



WJG 20<sup>th</sup> Anniversary Special Issues (6): *Helicobacter pylori*

## Vacuoles of *Candida* yeast as a specialized niche for *Helicobacter pylori*

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Received: October 15, 2013 Revised: January 9, 2014

Accepted: February 26, 2014

Published online: May 14, 2014

### Abstract

*Helicobacter pylori* (*H. pylori*) are resistant to hostile gastric environments and antibiotic therapy, reflecting the possibility that they are protected by an ecological niche, such as inside the vacuoles of human epithelial and immune cells. *Candida* yeast may also provide such an alternative niche, as fluorescently labeled *H. pylori* were observed as fast-moving and viable bacterium-like bodies inside the vacuoles of gastric, oral, vaginal and foodborne *Candida* yeasts. In addition, *H. pylori*-specific genes and proteins were detected in samples extracted from these yeasts. The *H. pylori* present within these yeasts produce peroxiredoxin and thiol peroxidase, providing the ability to detoxify oxygen metabolites formed in immune cells. Furthermore, these bacteria produce urease and VacA, two virulence determinants of *H. pylori* that influence phago-lysosome fusion and bacterial survival in macrophages. Microscopic observations of *H. pylori* cells in new generations of yeasts along with amplification of *H. pylori*-specific genes from consecutive generations indicate that new yeasts can inherit the intracellular *H. pylori* as part of their vacuolar content. Accordingly, it is proposed that yeast vacuoles serve as a sophisticated niche that protects *H. pylori* against the

environmental stresses and provides essential nutrients, including ergosterol, for its growth and multiplication. This intracellular establishment inside the yeast vacuole likely occurred long ago, leading to the adaptation of *H. pylori* to persist in phagocytic cells. The presence of these bacteria within yeasts, including foodborne yeasts, along with the vertical transmission of yeasts from mother to neonate, provide explanations for the persistence and propagation of *H. pylori* in the human population. This Topic Highlight reviews and discusses recent evidence regarding the evolutionary adaptation of *H. pylori* to thrive in host cell vacuoles.

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**Key words:** *Helicobacter pylori*; Intracellular; *Candida*; Vacuole

**Core tip:** *Helicobacter pylori* (*H. pylori*) have been observed within yeast vacuoles by light and fluorescence microscopy, and their presence has been confirmed by the detection of *H. pylori*-specific genes and proteins in yeast extracts, such as VacA subunits, UreA, peroxiredoxin and thiol peroxidase. Moreover, non-culturable *H. pylori* cells have been found in subsequent generations of yeasts, indicating the generational transmission of the bacteria is part of the transfer of vacuolar content. *H. pylori* are therefore well-equipped to establish in the vacuoles of yeast, which provide them with essential nutrients such as ergosterol for multiplication, as a pre-adaptation for invasion of human cells.

Siavoshi F, Saniee P. Vacuoles of *Candida* yeast as a specialized niche for *Helicobacter pylori*. *World J Gastroenterol* 2014; 20(18): 5263-5273 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i18/5263.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i18.5263>

## INTRODUCTION

It is currently unknown how *Helicobacter pylori* (*H. pylori*) persist in the environment and food supply. Moreover, the factors facilitating their entry to human gastric epithelium and the mechanism of their transmission from person to person are equally unclear. The ability to trace *H. pylori* from environmental sources to the human stomach is hampered by the fastidiousness of the bacterium, the chronic nature of bacterial infection, lack of outbreaks and a high prevalence of asymptomatic infected individuals. The failure to isolate *H. pylori* from environmental sources indicates the bacteria require a habitat that offers protection from environmental stresses, such as lack of nutrients and presence of biocides, for survival outside of the human stomach. Thus, the presence of an unknown environmental reservoir has been proposed, which could play a crucial role in the survival and spread of *H. pylori*<sup>[1]</sup>. Many bacteria, including pathogenic species, persist despite stressful conditions by establishing inside eukaryotic microorganisms. The intracellular life of prokaryotes inside eukaryotes is considered a significant evolutionary phenomenon that led to the adaptation of prokaryotes to a wide range of environmental niches, though details of this relationship have not been elucidated, largely due to the non-culturability of these intracellular bacteria<sup>[2]</sup>.

Human gastric epithelial and immune cells have been recognized as the sole specialized eukaryotic cells that host *H. pylori*<sup>[3,4]</sup>, with microscopic observations of *H. pylori* in the vacuoles of epithelial cells<sup>[5-7]</sup>, macrophages<sup>[8]</sup> and dendritic cells<sup>[9]</sup>. Thus, *H. pylori* have been described as facultative intracellular bacteria, which have evolved to utilize the vacuoles of eukaryotic cells as a protective niche, allowing the bacteria to multiply and persist for a long time<sup>[3,4,6]</sup>. However, there are no reports documenting a stable intracellular association of *H. pylori* with a eukaryotic microorganism. This review presents the research works concerning *H. pylori* inside *Candida* yeast. As *Candida* yeast are remarkably resistant to stressful conditions<sup>[10]</sup>, they could serve as an alternative host to shelter *H. pylori* against stressful conditions outside the human stomach, provide nutrients for its multiplication and act as a mediator for the spread of the bacterium in the environment and within human populations. In this regard, it is proposed that the yeast vacuole provides a unique and sophisticated niche for *H. pylori* that evolved when eukaryotic cells began to phagocytose prokaryotes as prey<sup>[11]</sup>.

## EVOLUTION OF INTRACELLULAR BACTERIUM

The intracellular establishment of bacteria inside fungi is regarded as an unusual evolutionary phenomenon, as the cell wall of fungi restricts endocytosis and bacterial uptake<sup>[12]</sup>. In contrast to other eukaryotes, such as protozoa, bivalves and insects<sup>[13]</sup>, there are a limited number of examples of fungi harboring intracellular bacteria<sup>[14]</sup>. The

most well-studied example involves the early evolutionary establishment of endobacteria in arbuscular mycorrhizal (AM) fungi<sup>[15]</sup>, a plant root-associated fungus that dates back to 300-400 million years ago<sup>[16]</sup>. Fungal endobacteria have been localized inside membrane-bound vacuoles using light, electron<sup>[17,18]</sup>, and confocal<sup>[15]</sup> microscopy, similar to other prokaryotic-eukaryotic endosymbioses<sup>[19]</sup>. The endosymbiotic bacteria of AM fungi have been identified as a new taxon based on ribosomal sequences, *Candidatus Glomeribacter gigasporarum*<sup>[17]</sup>, which is related to *Burkholderia cepacia*, a species of free-living bacteria with the potential to behave as saprophytes or pathogens. Similar to many other pathogenic bacteria, such as *Legionella*<sup>[20]</sup>, *Pseudomonas*<sup>[21]</sup>, and *Mycobacteria*<sup>[22]</sup>, *B. cepacia* could survive in the vacuoles of eukaryotic cells, ranging from free-living amoebae<sup>[23]</sup>, to macrophages<sup>[24]</sup>, epithelial cells<sup>[25]</sup>, and pneumocytes<sup>[26]</sup>. It is believed that this intracellular establishment protects the pathogenic bacteria against environmental stresses or the immune system of the host, and facilitates the transmission to a new host<sup>[27]</sup>. While the basis for the interaction between bacteria and AM fungi is not clear<sup>[16]</sup>, it is proposed that fungal vacuoles provide a nourishing and protective niche for the endosymbiotic bacterium, facilitating its replication and transmission to the next generation<sup>[28]</sup>.

## VACUOLES

The fungal vacuole is an acidic storage compartment with certain similarities to plant vacuoles and mammalian lysosomes. The various functions of vacuoles include glycoprotein turnover and hydrolysis, storage of Ca<sup>2+</sup>, phosphate and amino acids, pH and osmotic regulation, ion homeostasis and cytoplasmic detoxification<sup>[29,30]</sup>. Vacuoles also incorporate membranes from biosynthetic, endocytotic and autophagic cellular pathways<sup>[31]</sup>. Ergosterol in unicellular invertebrates, akin to cholesterol in vertebrates, is an important constituent of membrane lipids, and is implicated in several fungal cell processes, including plasma membrane fusion during mating and endocytosis<sup>[32]</sup>. Accordingly, a considerable amount of ergosterol is found in the membranes of vacuoles and other intracellular organelles<sup>[33]</sup>. Interestingly, a unique property of members of the genus *Helicobacter* is the incorporation of a large amount of ergosterol in their cell membrane. Ergosterol comprises up to 70% of cellular neutral lipids in *H. pylori*, much higher than in *Escherichia coli* (*E. coli*) (17%), which may have developed as a consequence of the symbiotic association with eukaryotic hosts<sup>[34]</sup>. The incorporation of cholesterol is important for *H. pylori* colonization of the host<sup>[35]</sup>, pathogenicity<sup>[36]</sup> and antibiotic resistance<sup>[37]</sup>.

## H. PYLORI AND CANDIDA

The relationship between bacteria and yeast has largely been focused on extracellular associations, such as those occurring in food materials<sup>[38]</sup> and human mucosal sur-

faces<sup>[39,40]</sup>. Although bacteria and yeast have co-existed for billions of years, the biological relevance of this inter-domain microbial interaction remains largely unknown<sup>[41]</sup>. The most well-studied interactions are in polymicrobial biofilms of *Candida albicans* (*C. albicans*) and bacterial species within the human host environment that exhibit resistance to the immune system and antimicrobials<sup>[42]</sup>. Shedding light on the details of such interactions could provide important information for the management of infectious diseases, especially those that exhibit resistance to antimicrobial therapy<sup>[43]</sup>.

Yeasts are highly sophisticated microorganisms with a remarkable ability for rapid change and adaptation to environmental stresses<sup>[44]</sup>, including antimicrobials, the host immune system and a change in body location or host physiology<sup>[45]</sup>. *C. albicans* mostly occur in association with humans, thriving on the mucosal surfaces of the gastrointestinal (GI) and genitourinary tracts and skin<sup>[39,40,45]</sup>. *H. pylori* are also found in the human GI tract, indicating that both microorganisms are well-adapted to this unique niche<sup>[46,47]</sup>. The interaction between these two microorganisms may have begun long ago and led to the intracellular establishment of *H. pylori* inside the yeast vacuole, as a pre-adaptation for the invasion of and persistence within human epithelial and immune cells. The resistance of intracellular bacteria to destruction in the phagosome may have originally occurred as a adaptation for survival within free-living amoebae<sup>[20]</sup>, reflecting a long co-evolutionary process that began more than one billion years ago<sup>[48]</sup>.

An association between *H. pylori* and *Candida* was first proposed in 1998 when yeast colonies were found as contaminants in gastric biopsy cultures on blood agar plates. Light microscopy revealed the presence of fast-moving bacterium-like bodies (BLBs) inside the vacuoles of 18 gastric yeasts cells that were purified and identified as *Candida* species based on their morphology and formation of blastoconidia on Sabouraud dextrose agar. The recruited yeasts were sub-cultured on yeast extract-glucose agar containing chloramphenicol several times to ensure the absence of bacterial contamination. Since BLBs from disrupted yeasts were not culturable, polymerase chain reaction (PCR) was used to reveal their bacterial nature. The *H. pylori*-specific *ureA* gene product, similar in size to control *H. pylori*, was amplified from 12/18 (67%) gastric yeast extracts. Furthermore, yeasts and pure cultures of control *H. pylori* were tested for tolerance to elevated temperatures, desiccation, acidic pH and biocides. The control *H. pylori* were inactivated upon exposure to stresses, however, yeasts were not inactivated and the intracellular *H. pylori* showed active movement<sup>[49]</sup>. There are many reports that indicate the tolerance of yeasts to stressful conditions<sup>[50,51]</sup>. Accordingly, it was proposed that concurrence of *H. pylori* and *Candida* in the human GI tract indicates the existence of a more intimate relationship, with the yeast serving to protect *H. pylori* from environmental stresses<sup>[49]</sup>.

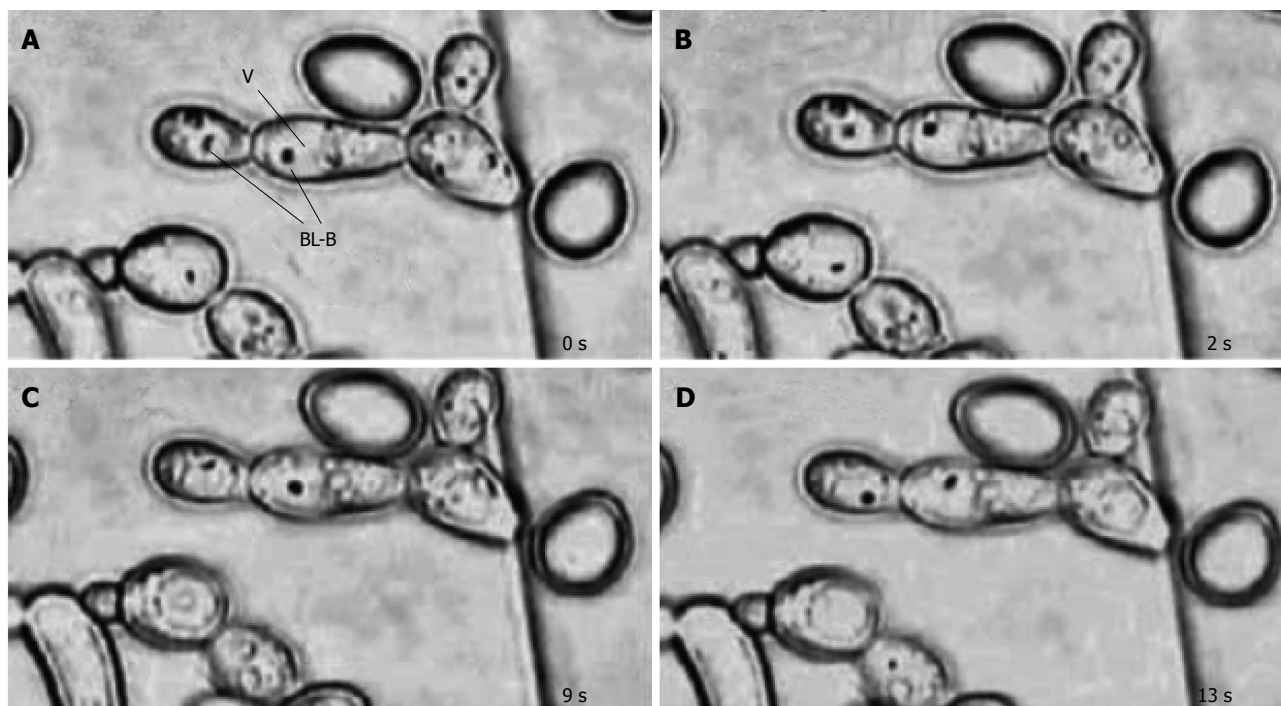
## H. PYLORI IN THE ORAL CAVITY

There are discrepancies concerning the permanence or transience of *H. pylori* in the oral cavity, and it is not clear whether the oral cavity creates sufficiently favorable conditions for *H. pylori* growth<sup>[52]</sup>. Bacterial DNA has been detected in the oral cavity<sup>[53,54]</sup>, dental plaque<sup>[55]</sup> and saliva<sup>[56]</sup>, thus implicating oral-oral and fecal-oral transmission modes of *H. pylori*<sup>[57]</sup>. However, the failure to cultivate *H. pylori* from the oral cavity and feces<sup>[58,59]</sup> indicates unfavorable survival conditions, likely due to antagonistic materials secreted by the microfloras of the oral cavity<sup>[60]</sup> and intestine<sup>[58]</sup>. On the other hand, persistence of *H. pylori* in the oral cavity after eradication therapy for gastric infection<sup>[61]</sup>, as well as the resistance of the bacteria against antimicrobial products of oral microflora, suggest that *H. pylori* must somehow be protected in the oral cavity<sup>[62]</sup>. Accordingly, it remains unclear whether the oral cavity serves as a potent reservoir for *H. pylori* gastric reinoculation<sup>[63]</sup>.

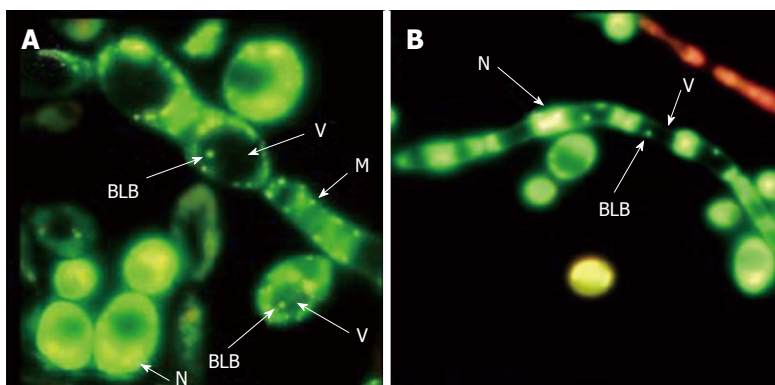
*Candida* spp. yeasts isolated from oral samples of dyspeptic patients were shown to contain fast-moving and non-culturable BLBs inside their vacuoles (Figure 1)<sup>[64]</sup>. *H. pylori*-specific *16S rRNA* and *cagA*<sup>[64]</sup>, as well as vacuolating cytotoxin A (*vacA*) and *ureAB*<sup>[65]</sup>, were amplified from these yeasts, showing 98% homology with those of the control *H. pylori*. These findings indicated that *H. pylori* are accommodated within the vacuoles of the *Candida* that thrive on the mucosa of the human oral cavity, and may explain the persistence of *H. pylori* in the oral cavity, increased risk for reinoculation of the stomach and spread of the bacterium from person-to-person. Accordingly, oral hygiene was suggested as a way to effectively reduce the yeast content in the oral cavity and control *H. pylori* transmission<sup>[64,65]</sup>.

## H. PYLORI IN FOOD SOURCES

Although it is well known that *H. pylori* are gastric colonizers that likely enter the human GI tract through intake of contaminated water or food<sup>[66]</sup>, culturable forms of *H. pylori* have not been recovered from water<sup>[56]</sup> or food<sup>[67,68]</sup> sources. There is no indication for long-term survival or growth of *H. pylori* in water<sup>[69]</sup> or food materials such as beef<sup>[70]</sup>, raw chicken, lettuce, milk<sup>[71]</sup> and yogurt<sup>[71,72]</sup>, due to the presence of oxidation and desiccation<sup>[71,73]</sup>, acidic pH<sup>[72]</sup>, unfavorable temperatures<sup>[74]</sup> and *H. pylori*-inhibitory products secreted by food microbiota<sup>[71]</sup>. Furthermore, it is not known whether the coccoid or non-culturable forms of *H. pylori* remain viable in food<sup>[75]</sup> or are able to infect humans<sup>[73,76]</sup>. Thus, the presence of *H. pylori* DNA in food and water sources has been thought to result from contamination with either naked DNA or dead bacteria<sup>[74,77]</sup>. However, reports have indicated that certain pathogenic bacteria, such as *L. pneumophila*<sup>[48]</sup>, *Vibrio cholera*<sup>[78]</sup>, *Listeria monocytogenes*<sup>[79]</sup>, *E. coli*<sup>[80]</sup>, *Campylobacter*



**Figure 1 Bacterial movement within yeast vacuoles.** Light micrographs taken at four time intervals (A, B, C and D) show fast-moving bacterium-like bodies (BLB) inside the vacuoles (V) of *Candida* yeast cells (magnification  $\times 1250$ )<sup>[64]</sup>.

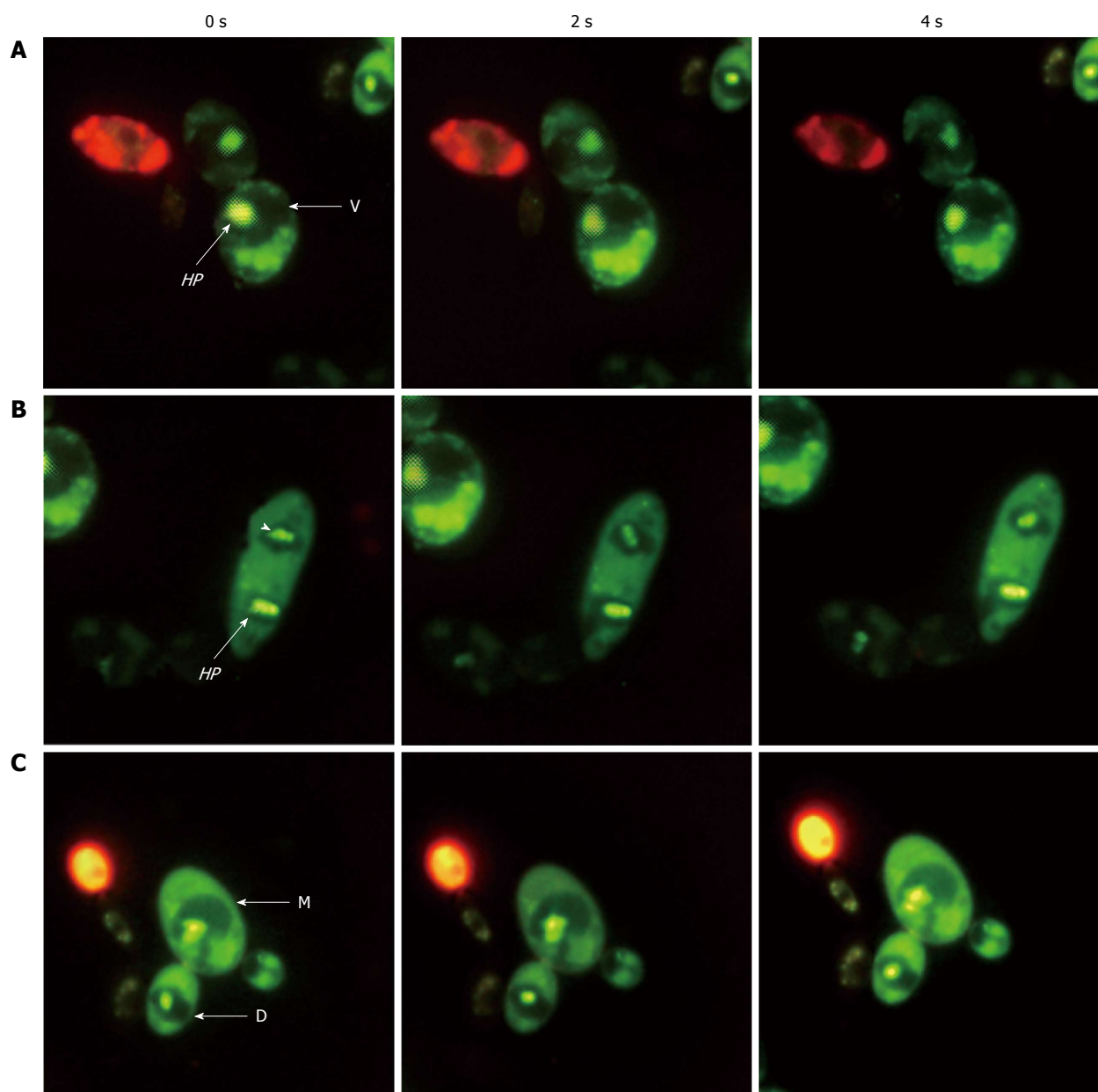


**Figure 2 Visualization of bacterium within yeast vacuoles.** Fluorescence micrographs of *Candida* yeast stained by a live/dead-BacLight kit show live (green) bacterium-like bodies (BLB) inside the vacuoles (V) of (A) yeast cells and (B) hyphae (N: Nucleus; M: Mitochondrion) (magnification  $\times 1000$ )<sup>[87]</sup>.

*jejuni*<sup>[81]</sup> and *H. pylori*<sup>[82]</sup>, have evolved to establish in the vacuoles of free-living amoebae, the inhabitants of water, soil and air, which resist environmental stresses by encysting<sup>[83]</sup>. Amoebae can protect bacteria against the stressful conditions, provide them with nutrients and serve as mediators for their spread in the environment and within hosts<sup>[84,85]</sup>. Accordingly, the presence of amoebae in water or the environment could be an important marker of contamination with these pathogenic bacteria<sup>[48]</sup>.

Yeast, another free-living microorganism that exhibits a remarkable resistance to environmental stresses<sup>[10,86]</sup>, can similarly accommodate pathogenic bacteria within vacuoles<sup>[87]</sup>. In this regard, foodborne yeasts, which are frequently recruited as primary tools for preparation of fermented foods or enter food as environmental contaminants<sup>[88,89]</sup>, may play a crucial role in the transmission

of *H. pylori*. Foodborne yeasts that occur in a variety of food materials such as dairy products<sup>[89]</sup>, fermented foods<sup>[90,91]</sup> and fruits<sup>[92]</sup> are able to withstand the stressful treatments applied in food processing, such as high temperatures, desiccation, acidic pH, high salt concentration and sanitization<sup>[10,93]</sup>. Accordingly, it was proposed that the intracellular establishment of *H. pylori* in the vacuoles of foodborne yeasts could protect the bacteria against these stressful conditions and play a crucial role in bacterial survival in food<sup>[87]</sup>. Indeed, *H. pylori*-specific genes *ureAB* and *babAB* were detected in *Candida* yeasts from Iranian traditional breads (Sangak, Taftoon and Barbary), yogurt, banana skin, grape juice and quince jam, which carried vacuolar fast-moving and non-culturable BLBs (Figure 2)<sup>[87]</sup>. Thus, foodborne yeasts originating from the environment, which were once considered as harmless



**Figure 3** *Helicobacter pylori* movement in dividing yeast. Fluorescence micrographs from three experiments (A, B and C) using a live/dead-BacLight kit showing live (green) fast-moving *Helicobacter pylori* (HP) cells (arrows and arrowhead) inside the vacuoles (V) of mother (M) and daughter (D) *Candida* cells taken at 0, 2 and 4 s (magnification  $\times 1000$ )<sup>[108]</sup>.

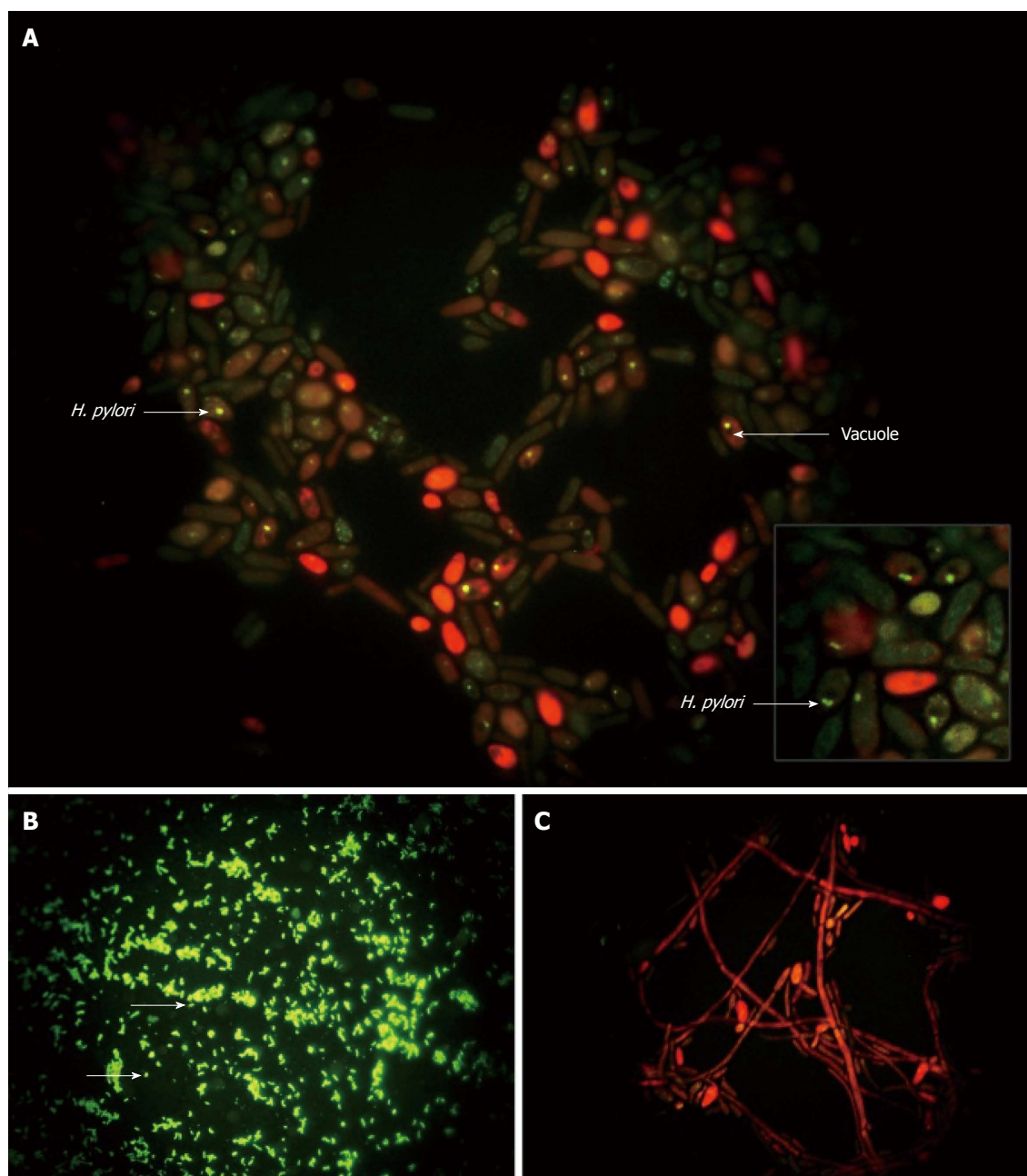
microorganisms when ingested through fermented foods such as dairy products<sup>[38,89]</sup>, including kefir and kumis<sup>[94,95]</sup>, could now be pinpointed as a public health problem source. In this regard, occurrence of yeast in food and environment can be considered as an important indicator of contamination with *H. pylori* and other pathogenic bacteria. Therefore, a key approach for the control of *H. pylori* infection may be to reduce the yeast content of foods through proper hygienic practice, especially by food handlers and during food processing<sup>[87]</sup>.

## MATERNAL TRANSMISSION OF *H. PYLORI*

Vertical transmission from mother to neonate during

vaginal delivery has been considered to be one important mechanism for *H. pylori* transmission. Although there are no published reports to show vaginal isolation of *H. pylori*<sup>[96]</sup>, it is plausible to propose that since *H. pylori* can inhabit the squamous epithelium of the oral cavity, it may therefore be able to survive on the vaginal mucosa<sup>[97]</sup>. The vagina supports the growth of a number of microaerophilic organisms, suggesting that a coexisting infection could provide conditions favorable for the growth of *H. pylori*<sup>[98-100]</sup>. In this regard, a mother's oral and vaginal yeasts were proposed to play a crucial role in the transmission of *H. pylori* to the neonate<sup>[101]</sup>.

The mucosal surfaces of the human oral cavity and vulva-vagina are the areas that are the first and second most frequently colonized with yeasts, respectively<sup>[146]</sup>. A



**Figure 4 Immunolabeling of *Helicobacter pylori*.** Immunofluorescence micrographs showing: A: Localization of *Helicobacter pylori* (*H. pylori*) (green) inside the *Candida* yeast, some of which appear red due to diffusion of the counterstain (magnification  $\times 4000$ ); B: Control *H. pylori* (green; arrows) (magnification  $\times 1000$ ); and C: Absence of bacteria in heat-killed yeast (magnification  $\times 1000$ )<sup>[108]</sup>.

yeast oral carriage rate of up to 75% has been reported in healthy individuals<sup>[102]</sup>, and a considerable proportion (5%-35%) of asymptomatic healthy women have positive vaginal cultures for *C. albicans*<sup>[103]</sup>, with the highest level of colonization in the vagina compared to other body sites<sup>[104]</sup>. As the incidence of vaginal yeast colonization during pregnancy can be up to 46%, *Candida* spp. in infants may be acquired vertically from the mother when passing through the birth canal *via* cutaneous contact or swallowing of fungi<sup>[105,106]</sup>. When examining yeasts from pregnant woman, vaginal yeasts were twice as likely to contain the *H. pylori*-specific genes *16S* rRNA and *vacA s1* when compared to oral yeasts<sup>[101]</sup>. Furthermore, the

carriage rate of oral yeast was found to be significantly higher in normally delivered neonates compared to those from a cesarean delivery. Moreover, a significant correlation was found between the frequency of *H. pylori* genes in vaginal yeasts and in oral yeasts of normally delivered neonates, indicating a common source<sup>[101]</sup>.

### ENDOSYMBIOTIC CHARACTERISTICS OF *H. PYLORI*

The inheritance of vacuoles by yeast daughter cells is a highly regulated process<sup>[107]</sup>. Fluorescence microscopy revealed the occurrence of *H. pylori* cells inside the vacuoles

of mother and daughter cells in consecutive subcultures of yeasts (Figure 3)<sup>[108]</sup>. Some yeasts contained more than one *H. pylori* cell, indicating the potential for endobacterial cells to multiply and transmit within the vacuole to the next generation of yeast. As a hallmark of endosymbiosis is that both partners need to be alive<sup>[109]</sup>, the mutual adaptation of intracellular bacteria and their eukaryotic hosts would often allow both partners to survive the entire lifespan with the endobacteria transmitted to the next generation<sup>[17,110]</sup>. Many intracellular bacteria have evolved to recruit certain proteins for protecting the membrane-bound vacuole and promoting intracellular partnership<sup>[111]</sup>. *H. pylori*-specific genes, encoding proteins such as VacA, urease and peroxiredoxin, have been detected in the extracts of oral and gastric yeasts<sup>[112]</sup>. Furthermore, western blotting performed on yeast extracts with antibodies raised against *H. pylori* revealed the presence of proteins with molecular weights of 56, 36, 32, 26 and 21 kDa, corresponding to the VacA large subunit, VacA small subunit<sup>[113,114]</sup>, urease A subunit<sup>[115]</sup>, peroxiredoxin and thiol peroxidase<sup>[116]</sup>, respectively. These proteins were detected with antibodies such as the IgY-Hp polyclonal antibody, which is used as a powerful tool for detection of *H. pylori* immunodominant proteins, such as the urease A and B-subunits, Hsp60, peroxiredoxin and thiol peroxidase<sup>[116]</sup>. Peroxiredoxin and thiol peroxidase may allow the bacteria to detoxify oxygen metabolites formed during processes such as the respiratory burst of immune cells<sup>[111]</sup>. The bacterial urease and VacA have been recognized as the two important *H. pylori* virulence factors that influence phago-lysosome fusion and bacterial survival in macrophages<sup>[46,117]</sup>.

The IgY-Hp antibody has also been used as a marker for localization of *H. pylori* inside vacuoles of yeast (Figure 4)<sup>[108]</sup>. Additionally, whole cell *H. pylori* and *H. pylori*-immunoreactivity have been observed in lamina propria of gastric biopsy specimens<sup>[118]</sup>. Furthermore, the specific identity and localization of immunoreactive *H. pylori* within defined membrane-bound vacuoles has been revealed with confocal<sup>[119]</sup> and ultrastructural<sup>[120]</sup> microscopy. The preserved ultrastructural morphology and presence of *H. pylori*-specific mRNA, detected with fluorescence *in situ* hybridization, indicated the viability of these intracellular bacteria<sup>[119,120]</sup>.

## CONCLUSION

Taken together, the data suggest that *H. pylori* are well-equipped for invasion of eukaryotic cells and survival within their vacuoles<sup>[108,112]</sup>. The intimate relationship between these two organisms suggests that yeasts are the unknown non-human source from which *H. pylori* were able to infect humans as early as 100000 years ago<sup>[121]</sup>. Establishment of *H. pylori* inside the ubiquitous yeast might explain why such a fastidious bacteria is able to survive outside the human stomach and remain highly prevalent in certain populations, with yeast acting as a Trojan horse, ferrying the potentially infectious *H. pylori* into the GI

tract environment. It is possible that the unique property of ergosterol dependence in *H. pylori* evolved as the result of adaptation to life inside the yeast vacuole, showing the crucial role of this organelle in the evolution and persistence of *H. pylori*. Further studies will elucidate how the intracellular life of *H. pylori* inside yeast influenced its adaptation for existence in the human stomach and long-term colonization of gastric epithelium, as well as provide insight regarding the remarkable heterogeneity of virulence determinants, and resistance to antibiotics and the immune system. Furthermore, examination of individuals within a population, with consideration of their yeast carriage, consumption of foods containing live yeast and *H. pylori* infection status, will guide understanding of the spread of *H. pylori* among humans.

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**P- Reviewers:** Arino J, Galgoczy L, Gurevich VV, Krishnan T  
**S- Editor:** Ma YJ **L- Editor:** A **E- Editor:** Ma S





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ISSN 1007-9327



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