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Monitoring of *Helicobacter pylori* in chicken products

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ABSTRACT

A total of 200 samples of chicken products collected randomly from Quesna and El-Bagour in Menoufia governorate. The collected samples were represented by chicken breast, thigh, gizzard and liver (50 of each) and subjected to bacteriological examination for detection of *Helicobacter* species particularly, *Helicobacter pylori*. Further, the effect of *L. rhamnosus* culture to control such serious pathogen contaminating chicken products was studied. *Helicobacter* spp., detected in 4%, 6%, 12% and 20% of breast, thigh, gizzard and liver, respectively and *H. pylori* represent 4%, 4% 6% and 10% from this examined samples, respectively. *H. pylori* inoculated chicken fillet (1×10^6 /g) which are not treated with *Lactobacillus rhamnosus* (control +ve) showed increase in prevalence of *H. pylori* 1.0×10^6, 1.3×10^6, 1.9×10^6 and 2.4×10^6 at zero time, 1st day, 2nd day and 3rd day, respectively. While chicken fillets treated with *L. rhamnosus* ($1x10^6$ /g) Effect on prevalence of *H. pylori* inoculated into chicken fillets with a reduction percent of 35%, 52% and 69% but *L. rhamnosus* ($1x10^7$ /g) showed remarkable reduction of *H. pylori* prevalence with a reduction percent of 54%, 67% and 81% at 1st day, 2nd day and 3rd day, respectively. *L. rhamnosus* effected on virulence genes of *H. pylori* isolated from treated chicken fillet as *L. rhamnosus* ($1x10^6$/g) destructed *cagA* gene and *L. rhamnosus* ($1x10^7$/g) destructed *cagA* and *vacA* genes so using of *Lactobacillus rhamnosus* culture as a method of control has a good effect on prevalence of *H. pylori* and its virulence genes in chicken fillets and *L. rhamnosus* ($1x10^7$ /g) more effective than *L. rhamnosus* ($1x10^6$ /g).

Introduction

In Egypt, chickens are a cheap and popular source of animal protein. Their edible offals are also in considerable demand because of their high biological value, reasonable cost, delectable taste, and simplicity of preparation. Since chicken is a common source of animal protein consumed by millions of people globally, hygienic ways of raising chickens are essential in maintaining public health (FAO, 2006).

One of the greatest dangers to food safety continues to be microbiological risk. Food must generally be wholesome, nourishing, and secure. Regulatory organizations and consumers have placed a lot of focus on the microbiological safety of chicken flesh and chicken giblets. Chicken products provide a highly favorable medium for a variety of contaminating microorganisms that may be dangerous to humans. *Helicobacter* was one of these (Momtaz *et al*. 2013).

It has been noted that *Helicobacter* species settle in the biliary tract and gut of numerous animals. *Helicobacter* species are Gram-negative, microaerophilic spiral bacteria that can be extremely harmful. These organisms are divided into two groups: stomach *Helicobacter* and enterohepatic *Helicobacter*, depending on where they prefer to colonize (Alfarouk *et al*., 2019). The human stomach serves as the primary location for *H. pylori* bacteria. Accordingly, foods derived from animals, particularly chicken meat, may have a significant role in the spread of *H. pylori* infections to people (Crowe, 2019).

Poultry meat contamination may occur at many points during the slaughter and processing processes (Smith, 2014; Mousavi *et al*., 2020). *H. pylori* can be found in chicken feces and has been proven in studies to be able to live in chicken gastrointestinal tracts (Hamada *et al*., 2018). Furthermore, in a separate study, found that *H. pylori* can survive in water (Ranjbar, 2018). If proper hygiene and sanitation practices are not followed during slaughter these bacteria can contaminate poultry meat. Consumption of poultry meat contaminated with *H. pylori* may increase the risk of gastrointestinal infections in humans (Zarinnezhad *et al*., 2021). Therefore, sanitary delivery methods for chickens produced for food are crucial for maintaining public health (Wong *et al*., 2017).

Probiotic bacteria are living microorganisms that, when present in high enough concentrations, contribute to the health of the host. In addition, probiotics and the metabolic products they produce are linked to a decreased risk of allergies, cancer, hepatic disease, *H. pylori* and urinary tract infections (Lollo *et al*., 2013; Nabavi *et al*., 2015).

Probiotic bacteria are thought to be vital for producing antimicrobial factors, which generally affects the activities of foodborne pathogens (Madureira *et al*., 2011). According to Claude *et al*. (2016), foodborne disease is a serious problem around the world, so this study was designed to identify *H. pylori* in chicken products and assess the impact of *Lactobacillus rhamnosus* culture on prevalence and virulence of *H. pylori* in chicken products as a method of control.

Materials and methods

Collection of samples

A total of 200 samples of chicken products collected randomly from Quesna and El-Bagour in Menoufia governorate. The collected samples were represented by chicken breast, thigh and gizzard and liver (50 of each) and subjected to bacteriological examination for detection of *Heli-*

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cobacter species particularly, *H. pylori*. Further, the effect of *L. rhamnosus* culture to control such serious pathogen contaminating chicken carcasses was studied.

Isolation of Helicobacter species (Stevenson et al., 2000)

Selective enrichment

Ten grams of the thoroughly mixed samples were weighed aseptically and inoculated into a sterile test tube containing 90 ml of selective enrichment broth *Helicobacter pylori* special peptone broth (HPSPB) containing selective supplement, which is Vancomycin 5.0 mg, Trimethoprim lactate 2.5 mg, Cefsulodin 2.5 mg and Amphotericin B 2.5 mg. The tubes containing the samples were homogenized using a vortex then incubated at 37°C for 48 h under microaerophilic condition in an atmosphere of 6% O_2, 10% CO_2 and 84% N2 by CO2 incubator.

Selective plating

From each enrichment culture, a loopful was streaked onto Columbia Blood Agar (CBA) plate supplemented with *Helicobacter* selective supplement and 5% defibrinated sheep blood. Streaked plates were incubated at 37°C for 3-5 days under appropriate microaerophilic conditions (6% O_2, 10% CO_2, and 84% N_2) with a maximum humidity by Co_2 incubator. After three to five days, all of the cultivated plates were examined. Suspected colonies grow slowly and weakly haemolytic on Columbia agar, small not exceeding 2 mm in diameter, translucent, circular and convex. All pure cultured colonies were subjected for further identification scheme.

Identification of Helicobacter species (Murray et al., 2003)

Morphological examination (ISO, 2013)

Films of pure suspected cultures were stained with grams stain and examined microscopically. Gram negative, none sporulating rod and coccoid shape (coccobacilli) were observed.

Biochemical identification

Biochemical tests for the differentiation between the various species of *Helicobacter* were shown in Table 1.

Experimental part

Accurately, 16 samples of chicken fillets (100g of each) were prepared. In general, *H. pylori* strain was obtained from Food Analysis Center, Faculty of Veterinary Medicine, Benha University, Egypt. Bacteria were subcultured on nutrient broth and incubated for 24 hours at 37°C. The cells were harvested by centrifugation (3000×g, 15 min), washed twice, and resuspended with sterile distilled water. For inoculation, 1 ml of the dense suspension of *H. pylori* was separately inoculated into the chicken fillet to limit of 1×10^6 /g and 1×10^7 /g, respectively. The samples were divided into 4 groups; the first was control negative, the second was control positive (inoculated with pathogen only, however, the third and fourth ones inoculated with *H. pylori* strain were treated with 1×10^6 /g and 1×10^7 /g. All tested groups were labeled and put in refrigerator at 4°C. Each group was subjected to bacteriological and sensory examination for 3 successive days at zero time (within 2 hours after treatment), 1, 2 and 3 days. The scheme was replicated for 3 times.

Table 1. Biochemical tests for identification of *Helicobacter* species.

Biochemical tests	*H. pylori*	*H. pullorum*	*H. cinaedi*
Oxidase test	+	+	+
Catalase test	+	+	+
Nitrate reduction	-	+	+
Alkaline Phosphatase test	+	-	-
Glutamyl trasferase test	+	-	-
Urease test	+	-	-
Growth at 42°C	-	+	-
Tolerance to 1% glycine	-	-	+
Resistance to Nalidixic acid	*R*	*S*	*S*
Resistance to Cephalothin	*S*	*R*	*I*

S: Sensitive; I: Intermediate; R: Resistanat

Sensory evaluation

The examined samples of chicken meat were examined according to procedures of the World's Poultry Science Association (1987). The suggested organoleptic approach of analyzing raw chicken meat was used by five skilled panelists. On a scale of 1 to 3, the distinct qualities were quantified. The sensory qualities that were examined on a sensory level included: the visual appearance (skin and meat color), meat firmness and suppleness, and odor.

Sensorial attribute values 3, 2, and 1 corresponded to raw chicken meat qualities evaluated as excellent, acceptable, and unacceptable.

Polymerase Chain Reaction (PCR)

Primer sequences of *Helicobacter pylori* were used for PCR system

The primers for detection of the virulence factors including endonuclease- replacing A (*hrgA*), cytotoxin- associated antigen (*cagA*) and vacuolating cytotoxin (*vacA*) genes of *Helicobacter pylori* were synthesized as follow (Table 2).

DNA Extraction using QIA amp kit (Chattopadhyay *et al*., 2004)

After overnight culture on nutrient agar plates, one or two colonies were suspended in an Eppendorf tube with 20 ml of sterile phosphate buffered saline and vortexed vigorously for 2 minutes. The tubes were then boiled in a water bath for 15 minutes, cooled in ice, and centrifuged at 13000 g for 1 minute. The supernatant was transferred to another tube from which 1 µl was used as the template for DNA amplification.

Table 2. Primers were used in the present study.

Target gene	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	References
hrgA (F)	5′ TCTCGTGAAAGAGAATTTCC ′3	594	Ando *et al*. (2002)
hrgA (R)	5′ TAAGTGTGGGTATATCAATC ′3		
cagA (F)	5′ GCGATTGTTATTGTGCTTGTAG ′3	499	Tiwari *et al*. (2007)
cagA (R)	5′ GAAGTGGTTAAAAAACAATGCCCC ′3		
vacA (F)	5′ ATGGAAATACAACAAACACAC ′3	259	Mughupadhyay *et al*. (2000)
vacA (R)	5′ CTGCTTGAATGCGCCAAAC ′3		

Amplification of virulence genes for *H. pylori* (Tiwari *et al*., 2007)

The Thermal Cycler was used for the amplification. (Master cycler, Eppendorf, Hamburg, Germany). Multiplex PCR was carried out in 25µl volumes using 10 ng of DNA, 1 U of Taq polymerase, 10 pmol of both oligonucleotide primers of all the selected genes (*hrgA*, *cagA* and *vacA*) per reaction, 0.25 mmol deoxynucleotide triphosphate and 2–3 mmol MgCl2 in standard PCR buffer for 35 cycles generally under the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 1 min, extension at 72°C for 1 min followed by final extension at 72°C for 7 min. DNA of *H. pylori* was used as positive control, while DNA isolated from non-pathogenic E. coli K12DH5α served as negative control. PCR products were electrophoresed in 20 g (w/v) agarose gel with 0.3% ethidium bromide in 10% Tris–borate–EDTA buffer. Gel was visualized under UV transilluminator. A 100 bp plus DNA Ladder was used to determine the fragment size.

Results

From the summarized results given in Table 3 it is evident that 4%, 6%, 12% and 20% of breast, thigh, gizzard and liver of chicken were contaminated with *Helicobacter* spp. The positive samples of breast were found to be 4% for *H. pylori*, while the positive samples of thigh were found to be 4% for of *H. pylori* and 2% for H. pullorum. Also, in the same table shows that gizzard sample was contaminated with *H. pylori*, H. pullorum and H. cinaedi with a percentage of 6%, 4% and 2%, respectively while liver samples was contaminated with *H. pylori*, H. pullorum, H. cinaedi and H. hepaticus with a percentage of 10%, 4%, 4% and 2% respectively.

In Table 4, the results recorded that *H. pylori* inoculated chicken fillets (1×10^6/g) which are not treated by *Lactobacillus rhamnosus* (control +ve) showed increase in *H. pylori* prevalence 1.0×10^6, 1.3×10^6, 1.9×10^6 and 2.4×10^6 at zero time, 1st day, 2nd day and 3rd day, respectively. While chicken fillets treated by *L. rhamnosus* ($1x10^6$ /g) showed decrease of *H. pylori* prevalence from 1.3×10^6 to 6.5×10^5 cfu/g , from 1.9×10^6 to 4.8×10^5 cfu/g and from 2.4×10^6 to 3.1×10^5 cfu/g with a reduction percent of 35%, 52% and 69% at 1st day, 2nd day and 3rd day, respectively. On the other hand chicken fillets treated by *L. rhamnosus* ($1x10^7$ /g) showed remarkable decrease of *H. pylori* prevalence from 1.3×10^6 to 4.6×10^5 cfu/g, from 1.9×10^6 to 3.3×10^5 cfu/g and from 2.4×10^6 to 1.9×10^5 cfu/g with reduced percentages of 54%, 67%, and 81% on the first, second, and third days, respectively.

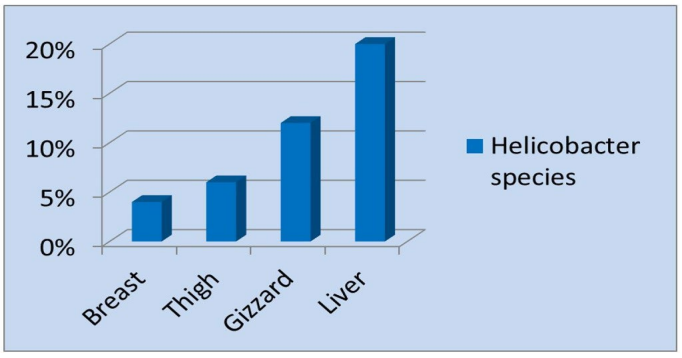

Fig. 1. Prevalence of *Helicobacter* species isolated from the examined samples of chicken products.

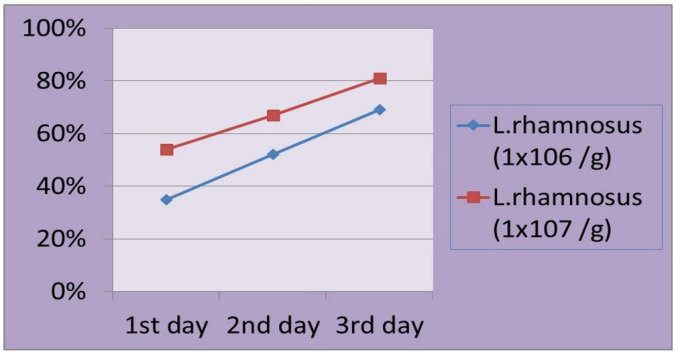

Fig. 2. Reduction % of *H. pylori* in chicken fillets treated by *L. rhamnosus*

Table 3. Prevalence of *Helicobacter* species isolated from the examined samples of chicken products (n= 50 of each).

Samples	Breast		Thigh		Gizzard		Liver		Total (200)	
Helicobacter sp.	No.	%	No.	%	No.	%	No.	%	No.	%
H. pylori	2	4	2	4	3	6	5	10	12	6
H. pullorum	0	0	1	2	2	4	2	4	5	2.5
H. cinaedi	0	0	0	0	1	2	2	4	3	1.5
H. hepaticus	0	0	0	0	0	0	1	2	1	0.5
Total	2	4	3	6	6	12	10	20	21	10.5

Table 4. Effect of *Lactobacillus rhamnosus* culture on viability of *H. pylori* inoculated into chicken fillets by intensity of 1×10^6 /g (n=3).

Storage time / Treatment	Control +ve	*L.rhamnosus* ($1x10^6$/g)	*L.rhamnosus* ($1x10^7$/g)
	Count	Count	R %
Zero time	$1.0\times10^6\pm0.1\times10^6$	$1.0\times10^6\pm 0.1\times10^6$	------
1st day	$1.3\times10^6\pm0.1\times10^{6\,A}$	$6.5\times10^5\pm 0.4\times10^{5\,B}$	35
2nd day	$1.9\times10^6\pm0.1\times10^{6\,A}$	$4.8\times10^5\pm 0.4\times10^{4\,B}$	52
3rd day	$2.4\times10^6\pm0.2\times10^{6\,A}$	$3.1\times10^5\pm 0.2\times10^{5\,B}$	69

Mean values with different superscripts in the same rows are significantly different at (P<0.05). R %= Reduction %

Table 5. Sensory characteristics of the examined samples of chicken fillets treated *Lactobacillus rhamnosus* culture.

Character / Treatments	External aspect (3)	Odor (3)	Color of skin (3)	Color of meat (3)	Meat elasticity (3)	Overall Score (15)	Sensorial Quality
Control –ve	2	1	2	3	1	9	Acceptable
L.rhamnosus ($1x10^6$/g)	3	1	2	2	2	10	Acceptable
L.rhamnosus ($1x10^7$/g)	3	2	2	2	3	12	Excellent

* 12-15: Excellent; 8-11: Acceptable; less than 8: Unacceptable.

Table 5 showed that treatment of chicken fillets by *L. rhamnosus* improve Sensory characteristics of it as Sensory characteristics of chicken fillets free from *H. pylori* (control –ve) and that treated with *L. rhamnosus* (1x10^6 /g) were acceptable but in samples treated by *L. rhamnosus* (1x10^7/g) were excellent.

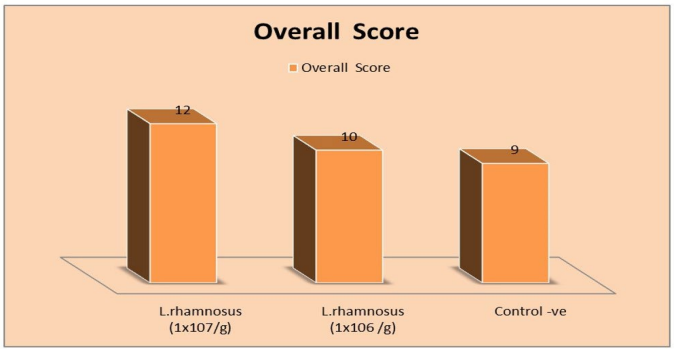

Fig. 3. Overall scores of sensory characteristics of control and treated chicken fillets with *L. rhamnosus* culture.

The Occurrence of the *hrgA* (594 bp), *cagA* (499 bp) and *vacA* (259 bp) genes in the *H. pylori* strains isolated from the examined samples of control and *L. rhamnosus* treated chicken fillets is showed in Fig. 4. Occurrence of *hrgA* gene was in control sample and sample treated by *L. rhamnosus* (1x10^6 /g) and *L. rhamnosus* (1x10^7 /g) but *cagA* gene present in *H. pylori* isolated from control chicken fillet only. Occurrence of *vacA* (259 bp) genes was in *H. pylori* strains isolated from control and chicken fillet treated by *L. rhamnosus* (1x10^6 /g).

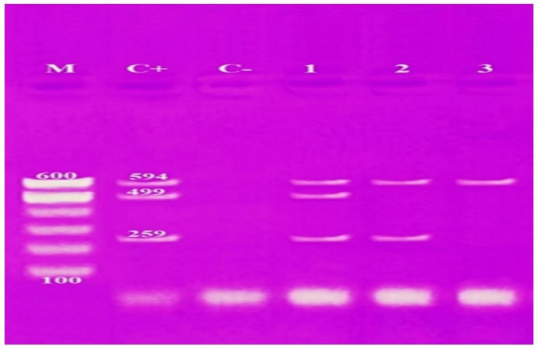

Fig. 4. Agarose gel electrophoresis of PCR of *hrgA* (594bp), *cagA* (499 bp) and *vacA* (259 bp) genes for characterization of virulence factors of *Helicobacter pylori* strains. Lane M: 100 bp ladder as molecular size DNA marker. Lane C+: Control positive *H. pylori* for *hrgA*, *cagA* and *vacA* genes. Lane C-: Control negative. Lane 1 (Untreated): Positive *H. pylori* for *hrgA*, *cagA* and *vacA* genes. Lane 2 (1x106 /g): Positive *H. pylori* for *hrgA* and *vacA* genes. Lane 3 (1x107 /g): Positive *H. pylori* for *hrgA* gene. *hrgA*: Endonuclease- replacing A gene. *cagA*: Cytotoxin- associated antigen gene. *vacA*: Vacuolating cytotoxin gene.

Discussion

Over the past few decades, there has been a lot of interest in the prevalence and survival of *H. pylori* in various foods. Studies addressing the occurrence of *H. pylori* in meat are rare, whereas the majority of published literature focused on milk and milk products (Quaglia and Dambrosio, 2018).

In current study, Results in Table 3 similar to that obtained by Gholami *et al.* (2015); El Dairouty *et al.* (2016); Hamada *et al.* (2018); Ali (2020); Ali (2020) and Sepehr *et al.* (2023).

On the other hand, Stevenson *et al.* (2000); Badr (2012) and Gholami *et al.* (2015) failed to detect *Helicobacter* species in chicken meat.

On contrast, higher results were recorded by Hassan *et al.* (2014); Ammar *et al.* (2015); Borges *et al.* (2015); Qumar *et al.* (2017) and Javed *et al.* (2019).

In chicken meat industry, cross-contamination of poultry meat is regarded as a major source of *H. pylori* infection; even though the actual cause of such a result is unknown but cutting, storing, and transporting poultry meat are the three main procedures that may raise the risk of *H. pylori* infection (Sepehr et el., 2023).

The results recorded in Table 4, indicated that *L. rhamnosus* (1x10^7 /g) had higher reduction effect on *H. pylori* than *L. rhamnosus* (1x10^6 /g).

In order to reduce *H. pylori* infection in the mouse model, probiotic bacteria such as L. acidophilus, *L. plantarum*, and *L. rhamnosus* may be effective (Asgari *et al.*, 2020).

Table 5 showed that treatment of chicken fillets by *L. rhamnosus* (1x10^7/g) improves Sensory characteristics of it.

Numerous researchers have discovered the various genotypes of *H. pylori* using molecular techniques; these genotypes are closely related to its epidemiology (Suerbaum and Josenhans, 2007). The genotyping and identification of conserved genes in *H. pylori* strains recovered from clinical samples often uses the Multiplex-PCR test (Espinoza *et al.*, 2011).

The present study evaluated effect of *L. rhamnosus* on the presence of some virulence genes in the *H. pylori* strains isolated from *L. rhamnosus* treated chicken fillets.

The possible connection between having *H. pylori hrgA /cagA/vacA* genotypes and the frequency of gastritis, gastric cancer and duodenal ulcers was proposed by Bibi *et al.* (2017).

Conclusion

Chicken products in this investigation were contaminated by different *Helicobacter* species particularly, *H. pylori* so raw chicken meat and giblets might be a source of *H. pylori* infection in human beings. Thus, good hygienic practices during slaughtering of chicken are very important and using *Lactobacillus rhamnosus* culture as a method of control has a good effect on prevalence of *H. pylori* and its virulence genes in chicken fillets and *L. rhamnosus* (1x10^7 /g) more effective than *L. rhamnosus* (1x10^6 /g).

Conflict of interest

The authors declare that they have no conflict of interest.

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