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# Activities of Muscadine Grape Skin and Polyphenolic Constituents against Helicobacter pylori

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# ACTIVITIES OF MUSCADINE GRAPE SKIN AND POLYPHENOLIC CONSTITUENTS AGAINST *HELICOBACTER PYLORI*

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by
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ABSTRACT

*Helicobacter pylori* is a microaerophilic, gram-negative bacterium and among the most persistent of all human pathogens. Its presence in the gastric environment is correlated with diseases such as gastritis, peptic ulcers, mucosa-associated lymphoid tissue lymphoma, and gastric carcinoma. Multiple factors are believed responsible for disease progression and outcome including infecting strain(s) genetic composition, age of acquisition, host immune response, environment, and diet. Due to the complexity of *H. pylori* infection, all possible host-pathogen interactions should be considered. One important factor frequently underplayed is host dietary habits which may serve a more important role during and immediately following infection with *H. pylori* than previously thought.

It has been shown that people consuming diets rich in fruits and vegetables have a lower incidence of *H. pylori* infection and severe gastric malignancies and that this may be in part due to natural bioactive compounds acting against *H. pylori*. We believe that alternative, diet-based strategies may have a significant impact on attenuating this bacterium when used alone or in combination with current antibiotic regimes; however, natural compounds should be better studied to understand their full therapeutic potential.

Muscadine grapes, because of their high levels of polyphenolic compounds and unique chemical makeup, are believed to be a proper candidate for study since these fruits have already been shown to exert anti-*H. pylori* activity *in vitro* with effects possibly related to inhibition of bacterial attachment and virulence factors. The objectives of this study were to 1) identify active phenolic compounds in muscadine grape skin responsible for anti-*H. pylori* activity, 2) use DNA microarrays to study gene expression upon

exposure to active compounds, and 3) explore the preventative potential of muscadine grape skin and identified polyphenols for ameliorating *H. pylori* infection and/or the *H. pylori*-induced inflammatory response in mice.

Our results show that muscadine grape skin and major phenolics quercetin and resveratrol exert strong anti-*H. pylori* activity; especially when in combination, and may have the potential to be incorporated into an effective, diet-based approach for the prevention and/or treatment of *H. pylori* infection.

# DEDICATION

I dedicate this work to my wife and family. This dissertation exists because of their love and support.

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TABLE OF CONTENTS

Page

TITLE PAGE ..... i

ABSTRACT ..... ii

DEDICATION ..... iv

ACKNOWLEGEDMENTS ..... v

LIST OF TABLES ..... viii

LIST OF FIGURES ..... ix

CHAPTER

1. LITERATURE REVIEW…….. 1

2. ACTIVITIES OF MUSCADINE GRAPE SKIN AND QUERCETIN AGAINST *HELICOBACTER PYLORI* INFECTION IN MICE…..... 44

   Abstract ..... 44
   Introduction ..... 45
   Materials and Methods ..... 47
   Results ..... 51
   Discussion ..... 53
   Acknowledgements ..... 57
   References ..... 57
   Figure legends ..... 61
   Tables and figures ..... 62

3. ACTIVITIES OF MUSCADINE GRAPE SKIN AND POLYPHENOLIC CONSTITUENTS AGAISNT *HELICOBACTER PYLORI IN VITRO* ..... 67

   Abstract ..... 67
   Introduction ..... 67
   Materials and Methods ..... 69
   Results ..... 77
   Discussion ..... 81
   Acknowledgements ..... 88
   References ..... 89

Table of Contents (continued)

Page

Figure legends ..... 94
Tables and figures ..... 95

4. GENE EXPRESSION OF *HELICOBACTER PYLORI* TREATED WITH MUSCADINE GRAPE SKIN EXTRACT OR ITS POLYPHENOLIC CONSTITUENTS QUERCETIN AND RESVERATROL ..... 106

Abstract ..... 106
Introduction ..... 107
Materials and Methods ..... 109
Results ..... 115
Discussion ..... 117
Acknowledgements ..... 129
References ..... 130
Figure legends ..... 135
Tables and figures ..... 136

5. CONCLUSION ..... 145

LIST OF TABLES


| Table | | Page |
|---|---|---|
| 2.1 | Minimal inhibitory concentrations of quercetin on *H. pylori* growth by agar dilution assay | 62 |
| 2.2 | Effects of muscadine grape skin powder on *H. pylori* infection in C57BL/6 mice. | 63 |
| 3.1. | Major phenolics in muscadine grape skin (MGS) extracts as analyzed by HPLC | 95 |
| 3.2. | *H. pylori* 26695 inhibition by major phenolic combinations by disk-diffusion | 96 |
| 3.3. | Visible effects of quercetin (256 µg/ml) on *H. pylori* 26695 by phase-contrast microscopy | 97 |
| 3.4. | Salt aggregation of *H. pylori* 26695 treatment with quercetin, resveratrol, or MGS extract | 98 |
| 4.1 | *H. pylori* genes and primer pairs selected for real-time RT-PCR | 136 |
| 4.2 | Differentially expressed genes of *H. pylori* 26695 upon exposure to MGS extract | 137 |
| 4.3 | Differentially expressed genes of *H. pylori* 26695 upon exposure to quercetin | 139 |
| 4.4 | Differentially expressed genes of *H. pylori* 26695 upon exposure to resveratrol | 141 |

LIST OF FIGURES


| Figure | | Page |
|---|---|---|
| 1.1 | Chemical structures of resveratrol (left) and quercetin (right) ..... | 21 |
| 2.1 | PCR amplification of *H. pylori* SS1 16S rRNA gene from *H. pylori*-infected control mice feces. ..... | 64 |
| 2.2 | Mice IFN-γ, IL-1β, and TNF-α levels following treatment with 5 or 10% muscadine grape skin (MGS) powder or quercetin (25 mg/kg body weight). ..... | 65 |
| 2.3 | Representative images of HE-stained sections of mouse gastric antrum mucosa ..... | 66 |
| 3.1 | Photograph of *H. pylori* 26695 plated on BHI/HS following treatment with standardized MGS (2mg/ml total phenolics) extracts by the disk-difusion method..... | 99 |
| 3.2 | Time-course bactericidal activity of quercetin (A), resveratrol (B) and amoxicillin (C) on *H. pylori* 26695..... | 100 |
| 3.3 | Synergistic activities of quercetin and resveratrol against *H. pylori* 26695 at 24 (A) and 48 (B) h. ..... | 101 |
| 3.4 | Uptake of NPN by *H. pylori* 26695 in the presence of EDTA, MGS, quercetin and resveratrol with or without $MgCl_2$ ..... | 102 |
| 3.5 | Uptake of quercetin into live *H. pylori* 26695 cells over time..... | 103 |
| 3.6 | Quercetin distribution following 3 h incubation of *H. pylori* 26695 with 256 µg/ml of quercetin..... | 104 |
| 3.7. | Phase-contrast microscopy images of *H. pylori* 26695 during exposure to quercetin (256 µg/ml) for 1 (A), 2 (B) , and 3 (C) hours. Magnification, 1,000. ..... | 105 |
| 4.1. | Distribution of functions of selected genes whose relatative transcript amounts were affected by treatement with MGS, quercetin, or or resveratrol. ..... | 143 |

List of Figures (continued)

Page

4.2. Changes in transcript levels upon exposure of *H. pylori* 26695 to to MGS extract (*cagA*, *ureA*, *atpC*) and quercetin (*hup*) determined by qRT-PCR..... 144

CHAPTER ONE

LITERATURE REVIEW

## Introduction

*Helicobacter pylori* is a gram-negative, spiral-shaped, microaerophilic bacterium and a resident of the human gastric environment. Since its discovery in 1983, countless studies have sought to better understand this microorganism and its role in human pathogenesis. In 1994, *H. pylori* was classified as a carcinogen by the World Health Organization (WHO) (International Agency for Research on Cancer meeting) and is currently recognized as the etiologic agent of peptic ulcers and gastritis and strongly linked to mucosa-associated lymphoid tissue (MALT) lymphoma and gastric carcinoma (Cover and Blaser, 1995). This pathogen infects more than half the world's population making it one of the most common bacterial infections in humans (Rothenbacher and Brenner, 2003). However, the majority of those infected develop asymptomatic gastritis while approximately 10-20% develop more severe diseases like peptic ulcers, atrophic gastritis, MALT or gastric carcinoma (0.1-4%) (Bourzac and Cuillemin, 2005). Because infection with *H. pylori* does not necessarily correlate with clinical symptoms, multiple factors are believed responsible for disease progression and outcome including infecting strain(s) genetic composition, age of acquisition, host immune response, environment, and diet (Blaser and Atherton, 2004).

Due to the complexity of *H. pylori* infection, all possible host-pathogen interactions should be considered. One important factor frequently underplayed is host dietary habits which may serve a more important role during and immediately following infec-

tion with *H. pylori* than previously thought (Testerman et al., 2001). Once *H. pylori* colonizes the gastric epithelium, although relatively safe from acidic conditions; protected by the thick gastric mucosa, it is still exposed and vulnerable to various solutes. Therefore, it is possible that host dietary compounds may play a beneficial role in *H. pylori* prophylaxis and/or treatment. Some studies have previously explored the link between *H. pylori* infection and host diet/nutrition with several associating high-salt diets with an increased risk and/or extent of disease (Tsugane et al., 1994; Fox et al., 1999; Willis et al., 1999; Gancz et al., 2008). On the other hand, consumption of fruits and vegetables rich in certain vitamins, antioxidants, and constitutive bioactive compounds (i.e. phytochemicals) has been shown to significantly reduce the incidence of *H. pylori* infection and/or ameliorate associated symptoms (Buiatti et al., 1990; Zhang et al., 1997, Bennedsen et al., 1999, Yamada et al., 1998; Fukai et al,. 2002, Yanaka et al., 2009).

Although many strains of *H. pylori* are susceptible to most currently used antibiotics (e.g. clarithromycin, metronidazole, amoxicillin) *in vitro*, treatment is increasingly challenging due to antibiotic resistance and reinfection in certain groups. Because the effective lifespan of any antibiotic is limited, the search for new antimicrobials has risen dramatically in recent years. In addition, increased public awareness of the overuse/misuse of prescription antibiotics coupled with the desire for greater autonomy over personal medical care has led to increased demand for natural, safe alternative medications. Therefore, novel, diet-based therapeutics for use where conventional antibiotic therapies have failed, are unavailable or rejected, and/or expensive have received considerable attention.

Numerous studies have investigated novel, naturally occurring, plant-derived substances as potential alternatives for *H. pylori* prevention or treatment (O'Gara et al., 2000; Bergonzelli et al., 2003; Lin et al., 2005; Paraschos et al., 2007; Yang et al., 2008; De et al., 2009; Martini et al., 2009; Pastene et al., 2010). In particular, studies have shown that grapes (*Vitis vinifera*) and their extractable bioactive compounds, especially polyphenols, have strong anti-*H. pylori* activity; inhibiting growth (Mahady and Penland, 2000; Mahady et al., 2003) in a variety of strains while reducing *H. pylori*-induced inflammation and gastritis in infected animal models (Tombola et al., 2003; Yahiro et al., 2005; Ruggiero et al., 2006; Ruggiero et al., 2007). We have previously reported that muscadine grapes (*Vitis rotundifolia*) are also a valuable source of anti-*H. pylori* compounds with activity against multiple strains *in vitro*. In addition, we believe that muscadine's reported biological activity is most likely due to major phenolic compounds (i.e. ellagic acid, myricetin, quercetin, *trans*-resveratrol, gallic acid) present in these fruits acting alone or in synergy (Brown et al., 2009). Although these polyphenols are ubiquitous in nature, the combination of ellagic acid, quercetin, and resveratrol is unique to muscadine species and may further suggest that these compounds are largely responsible for their reported biological activities (Mertens-Talcott and Percival, 2005) and be useful in the treatment of *H. pylori* infection.

## Background and Significance

Before *Helicobacter pylori*'s discovery (Marshall and Warren, 1984), the human stomach was considered to be sterile, or at most transiently populated by oropharyngeal bacteria (Blaser 1997). Today, *H. pylori* is considered a ubiquitous gastric pathogen and

firmly established as the causative agent of acute or chronic gastritis as well as a predisposing factor in peptic ulcer disease, gastric carcinoma, and B-cell MALT (Mitchell, 2001). In 1994, *H. pylori* was the first bacterium classified as a class I carcinogen by the WHO following epidemiological studies prompted by its association with severe gastric malignancies (e.g. MALT and stomach cancer).

*H. pylori* infects more than half the world's population making it one of the most common bacterial infections in humans (Rothenbacher and Brenner, 2003). Although infections occur worldwide, significant differences in the prevalence of infection exist with highest rates in developing countries—primarily attributed to the rate of acquisition in childhood (Mitchell et al., 1992). However, additional factors likely contribute to infection including low socioeconomic status, diets low in raw vegetables, fruits, and high fiber breads, unclean water supplies, overcrowding, etc. In addition, factors such as host genetics, immune response, etc. are also believed to play a critical role. It is generally agreed that, once infected, without treatment most persons remain infected for life (Pounder and Ng, 1995). Although infection is usually asymptomatic, with symptoms observed in only 15-20% of those infected; the organism is believed to be highly transmissible (presumably via the fecal-oral route) and damaging to the host, causing progressive destruction to the gastric mucosa over the lifetime of infection (Parosonnet, 1999).

Epidemiological studies have reported an overall decline in *H. pylori* transmission worldwide, most likely due to improved sanitation in recent decades. This trend suggests that *H. pylori* will eventually be eliminated from the U. S. population. However, without proper intervention, it is predicted that endemic *H. pylori* infection will remain in the

U.S. for at least another century and even longer in developing countries necessitating effective treatment (Rupnow et al., 2000; Suerbaum and Michetti, 2002).

### *Helicobacter pylori*

*Helicobacter pylori* is a gram-negative, spiral-shaped microaerophile and typically measures 2.5 to 5 µm long and 0.5 to 1 µm wide. Although most often observed in its spiral form, *H. pylo*ri is also capable of a spherical morphology resembling a coccoid and may be a stress response mechanism for minimizing contact with unfavorable environmental conditions (Hessey et al., 1990). *H. pylori* found in the environment are believed to exhibit this type of morphology; constricting themselves, decreasing their surface area, and entering into a viable-but-nonculturable physiological state. In this state, the organism can still infect but cannot be detected by culturing methods.

*H. pylori* is fastidious and microaerophilic, thus requiring an enriched medium and atmosphere with reduced oxygen (approximately 10% $CO_2$) and an optimum growth temperature of 37°C. The pH ranges from 5.5 to 8.5 with an optimal pH between 6.9 and 8. Growth is slow, typically requiring 3-5 days for colonies to be observed. When colonies are visible, they are approximately 1 mm in diameter and appear smooth and translucent; however, this characteristic may be strain dependent. *H. pylori* biochemical characteristics include positive tests for oxidase, catalase, urease, and acid and alkaline phosphatases (Rathbone and Heatley, 1992; Northfield and Mendall, 1994).

## Pathogenicity

Although the gastric mucosa is inherently well protected against most microbial attacks, *H. pylori* has successfully adapted to this extreme environmental niche. For *H.*

*pylori* to cause infection and subsequent disease in a host, various components are required for initial adherence, colonization, and cellular damage. This review will briefly discuss the most important factors necessary for *H. pylori*'s survival and virulence in the human stomach.

Motility

In order to establish a successful infection, *H. pylori* must first evade the highly acidic gastric secretions (pH 1.2-3.0) of the stomach, relying heavily on its ability to move quickly through the gastric mucosa. To do this, *H. pylori* uses its powerful flagella and spiral shape to corkscrew through the thick, viscous mucous layers to reach the underlying epithelial tissue. The flagella, typically four to five, are located at one pole of the cell and sheathed—believed to be an adaptation to the acidic conditions for moving through the mucosa thereby protecting the filaments from depolarization. Each flagellar filament contains two flagellin proteins, FlaA and FlaB encoded by the *flaA* and *flaB* genes (Kostrzynska et al., 1991). If one gene is removed, the organism exhibits decreased motility and if both are absent, the cell is immobile and unable to colonize (Josenhans et al., 1995; Ottemann and Lowenthal, 2002).

Urease

*H. pylori* urease is essential for colonization of the stomach as it allows survival under acidic conditions—accounting for up to 15% of the total protein synthesized by the organism (Prinz et al., 2003; Mobley et al., 1995). Urease is a $Ni^{2+}$-containing cytoplasmic enzyme that converts urea into bicarbonate and ammonia. These products generate a protective alkaline microenvironment and allow the organism to persist in this acid-

ic milieu (Mobley, 2001). Urease activity is regulated by pH-gated urea channels (UreI) in the inner membrane, unique to *H. pylori*, which open at low pH (< 6.5) and close under neutral conditions, regulating the influx of urea into the cell. Ammonia produced diffuses into, and thus buffers the periplasm (Prinz et al., 2003; Weeks et al., 2000; Weeks et al., 2001). The role of this enzyme in *H. pylori's* pathogenesis became evident after finding that strains with decreased urease activity were unable to initially colonize stomach epithelium resulting in no infection even while possessing all other virulence components (Eaton et al., 1991).

Adherence

Following initial colonization, *H. pylori* must securely attach to the stomach epithelial tissue to resist removal by peristalsis and gastric epithelial cell turnover. *H. pylori* is able to adhere, in part through binding to blood group antigens, expressed on gastric epithelial cell surfaces, with the most important being the Lewis b antigen (Atherton, 2006; Borén et al., 1993; Marshall, 1994). Other proteins including those found in the outer-membrane protein (Hop) family may also influence adhesion to epithelial cells and are briefly discussed below.

Adhesins. *H. pylori* binds to a variety of carbohydrate ligands on host epithelial cells, including sialic acid (Lelwala-Guruge et al., 1993), laminin (Valkonen et al., 1997), gastric mucin (Namavar et al., 1998) and fucosylated glycoproteins such as the Lewis b ($Le^b$) antigen (Borén et al., 1993; Guruge et al., 1998). Strains expressing the binding adhesin BabA, a 78-kD outer-membrane protein, encoded by the *babA2* gene, have the ability to bind to difucosylated $Le^b$ blood group antigens expressed on gastric epithelial

cells, functioning as receptor molecules (Gerhard et al., 1999; Monteiro et al., 1998). Lewis b is expressed on *H. pylori* cell surfaces with expression mimicking human cell surface glycolipids, causing a decreased anti-*H. pylori* T-cell response by the host (Sherburne and Taylor, 1995). This in turn generates increased host epithelial cell proliferation and inflammation while increasing the risk for gastric ulcer and cancer development (Atherton, 2006). However, even with Lewis b binding adhesins, some *H. pylori* strains may not bind to epithelial cells with $Le^b$ antigens. If this happens, the organism is able to utilize many of its other adhesins from Hop for cell attachment. Lewis a and Lewis x, mediated by SabA (sialic-acid-binding adhesin), are two other blood group antigens expressed on epithelial cells; however, these are not as important as $Le^b$. It was shown by Sheu et al. (2005) that mice expressing $Le^b$ developed gastritis while wild-type mice without $Le^b$ expression did not; supporting the importance of this ligand-receptor interaction for disease.

Another putative adhesin, part of the outer membrane, is the HopZ protein which mediates binding to gastric epithelial cells but does not function as a porin (Peck et al., 1999). Outer membrane proteins HopB and HopC may also function in a similar fashion but have yet to be extensively studied (Exner et al, 1995). In addition, cell-envelope proteins other than suspected porins have also been implicated as adhesins including Hpa, identified as a flagellar sheath lipoprotein. Despite extensive research on *H. pylori in vitro*, there is little agreement on which *H. pylori* adhesins are most important *in vivo* due to a multitude of possible host-pathogen interactions (Testerman et al., 2001).

Cellular Damage

After colonizing the gastric epithelium, *H. pylori* causes damage to epithelial cells; in turn promoting continual regeneration of the gastric epithelial layer. Damage is a result of multiple factors including bacterial toxin production, secretion of phospholipase and protease enzymes, inflammation, and promotion of a self-destructive host immune response.

Cytotoxin-associated genes. Of those infected with *H. pylori*, relatively few develop clinical symptoms. This phenomenon may be largely attributed to strain-specific differences with one of the most important being *cagA* status. *H. pylori* strains bearing a complete *cag* pathogenicity island (PAI), a 37-kb genome segment encoding about 30 genes, including *cagA*, are often associated with increased gastric inflammation and bacterial colonization, peptic ulcer disease, gastric cancer and higher induction of gastric epithelial cell cytokines responsible for recruiting/activating immune inflammatory cells (Peek et al., 1995; Blaser et al., 1995; Crabtree et al., 1995; Sharma et al., 1995, Figueiredo et al., 2005). These genes are used to encode products similar in composition to that of type IV secretion systems—allowing *H. pylori* to deliver the CagA effector protein into host cells—activating specific transcription factors and cell signaling pathways (Ding et al., 2007, Li et al., 1999; Keates et al., 1997).

CagA. CagA is a 128-kDa protein that elicits a strong immunological response and is used as a marker for the presence of a large pathogenicity island (PAI) encoding many proteins with several implicated in *H. pylori* pathogenesis (Censini et al., 1996; Prinz et al., 2003).

After entering the cell, multiple changes in host cell signaling are made, usually when the protein is phosphorylated and binds to SHP-2 tyrosine phosphatase (Higashi et al.,2002) triggering a growth factor-like cellular response and cytokine production by the host cell (Suerbaum and Michetti, 2002). This may support the organism's link to gastric cancer because of the activation of SHP-2, a protooncogene. CagA-positive strains have also been found to increase virulence and cell injury with studies showing *cagA*-positive infected patients with higher incidences of peptic ulcers and gastric cancer development (Gold, 1994; Blaser et al., 1995; Peek et al., 1997). In a related *in vitro* study, Smoot et al. (1999) supported the finding that *cagA*-positive strains were associated with greater cell injury than *cagA*-negative strains; however, less apoptosis of cell tissue was observed following exposure to *cagA*-positive strains than *cagA*-negative strains (Peek et al., 1997). These findings suggest that *cagA*-positive strains generate more cell damage but less apoptosis than those infected with *cagA*-negative strains. Because of this, gastric epithelial cells exposed to *cagA*-positive *H. pylori* may be allowed to continue their cellular cycle with an increased chance of replication with DNA damage (Smoot et al., 1999). This was later supported by Obst et al. (2000) who observed that gastric cells exposed to *cagA*-positive *H. pylori* extract did not show any change in the percentage of cells in the $G_2M$ phase of the cellular cycle but had increased levels of reactive oxygen species (ROS), DNA fragmentation and DNA synthesis.

Vacuolating cytotoxin. All *H. pylori* strains, regardless of *cagA* status, contain a copy of the *vacA* toxin gene. VacA toxin is an important virulence factor directly involved in gastric inflammation and ulceration (Ghiara et al., 1995; Eaton et al., 1997). Of

the various *vacA* allele types identified, the most important and extensively studied is s1/m1. Strains possessing this allele typically encode VacA proteins associated with a higher level of vacuolating cytotoxin activity compared to other allele types. In addition, *cagA*-positive strains are more likely to produce this exotoxin (Montecucco et al., 2001). VacA is composed of identical 87-kDa monomers assembled into flower-shaped oligomers. This toxin manipulates intracellular vesicular trafficking in host epithelial cells, generating cytoplasmic vacuoles (Reyrat et al., 2000), membrane channel formation, disruption of endosomal/lysosomal function, immunomodulation, and apoptosis (Figueiredo et al., 2005). Additional hypotheses for its activity have been proposed including formation of anion-selective membrane channels and interaction with a receptor-like protein tyrosine phosphatase (RPTPß); however, the primary mechanism is currently unknown. After reaching the endosome via internalization through a vacuole, it is not known what happens to VacA. However, it is proposed that once in the endosome, the toxin may inhibit energy activation and cause mitochondrial damage, leading to cell cycle impairment and cell death.

Most people in Western countries with severe gastritis are infected with VacA *H. pylori* variant strains; however, this association has not been found in Asia and has yet to be fully explained (Atherton et al., 1997). Strains with inactive *vacA* genes have been isolated from countless patients, indicating that VacA is not essential for colonization. However VacA-negative mutants were shown to be out competed by wild-type bacteria in one animal study, possibly suggesting that VacA-positive bacteria might be hardier and more competitive *in vivo* (Salama et al., 2001). Currently, it is still unknown whether

VacA causes epithelial cell death in humans. However, strong evidence using mice does exist showing oral administration of VacA does lead to gastric epithelium erosion, presumably involving cellular loss.

## Immune Response

A remarkable feature of *H. pylori* is its ability to persist throughout the life of the host despite significant immune responses aimed to destroy it. Once ingested, *H. pylori* must quickly move through the gastric mucosa to gain access to the underlying epithelium. While in the mucosa, nonspecific, antimicrobial molecules such as lactoferrin, lysozyme, and defensins are encountered; however *H. pylori* expresses mucolytic molecules (e.g. protease, lipase) which aid in evasion of these factors (Nakao et al,. 1997; O'Neil et al., 2000). In addition, gastric acid is also present; however, *H. pylori* urease effectively buffers its environment until it gains access to its target tissue (discussed earlier). Once situated adjacent to gastric epithelial cells, *H. pylori* adheres and begins colonization. Unfortunately, the gastric microenvironment is relatively absent of immune and inflammatory cells in uninfected, normal gastric mucosa (attributed to the fact that few microorganisms can handle gastric acid); therefore, this may provide the time required for *H. pylori* to expand and establish persistent infection. Having evaded innate responses, *H. pylori* then encounters adaptive responses mediated by B and T lymphocytes. T-cell responses will be discussed further.

Following colonization of the gastric epithelium by *H. pylori*, the induction of an inflammatory response, predominantly of the Th1 type occurs (Kuipers et al., 1995). This response is triggered by Th1 cells stimulating epithelial cells to produce proinflam-

matory cytokines [(e.g. tumor necrosis factor (TNF)-α, interleukin (IL)-1-β, interferon (IFN)-γ, and IL-8)] that recruit and activate host phagocytes (e.g. neutrophils and macrophages). In addition, release of reactive oxygen radicals (e.g. hydrogen peroxide, hydroxyl radicals, superoxide anions) during phagocytosis is also believed to contribute to the inflammatory process. In turn, this influx of proinflammatory cells is responsible for much of the destruction associated with acute phase gastritis and can impart an oxidative stress that causes damage to epithelial cells as well as to cellular DNA. Therefore, the Th1 response results in epithelial cell damage rather than removal of *H. pylori*. The ongoing presence of *H. pylori* thus causes a lifelong proinflammatory response coupled with host-responsible cellular damage. Finally, gastric T cells can also modulate B-cell responses, possibly leading to the production of antibodies (IgG) that can activate the complement cascade and contribute to immune-complex mediated inflammation (Wang et al., 2001).

The continuous source of oxidative stress and corresponding inflammation resulting from this long-term infection can give rise to DNA damage, thereby inducing the multiple mutations believed necessary for initiation of the "cancer cascade" (Correa et al., 1975; Kusters et al., 2006).

Interestingly, there is a marked difference in the ability of *H. pylori* to induce epithelial cytokines. Epithelial IL-8 (Crabtree et al., 1994) and associated NF-κB activation (Sharma et al., 1998) is only induced by strains carrying the *cag* PAI further supporting its role in the immune response and *H. pylori* pathogenesis. Following initial acute gastritis, active chronic gastritis occurs which can last a lifetime if infection remains un-

treated. However, the majority of *H. pylori*-positive subjects are unaware of this due to the lack of clinical symptoms.

## Conventional Treatment of *H. pylori* Infection

Currently, no vaccine for *H. pylori* infection exists. However, treatment is still relatively successful (> 80%) using a combination of antibiotics, i.e. 'triple therapy' consisting of two antibiotics plus either a proton-pump inhibitor (PPI, e.g. pantoprazole, omeprazole, rabeprazole) or bismuth compound (e.g. bismuth subsalicylate). Treatment time is usually one to two weeks with one of the following combinations: a PPI plus clarithromycin and amoxicillin, (2) a PPI plus clarithromycin and metronidazole, or (3) bismuth subsalicylate plus metronidazole and tetracycline (Howden and Hunt, 1998; Gaby, 2001).

Although treatment with multiple antibiotics increases eradication rates and reduces resistant *H. pylori* strain selection, various problems still arise through their use and has led to the still ongoing search for the most appropriate therapy (Mégraud et al., 2001; Suerbaum and Michetti, 2002). Depending on the patient and selected treatment, side effects such as diarrhea, nausea, vomiting, bloating, and abdominal pain frequently occur (Canducci et al., 2000). In addition, failure to complete prescribed treatments, most often because of these side effects, may further explain why treatments often fall short of effectively eliminating this pathogen and result in higher rates of reinfection following treatment (Del Giudice et al., 2001).

Eradication therapies are required to have cure rates of at least 80 percent with no significant side effects, and induce minimal bacterial resistance (Suerbaum and Michetti,

2002). With these strict guidelines, monotherapies are currently unable to achieve this result. Thus, drugs in combination as described above are recommended and most often employed. However, even with these treatment regimens, eradication rates still vary. This may be because of poor patient compliance, inappropriate evaluation by a health provider, and primary or secondary antibiotic resistance (Canducci et al., 2000). In addition, antibiotics themselves may not be able to penetrate the thick gastric mucosa enough to be effective or may be inactivated by the stomach acids before reaching its target (Kawase and Motohashi, 2004) and are dependent on the prescribed drug combination and degree of infection (Michetti et al., 1999).

Even with proper antibiotic therapy, the potential for antibiotic resistance development against commonly prescribed drugs like metronidazole, tetracycline, amoxicillin, and clarithromycin is on the rise (Graham, 1998; Yang et al., 2001). Resistance to amoxicillin and tetracycline is still relatively low for *H. pylori*. However, clarithromycin and metronidazole resistance is currently around 10 and 30 percent, respectively in the U.S. and even higher in some developing countries (Meyer et al., 2002). Such high levels of resistance may be partly attributed to the extensive use of antibiotics, especially nitroimidazoles, for treatment of other illnesses (Meyer et al., 2002).

No antimicrobial agent can cure all *H. pylori* infected individuals, and even the best fails in 5 to 10 percent of cases (Wu and Sung, 1999). Patient compliance and drug resistance are important elements to consider when treating this infection. Because of these factors, readily available, natural, alternative means are being studied to treat *H. pylori* infection.

## Natural Products for *H. pylori* Treatment

Because the effective lifespan of any antibiotic is limited, the search for new antimicrobials has risen dramatically in recent years. In addition, increased public awareness of the overuse/misuse of prescription antibiotics coupled with the desire for greater autonomy over personal medical care has led to increased demand for natural, safe alternative medications.

Since *H. pylori*'s discovery, numerous studies have investigated naturally occurring substances; mostly of vegetable and plant origin for anti-*H. pylori* activity. To date, a considerable amount of work has been done screening plant-derived compounds for their potential anti-*H. pylori* applications *in vitro* (O'Gara et al., 2000; Malekzadeh et al., 2001; Bhamarapravati et al., 2003; Bergonzelli et al., 2003; Adeniyi and Anyiam, 2004; Krausse et al., 2004; Lin et al., 2005; Ho et al., 2006; Paraschos et al., 2007; Yang et al., 2008; De et al., 2009; Pastene et al., 2010) and *in vivo* (Katoaka et al., 2001; Bergonzelli et al., 2003; Matsubara et al., 2003; Takabayashi et al., 2004; Ruggiero et al., 2006; da Mota Menezes et al., 2006; Gotteland et al., 2008; Lee et al., 2008) as well as beneficial microorganisms (e.g. *Lactobacilli* and *Bifidobacterium*) frequently consumed in the human diet (Canducci et al., 2000; Lorca et al., 2001; Chatterjee et al., 2003, Nista et al., 2004; Linsalata et al., 2004; Wang et al., 2004). These studies have evaluated a diverse variety of plant materials including licorice, cinnamon, cashew apples, mastic gum, ginger, and garlic extracts, along with numerous others with most reporting moderate bacteriostatic and/or -cidal activity against *H. pylori* (Fukai et al., 2002; Tabak et al., 1999; Kubo et al., 1999; Gaby, 2001; Iimuro et al., 2002; Chatterjee et al., 2004; O'Mahony et

al., 2005; Gaus et al., 2009). In addition to reduced cell viability, several studies have reported anti-inflammatory, -VacA, -urease, and -adhesive effects; suggesting the possibility of shared cellular targets or mode(s) of action between certain extracts or similar classes of constituent compounds (Pastene et al., 2010). Therefore, even if these substances do not completely eradicate *H. pylori*, their use still remains relevant as they may be useful in 1) the dietary management/amelioration of disease symptoms or 2) conjunction with current antibiotic treatments to possibly reduce required dosage levels (Koga et al., 2002; Hemaiswarya et al., 2008; Yang et al., 2008). In addition, it is believed that the complexity of bioactive compounds present in these products and their broad range of activity over a number of microorganisms may make it difficult for microbes to acquire resistance during treatment (Vattem et al., 2005).

Because of the diversity in plant materials evaluated, most studies prepare extracts using different methods and solvents. Therefore, when available, the use of single purified compounds has received increased scrutiny in order to eliminate variability. Extractable phenolic phytochemicals such as cinnamic acids, cinnamaldehydes, courmarins, phenolic acids, capsaicin, flavonoids and tannins have all shown some anti-*H. pylori* activity with promising results (Vattem et al., 2005). Berberine, a cationic alkaloid, found in goldenseal and Oregon grape was reported as having broad-spectrum antimicrobial activity (especially when used alongside multidrug pump inhibitors) (Tegos et al., 2002) as well as inhibiting the growth of *H. pylori in vitro* with moderate suppression *in vivo* (Gaby, 2001). Kaempferol (a flavonoid), isolated from indigo and also found in a variety of other plants, showed anti-*H. pylori* activity in infected Mongolian gerbils (Katoaka et

al., 2001). Curcumin, the major active constituent in *Curcuma longa* (i.e. turmeric), was recently reported to inhibit several clinical *H. pylori* isolates and be highly effective in eradicating *H. pylori* from infected mice as well as restoring *H. pylori*-induced gastric damage (De et al., 2009). Sulforaphane, a compound isolated from broccoli sprouts was shown to exert bacteriostatic and -cidal activity against multiple antibiotic-resistant *H. pylori* strains as well blocking gastric tumor formation in mice (Fahey et al., 2002).

Although these studies demonstrate the effectiveness of individual compounds against *H. pylori*, it is important to note that greatest antimicrobial activity is most likely achieved with the least risk of developing drug resistance is most likely achieved using combinations of compounds (in purified or natural forms) acting additive-ly/synergistically and should not be overlooked (Yang et al., 2008; Brown et al., 2009). Although many studies report promising findings, most do not reveal the active com-pounds responsible or the mechanism(s) by which these agents act against *H. pylori in vitro* or *in vivo*, leaving many questions unanswered (Elattar and Virji, 1999). Moreover, evidence has shown that populations which consume more fruits and vegetables in their daily diet do have lower incidences of gastric disease and that this may be in part because of natural compounds (phytochemicals) present in these plant materials (Yamada et al., 1998; Fukai et al., 2002). In the cancer cascade proposed by Kusters et al. (2006), host diet is recognized as a significant contributing factor (along with immune response and host genetics) influencing the outcome of *H. pylori* infection and disease progression. Therefore, an inexpensive, diet-based treatment possessing antibacterial, anti-

inflammatory, and anti-carcinogenic properties (all needed to counteract a multifaceted *H. pylori* infection) would be very appealing.

## Grapes for *H. pylori* Treatment

Of the numerous plant-based substances studied to date, grapes are considered a promising source of novel antimicrobial agents and a prime candidate for *H. pylori* treatment/prevention due to their safety, abundance, unique chemical profiles, high structural diversity, and extensive biological activity (Mertens-Talcott and Percival, 2005; Harwood et al., 2007; Xia et al., 2010). Grapes (*Vitis vinifera*) are widely consumed in the United States and a part of many popular foods as reported by the National Agricultural Statistics Service (2003). Previous studies have shown that consumption of these foods can inhibit carcinogenesis, protect against cardiovascular disease and other oxidation linked diseases, and be effective at inhibiting bacterial growth (Vattem et al., 2005; Steinmetz and Potter, 1996; Djousse et al., 2004; Jayaprakasha et al., 2003). In addition, extracts produced from grape seeds and pomace are considered waste by the wine and juice industries, so these by-products are readily available, inexpensive, and have already been exploited as natural antioxidants (Jayaprakasha et al., 2001).

Grape products contain high amounts of natural antioxidants with levels four to five times that of vitamin C or E; both of which were previously found to reduce *H. pylori*-induced gastritis in Mongolian gerbils because of their associated antioxidant levels (Shi et al., 2003; Sun et al., 2005). Further, grape seed extracts have continually shown better protection against free radicals and DNA damage than vitamins C, E, and β-carotene (Bagchi et al., 2000). Antioxidant levels are believed to be important for *H. py-*

*lori* treatment; especially since this organism produces oxygen-free radicals, e.g. superoxide anion and hydroxyl radicals; leading to increased oxidative damage and gastric inflammation. The free-radical scavenging ability of the grape extracts may work to prevent this damage (Chatterjee et al., 2004).

In addition to antioxidant capacity, grapes also possess numerous health-promoting properties due to their ability to produce large numbers of constituent polyphenolic phytochemicals including phenolic acids, flavonoids, and proanthocyanidins (Vattem et al., 2005). These compounds are divisible into single-ring phenolic acids (e.g. gallic acid), bisphenols including stilbenes (e.g. resveratrol), tricyclic phenols (e.g. flavonoids like rutin and quercetin) and their subclasses, and oligomeric and polymeric species (e.g. the proanthocyanidins and anthocyanidins, responsible for the major coloring components of red grapes) (Sovak, 2001). Anthocyanidins are the largest group of phenolic compounds consumed in the human diet and are important in maintaining health because of strong antioxidant activities with a positive relationship between total phenolic content and antioxidant capacity (Velioglu et al., 1998). The composition and levels of phenolic compounds found in grapes are dependent on the cultivar, year of production, geographic location, and degree of maturation (Shi et al., 2003). The color of grape also determines phenolic content (Lee and Jaworski, 1987).

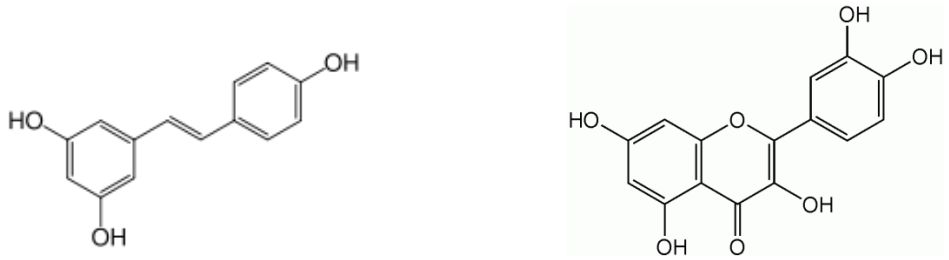


**Figure 1.1.** Chemical structures of resveratrol (left) and quercetin (right).

All the above molecules are synthesized naturally by the plant through simple condensation of phenylalanine in water (Sovak, 2001). These compounds can be extracted by different methods; however, organic solvents like ethanol, acetone, and methanol (used alone or in combination) are typically used in place of simple ethanol/water extraction (Sovak, 2001). Approximately, 60-70% and 28-35% of total extractable phenolics are found in the grape's seed and skin, respectively (Shi et al., 2003). Low levels are also found in the pulp; however, these amounts are insignificant relative to the seed and skin and are more difficult to extract in large quantities.

Of the polyphenols, flavonoids are the most extensively studied including quercetin (a flavonol in grape skin) and catechin (a flavonol in grape seeds). Flavonoids are hydroxylated phenolic substances occurring as a C6-C3 unit linked to an aromatic ring (Cowan, 1999). Because they are synthesized by plants in response to various stresses including microbial infection, it is not surprising that they have been found to be effective antimicrobial substances against a wide array or microorganisms (Dixon et al., 1983). This activity is believed to be due to their ability to act as antioxidants, suppressing inflammation, and ability to complex with extracellular and soluble proteins or bacterial

cell walls and components (i.e. surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes, as described for quinones (another well studied polyphenol in St. John's wort (*Hypericium perforatum*)) (Stern et al., 1996; Duke, 1985; Cowan, 1999). More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya et al., 1996). In addition, quercetin and green tea catechins have also been reported to inhibit DNA gyrase in *E. coli* by interaction with its ATP binding site (Gradišar et al., 2007) and reduce macrophage activation (Ramos et al., 2006; Okoko and Oruambo, 2009).

Although grapes, related fruits, and their compounds have been studied extensively for their antioxidant potential, relatively little information is available on the antimicrobial properties of grape extracts against *H. pylori*. Some studies have shown evidence that these products are effective against both Gram-negative and Gram-positive microorganisms (Jayaprakasha et al., 2003; Özkan et al., 2004). Other fruits such as cranberry have also demonstrated antibacterial and more importantly anti-*H. pylori* activity including inhibition of *H. pylori* adhesion to human gastric mucosa (Vattem et al., 2005; Burger et al., 2000) and urease production (Lin et al., 2005). A study by Dong and Shi (2003) reported that cranberry juice reduced *H. pylori* infection in mice with a clearance rate of 80%. In support of this, Zhang et al. (2005) found that approximately 15% of infected individuals who consumed cranberry juice for one month were no longer infected with *H. pylori*. In addition to these findings, various other berry extracts have been shown to inhibit *H. pylori* and even increase the organisms' susceptibility to antibiotics when used in conjunction with these fruits (Chatterjee et al., 2004).

Shi et al. (2003) reported that red wine may have anti-*H. pylori* properties since it contains approximately 63% of the total phenolics from grapes. Resveratrol, an active constituent present in high quantities in red wine was shown to have anti-*H. pylori* effects in addition to being highly anti-carcinogenic with superior superoxide radical trapping activity (Daroch et al., 2001; Mahady and Penland, 2000; Mahady and Penland, 2003; Jang et al., 1997).

Some studies have shown that grape/polyphenolic products may also act against *H. pylori* by specifically inhibiting VacA-induced gastric damage. Tombola et al. (2003) reported that red wine and green tea polyphenols inhibited *H. pylori* VacA-induced ion channel formation, urea conduction, and cell vacuolation and that the toxin was inhibited equally well at pH 4 or 7 upon exposure to these compounds; supporting their potential effectiveness *in vivo* since they would be unaffected by the stomach's high luminal acidity (Achtman and Suerbaum, 2001). Flavanoids and flavonoid glycosides were also reported as chemically stable under human stomach conditions, indicating that these products may also be ideal for the treatment of *H. pylori* infection (Gee et al., 1998). Later, using red wine, green tea, and pure polyphenols (tannic acid, *n*-propyl gallate, hop bract tannin) VacA-intoxicated mice were successfully treated following oral administration of VacA toxin (Yahiro et al., 2005; Ruggiero et al., 2006; Ruggiero et al., 2007); supporting Tombolo's study. When animals were infected with live *H. pylori* cells, gastric inflammation and erosion were reduced; however, no significant alternation in *H. pylori* colonization was observed, further supporting the hypothesis that VacA may be a key molecular target of these compounds (Ruggiero et al., 2006).

In addition to toxin inhibition, attachment of *H. pylori* to gastric epithelial cells may also be influenced by these compounds. Although the exact mechanism for this is currently unknown, it is hypothesized that it may be due to the high antioxidant activity generated by anthocyanins contained in the plant material (especially grape skins). This may be accurate since proanthocyanidin extracts from cranberries were previously found to decrease P-fimbriated *Escherichia coli* to uroepithelial-cell surfaces, possibly correlating with *H. pylori* (Howell et al., 1998). Procyanidins may also inhibit free radical production associated with inflammatory reactions by interacting with the synthesis and release of proinflammatory substances like histamine and leukotrienes and also hyaluronidase, an enzyme associated with tissue damage during inflammation (Hansen, 1995; Pearce et al., 1984).

## Muscadine Grapes for *H. pylori* Treatment

Muscadine grapes (*Vitis rotundifolia*) are native to the southeastern United States and can grow in warm, humid climates where *Vitis vinifera* do not grow as well. This grape type has significantly higher total phenolic contents than *Vitis vinifera*; distinguished by high ellagic, gallic, and flavonoid glycoside concentrations along with unique anthocyanin chemistries (Greenspan et al., 2005). Extremely high antioxidant activity has also been reported for muscadines (Patrana-Bonilla et al., 2003). In addition, muscadine grapes and their phenolics have shown strong anti-inflammatory and anticancer properties when tested in mice and with various cancer cell lines (Greenspan et al., 2005; Weiguang, et al., 2005). Furthermore, muscadine juice and phenolic extracts were recently reported to have significant antibacterial activity against *Salmonella, Listeria*, and

*Cronobacter* strains (Kim et al., 2010; Park et al., 2011). Although not extensively studied in relation to other grape types, muscadines are believed to not only possess more desirable properties (e.g. higher antioxidant and phenolic contents) but are superior to *Vitis vinifera* at inhibiting *H. pylori* as found in a recent study comparing these two grape types (Brown et al., 2009).

In addition to other studies reporting the antimicrobial and anti-*H. pylori* properties of phenolic compounds (Fahey et al., 2002; Mabe et al., 1999; O'Gara et al., 2000; Vattem et al., 2005; Yahiro et al., 2005), we have previously shown in our laboratory that phenolic-containing muscadine grape extracts may help weaken *H. pylori* adhesion to host cell surfaces or reduce cellular damage caused by this bacterium (Brown et al., 2009; Burger et al., 2000; Yahiro et al., 2005). Compounds such as ellagic acid and quercetin, high in some grape varieties, have also been shown to possess anti-cancer activities and reduce gastric epithelial cell death by inhibiting *H. pylori* VacA toxin (Smith et al., 2004; Shin et al., 2005).

## Rationale for Our Study

Muscadine grapes contain approximately five times the levels of total phenolic compounds as commercial red grapes (Pastrana-Bonilla et al., 2003). They are readily available, have no known toxicity, and no side effects. Muscadine grapes also contain many unique forms of compounds which may be effective, alone or in combination, in the dietary management of *H. pylori* infection as determined in previous studies conducted in our laboratory. We have previously demonstrated that muscadine grape extracts have significant but varied effects on *H. pylori* infection. Analysis of green fluo-

rescent protein-labeled *H. pylori* using confocal laser scanning microscopy revealed reduced attachment of the bacterium to host cell surfaces following treatment. Furthermore, earlier *in vitro* and *in vivo* studies suggest virulence factors associated with this organism (e.g. VacA) may be suppressed (as determined by MTS cell proliferation assays) following treatment with grape extracts or major phenolic compounds (Brown et al., 2009). However, additional factors are also believed to be affected. These results are in agreement with previous studies reporting reduced gastritis (linked to VacA) following administration of purified polyphenolic compounds or red wine to infected mice (Ruggiero et al., 2006; Ruggiero et al., 2007). In addition, anti-inflammatory responses may be responsible for reduced host cell damage as indicated by an animal study in which *H. pylori*-infect guinea pigs showed reduced *H. pylori* counts, gastric inflammation, and lipid peroxidation following administration of quercetin (Gonzalez-Segovia et al., 2008).

Since muscadine grapes are inexpensive, easily handled, and non-toxic, it is believed that consumption of the entire fruit or its extracted constituents may be beneficial to human health and an effective strategy for *H. pylori* treatment or prevention. An added benefit may be that these bioactive compounds may help limit erosion of the gastric mucosa caused by continuous inflammation (Greenspan et al., 2005). In addition, higher compliance and little to no side effects would further entice patients to be more accepting of treatment and complete prescribed therapies and not inadvertently select for antibiotic resistance by ending treatment prematurely. Finally, many of these compounds may be difficult for *H. pylori* to develop resistance against because of their suspected synergistic interactions and unique cellular targets; however, the means by which muscadine grapes

and their constituent compounds exert these effects have yet to be determined (Vattem et al., 2005).

Because of the available literature supporting the potential antimicrobial applications of grape products and associated compounds, as well as previous studies conducted in our laboratory, we believe that muscadine grape extracts and their chemical constituents may be beneficial in the treatment of *H. pylori* infection. Results from this study will not only provide valuable information on the therapeutic potential of muscadine grapes and related compounds against *H. pylori*, but also provide novel insights into the overall methodology for evaluating other natural compounds against *H. pylori* in the future.

The objectives of this study are to 1) determine the major phenolic compounds present in muscadine grape skin extracts responsible for anti-*H. pylori* activity 2) elucidate their possible mode(s) of action *in vitro* and 3) evaluate muscadine grape skin and these compounds as a prerequisite to the development of a diet-based strategy used to attenuate the disease process *in vivo.*

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CHAPTER TWO

# ACTIVITIES OF MUSCADINE GRAPE SKIN AND QUERCETIN AGAINST *HELICOBACTER PYLORI* INFECTION IN MICE


## Abstract

The aim of this study was to explore the preventative potential of muscadine grape skin and the single flavonoid, quercetin, as an alternative means for ameliorating *H. pylori* infection and/or the *H. pylori*-induced inflammatory response in mice.

The antimicrobial and anti-inflammatory properties of muscadine grape skin and quercetin, a major phenolic constituent, were evaluated against *H. pylori in vitro* and *in vivo*. The antimicrobial activity of quercetin was evaluated against 11 *H. pylori* strains *in vitro* with inhibition of all strains at 128 - 64 µg/ml. *In vivo* studies showed a moderate reduction in *H. pylori* counts following treatment with 5 and 10% muscadine grape skin or quercetin (25 mg/kg body wt) in addition to significantly reduced inflammatory cytokines (TNF-α, IL-1β, and IFN-γ) as compared to untreated mice.

Muscadine grape skin and quercetin did not significantly reduce *H. pylori* growth in a mouse model. However, these products were effective in regulating the inflammatory response to *H. pylori* infection.

Our results suggest that *H. pylori* infection may be reduced or prevented via the consumption of fruits rich in certain phenolic compounds (e.g. quercetin) such as muscadine grapes.

## Introduction

Infection with *Helicobacter pylori* can result in chronic gastritis, peptic ulcer disease, and an increased risk of developing mucosa-associated lymphoid tissue (MALT) lymphoma and gastric cancer (Cover and Blaser, 1995). Although this pathogen colonizes more than half the world's population, making it one of the most common bacterial infections in humans, only a subset of those infected display symptoms (Osaki et al., 1998). The severity of *H. pylori* infection is dependent on multiple factors like bacterial strain(s), age of acquisition, and immune response. Disease progression may also be influenced by additional factors such as host environment and diet. Host dietary factors in particular are thought to play a significant role in *H. pylori* disease outcome, especially during and immediately following colonization (Testerman et al., 2001).

It is well-documented that high-salt diets correlate with gastritis and gastric cancer and enhance the probability of developing severe disease following *H. pylori* infection. In support of this, high sodium chloride consumption has been found to alter *H. pylori* growth and increase expression of *cagA* and *vacA* genes *in vitro*, both encoding toxins key in *H. pylori* virulence (Gancz et al., 2008; Loh et al., 2007). In addition, people consuming salty foods may even enhance *H. pylori* colonization (Tsugane et al., 1994, Fox et al., 1999). On the other hand, people who consume more fruits and vegetables have a reduced risk of *H. pylori* infection and/or developing upper gastrointestinal disorders and related symptoms (Buiatti et al., 1990; Singh and Gaby, 1991). The means by which this protective effect occurs is currently unknown but may be associated with higher intake of bioactive compounds possessing strong antibacterial and antioxidant ac-

tivities (Tombola et al., 2003; O'Gara et al., 2000; Yahiro et al., 2005; Zhang et al., 1997; Bennedsen et al., 1999). Compounds present in these foods may act against *H. pylori* by reducing bacterial growth and/or interfering with attachment and/or virulence factors (e.g. urease, VacA, CagA). These compounds may also counteract destructive autoimmune-induced tissue damage via suppression of Th1 pro-inflammatory cytokines (e.g. IFN-γ, TNF-α) and associated oxidative damage triggered by *H. pylori* infection (Chatterjee et al., 2004; Ruggiero et al., 2006; Ruggiero et al., 2007).

Muscadine grapes (*Vitis rotundifolia*) are common in the southeastern U.S. and are characterized by their unique anthocyanin profiles and high flavonoid concentrations; contributing to their extremely high antioxidant levels and strong anti-inflammatory and anticancer potentials (Greenspan et al., 2005; Pastrana-Bonilla et al., 2003; Weiguang et al., 2005). Quercetin (3, 3′, 4′, 5, 6-pentahydroxyflavone) is a naturally-occurring dietary flavonol found in muscadine grape skin (Kühnau, 1976). Because of its high antioxidant capacity and anti-*H. pylori* potential, it was chosen for use in this study. In addition, these products are inexpensive, non-toxic, and previously shown to possess antibacterial properties; therefore, the fruit itself or its extractable chemical constituents may serve as an effective supplement for *H. pylori* suppression or treatment.

The objective of this study was to explore the preventative potential of muscadine grape skin and the single flavonoid, quercetin, as an alternative means for ameliorating *H. pylori* infection and/or the *H. pylori*-induced inflammatory response in mice.

## Materials and Methods

***H. pylori.*** Eleven *H. pylori* strains were used in this study. *H. pylori* strains G2-1, 26695, WV99, NB2-1 and 1324P-1 were obtained from Dr. Douglas Berg (Washington University, St. Louis, MO). *H. pylori* strains D5251, D5131, D5178, D5136, and D5135 were obtained from Dr. Ben Gold (Emory University and Centers for Disease Control and Prevention, Atlanta, GA). *H. pylori* SS1, a mouse-adapted strain, was provided by Dr. Kathryn Eaton (Department of Veterinary Biosciences, Ohio State University, Columbus, OH). *H. pylori* 26695, G2-1, 1324P-1, D5251, D5131, D5178, and SS1 were positive for the *cagA* gene, while all strains were *vacA* positive. Bacteria were grown on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, MI) (pH 7.4 ± 0.2) supplemented with 7% horse serum (HS) (Sigma Chemical Co., St. Louis, MO) at 37°C for 72 h under microaerophilic conditions (5% $O_2$, 10% $CO_2$, and 85% $N_2$) in a GasPak jar (BBL Microbiology Systems, Cockeysville, MD) (Jiang and Doyle, 2000).

For the animal challenge study, *H. pylori* SS1 was grown under the same conditions at 37 °C for 72 h. Plate growth was used to inoculate 30 ml of BHI broth (BHIB) in sterile media bottles. Cultures were then incubated overnight under the same conditions with shaking at 120 rpm. Cells were harvested by centrifugation at 3,500 x g for 5 min and resuspended in sterile BHIB for the animal challenge. Prior to infection, cultures were examined microscopically to confirm purity and motility. Serial dilutions of each inoculum were plated in triplicate onto antibiotic supplemented plates of BHI agar containing 7% HS and Dent antibiotic supplement [vancomycin (10 µg/ml), trimethoprim (5 µg/ml), cefsulodin (5 µg/ml), and amphotericin B (5 µg/ml)] (Oxoid, Basingstoke,

Hampshire, UK) and onto Tryptic Soy Agar (TSA) with 5% sheep blood (bioMérieux, Inc., Durham, NC) to confirm inoculum concentration and purity.

**Anti-*H. pylori* activity of quercetin.** For antimicrobial susceptibility testing, *H. pylori* was grown under the previously described conditions and spot-inoculated (ca. 1 x $10^5$ CFU/spot) onto pre-warmed BHI-HS agar containing 4.0 to 256 µg/ml quercetin (Sigma) in triplicate. Quercetin was prepared in dimethyl sulfoxide (DMSO) (MP Biomedicals, Solon, OH) and filter sterilized. Plates containing DMSO were used as a solvent control. The minimal inhibitory concentration (MIC) was determined as the lowest concentration required to completely inhibit the growth of *H. pylori* following 1 week incubation under microaerophilic conditions.

**Animals.** Six-week old, female, C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were specific-pathogen free and clear of *H. pylori* upon receipt. Mice were housed in groups of four in microisolater cages and fed a conventional rodent diet (Harlan Teklad, Madison, WI) with or without muscadine grape skin (MGS) powder [5 and 10% (w/w)] (Muscadine Products Corporation, Wray, GA) and sterile water ad libitum. Mice receiving quercetin alone were fed a non-supplemented diet. Quercetin treats (25 mg/kg body weight) (approximately 0.5 mg/mouse) were prepared by Bio-Serv (Frenchtown, NJ) and administered once daily. All diets were begun 1 week prior to infection with *H. pylori*. All procedures were approved by the Clemson University Institutional Animal Care and Use Committee.

**Mice challenge study.** Mice were fasted for 4 h and then orally administered 0.1 ml of sterile 0.5 M sodium bicarbonate (Fisher Scientific, Norcross, GA) immediately

prior to inoculation with *H. pylori*. Each mouse received 0.25 ml ($10^7$ to $10^8$ CFU/mouse) of *H. pylori* suspension by oral gavage using a 24 gauge needle; excluding the no *H. pylori* controls which received sterile BHIB. Mice were challenged three times at two day intervals over a 5-day period. In order to monitor *H. pylori* infection status throughout the study, a sensitive, non-invasive PCR assay was used. Following *H. pylori* infection, cages were changed weekly (i.e. mice transferred to new sterile cages containing fresh, sterile bedding, food, and water) and feces were collected using sterile forceps. Feces were kept frozen at -20 ºC until analysis. At 10 weeks post infection (p.i.), mice were anesthetized with Isoflurane and killed by cervical dislocation. A heart puncture was performed on each animal and serum was collected and stored at -80 ºC. Stomachs were aseptically removed and bisected along the greater and lesser curvatures with one-half used for *H. pylori* enumeration and the other immediately preserved in neutral buffered formalin for histological evaluation.

One-half of each stomach was weighed and immersed in brucella broth (BB) (Difco Laboratories, Detroit, MI) containing 10% HS. The tissue was homogenized using Dounce tissue grinders (1 ml) (Wheaton Industries, Inc, Millville, NJ) and serially diluted in phosphate-buffered saline (PBS) (pH 7.4). Tissue grinders were cleaned between samples by rinsing twice with 75% ethanol and then sterile BB. One-hundred microliters of each dilution were then spread-plated onto selective medium: BHI agar supplemented with 5% defibrinated sheep blood (Remel, Lenexa, KS), vancomycin (100 µg/ml), polymyxin B (3.3 µg/ml), bacitracin (200 µg/ml), amphotericin B (50 µg/ml), and nalidixic acid (10.7 µg/ml) (Sigma, St. Louis, MO). Plates were incubated microae-

robically at 37 ºC for 7 days. The identity of *H. pylori* was confirmed by Gram stain, oxidase, and catalase tests. Colonies were counted and expressed as CFU/g tissue.

**DNA extraction and purification.** *H. pylori* DNA was extracted from fecal samples using an UltraClean fecal DNA extraction kit from MO BIO Laboratories, Inc. (Carlsbad, CA) following the manufacturer's directions. To eliminate PCR inhibitors not removed during DNA extraction from fecal material, a modified approach of that described by Monteiro et al. (2001) was used. Briefly, after initial DNA extraction, 10 µl of DNA was electrophoresed in 1.5% (wt/vol) agarose gels in Tris-borate-EDTA (TBE) buffer at 50 V for 3 h. Following staining with ethidium bromide, DNA bands were excised with sterile scalpels and cleaned using an UltraClean GelSpin DNA purification kit (MO BIO Laboratories, Inc., Carlsbad, CA). Purified DNA was then used for subsequent PCRs.

**PCR.** PCR assays targeting the 16S rRNA gene were performed as described by Chisholm et al. (2001). Briefly, 20 µl reaction mixtures containing 2 µl of extracted DNA, 1.5 mM $MgCl_2$, 200 µM (each) dNTPs, 0.4 µM (each) primer HP1 (5′-CTG GAG AGA CTA AGC CCT CC) and HP2 (5′- ATT ACT GAC GCT GAT TGT GC), 1 U *Taq* polymerase in PCR buffer, was held for 5 min at 95 ºC, followed by 35 cycles of 30 sec each at 95 ºC, 60 ºC, and 72 ºC, and by 5 min. at 72 ºC. The amplification of a 110-bp fragment was confirmed by electrophoresis.

**Histological evaluation.** One half of each stomach was washed vigorously in sterile PBS, fixed in 10% buffered formalin, and sent to the Clemson University Veterinary Diagnostic Center, Columbia, SC for processing and evaluation. Samples were

stained with hematoxylin and eosin (HE) and modified Giemsa stain. All tissues were blindly scored by two pathologists based on the updated Sydney system (Dixon et al., 1996).

**ELISA.** Mouse stomach tissue homogenate and serum samples were analyzed by sandwich enzyme-linked immunosorbent assay (ELISA). Cytokines evaluated were tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin-1 beta (IL-1β). All samples were tested in duplicate following the manufacturer's instructions (eBioscience, Sandiego, CA). Total protein was measured by the Bradford method. Plates were read at 450 nm using a BioTek μQuant microplate spectrophotometer (Biotek Instruments, Inc., Winooski, VT). Cytokine levels were expressed as pg/ml of serum or pg/mg of total protein.

**Statistical Analysis.** The mean values of *H. pylori* cell count and ELISA data were submitted to one and two-way analysis of variance (ANOVA) and Tukey's tests to determine differences among treatments. All values were considered statistically significant at $P < 0.05$ using SAS 9.1 software (SAS Institute Inc., Cary, NC).

## Results

**Antibacterial Activity of Quercetin Against *H. pylori in vitro*.** Following antimicrobial susceptibility testing, all 11 *H. pylori* strains were determined to be sensitive to quercetin at concentrations ranging from 64 to 128 μg/ml (Table 2.1). DMSO had no effect on any of the strains tested.

**Anti-*H. pylori* Activity *in vivo*.** Mice were fed MGS (5 or 10% w/w) or quercetin (25 mg/kg) daily with infection status monitored weekly by PCR (eliminating the pos-

sibility of transient *H. pylori* infection). Final quercetin concentrations in treats were approximately 10 fold greater than quercetin levels previously determined in MGS powders (approximately 57 µg/g MGS dry weight) (unpublished data). After 10 weeks, *H. pylori* was still present in all treatment groups as detected by PCR, culturing and histological analysis. All mice (n=21) inoculated with *H. pylori* were successfully infected and remained colonized throughout the study as revealed by weekly analysis of stool samples by PCR (Figure 2.1). Of the mice given diets supplemented with MGS or quercetin, most showed a moderate reduction in *H. pylori* with cell counts ranging from $7.3 \times 10^5$ to $5.8 \times 10^6$ CFU/g of stomach tissue respectively, as compared with $1.7 \times 10^7$ CFU/g in no treatment controls ($P > 0.05$) (Table 2.2).

**Anti-inflammatory Activity of Muscadine Grape Skin and Quercetin.** Cytokines commonly associated with *H. pylori*-induced inflammation were tested to determine any effect diets supplemented with MGS (5% and 10%) or quercetin (~25 mg/kg body weight) had on reducing the inflammatory response. Following treatment with MGS, both IFN-γ and IL-1β levels were significantly reduced ($P < 0.05$) in infected mice (Figure 2.2). A dose-dependent response was observed when mice were fed either 5% or 10% MGS. TNF-α levels were also reduced ($P < 0.05$) following treatment, with uninfected and those receiving 10% MGS showing similar results with both below detection limits. Quercetin, when given alone to infected animals reduced IL-1β levels ($P < 0.05$) but did not lead to a significant reduction in TNF-α or IFN-γ ($P > 0.05$) in serum samples. In contrast, all cytokine levels were significantly reduced ($P < 0.05$) in gastric tissue homogenates of treated mice.

The majority of tissues sampled from the infected control group displayed moderate to diffuse infiltration of lymphocytes, plasma cells, neutrophils and eosinophils in the lamina propria with infiltrates especially intense at the submucosa interface (Figure 2.3). One control animal infected with *H. pylori* showed mild to moderate atrophy of chief cells accompanied by mucous metaplasia of cells in some gastric glands. Mice fed 10% muscadine grape skin showed no significant cellular atrophy with the antrum and corpus only mildly infiltrated by lymphocytes, plasma cells and fewer eosinophils despite containing relatively high numbers of *H. pylori* cells.

## Discussion

Because of the rise in antimicrobial resistance, an inexpensive, diet-based treatment against *H. pylori* infection would be of great interest. Numerous studies have examined the activities of natural, plant-based extracts and compounds against *H. pylori in vitro*; however, few have reported *in vivo* efficacy. We have previously shown that MGS extracts and their constituent polyphenolic compounds are capable of significantly reducing *H. pylori* growth *in vitro* and may affect bacterial attachment to host gastric cells, a key step in *H. pylori* colonization/virulence (Brown et al., 2009).

In this study, we aimed to determine if a diet supplemented with MGS or quercetin, the third highest major phenolic in MGS (Greenspan et al., 2005; Brown et al., 2009), could reduce or eliminate *H. pylori* from infected mice or protect against *H. pylori*-induced oxidative damage.

*In vitro* findings showed that quercetin inhibits the growth of all tested *H. pylori* strains suggesting that it may also be effective *in vivo*. Agar dilution results are in

agreement with our previous study (Brown et al., 2009) in which muscadine grape skin and seed extracts, as well as pure constituent compounds (e.g. resveratrol, ellagic acid) were found to be effective against the same *H. pylori* strains; suggesting that these compounds may work synergistically or additively against *H. pylori*.

Although *H. pylori* was not eradicated following treatment, bacterial numbers were lower (0.4 to 1.2 logs) in treatment groups as compared to infected controls; supporting a dose-dependent, albeit weak, antibacterial response. These findings are in agreement with similar studies in which other polyphenols failed to completely eradicate *H. pylori* when administered to infected animals while showing moderate reduction in *H. pylori* numbers (Ruggiero, 2006; Ruggiero, 2007). Quercetin, when given alone to infected animals, resulted in a decline in *H. pylori* counts similar to that seen in animals fed 10% MGS. This was surprising since an earlier hypothesis was that a synergistic action between phenolic compounds present in MGS may be necessary to effectively inhibit *H. pylori* growth *in vivo*. However, quercetin levels were higher in treats than those determined from MGS powder. This may suggest that quercetin, when administered alone at relatively high concentrations, may be equally effective in reducing *H. pylori* as 10% MGS. In support of this, González-Segovia et al. (2008) recently reported that quercetin significantly decreased *H. pylori* colonization in infected guinea pigs. However, the dosage of quercetin used in that study was four-fold higher than ours and directly administered twice a day for two weeks. Therefore, differences in *H. pylori* levels following treatment may be explained by higher quercetin concentrations. However, comparisons

between the two studies could not be made since no plate-count data was available and different animal models and *H. pylori* strains were used.

On the other hand, the anti-inflammatory properties of MGS appeared to be more notable, with gastritis in MGS treated animals only moderately higher than uninfected controls in addition to much reduced inflammatory cytokine levels. In animal models, both MGS and quercetin inhibited the induction of gastric damage from *H. pylori* infection as shown by ELISA and histological analyses. These findings are in agreement with other studies in which polyphenolic compounds from red wine and green tea were found to reduce overall gastritis in *H. pylori* infected animals while only moderately reducing bacterial load (Ruggiero et al., 2006). Although not tested in this study, the fact that MGS and quercetin reduced gastritis while only marginally affecting bacterial colonization may further support the protective nature of certain phytochemicals *in vivo* may be linked to VacA, CagA, or urease inhibition. In addition, the inherent anti-inflammatory activities of certain compounds aimed against destructive *H. pylori*-induced host inflammatory responses cannot be excluded (Ruggiero et al., 2006; Gonzalez-Segovia et al., 2008; Singh and Baby, 1991). Alternatively, MGS or quercetin may affect *H. pylori* growth by reducing iron availability, decreasing bacterial membrane stability, or inhibiting attachment mechanisms (Guo et al., 2007; Burger et al., 2000).

In this study, cytokine levels also appeared to be in agreement with gastric pathologies observed in stomach tissue samples with TNF-α, IL-1, and IFN-γ significantly lower in groups fed MGS ($P < 0.05$). Although quercetin was unable to significantly reduce TNF-α and IFN-γ levels in serum; it did reduce local cytokine levels in infected

stomach tissues. This may suggest that quercetin alone is insufficient in reducing inflammation and that a synergistic interaction may be taking place among phenolic compounds contained in MGS or quercetin's low bioavailability in its given form. Reduction in these inflammatory mediators may be due to decreased neutrophil leukocyte infiltration and associated free radical production. Decreased TNF-α and IL-1β production may result in reduced NF-kappaB levels, further contributing to the anti-inflammatory properties of MGS; however, additional factors cannot be excluded (Gonzalez-Segovia et al., 2008; Min et al., 2007; Ruiz et al., 2007).

In a previous study conducted in our laboratory, we reported that MGS extracts may also be effective at preventing and/or reducing *H. pylori* infection via inhibition of bacterial attachment mechanisms (Brown et al., 2009). Although *H. pylori* was able to successfully colonize mice; animals were given MGS for only one week prior to being challenged and fasted before infection which may have only slightly weakened the initial attachment of *H. pylori* to gastric epithelium. In addition, in previous experiments, highly concentrated MGS extracts were used, possibly resulting in a different observable effect.

In conclusion, both MGS and quercetin slightly inhibited *H. pylori* growth while significantly reducing inflammation and gastric damage in *H. pylori*-infected mice. Therefore, MGS and/or quercetin may be useful in preventing some of the gastric pathologies associated with *H. pylori* infection; however, further studies are necessary to determine MGS and quercetin's long-term applications for the prevention or treatment of *H. pylori* infection.

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## Figure Legend

**FIGURE 2.1.** PCR amplification of *H. pylori* SS1 16S rRNA gene from *H. pylori*-infected control mice feces. Lane 1, 50-bp DNA size marker (Promega Corp., Madison, WI); lane 2, negative control (no DNA template); lanes 3 to 12, DNA extracted from mice feces collected at week 1 to 10 p.i., respectively.

**FIGURE 2.2.** Mice IFN-γ, IL-1β, and TNF-α levels following treatment with 5 or 10% muscadine grape skin (MGS) powder or quercetin (25 mg/kg body weight). Panel A, serum (pg/ml). Panel B, supernatants of gastric tissue homogenates (pg/mg protein). Error bars represent standard error. The same letter represents no significant difference ($P > 0.05$) among treatments.

**FIGURE 2.3.** Representative images of HE-stained sections of mouse gastric antrum mucosa. A, negative control (i.e. no *H. pylori*); B, positive control (i.e. *H. pylori* with no treatment); C, *H. pylori*-infected mice receiving 10% muscadine grape skin powder. Mice infected with *H. pylori* showed an influx of inflammatory cells at the submucosa interface (arrowheads) whereas mice fed 10% MGSP were similar to no *H. pylori* controls. Scale bars, 50 μm.

**TABLE 2.1.** Minimal inhibitory concentrations of quercetin on *H. pylori* growth by agar dilution assay.

| Conc. (μg/ml) | *H. pylori* strains | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 26695 | G2-1 | WV99 | NB2-1 | 1324P-1 | D5251 | D5131 | D5178 | D5136 | D5135 | SS1 |
| 256 | –* | – | – | – | – | – | – | – | – | – | – |
| 128 | – | – | – | – | – | – | – | – | – | – | – |
| 64 | + | – | – | – | – | – | – | – | + | + | – |
| 32 | + | + | + | + | + | + | + | + | + | + | + |
| 16 | + | + | + | + | + | + | + | + | + | + | + |
| 8 | + | + | + | + | + | + | + | + | + | + | + |
| 4 | + | + | + | + | + | + | + | + | + | + | + |
| Control | + | + | + | + | + | + | + | + | + | + | + |

***** –, no growth; +, growth.

**TABLE 2.2.** Effects of muscadine grape skin powder on *H. pylori* infection in C57BL/6 mice.

| Treatment/diet | Mice infected | log CFU/g | Gastritis score[b] |
|---|---|---|---|
| no *H. pylori* control | 0/6 | ND [a] | 0.5 |
| *H. pylori* control | 3/3 | 7.2 ± 0.1 a | 2.3 |
| 10% MGSP | 7/7 | 5.9 ± 0.3 a | 1.0 |
| 5% MGSP | 7/7 | 6.8 ± 0.4 a | — |
| Quercetin | 4/4 | 5.9 ± 0.5 a | — |

[a] ND, not detected. The same letter within the column represents no significant difference ($P > 0.05$) among treatments.
[b] For gastritis scoring, "0" = no inflammation; "1" = mild inflammation; "2" = moderate inflammation; "3" = severe inflammation, and "-" not tested.

**FIGURE 2.1.**

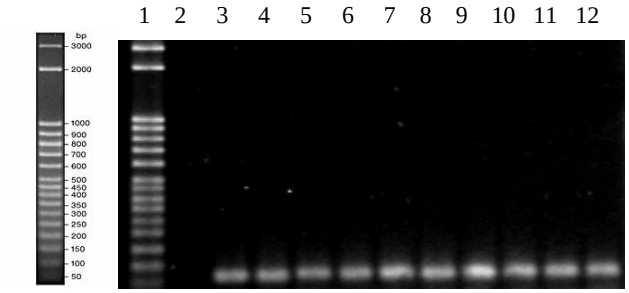

1 2 3 4 5 6 7 8 9 10 11 12
bp
3000
2000
1000
900
800
700
600
500
450
400
350
300
250
200
150
100
50

**FIGURE 2.2.**

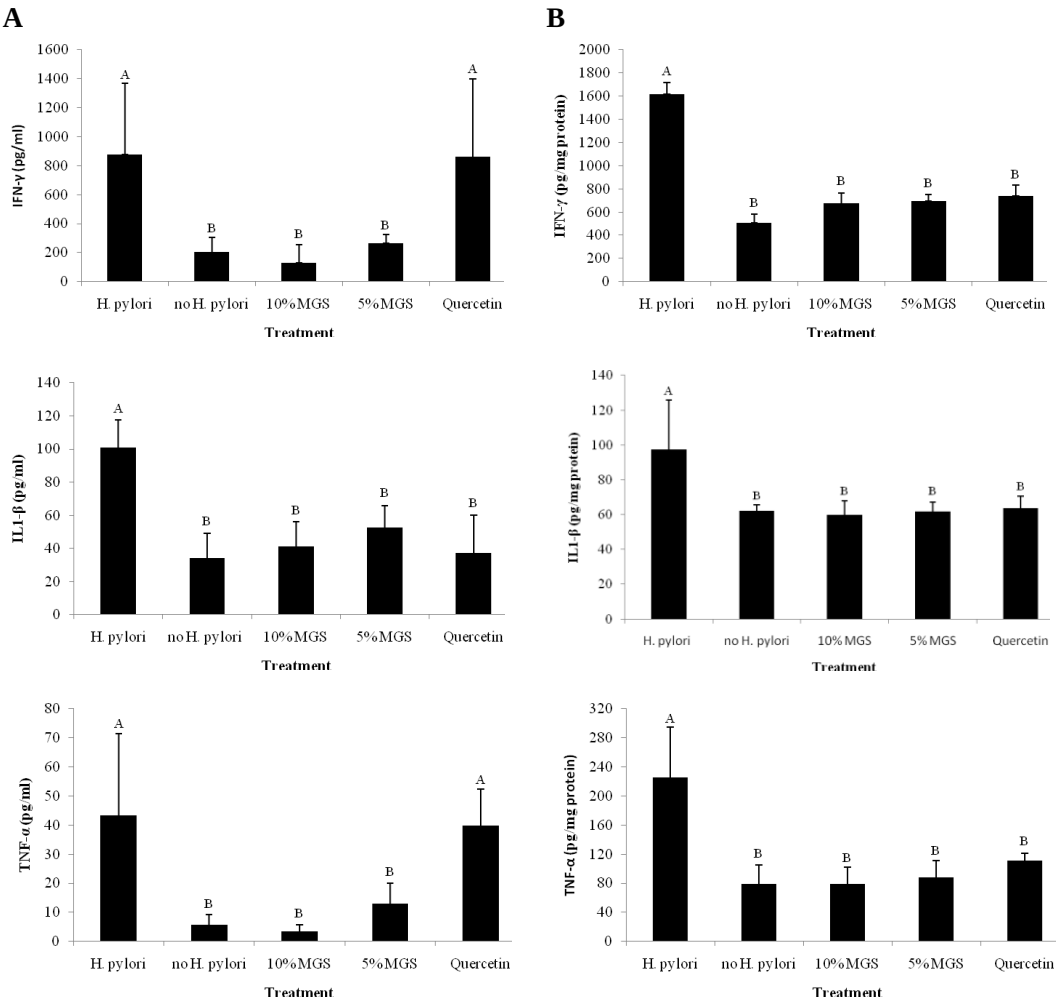
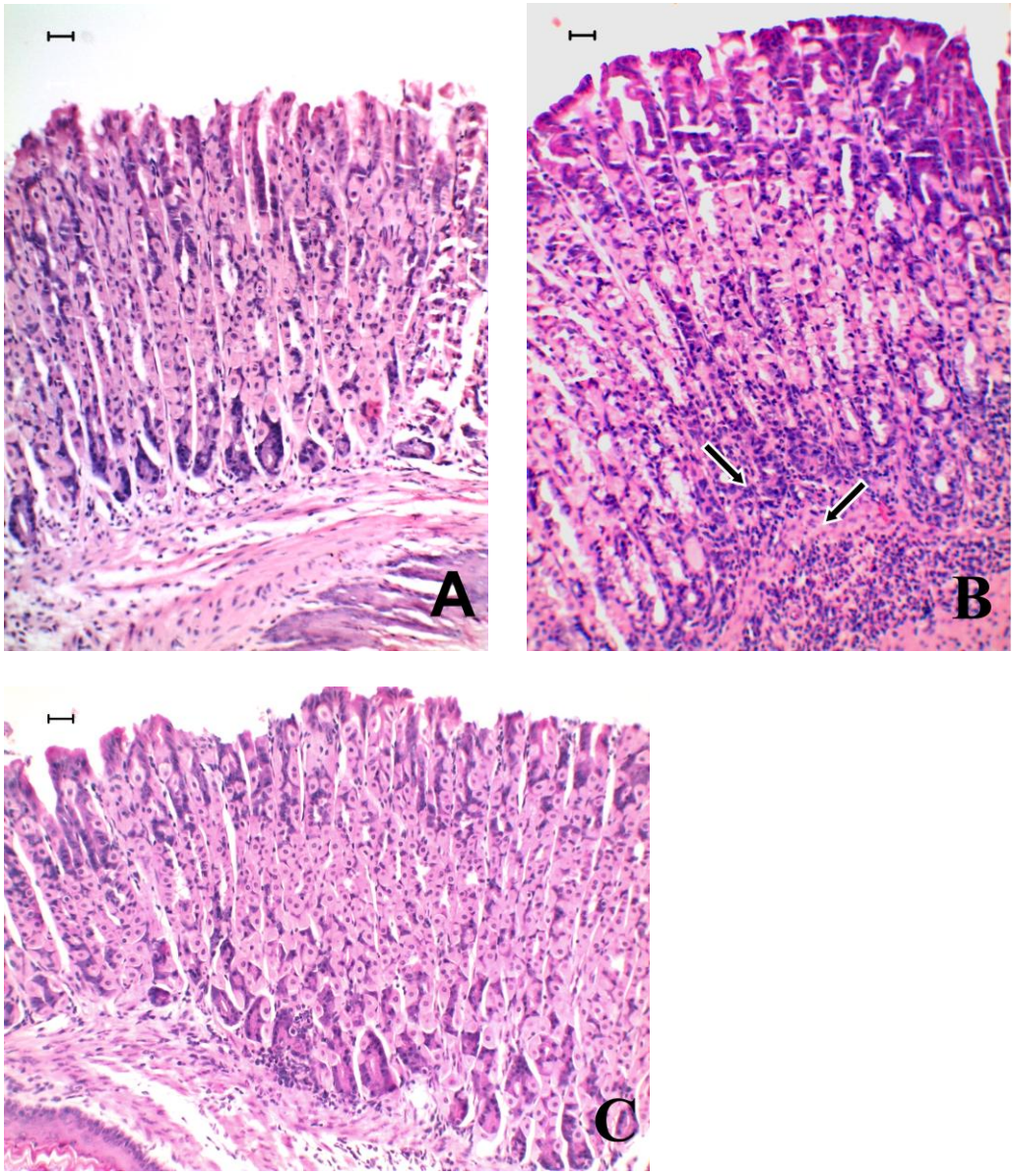

A
B
IFN-γ (pg/ml)
IFN-γ (pg/mg protein)
IL1-β (pg/ml)
IL1-β (pg/mg protein)
TNF-α (pg/ml)
TNF-α (pg/mg protein)
H. pylori
no H. pylori
10%MGS
5%MGS
Quercetin
Treatment

**FIGURE 2.3.**



CHAPTER THREE

# ACTIVITIES OF MUSCADINE GRAPE SKIN AND POLYPHENOLIC CONSTITUENTS AGAINST *HELICOBACTER PYLORI IN VITRO*


## Abstract

The inhibitory effects of quercetin and resveratrol, active polyphenols identified in muscadine grape skin (MGS) extracts, against *Helicobacter pylori* were investigated. Quercetin and resveratrol significantly reduced *H. pylori* growth regardless of pH (neutral and acidic) with minimal bactericidal concentrations (MBCs) of 256 and 128 µg/ml, respectively, while displaying synergistic activity when in combination. MGS extracts displayed the highest efficacy suggesting additional unidentified compounds not detected in this study. Time course viability experiments and phase-contrast microscopy showed a dose-dependent anti-*H. pylori* response to quercetin and resveratrol. Interestingly, neither quercetin nor resveratrol affected *H. pylori* outer membrane integrity as determined by 1-*N*-phenylnapthylamine (NPN) uptake assays. However, quercetin was found to enter *H. pylori* as determined by HPLC analysis supporting intracellular drug accumulation. The anti-*H. pylori* effects of quercetin and resveratrol, alone and in combination, suggest that these compounds may be useful in the dietary prevention and/or treatment of *H. pylori* infection.


## Introduction

*Helicobacter pylori* is a well-known human pathogen and the etiology of various gastric diseases including peptic ulcers, gastritis, and stomach cancer (Cover and Blaser, 1995; Dunn et al., 1997). While infection is common, disease outcome is likely multifac-

torial with virulence of infecting strain, host age, genetic constitution, and environment all contributing to disease progression (Bergonzelli et al., 2003). Because of the complexity of *H. pylori* infection, all possible host/pathogen interactions should be considered. One important factor frequently underplayed is host dietary habits which may serve a more important role during and immediately following infection with *H. pylori* than previously thought (Testerman et al., 2001). Some studies have explored the link between *H. pylori* infection and host diet/nutrition with several associating high-salt diets with increased risk and/or extent of disease (Tsugane et al., 1994; Fox et al., 1999; Willis et al., 1999; Gancz et al., 2008). On the other hand, consumption of fruits and vegetables rich in certain vitamins, antioxidants, and constitutive bioactive compounds (e.g. phytochemicals) has been shown to significantly reduce the incidence of *H. pylori* infection and/or ameliorate associated symptoms (Buiatti et al., 1990; Zhang et al., 1997, Bennedsen et al., 1999, Yamada et al., 1998; Fukai et al,. 2002, Yanaka et al., 2009).

Although many strains of *H. pylori* are susceptible to most currently used antibiotics (e.g. clarithromycin, metronidazole, amoxicillin) *in vitro*, treatment is increasingly challenging due to antibiotic resistance and reinfection in certain groups. Therefore, novel, diet-based therapeutics for use where conventional antibiotic therapies have failed, are unavailable, and/or expensive have received considerable attention.

Numerous studies have investigated naturally occurring plant-derived substances as potential alternatives for *H. pylori* prophylaxis or treatment (O'Gara et al., 2000; Bergonzelli et al., 2003; Lin et al., 2005; Paraschos et al., 2007; Yang et al., 2008; De et al., 2009; Pastene et al., 2010). In particular, studies have shown that grape polyphenols have

strong anti-*H. pylori* activity; inhibiting growth (Mahady and Penland, 2000; Mahady et al., 2003) while reducing *H. pylori*- and vacuolating cytotoxin-induced gastritis in animal models (Tombola et al., 2003; Yahiro et al., 2005; Ruggiero et al., 2006; Ruggiero et al., 2007). We have previously reported that muscadine grapes (*Vitis rotundifolia*) are a valuable source of anti-*H. pylori* compounds with activity against multiple strains *in vitro* and *in vivo* with effects most likely due to major phenolic compounds (i.e. ellagic acid, myricetin, quercetin, *trans*-resveratrol, gallic acid) acting alone or in synergy (Brown et al., 2009; Brown et al., 2010). Polyphenols are naturally found in fruits and vegetables, especially grapes; however, the combination of ellagic acid, quercetin, and resveratrol is unique to muscadine species which may suggest that these compounds are largely responsible its reported biological activities (Mertens-Talcott and Percival, 2005).

Our earlier studies demonstrating the effectiveness of muscadine grapes and the pure flavonoid quercetin against *H. pylori* prompted us to further explore these products. The objective of this study was to identify active phenolic constituents in muscadine grape skin (MGS) extracts and determine if any synergism exists among compounds while further exploring their anti-*H. pylori* potential *in vitro*.

## Materials and Methods

**Chemicals.** Pure standards of ellagic acid, gallic acid, myricetin, quercetin, *trans*-resveratrol and amoxicillin were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO), ethanol, acetonitrile, methanol, acetic acid, EDTA, and water (HPLC grade) were purchased from Fisher Scientific (Norcross, GA).

**Muscadine grape skin extraction.** Four muscadine grape cultivars, namely, two purple (Carlos and Woodruff) and two bronze (Noble and Cowart) provided by the Clemson University Pee Dee Research and Education Center (Florence, SC) were used in this study. Fresh muscadine grapes were washed in distilled water to remove debris. Skins were collected and dried in a gravity convection oven (Fisher Scientific, Gaithersburg, MD) at 60 °C for 24 h, and ground into a fine powder. A modified method of Jayaprakasha et al. (2003) was used for extraction of total phenolics. Briefly, 5 ml of acetone:water:acetic acid (90:9.5:0.5, v/v/v) was added to 1 g dried MGS each in 10 ml screw-capped glass tubes, vortexed for 30 sec, and incubated in a 60 °C waterbath for 8 h with shaking. Samples were centrifuged at 4,500 x g for 10 min to remove debris. The supernatant was collected and the solvent was removed using a SpeedVac (Thermo Fisher Scientific, Waltham, MA) at 43 °C. Dried extracts were stored at -80 °C under nitrogen or resuspended in DMSO and used immediately.

**Total phenolics determination.** The Folin-Singleton colorimetric method was used to determine total soluble phenolic content in the extracts (Singleton and Rossi, 1965). Two hundred microliters of appropriately diluted extract were mixed with Folin-Ciocalteu phenol reagent (1:10, v/v). After 3 min, 800 µl of 7.5% $Na_2CO_3$ was added to the reaction mixture, vortexed for 5 sec., and allowed to stand at room temperature, in the dark, for 60 min. Absorbances were read at 750 nm using a BioTek µ-Quant microplate reader (BioTek Instruments, Inc. Winooski, VT). The optical density values (O.D.) were converted to total phenolics, and expressed as milligram gallic acid equivalents (GAE)/100 ml and then to mg GAE/g dry weight (DW). Each sample was measured in

triplicate and GAEs were calculated from the standard curve. Total soluble phenolic standard curves were prepared before each experiment using various concentrations of gallic acid (100, 200, 300, and 400 mg/l) in methanol.

**Major phenolics determination.** Major phenolics were determined using conditions described by Pastrana-Bonilla et al. (2003). Briefly, extracted samples were filtered through a 0.2 μm nylon syringe filter (VWR International, West Chester, PA) and injected into a Hewlett-Packard (Avondale, PA) HP 1090 HPLC (high-performance liquid chromatography) system with diode array and fluorescence detectors. The mobile phases were, solvent A, methanol/acetic acid/water (10:2:88, v/v/v); solvent B, acetonitrile; and, solvent C, water. The linear gradient used for phenolic separation was as follows: at 0 min., 100% solvent A; at 5 min. 90% solvent A and 10% solvent B; and at 25 min., 30% solvent A and 70% solvent B, with 5 min. postrun with 100% solvent C. The flow rate was 1 ml/min. The Regis REXCHROM C18 ODS 4.6 x 250 mm column was used with a column temperature of 45 °C. The sample injection volume was 20 μl. All extractions were performed under reduced lighting to protect phenolic compounds from degradation.

***Helicobacter pylori* strains and growth.** Two *cagA*- and *vacA*-positive *H. pylori* strains, 26695 and D5178 (a clinical isolate), were used in this study. Both strains were routinely grown on brain heart infusion (BHI) agar (Difco Laboratories, Detroit, MI) (pH 7.2 ± 0.2) supplemented with 7% horse serum (HS) (Sigma) at 37°C for 72 h under microaerophilic conditions (5% $O_2$, 10% $CO_2$, and 85% $N_2$) in a GasPak jar (BBL Microbiology Systems, Cockeysville, MD) (Jiang and Doyle, 2000). Plate-grown bacteria was

subcultured in BHI/HS broth (pH 7.2) and grown overnight at 37 °C in conical flasks, with shaking (120 rpm), under microaerophilic conditions.

**Active phenolics determination from MGS extracts.** To determine active phenolics, *H. pylori* 26695 was grown as described, washed twice in phosphate buffered saline (PBS, pH 7.4) and inoculated onto BHI-HS agar plates (ca. 5 x $10^7$ CFU/plate) using an Autoplate 4000 spiral plater (Spiral Biotech, Inc., Norwood, MA). Twenty-five microliters of each extract, adjusted to 2 mg/ml total phenolics, was added to sterile cotton disks (6 mm in diameter) (Becton, Dickenson and Company, Franklin Lakes, NJ), placed onto freshly inoculated plates, and incubated under microaerophilic conditions for 72 h or until visible growth occurred. Each test was performed in triplicate. Following incubation, zones of inhibition were measured to the nearest millimeter and visually compared to determine any differences among MGS extract effectiveness. After determining phenolic concentrations by HPLC for each extract, single phenolics and all possible combinations were tested against the same strain under identical conditions to deduce which phenolic(s) were most likely responsible for anti-*H. pylori* activity.

***In vitro* analysis of active compounds in combination against *H. pylori*.** Combination effects of quercetin and resveratrol were tested using a two-dimensional checkerboard assay (McLaren, 1997) in 96-well cluster trays (Corning Costar Corp., Cambridge, MA). Briefly, quercetin and resveratrol were suspended in DMSO, filter-sterilized, and added to BHI/HS broth yielding final concentrations ranging from 8 to 512 and 2 to 512 µg/ml, respectively. *H. pylori* was grown as previously described and added to wells containing compounds or broth plus DMSO (control) for a final concentration of

approximately 1 x $10^7$ CFU/ml. Plates were incubated under microaerophilic conditions, without shaking, for 24 h and 48 h. At each time point, wells were sampled using a replica plater (Sigma) and aseptically transferred (ca. 2 µl/pin) to BHI/HS agar plates supplemented with vancomycin (100 µg/ml), polymyxin B (3.3 µg/ml), bacitracin (200 µg/ml), amphotericin B (50 µg/ml), and nalidixic acid (10.7 µg/ml) (Sigma, St. Louis, MO) and incubated microaerobically for up to 1 week. Plates were scored by assessing the amount of growth on each spot. No growth = 0; 1-5 colonies = 1; 6-10 colonies = 2, 11-15 colonies = 3; 16-20 colonies = 4; > 20 colonies = 5; confluent growth = 6. Results were then analyzed by calculating the fractional bactericidal concentration (FBC) and consequent FBC index and mean FBC index. Synergy = mean FBC index of < 0.5; an additive effect = mean FBC index of 0.5-1; and antagonism = mean FBC index of > 1. All tests were performed in triplicate.

**Rate-of-kill analysis of compounds against *H. pylori.*** The bactericidal activity of quercetin, resveratrol, and amoxicillin were determined using a modification of the Miles-Misra method (McLaren, 1997). Briefly, *H. pylori* 26695 and D5178 was grown as previously described and resuspended in BHI/HS broth to a final concentration of ca. 5 x $10^6$ CFU/ml. Time-course killing effects were determined at both neutral (pH 7.4±0.2) and acidified (pH 5.8±0.2) conditions (the latter reflecting pH conditions in the human gastric juxtamucosal environment) (Fahey et al., 2002). Quercetin, resveratrol, and amoxicillin were tested at 0.5x, 1x, 2x, and 4x the MIC determined previously (Brown et al., 2010, data not shown). After 0, 1, 3, 6 and 24 h of incubation at 37°C under static, microaerophilic conditions, samples were serially diluted in 0.85% saline and plated onto

BHI/HS supplemented with the antibiotic mixture previously described. Untreated *H. pylori* was plated as a control. Tests were performed in triplicate and expressed as mean $\log_{10}$ CFU/ml.

**Uptake of 1-*N*-phenylnapthylamine (NPN) by *H. pylori*.** NPN uptake assays were performed as described by Bina et al. (2000) with some modifications. Briefly, overnight liquid cultures of *H. pylori* 26695 were harvested by centrifugation (3,500 x g, 10 min), washed twice in 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.4) containing 10 µM $MgCl_2$, and resuspended to an O.D. at 600 nm of 0.5 in 5 mM HEPES-5 mM glucose. Cell suspensions were aliquoted and used immediately in the uptake assays. NPN fluorescence was measured using black 96-well plates (Greiner Bio-One Inc., Monroe, NC). Aliquots (100 µl) of the cell suspension were added to each well containing 50 µl of quercetin (40 µM, final concentration), resveratrol (40 µM), EDTA (1 and 0.5 mM) or HEPES buffer (with and without $MgCl_2$) as a control. Fluorescence measurements were performed using a SPECTRAmax GEMINI microplate spectrofluorometer (Molecular Devices, Inc., Sunnyvale, CA) with an excitation wavelength of 350 nm and an emission wavelength of 420 nm. Following the addition of NPN (10 µM final concentration), measurements were taken following 30 min incubation at 37 ° C, with shaking before each read. All NPN assays were performed in triplicate.

**Uptake of quercetin and resveratrol by *H. pylori*.** To determine quercetin uptake, *H. pylori* 26695 was grown in BHI/HS broth overnight to mid-logarithmic phase (15-16 h) determined by a previous growth curve (data not shown). Cells were pelleted

by centrifugation (3,500 x g, 10 min), washed once in sterile PBS (pH 7.4±0.2), and re-suspended in BHI broth (without HS) to a final concentration of ca. 1 x $10^8$ CFU/ml. Heat-inactivated controls were prepared by boiling cells (100 °C) for 15 min. After cooling to room temperature, quercetin was added to live and heat-inactivated cell suspensions at a final concentration of 256 µg/ml. In addition, quercetin (in DMSO) was added to broth as a cell-free control. Cells were incubated microaerobically at 37 °C for 0, 1, 2, and 3 h. At each time point, *H. pylori* counts were determined by serially diluting samples in 0.85% saline, plating onto BHI/HS, and incubating microaerobically for up to 1 week. Additional samples were also collected at each time point and analyzed by HPLC under the described conditions. For this, 5 ml aliquots were collected and centrifuged at 12,000 x g for 10 min at 4°C. Supernatants were removed and stored at -20 °C until analysis. Bacterial pellets were washed twice in sterile PBS (pH 7.4) and resuspended to 1 ml in the same buffer. Cells were then subjected to ultrasound for 8 min at 30 sec intervals with a 1 min cooling period between pulses. All samples were kept on ice during cell lysis. Sonicates from each collection time were plated onto selective media described previously to verify total cell lysis. Cell material was pelleted by centrifugation at 4,000 x g for 10 min at 4 °C and the supernatant was collected for immediate HPLC analysis using the same conditions described above.

**Salt aggregation assay.** Salt aggregation assays were done to determine the *H. pylori* cell surface hydrophobicity in the presence of quercetin, resveratrol, or MGS. Procedures were performed as described by Annuk et al. (1999) with some modifications. Briefly, overnight liquid cultures of *H. pylori* 26695 were harvested by centrifugation

(3,500 x g, 10 min) and resuspended in 0.01 M $NaPO_4$ (pH 7.4) with 0.15 M NaCl and adjusted to an O.D. at 600 nm of 1.0. Fifty-microliters of ammonium sulfate diluted in 0.02 M $NaPO_4$ buffer (0.05 M-3.0 M) was mixed with an equal volume of *H. pylori* cell suspension (control) or suspensions treated with quercetin, resveratrol, or MGS extract for 15 min at room temperature and added to U-shaped microwells (Nalge Nunc International, Rochester, NY). Plates were incubated for 3 h at room temperature under microaerophilic conditions and visually compared to determine aggregation. Salt aggregation was defined as positive if bacterial aggregation was clearly visible and negative if no aggregation was observed. The salt aggregation titer was defined as the lowest concentration of ammonium sulfate at which bacteria still showed cell aggregation. All tests were performed in duplicate.

**Phase-contrast microscopy of *H. pylori.*** For phase-contrast microscopy, scoring criteria described by O'Gara et al. (2000) were used with some exceptions. Briefly, *H. pylori* 26695 was grown in BHI/HS broth overnight as described, washed once in 0.85% saline, and resuspended in BHI/HS broth to ca. 1 x $10^8$ CFU/ml. Cell suspensions were treated with quercetin (256 µg/ml) or DMSO (control) and incubated at 37 °C under microaerophilic conditions. At 0, 1, 3, 6 and 24 h, cells were collected and observed using a Leica DM E microscope with phase-contrast settings (Leica Microsystems GmbH, Wetzlar, Germany) to compare time-course bactericidal data with real-time phenotypic changes. For scoring, the average of at least five fields of view per time point was used. All experiments were performed in duplicate.

**Statistical Analysis.** One and two-way ANOVA tests were used to determine differences between data. All values were considered statistically significant at $P < 0.05$ using SAS 9.1 software (SAS Institute Inc., Cary, NC).

## Results

**Major and active phenolics determination from MGS extracts.** Total phenolics were determined from four MGS samples following extraction and ranged from 67.9 to 109.8 mg GAE/g DW of starting material. Standardized extracts (2 mg total phenolics/ml) were then evaluated for antimicrobial activity (Figure 3.1) and subjected to HPLC. This was done to reveal any measurable difference in major phenolic profiles and determine selected phenolic concentrations (ellagic acid, myricetin, quercetin, *trans*-resveratrol, gallic acid). These compounds were chosen because they constitute much of the total phenolics present in MGS as determined previously, have been chemically characterized, are commercially available, and have previously been reported to possess antimicrobial properties against a wide variety of organisms, including *H. pylori*.

Major phenolics were identified by their retention times and characteristic spectra from HPLC chromatograms. Quantification was made by comparing peak areas to those obtained from phenolic standards at known concentrations. Selected compounds were also verified by UV-Vis spectrometry. Table 3.1 shows the major phenolics identified and quantified in selected muscadine cultivars.

Following chemical analysis, pure phenolics at equivalent concentrations to those in Table 3.1 were tested alone and in combinations against *H. pylori* 26695 by disk-diffusion in order to elucidate the most active phenolic(s). Inhibition zones were meas-

ured and compared to those using MGS extracts to determine which compound(s) was most effective against *H. pylori* growth and if any additive/synergistic effect was evident between compounds (Table 3.2).

Based on disk diffusion results, MGS extracts were the most effective against *H. pylori* growth; suggesting possible synergistic action among compounds present in MGS preparations. To further support this hypothesis, all major phenolic compounds were then combined at concentrations equivalent to those determined previously by chemical analyses (Table 3.2). However, the same result (i.e. inhibition diameter) could not be obtained; suggesting the presence of additional unidentified antimicrobial components in MGS not determined in this study. Although we were unable to determine the definitive compound(s) responsible for activity using MGS, quercetin repeatedly showed the highest anti-*H. pylori* activity with large zones of inhibition, clearly defined edges, and no observable tailing. When combined with resveratrol, quercetin showed a marked increase in activity. Therefore, quercetin and resveratrol were selected for further evaluation.

**Bactericidal activity of quercetin and resveratrol alone and in combination.** Having established the bacteriostatic activity of quercetin previously (MIC, 32-64 µg/ml) and resveratrol (MIC, 16-32 µg/ml) against multiple *H. pylori* strains (Brown et al., 2009; Brown et al., 2010), we next evaluated the compounds' bactericidal potency by using a time-to-kill assay with the reference strain (26695) and a clinical isolate (D5178). All experiments were run in parallel at pH 7.4 (neutral) and 5.8 (acidic) since most *H. pylori* colonize the mucous layer and gastric pits of the antrum where the pH is believed to be

~5.5 (Fahey et al., 2002). Bactericidal activity was defined as a reduction in plate counts of ≥1,000 CFU/ml (Fahey et al., 2002). In addition to quercetin and resveratrol, amoxicillin was chosen as a reference drug because of its low MIC (MIC, 0.25 µg/ml) and since resistance has not been extensively reported in *H. pylori*. In time course studies with quercetin and resveratrol (Figure 2), similar dose-dependent patterns upon *H. pylori* 26695 and D5178 were observed at all concentrations and the MICs and MBCs corresponded appropriately with those obtained in additional 24-h MIC and MBC experiments. Lag phages of 1-3 h were observed. Anti-*H. pylori* activity was greater (2-fold-lower MIC and MBC) for resveratrol than quercetin. There was no effect of changes in pH over the range examined with either strain or any of the compound concentrations tested. At all of the subinhibitory concentrations (1X and 0.5X the MIC) of quercetin and amoxicillin tested against strains 26695 and D5178, significant reductions ($P > 0.05$) in viable cell counts were not observed at any time during the assay. Interestingly, 2X concentrations of resveratrol did not result in significant reductions ($P > 0.05$) over the 24 h treatment period (similar to lower doses); suggesting that a concentration threshold must be achieved before resveratrol activity is bactericidal against *H. pylori*.

Since both quercetin and resveratrol showed a concentration- and time-dependent effect on *H. pylori* ($P < 0.05$) and preliminary tests suggested that quercetin and resveratrol may act synergistically or additively against *H. pylori* growth, both compounds were tested using a checkerboard assay for 24 and 48 h with results shown in Figure 3.3.

From checkerboard assays, quercetin and resveratrol together showed synergistic activity against *H. pylori* 26695 with an FBC index of ≤ 0.5 at both 24 and 48 h. Minim-

al bactericidal concentrations of quercetin and resveratrol required to completely kill *H. pylori* were lower with extended treatment time. The lowest combined concentrations of quercetin and resveratrol required to completely kill *H. pylori* was 16 µg and 128 µg/ml (24 h) and 8 µg and 64 µg/ml (48 h), respectively.

**Effect of quercetin and resveratrol on *H. pylori* membrane permeability.** Figure 3.4 shows the results of NPN uptake with quercetin, resveratrol, or EDTA (a permeabilizer acting by chelation) with or without $MgCl_2$. EDTA weakened the outer membrane of *H. pylori* as indicated by strong increases in NPN uptake. Neither quercetin nor resveratrol enhanced NPN uptake suggesting that their effects on *H. pylori* are likely not associated with reducing outer membrane integrity. The addition of $MgCl_2$ had no significant ($P > 0.05$) impact on fluorescence levels with any treatment other than EDTA.

**Quercetin uptake by *H. pylori*.** Quercetin and resveratrol did not affect *H. pylori* outer membrane integrity as revealed by NPN assays. Therefore, we next set out to determine the degree of quercetin uptake by *H. pylori* as reported in other cell types. To do this, *H. pylori* 26695 was treated with quercetin for 1, 2, and 3 h under conditions described previously. HPLC was then used to measure quercetin uptake in live, metabolically active *H. pylori* cells while measuring overall flavonoid losses in culture supernatant, inactive cell biomass (i.e. cell membranes/protein) and cell-free controls (to account for possible oxidation losses) to confirm active drug uptake and validate our approach.

Quercetin levels in live *H. pylori* cells were highest following 1 h incubation with 20.2 µg/ml detected in cell lysates. At 2 and 3 h, quercetin levels decreased to 14.7 and 11.5 µg/ml, respectively, while at the same time reducing *H. pylori* counts approximately

0.2 logs (Figure 3.5). Of the initial quercetin amount added (256 µg/ml), inactive cell biomass accounted for 3.5% of quercetin losses whereas culture supernatants and cell-free controls accounted for losses of 24.1 and 18.6%, respectively (Figure 3.6).

**Morphological and physiological changes in *H. pylori* following quercetin treatment.** Phase-contrast microscopy revealed noticeable changes in both the behavior and morphology of *H. pylori* cells treated with quercetin (Table 3.3 and Figure 3.7). Within 1 h of exposure to quercetin (256 µg/ml), vigorous motility declined and 10 to 15% of cells, previously spiral rod-shaped, appeared as coccoidal forms. In addition, cell clumping was clearly visible and increased with time, especially following 3 h treatment. Within 6 h, viability dropped approximately 2 logs with the number of spiral rods below 10%. At 24 h, all cells were coccoidal and non-motile with large amounts of cellular debris clearly visible.

**Cell surface hydrophobicity following quercetin, resveratrol, or MGS treatment.** To determine *H. pylori* cell surface hydrophobicity (CSH), the salt aggregation (SA) assay was performed. *H. pylori* 26695 in phosphate buffer did not aggregate at or below 1.5 mM ammonium sulfate during incubation. Quercetin and resveratrol (alone and in combination) slightly enhanced cell aggregation while MGS extract showed the greatest effect with cell aggregation observed at the lowest salt concentration tested (Table 3.4).

## Discussion

Polyphenols possess significant anti-*H. pylori* activity as shown previously (Brown et al., 2009; Brown et al., 2010). However, the primary bioactive component(s)

in MGS extracts against *H. pylori* has yet to be definitively identified but is believed to be a major phenolic based on earlier reports and established chemical profiles (ellagic acid > myricetin > quercetin > resveratrol > gallic acid). Therefore, in this study, further work was done to elucidate the most active chemical constituent(s) in MGS responsible for anti-*H. pylori* activity and explore some of the proposed interactions between these compounds and *H. pylori*. Following extraction and determination of phenolic concentrations among four muscadine grape varieties, and subsequent *in vitro* screening, it was found that quercetin, while in association with resveratrol, yielded the greatest anti-*H. pylori* activity.

Quercetin (3, 3′, 4′, 5, 6-pentahydroxyflavone) is a naturally-occurring dietary flavonol found in MGS and one of the most widely studied phenolics known. Flavonols exhibit numerous biological and pharmacological effects, including antioxidant, chelation, anti-carcinogenic, cardioprotective, antibacterial, and secretory properties (Gross et al., 1996, Middleton et al., 2000). Resveratrol (3, 5, 4′-trihydroxystilbene) is a naturally occurring stilbene found in grapes and red wine and also exhibits many physiological effects associated with health benefits.

When evaluating antimicrobial agents against *H. pylori*, several important factors must be taken into consideration given its basic physiology and environmental niche. When assessing the suitability of an agent for the reduction/eradication of *H. pylori*, speed of kill (i.e. bactericidal activity) is critical as most agents have a short lifespan in the stomach (Millar and Pike, 1992). In addition, acid stability and emergence of resistant variants must also be evaluated. Following time-course viability studies, quercetin

and resveratrol both demonstrated cidal modes of action against *H. pylori* regardless of pH conditions. Although MBCs were high compared to amoxicillin, neither quercetin nor resveratrol have any reportable toxicity and are generally regarded as safe at high levels. To analyze drug interactions, a checkerboard assay was used and revealed a synergistic mode of action against *H. pylori*. MBCs of both quercetin and resveratrol were significantly lower when used in combination suggesting that these compounds would be most effective when taken together. Finally, since quercetin and resveratrol are believed to affect unique cellular targets simultaneously, the possibility of drug resistance may be reduced.

Polyphenols have been reported to interact with various biological components such as cell membrane proteins and DNA (Kaldas et al., 2005; Walle et al., 2003). Because of its hydrophobic nature, the flavonoid quercetin may also interact with sites such as the lipid bilayer of cell membranes (LoCascio et al., 2006) disrupting normal proton pump function (i.e. electron transport and $H^+$-ATPase) resulting in loss of proton motive force. In addition, polyphenols may interact with cellular (e.g. transmembrane) and/or secreted proteins (55- to 80-kDa) (Kaldas et al., 2005); altering their conformation/activity, destabilize membranes causing cells to become 'leaky', or simply accumulate in the plasma membrane resulting in loss of function and cell death. We previously reported that MGS extracts and constitutive polyphenols may also affect *H. pylori* attachment while reducing VacA-induced vacuolation in AGS (CRL-1739) cells (Brown et al., 2009). Other studies have also shown the effectiveness of polyphenols against *H. pylori* growth, toxicity, urease production, and adherence (Yahiro et al., 2005; Lin et al.,

2005; Burger et al., 2000; Pastene et al., 2011); thereby supporting results from our earlier studies and emphasizing that these compounds, although diverse in origin, may share some common structural feature(s) which allow them to exert similar activities against *H. pylori*.

In order to determine the some of the possible interactions between quercetin and *H. pylori*, two different approaches were used. To determine whether quercetin or resveratrol affect *H. pylori* via disruption of its outer membrane (OM), 1-*N*-phenylnaphthylamine (NPN) uptake in *H. pylori* 26695 was measured following exposure to these compounds or EDTA in the presence or absence of $MgCl_2$. NPN is a small hydrophobic fluorescent probe used to determine the hydrophobic and self-promoted uptake pathways across the outer membranes of gram-negative bacteria (Bina et al., 2000). Fluorescence results from this probe associating with a glycerophospholipid environment such as the lipid bilayers of the OM interior. Therefore, increased fluorescence values indicate weakening of the biological membrane. NPN is normally excluded from the OM by the outer monolayer comprised of the polyanion lipopolysaccharide, stabilized by divalent cross bridging and fluoresces only weakly in the extracellular aqueous environment (Bina et al., 2000). Although many bacteria require destabilization of the OM before uptake of hydrophobic antibiotics, usually through a destabilizing polycation such as polymyxin B, *H. pylori* has been reported to demonstrate relatively high intrinsic uptake of NPN compared to other bacterial types.

Neither quercetin nor resveratrol increased NPN fluorescence levels in *H. pylori* compared to no-treatment controls, regardless of time or concentration; suggesting a lack

of damage to the OM. However, the possibility of active NPN efflux could not be completely ruled out. Observations of *H. pylori* by phase-contrast microscopy revealed that cells in the presence of quercetin, with glucose (5 mM), appeared to become deenergized over time via reduced motility possibly suggesting the absence of active NPN efflux. This may also suggest that the plasma membrane is affected; however, this was not determined.

Since neither quercetin nor resveratrol affected OM integrity, we next chose to examine quercetin uptake by *H. pylori*. Quercetin has been reported to rapidly accumulate in the cytosol and mitochondria of eukaryotes (Fiorani et al., 2005; Walgren et al., 2000); however, its transport into bacteria has yet to be fully established. Quercetin was previously found to enter both aerobic and anaerobic, gram-positive bacteria and *Saccharomyces cerevisiae* while in resting states (Braune et al., 2001; Schoefer et al., 2003; Lo-Cassio et al., 2006). To date, no reports exist regarding quercetin uptake by *H. pylori*. However, given its relatively high sensitivity to hydrophobic antimicrobials, including numerous plant-derived compounds (many with low water solubility) (and NPN as discussed above), we believe that quercetin may enter the cell via non-specific OM porins such as HopE, forming large channels compared to less sensitive gram-negatives. This may permit passage into the periplasm and subsequent accumulation of quercetin in the cytosol via passive or active transport systems.

To determine quercetin uptake, *H. pylori* cells and culture supernatants were measured to determine intracellular compound accumulation. Because quercetin may oxidize and degrade during extended incubation, cell-free negative controls were in-

cluded for each trial. In addition, because quercetin may passively bind to various components in the cytoplasmic and/or OM, basal levels of quercetin absorption onto metabolically inactive cell biomass were determined using heat-inactivated *H. pylori* controls. Therefore, any difference between quercetin detected in extracts of live cells and heat-inactivated cells, cell-free controls, or culture supernatants would indicate active uptake. Because bacterial numbers declined during treatment, accumulation rates and drug efflux could not be determined. However, if quercetin was pumped out of the cell, levels should have increased over time in cell supernatants; minus oxidation and losses due to washing and/or extraction (lysis efficiency was 100% since no detectable growth following sonication). Differences in quercetin levels gained or lost may be attributed to its intracellular degradation by metabolically active *H. pylori*. This may further explain why quercetin levels decreased over time in cell pellets but were not detected in corresponding supernatants; however, no metabolites were revealed by our chromatographic method.

Following treatment, relatively high levels of quercetin were detected in live *H. pylori* extracts. Therefore, four possibilities were proposed. Quercetin may have 1) intercalated in the hydrophobic region of the cell membrane lipid bilayer, 2) passively diffused through the cell membrane into the cell cytosol, 3) interacted with cell membrane proteins, or 4) been actively imported into the cytosol. Also, it should be noted that a combination of these factors may have contributed to quercetin detection. However, if quercetin did passively intercalate within the cell membrane or associate with cell membrane proteins, similar amounts should have been detected in heat-inactivated controls as

live, metabolically active cells. Because quercetin levels were low in controls compared to live cell extracts, we believe that quercetin must have entered the cytosol.

By tracking *H. pylori* cells exposed to high concentrations of quercetin via phase microscopy, there appeared to be an increase in coccoidal cell morphology and reductions in spiral and rod forms over time. This result corresponded well with overall reduced cell viability and cell motility, possibly indicating a loss of proton motive force and/or active drug efflux. Increases in cellular debris following 24 h exposure indicates progressive cellular destruction, suggesting that quercetin and other polyphenols may interfere with the bacterial cell envelope, causing cell lysis, or with cell metabolism, triggering autolysis. Based on uptake assays (NPN and HPLC), the latter is more likely; however, this has yet to be fully determined.

Pathogenic bacteria commonly attach to target tissues via species-specific adhesion receptor mechanisms. However, microbial cell surface hydrophobicity (CSH) is also associated with binding to the specific cell and tissue receptors of the host mucosal surface. Given the results from our initial study (Brown et al., 2009) and phase-contrast microscopy, *H. pylori* cells appeared to aggregate following treatment with quercetin. *H. pylori* does not possess high CSH as shown by the SA assay. However, some strains have been found to have high CSH as reported following hydrophobic interaction chromatography (Smith et al., 1990). For many bacterial infections, adhesive strains often possess high CSH values as determined by SA and other methods. It has been proposed that the relatively low CSH of *H. pylori* may allow the organism to attach and then penetrate the gastric mucus layer. Following treatment with quercetin, resveratrol, and MGS

extract, only moderate increases in CSH were measured for both quercetin and resveratrol. However, MGS extract treatment greatly increased CSH (corresponding cell aggregation at low salt concentration) and may suggest other polyphenols may also interfere with initial attachment of *H. pylori*.

In this study, we examined the anti-*H. pylori* properties of different muscadine grape cultivars and determined that the major phenolics quercetin and resveratrol located in the skin have the strongest anti-*H. pylori* activity *in vitro*, especially when used in combination, and are independent of pH. Neither quercetin nor resveratrol were found to affect *H. pylori* OM integrity or significantly alter CSH as previously hypothesized. However, treatment with MGS did increase NPN uptake; indicating OM destabilization, and increase CSH possibly by additional unknown components not revealed in this study. Furthermore, quercetin was found to decrease *H. pylori* motility while being transported into the cell cytosol suggesting its primary anti-*H. pylori* activity may be linked to its interaction with intracellular components; however this has yet to be fully determined. Our results indicate that MGS extracts and constitutive phenolics quercetin and resveratrol exert strong anti-*H. pylori* activity and may have the potential to be incorporated into an effective, diet-based approach for prevention and/or treatment of *H. pylori* infection.

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## Figure Legend

**FIGURE 3.1.** Photograph of *H. pylori* 26695 plated on BHI/HS following treatment with standardized MGS (2 mg/ml total phenolics) extracts by the disk-diffusion method.

**FIGURE 3.2.** Time-course bactericidal activity of quercetin (A), resveratrol (B) and amoxicillin (C) on *H. pylori* 26695 after exposure to 0.5x, 1x, 2x, and 4x the MIC at pH 7.4 (a) and pH 5.8 (b). MICs were 32 and 16 µg/ml for quercetin and resveratrol, respectively.

**FIGURE 3.3.** Synergistic activities of quercetin and resveratrol against *H. pylori* 26695 at 24 (A) and 48 (B) h. FBC index $\leq 0.5$.

**FIGURE 3.4.** Uptake of NPN by *H. pylori* 26695 in the presence of EDTA, MGS, quercetin (40 µM, 13.3 µg/ml) and resveratrol (40 µM, 9.1 µg/ml) with or without $MgCl_2$. MGS, muscadine grape skin extract (~1 mg total phenolics/ml); RFU, relative fluorescence units.

**FIGURE 3.5.** Uptake of quercetin into live *H. pylori* 26695 cells over time.

**FIGURE 3.6.** Quercetin distribution following 3 h incubation of *H. pylori* 26695 with 256 µg/ml of quercetin.

**FIGURE 3.7**. Phase-contrast microscopy images of *H. pylori* 26695 during exposure to quercetin (256 µg/ml) for 1 (A), 2 (B), and 3 (C) hours. Magnification, x1000.

**TABLE 3.1.** Major phenolics in muscadine grape skin (MGS) extracts as analyzed by HPLC.

| | Major phenolic (µg/ml extract)[a] | | | | |
|---|---|---|---|---|---|
| Cultivar | ellagic acid | myricetin | quercetin | resveratrol | gallic acid |
| Carlos | 728.7 | 230.0 | 96.0 | 24.4 | 119.8 |
| Woodruff | 764.1 | 70.6 | 125.8 | 69.9 | 102.3 |
| Noble | 411.0 | 133.0 | 21.4 | 83.3 | 125.7 |
| Cowart | 739.8 | 113.3 | 210.4 | 181.1 | 34.5 |

[a]Approximate concentration calculation based on 200 µg/ml standards. All MGS samples contained ~2 mg/ml of total soluble phenolics.

**TABLE 3.2.** *H. pylori* 26695 inhibition by major phenolic combinations by disk-diffusion.

| phenolic combination | Total phenolics (µg/ml) | Zone diameter (mm)[a] |
|---|---|---|
| ellagic acid | 739.8 | 9 |
| ellagic acid, myricetin | 853.1 | 9 |
| ellagic acid, quercetin | 950.2 | 11 |
| ellagic acid, resveratrol | 920.9 | 10 |
| ellagic acid, gallic acid | 774.3 | 9 |
| myricetin | 113.3 | 10 |
| myricetin, quercetin | 323.7 | 13 |
| myricetin, resveratrol | 294.4 | 12 |
| myricetin, gallic acid | 147.8 | 11 |
| quercetin | 210.4 | 13 |
| quercetin, resveratrol | **391.5** | **16** |
| quercetin, gallic acid | 244.9 | 13 |
| resveratrol | 181.1 | 12 |
| resveratrol, gallic acid | 215.6 | 11 |
| gallic acid | 34.5 | 8 |
| ellagic acid, myricetin, quercetin, resveratrol, gallic acid | 1279.1 | 17 |
| MGS extract | **2000.0** | **20** |
| DMSO | 0 | 0 |

[a]Average of three independent experiments; MGS, Cowart muscadine grape skin; DMSO, solvent control.

**TABLE 3.3.** Visible effects of quercetin (256 µg/ml) on *H. pylori* 26695 by phase-contrast microscopy.

| Time (h) | No. of spiral rods[a] | Clumping[b] | Cocci (%) | Motility[c] | Viable count $\log_{10}$ |
|---|---|---|---|---|---|
| 0 | >25 | 0 | 0 | +++ | 7.8 |
| 1 | 15-25 | 1 | 10-15 | ++ | 7.5 |
| 3 | 1-10 | 2 | 25-30 | + | 7.2 |
| 6 | <10 | 3 | 50-75 | - | 5.4 |
| 24 | 0 | 3 | 100 | - | 3.5 |

[a]Number of rods observed per field of view.
[b]Clumping rated on a scale of 1 to 4 as follows: 1, motile clumps containing ≤10 cells; 2, nonmotile clumps containing 10 to 15 cells; 3, nonmotile clumps containing 15 to 20 cells; 4, nonmotile clumps containing ≥20 cells.
[c] -, no movement; +, slow; ++, moderate; +++ fast movement.

**TABLE 3.4.** Salt aggregation of *H. pylori* 26695 treatment with quercetin, resveratrol, or MGS extract.

| Treatment | Ammonium sulfate concentration (M) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 3.0 | 1.5 | 1.0 | 0.5 | 0.25 | 0.1 | 0.05 | Control[a] |
| *H. pylori* | + | - | - | - | - | - | - | - |
| Quercetin | + | + | + | - | - | - | - | - |
| Resveratrol | + | + | - | - | - | - | - | - |
| Synergy[b] | + | + | + | - | - | - | - | - |
| MGS[c] | + | + | + | + | + | + | + | - |

[a] Control, *H. pylori* treated with DMSO in phosphate buffer without $(NH_4)_2SO_4$.
[b] Synergy, quercetin (256 µg/ml) and resveratrol (128 µg/ml).
[c] MGS, muscadine grape skin (2 mg GAE phenolics/ml).
[d] +, cell aggregation
[e] -, no cell aggregation

**FIGURE 3.1.**

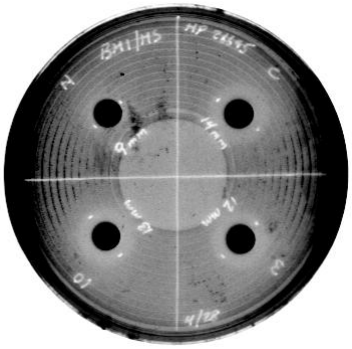
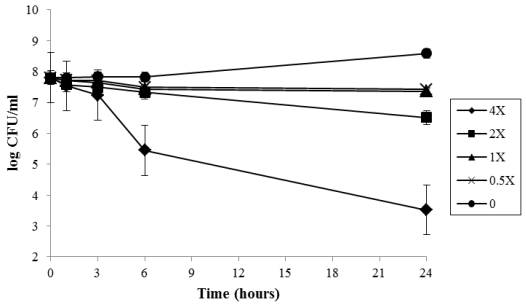
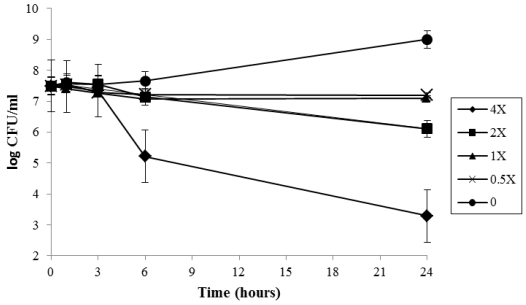


**FIGURE 3.2.**

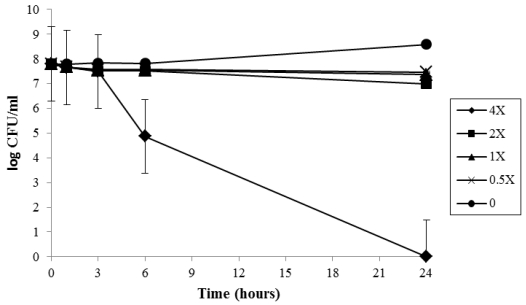
**Aa**



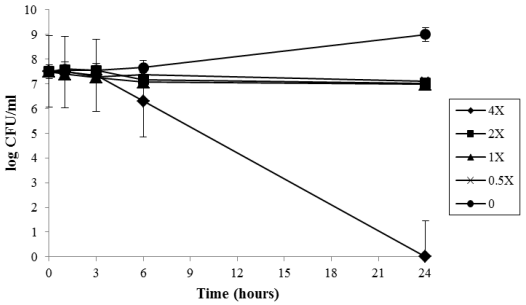

**Ab**



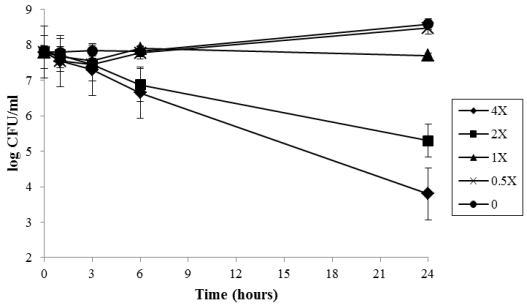

**Ba**




**Bb**




**Ca**




**Cb**

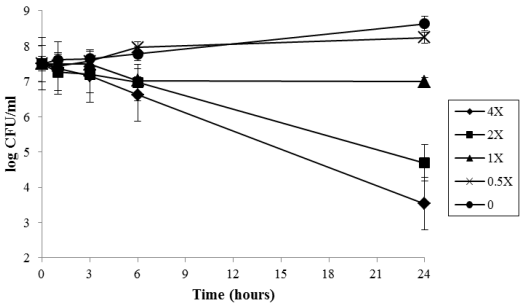
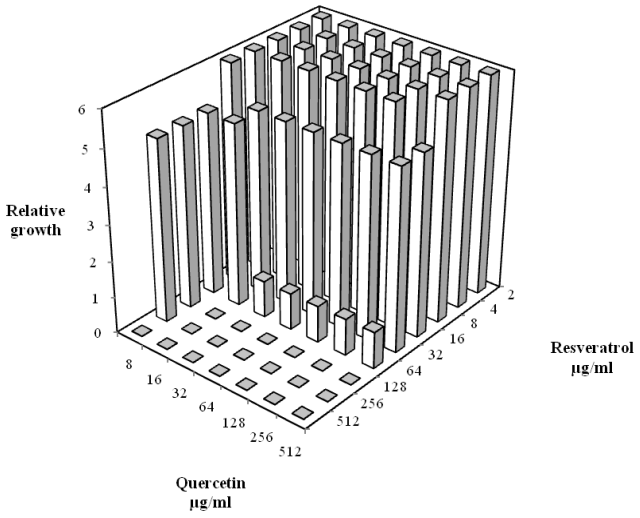


**FIGURE 3.3.**

**A**


Relative growth
0
1
2
3
4
5
6
8
16
32
64
128
256
512
Quercetin µg/ml
2
4
8
16
32
64
128
256
512
Resveratrol µg/ml

**B**

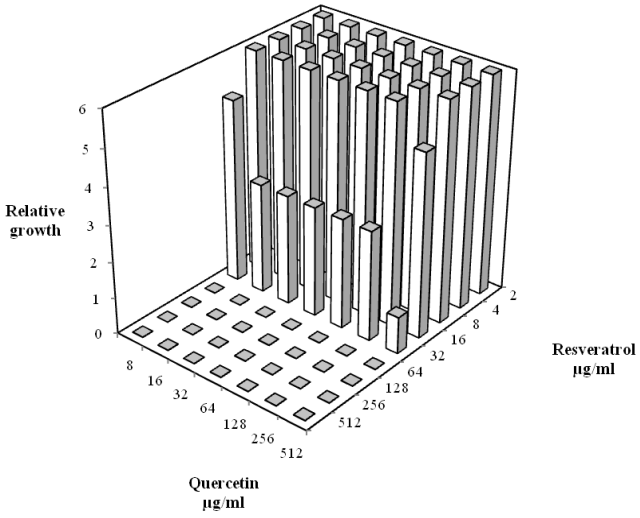

Relative growth
0
1
2
3
4
5
6
8
16
32
64
128
256
512
Quercetin µg/ml
2
4
8
16
32
64
128
256
512
Resveratrol µg/ml

**FIGURE 3.4.**

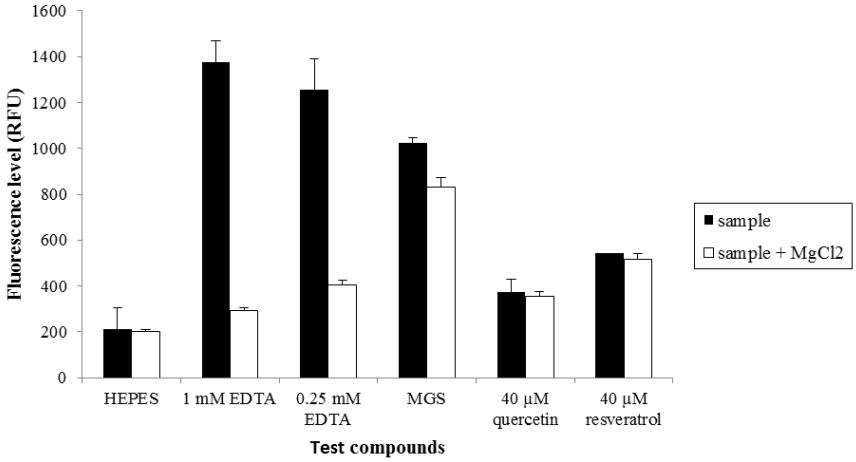

1600
1400
1200
1000
800
600
400
200
0
Fluorescence level (RFU)
HEPES
1 mM EDTA
0.25 mM EDTA
MGS
40 µM quercetin
40 µM resveratrol
Test compounds
sample
sample + MgCl2

**FIGURE 3.5.**

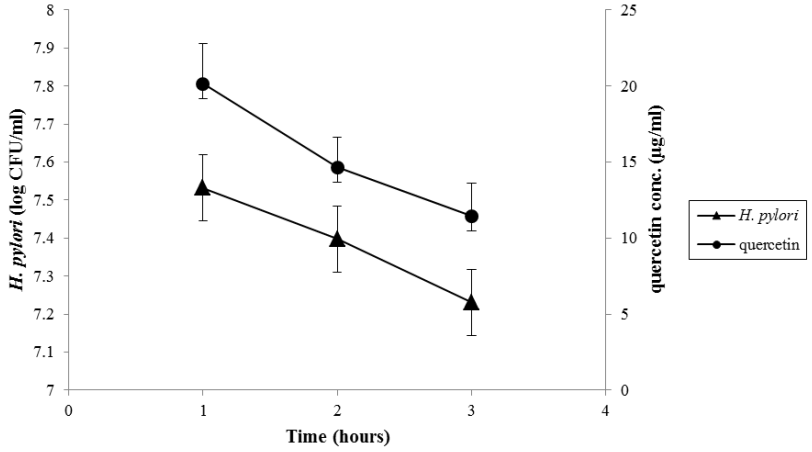

8
7.9
7.8
7.7
7.6
7.5
7.4
7.3
7.2
7.1
7
H. pylori (log CFU/ml)
25
20
15
10
5
0
quercetin conc. (µg/ml)
0
1
2
3
4
Time (hours)
H. pylori
quercetin

**FIGURE 3.6.**

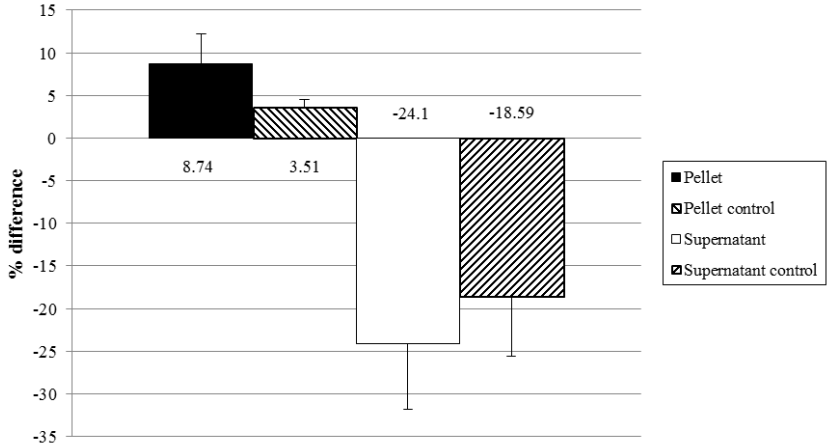
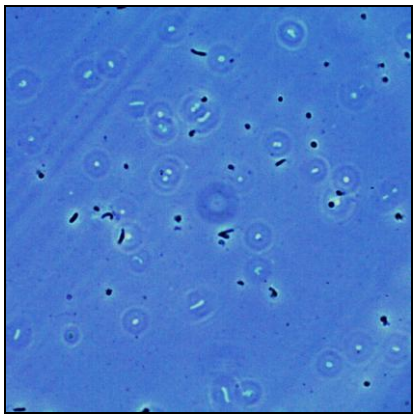
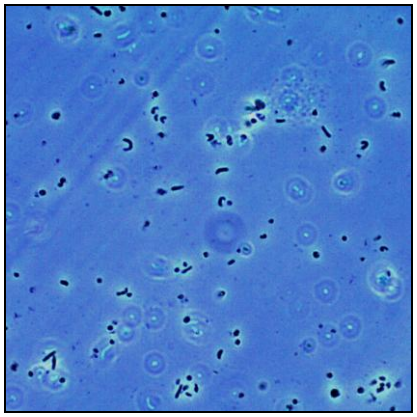

15
10
5
0
-5
-10
-15
-20
-25
-30
-35
% difference
8.74
3.51
-24.1
-18.59
Pellet
Pellet control
Supernatant
Supernatant control

**FIGURE 3.7.**

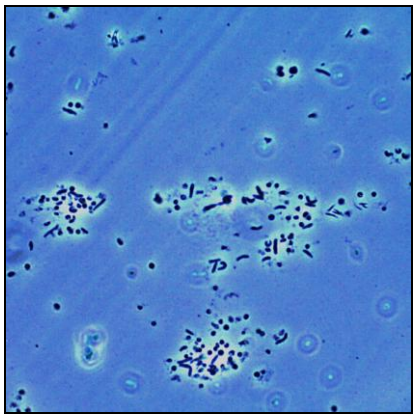
**A**



**B**



**C**



# CHAPTER FOUR

# GENE EXPRESSION OF *HELICOBACTER PYLORI* TREATED WITH MUSCADINE GRAPE SKIN EXTRACT OR ITS POLYPHENOLIC CONSTITUENTS QUERCETIN AND RESVERATROL


## Abstract

The effects of sub-inhibitory concentrations of muscadine grape skin extract (MGSE) and constitutive polyphenols quercetin and resveratrol on *Helicobacter pylori* transcripts were determined by microarray analyses. Following 15 min exposure, 63, 39, and 29 genes were found to be differentially expressed in MGSE-, quercetin- and resveratrol-treated cells, respectively, with only one overlapping gene detected between resveratrol and quercetin treatments. Interestingly, ATP synthase was induced upon quercetin and MGSE exposure while shikimate 5-dehydrogenase was induced in quercetin-treated cells. In addition to highlighting possible primary targets of quercetin and resveratrol, *cagA* and *ureA* genes were significantly repressed as confirmed by quantitative reverse transcriptase PCR (qRT-PCR). This may further support the potential use of plant-based polyphenolic compounds for *H. pylori* prevention and/or treatment. This study also demonstrates that with low inhibitor concentration and short incubation periods, relatively few genes show a clear change in expression following antimicrobial treatment and may be optimal for the analysis of primary response mechanisms in *H. pylori* exposed to uncharacterized natural compounds. Results from this study may be useful in determining quercetin or resveratrol's primary mode(s) of action against *H. pylori* in the future; however, additional studies are necessary to confirm these predicted targets.

## Introduction

*Helicobacter pylori* is a microaerophilic, gram-negative bacterium and among the most persistent of all human pathogens. Its presence in the gastric environment is correlated with diseases such as gastritis, peptic ulcers, mucosa-associated lymphoid tissue lymphoma, and gastric carcinoma (Cover and Blaser, 1995; Rothenbacher and Brenner, 2003). The continued emergence of antibiotic-resistant strains emphasizes the need to identify new therapeutic targets for the treatment and/or prevention of *H. pylori* infection. It has been shown that people consuming diets rich in fruits and vegetables have a lower incidence of infection and severe gastric malignancies and that this may be due to constitutive bioactive compounds acting against *H. pylori* (Testerman et al., 2001).

We believe that alternative, diet-based strategies may have a significant impact on attenuating this bacterium when used alone or in combination with current antibiotic regimes; however, natural compounds used should be better studied to understand their full therapeutic potential. In addition, cellular targets should be elucidated prior to use to determine possible interactions they may elicit on their conventional antimicrobial counterparts. Muscadine grapes, because of their high levels of bioactive compounds (e.g. ellagic acid, myricetin, quercetin, resveratrol, etc.) and unique chemical makeup, are believed to be a proper candidate for study since these fruits have already been shown to exert anti-*H. pylori* activity both *in vitro* and *in vivo* with effects possibly related to bacterial attachment or virulence factors (Brown et al., 2009; Brown et al., 2010).

Previous studies have reported the anti-*H. pylori* activity of quercetin and resveratrol as well as numerous plant-based extracts (Gonzalez-Segovia et al., 2008; Mahady et

al., 2003). Although it has been proposed that polyphenols may act against *H. pylori* (and other bacteria) via their interactions with cell membrane proteins and DNA (Tsuchiya et al., 1996; Cowen, 1999; Chan, 2002), these targets have yet to be fully established. One exception is that quercetin's antimicrobial activity can be attributed in part to its inhibition of DNA gyrase and ATP synthase activity in *Escherichia coli* (Plaper et al., 2003; Dadi et al., 2009). Myricetin has also been reported as having activity against *E. coli*; inhibiting helicase but not primase (Griep et al., 2007); however, these interactions have not been determined in *H. pylori*. In addition, no report exists on resveratrol's possible antimicrobial mode of action.

Although the above studies used membrane preparations and purified protein, they do support that different polyphenols are able to interact with multiple targets—contributing to their overall antimicrobial activity. However, these effects have yet to be determined using intact, viable cells or with *H. pylori* for that matter. Furthermore, no studies to date have examined *H. pylori* gene expression changes following treatment with any natural compounds. Although DNA microarrays have been used in studying antibiotic-induced gene expression in some bacteria, this technology has yet to be used on *H. pylori*.

Therefore, the objective of this study was to use DNA microarrays to determine the patterns of relative transcript amounts in cells exposed to sublethal concentrations of two well-known but incompletely characterized polyphenols, quercetin and resveratrol, as well as muscadine grape skin extract (MGSE) in an attempt to determine their possible mechanism(s) of action against *H. pylori* 26695.

## Materials and Methods

**Strain and growth conditions.** *H. pylori* strain 26695 was streaked from frozen stock onto Brain Heart Infusion (BHI) agar supplemented with 7% (vol./vol.) horse serum (HS) and grown at 37 °C for 72 h in a GasPak jar (BBL Microbiology Systems, Cockeysville, MD) under microaerophilic conditions (5% $O_2$, 10% $CO_2$, 85% $N_2$). Liquid cultures were grown in BHI broth containing 7% HS in 100 ml conical flasks at 37 °C, with shaking (120 rpm), under microaerophilic conditions.

**Experimental design.** Plate-grown *H. pylori* was harvested and inoculated into BHI broth with HS, in duplicate, to an optical density at 600 nm ($O.D._{600}$) of 0.05 and incubated under microaerophilic conditions, with shaking, at 37°C for 15-16 h to reach exponential, mid-logarithmic growth as determined by established growth curves (data not shown). Following incubation, cells were pelleted by centrifugation (3,500 x g, 5 min), washed once in sterile phosphate buffered saline (PBS, pH 7.4±0.2), and resuspended to an $O.D._{600}$ of 0.5 in BHI/HS broth. Two-milliliter aliquots were then distributed to sterile 5 ml test tubes containing sub-lethal concentrations of MGS extract, quercetin, or resveratrol in dimethyl sulfoxide (DMSO) at final concentrations of 0.1 mg total phenolics/ml, 32 µg/ml, and 16 µg/ml(pH 6.9 to 7.3), respectively. Test compound concentrations and treatment times were determined previously (unpublished data). Cells with DMSO (1%) served as negative controls. At 15 min, samples were collected for RNA extraction and plated onto BHI-HS to verify bacterial survival and concentration.

**RNA isolation.** Following treatment, 1 ml aliquots of *H. pylori* cells were pelleted at 12,000 x g for 30 sec and immediately resuspended in RNA*later* solution (Ap-

plied Biosystems, Austin, TX). Total RNA was extracted and purified using a RiboPure-Bacteria Kit (Applied Biosystems, Austin, TX) as described by the manufacturer and treated with supplied DNase to remove contaminating genomic DNA. Following purification, RNA was precipitated overnight at -80°C with 1/10 volumes of sodium acetate (3 M, pH 5.2) (Ambion) and 2.5 volumes of molecular grade ethanol (Sigma). Following precipitation and immediately before use, RNA was recovered by centrifugation at 16,000 x g for 15 min at 4°C, washed twice with 70% ethanol, and resuspended in TE buffer (Ambion). RNA concentration and purity/integrity was verified using standard spectrophotometric methods (NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA) and an Agilent 2100 Bioanalyzer (Santa Clara, CA). RNA was stored at -80°C until use.

**cDNA synthesis.** cDNA probes for microarray experiments were prepared as described. Briefly, 2 µg of total RNA was incubated at 42 °C for 18 h in a reaction mixture containing 6 µg of random hexamers (Invitrogen, Carlsbad, CA); 0.1 M DL-dithiothreitol; an amino-allyl-deoxynucleoside triphosphate mixture containing 25 mM (each) dATP, dCTP, and dGTP, 15 mM dTTP, and 10 mM amino-allyl-dUTP (Sigma); 5x first strand reaction buffer (Invitrogen); and 400 units of SuperScript III reverse transcriptase (Invitrogen). Following incubation, RNA template was hydrolyzed by incubating the reaction mixture at 95 °C for 5 min, snap cooling for 30 sec, adding 1 M NaOH (0.3 M final concentration) and incubating at 65 °C for 15 min. Solutions were neutralized with 1 M HCl (50:50, v/v). One-tenth volume (relative to the neutralized solution) of 3 M sodium acetate (pH 5.2) was added and the mixture was brought to 100 µl with nuclease-free $H_2O$. Unincorporated amino-allyl-dUTP was removed using MinElute

PCR purification columns (Qiagen, Valencia, CA) with some modifications. Briefly, kit-supplied wash buffer was replaced with 75% EtOH and cDNA probes were eluted with phosphate buffer (4 mM $KPO_4$, pH 8.5, in nuclease-free water), dried under vacuum at 37 °C using a SpeedVac (Savant), and resuspended in 0.1 M sodium carbonate buffer (pH 9.3 ± 0.1). To couple the amino-allyl cDNA with fluorescent labels, equivalent volumes of normal human serum-Cy3 or normal human serum-Cy5 (Amersham Biosciences, Piscataway, NJ) (in DMSO) was added to resuspended cDNA and incubated at room temperature, in the dark, for 2.5 h. Following incubation, uncoupled label was removed using MinElute PCR columns with the exception that prior to elution, cDNA was washed once with 35% guanidine hydrochloride to enhance removal of unincorporated dye. Analysis of each labeling reaction was performed using a NanoDrop (Thermo Fisher) and the following absorbance calculations:

$$\text{pmol nucleotides} = \frac{[OD_{260} \times \text{volume (µl)} \times (37\ \text{ng/µl}) \times (1000\ \text{pg/ng})]}{(324.5\ \text{pg/pmol})}$$

$$\text{pmol Cy3} = \frac{OD_{550} \times \text{volume (µl)}}{0.15}$$

$$\text{pmol Cy5} = \frac{OD_{650} \times \text{volume (µl)}}{0.25}$$

$$\text{\#nucleotides/dye incorporated} = \frac{\text{pmol cDNA}}{\text{pmol cy dye}}$$

**Microarray design.** *H. pylori* 26695-specific aminosilane-coated microarrays were provided by the Pathogen Functional Genomics Resource Center at the Institute for

Genomic Research (Rockville, MD). Microarray slides contained 13,872 elements, including oligonucleotide controls (500 *Arabidopsis thaliana* 70-mers). Oligonucleotide design was based on 2,572 unique open reading frames (ORFs) (four replicates of each) with sequences derived from *H. pylori* strains 26695 and J99.

**Hybridization and scanning of microarrays.** Aminosilane-coated microarray slides were prehybridized in Coplin jars containing 5X SSC (Sigma), 0.1% sodium dodecyl sulfate (SDS) (Ambion), 1% bovine serum albumin (BSA) (Sigma) and nuclease-free $H_2O$ at 42 °C for 1.5 h. Following prehybridization, slides were washed four times in millipure $dH_2O$, dipped 5-6 times in molecular grade isopropanol (Sigma), spun at maximum speed in a microfuge slide centrifuge (VWR) until dry, and cleaned with filtered (0.22 µm) compressed air immediately before use. For each slide hybridized, equal volumes of the appropriate Cy3- and Cy5-labeled probes were combined (yielding 75-100 pmol of each target), dried under vacuum at 42 °C (Savant), and resuspended in 60 µl of hybridization buffer containing 40% formamide, 5X SSC, 0.1% SDS, and 0.6 µg/ml sheared salmon sperm DNA. Resuspended probes were denatured by heating to 95 °C for 10 min immediately prior to hybridization. LifterSlip coverslips (25 x 60 mm) (Erie Scientific, Portsmouth, NH) were placed over arrays and probe mixtures were added. Slides were hybridized for 18 h at 42 °C in hybridization chambers (Argos Technologies, Elgin, IL). A total of 11 hybridizations were performed including 3 biological replicates from MGSE-treated cells and 8 biological replicates from quercetin- and resveratrol-treated cells, respectively. Following hybridization, slides were removed, placed in glass staining dishes (Wheaton) and washed sequentially, with shaking (100 rpm) in 1X SSC-

0.2% SDS (53 °C, 10 min), 0.1X SSC-0.2% SDS (room temperature, 10 min), and 0.1X SSC (room temperature, 10 min), dried by centrifugation, and scanned with an Agilent G2505B microarray scanner (Santa Clara, CA) .

**Microarray normalization and data analysis.** Microarray data were normalized as described by Schmid et al. (2005). Briefly, scanned TIFF images were processed with TIGR Spotfinder software (http://www.tigr.org/software/tm4). Raw intensity values of processed data were normalized by LocFit (LOWESS) normalization in block mode using TIGR MIDAS software (http://www.tigr.org/software/tm4). Signal intensities of < 7,500 were removed prior to analysis to avoid false signal intensity measurements. Each microarray slide contained four in-slide replicates of each oligonucleotide. Median signal intensities of each set of in-slide replicates and experimental replicates were used to calculate $\log_2$ and *x*-fold changes in gene expression.

**Statistics and bioinformatics for identification of differentially expressed genes.** The fold change for gene expression was calculated as the average ratio of copy number of the genes from treatment samples divided by that from control samples. The fold changes were then transformed into logarithmic scale. To select the genes that were differentially expressed, the concept of false discovery rate (FDR) was utilized (Efron and Tibshirani, 2002; Storey and Tibshirani, 2003). The purpose of FDR is to control for the expected fraction of false rejection among the total number of rejections. FDR maintains a balance between the number of false positives and true positives (Storey and Tibshirani, 2003) and is equivalent to the family-wise error but with a gain in power (Bengamini and Hochberg, 1995). The strength of the FDR lies on its 'adaptive' nature, i.e.

the cut-off *P* value is selected based on the available information in the data (Cui and Churchill, 2003; Sabatti et al., 2003). The significant DNA genes were selected by a popular method, Significance Analysis of Microarrays (SAM) (Tusher et al., 2001). SAM implements a modified *t*-test by assigning a score to each marker on the basis of change and utilizes permutations to estimate the percentage of markers identified by chance (FDR) (Tusher et al., 2001). The threshold for the false discovery rate was set at 0.10, i.e. an estimated 10% of the genes called significant could be false. Such algorithms enable the identification of differentially regulated genes even if the fold change is low.

**Primer design for quantitative reverse transcription-PCR (qRT-PCR).** Oligonucleotide primer pairs were designed using OligoPerfect Designer software (Invitrogen, Carlsbad, CA) and the *H. pylori* 26695 genome sequence (Tomb et al., 1997). All primer pairs had 100% template homology, calculated melting temperatures of 59.8 to 60.5 °C, and amplified products ranging from 81 to 174 bp. Prior to RT-PCR, each primer pair was first used to amplify genomic DNA from *H. pylori* 26695 to confirm target amplification and amplicon size; determined by agarose gel electrophoresis.

**qRT-PCR validation of microarray data.** qRT-PCR was performed using the gene-specific primer pairs (Table 4.1) of four ORFs determined to be differentially expressed from microarray experiments. cDNA was synthesized as previously described using 1 µg of total RNA. No contaminating DNA was detected by PCR. Amplification of cDNA was performed, in triplicate, for 40 cycles at 94 °C, 52 °C, and 72 °C for 30 sec each with an iCycler (Bio-Rad). Accumulation of PCR product was detected during each cycle by excitation of SYBR green (Bio-Rad, Hercules, CA) at 490 nm followed by run-

ning melt curves to reveal non-specific amplification. Relative fluorescence was determined by a cycle threshold ($C_t$) value, defined as the crossover point of the kinetic curve with an arbitrary fluorescence level set at 150 relative fluorescence units. Expression of 16S rRNA was monitored for sample normalization.

**PCR amplification efficiency.** Because even primer pairs with 100% homology to their template can amplify with significantly different efficiencies (Pfaffl, 2001), primer pairs were first evaluated using *H. pylori* 26695 DNA. The efficiency of each primer pair was determined using the slopes calculated from standard curves prepared from corresponding $C_t$ values determined using 10-fold serial dilutions of starting DNA, and defined as:

$$\text{primer amplification efficiency (E)} = 10^{(-1/\text{slope})}$$

**Calculation of gene expression.** Using $C_t$ values of both control and treated samples and the formulas below (Pfaffl, 2001), gene expression was determined, corrected for the internal 16S rRNA standard, and reported as fold change relative to no treatment controls.

$$\text{gene fold change} = (E)^{\text{avg. } Ct \text{ control} - \text{avg. } Ct \text{ treated}}$$

$$\text{absolute gene expression} = \frac{(E_{\text{target}})^{\Delta Ct \text{ target}}}{(E_{\text{16S rRNA}})^{\Delta Ct \text{ 16S rRNA}}}$$

## Results

**Significant changes in *H. pylori* gene expression after 15 min exposure to MGSE, quercetin, and resveratrol.** Microarray analysis was used to determine the pat-

terns of relative transcript amounts in cells exposed to sub-inhibitory concentrations of quercetin, resveratrol, and MGSE in an attempt to determine/highlight their possible mechanism(s) of action against *H. pylori* 26695.

The amount of compound used in each experiment was determined previously by microbroth and agar dilution assays and represented concentrations two-fold lower than the average minimal inhibitory concentration recorded. In this study the concentrations of quercetin, resveratrol, and MGSE were 32 µg/ml, 16 µg/ml, and 0.1 mg total phenolics/ml, respectively. Total RNA was extracted from each culture 15 min after addition of each compound/extract or DMSO (control). This short time period was chosen to minimize possible secondary and downstream/bystander effects that might arise during prolonged treatment; thus interfering with analysis and interpretation of results. Hybridization intensity for each gene on the array was compared for the compound/extract-treated and DMSO control samples (Tables 4.2-4.4).

Of the total number of ORFs subjected to data analysis from each treatment, few transcripts were significantly altered with 63, 39, and 29 genes differentially expressed upon 15 min exposure to MGS extract, quercetin or resveratrol, respectively. Of the genes showing altered transcript amounts in response to each treatment, ~30% encoded hypothetical proteins without known functions, 22% encoded proteins involved in transcription/translation, 15% encoded proteins involved in energy production/metabolism, whereas genes related to other cellular processes, amino acid biosynthesis, etc. accounted for the remainder, ~34% (Figure 4.1).

Of the genes involved in transcription/translation, 20 code for ribosomal proteins (3 induced, 17 repressed). Interestingly, histone-like DNA binding protein (*hup)* (HP0835), shikimate 5-dehydrogenase (*aroE*) (HP1249), and ATP synthase F1 subunit A (*atpA*) (HP0828) were induced upon exposure to quercetin. In resveratrol-treated cells, translation initiation factor 2 (*infB*) (HP1048) was induced. In addition, *cagA* and *ureA* were repressed in MGSE-treated cells whereas *cagA*, *ureE* and *I*, were repressed following quercetin and resveratrol treatment, respectively.

**Validation of microarray results.** To validate the ability of *H. pylori* microarrays to determine significant changes in gene expression, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed. Four representative genes from those found to change significantly following treatment were chosen for further analysis. To optimize PCR analysis, the efficiency of each primer pair was determined prior to testing and ranged from 87.7 to 94.0 %. As shown in Figure 4.2, transcript levels of *cagA* and *ureA* were repressed following MGSE treatment while *atpC* and *hup* were induced following MGS and quercetin treatment, respectively. Thus, qRT-PCR confirms the ability of microarray to detect changes in selected gene expression.

## Discussion

DNA microarrays have been increasingly used to measure changes in gene expression levels. For example, antibiotic-induced gene expression has previously been studied using microarray hybridization (Wilson et al., 1999; Gmuender et al., 2001). However, unlike these studies reporting the effects of low concentration antibiotic exposure on *Mycobacterium tuberculosis* and *Haemophilus influenzae* transcript patterns,

quercetin and resveratrol's primary target(s) against *H. pylori* are unknown. In those studies, only 14 and 5 genes were differentially expressed, respectively, with a majority directly associated with each drug's reported mode of action. Therefore, using a similar approach, we chose to treat *H. pylori* 26695 with sub-inhibitory concentrations of each compound for a short time period with the goal of revealing each compound's likely primary target(s). Although fewer genes were differentially expressed given these conditions, we believe that by using this strategy, the most interpretable results were obtained and likely represent the compounds' primary mechanism(s) of action. Alternatively, use of high inhibitor concentrations for extended periods of time changes the expression of a large set of genes; however, these effects may be secondary to the action of the drug (Palzkill, 2001). In addition to highlighting suspected primary targets, determination of the function of unknown genes induced in the primary response to antibiotic exposure may uncover potential new targets for future antimicrobial development.

The data presented here indicate that *H. pylori* responds to resveratrol and quercetin quite differently with only one overlapping gene (outer membrane protein, *omp30*) (HP1395) detected for the two treatments. This was not surprising given these compounds belong to different chemical classes; quercetin being a flavonoid and resveratrol a stilbene, with highly different structures (Pastrana-Bonilla et al., 2003). This trend of non-overlapping gene expression has also been reported when examining transcription patterns between other antimicrobials with different modes of action such as triclosan (a possible fatty acid biosynthesis inhibitor) and tetracycline (translation inhibitor) in *Streptococcus pneumoniae* (Ng et al., 2003). Furthermore, antibiotics acting on the same bac-

terial target but by different mechanisms have also been reported to result in different gene expression profiles (Gmeunder et al., 2001). In addition, we have previously shown that resveratrol and quercetin act synergistically against *H. pylori in vitro* further supporting their different mode(s) of action.

Following array analysis, some commonality in expression of selected genes was found between MGSE and quercetin albeit at different levels (most likely due to concentration differences and additional unknown factors). Because MGSE was previously determined to contain relatively high levels of both quercetin and resveratrol by HPLC; which were found to be the most active major phenolics following susceptibility testing (Chapter 3), we hypothesized that expression patterns of selected genes may be similar between purified and crude preparations. However, due to the overall complexity of the MGSE (i.e. the numerous additional unknown components undoubtedly present), natural synergism, and inability to standardize quercetin and resveratrol levels, this was not achievable. However, some degree of overlap in expression was observed and rather surprising. Of the similar changes detected, the most interesting were secondary effects involving genes encoding for known *H. pylori* virulence factors (i.e. CagA and urease) and are discussed later.

**Muscadine grape skin extract.** Of the genes significantly altered following MGSE exposure, the majority are diverse in function and not clearly indicative of any individual target; likely reflecting the complex synergistic antimicrobial action found naturally in MGSE. However, some are in support of *H. pylori* cell membrane protein inhibition/damage previously hypothesized for this extract including ATP synthase F1 sub-

unit C, cytochrome c oxidase, and cytochrome c551 peroxidase (all induced). In addition, the *clpA* gene, coding for the ATP-dependent caseinolytic protease (Clp), was also induced upon MGSE exposure. Interestingly, these proteins are important in resistance against environmental stress, antibiotic treatment, and host immune defenses for a number of bacteria (Loughlin et al., 2009). While ClpP is the proteolytic subunit, ClpA acts as both a chaperone and an ATPase driving the degradation of damaged or mismade proteins. Because polyphenols are suspected to cause protein damage in *H. pylori*, induced expression of *clpA* may implicate the organism's attempt to degrade/remove damaged protein.

*H. pylori* gene *yihK* (HP0480) coding for protein elongation factor (EF-G) (homolog) and *pheT* (HP0402), aminoacyl-tRNA synthetase, were also induced; suggesting possible targeting of these proteins resulting in reduced protein synthesis. In line with this, 10 transcripts for ribosomal proteins were repressed (Table 1). Ribosomal proteins, known for their essential role in translation, also act as sensors in response to environmental stimuli such as temperature change and antibiotic treatment with corresponding expression of heat shock genes (VanBogelen and Neidhardt, 1990; Jones et al., 1992). Following treatment, a definite heat shock response was observed; however, these genes (*groEL*, *dnaK*, and *grpE*) (HP0010, HP0109, HP0110) were also repressed and may only be explained by the corresponding reduction in ribosomal protein.

*H. pylori* cell binding factor-2, a secretory protein, was repressed following treatment and contributes to activation of epidermal growth factor receptor and activation of vascular endothelial growth factor, important in *H. pylori* pathogenesis (Basu et al.,

2008). Serine protease was also repressed. Although primarily known for its role in protein quality control, it was recently identified as a new secreted virulence factor as it cleaves the ectodomain of the cell-adhesion protein E-cadherin; disrupting epithelial barrier functions which allow *H. pylori* to access the intercellular space (Hoy et al., 2010). Catalase was also repressed following treatment. Despite being nonessential for *in vitro* viability, this enzyme is of key importance in *H. pylori*'s antioxidant defense mechanisms upon colonization and may support earlier *in vivo* findings (Harris et al., 2002).

Although difficult to interpret given the complex nature of MGSE treatment, these results may partially support our earlier findings on the anti-*H. pylori* properties of MGSE (Brown et al., 2009) while further emphasizing the need to determine the most active compound(s) in plant-based extracts prior to gene expression studies.

**Resveratrol.** Following treatment with resveratrol, a transcript profile similar to that of the bacterial stringent response was observed (Durfee et al., 2008). Of the transcripts significantly altered following treatment, translation initiation factor 2 (*infB*) was induced whereas GTP-dependent nucleic acid-binding protein was repressed. In addition, respiration-related gene expression was affected with NADH dehydrogenase enzymes and cytochrome c oxidoreductase levels significantly reduced. Not only are these changes in line with the stringent response process but have also been observed in other microorganisms following treatment with translation inhibitors (Ng et al., 2003). In addition, neither quercetin nor MGS-treated cells exhibited this response even though samples were prepared under identical conditions. Therefore, these results may suggest that resveratrol's primary mode of action against *H. pylori* is related to translation inhibition.

Translational IF-2 is highly conserved among prokaryotes and essential for the GTP-dependent binding of fMet-tRNA to ribosomes to form the 70S initiation complex (Kyrpides and Woese, 1997; Gualezi and Pon, 1990). IF-2 catalyzes the ribosome-dependent hydrolysis of GTP to GDP and inorganic phosphate ($P_i$) concomitant with the formation of the 70S initiation complex. It also catalyzes the uncoupled ribosome-dependent hydrolysis of GTP in the absence of other components required for imitation. Molecules like thiostrepton (Brandi et al., 2004), some structural analogs of fMet-adenosine/NacPhe-adenosine (Delle Fratte et al., 2002), and guanosine-3′-5′-bis (diphosphate) (ppGpp) (Milon et al., 2006), capable of inhibiting IF-2 activity, have been found. However, no antibiotic selectively targeting its functions has been reported to date. Thus, IF-2 may be regarded as an ideal target for new bacteria-specific drugs for which no resistance mechanisms have yet been developed (Letizia Brandi et al., 2007).

A number of antibiotics that inhibit bacterial protein synthesis are thought to act by stabilizing particular conformations of the ribosome; preventing structural changes that occur during translation. Thiostrepton and micrococcin are two examples which bind to the L11-bindng region of the 23S rRNA (the GTPase center of the ribosome). These molecules are believed to interfere with the function of the factors that interact with this region by stabilizing the L11-binding region so that it is unable to undergo a necessary conformational change (Cameron et al., 2002)

Given the possibility of IF-2 inhibition, induction of *infB* would be expected, supporting our hypothesis. However, although strongly indicative of translation inhibition, further studies are necessary to confirm resveratrol's effect on IF-2. Tests to determine

resveratrol's interaction with some of the main activities of this factor, such as 1) recognition and binding of the initiator tRNA; 2) codon-dependent ribosomal binding of fMet-tRNA leading to the formation of a 30S or 70S initiation complex; 3) ribosome dependent hydrolysis of GTP; and 4) accommodation of fMet-tRNA in the ribosomal P-site and formation of the first peptide bond (initiation dipeptide formation) may be useful in determining resveratrol's precise activity against *H. pylori*.

**Quercetin.** Of the transcripts significantly altered following quercetin treatment, three were selected for further discussion; shikimate 5-dehydrogenase (*aroE*), histone-like DNA binding protein (HU) (*hup*) and F0F1 ATP synthase subunit A (*atpA*), as these may play key roles in *H. pylori*'s response to quercetin and/or this compound's primary anti-*H. pylori* activity.

Previous studies have shown that the shikimate pathway is essential for the synthesis of key metabolites such as folic acid, aromatic amino acids, and ubiquinone in bacteria (Parish and Stoker, 2002). Recently, enzymes involved in this pathway; including shikimate dehydrogenase (SDH), the fourth enzyme in the pathway responsible for catalyzing the NADPH-dependent reduction of 3-dehydroshikimate to shikimate, have received increased attention in the development of nontoxic antimicrobial agents (Coggins et al., 2003). In support of this, Han et al. (2006) reported curcumin (diferuloylmethane), regarded as the most active constituent of *Curcuma longa* (i.e. turmeric), to be a non-competitive inhibitor of SDH. More specifically, the antimicrobial action of curcumin against multiple *H. pylori* isolates *in vitro* and during infections in mice was recently re-

ported; further supporting the potential use of polyphenols against *H. pylori* infection (De et al., 2009).

In this study, we found that quercetin caused an increase in *aroE* transcripts. We hypothesize that this may have been due to quercetin binding to SDH allosteric sites, much like curcumin, thereby reducing SDH activity and inducing *aroE* expression in order to compensate for this loss. This result may suggest that different natural compounds may share antimicrobial targets. However, quercetin's interaction with SDH and *aroE* should be explored further.

In addition to *aroE*, *hup* was also induced upon exposure to quercetin. The *hup* gene codes for HU which is important in bacterial genome compaction (i.e. introduction of negative DNA supercoiling) and possibly specific regulatory functions in *H. pylori* (Chen et al., 2004). Supercoiling and topoisomerases influence a variety of DNA activities such as transcription, transposition, recombination and DNA replication (Matthews, 1992). DNA gyrase, like HU, introduces and maintains negative supercoils, also contributing to DNA compaction. (Drlica and Snyder, 1978). Because HU stimulates DNA gyrase and lowers the activity of topoisomerase I, this facilitates the maintenance of intracellular supercoiling; yet the specific HU-gyrase interactions are not currently clear; especially in *H. pylori* (Malik et al., 1996).

Quercetin was previously found to inhibit DNA gyrase and introduce DNA cleavage in *E. coli* via its interaction with DNA or gyrase's ATP binding site on the 24 kDa fragment of gyrase B, inhibiting gyrase B ATPase activity (Plaper et al., 2003). The later mechanism is similar to novobiocin (a coumarin) which inactivates the enzyme without

introducing DNA strand breaks (Gmuender et al., 2001). Later, green tea catechins were also found to inhibit gyrase activity by the same mechanism (Gradišar et al., 2007). Therefore, we expected to see changes in *H. pylori* gene expression consistent with these findings. However, analysis of array data did not reveal DNA gyrase (induction) or topoisomerase (repression) gene products, possibly due to poor labeling or hybridization. Although we may have missed expression level changes in DNA gyrase and topoisomerase I, *hup* was clearly induced. Therefore, given HU's close association with DNA gyrase, one may argue that if *H. pylori* DNA gyrase is in fact inhibited by quercetin, an increase in HU (and DNA gyrase for that matter) may be the cell's attempt to maintain an optimal supercoiling degree to compensate for this deficiency. Additional work may be necessary to further verify these results; however, our data appear to be in agreement with other studies, supporting quercetin's possible mode of action against *H. pylori*.

Recently, the inhibition of ATPase activity of *E. coli* ATP synthase by polyphenols was reported (Dadi et al., 2009). In that study, quercetin inhibited ATPase activity ca. 80% at low concentration ($IC_{50}$ = 33µM) with maximal inhibition achieved within 15 min, and was reversible upon removal of inhibitor. Interestingly, our results showed an increase in *atpA* expression following quercetin treatment. Furthermore, previous phase-contrast microscopy experiments showed reduced *H. pylori* motility in the presence of quercetin indicating a possible reduction in proton-motive force—further supporting quercetin's interaction with this enzyme in *H. pylori*.

In addition to the three genes discussed above, 36 other genes also showed a changed expression rate. Whether these are primary or secondary effects of quercetin

remains to be determined. However, it is unlikely that these differences are artifacts derived from culture conditions as all cells were grown under identical conditions and may be important in understanding quercetin's full anti-*H. pylori* activity. Finally, hypothetical proteins differentially expressed following quercetin treatment are noteworthy and may be subject for future detailed functional genomic studies.

***H. pylori* virulence factors**. In this study, both quercetin and MGSE-treated cells showed reduced *cagA* levels. CagA is known to elicit a strong immunological response and implicated in *H. pylori* pathogenesis through its interruption of host cell signal transduction, actin cytoskeleton rearrangement, and interleukin-8 production—promoting inflammation and subsequent host cell damage (Sharma et al., 1995; Censini et al., 1996; Prinz et al., 2003). We have previously reported that MGSE is effective at reducing host cell damage/death in a gastric epithelial cell line (ATCC CRL-1739) which may be partially due to inhibition of CagA toxin production (Brown et al., 2009). We have also shown using *H. pylori*-infected mice, that both MGS powder (5-10%) and quercetin significantly reduced gastric inflammation and host cell damage following prolonged treatment, even while *H. pylori* levels remained relatively unchanged; also suggesting the possibility of CagA inhibition.

In addition to *cagA*, genes associated with *H. pylori* urease were also repressed following treatment with MGSE. However, unlike *cagA*, no changes were detected in quercetin-treated cells but were detected following resveratrol treatment. Genes differentially expressed were non-overlapping; representing two operons (*ureAB* and *ureIEFGH*), which was surprising given that resveratrol was also detected in MGSE. However, dif-

ferences in resveratrol concentration and/or additional unknown factors may have contributed to this result.

In MGSE-treated cells, urease subunit A (*ureA*) (HP0073) was significantly repressed. UreA is one of two structural subunits (*ureAB*) forming the urease enzyme with a predicted molecular mass of 26.5 kDa (Mobley, 2001). Urease is a $Ni^{2+}$-containing cytoplasmic enzyme that converts urea into bicarbonate and ammonia. *H. pylori* urease is essential for colonization of the stomach as it allows survival under acidic conditions—accounting for up to 15% of the total protein synthesized by the organism (Prinz et al., 2003; Mobley et al., 1995). These products generate a protective alkaline microenvironment (optimal pH of 7.0) and allow the organism to persist in the gastric environment (Mobley, 2001). Interestingly, other studies evaluating the anti-*H. pylori* activities of various plant-based extracts have also reported urease inhibition (Lin et al., 2001; Xiao et al.., 2007) further supporting results from this study.

In resveratrol-treated cells, *ureI* (HP0071) and *ureE* (HP0070) transcripts were significantly repressed but *ureA* and *B* were not. The *ureI* gene, unique to *H. pylori*, encodes for an integral cytoplasmic protein involved in the formation of an $H^+$-gated, urea-specific pore; allowing entry of urea at low pH (Ang et al., 2001). Previous studies have concluded that urease-dependent adaptation to acidity *in vitro* depends on the transport function of UreI (Mobley et al., 2001). Furthermore, deletion mutants are unable to colonize mice, supporting the role of UreI in gastric acid adaptation and survival *in vivo* (Sachs et al., 2001; Skouloubris et al., 1998). The *ureE* gene, also repressed following resveratrol treatment and closely associated with *ureI* contributes (alongside remaining

accessory genes *ureF*, *ureG*, and *ureH*) to production of the active product for nickel incorporation into the enzyme's active site during the final step of urease biosynthesis. Like *ureI*, for synthesis of a catalytically active urease enzyme, *ureE* (in addition to accessory genes) must also be expressed (Mobley et al., 2001).

Although *vacA* was detected during microarray analysis, its expression was not repressed at a significant level following any treatment and was therefore not reported. However, other studies have shown the anti-VacA activities of polyphenols; especially those from red wine, green tea, and even apple peel (Ruggiero et al., 2006; Tombolo et al., 2003, Pastene et al., 2010). Although *vacA* expression levels were not changed, this does not negate the role of currently unknown polyphenolic constituents against VacA toxin. Based on previous studies reporting VacA inhibition by polyphenols, MGSE may also neutralize VacA by 1) direct VacA-polyphenol interaction precluding the insertion of VacA into the eukaryotic membrane or 2) interference with vacuolization or channel formation caused by VacA toxin. However, this has yet to be fully studied with MGSE but does support our earlier findings showing reduced gastric epithelial cell damage (primarily caused by VacA) following MGSE treatment (Brown et al., 2009). In addition, anti-adhesive properties of polyphenols (including those in MGSE) have also been proposed (Burger et al., 2002) but were not revealed in this study.

Collectively, quercetin, resveratrol, and MGSE repressed *cagA*, *ureA*, *ureE*, *ureI*, and *vacA* (not significant) expression. Although the exact regulatory mechanism behind these changes were not elucidated, our results do support that phenolic compounds may be effective at reducing *H. pylori* virulence via repression of these genes; however, addi-

tional studies are necessary to fully understand these interactions. Furthermore, the precise antibacterial mechanism(s) of quercetin and resveratrol were not confirmed in this study, but results are in line with previous findings obtained in our laboratory and other groups on these compounds and support future, more specific studies into the exact mechanism(s) by which these compounds exert their anti-*H. pylori* activity.

In conclusion, this study shows that microarrays may be useful tools in the characterization of novel plant-based antimicrobials. This is the first report describing *H. pylori* gene expression change upon treatment with any natural compound. Results also support previous findings on the anti-*H. pylori* activity of polyphenols quercetin and resveratrol. Additionally, this study demonstrates that with low inhibitor concentration and short exposure, only a few genes show a clear change in expression and may be optimal for the analysis of primary response mechanisms. However, these may be concentration- and/or time-dependent and require additional preliminary work to optimize conditions, but may be useful in eliminating many secondary effects commonly seen in array studies. It is also our opinion that using crude extracts may not be suitable in the screening process for specific antimicrobial targets of polyphenolic compounds as results are complicated given numerous uncontrollable factors.

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## Figure Legend

**FIGURE 4.1.** Distribution of functions of selected genes whose relative transcript amounts were affected by treatment with MGS, quercetin, or resveratrol. The relative abundance is expressed as a percentage of the total number of genes whose relative transcript amount was affected by each treatment (Tables 2-4) and includes both increased and decreased transcript amounts.

**FIGURE 4.2.** Changes in transcript levels upon exposure of *H. pylori* 26695 to MGS extract (*cagA*, *ureA*, *atpC*) and quercetin (*hup*) determined by qRT-PCR. The mean $\log_2$ expression and standard deviation is reported for three independent replicates. The 16S rRNA gene was used for normalization.

**TABLE 4.1.** *H. pylori* genes and primer pairs selected for real-time RT-PCR.

| Gene | HP no.[a] | Forward primer | Reverse primer |
|---|---|---|---|
| *ureA* | HP0073 | CCTTCTTGCATCAATTCAGCCGCA | AGCGGTAGCTTTGATTAGTGCCCA |
| *hup* | HP0835 | GCGATCAGTGCCTTTACTTTGGCA | TCGCTTCCTGGCACTTTACCTTCT |
| *F0F1* | HP1212 | ATCACTTGCGCTTCAATCATCGCC | ATTACAGGCACAGCGAGAAACCCA |
| *cag26* | HP0547 | TGTTGAGGGAGTCGCTGACATTGA | GTGGCGTTCCAATCGTGTTTGTCT |
| 16S rRNA | | ATGGATGCTAGTTGTTGGAGGGCT | TTAAACCACATGCTCCACCGCTTG |

[a] *H. pylori* gene designation (Tomb et al., 1997)

**TABLE 4.2.** Differentially expressed genes of *H. pylori* 26695 upon exposure to MGS extract.

| Gene set | Category and TIGR no. | Symbol | Putative function | *n*-fold |
|---|---|---|---|---|
| | | | | |
| Induced genes | *Protein degradation* | | | |
| | HP0033 | *clpA* | Chaperone and ATPase | 1.2 |
| | HP0363 | *pcm* | Protein-L-isoaspartate O-methyltransferase | 1.8 |
| | | | | |
| | *Unknown function* | | | |
| | HP0412 | | Hypothetical protein | 1.2 |
| | HP0563 | | Hypothetical protein | 1.6 |
| | HP0571 | | Hypothetical protein | 1.6 |
| | HP0637 | | Hypothetical protein | 2.1 |
| | HP0813 | | Hypothetical protein | 1.0 |
| | HP1083 | | Hypothetical protein | 1.5 |
| | HP1175 | | Hypothetical protein | 1.3 |
| | HP1217 | | Hypothetical protein | 1.5 |
| | | | | |
| | *Motility* | | | |
| | HP1377 | *fliW* | Flagellar assembly protein | 1.1 |
| | | | | |
| | *Transcription/translation* | | | |
| | HP0480 | *yihK* | GTP-binding protein, *fusA* homolog | 1.1 |
| | | | | |
| | *Energy metabolism* | | | |
| | HP1212 | *atpC* | ATP synthase F1, subunit C | 1.6 |
| | HP0147 | *fixP* | Cytochrome c oxidase | 1.1 |
| | HP1461 | | Cytochrome c551 peroxidase | 2.1 |
| | | | | |
| | *Cell envelope* | | | |
| | HP1395 | *omp30* | outer membrane protein | 1.2 |
| | | | | |
| | *Amino acid biosynthesis* | | | |
| | HP1210 | *cysE* | serine acetyltransferase | 1.5 |
| | | | | |
| | *Fatty acid and phospholipid metabolism* | | | |
| | HP1052 | *lpxC* | N-acetylglucosamine deacetylase | 1.1 |
| | HP0215 | *cdsA* | CDP-diglyceride synthetase | 1.3 |
| | | | | |
| | *Protein biosynthesis* | | | |
| | HP0402 | *pheT* | Aminoacyl-tRNA synthetase, ligase | 2.0 |
| | | | | |
| | *Biosynthesis of cofactors* | | | |
| | HP0306 | | Porphyrin biosynthetic protein | 1.0 |
| | HP0625 | | Isoprene biosynthetic protein | 2.1 |
| | HP0020 | *nspC* | Norspermidine biosynthetic protein | 1.2 |
| | | | | |
| | *Purine biosynthesis* | | | |
| | HP1218 | *purD* | Glycinamide ribonucleotide synthetase | 1.3 |
| | HP1193 | *akR* | Aldo-keto reductase, putative | 1.0 |
| | | | | |
| Repressed genes | *Transcription/translation* | | | |
| | HP1196 | *rps7* | Ribosomal protein S7, 30S | -1.1 |
| | HP1197 | *rpsL* | Ribosomal protein S12, 30S | -1.4 |
| | HP1200 | *rplJ* | Ribosomal protein L10, 50S | -1.0 |

| | | | | |
|---|---|---|---|---|
| | HP1244 | *rpsR* | Ribosomal protein S18, 30S | -1.1 |
| | HP1245 | *ssb* | Single-stranded DNA binding protein | -1.2 |
| | HP1303 | *rplR* | Ribosomal protein L18, 50S | -1.5 |
| | HP1305 | *rpsH* | Ribosomal protein S8, 30S | -1.3 |
| | HP1306 | *rpsN* | Ribosomal protein S14, 30S | -0.9 |
| | HP1308 | *rplX* | Ribosomal protein L24, 50S | -1.1 |
| | HP1309 | *rplN* | Ribosomal protein L14, 50S | -1.2 |
| | HP1311 | *rpmC* | Ribosomal protein L29, 50S | -1.5 |
| | HP1312 | *rplP* | Ribosomal protein L16, 50S | -1.4 |
| | HP1019 | *htrA* | Serine protease | -1.2 |
| | HP0569 | *engD* | GTP-dependent nucleic acid-binding protein, putative translation- associated GTPase | -0.9 |
| | | | | |
| | *Cellular processes* | | | |
| | HP0547 | *cagA* | Cytotoxin-associated gene A | -1.5 |
| | HP0010 | *groEL* | Chaperone and heat shock protein | -1.0 |
| | HP0109 | *dnaK* | Chaperone and heat shock protein, 70 kDa | -1.1 |
| | HP0110 | *grpE* | Cochaperone and heat shock protein, 24 kDa | -1.3 |
| | HP0175 | | Cell binding factor 2 | -1.8 |
| | | | | |
| | *Central intermediary metabolism* | | | |
| | HP0073 | *ureA* | Urease alpha subunit | -2.3 |
| | HP0875 | | Catalase | -1.0 |
| | | | | |
| | *Cell envelope* | | | |
| | HP0317 | *hopU* | Outer membrane protein | -0.9 |
| | HP1243 | *omp28* | Outer membrane protein | -0.6 |
| | | | | |
| | *Unknown function* | | | |
| | HP0102 | | Hypothetical protein | -1.0 |
| | HP0185 | | Hypothetical protein | -1.9 |
| | HP0367 | | Hypothetical protein | -1.0 |
| | HP0659 | | Hypothetical protein | -1.0 |
| | HP0697 | | Hypothetical protein | -1.3 |
| | HP0719 | | Hypothetical protein | -0.7 |
| | HP0720 | | Hypothetical protein | -1.5 |
| | HP0721 | | Hypothetical protein | -1.5 |
| | HP0731 | | Hypothetical protein | -2.1 |
| | HP0733 | | Hypothetical protein | -1.3 |
| | HP1124 | | Hypothetical protein | -1.8 |
| | | | | |
| | *Energy metabolism* | | | |
| | HP0695 | *hyuA* | Hydantoin utilization protein A | -1.3 |
| | HP0027 | *Icd* | Isocitrate dehydrogenase | -1.1 |
| | HP0690 | *fadA* | Acetyl coenzyme A acetyltransferase (thiolase) | -1.1 |
| | | | | |
| | *Transport and binding* | | | |
| | HP1172 | *glnH* | Glutamine ABC transporter, periplasmic glutamine-binding protein | -1.3 |

**TABLE 4.3.** Differentially expressed genes of *H. pylori* 26695 upon exposure to quercetin.

| Gene set | Category and TIGR no. | Symbol | Putative function | *n*-fold |
|---|---|---|---|---|
| | | | | |
| Induced genes | *Transcription/translation* | | | |
| | HP0835 | *hup* | Histone-like DNA-binding protein | 0.5 |
| | HP1302 | *rpsE* | Ribosomal protein S5, 30S | 0.5 |
| | HP1447 | *rpmH* | Ribosomal protein L34, 50S | 0.6 |
| | | | | |
| | *Unknown function* | | | |
| | HP0031 | | Hypothetical protein | 0.4 |
| | HP0129 | | Hypothetical protein | 0.6 |
| | HP0149 | | Hypothetical protein | 0.4 |
| | HP0199 | | Hypothetical protein | 0.4 |
| | HP0565 | | Hypothetical protein | 0.4 |
| | HP0641 | | Hypothetical protein | 0.4 |
| | HP0891 | | Hypothetical protein | 0.6 |
| | HP0920 | | Hypothetical protein | 0.4 |
| | HP0947 | | Hypothetical protein | 0.7 |
| | HP1289 | | Hypothetical protein | 0.5 |
| | | | | |
| | *Fatty acid and phospholipid metabolism* | | | |
| | HP0559 | *acpP* | Acyl carrier protein | 0.3 |
| | HP1375 | *lpxA* | UDP-N-acetylglucosamine acyltransferase | 0.5 |
| | | | | |
| | *Cell envelope* | | | |
| | HP1395 | *omp30* | Outer membrane protein | 0.6 |
| | HP1512 | *frpB* | Iron-regulated outer membrane protein | 0.5 |
| | | | | |
| | *Motility* | | | |
| | HP1240 | *maf* | Motility accessory factor-like protein | 0.4 |
| | | | | |
| | *Energy metabolism* | | | |
| | HP0824 | *trxA* | Thioredoxin reductase | 0.4 |
| | HP1458 | | Thioredoxin, putative | 0.6 |
| | HP0857 | | Phosphoheptose isomerase | 0.4 |
| | HP1100 | | Phosphogluconate dehydratase | 0.3 |
| | HP1249 | *aroE* | Shikimate 5-dehydrogenase | 0.7 |
| | HP0828 | *atpA* | ATP synthase F1, subunit A | 0.5 |
| | HP0145 | | Cbb3-type cytochrome c oxidase subunit II | 0.5 |
| | HP0510 | | Dihydrodipicolinate reductase | 0.5 |
| | | | | |
| | *Cellular processes* | | | |
| | HP0542 | *cag21* | cag pathogenicity island protein | 0.5 |
| | | | | |
| Repressed genes | *Transcription/translation* | | | |
| | HP1200 | *rplJ* | Ribosomal protein L10, 50S | -0.4 |
| | HP1308 | *rplX* | Ribosomal protein L24, 50S | -0.5 |
| | HP1309 | *rplN* | Ribosomal protein L14, 50S | -0.8 |
| | HP1316 | *rplB* | Ribosomal protein L2, 50S | -0.4 |
| | HP1293 | | DNA-directed RNA polymerase subunit alpha | -0.5 |
| | | | | |
| | *Unknown function* | | | |
| | HP0102 | | Hypothetical protein | -0.5 |

| | | | | |
|---|---|---|---|---|
| | HP0697 | | Hypothetical protein | -0.6 |
| | *Fatty acid and phospholipid metabolism* | | | |
| | HP0561 | *fabG* | 3-ketoacyl-(acyl-carrier-protein) reductase | -0.6 |
| | | | | |
| | *Energy metabolism* | | | |
| | HP0695 | *hyuA* | Hydantoin utilization protein | -1.1 |
| | HP0696 | | N-methylhydantoinase | -0.5 |
| | | | | |
| | *Cell envelope* | | | |
| | HP0743 | *mreB* | Rod shape-determining protein | -0.5 |
| | | | | |
| | *Cellular processes* | | | |
| | HP0547 | *cagA* | Cytotoxin-associated gene A | -0.8 |

**TABLE 4.4.** Differentially expressed genes of *H. pylori* 26695 upon exposure to resveratrol.

| Gene set | Category and TIGR no. | Symbol | Putative function | *n*-fold |
|---|---|---|---|---|
| | | | | |
| Induced genes | *Cellular processes* | | | |
| | HP0323 | *nucT* | DNA transformation | 0.6 |
| | HP0531 | *cag11* | cag pathogenicity island protein | 0.5 |
| | | | | |
| | *Cell envelope* | | | |
| | HP0896 | *omp19* | Outer membrane protein | 0.4 |
| | HP1395 | *omp30* | Outer membrane protein | 0.4 |
| | | | | |
| | *Transcription/translation* | | | |
| | HP1048 | *infB* | Translation initiation factor IF-2 | 1.0 |
| | HP1203 | *nusG* | Transcription antitermination protein | 0.6 |
| | | | | |
| | HP0514 | *rpll* | Ribosomal protein L9, 50S | 0.8 |
| | | | | |
| | *Unknown function* | | | |
| | HP0129 | | Hypothetical protein | 0.4 |
| | HP0217 | | Hypothetical protein | 0.8 |
| | HP0222 | | Hypothetical protein | 0.6 |
| | HP1005 | | Hypothetical protein | 0.9 |
| | HP1049 | | Hypothetical protein | 0.6 |
| | HP1074 | | Hypothetical protein | 0.5 |
| | HP1122 | | Hypothetical protein | 0.5 |
| | HP1242 | | Hypothetical protein | 0.4 |
| | | | | |
| | *Amino acid metabolism* | | | |
| | HP1050 | *trhB* | Homoserine kinase | 0.7 |
| | | | | |
| | *Energy metabolism* | | | |
| | HP1099 | *eda* | 2-keto-3-deoxy-6-phoshogluconate aldolase | 0.6 |
| | | | | |
| Repressed genes | *Transcription/translation* | | | |
| | HP0569 | *engD* | GTP-dependent nucleic acid-binding protein, putative translation- associated GTPase | -0.7 |
| | HP1310 | *rpsQ* | Ribosomal protein S17, 30S | -0.5 |
| | HP1317 | *rplW* | Ribosomal protein L23, 50S | -0.4 |
| | | | | |
| | *Unknown function* | | | |
| | HP0080 | | Hypothetical protein | -0.4 |
| | | | | |
| | *Transport and binding* | | | |
| | HP1400 | *fecA* | Iron (III) dicitrate transport protein | -0.6 |
| | HP0490 | | Potassium channel protein, putative | -0.6 |
| | | | | |
| | Energy metabolism | | | |
| | HP1272 | | NADH dehydrogenase subunit M | -0.6 |
| | HP1273 | | NADH dehydrogenase subunit N | -0.7 |
| | HP1539 | *fbcH* | Ubiquinol cytochrome c oxidoreductase, cytochrome b subunit | -0.6 |
| | | | | |
| | *Cellular processes* | | | |
| | HP1160 | | Metolloprotease (putative) | -0.6 |

| | | | | |
|---|---|---|---|---|
| | *Central intermediary metabolism* | | | |
| | HP0070 | *ureE* | urease accessory protein | -0.6 |
| | HP0071 | *ureI* | urease accessory protein | -0.7 |

**FIGURE 4.1.**

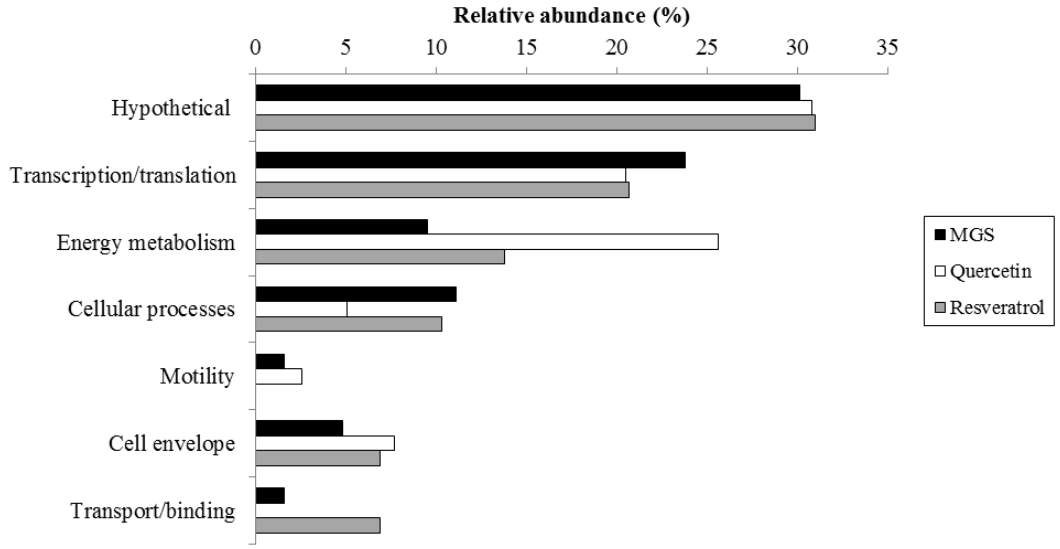
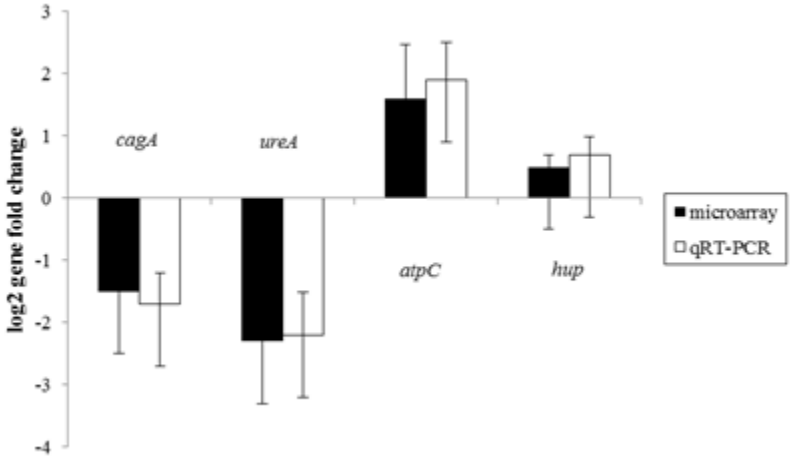

Relative abundance (%)
0
5
10
15
20
25
30
35
Hypothetical
Transcription/translation
Energy metabolism
Cellular processes
Motility
Cell envelope
Transport/binding
MGS
Quercetin
Resveratrol

**FIGURE 4.2.**


log2 gene fold change
3
2
1
0
-1
-2
-3
-4
cagA
ureA
atpC
hup
microarray
qRT-PCR

# CHAPTER FIVE

# CONCLUSION

In this study, we examined the properties of muscadine grape skin and constitutive polyphenolic compounds against *Helicobacter pylori*. Both muscadine grape skin and quercetin inhibited bacterial growth *in vitro* and *in vivo* while significantly reducing inflammation and gastric damage in *H. pylori*-infected mice. The major phenolics quercetin and resveratrol, located in the skin, have the strongest anti-*H. pylori* activity *in vitro*, especially when used in combination, and are independent of pH. Neither quercetin nor resveratrol affected *H. pylori* outer membrane integrity or significantly altered cell surface hydrophobicity; however, muscadine grape skin did suggesting additional unknown factors not revealed in this study. In addition, quercetin reduced *H. pylori* motility while being transported into the cell cytosol. Gene expression studies showed that microarrays may be useful in the characterization of novel plant-based antimicrobials while supporting previous findings on the anti-*H. pylori* potential of polyphenols quercetin and resveratrol. Furthermore, this study demonstrates that with low inhibitor concentration and short exposure, only a few genes show a clear change in expression and may be optimal for the determination of primary response mechanisms. However, these may be concentration- and/or time-dependent and require additional preliminary work to optimize conditions, but may be useful in eliminating many secondary effects commonly seen in array studies. It is also our opinion that using crude extracts are not suitable in the screening process for specific antimicrobial targets of polyphenolic compounds as results are complicated given numerous uncontrollable factors.

Our results indicate that muscadine grape skin and constitutive polyphenolics quercetin and resveratrol exert strong anti-*H. pylori* activity and may have the potential to be incorporated into an effective, diet-based approach for prevention and/or treatment of *H. pylori* infection.