



ASSESSING SENSITIVITY OF *CAMPYLOBACTER JEJUNI* TO ANTI-MICROBE EFFECTS TO REDUCE RISKS OF FOOD PRODUCTS CONTAMINATION WITH *CAMPYLOBACTERIOSIS* AGENTS

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We aimed to assess efficiency of various anti-microbe effects on pathogenic microflora; to do that, we performed comparative study on how sensitive Campylobacter jejuni test strains were to ultraviolet radiation and biocides based on peracetic acid (PAA). PAA-based biocides suppressed significant numbers of Campylobacter; however, preset treating modes didn't allow achieving complete inactivation of the test strains. Efficiency of effects produced by ultraviolet radiation on C.jejuni strains depended on exposure duration: 20-minute treatment of plates with broth bacterial suspensions caused a decrease in a number of viable cells which was equal to 1.5–2,0 logarithmic orders; 60-minute treatment resulted in a decrease in C.jejuni contents that amounted to less than 200 CFU/cm³.

Polypeptide antibiotics produced by lactic-acid bacteria (bacteriocins, nisin, etc.) have some useful properties and it makes them applicable for suppressing adverse microflora in food products manufacture. We applied an 'associate growth' model to examine peculiarities related to C.jejuni growth in milk that, beside this pathogen, was simultaneously inoculated with mesophilic lactic-acid lactococci or thermophilic lactic-acid bacteria that were bacteriocins producers. Depending on quantity of introduced lactic-acid bacteria, such as Lactobacillus plantarum, Lactobacillus lactis and Lactococcus lactis, C.jejuni growth was substantially inhibited. We revealed a relationship between anti-bacterial activity and nature of inhibiting effects and concentrations of the above-mentioned lactic-acid microorganisms, temperature, an amount of time during mixed cultures were cultivated together, and properties of used strains which became the most apparent when lactobacteria were introduced in a dose equal to 10⁸ CFU/cm³.

Our study on an ability of C.jejuni to survive under exposure to an adverse environment revealed that microbe populations had variable physiological properties, Campylobacter were greatly resistant, and it was difficult to make a relevant choice on efficient tools and procedures for anti-microbe treatment.

Key words: *Campylobacter jejuni*, in vitro model, biocides, anti-microbe effects, ultraviolet radiation, contamination, biofilms, lactic-acid bacteria.

Bacteria from *Campylobacter* stem have considerable epidemiological significance and it makes it necessary to establish whether these microorganisms are able to survive contemporary technological processes in food products manufacturing under exposure to various technogenic or biological factors. It is advisable to examine how bacteria from *Campylobacter* stem develop their stress tolerance as it allows more efficient assessment of microbiological risks and more efficient monitoring over food products

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contamination with campylobacteriosis-inducing agents.

Bacteria from *Campylobacter* stem are widely spread in the environment and can be found on surfaces of any equipment and tools applied at food manufacturing enterprises [1, 2]. As objects located within production workshops are frequently contaminated with live *Campylobacter* bacteria, there is a potential hazard that they can be additionally infected due to non-cultivated forms and biofilms occurring under exposure to technological stress factors such as sub-lethal doses of antibiotics, acids, disinfectants, biocides, etc. [2–4]. This process is seen as a mechanism microorganisms apply to adapt and survive in natural and artificial ecosystems [5–7]. Irradiation that is widely used to perform bactericidal treatment of objects at production facilities and packing materials can also stimulate a stress response in bacteria; this response not only induces tolerance in bacteria to such exposure but also make them resistant to other techniques applied to treat raw materials and finished products [8, 9].

Influence exerted by adverse environmental factors on vital functions performed within a bacterial cell occurs at various regulatory levels and it can be accompanied with induced tolerance of microorganisms to exposure to these or those bactericidal factors. Bacteria can become tolerant in nature or during sanitary treatment of water or equipment under exposure to different antibacterial agents including chlorine, acids, alkali, preservatives, antioxidants, bacteriophages, colicins, acrylates, and metal ions [10–12].

At present treatment with chlorine-containing substances is quite often used in food production as a tool to decrease contamination with pathogenic microorganisms [8, 9, 13, 14]. Free chlorine and substances that evolve it (sodium hypochlorite, calcium hypochlorite, magnesium hypochlorite, chlorinated lime, chloramines, chlorine dioxide, sodium dichloroisocyanurate, and calcium dichloroisocyanurate) have substantial anti-microbe properties and are efficient against most

pathogenic bacteria; they are widely used as disinfectants in medicine and veterinary, to treat drinking water and sewage waters, as well as in food production where they are applied to treat equipment and even to reduce microbe contamination on surfaces of raw materials [13–15].

Treatment with chlorine-containing biocides prevents cross contamination of products with infectious agents that cause alimentary infections and toxic infections and allows achieving longer shelf life for food products. However, application of chlorine also produces certain adverse effects such as occurrence of trihalomethanes that are toxic and carcinogenic, for example, chloroform, dichlorobrommethane, di bromchloromethane, and bromoform [6, 13]. In general, when such compounds are found in residual quantities conforming to maximum permissible levels, it allows avoiding direct health risks such as toxic, allergic or any other reactions when people consume food products that contain such residual quantities. However, at present it is proven that potentially microorganisms that contaminate food and drinks can have both acquired lower sensitivity to biocides and co-resistance to anti-microbe preparations [10, 16]. Experts also think that biocides application can have several negative consequences, for example, making bacterial pathogens evolve faster and causing occurrence of new infections that are hazardous for people [11, 12].

These phenomena indicate that remote risks related to application of anti-microbe means in technological processes are somewhat underestimated. Such issues as whether conventional concentrations of biocides applied in food industry are safe or various contaminants becoming tolerant to them as well as changes in phenotypic properties of the most significant microorganisms groups haven't been studied in depths.

To reduce risks caused by the given adverse impacts, it is vital to make a well-substantiated choice on efficient and safe tools for decontamination of raw materials and sani-

tary treatment of equipment used in food manufacturing.

Data and methods. We assessed how efficient various anti-microbe preparation were in terms of suppressing pathogenic microflora; to do that, we comparatively analyzed how sensitive test-strains of *Campylobacter* and salmonella (bacteria-indicators of food pathogens) were to exposure to biocides based on hypochlorite and technological auxiliary agents “Food Clean Peroxy” and “Kriodez” based on peracetic acid (PAA). We applied a previously developed laboratory model *in vitro* [17] to determine how sensitive *C. Jejuni* bacteria were to adverse impacts; to do that, we simultaneously cultivated several sub-populations of the examined strains under different parameters of gas medium and incubation regimes. We determined a ratio of viable colony-forming units (CFU) to overall number of cells in a population via calculating genome DNA contents in samples with qualitative real-time PCR [18].

We assessed efficiency of anti-microbe effects produced by active chlorine solutions on salmonella as per occurrence or absence of growing test-strains in dextrose-peptone medium with bromthymol blue indicator after a biocide had been introduced and samples had been cultivated for 18 hours under 37 °C. To vary two factors, namely biocide concentration and bacterial suspension density, we applied 96-well sterile immunologic plates [19]. A medium becoming turbid and changes in its color from blue-green to yellow indicated there was growth. We measured optical density of media with “Sunrise” automated plate spectrophotometer.

We determined *C. jejuni* strains ability to create biofilms with a previously developed *in vitro* laboratory model [20]. Strains were cultivated on polystyrene 96- and 24-well plates; we applied Mueller-Hinton broth as a cultivating medium with 5 % defibrinated blood being added into it as well as growth additives “Mersk” and “HiMedia” that contained sodium pyruvate, sodium metabisulfite, and iron sulfate. We determined film

creation intensity with chromogenic technique using a plate spectrophotometer.

Obtained results were statistically treated with Student’s t-test and Mann-Whitney non-parametric rank test. Discrepancies were considered to be authentic at $P < 0.05$. We made all the calculations with EXCEL and SPSS 18.0 software packages.

Results. We assessed inhibiting effects produced by disinfectants as per intensity of test strains growth depending on biocides concentrations and bacterial population density (Table 1).

Figure 1 shows assessment of effects produced by active chlorine on salmonella in a chromogenic model *in vitro*.

“Kriodez” had weaker effects on campylobacter in comparison with “Food Clean Peroxy” and its application resulted in a fall in test-strains quantity that was equal only to 1–2 logarithmic orders (from 1.0×10^9 CFU/cm³ to $1.0 \times 10^{7-8}$ CFU/cm³). Results obtained via our comparative assessment of anti-microbe effects produced by PAA-based biocides prove that it is necessary to optimize modes of contact cooling that are applied to cool raw materials at poultry-processing enterprises; it is also necessary to make relevant selection on working concentrations of disinfectant solutions.

After *S. enteritidis* and *S. typhimurium* strains were treated with chlorine, we detected that there was no film-creating whereas *C. jejuni* tended to have stronger ability to create biofilms (Figure 2).

So, as we concluded, application of chlorine-contained disinfectants didn’t provide proper and efficient treatment of substrates contaminated with campylobacter; therefore, we tested other anti-microbe impacts. To do that, we examined how sensitive campylobacter test strains were to UV-radiation as compared with affects produced by PAA-based biocides.

We assessed inhibiting effects produced by bactericidal means as per intensity of test strains growth depending on UV treatment duration and PAA concentration (Table 2).

Table 1

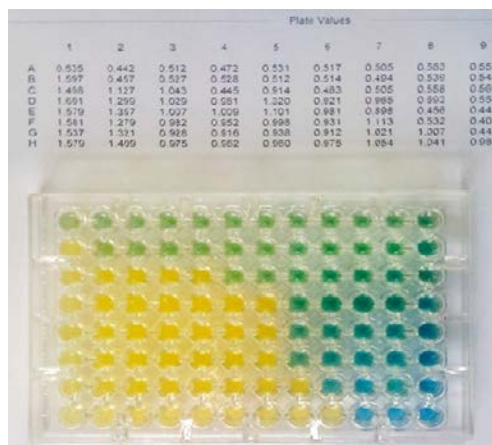
Effects produced by biocides on *C. jejuni* and *Salmonella* spp.

Test strains	Cultivation of initial test-cultures suspension *									
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	10 ⁻¹⁰
Control (without biocides)										
<i>Salmonella enteritidis</i>	+	+	+	+	+	+	+	+	+	+
<i>Salmonella typhimurium</i>	+	+	+	+	+	+	+	+	+	-
<i>C.jejuni</i> 13p	+	+	+	+	+	+	+	+	+	-
<i>C.jejuni</i> 5p	+	+	+	+	+	+	+	+	-	-
Hypochlorite (active chlorine concentration is 50 mg/dm ³ , exposed for 25 minutes at +2°C)										
<i>Salmonella enteritidis</i>	+	+	+	+	+	-	-	-	-	-
<i>Salmonella typhimurium</i>	+	+	-	-	-	-	-	-	-	-
<i>C.jejuni</i> 13p	+	+	+	-	-	-	-	-	-	-
<i>C.jejuni</i> 5p	+	+	-	-	-	-	-	-	-	-
"Food Clean Peroxy" (0.04%-solution, exposed for 25 minutes at +2°C)										
<i>Salmonella enteritidis</i>	-	-	-	-	-	-	-	-	-	-
<i>Salmonella typhimurium</i>	-	-	-	-	-	-	-	-	-	-
<i>C.jejuni</i> 13p	+	+	+	+	-	-	-	-	-	-
<i>C.jejuni</i> 5p	+	+	+	-	-	-	-	-	-	-
"Kriodez" (0.04%-solution, exposed for 25 minutes at +2°C)										
<i>Salmonella enteritidis</i>	-	-	-	-	-	-	-	-	-	-
<i>Salmonella typhimurium</i>	-	-	-	-	-	-	-	-	-	-
<i>C.jejuni</i> 13p	+	+	+	+	+	+	+	-	-	-
<i>C.jejuni</i> 5p	+	+	+	+	+	+	-	-	-	-

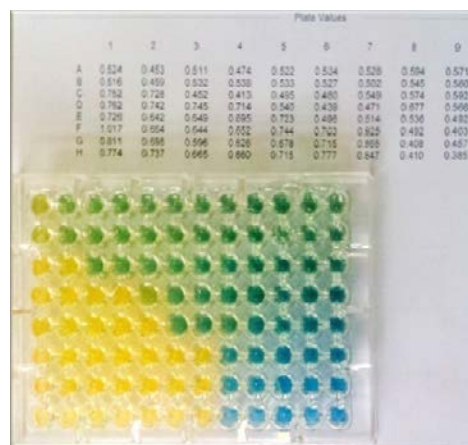
Note:

+/- means absence or occurrence of growth in relevant cultivated test-strains suspensions after exposure to a biocide;

* means that initial broth culture contains bacteria in a quantity not less than 1 x 10⁹ CFU/cm³.



S. enteritidis



S. typhimurium

Figure 1. Growth in collection salmonella test strains under exposure to active chlorine within concentrations range equal to 10–200 mg/dm³ in an experimental chromogenic model

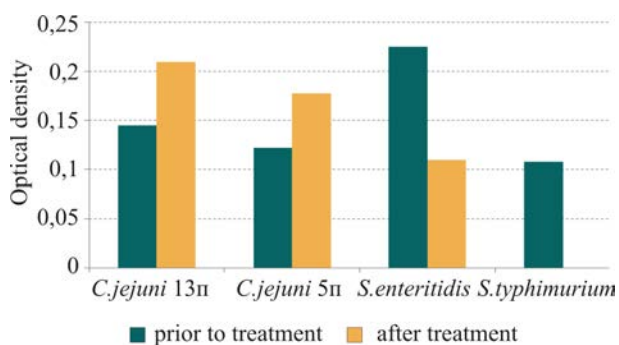


Figure 2. Ability of campylobacter and salmonella strains to form biofilms

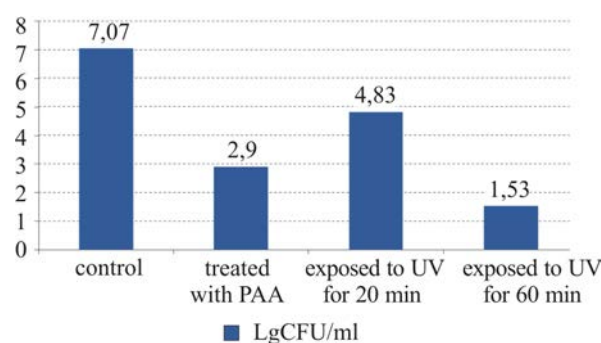


Figure 3. Bactericidal impacts on *C.jejuni* survival

PAA-based biocides with working solution concentration being equal to 0.04% inhibited a considerable number of *C.jejuni* bacteria (from $10^{6.5-8}$ to 10^{2-3} CFU/cm³); however, preset treatment modes didn't make test-strains completely inactive. Efficiency of impacts exerted by UV-radiation on campylobacter strains depended on exposure dura-

tion; after plates with broth bacterial suspension were treated for 20 minutes, quantity of viable cells went down by 1.5–2 logarithmic orders from their initial concentration which was equal to 10^7-10^8 CFU/cm³; after 60-minute treatment *C.jejuni* concentration decreased drastically and amounted to less than 200 CFU/cm³ (Figure 3).

Table 2

Growth of *C.jejuni* strains under exposure to different anti-microbe impacts

Determined parameters	Number of strains / repetitions				Mean values, $M \pm m$ (test)
	NCTC 11,168 (control) $n = 3$	17p $n = 3$	23p $n = 3$	5.2 $n = 3$	
Test strains cultivation at +37°C under microaerophilic conditions (control)					
Bacterial suspension density, lg CFU/cm ³	7.99	6.9	6.8	6.6	7.07±0.31
PCR results / (Ct)	+ (14.5)	+ (14.8)	+ (17.5)	+ (16.3)	(15.78 ± 0.7)
Treatment with PAA (0.04%-solution, exposed for 25 min at +2°C)					
Bacterial suspension density, lg CFU/cm ³	3.6	2.6	2.1	3.3	2.90 ± 0.34
PCR results / (Ct)	+ (21.6)	+ (17.3)	+ (23.1)	+ (21.0)	(20.75 ± 1.2)
Inhibition, lg orders from initial level	4.4	4.3	4.7	3.3	4.1
Exposed to UV-radiation for 20 min					
Bacterial suspension density, lg CFU/cm ³	4.3	5.6	5.1	4.3	4.83 ± 0.32
PCR results / (Ct)	+ (28.8)	+ (29.8)	+ (28.6)	- (32.7)	(29.07±0.37)
Inhibition, lg orders from initial level	3.7	2.3	1.7	2.3	2.1
Exposed to UV-radiation for 60 min					
Bacterial suspension density, lg CFU/cm ³	1.9	2.1	1.1	<1.0	1.53 ± 0.28
PCR results / (Ct)	+ (29.0)	+ (26.2)	+ (27.1)	+ (30.7)	(28.25 ± 1.0)
Inhibition, lg orders from initial level	6.09	4.8	5.7	5.6	5.4

Table 3

Detection of *C.jejuni* during souring of experimentally contaminated raw milk

Lactic-acid microorganisms	Lactic-acid microorganisms concentration, cells/cm ³	<i>C.jejuni</i> concentration, cells/cm ³					
		10 ²		10 ³		10 ⁴	
		6 h	24 h	6 h	24 h	6 h	24 h
<i>Lactobacillusplantarum</i>	10 ⁴	+	–	+	+	+	+
	10 ⁶	–	–	+	+	+	+
	10 ⁸	–	–	–	–	+	–
<i>Lactobacilluslactis</i>	10 ⁴	–	–	+	–	+	+
	10 ⁶	–	–	+	–	+	+
	10 ⁸	–	–	–	–	+	–
<i>Lactococcuslactis</i>	10 ⁴	+	–	+	+	+	+
	10 ⁶	–	–	+	–	+	+
	10 ⁸	–	–	–	–	–	–

Note: “+” means *C.jejuni* grow, “–” means *C.jejuni* don’t grow.

We performed comparative examinations aimed at assessing anti-microbe impacts on bacteria from *Campylobacter* stem; the results indicate it is necessary to select up-to-date and more efficient means for decontaminating industrial environment at food-making productions.

Available data on impacts exerted by physical, chemical, and biological factors on bacteria from *Campylobacter* stem stimulated research on abilities possessed by certain microorganisms, namely their ability to demonstrate symbiotic or antagonistic properties within complicated biocenosis with food pathogens. The greatest attention here is paid to lactic-acid bacteria with apparent anti-microbe effects and substances extracted from them. Antagonism that lactic-acid bacteria seem to have is predominantly explained by specific antibiotic substances with polypeptide essence; these substances can produce certain anti-microbe effects and have different chemical structure. Bacteriostatics produced by lactic-acid bacteria (bacteriocin, nisin, etc.) have a set of benign features and it gives an opportunity to use them for inhibiting adverse microflora in food production.

Data on interactions between campylobacter and lactic-acid microorganisms are

scarce due to difficulties related to simulating a natural microbial background of this complicated biotechnological process in experimental conditions. A model of “associated growth” was applied to examine peculiarities of *C.jejuni* reproduction in milk that was, in addition to this pathogen, simultaneously inoculated with either mesophilic lactic-acid lactococcus or thermophilic lactobacillus. *Campylobacter* reproduction was inhibited considerably depending on a quantity of lactic-acid bacteria introduced into a culture and incubation temperature (Table 3).

Anti-bacterial impacts exerted by *Lactobacillusplantarum* cultures were similar to those produced by bacteriocin; they were detected in experiments *in vitro*, but *C.jejuni* bacteria didn’t die, that is, the effect was only bacteriostatic. Growth of pathogenic campylobacter strains was also detected when they were cultivated in milk together with *Lactobacilluslactis* and lactic-acid lactococcus *Lactococcuslactis*. *C.jejuni* were inactivated after 6–24 hours of incubating in mixed populations as their initial level dropped by 1–3 lg. Anti-bacterial activity and essence of inhibiting effects were established to depend on concentrations of the above-mentioned lactic-acid microorganisms, temperature, a pe-

riod during which mixed cultures were cultivated, and properties of used strains which became the most apparent when lactic-acid bacteria were introduced in a dose equal to 10^8 CFU/cm³.

Studies on *C. Jejuni* ability to survive under adverse environmental exposure revealed that physiological properties of microbe populations were variable and bacteria from *Campylobacter* stem were highly resistant; therefore, it was difficult to select relevant and efficient means and procedures for anti-microbe treatment. So any further research should concentrate on transferrable resistance of bacteria from *Campylobacter* stem and influence exerted on their survival and transfer into non-cultivated forms by stress factors in industrial environment; it is also important to examine specific mechanisms of phenotypic adaptation (tolerance) to sub-lethal concentrations of anti-microbe agents.

Conclusion. General protective properties possessed by *Campylobacterspp.* can become apparent via different interactions between bacteria and the environment thus making for occurrence of resistant microorganisms both *in vitro* and in real industrial conditions under exposure to stress technogenic or biological factors.

The most apparent trend in changes that occur in *C. jejuni* properties is an increase in

their resistance to bactericidal effects caused by wide use of anti-microbe preparations (AMP), including antibiotics and biocides.

A model of “associated growth” was applied to show dependence between antibacterial activity and inhibiting effects and concentrations of lactic-acid bacteria

Lactobacillusplantarum, *Lactobacilluslactis* and *Lactococcuslactis*, temperature, a period during which mixed cultures were cultivated, and properties of used strains that became the most apparent when lactic-acid bacteria were introduced in a dose equal to 10^8 CFU/cm³.

To assess efficiency of impacts exerted by pathogenic microflora by various anti-microbe means, we conducted a comparative study on how sensitive campylobacter test-strains were to UV-radiation and PAA-based biocides. PAA-based biocides inhibited a considerable number of *C.jejuni* bacteria; however, preset treatment modes didn't make test-strains completely inactive. Efficiency of impacts exerted by UV-radiation on campylobacter strains depended on exposure duration and anti-microbe exposure conditions.

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Conflict of interests. The authors declare there is no any conflict of interests.

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