Journal of Food Safety and Food Quality 65, Heft 1 (2014), Seiten 1–28

Arch Lebensmittelhyg 65,
4–9 (2014)
DOI 10.2376/0003-925X-65-4

ISSN 0003-925X

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Effect of electrolyzed oxidizing water on reducing *Campylobacter* spp. in broiler chikken at primary production

Einfluss von elektrochemisch aktiviertem Wasser auf das Vorkommen von Campylobacter spp. bei Masthähnchen in der Primärproduktion

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Summary

In addition to numerous methods of controlling *Campylobacter* spp. during slaughter and processing, there are efforts to develop reduction strategies in primary production. In the present study drinking water was treated with a 3 % solution of neutral electrolyzed oxidizing water as water additive in two naturally *Campylobacter* colonized farms. The experiment was performed from day zero until main catching. On each farm three rearing periods were included in the study. Carcasses were examined for contamination after batch depletion and after main catching. In none of the treated water samples *Campylobacter* spp. could be detected. Drinking water samples in all control groups were positive on day 35 of the rearing period. In one case *Campylobacter* spp. could be detected in the water sample of a control group already at day 28. After main catching significant lower numbers of *Campylobacter* spp. were isolated from the carcasses in the test group at both farms. The permanent addition of electrochemically activated water seems to be an opportunity to reduce the carriage of *Campylobacter* spp. in poultry drinking water and appears to affect counts on carcasses.

Keywords: Electrolyzed oxidizing water, *Campylobacter*, broiler chicken, drinking water, carcasses

Zusammenfassung

Neben zahlreichen Verfahren zur Bekämpfung von *Campylobacter* spp. während der Schlachtung und Verarbeitung gibt es mehr und mehr Bemühungen, Strategien zur Senkung des Erregervorkommens in der Primärproduktion zu entwickeln. In diesem Feldversuch wurde in zwei Hähnchenmastbetrieben, bei jeweils auf natürliche Weise mit *Campylobacter* spp. kolonisierten Herden, Tränkwasser mit einer 3%igen Lösung von neutralem, elektrochemisch aktiviertem Wasser als Tränkwasserzusatz eingesetzt. Die Behandlung erfolgte von Tag Null der Mastperiode bis zum Hauptfang. Als Kontrollgruppe diente eine unter gleichen Bedingungen gemästete Herde gleicher Herkunft. In jedem Betrieb wurden 3 Mastdurchgänge in die Studie einbezogen. Nach dem Vorfang sowie nach dem Hauptfang wurden ganze Schlachtkörper hinsichtlich einer Kontamination des Endproduktes untersucht. In keiner der Wasserproben der Versuchsgruppen wurden *Campylobacter* spp. nachgewiesen. Die Tränkwasserproben in allen Kontrollgruppen waren am Tag 35 der Aufzucht positiv. In einem Fall wurden *Campylobacter* spp. bereits am 28. Masttag im Tränkwasser der Kontrollgruppe nachgewiesen. Nach dem Hauptfang konnte die Anzahl der *Campylobacter* spp. auf den Karkassen der Versuchsgruppe beider Betriebe signifikant gesenkt werden. Somit scheint die permanente Zugabe von elektrochemisch aktiviertem Wasser zum Tränkwasser eine Möglichkeit darzustellen, die *Campylobacter*last im Tränkwasser und auf Karkassen von Masthähnchen zu reduzieren.

Schlüsselwörter: Elektrochemisch aktiviertes Wasser, *Campylobacter*, Masthähnchen, Tränkewasser, Karkassen

Introduction

Poultry seems to be the main source of human infection with *Campylobacter* spp. (Hermans et al., 2012b). In 2011 more than 220 000 humans in the European Union were infected. This number is rising since 2005. In most cases the infection of humans took place through contact of fecal contaminated fresh broiler meat (EFSA, 2012). An important measure for consumers would be a more intensive compliance with the handling of poultry meat. Due to the sharp increase in human campylobacteriosis there are other measures needed to reduce the prevalence of *Campylobacter* spp. in meat processing and at farm level (Klein, 2010). In primary production strict biosecurity measures should minimize the entry sources. This does not seem to suffice. (Hermans et al., 2011). Some studies deal with the use of non-biosecurity measures. The use of probiotics (Ghareeb et al., 2012) or organic acids (Jansen, 2012) in drinking water of chicken seem to be effective to stop or reduce the entry and colonization of *Campylobacter*. Electrolyzed oxidizing (EO) water as water additive might be promising because investigations for reduction of *Campylobacter* on eggshells, wash water of carcasses and in poultry processing have shown a bactericidal effect of EO water (Park H. et al., 2002; Kim et al., 2005; Fasenko et al., 2009). EO water is a nontoxic sanitizer (Russell, 2003). The method is based upon an electrolytic process which takes place in a special made generator (Fig. 1). Before the generator starts, a reverse osmosis process for water softening is conducted. To this softened water sodium chloride (NaCl) is added. The solution passes through an electrochemical cell containing an anode and a cathode. The two poles are separated by a ceramic diaphragm. By applying a direct current voltage, two different solutions can be obtained. From the cathode side, a solution with pH 10–11.5 and an oxidation-reduction potential (ORP) between –800 and –900 mV is produced. From the anode side, acidic EO water with pH between 2.3 and 2.7 and a high ORP (> 1000 mV) is generated (Hsu, 2005). The chlorine concentration depends on the amperage applied. Increasing voltage and NaCl concentration results in a lower pH, higher ORP and higher residual chlorine of the acidic EO water. Increasing the electrolyte flow rate has the reverse effect of these trends because of shorter residence time in the electrolytic cell (Ezeike and Hung, 2004). Hypochlorous acid (Len et al., 2000) and the ORP (Liao et al., 2007) seem to be the main active components and closely related to the bactericidal effect of EO water. Numerous studies demonstrate that the acidic EO water is a promising possibility for disinfection of surfaces and objects in the food production (Hricova et al., 2008). It provides an opportunity for decontamination of eggshells in the hatchery, lettuce, spinach, cattle hides as well as fresh pork (Bialka et al., 2004; Fabrizio and Cutter, 2004; Bosilevac et al., 2005; Park E. J. et al., 2008) and shows its effectiveness at spraying carcasses in poultry processing (Northcutt et al., 2007). The disadvantage of using acidic EO water is especially the corrosive effect on processing equipment.

The use of neutral EO water does not cause these disadvantages (Ezeike and Hung, 2004; Ayebah and Hung, 2005). The state of solution is more stable than the acid variant because chlorine loss is significantly reduced at pH 6–9 (Len et al., 2002). Deza et al. (2003) showed that washing tomatoes inoculated with *Escherichia coli, Salmonella enteritidis* and *Listeria monocytogenes* with neutral EO water reduced the bacterial number without prejudice for the surface of tomatoes and without sensory deviations. A bactericidal effect could also be achieved in the cleaning of kitchen cutting boards (Deza et al., 2007). Some other studies have been conducted in evaluating the effects of EO water in animal production systems. Using neutral EO water as spray in the air of a layer breeding house resulted in dust retention, lower temperatures and lower mortality of chickens near the EO spray device (Zheng et al., 2012). Furthermore Jirotková et al. (2012) showed that neutral EO water has no negative effects regarding color, pH and loss of water on poultry carcasses after adding it to drinking water in chicken houses while fattening.

Until now there have been no studies conducted to determine the influence of EO water for reduction of *Campylobacter* across different production levels. The aim of this study was therefore to investigate the influence of electrolyzed oxidizing water as water additive, on the occurrence of *Campylobacter* in broiler farms and its effects on the carcasses.

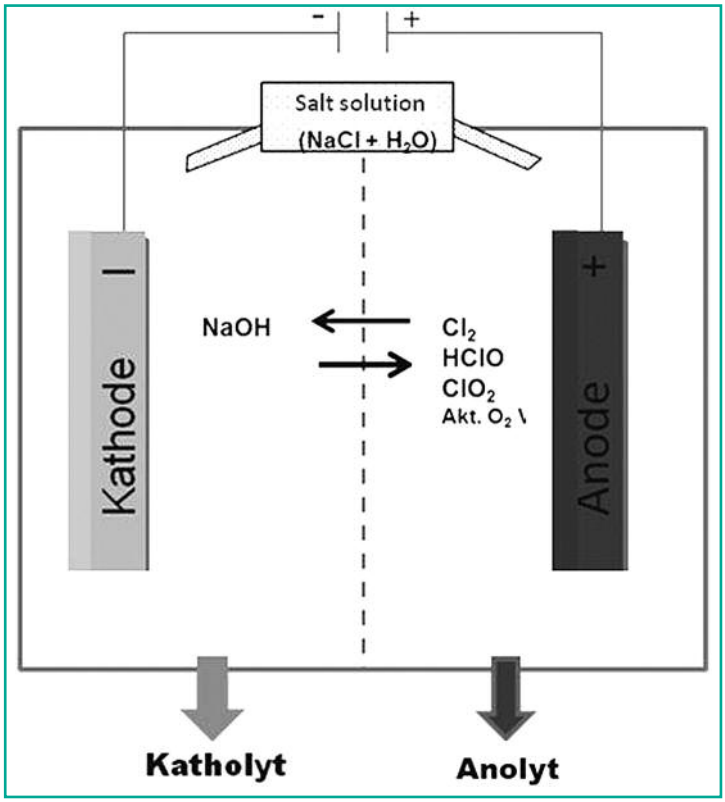

FIGURE 1: *Schematic diagram of a generator for the production of electrochemical activated water*

Material and Methods

Rearing farms

As part of a field trial, two broiler farms were examined for three rearing periods under conventional production conditions. The selection of farms was carried out according to criteria regarding type of chicken house, animals and biosecurity. Chicken houses on each farm were built in the same year and identical in size. Equipment for water and feed was the same and could be individually operated. Two chicken flocks (Ross 308, unsexed) were examined from the same breeder flock. In both houses, chicken received consi-

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stently the same feed. Another criterion was natural colonization of *Campylobacter* before the experiment, which was confirmed by the Status Quo investigations. These investigations took place in the period before starting the experiment to the same extent as in the test series. Farm A was rearing for integration A. Chickens were slaughtered at slaughterhouses A and B after batch depletion and at slaughterhouse C after main catching. The farm had two identical stables, which were connected via a shared entrance hall. Within a radius of 3 km there was no other broiler farm. The access road was paved. Both houses housed 40 000 chicken. For gaining access to the entrance hall a hygiene sluice which required a change of clothes and shoes had to be passed though. The supply with drinking water in flock 1 occurred with water from an own well, which was made free from iron by a deferrization unit (Remotector 2000; Remon water treatment, Marum, Netherlands). Flock 2 got drinking water supplemented by 3 % of neutral electrochemical activated water as water additive. Farm B was rearing for integration B. Chicken were slaughtered at slaughterhouse D and E after batch depletion and at slaughterhouse F after main catching. The farm had two identical stables, which were connected via a shared anteroom. For gaining access to the anteroom a hygiene sluice which required a change of clothes and shoes had to be passed trough. There were three other broiler farms and one turkey farm within a radius of 4 km. The access road was not paved. The area in front of the barn (20 m x 80 m) was concreted. Both houses housed 35 000 chickens. The supply with drinking water occurred with water from an own well, which was made free from iron by a deferrization unit (Remotector 2000; Remon water treatment, Marum, the Netherlands). Flock 1 served as a control group and flock 2 received drinking water supplemented with 3 % neutral electrochemically activated water as a water additive.

Production of electrochemical activated water

Neutral EO water was generated using an Agrilyt-Generator (Schulz Systemtechnik GmbH, Visbek, Germany) equipped with a DEA-30 electrolytic cell operating at 24 V DC, 10 A and 30 l/h (Elliod GmbH, Berlin, Germany). The cell was divided into two chambers by a ceramic diaphragm for producing an acidic and a basic solution. To produce a salt solution NaCl was used (8 kg/m^3 Agrilyt). In order to produce neutral Agrilyt, five to ten percent of the full amount of catholyte was mixed with anolyte. The generator consisted of a control cabinet for the electrical component, a control cabinet for the hydraulic components and a reverse osmosis system. It was installed by the manufacturer, and remained in the anteroom throughout the entire duration of the experiment. The device was directly connected to the dispenser at the water supply line, which was normally used for drug administration. The solution was thus produced on site and was added at a concentration of three percent directly into the drinking water. The neutral EO water solution had a pH of 6.2 to 7.5 and an ORP of 800–1100 mV. The amount of residual chlorine was measured using an ExStik CL200 chlorine tester (Extech instruments corporation, USA) pH and ORP were measured using an Exstik PH100 pH meter and ExStik RE300 ORP tester (Extech instruments corporation, USA).

Presence of *Campylobacter* spp.

To acknowledge the presence of *Campylobacter* spp. in each group in every rearing period water samples before influent to the drinking line (n=1), sock swabs (n=2), cloacal swabs at day 25 (n=15) and cloacal swabs at day 35 (n=15) were taken. The samples were examined for thermophilic *Campylobacter* qualitatively according to ISO 10272-1:2006. Before the start of each rearing period water samples (1 liter) were taken before influent to the drinking line. The water was examined for *Campylobacter* by transferring the water sample in sterile 100 ml MicroFunnel (PALL Life Science, Germany) using membrane filters made of mixed cellulose esters with a pore size of 0.45 µm (GN-6 Metricel, PALL Life Sciences). The MicroFunnel was placed on the aluminium manifold (PALL Life Science) and a peristaltic pump drew the whole water sample (1 liter) through an integrated system. Subsequently the membrane filter was cultured in 90 ml of Bolton broth (CM 0983, supplement SR 0183 and SR 048; Oxoid, Germany). The broth was incubated for 48 h ± 2 h at 42 °C ± 0,5 °C under microaerophilic conditions (5 % O_2, 10 % CO_2, 85 % N_2) in gas jars (Campygen 2.5 L; Oxoid). After enrichment, 10 µl of the solution was streaked on mCCDA and Karmali plates and incubated for 48 h ± 2 h at 42 °C ± 0,5 °C under microaerophilic conditions (5 % O_2, 10 % CO_2, 85 % N_2) in gas jars (Campygen 2.5 L; Oxoid). The presumptive colonies were confirmed as described below.

On day 21 of every rearing period two pairs of sock swabs were taken from control and tested group. Sock swabs were composed of disposable shoes made of gauze which cover plastic disposable shoes. Directly before use, the sock swabs were moisturized with sterile NaCl-Peptone-Water solution (85 % NaCl, 0,1 % peptone, 14,9 % water). Each pair of sock swabs was worn for 100 steps in the stable. On this way droppings and litter adhered and arrived at the laboratory. Samples were arranged paired in transport bags. At the laboratory 250 ml Bolton broth (CM 0983, supplement SR 0183 and SR 048, Oxoid) was filled in each bag. The bag was kneaded manually for two minutes and subsequently incubated for 48 h ± 2 h at 42 °C ± 0,5 °C under microaerophilic conditions (5 % O_2, 10 % CO_2, 85 % N_2) in gas jars (Campygen 2.5 L; Oxoid). After enrichment, 10 µl of the solution was streaked on mCCDA and Karmali plates and incubated for 48 h ± 2 h at 42 °C ± 0,5 °C under microaerophilic conditions (5 % O_2, 10 % CO_2, 85 % N_2) in gas jars (Campygen 2.5 L; Oxoid).

The presumptive colonies were confirmed as described below.

For sampling cloacal swabs chicken were picked out randomly. A sterile swab was inserted in the chloaca and faeces material was sampled by rotary movements. In each rearing period in control and test group 15 cloacal swabs were collected on day 25 and after batch depletion on day 35. They were kept in Cary-Blair medium at 4 °C during transportation to the laboratory for 2 to 4 h. Each swab was enriched in a single test tube with 9 ml of Bolton broth at 48 h ± 2 h at 42 °C ± 0,5 °C under microaerophilic conditions (5 % O_2, 10 % CO_2, 85 % N_2) in gas yars (Campygen 2.5 L; Oxoid). After enrichment, 10 µl of each single test tube was streaked on mCCDA (Oxoid) and Karmali (Oxoid) plates and incubated for 48 h ± 2 h at 42 °C ± 0,5 °C under microaerophilic conditions (5 % O_2, 10 % CO_2, 85 % N_2) in gas jars (Campygen 2.5 L; Oxoid). The presumptive colonies were confirmed as described below.

Identification and storage of *Campylobacter* spp.

Identification of *Campylobacter* spp. occurred according to ISO10272-1:2006. At least five presumptive colonies from

each sample were streaked on Columbia-blood plates (Oxoid) and incubated for 48 h ± 2 h at 42 °C ± 0,5 °C under microaerophilic conditions (5 % O_2, 10 % CO_2, 85 % N_2) in gas jars (Campygen 2.5 L; Oxoid). Subsequently *Campylobacter* was identified with phase contrast microscopy, gram staining, positive oxidase tests as well as lack of growth at 25 °C under microaerobic conditions. For future typing the confirmed *Campylobacter* strains were stored in a cryopreservation system (Mast diagnostic, Cryo series).

Quantitative analyses of *Campylobacter*

Water samples of the drinking lines were examined quantitatively as a variation to the recommendations of ISO 10272-2:2006. At day 0, 7, 14, 21, 28 and 35 samples (1 liter) were taken from the drinking lines in both chicken houses. The 1 liter sample was a pooled sample of each 250 ml which were taken on the same day from four different drinking lines. pH and ORP levels were measured and numbers of campylobacters in samples were determined by plating 10-fold dilutions of each sample prepared using Maximum Recovery Diluent (MRD; CM733, Oxoid) onto mCCDA and Karmali plates. The plates were incubated 48 h ± 2 h at 42 °C ± 0,5 °C under microaerophilic conditions (5 % O_2, 10 % CO_2, 85 % N_2) in gas jars (Campygen 2.5 L; Oxoid). Presumptive colonies were counted, and 5 colonies per plate were confirmed as described above. After batch depletion and at the end of every rearing period five carcasses from control and test group were examined for *Campylobacter* quantitatively according to ISO 10272-2:2006.

Carcasses were taken at the slaughterhouse from the processing line after evisceration and chilling and put into sterile bags. The samples were sent to the laboratory in an insulated box, within 24 - 48 h. At the laboratory each carcass was put into a sterile plastic bag with 500 ml of 0.9 % NaCl peptone solution. The bag was shaken 1.5 minutes in every direction whereby the whole surface of the carcass came in contact with the solution. 100 µl of this solution were spread plated in duplicate onto mCCDA (Oxoid) and Karmali (Oxoid) plates. Furthermore a serial 10-fold dilution with MRD (Maximum Recovery Dilution, Oxoid) was made and plated onto selective agar plates (mCCDA and Karmali). The plates were incubated as described above. The presumptive colonies were counted and confirmed.

Statistical analyses

Differences of *campylobacter* counts on carcasses were analyzed using an independent t-test, comparing the means of control and test group for differences in *Campylobacter* numbers. Drinking water samples from control and test group were analyzed using a 2-factorial analysis of variance. The results of cloacal swabs were determined using a fisher s exact test for showing differences between the colonization of control and treated flock. The significance level for all data was set at $P = 0.05$. All analyses were carried out with SAS 9.3 (Statistical Analysis System), Cary, USA.

Results and Discussion

Previous studies have shown that bacterial pathogens like *Escherichia coli* and *Staphylococcus aureus* could be reduced or elimated by the use of neutral EO water (Liao et al., 2007; Zeng et al., 2010). Park H. et al. (2002) compared the effect of chlorine water and EO water on the reduction of *Campylobacter jejuni* during poultry washing. It was demonstrated that EO water is very effective for inactivating *Campylobacter jejuni* on surfaces of poultry during the slaughter operation. The mean population of *Campylobacter jejuni* treated with EO water was reduced to less than 1.0 log10 CFU/ml and the chlorine water was less effective than the EO water.

In chicken houses drinking water is a known source or even a vector for colonization of flocks with *Campylobacter* (Pearson et al., 1993; Herman et al., 2003; Messens et al., 2009). For this reason the use of neutral EO water as water additive seemed to be promising. In this study the drinking water was treated with a 3 % solution of neutral EO water as water additive from day zero until slaughtering. A control group didn't receive the water additive. At farm A in control groups water pH was 6.92 and ORP level was 489 mV on average of 18 water samples from the drinking lines within three rearing periods. In treated groups of farm A a pH of 6.73 and an ORP level of 803 mV were detected on average of 18 water samples. At farm B a pH of 7.9 and an ORP of 631 mV on average of 18 water samples were measured from drinking lines within three rearing periods. In treated groups of this farm the water pH was 7.5 and the ORP value ORP increased by 814 mV on average of 18 water samples. Values of ORP were considerably higher in EO water treated groups at both farms. The increase of ORP probably was the reason for a bactericidal effect to the drinking water. Liao et al. (2007) showed that high ORP values in EO water lead to oxidation of glutathione-synthetase and destroyed membrane structures and functions of *Escherichia coli.* Similar results were shown by Zeng et al. (2010). The survival of *Campylobacter* in water is depended on the species, a low temperature, absence of light, a low oxygen concentration, low numbers of indigenous bacteria and the existence of biofilms (Pitkänen, 2013). High environmental temperatures at the beginning of the rearing period and the damage of microbial structure by EO water therefore decreased the survival of *Campylobacter*. Consequently *Campylobacter* spp. were not detected in any of the treated water samples. Drinking water samples in control stables were positive on day 28 at the earliest but in any case on day 35 ($p \leq 0.01$) (Tab. 1).

TABLE 1: *Campylobacter numbers in drinking water provided to chickens.*

	Farm A (log10 cfu/l)		Farm B (log10 cfu/l)	
	Control	EO water	Control	EO water
Day 0	NF	NF	NF	NF
Day 7	NF	NF	NF	NF
Day 14	NF	NF	NF	NF
Day 21	NF	NF	NF	NF
Day 28	NF	NF	2.09*	NF
Day 35	2.28**	NF	2.29**	NF

Values are the means of three replicated rearing periods. NF = not found; *: significant difference at P ≤ 0.05; **: significant difference at P ≤ 0.01.

The results of the drinking water samples are involved by the results of cloacal swabs (Tab. 2). Both farms were naturally colonized with *Campylobacter* in all fattening periods. The colonization could be determined after at least day 25 in cloacal swabs. Most positive swabs were

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TABLE 2: *Prevalence of Campylobacter at farm level.*

	RP	water (before influent to the chicken house)		sock swabs		cloacal swabs day 25		cloacal swabs day 35	
		Control	EO water	Control	EO water	Control	EO water	Control	EO water
Farm A	I	0/1[a]	0/1	0/2	0/2	4/15	5/15	9/15	6/15
	II	0/1	0/1	0/2	0/2	2/15	3/15	4/15	2/15
	III	0/1	0/1	0/2	0/2	2/15	1/15	6/15	0/15
Farm B	I	0/1	0/1	0/2	0/2	1/15	4/15	0/15	2/15
	II	0/1	0/1	0/2	0/2	1/15	8/15	11/15	1/15
	III	0/1	0/1	0/2	0/2	0/15	0/15	14/15	6/15

RP: rearing period, [a]: number of positive samples/total number of samples taken

found in control groups of farm A and B at day 35. At this day of rearing in both farms counts of positive cloacal swabs in the test group were significantly lower ($p < 0.05$). The results showed that although the water was free of *Campylobacter*, the flocks were still colonized. This is consistent with previous results and it is suggested that the fecal-oral transmission played an important role in the spread of *Campylobacter* between the animals of a flock (Hermans et al., 2012a). By this fecal-oral transmission, drinkers were contaminated and a tracking up effect of *campylobacter* into the water supply system leads to the detection in water samples (Newell et al., 2011).

However through the use of neutral EO water as water additive, drinking water as vector seemed to be no longer a risk for colonization of *Campylobacter*. Against this background the use of neutral EO water may thus be a way to prevent the entry of *Campylobacter* into the rearing environment from well water as described by Pearson et al. (1993) in broiler flocks and by Perez-Boto et al. (2010) in broiler breeder flocks. In this field trial this could not be demonstrated because all of the well water samples were tested negative for *Campylobacter*.

Besides samples at farm level, carcasses were investigated after batch depletion and after main catching (Tab. 3). At the slaughterhouse after evisceration and chilling samples were taken from the processing line and analyzed for *Campylobacter* spp. The used analytical method showed the numbers of *Campylobacter* spp. on carcasses after chilling but before cutting. At farm B in the second rearing period after batch depletion *Campylobacter* numbers on carcasses of the test group show lower levels of contamination. In all other rearing periods *Campylobacter* could be not found in both groups after batch depletion. This results could be explained by the identity of the slaughterhouse which was a different one (E) in comparison to the other batch depletion slaughterhouses (A,B,D). Similar observations were made by Herman et al. (2003) which emphasized the importance of hygiene during slaughter. In addition previous studies demonstrated a high risk of cross-contamination at the abattoir. When chicken arrived at the slaughterhouse with a negative *Campylobacter* status, carcasses sampled after chilling were contaminated with the bacteria (Slader et al., 2002; Herman et al., 2003). However, the numbers of *Campylobacter* spp. on carcasses seemed to be even affected by the prevelance of the flock. Allen et al. (2007), showed significant higher numbers of *Campylobacter* spp. on carcasses of flocks with high prevalence at farm level. This leads to the consequence that a lower *Campylobacter* colonization on carcasses could be correlated to a lower concentration in caeca content at farm level. As described before, the EO water treated group showed a lower prevalence at farm level and a lower number of *Campylobacter* numbers on carcasses in this study. In the EO water treated flock the reduction of the colonization of *Campylobacter* resulted apparently in reducing the numbers on carcasses of this flock. However, it was not possible to establish a direct relationship between the prevelance of a flock and numbers of *Campylobacter* on carcasses because the extent to which carcasses originating became contaminated from these flocks is not clear (Allen et al., 2007).

This study showed that EO-water treatment of drinking water in chicken houses seemed to be an opportunity to exclude drinking water as a risk factor for *Campylobacter* colonization within a flock. Additional investigations on numbers of *Campylobacter* on carcasses, which maybe provide a larger amount of samples, are necessary to analyze the effect of EO water treatment. In this study it provides a decline of *Campylobacter* counts.

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TABLE 3: *Campylobacter counts on carcasses after processing (log10 cfu/ml).*

	RP	Batch depletion			Main catching		
		Control	EO Water	Slaughter-house	Control	EO Water	Slaughter-house
Farm A	I	NF	NF	A	4.06 ± 1.09	NF**	C
	II	NF	NF	B	1.70 ± 1.86	NF	C
	III	NF	NF	A	4.46 ± 0.52	1.55 ± 1.69*	C
Farm B	I	NF	NF	D	4.39 ± 0.70	2.63 ± 1.26*	F
	II	3.13 ± 1.11	1.85 ± 0.15	E	2.92 ± 0.61	1.64 ± 1.14*	F
	III	NF	NF	D	–	–	–

RP: rearing period; NF: not found; * significant difference at $P \leq 0.05$; ** significant difference at $P \leq 0.01$

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