Combinations of plant-derived compounds against *Campylobacter* in vitro

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Primary Audience: Quality Assurance Personnel and Food Safety Researchers

SUMMARY

Campylobacter occur in fresh retail poultry products as a result of their colonization of the gastro-intestinal tract of chickens during growth. Feed additives could be used for suppression of *Campylobacter* levels in the chickens prior to slaughter. To address this opportunity, feed manufacturers are targeting natural antimicrobials from plant material as new forms of consumer-accepted feed additives. However, to be practical, these natural antimicrobials must be effective at low concentrations. The current study has validated an improved laboratory method to study minimal inhibitory concentrations of plant compounds and their combinations against *Campylobacter*. The assay was shown to be valid for testing lipid-soluble and water-soluble plant extracts and byproducts from the food industry. The study screened 29 extracts or plant-derived compounds and their mixtures for anti-*Campylobacter* activity using a laboratory assay. Combinations of oregano, lactic acid, and sorghum byproduct showed effective synergy in anti-*Campylobacter* activity. The synergies allowed a large reduction in the concentration of the individual compounds needed to kill the bacteria with an 80% reduction in concentration being achieved for oregano essential oil. The assay gives rise to further opportunities for the testing of a greater range of combinations of plant-derived compounds and other natural antimicrobials. The method is robust, simple, and easily automated, and it could be used to adjust the cost of feed formulations by reducing costs associated with antimicrobial feed additives.

Key words: antimicrobial, plant extracts, essential oils, synergy, *Campylobacter*

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DESCRIPTION OF PROBLEM

Campylobacteriosis has been the most commonly reported bacterial-derived food-borne disease in developed countries during the last decade [1, 2] with a reported annual cost of 2.4 billion Euros in the European Union [3]. Chicken meat is the main source of infection in humans [4]. Consequently, the control of *Campylobacter* infections in poultry has become the main target to reduce the incidence of human campylobacteriosis [5–7]. The poultry industry has been trying to reduce *Campylobacter* colonization by improving biosecurity measures in production sites to avoid contamination of production facilities and cross contamination by infected birds [8, 9]. In addition, research related

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to the development of vaccines [10–12], specific bacteriophages [13] and bacteriocins [14, 15] targeting *Campylobacter* colonization in commercial avian species has advanced significantly. New feed additives could also be part of the solution, but consumers have concerns regarding the use of synthetic additives in animal feed and their potential residues in meat, leading to pressure for the development and use of natural additives [16, 17]. Many essential oils and plant extracts are generally recognized as safe (www.accessdata.fda.gov) or the equivalent classification in different countries and can be used in foods. In addition, plant byproducts of the food industry, including those resulting from microbial fermentation, can have good antimicrobial activity and potential as natural alternatives to synthetic antimicrobials [18]. In recent years numerous studies have shown that *Campylobacter* species are sensitive to a wide variety of plant extracts (e.g., *Acacia decurrens*, *Alpinia katsumada*, basil, capsicum, cinnamon bark, clove, garlic, laurel, lemon, lemon grass, lemon myrtle, mandarin, bitter and sweet orange, oregano, rosemary, sage, and thyme), plant-derived compounds (e.g., anethole, carvacrol, cinnamaldehyde, citral, curcumin, eugenol, thymol, and vanillin) [19–31]. The mechanism by which this toxicity occurs in bacteria is still unclear. Recently, Nowotarska et al. [32], using model membranes, were able to associate the phenolic group of carvacrol with the penetration of the compound into the lipid molecules of the bacterial membrane. With *Campylobacter*, carvacrol has been linked to a reduction in motility of the bacterium, with a consequent reduction of its infectivity [23]. In other pathogens like *Bacillus cereus*, *Staphyloccocus aureus*, and *Pseudomonas aeruginosa*, it has been shown that carvacrol and oregano affect the essential metabolic process in the cell which leads to death [33–34].

Sorghum byproducts available from the bioethanol industry in Australia are available on a large-scale [35]. In this industrial process, sorghum is fermented using the yeast *Saccharomyces cerevisiae* to produce ethanol. Sorghum syrup is a byproduct of this extraction process and contains bioactive compounds. Other fermented products from sorghum, like porridge, are reported to exhibit higher antimicrobial activity than the original nonfermented extract although the content of most phenolic acids decreased after fermentation [36–37].

However, as showed in Table 1, it is difficult to compare the antimicrobial results of plant-derived compounds and organic acids between publications [19, 23–27, 38–40], because different techniques have been used to determine the minimum inhibitory concentration (**MIC**).

Disk diffusion antimicrobial assays have been widely used, but are laborious and the solubility and diffusion of the substances can be variable and affect the results [41]. The use of methods based on microdilutions has been shown to be more appropriate for the determination of MIC values [41]. Broth dilution assay using 96-well microtiter plates have been applied to detect the antimicrobial activity of many substances [42–44]. The microplate assays are simple, rapid, reproducible, and inexpensive, and can be automated to screen a large number of samples and bacterial strains [45]. However, studies on the use of microplate assays to study anti-*Campylobacter* activity of plant-derived compounds are limited. The objective of this study was to adapt and validate a 96 microwell assay to measure the anti-*Campylobacter* activity of both lipid-soluble and water-soluble natural plant-derived substances in one universal assay format. The ability to compare and combine different types of compounds and extracts was then used to study combinations of samples to look for synergies that could lower the overall concentrations and costs of active compounds needed to achieve microbial activity in poultry feed.

MATERIALS AND METHODS

Microplate Assay for Campylobacter

Anti-*Campylobacter* activities were tested in a 96-well microplate assay system based on the work of Bishop-Hurley et al. [46]. The assay determines the MIC values and correlates the concentration of the substances tested with the degree of inhibition of bacterial growth.

In order to screen a variety of plant extracts and essential oils that vary in their solvent solubilities, specific adaptations to the method were developed following the procedures of Sultanbawa et al. [47]. To explore the interactions and

Table 1. Antimicrobial effects of essential oil, pure compounds, and organic acids on *Campylobacter* spp. reported in the literature.

Essential Oils, Plant-Derived Compounds, and Organic Acids	Anti-*Campylobacter* Activity Reported (%)[1]	References
Cinnamon bark	MIC 0.05	[27]
	MIC 0.42 to 1	[23]
	BA50 0.021	[19]
Clove bud oil	MIC 0.05	[27]
	MIC 0.06 to 0.25	[23]
	BA50 0.016	[19]
Garlic	MIC >1	[27]
	MIC 0.25 to 1	[23]
Lemon	BA50 0.045	[19]
	MIC >4	[25]
Lemon grass	BA50 0.018	[19]
	MIC 0.42 to 1.33	[23]
Oregano	MIC 0.002 to 0.125	[26]
	MIC 0.0066	[24]
	BA50 0.01	[19]
	MIC 0.001 to 0.07	[31]
	MIC 0.2	[30]
Rosemary	MIC 0.5	[27]
	BA50 0.06	[19]
Sage	MIC >1	[27]
	MIC 0.5	[44]
Thyme	MIC 0.04	[27]
	BA50 0.022	[19]
Eugenol	MIC 0.12	[38]
	BA50 0.022	[19]
Carvacrol	MIC 0.2	[30]
	MIC 0.12	[38]
	MIC 0.006	[28]
Cinnamaldehyde	BA50 0.0028	[19]
	MIC 0.2	[30]
Thymol	BA50 0.024	[19]
	MIC 0.47	[38]
Benzoic	BA50 >0.67	[40]
	MIC 0.38	[38]
	IC50 0.7 to 2.5	[39]
Citric	MIC 19.2	[38]
	IC50 0.23 to 0.57	[39]
Lactic	MIC >9	[38]
	IC50 0.9 to 4.6	[39]
Malic	MIC 3.35	[38]
	IC50 0.1 to 0.6	[39]

[1]MIC = Lowest concentration of the compound resulting in 100% inhibition of bacterial growth. BA50 defined as 50% decrease in the number of cfu. IC50 defined as concentration that decreased bacterial DNA synthesis to 50%.

determine the possible synergies between the natural substances, a central composite design [48] was used to generate the checkerboard table of possible combinations.

Bacteria

Eleven strains of *Campylobacter* [8 *Campylobacter jejuni* (1016, 1065, 1110, 1119, 1122,

1132, 1201, and 1209) and 3 *Campylobacter coli* (C1951, C1954, C1955)], isolated from chicken fecal droppings collected from 2 commercial Australian poultry producers, were used to validate the assay. These strains were stored on cryo-beads in a −80°C freezer. For every experiment, one bead was aseptically transferred to a blood agar plate [49] supplemented with 7% defibrinated sheep blood [50]. The plates were prepared with defibrinated sheep blood before each experiment and stored in the refrigerator. The plates were incubated at 42°C under 5% CO_2 [51–52] and 95% air, atmosphere provided within a CB150 incubator [53]. This capnophile condition allows the growth of *Campylobacter* and has been extensively utilized in our laboratory. At 24 h, an inoculum of the culture was recovered with a sterile loop and placed in Nutrient Broth No. 2 [54] enriched with 0.4% *Campylobacter* growth supplement [55]. The inoculum was resuspended using the same enriched nutrient broth to an optical density of 0.2 A at 595 nm wavelength [56]. The culture was further diluted to obtain 10^4 to 10^5 cfu/mL, using the same supplemented nutrient broth. To confirm the bacterial count, an aliquot of this diluted culture was further diluted and plated onto sheep blood agar. The inoculum was used in the 96-well plate assay. Each compound/strain was replicated 9 times.

Essential Oils, Plant-Derived Extracts, and Organic Acids

The compounds used to develop and validate the assay against *Campylobacter* strains are listed in Tables 2 and 3. All the essential oils and lipid-soluble compounds (carvacrol, citral, eugenol, cinnamaldehyde, and anethole) were diluted using a water solution (0.2% wt/vol) of agar [57]. The sloppy agar solution provides a stable homogeneous dispersion of the oils. The agar was prepared, autoclaved, and cooled at room temperature to form a sloppy agar. Thymol powder was presolubilized with a 10% (vol/vol) ethanol solution before dilution in the agar solution. The water-soluble compounds (organic acids and sorghum syrup) were diluted in sterile MilliQ-water [58]. The sorghum syrup was obtained as a byproduct from Dalby Bio-Refinery Ltd [59]. To extract active compounds from sorghum syrup by-product, accelerated solvent extraction was performed using the Dionex ASE 200 [60] system. Aliquots of 1.0 g freeze-dried sorghum syrup was mixed with diatomaceous earth and placed in a 10-mL stainless steel extraction cell, fitted with a 27-mm cell filter in the bottom end. The use of a dispersion agent, such as diatomaceous earth, is recommended to reduce the solvent volume used for the extraction. Five different extraction solvents were used: acetone, ethanol, hexane, methanol (each at a concentration of 100% wt/wt, analytical grade), and distilled water. The cells containing samples were prefilled with the extraction solvents, pressurized, heated (preheating period = 5 min), and subjected to 6 extraction cycles, at 60°C (for acetone, ethanol, hexane, and methanol) or 80°C (for water) and 1,000 psi. The cells were rinsed with fresh extraction solvent (60% extraction cell volume) and purged with a flow of nitrogen (150 psi during 90 s). The extract was collected into 60-mL amber glass vials. The collected extracts were concentrated in a miVac sample concentrator [61] at 45°C. The concentrated extracts were weighed and stored at −20°C until use.

Accelerated solvent extraction extracts listed in Table 3 were resuspended in 10% (vol/vol) ethanol and the sorghum concentrate was solubilized with 10% Dimethylsulfoxide solution. All the dilutions were homogenized for 3 min at 3,000 rpm using a vortex mixer [62]. Two-fold serial dilutions of the treatments were prepared to determine the MIC inhibition curves (Tables 4 and 5). The compounds with the lowest MIC values were then selected and retested in isolation as well as combined at different concentrations to assess potential synergies (Table 6).

Determination of the MIC and Fractional Inhibitory Concentrations

The screening assay was carried out using flat bottom 96-well sterile microtiter plates with lids [63] to prevent cross contamination. The upper and bottom rows of wells in each plate contained the sterilized medium (negative control) and the *Campylobacter* strains without inhibitory substances (positive control), respectively. Intermediate rows contained the different concentrations of the plant compounds and their combinations

Table 2. Essential oils, pure compounds, and organic acids used in this study.

Essential Oils of Plants	Essential Oil Main Component
Anise myrtle (*Syzygium anisatum*)[1]	Anethole
Blue gum (*Eucalyptus globulus*)[3]	1,8 Cineole
Blue mallee (*Eucalyptus polybractea*) [3]	1,8 Cineole
Cinnamon bark (*Cinnamommum zeylanicum*)[2]	Cinnamaldehyde
Clove bud oil (*Eugenia caryophyllate*)[2]	Eugenol
Garlic (*Allium sativum*)[2]	Allicin
Grapefruit (*Citrus paradisi*)[2]	Limonene
Lemon (*Citrus limonum*)[2]	Limonene
Lemon grass (*Cymbopogon citratus*)[2]	Citral
Lemon myrtle (*Backhousia citriodora*)[1]	Citral
Mandarin (*Citrus reticulata*)[2]	Limonene
Narrow-leaved peppermint (*Eucalyptus radiata*)[3]	1,8 Cineole
Niaouli (*Melaleuca quinquenervia ct*)[3]	1,8 Cineole
Oregano (*Origanum vulgare*)[2]	Carvacrol
Bitter orange (*Citrus aurantium spp amara*)[2]	Limonene
Navel orange (*Citrus sinensis*)[2]	Limonene
Rosemary (*Rosmarinus officinalis*)[2]	Camphor/1,8 cineole
Sage (*Salvia lavandulifolia*)[2]	Camphor/α thujone
Tasmanian native pepper (*Tasmannia lanceolata*)[4]	Eugenol
Thyme (*Thymus vulgaris*)[2]	Thymol
Pure compounds[5]	
Anethole	
Eugenol	
Carvacrol	
Cinnamaldehyde	
Citral	
Thymol	
Organic acids[5]	
Benzoic	
Citric	
Formic	
Lactic	
Malic	
Tartaric	

[1]Essential oils supplied by Byron Bay Essential Oils Inc., Tyagarah, NSW 2481, Australia.

[2]Australian Botanical Company, Hallam VIC 3803 Australia.

[3]Essentially Australia, Byron Bay, NSW 2481 Australia.

[4]Essential Oils of Tasmania Pty Ltd, Kingston, TAS 7051, Australia.

[5]Sigma–Aldrich, Castle Hill, NSW 1765, Australia.

Table 3. Description of the byproducts of sorghum bio-ethanol production used in this study.

Sorghum Byproducts	Total Polyphenols (Gallic Acid Equivalents, mg/L)
Sorghum syrup[1]	1,756
Sorghum syrup concentrated	Not measured
Solvent extraction of sorghum syrup[2]	
Water[2]	1,010
Methanol[2]	579
Ethanol[2]	307
Acetone[2]	55
Hexane[2]	3

[1]Sorghum syrup samples were a gift from Dalby Bio-refinery Ltd, Dalby, QLD, Australia.

[2]Accelerate solvent extraction method.

Table 4. Minimum inhibitory concentration of essential oils, pure compounds, and organic acids against *Campylobacter* spp[1].

Essential Oils	MIC (%, vol/vol)	Inhibition (%)	Essential Oils	MIC (%, vol/vol)	Inhibition (%)
Anise myrtle	0.125	132 ± 16	Tasmanian native pepper leaf	0.012	103 ± 6
Blue gum	0.2	102 ± 4	Thyme	0.006	102 ± 4
Blue mallee	0.2	101 ± 2			
Cinnamon bark	0.050	101 ± 11	Pure Compounds		
Clove bud oil	0.020	108 ± 3	Anethole	0.030	103 ± 14
Garlic	0.050	103 ± 9	Eugenol	0.020	101 ± 6
Grapefruit	0.25	115 ± 15	Carvacrol	0.004	101 ± 8
Lemon	0.25	108 ± 12	Cinnamaldehyde	0.050	100 ± 8
Lemon grass	0.125	107 ± 3	Citral	0.010	103 ± 12
Lemon myrtle	0.012	103 ± 13	Thymol	0.006	102 ± 5
Mandarin	0.25	109 ± 18			
Narrow-leaved peppermint	0.1	102 ± 5	Organic Acids[2]		
Niaouli	0.1	107 ± 4	Benzoic	0.060	100 ± 0.3
Oregano	0.0037	103 ± 2	Citric	0.050	102 ± 8
Bitter orange	0.25	116 ± 17	Formic	0.025	101 ± 1
Navel orange	0.25	119 ± 15	Lactic	0.050	103 ± 3
Rosemary	0.015	103 ± 8	Malic	0.050	101 ± 1
Sage	0.150	106 ± 9	Tartaric	0.050	101 ± 0.4

[1]The MIC values were obtained against 8 strains of *Campylobacter jejuni* (1016, 1065, 1110, 1119, 1122, 1132, 1201, and 1209) and 3 strains of *Campylobacter coli* (C1951, C1954, and C1955) using the broth microdilution assay (n = 9 for every strain and every treatment).

[2]The pH of the dilution of organic acids ranged from 4.83 to 5.72.

Table 5. Minimum inhibitory concentration of sorghum syrup and sorghum syrup concentrate against *Campylobacter* spp.

Sorghum Byproducts[1]	MIC (%, vol/vol)	Inhibition (%)[3]
Sorghum syrup	1	105 ± 3
Sorghum syrup concentrated	0.25	100 ± 4
Solvent extraction of sorghum syrup[2]		
Water	4	100 ± 0.5
Methanol	4	100 ± 2
Acetone	>4	ND
Hexane	>4	ND
Ethanol	>4	ND

[1]*Campylobacter jejuni* (1016, 1065, 1110, 1119, 1122, 1132, 1201, and 1209) and *Campylobacter coli* (C1951, C1954, and C1955) using the broth microdilution assay (n = 9).

[2]*Campylobacter jejuni* 1016 and 1065 and *Campylobacter coli* 1955 using the broth microdilution assay (n = 9).

[3]ND = Not determined.

under study. Every treatment was replicated 3 times within a plate and every plate, in turn, was also replicated 3 times.

Each test well contained 50 μL treatment solution, 100 μL nutrient broth, and 50 μL inoculum. Each negative or sterile control well contained 200 μL nutrient broth alone. Each positive control well contained 50 μL bacterial inoculum and 150 μL nutrient broth. The treatment was placed first in the wells followed by the nutrient broth. The bacteria were added at the end of the procedure.

The plates were shaken for 1 min with a microplate shaker Titertek [64] immediately before determining the initial optical density of the wells measured at 595 nm wavelength (**OD_{595}**) by light spectrophotometry [65]. The OD_{595} was measured again using the same protocol at 24

Table 6. Evaluation of the interactions of oregano with other plant-derived compounds and organic acids against *Campylobacter jejuni* 1016, 1065, 1119, and 1132, and *Campylobacter coli* 1955.

Oregano Essential Oil Combinations	Lowest Oregano Concentration (a)[1] in Combination with Other Substances (b/c)[2,3] That Results in MIC (%) n = 9 against *Campylobacter* spp.	Inhibition (%)	FICa[4,5]	FICb[4,6]	FICc[4,7]	FIC Index[8]	Interpretation
Oregano (a)[1] Plus Other Essential Oil (b)[2]							
Oregano/thyme	0.0010/0.001	104 ± 2	0.25	0.16	NA	0.40	Synergy
Oregano/cinnamon bark	0.0010/0.007	104 ± 3	0.25	0.15	NA	0.40	Synergy
Oregano/ lemon myrtle	0.0015/0.037	107 ± 7	0.42	0.75	NA	1.17	Additive
Oregano/Tasmanian native pepper leaf	0.0015/0.037	112 ± 10	0.42	0.75	NA	1.17	Additive
Oregano/clove bud	0.0015/0.015	101 ± 0.1	0.42	0.75	NA	1.17	Additive
Oregano/orange	0.0015/0.019	101 ± 1	0.42	0.79	NA	1.18	Additive
Oregano/grapefruit	0.0015/0.015	101 ± 2	0.42	0.60	NA	1.02	Additive
Oregano (a)[1] Plus Organic Acid (b)[2,4]							
Oregano/formic acid	0.0008/0.007	100 ± 1	0.20	0.28	NA	0.48	Synergy
Oregano/citric acid	0.0020/0.035	101 ± 1	0.65	0.70	NA	1,35	Indifferent
Oregano/lactic acid	0.0015/0.020	102 ± 2	0.30	0.40	NA	0.70	Additive
Sorghum Syrup (b)[2]							
Oregano/sorghum syrup	0.001/0.300	111 ± 6	0.25	0.30	NA	0.55	Additive
Oregano (a) Plus Sorghum Syrup (b) Plus Organic Acid (c)[9]							
Oregano/sorghum syrup/lactic acid	0.0005/0.150/0.010	107 ± 12	0.12	0.15	0.20	0.47	Synergy
Oregano/sorghum syrup/formic acid	0.0005/0.150/0.010	102 ± 7	0.12	0.15	0.59	0.86	Additive
Oregano/sorghum syrup/citric acid	0.001/0.250/0.020	108 ± 8	0.27	0.25	0.40	0.92	Additive

[1]Letter a represents the oregano in the combination.

[2]Letter b represents the second compound in the combination.

[3]Letter c represents the third compound in the combination.

[4]The pH of the solution ranged from 5.70 to 6.71.

[5]FICa = MICa combination/MICa alone.

[6]FICb = MICb combination/MICb alone.

[7]FICc = MICc combination/MICc alone. NA = Not applicable.

[8]FICindex = FICa + FICb + FICc.

[9]The pH of the solution ranged from 6.12 to 6.88.

and 48 h of incubation in capnophile conditions (5% CO_2, 42°C).

The growth inhibitory capacities of the compounds tested were calculated using the following formula: percentage of inhibition = [1−(T48 OD_{595} − T0 OD_{595})/(C48 OD_{595} − C0 OD_{595})] × 100, where T0 and T48 refer to the treatment wells at the start and 48 h respectively, whereas C0 and C48 refers to positive control wells at the start and 48 h, respectively. The MIC was defined for each compound as the lowest concentration of the compound resulting in 100% inhibition of bacterial growth. To confirm complete inhibition, an inoculum of the tested well (100 μL) was plated in sheep blood agar and incubated for 48 h in microaerophilic conditions described above. No growth during 48 h confirmed complete inhibition.

Interactions between the natural antimicrobials were evaluated by the checkerboard method [66]. The fractional inhibitory concentration (**FIC**) index of each combination was calculated based on the MIC values of the compound in combination relative to the MIC value of the compound alone (FIC of compound "a" is referred to as FICa = MICa combination/MICa alone). The FIC index of the combination is obtained following the formula: FICab = FICa + FICb [67]. Based on the FIC values the combined effects of the plant compounds were categorized as either synergy or, addition or indifference or antagonism according to the following scale: FIC ≤ 0.5, synergy; FIC = 0.51 to 1, additive; FIC = 1.01 to 2, indifference; FIC > 2, antagonism.

The results were analyzed for statistical differences using ANOVA and Fisher's LSD procedure of XLSTAT [68] at a 5% significance level. The CV was calculated and it was less than 10% for all the calculations where 9 replicates were done for each treatment against the selected *Campylobacter* strains.

RESULTS AND DISCUSSION

The main focus of the study was the study of natural antimicrobials against *Campylobacter* spp. to determine potential usefulness as active compounds to reduce gut colonization in the chicken. Sixteen essential oils, 7 pure essential oil compounds, 6 organic acids, and 7 sorghum industry byproducts were tested against 11 *Campylobacter* strains using flat bottom 96-well sterile microtiter plates. Levels of phenolic compounds were also obtained in the sorghum syrup and its extractions (Table 3). All tested compounds showed a dose-dependent response against *Campylobacter* indicating higher the dose tested the higher the inhibition. However, the MIC was independent of the strain of *Campylobacter jejuni* or *Campylobacter coli* used ($P > 0.005$). The MIC values of each product tested are given in Tables 4 and 5. Oregano and thyme essential oils resulted in the highest anti-*Campylobacter* activity (MIC of 0.0038 and 0.006%, respectively) only matched by their main active components carvacrol for oregano and thymol for thyme. Very low anti-*Campylobacter* MIC values below 0.02% were also identified for citral, eugenol, lemon myrtle, rosemary, and Tasmanian native pepper leaf. A group of compounds from clove, anethole, cinnamon bark, garlic, and lemon myrtle as well as cinnamaldehyde showed middle range MIC values between 0.020 and 0.05%. The essential oils of anise myrtle, blue gum, blue mallee, lemon grass, lemon, mandarin, narrow-leaved peppermint, niaouli, sage, grapefruit, bitter orange, and navel orange all had MIC values between 0.125 and 0.25%. The most active organic acid against *Campylobacter* was formic acid, with an MIC of 0.025%. The MIC value for citric, lactic, malic, and tartaric acids was 0.05%, while that for benzoic acid was 0.06%. The pH range for these organic acids was in the range 4.83 to 5.72. At acidic pH most of the organic acid would be in the undissociated form, which is lipophilic and enables the diffusion of the acid through the bacterial cell membrane [69]. This clearly indicates that growth inhibition of *Campylobacter* in this study is due to the low pH and not the organic acid. Seven sorghum byproducts from bioethanol production were also active against *Campylobacter* with MIC values ranging from 0.25% for the rotatory concentrated sorghum syrup up to 4% for the methanol and water extractions.

As oregano essential oil showed the strongest anti-*Campylobacter* activity (MIC 0.0038%), it was retested at a wide range of concentrations. Oregano showed some activity from 0.0005%

level with increased inhibition of *Campylobacter* growth up to 0.0038% that showed 100% inhibition in all the *Campylobacter* strains tested in this assay.

The effectiveness of synergistic interaction of plant-derived antimicrobials has been known for a long time [17, 70], and is based on the principle that the combinations of active compounds enhances the efficacy, while reducing the toxicity and quantity of substance in the final formula. However, existing literature addressing interactions between plant-derived antimicrobial compounds is scattered and difficult to interpret [71]. In addition, there is very little comprehensive information available regarding plant-derived compound synergies against *Campylobacter*. The final goal of this study was to determine combinations of compounds showing the strongest synergies at the lowest concentrations possible. Oregano had the lowest MIC values against the *Campylobacter* strains tested and was selected as the base compound to test binary and ternary combinations. The results presented in Table 6 are expressed using the FIC index previously described in the Materials and Methods section. The binary combination of oregano with thyme and oregano with cinnamon bark resulted in a synergistic increase of MIC efficacy for both compounds (Table 6). Combinations of oregano with lemon myrtle, Tasmanian native pepper leaf, clove bud, orange, or grapefruit only showed additive effects. Synergy effects were also uncovered for binary combinations of oregano with formic acid, while additives effects were revealed for binary combination with lactic acid. There was a slight synergistic interaction between oregano and the sorghum syrup byproduct with an FIC index of 0.55, which was slightly over the synergy threshold value of 0.5. Although formic acid showed the best results, lactic acid was studied in tertiary combinations, because of its common occurrence and antimicrobial use in foods. Lactic acid shows high activity against *Campylobacter* and the ability to inhibit the growth of many other Gram–negative species of the *Enterobacteriaceae* bacteria [72]. In addition to its antimicrobial effect by lowering of the pH, lactic acid also acts as a permeabilizer of the Gram-negative bacterial outer membrane [73]. Lactic acid is related to the development of nonpathogenic competitive species such as *Lactobacillus* [14], which are one of the predominant bacterial populations in the chicken ceca [74]. When lactic acid was added to the formula in a ternary combination, i.e., oregano, sorghum syrup, and lactic acid, it showed significant synergy in antimicrobial activity at the concentration of 0.0005, 0.015, and 0.01%, respectively.

Oregano (*Origanum vulgare*) essential oil is one of the most studied essential oils exhibiting antibacterial activity against a broad range of bacteria, both Gram-negative and Gram-positive[19], and recently has been shown to have antivirus properties as well [75]. Although the antimicrobial mechanism is still unclear, it has been speculated that the lipophilic character of the main oregano constituents (carvacrol, thymol, and *p*-cymene) interacts with the lipid membranes of the bacteria [76], and may produce a bacterial destabilization [33, 77].

To the best of our knowledge, this is the first time that a significant antimicrobial activity has been reported in sorghum syrup a byproduct from large-scale bio-ethanol production.

Svensson et al. [36] reported on the conversion of phenolic acid present in the sorghum grain to flavonoid aglycones during fermentation, of sorghum porridges, which could compensate for the decrease in phenolic acid content and increase in the antimicrobial activity.

Recently, Borges et al. [37] have verified that phenolic acids from sorghum, changed bacterial hydrophobicity, affecting the physicochemical surface properties of bacterial cells [37]. These authors hypothesize that the antibacterial activity of phenolic acids is associated with both the affinity for the lipid bilayer and the disruption of the membrane. We suggest that the combination of lipophilic antimicrobial compounds from oregano essential oil and hydrophilic compounds from sorghum syrup can affect the integrity of *Campylobacter* membrane in a complementary way. Lactic acid in the plant-derived combination may complement the antimicrobial effect in the destabilization of the outer membrane of *Campylobacter* and potentiate the lethality by acidifying the cell cytoplasm.

CONCLUSIONS AND APPLICATIONS

The 96-well microplate assay in this study was used to test combinations of oregano with organic acid and other plant-derived byproducts like sorghum syrup from bio-ethanol production. New plant-derived antimicrobials and their combinations against *Campylobacter* could also be screened using this assay. This could lead to the development of potent cost-effective combinations that can be used to reduce *Campylobacter* colonization in chickens.

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