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Modification of *Campylobacter jejuni* Broiler Colonization by a Feed Additive Composed of Encapsulated Organic Acids and Essential Oils

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Abstract: The objective of this study was to evaluate the effect of a novel feed additive on chicken intestinal colonization and carcass contamination by *Campylobacter jejuni*. The feed additive was composed of microencapsulated organic acids and essential oils (OA/EO). The feed additive tested was provided by Jefo Nutrition Inc., St-Hyacinthe, Quebec, Canada. Day-old birds were separated into two rooms and subdivided into two groups. Chicken were fed with OA/EO or not fed with OA/EO until they reached 35 d of age. At 14 d of age, chickens received an oral suspension of two well characterized *C. jejuni* strains, depending on the room they were housed in. The levels of *C. jejuni* were periodically monitored in the caecum and on the carcasses. *C. jejuni* colonization was further characterized by the use of high-resolution melt analysis of the *C. jejuni fla*A gene (HRM-*fla*A). The effect of the feed additive was strain-dependent. In room two, the feed additive had no effect on the caecal counts. In room one, at 35 d of age, caecal *C. jejuni* counts were higher with OA/EO, as opposed to carcasses counts which were lower in the treated group. The HRM-*fla*A analysis showed that an amplification profile was predominant in birds fed with OA/EO at 35 d of age in room one, suggesting the selection of a *C. jejuni* strain. In conclusion, the OA/EO seemed to be effective to reduce *C. jejuni* levels but this effect appeared strain dependent.

Key words: *C. jejuni* control, feed additive, HRM, organic acids and essential oils, chicken colonization.

1. Introduction

One of the most important worldwide foodborne bacterial pathogen is *Campylobacter jejuni* [1]. It causes campylobacteriosis in humans, a gastrointestinal disease that may lead to serious autoimmune disorders like the Guillain-Barré syndrome [2]. Consumption and inappropriate handling of contaminated poultry meat products have been identified as major sources of *C. jejuni* for humans [3]. One way to reduce human exposure to *C. jejuni* is by controlling the pathogen at the farm level [4]. It is estimated that the fecal matter of a colonized chicken contains approximately 10^6 CFU/g of *C. jejuni* [5].

Great *C. jejuni* genetic diversity is present in the chicken flocks, making it difficult to implement comprehensive and effective control measures. First, chicken farms may be exposed to environmental *C. jejuni* where high diversity is observed [6] leading to different farms being colonized by unrelated *C. jejuni* strains [7]. On the other hand, on the same farm, isolated strains are often highly clonal [7], probably as a result of strains competing for the same ecological

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niche [8, 9].

Strategies used on the farms could also have an impact of diversity during processing. It is known that carcass *C. jejuni* genetic diversity is higher than caecal diversity [10, 11]. This probably results from the cross-contamination between slaughtered flocks [10, 11] and processing-related selection [12].

Numerous control strategies have been tested to reduce *C. jejuni* colonization in poultry, including the maintenance of high farm biosecurity, vaccination, phage therapy, competitive exclusion microflora and the use of feed additives [13]. Interest in feed additives, especially non-antibiotic ones, is increasing as they represent an alternative to the antibiotics used in the poultry industry to promote health and birds productivity. Also, the use of antibiotic agents for non-therapeutic purposes was stopped in some countries [14]. Some non-antibiotic feed additives, such as organic acids and essential oils, were already tested for their action on *C. jejuni*, showing varying efficacy. Essential oil extracts, such as *Eucalyptus occidentalis*, Valencia orange and cinnamon, are very potent against *C. jejuni in vitro* [15, 16]. Trans-cinnamaldehyde, eugenol, carvacrol and thymol were also found effective at various concentrations even in the presence of caecal matter [17]. On the other hand, essential oils active *in vitro* may lose their efficacy *in vivo*, as was shown with encapsulated trans-cinnamaldehyde [18]. Organic acids such as caprylic acid, formic acid, sorbate, lactic and acetic acid are bactericidal against *C. jejuni in vitro* and are also effective at some degree *in vivo* [19-22]. It was also shown that the combination of formic acid and sorbate could prevent *C. jejuni* chicken colonization whereas their separate use could not [21]. That being said, the relative capacity to reduce *C. jejuni* chicken colonization using organic acids is still debated. Therefore, the variable efficacy of organic acids and essential oils as feed additives commands more research to confirm their individual or combined effects on *C. jejuni* chicken colonization. These studies should also take into account the strain diversities encountered by chickens during industrial rearing to limit interpretations based on single-strain trials.

This study was meant to periodically quantify the effect of a new feed additive, composed mostly of microencapsulated sorbic acid and phenolic essential oils (OA/EO), both on the caecal colonization and carcass contamination. Also, for the first time, this study used the high-resolution melting analysis of the *C. jejuni fla*A gene (HRM-*fla*A) to follow specific strains colonization throughout the study.

2. Materials and Methods

2.1 Experimental Design

All animal experiments were approved by the Ethics Committee (CEUA) of the Faculty of Veterinary Medicine of the University of Montreal. The birds (200 female Ross chickens), purchased from a local hatchery, were raised on wood shavings in floored pens. The chickens were given water and fed with *ad libitum*. Upon reception, the day-old birds were divided into two rooms containing 100 chickens each and designated to receive different *C. jejuni* strains. The 100 chickens were then subdivided into two groups in each room. From the beginning of the trial to the end, for each room, one chicken group received a standard commercial feed (control group, $n = 50$ birds) and the others received the same feed but were supplemented with the experimental microencapsulated organic acid and phenolic essential oil blend (mainly constituted of fumaric acid, sorbic acid and thymol) at a concentration of 500 ppm (OA/EO group, $n = 50$ birds) and mixed manually at the avian research center (CRA). For all groups, a standard starter diet composing of 18% protein, 3.85% fiber, 2.84% fat, 0.83% calcium, 0.61% phosphorous, 0.19% sodium, vitamin A 6,238 UI/kg, vitamin D_3 2,275 UI/kg and vitamin E 20 UI/kg was used from 0 d to 21 d of age, and a finishing diet composing of 15% protein, 5.32% fiber, 3.82% fat, 0.81% calcium,

0.55% phosphorous, 0.15% sodium, vitamin A 3,900 UI/kg, vitamin D3 1,425 UI/kg and vitamin E 13 UI/kg was used for the remainder of the study.

Two days prior to the *C. jejuni* inoculation, fresh caecal droppings and feed samples were tested to confirm the absence of *C. jejuni*. At 14 d of age, each chicken was orally inoculated with two different *C. jejuni* strains simultaneously. The birds received a suspension containing approximately 10^5 CFU of each strain, depending on the room they were housed in (Table 1). These strains possessed different phenotypic properties and were originated from distinct broiler farms (Table 1). The strains were characterized in a previous study for their autoagglutination, chemotaxis and epithelial cell adhesion/invasion properties. The inoculating suspension was obtained as follows: a -80 °C frozen aliquot was cultured on mCCDA (Innovation Diagnostic Inc., Montreal, Canada,) for 24 h at 42 °C in a microaerobic atmosphere, using the *Campylobacter* gas generation kit (Oxoïd, Nepean, Ontario, Canada). The strains were then transferred onto tryptic soy agar (TSA) containing 5% (v/v) defibrinated sheep's blood (Fisher sci., Ottawa, Ontario, Canada). Each strain was suspended in 1 mL of trypton salt (TS) (Innovation Diagnostic Inc.) to obtain an optic density (630 nm) of 1.0, corresponding to approximately 10^8 CFU/mL. This suspension was further diluted to approximately 10^5 CFU/mL and then mixed with an equal volume of the second strain to form the final inoculation suspension (Table 1). All suspensions were enumerated by culture on Mueller-Hinton Agar (Innovation Diagnostic Inc.).

On day 7, 14 and 21 post-inoculation (PI), the chickens were euthanized and processed in the experimental facility using procedures similar to commercial poultry production. For each group, small subgroups of four birds were stunned using electronarcosis, euthanized by bleeding, scalded (Syri Ltd., Saint-Félix-de-Valois, QC, CA) at 60 °C for 110 s, mechanically plucked (Syri Ltd.) for 40 s to 60 s and manually eviscerated. Immediately after the evisceration, the caeca, liver and spleen were individually collected for microbial analysis. The whole carcasses were then rinsed in 400 mL of peptone buffered water (Canadian Food Inspection Agency, Meat Hygiene Manual Procedure, Chapter 11, Appendix U). All samples were kept on ice and processed within 4 h to 6 h.

2.2 Sample Processing

All *C. jejuni* incubations were conducted in a microaerobic atmosphere at 42 °C. Detection of *C. jejuni* from three samples of 50 g of starter feed and grower feed and three samples of 10 g of pooled fresh droppings recovered from each group before the bird inoculation, was done using *Campylobacter* Enrichment Broth (Innovation Diagnostic Inc.) at a concentration of 1:9 (w/v), followed by mCCDA inoculation. After the incubation, the absence of typical

Table 1 Phenotypical properties of the strains used in the chicken colonization model.

Strain	Autoagglutination	Chemotaxis	Adhesion	Invasion	Inoculation dose (log CFU/mL)	Room
Strain 1	65	0.493	0.406	0.409	4.7	1
Strain 2	78	0.476	0.500	0.277	4.4	
Strain 3	62	0.411	0.222	0.247	5.6	2
Strain 4	63	0.115	0.244	0.211	4.2	

Autoagglutination (optic density 630 nm after 3 h of incubation at room temperature/optic density 630 nm initial bacterial suspension × 100); adhesion, invasion and chemotaxis: -1/log (recovered bacteria after test/initial bacteria). Strains 1 and 2 were co-administered to all chickens in room one; strains 3 and 4 were co-administered to all chickens in room two. The strains were originated from distinct broiler farms. Strain 1 and strain 2 were in our strain collection, strains possessing higher overall phenotypic properties whereas strain 3 and 4 possessed lower phenotypic properties.

colonies was verified on the agar.

Samples from euthanized birds were processed for *C. jejuni* enumeration and the total aerobic bacteria in the caeca or post-evisceration carcasses. For each bird, the caecal content (1 g) and carcass rinses were diluted in TS and plated on CASA agar (Innovation Diagnostic Inc.) for *C. jejuni* enumeration [23]. The first caecal content TS dilutions were supplemented with 10% (v/v) glycerol and aliquots were kept at -80 °C for total DNA extraction. Presence of *C. jejuni* in the organs was also investigated for one out of two birds. The liver (10 g) and spleen (whole) were dipped in 70% ethanol for 15 s, flamed and then homogenized in TS (1:9) with a stomacher for 30 s. Each organ suspension was then directly plated on CASA agar. Organs were considered positive if at least one typical *Campylobacter* colony was detected for the spleen or liver sample. Half of the carcass rinses were also plated on total aerobic bacteria Pétrifilm (3M, Saint-Paul, Minnesota, USA) and incubated at 37 °C prior to enumeration.

2.3 DNA Extraction

DNA was extracted from a -80 °C frozen samples kept during the bird's necropsy. Then, 100 mg of glass beads were added to each sample. Suspensions were vortexed for 15 s, heated at 95 °C for 10 min, put on ice for 5 min and mixed using a vortex mixer again for 15 s. Samples were then centrifuged at 18,000 ×g for 10 min and the supernatant kept for a standard phenol: chloroform DNA extraction [24]. The final concentration and purity of the DNA was assessed by NanoDrop (ND-1000, NanoDrop, Wilmington, USA) and standardized to 20 ng/μL in sterile water.

2.4 HRM-flaA Typing

The genetic characterization of the DNA extracts from the strains and caecal contents was conducted using HRM-*fla*A analysis, as previously described in Ref. [25], on an ECO Real-Time PCR (qPCR) (Illumina, Montreal Biotech Inc., Montreal). Then, 40 ng of DNA was subjected to amplification in a 20 μL reaction containing 1× qPCR master mix (MBI EVOlution EvaGreen (R), Montreal Biotech Inc.) and 350 nmol of each primer. Each PCR run contained a no-DNA control, the two inoculated strains, reference strain 81-176 and eight caecal DNA samples. All samples were run in triplicate in two different PCR runs. The HRM-*fla*A data analysis was conducted using ECO software version 4.0 with the pre-melt region set between 72 °C and 73 °C and the post-melt region set between 91 °C and 92 °C. The curves obtained were normalized using reference strain 81-176. For each plate, sample curves were assigned a profile depending on the graphical comparison with the inoculated strain curves.

2.5 Statistical Analysis

The statistical analysis was conducted using GraphPad Prism 5 (GraphPad, La Jolla, CA, USA) with an alpha set to 0.05. The caecal and carcass bacterial levels between the groups in rooms one and two were compared using the Kruskal-Wallis test and Dunn's multiple comparisons post-test. A one-way ANOVA was conducted to analyze the combined data and a Tukey post-test to compare the groups. The Chi square and Fisher exact tests were respectively used to assess the effect of the additive on the *C. jejuni* organ occurrence and HRM-*fla*A profile selection.

3. Results

3.1 Caecal Campylobacter Levels

Before the inoculation, all chickens and feeds were free of *Campylobacter*. The treated and control chickens were colonized at 7 d post-inoculation (Table 2) in both rooms but differences in the level of *C. jejuni* were observed in the caecum of the inoculated chickens ($P < 0.001$).

In room one, at 21 PI, the total *C. jejuni* counts were higher in the OA/EO group than the control group by a mean difference of 1.4 log CFU/g. Also,

Table 2 OA/EO supplementation effects on mean caecal *C. jejuni* counts at different time post-inoculation.

Room	Group	7 PI (log CFU/g)	14 PI (log CFU/g)	21 PI (log CFU/g)
1	Ctl.	7.3 (0.2)^{B}	7.0 (0.1)	6.5 (0.2)^{D}
	OA/EO	7.1 (0.3)	7.0 (0.2)	7.9 (0.2)^{AC}
2	Ctl.	8.9 (0.1)^{AC}	6.6 (0.2)	6.6 (0.2)
	OA/EO	7.6 (0.2)^{D}	6.6 (0.1)	6.6 (0.3)^{B}
Combined	Ctl.	8.1 (0.2)^{C}	6.8 (0.1)	6.6 (0.1)^{D}
	OA/EO	7.3 (0.2)^{D}	6.8 (0.1)	7.2 (0.2)^{C}

Standard deviation (SEM); "A" being statistically different from "B" between the rooms for the same sampling time; "C" being statistically different from "D" between the groups for the same sampling time and within a room; $n = 16$ for 7 PI and 14 PI; $n = 18$ for 21 PI; PI = post-inoculation time; chickens inoculated at 14 d of age.

in the room one control group, mean levels of *C. jejuni* decreased from 7 PI to 21 PI (0.8 log CFU/g), contrary to the OA/EO group where an increase (0.8 log CFU/g) was observed for the same period. In room two, counts decreased by a mean of 2.3 log CFU/g for the control group between 7 PI and 14 PI.

Count differences between the two rooms could also be observed. In room two, control group strains showed a better colonization of chickens than their counterpart in room one by 1.6 log CFU/g at 7 PI. In room one, the treated chickens had 1.3 log CFU/g more *C. jejuni* than their counterparts in room two at 21 PI.

When room results were combined, differences between treatments could still be observed ($P < 0.001$) on caecal *C. jejuni* loads. At 7 PI, the additive reduced counts by 0.8 log CFU/g but increased them by 0.6 log CFU/g at 21 PI.

3.2 Carcass Campylobacter Levels

Total *C. jejuni* counts of the post-evisceration carcasses differed between the chicken groups in each room ($P < 0.001$, Table 3). In room one, at 21 PI, *C. jejuni* levels found on the OA/EO group were lower by a mean of 1.4 log CFU/carcass compared to the levels found on the control carcasses. Mean counts in the treated group decreased by 1.1 log CFU/carcass (7 PI > 21 PI) throughout the study but increased by 1.3 log CFU in the control group (14 PI < 21 PI). In room two, counts for the control group decreased by 0.8 log CFU/carcass between 7 PI and 14 PI.

Count differences between the two rooms could also be observed. The control carcasses from room one were less contaminated by 0.8 log CFU/carcass compared to their room two counterparts at 7 PI and treated bird carcasses were less contaminated by 1 log CFU/carcass at 21 PI.

When the room results were combined, differences could still be observed between treatments ($P < 0.001$) on carcass contamination levels. At 21 PI, the feed additive decreased the count by 0.9 log CFU/carcass.

3.3 Total Aerobic Bacterial Counts on Carcasses and C. jejuni Organ Occurrence

Throughout the study, the total aerobic bacterial counts of the carcasses ranged from 8 log CFU/carcass to 9 log CFU/carcass but were not statistically different ($P = 0.161$). No differences could be observed when comparing groups ($P = 0.161$). For organ contamination, in room one, the livers or spleens of the tested chickens were found positive regardless of their group (Table 4). But overall, *Campylobacter* was found in fewer organs at 21 PI as compared to those at 7 PI ($P = 0.009$). The OA/EO supplementation reduced the number of positive birds from six at 7 PI to one at 14 PI compared to the control feed where an increase of one positive bird was observed for the same period. When using time as a variable, it was found that the use of feed additive significantly lowered the number of positive birds ($P = 0.007$) between 7 PI and 14 PI. This was not the case for the control group ($P = 0.076$). In room two,

Table 3 OA/EO supplementation effects on mean carcass *C. jejuni* counts at different time post-strain inoculation.

Room	Group	7 PI (log CFU/carcass)	14 PI (log CFU/carcass)	21 PI (log CFU/carcass)
1	Ctl.	4.6 (0.2)^{B}	4.0 (0.1)^{A}	5.3 (0.3)^{C}
	OA/EO	5.0 (0.2)	4.0 (0.1)^{A}	3.9 (0.1)^{BD}
2	Ctl.	5.4 (0.1)^{A}	4.6 (0.2)^{B}	5.4 (0.1)
	OA/EO	5.2 (0.1)	4.6 (0.2)^{B}	4.9 (0.1)^{A}
Combined	Ctl.	5.0 (0.1)	4.3 (0.1)	5.3 (0.2)^{C}
	OA/EO	5.1 (0.1)	4.3 (0.1)	4.4 (0.1)^{D}

Standard deviation (SEM); "A" being statistically different from "B" between the rooms for the same sampling time; "C" being statistically different from "D" between the groups for the same sampling time and within a room; $n = 16$ for 7 PI and 14 PI; $n = 18$ for 21 PI; PI = post-inoculation time; chickens inoculated at 14 d of age.

Table 4 OA/EO supplementation effects on *Campylobacter*-positive organs.

Room	Group	7 PI $n = 8$	14 PI $n = 8$	21 PI $n = 9$
1	Ctl.	4	5$^{\mathbf{C}}$	1$^{\mathbf{D}}$
	OA/EO	6$^{\mathbf{A}}$	1$^{\mathbf{B}}$	5
2	Ctl.	8	6	6
	OA/EO	5	4	3
Combined	Ctl.	12	11	7
	OA/EO	11$^{\mathbf{A}}$	5$^{\mathbf{B}}$	4

All results are expressed as number of positive birds; "A" being statistically different from "B" between the sampling time within one room; "C" being statistically different from "D" between the sampling time within one room ; PI = post-inoculation time; chickens inoculated at 14 d of age.

no difference was observed.

When all the data were combined, it was found that the number of positive birds significantly decreased from 11 to 5 ($P = 0.015$) from 7 PI to 14 PI in the treated group, whereas the number in the control group did not.

3.4 HRM-flaA Profiles

An analysis was performed at 21 PI but only in room one where caecal counts differed between the control and treated group. For the caecal contents of the control birds at 21 PI, three different profiles could be seen (Fig. 1). In the OA/EO group, profile 1 dominated ($P = 0.042$) over profiles 2 and 3. Each inoculated strain had a different and reproducible HRM-*fla*A profile (profile 1 for strain 1 and profile 2 for strain 2, Fig. 2) that could be related to the profiles seen in the caecal contents. Profile 3 was successfully reproduced *in vitro* by conducting a HRM-*fla*A analysis on a sample containing the DNA of both inoculated strains in different proportions (Fig. 2).

4. Discussion

4.1 C. jejuni Broiler Chicken Colonization Model

This study evaluated the potential of using an in-feed additive as a strategy to reduce *C. jejuni* chicken colonization. The colonization model developed in this study accurately replicated natural chicken colonization. Indeed, the caecum *C. jejuni* levels obtained in both rooms were similar to the levels recorded in the field as we observed a mean colonization of 7.1 log CFU/g for all birds throughout the study while the literature reports a mean of 4 log CFU/g to 10 log CFU/g [26, 27]. We also obtained a mean *C. jejuni* carcass contamination level of 4.7 log CFU/carcass, as it is reported that carcasses in commercial conditions can be contaminated with 1.8-8 log CFU/carcass [26, 28]. The *C. jejuni* caecal and carcass counts obtained in this study therefore reflect what can be observed in commercial processing plants,

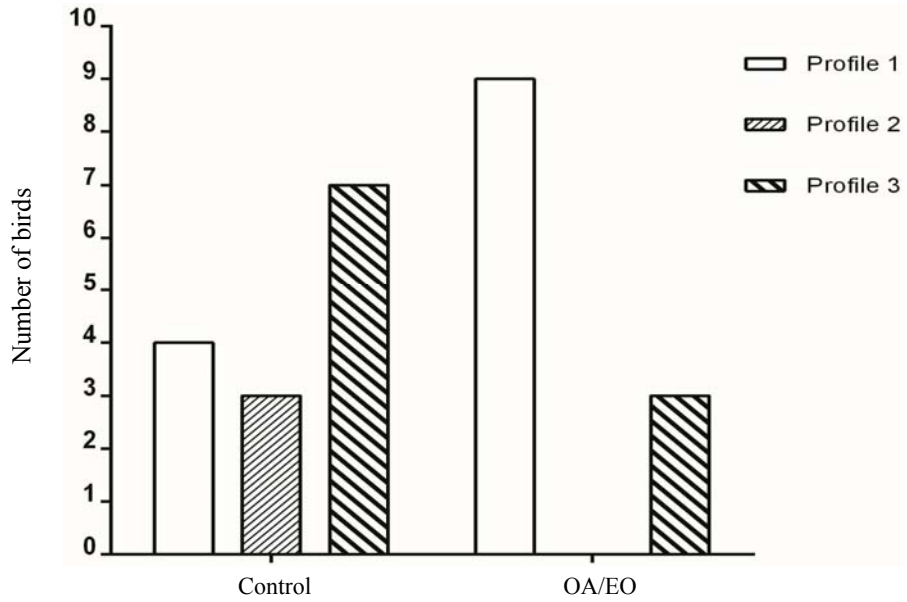

Fig. 1 OA/EO effects on HRM-*fla*A caecal content profiles of broilers at 21 PI.

The total DNA extracted from the feces of the birds in room one at 21 PI was also subjected to a HRM-*fla*A analysis. In OA/EO birds, profile 1 was found in more birds than profiles 2 and 3 ($P = 0.042$). A total of 14 birds were in the control group, while 12 were in the OA/EO group.

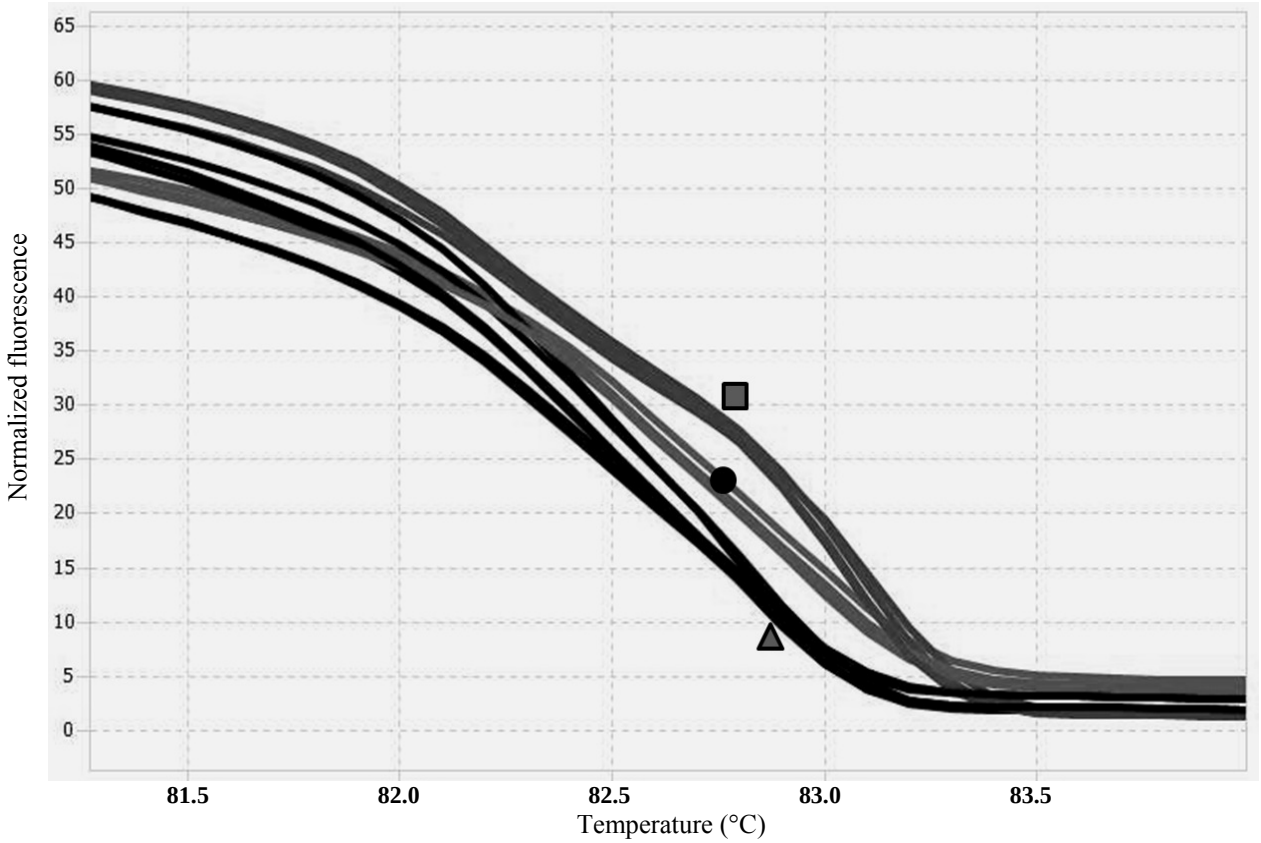

Fig. 2 HRM-*fla*A profiles of inoculated *C. jejuni* strains obtained *in vitro*.

Three different profiles were obtained from the HRM-*fla*A analysis of the inoculated strains from room one using DNA extracted from fresh culture: strain 1 alone (circle), strain 2 alone (square), a mix of their DNA in a 1:9 or 1:99 proportion (triangle).

indicating that our *C. jejuni* colonization model was appropriate to study the effect of OA/EO on both *C. jejuni* caecal and carcass populations. Bird processing up to manual evisceration was reproducible during the whole study as shown by the stable total aerobic bacteria counts on the carcasses (Table 4). No evisceration-related event affected the results as shown by the homogeneous bacterial counts on the carcasses studied.

4.2 Feed Additive Effects on C. jejuni Caecal Colonization

In the specific conditions of this study, the administration of the microencapsulated organic acids and essential oils did not prevent *C. jejuni* chicken colonization as observed in other studies [19-22], regardless of the strain used.

The effect of OA/EO was strain dependent. In room one, the OA/EO increased the counts in the chicken caecum near the end of the production cycle of a broiler chicken (21 PI, 35 d of age) while in room two, the feed additive only reduced chicken colonization at 7 PI.

The different phenotypic properties of the strains used seem to influence chicken caecal colonization at some degree. The inoculated strains of room two had lower adhesion/invasion properties and seemed slightly better for colonizing chickens, but only a few days after inoculation. It has been demonstrated that strains with lower invasion capabilities are poorer chicken colonizers [29] as opposed to observations made in this study. In our study, colonization may have been counterbalanced by other phenotypic properties of the tested strain or by differences in the chicken colonization model used. The differences observed at 7 PI and 14 PI between the two rooms could also be the results of the birds' unequal inoculation.

In room one, the OA/EO increased the *C. jejuni* counts in the chicken caeca, contrary to other studies that reported the opposite effect [19-22]. These differences could stem from the novel use of a blend of organic acids and essential oils compared to the use of individual molecules in other studies. More studies on the individual substances contained in the feed additive would be necessary to identify their independent action on *C. jejuni*.

In this study, the unexpected increase in caecal *C. jejuni* level by OA/EO could be due to the selection of a strain, enabling it to better colonize the chicken. In other studies, thymol and cinnamaldehyde were found to modify the chicken gut microflora by increasing the caecal *Lactobacillus* and *Escherichia coli* populations [30]. Essential oils such as thymol and cinnamaldehyde also modified butyrate, isobutyric acid, propionic acid and isovaleric acid caecal proportions [30, 31]. *C. jejuni* can metabolize different organic acids such as citric, fumaric, lactic, malic, succinic [32], aspartic, glutamic acids and ketoglutarate [33]. These organic acids can also act as a chemoattractant for *C. jejuni* [34]. Therefore, an increase in these organic acids in the gut concentration by the feed additive itself or by a modified microbiota may be beneficial for *C. jejuni*. Formic acid was also found to improve the intestinal mucosa physiology of chickens [35]. Intestinal mucus can neutralize the bactericidal effect of organic acids [36]. *C. jejuni* is also attracted to mucins [37] and colonizes the caecal mucosa [1]. An improvement in the chicken gut mucus layer may lead to an increase in *C. jejuni* colonization for some strains. Modification of the chicken gut environment by the tested feed additive remains to be studied. In this study, we hypothesize that the modifications caused by the OA/EO experimental feed additive may have impacted the inoculated strains in a strain-dependent manner, favoring one strain over another and therefore increasing the *C. jejuni* caecal count at 21 PI.

This *C. jejuni* strain modulation is supported by the chicken caecal content HRM-*fla*A analysis results, where profile 1 seemed omnipresent when the feed additive was used. In recent studies, HRM-*fla*A was

successfully shown to discriminate between *C. jejuni* strains distinguishable by *fla*A sequencing [25] and could successfully be used for multi-locus sequence typing (MLST) [38]. To our knowledge, this study is the first to report the use of HRM analysis directly with chicken caecal content. The reproducible results obtained suggest that the HRM technology could be useful for the direct analysis of caecal content contaminated by *C. jejuni*. This is also, to our knowledge, the first observation of the modulation of competing *C. jejuni* chicken for gut colonization by organic acids and essential oils. The exact mechanism that would make this selection possible remains to be investigated.

4.3 Feed Additive Effects on Carcass C. jejuni Contamination

Carcass contamination also seemed to be strain dependent as it was different between the two rooms. In room one, a significant *C. jejuni* reduction (1.4 log CFU/carcass) was achieved at 21 PI when using OA/EO. It also reduced the value of the carcass contamination by 0.5 log CFU/carcass at 21 PI in room two. These carcass *C. jejuni* reductions could result in an appreciable effect of OA/EO on public health. Indeed, some authors suggested that the reduction of carcass contamination by 1 log CFU/carcass could reduce human health risks by 50% to 90%, depending on the pre-existing production conditions [3].

This carcass count reduction is unexpected as the opposite effect was seen in the caecum in room one. In commercial settings, high *C. jejuni* caecal counts were found to be correlated with high carcass contamination [26]. That being said, this is not always the case as the caecal colonization level is not always a good predictor of carcass contamination [28], as observed in this study.

This study is one of the few that is evaluating the effect of OA/EO on *C. jejuni* carcass contamination. It was reported that citric acid decreased ileal coliform levels but increased carcass populations of *Staphylococcus*, *Campylobacter* and lactic acid bacteria [39], in opposition to the results of this study. Nevertheless, in another study [40], a decrease of *C. jejuni* post-chill carcass contamination with the use of in-feed sub-therapeutic doses of macrolide was observed.

The mechanisms explaining the impact of feed additives on carcass contamination should be defined carefully. Processing seems to affect the type of *Campylobacter* found on the poultry carcasses [12, 41]. Moreover, *C. jejuni* strain-dependent stress resistance can play a major role in this observation [42]. If such mechanism is confirmed, using feed additives to favor chicken colonization and subsequently carcass contamination by strains more susceptible to the stresses encountered during processing may help to lower the number of remaining *C. jejuni* on the carcasses, thus possibly diminish the consumer exposition to *C. jejuni*. Much work needs to be done to understand the effects of feed additives on carcass microbiology.

4.4 Feed Additive Effects on C. jejuni Organs Positivity

This study also showed that birds gradually cleared *C. jejuni* from their organs in both rooms. In room one, and when combining all results, this clearance was faster for the OA/EO treated group. This is, to our knowledge, the first time that this observation is reported. It has been suggested that the adaptive immune response might be responsible for chicken *C. jejuni* organ clearance [43]. Chicken immunity modulation by the feed additive could also explain this result. For example, in another study, the organic acid butyrate was found to activate chicken host defense peptides *in vitro* in chicken tissue explants as well as monocytes and macrophages. Butyrate also increased response against Salmonella Enteritidis, both *in vitro* and *in vivo*, when added as a feed additive (1 log CFU/g caecal reduction) [44]. Immune stimulation by OA/EO used in this study remains to be demonstrated.

5. Conclusions

Overall, OA/EO did not prevent *C. jejuni* chicken colonization at 14 d of age. The effects of the feed additive seemed strain dependent. For room one and when combining both room results, the feed additive moderately lowered the *C. jejuni* levels found on the carcasses at 21 PI, which could potentially lead to a lower exposure of consumer to the pathogen, although caecal levels were unexpectedly increased for treated chickens in room one. Using the HRM-*fla*A analysis, our results also suggest that the type of feed given to the chickens may modulate the *C. jejuni* chicken colonization dynamic, which could have led to the chicken colonization increase observed in room one. The positivity of the organs of the birds fed OA/EO also decreased faster than that of the control group for room one, and when combining room 1 and room 2 results, it is indicated that there is a possible immunostimulatory effect of OA/EO. Further studies on more *C. jejuni* strains should be carried out to confirm this. Based on these results, we hypothesize that the OA/EO additive tested could modify the chicken gut environment, thus preferentially benefiting the caecal colonization of some *C. jejuni* strains and possibly affecting the strains fitness under processing conditions. This remains to be validated in further studies.

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