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Effect of thyme extract on some *Enterobacteriaceae* isolated from some meat products in Assuit city

Abstract

The present study was conducted to investigate the prevalence and numbers of *Enterobacteriaceae* in a total of 80 samples including 20 samples each of frozen beef burgers, frozen sausages, beef burger sandwiches and sausage sandwiches. The samples were randomly collected from retail supermarkets and restaurant in Assiut, Egypt. In addition, 20 stool cultures collected from hospitalized children admitted in Assiut Pediatric University Hospital with history of Diarrhea or fever. *Enterobacteriaceae* was detected in 16(80%), 20(100%), 8(40%) and 10(50%) of frozen beef burgers, frozen sausages, beef burger sandwiches and sausage sandwiches, respectively with mean values of 4.4×104, 6.3×104, 3 ×104 and 0.95×104CFU/g, respectively. The most prevalent isolates of *Enterobacteriaceae* were *Escherichia coli*, *Klebsiella pneumonia, Enterobacter cloacae, Citrobacter freundii and Enterobacter aerogenes*. Furthermore, the ethanol extracts of thyme were shown to have an inhibitory effect against *Escherichia coli*, *Enterobacter aerogenes* and *Klebsiella pneumoniae*. *Escherichia coli* were the most sensitive while *Klebsiella pneumoniae* was the most resistant. In conclusion, Natural substances that extracted from plants have applications in controlling pathogens in foods.

Keywords: Enterobacteriaceae, thyme, beef burgers, sausages, children stools, antimicrobial activity, pathogenic microbes, Enterobacteriaceae, isolation, pre-enrichment, biological safety

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Abbreviations: WHO, world health organization; RTE, ready-to-eat; RV, rappaport-vassiliadis; DHL, deoxycholate hydrogen sulfide lactose; SS, salmonella-shigella; TSI, triple sugar iron; LIA, lysine iron agar; DMSO, dimethyl sulphoxide; MIC, minimal inhibitory concentrations

Introduction

Food-borne infections still remain as one of the important concerns of public health worldwide. World Health Organization (WHO) has reported that 50million children under five years of age get diarrhoeal diseases each year from which nearly three million die and 40% to 60% of these diseases have been reported to be due to contaminated water and foodstuffs.[1]

Microbial contamination of raw and ready-to-eat (RTE) meat products by human pathogens is a consequence of a wide array of pre-harvest, harvest, and post-harvest processes. During slaughter, pathogenic bacteria may contaminate the carcass and subsequently be distributed via cut meat or raw meat materials intended for further processing into meat products.[2] Although through cooking kills the pathogens, cooked meat may become re-contaminated by food handlers during processing or from bacteria harbored in the environment. This represents a significant challenge in the RTE meat industry because consumers are not likely to sufficiently reheat these products to kill microbial pathogens.[3]

Fresh meat and meat products can be easily contaminated with microorganisms and, if not properly handled and preserved, support growth of spoilage and pathogen bacteria, leading to loss of quality and potential public health problems.[4] Refrigeration storage is usually the most common preservative method of fresh meat and meat products. In order to extend refrigerated storage time, antimicrobial and antioxidant additives especially of synthetic origin, are added to muscle foods. However, consumers increasingly demand use of natural products as alternative preservatives in foods, as the safety of synthetic additives has been questioned in last year's.[5]

Thyme (*Thymus vulgaris* l), an aromatic plant of the Labiateae family, has been long used in foods for culinary purposes. The most important compounds of thyme EO are the phenols thymol (44-60%) and carvacrol (2.2-4.2%), which constitute the major and more active constituents Di Pasqua et al.,[6] as well as the monoterpene hydrocarbons ρ-cymene (18.5-23.5%) and γ-terpinene (16.1-18.9%).[7] *In vitro* studies showed that these compounds posses antimicrobial activity against a broad spectrum of gram negative or positive bacteria.[8] The aim of this work was to assess to what extent frozen and ready-to- eat (RET) meat products and children stool in Assuit Governorate are contaminated by *Enterobacteriaceae* group, and to determine the antimicrobial activity of ethanolic extracts of thyme on selected pathogenic microbes isolated from the samples.

Materials and methods

Collection of samples

The food samples examined were obtained from different supermarkets and shops selling ready-to-eat meat in Assiut Governorate. The samples were grouped in three categories. The first category consisted of 40 frozen meat product samples (20 samples each of beef burgers and sausages). The second category consisted of 40 thermally processed sandwiches (20 samples each of beef burgers

and sausages). The third category consisted of 20 stool samples collected from diarrheic children from different clinical laboratories and hospitals in Assiut Governorate. Samples were transferred directly without delay to the laboratory in an ice box for bacteriological examination.

Preparation of samples

At the laboratory, fresh samples were processed upon delivery. The frozen samples were thawed by overnight refrigeration; each sample was aseptically and carefully freed from its casing and mixed thoroughly in sterile mortar.

Enumeration of Enterobacteriaceae

Meat samples were analyzed using the method described in the British Standards BS5763. Samples were well homogenized, ten grams of homogenate were stomached (230rpm/1min) with 90ml buffered peptone water (Himedia, Mumbai), to make the sufficient ten-fold dilutions.1ml aliquots of each dilution was pipetted into sterile Petri dishes. They were then pour plated in duplicate using violet red bile glucose agar (VRBGA) (Oxoid) and overlaid with the same media. Plates were incubated at 37°C for 24h after which those plates containing typical *Enterobacteriaceae* colonies (pink or red, with or without precipitation haloes) were counted.

Isolation and identification of Enterobacteriaceae

Pre-enrichment procedure: Twenty five grams of each meat product samples were homogenized in 225ml Lactose broth (Oxoid, CM137) using a stomacher bag for at least 2min. into a stomacher (Colworth, 400) and then they were incubated at 37°C for 24 h.

Enrichment procedure: One tenth ml and 1ml of the incubated pre-enrichment homogenate as well as swabs from children stools were aseptically added to 10ml Rappaport-Vassiliadis broth (RV) (Oxoid) and 10ml of MacConkey broth (Oxoid, CM5) as selective enrichment, respectively. RV broth incubated at 42°C for 24h and MacConkey broth incubated at 37°C for 24h.

Selective plating: Culture from each enrichment broth was separately streaked on plates of MacConkey Agar (Oxoid, CM7), Deoxycholate Hydrogen Sulfide Lactose (DHL) agar (Eiken Kizai, Tokyo, Japan) and Salmonella-Shigella (SS) agar and incubated at 37°C for 24h.

Confirmation: Characteristic colonies from each selective medium were picked, purified and tested biochemically on triple sugar iron agar (TSI) slant (Oxoid), lysine iron agar (LIA) slant (Merck), urea agar (Merck) , simmons citrate agar (Oxoid) and SIM Medium (Oxoid). Presumptive isolates were further confirmed by using API 20E (BioMerieux, France) identification system as described by the manufacturer.

Determination of antimicrobial activity of thyme

Preparation of the plant extract: Thyme was purchased from local retail markets in Assiut city. It was first cleaned using tap water and using sterile distilled water, then dried in laminar flow biological safety cabinet. The dried plants were crushed immediately before assay using an electric grinder. Organic extracts were prepared by soaking 50gm of the dried powder separately in 200ml of analytical organic solvents (Ethanol 70%), using a conical flask plugged. The mixture was kept at 20°C over night under continuous shaking at 130rpm, and were filtered through Whatman filter paper (No.2). The filtrates were evaporated using vacuum rotary evaporator. Stock solutions of crude ethanolic extracts were prepared by diluting the dried extracts with 10% Dimethyl Sulphoxide (DMSO) solution to obtain a final concentration of 10mg/ml.[9]

Antimicrobial test

Standard strain inocula: *E. coli, Enterobacter aerogenes* and *Klebsiella pneumoniae* were isolated from these studies. The strain was suspended in sterile nutrient broth at 37°C for overnight and 0.1ml of inoculum was added to sterile saline(0.85% sodium chloride) to bring the turbidity to 0.5 Mcfarland standard. The standard cell suspension containing approximately 1×10 cfu/mL was used for antimicrobial study.[1]

Screening of the plant extracts: plant extract was diluted (twofold serial dilution) using sterile saline supplemented with 0.2% Tween 80 according to the method described by Bagamboula CF et al.[11]

Minimal Inhibitory Concentrations (MIC): This test was performed with 12 different concentrations of plant extract (50%, 25%, 12.5%, 6.25%, 3.12% 1.56%, 0.78%, 0.39%, 0.2%, 0.1% 0.05% and 0.02%). The MIC was defined as the lowest concentration (highest dilution) of the of the extract that inhibited the visible growth (no turbidity), when compared to the control. It was obtained according to the method described by Quinn et al.[10]

Results and discussion

Food born infections are an important public health concern worldwide. According to reports of WHO,[12] every year a large number of people are affected by diseases due to contaminated food consumption. Wide spectrums of pathogens play a role in food borne disease. Foods of animal origin are considered major vehicles of food borne infections.[13]

Enterobacteriaceae group has an epidemiological interest and importance as some of them are pathogenic and may cause serious infections and/or food poisoning. The obtained data posed high contamination level of *Enterobacteriaceae* in frozen sausage (100%) and frozen beef burger samples (80%), with lower isolation frequencies from sausage sandwiches (50%) and beefburger sandwiches (40%). Our obtained results are in harmony with those obtained by Zaghloul et al.[14] who showed that, 50% of sausage sandwiches and 60% of burger sandwiches were contaminated with *Enterobacteriaceae*.

Enterobacteriaceae count used to assess the general hygiene status of a food product and their presence in heat treated food indicates inadequate cooking or post processing contamination.[15] It is also could indicates time/temperature abuse during handling or inadequate storage and displaying conditions during sale. As these microbial groups are safety indicators, the presence of high counts may indicate possible presence of pathogens.[16]

The obtained results in Table 1 showed that the higher average of *Enterobacteriaceae* count in the examined samples was recorded in frozen sausage samples (6.3×104cfu/g), while the lower one was in the examined sausage sandwiches samples (9.5×103cfu/g). Moreover, the average of *Enterobacteriaceae* count in the examined samples of frozen beef burgers and beef burger sandwiches were 4.4×104 and 3×104cfu/g. The current results were relatively agree to that obtained by Shaltot et al.[17] who found that the mean value of *Enterobacteriaceae* count in street vended sausage was 9.91×103cfu/g, and Al-Mutairi[18] who detected that the mean *Enterobacteriaceae* count was 5×104cfu/g

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in sausage. while lower results were recorded by Zaghloul et al.[19] who found that the mean value of street vended burger and sausage sandwiches were 31×102 and 35×102 cfu/g, respectively, and Gaafar et al.[20] revealed that the mean Enterobacteriacae count of hamburger and sausage was 5.8x103and 3.9 x104CFU/g, respectively.

Based on the microbiological guidelines of ready-to-eat food[15] out of 40 examined samples of ready-to-eat meat products 12.5% were of unsatisfactory quality due to the high level of *Enterobacteriaceae* count which exceeds the recommended limit (total count ≥104) as declared in Table 2. Regarding the results in Table 3, it is obvious that different species of *Enterobacteriaceae* were isolated in low incidence (5 %). While, the most prevalent isolates of *Enterobacteriaceae* were *Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Citrobacter freundii* and *Enterobacter aerogenes*. Furthermore, it follows from the foregoing that contamination of the examined food samples in this study with *Enterobacteriaceae* could be taken as an index of fecal pollution and could be attributed to the unsanitary practices and poor hygienic quality of ingredients used. It has been hypothesized that the reservoir for *Klebsiella oxytoca, K. pneumoniae, E. cloacae and Citrobacter species*, may be primarily environmental and from plant materials.[21]

Plants are used in different ways, e.g., as medicine and as food preservatives. Some authors indicated that their intake may be beneficial as food additives.[22,23] In this study, the antimicrobial activity of ethanol extracts of thyme against *Enterobacter aerogenes*, *Escherichia coli* and *K. pneumoniae* was evaluated. Investigation on the crude ethanol extracts of thyme showed different degrees of growth inhibition (Table 4), *Escherichia coli* proved to be the most susceptible organism followed by *Enterobacter aerogenes* and *K. pneumoniae*. Similar result of thyme was reported by Niculae et al.[24] who found that the ethanolic extract of thyme showed the highest antibacterial activity against *Escherichia coli* (0.125mg/ml). On the other hand Tirmidhi et al.[25] reported the antimicrobial activity of thyme was 25mg/ml for *Escherichia coli* and *K. pneumoniae.*

Table 1 Prevalence of *Enterobacteriaceae spp* from different meat product samples (n=20)

Type of samples	Positive samples		Min. ($\times10^1$)	Max. ($\times10^4$)	Mean±S.E.($\times10^4$)
	No.	%			
Frozen beef burgers	16	80	1	36	4.4±2.3
Frozen sausages	20	100	4	5.9	6.3±3.8
Beef burger sandwiches	8	40	1	58	3±2.9
Sausage sandwiches	10	50	2	58	0.95±0.33

Table 2 Acceptability of the examined ready-to-eat meat products according to the microbiological guidelines, (CFS, 2014) for Enterobacteriaceae counts (n=20)

	Range	Beef burger		Sausage		Total	
Categories	CFU/g	No.	%	No.	%	No.	%
Satisfactory	$<10^2$	14	70	12	60	26	65
Borderline	10^2-$<10^4$	5	25	4	20	9	22.5
Unsatisfactory	$\geq 10^4$	1	5	4	20	5	12.5

Table 3 Frequency distribution of Enterobacteriaceae species isolated from different meat product and children stool samples (n=20)

Enterobacteriaceae species	Frozen beef burgers		Frozen sausages		Beef burger sandwiches		Sausage sandwiches		Children stools	
	No.	%	No.	%	No.	%	No.	%	No.	%
Citrobacter diversus	_	_	1	5	_	_	_	_	_	_
Citrobacter freundii	4	20	4	20	_	_	3	15	6	30
Enterobacter aerogenes	1	5	_	_	4	20	2	10	_	_
Enterobacter agglomerans	_	_	_	_	1	5	1	5	_	_
Enterobacter claocae	4	20	3	15	6	30		20	4	20
Enterobacter gergoviae	_	_	_	_	4	20	4	_	_	_
Escherichia coli	9	45	6	30	1	5	_	5	12	60
Klebsiella pneumoniae	5	25	4	20	6	30	1	25	1	5
Klebsiella ornithinolytica	1	5		5	_	_	5	_		
Klebsiella oxytoca	1	5	1	15	2	10		5	_	_
Klebsiella ozaenae	1	5	3	5	2	10	_	5	_	_
Klebsiella rhinoscleromatis	_	_		_	1	5	1	_	_	_

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Table Continued

***Enterobacteriaceae* species**	**Frozen beef burgers**		**Frozen sausages**		**Beef burger sandwiches**		**Sausage sandwiches**		**Children stools**	
morganella morganii	2	10	1	_	_	_	1	_	_	_
proteus mirabilis	_	_	_	10	1	5		20	_	_
proteus vulgaris	_	_	_	_	_	_	_	_	2	10
providencia rettgeri	1	5		_	_	_	_	_	1	5
providencia stuartii	_	_	2	_	_	_	4	_	_	_
pseudomonas	1	5	_	_	_	_	_	_		5
salmonella spp.	1	5	_	5	_	_		5	1	_
Serratia marcescens	_	_	_	_	2	10	_	_		5
Serratia rubidaea	_	_	_	_	_	_	_	5	_	_
			1				_		1	_
			_				1		_	
			_				_		_	
							1			

Table 4 Determination of MIC (mg/ml) values of ethanolic extracts of thyme against tested bacteria

Organisms	**MIC**
Enterobacter aerogenes	6.25
Escherichia coli	0.4
Klebsiella pneumoniae	25

Conclusion

Increased knowledge of concern over leading to raise pointer to the effect of medical plant, herbs and their extracts in improving keeping quality of food as a preservative and their bacteriostatic and bactericidal against food borne microorganisms. To fulfill this only a small quantities would be required for this effect. This study opens up the possibility for the search of new antimicrobials as an alternative to the antibiotics. It is hope that this study positively participate in solving the problem of food contamination.

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Conflict of interest

The author declares no conflict of interest.

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