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REGULAR ARTICLE

BIOCHEMICAL CHARACTERIZATION OF BACTERIA AND FUNGI ISOLATES ASSOCIATED WITH POST-HARVEST SPOILAGE OF AVOCADO PEAR (*PERSEA AMERICANA)* SOLD IN TWO FRUIT MARKETS IN THE BENIN CITY METROPOLIS, NIGERIA

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ABSTRACT

Persea americana is a major and cheap source of nutrients-containing protein fruit and commonly referred to as Avocado pear. It is a green-skin, fleshy body and may be spherical or pear-shaped and ripens easily after harvest, significantly reducing its shelf-life. The average storage time before spoilage is 3-6 days. The fruit is highly cherished by many and as such a significant dietary contribution, in developing countries. However, the poor shelf-life of the fruit has led to its high perishability, huge post-harvest losses and market glut during harvest. In this study, fresh, undamaged, firm, healthy-looking, ripe avocado fruits purchased from Oba Market and New-Benin market were left free of dust and insects under room temperature for between 5-6 days to undergo a natural process of spoilage. A homogenate of each of the sample was achieved by blending 25 grams of the sample in 225 ml of sterile 1.5 % peptone water with a sterile glass blender. Serial dilutions of up to 10^{-1} - 10^{-5} were made and 1 ml of each of the dilutions were transferred into sterile Petri dishes and respectively mixed with 15 ml of an appropriate sterile media and incubated at a temperature of 37 ºC for 48 hours, while the Sauboraud dextrose agar (SDA) was left at room temperature for 5 days. After incubation, bacterial and fungal colony-forming units were counted and used to determine the total aerobic viable counts (TAVC), total coliform counts (TCC), *Escherichia coli* counts (EC), *Staphylococci* counts (SC) and total fungi counts (FC). Representative colonial isolates were subsequently subcultured on nutrient agar slants and stored at a temperature of 4 ºC prior to characterization. Phenotypic identification of microbes was performed according to standard methods. The present study revealed that the coliform bacteria (TCC = 2.42 x 10^3 cfu/g and 2.06 x 10^3 cfu/g) accounted for a significant fraction of the total bacterial population (TAVC = 2.75 x 10^4 cfu/g and 9.68 x 10^3 cfu/g) isolated from spoiled pear produce. Hence, *Erwinia and Klebsiella aerogenes* of genus of Enterobacteriaceae, were the main spoilage bacteria; while *Phytophthora*s species (FC = 1.73 x 10^4 cfu/g and 1.02 x 10^4 cfu/g) was the main spoilage fungus of pear produce sold in the two Nigerian markets. The isolation of pathogenic organisms also calls for a public health concern.

Keywords: Avocado Pear, Microbial, Post-Harvest Spoilage, *Persea americana*, Biochemical Characterization

INTRODUCTION

Persea americana commonly called Avocado pear is a tree native to Mexico and Central America **(Chen, Moriell, Ashworth, De La Cruz, & Clegg, 2008**) and a member of the family Lauraceae, which are mainly shrubs and trees that yield resinous aromatic gum from their cut bark (**Wogu & Ighile, 2014**). It is among the well-known indigenous fruit trees in the tropical and subtropical rain forest zone of the Southern regions of West Africa (extending eastward from Sierra Leone to Nigeria and Western regions of Central Africa, which includes Cameroon, Equatorial Guinea, Gabon, Democratic Republic of Congo, Congo Brazzaville, and Angola. Avocado or alligator pear which also refers to the fruit is a large berry that contains a single seed (**Wogu & Ighile, 2014**).

Avocados have a green-skin, fleshy body that may be pear-shaped or spherical and they easily ripen after harvesting. The trees are partially self-pollinating and are often propagated through grafting to maintain a predictable quality and quantity of the fruit (**Eze & Chimaeze, 2014**). Avocado fruit is a major and cheap source of nutrients containing protein (2 g), moisture (72.23 g), fibre (6.7 g), fat (14.66 g) and carbohydrate (8.53 g) and high energy value of 160 kcal per 100 g. They are also rich in fatty acids, amino acids, potassium, B-vitamins, vitamins K and E. Avocado fruit is much cherished by many people and it makes a significant dietary contribution, as it improves the food problems in developing countries. Besides, it is available at most seasons including strategic periods of the year when conventional staples that are difficult to store are scarce (**Okafor, 1975 in Wogu and Ighile, 2014**). The oils from the pulps and seeds are used in foods, pharmaceuticals and cosmetics manufacturing as well as numerous industrial uses. They are rich in monounsaturated fatty acids and are comparable to other currently used vegetable oils (**Lopez, *et al.*, 1996)**.

Avocados are commercially valuable and are picked hard and green and kept in coolers at 3.30 to 5.6 ºC until they reach their destination. Once picked, avocados ripen in a few days at room temperature. The fruit has a very short shelf-life and can averagely be stored 3-6 days before spoilage. The poor shelf life of the fruit has led to its high perishability, huge post-harvest losses and market glut during harvest as noticed by large heaps of unsold rotten fruits in the refuse dump of the village and urban markets. These characteristics of Avocado fruits are a serious setback for export market as well as industrial uses, as it does not offer flexibility throughout the market channels **(Wogu & Ighile, 2014**). It is estimated that one-fourth of all Avocado fruits harvested are not consumed before spoilage. Spoilage of fresh avocado fruits usually occurs during storage and transport and while waiting to be processed unlike many other fruits (**Eze & Chimaeze, 2014**).

The avocado fruit is vulnerable to bacterial, viral, and fungal diseases which lead to its spoilage. Disease and microorganisms can affect the fruit causing spotting, rotting, cankers, pitting and discolouration **(Abbott, 1999**). Numerous species of microorganisms easily attack the fruit. The composition of the avocado fruit influences the likely type of spoilage (**Eze & Chimaeze, 2014**). The high spoilage rate of Avocado fruit coupled with its high nutritional contents pre-supposes that an array of microorganisms may be involved in its spoilage of Avocado fruits.

The issue of food safety cannot be overemphasized when it comes to the consumption of food or fruits. Hence, in the purchase and consumption of fresh fruits, spoilage is often an issue of concern. Also, consumption of fruits spoilt by spoilage microorganism can lead to foodborne diseases such as infections and intoxications. Also, depending on the type of spoilage microorganism and the severity of the foodborne disease, death may occur.

Although, some researchers have worked on the microbiological and nutritional qualities of avocado pear, however, limited studies exist regarding the characterization of bacteria and fungi isolates associated with the post-harvest spoilage of avocado fruits sold in two popular fruit markets in the Benin City Metropolis. Hence, this research on the characterization **of bacteria and fungi Isolates associated with** Post-Harvest Spoilage **of Avocado fruits.**

MATERIALS AND METHODS

Sample Collection

Two samples each were purchased from two different markets (Oba Market and New-Benin Market). The avocado pear samples collected were fresh, undamaged, firm, healthy and ripe. The samples were dispensed into sterile bags and then brought to the laboratory. The samples were left free of dust, insect and

kept under room temperature for between 5-6 days to undergo a natural process of spoilage before being used in the study.

Isolation and Enumeration of Bacteria and Fungi

A homogenate of each sample was made by blending 25 grams of the sample in 225 ml of sterile 1.5 % peptone water with a sterile glass blender (**Public Health England, 2014**). Serial dilutions of up to 10^{-}-10^{-5} were made and 1 ml of each of the dilutions were transferred in to sterile Petri dishes and were respectively mixed with 15 ml of sterile molten nutrient agar medium, MacConkey agar medium, eosin methylene blue (EMB) agar medium, mannitol salt agar medium, and Sauboraud dextrose agar (SDA) medium amended with entrancing antibiotic. The inoculated nutrient, MacConkey, EMB, and mannitol salt agar Petri plates were then incubated at a temperature of 37 ^{o}C for 48 hours, while the SDA was left at room temperature for 5 days. After incubation, bacterial and fungal colony-forming units were counted and counts were used to deducing the total aerobic viable counts (TAVC); total cruciform counts (TCC), *Escherichia coli* counts (EC), *Staphylococci* counts (SC), and total fungi counts (FC).Representative colonial isolates were subsequently subcultured on nutrient agar slants and stored in the refrigerator at a temperature of 4 ^{o}C for characterization (**Bergey's Manual, 1984;1986**).

Identification and Characterization of the Microbial Isolates

Phenotypic identification of microbes was performed according to standard methods **(Bergey's Manual, 1984; Bergey's Manual, 1986)**.Morphological traits examined include the orientation, size, margins, shapes, and pigmentation (colour) which were performed by visual examination of microbial isolates on culture media, as well as cell wall characteristics which were performed by Gram staining of the isolates. The biochemical tests employed include: the production of coagulase enzyme (coagulase test);the production of catalase enzyme (catalase test),the ability of isolates to produced cytochrome oxidase (oxidase test),the production of urease enzyme (urease test), biodegradation of tryptophan to produce indole (indole test), utilization of citrate as a sole carbon source (citrate test), production of stable acids from glucose fermentation (methyl red test), production of acetoin as the main end product with small quantities of mixed acids from glucose metabolism (Voges Proskauer test), fermentation of mannitol (Mannitol test), the fermentation of lactose (Lactose test) and Microdase a modified oxidase test to that detects the enzyme oxidase.

RESULT

Microbial Load Obtained in Avocado Produce

Table 1 represents the microbial concentration of microbes associated with Avocado spoilage obtained from two markets in Benin City metropolis. The pear produce obtained from Oba market had the highest total aerobic viable counts (TAVC) ($2.75x10^4$cfu/g), while the lowest TAVC value was recorded in the pear produce obtained from New Benin Market ($9.68x10^3$) cfu/g). Total coliform counts were $2.42x10^3$ cfu/g and $2.06x10^3$ cfu/g for Oba and New Benin Market respectively No *Escherichia coli* count (ECC) was recorded for both New Benin and Oba Markets. *Staphylococcus* count (SC) was lowest ($0.7x10^1$cfu/g) in the New Benin Market and highest ($2.4x10^1$cfu/g) in the Oba market. Fungi count (FC) was also lowest ($1.02x10^4$cfu/g) in Oba market.

Table 1 Concentration of Microbes Associated with Avocado spoilage

Parameter	Sample (Oba Market)	Microbial Load (cfu/g)	Mean Microbial Load (cfu/g)	Sample (New Benin)	Microbial Load (cfu/g)	Mean Microbial Load (cfu/g)
TAVC	1	$4.5x10^4$	$2.75x10^4$	1	$3.25x10^3$	$9.68x10^3$
	2	$3.65x10^3$		2	$3.95x10^3$	
	3	$3.45x10^3$		3	$3.25x10^4$	
	4	$4.65x10^4$		4	$4.00x10^3$	
	5	$3.90x10^4$		5	$4.70x10^3$	
TCC	1	$4.7x10^3$	$2.42x10^3$	1	$3.50x10^2$	$2.06x10^3$
	2	$3.15x10^3$		2	$3.15x10^3$	
	3	$4.70x10^2$		3	$4.70x10^2$	
	4	$4.70x10^2$		4	$3.30x10^3$	
	5	$3.30x10^3$		5	$3.05x10^3$	
ECC	1	0	0	1	0	0
	2	0		2	0	
	3	0		3	0	
	4	0		4	0	
	5	0		5	0	
SC	1	$1.50\ x10^1$	$2.4x10^1$	1	$0.50x10^1$	$0.7x10^1$
	2	$3.50\ x10^1$		2	$0.00x10^1$	
	3	$4.50x10^1$		3	$2.00x10^1$	
	4	$1.50x10^1$		4	$1.00x10^1$	
	5	$1.00x10^1$		5	$0.00x10^1$	
FC	1	$1.75x10^4$	$1.73x10^4$	1	$1.20x10^4$	$1.02x10^4$
	2	$9.00x10^3$		2	$9.00x10^3$	
	3	$2.30x10^4$		3	$1.50x10^4$	
	4	$2.15x10^4$		4	$6.50x10^3$	
	5	$1.55x10^4$		5	$8.50x10^3$	

Key: TAVC: Total Aerobes Viable count; TCC: Total Colony Count; ECC: Escherichia Coli Count; SC: Staphylococcus Count; FC: Fungi Count; cfu/g: Colony Forming Unit per gram.

Morphological Identification of Microbial Isolates

Table 2 represents the phenotypic characterization of microbial isolates using morphological and biochemical methods. Six bacteria colonies with unique characteristics were respectively isolated from pear produce collected from Oba and New Benin markets respectively. Phenotypic characterizations identified *Erwinia* species, *Bacillus cereus* and *Staphylococcus aureus* as spoilage bacteria in the pear produce collected from both Oba and New Benin markets. *Pseudomonas aeruginosa* was seen only in pear produce obtain from Oba market. *Phytophthora* species was the main spoilage fungi in the pear produce collected from both Oba and New Benin markets.

Table 2 Phenotypic Characterization of Microbial Isolates Obtained from **Associated with The Spoilage of Avocado Pear**

Location	Microbial type	Isolates	Colonial characteristics	Microscopic characteristic	Biochemical tests											Probable Organism
					Co	Ca	Ox	Ur	Ci	In	Mr	Vp	Ma	La	Mi	
Pear produce from Oba market	Bacteria	1	Spreading mucoid colony on nutrient agar plate	Gram negative rods	-	+	-	-	+	-	-	+	-	+	Np	*Erwinia species*
		2	Seriated dry colony on nutrient agar plate	Gram positive rods	-	+	-	-	+	-	-	+	-	-	Np	*Bacillus cereus*
		3	Yellow pigmented colony on nutrient agar plate	Gram positive cocci in pairs and tetrads	-	+	+	+	-	-	+	+	-	+	+	*Micrococcus Species*
		4	Yellow pigmented colony on nutrient agar plate	Gram positive cocci in clusters	+	+	-	+	-	-	+	+	+	+	-	*Staphylococcus aureus*
		5	Mucoid colony on MacConkey agar plate	Gram negative rods	-	+	+	-	+	-	-	-	-	-	Np	*Pseudomonas aeruginosa*
		6	Yellow mucoid colony on mannitol salt agar plate	Gram positive cocci in clusters	+	+	-	+	-	-	+	-	+	-	-	*Staphylococcus aureus*
	Fungi	1	White fluffy and spreading colony that turned black with age	Coenocytic hyphae with intercalating chlamydospore	Np	Np	Np	Np	Np	Np	Np	Np	Np	Np	Np	*Phytophthora species*
Pear produce from New Benin market	Bacteria	1	Yellow mucoid colony on mannitol salt agar plate	Gram positive cocci in clusters	+	+	-	+	-	-	+	-	+	-	-	*Staphylococcus aureus*
		2	Spreading mucoid colony on nutrient agar plate	Gram negative rods	-	+	-	-	+	-	-	+	-	+	Np	*Erwinia species*
		3	Seriated dry colony on nutrient agar plate	Gram positive rods	-	+	-	-	+	-	-	+	-	-	Np	*Bacillus cereus*
		4	Pink mucoid colony on MacConkey plate	Gram negative rods	-	+	-	-	+	-	-	+	+	+	Np	***Klebsiella aerogenes*
		5	Yellow pigmented mucoid colony on nutrient agar plate	Gram positive cocci in clusters	+	+	-	+	-	-	+	-	+	-	-	*Staphylococcus aureus*
	Fungi	1	White fluffy and spreading colony that turned black with age	Coenocytic hyphae with intercalating chlamydospore	Np	Np	Np	Np	Np	Np	Np	Np	Np	Np	Np	*Phytophthora species*

Key: Co: coagulase; Ca: Catalase; Ox: Oxidase; Uri: Urease; Ci: Citrate; In: Indole; Mr: Methyl Red; Vp: Voges Proskauer; Ma: Mannitol; La: lactose; Mi: Microdase Test; Np: Not Performed; **previously *Enterobacter aerogenes*

DISCUSSION

The high concentration of bacteria and fungi in the spoilt avocado pear samples (Table 1) indicates that these microbes were the main cause of spoilage. Bacteria and fungi were found in high numbers mainly because of the indiscriminate exposure of the fruit's outer surface to the environment at the farms and in the market **(Buck, Walcott, & Beuchat, 2003**). This indiscriminate exposure coupled with the high nutritional content of the pear often resulted in an increased likelihood of contamination of the avocado pear that was propagated by flies, airborne dust, unhygienic human contacts, and damages to the fruit's outer surface (**Dreher & Davenport, 2013)**. The implicated spoilage bacteria and fungi were *Erwinia* species, *B. cereus*, *P. aeruginosa*, *S. aureus*, *Micrococcus* species, *Phytophthora* species and *Klebsiella aerogenes* (Table 2). **Eze & Chimaeze (2014)** also reported the presence of some of these spoilage organisms in avocado pear. The presence of *staphylococcus aureus* in virtually all the pear produce examined is an indication of human contamination of the pear produce from handling. *S. aureus* has been shown to produce enterotoxins that are extremely potent gastrointestinal toxins that can cause symptoms of intoxication when ingested. The wide spectrum of bacteria found in the spoilt avocado pear compared to fungi could be due to the low carbohydrate and high moisture contents of the pear (**Ihekoronye & Ngoddy, 1985**). Pre-harvest and post-harvest factors, consisting of the farm soil-type, storage conditions, and handling practices, maybe the likely source of these microbes **(CFS, 2006; Leff & Fierer, Bacterial Communities Associated with the Surfaces of Fresh Fruits and Vegetables, 2013**).

CONCLUSION

The present study revealed that the coliform bacteria accounted for a significant fraction of the total bacterial population isolated from spoiled pear produce. Hence *Erwinia and Klebsiella aerogenes*, of the genus of Enterobacteriaceae, were the main spoilage bacteria; while *Phytophthora* species was the main spoilage fungus of pear produce sold in the Nigerian markets. The isolation of several other pathogenic organism is a huge public health concern.

Author Contributions

O.A. designed the study and wrote the manuscript; O.S. conducted the experimental work and critical revisions; T. E Contributed to the manuscript

writing and Revisions; M.U contributed to revisions of the manuscript and study design.

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REGULAR ARTICLE

OCCURRENCE AND GENETIC IDENTIFICATION OF *ARCOBACTER BUTZLERI* IN DIFFERENT CHEESE TYPES

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ABSTRACT

The objectives of this study were to investigate the presence of foodborne pathogen *Arcobacter butzleri (A. butzleri*) in different types of cheese (Talaga, Mozzarella and Roumy cheese - 30 each) retailed in Assiut dairy shops, Egypt and to test the antimicrobial effect of thyme (*Thymus vulgaris*) and its essential oil (EO) against isolated strains. The highest percentage of *A. butzleri* existence was noticed in Talaga cheese samples and confirmed by PCR using *A. butzleri. 16S rRNA* gene which at the same time, the existence of it in Roumy cheese after detected biochemically. To exam the effect of *Thymus vulgaris* and its EO on *A. butzleri*, were added at 2 and 4% concentratins during cheese manufacturing with addition of *A. butzleri* at 10^5 and the consumer accepatability was studied. The plant could prohibit the growth of organism in cheese nearly at 6^{th} day. These results suggest that 2% of thyme plant have sufficient effect on *A. butzleri* with good consumer satisfaction so, it is recommended that e thyme should be used as natural food additive in dairy product in Egypt factories.

Keywords: *Arcobacter butzleri; Thymus vulgaris;* Talaga; Mozzarella and Roumy cheese

INTRODUCTION

Arcobacter spp. considered as emerging food- and waterborne zoonotic pathogens (**Hänel *et al.*, 2016**), aero-tolerant campylobacters' (**Levican and Figueras, 2013**). It's ability to grow between 15 to 30 °C temperature aerobically and need of microaerophilic condition for primary isolation (**Ferreira *et al.*, 2016**), are nearly similar to *Campylobacter* spp. in biochemical reaction which complicates their phenotypic differentiation. Hence, polymerase chain reaction (PCR)-based methods are more commonly used for specific detection and identification purposes (**Douidah *et al.*, 2010**) and currently *Arcobacter* spp. includes 21 species (**Giacometti *et al.*, 2015**). Among these species, *A. butzleri*, *A. cryaerophilus* (with two subgroups) and *A. skirrowii* which classified as serious hazards to human health by the International Commission on Microbiological Specifications for Foods (ICMSF) (ICMSF, 2002) as it associated with various illnesses such as gastroenteritis, abdominal pain, nausea, vomiting, bacteremia and sepsis in humans, mastitis, diarrhea, abortion, and other reproductive disorders in animals (**Vanderberg *et al.*, 2004; D'Sa and Harrison, 2005 and Girbau *et al.*, 2015)**.

In the Mediterranean countries, *A. butzleri* is a widespread in raw milk cheese production, including, particularly soft cheese (**Serraino and Giacometti, 2014**) and the organism also survived during processing and storage of water buffalo mozzarella cheese, fresh village cheese and sheep ricotta cheese **(Serraino *et al.*, 2013)**. The ability of this microorganism to survive in food products and water strengthened by its resistance to stress created during food storage and processing (**Ferreira *et al.*, 2019**), biofilms formation (**Assanta et al., 2002**) and can survive in pipe and food-processing surfaces (**Douidah *et al.*, 2010**). So, dairy researchers have found that selected plant Essential Oils (EO) can act as inhibitors of spoilage microorganisms in food products (**Smith *et al.*, 2001 and Conte *et al.*, 2007**) Especially with increasing the reports of resistance to current antibiotic employed in treatment of *Arcobacter* related infections there is need to develop new or alternative antimicrobial agents effective against it (**Smith *et al.*, 2003**).

The thyme plants belongs to the family of Labiatae (**Selmi and Sadok, 2008**) and EO or plant extracts originating from common or garden thyme (*Thymus vulgaris*) (**Wiese *et al.*, 2018**) is utilized as a flavor enhancer in a wide variety of foods, beverages and confectionery products (**Boskovic *et al.*, 2013**). It possesses some antiseptic, antispasmodic and antimicrobial properties that make it popular as a medicinal herb and as a preservative for foods (**Cosentino *et al.*, 1999**).

So, in this study focused on isolation of *A. butzleri* from different types of cheeses retailed in Assiut city markets, Egypt, with studying the effect of *Thymus vulgaris* and its EO at different concentrations on isolated *A. butzleri* .

MATERIAL AND METHODS

Sample collection

A total of 90 samples including random samples of different types of cheese soft cheese (Talaga cheese), semisoft (Mozzarella cheese) and hard one (Roumy cheese) 30 each, were collected from different markets and dairy shops in Assiut City, Egypt. The samples were collected in package as marketed to the consumer and sent to the laboratory in an insulated box with a minimum of delay to be examined.

Isolation of A. butzleri

The samples were homogenized by stomacher and prepared for Arcobacter isolation by taking 25 g of these samples and aseptically inoculated in a 1:10 ratio in Arcobacter enrichment broth (oxiod, UK) supplemented with Cefoperazone, Amphotericin B and Teicoplanin (CAT) selective supplement (SR0174, Oxiod, Uk), then incubated at 30°C under microaerophilic condition for 48 h. (Mottola et al., 2016). Then streaked onto Arcobacter selective media supplemented with 5% sheep blood and with CAT (Oxiod, Uk), the agar plates were incubated for 48 h at 30°C and samples of no growth were incubated for another 48h. (Aydin et al., 2007 and Ramees et al., 2014). Subsequently, presumptive Arcobacter colonies (small colourless, translucent, convex with an entire edge) were picked, sub-cultured onto blood agar and incubated at 30°C for 48h. Purified isolates were further confirmed morphologically by Gram staining and biochemical analysis (catalase, oxidase, urease tests and motility, indoxyl acetate hydrolysis, salt tolerance and growth on McConkey agar). The isolates referable at Arcobacter genus (Gram negative, spiral shaped, motile, oxidase and catalase positive, urease negative), were stored in 20% (v/v) nutrient broth glycerol at −80°C, after molecular identification (Ferreira et al., 2016 and Salas-Masso et al., 2016)

PCR confirmation

This part was done in molecular biology department (authorized by EGAC, ISO17025:2017) at Animal Health Research Institute, Dokki, Giza, Egypt.

The isolates were performed using the QIAamp DNA Mini kit (Catalogue no.51304, Qiagen, Germany, GmbH) with modifications from the manufacturer's recommendations. The spin-column procedure does not require mechanical homogenization, so total hands-on preparation time is only 20 minutes. Primers used were supplied from Metabion (Germany) are listed in table (I).

Table (I): Primers, sequences, target genes, and amplicon sizes

Target gene	Primer sequence (5'-3')	Length of amplified product	Reference
Arcobacter butzleri. 16S rRNA	CGTATTCACCGTAGCATAGC CCTGGACTTGACATAGTAAGAATGA	401 bp	**Lehmann *et al.*, (2015)**

Primers were utilized in a 25- µl reaction containing 12.5 µl of Emerald Amp Max PCR Master Mix (Takara, Japan), 1 µl of each primer of 20 picomole concentrations, 4.5 µl of water, and 6 µl of DNA template. The reaction was performed in an applied bio-system 2720 thermal cycler table (II).

Table (II): thermo cycling conditions

Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Arcobacter butzleri. 16S rRNA	94˚C 5 min.	94˚C 30 sec.	61˚C 40 sec.	72˚C 45 sec.	35	72˚C 10 min.

Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20 µl of the products was loaded in each gel slot. Gelpilot 100 bp ladder (cat. no. SM0243) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Determination of anti-*A. butzleri* activity of the Thymus vulgaris plants and its extraction

Thymus vulgaris used in this research was obtained from Plant Department, Faculty of Agriculture, Al Azhar University, Assiut branch, Egypt. The plant was washed, dried, and ground in a mortar to be used in Talaga cheese preparation. The alcohol extraction was done by maceration in 70% alcohol for 24 hours. This process was repeated three times. The combined alcoholic EO was filtered and then evaporated under reduced pressure at a temperature not exceeding 50ºC until a semisolid residue was obtained **(Ibraheim and Boulatova, 2002).**

Preparation of *A. butzleri* standard inoculum.

A. butzleri subcultures were first prepared from the stock cultures on Brain Heart Infusion (BHI) agar supplemented with 5% yeasts and 7% sheep blood. BHI agar plates were incubated at 37 ºC in microaerophilic atmosphere (Vandamme et al., 1991). Arcobacter inoculum was prepared by collecting bacterial colonies from BHI agar plates at the exponential growth phase and diluting in 0.85% saline. The resulting bacterial suspension was then standardized by McFarland nephelometry to 10^5 CFU/ml. (**Adesiji *et al.*, 2012**)

Manufacture and treatment of Talaga cheese.

Talaga cheese was prepared from whole raw milk that was pasteurized at 63ºC for 30 minutes. The inoculated milk was salted to a concentration of 5%. Rennet was added, the milk was divided into five equal portions, and each was subjected to the following treatments: two portions for addition of 2% and 4% Thymus vulgaris and another two portions for its EO and 5th portion as a control block (free from *A. butzleri*). The treated milk and the control one were incubated at 30ºC for overnight until coagulation and cheese was obtained. Treated cheese as well as control samples were stored at refrigeration temperature (4±2 ºC). Counts were calculated from the finished cheese after curdling, first, second day and every 3 days for *A. butzleri* count and pH measurement.

Sensory evaluation of Talaga cheese manufactured.

Talaga cheese was prepared as previously mentioned and divided into 5 equal portions; each was subjected to the previous treatments (without adding *A. butzleri*). Samples were stored at refrigeration temperature (4 ± 2 ºC). Twenty-three consumers were selected in teams of different ages, sex and education to taste the samples. The perception of consumers toward cheese with various treatments was studied with respect to three different attributes (flavor, appearance and palatability). The level of agreement was scored as strongly agree (SA), agree (A), disagree (D), and strongly disagree (SD) **(Nelson and Torut, 1981**).

Statistical Analysis

The statistical analysis was performed using programs GraphPadPrism 5.04 (GraphPad, Inc., San Diego, USA) and Statistical 12.0 (Dell, Inc., Tulsa, USA). Least significant differences were used at $p < 0.05$. The data represented by using the Microsoft Excel Spreadsheet.

RESULTS AND DISCUSSION

A.butzleri is considered as one of the most important foodborne pathogen causing sever gastrointestinal disease, with persistent diarrhea in human (**Collado and Figueras, 2011**) and in dairy chain could be isolated from fecal samples of dairy animals (**Shah *et al.*, 2013**), in-line milk filters (**Serraino *et al.*, 2013**), cow and water buffalo milk (**Yesilmen *et al.*, 2014**) and from different localities in dairy industry (**Serraino and Giacometti, 2014**).
In this study soft (Talaga), semisoft (Mozzarella) and hard (Roumy) cheeses examined for existence of *A.butzleri* and could be isolated in percentages of 16.67, 10 and 6.67%, respectively (**Figure 1**). From a previous study, in the same city, *A.butzleri* could be isolated in different percentages from food samples collected from Assiut city, Egypt (6.67%) by **Elsherif and Amin (2012)** and (5%) by **Ammar and AL-Habaty (2015).** Also, confirmed the ability to contaminate the cheese processing plants (**Ferreira *et al.*, 2019**) through, its ability to survival for long time and good growth in milk **(Giacometti *et al.*, 2014),** food surfaces, instruments (**Ferreira *et al.*, 2019**), resistant to several substrates **(D'Sa and Harrison, 2005)** and able to tolerate sodium hypochlorite concentrations close to working solutions used for sanitizing in food processing plants **(Rasmussen *et al*., 2013).**

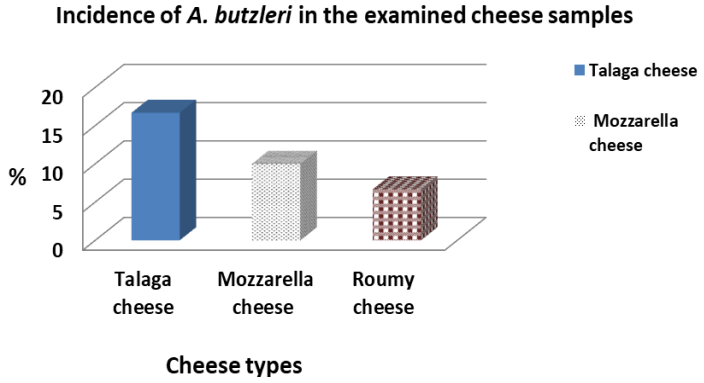


Figure 1 Percentages of isolation of *A. butzleri* from different types of cheese samples (n=30 for each)

The differentiation of *Arcobacter* species from related organisms using laboratory biochemical reaction after selective culturing may be performed the purpose, but these techniques are cumbersome to perform, time-consuming and

highly limited in specificity that because its relatively biochemically inert and morphologically similar to campylobacters, factors that may contribute to incorrect detection and identification of these organisms when relying on agar plating or phenotypic tests **(Prouzet-Mauleon *et al.* 2006)**. In view of culture failure and misidentification, nucleic acid approaches, particularly PCR-based methods, are increasingly being considered for detection, identification, and monitoring of arcobacters in foods (**Prouzet-Maule´on *et al.*, 2006 and Gonza´lez *et al.*, 2007**). So, as shown in **Figure** (2) *A.butzleri* isolates could be confirmed by using *16S rRNA* in Talaga (2 isolates) and Mozzarella (1 isolate) cheese samples. A study included detection using culturing and molecular method in parallel reported that 1.4%of the samples positive by culturing, and 0.7% by molecular detection (**Collado and Figueras, 2011**).

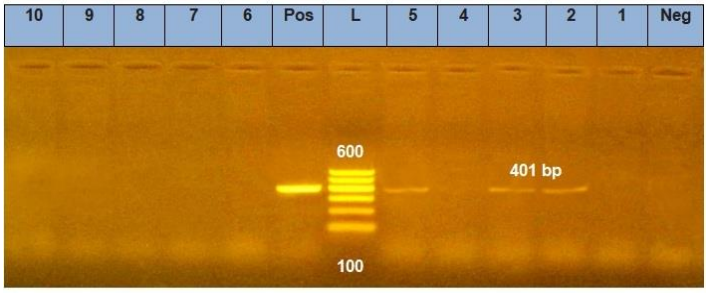


Figure 2 The amplified 16S rRNA gene of *A. butzleri* recovered from different types of cheese samples.
Lane L: Molecular marker; **Lane pos.:** Positive control; **Lane Neg.**: Negative control; **Lanes 1, 4,7-10**: negative isolates; **Lane 2, 3, 5**: positive isolates (Talaga and Mozzarella cheese samples).

The use of herbal and its extracts as alternative medicine, natural therapies (**Adesiji *et al.*, 2012**), food additives and as food preservatives has been documented for ages especially with distribution of antibiotic resistant genes (**Satyanarayana *et al.*, 2004 and Gutierrez *et al.*, 2008**). Thyme oil and *Thymus vulgaris* are widely used as food relish in Egypt nowadays and in an ancient age in embalming (**Beth, 2013**). In this study, the antibacterial effect of *Thymus vulgaris* and its EO against *A.butzleri* was evaluated as showed **Figure** (3, 4), the plant can decrease the count of it throughout the storage time until became couldn't be detected at 10th day specially at concentration 4%, showing significance difference when use the thyme EO at 4% *A.butzleri* undetectable at 8th day. Subsequently, the count stabilized over the remaining period of storage in untreated cheese (positive control samples), a slight decrease in the count of *A.butzleri* was noted toward the end of the 12th day of storage. Thyme belongs to the family of Labiatae and as an aromatic agent is widely used in many cooked dishes, the antimicrobial mechanism of thyme and thyme extract is based on their ability to disintegrate the outer membrane of bacteria, releasing lipopolysaccharides, increasing the permeability of the cytoplasmic membrane to ATP (**Lacroix *et al.*, 1997; Lampert et al., 2001 and Justesen and Knuthsen, 2001**), antioxidants effect based essentially on polyphenolic compounds as flavonoids (**Selmi and Sadok, 2008**). It is also well known that essential oil of this plant is a rich source of thymol and carvacrol which has been reported to possess a high antioxidant activity. Such, essential oils degrade the cell wall, interact with the composition, disrupt cytoplasmic membrane (**Lampert *et al.*, 2001**), damage membrane protein, interfere with membrane-integrated enzymes, cause leakage of cellular components, coagulate cytoplasm, and influence the synthesis of DNA and RNA (**Tannguchi *et al.*, 1988 and Rauha *et al.*, 2000**). Therefore, it is necessary to investigate further to understand the relationship between antibacterial activity and chemical structure of plants.

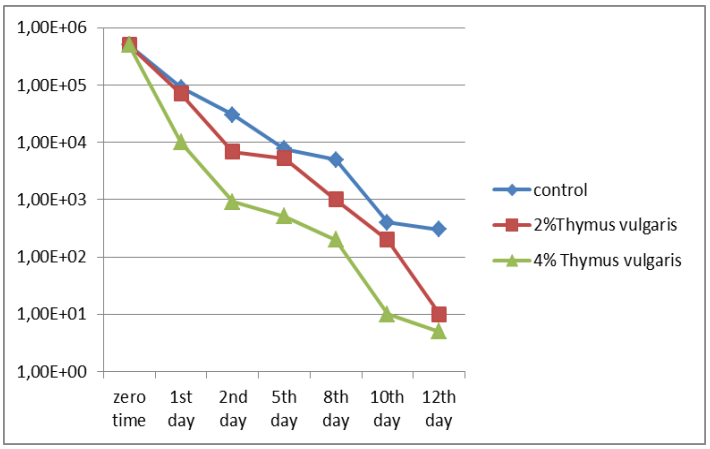


Figure 3 Effect of *Thymus vulgaris* at different concentrations on inoculated *A. butzleri* in manufactured Talaga cheese during refrigeration storage

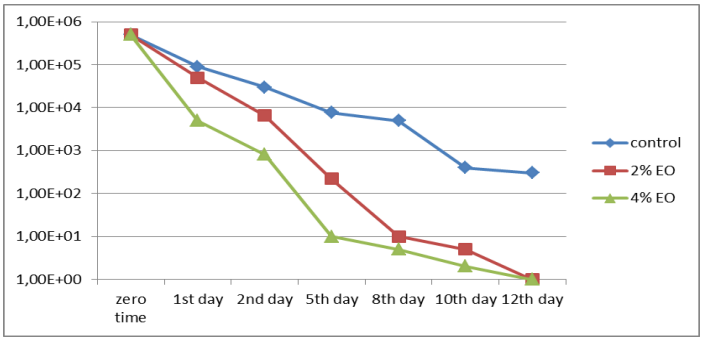
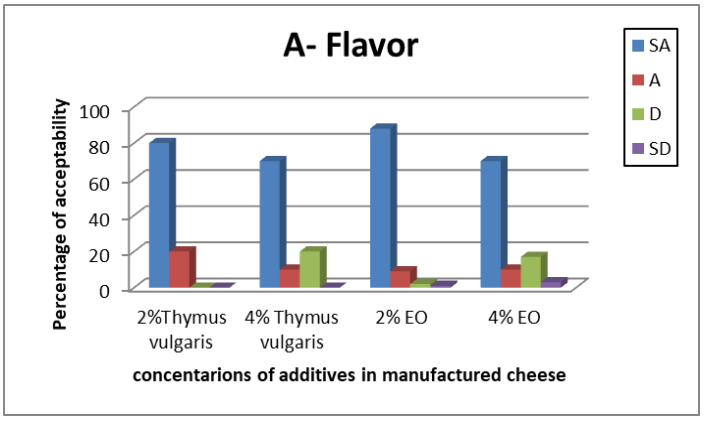
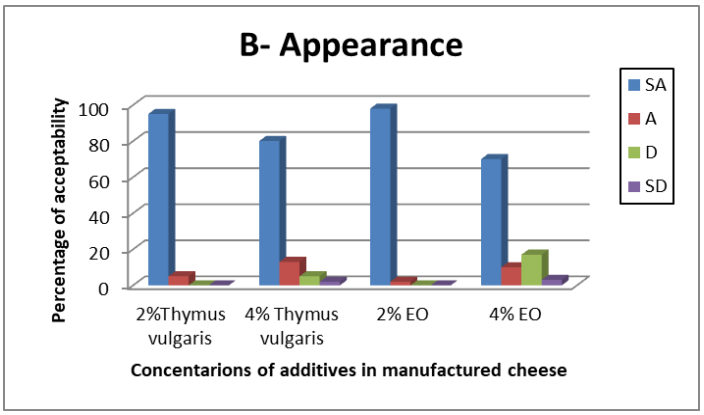


Figure 4 Effect of Thyme EO at different concentrations on inoculated *A. butzleri* in manufactured Talaga cheese during refrigeration storage
*Significance (P<0.05) Sig. difference between 4% EO and control P<0.003

Although the acceptability of consumer to cheese with 0, 2, 4% of thyme plant and its extract investigated based on inner and outer appearance, flavor, palatability and texture also, define the additives (**Figure 5)**. 80% were strongly agree to cheese with 2% *Thymus vulgaris*, 60% accept the palatability at 4% and 77, 65% for 2, 4% extract, respectively with no significant different between trials. These acceptance returned to that thyme plant considered as one of main spices in Egyptian kitchen so, its taste, flavor and palatability considered familiar but the difference in percentages depend on appearance and individual variations.





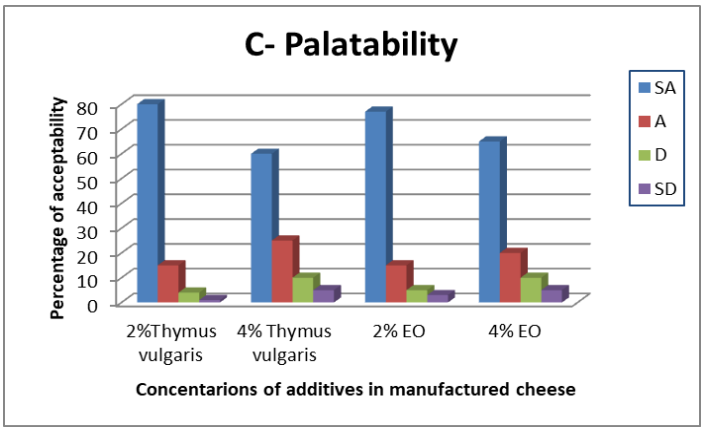


Figure 5 Sensory evaluation of manufactured Talaga cheese with different concentrations of additives according to flavor (A), appearance (B), and palatability (C).

* SA: Strongly Agree A: Agree D: Disagree SD: Strongly Disagree

CONCLUSION

The present study concluded that Thymus vulgaris and its extract have antibacterial activity against *A.butzleri*, which isolated from different types of cheese, in contrast that 4% of plant added to cheese have strongly effect on isolated strains with no really difference with 4% EO and achieved significant antimicrobial effect. Moreover 2% of Thymus vulgaris or its EO were mostly accepted to the consumers and so, it is recommended to add Thymus vulgaris in cheese, to improve the quality of product and increase the benefits from it.

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