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Proceeding Paper

Investigating the Inhibitory Effect of Lactic Acid on Biofilm Production by Raw Chicken *Campylobacter* spp. Isolates in Pure and Mixed Cultures †

Dimitra Kostoglou *, Athina Vass and Efstathios Giaouris

Laboratory of Food Microbiology and Hygiene, Department of Food Science and Nutrition, School of the Environment, University of the Aegean, 81400 Myrina, Lemnos, Greece; athinavass.fns@gmail.com (A.V.); stagiaouris@aegean.gr (E.G.)

* Correspondence: fnsd21001@fns.aegean.gr

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Abstract: *Campylobacter* spp. are the main cause of foodborne gastroenteritis worldwide and biofilm growth mode seems to play a key role in their prevalence. In this work, the effect of lactic acid (LA) on planktonic growth and biofilm production by eight *Campylobacter* spp. raw chicken isolates, was investigated using polystyrene and stainless steel as the abiotic substrata. Results revealed that the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum biofilm inhibitory concentration (MBIC) values of LA against the *Campylobacter* isolates ranged from 1024 to 4096 μg/mL depending on the isolate, mode of growth (planktonic *vs* biofilm; single *vs* mixed culture), and the growth medium. Overall, the results of this work offer insight into biofilm control of a pathogen of public health importance.

Keywords: lactic acid; *Campylobacter jejuni*; *Campylobacter coli*; biofilms; food safety; polystyrene; stainless steel

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1. Introduction

Campylobacter spp. are zoonotic agents with broiler chickens and their products, as well as unpasteurized milk being their main reservoirs [1,2]. Chickens (*Gallus gallus domesticus*) hence act as their most common hosts, and in combination with incorrect handling of these products (usually insufficient cooking, as well as cross-contamination events), the pathogenic bacteria can end up on the consumer's plate [3]. Campylobacteria are Gram-negative microaerophilic bacteria with a spiral (curved) or rod-like shape [4]. According to the European Food Safety Authority (EFSA) *Campylobacter jejuni* and *Campylobacter coli* are the most reported species in cases of campylobacteriosis [2].

Campylobacter spp. are fragile and fastidious in their growth requirements but, paradoxically, they can be easily transmitted from animals to human through the food production chain [5]. Biofilm mode of growth is suggested as a key survival and persistence mechanism used by them [6]. Interestingly, *Campylobacter* spp. cannot grow and multiply in food during processing and storage as it happens with most other foodborne pathogenic bacteria, since the prevailing conditions (e.g., aerobiosis, temperature) are unfavorable for them [7]. An important role in their survival and eventual dominance against other pathogens should thus be played by their ability to attach to food-related surfaces and their inclusion in multi-species biofilms [8–10]. Macromolecules such as food constituents can influence the attachment of bacteria to surfaces, whereas it has been reported that the presence of proteins such as albumin, gelatin and casein can inhibit the initial attachment of bacteria to some surfaces [11–13]. However, it seems that there are no

sufficient relevant data regarding *Campylobacter* spp. in single or mixed cultures. Additionally, chicken juice has been suggested to promote the attachment of *C. jejuni* by creating a conditioning film on the abiotic surface, while at the same time it seems like a suitable laboratory model to study *Campylobacter* biofilm formation as it mimics the conditions being present in the environment of slaughterhouses [14,15].

Although it is difficult if not impossible to completely get rid of campylobacteria, there are still many physical and chemical strategies that can be used to limit their prevalence. These can be employed at different stages of the food production chain [16]. During food animal processing, for instance, organic acids may be used to remove pathogens from carcasses and decrease their microbial load. Organic acids are also used as acidifiers in poultry drinking water and as antimicrobial feed additives, having at the same time a positive effect on the good functioning of the poultry digestive system. Noteworthily, poultry carcass treatment with 2% *w*/*v* (20 mg/mL) lactic acid (LA) is estimated to reduce the risk of *Campylobacter* infection in humans between 37–56% [17].

In this work, the inhibitory effect of LA on planktonic growth and biofilm production by eight *Campylobacter* spp. raw chicken isolates (five *C. jejuni* and three *C. coli*) on model polystyrene (PS) surfaces, was investigated. The inhibitory effect of this acid on mixed culture biofilms (composed of three different isolates) was also determined using 6-well PS microplates and stainless steel (SS) coupons as the abiotic substrata.

2. Methods

2.1. Preparation of Sterile Chicken Juice (CJ)

Minced chicken (≈ 300 g) was purchased from a local supermarket and immediately transported to the laboratory. In a stomacher bag, 250 g of minced meat were weighed and 250 mL of sterile deionized water were then added (1:1 dilution). The mixture was homogenized in a stomacher (BagMixer® 400; Interscience, Saint Nom la Bretêche, France) for 3 min and then aliquoted into 50 mL plastic Falcon tubes and centrifuged at 7000× *g* for 12 min at 4 °C (to remove animal tissue sediment). Following this, the supernatants were carefully removed from each tube and collected together to a glass beaker. The aqueous mixture was initially filtered through paper filters (200 g/m^2; diameter 55 mm; Munktell Filter AB, Falun, Sweden) using a Buchner funnel to remove the largest aggregates. The filtrate was then aseptically filtered through microbiological filters (pore diameter 0.22 μm; SFCA-22E-050, Labbox Labware S.L., Barcelona, Spain) and stored at −80 °C.

2.2. Campylobacter Isolates and Preparation of Their Working Cultures

The raw chicken Campylobacter isolates used in this work belonged to the species *C. jejuni* (n = 5) and *C. coli* (n = 3). Some other critical information on these isolates is provided in Table 1. Before their experimental use, the isolates were long-term stored at −80 °C in Muller–Hinton (MH) broth (CM0405, OXOID, Freiburg, Baden-Wurttemberg, Germany) supplemented with 5% *v*/*v* laked horse blood (HB) (SR0048C, Thermo Fisher Diagnostics B.V., The Netherlands) and 20% *v*/*v* glycerol. When needed for the assays, each isolate was streaked on the surface of MH agar (AGMH-00P-500, Labbox Labware, S.L., Barcelona, Spain) and incubated at 42 °C for 24 h under microaerophilic conditions (6.2–13.2% O_2, 2.5–9.5% CO_2; Oxoid CampyGen 2.5L Sachet; CN0025A, Thermo Fisher Diagnostics B.V., The Netherlands) (primary precultures). Secondary precultures were prepared by inoculating a biomass of 5 to 10 colonies from each primary preculture into 2 mL of fresh MH–HB broth and then incubating at 42 °C for 24 h under microaerophic conditions. Working cultures were prepared by transfering 200 μL of each secondary preculture to 1800 μL of fresh MH–HB and then incubating at 42 °C for 24 h under microaerophic conditions (thereby achieving a final concentration of ca. 10^8 CFU/mL).

Table 1. Campylobacter raw chicken isolates used in this study and their relevant info.

Isolate Code	Species	Other Information	Poultry Isolation Origin
CAMP [1]_005	*C. coli*	strong BP [2], MDR [3]	wings
CAMP_022	*C. jejuni*	strong BP, MDR	minced meat
CAMP_025	*C. coli*	strong BP, MDR	neck
CAMP_048	*C. jejuni*	strong BP	souvlaki
CAMP_083	*C. coli*	weak BP, high resistance to ERY [4]	thigh
CAMP_091	*C. jejuni*	weak BP, high resistance to ERY	wings
CAMP_114	*C. jejuni*	moderate BP, MDR	neck
CAMP_130	*C. jejuni*	weak BP, MDR	wings

[1] *Campylobacter*; [2] Biofilm producer; [3] Multidrug resistance; [4] Erythromycin.

2.3. Determination of Minimum Inhibitory and Bactericidal Concentrations (MICs, MBCs) of LA against Planktonic Campylobacter Bacteria

The MIC of LA against the planktonic cells of each Campylobacter isolate was determined using the broth microdilution method as previously described with slight modifications [18]. Briefly, bacteria from the final working cultures (ca. 5×10^5 CFU/mL) were incubated in two different nutrient broths i.e., either MH or MH–HB broth at 42 °C for 48 h under microaerophilic conditions. For each broth, seven different concentrations of LA were tested ranging from 4096 to 64 μg/mL (two-fold dilutions). The MIC of LA was considered as its lowest concentration resulting in no visible bacterial growth. The absence of growth was confirmed through no increase in the absorbance of the medium in the case of MH broth, whereas in the case of MH–HB broth this was verified by the naked eye through observing change of color of the medium from red to brown. Resazurin sodium salt (B21187; Alfa Aesar; Massachusetts, United States) was also used at a concentration of 0.01% *w*/*v*, as an indicator of metabolic activity in the case of MH broth [19]. To calculate the MBC, 10 μL of broth cultures were aspirated from all the non-growth wells of the MIC assay and spotted (in duplicate) on MH agar plates, which were then incubated at 42 °C for 48 h under microaerophilic conditions. For each bacterial isolate, the MBC of the LA was determined as its lowest concentration that reduced the initial inoculum (ca. 5×10^5 CFU/mL) by more than 99.9% (no appearance of colonies). These experiments were repeated three times starting with independent bacterial cultures.

2.4. Determination of Minimum Biofilm Inhibitory Concentrations (MBICs) of LA against Singe and Mixed Campylobacter Cultures

2.4.1. Determination of MBICs of LA against Single Cultures

The MBIC of LA against the biofilm growth of each Campylobacter isolate was determined by the crystal violet (CV) staining assay as previously described [20]. For this, bacteria were initially left to form biofilms on 96-well PS microtiter plates (transparent, flat, Cat. No. 30096, SPL Life Sciences, Gyeonggi-do, Korea) for 48 h in MH broth supplemented with 5% (*v*/*v*) chicken juice (MH–CJ) at 42 °C under microaerophilic conditions and in the presence of seven different LA concentrations (two-fold dilutions ranging from 4096 to 64 μg/mL). At the end of incubation, for each bacterial isolate and LA concentration, the accumulated biomass in each well was quantified following its staining with CV (0.1% (*w*/*v*)), solubilization of the bound dye with an ethanol-acetone mixture (80:20, *v*/*v*), and absorbance measurements of the resulting solution at 590 nm (A_{590nm}) using a multimode microplate reader (Tecan Spark®, Tecan Group Ltd., Männedorf, Switzerland). As positive control for biofilm growth, wells containing inoculated MH–CJ without LA were used, whereas wells containing uninoculated MH–CJ were used for the negative control. For each bacterial isolate, the MBIC of the LA was determined as its lowest concentration that completely inhibited biofilm formation (biomass accumulated was not significantly different from that of the negative control). These experiments were repeated three times starting with independent bacterial cultures.

2.4.2. Determination of MBICs of LA against Mixed Cultures

The procedure described in Section 2.4.1. was also followed to determine the MBICs of LA against three different mixed Campylobacter cultures (consortia), each composed of three isolates (Table 2). In this case, nine different LA concentrations (two-fold dilutions ranging from 16,384 to 64 μg/mL) were tested. The selected Campylobacter isolates (n = 6) were divided into three different groups based on MDR character (Group A), high ERY resistance but not MDR (Group B), and strong biofilm production capacity (Group C). In addition, isolates with different macroscopic colony characteristics were selected per group.

Table 2. The three different Campylobacter consortia, each composed of three isolates. The six different isolates of these consortia were divided into three different groups (A–C) depending on their drug resistance and biofilm-forming phenotypes.

Consortium Code	Group A [1]	Group B [2]	Group C [3]
CONS1	CAMP_130	CAMP_083	CAMP_048
CONS2	CAMP_130	CAMP_091	CAMP_022
CONS3	CAMP_130	CAMP_083	CAMP_005

[1] MDR; [2] No MDR, with high-level resistance to ERY; [3] Strong biofilm producing capacity.

2.5. The Inhibitory Effect of LA on Biofilm Production by Mixed Campylobacter Cultures on PS and SS Surfaces

The inhibitory effect of LA against a selected mixed Campylobacter culture (i.e., CONS1) was further tested following the procedure described thereafter. In this treatment, 6-well PS microplates and SS coupons (30 × 10 × 1 mm, type AISI 304; placed vertically into glass tubes), were used as the abiotic substrata. For both types of substrata, 5 mL of growth medium (MH–CJ) were used to fill either each PS well or each glass tube. Four different LA concentrations were examined (two-fold dilutions ranging from 4096 to 512 μg/mL). After 48 h of static incubation at 42 °C under microaerophilic conditions, planktonic and biofilm cells were quantified by serial decimal dilutions in quarter-strength Ringer's solution (Lab M, Heywood, Lancashire, UK) and subsequent inoculation through spreading of MH-HB agar plates. More specifically, to detach and enumerate the biofilm cells, following the removal of planktonic suspension, each well was washed twice with quarter-strength Ringer's solution and after being filled with 5 mL of the same solution, it was thoroughly scratched with a plastic sterile pipette tip to remove the biofilm cells. Concerning the SS coupons, each of them was also washed twice with quarter-strength Ringer's solution, and it was then placed into a Falcon tube containing 5 mL of the same solution and 10 glass beeds (3 mm diameter), and vortexed for 2 min.

3. Results and Discussion

The inhibitory and lethal effects of LA against planktonic populations of eight wild-type *Campylobacter* isolates, grown in MH broth supplemented or not with 5% laked horse blood, under microaerobic conditions for 48 h at 42 °C, were initially tested in this study. The reason for selecting two different broths was because *Campylobacter* spp. usually grow better in nutrient media supplemented with blood, however there is no standardized protocol for the use of blood in the broth microdilution method for MIC determination. It should also be noted that in the case of LA treatments in the presence of blood, it was not possible to obtain reliable results from the spectrophotometric data. However, in this case, we were still able to accurately determine the endpoint MICs at those LA concentrations where a change in broth color from red to brown was observed (always comparing to the negative control). Table 3 presents the results of MIC/MBC determination for each one of the eight *Campylobacter* isolates. These results reveal that in general the presence of blood seems to favor the resistance of campylobacteria to LA treatment, with MIC values

recorded equal to 1024 μg/mL for six of the eight tested isolates incubated in blood-free MH broth and on the other hand MIC values equal to 2048 μg/mL were recorded for seven of the eight tested isolates incubated in MH broth with blood. However, these observed differences in MIC values are probably without any important practical effect, considering that LA is commonly applied at much higher concentrations (> 5000 μg/mL; 0.5% *v*/*v*) in antimicrobial treatments encountered in poultry processing [17,21]. For almost all the *Campylobacter* isolates, the MICs of LA were equal to the MBCs, indicating its strong bactericidal action.

Table 3. MIC, MBC and MBIC values of LA against the eight Campylobacter isolates and the three different consortia.

***Campylobacter*/Consortium Code**	**Species/Isolates**	**MIC [2]**	**MBC [3]**	**MIC**	**MBC**	**MBIC [4]**
		μg/mL				
		in MH [5]		**in MH -HB [6]**		**in MH -CJ [7]**
CAMP [1]_005	*C. coli*	1024	1024	2048	2048	1024
CAMP_022	*C. jejuni*	1024	1024	2048	2048	1024
CAMP_025	*C. coli*	1024	1024	1024	1024	1024
CAMP_048	*C. jejuni*	2048	2048	2048	2048	1024
CAMP_083	*C. coli*	1024	2048	2048	2048	2048
CAMP_091	*C. jejuni*	2048	2048	2048	2048	1024
CAMP_114	*C. jejuni*	1024	1024	2048	2048	1024
CAMP_130	*C. jejuni*	1024	1024	2048	2048	2048
CONS1	CAMP_048/083/130					4096
CONS2	CAMP_022/091/130					4096
CONS3	CAMP_005/083/130					4096

[1] *Campylobacter*; [2] Minimum Inhibitory Concentration; [3] Minimum Bactericidal Concentration; [4] Minimum Biofilm Inhibitory Concentration; [5] Muller—Hinton broth; [6] MH with 5% *v*/*v* laked horse blood; [7] MH broth with 5% *v*/*v* chicken juice.

Regarding the inhibitory effect of LA on biofilm production by the *Campylobacter* isolates grown in monoculture, the recorded MBIC values were 1024 μg/mL for six of the eight isolates, while for the other two of them (CAMP_083 and CAMP_130), the required LA concentration to inhibit their biofilm growth was double and equal to 2048 μg/mL (Table 3). It is worth to be noted that these results do not reveal any relationship between biofilm-forming capacity (weak, moderate, strong) of a given isolate and LA biofilm-inhibitory action against it. In the case of mixed *Campylobacter* biofilm cultures (CONS1, CONS2, CONS3), a LA concentration of 4.096 μg/mL was always required to inhibit the growth of biofilms for all three consortia (Table 3). Alarmingly, this denotes the favoring effect of inter-strain interactions on the resistance of mixed-culture biofilms to LA.

Figure 1 presents the biofilm populations (Log_{10}CFU/mL) for each one of the three isolates (CAMP_130, CAMP_083 and CAMP_048) of the mixed *Campylobacter* culture CONS1 incubated in MH-CJ in the presence of four LA concentrations, on either PS (6-well microplates) or SS (coupons) surfaces, under microaerophilic conditions for 48 h at 42 °C. For both surfaces CAMP_130 and CAMP_083 isolates appeared to dominate over CAMP_048 isolate at the two highest LA concentrations that were applied (2048 and 4096 μg/mL). This is an interesting observation and is probably due to the fact that the MBIC value of LA against CAMP_048 was lower than that observed against the two other isolates, thus indicating its higher sensitivity to LA. In addition, the competition that may develop between the different isolates under the mixed culture conditions, mainly for

available nutrients, may also account for this observation, as it has been previously reported for some other bacterial species [22].

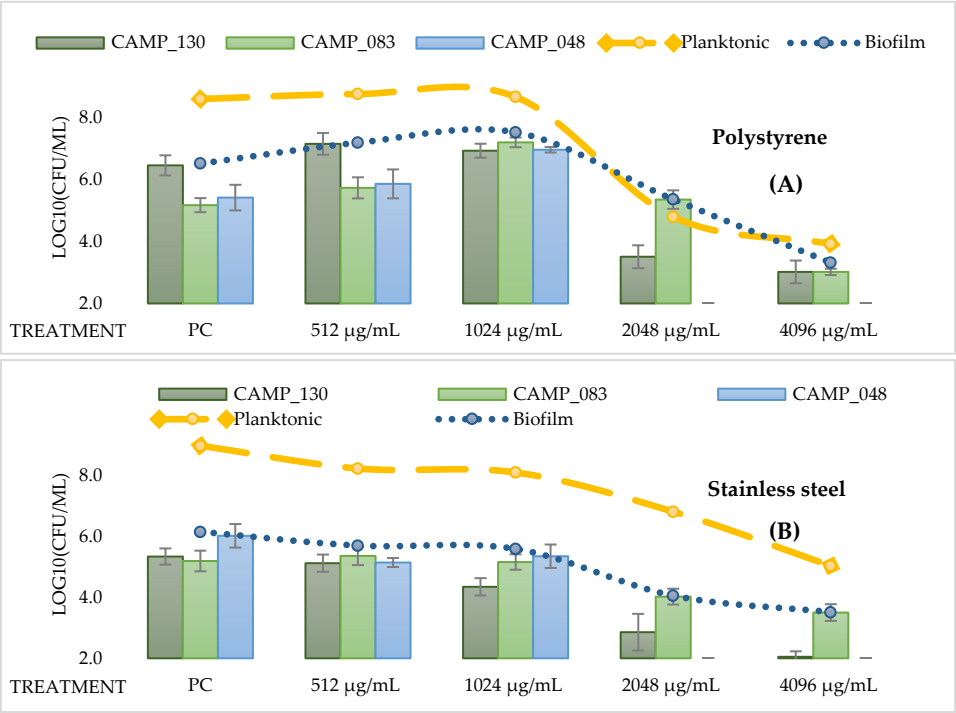

Figure 1. Biofilm populations (log_{10} CFU/mL) for each isolate of the mixed Campylobacter culture (two C. jejuni isolates, i.e., CAMP_130 and CAMP_048, and one C. coli isolate, i.e., CAMP_083) on the PS surface of the 6-well microplates (**A**) and the SS surface of the coupons (**B**), in the presence of four different LA concentrations (two-fold dilutions ranging from 4096 to 512 μg/mL). The biofilm populations of the positive control (PC; without LA treatment) are also shown. The bars represent the mean values ± standard deviations. The detection limit was 2 log_{10} CFU/mL. The total biofilm populations for each treatment are also shown as blue dots (dotted curved line), while the total planktonic populations found in the wells/tubes at the time of sampling (48 h) are also shown for each treatment (as yellow dotted curved lines).

4. Conclusions

Overall, the results of this work offer insight into biofilm control of a pathogen of public health importance.

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Conflicts of Interest:

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