Evaluation of the sanitary status and prevalence of shiga-toxin producing *E. coli* in chicken meat products with a reduction trial using organic acids

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ABSTRACT

This study was undertaken to investigate the sanitary status of three chicken meat products namely, chicken fillet, chicken nuggets and chicken mince. Evaluation of the hygienic status of these products were done via estimation of total mesophilic count, total psychrophilic count, most probable number (MPN) of coliforms and *E. coli*. Isolation and identification of shiga-toxin producing *E. coli* were conducted. A trial for improvement of the sanitary status of the chicken fillets was performed using diluted acetic acid, diluted lactic acid and acid cocktail contains equal volumes of both acetic acid and lactic acids. The achieved results in this study declared unsatisfactory sanitary status for the chicken meat products marketed in Sharkia province, Egypt in terms of high mesophilic, psychrophilic, MPN of coliforms and *E. coli* counts. In particular, chicken mince had significantly the highest counts followed by chicken fillet and finally chicken nuggets. The contamination percentages of the examined chicken meat product samples with *E. coli* were 30%, 20% and 40% in chicken fillets, nuggets and mince, respectively. The isolated *E. coli* were further identified into four serotypes namely *E. coli* O2:H6, *E. coli* O26:H11, *E. coli* O119:H4 and *E. coli* O55:H7. The identified serogroups were further screened for shiga-toxin producing genes (stx1 and stx2) using multiplex PCR. A clear and significant reduction for the microbial load was achieved after dipping of the chicken fillets at the acid solutions, in particular at the acid cocktail 2%.

Keywords: shiga-toxin, *E. coli*, organic acids, chicken meat products

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1. INTRODUCTION

Chicken meat and meat products are considered among major sources of animal derived protein. Moreover, they supply humans with vast array of minerals, vitamins and polyunsaturated fatty acids. The industry of chicken meat products had increased worldwide, particularly in Egypt to fill the gap of shortage of the red meat (Fratianni et al., 2010). Foodborne illnesses due to bacterial pathogens and toxins are well known all over the world especially when foodborne diseases become correlated to the consumption of chicken meat and its products. The incidence of food-borne diseases is a direct indicator of the hygienic quality of food. Food contamination may occur during food processing or may be due to the use of contaminated raw material, since some bacteria that form part of the natural flora of poultry and cattle are pathogenic for humans as well (Darwish et al., 2015). Therefore, there is a special attention paid to the hygienic practices during production, storage and distribution (Aberle et al., 2001). *Escherichia coli (E. coli)* is a major component of the normal intestinal flora of animals which, if found in food of animal origin indicates fecal contamination. At the same time, *E. coli* is one of the food-borne pathogens, associated with several human illness outbreaks. Currently, *E. coli* strains are classified into six main pathotypes based on their distinct virulence determinants and pathogenic features, including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorragic *E. coli* (EHEC), Shiga toxin-producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and

diffusively adherent *E. coli* (DAEC) (Ruttler et al. 2006; Xia et al. 2010). Shiga toxin-producing *E. coli* are classified into two broadly categorized as shiga toxin producing *E. coli* (STEC) O157 and non-O157 STEC. While more than 100 serotypes of STEC have been associated with human disease, *E. coli* O157 causes 50 to 90% of cases, with most of the remaining cases caused by just six serotypes: O26, O45, O103, O111, O121 and O145 (Scallan et al., 2011). Organic acids such as acetic, propionic, citric and lactic acids are generally recognized as safe interventions and are used extensively by the meat and poultry industries to reduce bacterial contamination on carcass surfaces. These interventions have been found to be effective for immediately reducing foodborne pathogens such as *E. coli* O157:H7, *Salmonella Typhimurium*, and *Listeria monocytogenes* associated with meat or poultry surfaces (Fabrizio et al., 2002).

Therefore, this study was undertaken to investigate the sanitary status of three chicken meat products namely, chicken fillet, chicken nuggets and chicken mince. Evaluation of the hygienic status of these products were done via estimation of total mesophilic count, total psychrophilic count, most probable number (MPN) of coliforms and *E. coli*. Isolation and identification of shiga-toxin producing *E. coli* were further conducted. A trial for improving the sanitary status of the chicken fillets was performed using diluted acetic acid, diluted lactic acid and acid cocktail contains equal volumes of both acetic acid and lactic acids. Public health importance of the isolated organism was also discussed.

2. MATERIALS AND METHODS

2.1. Collection of Samples:

One hundred and twenty samples were collected randomly and equally from three chicken meat products marketed at retail shops in Sharkia province, Egypt. The examined chicken meat products were chicken fillet, chicken nuggets and chicken mince (n = 40 each, each sample weighs 100 g). The collected samples were transferred cooled directly without delay to the Microbiology laboratory at Food Control Department, Faculty of Veterinary Medicine, Zagazig University for bacterial isolation and identification.

2.2. Organoleptical examinations:

Organoleptical examination for the examined samples was conducted using the method of Varnam and Sutherland (1995). Samples with light pink color (with light yellow coating in case of nuggets), fresh odor and firm consistency were considered normal.

2.3. Microbiological examinations:

Samples were prepared according to the technique recommended by APHA (2001) as follows: under aseptic conditions, 25 g of each sample weighed and homogenized with 225 ml of 0.1% of sterile buffered peptone water (LAB104, LAB M, UK) for 1-2 minutes at 2000 rpm using sterile homogenizer (type M-p3- 302, mechanic, precyzina, Poland), such homogenate represents the dilution of 10^{-1}. The homogenate was allowed to stand for 5 minutes at room temperature. One ml from the original dilution (10^{-1}) was aseptically transferred to another sterile tube containing 9 ml of sterile 0.1% buffered peptone water and further tenfold decimal serial dilutions were prepared.

2.3.1. Total mesophilic Count (TMC):

Total mesophilic count was estimated using the method of APHA (2001). In brief, pipet one ml from each dilution to clean and sterile Petri dish. Add 12-15 ml of plate count agar (Difco Laboratories, Detroit, Michigan, USA) cooled to 45 ± 1 ºC for each Petri dish, mix well, then leave to solidify and incubate at inverted position for 48 h at 35 ± 2 ºC. Record all colonies including pinpoint size colony forming units as TBC in plates with 25-250 colonies per dish.

TMC/g = average No. of colonies × reciprocal of dilution

Counted colonies expressed as log cfu/g.

2.3.2. Determination of total psychrophilic count (TPC):

The pour plate technique recommended by APHA (2001) was applied using standard plate count agar medium and incubated at 7 ºC for 10 days. Results were calculated and recorded in the same way as TMC. Counted colonies expressed as log cfu/g.

2.3.3. Determination of most probable number (MPN) of Coliforms:

Three tubes most probable number (MPN) method (APHA, 2001) was adopted. In brief, one ml of each dilution was used to inoculate separately into three test tubes containing MacConkey broth with inverted Durham's tubes. The inoculated tubes were incubated at 37 ºC for 24-48 hrs. Positive tubes showing acid (yellow color) and gas production in inverted Durham's tubes were recorded. The most probable number of coliforms was calculated according to the recommended tables.

2.3.4. Determination of MPN of E. coli:

Loopfuls from previously positive tubes showing acid and gas productions were inoculated

into previously warmed tubes containing 7 ml *E. coli* (EC) broth (Himedia, Mumbai) at 44.5° C and incubated at 44.5°C for 24-48 hrs. Positive tubes, showing gas production, were used to calculate MPN of *E. coli* according to the recommended tables.

2.3.5. Isolation of Escherichia coli:

A loopful from each positive tube (acid and gas) of EC broth was streaked onto Eosin Methylene blue (EMB) agar (APHA, 2001). The inoculated plates were incubated at 37 °C for 24 hrs. Typical colonies of *E. coli* appear greenish, metallic with dark purple center. Suspected colonies were purified and subcultured onto nutrient agar slope and incubated for further investigations. Identification of isolates was done according to the method described before (APHA, 2001) based on staining and biochemical tests (Catalase, Oxidase, Indol, Methyl Red, VP test, Citrate utilization, Nitrate reduction, Urease, H2S production, Gelatin liquefaction and Eijkman test).

2.3.6. Serodiagnosis of E. coli:

The confirmed *E. coli* isolates were serologically identified according to Kok *et al.* (1996) by using rapid diagnostic *E. coli* antisera sets (Difco, Detroit, MI, USA) for diagnosis of the Enteropathogenic types.

2.3.7. DNA preparation:

Bacterial DNA was extracted from each of glycerol stock serodiagnosed *E. coli* according to the method described before (Darwish et al, 2015).

2.3.8. Detection of shiga-toxin producing genes in the identified isolates:

Detection of shiga-toxin producing genes in the identified *E. coli* isolates was carried out using multiplex polymerase chain reaction (multiplex PCR). Primer sequences and amplified products sizes were shown in table 1. The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). PCR assays were carried out using the method of Dhanashree and Mallya (2008) in 1 ml of nucleic acid template prepared by using reference EHEC isolates (approximately 30 ng of DNA), 10 mM Tris-HCl (pH 8.4), 10 mM KCl, 3 mM MgCl2; 2 mM concentrations of each primer, 0.2 mM concentrations of each 29-deoxynucleoside 59-triphosphate, and 4 U of AmpliTaq DNA polymerase (Perkin-Elmer). Amplification conditions consisted of an initial 95 °C denaturation step for 3 min followed by 35 cycles of 95 °C for 20 secs, 58 °C for 40 s, and 72 °C for 90 secs. The final cycle was followed by 72 °C incubation for 5 min. The reference strains were *E. coli* O157:H7 Sakai (positive for stx1& stx2) and *E. coli* K12DH5α (a nonpathogenic negative control strain) that does not possess any virulence gene.

Table 1. Primer sequences of Shiga-toxin producing genes

Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
stx1 (F)	5′ ACACTGGATGATCTCAGTGG ′3	614	Dhanashree and Mallya (2008)
stx1 (R)	5′ CTGAATCCCCCTCCATTATG ′3		
stx2 (F)	5′ CCATGACAACGGACAGCAGTT ′3	779	Dhanashree and Mallya (2008)
stx2 (R)	5′ CCTGTCAACTGAGCAGCACTTTG ′3		

Amplified DNA fragments were analyzed by 2% of agarose gel electrophoresis (Applichem, Germany, GmbH) in 1x TBE buffer stained with Ethedium bromide and captured as well as visualized on UV transilluminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes.

2.3.9. Improvement of the bacteriological status of chicken fillet using acid solutions:

In a trial for reduction of the bacterial load of chicken fillet samples, diluted acids were used. Three sets of experiments were conducted to investigate the effect of different concentrations of each of acetic acid, lactic acid and acid cocktail (equal parts of acetic acid and lactic acid 1:1) on total mesophilic count, total psychrophilic count, MPN of coliforms and MPN of *E. coli*. Five chicken fillet samples (weigh 200 g/each) were used in each experiment. Each fillet was divided in to 4 equal pieces (50g/each). Each piece was immersed in either 0 (DDW), 0.5, 1.0 or 2% acid solution in distilled water for 30 min. Microbiological examination was conducted as mentioned before.

2.4. Measurement of pH:

Ten grams of each chicken fillet sample either immersed in the diluted acid solutions or control were homogenized with 40 ml distilled water in a blender for 30s. The homogenate was filtered and the pH value of the filtrate was determined using a digital pH meter (HI 98128; EC/ TDS., Hanna, Italy) standardized at pH 4 and 7 (Sallam and Samejima 2004).

2.5. Statistical analysis:

All values are expressed as means ± SD, and all measurements were carried out in duplicates. Bacterial counts were converted into base logarithms of colony forming units per g (log cfu/g). Statistical significance was evaluated using the Tukey–Kramer HSD test. In case of reduction experiments, measurements were compared with that of the control (DDW) using Dunnett's test (Gomez and Gomez, 1984). In all analyses, $P < 0.05$ was taken to indicate statistical significance using JMP statistical package; SAS Institute Inc., Cary, NC.

3. RESULTS

Organoleptical examination of the collected samples revealed that all samples had normal sensory parameters. The achieved results for microbiological examination of the collected samples in this study declared that the mean values of the total mesophilic counts were 5.25 ± 0.28, 3.59 ± 0.15 and 6.50 ± 0.21-log cfu/g in the examined chicken fillets, nuggets and mince, respectively (Figure 1A). The mean concentrations of the total psychrophilic counts in the examined chicken fillets, nuggets and mince were 3.69 ± 0.11, 2.60 ± 0.22 and 4.69 ± 0.18-log cfu/g, respectively (Figure 1B). By comparing these values with the maximum permissible limits (MPL) of total mesophilic and psychrophilic counts ($<10^4$ for both) decided by Egyptian Organization for Standardization (EOS, 2005), it is noted that 80%, 70% and 100% of the examined chicken fillets, nuggets and mince, respectively exceeded MPL of total mesophilic counts and 60%, 40% and 80% of these samples exceeded MPL of total psychrophilic counts (Table 2). Most probable number of coliforms (MPN) was estimated in the collected samples, the achieved results declared that the mean values of MPN of coliforms were 4.21 ± 0.14, 3.47 ± 0.25 and 4.85 ± 0.21-log MPN/g in the examined chicken fillets, nuggets and mince, respectively (Figure 2A). The most probable number of *E. coli* was further estimated using EC broth. The achieved results declared that MPN of *E. coli* in the examined samples were 3.25 ± 0.18, 2.11 ± 0.22 and 4.15 ± 0.19-log MPN/g in the examined chicken fillets, nuggets and mince, respectively (Figure 2B). It is recorded that all examined samples exceeded MPL of coliforms and *E. coli* in this study (Table 2). The incidence of *E. coli* in the examined samples was also screened, the achieved results declared that the contamination percentages of *E. coli* in chicken fillets, nuggets and mince were 30%, 20% and 40%, respectively (Figure 3A). The isolated *E. coli* were further identified into four serotypes namely *E. coli* O2:H6, *E. coli* O26:H11, *E. coli* O119:H4 and *E. coli* O55:H7. The isolation percentages for these serotypes were 22%, 45%, 11% and 22% respectively (Figure 3B). We further screened the identified serotypes for harboring shiga-toxin producing genes using multiplex PCR. The achieved results declared that *E. coli* O2:H6 expressed only stx1; *E. coli* O26:H11 and *E. coli* O55:H7expressed only stx2; however, *E. coli* O119:H4 harbored virulent genes associated with the production of both stx1 and stx2 (Figure 4). In a trial to improve the sanitary status of chicken fillet samples, diluted acetic acid, lactic acid, and acid cocktail were used. The achieved results declared that the total mesophilic count (Figure 5 A), total psychrophilic count (Figure 5 B), most probable number of coliforms (Figure 6 A) and most probable number of *E. coli* (Figure 6 B) and pH values (Figure 7) were significantly reduced ($p<0.05$) after immersion in either acetic or lactic acids especially at 2%. The highest reduction for the bacterial load percentage was achieved after incubation at the acid cocktail 2% ($p<0.001$).

Table 2: Incidence percentages of the examined samples exceeding permissible limits of Egyptian Organization for Standardization (EOS, 2005).

Parameter	EOS limit	Chicken fillet	Chicken nuggets	Chicken mince
Total mesophilic count	$< 10^4$	80%	70%	100%
Total psychrophilic count	$< 10^4$	60%	40%	80%
MPN of coliforms	$< 10^2$	100%	100%	100%
E. coli	0	30%	20%	40%

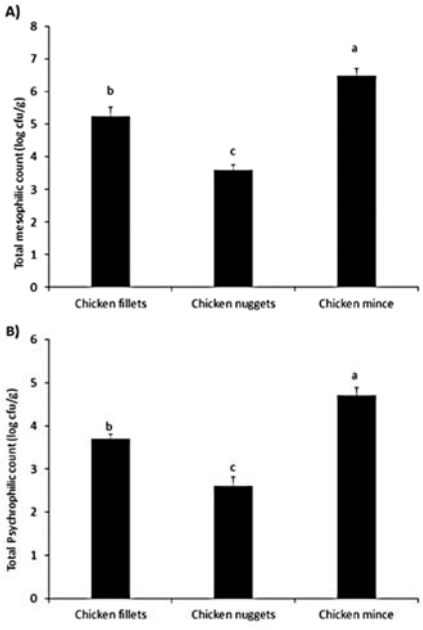

Fig. 1

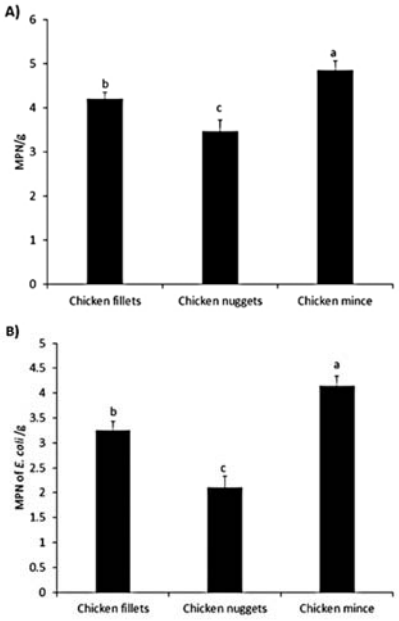

Fig. 2

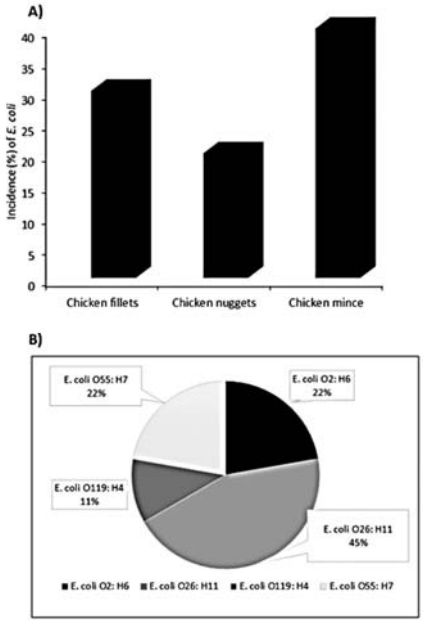

Fig. 3

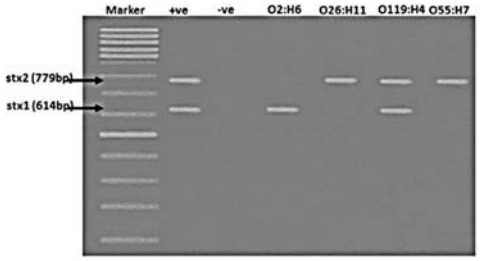

Fig. 4

Figure 1: Total mesophilic and psychrophilic counts in chicken meat products. A) Total mesophilic count, B) Total psychrophilic count. Values represent means ± SE (Log cfu/g) of forty samples from each of chicken fillet, nuggets and mince. Columns carrying different superscript letter differ significantly among examined samples at $P < 0.05$.

Figure 2. Most probable number of coliforms and *E. coli* in chicken meat products. A) Most probable number of coliforms B) Most probable number of *E. coli*. Values represent means ± SE (Log MPN/g) of forty samples from each of chicken fillet, nuggets and mince. Columns carrying different superscript letter differ significantly among examined samples at $P < 0.05$.

Figure 3. Prevalence of *E. coli* in chicken meat products. A) Incidence (%) of *E. coli* in chicken fillet, nuggets and mince (n=40 each) B) Incidence (%) of different *E. coli* serotypes identified in this study

Figure 4. DNA expression of shiga-toxins producing genes (stx1 & stx2) in the identified *E. coli* serotypes isolated from chicken meat products

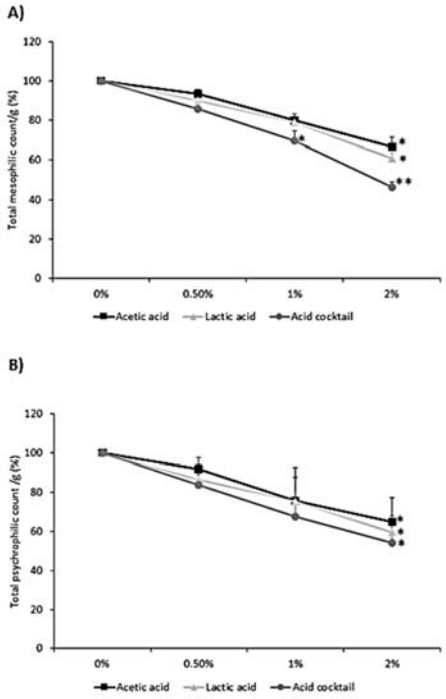

Fig. 5

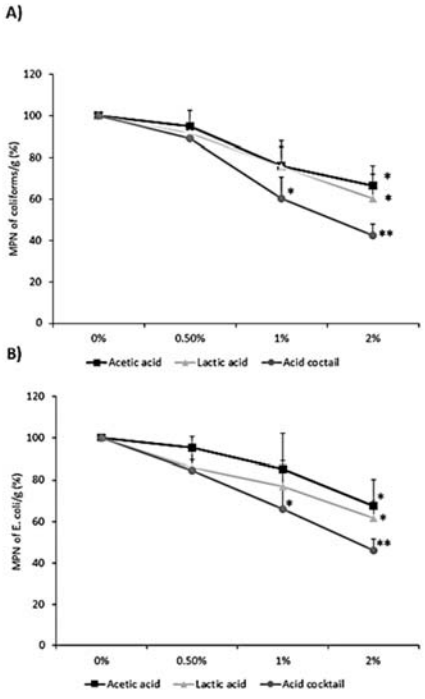

Fig. 6

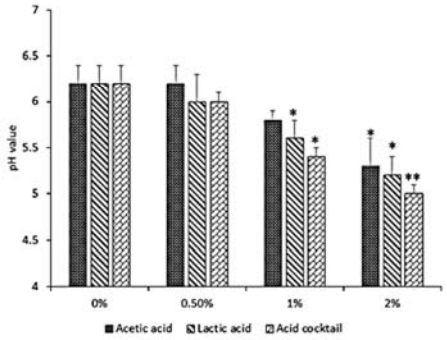

Fig. 7

Figure 5: Effect of organic acids on total mesophilic and psychrophilic counts in chicken fillet. Effect of different concentrations of acetic acid, lactic acid, and acid cocktail (equal volumes of acetic and lactic acids) on A) Total mesophilic count, B) Total psychrophilic count. Values represent mean ± SE (%) of five reduction trials for each concentration of the used organic acids. Markers carrying star mark is significantly different with the control (0%). * Significant at $p<0.05$; ** significant at $p<0.001$.

Figure 6: Effect of organic acids on most probable number of coliforms and *E. coli* in chicken fillet. Effect of different concentrations of acetic acid, lactic acid, and acid cocktail (equal volumes of acetic and lactic acids) on A) most probable number of coliforms, B) most probable number of *E. coli*. Values represent mean ± SE (%) of five reduction trials for each concentration of the used organic acids. Markers carrying star mark is significantly different with the control (0%). * Significant at $p<0.05$; ** significant at $p<0.001$.

Figure 7: Effect of different concentrations of organic acids on pH of chicken fillet. Effect of different concentrations of acetic acid, lactic acid, and acid cocktail (equal volumes of acetic and lactic acids). Values represent mean ± SE of five reduction trials for each concentration of the used organic acids. Columns carrying star mark is significantly different with the control (0%). * Significant at $p<0.05$; ** significant at $p<0.001$.

4. DISCUSSION

The sanitary status of the marketed chicken meat products reflects the hygienic conditions adopted during preparation and processing of such products and affect the entire shelf life and microbiological quality of the end products. Thus, it is highly recommended to conduct screening studies to investigate the sanitary status of the chicken and meat products on a regular basis.

There are many microbial indicators for the sanitary status of chicken meat products such as total mesophilic counts, total psychrophilic counts, most probable number (MPN) of coliforms and *E. coli*. These indicators give a clear image about the hygienic practices and measures adopted during product handling and processing (Mossel et al., 1995).

The achieved results in this study declared unsatisfactory sanitary status for the chicken meat products marketed in Sharkia province, Egypt in terms of high mesophilic, psychrophilic, MPN of coliforms and *E. coli* counts. In particular, chicken mince had significantly ($p<0.05$) the highest counts followed by chicken fillet and finally chicken nuggets. In agreement with the recorded results in this study, Kozacinski et al. *(*2006) found that the total mesophilic count in chicken breast fillets varied from 2.30 to 5.41-log cfu/g with an average of 4.72 ± 0.38-log cfu/g, in Crotia. Similarly, AL-Dughaym and Altabari (2010) tested 10 samples for each of breast fillet and nuggets collected from AL-Ahsa markets, Saudi Arabia. The examined chicken fillet samples had higher total mesophilic count compared with chicken nuggets. The mean values of the total mesophilic count were 6.79 and 4.43-log cfu/g in the chicken fillet and nuggets respectively. The high bacterial counts in the chicken mince compared with the nuggets recorded in this study seem reasonable as mincing process of chicken meat with ingredients other than the meat itself may lead to increasing the microbiological load of the produced chicken mince. Furthermore, mincing machine is considered as a possible source of transferring food-borne organisms from contaminated meat to non-inoculated ones (Papadopoulou et al., 2012). Unlikely, in case of nuggets, chicken meat is subjected to heat treatment during the manufacture process of the nuggets, which lead to reduction of the count of the indicator bacteria. However, despite having significantly, lower counts compared with chicken fillets and mince, presence of indicator organisms in chicken nuggets indicate post-processing contamination. In general, these results reflect the bad hygienic measures adopted during the manufacture process of the examined chicken meat products.

E. coli is considered major food-borne pathogenic bacteria responsible for hospitalization and deaths especially among children and elderly. For instance, in May 2011, an enteroaggregative *E. coli* O104:H4 outbreak occurred in Germany infecting more than 3000 peoples with 50 people dead (Frank et al. 2011). In 2014, a total of 12 persons infected with the outbreak strain of shiga toxin-producing *E. coli* O157:H7 in four of United States. This outbreak was linked to ground beef ingestion (CDC 2014a). In addition, another 19 persons were infected with the outbreak strain of shiga toxin-producing *E. coli* O121, that case was reported in six of United States due to raw clover sprouts (CDC 2014b). The incidence of *E. coli* in the examined samples was considered high and exceeding EOS (2005) recommendations as the contamination percentages of *E. coli* in chicken fillets, nuggets and mince were 30%, 20% and 40%, respectively. The isolated *E. coli* were further identified into four serotypes namely *E. coli* O2:H6, *E. coli* O26:H11, *E. coli* O119:H4 and *E. coli* O55:H7. We further screened the identified serotypes for harboring shiga-toxin producing genes and the achieved results declared that *E. coli* O2:H6 expressed only stx1; *E. coli* O26:H11 and *E. coli* O55:H7expressed only stx2; however, *E. coli* O119:H4 harbored both stx1 and stx2. In agreement with our results, Zhao et al. (2001) reported that of the 212 examined chicken samples, 82 (38.7%) yielded *E. coli* in Greater Washington area, USA. Furthermore, Dambrosio et al. (2007) identified that shiga toxin producing *E. coli* of non-O157 serogroups (O26, O103, O111) as the most substantial food poisoning pathogen groups, especially O26 that able to cause wide range of illness in human. In addition, Konishi et al. (2011) reported that the chief serogroups of ETEC isolated from outbreaks in Tokyo, Japan were O6, O27, O148 and O159, which were continuously detected after 1970s. Contamination of chicken meat products with *E. coli* could be resulted from mishandling of chicken carcasses, cross contamination from soil, cutting instruments, use of contaminated water for washing purpose or post-processing contamination (Chang et al., 2013).

In a trial to reduce the microbial load in chicken fillets, diluted acetic, lactic and acid cocktail were used. Interestingly, a clear and significant reduction for the microbial load was achieved, in terms of reduction of the total mesophilic, psychrophilic counts and MPN of coliforms and *E. coli*. The highest reduction for the bacterial load percentage was achieved after dipping in the acid cocktail 2%. It notes worthy to report that the used acid

concentrations did not alter the sensory characters of the chicken fillet examined. In agreement with this result Menconi et al. (2013) evaluated different combinations of organic acid (OA) wash solutions (acetic, citric, and propionic acid) for their ability to reduce bacterial contamination of raw chicken skin and to inhibit growth of spoilage bacteria and pathogens on skin during refrigerated storage. A significant ($P < 0.05$) differences were observed when skin samples were treated with the OA wash solution and no spoilage organisms were recovered at any given time point. Their results suggested that 0.2 to 0.8% concentrations of an equal-percentage mixture of this OA combination may reduce pathogens and spoilage organisms and improve food safety properties of the raw poultry. One possible explanation for the reduction in the microbial load of the chicken fillet using diluted acid solutions is due to lowering the pH value of the chicken meat products producing unfavorable media for growth and multiplication of the bacteria as clear in figure 7. In line with the recorded results in this study, Koutsoumanis et al. (2006) recorded a significant effect of meat pH on the growth kinetics of *pseudomonads, B. thermosphacta*, and *Enterobacteriaceae*. Although the reduction in the pH value was narrow (6.2 to 5.0) but it significantly reduced the bacterial load. Future studies should be taken to examine the effect of diluted acids on the lactic acid producing and acid tolerant bacteria. In conclusion, strict hygienic precautions should be adopted during processing, transportation and distribution of chicken meat products. Additionally, dipping of chicken products in diluted acid solutions can reduce the microbial load in such products.

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