Influence of Using Probiotics on Microbial Status of Chicken Carcasses

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Abstract

There is currently a world trend to reduce using of antibiotics in poultry industry and replacing it by natural alternatives such as probiotics to obtain healthy and antibiotic-free chicken meat. Therefore, this study evaluated firstly the microbial status of chicken carcasses in Dakahliya Governorate, Egypt. Then, the effect of using probiotics before slaughtering by 48 hours on the microbial status of chicken carcasses was experimentally investigated. Microbiological examination in this study included general microbial indicators (Aerobic plate count, Enterobacteriaceae count and Most Probable Number of coliforms), isolation and identification of *Campylobacter spp*. In addition, multiplex PCR was used to detect the virulence-associated genes of *Campylobacter jejuni* including cdtA, cdtB and cdtC toxin-producing genes. The achieved results revealed high microbial load of both commercial and untreated chicken carcasses compared with the probiotics-treated carcasses, which showed significant improvement, in terms of the microbiological quality of the chicken carcasses.

Keywords: Probiotics; Microbial status; Campylobacter spp; Chicken carcasses

Introduction

Chicken meat is considered as a good source of animal-derived protein, low in carbohydrates and calories contain certain essential fatty acids like linoleic, linolenic and arachidonic. It also contains a lot of minerals as potassium, calcium, iron, phosphorus and iodine beside traces of vitamins like B12, niacin, riboflavin and thiamine [1]. In recent years, the poultry industry has experienced an unrivalled rate of growth. This can be attributed to relatively low production costs, high nutritional values, rapid growth rates, and a great number of further-processed products [2]. Although chicken meat is highly nutritious, but it may be come harmful when it is soiled during preparation, handling, distribution and storage.

In developing countries like Egypt, foodborne diseases occur commonly because of inadequate food safety laws, weak regulatory systems, lack of financial resources and lack of food-handlers education [3]. Several bacterial indicators are used to evaluate chicken carcasses like monitoring aerobic bacterial counts, Enterobacteriaceae counts and most probable number of coliforms [4]. *Campylobacter spp*. is considered as a potential source for human infection and bacterial enteric illness in industrialized countries that occur through consumption of contaminated chicken carcasses [5]. There is currently a world trend to reduce the use of antibiotics as animal feed additives due to the possible contamination of meat products with antibiotic residues [6].

Competitive exclusion of beneficial bacteria (probiotics) in poultry intestine and caecum can provide best opportunity to reduce human pathogens like *Campylobacter spp*. in chicken carcasses [7]. Probiotics, which are one of the oldest feed additives, are live microorganisms, that if supplemented in the right way, they can beneficially affect the host's health by making a balance between commensal and pathogenic microbiota in the gastrointestinal tract [8]. Therefore, this study was conducted firstly to evaluate the hygienic status and the prevalence of *Campylobacter spp*s in chicken carcasses marketed in Egypt as a survey part. Secondly, in an experimental model, the influence of using probiotics on the microbial status and incidence of *campylobacter spp*. in chicken carcasses was investigated.

Materials and Methods

Collection of samples

One hundred and eighty random and equal samples (n=60) from each of commercially fresh chicken carcasses (ready for cooking) (Survey part), probiotics-untreated chicken carcasses, and treated chicken carcasses with multispecies probiotic (Poultry star®) which composed of (*Enterococcus faecium*, *Bifidobacterium* animalis, *Pediococcus acidilactici*, *lactobacillus reuteri*, *lactobacillus salivarius*) (Experimental part). The probiotics-untreated and treated groups were designed as an experimental study, as treated-birds received probiotics in their drinking water for 48 h before slaughter, followed by slaughtering, mechanical scalding and evisceration in the same way followed at the butchery shop. All samples were collected from Dakahliya governorate, Egypt. Samples were rapidly transferred in a cooled condition (4°C) to Food Control Laboratory, Faculty of Veterinary medicine, Zagazig University, Egypt for aerobic plate count (APC), Enterobacteriaceae count (EC), MPN of coliforms and isolation and identification of *Campylobacter spp*.

Preparation of samples, enumeration and isolation procedures

Twenty-five grams of each sample were aseptically homogenized in 225 mL of 1% sterile peptone water (Oxoid CM9) to make a dilution of 10^{-1} then were allowed to stand for 5 minutes, then 1 mL was transferred aseptically to a test tube containing 9 mL sterile 0.1%

buffered peptone to prepare tenfold decimal serial dilution up to 10^{-7} dilution [9].

For aerobic plate count, one ml from each of the previously prepared dilution was transferred into two separate sterile Petri-dishes to which approximately 15 ml of sterile melted and tempered plate count agar (45°C) were added. After thorough mixing, the inoculated plates were allowed to solidify before being incubated at 37°C for 24 h. The aerobic plate count (APC) per gram was calculated on plates containing 30-300 colonies and each count was recorded separately.

For Enterobacteriacae count, violet red bile glucose agar medium (VRBG) was used as culture medium. Accurately, the plates were inoculated by spreading technique with 0.1 ml of each decimal dilution under complete aseptic conditions. The plates were inverted and incubated at 37°C for 24 h. All purple colonies were then counted and the average number of colonies was determined as Enterobacteriacae count/g.

For coliform count MPN/g, one ml from each decimal dilution was inoculated into 3 fermentation tubes containing 5 ml of lauryl sulphate tryptose (LST) broth and inverted Durham's tubes. The inoculated and control tubes were incubated at 37°C for 24 h. The positive tubes showing gas production were recorded. Further, a loopful from each positive tube was transferred into another fermentation tube containing brilliant green bile lactose broth (2%) and incubated at same conditions. The inoculated positive tubes showing gas production were recorded. According to MPN tables, the results were recorded as the presumptive MPN of coliforms/g.

For *Campylobacter spp.*, pre-enrichment by transferring twenty five grams of each sample were aseptically moved to sterile stomacher bag containing 225 ml Bolton selective enrichment broth with 5% (v/v) lysed horse blood and antibiotics supplement: (cefoperazone 20 μg/ml, vancomycin 10 μg/ml, trimethoprim 10 μg /ml and cycloheximide 50 μg/ml) [10]. The bag contents were homogenized using a stomacher (Seward stomacher BA 7021, England) for 1 min. The content was aseptically transferred to a sterile 250 ml flask and incubated for 4 h at 37°C, followed by further incubation at 42°C for 48 h under microaerophilic condition (5% oxygen, 10% carbon dioxide and 85% nitrogen). Loopfulls from the previously incubated broth cultures were streaked on modified *Campylobacter* charcoal deoxycholate Agar (CCDA, Biolife Italiana) base plates supplemented with 10 mg/L of amphotericin B and 32 mg/L of cefoperazone (Biolife, Italiana). The inoculated plates were incubated for 48 h at 42°C under microaerophillic conditions, suspected *Campylobacter* colonies were appeared as round to irregular with smooth edges, showing thick translucent white growth to spreading film-like transparent growth. *C. jejuni* strains produced grey, moist flat spreading colonies (some strains had a green hue or a dry appearance, with or without a metallic sheen). However, *C. coli* strains tend to be creamy-grey in color, moist, slightly raised and produced discrete colonies. The obtained purified isolates were identified microscopically by phase-contrast microscope

for characteristic spiral or curved slender rods with a corkscrew-like motility, for Gram's staining *Campylobacter spp* are Gram's negative, typically curved or "S" shaped rods (gull wings). Biochemical identification was proceeded using catalase, oxidase, triple sugar iron test, lead acetate strip, growth in the presence of 1% glycine, nitrate reduction, hippurate hydrolysis test, nalidixic acid resistance and growth temperature tolerance (Table 1).

Test	C. jejuni	C. coli
Growth at 25°C	-	-
Growth at 35°C	+	+
Growth at 42°C	+	+
Nitrate reduction	+	+
H_2S- lead acetate strip	+	+
H_2S- TSI	-	-
Catalase test	+	+
Oxidase test	+	+
Growth in 1% glycine	+	+
Hippurate hydrolysis	+	-
Resistance to Nalidixic acid	S	S

Table 1: Biochemical tests for identification of Campylobacter spp. (-): negative growth/negative reaction, (+): positive growth/positive reaction, S: Susceptible.

Serotyping was performed according to Oyarzabal et al. [11] with commercial latex agglutination kits namely Dry spot *Campylobacter* (Oxoid, Basingstoke, Hampshire, England).

Genomic DNA extraction and PCR analysis

Genomic DNA extraction was done using bacterial DNA extraction Kit (Spin column) (BioTeke Corporation, China) in Dokki National Research Centre, Dokki, Cairo, Egypt. The PCR amplification for Cytolethal distending toxins (cdtA, cdtB and cdtC) was performed according to El-Jakee et al. [12] by using specific primers (Pharmacia Biotech) which were described in Table 2.

Statistical analysis

Using SPSS-14, one-way analysis of variance (ANOVA) was performed to compare the samples while differences among individual means were compared by Tukey HSD test at 95% level of confidence; $P < 0.05$ was considered as significant.

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)
cdtA (F)	5′ AGGACTTGAACCTACTTTTC ′3	
cdtA (R)	5′ AGGTGGAGTAGTTAAAAAC ′3	631
cdtB (F)	5′ ATCTTTTAACCTTGCTTTTGC ′3	
cdtB (R)	5′ GCAAGCATTAAAATCGCAGC ′3	714

cdtC (F)	5′ TTTAGCCTTTGCAACTCCTA ′3	
cdtC (R)	5′ AAGGGGTAGCAGCTGTTAA ′3	524

Table 2: Primer sequences of virulence associated genes of *C. jejuni* tested in the present study.

Results

In this study, APC, EC, MPN of coliforms were used as indicators for the hygienic status of commercial and probiotics-treated and untreated chicken carcasses. The achieved results declared that the ranges (log cfu/g) of APC in the examined chicken carcasses were 3.99 to 5.94 (commercial), 3.70 to 5.71 (untreated) and 3.70 to 3.99 (treated) (Table 3). The recorded mean counts (log cfu/g) were 5.11 ± 0.15, 4.92 ± 0.15 and 3.83 ± 0.02 in the commercial, probiotics-untreated and treated chicken carcasses, respectively. EC of these three groups ranged from 3.15 to 4.86 (commercial), 2.96 to 4.77 (untreated) and 0.00 to 3.42 (treated), respectively. The mean values of the EC were 4.07 ± 0.11, 3.86 ± 0.13 and 2.61 ± 0.22-log cfu/g in the commercial, probiotics-untreated and treated chicken carcasses, respectively. MPN of coliforms (log MPN/g) ranged from 2.64 to 4.53 (commercial), 2.46 to 4.30 (untreated) and 0.00 to 2.53 (treated), respectively with mean values of 3.60 ± 0.14, 3.35 ± 0.16 and 1.05 ± 0.30 log MPN/g in these three groups, respectively (Table 3).

Campylobacter spp. were isolated from 4 out of 60 (6.7%) from both commercial, and probiotics-untreated chicken carcasses, while probiotics-treated chicken were free from *Campylobacter spp.* (Table 3). Serological identification of the isolated *Campylobacter spp.* revealed that *C. jejuni* is the predominant strain in both commercial (6.7%) and untreated chicken carcasses (5.0%), while *C. coli* was identified only in untreated chicken carcasses at 1.7% (Table 4).

A multiplex PCR was designed to confirm the expression of toxin-associated genes (cdtA, cdtB and cdtC) in *C. jejuni*. It was found that the identified *C. jejuni* isolates expressed at least one of these toxin-associated genes (Figure 1).

Samples	Aerobic plate count (APC)	Enterobacteriaceae count (EC)	Coliform (MPN)	Campylobacter spp
	Mean ± SE	Mean ± SE	Mean ± SE	Prevalence
Commercial	5.11 ± 0.15a	4.07 ± 0.11a	3.60 ± 0.14a	4 (6.7%)
Untreated	4.92 ± 0.15a	3.86 ± 0.13a	3.35 ± 0.16a	4 (6.7%)
Treated	3.83 ± 0.02b	2.61 ± 0.22b	1.05 ± 0.30b	ND

Table 3: Microbial status of commercial, probiotics-untreated and treated chicken carcasses. Counts are in log cfu/g; n=60 each, SE is the standard error of mean, ND: Campylobacter is not detected in probiotics-treated samples but detected in both commercial and untreated samples, a-b Means within the same column carrying different superscript are significantly different at P<0.05.

Sample	Latex Agglutinating Kit Observation	Identified strain	Positive samples			
			Biochemical		Latex agglutinating	
			No.	%	No.	%
Commercial	Agglutination clumps	*C. jejuni*	4	6.7	4	6.7
Untreated	Agglutination clumps	*C. jejuni*	3	5	3	5
		C. coli	1	1.7	1	1.7
		Total	4	6.7	4	6.7
Treated	Agglutination clumps	–	–	–	–	–

Table 4: Latex Agglutination test for identification of *Campylobacter* serotypes isolated from the examined commercial, probiotics-untreated and treated chicken carcasses.
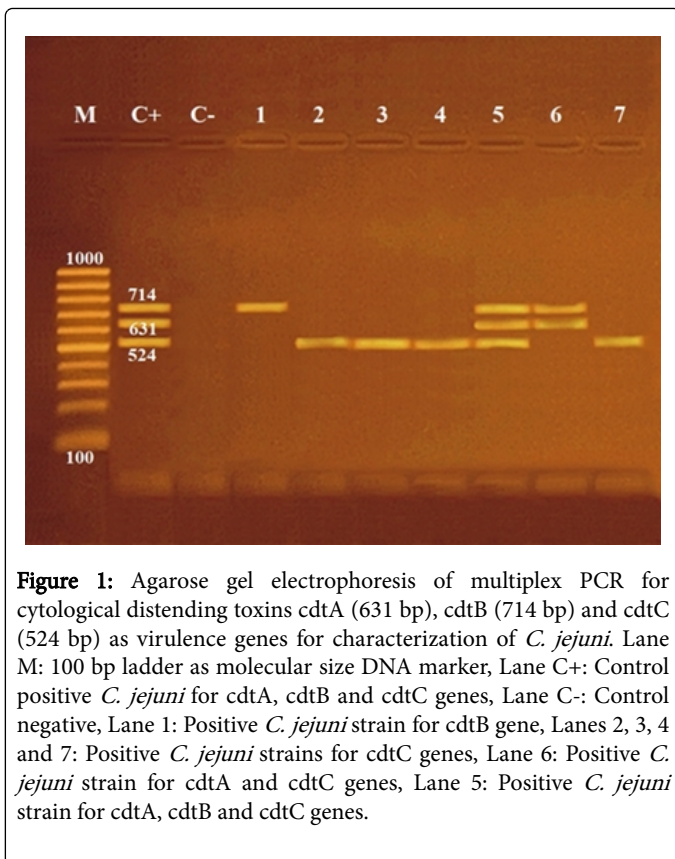
Figure 1: Agarose gel electrophoresis of multiplex PCR for cytological distending toxins cdtA (631 bp), cdtB (714 bp) and cdtC (524 bp) as virulence genes for characterization of *C. jejuni*. Lane M: 100 bp ladder as molecular size DNA marker, Lane C+: Control positive *C. jejuni* for cdtA, cdtB and cdtC genes, Lane C-: Control negative, Lane 1: Positive *C. jejuni* strain for cdtB gene, Lanes 2, 3, 4 and 7: Positive *C. jejuni* strains for cdtC genes, Lane 6: Positive *C. jejuni* strain for cdtA and cdtC genes, Lane 5: Positive *C. jejuni* strain for cdtA, cdtB and cdtC genes.

Discussion

Broiler chicken carcasses are liable to contamination with various kinds of spoilage microorganisms during handling, transportation and processing. Such contamination may render the chicken meat unsafe to consumer or impair its quality. In the present study, microbial examination of broiler chicken carcasses served in Dakahliya governorate, Egypt revealed high microbial load indicated by the high APC, EC and MPN of coliforms in commercial samples which collected from different markets and from probiotics-untreated chicken carcasses (experimental group). This may indicate poor hygienic standards inside the markets and unsanitary measures during meat preparation, handling, transportation and processing. Administration of probiotics 48 h prior to slaughter has a great effect on reduction of the microbial load in the chicken carcasses by competitive exclusion of pathogenic microorganisms in the intestinal tract of the live birds. In agreement with such results Kabir [13] and Ebeid et al. [14] reported lower values of the microbial population of broiler carcasses after administration of probiotics and significant improvement in the carcass quality.

Campylobacter is one of the most common causes of human bacterial enteritis worldwide; it is a natural inhabitant in the intestinal tract of live birds and can contaminate chicken carcasses via cross contamination from the intestinal tract due to low hygienic standards. In our study, *Campylobacter spp* was detected in the commercial and probiotics-untreated chicken carcasses. Isolation of *Campylobacter spp*. from chicken carcasses is an indication of bad hygienic measures during carcass preparation and evisceration as poultry is the main source of Campylobacteriosis. Strains of *C. jejuni* harboring toxin-producing genes are among the most common causes of acute gastroenteritis in humans in both developed and developing countries, particularly, in children and immunocompromised patients. In the present study *C. jejuni* isolates a harbored toxin producing gene which represents a major risk for consumers of such contaminated meat. Interestingly, administration of probiotics successfully eliminated *Campylobacter* species in the carcasses of the probiotics-treated group. Such results go in agreement with previously published reports [15-17], which indicated the value of the probiotics in reducing the colonization of *Campylobacter spp*. In the chicken intestinal tract.

Conclusion

The results achieved in this study revealed unsatisfactory hygienic measures followed during preparation of the chicken carcasses as indicated by the high microbial load of APC, EC, MPN of coliforms and isolation of *Campylobacter spp*. in both commercial and probiotics-untreated chicken carcasses in Dakahliya governorate, Egypt. On the other hand, probiotics-treated chicken carcasses with multispecies probiotic 48 before slaughtering showed a significant improvement in the microbial quality of the treated carcasses as indicated by the high reduction in the microbial load and the abscence of *Campylobacter spp*. Thus, application of probiotics during the life cycle of the chicken, particularly 24 h prior to slaughter is highly recommended.

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References

1. Bordoni A, Danesi F (2017) Poultry meat nutritive value and human health. J Sci Direct 11: 279-290.

2. Barbut S (2002) Poultry meat processing and product technology. Poultry Products Processing, pp: 1-29.

3. World Health Organization (WHO) (2004) Regional office for Africa: Developing and maintaining food safety for change, Second FAO/WHO global forum of food safety regulators. Bangkok, Thailand, pp: 12-14.

4. Ghafir Y, China B, Dierick K, De Zutter L, Daube G (2008) Hygiene indicator microorganisms for selected pathogens on beef, pork, and poultry meats in Belgium. J Food Protect 71: 35-45.

5. Carvalho A, Ruiz-Palacios G, Ramos-Cervantes P, Cervantes L, Jiang X, et al. (2001) Molecular characterization of invasive and noninvasive Campylobacter jujeni and Campylobacter coli isolates. J Clin Microbiol 39: 1353-1363.

6. Menten JFM (2001) Alternative additives in poultry nutrition: probiotics and prebiotics. Animal production in the view of Brazilians, Piracicaba, pp: 141-157.

7. Stern NJ, Cox NA, Bailey JS, Berrang ME, Musgrove MT (2001) Comparison of mucosal competitive exclusion and competitive exclusion treatment to reduce Salmonella and Campylobacter spp. colonization in broiler chickens. Poult Sci 80: 156-160.

8. Fellah JS, Jaffredo T, Nagy N, Dunon D (2014) Development of avian immune system. Avian Immunology. 2nd edn. Academic Press, San Diego, USA, pp: 45-65.

9. American Public Health Association (APHA) (2001) Compendium of methods for the microbiological examination of food. 4th edn. Washington.

10. Bolton FJ, Coates D, Hutchinson DN (1984) The ability of Campylobacter supplements to neutralize photochemically induced toxicity and hydrogen peroxide. J Appl Bacteriol 56: 151-157.

11. Oyarzabal O, Backert S, Nagaraj M, Miller R, Hussain S, et al. (2007) Efficacy of supplemented buffered peptone water for the isolation of Campylobacter jejuni and Campylobacter coli from broiler retail products. J Microbiol Methods 69: 129-136.

12. El-Jakee J, Atta N, Hakim A, Syame S, Omara SH (2008) Validation of PCR for detection of Campylobacter isolated from chicken. American-Eurasian J Agri Environ Sci 3: 748-753.

13. Kabir S (2009) Effect of probiotics on broiler meat quality. African J Biotechnol 8: 3623-3627.

14. Ebeid AT, Fathi MM, Al-Homidan I, Ibrahim Z, Al-Sagan A (2018) Effect of dietary probiotics and stocking density on carcass traits, meat quality, microbial populations and ileal histomorphology in broilers under hot-climate conditions. Animal Production J.

15. Ghareeb K, Awad WA, Mohnl M, Porta R, Biarnes M, et al. (2012) Evaluating the efficacy of an avian-specific probiotic to reduce the colonization of Campylobacter jejuni in broiler chickens. Poult Sci 91: 1825-1832.

16. Smialek M, Burchardt S, Koncicki A (2018) The influence of probiotic supplementation in broiler chickens on population and carcass contamination with Campylobacter spp. Field study. J Vet Sci 118: 312-316.

17. Willis WL, Reid L (2008) Investigating the effects of dietary probiotic feeding regimens on broiler chicken production and Campylobacter jejuni presence. Poult Sci 87: 606-611.