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Helicobacter pylori growth pattern in minimally processed products of vegetable origin

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ABSTRACT

Helicobacter pylori is a concerning emergent foodborne pathogen which entrance into the food chain has been recently related with the possible contamination of raw or minimally processed vegetables. The present study registered the growth kinetics of the bacterium at 5, 20 and 37 ºC, in reference media and vegetable substrates, to be fitted to the Gompertz equation. *H. pylori* was able to grow at 37 ºC and 20 ºC, but not at refrigeration temperature. Incubation temperature decrease significantly (p-value < 0.05) affected growth kinetic parameters, with the elongation of lag phase duration (λ) and the reduction of the maximum specific growth rate (μ_{max}) (0.10 $\log_{10}$(CFU/ml)/h at 37 ºC; 0.04 $\log_{10}$(CFU/ml)/h at 20 ºC). In vegetable extracts, the microorganism remained in a viable culturable (VC) form for a maximum of 5 days (20 ºC), being not able to significantly grow in chard, spinach and in kale. Contrary, in lettuce *H. pylori* achieved close to 1 $\log_{10}$ cycle of growth (after 5 days at 20 ºC) (μ_{max} 0.79 $\log_{10}$(CFU/ml)/d).

The present study provides by the first time the kinetic parameter values describing the growth behavior of *H. pylori* at the optimum growth temperature of the bacterium and, also studying the most interesting exposure temperatures of minimally processed products: commercial distribution (room temperature 20ºC), and refrigeration temperature.

1. INTRODUCTION

Nowadays, the consumption of minimally processed vegetables is gaining relevance, due to the importance to include as natural as possible components into the diet, richest in vitamins, minerals and fibers, to prevent chronic diseases and the oxidative stress consequences (Al-Mamun, et al., 2016). Cruciferous vegetables (Zhang et al., 2011), moreover to citrus and red fruits (He & Giusti, 2010; Oikeh, et al., 2016), have been related to potent antioxidant and antimicrobial bioactivities (Liu, 2013).

In spite of their nutritional and healthy value, food diseases outbreaks worldwide caused by bacteria, viruses, and parasites have been associated in large with a wide range of vegetables, among them lettuce, spinach, tomato, and sprouts (Buchholz et al. 2011; FDA, 2016). Although *Listeria monocytogenes* (Greig & Ravel, 2009), *Salmonella* spp. (Oliveira et al., 2010), and *Escherichia coli* (Abu-Duhier, 2015; Buchholz et al., 2011) are the major bacterial challenges for the food safety in several types of ready-to-eat and fresh products (vegetables, fresh - cut fruits, and unpasteurized juices), new emerging pathogens are increasing the scientific community concern due to the drastic consequences associated to their infection. Among them, *Helicobacter pylori* is one of the most relevant emergent foodborne pathogens, the unique classified up to date as carcinogenic agent level I by the World Health Organization (IARC-WHO, 2014). This Gram-negative, microaerophilic, spiral-shaped bacterium is the causative agent of upper gastrointestinal tract diseases, mainly gastritis, peptic and duodenal ulcers, and in fatal cases gastric cancer (WHO, 2017).

Up to date, some food products have been related to *H. pylori* possible contamination, including milk (Mousavi et al., 2014), meat (Stevenson et al., 2000) and vegetables (Atapoor et al., 2014). The use of faecal irrigation water and the permanent contact with possibly contaminated soils are the main factors suggested to lead the contamination of fruits and vegetables with these

76 pathogenic bacteria (Nutt et al., 2003; Atapoor et al., 2014). The contamination of vegetables
77 with *H. pylori* (Yahaghi et al., 2014) and the subsequent introduction of these minimally –
78 processed vegetables into the food chain have been suggested to be one of the contributing
79 factors to the transmission of the bacterium to humans, increasing the prevalence of this
80 concerning infection (Atapoor et al., 2014, Yahaghi et al., 2014).
81
82 In recent years, several fruits and vegetables have emerged as "*superfoods*", based on their
83 functional properties (Machado et al., 2016; Segura-Campos et al., 2014). Among the vegetable
84 trendy foods, special attention should be paid on the *Brassica oleracea var.* sabellica, named
85 kale, a very nutritional vegetable rich in vitamin C, β-carotene, kaempferol and quercetin, with
86 high recognized anti-carcinogenic potential (Nasri et al., 2015; Okada & Okada, 2013). Kale has
87 been named as the "beef of the future" because it is highly richness in iron (per calorie, kale has
88 more iron than beef). Moreover, kale contains more calcium per calorie than milk (90 grams per
89 serving), being also rich in omega 3 and 6 fatty acids (121 mg of omega-3 fatty acids and 92.4
90 mg of omega-6 fatty acids per serving) (Sikora & Bodziarczyk, 2012). This vegetable is mainly
91 consumed raw in salads, or minimally cooked, to be introduced in warm dishes.
92
93 The processing history of raw vegetables is significantly affecting the capability of contaminant
94 microbiota to grow (Nutt et al., 2003; Sant'Ana, Franco, Schaffner, 2012). Factors such as
95 temperature, nutritious value of the leafy vegetables and the possible epidermal barrier
96 breakage by physical damage, such as punctures or bruising, could affect the growth kinetics of
97 the bacterium, modifying as a consequence the final levels at the time of consumption. This fact
98 could significantly increase the public health risk, mainly associated to only washed or uncooked
99 consumed vegetables.
100

To characterize the risk associated to the consumption of minimally processed vegetables regarding *H. pylori* contamination, an estimation of final microbial levels should be carried out. These final bacterial counts are conditioned by initial contamination levels, the growth kinetics of the bacterium, and the incubation – storage conditions to which the binomial [food product-microorganism] is exposed.

The present study aims to contribute to increase the required knowledge to apply in future quantitative microbiological risk assessment (QMRA) conducted for *H. pylori,* by studying the growth kinetics of the bacterium in reference, conventional and *superfood* minimally processed vegetables. Gompertz equation has been used as predictive mathematical model to fit growth/no growth kinetics results.

2. MATERIAL AND METHODS

2.1. Bacterial culture

Reference strain *H. pylori* 11637 NCTC, provided by the United Kingdom National Collection of Type Cultures, was included into the present study. *H. pylori* innocula were prepared from seed Columbia Blood Agar (CBA, Difco, Franklin Lakes, New Jersey, USA) plates supplemented with 10 % defibrillated sterile horse blood (HB, Oxoid, UK). Plates were incubated under microaerobic conditions (5% oxygen, 10% carbon dioxide, and 85% nitrogen) in anaerobic jars (Campy Gas Pak system; Oxoid, Basingstoke, UK) at 37 °C for 5-7 days. Five-day-old cultures were harvested by scraping the bacterial growth with a sterile swab. The cells were once washed with sterile filtered (pore size, 0.2 µm; Sigma Aldrich sterile syringe filter) PBS 1X (130 mmol/L sodium chloride, 10 mmol/L sodium phosphate, pH 7.2) and adjusted to a density of 0.5 to 1 McFarland

126 standard to be finally serially diluted (final inoculum levels ranged from 1.5 x 10^4 to 4 x 10^4 CFU
127 per mL).
128
129
130 **2.2. Preparation of matrices: REFERENCE MEDIA and FOOD SAMPLES**
131
132 ***2.2.1. Reference media***. *H. pylori* was inoculated in Brucella Broth (BB) supplemented with 5 %
133 (v/v) fetal bovine serum (FBS, Bayona-Rojas, 2013; Douraghi et al., 2010) (BB - 5 % FBS) to be
134 used as a liquid substrate for the growth kinetic analysis.
135
136 ***2.2.2. Vegetable extracts***. Fresh samples of leafy commonly consumed vegetables (LV), in this
137 case lettuce (LT), chard (CH) and spinach (SP), were obtained from a local market. In the same
138 way, minimally processed packaged curly leaves of kale (*Brassica oleracea var.* Sabellica) (CLK)
139 were also acquired from the local market to be included into the study as a representative
140 vegetable of the recently named "*superfoods*". For each leafy vegetable, external parts were
141 removed and internal ones were separated individually. Leafy vegetables were washed by
142 immersion into 10 % sodium hypochlorite solution (NaClO, Sigma Aldrich) for 2 min and re-
143 washed afterwards with chlorinated flow water. Each vegetable (conventional and superfood)
144 was exposed to UV radiation (λ=253.7 nm) for 20 min, in a safety cabinet hood (level II).
145 Afterwards, treated samples were sliced into homogenous pieces, and additionally exposed to
146 UV treatment for 20 min prior to its fractionation into equal weightened samples of 10 g.
147 Sterilized samples were disposed into sterile stomacher bags. Samples were diluted with 20 ml
148 of PBS 1X (130 mmol/L sodium chloride, 10 mmol/L sodium phosphate, pH 7.2). The bags were
149 placed into a stomacher machine (Lab-Blender-400 Seward Medical) and homogenized during 2
150 min. Homogenized samples were taken and processed by centrifugation in 50 ml screw cap
151 individual tubes for 6 min at 7000 rpm (Nutt et al., 2003). After centrifugation, the supernatant

was collected and sterilized by filtering (0,2 µm). Sterile extracts were freeze up to the moment of use.

2.3 Inoculation and Culture conditions

H. pylori 10^4 CFU/mL suspensions were inoculated into 10 ml of duplicate tubes of reference and vegetable extracts, to provide a final load of 3-4 x 10^3 CFU/mL per tube.

Inoculated and non-inoculated (control) samples in BB - 5% FBS were incubated at three temperatures, 5, 20 and 37 ºC, during a time period of 14 d, 10 d, and 7 d, respectively, and maintained under microaerobic conditions.

Also, LV and CLK vegetable extracts were inoculated to final load of 3-4 x 10^3 CFU/mL and incubated at temperature of commercial distribution, 20 ºC, during 10 days under microaerobic conditions. Growth kinetics of the bacterium were registered under the studied conditions considering non-inoculated extracts as control samples.

2.4. *Helicobacter pylori* growth measurements

Growth was measured in reference media (BB - 5% FBS) by means of the registration of culture absorbance at 550 nm on a Visible Light Spectrophotometer (Spectronic Instrument (BioMate™ 3S ThermoScientific)). Readings were taken at regular intervals until the stationary phase was reached, after 20 seconds of vigorous agitation. On the basis of the incubation temperature it was established the time interval between readings, ranging between 24 h at 37 ºC, up to 72 h at 5 ºC. Non-inoculated reference substrate tubes were used as negative control for growth and blank for the spectrophotometer.

177 Aliquots of vegetable inoculated/non-inoculated extracts were measured daily, regarding the
178 optical density (OD) at 550 nm, after 20 seconds of vigorous agitation. Non-inoculated extracts
179 were used as negative control for growth and blank for the spectrophotometer.
180
181 Additionally, 100 µl aliquots of reference and vegetables inoculated/non-inoculated samples
182 were collected at the considered time intervals and analyzed for *H. pylori* viability by plate count.
183 Viable counts (numbers of CFU per milliliter) were determined in triplicate for each condition
184 under study, by seeding 100 µl of diluted aliquots (1:10; 1:100; 1:1000) on CBA plates
185 supplemented with 10 % (v/v) HB. Non-inoculated matrices of both, reference and vegetable
186 substrates, were also plated to register the possible presence contamination. Seeded agar plates
187 were incubated at 37 ºC (microaerobic conditions), and colonies on agar surfaces were counted
188 after 5-7 days. The results obtained for each one of the [substrate-temperature] growth assays
189 were estimated as the average of three independent repetitions (including three replicates per
190 each independent trial). Colony counts were converted into $\log_{10}$ (CFU/mL).
191
192 **2.5 Real Time - Quantitative Polymerase Chain Reaction (qRT-PCR) based on SYBR green I**
193 **fluorescence in vegetable extracts**
194 Parallel to previously described assays, one milliliter aliquots from LV and CLK samples were
195 collected at considered intervals for further processing by qRT-PCR, in order to accurately
196 estimate the final *H. pylori* load in vegetable extracts. The GeneJet™ genomic DNA purification
197 kit (Fermentas, Baden-Württemberg, Germany) was used for the extraction of nucleic acids,
198 following the mammalian tissue protocol, according to the manufacturer´s instructions.
199 *Helicobacter* DNA was detected using a LightCycler® 2.0 Instrument (Roche Applied Science,
200 Spain) according to Santiago et al. (2015): optimized qRT-PCR mixture (2 µl SYBR green real-time
201 PCR master mix, Roche Applied Science, Barcelona, Spain), 0.5 µl of each primer (20 mM), 1.6
202 µL $MgCl_2$ (50 mM), and 2 µL of DNA, in a final volume of 20 µL. Specific oligonucleotide primers

to amplify a 372 bp fragment of *H. pylori* vacuolating cyctotoxin gene (*vacA*) were used. The conditions applied for qRT-PCR were as follows: an initial cycle of DNA denaturation at 95 °C for 10 minutes followed by an amplification process consisting on: 40-cycles: 95 °C for 10 seconds, 62 °C for 5 seconds, 72 °C for 16 seconds; a cycle of extension at 72 °C for 15 seconds, and a finalization cycle of 40 °C for 30 seconds. A negative control was used, replacing DNA by an equal volume of sterile water. Known pure DNA concentration from *H. pylori* NCTC 11638 (2.34×10^3 CFU/mL) was included into each trial as positive control. Assays were carried out by triplicate for each time point in the kinetic assay (0, 1, 2, 3, 5, 7, 10 d), for each one of the studied vegetable extracts (LT, CH, SP, CKL).

Quantitative results were expressed by determination of the crossing point (Cp) and melting curves. The melting temperature (Tm) for the vacA primers was 85 °C. A standard curve across six log of DNA concentration, in the range 2.16×10^1 to 1.79×10^6 genomic units (GU), was built based on *H. pylori* NCTC 11638 DNA, corresponding to cycle threshold (Cp) media values ranged from 34 to 15.62 ($Cp = -3.733 \cdot Log_{10}(GU) + 38.98$; $R^2 = 1$) (Santiago et al. 2015).

2.6 Modelling Growth Kinetics: Gompertz equation

The log_{10} (CFU/mL) obtained data were fitted to a primary growth model, the Gompertz equation, whose mathematical expression is detailed below:

$$Log_{10}(N_t) = A + C\, e^{-e^{-Bx(t-M)}} \quad \text{Eq. 1}$$

Where, N*t* represents the final load of microorganisms at time *t* (CFU/mL); the A parameter corresponds to the log_{10} of the initial count (log_{10} (CFU/mL)); C parameter is the difference between asymptotes N_{max} and N_0 (log_{10} (CFU/mL)); B is the relative growth rate when t = M (log_{10} (CFU/mL)/h); and M is the parameter corresponding to the elapsed time until the maximum growth rate is reached (h).

The data were fitted to the mathematical model using the statistical software Statgraphics Centurion XV (Statpoint Inc. Viriginia, USA) by non-linear regression. According to Zwietering et al. (1990) a Marquardt algorithm was employed minimizing the residual sum of squares in the estimation of the model parameters.

The A, B, and C parameters of the Gompertz equation were used to calculate the kinetic parameters lag time (λ, h) and maximum specific growth rate (μ_{max}, (log_{10} (CFU/mL))/h)), according to the following equations (McMeekin et al., 1993):

$$\lambda = M - \left(\frac{1}{B}\right) + \frac{log_{10}(N0) - A}{\mu_{max}} \quad \text{Eq. 2}$$

$$\mu_{max} = \frac{B\ x\ C}{e} \quad \text{Eq. 3}$$

2.7 Accuracy of the mathematical fitting

The accuracy of the fit was determined by using the adjusted determination coefficient (R^2 - adjusted) and the Root Mean Square Error (RMSE), according to Belda-Galbis et al. (2014). The Accuracy factor (A*f*) (Eq. 4) and Bias factor (B*f*) (Eq. 5) were calculated to determine the goodness of the mathematical Gompertz equation to predict the experimental observed data:

$$A_f = 10^{\left(\frac{\sum|log10(\frac{predicted}{observed})|}{number\ of\ observations}\right)} \quad \text{Eq. 4}$$

$$B_f = 10^{\left(\frac{\sum log10(\frac{predicted}{observed})}{number\ of\ observations}\right)} \quad \text{Eq. 5}$$

Closest values to 1 for A*f* and B*f* are indicating that the model produces a perfect fit to data, meanwhile the larger the A*f*, and the lower the B*f*, the less accurate is the average estimated value with respect to the observed one, under each studied condition.

2.8. Statistical analysis

An analysis of variance (ANOVA) was done to evaluate the significance of temperature effect on the growth behavior of the pathogen in reference media (three levels: 37, 20 and 5 °C). A qualitative analysis was also carried out to determine in which measure vegetable extracts significantly affect (positively or negatively) the *H. pylori* growth capability at 20°C.

3. RESULTS AND DISCUSSION

3.1 *Helicobacter pylori* growth/no growth in reference substrate

Optical density ($OD_{550\ nm}$) registration was carried out periodically in the course of the *H. pylori* growth at 5, 20 and 37 °C. The absorbance values recorded were faced to the $\log_{10}$ of CFU per mL values obtained from bacterial counts under the studied growth conditions. The calibration curve OD *versus* $\log_{10}$ (CFU/mL) was validated in this reference substrate, BB – 5 % FBS, with an $R^2\text{-}_{adjusted}$ value of 0.97 ($\log_{10}$ CFU/mL = 4.034*(OD) + 3.54; MSE: 0.025).

The growth curves for *H. pylori* in BB - 5% FBS at 5, 20 and 37 °C are presented in Figure 1. The effect of temperature reduction in the range [37-20] °C, impacted the *H. pylori* growth pattern, which is graphically reflected by a significant elongation of the lag phase (λ, days), and the slowdown of the maximum specific growth rate (μ_{max}, ($\log_{10}$ (CFU/mL))/h) detected in a lower

278 slope of the exponential phase. The bacterium entered into the stationary phase after 3 days at
279 37 °C, and after 6 days at 20 °C. As can be seen graphically, no decrease in *H. pylori* cells
280 culturability was observed during the studied 7 days of incubation at 37 °C. *H. pylori* remains in
281 a viable culturable stage (VC) at 37 °C along the considered period, increasing the bacterial load
282 from the initial artificially inoculated levels (10^4 CFU/mL) up to final levels close to 10^7 CFU/mL,
283 achieved at the end of the incubation period (7 days). However, higher final bacterial load levels
284 than the obtained ones in the present study, were reached by Vega et al. (2003) (10^9 CFU/mL)
285 in Muller Hinton Broth supplemented with fetal calf serum (FCS) or Cyanobacterial extract (CE),
286 starting with an initial inoculum of 10^4 CFU/ml, and after a complete incubation period of 120 h
287 at 37 °C, under 120 rpm agitation. In the present study, lower final biomass levels have been
288 obtained, possible due to the influence of several factors, such as different reference substrate,
289 different supplement, absent support of agitation, and also the 1 $\log_{10}$ cycle lower starting load
290 in our study.
291
292 Our study is in agreement with the results obtained by Douraghi et al. (2010) that registered the
293 growth kinetics of *H. pylori* at 37 °C in liquid media, comparing several supplemented/not
294 supplemented reference substrates. Douraghi et al. (2010) concluded that FBS supplementation
295 significantly increased the *H. pylori* growth rate in the 24 - 48 h first hours of incubation in
296 relation to the other studied supplements (e.g. β-cyclodextrin).
297
298 One of the most interesting fields of study regarding this pathogen is the possible conversion of
299 bacillary *H. pylori* cells in a coccoid viable but not culturable stage (VBNC). According to the
300 studies of Piqueres et al. (2006), *H. pylori* cells in a VBNC stage were detected in the 35 % of
301 water samples analyzed using the procedure of direct viable count method (DVC) combined with
302 fluorescent in situ hybridization (FISH) for the specific detection of viable cells of *H. pylori* (DVC–
303 FISH). Taking into account the consolidated hypothesis regarding the potential of this VBNC

304 forms to remain infective (Cellini et al., 1994; She et al., 2003) it seems very relevant to study
305 how this bacterium change into coccoid form under sub-optimal growth conditions. The *in vivo*
306 studies carried out by She et al. (2003) conclude that *H. pylori* could revert from VBNC stage to
307 a vegetative form in mice, being also protein synthesis detected after even 3 months of storage
308 in phosphate-buffered saline (PBS) at 4 ºC.
309
310 A viability analysis of *H. pylori* cells incubated in liquid media at 37 ºC was carried out by Douraghi
311 et al. (2010), indicating that a loss of viability was observed after 72h of incubation under
312 microaerobic conditions, increasing the conversion of helical forms into coccoid in the course of
313 time [0-72] h, from 19 % coccoid forms after 24h, up to 45 % coccoid forms, after 72 h, at 37 ºC.
314 Contrary to these authors, and according to our results, no significant viability lost was observed
315 in the growth pattern of the bacterium after 7 days of incubation at 37 ºC.
316
317 At 20 ºC, similar viability pattern was observed for *H. pylori* cells in reference media. After 7 days
318 of incubation the bacterium remains in a culturable form. A decrease in the culturability levels
319 was detected after 10 days of incubation, being the viability lost close to 1 $\log_{10}$ cycle (see Figure
320 2). These results are in agreement with those published by Xu et al. (1999) according to which
321 the bacterium remained in a spiral - culturable form for 8 days of incubation at room
322 temperature, becoming in a coccoid stage after this period.
323
324 When the incubation temperature is reduced to refrigeration values (5 ºC) no significant
325 increment in the *H. pylori* bacterial counts (close to 10^3 CFU/mL) is observed during the course
326 of the first 10 days of incubation (p-value > 0.05). Moreover, at refrigeration temperature, the
327 bacterial viability is compromised, being *H. pylori* plate counts reduced after 3 days of incubation
328 (0.72±0.08 $\log_{10}$ cycles of viability lost) (see Figure 2). The bacterium enters in a viable but non-

culturable stage (VBNC) after 15-20 days of incubation (VBNC at 5 °C, >10 d) being non-detected any CFU by plate count after this incubation period.

An ANOVA analysis was carried out to determine in which measure temperature is affecting the growth behavior of the bacterium. According to the obtained results, temperature is revealed as a significant factor (p-value < 0.05) that influences the growth/no growth pattern of this microorganism, at three levels under study, 37, 20 and 5 ° C. Additionally, it seems that temperature values close to 5 ° C are at the boundary of the no-growth of the bacterium that remains, in spite of this, in a VC stage during the first 10 days of incubation in BB - 5 % FBS.

Artificially inoculated food matrices (liquid and solid matrices) were studied by Poms and Tatini (2001) in terms of *H. pylori* proliferation, during a refrigerated storage at 4 °C. According to Poms and Tatini (2001) results, the liquid phase of studied substrates seems to protect the viability of the bacterium (increased viability observed in tofu storage water and skim milk than in lettuce, yogurt, and chicken). Their results indicate that *H. pylori* was unable to grow in any of the studied food substrates (solid or liquid) at this temperature. Even more, the culturability of *H. pylori* cells decreased 1 $\log_{10}$ cycle after 4 days of incubation at 4 °C, on tofu, tofu storage water and skim milk. A complete viability lost of *H. pylori* cells was detected after 5 d of inoculation at 4 °C in milk. Contrary, in solid substrates such as lettuce leaves and chicken meat, *H. pylori* remains in a VC stage only for up to 2 days after inoculation, possible due to the lack of protection against oxygen and desiccation (Poms and Tatini, 2001).

According to our results, bacterial initial load remained unalterable during the first 3 days of incubation at 5 °C in reference media (BB – 5% FBS), and afterwards, a significant reduction in the *H. pylori* viability was observed, which is in agreement with the previous results of Poms and Tatini (2001) in liquid food substrates (4 days, 1 $\log_{10}$ cycle viability lost in tofu storage water,

355 and 5 days - complete viability lost in skim milk). However, in our study, a remaining bacterial
356 viable population of cells was observed up to 10 days of incubation at refrigeration temperature.
357 These findings are in agreement with the obtained results by Quaglia et al. (2007), according to
358 which *H. pylori* artificially inoculated in milk was able to survive into a viable culturable form up
359 to 9 – 12 days in pasteurized and UHT milk, respectively. According to Quaglia et al. (2007) no
360 changes in bacterial initial load were observed during the first 3 days of milk storage at 4 ºC. On
361 the fourth day, and according to our results, a 1 - $\log_{10}$ cycle reduction was observed in both
362 pasteurized and UHT inoculated milk samples. Bacterial load in both substrates remained
363 invariable up to the 8th day of incubation, being undetectable by plate count after a maximum
364 of 12 days. In our study, the bacterial load of *H. pylori* 11637 NCTC remained in a VC invariable
365 levels from 4th to 10th days of incubation at 5 ºC, being undetectable by plate count after 15 days
366 of incubation at this temperature.
367
368
369 **3.1.1. Modified Gompertz Equation fitting *Helibocater pylori* growth in reference media**
370 The obtained growth curves at 20 and 37 ºC were fitted to the modified Gompertz equation and
371 the kinetic growth parameters, λ and μ_{max}, were obtained (see Table 1). The accuracy of the
372 Gompertz model (A*f*, B*f*, RMSE, R^2-adjusted) to predict the *H. pylori* growth at 20 and 37 ºC is
373 presented in table 1. According to the obtained kinetic parameters, when temperature is
374 reduced from 37 to 20 ºC, the values of the lag phase λ duration are doubled. In the same way,
375 a significant reduction in the maximum specific growth rate μ_{max} was observed between 37 and
376 20 ºC, in this case μ_{max} value is reduced in more than a half ($\log_{10}$(CFU/ml)/h) due to temperature
377 reduction from 37 ºC to 20ºC. At the optimum growth temperature of the bacterium (37 ºC), in
378 BB - 5 % FBS, the lag phase duration obtained in the present study is close to 22 h. The previous
379 studies of Vega et al. (2003) revealed that in Mueller-Hinton broth (MHB) the lag period of
380 several *H. pylori* clinical isolates was in values between 8 to 12 h. Generation time values (GT)

ranging from 2.39 h to 4.62 h were previously obtained by Vega et al. (2003) for clinical isolates of *H. pylori* suspended in liquid MHB supplemented with 0,7 % of a Cyanobacterial Extract (CE) and incubated at 37 ºC for 120h. In the present study, higher GT values have been obtained, being in the range [10-19] h corresponding to temperatures of 37 and 20 ºC, respectively.

Scarce information has been published regarding the kinetic growth behavior of this microorganism in reference media (Douraghi et al., 2010; Jiang & Doyle, 2000, Kitsos, et al., 1998; Vega et al., 2003; Walsh & Moran, 1997). The reference substrate used (among them, Muller Hinton broth (MHB), Brain Heart Infussion (BHI), Brucella Broth (BB)), the supplements added to the media (fetal calf serum, fetal bovine serum, cyanobacterial extract, sodium piruvate and/or mucin), and also the bacterial strains included into the study, are conditioning factors affecting the kinetic parameters values obtained under the studied conditions.

Up to date, the published articles related to *H. pylori* growth pattern in reference media have been mainly carried out at the optimum growth temperature of the bacterium. The present study provides by the first time, the kinetic parameters values of this concerning pathogen not only at 37 ºC, but also studying the most interesting temperatures of minimally processed products exposure: the temperature of commercial distribution (room temperature 20ºC), and also the refrigeration temperature applied to ready-to-eat and minimally processed vegetables) in a highly extended reference substrate in laboratory BB – 5 % FBS, as a baseline for future QMRA studies in liquid and solid real food matrices.

3.2. *Helicobacter pylori* growth / no growth kinetics in vegetable extracts

According to the previous studies of Yahaghi et al. (2014) alarming percentages of raw vegetables could be contaminated with *H. pylori.* The growth of these foodstuffs in contact with soil and manure promotes the possible contamination of these highly aqueous substrates that

afterwards, are submitted to minimum preservation processes, holding the capability to act as vehicles of *H. pylori* infection, in major or minor degree, depending on the initial level of contamination and the manufacturing practices applied. According to Yahaghi et al. (2014) results close to 14 % of analyzed vegetable samples (among them leek, basil, parsley, spinach, lettuce, cabbage, pepper, garlic, and broccoli) were positive for *H. pylori* by PCR, based on the *UreB* gene specificity.

Taking into account the *H. pylori* growth results obtained in the present study by using the BB - 5 % FBS as a reference substrate, our research group has selected 4 of the most common and valuable consumed vegetables nowadays, to be studied in terms of *H. pylori* growth support at 20 ºC. Optical measurements were registered along the incubation period (10 days), and also bacterial counts were determined by plate in CBA – 10 % HB incubated at 37 ºC under microaerobic conditions. Non-inoculated vegetable extracts were used as control samples.

H. pylori was able to remain in a VC form during the first 5 days of incubation at 20 ºC, independently of the vegetable extract considered. No VC forms of *H. pylori* were detected after 7 - 10 days of incubation at the studied conditions in no one of the studied vegetable extracts. Although in the reference substrate BB – 5 % FBS the bacterium was able to remain in a VC form during 7 days at 20 ºC, in the studied vegetable extracts the microorganism lost viability after 5 days of incubation at this temperature.

Growth/no growth graphic bars are presented in figure 3. As can be seen, after 24h of incubation, a slightly growth was observed in all of the studied vegetable extracts. The increments in *H. pylori* bacterial load in lettuce and chard achieved values close to 1 $\log_{10}$ cycle at 20º C ([0.82-0.94] $\log_{10}$ cycles, respectively). In spinach and kale, the bacterial load increments after 24 h of incubation achieved values of 0.65-0.43 $\log_{10}$ cycles, respectively. No significant *H.*

433 *pylori* growth was observed (CFU/mL) during the course of incubation period between 24 - 120
434 h, considering chard, spinach and kale. An additional extra 0.30 log_{10} cycles of growth were
435 achieved in lettuce in the period comprised between 24 – 120 h of incubation at 20 °C.
436
437 According to the studies of Yahaghi et al. (2014) the incidence and survival of *H. pylori* in
438 vegetables is highly dependent on the amount of activated water (AW), pH, and the hygienic
439 conditions followed during processing of vegetables and ready-to-eat samples. The differences
440 observed in *H. pylori* growth in the present study could not be forthright related with the
441 nutritional and physico-chemical propierties inherent to the studied substrates (see table 2). No
442 relation could be established between the most nutritional value of kale and the highest levels
443 of *H. pylori* growth that were found in lettuce. Regarding the pH value of the considered extracts,
444 kale was the closest one to neutrality.
445
446 The sterility of vegetable extracts was maintained intact during 7 days in kale, being detected
447 background microflora that difficulted the *H. pylori* plate detection from the 7^{th} up to the 10^{th}
448 day of incubation in spinach, chard and lettuce, possibly due to the initial different levels of
449 contamination, and the processing conditions applied after harvest.
450
451 The quantitative results obtained by plate count were complemented by means of the qRT-PCR
452 analysis of the aliquots, taken at the considered time intervals (0, 1, 2, 3, 5, 10 days, 20 °C) using
453 the *H. pylori VacA* gene specificity. A comparison between *H. pylori* bacterial count levels
454 between culture and qRT-PCR assays is presented in figure 4. A good correspondence exists
455 between both methods regarding the quantification of *H. pylori* bacterial load in the studied
456 vegetable extracts during the first 24 h of study. After that, the enumerations obtained using
457 the plate count method remained below the qRT-PCR values, confirming a marked lost in

458 viaibility during this period, with maximum values from 0.60 log_{10} cycles after 3 days in lettuce,
459 up to 4.5 log_{10} cycles of viability lost after 10 days of incubation at this temperature.
460
461 The qRT-PCR detected growth of *H. pylori* in lettuce extract (10 days, 20 °C) was fitted to the
462 modified Gompertz equation in order to obtain the kinetic parameters defining the growth
463 behavior of the bacterium under the studied conditions. The kinetic results are presented in
464 table 3. The kinetic parameters values defining *H. pylori* growth in lettuce differ significantly
465 from the obtained ones in reference media. The lag phase λ duration (3.91±0.24 days) was
466 significantly higher than the value obtained in BB – FBS 5 % (λ ≈ 2 days). Contrary, the maximum
467 specific growth rate μ_{max} (0.79±0.06 log(CFU/ml)/d) was lower than the value obtained in BB –
468 FBS 5 % (μ_{max} 0.96 log(CFU/ml)/d)) under the same incubation conditions. The lack of nutritional
469 factors required for the optimal growth of the bacterium, as well as the possible presence of
470 antibacterial compounds in lettuce (Lucera et al., 2012), could be influential factors reducing the
471 μ_{max} and increasing the lag phase duration at this temperature. Some research articles are
472 supporting the reduced growth capability of other Gram negative bacteria in real food matrices
473 in comparison with their growth in reference substrates, even under optimal incubation
474 conditions (Pina-Pérez et al., 2012; Sanz-Puig et al., 2016).
475
476 According to the studied factors defining the goodness of the model fit to experimental data (R^2-
477 adjusted, RMSE, A*f*, B*f*), it could be concluded that Gompertz equation is a very useful tool to
478 accurately estimate the slight growth of the bacterium in real vegetable food matrices (close to
479 1 log_{10} cycle in lettuce) contributing in this way to the future development of QMRA approaches.
480
481
482 **Conclusions**

The present research contributes to the *state of the art* advancing in the *H. pylori* growth pattern knowledge, both in reference media and in real food substrates. Under sub-optimal growth conditions (20 °C) the kinetic parameters of the bacterium were significantly affected, increasing the lag phase duration (λ) (from 22 h at 37 °C to 48 h at 20 °C) and decreasing the maximum specific growth rate (μ_{max}) values (from 0.10 (log(CFU/ml)/h at 37 °C, to 0.04 (log(CFU/ml)/h at 20 °C). Even more, among the studied vegetable substrates, *H. pylori* was able to grow (20 °C) only in lettuce (λ = 3.71 d; μ_{max} = 0.79 (log(CFU/ml)/d). Although there is scarce literature regarding the role of vegetable raw products in the transmission of *H. pylori* infection, this premise cannot be completely discarded, considering the possible initial contamination of vegetables and the survival, even the slight growth of this bacterium under common marketed conditions (limited time < 10 d; temperature 5-20 °C). The present research work provides valuable insights to further carried out quantitative exposure assessment studies determining the most probable values of *H. pylori* contamination in different simulated scenarios and including several nutritionally profiled vegetables.

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References

Abu-Duhier, F.M. (2015). Escherichia coli contamination of selected vegetables and fruits from markets of Tabuk city, Saudi Arabia: An anticipatory surveillance using real-time PCR for the presence of pathogenic strain *E. coli* O104:H4. *International J. of Healthcare and Biomedical Research, 4*(1), 126–134.

Al-Mamun, M.A., Husna, J., Khatun, M., Hasan, R., Kamruzzaman, M., Hoque, K.M.F., Abu Reza, M., Ferdousi, Z. (2016). Assessment of antioxidant, anticancer and antimicrobial activity of two vegetable species of *Amaranthus* in Bangladesh. *BMC Complement. Alternative Medicine*, *16*(157), 2–11.

Atapoor, S., Safarpoor Dehkordi, F., Rahimi, E. (2014). Detection of *Helicobacter pylori* in Various Types of Vegetables and Salads. *Jundishapur* Journal of *Microbiology,* 7(5), 1–4.

Azevedo, N.F., Almeida, C., Cerqueira, L., Dias, S., Keevil, C.W., and Vieira, M.J. (2007). Coccoid Form of *Helicobacter pylori* as a Morphological Manifestation of Cell Adaptation to the Environment. *Applied & Environmental Microbiology, 73*(10), 3423–3427.

Bayona Rojas, M.A. (2013). Microbiological conditions for culturing *Helicobacter Pylori. Revista Colombiana de Gastroenterologia, 28* (2), 92–97.

Belda-Galbis, C.M., Pina-Pérez, M.C., Espinosa, J., Marco-Celdrán, A., Martínez, A., Rodrigo, D., (2014). Use of the modified Gompertz equation to assess the Stevia rebaudiana Bertoni antilisterial kinetics. *Food Microbiology, 38*, 56–61.

Cellini L, Allocati N, Angelucci D, Iezzi T, Di Campli E, Marzio L, Dainelli B., 1994. Coccoid Helicobacter pylori not culturable in vitro reverts in mice. *Microbiology and Immunology, 38*(11), 843–850.

Buchholz, U., Bernard, H., Werber, D., Böhmer, M.M., Remschmidt, C., Wilking, H., Deleré, Y., an der Heiden, M., Adlhoch, C., Dreesman, J., Ehlers, J., Ethelberg, S., M., Frank, C., Fricke, G.,

Greiner, M., Höhle, M., Ivarsson, S., Jark, U., Kirchner, M., Koch, J., Krause, G., Luber, P., Rosner, B., Stark, K., and Kühne, M. (2011) German Outbreak of *Escherichia coli* O104:H4 Associated with Sprouts. *New England Journal of Medicine, 365*, 1763–1770.

Douraghi, M., Saberi Kashani, S., Zeraati, H., Esmaili, M., Oghalaie, A., Mohammadi, M. (2010). Comparative Evaluation of Three Supplements for Helicobacter pylori Growth in Liquid Culture. *Current Microbiology, 60*, 254–262

Food and Drug Administration (FDA). (2016). Analysis and Evaluation of Preventive Control Measures for the Control and Reduction/Elimination of Microbial Hazards on Fresh and Fresh-Cut Produce: Chapter IV. Outbreaks Associated with Fresh and Fresh-Cut Produce. Incidence, Growth, and Survival of Pathogens in Fresh and Fresh-Cut Produce. Available at: *http://www.fda.gov/Food/FoodScienceResearch/SafePracticesforFoodProcesses/ucm091265.htm*

Greig, J.D., and Ravel, A. (2009). Analysis of foodborne outbreak data reported internationally for source attribution. *International Journal of Food Microbiology, 130*, 77–87.

He, J., Giusti, M.M. (2010). Anthocyanins: natural colorants with health-promoting properties. *Annual Review of Food Science and Technology, 1,* 163–187.

IARC-WHO (International Agency for Research on Cancer- World Health Organitation) (2014). *Helicobacter pylori* Eradication as a Strategy for Preventing Gastric Cancer. Working Group Reports (Vol. 8) 190 pag. Available from: https://www.iarc.fr/en/publications/pdfsonline/wrk/wrk8/HelicobacterpyloriEradication.pdf. Mayo 2016

561

562 Jiang, X., and M. P. Doyle. (2000). Growth supplements for *Helicobacter pylori. Journal of Clinical*
563 *Microbiology, 38,* 1984–1987.

564

565 Kitsos, C. M., T. K. Christian, and H. Stadtlander. (1998). *Helicobacter pylori* in liquid culture:
566 evaluation of growth rates and ultrastructure. *Current Microbiology, 37*, 88–93.

567

568 Liu, R.H. (2013). Health-promoting components of fruits and vegetables in the diet. *Advances in*
569 *Nutrition 4*(3), 384–392.

570

571 Lucera, A., Costa, C., Conte, A., Del Nobile, M.A. (2012). Food applications of natural
572 antimicrobial compounds. *Frontiers in Microbiology, 3* (287), 1–13.

573

574 Machado, A.K., Andreazza, A.C., da Silva, T.M., Boligon, A.A., do Nascimento, V., Scola,
575 G., Duong, A., Cadoná, F.C., Ribeiro, E.E., da Cruz, I.B. (2016). Neuroprotective Effects of Açaí
576 (Euterpe oleracea Mart.) against Rotenone In Vitro Exposure. *Oxidative Medicine and Cellular*
577 *Longevity,* 1–14.

578

579 McMeekin, T.A., Olley, J.N., Ross, T. and Ratkowsky, D.A. (1993). Predictive microbiology –
580 Theory and application. Predictive microbiology - Theory and application. Somerset (UK):
581 Research Studies Press Ltd; 1993.

582

583 Mousavi, S., Safarpoor Dehkordi, F., & Rahimi, E. (2014). Virulence factors and antibiotic
584 resistance of Helicobacter pylori isolated from raw milk and unpasteurized dairy products in Iran
585 *Journal of Venomous Animals and Toxins including Tropical Diseases, 20*(51), 1–7.

586

Nasri, H., Baradaran, A., Shirzad, H., Rafieian-Kopaei, M. (2015). New Concepts in Nutraceuticals as Alternative for Pharmaceuticals. *International Journal of Preventive Medicine, 5*(12), 1487–1499.

Nutt , J.D., Li, X., Woodward, C.L., Zabala-Díaz, I.B., Ricke, S.C. (2003). Growth kinetics response of a *Salmonella typhimurium* poultry marker strain to fresh produce extracts. *Bioresource Technology, 89,* 313–316

Okada, Y., Okada, M. (2013). Protective effects of plant seed extracts against amyloid β-induced neurotoxicity in cultured hippocampal neurons. *Journal of Pharmacy And Bioallied Sciences, 5*(2), 141–147.

Oikeh, E.I., Omoregie, E.S., Oviasogie, F.S., Oriakhi, K. (2016). Phytochemical, antimicrobial, and antioxidant activities of different citrus juice concentrates. *Food Science and Nutrition, 4(*1), 103–109.

Oliveira, M.A., Abeid Ribeiro, E.G., Morato Bergamini, A.M., Martinis, E.C.P. (2010). Quantification of *Listeria monocytogenes* in minimally processed leafy vegetables using a combined method based on enrichment and 16S rRNA real-time PCR. *Food Microbiology, 27*, 19–23.

Pina-Pérez, M.C., Rodrigo, D., Martinez, A. (2012). *Cronobacter sakazakii* growth prediction in Reconstituted Powdered Infant Formula Milk. *Archiv für lebensmittelhygiene, 63*(6), 168–174.

612 Piqueres, P., Moreno, Y., Alonso, J.L., Ferrús, M.A. (2006). A combination of direct viable count
613 and fluorescent in situ hybridization for estimating *Helicobacter pylori* cell viability. *Research in*
614 *Microbiology, 157*(4), 345–349.
615
616 Poms, R.E. & Tatini, S.R. (2001). Survival of *Helicobacter pylori* in ready-to-eat foods at 4ºC.
617 *International Journal of Food Microbiology, 63*, 281–286.
618
619
620 Quaglia, N.C., Dambrosio, A., Normanno, G., Parisi, A., Firinu, A., Lorusso, V., Celano, G.V. (2007).
621 Survival of Helicobacter pylori in artificially contaminated ultrahigh temperature and
622 pasteurized milk. *Food Microbiology*, 24, 296–300.
623
624 Sanz-Puig, M., Pina-Pérez, M.C., Martínez-López, A., Rodrigo, D. (2016). *Escherichia coli* O157:H7
625 and *Salmonella Typhimurium* inactivation by the effect of mandarin, lemon, and orange by-
626 products in reference medium and in oat-fruit juice mixed beverage. *LWT - Food Science &*
627 *Technology, 66*, 7–14.
628
629 Sant'Ana, A.S., Franco, B.D.G.M., Schaffner, D.W. (2012) Modeling the growth rate and lag time
630 of different strains of *Salmonella enterica* and *Listeria monocytogenes* in ready-to-eat lettuce.
631 *Food Microbiology, 30*(1), 267–273
632
633 Santiago, P., Moreno, Y., Ferrús, M.A. (2015). Identification of Viable *Helicobacter pylori* in
634 Drinking Water Supplies by Cultural and Molecular Techniques. *Helicobacter, 20*(4), 252–259.
635

636 Segura-Campos, M.R., Ciau-Solís, N., Rosado-Rubio, G., Chel-Guerrero, L., Betancur-Ancona, D.,
637 (2014). Chemical and Functional Properties of Chia Seed (*Salvia hispanica* L.) Gum. *International*
638 *Journal of Food Science,* 1–5.
639

640 She, F.-F., J.-Y. Lin, J.-Y. Liu, C. Huang, and D.-H. Su. (2003). Virulence of water-induced
641 coccoid *Helicobacter pylori* and its experimental infection in mice. *World Journal of*
642 *Gasteroenterology, 9,*516–520.
643

644 Sikora, E., Bodziarczyk, I. (2012). Composition and antioxidant activity of kale (Brassica oleracea
645 L. var. acephala) raw and cooked. *Acta Scientiarum Polonorum Technologia Alimentaria, 11*(3),
646 239–48.
647

648 Stevenson, T.H., Bauer, N., Lucia, L.M., Acuff, G.R. (2000). Attempts to isolate Helicobacter from
649 cattle and survival of Helicobacter pylori in beef products. *Journal of Food Protection, 63*(2), 174–
650 8.
651
652

653 Vega, A.E., Cortinas, T.I., Mattana, C.M., Silva, H.J., and Puig de Centorbi, O. (2003) Growth of
654 Helicobacter pylori in Medium Supplemented with Cyanobacterial Extract. *Journal of Clinic*
655 *Microbiology, 41*(12), 5384–5388.
656

Walsh, E. J., and A. P. Moran. (1997). Influence of medium composition on the growth and antigen expression of Helicobacter pylori. *Journal of Applied Microbiology, 83,* 67–75

WHO. 2017. WHO priority pathogens list for R&D of new antibiotics. Available at: *http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/*

Xu, Z.M., Zhou, D.Y, Pan, L.J. and Song, S. (1999) Transformation and reversion of *Helicobacter pylori in vitro*. *Shijie Huaren Xiaohua Zazhi, 7,* 215–217.

Yahaghi, E., Khamesipour, F., Mashayekhi, F., Safarpoor Dehkordi, F., Sakhaei, M.H., Masoudimanesh, M., Khayyat Khameneie, M. (2014). *Helicobacter pylori* in Vegetables and Salads: Genotyping and Antimicrobial Resistance Properties. *BioMed Research International,* 1–12.

Zhang, X., Shu, X.O., Xiang, Y.B., Yang, G., Li, H., Gao, J., Cai, H., Gao, Y.T., Zheng, W. (2011). Cruciferous vegetable consumption is associated with a reduced risk of total and cardiovascular disease mortality. *American Journal of Clinical Nutrition, 94*(1), 240–246.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M. and van't Riet, K. (1990). Modeling of the bacterial growth curve. *Applied and Environmental Microbiology, 56*, 1875–1881.