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Using Food to Reduce *H. pylori*-associated Inflammation

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Inflammation is widely recognized as a risk factor for gastric *H. pylori*-associated disease and disruption of this process provides a potential target for intervention. Using an *in vitro* system, broccoli sprouts, manuka honey and omega-3 oil, singly and in combination, were screened for their ability to limit *H. pylori*-associated inflammation. Each food significantly attenuated the release of IL-8 by *H. pylori*-infected cells, although the magnitude of this effect was variable. Only broccoli sprouts (0.125 mg/mL, w/v) were able to inhibit IL-8 release in response to TNFα, suggesting it acted by a different mechanism to the other two foods. The combination of manuka honey (1.25%, v/v) with omega-3 oil (0.006%, v/v) failed further to reduce IL-8 levels below those observed with honey alone, but the same concentrations of omega-3 oil and manuka honey independently enhanced the antiinflammatory effect of the isothiocyanate-rich broccoli sprouts. The results suggest that in the future certain foods may find increased clinical use as a non-antimicrobial approach for reducing the inflammation that is a major risk factor for *H. pylori*-associated disease, notably gastric cancer.

Keywords: Helicobacter pylori; inflammation; food; gastric cancer.

INTRODUCTION

Worldwide, gastric cancer is the second most frequent cancer, and *H. pylori* has an integral role in disease progression (Uemura *et al*., 2001). However, despite this, there is a growing awareness that eradication of these bacteria from the stomach may not be entirely beneficial for the human host. Mounting evidence of an increase in more proximal diseases such as gastro-oesophageal reflux, Barrett's oesophagus and adenocarcinomas of the lower oesophagus (de Martel *et al*., 2005) as *H. pylori* infection rates fall, notably in developed countries, suggests a protective role for these bacteria against these diseases (Blaser, 2005). Therefore, elimination of *H. pylori* from the human stomach may have long-term risks as well as benefits for human health.

Inflammation that increases the risk of atrophy and intestinal metaplasia is widely recognized as a risk factor for gastric *H. pylori*-associated disease. Pharmaceutical-based reduction of this inflammation is possible but the approach may be limited by the fact that *H. pylori* infect more than half the world's population (Parsonnet, 1995). *In vitro* studies provide evidence of anti-*H. pylori* activity in certain foods, including broccoli sprouts, honey and oils (Fahey *et al*., 1997; Frieri *et al*., 2000; Keenan *et al*., 2010). There is little evidence of these *in vitro* studies translating into therapeutic clearance of *H. pylori* from the human stomach (Galan *et al*., 2004; McGovern *et al*., 1999; Frieri *et al*., 2000), but it is possible that consumption of these foods may attenuate the host inflammatory response to infection (Prakash *et al*., 2008; Hudert *et al*., 2006). Intriguingly, in 1986, when the discovery of *H. pylori* was relatively new, a hypothesis was put forward that increased consumption of vegetable oils over animal fats was a contributing factor in the decline of peptic ulcer disease in both the United States and in England and Wales in the preceding 20 years (Hollander and Tarnawski, 1986). There is also evidence that some human dietary adjustments can result in regression of intestinal metaplasia and atrophy (Correa, 2000) and this concept is strengthened by a recent study reporting that daily intake of sulforaphane-rich broccoli sprouts is associated with reduced gastric inflammation in humans and mice (Yanaka *et al*., 2009).

Research has shown that contact of *H. pylori* with epithelial cells results in the expression of a potent neutrophil-attracting chemokine, interleukin (IL)-8 via activation of the nuclear factor (NF)-κB pathway (Lamb *et al*., 2009). Increased IL-8 is found in *H. pylori*-infected gastric tissue (Noach *et al*., 1994) and disruption of cytokine levels provides a target for intervention to reduce *H. pylori*-associated inflammation. To explore this hypothesis, it was investigated whether broccoli sprouts, honey and omega-3 oil, singly and in combination, were able to affect the release of IL-8 from AGS cells, a cultured gastric epithelial cell line that is widely used for *in vitro* analysis of *H. pylori* pathogenesis. It was found that each food significantly attenuated the release of IL-8, and importantly, that omega-3 oil or manuka honey in combination with broccoli sprouts potentiated the antiinflammatory effect of these isothiocyanate-rich cruciferous seedlings.

MATERIALS AND METHODS

Food preparation. Each food was prepared and diluted in F12 Nutrient Mixture (HAM) (Invitrogen, Auckland, New Zealand) supplemented with 10% (v/v) fetal

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bovine serum (FBS; Gibco BRL, Auckland NZ) without antibiotics. Commercially grown broccoli sprouts, which are the 3-day old germinated seeds of broccoli (*Brassica oleracea)*, were purchased from a supermarket chain and lyophilized. Prior to use, the lyophilized sprouts were reconstituted in medium, homogenized and centrifuged to remove particulate matter before being passed through a 0.2 μm filter, giving a sterile stock solution that was subsequently diluted for use. Manuka honey (UMF20; Comvita, New Zealand) was dissolved in medium, centrifuged to remove any particulate material and used immediately, without filtration. The UMF 20 rating of this honey indicates antibacterial strength of the honey comparable to a 20% standard phenol solution. The chemical composition of the omega-3 oil (Sea Dragon Marine Oils Ltd, New Zealand) used was 41.4% total omega-3 and included 27.5% docosahexaenoic acid (DHA) and 8.5% eicosapentaenoic acid (EPA). The source of the oil was *Macruronus novaezelandiae*. The oil was dispersed in 95% ethanol before being diluted to give a 1% (v/v) stock solution in medium (with a final concentration of 2.5% ethanol). This stock solution was then serially diluted in medium without ethanol. For bacterial growth assays (see below), each food was prepared and diluted in Brucella broth (BB; Difco, Detroit, MI) with 5% (v/v) FBS.

Bacterial strain and culture. Each food was tested against the well characterized *H. pylori* clinical isolate 60190 (ATCC 49503) (Cover *et al*., 1990), a cag PAI + strain associated with enhanced IL-8 production by AGS cells (Sharma *et al*., 1995). The bacteria were grown overnight in BB supplemented with 5% (v/v) FBS at 37 °C under micro-aerobic conditions on a rotating platform (100 rpm). The culture density was monitored by optical density (650 nm) and bacterial numbers adjusted to give approximately 8.5×10^8 per mL. Cultures were assessed by light microscopy and only used when the majority of bacteria were helical-shaped and highly motile.

Cell culture. The AGS cell line (ATCC CRL-1739) derived from a human gastric adenocarcinoma was used in this study. The AGS cells were cultured routinely in F-12 Nutrient Mixture (HAM) supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin-glutamine supplement (all from Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were seeded on 24-well plates and cultured overnight. The resulting cell monolayers were washed twice with antibiotic-free medium before the addition of *H. pylori*.

Cell viability. The cell viability was assessed by propidium iodide staining after 24 h incubation with *H. pylori* and/or the foods. Culture medium from two duplicate wells was removed and pooled. The adherent AGS cells were detached with 1% trypsin–EDTA, the well washed with PBS, and combined fractions added to the pooled medium. The cells were incubated with 2 μg/mL propidium iodide for 10 min, and the fluorescence of 10000 cells was measured using a fluorescence-activated cell sorter (FACS) vantage flow cytometer (Beckman Coulter Cytomics FC 500 MPL, Australia) and CXP software (Beckman Coulter 2005). Cytotoxicity was expressed as the percentage of propidium iodide-positive (dead) cells.

IL-8 expression by AGS cells. Foods were tested for their ability to reduce IL-8 expression by *H. pylori*- or TNFα (R & D Systems, Minneapolis, MN)-stimulated AGS cells. Each food was prepared and serially diluted (in F12 Nutrient Mixture supplemented with 10% (v/v) FCS and 1% glutamine but no antibiotics), starting at a dilution four-fold below the minimal inhibitory concentration (MIC) determined for each individual food against *H. pylori* (below). A positive control culture (*H. pylori* alone) and a negative control (medium alone) were included on every plate. The amount of IL-8 secreted into cell culture medium following 24 h incubation was determined by ELISA using R & D Systems Quantikine kit (Minneapolis, MN), according to the manufacturer's instructions (Ismail *et al*., 2003).

Bacterial growth. The effect of each food on *H. pylori* growth was determined using an assay that measures changes in absorbance over time (Keenan *et al*., 2010). Briefly, *H. pylori* were cultured in a 96-well plate in the presence of each food serially diluted in BB with 5% (v/v) FBS (under sterile conditions) to give a range of concentrations. Additional wells (without the addition of *H. pylori*) acted as absorbance controls for each food dilution. The positive control consisted of wells inoculated with growth medium seeded with *H. pylori* at the same rate as the test wells. The optical density (650 nm) of each of the wells was read at regular intervals (up to 48 h) and the mean absorbances of each food dilution (test wells minus absorbance controls) calculated. The MIC was defined as the lowest concentration that resulted in non-detectable changes in absorbance (and therefore no growth) after 48 h of incubation at 37 °C under microaerophilic conditions (Fahey *et al*., 2002). Foods were also tested together using a chequerboard assay to determine if their ability to inhibit *H. pylori* growth was more effective in combination than similar concentrations of the same foods tested individually (Keenan *et al*., 2010).

Labelling of bacteria. Broth grown bacteria were pelleted and resuspended in 1.0 mL of 0.15 M NaCl and 0.1 M Na_2CO_3, pH 9.0. Ten microlitres of freshly prepared 1% fluorescein isothiocyanate (FITC, Sigma) in dimethyl sulfoxide (DMSO) was added to the suspension, which was then incubated for 1 h at room temperature in the dark. Bacteria were recovered by centrifugation ($3000 \times \mathbf{g}$ for 5 min), resuspended in 1 mL of PBS-Tween 20 (0.05%, v/v) and pelleted by centrifugation as above. The wash cycle was repeated three times. Bacterial numbers were adjusted to give approximately 3×10^7 per mL.

Adherence assay. The AGS cells were cultured overnight and then washed once with PBS. The medium was replaced with antibiotic-free medium prior to the addition of FITC-labelled bacteria (MOI 100:1) for 24 h. To investigate a role for manuka honey in blocking *H. pylori* adherence, solutions of honey diluted in antibiotic-free cell culture medium were added to the cells at the same time as FITC-labelled bacteria. Control cells were incubated with bacteria alone. After incubation, the cells were washed three times to remove unbound

bacteria and lifted with trypsin/EDTA (Invitrogen). Fluorescence measurements were made using a FACS flow cytometer, with a total of 10000 events collected for each sample. Mean fluorescence intensity (MFI) values of cells incubated in the absence of manuka honey were subtracted from the values of bacteria and honey-treated cells.

Statistical analysis. Results are the mean ± standard error of the mean (SE) of at least three independent experiments. Data were analysed by one-way analysis of variance (ANOVA) using GraphPad Prism (Version 5.01). If the ANOVA p value was < 0.05, this was followed by Tukey's post-hoc test.

RESULTS AND DISCUSSION

The aim of this study was to determine whether broccoli sprouts, manuka honey and omega-3 oil, singly or in combination, might attenuate inflammation by blocking the release of IL-8 from gastric epithelial cells infected with *H. pylori*. The AGS cells are a gastric epithelial cell line that does not constitutively produce IL-8 to significant levels (Sharma *et al*., 1995) and therefore an ideal model to investigate the ability of foods to abrogate an inflammatory response. Recently, it was reported that the minimal concentrations of broccoli sprouts, UMF 20 manuka honey and omega-3 oil required to inhibit *H. pylori* growth were 0.5 mg/mL, 5% (w/v) and 0.025% (v/v), respectively (Keenan *et al*., 2010). Each of these foods was tested for its ability to inhibit IL-8 release from *H. pylori*-stimulated AGS cells, starting at a maximum concentration four-fold below the MIC for *H. pylori* growth. A multiplicity of infection (MOI) of 50:1 was used and IL-8 levels were measured after 24 h incubation.

Broccoli sprouts, manuka honey and omega-3 oil each reduced IL-8 release by *H. pylori*-infected AGS cells. Broccoli sprouts had the greatest effect, with 60% inhibition at 0.125 mg/mL (Fig. 1A). In contrast, manuka honey was able to reduce IL-8 output by 30–40% (Fig. 1B) and omega-3 oil by only 10% (Fig. 1C). The study also compared the response of broccoli sprout, manuka honey and omega-3 oil-treated AGS cells to TNFα-stimulation. TNFα (20 ng/mL) stimulated AGS cells to express similar levels of IL-8 over a 24 h period as *H. pylori* strain 60190 added at a MOI of 50:1 (not shown), supporting evidence that TNFα promotes an intracellular signalling pathway convergent to that initiated by *H. pylori* in AGS cells (Lamb *et al*., 2009). Using the same concentrations of foods as above, it was determined that co-incubation with broccoli sprouts resulted in a dose-dependent decrease in IL-8 expression by AGS cells co-cultured with TNFα, similar to that observed in the presence of *H. pylori* (Fig. 1D). In contrast, manuka honey (Fig. 1E) and omega-3 oil (Fig. 1F) failed to limit IL-8 output by TNFα-treated AGS cells. These foods had no effect on AGS cell viability

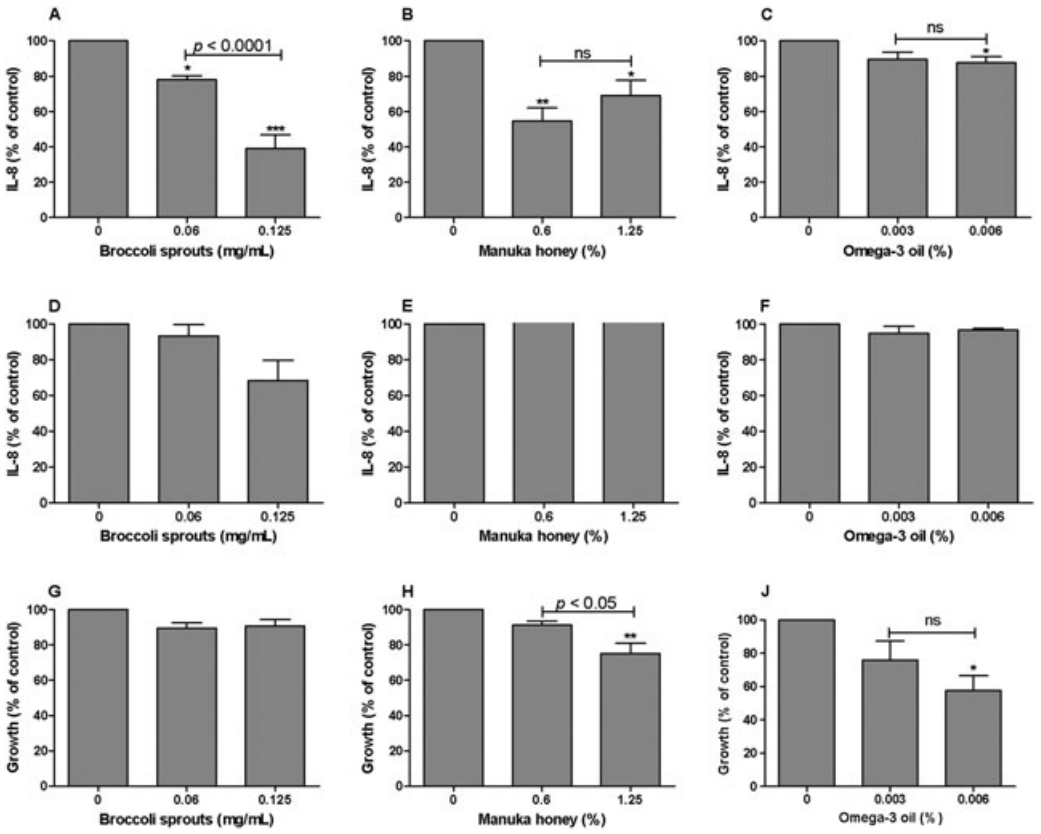

Figure 1. Antiinflammatory and antibacterial effect of foods. The AGS cells were incubated *H. pylori* (top) or TNFα (middle) alone or with increasing concentrations of (A, D) broccoli sprouts, (B, E) manuka honey or (C, F) omega-3 oil for 24 h. Supernatants were collected and IL-8 levels measured by ELISA. Additionally, *H. pylori* were incubated alone (control) or with increasing concentrations of (G) broccoli sprouts, (H) manuka honey or (J) omega-3 oil and the change in bacterial growth was detected by measuring the absorbance at 650 nm after 24 h (bottom). Inhibition of IL-8 release or bacterial growth in the presence of each food is reported as a percentage of the respective controls. Results are the mean ± SE of three independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; versus control (broccoli sprouts, manuka honey, omega-3 oil, respectively).

(Table 1), which suggests that the inhibitory effects of manuka honey and omega-3 oil are limited to *H. pylori*-dependent stimulation. In support of this hypothesis, manuka honey (Fig. 1H) and omega-3 oil (Fig. 1J) each significantly attenuated *H. pylori* growth, despite being diluted to a maximum concentration four-fold below the concentration required completely to inhibit bacterial growth (Keenan *et al.*, 2010).

Next, the study tested these three foods in pairs to determine their combined effect on IL-8 release. In these experiments, the results were normalized to the IL-8 levels released by the *H. pylori*-treated AGS cells (100%). It was observed that manuka honey increased the inhibitory effect that broccoli sprouts had on IL-8 output (Fig. 2A). Moreover, manuka honey added to 0.06 mg/mL broccoli sprouts reduced IL-8 output to 40% of the control level, providing evidence of an additive effect between these two foods. Omega-3 oil, which only had a very small effect on IL-8 release from *H. pylori*-infected AGS cells when tested singly, also demonstrated a additive effect when added to broccoli sprouts, reducing IL-8 output by 77% (Fig. 2B). In contrast, omega-3 oil had no additive effect on the existing antiinflammatory activity of manuka honey (Fig. 2C). The combined effect of broccoli sprouts with omega-3 oil or manuka honey on reducing IL-8 release did not correlate with reduced AGS cell viability (Table 1) and/or significant inhibition of bacterial growth (Keenan *et al.*, 2010).

A daily intake of sulforophane-rich broccoli sprouts is associated with reduced gastric inflammation in humans and mice (Yanaka *et al.*, 2009). There is also evidence of manuka honey and omega-3 oil reducing colonic inflammation in experimentally induced inflammatory bowel disease in rats (Prakash *et al.*, 2008; Hudert *et al.*, 2006). The antiinflammatory activity in the broccoli sprouts likely reflects high levels of glucoraphanin, a naturally occurring glucosinolate precursor of the isothiocyanate sulforaphane that is found in these 3-day old broccoli seedlings (Fahey *et al.*, 1997). Sulforaphane is known to act on the NF-κB pathway to suppress IL-8 release (Heiss *et al.*, 2001) and our preliminary data suggests that broccoli sprouts affect the same pathway in AGS cells (Salm, unpublished data).

The antiinflammatory activity of honey is largely attributed to the presence of polyphenols that include flavonoids, phenolic acids and phenolic acid derivatives. These dampen inflammation by mechanisms that include the suppression of NF-κB activation (Abdel-Latif *et al.*, 2005). However, our finding that IL-8 levels in TNFα-treated cells were unchanged in the presence of manuka honey (Fig. 1E) suggests that the effect of the honey might be antibacterial rather than antiinflammatory at the concentrations used in our experiments. *H. pylori* adherence is a precursor of CagA translocation and CagA-induced release of IL-8 in AGS cells (Lai *et al.*, 2008) and there is evidence that carbohydrate-rich foods interfere with this process (Lengsfeld *et al.*, 2004). Honey is essentially a complex mixture of sugars and our observation of significantly reduced IL-8 levels in the presence of 0.6% (v/v) manuka honey despite near normal bacterial growth (Fig. 1H) suggested that honey might affect the ability of *H. pylori* to adhere to AGS cells. However, despite evidence of a dose-dependent effect of manuka honey on *H. pylori* adherence to AGS cells, this effect was not significant

Table 1. The effect of individual, and combined, foods on AGS cell viability

Food (concentration)	% Dead cells
Broccoli sprouts (0.125 mg/mL, w/v)	1.8 ± 0.1
Manuka honey (1.25%, w/v)	2.0 ± 0.2
Omega-3 oil (0.006%, v/v)	5.1 ± 0.1
Broccoli sprouts (0.125 mg/mL, w/v) and manuka honey (1.25%, w/v)	2.3 ± 0.4
Broccoli sprouts (0.125 mg/mL, w/v) and omega-3 oil (0.006%, v/v)	6.2 ± 0.9

Viability control, 1.7% dead cells.

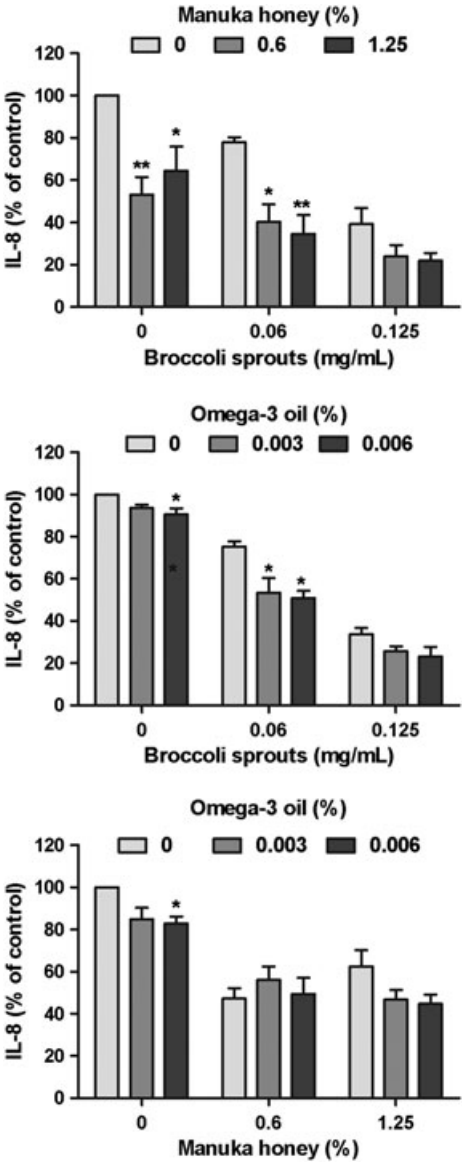

Figure 2. Manuka honey and omega-3 oil significantly enhance the antiinflammatory effect of broccoli sprouts. The AGS cells were incubated with bacteria alone or with increasing concentrations of combinations of broccoli sprouts, manuka honey and omega-3 oil for 24 h. IL-8 levels were measured in the supernatants and inhibition of IL-8 release in the presence of these food combinations reported as a percentage of the control. Results are the mean ± SE of three independent experiments. $*p < 0.05$; $**p < 0.01$; versus control (broccoli sprouts in the absence of manuka honey and omega-3 oil, respectively).

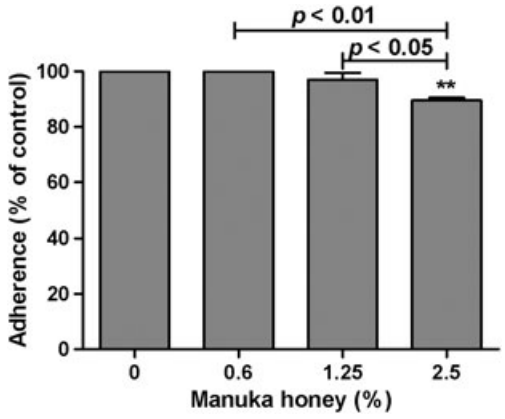

Figure 3. Manuka honey inhibits *H. pylori* adherence to AGS cells. The AGS cells were incubated with FITC-labelled bacteria alone or with increasing concentration of manuka honey. Bacterial adherence was measured by FACS flow cytometry and mean fluorescence intensity (MFI) values of cells incubated in the absence of manuka honey were subtracted from the values of bacteria and honey-treated cells. Results are the mean ± SE of three independent experiments. $**p < 0.01$; versus control (bacteria in the absence of manuka honey).

at the concentrations of manuka honey used in this study (Fig. 3). Thus, the mechanism(s) behind the ability of manuka honey to reduce IL-8 expression by *H. pylori*-infected gastric epithelial cells remains unclear. It may reflect a combination of antibacterial, antiadhesive and antiinflammatory activity.

The mechanism underlying the significant fall in IL-8 levels when omega-3 oil is added to broccoli sprouts is also unclear since the concentration-dependent effect of omega-3 oil on bacterial growth (Fig. 1J) was lost when the oil was added with broccoli sprouts to *H. pylori*-infected AGS cells (Keenan *et al*., 2010). Omega-3 oil is rich in long chain polyunsaturated fatty acids (PUFAs) that reportedly perturb host and bacterial cell membranes. In bacteria, this can result in cell lysis (Thompson *et al*., 1994; Khulusi *et al*., 1995), whereas in host cells, incorporation of omega-3 PUFAs into the phospholipid membrane can cause a loss of membrane fluidity that may modulate lipid raft assembly and/or function (Siddiqui *et al*., 2007) that are required for *H. pylori* induction of the NF-κB pathway and expression of IL-8 (Hutton *et al*., 2010). Additionally, there is growing evidence of a role for the metabolic products of omega-3 PUFAs in the resolution of inflammation via mechanisms that include counter-regulating the production of pro-inflammatory prostaglandins and leukotrienes (Kohli and Levy, 2009), binding to the nuclear peroxisome proliferator-activated receptor (PPAR) γ and interfering with the activation of NF-κB, thereby potentially inhibiting IL-8 expression (Groeger *et al*., 2010).

Peroxisome proliferator-activated receptor γ is present in AGS cells (Cha *et al*., 2011) and the omega-3-related reduction in IL-8 levels seen in our study may involve PPAR γ and interference with NF-κB. However, this mechanism seems unlikely given our finding that the combined effect of omega-3 oil (0.006%) and 0.06 mg/mL broccoli sprouts on IL-8 output was no greater that that observed when TNFα-treated AGS cells were treated with broccoli sprouts alone (not shown). A significant disruption of lipid rafts in our assay system is also unlikely, again given our finding that IL-8 levels in TNFα-treated cells were unchanged in the presence of the omega-3 oil at the concentrations used here. Thus, in our assay system, the effect of omega-3 oil on IL-8 output by *H. pylori*-infected AGS cells likely reflects a predominantly antibacterial effect. However, it is important to note that the concentrations of omega-3 oil and manuka honey used in our study were much lower than normally used in dietary studies.

In conclusion, three foods were screened (singly and in combination) for their ability to reduce the inflammatory response of a gastric epithelial cell line to *H. pylori*. These preclinical findings do not provide evidence that consumption of these foods will have a similar effect *in vivo*, but there are studies that support this concept (Yanaka *et al*., 2009; Frieri *et al*., 2000; Galan *et al*., 2004). It is proposed that in the future there may be an increased clinical use of certain foods for reducing the inflammation that is a major risk factor for *H. pylori*-associated disease, notably gastric cancer.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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