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1 ***Helicobacter pylori*'s cholesterol uptake impacts resistance to**
2 **docosahexaenoic acid**

3 Marta Correia^{1,2,3}, Susana Casal⁴, João Vinagre^{1,2,5}, Raquel Seruca^{1,2}, Ceu
4 Figueiredo^{1,2}, Eliette Touati³, José C Machado^{1,2#}

5

6 ¹Institute of Molecular Pathology and Immunology of the University of Porto,
7 Portugal. Rua Dr. Roberto Frias s/n 4200-465-Porto, Portugal;

8 ²Faculdade de Medicina da Universidade do Porto, Portugal. Al. Prof. Hernâni
9 Monteiro 4200 - 319 Porto Portugal.

10 ³Institut Pasteur, Unité de Pathogénèse de *Helicobacter*, Paris, France. 25,28 rue
11 du Docteur Roux 75724 Paris CEDEX 15 – France.

12 ⁴REQUIMTE/Laboratório de Bromatologia e Hidrologia, Faculdade de Farmácia do
13 porto Rua de Jorge Viterbo Ferreira, 228, 4050-313, Porto, Portugal.

14 ⁵Institute of Biomedical Sciences Abel Salazar of the University of Porto (ICBAS),
15 4099-003 Porto, Portugal.

16 **#Corresponding author**

17 - Dr. JC Machado, IPATIMUP, Rua Dr. Roberto Frias, s/n, 4200-465 Porto,
18 Portugal;Tel:00351225570700;Fax:00351225570799,josem@ipatimup.pt.

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20 Bactericidal agents; *Helicobacter pylori*; Mechanisms of resistance; Cholesterol;
21 PUFAs

22

23 **Abstract** *Helicobacter pylori* colonizes half of the world population and is associated
24 with gastric cancer. We have previously demonstrated that docosahexaenoic acid
25 (DHA), an n-3 polyunsaturated fatty acid known for its anti-inflammatory and
26 antitumor effects, directly inhibits *H. pylori* growth in vitro and in mice. Nevertheless,
27 the concentration of DHA shown to reduce *H. pylori* mice gastric colonization was
28 ineffective in vitro. Related to the auxotrophy of *H. pylori* for cholesterol, we
29 hypothesize that other mechanisms, in addition to DHA direct antibacterial effect,
30 must be responsible for the reduction of the infection burden. In the present study
31 we investigated if DHA affects also *H. pylori* growth, by reducing the availability of
32 membrane cholesterol in the epithelial cell for *H. pylori* uptake. Levels of cholesterol
33 in gastric epithelial cells and of cholesteryl glucosides in *H. pylori* were determined
34 by thin layer chromatography and gas chromatography. The consequences of
35 epithelial cells' cholesterol depletion on *H. pylori* growth were assessed in liquid
36 cultures. We show that *H. pylori* uptakes cholesterol from epithelial cells. In addition,
37 DHA lowers cholesterol levels in epithelial cells, decreases its de novo synthesis,
38 leading to a lower synthesis of cholesteryl glucosides by *H. pylori*. A previous
39 exposition of *H. pylori* to cholesterol influences the bacterium response to the direct
40 inhibitory effect of DHA. Overall, our results suggest that a direct effect of DHA on
41 *H. pylori* survival is modulated by its access to epithelial cell cholesterol, supporting
42 the notion that cholesterol enhances the resistance of *H. pylori*. The cholesterol-
43 dependent resistance of *H. pylori* to antimicrobial compounds raises new important
44 aspects for the development of new anti-bacterial strategies.

45 **Introduction**

46 *H. pylori* colonizes half of the world population and is associated with dyspepsia,
47 chronic gastritis, ulcer disease and gastric cancer. (Peek and Crabtree, 2006)
48 Eradication of this infection leads to a reduction in the recurrence of peptic ulcer
49 disease and prevents the development of gastric precancerous lesions (Talley et al.,
50 2008), highlighting the need for new therapeutic strategies to treat *H. pylori* carriers.
51 Usually, the first-line option for eradicating *H. pylori* infection is a triple treatment that
52 consists of a proton pump inhibitor (PPI) and two antimicrobial agents. (Malfertheiner
53 et al., 2007) However, the increasing incidence of bacteria strains resistant to these
54 antibiotics is partially responsible for failure of treatment reported for triple therapy.
55 (Gisbert et al., 2010) Thus, new alternative therapies that improve the eradication
56 rate of *H. pylori* and that circumvent the antibiotics resistance are of great interest.
57 According to a previous study (Thompson et al., 1994), we have demonstrated that
58 polyunsaturated fatty acids (PUFAs) and in particular docosahexaenoic acid (DHA),
59 which is a n-3 PUFA, hold an anti-*H. pylori* growth action. DHA inhibits *H. pylori*
60 growth in a concentration dependent manner in vitro, and this was confirmed in a
61 murine model.(Correia et al., 2012) Our previous data also suggest that this
62 inhibitory effect involves the incorporation of DHA into the bacterium's cell wall,
63 inducing changes in the components of the cell wall and facilitating bacteria lysis.
64 (Correia et al., 2012) Nevertheless, the concentration of 50 μ M of DHA shown to
65 reduce *H. pylori* mice gastric colonization was not effective in vitro. (Correia et al.,
66 2012) These results are in line with the fact that other mechanisms in addition to a
67 DHA direct antibacterial effect on *H. pylori*, must be responsible for the reduction of

68 *H. pylori* load in the stomach of infected mice treated with DHA. In this context, we
69 cannot exclude that the direct action of DHA on epithelial cells impacts its anti-
70 *Helicobacter* effect besides affecting bacteria. In fact, it is well recognized that DHA
71 is incorporated into cell membrane of eukaryotic cells leading to alteration of
72 membrane physical properties such as permeability, lateral diffusion, lipid packing,
73 domain formation and cholesterol availability/distribution.(Shaikh et al., 2004)
74 Interestingly, cholesterol rich-platforms within cell membranes have been implicated
75 as cell gates for pathogens.(Xiong et al., 2009) Moreover, the genus *Helicobacter*
76 has the unique feature of assimilating free cholesterol (FC) extracted from epithelial
77 lipid rafts into its bacterial outer membrane, allowing its enzymatic
78 glycosylation.(Wunder et al., 2006) The *cgt H. pylori* mutant, which lacks the ability
79 to biosynthesize the glycosyl cholesterol and retains FC without glycosylation, is
80 easily phagocytized by macrophages and induces activation of antigen-specific T
81 cells. These features highlight the importance of cholesterol metabolism in the
82 bacteria survival and ability to colonize.(Shimomura et al., 2004; Wunder et al., 2006)
83 Moreover, the translocation of *H. pylori* virulence factors, such as vacuolating
84 cytotoxin A (VacA) and cytotoxin associated gene A (CagA), via the type IV secretion
85 system (TFSS), depends on the FC level of epithelial cell, as they are blocked by
86 cholesterol depletion (ex: cyclodextrins).(Lai et al., 2008; Patel et al., 2002) The
87 glycosyl cholesterol also modifies lateral-phase segregation in epithelial cell
88 membranes, reorganizing the architecture of the membrane and promoting the
89 formation of a functional TFSS.(Wang et al., 2012) Hence, glycosylation of
90 cholesterol plays an important role in *H. pylori* survival.

91 Given the known ability of n-3 PUFAs, and DHA in particular, to interfere with
92 cholesterol's distribution in the epithelial cell membrane and given the auxotrophy of
93 *H. pylori* for cholesterol, we hypothesized that besides its direct antibacterial effect,
94 DHA holds an indirect effect on *H. pylori* growth. This indirect effect might be
95 achieved through mechanisms that involve DHA ability to interfere with cholesterol
96 in epithelial cells. Additionally, statins, that as DHA, affect cholesterol metabolism,
97 increase the eradication of *H. pylori* infection when used as co-adjuvant to standard
98 triple therapy (Nseir et al., 2012). In this study we aim to further characterize the
99 mechanisms by which DHA holds an anti-*H. pylori* activity. We investigate if in
100 addition to its direct bactericidal action, DHA exerts an indirect effect on *H. pylori* by
101 modulating epithelial cells' cholesterol uptake.

102 MATERIAL AND METHODS

103 Fatty acids, *H. pylori* strains and cell culture conditions

104 DHA was obtained from *Cayman Chemical Company* (Michigan, USA) with a degree
105 of purity of 99% in ethanol 0.06% (v/v). The *H. pylori* strains used were: SS1 (Lee et
106 al., 1997) 26695 (ATCC 700392) obtained from the American Type Culture
107 Collection (ATCC, Rockville, MD). *H. pylori* was grown on blood agar base 2 plates
108 (Oxoid, Lyon, France) supplemented with 10% defibrinated horse blood (bioMérieux,
109 Marcy l'Etoile, France). Plates were incubated at 37°C for 48h under microaerobic
110 conditions (7% O₂, 10% CO₂; anoxomat system). Cell infections were performed with
111 *H. pylori* grown in liquid cultures. Briefly, bacteria were inoculated to an initial optical
112 density of 0.03 at 600 nm into liquid Brucella broth (BB) (Oxoid) supplemented with
113 10% fetal calf serum (FCS). *H. pylori* grown for 18-20h yields approximately 5x10⁸
114 colony forming units (CFU)/ml. Bacterial cholesterol analysis was performed with *H.*
115 *pylori* strain 26695, due to the fact that this strain has a functional TFSS, described
116 to be sensitive to host epithelial cholesterol. (Wang et al., 2012)

117 Human gastric epithelial cells (AGS: gastric adenocarcinoma, CRL-1739, ATCC-
118 LGC®) were grown in RPMI medium 1640 supplemented with 10% FCS and 1% of
119 penicillin-streptomycin under 37°C and under 5% CO₂ in humidified air. For cell
120 viability assays, cells were trypsinized and counted using trypan blue (Sigma-
121 Aldrich) staining. Liquid cultures of *H. pylori* were added to AGS cells in serum free
122 medium for at least 18h, at a multiplicity of infection (MOI) of 100:1 and kept under
123 agitation for 1h, 6h or 24h at 37° C. Treatment with cholesterol rich-microdomains
124 disruptors in AGS cells was performed with methyl-β-cyclodextrin (MBCD; Sigma-

125 Aldrich). Briefly, AGS cells were washed with PBS three times and treated with 2.5
126 mM of MBCD for 1h at 37°C. To remove MBCD cells were washed with PBS three
127 times.

128

129 **Incubation of AGS cells with 25-benz-oxa-diazol- (NBD)-labeled cholesterol** 130 **and with DHA**

131 Stock solutions of 25-benz-oxa-diazol-labeled cholesterol (NBD-cholesterol) (Avanti
132 Polar Lipids) were solubilized in ethanol and stored at -20°C, at a final concentration
133 of 5 µg/ml NBD-cholesterol. AGS cells were used as cholesterol suppliers for *H.*
134 *pylori* uptake, because of previous demonstration that the bacteria extracts
135 cholesterol from epithelial cells in a more efficient way than it extracts from liquid
136 medium. (Wunder et al., 2006). In order to verify that epithelial cells had cholesterol
137 available for *H. pylori* uptake, confluent AGS monolayers cells grown in a serum-free
138 RPMI medium for 3h, were labeled with NBD-cholesterol (5 µg/ml NBD-cholesterol
139 and ethanol concentration 0.25% v/v). Cells were then washed with PBS to remove
140 unbound NBD-cholesterol and treated with DHA 50µM in RPMI medium for 12h. This
141 concentration was shown to be within the range of non-toxic concentrations
142 according to trypan blue exclusion test (data not shown). After DHA treatment, AGS
143 cells were vigorously washed with PBS and put into a DHA-free fresh medium for
144 18h to exclude other sources of cholesterol than the ones present in epithelial cell
145 membranes. Cells were then infected with *H. pylori* at MOI of 100:1 for 3h.

146

147 **DHA treatment of *H. pylori* cultures**

148 Stock solutions of DHA were diluted in BB enriched with 10% FCS and used at
149 concentrations of 100µM and 250µM. To establish *H. pylori* growth curves, 18-20h
150 bacteria cultures were diluted 100-fold in 10 ml of medium with or without DHA to an
151 initial OD of 0.03. Each experiment consisting in *H. pylori* culture incubated with DHA
152 at 100 µM and 250 µM and a control non-treated culture were performed in triplicate.
153 Cultures were incubated under microaerophilic conditions, as described above.
154 Every 12h, during a 48h period, a 200 µL sample of each bacterial culture was
155 isolated, the OD measured and aliquots serially diluted and plated on blood agar
156 petri dishes. After 48h of incubation, the number of viable bacteria was determined
157 by colony forming units (CFU) counting.

158

159 **Analysis of DHA and n-3 PUFAs composition in AGS cells**

160 AGS cells used for fatty acid composition experiments grew in 75-cm² flasks as
161 previously described. Lipids were trans-esterified directly from the membrane pellets
162 by acid-catalysed transmethylation with methanolic-HCl (0.6M, 80°C, 2h), using
163 tricosanoic acid as internal standard (Sigma). Fatty acid methyl esters (FAME) were
164 extracted with hexane, taken to dryness under a nitrogen stream, then resuspended
165 in dichloromethane and stored at -20°C. The chromatograph (Chrompack, CP-9001,
166 the Netherlands) was equipped with a flame ionisation detector (250°C) and CP-Sil
167 88capillary column (Varian), with temperature gradient and splitless injection. EPA
168 and DHA were quantified from calibration curves with internal standard, submitted
169 to the entire analytical protocol, being expressed on a mass per cell count
170 basis.(Ranjekar et al., 2003)

171

172 **Free and total cholesterol quantification**

173 AGS cells used for cholesterol profile analysis experiments grew in 75-cm² flasks as
174 previously described. Cells were then washed with PBS and the gastric cell pellets
175 were extracted with 2:1 (v/v) chloroform-methanol (50 mg/L BHT), sonicated for 5
176 minutes and gently shaken overnight. Aqueous NaCl solution (0.9%) was added in
177 accordance with Folchet al.(Riff et al., 2005) The lower chloroform phase was
178 collected, dehydrated with anhydrous sodium sulphate, taken to dryness under a
179 gentle N₂ stream and reconstituted with hexane. Based on the initial cell counts, an
180 accurate volume of lipid solution corresponding to 100,000 cells (5 to 10 µL) was
181 spotted on a silica 60 HPTLC plate (Merck). A mixture of hexane: diethyl ether; acetic
182 acid (70:30:1) was used for elution and 13mM α -naphthol in sulphuric acid/methanol
183 (3/97; v/v; at 160 °C) for staining.(Riff et al., 2005) Free and esterified cholesterol
184 from Sigma (USA) was used for band identification.

185 From the remaining lipid solution, a 150 µL portion was transferred to a glass micro-
186 vial and 5- α -cholestane (Sigma) was added as internal standard. An accurate portion
187 was used for FC quantification, being taken to dryness (dessicated), derivatized with
188 N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane
189 (TMCS) (80 µl; 80°C, 1h) (Fluka, Spain) and directly evaluated by gas
190 chromatography-mass spectrometry GC-MS (details below). The remaining volume
191 was used for the analysis of total cholesterol, with a previous hydrolysis (0.36M KOH;
192 70°C; 30'+30') as described by Pelletier et al. and the lipid extract derivatized as for
193 free cholesterol.(Pelletier et al., 1987)

194 GC–MS analyses were performed on an Agilent (USA) gas chromatograph 6890
195 interfaced (280 °C) to a MSD-5973N mass selective detector and equipped with a
196 DB-5MS column (30 m × 0.25 mm I.D., 0.25 µm; J&W Scientific, USA). Electron
197 impact mass spectra were measured at acceleration energy of 70 eV. Data
198 acquisition was performed in the full-scan mode during the optimization of the
199 method (mass range 50–600). Quantification was carried out in selective ion
200 monitoring (SIM) mode with a dwell time of 30 ms and using the *m/z* 129, 329, 353,
201 268, 443, and 458 for cholesterol, and *m/z* 217, 357 and 372 for the internal
202 standard. AGS cells protein concentration was determined by Lowry’s method.
203 (Lowry et al, 1951)

204

205 **Cholesteryl glucosides analysis**

206 Lipids were extracted as described above for the cholesterol analysis and
207 reconstituted in chloroform:methanol (2:1). Each sample was applied sequentially in
208 a 20x20 silica 60 glass plate (Merck), eluted with chloroform/methanol/ammonia
209 (40/10/0.4, vol/vol) and stained with 13mM α -naphthol in sulphuric acid/methanol
210 (3/97; v/v; at 160 °C) as described by Wunter *et al.*(Wunder et al., 2006) Glycolipids
211 isolated from *H. pylori* lipids were used as reference, after fractionation by column
212 chromatography on silicic acid and confirmation by HPTLC.(Hirai et al., 1995;
213 Shimomura et al., 2004)

214

215 **Cholesterol metabolism in AGS cells upon DHA and *H. pylori* infection**

216 We were interested in assessing if DHA and *H. pylori* infection in AGS cells impaired
217 cholesterol metabolism. 3-hydroxy-3-methylglutaryl coenzyme A reductase
218 (HMGCoA-r) is the rate-limiting enzyme in cholesterol biosynthesis that catalyses
219 mevalonate formation; Low Density Lipoprotein receptor (LDLr) is the preferential
220 receptor for cholesterol uptake in epithelial cells. (Go, W. and Mani, A., 2012) (Kaur,
221 G. et al, 2012) We have assessed both their expressions by immunoblotting. Anti-
222 HMGCoA-r (Upstate, 1:200), anti-LDL-r (Abcam, 1:200) and anti- α -tubulin (Sigma,
223 1:10000) as loading control were used on protein extracts from AGS cells treated
224 with DHA for 24 hours and infected with *H. pylori* strain 26695. Briefly, cells were
225 lysed in cold Catenin lysis buffer 1% Triton X-100 (Sigma) in PBS enriched with a
226 protease inhibitor cocktail (Roche) and a phosphatase inhibitor cocktail (Sigma). The
227 proteins were quantified using a modified Bradford assay (Bio-Rad). Thirty- μ g of
228 proteins were eluted in sample buffer and loaded in 7.5% or 12% SDS-PAGE,
229 depending on the molecular mass of molecules of interest. The proteins were then
230 electroblotted onto a Hybond ECL membrane (Amersham Biosciences). Membranes
231 were blocked with 5% non-fat milk and 0.5% Tween-20 in PBS and immunoblotted.
232 Donkey anti-rabbit (Amersham Biosciences) or sheep anti-mouse (Amersham
233 Biosciences) HRP-conjugated secondary antibodies were used as required,
234 followed by ECL detection (Amersham Biosciences). Immunoblots were quantified
235 with the Quantity One Software (Bio-Rad).

236

237 **Statistical Analysis**

238 Data are expressed as mean \pm standard deviation (SD). Student's *t*-test was used

239 to assess differences in cell lipid composition profile. All statistical tests were two-
240 sided. Differences were considered significant when $P < 0.05$.

241

242 **RESULTS**

243 **DHA is incorporated into AGS cells**

244 Our first goal was to test if DHA was incorporated into AGS cells upon medium
245 supplementation. Cell cultures were treated with 50 μM of DHA for 12h, a
246 concentration shown to be non-toxic according to trypan blue exclusion test (data
247 not shown). Gas chromatography was performed to assess DHA content and n-3
248 PUFAs (DHA+EPA) content in cells. As shown in Figure 1, DHA supplementation in
249 AGS cells medium significantly increased total n-3 PUFA content (4-fold compared
250 to non-DHA treated cells) and DHA content (9-fold compared to non-DHA treated
251 cells) in AGS cells, $P < 0.05$. These results confirm that DHA present in the medium
252 was incorporated into AGS cells.

253

254 **DHA decreases cholesterol availability to *H. pylori* in AGS cells**

255 We postulated that DHA incorporation into AGS cells induces changes in cholesterol
256 levels. Cholesterol can be present either in a free form or esterified, depending on
257 epithelial cell dynamic and cholesterol metabolism.(Shimomura et al., 2004)
258 Fractions of total and FC were assessed by gas chromatography analysis. Results
259 presented in figure 2 correspond to the 24h time-point, but all the other time-points
260 assessed reflect the same relation in cholesterol levels. DHA treatment decreased
261 total cholesterol levels (4.4 ± 0.8 vs 7.1 ± 0.9 μg per mg of cell protein), $P < 0.05$ (Figure

262 2A). Similarly, the cholesterol free fraction, which is the preferential form of
263 cholesterol extracted by *H. pylori* (Shimomura et al., 2004), is significantly
264 decreased from AGS cells upon DHA treatment (5.4 ± 0.4 vs 2.8 ± 0.5 μg per mg of cell
265 protein, $P<0.05$, (Figure 2A).

266 To confirm that *H. pylori* is indeed able to extract cholesterol from epithelial cells, we
267 incubated AGS epithelial cells with *H. pylori* and then analysed FC amounts in cells
268 after 24h of infection. Both total and FC cellular levels were significantly decreased
269 by *H. pylori* infection comparatively to non-infected cells (3.3 ± 0.7 vs 7.1 ± 0.9 μg per
270 mg of cell protein, $P<0.01$, and 2.1 ± 0.6 vs 5.4 ± 0.4 μg per mg of cell protein,
271 respectively, $P<0.05$ (Figure 2A). These results were confirmed by TLC analysis
272 showing that FC was significantly lower in AGS cells treated with $50\mu\text{M}$ of DHA or
273 incubated with *H. pylori* 26695 when compared with control cells, $P<0.05$ (Figures
274 2B). In conclusion, our results indicate that DHA incorporation into AGS cells leads
275 to a decrease in the levels of total and free forms of cholesterol.

276

277 **DHA treatment of cells results in a decreased cholesterol glycosylation by *H.*** 278 ***pylori***

279 It was demonstrated that *H. pylori* glycosylates cholesterol by an enzymatic reaction
280 following its extraction from epithelial cells (Wunder et al., 2006). In order to
281 determine if the reduction of FC observed in DHA treated and infected cells results
282 in a decrease of cholesterol uptake due to *H. pylori*, we performed a TLC analysis to
283 measure cholesterol α -glycosylation by *H. pylori* on AGS cells either pre-treated or
284 not with DHA. TLC results show that cholesterol α -glycosylation occurs in a time

285 dependant manner (Figure 3). Pre-treatment of AGS cells with 50 μ M of DHA for 12h
286 before the co-culture with *H. pylori* significantly decreased the synthesis of α -
287 glucoside cholesterol by bacteria. These results support the hypothesis that DHA
288 leads to a reduction in the availability of cell cholesterol for *H. pylori* uptake.

289

290 **Previous exposition of *H. pylori* to cholesterol decreases DHA anti-bacterial**
291 **effect**

292 It was previously shown that *H. pylori* resistance to antibiotics is enhanced in the
293 presence of cholesterol. (McGee et al., 2011) Following this line of thought, we
294 decided to investigate if the sensitivity of *H. pylori* to DHA could also be dependent
295 on its access to epithelial cells' cholesterol. Briefly, AGS cells were incubated with
296 NBD-cholesterol (NBD was used for fluorescent staining of cholesterol) and treated
297 in three alternative ways: 1) incubation with DHA; 2) incubation with MBCD
298 (cholesterol removal agent); 3) no incubation, as summarized in Figure 4A. Liquid
299 cultures of *H. pylori* 26695 and SS1 were added to AGS cells at MOI of 100:1 for 3h.
300 Bacteria were then retrieved and let to grow for 48h in liquid cultures either in DHA
301 free medium or in the presence of 100 μ M or 250 μ M DHA; these concentrations
302 were chosen because they are known to inhibit *H. pylori* growth (Correia et al., 2012).
303 Aliquots were collected every 12h to analyse bacteria viability. As illustrated in Figure
304 4B, NBD-cholesterol was efficiently incorporated into AGS cells. *H. pylori* growth rate
305 and viability were neither affected by NBD-cholesterol or by MBCD treatment (data
306 not shown).

307 The effects of DHA on the growth of *H. pylori* following exposure to cells' cholesterol
308 were analysed on strain 26695 (Figure 4C) and SS1 (Figure 4D). When *H. pylori*
309 uptakes NBD-cholesterol from non-treated AGS cells, the presence of 100 μ M of
310 DHA in the bacterial culture did not affect the bacteria growth. However, if cholesterol
311 is previously removed from AGS cells by treatment with DHA or MBCD, 100 μ M of
312 DHA significantly decreases *H. pylori* growth in both strains, $P < 0.05$. Similar effects
313 were obtained with 250 μ M of DHA, $P < 0.05$. We thus observed that the sensitivity of
314 *H. pylori* to DHA treatment is higher when bacteria are previously in contact with
315 cholesterol depleted cells, compared to cells with a normal level of cholesterol.
316 Hence, our results show that the direct inhibitory effect of DHA on *H. pylori* growth
317 can be modulated by the accessibility of *H. pylori* to cholesterol from the host
318 epithelial cells.

319

320 **Cholesterol metabolism in AGS cells upon DHA and *H. pylori* infection**

321 We have seen that DHA treatment in AGS cells regardless *H. pylori* infection
322 significantly decreased the expression of both HMG-CoA reductase and LDL-
323 receptor. MBCD did not hold a significant effect in the expression of these proteins.
324 These results show that DHA decreases cholesterol levels through an interference
325 in cholesterol metabolism *de novo* synthesis.

326 **Discussion**

327 N-3 PUFAs were identified as molecules holding an anti-*H. pylori* effect through
328 mechanisms that interfere with the bacteria's cell wall integrity.(Thompson et al.,
329 1994) We have demonstrated that high concentrations of DHA, an n-3 PUFA, directly
330 inhibit *H. pylori* growth in vitro and its adherence to AGS cells. (Correia et al., 2012;
331 Correia et al., 2013) However, the amount of DHA shown to reduce *H. pylori* mice
332 gastric colonization was lower, suggesting that other mechanisms, in addition to the
333 DHA direct antibacterial effect on *H. pylori*, should be accountable for the reduction
334 of *H. pylori* load in the stomach of infected mice treated with DHA. Hence we
335 hypothesized that DHA might modify key properties of epithelial cells that would
336 challenge *H. pylori* infection. According to the auxotrophy of *H. pylori* for cholesterol,
337 we aimed to determine if DHA, besides its direct bactericidal effect, holds an indirect
338 action on *H. pylori* growth through modulation of cholesterol levels in the cellular
339 membrane of epithelial cells. As reported in the literature, DHA can influence lipid
340 properties of epithelial cells.(McMurray et al., 2011) It is advocated that the unique
341 hairpin structure of DHA is incompatible with cholesterol presence. Therefore, the
342 treatment of epithelial cells with DHA leads to its incorporation into membrane
343 phospholipids which will cluster unevenly into microdomains DHA-rich and DHA-
344 poor. As the DHA amount increases in cell membrane, cholesterol will be effluxed,
345 since DHA and cholesterol are chemically incompatible. (Dusserre et al., 1995;
346 McMurray et al., 2011) DHA is selectively incorporated into closely packed
347 phospholipids, concentrated in the inner leaflet of the cell membrane, which will
348 exclude cholesterol from the inner towards the outer leaflet of the membrane by

349 negative association.(Applegate and Glomset, 1986; Knapp et al., 1994) Cholesterol
350 from the outer layer can be adsorbed by lipoproteins or other molecules and the
351 efflux increases in epithelial cells.(Dusserre et al., 1995) The ability of DHA to affect
352 membrane composition and structure raised the hypothesis that DHA could have an
353 indirect effect on the ability of bacteria to survive and to colonize efficiently the gastric
354 mucosa, by modulating the availability of cholesterol to *H. pylori* uptake in gastric
355 epithelial cells, although not as efficient as MBCD that is described to remove more
356 than 80% of cell membrane cholesterol. (Francis et al., 1999) These data led us to
357 further analyse if the direct inhibitory effect of DHA on *H. pylori* growth could be
358 influenced by accessibility of bacteria to the cells' cholesterol.

359 Most interestingly, molecules that are known to lower cholesterol levels, such as
360 statins, have been shown to inhibit *H. pylori* eradication when used as co-adjuvants
361 to standard triple therapy. This occurs through a mechanism not yet elucidated. Both
362 n-3 PUFAs and statins have been shown to reduce bacterial growth in vivo, at lower
363 concentration as compared to their in vitro effect. (Bergman et al., 2011) Recently,
364 simvastatin was reported to increase the efficacy of triple therapy to eradicate *H.*
365 *pylori*. (Nseir et al., 2012) Altogether, these observations support that their anti-*H.*
366 *pylori* properties might be associated to their ability to interfere with cholesterol,
367 rather than their bactericidal action *per se*. If confirmed, this could be a major
368 breakthrough in terms of *H. pylori* infection treatment, since the involvement of
369 cholesterol metabolism as a major target for *H. pylori* therapy opens new possibilities
370 and strategies to manage the infection.

371 In this study we show that *H. pylori* uptakes cholesterol from epithelial cells and that
372 DHA effectively lowers total cholesterol and FC in epithelial cells.(Dusserre et al.,
373 1995; Wunder et al., 2006) We have also demonstrated that according to this lower
374 level of cholesterol in epithelial cells, *H. pylori* uptakes less FC and consequently
375 produces less cholesteryl glucosides. Low levels of cholesterol in the epithelial cell
376 membrane do indeed result in less cholesterol availability to *H. pylori* uptake. This
377 feature is of utmost importance, as *H. pylori* is auxothrophic for cholesterol. It
378 extracts FC (non-esterified) from host epithelial cells, modifies it by α -glycosylation
379 and incorporates the modified cholesteryl glucosides into its membrane.(Lee et al.,
380 2008; Patel et al., 2002; Shimomura et al., 2004; Wunder et al., 2006) This
381 dependence on cholesterol is very well illustrated by the fact that *cgt* knockout *H.*
382 *pylori* mutants that are unable to α -glycosylate cholesterol, do not colonize efficiently
383 the gerbil model.(McGee et al., 2011)

384 We have also observed that DHA interferes with cholesterol metabolism *de novo*
385 synthesis. Indeed, DHA is able to lower LDL-r and HMGCoA reductase (which
386 catalyses a critical step of converting HMG-CoA to mevalonic acid in the cholesterol
387 synthesis pathway) which might explain its effect in lowering cholesterol levels. This
388 reduction was already identified by others in epithelial cells, were incubation of
389 epithelial cells with DHA down-regulated the expression levels of important players
390 in cholesterol metabolism. (Goldstein, J, Brown, M, 1990)

391 Our observations propose that a previous exposition of *H. pylori* to cholesterol
392 renders the bacterium more effective in resisting the direct inhibitory effect of DHA.
393 However, if the cholesterol is previously removed by DHA or MBCD, *H. pylori*

394 becomes more sensitive to DHA exposure. These results indicate that *H. pylori*
395 accessibility to cholesterol improves the resistance of the bacteria to the inhibitory
396 growth effect of DHA. This increased resistance was also shown with antibiotics. In
397 the same sense MBCD, which depletes epithelial cell cholesterol, is known to
398 abrogate *H. pylori* infection both in vitro and in vivo, (Hildebrandt and McGee, 2009;
399 Lai et al., 2008) Although the physiologic significance of incorporation of cholesterol
400 and its derivatives into the *H. pylori* membrane is still not well established, our results
401 support the hypothesis that cholesterol assimilation is important for *H. pylori*'s
402 survival and growth.(Shimomura et al., 2004; Trainor et al., 2011)

403 In conclusion, in the present study, we have shown that DHA is efficiently
404 incorporated into epithelial cells and is able to decrease cell membrane cholesterol
405 levels, which confirms its recognized role in modulating the lipid profile of the cell
406 membrane.(Rockett et al., 2011) DHA also decreases cholesterol de novo synthesis
407 which overall can explain the decrease in cholesterol levels. Our findings also
408 demonstrate that DHA decreases cholesterol uptake by *H. pylori* from epithelial cells,
409 and that *H. pylori* sensitivity to DHA can be influenced by a previous exposure to
410 cholesterol. These results are in line with reported observations showing that *H.*
411 *pylori* that grows with cholesterol is up to thousand-fold more resistant to amoxicillin
412 treatment than *H. pylori* grown in cholesterol-depleted medium.(McGee et al., 2011)
413 *H. pylori* cholesterol-dependent resistance to some anti-*Helicobacter* molecules
414 such as bile salts, is also documented.(Trainor et al., 2011) Previous studies have
415 implicated cholesterol as a crucial component for antibiotics resistance, which would
416 enable it to modulate therapies efficiency. This concept may be very important for

417 the management of *H. pylori* infection since it supports the important notion that the
418 resistance of *H. pylori* to some antimicrobial compounds, which are or could be used
419 clinically to eradicate the infection, can be modulated by cholesterol uptake.
420 (Testerman et al., 2006; Trainor et al., 2011)

421

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432 **References**

- 433 Applegate, K.R., Glomset, J.A., 1986. Computer-based modeling of the conformation and
434 packing properties of docosahexaenoic acid. *Journal of lipid research* 27, 658-680.
- 435 Bergman, P., Linde, C., Putsep, K., Pohanka, A., Normark, S., Henriques-Normark, B.,
436 Andersson, J., Bjorkhem-Bergman, L., 2011. Studies on the antibacterial effects of statins--
437 in vitro and in vivo. *PloS one* 6, e24394.
- 438 Correia, M., Michel, V., Matos, A.A., Carvalho, P., Oliveira, M.J., Ferreira, R.M., Dillies,
439 M.A., Huerre, M., Seruca, R., Figueiredo, C., Machado, J.C., Touati, E., 2012.
440 Docosahexaenoic Acid Inhibits Helicobacter pylori Growth In Vitro and Mice Gastric
441 Mucosa Colonization. *PloS one* 7, e35072.
- 442 Correia, M., Michel, V., Osorio, H., El Ghachi, M., Bonis, M., Boneca, I.G., De Reuse, H.,
443 Matos, A.A., Lenormand, P., Seruca, R., Figueiredo, C., Machado, J.C., Touati, E., 2013.
444 Crosstalk between Helicobacter pylori and Gastric Epithelial Cells Is Impaired by
445 Docosahexaenoic Acid. *PloS one* 8, e60657.
- 446 Dusserre, E., Pulcini, T., Bourdillon, M.C., Ciavatti, M., Berthezene, F., 1995. Omega-3 fatty
447 acids in smooth muscle cell phospholipids increase membrane cholesterol efflux. *Lipids* 30,
448 35-41.
- 449 Francis, S.A., Kelly, J.M., McCormack, J., Rogers, R.A., Lai, J., Schneeberger, E.E., Lynch,
450 R.D., 1999. Rapid reduction of MDCK cell cholesterol by methyl-beta-cyclodextrin alters
451 steady state transepithelial electrical resistance. *Eur J Cell Biol* 78, 473-484.
- 452 Gisbert, J.P., Calvet, X., O'Connor, A., Megraud, F., O'Morain, C.A., 2010. Sequential
453 therapy for Helicobacter pylori eradication: a critical review. *J Clin Gastroenterol* 44, 313-
454 325.
- 455 Go, G. W., Mani, A. 2012. Low-density lipoprotein receptor (LDLR) family orchestrates
456 cholesterol homeostasis. *Yale J Biol Med*, 85(1):19-28.
- 457 Goldstein, J, Brown, M. 1990. Regulation of mevalonate pathway. *Nature*, 343 (6257):425-
458 430
- 459 Hildebrandt, E., McGee, D.J., 2009. Helicobacter pylori lipopolysaccharide modification,
460 Lewis antigen expression, and gastric colonization are cholesterol-dependent. *BMC*
461 *microbiology* 9, 258.
- 462 Hirai, Y., Haque, M., Yoshida, T., Yokota, K., Yasuda, T., Oguma, K., 1995. Unique
463 cholesteryl glucosides in Helicobacter pylori: composition and structural analysis. *Journal of*
464 *bacteriology* 177, 5327-5333.
- 465 Kaur, G., Sinclair, A. J., Cameron-Smith, D., Barr, D. P., Molero-Navajas, J. C.,
466 Konstantopoulos, N.2011. Docosapentaenoic acid (22:5n-3) down-regulates the expression
467 of genes involved in fat synthesis in liver cells. 85(3-4):155-61.
- 468 Knapp, H.R., Hullin, F., Salem, N., Jr., 1994. Asymmetric incorporation of dietary n-3 fatty
469 acids into membrane aminophospholipids of human erythrocytes. *Journal of lipid research*
470 35, 1283-1291.
- 471 Lai, C.H., Chang, Y.C., Du, S.Y., Wang, H.J., Kuo, C.H., Fang, S.H., Fu, H.W., Lin, H.H.,
472 Chiang, A.S., Wang, W.C., 2008. Cholesterol depletion reduces Helicobacter pylori CagA
473 translocation and CagA-induced responses in AGS cells. *Infect Immun* 76, 3293-3303.
- 474 Lee, A., O'Rourke, J., De Ungria, M.C., Robertson, B., Daskalopoulos, G., Dixon, M.F.,
475 1997. A standardized mouse model of Helicobacter pylori infection: introducing the Sydney
476 strain. *Gastroenterology* 112, 1386-1397.

477 Lee, H., Wang, P., Hoshino, H., Ito, Y., Kobayashi, M., Nakayama, J., Seeberger, P.H.,
478 Fukuda, M., 2008. Alpha1,4GlcNAc-capped mucin-type O-glycan inhibits cholesterol alpha-
479 glucosyltransferase from *Helicobacter pylori* and suppresses *H. pylori* growth. *Glycobiology*
480 18, 549-558.

481 Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with
482 the Folin phenol reagent. *J Biol Chem*, 193(1):265-75.

483 Malfertheiner, P., Megraud, F., O'Morain, C., Bazzoli, F., El-Omar, E., Graham, D., Hunt,
484 R., Rokkas, T., Vakil, N., Kuipers, E.J., 2007. Current concepts in the management of
485 *Helicobacter pylori* infection: the Maastricht III Consensus Report. *Gut* 56, 772-781.

486 McGee, D.J., George, A.E., Trainor, E.A., Horton, K.E., Hildebrandt, E., Testerman, T.L.,
487 2011. Cholesterol enhances *Helicobacter pylori* resistance to antibiotics and LL-37.
488 *Antimicrob Agents Chemother* 55, 2897-2904.

489 McMurray, D.N., Bonilla, D.L., Chapkin, R.S., 2011. n-3 Fatty acids uniquely affect anti-
490 microbial resistance and immune cell plasma membrane organization. *Chem Phys Lipids*
491 164, 626-635.

492 Nseir, W., Diab, H., Mahamid, M., Abu-Elheja, O., Samara, M., Abid, A., Mograbi, J., 2012.
493 Randomised clinical trial: simvastatin as adjuvant therapy improves significantly the
494 *Helicobacter pylori* eradication rate--a placebo-controlled study. *Aliment Pharmacol Ther*
495 36, 231-238.

496 Patel, H.K., Willhite, D.C., Patel, R.M., Ye, D., Williams, C.L., Torres, E.M., Marty, K.B.,
497 MacDonald, R.A., Blanke, S.R., 2002. Plasma membrane cholesterol modulates cellular
498 vacuolation induced by the *Helicobacter pylori* vacuolating cytotoxin. *Infect Immun* 70,
499 4112-4123.

500 Peek, R.M., Jr., Crabtree, J.E., 2006. *Helicobacter* infection and gastric neoplasia. *J Pathol*
501 208, 233-248.

502 Pelletier, O., Wright, L.A., Breckenridge, W.C., 1987. Isotope dilution/mass spectrometry of
503 serum cholesterol with [3,4-¹³C]cholesterol: proposed definitive method. *Clinical chemistry*
504 33, 1403-1411.

505 Ranjekar, P.K., Hinge, A., Hegde, M.V., Ghate, M., Kale, A., Sitasawad, S., Wagh, U.V.,
506 Debsikdar, V.B., Mahadik, S.P., 2003. Decreased antioxidant enzymes and membrane
507 essential polyunsaturated fatty acids in schizophrenic and bipolar mood disorder patients.
508 *Psychiatry research* 121, 109-122.

509 Riff, J.D., Callahan, J.W., Sherman, P.M., 2005. Cholesterol-enriched membrane
510 microdomains are required for inducing host cell cytoskeleton rearrangements in response to
511 attaching-effacing *Escherichia coli*. *Infect Immun* 73, 7113-7125.

512 Rockett, B.D., Franklin, A., Harris, M., Teague, H., Rockett, A., Shaikh, S.R., 2011.
513 Membrane raft organization is more sensitive to disruption by (n-3) PUFA than nonraft
514 organization in EL4 and B cells. *J Nutr* 141, 1041-1048.

515 Shaikh, S.R., Dumaul, A.C., Castillo, A., LoCascio, D., Siddiqui, R.A., Stillwell, W.,
516 Wassall, S.R., 2004. Oleic and docosahexaenoic acid differentially phase separate from lipid
517 raft molecules: a comparative NMR, DSC, AFM, and detergent extraction study. *Biophys J*
518 87, 1752-1766.

519 Shimomura, H., Hayashi, S., Yokota, K., Oguma, K., Hirai, Y., 2004. Alteration in the
520 composition of cholesteryl glucosides and other lipids in *Helicobacter pylori* undergoing
521 morphological change from spiral to coccoid form. *FEMS Microbiol Lett* 237, 407-413.

522 Talley, N.J., Fock, K.M., Moayyedi, P., 2008. Gastric Cancer Consensus conference
523 recommends *Helicobacter pylori* screening and treatment in asymptomatic persons from
524 high-risk populations to prevent gastric cancer. *The American journal of gastroenterology*
525 103, 510-514.

526 Testerman, T.L., Conn, P.B., Mobley, H.L., McGee, D.J., 2006. Nutritional requirements and
527 antibiotic resistance patterns of *Helicobacter* species in chemically defined media. *Journal of*
528 *clinical microbiology* 44, 1650-1658.

529 Thompson, L., Cockayne, A., Spiller, R.C., 1994. Inhibitory effect of polyunsaturated fatty
530 acids on the growth of *Helicobacter pylori*: a possible explanation of the effect of diet on
531 peptic ulceration. *Gut* 35, 1557-1561.

532 Trainor, E.A., Horton, K.E., Savage, P.B., Testerman, T.L., McGee, D.J., 2011. Role of the
533 HefC efflux pump in *Helicobacter pylori* cholesterol-dependent resistance to ceragenins and
534 bile salts. *Infection and immunity* 79, 88-97.

535 Wang, H.J., Cheng, W.C., Cheng, H.H., Lai, C.H., Wang, W.C., 2012. *Helicobacter pylori*
536 cholesteryl glucosides interfere with host membrane phase and affect type IV secretion
537 system function during infection in AGS cells. *Mol Microbiol* 83, 67-84.

538 Wunder, C., Churin, Y., Winau, F., Warnecke, D., Vieth, M., Lindner, B., Zahringer, U.,
539 Mollenkopf, H.J., Heinz, E., Meyer, T.F., 2006. Cholesterol glucosylation promotes immune
540 evasion by *Helicobacter pylori*. *Nat Med* 12, 1030-1038.

541 Xiong, Q., Lin, M., Rikihisa, Y., 2009. Cholesterol-dependent *Anaplasma phagocytophilum*
542 exploits the low-density lipoprotein uptake pathway. *PLoS Pathog* 5, e1000329.

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546 **Figures legend**

547 **Figure 1** Quantification of n-3 PUFA and DHA in gastric epithelial cells. AGS cells
548 were incubated with DHA 50 μ M for 12h. Incorporation of n-3 PUFA and DHA was
549 quantified by Gas Chromatography as described in material and methods. Student's
550 t-test was performed to assess differences between AGS control cells and DHA
551 treated cells. DHA content and n-3 PUFAs content in AGS cells were significantly
552 different in DHA treated cells compared to non-treated cells, $p < 0.05$. Results are
553 expressed as fold-increase compared with normalized control levels. Data are
554 expressed as the mean \pm standard deviation and are representative of three
555 independent experiments.

556

557 **Figure 2:** Cholesterol levels in DHA-treated and *H. pylori*-infected AGS cells for 24
558 hours. A) Both DHA treatment and *H. pylori* infection decreased the levels of total
559 cholesterol and free cholesterol in AGS cells. Results are expressed as μ g of
560 cholesterol per mg of cell protein. B) Free and esterified cholesterol levels in AGS
561 cells treated with DHA and incubated with *H. pylori* 26695 were assessed by TLC.
562 C) Cholesterol metabolism in AGS cells in terms of expression of LDL-receptor and
563 HMG-CoA reductase by Western Blot analysis.

564

565 **Figure 3:** *H. pylori* enzymatic glycosylation of cholesterol assessed by Thin Layer
566 Chromatography (TLC).

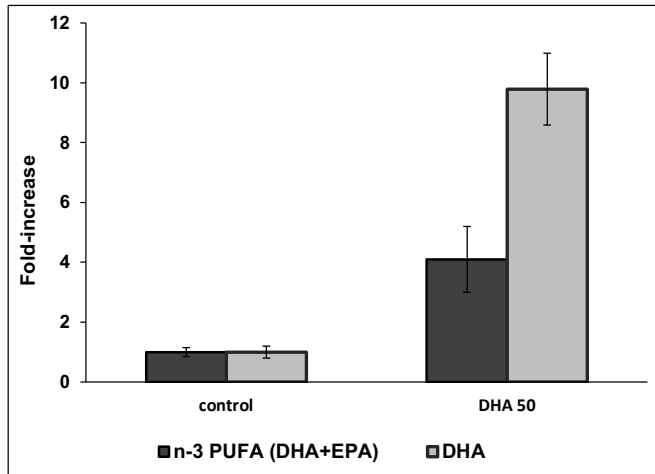
567 Liquid cultures of *H. pylori* 26695 were added to AGS cells previously treated or not
568 with 50 μ M of DHA. Cholesterol α -glycosylation significantly increased with *H. pylori*

569 incubation time ($p < 0.05$) in the absence of DHA treatment. Cholesterol α -
570 glycosylation was significantly decreased in AGS cells treated with 50 μM of DHA in
571 comparison with AGS cells not treated with DHA in all time points (one, six and 24h
572 of *H. pylori* incubation), $p < 0.01$.

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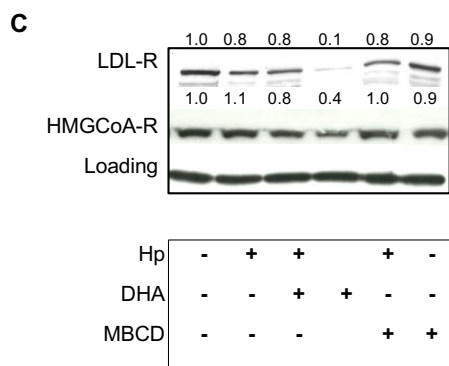
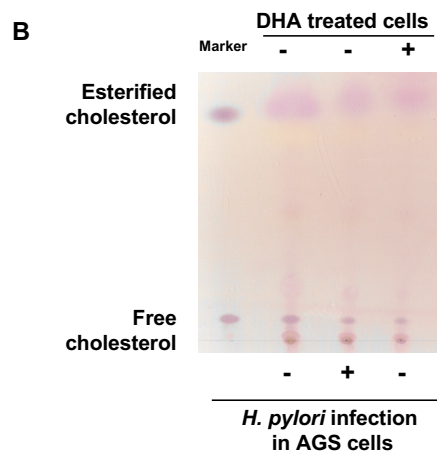
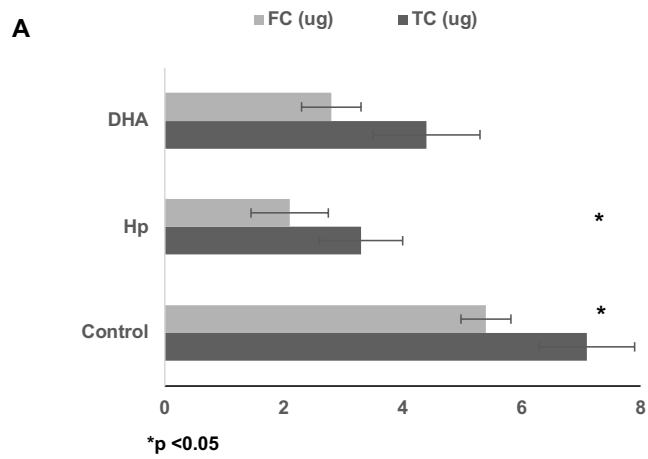
574 **Figure 4:** DHA efficiency in inhibiting *H. pylori* growth depends on the previous
575 uptake of cholesterol by *H. pylori*. A) Description of the experiment: AGS cells were
576 grown as described until reaching monolayers of cells, incubated with NBD-
577 cholesterol for 3h and treated in three alternative ways: 1) incubation with DHA for
578 12 hours; 2) incubation with MBCD for 1 hour; 3) no incubation. Then, liquid cultures
579 of *H. pylori* 26695 and SS1 were added to the AGS cells for 3h; the pelleted *H. pylori*
580 was then grown in liquid cultures without DHA, with 100 μM of DHA, or with 250 μM
581 of DHA, for 48h. B) AGS cells were grown on glass coverslips. AGS cells were
582 preloaded with NBD-cholesterol. Samples were analyzed by laser scanning
583 microscopy (magnification 40x). The experiment shows that NBD-cholesterol was
584 effectively incorporated into AGS cells following a 3h incubation period (green
585 staining represents NBD-cholesterol in AGS cells). C) Impact of cholesterol previous
586 uptake on the inhibitory growth effect of DHA on *H. pylori*. Data are expressed as
587 the mean \pm standard deviation and are representative of three independent
588 experiments. *(asterisk) Refers to significant differences in *H. pylori* growth between
589 cells exposed to cholesterol and DHA-treated, MBCD-treated and AGS cells non-
590 treated.

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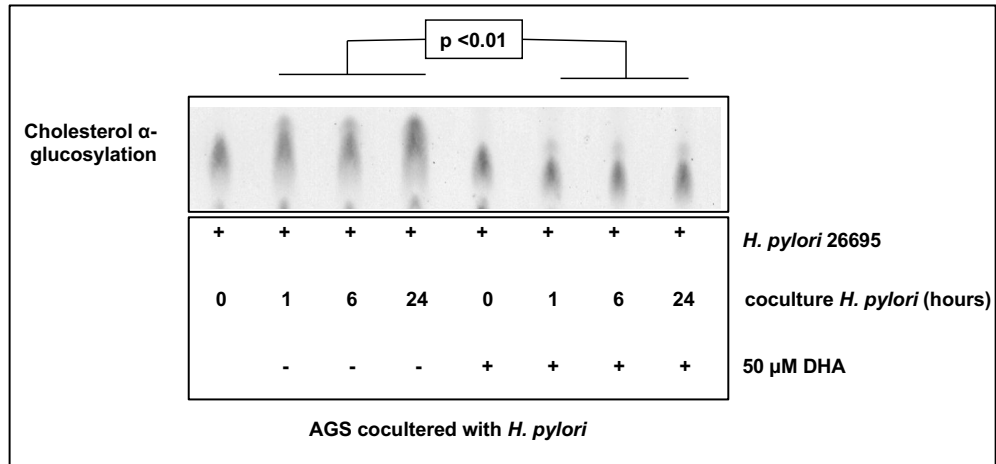


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593 **Figure 1**

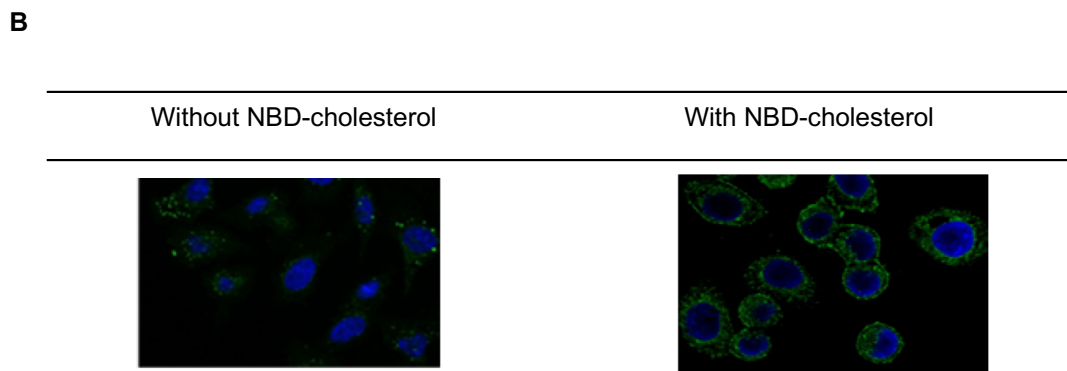
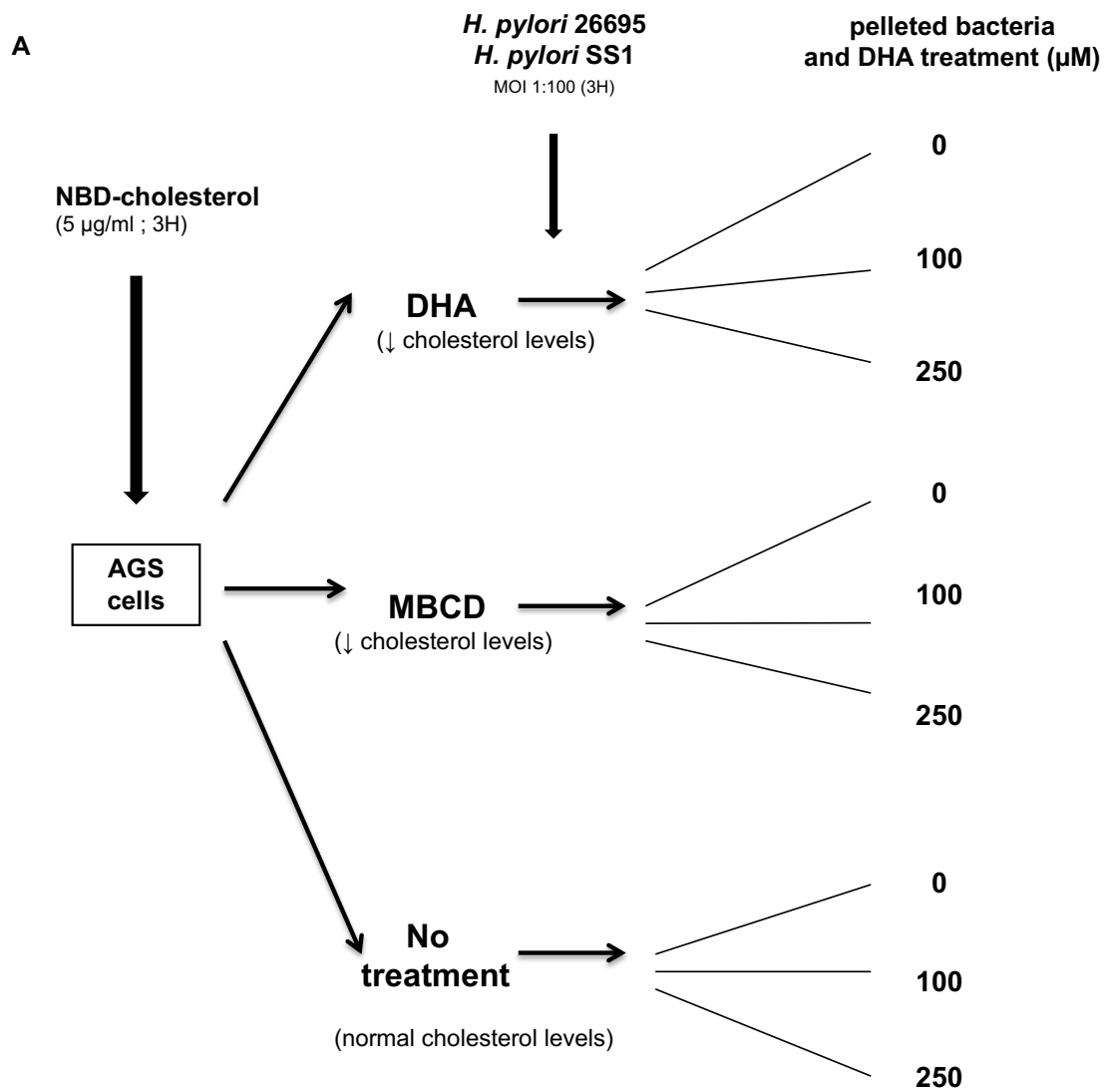


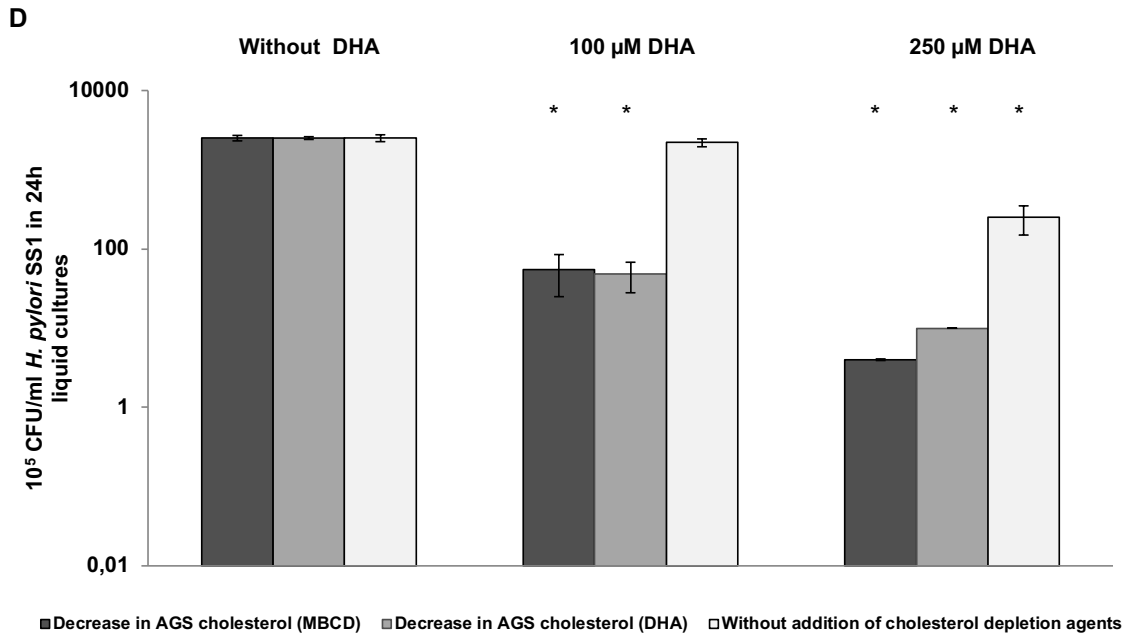
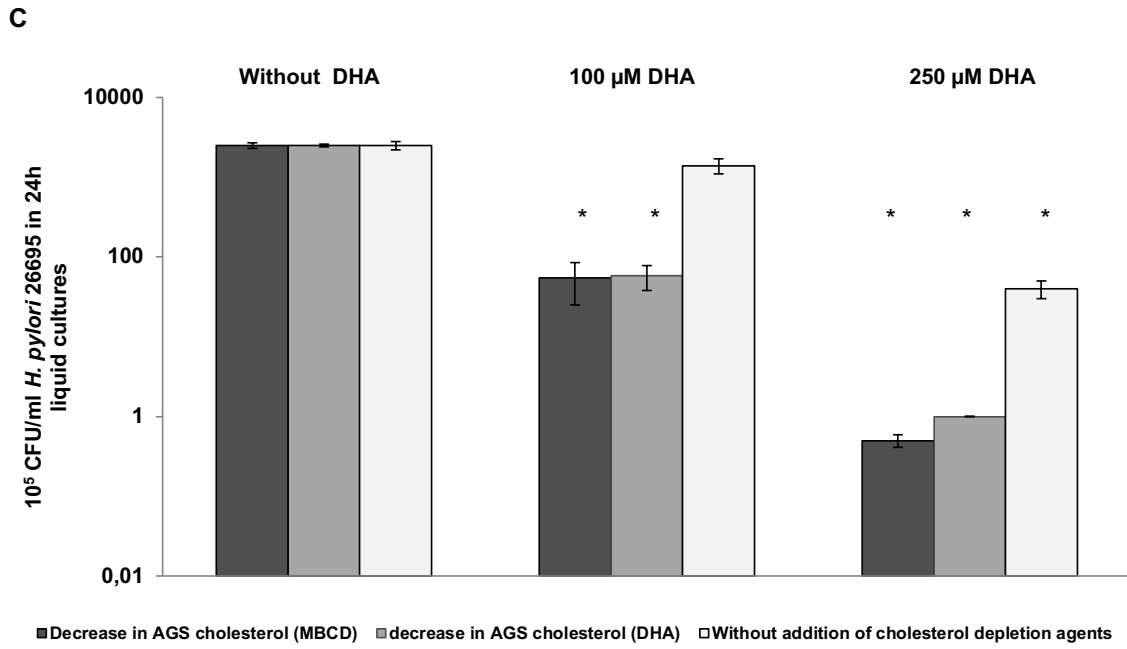
594
595 **Figure 2**



597
598 **Figure 3**

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603 **Figure 4**