting of bacterial spores, yeasts, and molds. Yeasts and molds do not usually survive the main steps of sugar manufacturing operations, which involve high temperatures and reduced water activity. However, airborne yeasts or those that occur on the surface of refinery equipment can recontaminate the raw sugar in the final steps, multiply, and increase their population to 104-106 per gram of sugar.[23] Accordingly, most of the yeast populations in sugar products are postproduction contaminants.[24]

In this study, yeast isolates from sugar-rich foods, fresh fruits, dried fruits, commercial unprocessed and processed sweet foods, and miscellaneous foods were examined for the occurrence of intracellular *H. pylori* and *Staphylococcus* spp. by molecular and microscopic methods. Specific primers were used for the detection of *H. pylori* and *Staphylococcal* 16S rDNA in the total DNA of yeasts. Light and fluorescence microscopes were used for observing the live and moving bacteria inside the yeasts. Moreover, FITC (Fluorescein isothiocyanate) conjugated antibodies were used for immunodetection of *H. pylori* and *Staphylococcus* spp. inside the vacuole of yeast cells. The rationale of the study was to demonstrate that yeasts in popular sugar-rich foods may serve as reservoirs of *H. pylori* and *Staphylococcus*, facilitating their spread within human populations.

MATERIALS AND METHODS

Collection and culture of samples

Sixty samples were collected from high-sugar foods

and classified into four groups: fresh fruits (×15): blackberry, apple, grape, persimmon, peach, fig, banana, white dragon, red dragon, Saturn peach, strawberry, carrot, plum, and cantaloupe; dried fruits (×15): whole date, heart of palm, date cube, raisin, and dried apricot; processed and unprocessed commercial foods (×22): brown sugar, icing sugar, quince jam, date syrup, sugarcane syrup, sugarcane foam, cooked beets, kombucha tea, white sugar, sugar cube, cinnamon-flavored sugar cube, low-calorie sweetener, Gaz (traditional Persian sweet), rock candy, black grape syrup, white grape syrup, plain biscuit, and Iranian delight; miscellaneous foods (×8): pistachio, almond, cashew nut, walnut, old pickled garlic, and old pickled garlic syrup. One gram of each food material was inoculated into 3 mL of brain heart infusion (BHI) broth (Merck, Germany) and incubated at 30ºC for 7-10 days. A 50-µL volume of each BHI broth was surface inoculated on YGC (0.5% yeast extract, 2% glucose, 0.01% chloramphenicol, and 1.5% agar) and observed for the growth of yeast after 24-48 hours of incubation at 30ºC.

Isolation of yeasts

A single colony was selected from each of the 32 yeast-positive cultures and sub-cultured more than 10 times on YGC agar to ensure the absence of bacterial contamination. Fresh cultures of yeasts were used for gram staining and observation of the typical morphology of yeasts by light microscopy.

PCR-restriction fragment length polymorphism (RFLP) of the internal transcribed spacer (ITS) region in 5.8S rDNA

Fresh cultures of yeasts were used for the extraction of DNA.[25] The primer pair used to amplify the ITS region was ITS-1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3').[26] Amplification was performed with yeast DNA as a template and initial denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, and extension at 72ºC for 2 min, and a final extension at 72ºC for 10 min. PCR products were electrophoresed using 1% agarose gel in Tris-borate-EDTA (TBE) buffer (0.5x) and digested without further purification, using restriction endonucleases HhaI (Promega, USA), HaeIII and HinfI (Bioron, Germany). Restriction fragments were electrophoresed using 2% agarose, and the size of fragments was determined according to a 50-1500 bp molecular ladder. Yeasts were classified into 13 groups according to their RFLP pattern.[26]

Amplification and sequencing of 26S rDNA

Amplification of the D1/D2 region of 26S rDNA of the 32 yeasts was carried out using primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3').[27] PCR was performed with initial denaturation at 94º C for 1 min followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 50ºC for 1 min, and extension at 72ºC for 2 min with final extension at 72ºC for 5 min. After electrophoresis, PCR products of representatives of the 13 RFLP groups with a size of 600 bp were purified, sequenced, and matched with published sequences in GenBank by using the BLAST program (https://blast.ncbi.nlm.nih.gov).

Light and fluorescence microscopy for observation of intracellular bacteria

Wet mounts were prepared from fresh cultures of the 32 yeasts on YGC agar and examined by light microscopy to observe the moving bacteria inside the vacuoles of yeasts. Furthermore, to find out whether bacteria were alive, a fresh culture of a yeast isolate was used for staining with the LIVE/DEAD BacLight Bacterial Viability Kit (L-7012; Molecular Probes, USA) according to the manufacturer's instructions. A wet mount was examined by a fluorescence microscope (Olympus, Tokyo, Japan), and photographs were taken at different time intervals.

Detection of *H. pylori*-specific 16S rDNA in yeasts

Total DNA from 32 yeast isolates was examined for the presence of *H. pylori*-specific 16S rDNA. PCR was carried out using primers HP1: 5'-GCAATCAGCGTCAGTAATGTTC-3' and HP2: 5'-GCTAAGAGATCAGCCTATGTCC-3'.[28] A clinical isolate of H. pylori that was previously identified by amplification and sequencing of *H. pylori*-specific 16S rDNA was used as a positive control. PCR reaction mixture without template was used as a negative control. PCR was started with 94°C for 3 min and 33 cycles of 94°C for 45 s, 57°C for

1 min and 72°C for 1 min, followed by 72°C for 5 min. PCR products were electrophoresed using 1% agarose gel, and their size was determined using a 50-1500 bp DNA ladder.

Detection of *Staphylococcus*-specific 16S rDNA in yeasts

Amplification of *Staphylococcus*-specific 16S rDNA was performed using the primers 16S-F 5-AACTCTGTTATTAGGGAAGAACA-3,[29] and 16S-R 5'-CCACCTTCCTCCGGTTTGTCACC-3.[30] The PCR program consisted of an initial denaturation step at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 45s, annealing at 54°C for 45s and extension at 72°C for 75s, and a final extension step for 10 min at 72°C. A clinical isolate of *Staphylococcus* aureus, which was previously identified by amplification and sequencing of *Staphylococcus*-specific 16S rDNA, was used as a positive control. PCR reaction mixture without template was used as a negative control. The size of PCR product was determined as above. The PCR product amplified from the Candida parapsilosis isolate was purified, sequenced, and matched with published sequences of *Staphylococcus* spp. in GenBank. Results of sequence analysis showed 99% similarity to *Staphylococcus* succinus.

Localization of *H. pylori* and *Staphylococcus* spp. inside the yeast vacuole using direct immunofluorescence assay

Localization of *H. pylori* and *Staphylococcus* spp. inside the vacuole of C. parapsilosis was performed by direct immunofluorescence (IF) assay. FITC-conjugated IgY-HP prepared against *H. pylori* in hen,[31] and FITC-conjugated IgG-ST prepared against *Staphylococcus* spp. in rabbit,[32] were used for detection of *H. pylori* and *Staphylococcus* spp. inside the yeast's vacuole. IF assay was performed according to Hašek.[33] A fresh culture of yeast in YG (0.5% yeast extract and 2% glucose) broth, was fixed with 7.4% paraformaldehyde for 120 min while shaking. After washing with 0.1 M potassium phosphate citrate buffer (KCP), fixed cells were permeabilized using lyticase (L4025; Sigma) and Triton X-100. Cells were washed and resuspended in 0.4 M PIPES buffer containing FITC-labeled antibodies and 0.01% evans blue solution (for color contrast) and incubated at room temperature for 60 min. After washing three times with PIPES, a 10-µL volume of yeast suspension was smeared onto a glass slide, air-dried, covered with mounting oil (Invitrogen, USA) and examined by fluorescence microscopy. Fresh cultures of *H. pylori* and *Staphylococcus* aureus were used as positive controls. A negative control yeast (a yeast with no amplification of *H. pylori* and *Staphylococcus* 16S rDNA) was used to demonstrate the lack of non-specific interaction of antibodies.

RESULTS

Isolation of yeasts

Microscopic examination of gram-stained smears of yeast colonies on YGC agar showed typical yeast morphology. Of 60 samples, 32 (53.3%) were positive for yeast growth. Yeast-positive samples included 10 of 15 (66.6%) fresh fruits, 8 of 15 (53.3%) dried fruits, 11 of 22 (50%) commercial foods, and 3 out of 8 (37.5%) miscellaneous foods.

Molecular identification of yeasts

Amplification of the ITS region of 5.8S rDNA from the 32 yeasts revealed bands with a size of 370 to 880 bp, which were digested with restriction endonucleases. Yeasts were classified into 13 groups according to their PCR-RFLP pattern. RFLP group 12 with five isolates of *C. albicans*, group 8 with four isolates of *Meyerozyma guilliermondii*, group 5 with four isolates of *Candida diversa*, and group 1 with four isolates of *Pichia kudriavzevii* contained the highest number of yeast isolates. In the fresh fruits group, isolated yeasts included *Zygosaccharomyces bailii* (×2), P. *kudriavzevii* (×2), *Pichia pastoris* (×1), *Zygosaccharomyces mellis* (×1), *Metschnikowia pulcherrima* (×1), *C. diversa* (×1), and *Candida catenulata* (×2) (table 1).

In the dried fruits group, isolated yeasts included *P. kudriavzevii* (×1), *Meyerozyma guilliermondii* (×3), *Saccharomyces cerevisiae* (×1), *C. parapsilosis* (×2), and *Wickerhamomyces anomalus* (×1) (table 2). In the commercial foods group, isolated yeasts included C. albicans (×3), *Meyerozyma guilliermondii* (×1), *P. kudriavzevii* (×1), *Saccharomyces cerevisiae* (×2), *C. diversa* (×2), *C. parapsilosis* (×1), and *Yarrowia lipolytica* (×1) (table 3). In the miscellaneous foods, isolated yeasts included *C. albicans* (×2) and *C. diversa* (×1) (table 4).

Table 1: **Frequency of *Helicobacter pylori* 16s rDNA and *Staphylococcus* 16s rDNA in 10 yeast isolates from fresh fruits**

Samples	RFLP group	Sequencing result (26S rDNA)	*H. pylori*-16S rDNA	*Staphylococci*-16S rDNA
Blackberry 2	1	*P. kudriavzevii*	-	-
Strawberry	2	*Z. bailii*	+	-
Grape	3	*Z. mellis*	-	-
Persimmon	4	*M. pulcherima*	-	-
Peach	2	*Z. bailii*	+	-
Fig	5	*C. diversa*	+	-
Banana	6	*P. pastoris*	+	-
White dragon	7	*C. catenulata*	+	-
Red dragon	7	*C. catenulata*	+	-
Carrot	1	*P. kudriavzevii*	+	-

Table 2: **Frequency of *Helicobacter pylori* 16s rDNA and *Staphylococcus* 16s rDNA in eight yeast isolates from dried fruits**

Samples	RFLP group	Sequencing result (26S rDNA)	*H. pylori*-16S rDNA	*Staphylococci*-16S rDNA
Date 2	1	*P. kudriavzevii*	-	-
Date 3	8	*M. guilliermondii*	-	-
Date 4	9	*S. cerevisiae*	+	+
Date 5	8	*M. guilliermondii*	+	+
Date 6	10	*W. anomalus*	+	+
Date 7	11	*C. parapsilosis*	+	+
Date 8	11	*C. parapsilosis*	+	+
Heart of palm	8	*M. guilliermondii*	+	-

Table 3: **Frequency of *Helicobacter pylori* 16s rDNA and *Staphylococcus* 16s rDNA in 11 yeast isolates from processed and unprocessed commercial foods**

Samples	RFLP group	Sequencing result (26S rDNA)	*H. pylori*-16S rDNA	*Staphylococci*-16S rDNA
Brown sugar 1	12	*C. albicans*	+	-
Brown sugar 2	12	*C. albicans*	+	-
Icing sugar	8	*M. guilliermondii*	-	+
Quince jam	1	*P. kudriavzevii*	+	-
Date syrup	13	*Y. lipolytica*	+	-
Sugarcane syrup	12	*C. albicans*	+	-
Sugarcane foam	9	*S. cerevisiae*	+	-
Cooked beet 1	5	*C. diversa*	-	+
Cooked beet 2	11	*C. parapsilosis*	+	-
Cooked beet 3	5	*C. diversa*	+	-
Kombucha tea	9	*S. cerevisiae*	+	+

Table 4: **Frequency of *Helicobacter pylori* 16s rDNA and *Staphylococcus* 16s rDNA in three yeast isolates from miscellaneous samples**

Samples	RFLP group	Sequencing result (26S rDNA)	*H. pylori*-16S rDNA	*Staphylococci*-16S rDNA
Pistachio	5	*C. diversa*	+	-
Old pickled garlic	12	*C. albicans*	-	-
Old pickled garlic syrup	12	*C. albicans*	-	-

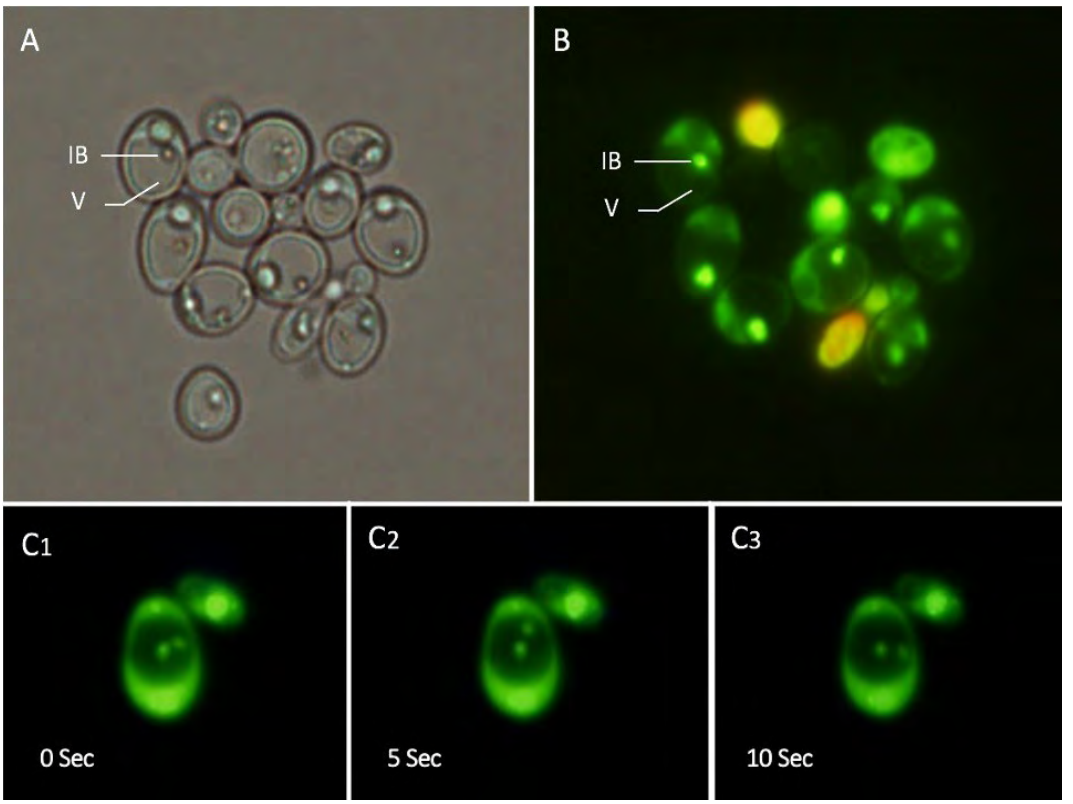

Fig.1: Light and fluorescence microscopy of yeast. A) Light microscopy of yeast cells shows intracellular bacteria (IB) inside yeast's vacuole (V). B) Live intracellular bacteria (IB) appeared as green spots in the vacuole (V) of stained yeast cells. C1-C3) Photographs taken at three-time intervals (0, 5, and 10 seconds) show the moving bacteria. Original magnification x 1000.

Light and fluorescence microscopy of yeast

Light microscopic examination of wet mounts prepared from cultures of the 32 isolated yeasts showed the occurrence of bacteria inside the vacuole of all the yeast cells (Fig.1 A). Live/Dead staining of yeast cells confirmed the viability of intracellular bacteria (Fig.1 B). Photographs taken from a stained wet mount of yeast, at three-time intervals, showed live and moving bacteria inside the yeast cell vacuole (Fig.1 C1-C3).

Detection of *H. pylori*- and *Staphylococcus*-specific 16S rDNA in yeasts

The amplified product of *H. pylori*-specific 16S rDNA with a size of 521 bp was detected in 23 of 32 (71.8%) yeast isolates. The frequency of *H. pylori*-positive yeasts in different groups of samples was determined to be 70% (7out of 10) in the fresh fruits group: *Z. bailii* (×2), *C. diversa* (×1), *P. pastoris* (×1), *C. catenulate* (×2), and *P. kudriavzevii* (×1) (table 1); 75% (6 out of 8) in the dried fruits group: *Meyerozyma guilliermondii* (×2), *C. parapsilosis* (×2), *W. anomalus* (×1) and *Saccharomyces cerevisiae* (×1) (table 2); 81.8% (9 out of 11) in the commercial foods group: C. albicans (×3), *P. kudriavzevii* (×1), *Y. lipolytica* (×1), *Saccharomyces cerevisiae* (×2), *C. diversa* (×1), and *C. parapsilosis* (×1) (table 3); and 33.3% (1 out of 3) in miscellaneous foods: *C. diversa* (×1) (table 4). *Staphylococcus*-specific 16S rDNA with a size of 750 bp was detected in 22.2% (8 out of 36) of yeast isolates. The frequency of *Staphylococcus* spp.-positive yeasts was determined to be 62.5% (5 out of 8) in the dried fruits group (Saccharomyces cerevisiae, *Meyerozyma guilliermondii*, *W. anomalus*, and 2× *C. parapsilosis*) and 27% (3 out of 11) in the commercial foods group (Saccharomyces cerevisiae, M*eyerozyma guilliermondii* and *C. diversa*) (tables 2 and 3). Yeast isolates from fresh fruits, and miscellaneous foods did not carry *Staphylococcus*-specific 16S rDNA (tables 1 and 4). The frequency of yeasts containing both *H. pylori* and

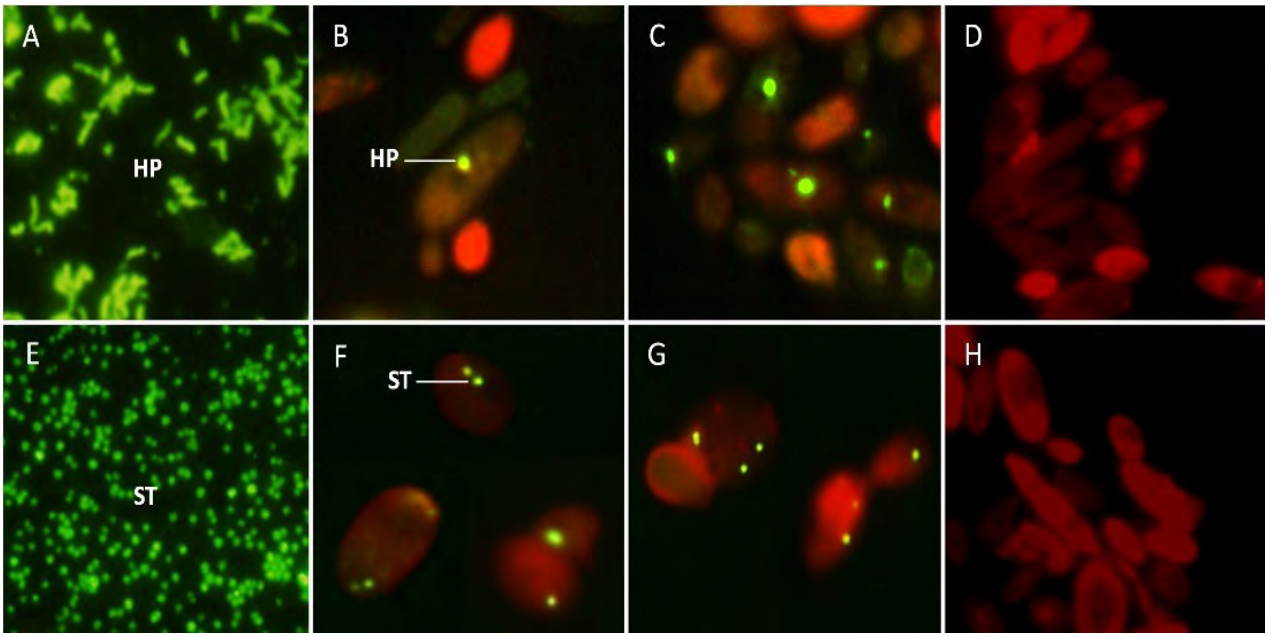

Fig.2: Direct immunofluorescence assay on C. *parapsilosis* for localization of *H. pylori* and *Staphylococcus spp* inside the yeast vacuole. Photographs show specific interaction of FITC-labeled IgY-HP with intracellular *H. pylor*i (HP) (B and C) and specific interaction of FITC-labeled IgG-ST with intracellular *Staphylococcus* (ST) (F and G). Photographs A and E show positive interaction of labeled antibodies with pure culture of *H. pylori* and *Staphylococci*, respectively. Negative control yeast with the dark vacuole, showing the lack of non-specific interaction of antibodies (D and H). Original magnification x 1000.

Staphylococcus spp. was 62.5% in dried fruits and 9% in commercial foods. Altogether, among the 32 isolated yeasts, 17 (53%) were *H. pylori*-positive only, two (6%) were *Staphylococcus* spp.-positive only, six (18.7%) were positive for both *H. pylori* and *Staphylococcus* spp., and seven (21.8%) were negative for both.

Localization of *H. pylori* and *Staphylococcus spp*. inside the yeast vacuole using direct immunofluorescence assay

Specific interaction of FITC-IgY-HP (Fig.2, B and C) or FITC-IgG-ST (Fig.2, F and G) with intracellular bacteria confirmed the identity of bacteria as *H. pylori* or *Staphylococcus spp*. and their localization inside yeast's vacuole. Dark vacuole of negative control yeast without fluorescent spots indicated the lack of non-specific interaction of antibodies (Fig.2, D and H).

DISCUSSION

Carbohydrates are the most popular foods consumed by humans worldwide. In addition to be a major carbon source for building living cells, their sweet taste, and energy provision afford these compounds a very special place in the human diet. Sweet foods are recognized as comfort foods because their consumption leads to elevation of serotonin, the known anti-depressant neurotransmitter that reduces pain and regulates sleep and the biological clock.[34] Sugars are also used to improve the quality of foods due to their functional properties.[35] Furthermore, sugar and salt are the oldest preservatives that, when added to food materials, protect them against microbial spoilage by producing high osmotic pressure. Accordingly, sugars are frequently added to a variety of foods and beverages. Bacteria cannot withstand the osmotic stress of sugar-rich environments and die, while osmotolerant yeasts survive and even multiply.[36]

Fresh and dried fruits, fruit juice, and refined sugar products are sugar-rich environments of plant origin that cause stress to microbial cells by reducing water activity, changing cell turgor pressure, and destabilizing macromolecules.[15] This might indicate that floral nectar and fruits have evolved to accumulate high concentrations of sugar to protect the fertile parts of plants from microbial attack.[37,38] However, osmotolerant yeasts that show max-

imum fitness in high concentrations of sugars,[39] increase their population and become established as the normal microflora of such sugar-rich plant environments, including floral nectar and fruits.[40] These symbiotic yeasts, while feeding on plants, stimulate plant metabolism, and inhibit phytopathogens.[41,42] Insects, which play an important role in pollination and reproduction of plants, feed on yeasts and carry them from the soil to plants and disperse them within plants during pollination.[43] It appears that symbiosis of yeasts with insects and plants is an important and inevitable evolutionary event.[44] Accordingly, yeasts as permanent associates of plants, enter the human digestive system through the consumption of sugar-rich and plant-derived food products.

Yeasts with high potential for genotypic [45,46] and phenotypic [47] plasticity are permanent symbiotic inhabitants of plants,[41,42] insects,[48] animals,[49] and humans [50] in a wide range of environments.[37,51] Accordingly, it is not surprising that fungi with these sophisticated properties have evolved to serve as a unique niche for sheltering the endosymbiotic bacteria.[52] In our previous studies, *H. pylori*-specific genes were detected in oral,[53] gastric,[54] vaginal,[55] and foodborne 7 yeasts. Furthermore, *H. pylori*-specific proteins were detected in the protein pool of gastric yeasts by western blot technique,[56] and intracellular *H. pylori* was localized in the vacuole of Candida yeast by FITC-IgY-HP.[31] Results of similar studies performed in our lab showed the detection of *Staphylococcus*- specific genes [57] and proteins [32] in gastric yeasts as well as staphylococcal localization inside the vacuole of gastric yeast by immunodetection and FISH methods.[32] It was proposed that inside the vacuole of *Candida yeast*, *H. pylori*, or *Staphylococcus* are protected from environmental stresses and provided with nutrients for survival and multiplication. Accordingly, yeast was suggested as a potent reservoir of *H. pylori* and *Staphylococcus*.[7,53,54,58]

Among the 32 yeasts isolated in this study, 17 (53%) were *H. pylori*-positive only, two (6%) were *Staphylococcus* spp.-positive only, six (18.7%) were positive for both *H. pylori* and *Staphylococcus* spp., and seven (21.8%) were negative for both bacteria. Among the sugar-rich foods studied, dates showed the greatest potential for supporting intracellular *H. pylori* (75%) and *Staphylococcus* spp. (62.5%), or both (62.5%) in yeasts.

The frequency of *H. pylori*-positive yeasts in fresh fruits, dried fruits, and commercial foods was 70–81.8%. Fresh fruits contain high levels of sugars, other nutrients, and intermediate water activity that favor microbial growth. However, acidic pH eliminates bacteria and provides appropriate conditions for fungal growth. The natural microbiota of fruits is commonly composed of yeasts such as *Candida*, *Pichia*, *Saccharomyces*, *Hanseniaspora*, and *Zygosaccharomyces*.[21] Dried fruits such as date fruits, in addition to sugar, contain salts and minerals, fatty acids, amino acids, proteins, and vitamins, including B1, B2, and B3. Furthermore, dates are rich in different kinds of sterols [59,60] that are precursors of ergosterol involved in the synthesis of yeast membranes.[61] It is noteworthy that sterols also serve as precursors of cholesterol, an important constituent of *H. pylori* cell membrane.[62] Commercial foods such as sugar cubes, granulated white and brown sugars, and other related high-sugar products are frequently used as sweet additives to tea, coffee, and sherbets or to formulated foods such as desserts and pastries. These compounds with high sugar content could carry yeasts either of plant origin or introduced as post-operation contaminants.[63]

Among the miscellaneous foods investigated, the two *C. albicans* isolates from old pickled garlic, and old pickled garlic syrup did not contain *H. pylori* or *Staphylococcus* spp. However, the three *C. albicans* isolates from commercial foods, and eight out of nine *Candida* spp. from other foods carried *H. pylori*. This might indicate that long storage of pickled garlic in vinegar, although favoring the survival of *C. albicans yeasts*, could exert a negative effect on the survival of intracellular bacteria, leading to a reduction in bacterial copy number such that bacterial DNA was not detectable by PCR. Negative PCR results have been suggested to result from failure in the detection of bacterial genes due to low bacterial copy number, inadequate amount of extracted DNA,[64] or lack of primer recognition sites in bacterial DNA due to variation in the target sequence.[65]

Results of this study showed that sugar-rich foods, whether naturally sweet or containing added sugar, are carriers of osmotolerant yeasts that could contain *H. pylori*

and/or *Staphylococcus* spp. Detection of *H. pylori*- and/or *Staphylococcus*-specific genes shows the probable occurrence of multiple endosymbiotic bacteria in the vacuoles of yeasts with different frequencies. Microscopic observations of bacteria in new generations of yeasts along with amplification of *H. pylori*- and *Staphylococcus*-specific genes from consecutive generations indicate that new yeast cells can inherit the intracellular bacteria as part of their vacuolar content. Extensive studies on the intracellular existence of non-culturable bacteria inside arbuscular mycorrhizal fungi indicated that the fungal vacuole provided a nourishing and protective niche for the endosymbiotic bacterium '*Candidatus* Glomeribacter g*igasporarum*' (CaGg), facilitating its replication and transmission to the next generation.[52] Moreover, the occurrence of two types of endosymbiotic bacteria has been reported in arbuscular *mycorrhizal fungi*: the gram-negative beta *proteobacterium* CaGg, and a gram-positive molicutes-related *endobacterium*.[66] It is noteworthy that microscopic observation of bacterial structures inside the vacuoles of yeast isolates with negative results for amplification of *H. pylori* or *Staphylococcus* genes suggests the likelihood of the occurrence of other intracellular bacteria yet to be identified.

Yeasts enter the food cycle of animals, including humans, through the consumption of sugar-rich and plant-derived foods. Yeasts are able to survive in the human gastro-intestinal tract due to their high potential to adapt to different stressful conditions and return to natural soils when excreted. It can be concluded that different yeasts that occur in the soil are more or less representatives of the yeast populations of plant and animal life above the soil surface. In this fashion, yeasts establish more or less similar populations in soils, plants, and animals, including humans. In all the steps of the food cycle, yeast may carry its intracellular bacteria and spread it to different hosts such as insects, plants, and animals. Accordingly, the intracellular occurrence of *H. pylori*, *Staphylococcus* spp. and probably other bacteria inside the yeast could be regarded as a sophisticated survival strategy of bacteria that evolved along the evolutionary path. Overall, yeasts may be regarded as permanent reservoirs of bacteria, and thus bacteria will exist as long as yeasts persist.

ETHICAL APPROVAL

There is nothing to be declared.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to this work.

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