ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 11, Issue, 02, pp.1490-14894, February, 2019

DOI: https://doi.org/10.24941/ijcr.34392.02.2019

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

ANTIMICROBIAL ACTIVITY OF SODIUM CARBOXYLATES AGAINST *ARCOBACTER* SP. *Y CAMPYLOBACTER* SP.

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ARTICLE INFO

Article History:
Received 10th November, 2018
Received in revised form
24th December, 2018
Accepted 20th January, 2019
Published online 28th February, 2019

Key Words:
Arcobacter, Campylobacter,
Sodium carboxylates,
Microbial inhibition, Poultry

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ABSTRACT

Arcobacter and *Campylobacter* genres are pathogenic microorganisms associated with gastrointestinal diseases and their main transmission route is through food and water. Both genres have been strongly associated to poultry, these animals have been signed as reservoirs and contamination sources. Due to this characteristic, industries associated to poultry production had to establish control measures for these bacteria, being hygiene and disinfection one of the most important ones at processing plant level. The use of chemical agents for the control of pathogenic and spoilage microorganisms is one of the most used strategies in food industry, nevertheless, the inadequate use of these substances and the transmission of resistance genes between strains has motivated an increase in the appearance of resistant microorganisms. This has led to the search of new chemical agents that accomplish with the characteristics requested by food industry, including to be non-toxic for consumers, food compatible and active at concentrations similar to routinely used. The main objective of this study was to evaluate the antimicrobial activity of eight different sodium carboxylates against different *Arcobacter* and *Campylobacter* strains. Growth of these bacteria was determined at different concentrations of the salts. For *Arcobacter*, the inhibitory effect of the corresponding sodium salts was as follows: butanoate >gallate – 4-bromobenzoate - decanoate>benzoate>octanoate>caffeate> ascorbate; whereas for *Campylobacter* was: gallate – 4-bromobenzoate >decanoate – octanoate – butanoate – benzoate >caffeate> ascorbate. Sodium gallate and sodium 4-bromobenzoate were the two salts that presented inhibitory effect at lower concentrations for both bacteria (MIC = 31 µg/mL), butanoate presented a MIC ever lower but only against *Arcobacter* strains (< 8µg/mL).

***Citation: Lucía Acuña Díaz, Melissa Umaña Solano, Jorge Cabezas Pizarro, Mauricio Redondo Solano, Carolina Chaves Ulate and María Laura Arias Echandi,* 2019.** "Antimicrobial Activity of Sodium Carboxylates *Against Arcobacter sp. y Campylobacter sp.", International Journal of Current Research,* 11, (02), 1490-14894.

INTRODUCTION

Arcobacter and *Campylobacter* are zoonotic pathogens mainly associated to poultry consumption. This situation has led to the implementation of control measures for these bacteria at different levels of poultry production chain. *Arcobacter* is a bacterial genre of growing importance in public health, the increase in the data of incidence and prevalence of associated cases has positioned it as an emerging pathogen, with zoonotic potential and a strong association to food transmition (Calvo *et al.,* 2013), reason why the International Commission for the Microbiological Specifications in Food (ICMSF) has classified it as a risk for human health (Ramees *et al.,* 2017). This bacterium has been isolated from multiple domestic and wild animal hosts, the biggest incidence is described for poultry, recognized as the biggest reservoir and infection source. Nevertheless, pork and cattle are also important sources of infection (Calvo *et al.,* 2013). *Arcobacter butzleri, A. cryiaerophilus* and *A. skirowii* are the species most commonly involved in the generation of clinic conditions (Calvo *et al.,* 2013). Since poultry is described as the principal reservoir of *Arcobacter,* this industry has begun to give importance to this pathogen in their facilities, animals and products. It has been proposed that this microorganism refugees in the intestines of birds and these contaminate production plants when carcasses are processed, spreading in the meat. Isolation of this bacterium has been reported in slaughterhouses even after disinfection processes (Calvo *et al.,* 2013). These bacteria have shown resistance to high concentrations of sodium chloride; ability to grow at refrigerating temperatures, survival at scalding temperatures and a low susceptibility to desiccation, characteristics that make difficult its control within food industry. Same time, the presence and survival of *Arcobacter* in water has been demonstrated, and it depends on the availability of organic material in the environment and an adequate temperature (Collado and Figueras, 2011). Same way, species of the Campylobacter genre have clinical importance since they are considered food borne pathogens, especially at industrialized zones as United States and Europe, where cases reported vary between 31-151/100000 inhabitants (Skřivanová *et al.,* 2011). Principal pathogenic agents of this genre include *Campylobacter jejuni, C. coli, C lari* and *C.*

upsaliensis. These are naturally found in the gastrointestinal tract of diverse animal species, both from domestic and wild life (Blackburn and McClure, P. 2009). Exposure of human beings to these bacteria might be by direct contact with animals or the ingestion of contaminated food and water (Skarp *et al.,* 2015). Campilobacteriosis is characterized as an acute and auto limited enterocolitis, with bloody or aqueous stools that can last even for a week. Poultry is the principal reservoir and contamination source of *Campylobacter* through the processing chain of meat products, being the breeding farms the production spot where the greatest contamination with these bacteria occurs. Also, the contamination of carcasses with this pathogen occurs during sacrifice and evisceration of the animal (Skarp *et al.,* 2015). For the control of these bacteria with disinfecting agents, products such as sodium hypochlorite and peracetic acid have been used, nevertheless, the application of chlorine at 50 ppm concentration at poultry carcasses has not been effective (Blackburn and McClure, 2009). Since breeding farms are the principal contamination point in the production chain, the introduction of control steps for these bacteria are important (European Food Safety Authority, 2011; Skarp *et al.,* 2015). Given this situation, in the last years several alternative chemical strategies have been proposed, such as the use of acidified sodium chloride, cetilpiridechrolide, ozone, trisodic phosphate, (Oyarzabal, 2000) and organic acids (Meredith *et al.,* 2013). Because of the appearance and increase of resistance to common useantimicrobial agents, food industry has been required to search and develop new non-toxic agents for the control of these pathogens (Cabezas-Pizarro *et al.,* 2016). The use of chemical agents at chiller phase of production chain has emerged as an alternative for the control of pathogens, where further than inhibiting or diminishing remnant microbial growth rate by the immersion of carcasses in water at a 0°C temperature, the addition of chemical agents such as chlorine reduces even more the microbial load. Nevertheless, recent reports have indicated that both *Campylobacter* and *Arcobacter* have presented resistance to common use chemical agents. Because of the huge growth of poultry industry, the increase in the number of reported cases related to these pathogens and the resistance to common use chemical agents shown, the need of new control agents emerges. These shall benon-toxic, chemically compatible with food and with an activity at concentrations similar to routine ones. The aim of this work was to evaluate the antimicrobial activity of eight different sodium carboxylates against at least 10 *Arcobacte* rsp and 10 *Campylobacter* sp strains, isolated from Costa Rican poultry.

MATERIALS AND METHODS

General Information: All glassware and syringes were dried in an oven overnight at 140^o C, assembled while hot, flushed and cooled under nitrogen immediately prior to use. All reactions were carried out under a positive pressure of nitrogen. Nitrogen was passed through a Drierite gas-drying unit prior to use. Tetrahydrofuran was refluxed and freshly distilled from potassium /benzophenone ketyl, under nitrogen atmosphere. Sodium hydride was weighted out in a glove bag under nitrogen.

Preparation of sodium carboxylic acid salts: To a round bottom flask, under nitrogen, was added NaH (0.75 g, 60% in mineral oil). Dry THF was added to the flask, stirred for a few minutes and then the NaH was allowed to settle down. The supernatant liquid was removed with a double-tipped needle, under pressure of nitrogen, and the procedure repeated. The remnant THF was evaporated *in vacuo*, and the flask filled with nitrogen, to obtain NaH as a dry white solid (0.542 g, 22.6 mmol). Dry THF (~20 mL) was added to the pure NaH, obtained as describe above, and the temperature was lowered to 0^o C. The corresponding carboxylic acid (22.6 mmol), dissolved in THF (20 mL), was added drop wise with stirring in about 15 minutes. The ice bath was removed and the suspension stirred overnight under nitrogen atmosphere. The solvent was removed in a rotavapor and the solid salt was obtained as a white solid.

***Salts*:** Carboxylic salts, including sodium decanoate, sodium octanoate, sodium butanoate, sodium benzoate, sodium bromobenzoate, sodium gallate, sodium caffeate and sodium ascorbate in concentrations ranging from 100 mg/mL to 8 µg/mL were prepared.

***Strains*:** 10 strains previously characterized of *A. butzleri* and *C jejuni* were used. *Arcobacter* strains were kindly supplied by the Water Microbiology Lab, Faculty of Microbiology, Universidad de Costa Rica and *Campylobacter* strains by the Veterinary Faculty of the Universidad Nacional.

Suspension preparation: Each microorganism was inoculated into blood agar (Oxoid®) and cultured at 37 °C in aerobic atmosphere for *Arcobacter* and microaerophilic atmosphere for *Campylobacter* for 24-48 h. The bacterial suspension to be cultured was equivalent to 0.5 McFarland standard, (1.5×10^8 CFU/mL).

Bactericidal assays: 96 well tissue culture microtiter plates (Nalge, Nunc International, Rochester, NY) were used for each experiment. An assay mixture with a final volume of 150 µL was done as described below:

Negative control: 50 µL of the acidic salt in a pre-established concentration for each specific assay+ 100 µL solvent (dimethyl sulfoxide DMSO or distilled water).

Positive control: 50 µL yeast TSB (tripticase soy broth) +, 50 µL bacterial suspension with a concentration similar to 0,5 McFarland, 50 µL solvent. Trial 50 µL bacterial suspension in a concentration similar to 0,5 McFarland + 50 µL yeast TSB + 50 µL of the acidic salt in the pre-established concentration to evaluate. Three wells were used as positive growth controls, 3 wells as negative controls and 3 wells for trials. Plates were incubated at 37°C under aerobic atmosphere for *Arcobacter* and microaerophilic atmosphere for *Campylobacter* for a maximum of 96 h. Growth was determined using the Biotek Synergy HT multi detection reader (Vermont, US). Protocol followed included readings at 600 nm.

Data analysis: All experiments were performed in triplicate. Data was analyzed using the SPSS-PC program (Statistical Package for the Social Sciences) with a 95% ($p \leq 0.05$) confidence level.

RESULTS

The antimicrobial activity of different sodium carboxylates against 10 *Arcobacter* sp and 10 *Campylobacer* sp. strains were evaluated in order to obtain the minimal inhibitory concentration (MIC) (Tables I and II).

Table I. Minimal Inhibitory Concentration (MIC) of different aromatic sodium carboxylates tested against *Arcobacter* sp. and *Campylobacter* sp.

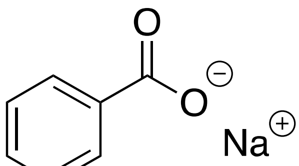
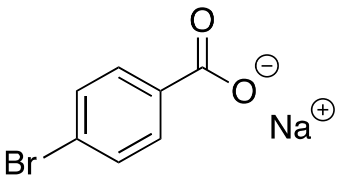
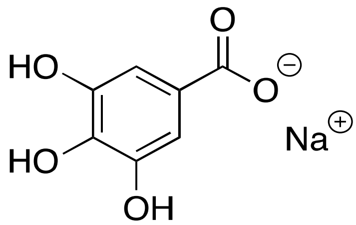
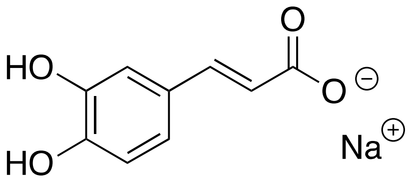
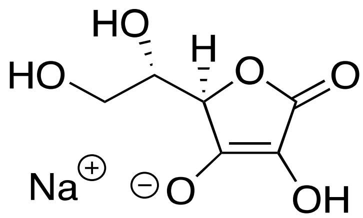
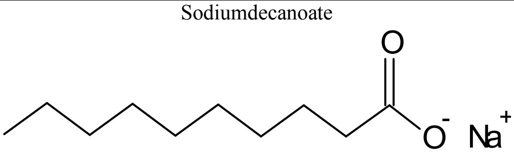
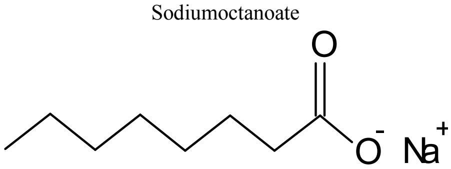
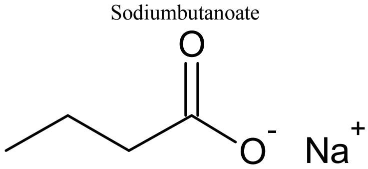
Sodium Carboxylates	Molecular weight (g/mol)	Minimal Inhibitory Concentration (MIC) µg/mL	
		***Arcobacter* sp.**	***Campylobacter* sp.**
Sodiumbenzoate 	144,11	62	625
Sodium 4-bromobenzoate 	224,01	31	31
Sodiumgallate 	192,11	31	31
Sodiumcaffeate 	202,14	25 000	25 000
Sodiumascorbate 	198,11	>25 000	>25 000

Table II. Minimal Inhibitory Concentration (MIC) of the different aliphatic chain salts tested against *Arcobacter* sp. and *Campylobacter* sp.

Sodium Carboxylates	Molecular weight (g/mol)	Minimal Inhibitory Concentration (MIC) µg/mL	
		***Arcobacter*sp.**	***Campylobacter*sp.**
Sodiumdecanoate 	194,25	31	625
Sodiumoctanoate 	166,20	2500	625
Sodiumbutanoate 	110,09	≤ 8	625

For *Arcobacter*, the inhibitory effect of the corresponding sodium salts was as follows: butanoate >gallate – 4-bromobenzoate - decanoate> benzoate >octanoate>caffeate> ascorbate; whereas for *Campylobacter* was: gallate – 4-bromobenzoate >decanoate – octanoate – butanoate – benzoate >caffeate> ascorbate (Tables I and II). All data compared are reported with a ≤0,05 significance level. For aliphatic salts, the inhibitory effect against *Arcobacter* was butanoate>decanoate>octanoate. For *Campylobacter* all salts had a similar effect, all presented a MIC of 625 µg/mL (Table II). For both bacteria, sodium caffeate and sodium ascorbate were the salts that needed the highest concentrations in order to exert an inhibitory effect; sodium ascorbate did not show any inhibition effect event at the highest concentration evaluated, (25 mg/mL). Contrasting, sodium decanoate and sodium 4-bromobenzoate presented an important inhibitory activity against *Arcobacter*, having a MIC of 31 µg/mL, and sodium butanoate presented an MIC even lower (8 µg/mL)

DISCUSSION

Bird meat is considered as one of the main reservoirs of *Arcobacter* and *Campylobacter* genre, and since their meat is one of the principal products of human consumption, its contamination is of great concern for poultry industry worldwide. In Costa Rica, Valverde-Bogantes *et al.* (2015) studied the contamination of retail selled poultry viscera with these bacteria, reporting a 17% contamination. Zumbado-Gutierrez and Romero-Zúñiga (2016) reported a 59, 37% prevalence of *Campylobacter* in Costa Rican market and industry. For the control of these microorganisms, Zumbado (2013) reported that all processing plants included in her study, except one, used chlorine in concentrations ranging 20 and 50 ppm in the water of chillers, also, some plants included the use of peraceticacid in concentrations up to 240 ppm. Since there are few commercially available chemical agents that present a broad antimicrobial efficacy, ease of application and of low cost, chlorine keeps on being extensively used in food industry (Ramos *et al.,* 2013). Nevertheless, its use has been widely questioned since it is corrosive and can promote the formation of potentially toxic substances (carcinogenic) when combined with organic material (European Food Safety Authority, 2011). Also, the relationship between disinfection with chlorine and the presence of common use antibiotic resistance genes has been described because of co-selection factors (Khan *et al.,* 2016; Liu *et al.,* 2018). Although chlorine has been described as effective in the inactivation of *Campylobacter* in water, a previous study done by Northcutt *et al.* (2005) determines that the efficacy of this agent against *Campylobacter* present in poultry carcasses is low. Blackburn and McClure (2009) also report the resistance of these bacteria to 50 ppm concentrations. In regards to *Arcobacter,* a previous study of its prevalence in poultry carcasses in processing plants in Costa Rica suggests that the use of chlorine and peracetic acid does not warranty the complete removal of these microorganisms (Barboza *et al.,* 2017). Search for new and alternative decontaminating methods is an actual challenge for poultry industry, and organic acids represent a promising option since they are generally recognized as toxicologically safe (Hauser *et al.,* 2016) and have demonstrated to have an inhibitory effect against different bacteria including *Campylobacter jejuni* and *Arcobacterbutzleri* (Oyarzabal, 2000; Skřivanová, *et al.,* 2011). Results obtained in this study demonstrate the important antimicrobial activity of bromobenzoate, gallate and butanoate sodium salts against *Arcobacter* sp and of the first two salts against *Campylobacter* sp., suggesting that its incorporation as disinfecting and decontaminating agents in the production lines of poultry industry might be effective in the control of both bacteria, since the minimal inhibitory concentrations achieved are lower than the ones used for chlorine and peracetic acid. The caffeate and ascorbate sodium salts did not result in good candidates for their incorporation in poultry industry, since they required significantly high concentrations than other salts evaluated for exerting an important inhibitory effect over both bacteria. These results correlate with the ones reported by Cabezas-Pizarro *et al.,* (2016) in which they report that for the complete inhibition of the microorganisms evaluated they required concentrations greater than 100 µg/mL. *Arcobacter* showed a greater susceptibility than *Campylobacter* for all the salts tested, except for sodium octanoate. This difference has been described also by Skrivanová *et al.* (2011) nevertheless, there is no clear explanation for these differences. Inhibitory effect of decanoate, octanoate, butanoate and benzoate acidic salts over *Campylobacter* sp. has also been described by Molatová *et al.* (2010). Although there are methodological differences, results obtained in both studies correlate, demonstrating the capacity of these substances to inhibit pathogen's growth. Contrastively, data obtained disagree with results reported by Skrivanová *et al.* (2011) in which the inhibitory effect shown by butyric acid is lower than the one demonstrated by capric (decanoic) and caprylic (octanoic) acids. For *Arcobacter*, results obtained correlate partially with the proposed by Cabezas-Pizarro *et al.* (2016), that establish an inverse relationship between the number of carbon atoms present in the molecular structure and its inhibitory effect. In this study, sodium butanoate was the aliphatic salt with lower number of carbons in its structure tested, and presented the lower MIC for this bacteria. Nevertheless. Sodium decanoate presented a discordant result, since it has a MIC lower than octanoate. For *Campylobacter,* the three aliphatic salts tested presented the same MIC (625 µg/mL), a situation that suggests that this kind of salts has little affinity for the lipidic membrane of this bacteria, reason why they cannot penetrate it easily. The aromatic sodium carboxylates studied did not show an effectivity trend for the inhibition of both bacteria, what is obvious is that the addition of a bromide might confer a bigger inhibitory effect for these carboxylates. Results obtained in this study show that the addition of organic salts might be considered an important alternative for common use disinfecting agents, including chlorine, for the control of these bacteria on poultry industry. Further research might include the analysis of a synergetic use of both salts and chlorine in the control of pathogenic bacteria present in poultry in order to achieve a greater inhibition using the lowest possible concentrations of control agents.

Acknowledgement: This study was financed by the University of Costa Rica, through the project 803 B7 119 and Fundación UCR, project 217

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