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The American University in Cairo

School of Sciences and Engineering

Chemistry Department

Antimicrobial activity of grape seed and skin extracts coated on Corona treated LDPE and PET films

A Thesis Submitted to

The Food Chemistry Master's Program

In partial fulfilment of the requirements for

The degree of Master of Science

By

Nahla M. Khalil

Under the supervision of

Dr. Tamer Shoeib
Dr. Michael Kontominas

August, 2017

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Dedication

To my late mother who used to encourage me to gain knowledge as long as I live…….

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ABSTRACT

Consumer demand for ready-to-eat 'fresh' and safe food products with less synthetic preservatives together with well documented food-borne microbial outbreaks drive both research and food industry toward new innovative methods for microbial growth inhibition while keeping food freshness, quality, and safety. Incorporation of natural bioactive agents in the packaging material to increase the shelf life of meat products is a promising technology. Grapes are of special interest because of their high content of phenolic compounds that showed antimicrobial and antioxidant effects. The aim of the present work was to investigate grape seed (GSE) and skin (GSKE) extracts' antibacterial activity and developing bioactive LDPE/PET films that could be used as food packaging for poultry and meat products. Commercial corona treated LDPE and PET were coated with either grape seed or grape skin extract. Agar plate diffusion method was used for the investigation of the microbial properties of both extracts' coated films against *E.coli* as a Gram-negative bacterium and *Staphylococcus aureus* as a Gram-negative one. LDPE and PET films coated with GSE showed inhibition zones of *E.coli* growth in the range of 16-25mm, while *Staph.aureus* growth inhibition zones were in the range of 15-20mm. For LDPE corona films coated with grape seed extract, the minimum inhibitory concentration (MIC) was 0.002g/1cm^2 for *E.coli* and 0.003g/1cm^2 for *Staph.aureus*. While for corona treated PET films/GSE, the MIC for both *E.coli* and *Staph.aureus* was 0.002g/1cm^2. Corona treated LDPE and PET coated with GSKE showed inhibition zone range of 13-16.3mm with *E.coli* and 12-20mm with *Staph.aureus*. For LDPE corona films/GSKE, the minimum inhibitory concentration (MIC) was 0.0009g/1cm^{2}for *E.coli* and 0.003g/1cm^{2}for *Staph.aureus*. While for corona treated PET films/GSKE, the MIC was 0.002g/1cm^2 for *E.coli* and 0.003g/1cm^2 for *Staph. aureus*. The Total Phenolic Content of both GSE and GSKE was determined using the Folin- Ciocalteu methodology to be 315.32g (GAE)/kg, and 265.326g (GAE)/kg for GSE and GSKE respectively. The coated films; LDPE/GSE or GSKE, were used to wrap fresh chicken fillets, TVC, *Pesudmonads*, *Brochothrix thermosphacta*, Lactic acid bacteria and Enterobacteriaceae counts were determined during the storage period; 10 days for test samples and 8 days for controls. Microbiological analysis for tested samples was done on day 0, 2,4, 6, 8, and10, while for control till day 8. There was a reduction in the populations of the examined bacteria in the range of 0.2-

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LIST OF ABBREVIATIONS

ABTS: 2, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid radical cation

AFM: Atomic force microscopy.

AM: Antimicrobial systems.

APET: Amorphous PET

AP: Active packaging

AV: p-anisidine value.

BHT: Butylated-hydroxytoluene

BOPP: Biaxially oriented polypropylene

CD: Conjugated dienes.

DPPH: 2, 2-diphenyl-1-picrylhydrazyl.

DMT: Dimethyl ester of TPA

EG: Ethylene glycol

ESC: Environmental stress crack

ESCA: Electron spectroscopy for chemical analysis.

EVOH: ethylene vinyl alcohol

FFA: Free fatty acids.

FRAP: Ferric reducing/antioxidant power.

GAE: Gallic acid equivalents.

GSE: Grape seed extract

GSKE: Grape skin extract

GTE: Green Tea Extract.

HDPE: High density polyethylene

IR / ATR: Infrared spectroscopy / Attenuated total reflectance.

LCBs: long-chain branches

LDPE: Low density polyethylene

LLDPE: Linear low density polyethylene

MIC: Minimal inhibitory concentration

MBC: minimal bactericidal concentration

NABH: Natural antioxidants derived from barley husks.

PC: Polycarbonate

PET: Polyethylene terephthalate

PE: Polyethylene

PMMA: Polymethyl methacrylate

PP: Polypropylene

PVC: Polyvinyl chloride

PVDC: Polyvinylidene chloride

PS: Polystyrene

PUFAs: Polyunsaturated fatty acids.

PV: Peroxide value.

TBARS: Thiobatbituric acid reactive substances.

TEAC: The Trolox equivalent antioxidant capacity

TH: Conjugated triene hydroperoxides.

TPA: Terephthalic acid

TPC: Total phenolic content.

TVC: Total Viable Count.

Chapter 1
Introduction

1. Introduction

The demand for minimally processed, easily prepared and ready-to-eat ‘fresh ’food products, globalization of food trade, and distribution from centralized processing pose major challenges for food industry in terms of food safety and quality (Lucera *et al*., 2012). Well documented food spoilage as well as recent food-borne microbial outbreaks are driving both research and the food industry toward innovative ways to inhibit microbial growth in foods while maintaining quality, freshness, and safety (Appendini *et al*., 2002).

One option is to use packaging to provide an increased margin of safety and quality. The food package may include materials with antimicrobial and /or antioxidant properties. Such packaging technologies may play a key role in extending shelf –life of foods and reducing the risk of pathogens. A promising technology of ‘active’ packaging consists of the incorporation of functional additives in the packaging material (Kerry *et al*., 2006; Kontominas, 2016).

This approach combines the principles of food technology and packaging science, aiming to satisfy consumer demand for ‘fresh like’ products (Miltz *et al*., 1997; Kerry *et al*., 2006). The feasibility of the technology has been demonstrated by incorporating or immobilizing antimicrobial/antioxidant agents in or onto packaging materials (Abugroun *et al*., 1993; Miller *et al*., 1993; Hotchkiss, 1995; Ouattara *et al*., 1997; Ouattara *et al*., 2000; Suppakul *et al*., 2003; Mauriello *et al*., 2004; Conte *et al*., 2007; Marini *et al*., 2007; Farghal, 2016; Farghal *et al*., 2017).

A contemporary approach to the above problem known as **bioactive packaging** is based on the rationale that the bioactive agent added to a packaging material will a) prevent lipid oxidation / microbial growth in packaged food and b) will exert a beneficial health effect to the host (consumer) through its migration into the packaged food (Kontominas, 2016).

Antimicrobial packaging, besides protecting the product from the external environment, inhibits or retards microorganism growth in foods, minimizing direct addition of chemical preservatives and satisfying the actual demand of consumers for healthier foods, containing less synthetic additives.

Grapes have a long rich history, in ancient Greek and Roman civilizations being used both as table grapes and in winemaking. Today, French hybrids, European grapes (*Vitis vinifera)* and North American grapes (Vitis labrusca and Vitis rotundifolia) are the three major grape cultivated species. Because of their biological activity and health-promoting benefits, polyphenols are the most significant phytochemicals in grapes (Xia et al., 2010).

In general, grape phenolics are distributed in the stem, skin, seed and leaf, rather than the grape pulp. The phenolic compound concentrations in seed, skin, flesh, and leaf of fruits of 10 cultivars of muscadine grapes (five bronze skin and five purple skin) grown in southern Georgia, were 2178.8, 374.6, 23.8, and 351.6 mg/g GAE (gallic acid equivalent) respectively (Eduardo *et al*., 2003). Factors that influence grape total phenolic content are: cultivar, geographical origin, climate, soil composition, and cultivation practices (Xia *et al*., 2010).

The phenolic compounds principally consist of flavanols, stilbenes (resveratrol), phenolic acids, anthocyanins and flavonols. The most important grape polyphenols are anthocyanins,

flavanols, flavonols and resveratrol because they possess antioxidant, cardioprotective, anticancer, anti-inflammatory, anti-aging and antimicrobial properties. While anthocyanins are pigments that mainly exist in grape skins, flavonoids are distributed mainly in seeds and stems. Flavonoids principally include procyanidin polymers, (+)-catechins and (−)-epicatechin. The essential polyphenolics in red grapes are anthocyanins, while in white varieties flavan-3-ols are more predominant (Xia *et al*., 2010).

In this work commercial grape seed and skin extracts were used as antimicrobial agents. These are natural substrates, rich in phenolic compounds, to be coated on corona treated Low density polyethylene (LDPE) and Polyethylene terephthalate (PET) films in order to prepare experimental food packaging materials with antimicrobial properties. Experimental coated films were evaluated for their antimicrobial properties against different spoilage and pathogenic bacteria commonly found in poultry meat.

Chapter 2
Literature review

2. Literature review

The literature review will be categorized under the following subchapters:

2.1) Food Spoilage (Focusing on meat products)

2.2) Preservation using packaging

2.3) Chemical vs. natural preservatives

2.4) Corona treatment of films

2.5) Antimicrobial and antioxidant activity of grape seed/skin extracts

2.1. Food Spoilage: Focus on meat and poultry products

Food spoilage is a complex process involving physical, chemical/biochemical as well as microbiological changes that occurs in foods with time and storage conditions. As a result of above changes, food sensory properties deteriorate to the point that food becomes unacceptable for consumption. Despite the usage of modern preservation techniques even today, an excessive amount of foods is lost before reaching the consumer. Factors controlling degree of food deterioration include: temperature, pH, $a_{w,}$ light and atmosphere as well as food composition. Based on the knowledge of previous parameters, detailed sensory, microbiological and chemical analysis is carried out to evaluate food quality and safety. This subchapter will further focus on the microbiological aspect of food quality and safety deterioration (Gram *et al*., 2002). Table 1 gives examples of typical spoilage substrates and metabolites found in microbiologically spoiled foods.

Every food product has its specific microflora which is a function of particular food composition, processing, preservation and storage conditions. Specific spoilage organisms (SSO) are those

microorganisms that cause spoilage to particular food commodities such as meat, fish, fruit, vegetables, dairy products, etc. (Gram et *al*., 2002).

The term “metabiotic spoilage association’’ was introduced by Jørgensen et al. (2000b) to describe cases where more than one microbial species is involved in the spoilage of a food product through metabolite or nutrient exchange. The term ‘‘specific spoilage organisms’’ can cover this scenario where a group of microorganisms interact to spoil the food product. Methods for the characterization of specific spoilage organisms are given in Table 2 (in’t Veld, 1996).

‘Microorganism spoilage potential’ is defined as the pure culture capability to produce metabolites that contribute to the spoilage of a specific food product. The microbial activity of different genera of microorganisms is the main cause of the spoilage of both foods and beverages. The specific type of microbial flora developing during storage in a particular foodstuff relies on intrinsic parameters (e.g. pH, water activity, nutrients, redox potential, antimicrobial compounds etc.), extrinsic factors (e.g. humidity, temperature, atmosphere etc.), as well as on methods of processing and preservation, and implicit parameters (e.g. direct and indirect interactions of microorganisms) (Van Der Vossen and Hofstra, 1996).

Table 1. Examples of typical spoilage substrates and metabolites found in microbiologically spoiled foods.

Sensory impression	Spoilage product	Spoilage substrate	Food Product	Specific spoilage organism
Slime	EPS (dextran)	sucrose	kimchi	*Leuconostoc*
			turkey breast	*Leuconostoc*
		sugars	wine	*Pediococcus damnosus*
		sugars	bread	*Bacillus*
Slime	hydrolysed polymer	pectin	vegetables	*Erwinia*, *Pseudomonas*
Fishy off-odour	trimethylamine (TMA)	trimethylamine oxide (TMAO)	fish	*S. putrefaciens*
				P. phosphoreum
				Aeromonas spp.
Ammonia, putrid	NH_3	amino acids	proteinaceous foods	many microorganisms
	Biogenic amines	amino acids	meat	Enterobacteriaceae and LAB
			fish[a]	Enterobacteriaceae, LAB
			fish[a]	*P. phosphoreum*
Sulphidy off-odour	H_2S	cysteine	fish, meat	*S. putrefaciens*
				Enterobacteriaceae
				L. saké, *L. curvatus*
Greening	H_2S	cysteine	meat	*L. plantarum*
Sulphydryl off-odours	$(CH_3)_2S_2$	methionine	fish, meat	*Pseudomonas* spp.
				Enterobacteriaceae
Acid off-odour	acetic acid L,D-lactic acid	glucose, ribose, other CHO	meat	LAB
"Sweet curdling"	proteinaceus fat particles	phospholipid	milk	*B. cereus*
Fruity off-odour	esters		fish	*P. fragi*
			milk	*P. fragi*, *P. putida* and *Y. intermedia*
Cheesey off-odour	acetoin, diacetyl, 3-methylbutanoyl	glucose	meat	*B. thermosphacta*
				Enterobacteriaceae
				homofermentative LAB
Medicine off-odour	2-methoxy-phenol, sediment	sugars	juice	*A. acidoterrestris*
Musty odour	trichloroanisol	2,4,6 trichlorophenol	wine	*P. brevicompactum*, *A. flavus*

(From Gram *et al*., 2002)

Table 2. Methods for the characterization of specific spoilage organisms

Method	Comparison of results from product and model substrate experiments
Spoilage potential (qualitative)	Microorganisms are isolated from products at sensory rejection and the ability of isolates to produce off-odours is determined by inoculation of substrates
Spoilage activity (quantitative)	The concentration of groups of bacteria is determined at the time of sensory rejection and the concentration of these bacteria at the time of off-odour detection is determined in model substrates
Yield factor determination (quantitative)	Numbers of groups of bacteria and the concentration of selected metabolites are determined in products and the increase in concentration of these bacteria and of selected metabolites are then determined in model substrates
Chemical spoilage profiles (qualitative or quantitative)	Chemical spoilage profiles of naturally spoiled products compared to chemical spoilage profiles of isolated microorganisms grown in model substrates

(From In't Veld, 1996)

Spoilage of poultry and meat products

Approximately 1.3 billion tons per year of food produced for human consumption is wasted globally (Gustavsson *et al*., 2011). Meat and meat product losses comprise nearly 21% of these food losses (Höll and Vogel, 2016). It has been documented that one-fourth of the world's food losses is through microbial activity (Anonymous, 1985).

Regarding spoilage and prediction of minimum shelf life, meat is one of the most sensitive food products. Meat microflora is mainly influenced by meat type, processing hygiene, distribution, and storage conditions. Concerning storage, temperature variations and packaging atmosphere are the main factors that influence the growth dynamics and the composition of microbiota. Poultry meat, particularly parts that contain skin, have a higher initial contamination rate than meat muscle. It has been reported that proteinaceous foods as meat, poultry, fish, shell fish and some dairy products have similar microbial spoilage pattern (Fig.1).

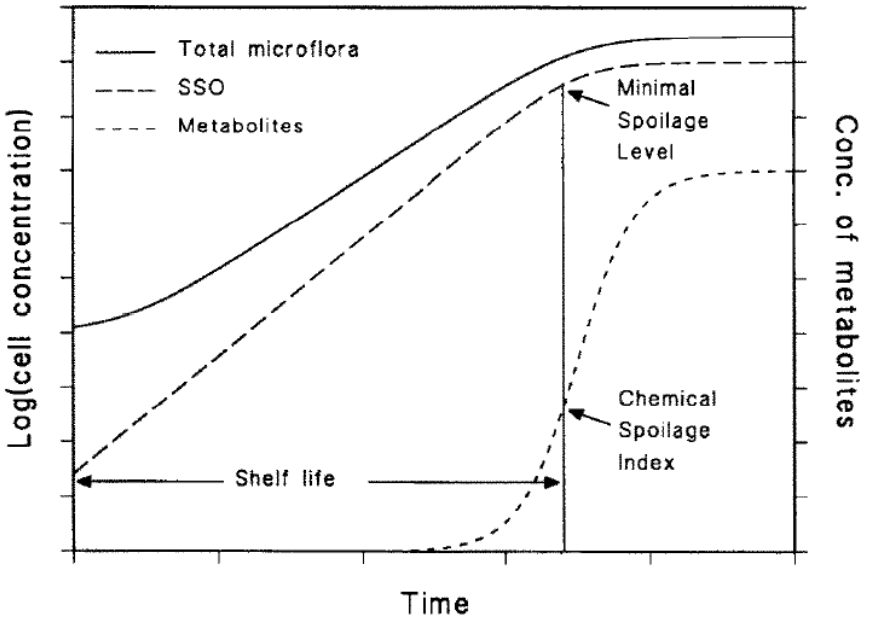


Figure 1. General Pattern of microbial spoilage. SSO, specific spoilage organisms; MSL, minimal spoilage level; CSI, chemical spoilage index (From in't Veld, 1996).

Firstly, SSO represent only a small portion of the natural microflora, then during storage they grow much faster than the rest of microflora with the production of the metabolites that cause off-flavors, off-odors or slime and sensory rejection. These foods have a high moisture content, are highly nutritious and possess a neutral to acidic pH which permits the growth of various microorganisms (in'Veld, 1996).

Meat products are differentiated, based on their pH and water activity, into "easily perishable", "perishable" and "shelf-stable". Meat products that have a pH>5.2 and a_w >0.95 are the "easily perishable" and should be stored at or below +5 ^{0}C. The ''perishable'' meat products have either a pH of 5.2–5.0 (inclusive) or an a_w of 0.95–0.91(inclusive) and must be stored at or below +10 ◦C. Meat products that have a pH <5.2 and an a_w<0.95 or only pH<5.0 or aw<0.91; these products are "shelf-stable" and need no refrigeration; the shelf-life of these meat products is not limited by bacteria but by chemical or physical spoilage, specifically rancidity and discoloration

(Ambrosiadis *et al*., 2004). Figure 2 illustrates the development of off-odor and slime on dressed chicken and packaged beef during storage at 5◦C.

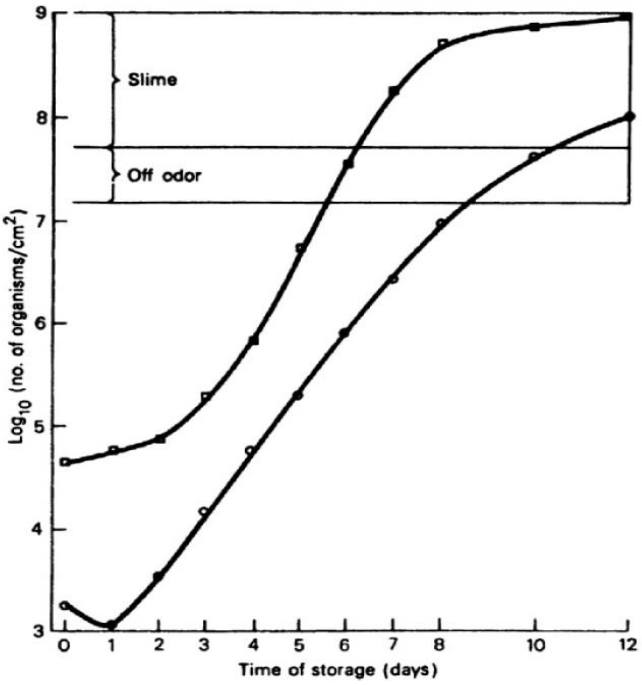


Figure 2. The development of off-odor and slime on dressed chicken (squares) and packaged beef (circles) during storage at 5◦C. (From Ayres, 1960)

Mainly, meat and poultry microbiological spoilage is due to psychrotrophic microorganisms' activity that produce undesirable appearance, off-odors and off-favors at refrigeration temperatures. Proportions of psychrotrophic spoilage microorganisms isolated from chicken carcasses are shown in Table 3 (Doyle, 2009).

There are four factors that can control meat and poultry microbiological spoilage if effectively applied: (1) Good sanitation during slaughter and processing limits the initial contamination; (2) Removal of microorganisms that cause spoilage; (3) Maintaining low temperature while processing, storage and transportation reduces the rate of spoilage microorganisms' growth; (4) Knowledge of time-temperature response limitations for keeping product quality (Bailey, 1986).

Table 4 and 5 illustrate the major genera of bacteria, yeasts and molds found in meat and poultry products before spoilage (Appendix D) (Jay *et al*., 2005).

Table 3. Proportions of psychrotrophic spoilage microorganisms isolated from chicken carcasses.

Type of microorganisms	Percentage of population
Pseudomonads	30.5
Acinetobacter spp.	22.7
Flavobacterium spp.	13.9
Corynebacterium	12.7
Yeasts, enterics, and others	20.2

(From Doyle, 2009)

2.1.1. Microbial spoilage

Meat and meat products are excellent media for the growth of a variety of microflora (bacteria, molds and yeasts) some of which are pathogens (Jay *et al*., 2005). The principle sources of these microorganisms are the intestinal tract and animal skin. The microbial growth curve is shown in Figure 3.

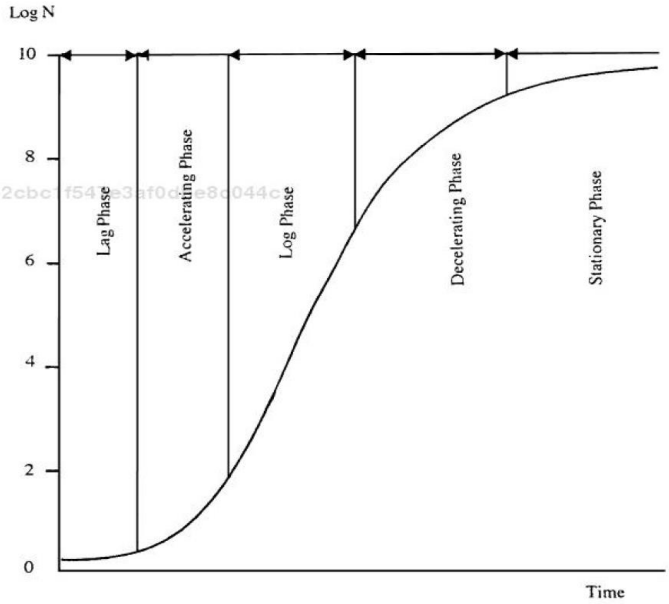


Figure 3. The microbial growth curve (From Adams and Moss 2007)

Many factors determine microflora composition in meat and poultry: (a) husbandry practices of pre-slaughtering (free range vs. intensive rearing), (b) time of slaughtering and age of animal, (c) handling during slaughtering and processing, (d) control of temperature during slaughtering, processing and distribution, (e) method of preservation, (f) packaging type and (g) consumer handling and storage (Cerveny *et al*., 2009).

Surface microbial contamination is the principle cause of meat spoilage and it is the reason of off-flavors development. The first signs of spoilage are associated with the production of fruity, sweet-smelling esters, then production of putrid sulphur compounds follows. Pseudomonads were reported to be the main bacterial genus contamination (Dainty *et al*., 1983). It was confirmed that protein and amino acid decomposition by anaerobic bacteria is the main cause of various putrid odors. Off-odor volatiles include methanethiol, indole, ammonia and dimethyl disulphide (Dainty, 1996).

Species of molds found on meat and poultry before spoilage include *Penicillium*, *Sporotrichum*, *Cladosporium*, *Mucor* and *Geotrichum* while yeasts species include *Cryptococcus* spp., *Candida* spp. And *Rhodotorula* spp. Bacterial species include *Escherichia*, *Micrococcus*, *Sarcina*, *Salmonella*, *Bacillus*, *Streptococcus* and *Clostridium* (Dave and Ghaly, 2011) (Tables 4 and 5 Appendix D). In the state of Iowa, *Enterococcus spp.* was the major dominant bacteria on 971 of the 981 samples of all meat types; chicken, pork, beef and turkey (Hayes *et al*., 2003).

2.1.1.1. Intrinsic and Extrinsic Parameters of Foods That Affect Microbial Growth

There are many factors affecting the development of microbial associations in different food products. Intrinsic parameters (e.g. pH, water activity, nutrients, redox potential, antimicrobial compounds etc.), extrinsic factors (e.g. humidity, temperature, atmosphere etc.), as well as on methods of processing and preservation, and implicit parameters (e.g. direct and indirect interactions of microorganisms) (Van Der Vossen and Hofstra, 1996).

2.1.1.1.1. Intrinsic Parameters

The inherently found parameters of animal and plant tissue are known as intrinsic parameters, which are water activity, nutrient content, pH, oxidation-reduction potential, biological structures and antimicrobial constituents (Jay *et al*., 2005).

a) pH

Most microorganisms favorably grow at pH values 7.0 (6.6-7.5), while few of them prefer pH below 4.0 for best growth (Fig. 4). Regarding pH, bacteria are more sensitive to pH changes than molds and yeasts; pathogenic bacteria are the most fastidious towards pH changes. In general, bacteria grow fastest in the range 6.0-8.0, yeasts 4.5-6.0 and filamentous fungi 3.5-4.0. Approximate pH Values of Dairy, Meat, Poultry, and Fish Products are illustrated in Table 6 (Jay *et al*., 2005).

Table 6. Approximate pH Values of Dairy, Meat, Poultry, and Fish Products

Product	*pH*	*Product*	*pH*
Dairy products		**Fish and shellfish**	
Butter	6.1–6.4	Fish (most species)*	6.6–6.8
Buttermilk	4.5	Clams	6.5
Milk	6.3–6.5	Crabs	7.0
Cream	6.5	Oysters	4.8–6.3
Cheese (American mild and cheddar)	4.9; 5.9	Tuna fish	5.2–6.1
		Shrimp	6.8–7.0
Meat and poultry		Salmon	6.1–6.3
Beef (ground)	5.1–6.2	White fish	5.5
Ham	5.9–6.1		
Veal	6.0		
Chicken	6.2–6.4		
Liver	6.0–6.4		

*Just after death.

(From Jay *et al*., 2005)

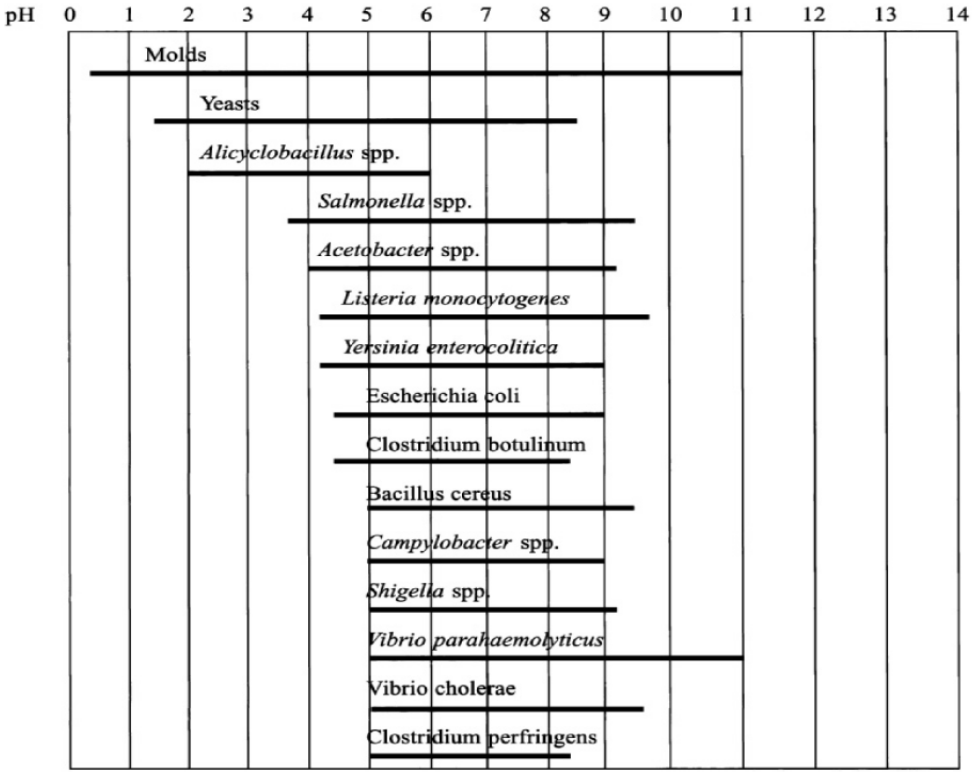


Figure 4. Approximate pH growth ranges for some foodborne organisms. The pH ranges for *L. monocytogenes* and *S. aureus* are similar. (From Jay *et al*., 2005)

b) Nutrient Content

Foods function as sources of nutrients and energy for microorganisms. The essential molecules for growth that microorganisms cannot synthesize are derived from food. In microbial media

food products like meat, meat infusions, casein digests (peptone and tryptone), sugar and starch are very suitable for this purpose.

The principle needs for microorganism growth are: water, energy, nitrogen sources, minerals, vitamins and other growth factors. Microorganism inability of utilizing certain food material limits its growth and allows the opportunity for its competitors to flourish on this food material. Thus, the rate of microbial growth is somehow determined by the concentration of key nutrients (Adams and Moss, 2007; Jay *et al*., 2005).

c) Oxidation–Reduction Potential

Microorganisms show different degrees of sensitivity to oxidation-reduction potential (O/R, Eh) of their growth medium. In general, substrate O/R potential is defined as the ease with which substrate gains or loses electrons. The substrate is oxidized after losing electrons and reduced after gaining them: (Jay *et al*., 2005)

$$Cu \underset{\text{reduction}}{\overset{\text{oxidation}}{\rightleftharpoons}} Cu + e \qquad (Cu^{+} + e)$$

Oxidation is also accomplished by adding oxygen as shown in the next reaction:

$$2Cu + O_2 \rightarrow 2CuO$$

'Eh' is the symbol which represents the O/R potential of a system. Aerobic microorganisms need positive Eh values (oxidized) for their growth, while anaerobes seek negative Eh values (reduced). Increasing the access of air to a food material by chopping, grinding or mincing will increase Eh. Similarly, exclusion of air as in modified atmosphere packaging or vacuum packaging or canning will reduce Eh. Microbial growth in a food reduces its Eh (Jay *et al*., 2005).

d) Water Activity

Living organisms totally depend on water in its liquid state. In the cytoplasm, reactions occur in an aqueous environment, the membrane which surrounds the cytoplasm is water permeable, so, there is a dynamic two way flow of water molecules in and out the living cell. There is an equilibrium state that prevents plasmolysis (more water flow out the cell), or membrane rupture if there is more water flow into the cell, which is prevented by cell wall in fungi and bacteria. For a given food substrate, water activity is the ratio of water partial pressure in the atmosphere in equilibrium with substrate, P, compared to the atmosphere partial pressure in equilibrium with pure water at the same temperature, P◦ (Adams and Moss 2007). Table 7 gives the minimum a_w requirements for microorganism growth.

Table 7. Minimum water activities at which active growth can occur

Group of micro-organism	*Minimum a_w*
Most Gram-negative bacteria	0.97
Most Gram-positive bacteria	0.90
Most yeasts	0.88
Most filamentous fungi	0.80
Halophilic bacteria	0.75
Xerophilic fungi	0.61

(From Adams and Moss 2007)

e) Biological structures

Excellent protection against invasion of spoilage microorganism into food materials provided by natural coatings such as seed testa, fruit outer peel, nut shell, egg shells and animal hide. The shell of nuts and the outer shell of eggs if intact, prevent the invasion of almost all types of microorganisms if they stored under appropriate conditions of temperature and humidity. Likewise, fish skin prevents spoilage and contamination of such food (Jay *et al*., 2005).

f) Antimicrobial constituents

Some foods have naturally existing substances that have antimicrobial activity which contribute to their defense mechanism against microorganism attacks (Jay *et al*., 2005). The Lactoperoxidase system is an example of a naturally inhibitory system found in bovine milk; it consists of three components, all of them required for its antimicrobial effect: lactoperoxidase, thiocyanate, and H_2O_2, i.e. *Pseudomonads* as Gram-negative psychrotrophs are very sensitive to *Lactoperoxidase* effects (Björck, 1978).

2.1.1.1.2. Extrinsic Parameters

Extrinsic parameters are independent of substrate; they are the main characteristics of storage environment which influence foods and their microflora. The most important of them are: storage temperature, environment relative humidity, co-existence of other microorganisms as well as the existence of specific gases in the immediate food environment (Jay *et al*., 2005).

a) Temperature of Storage

Microorganisms generally grow over a broad range of temperatures as an individual microorganism or as group. For proper selection of the storage temperature of various food types, microorganism temperature growth ranges should be considered. It has been confirmed that the lowest temperature for microorganism growth is -34◦C, while the highest is over 100◦C. According to temperature growth ranges, microorganisms can be classified into: *psychrophiles* ; they grow at as low as -5 ^{0}C and their optimum is 12-15 ^{0}C, *psychrotrophs*; they grow at or below 7◦C and their optimum 20◦C to 30◦C, *mesophiles*; grow between 20◦ and 45◦C with optimum between 30◦C and 40◦C, and *thermophiles;* grow at and above 40◦C and and their optimum growth temperature is between 55◦C and 65◦C. Species and strains of *psychrotrophs* are among the following genera: *Alcaligenes, Shewanella, Brochothrix, Corynebacterium, Flavobacterium, Lactobacillus, Micrococcus, Pectobacterium, Pseudomonas, Psychrobacter, Enterococcus,* and others. The most common *psychrotrophs* found in foods are those of the

genera *Pseudomonas* and *Enterococcus;* they grow well at refrigerator temperatures causing spoilage of meats, poultry, eggs, fish and other foods at 5-7◦C (Jay *et al*., 2005).

b) Relative Humidity of Environment

Storage under conditions of low RH is necessary for foods that are subject to surface spoilage from bacteria, molds and yeasts. Chicken and beef cuts usually suffer surface spoilage in the refrigerator prior to deep spoilage due to the high refrigerator RH and the aerobic nature of meat-spoilage microflora (Jay *et al*., 2005). Figure 5 gives ranges of a_w values associated with a number of food commodities.

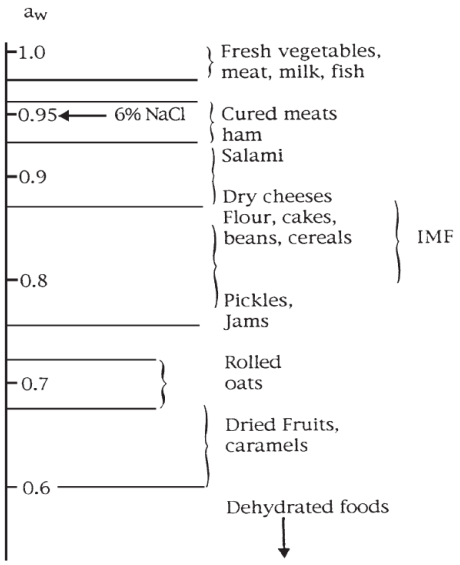


Figure 5. Range of a_w values associated with a number of food commodities
(From Adams and Moss 2007)

c) Presence and Concentration of Gases in the Environment

The most important gas used to control microorganisms in foods is Carbon dioxide (CO_2) (Parekh and Solberg, 1970; Clark and Lentz, 1973). CO2, Nitrogen and O2 are the most important gases in modified atmosphere packaging (MAP) of food commodities. The other atmospheric gas which has antimicrobial activity is ozone (O_3), it has been used to extend certain foods' shelf life because of its effectiveness against various microorganisms (Burleson *et al*., 1975).

d) Presence and Activities of Other Microorganism:

Foodborne organisms may produce substances that inhibit or cause the death of other microorganisms that are found on the same food product e.g., bacteriocins, organic acids, antibiotics and hydrogen peroxide (Jay *et al*., 2005).

2.1.2. Chemical Spoilage

Despite the fact that chemical and physical food spoilage are interrelated, flavor and color changes due to oxidation, irradiation, lipolysis and heat are principal contributors to food spoilage. The previous changes could be stimulated by metal ions, light or temperature abuse during processing or storage. Physical changes such as gelation, colour change, elevated viscosity or sedimentation may result from chemical reactions (in't Veld, 1996).

2.1.2.1. Lipid Oxidation

Lipid oxidation is most often responsible for food quality deterioration. Unsaturated fats are oxidized via free radical autoxidation. The mechanism of lipid auto-oxidation involves three steps: initiation, propagation and termination (Dave and Ghaly, 2011). Rate of and susceptibility to oxidation depend on the number of fatty acid double bonds; as the number of double bonds increases, susceptibility to and rate of oxidation increase. Initiation of oil oxidation may occur by photosensitizers or lipoxygenase. Lipid autoxidation and free radical production lead to oxidative meat deterioration and off-flavor production.

Lipid oxidation is inhibited by antioxidants which function as hydrogen or electron donors that interrupt the radical chain reaction through the formation of non-radical compounds which will block the radical reaction propagation (King *et al*., 1993). Many foods (fruits, plants, roots and meats) have compounds with antioxidant activity i.e. ascorbic acid, vitamin E (tocopherols), flavonoids and carotenoids that inhibit auto-oxidation.

2.1.2.2. Discoloration

Because of different oxygen partial pressure at the surface of meat, there are different forms of myoglobin different in color, purple, bright red or brown that exist. Oxygen penetration into meat tissues determines the depth of the oxymyoglobin layer which is responsible for meat bright red color (Belitz and Grosch, 1987).

2.1.2.3. Protein Hydrolysis

Various food spoilage bacteria produce proteolytic enzymes which cause protein hydrolysis in foods such as meat, poultry, milk, and seafood products. Food noxious putrefaction can result from *Clostridium* spp. anaerobic proteolysis. The *Pseudomonads* can exceed proteolysis by one step through amino acid metabolism and the production of foul-smelling products e.g. cadaverine and putrescine (Doyle, 2009).

2.1.2.4. Lipolysis

Lipolysis is an enzymatic fat hydrolysis by lipases occurring in lipid containing foods. Reaction products' accumulation, specifically, free fatty acids is the cause of off-flavor or the so called rancidity development in a fat containing foods (Muir *et al*., 1979).

2.2. Preservatives using packaging

Packaging has become an important factor in the process of food manufacturing. It helps food retain its quality and safety protecting it from microorganisms, chemical and biological changes resulting in food longer shelf life (Skandamis and Nychas, 2002). Factors affecting the choice of food packaging materials include: processability, barrier properties, mechanical properties (tear strength, friction, elongation, burst strength, puncture resistance, tensile strength, etc.), interaction with food product and chemical resistance (Rahman, 2007).

When selecting packaging materials, environmental factors such as relative humidity, temperature, intensity of light to which the product may be exposed to during storage and

distribution must be taken into consideration. Transportation also must be considered when selecting packaging materials; proper packaging could reduce the extent of mechanical damage occurring during transportation. Packaging materials are divided into those with flexible structures such as plastic film, paper, foil, and textiles, and those with rigid structures such as wood, rigid plastics, glass and metals. The historical developments of plastics are presented in Table 8 (Rahman, 2007; Kontominas, 2016).

Table 8. Historical Developments of Plastics

Year	Plastic Type
1843	Malayan *gutta percha*, a shellac molding material, was the first seminatural plastic.
1870	Searching for a substitute for ivory for constructing billiard balls, Hyatt made pyroxylin from cotton and nitric acid and then reacted this with camphor, producing celluloid.
1909	Leo Baekeland reacted phenol with formaldehyde with a catalyst, hexemethyleneteramine, under pressure (to stop foaming), producing the first synthetic resin, called bakelite.
1919	Casein was developed as a film.
1927	Cellulose acetate and polyvinyl chloride were developed.
1935	ICI reacted with ethylene under high pressure with trace 02, giving LDPE, which suited the newly developed technique of blow molding.
1953	Karl Zeigler produced HDPE from ethylene using catalysts, titanium tetrachloride and triethyl aluminum.

(From Rahman, 2007)

In the packaging industry, plastic films are applied alone or in combination with metal and/or paper to perform packaging essential functions for the delivery of high quality food products to the consumer (Abdel-Bary, 2003). Polymers or plastics (the commercial products of polymers) are made of various repeating units (monomers) and combined together by means of a chemical reaction (Rahman, 2007). Plastics are the fastest-growing group of food packaging materials. Broad spectrum of properties and wide diversity is the unique advantage of synthetic polymers.

Plastics are inexpensive, light, easily shaped and sealed. Their permeability to gases and vapors and the possibility of their interaction with food product are often among their disadvantages.

The chemical and physical nature of polymers used in the manufacturing of plastic containers determine their properties; polymer characteristics are mainly ruled by their molecular weight, molecular structure, chemical composition and degree of crystallinity. In turn, these factors influence polymer density and the temperatures at which polymers undergo physical transitions. Regarding chemical composition, polymers are divided into two broad categories; homopolymers: they have similar repeating building-block units throughout their macromolecules, and heteropolymers: they have two or more different building- block units distributed regularly or irregularly throughout their macromolecules (Robertson, 2016).

2.2.1. Food packaging plastic materials

Polyolefins are the mostly used polymers for food packaging because of their low cost, good barrier properties to moisture and heat sealing properties. They are thus, used to line the interior of plastic or paperboard containers and flexible pouches (Stollman *et al*., 1994). Polyolefins include mainly Polyethylene (PE) and Polypropylene (PP). Polyethylene (PE), is the most widely used polyolefin in packaging. Other widely used plastics include: polyvinyl chloride (PVC), polyamide (Nylon), polyesters i.e. polyethylene terephthalate (PET), polyvinylidene chloride (PVDC), polystyrene (PS), polycarbonate (PC), ethylene vinyl alcohol (EVOH), etc. (Abdel-Bary, 2003). Other desirable characteristics of plastic packages are: easy to open, reclosable, safe to consumers, tamper-proof and environmentally friendly (Rahman, 2007).

2.2.1.1. Polyethylene (PE) is a polymer produced from the polymerization of ethylene gas. Ethylene (C_2H_4) molecule can produce highly inert long-chain macromolecules by combining

repeating ethylene monomers through breaking of carbon-carbon double bond. PE molecular formula is (-CH_2-CH_2-) n. Four main types of polyethylene that differ in structure, properties and processes of manufacturing are commercially available. They are: Low density polyethylene (LDPE), Medium density polyethylene (MDPE), High density polyethylene (HDPE) and Linear low density polyethylene (LLDPE) (Stollman *et al*., 1994). Typical properties of polyethylene films are shown in Table 9 (Abdel-Bary, 2003).

Table 9. Typical properties of polyethylene films

Property	Polymer		
	LDPE	LLDPE	HDPE
Glass transition temperature (T_g; °C)	–120	–120	–120
Melting temperature (T_m; °C)	105-115	122-124	128-138
Heat distortion temperature, at 455 kPa (°C)	40-44		62-91
Density (g/cm^3)	0.915-0.940	0.915-0.935	0.94-0.97
Tensile modulus (GPa)	0.2-0.5		0.6-1.1
Tensile strength (MPa)	8-31	20-45	17-45
Elongation (%)	100-965	350-850	10-1200
WVTR* at 37.8 °C and 90% RH (g µm/m^2 d)	375-500		125
O_2 permeability, at 25 °C (10^3 cm^3 µm/m^2 d atm)	160-210		40-73
*WVTR: Water vapour transmission rate (d = day, 24 h) RH: relative humidity			

(From Abdel-Bary, 2003)

a) LDPE is the type of polyethylene most widely used in packaging. It is almost chemically inert with high permeability to gases, very low permeability to water vapor, and poor odor barrier properties. The advantages of LDPE are: low price, flexibility, containing few additives, good processability and heat sealability, toughness and ease of coating onto other materials e.g.

aluminum and paper. It can be extrusion-coated, laminated, or coextruded (Stollman *et al*., 1994).

b) HDPE in comparison to LDPE, it is less transparent, stiffer, and more resistant to greases and oils. It has lower permeability to water vapor and a higher softening point compared to LDPE (Stollman *et al*., 1994).

c) LLDP contains many short side chains. Compared to LDPE, LLDPE is stiffer, less transparent and more crystalline because of its molecules linearity. While branching causes toughness, linearity causes strength. LLDPE has many advantages over LDPE such as better heat sealing properties, better chemical resistance and performance at different range of temperatures, better resistance to environmental stress crack (ESC), higher strength and higher surface gloss. LLDPE in the form of film has higher tear resistance properties and elongation than LDPE, also, better puncture resistance and higher tensile strength. LLDPE film manufactured using metallocene catalysts has high-clarity and used widely for food packaging films and bottle blow molding. LLDPE replaces LDPE and HDPE in many applications because of its superior properties (Robertson, 2016). Schematic representation of branched and linear polymers is illustrated in Figure 6.

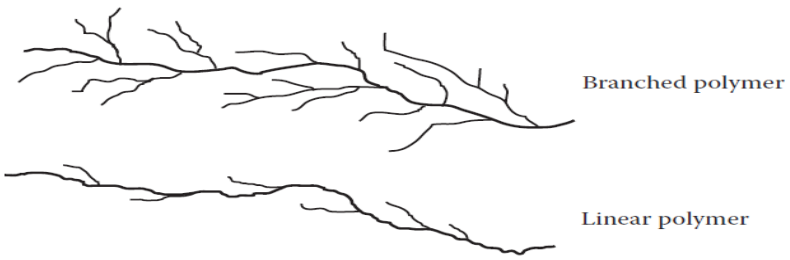


Figure 6: Schematic representation of branched and linear polymers showing the larger volume swept out by the branched structure, resulting in its lower density. Branches of the main backbone are indicated by narrower lines; they have no direct proportional relationship to cross-sectional dimensions. (From Brown, W.E., Plastics in Food Packaging: Properties, Design and Fabrication, Marcel Dekker, New York, p. 106, 1992)

2.2.1.3. Polyethylene Terephthalate (PET)

PET production involves an esterification reaction; ethylene glycol (EG) reacts with terephthalic acid (TPA). Also, it can be produced by a preferred, more controllable reaction where (EG) reacts with the dimethyl ester of TPA (dimethyl terephthalate or DMT) in a *trans*-esterification reaction. The by-product of the first reaction is water, while the second reaction's by-product is methanol. PET is a strong, linear, transparent thermoplastic polymer. In the glassy state, it is tough, stiff and ductile. Films and bottles made of PET are highly amorphous (APET) with small crystallites and exceptional transparency.

As a food packaging material, PET films have superior characteristics such as their great tensile strength, light weight, remarkable chemical resistance and elasticity. An important property of PET films also, is their stability over a broad temperature range of (−60°C to 220°C) which justifies their use as "boil-in-the-bag" products, bags for frozen products and oven bags, as they can tolerate high temperatures without decomposing. PET is often laminated to or extrusion coated to LDPE and is essentially the outer and primary support of such multilayered materials. Reaction scheme for formation of poly (ethylene terephthalate) (PET) is shown in Figure 7 (Robertson, 2016). Typical properties of polyethylene terephthalate (PET) films are illustrated in Table 10 (Abdel-Bary, 2003).

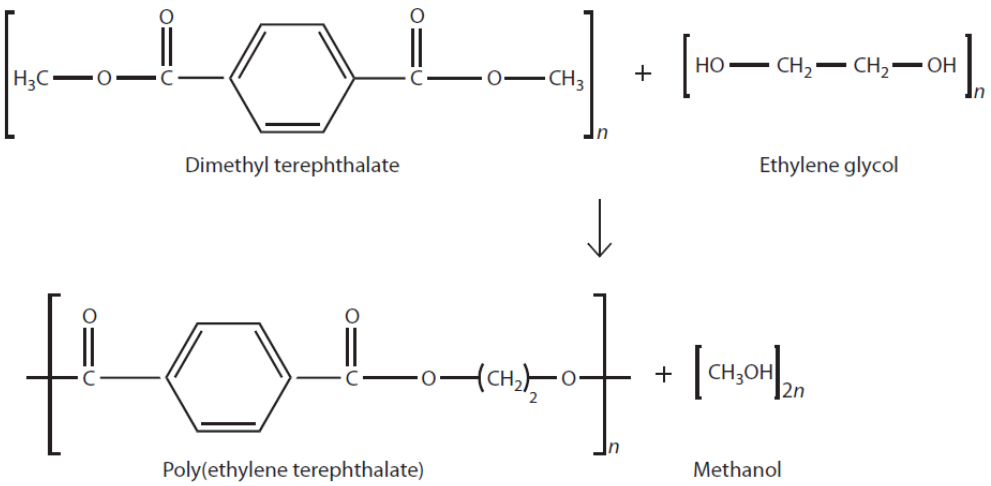


Figure 7: Reaction scheme for formation of poly (ethylene terephthalate) (PET) (From Robertson, 2016).

Table 10.Typical properties of polyethylene terephthalate (PET) films

Property	PET polymer	
	Unoriented	Oriented
T_g (°C)	73-80	73-80
T_m (°C)	245-265	245-265
Heat distortion temperature, at 455 kPa (°C)	38-129	
Density (g/cm^3)	1.29-1.40	1.40
Tensile modulus (GPa)	2.8-4.1	
Tensile strength (MPa)	48-72	220-270
Elongation (%)	30-3,000	70-110
WVTR, at 37.8 °C and 90% RH (g µm/m^2 d)	390-510	440
O_2 permeability, at 25 °C (10^3 cm^3 µm/m^2 d atm)	1.2-2.4	1.1

(From Abdel-Bary, 2003)

2.2.3. Smart, interactive and active packaging

Although storage of food products using various preservation technologies (vacuum packaging, modified atmosphere packaging, etc.) extends the shelf life of refrigerated products, there is still concern regarding the potential survival of anaerobic and/or microaerophilic psychrotrophic pathogens. Thus, the use of additional hurdles is required in order to guarantee such products'

safety. The terms: smart, interactive and active packaging, have been used to describe the innovative concept of package structures that maintain food quality, improve safety, sensory properties and extend products' shelf life through interaction of the packaging material with the contained foodstuff (Skandamis and Nychas, 2002).

According to European Union (EU) regulations 1935/2004/EC and 450/2009/EC, active packaging materials are used to maintain or enhance safety and quality of packaged food or extend its shelf life. This can be obtained through the incorporation of certain additives that release or absorb substances into or from the environment surrounding the food or from the packaged food itself. The principle of active packaging has been applied to the use of antimicrobials, antioxidants and other synthetic and naturally occurring substances added to the packaging material resulting to the inhibition of microbial growth and lipid oxidation respectively (Kontominas, 2016; Farghal *et al*., 2017).

As a result of market trends and consumer demand, active packaging (AP) is increasingly becoming an important area of research. AP main systems involve moisture absorption and control, oxygen scavenging, antioxidant/antimicrobial (AO/AM) migrating and non-migrating systems, carbon dioxide and ethanol generation, etc... Of these, AM systems are of utmost importance (Suppakul *et al*., 2003).

Antimicrobial packaging may substantially influence the shelf life and safety of meat and meat products. Antimicrobial substances may provide high quality and safe products through controlling the microbial population by targeting specific microorganisms for each product. Incorporation of various antimicrobial compounds in synthetic polymers and edible films has

been evaluated using enzymes, organic acids and their salts, bacteriocins, miscellaneous compounds like triclosan, silver zeolites, fungicides, etc. as antimicrobial active agents (Quintavalla and Vicini, 2002). In general, food packaging systems include: 1) package/food systems and 2) package/headspace/food systems (Fig.8) (Han, 2000).

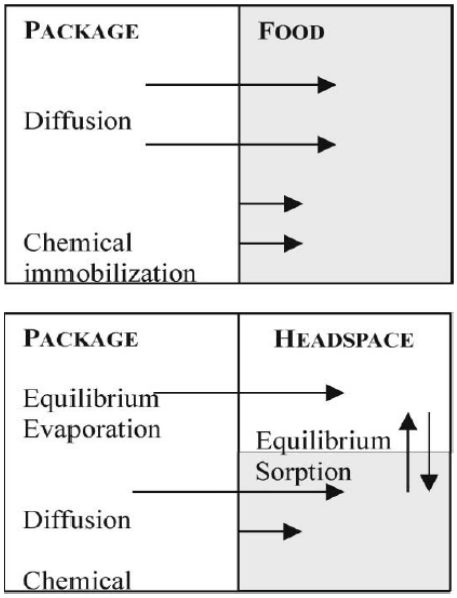


Figure 8. Food packaging systems and relative behavior of active substances. (From Han, 2000)

A package/food system may be a liquid or solid food product in contact with the packaging material, without headspace. In this system, the migration phenomena are described by diffusion occurring between food and the packaging material and the food and partitioning at the interface. In this situation, antimicrobial agents may be incorporated into the packaging materials and then, by diffusion and partitioning they will migrate into the food. Migration of active substances in different applications of antimicrobial packaging systems are shown in Figure 9. In package/headspace/food systems, the distribution of the antimicrobial agent among packaging material, headspace and/or food must be considered to evaluate the interfacial distribution of the used antimicrobial substance (Quintavalla and Vicini, 2002).

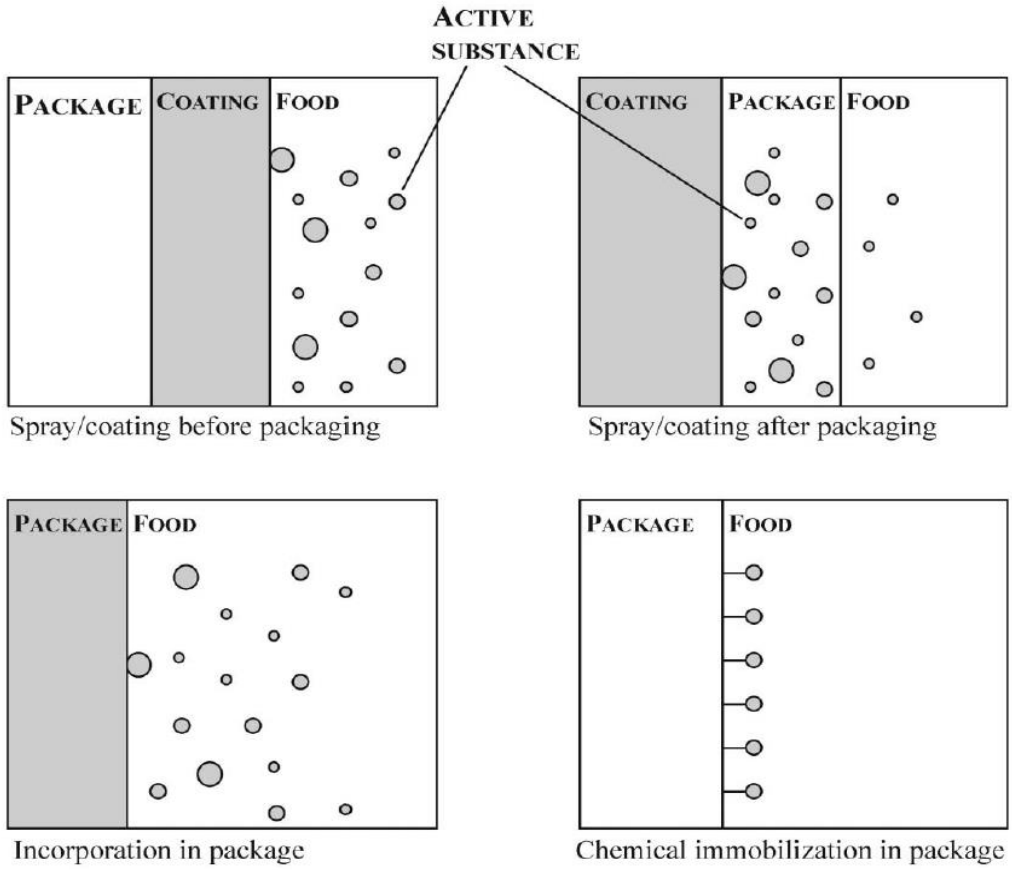


Figure 9. Migration of active substance in different applications of antimicrobial packaging systems. (From Han, 2000)

The antimicrobial effect of nisin (NI), natamycin (NA) and their combination (NI+NA), incorporated into cellulose was investigated by Pires *et al*.,(2008).The results showed that, in vitro, NI films exhibited an antimicrobial effect against *Staphylococcus aureus* and *L. monocytogenes*, while NA films showed antimicrobial activity against *Geotrichum sp*. and *Penicillium sp.* isolated from mozzarella cheese slices. No synergistic effect for antimicrobial activity was shown by NI+NA films when tested in cheese. During a storage period of 9 days, NA and NI+NA containing films inhibited the growth of yeasts and moulds in mozzarella cheese slices, extending product the shelf life by 6 days compared to control samples. NI and NI+NA containing films when tested against *Staphylococcus sp*. in sliced mozzarella, showed a limited

effect, while films containing NI were able to delay the growth of psychrotrophic bacteria in the cheese by 6 days. NA containing films decreased the count of mozzarella cheese yeasts and moulds by 2 log units by the ninth day of storage compared to control films. The study concluded that NA containing films could be used as antimicrobial active food packaging for mozzarella cheese. Effect of antimicrobial films against yeasts and moulds in sliced mozzarella is illustrated in Figure 10.

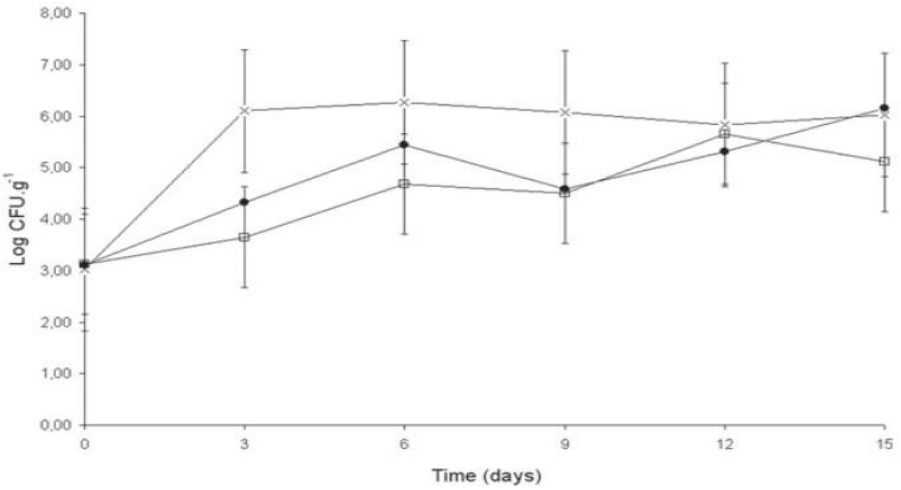


Figure 10. Effect of antimicrobial films against yeasts and moulds in sliced mozzarella. (×) Control; (□) film containing 8% commercial NA (50% w/w of purity); (•) film containing 50% commercial NI (2.5% w/w of purity) + 8% commercial NA (50% w/w of purity). (From Pires *et al*., 2008)

In a study by Guerra et al., (2005), an active packaging was developed using nisaplin as a biopreservative agent, coated or adsorbed to cellophane for the preservation of chopped meat. First, nisin adsorption to cellophane, surface was examined at 8, 25, 40, 60◦C using nisin at different concentrations. Then, the determination of antimicrobial activity effectiveness of adsorbed nisin to cellophane for the preservation of fresh veal meat was evaluated by the reduction of the total aerobic bacteria. The results showed that nisin adsorption was higher at 8◦C to cellophane. The nisin-cellophane active packaging remarkably reduced the total aerobic bacteria by ca 1.5 log units during a storage period of 12 days at 4◦C. This study suggested that

the developed active nisin-cellophane packaging could efficiently control the microbial growth of chopped meat and extend its shelf life at refrigeration temperatures. Additionally, more enhanced microbial stability of fresh meat may be provided by the combination of nisin with refrigeration and storage of meat under vacuum or modified atmosphere.

2.2.4. Bioactive packaging

Recently, the incorporation of biologically active and functional additives into the food packaging material has been devised. Such biologically active substances exert a beneficial health effect to the consumer. This comprises the principle of **bioactive packaging**. Bioactive substances migrate from the packaging material into the packaged food and exert their beneficial health function. Since bioactive additives used in packaging migrate into the packaged food, they should be authorized as indirect food constituents. As a rule, the difference between active and bioactive packaging, is that the former maintains packaged food safe and protected from microbial, chemical and physical changes, while the latter functions as active packaging besides exerting a beneficial health effect to the consumer. Exceptions to this rule exist i.e. the incorporation of a natural substance into the packaging material which upon migration into the packaged food will provide for example a cholesterol lowering effect to the consumer. In this case the additive does not affect quality and/or safety of the packaged food (Kontominas, 2016).

2.3. Chemical vs. natural preservatives

Preservation of food is the process by which food maintains its desirable properties at their maximum level for consumer's benefit. Food properties, in general, are influenced by each step of handling, processing, storage and distribution. Thus, in food preservation, understanding of the effects of each preservation process and handling method is highly important. The food quality level desired and length of preservation are main points that should be considered in food

preservation. In Figure 11 different food production stages are shown. At any stage, quality loss may be diminished with proper control; thus quality mainly relies on the control of the whole food processing chain. The main quality-loss mechanisms and consequences are illustrated in Table 11 and Figure 12 (Rahman, 2007).

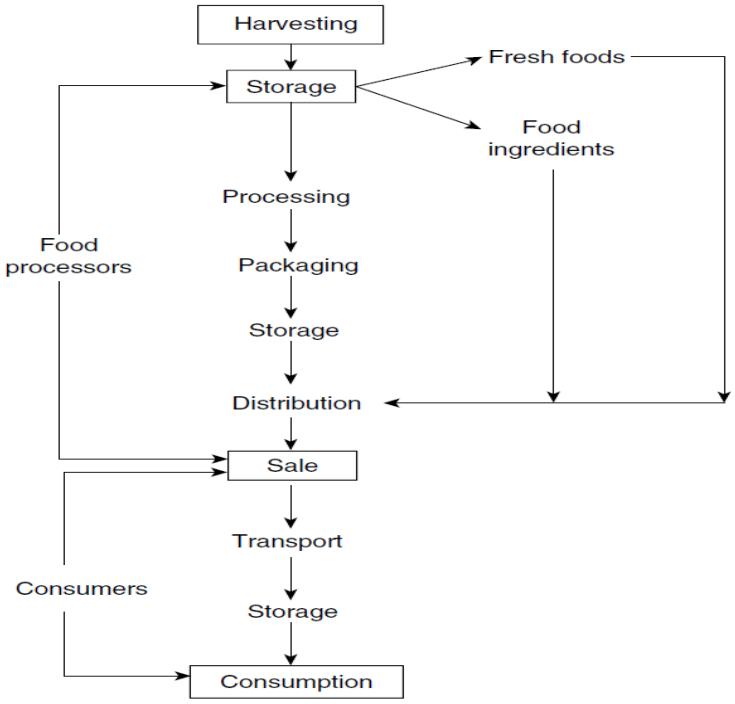


Figure 11: Various stages of food production, manufacture, storage, distribution, and sale. (From Rahman, 2007)

Table 11. Major Food Quality-Loss Mechanisms

Microbiological	Enzymatic	Chemical	Physical	Mechanical
Microorganism growth	Browning	Color loss	Collapse	Bruising due to vibration
Off-flavor	Color change	Flavor loss	Controlled release	Cracking
Toxin production	Off-flavor	Nonenzymatic browning	Crystallization	Damage due to pressure
		Nutrient loss	Flavor encapsulation	
		Oxidation–reduction	Phase changes	
		Rancidity	Recrystallization	
			Shrinkage	
			Transport of component	

(From Rahman, 2007)

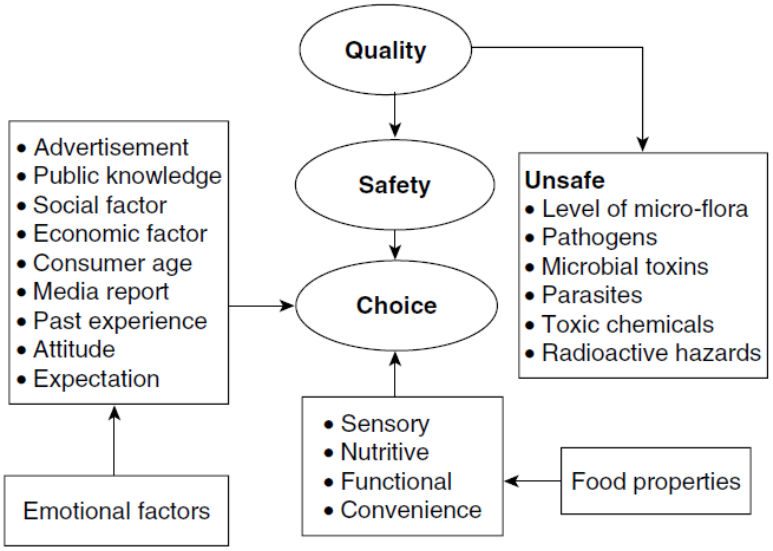


Figure 12: Factors affecting food quality, safety, and choice. (From Rahman, 2007)

Antimicrobial compounds used as food preservatives may be divided into: conventional (chemical) and naturally occurring.

2.3.1. Conventional antimicrobials

Conventional antimicrobials include chemical compounds with documented antimicrobial activity i.e. SO_2 (H_2SO_3), $NaNO_2$, $NaNO_3$, CO2 (H_2CO_3), synthetically produced organic acids (benzoic, propionic, lactic, acetic and sorbic acids) etc. as well as enzymes possessing antimicrobial activity i.e. lysozyme, lactoferrin etc. i.e. for the inhibition of *Escherichia coli* O157:H7 growth in ground roasted beef slurries, acetic acid was the most effective antimicrobial additive compared to lactic or citric acid. As a conventional food additive, sorbate could be applied to food by dipping, direct addition, spraying or by incorporation into the packaging material (Davidson *et al*., 2013).

In processed meats, sodium nitrite (as an antimicrobial agent) inhibits *Clostridium botulinum* growth and production of its neurotoxin. Lysozyme is among the most active antimicrobial agents against gram-positive bacteria, because of the specific construction of G (+) bacterial cell wall (peptidoglycan layer). In meat products, sulfites may be used for the inhibition

of acetic acid-producing bacteria, lactic acid bacteria, and spoilage bacteria. The mechanism by which organic acids function inside the microbial cell is illustrated in (Fig.13). In their un-dissociated form, organic acids can penetrate the microbial cell membrane, then acid dissociates when encountering a near-neutral pH environment. It dissociates into free proton and acid anion that acidifies the cell interior (Davidson *et al*., 2013).

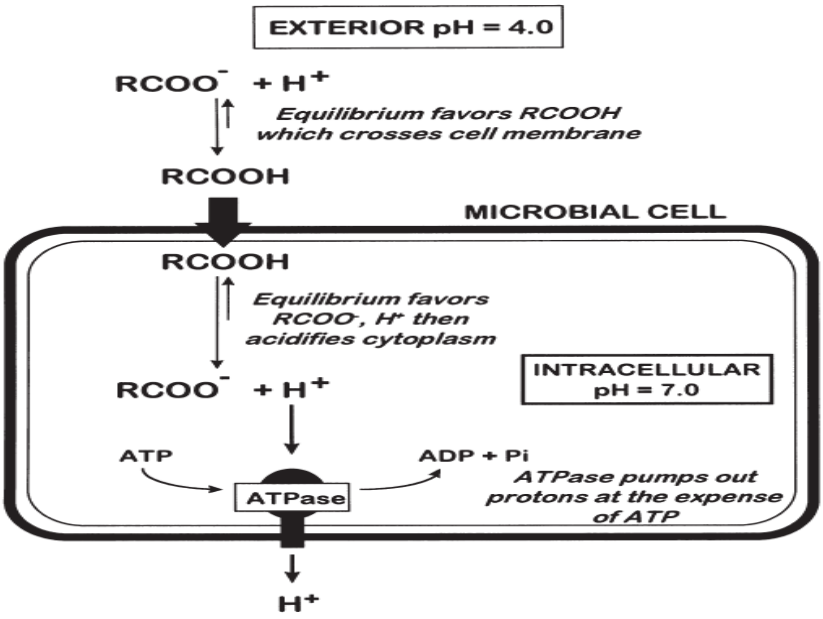


Figure 13: Fate of an organic acid (RCOOH) in a low-pH environment in the microbial cell (From Davidson *et al*., 2013)

2.3.2. Naturally occurring antimicrobial compounds (natural preservatives)

Despite the wide range of food preservation techniques such as fermentation, drying, sterilization, freezing and use of conventional preservatives food spoilage and poisoning by microorganisms is yet a problem for the food industry in its effort to supply high quality and safe food to consumers. Food manufacturers progressively depend on milder preservation techniques to satisfy consumer demands for less processed, more natural foods of high quality and nutritional value as opposed to that achieved by severe food preservation techniques including those using chemical and synthetically derived preservatives. As a result of the natural defense

mechanisms or competition, living organisms (plants, animals, insects, etc.) produce different natural antimicrobial compounds which may be used in food preservation (Rahman, 2007).

2.3.2.1. Natural Antimicrobials of Plant Origin:

Most of plant antimicrobial compounds are classified as secondary metabolites of terpenoid or phenolic biosynthetic origin and the rest are proteins and hydrolytic enzymes (glucanases and chitinases) that act by invading microorganism membranes exerting specific antimicrobial activity (Rahman, 2007).

a) Phytoalexins

They are broad-spectrum antimicrobial compounds; their production in plants is induced by microbial infection. They are known as host-synthesized usually having low-molecular-weight. In more than 20 plant families, more than 200 types of phytoalexins have been discovered. Phytoalexins have antimicrobial activity against fungi, gram-positive and gram-negative bacteria. It was found that gram-positive bacteria are more sensitive to the effect of phytoalexins than gram-negative bacteria. Phytoalexins from *Pisum sativum* and *Solanum tuberosum* are shown in Figure 14 (Nychas, 1995; Rahman, 2007).

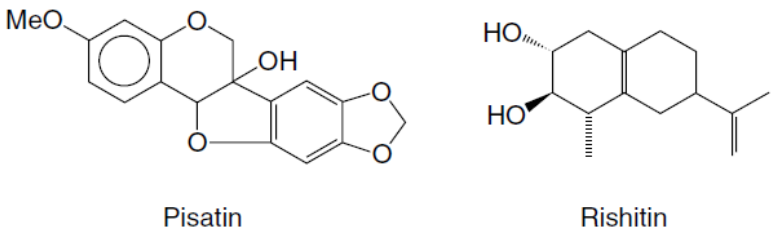


Figure 14: Phytoalexins from *Pisum sativum* (left) and *Solanum tuberosum* (right). (From Rahman, 2007)

b) Organic Acids

Organic acids like acetic, propionic, lactic, malic, citric, succinic and tartaric and their derivatives are used as antimicrobial agents. Malic, citric, succinic and tartaric are found in grapes, pineapples, citrus fruit and vegetables (e.g. oranges, lemons, carrots, broccoli, etc.).

Malic acid occurs in various fruits mainly in apples. The antimicrobial activity of lactic and propionic acids is well documented. They are active against a broad spectrum of microorganisms and specifically target cell membranes, cell walls, metabolic enzymes, genetic material and protein synthesis systems (Nychas, 1995).

c) Phenolic Compounds

The vast majority of plant phenolic compounds contribute to the plant defense mechanism as well as its sensory attributes (odor, taste, and appearance). Structurally, phenolic compounds can be simple i.e. phenolic acids, or highly polymerized compounds as tannins. In general, phenolics are composed of an aromatic ring with one or more hydroxyl groups on it. Flavonoids are the most important group of phenolics in food, including catechins, proanthocyanins, anthocyanidins, and flavons, flavonols, and their glycosides. Of plant phenolics, tannins are an important group. Plant phenolics are found in vegetables, fruits, cereals, legumes, olive oil, tea, wine, etc. The antimicrobial activity of natural phenolics of olives, tea, and coffee have been investigated in many studies more than phenolics from other sources. Spice phenolics such as gingerol, capsaicin, and zingerone have been documented to inhibit bacterial spore germination (Rahman, 2007). Naturally occurring plant phenolics although rarely used as food preservatives, exhibit an impressive antimicrobial spectrum and are important food preservatives (Rahman, 2007; Kontominas, 2016).

d) Essential Oils

Essential oils are aromatic, volatile liquids extracted from plants. Essential oils are secondary metabolites possessing antimicrobial properties and hence play a role in plant defense mechanism. Because of consumers' negative perception of synthetic preservatives, recently the interest in application of essential oils in food preservation has increased. Essential oils and their constituents' antimicrobial activity have been broadly documented. The major hurdle for

applying essential oils as food preservatives is that they are not strong enough if used alone, and if added in adequate amounts to provide a desirable antimicrobial effect they result in organoleptic food quality deterioration. A solution to this problem is taking advantage of synergies between small amounts of essential oils added to food along with other additives. Chemical structures of selected essential oil constituents are illustrated in Figure 15(Appendix D) (Hyldgaard *et al*., 2012).

For the investigation of synergistic combinations of different food antimicrobials, carvacrol and thymol were evaluated separately and in combination with other food preservatives such as acetic acid, EDTA, lactic acid, and citric acid against *Salmonella Typhimurium*. In the form of 22 combinations, six antimicrobial agents were used (Fig.16) (Appendix D). Results showed that *Salmonella Typhimurium* growth was outstandingly inhibited in Mueller-Hinton broth containing thymol(400 mg/l) carvacrol (400 mg/l), EDTA (300 mg/l), acetic acid 0.2%(vol/vol), lactic acid 0.2% (vol/vol), or citric acid 0.2% (wt/vol) at the mentioned concentrations. Different antimicrobial combinations showed a remarkable reduction in *Salmonella Typhimurium* populations. There were synergistic effects in samples treated with combinations compared to samples treated with citric acid, EDTA, carvacrol, thymol, or acetic acid alone. There was no synergistic effect in the combination of lactic acid with carvacrol or thymol against *Salmonella Typhimurium.* The study concluded that some organic acids could be useful food preservatives and produce a sufficient antimicrobial effect if used in combination with reduced concentrations of both carvacrol and thymol (Zhou *et al*., 2007).

2.3.2.2. Natural Antimicrobials of Animal Origin:

a) Lactoperoxidase

It is a glycoprotein enzyme naturally found in saliva, colostrum, raw milk and other biological secretions such as tears (Davidson *et al*., 2013).

b) Chitosan

Chitosan, (1-4)-2-amino-2-deoxy-b-d-glucan, is a natural component found in cell walls of fungi, derived from chitin; a shellfish processing by-product.

Chitosan includes a series of polymers with different ratios of glucosamine and N-acetyl glucosamine. Chitosan can efficiently inhibit the growth of foodborne bacteria and fungi (Davidson *et al*., 2013). Chitosan, besides its antimicrobial action, is also known to possess antioxidant activity.

c) Avidin

It is a glycoprotein present in egg albumen. Its concentration differs with the hen's age but comprises approximately 0.05% of the total albumen protein (Davidson *et al*., 2013).

d) Ovotransferrin

It is found naturally in egg albumen, called also conalbumin, it is an iron-chelating protein (Davidson *et al*., 2013).

2.3.3. Applications of natural food preservatives

In a study regarding the preservation of whole, refrigerated, air-packaged crucian carp, both tea polyphenols and rosemary extract as potential natural food preservatives were used. Results showed that the shelf-life of untreated (control) crucian carp was 7–8 days, 13-14 days for samples treated with tea polyphynols and 15-16 days for samples treated with rosemary extract according to the results of sensory attributes assessment. Increased shelf-life was confirmed also by microbiological assessment tests. In both groups of samples that were treated with tea polyphynols or rosemary extract, the increase of TBA, K-value, TVB-N and pH was markedly postponed compared to the control group. This study suggested that both rosemary extract and tea polyphynols could be used as natural food preservatives for the extension of crucian carp shelf-life during refrigerated storage (Li *et al*., 2012).

For preservation purposes, antimicrobial packaging as a promising form of active food packaging, can be efficiently applied. In this kind of food packaging antimicrobial agents are incorporated into a polymeric packaging material to prevent microbial growth. This application could be effectively used in the form of films, containers and utensils. Edible antimicrobial films are also a promising development in using active packaging as a preservation technique (Rahman, 2007).

Salmon as a perishable fish, contains high levels of polyunsaturated fatty acids (PUFAs) that affect human health positively. The content of PUFAs is very much vulnerable to oxidation. Incorporation of antioxidants into new food packaging films could highly improve food shelf life and as a consequence increase safety and health of consumer. For investigating lipid oxidation in salmon samples during frozen storage, peroxide value (PV), free fatty acids (FFA), thiobatbituric acid index (TBARS), conjugated dienes (CD), conjugated triene hydroperoxides (TH) and p-anisidine value (AV) were determined. The results confirmed that natural antioxidants, derived from barley husks (NABH) were effective in delaying lipid hydrolysis and increasing salmon oxidative stability. The study highly demonstrated the advantage of using natural antioxidants in the development of active packaging films for food preservation purposes (de Abreu *et al*., 2010).

2.4. Corona treatment of films

Many plastic films due to their inherently low surface energy structure have poor surface adhesion which makes it difficult for different adhesives and coatings e.g., inks and paints to adhere to their surfaces. Suitable surface modification of these plastic films will improve surface adhesion properties through increasing plastic films' surface energy (Wolf, 2015). There are

many surface modification techniques that used for the improvement of inert non-porous structure of plastic films surfaces. Their classification is as follows:

Chemical Methods Used for Surface Modification

- Fluorination; Direct Fluorination, Indirect Fluorination
- Chlorination
- Bromination
- Sulfonation
- Grafting
- Chemical etching

Physical Methods Used for Surface Modification

- Plasma Treatment
- Flame treatment
- Corona Treatment (Abdel-Bary, 2003)

Modifications of plastic films:

Improvement of physical, chemical or mechanical properties of plastic films is the target of different plastic films modifications, so that plastic films become suitable for different applications e.g. decorating, printing, coating, lamination and wetting. This can be done by subjecting plastic films to either physical, chemical or mechanical treatments e.g., orientation, crystallization, crosslinking**,** which modify surface and bulk topography, crystalline morphology, remove contaminants and most importantly, increase surface energy. Good adhesion of plastic films surfaces to other substrates requires the removal of different contaminants and 'roughening' of the film surface (Abdel-Bary, 2003).

2.4.1. Chemical Modifications

Polyolefins are very popular packaging materials as they have excellent water barrier properties besides their low cost, processability and toughness. In order to modify the surfaces of polyolefins without changing their bulk properties, chemical reactions of those surfaces with gas are used. For this kind of chemical modification of the surface, hydrogen fluoride, fluorine, sulfur tetrafluoride, bromine and chlorine have been investigated (Abdel-Bary, 2003).

2.4.1.1. Fluorination

Fluorination of polymers may be direct or indirect (Abdel-Bary, 2003).

2.4.1.1.1. Direct fluorination:

This surface modification is a treatment of the polymer surface with gaseous fluorine mixtures spontaneously at room temperature (Kharitonov and Kharitonova, 2009). The polymeric material is totally converted to a fluorocarbon polymer by means of highly active agents such as hydrogen fluoride, fluorine, or sulfur tetrafluoride. The low dissociation energy of fluorine makes it a highly active fluorinating agent. With carbon, it forms exceedingly stable bonds (Abdel-Bary, 2003). Experimentally, it was shown that direct fluorination enhances polymer articles' commercial properties, such as adhesion and printability, barrier properties of polymer vessels, gas-separation and mechanical properties of polymer membranes and polymer-based composites (Kharitonov and Kharitonova, 2009).

Polymer fluorination using fluorine is divided into: **bulk fluorination and surface fluorination**. In surface fluorination of polycarbonate (PC), polytstyrene (PS) and polymethyl methacrylate (PMMA) using F_2 diluted with He or N_2, the extent and depth of fluorination increase with temperature, reaction time and gas pressure of F_2 (Abdel-Bary, 2003). The dissociation of

moleculer fluorine F2 → F• + F• is usually considered as the initiation process of polymer fluorination, this reaction being highly endothermic. However, there are also other exothermic reactions that could be regarded as initiation processes as the first reaction does not fulfil this requirement (Fig.17) (Kharitonov and Kharitonova, 2009).

$$F_2 + –CH_2– \rightarrow F^{\bullet} + –CH^{\bullet} – + HF$$
$$F_2 + –CH_2–CH=CH–CH_2–CH_2– \rightarrow F^{\bullet} + –CH_2–CH=CH–CH^{\bullet} –CH_2– + HF$$
$$F_2 + –CH_2–CH_2–C(=CH_2)–CH_2– \rightarrow F^{\bullet} + –CH_2–CH^{\bullet} –C(=CH_2)–CH_2– + HF$$
$$F_2 + –CH=CH– \rightarrow –CHF–CH^{\bullet} + F^{\bullet}$$

Figure 17: Direct fluorination reactions (From Kharitonov and Kharitonova, 2009)

2.4.1.1.2. Indirect fluorination

This type of fluorination is used to avoid the disadvantages of current fluorinating agents like F_2, SF_4, or HF. Nontoxic sulfur hexafluoride, fluorocarbons and chlorofluorocarbons are used. When these gases are exposed to high-energy environments such as glow discharge, plasma, or gamma radiation, they produce active fluorinating agents. Surface fluorination is preferred than bulk fluorination of plastic items because it is more cost-effective. A surface coating of fluorinated polymer (0.1mm thickness) can be given to large fabricated plastic items making them impenetrable to most solvents while maintaining acceptable chemical, water and solvents resistance. Accordingly, fluorinated plastic containers are used to package paint, gasoline, motor oil and turpentine (Abdel-Bary, 2003).

2.4.1.2. Chlorination:

In a study on gas-phase chlorination of LDPE surface, both in the presence of ultraviolet (UV) radiation and under ambient light, it was reported that the surface consisted of C-Cl and C-Cl_2 moieties. Formation of vinyl chloride and allyl chloride moieties is also the result of PE surface

chlorination. The chlorination reaction results in good barrier characteristics with more flex resistance (Abdel-Bary, 2003).

2.4.1.3. Bromination:

PE, PP and PS film surface gas-phase bromination using a free-radical photochemical pathway occurs with high regioselectivity. The introduction of Br moieties on the surface of polyolefin promotes the introduction of many specific functional groups on the surface by means of nucleophilic substitution (of Br moieties by different nucleophiles). Formation of conjugated double bond long sequences is the result of surface bromination that is accompanied by concurrent dehydro-bromination. Thus, the surface of brominated polyolefins includes (Br) moieties in different chemical environments (Abdel-Bary, 2003).

An example, the free-radical mechanism for the bromination of PE film surface follows. The first step is the cleavage of the homolytic bond of the bromine molecule into two bromine radicals upon exposure to radiation, [equation (1)]. The second step is the elimination of a hydrogen atom from LDPE methylene unit by the bromine radical leading to the formation of a radical center on the LDPE chain. Then, a C-Br moiety and a bromine radical are formed when this radical center reacts with a bromine molecule, [equations (2) and (3)]. Then, the bromine radical reacts with another –CH2– unit and this chain reaction continues, [equation(4)] (Abdel-Bary, 2003).

$$Br_2 \xrightarrow{UV} 2Br^{\bullet} \qquad (1)$$

$$-{}^{\bullet}CH-CH_2- + Br_2 \rightarrow -CHBr-CH_2- + Br^{\bullet} \qquad (2)$$

$$-CH_2-CH_2- + Br^{\bullet} \rightarrow -{}^{\bullet}CH-CH_2- + HBr \qquad (3)$$

$$-CHBr-CH_2- + Br^{\bullet} \rightarrow -CHBr-{}^{\bullet}CH- + HBr \quad (4)$$

2.4.1.4. Sulfonation

This chemical modification involves the exposure of the surface of the polymer to SO_3/air followed by neutralization using NH_4OH, NaOH, or LiOH. “Reductive metallization” is the name of chemical reduction of silver, tin, or copper that results from the neutralization process that follows sulfonation. Proton conducting polymer electrolytes used in fuel cells can be obtained by sulfonation of polystyrene and aromatic polymers. Sulfonation of aromatic polymers can be easily achieved using chlorosulfonic acid, or concentrated sulfuric acid, or pure or complexed sulfur trioxide, or acetyl sulfate. The result of such a modification is that it creates excellent gas barrier properties under dry conditions, it does not influence polymer mechanical stability and it is simple (Abdel-Bary, 2003).

2.4.1.5. Grafting

Grafting is one of the promising methods of polymer surface modifications. Various functional groups are imparted to a polymer by means of graft co-polymerization. This modification could be initiated by photo-irradiation, chemical treatment, high-energy radiation, etc. (Bhattacharya and Misra, 2004).

2.4.1.6. Chemical Etching

This method of surface modification is used generally for uneven large articles when other surface modifications techniques are not suitable. In this method the LDPE container is immersed in an etchant solution such as sulfuric acid, chromic acid permanganate or chlorosulfonic acid. Extensive chemical changes on the LDPE surface are shown by reflection infrared studies; introduction of –OH, >C=O and SO_3H groups expressed in the form of new (IR)

bands. It was found that polymer surface energy of adhesion increases together with surface density of hydrophilic sites that are formed as a result of oxidation (Abdel-Bary, 2003).

2.4.2. Physical Methods Used for Surface Modification

2.4.2.1. Plasma Treatment

The term plasma means an ionized gas that contains free ions, electrons and neutral species (atoms and molecules) also known as the "4th state of matter". Plasma has specific chemical and physical characteristics that differ from solids, liquids and gases; it is electrically conductive, and contains both excited and chemically reactive species. It responds to electromagnetic field and emits electromagnetic radiation in many wave length regions (Schiorlin *et al*., 2015).

Plasma treatment is one of the most effective methods of polymer surface modification. First, gas molecules, such as nitrogen and oxygen are activated by the plasma. Then, activated species interact with the surface of the polymer forming functional groups like carbonyl, hydroxyl, carboxyl and amide groups on the polymer surface. Thus, plasma implantation reactions cause considerable changes in the properties of the polymer surface; polymers change from hydrophobic to hydrophilic. Generally, 'Plasma treatment' is used when improvement of polymeric material wettability and adhesion are required. (Abdel-Bary, 2003).

In a study focusing on the surface modification of biaxially oriented polypropylene (BOPP) to improve its wetting and hydrophilic properties, a low-pressure and low-temperature oxygen plasma, were used for polymer surface treatment. It was shown using Scanning electron microscopy that plasma treatment changes the film surface physically through creating microcraters and roughness on the polymer surface and increasing surface friction and energy.

Oxygen-containing groups such as –OH and C=O were formed and identified at 3513$cm^{-1}$and 1695cm^{-1}respectively by attenuated total reflectance infrared spectrometry. It was also indicated by microscopic investigations of water droplets on the surface of the examined polymer (BOPP) that interfacial adhesion of treated surface increased (Yousefi *et al*, 2003).

2.4.2.2. Flame treatment

For surface modification, flame treatment has been used to allow the addition of different coatings. The action of this method is hydrophilic species production on the surface of the plastic film resulting in increased surface wettability. Plastics separation by froth flotation (process separating hydrophilic from hydrophobic materials) also requires hydrophilic plastic surfaces production. In the separation of PVC and PET, flame treatment was very effective in the production of a hydrophilic surface on both plastics (Pascoe and O'Connell, 2003).

A flame (1000°C–2800°C) produced by hydrocarbon (HC) combustion is used in flame treatment, and surface of treated polymer is allowed to pass directly through these flame tips where an O_2-rich plasma has formed. By a mechanism similar to that of corona discharge, but more difficult to be controlled, an oxidized layer on the surface of the polymer is produced. It is known that this surface treatment generates high surface energy levels with longer lasting treatment than that of corona discharge (Robertson, 2016).

2.4.2.3. Corona Treatment:

As mentioned earlier, surface modifications of polyolfin films are very important in order to enhance surface wettability and adhesion properties in several applications such as printing, heat sealing, adhesive bonding, extrusion coating, composites, and metallized polyolfins. Among the numerous surface treatments that have been developed to modify polymer surface is corona treatment, which is widely used in industry. There are many theories that explain the increased

adhesion of corona-treated polyolefin films surfaces such as : 1) Polar group production as a result of oxidation, 2)Weak boundary layers elimination, 3)Increase of polymer surface roughness due to pitting.

Currently, the most accepted theory, is that corona discharge treatment causes surface energy increase through introduction of polar groups on the treated polymer surface, which in turn improves wettability and adhesion characteristics of the treated film. Most studies on film surface corona treatments are based on polyethylene (PE) and polypropylene (PP), as they are the most widely used plastic films in the world (Zhang *et al*., 1998).

Corona Effect

"Corona" is a term used to describe the situation of gas, mostly air between two electrodes. Air is a well-known electrical insulator, but in the presence of strong electric field it breaks down into ionized molecules that conduct electricity. This gives rise to a sudden electric discharge that turns to an arc or sparks between two electrodes. The discharge of the air at atmospheric pressure is the basis of corona treatment (Zhang *et al*., 1998). In this technique, application of adequately high-voltage electrical discharge to a moving sheet or film takes place. Required pretreatment of films is usually done during film extrusion. It has been shown that if the film is extruded and left for a while before treatment, its additives bloom to its surface and uneven treatment is achieved.

In one method, film is passed between metal blades (first electrode) which is connected to a high-voltage, high-frequency generator. An earthed roller (second electrode) is distanced from the first high-voltage electrode by a small gap. To avoid direct discharge of the roller, the metal electrode must be lightly narrower in width than the film to be treated. The level of corona treatment is controlled by the speed of throughput together with the generator output. A corona

discharge treatment facility is illustrated in Figure 18 (Abdel-Bary, 2003). A **close**-up of the corona treatment discharge is shown in Figure 19.

The corona effect begins with some stray electrons situated in the gas between the two electrodes as a result of cosmic rays or any background radiation. Upon application of high voltage, creation of a strong electric field occurs that accelerates the stray electrons towards the positive electrode, striking gas molecules in their path. As a result, gas becomes full of positive ions, electrons and excited molecules. Because of the instability of the excited molecules, they automatically decompose forming radicals, ions and photons. Oxidation is the principal chemical mechanism of corona treatment. Additionally, on the treated surface, crosslinking of the molecules occurs, which limits surface molecule mobility and causes molecular weight increase as well as increase in the treated film cohesive strength (Zhang *et al*., 1998).

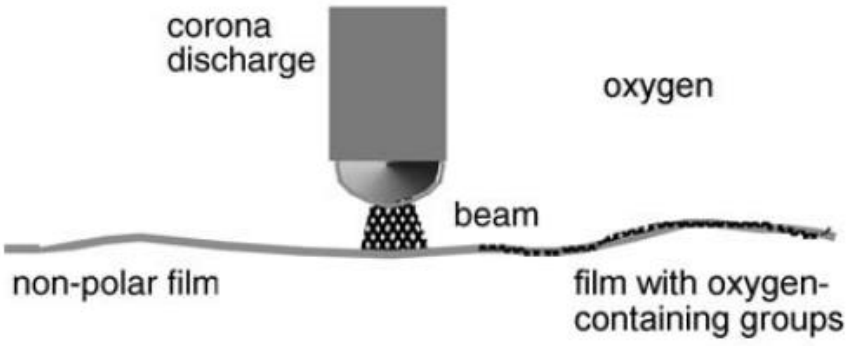


Figure 18: Corona discharge surface treatment system (From Abdel-Bary, 2003).

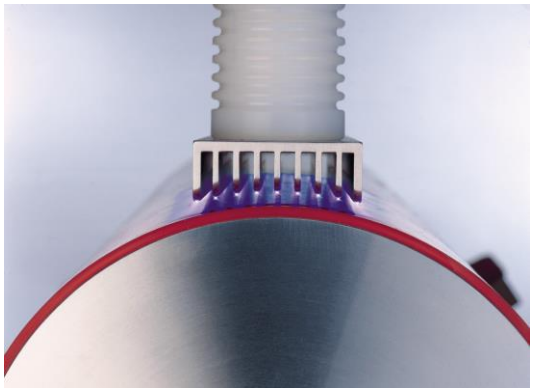


Figure 19: Close-up of the Corona treatment discharge
(From www.vetaphone.com/Corona_treater/High_dyne_level)

It has been documented that corona treatment changes the treated surface both chemically and physically. Many analytical techniques have been used for the identification of the chemical functional groups that are produced as a result of corona treatment. Of them, electron spectroscopy for chemical analysis (ESCA or XPS) and infrared spectroscopy (IR or ATR) are widely used (Zhang *et al*., 1998). Using XPS, the presence of hydroperoxide, ether, hydroxyl, ester, carbonyl or carboxylic, functional groups in corona discharge treated polyofins may be identified (Abdel-Bary, 2003).

Also, in a study investigating the changes occurring on the surface composition of corona discharge treated polypropylene film (PP) oxidized polar groups such as C=O, C-O and C-OH were identified on the film surface by infrared spectroscopy (FTIR/ ATR). Additionally, a dramatic morphology change was revealed by atomic microscopy (AFM). This change gave rise to a new chemical composition of the PP surface. Due to the polar groups formed after corona treatment, the contact angle (q) of the PP decreased, indicating an increase in the wettability which is the reason for the improvement of polymer adhesion performance (Sellin *et al*., 2003).

It was shown by XPS spectroscopic studies that oxygen is the main element incorporated into the treated film surface during corona discharge treatment using other gases other than oxygen.

Thus, oxidation is the principle mechanism by which corona treatment functions to impart good adhesion to the treated polymer surface (Zhang *et al*., 1998).

The effect of corona treatment depends on gas composition between electrodes, the treated film and its additives and relative humidity. The higher the surface humidity the longer the time required for corona discharge treatment. The higher the power used in corona discharge, more ions form and as a consequence, the higher the average kinetic energy of the particles. Temperature and time of corona treatment are also important parameters. It has been shown that corona treatment of polyolefin films for long periods of time at high temperatures shows more loose surface materials and decreased bond strength than films that are corona-treated modestly (Zhang *et al*., 1998). The corona treatment effect decreases with time, and the treated polymer surface is sensitive to both handling and dust pickup (Abdel-Bary, 2003).

2.5. Antimicrobial and antioxidant activity of grape seed/skin extracts

Grape seed (GSE) and skin (GSKE) extracts have been known to be rich sources of phenolic compounds; both flavonoids and non-flavonoids. The most important grape phenolic compounds are resveratrol, anthocyanins, flavanols and flavonols. These bioactive polyphenols have a variety of biological activities such as, antimicrobial, antioxidant, anti-inflammatory, cardio-protective, antiaging, and anticancer properties. Anthocyanins are pigments found primarily in the skins of grapes, while flavonoids, containing procyanidin polymers, (+)-catechins and (−)-epicatechin, are distributed in grapes, in stems and seeds specifically. In red grapes the main polyphenolics are anthocyanins, while white grapes are rich in flavan-3-ols. In general, phenolic compounds (Fig.20) are considered as natural food preservatives against lipid oxidation and

microbial growth. The phenolic compounds in different parts of grape and its products are shown in Table 12 (Xia *et al*., 2010).

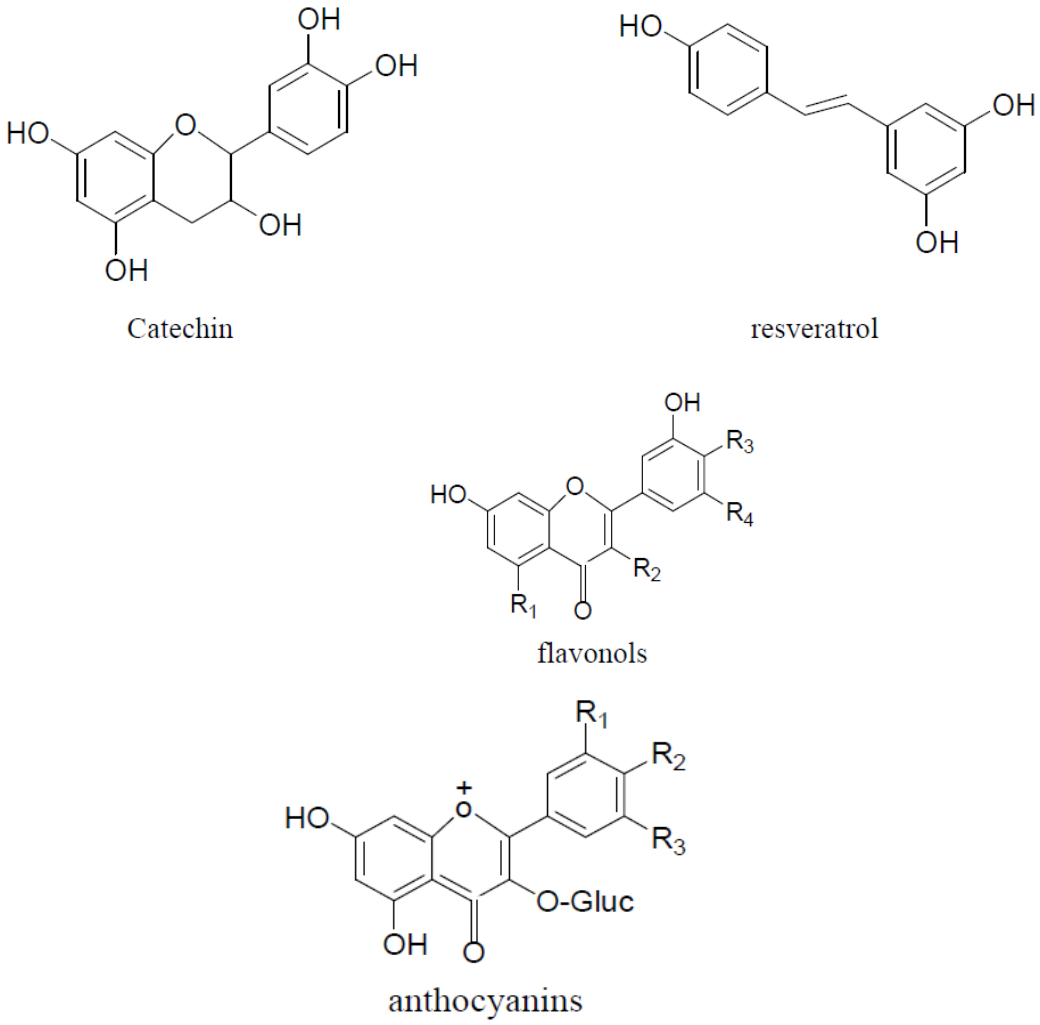


Figure 20. The chemical structures of some phenolic compounds found in grapes (From Xia *et al*., 2010)

Table 12. The phenolic compounds in different parts of grape and its products.

Resource	Phenolic compounds
seed	gallic acid, (+)-catechin, epicatechin, dimeric procyanidin, proanthocyanidins
skin	Proanthocyanidins, ellagic acid, myricetin, quercetin, kaempferol, trans-resveratrol
leaf	myricetin, ellagic acid, kaempferol, quercetin, gallic acid
stem	rutin, quercetin 3-O-glucuronide, trans-resveratrol, astilbin
raisin	hydroxycinnamic acid, hydroxymethylfurfural
red wine	malvidin-3-glucoside, peonidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, catechin, quercetin, resveratrol, hydroxycinnamic acid

(From Xia *et al*., 2010)

Grape phenolic compounds' concentration is variety dependent. Grape skin total phenolics include: catechins, flavonoids, flavanols and individual polyphenols e.g., quercetin glucoside (flavonol), ((+)-catechin, (-)-epicatechin, epicatechin gallate, astringin (stilbenes), procyanidin B1, procyanidin B2, piceid and cis- and trans resveratrol monomers (Katalinić *et al*., 2010). While the phenolic profile of grape seed extract mainly consists of flavonols, phenolic acids, catechins, proantho-cyanidins, and anthocyanins. Among them, catechins and proanthocyanidins are the major compounds, representing 77.6% of total phenolic compounds determined (Silván *et al*., 2012). **The Mechanism of the antibacterial effect of polyphenols** may be through the formation of complexes with polysaccharides and proteins (Haslam, 1996), or by binding to surface components rather than entering the cell and inhibiting extracellular or cell-bound enzymes, e.g. membrane transport proteins. Also, polyphenols bind metal ions and metal ion reduction by complexation with polyphenols may cause bacterial growth inhibition (Scalbert, 1991). Procyanidin polymers have more sites for metal ion chelation, causing greater growth inhibition (Smullen *et al.*, 2007).

The antimicrobial properties of GSE were investigated against different Campylobacter strains. Growth inhibition was in the range from 5.08 to 6.97 log CFU/ml, demonstrating the strong capacity of the GSE to inhibit Campylobacter growth. Serial dilution of the extract showed a minimal inhibitory concentration (MIC) of 20 mg/l and a minimal bactericidal concentration (MBC) of 60 mg/l against *Campylobacter jejuni*. GSE was fractionated and phenolic composition was determined by HPLC-DAD and HPLC-MS. The analysis of the antibacterial activity against *C. jejuni* of collected fractions showed that phenolic acids, catechins and 5-proanthocyanidins were mainly responsible for the antimicrobial activity observed (Silván *et al*., 2012).

In order to investigate the antibacterial activity of GSE and to determine its total phenolic content, petroleum ether was first used for the removal of fatty materials from powdered grape seeds and bagasse. The total phenolic content of GSE was 627.98 mg gallic acid equivalent (GAE)/g with the re-extraction solvent mixture; acetone: water: acetic acid(90: 9.5 : 0.5), while the phenolic compounds of bagasse extracts with the same re-extraction solvent mixture was 45.44 mg GAE/g. With the solvent mixture; ethyl acetate: methanol: water (60: 30: 10), the total phenolics of both GSE and bagasse extract were 667.87mg GAE/g and 29.55mg GAE/g respectively. The paper disc diffusion test was used for the determination of the antibacterial activity of both extracts at 1%, 2%, 4% and 20% concentrations against some pathogenic and spoilage food bacteria e.g., *Bacillus megaterium*, *Escherichia coli*, *Listeria monocytogenes*, and *Pseudomonas aeruginosa*. Results showed that acetone: water: acetic acid (90: 9.5: 0.5) extract was effective against most of the examined bacteria at 4% and 20% of GSE concentration. The tested bacteria were not inhibited by the grape bagasse. The result showed that GSE could serve

as a good antibacterial agent for preventing food deterioration at 4% and 20% concentrations (Baydar *et al*., 2004).

In order to investigate the antioxidant activity of red grape marc (**peels and seeds)**, its ethanolic extract was prepared and classes of phenolic compounds were determined. The b-carotene bleaching test was used to evaluate the antioxidant activity of red grape marc to examine its potential use as a natural antioxidant. This study showed that red grape marc was a rich source of polyphenols with effective antioxidant activities. The extracts showed an antioxidant activity higher than 43% at very low concentration (20 ppm) in total phenols; moreover all the fractions at higher concentration (80-160 ppm) showed antioxidant activity almost as effective as Butylated-hydroxytoluene (BHT). The study assumes that grape seeds had the highest contribution to the antioxidiant activity of red grape as they contain high amounts of proanthocyanidines that known for its antioxidant characteristics. The results concluded that polyphenolic compounds with an antioxidant activity comparable to that of BHT could be obtained from grape processing by-products. This study suggested that polyphenolic compounds from grape processing by-products (GSE and GSKE) can be used as natural antioxidant sources (Negro *et al*., 2003).

A standard susceptibility agar dilution technique was used in a study for the determination of the antibacterial activity of commercially available and 70% aqueous propanone (P70) extracts from different plants that are known for their high polyphenol content on *Streptococcus mutans* and other bacteria. The extracts used were **seed and skin extracts** of red and white grapes, green tea, cocoa, Pynogenol, and sloe berry. The results concluded that the minimal inhibitory concentration (MIC) of P70 extract of red grape seeds (*Vitis vinifera*) was 0.5 mg ml^{-1}, the lowest

MIC of all P70 extracts, followed by green tea and sloe berry skin (2 mg ml $^{-1}$) ; while for other P70 extracts, MIC was ≥ 2 mg ml^{-1}. This study also showed that both red and green grape extracts possessed higher activity than other tested fruits. Grape (red and green) seed P70 extracts were more active than their skin P70 extract counterparts. Finally, red grape seed P70 extract showed the highest activity (Smullen *et al*., 2007).

Also, in a study to examine the polyphenolic composition of grape skin extracts from 14 grape varieties (seven red and seven white), both antimicrobial and antioxidant properties, related to the polyphenolic content were evaluated. The broth microdilution test was used for the examination of the antimicrobial properties. *Bacillus cereus* and *Staphylococcus aureus* were used as Gram-positive bacteria, while *Campylobacter coli*, *Salmonella infantis* and *Escherichia coli O157:H7* were used as Gram-negative bacteria. The antimicrobial properties of grape skin extracts of the 14 grape varieties were evaluated against all the examined bacteria with a minimal inhibitory concentration range 0.014–0.59 mg of gallic acid equivalents (GAE)/ml. Regarding the antioxidant activity, this was determined as ferric reducing/antioxidant power (FRAP), DPPH radical-scavenging ability (IC_{50}), Fe^{2+}chelating activity (IC_{50}), and b-carotene bleaching assay. The ethanolic extracts of grape skin, red and white grape varieties interacted quickly and efficiently with the free DPPH radicals with IC_{50} of 148 ±70.1 mg GAE/l. Grape skin extracts reducing power (FRAP)when determined as FRAP the results were 10.5 ± 5.41 mM TE and 3.50 ±1.80 mM TE for red and white cultivars respectively. The results were corrected for dilution and expressed in mmol Trolox equivalents (TE) (Katalinić *et al*., 2010).

For red and white grapes, the high antioxidant activity was reported to be related to the amount of polyphenolic compounds that have antioxidant properties (Katalinić *et al*., 2010). Antioxidant properties for grape skin extracts of 14 *Vitis vinifera L*. varieties are shown in Table 13, while

Figure 21 illustrates the correlation between total phenolic content (TPC) and related FRAP (ferric reducing ability/antioxidant power) of phenolic extracts from grape skins of 14 *Vitis vinifera L.* varieties (Katalinić *et al*., 2010).

Table 13. Antioxidant properties for grape skin extracts of 14 *Vitis vinifera L.* varieties determined as DPPH radical-scavenging capacity, Fe2+-chelating ability, ferric reducing/antioxidant power (FRAP) and efficiency of investigated grape skin extract in protecting the oxidation of emulsified linoleic acid (CAA). Results are expressed as mean ±SD.

Grape variety	DPPH radical-scavenging activity IC_{50} (mg GAE/l)*	Fe^{2+}-chelating ability IC_{50} (mg GAE/l)*	FRAP (mM TE)**	C_{AA} (%)
White varieties				
Kujundžuša	79.3 ± 4.04	151 ± 8.32	5.67 ± 0.10	62.4 ± 9
Rkaciteli	52.8 ± 3.33	21.5 ± 3.11	1.52 ± 0.03	72.9 ± 0.2
Zlatarica	133 ± 5.17	75.5 ± 3.31	5.79 ± 0.05	69.7 ± 7
Medna	236 ± 8.08	21.0 ± 3.61	2.46 ± 0.04	n.d.
Kuč	192 ± 3.00	21.7 ± 2.89	3.41 ± 0.04	81.2 ± 2
Maraština	291 ± 4.51	70. 7 ± 3.78	1.48 ± 0.03	69.4 ± 6
Debit	159 ± 6.11	129 ± 4.68	4.19 ± 0.11	50.2 ± 13
Red varieties				
Vranac	156 ± 6.76	475 ± 5.51	15.3 ± 0.52	87.4 ± 4
Trnjak	209 ± 6.54	482 ± 15.3	15.5 ± 0.15	87.8 ± 7
Rudežuša	239 ± 4.16	655 ± 7.07	16.4 ± 0.11	88.0 ± 4
Merlot	153 ± 3.79	300 ± 9.41	9.88 ± 0.28	89.7 ± 4
Babić	58.0 ± 3.00	235 ± 10.0	8.24 ± 0.09	85.9 ± 0.2
Lasin	64.2 ± 4.25	52.0 ± 2.65	2.68 ± 0.04	75.5 ± 3
Plavina	158 ± 3.18	102 ± 3.21	5.49 ± 0.12	87.8 ± 0.5

* IC50 – sample concentration in mg GAE per l of grape skin extracts providing 50% inhibition. ** TE – Trolox equivalents. (From Katalinić *et al*., 2010)

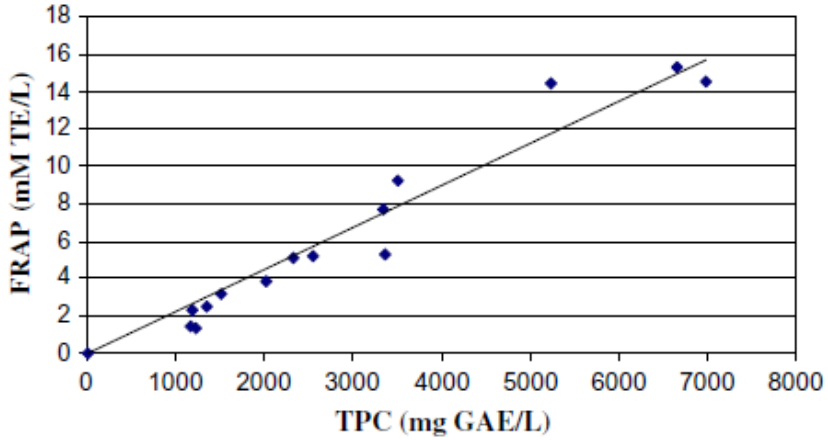


Figure 21. Correlation between total phenolic content (TPC) and related FRAP (ferric reducing ability/antioxidant power) of phenolic extracts from a grape skins of 14 *Vitis vinifera L.* varieties (y = 0.0022 x; r2 = 0.9456) (From Katalinić *et al*., 2010)

In a comparison study done by Arnous et al.(2008) between grape (*Vitis vinifera L.*) and apple (*Malus domestica*) skins, extraction of the examined samples' skins showed that grape skins had almost fivefold total phenolic content than apple samples' skins. The study concluded that grape

skin is a rich source of anthocyanin pigments, especially the 3-glucosides of cyandin and malvidin.

In a study to investigate the antibacterial and antioxidant properties of grape seed extract (GSE), Furiga et al. (2009) found that GSE had an effective antibacterial action on two oral anaerobes associated with periodontal diseases as illustrated in Table 14 and (Fig.22). The macro dilution broth technique was used to determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) in order to characterize the antimicrobial activity of GSE for the two oral anaerobes. Antioxidant activity of grape seed extract was evaluated according to the ability of a sample to scavenge the ABTS radical cation in comparison with a standard antioxidant (Trolox). Among the tested samples; Ascorbic acid, Chlorhexidine, and grape seed extract, GSE had the highest antioxidant activity, proving its ability to scavenge the ABTS radical cation (Table15).

Table 14. Antibacterial activity of grape seed extract (GSE)

	MIC (µg/ml)	MBC (µg/ml)
P. gingivalis	4000	8000
F. nucleatum	2000	8000

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) after 24 h incubation with bacteria (From Furiga *et al*. 2009).

Table15. Antioxidant activity of grape seed extract (GSE)

Compounds	TEAC
Ascorbic acid	5.73 ± 0.06
Chlorhexidine	0.02 ± 0.03
GSE	7.01 ± 0.18

The Trolox equivalent antioxidant capacity (TEAC) corresponds to the µmolar concentration of Trolox equivalent to a 1 µg/ml solution of sample (the higher the more effective). Each value corresponds to the mean and standard deviation of the triplicate of three separate concentrations within the linear interval (n = 3) (From Furiga et al. 2009).

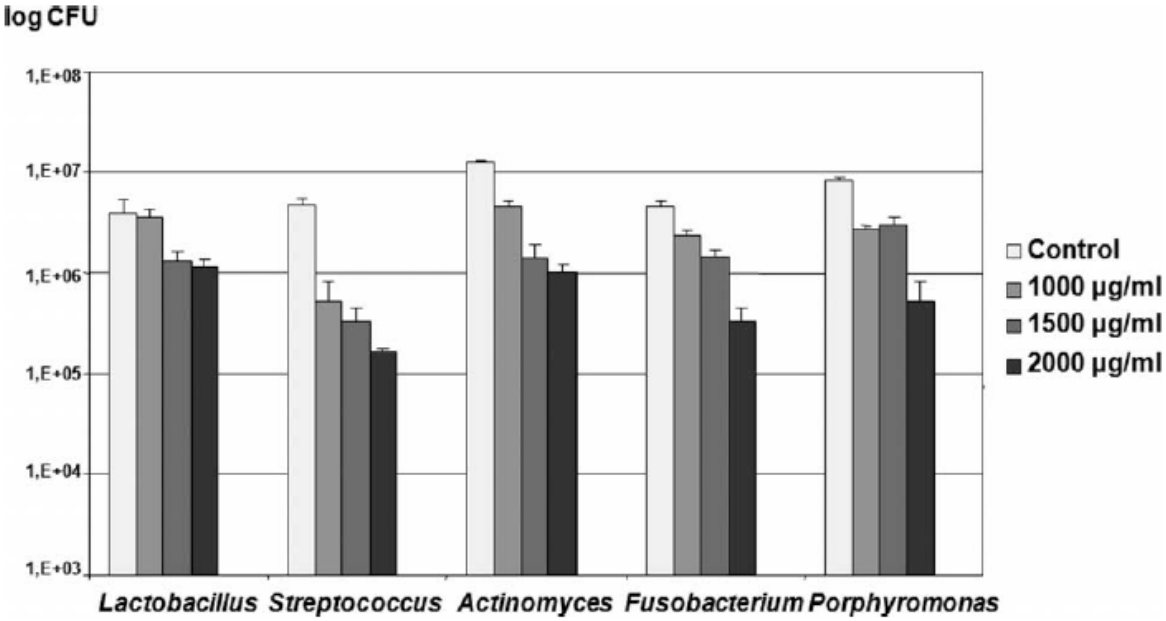


Figure 22. Effect of different concentrations of grape seed extract (GSE) on bacteria composing the multi-species biofilm. Results are expressed as means and standard deviations of triplicate experiments. Statistical differences (*, $p < 0.05$; **, $p < 0.01$ and ***, $p < 0.001$) between test compound and control ($n = 3$) (From Furiga *et al*. 2009).

Crude extracts of grape seeds were prepared using Soxhlet extraction with petroleum ether (60–80 ◦C) for 6 h, then re- extracted separately with two extraction solvent mixtures; acetone: water: acetic acid(90: 9.5: 0.5) and methanol: water: acetic acid(90: 9.5: 0.5). Then extracts were filtered and concentrated under vacuum (Buchi, Switzerland) to get crude extracts. Using high performance liquid chromatographic analysis with UV detection at 280 nm, the major extract compound was monomeric procyanidin at 48 and 40% in the first and second extraction solvent mixture respectively. The pour plate method was used to investigate the antibacterial properties of these extracts against *Pseudomonas aeruginosa, Bacillus coagulans, Bacillus cereus, Bacillus subtilis, Escherichia coli* and *Staphylococcus aureus.* Complete inhibition of Gram-positive bacteria occurred at 850-1000ppm, while inhibition of Gram-negative bacteria was achieved at 1250-1500ppm. It was observed that better radical scavenger activity was found using the first

extraction solvent mixture extract; acetone: water: acetic acid (90:9.5:0.5) (Jayaprakasha *et al*., 2003).

Plant tissues such as grape skins and seeds, contain natural antimicrobial substances in order to avoid plant microbial infections. These natural substances, mainly phenolics, could be used as natural preservatives for maintaining food safety and quality (Serra *et al*., 2008). The use of grape seed extract is a feasible alternative as an antibacterial and antioxidant agent to prevent the deterioration of stored foods by bacteria and oxidation (Delgado Adámez *et al*., 2012).

For the evaluation of grape seed (GSE) and Green tea (GTE) extracts as potential natural food preservatives, these were tested for extending low sulphite containing raw beef shelf life. Both extract antimicrobial and antioxidant activities were compared to that of ascorbate. The study concluded that low sulphite raw beef could be preserved using SO_2 - vegetable extract combination; SA (100 SO2 + 400 sodium ascorbate), ST (100 SO2 + 300 GTE) and SG (100 SO2 + 300 GSE) (mg/kg of meat). SA, ST, and SG not only delayed microbial spoilage, lipid oxidation, and redness loss, resulting in shelf life extension of the raw low sulphite beef by 3 days, but also rancid flavors in cooked patties were delayed with no effect on the sensory attributes of the raw beef caused by all three extracts. The study suggested that added SO_2 amount could be decreased if combined with GSE or GTE for healthier meat products (Banon *et al*., 2007).

Baydar et al. (2006), investigated the relationship between the antimicrobial activity of grape seed extract and its phenolic content. Grape seed extracts from three different grapes were used; Emir, Hasandede and Kalecik Karasi cultivars. The total phenolic contents of the three grapes, using acetone: water: acetic acid (90:9.5:0.5) as the extracting solvent were 589.09 (Hasandede), 506.60 (Emir) and 549.54 (Kalecik Karasi) mg gallic acid equithvalents (GAE)/ g. The three

grape seed extracts were tested against fifteen pathogenic and spoilage bacteria; *Yersinia enterocolitica, Pseudomonas aeruginosa, Pseudomonas fluorescens, Staphylococcus aureus, Salmonella Typhimurium, Salmonella enteritidis, Escherichia coli, E. coli O157:H7, Proteus vulgaris, Aeromonas hydrophila, Bacillus cereus, Enterobacter aerogenes, Enterococcus faecalis, Mycobacterium smegmatis* and *Klebsiella pneumonia.* All the three extracts at 1%, 2.5%, 5% and 10% concentrations inhibited all the examined bacteria using the agar well diffusion method. *Aeromonas hydrophila* was the most sensitive bacterium and the Hasandede grape seed extract was the most effective against the tested bacteria. The study concluded that grape seed extracts at low concentrations can be used in both food and beverage preservation.

Ahn et al. (2007), investigated the effects of different plant extracts on the microbial growth, lipid oxidation, and color change in cooked beef. Grape seed extract (ActiVin ^{TM}) was tested with the examined plant extracts. Compared to the control, 1.0% of grape seed extract (ActiVin^{TM}) clearly decreased the bacterial growth of *Salmonella Typhimurium* and *Escherichia coli* O157:H7 and retarded the growth of both *Aeromonas hydrophila* and *Listeria monocytogenes*. The color of cooked beef treated with Grape seed extract was more red (a*), less yellow (b*), and less light (L*) than samples treated with other extracts; BHA/BHT, Pycnogenols (pine bark extract), and Herbalox (oleoresin rosemary). ActiVin ^{TM} delayed thiobarbituric acid reactive substances (TBARS) formation by 92% after 9 days of refrigerated storage with significantly lower hexanal content than the control during storage. The study concluded that both ActiVin^{TM} and Pycnogenol are potential preservatives of cooked beef.

Antibacterial and antioxidant activities of five spice and herb extracts were examined as natural preservatives of raw pork. The five extracts were grape seed extract, oregano, pomegranate peel,

clove, and cinnamon sticks. All five natural extracts were effective against *Staphylococcus aureus, Salmonella enterica,* and *Listeria monocytogenes* at room temperature (∼20 ◦C). Because of the high levels of bioactive phenolic compounds these natural extracts contain, they inhibited the above foodborne bacteria and retarded lipid oxidation. The study showed that these natural extracts can be used as natural preservatives of meat products (Shan *et al*., 2009). Additionally, in a study focusing on the antimicrobial activities of two natural extracts; olive- and grape-based extracts, both rich in polyphenols, against several food-borne pathogenic microorganisms, the two natural extracts were obtained through extraction with biocompatible solvents followed by a membrane-based process. Isolation of grape extract (GE) was carried out from white wine production residues, particularly grape seeds and skins. Preparation of Grape aqueous extract was obtained from grape residues (grape skins and seeds) of Arinto variety (Bucelas, Portugal) at room temperature. The obtained liquid was centrifuged at 9000 rpm at 20 °C for 15 min while a rotary evaporator was used for its concentration. The extract was then filtered and stored at −20 °C (Serra *et al*., 2008). Grape residues extract total phenolics content was very high; 3400mg GAE/L, compared to 400mg GAE/L for the total phenolic content of olive extract.

The chromatogram presented in Figure 23 was obtained for grape natural extract using UV–VIS detection. Kaempferol, quercetin glycosides and resveratrol were identified in the grape extract (Fig. 23). The mentioned extracts were tested along with three standard antioxidants (oleuropein, quercetin and hydroxytyrosol) against five bacterial species (*Candida albicans, Bacillus cereus, Escherichia coli, Salmonella poona* and *Saccharomyces cerevisiae*). The microplate photometer assay was used. Antimicrobial activity of examined natural extracts was higher than that of antioxidants/antimicrobials alone against all the selected bacteria. The results concluded that

grape natural extract (seeds and skins) was the most effective antibacterial agent. This work suggested that the use of natural extracts has great future potential as antimicrobial agents for the food industry (Serra *et al*., 2008).

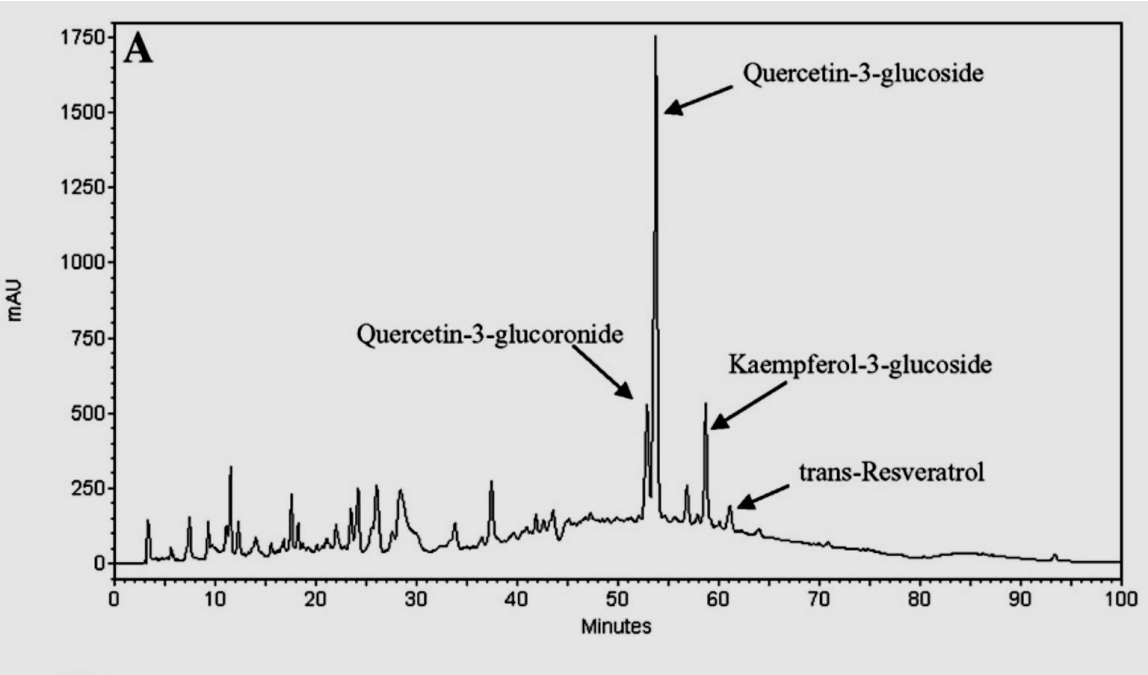


Figure 23. HPLC profiles at 280 nm of natural grape extract (From Serra *et al*., 2008).

Rhodes et al. (2006) investigated the antibacterial activity of *Vitis vinifera* var. *Ribier* grape commercial juice and of fresh Ribier grape extracts; seed, skin, and pulp extracts on *Listeria monocytogenes*. The commercial dark red grape juice was preservative free; it was checked (for the absence of preservatives) by analysis for sulpher dioxide, benzoic acid, and sorbic acid using standarad procedures (AOAC International, 2000). For the preparation of *Ribier* grape extracts, grapes were washed with 70% (v/v) ethanol and then by sterile osmosis treated water followed by air-drying at 20 ^{0}C. After separating the grape skins and seeds, 1ml of 10% (v/v) ethanol was added/gram of skin and pulp, while for every gram of seeds 2ml of 10% (v/v) ethanol was added. To facilitate extraction, the grape components were macerated using a blender and then stored at 4 ◦C for 72 h in the dark. After extraction and before freezing of skin, seed and pulp extracts, an

aliquot of each was adjusted to pH 3.5 with sterile 10mM HCl and then pasteurized at 63 ^{0}C for 30 min. The results of the study showed that commercial grape juice was highly inhibitory to *L. monocytogenes* serotypes 1/2a and 4b, the cause of foodborne listeriosis, and to all tested *Listeria* species. Its effect was so rapid that it decreased the number of *L. monocytogenes* colonies from 10^{6}–10^{7} CFU/ml to non-detectable within 10 min. Regarding the fresh extracts of *Ribier* grape skin, seed and pulp, skin and seed extracts had strong antilisterial activity, while the grape pulp had no inhibitory activity (Fig.24). Inhibition of tested *Listeria* species by fresh grape skin and seed extracts was similar to the inhibitory effect occurred by commercial grape juice. Grape skin extract caused reduction in *L. monocytogenes* numbers by 1-log, while grape seed extract caused the reduction in *L. monocytogenes* numbers by 2-log after a 1 min exposure. The study showed that two active compounds were identified to have the strongest antilisterial activity: from juice and skin; red-pigmented polymeric phenolics which showed pH-dependent antilisterial activity, and from the grape seed; unpigmented polymeric phenolics which had pH- independent antilisterial activity.

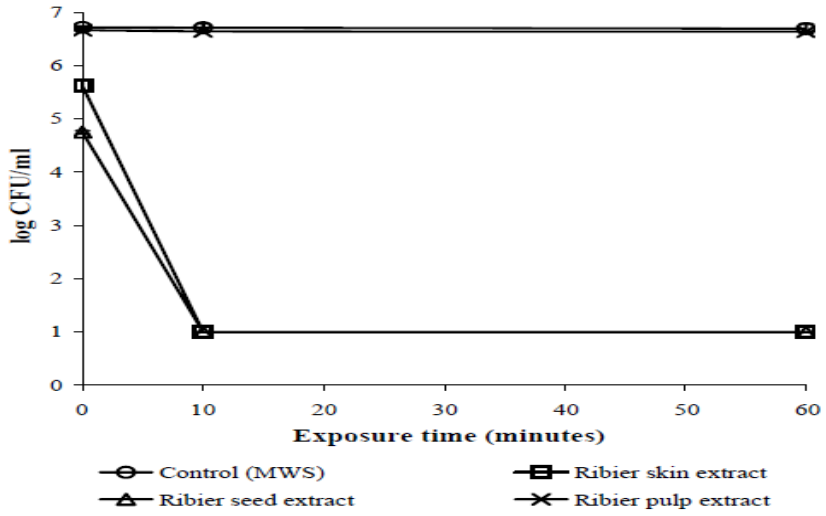


Figure 24. Survival of *Listeria monocytogenes* in *Vitis vinifera var. Ribier* grape skin, seed, and pulp extracts. Error bars (too small to be observed for some points) represent the standard deviation of the mean of two counts. The detection limit of the assay is 1-log or 10 CFU/ml. (From Rhodes *et al*. 2006)

Chapter 3
Materials and Methods

3. Materials and methods

3.1. Materials

Grape skin and seed extracts were purchased from Xi'an Xiao Cao Botanical Development Co., Ltd China; distilled water; LDPE and PET corona treated films were donated by “Three brothers” Co, Cairo; Corona test pen was donated by SOFTAL Co. in Egypt. Polyethylene pellets were donated by the Mechanical Engineering Dept. (MED), AUC. Thermoforming press was that of MED. Fresh cultured *E.coli* and *Staphylococcus aureus* cultures were prepared and supplied by the Biology Department, AUC. “Gallenhamp Colony counter” was used for enumeration of bacterial colonies (Ioannina University, Ioannina, Greece).

Dehydrated Culture Media used were; nutrient agar (Oxoid Code CM0003, UK), Pseudomonas agar base (Oxoid Code CM0559, UK), Pseudomonas “C-N” Selective Supplement)(Box of 10 Vials) (Oxoid Code SR0102, UK), Violet Red Bile Glucose (VRBG) AGAR (Oxoid Code CM0485, UK), M.R.S. Agar (de man, Rogosa, Sharpe, Oxoid code CM0361, UK), Plate Count Agar (LAB M code LAB149, UK), and Buffered Peptone water (ISO) (LAB M code LAB204, UK) were purchased through “Mecca Trade”, “Healthy Family”, and “New Star” companies, Cairo, and STAA; *Brochothrix thermosphacta* selective medium supplement from Oxoid (Code: SR0151, UK) through the Ioannina University’s suppliers, Ioannina, Greece.

Magnesium sulfate-Heptahydrate (MERCK, Germany), Agar-Agar, Danish, Potassium dihydrogen Phosphate (MERCK, Germany), and Yeast Extract Powder (LAB M Code: MC001 UK) were donated by Chemistry Department, Ioannina University, Ioannina, Greece. Glass molds were purchased from a local glass store in Ioannina, Greece. The fresh slaughtered chicken breast fillets were donated by the Pindos poultry processing plant, Ioannina, Greece.

3.2. Methods

3.2.1. Preparation of thermoformed polyethylene sheets using GSE

Initial work in the Mechanical Engineering Department, AUC, involved the preparation of Polyethylene sheets incorporating GSE in the polymer matrix by thermo-forming. 45gm of polyethylene powder were mixed with 5gm GSE powder to obtain a polyethylene sheet with 10% w/w concentration of GSE. From the above mixture, 3gm were placed in the thermoforming press mold after greasing it with silicon grease. The pressure of the device was adjusted to be 3.5 bar and temperature controlled between 121-131°C. The thickness of the resulted sheet was 210 microns. The thermoformed sheet of polyethylene with 10% GSE concentration was tested against both *E.coli* and *Staph. aureus* by the agar diffusion test. Fresh cultures of *E.coli* and *Staph. aureus* (population of cultures; *E.coli* is 4.7 $X10^7$ CFU/ml and *Staph.aureus* is 6.5$X10^7$ CFU/ml)(Peñuelas-Urquides *et al*., 2013) were spread on two separate agar plates. Upon solidification of the agar, 1x1 cm sheets were placed in each plate and plates incubated for 24 hours at 37 °C (Fig. 25).

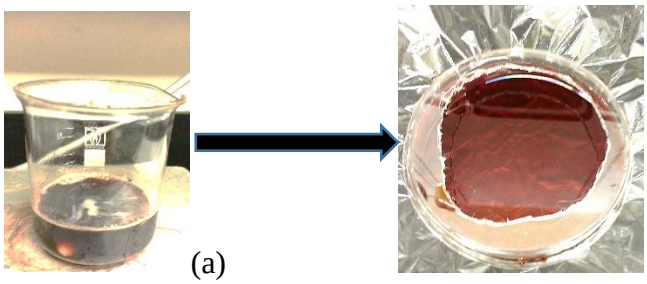
3.2.2. Determination of antimicrobial activity of GSE extract

For the determination of the antibacterial activity of grape seed extract, 5 g of GSE were dissolved in 100 ml distilled water. This 5% GSE solution was added using a (100µl) pipette directly to a fresh culture of *Staph. aureus* and *E.coli* as shown in (Fig. 26) for *Staph. aureus.* A positive control plate was also prepared using agar with ampicillin (commercial antibiotic) and tested bacteria (*E.coli*). As shown in (Fig. 27) an area of inhibition (no bacterial growth) was observed around the antibiotic disc.

3.2.3. Preparation of LDPE and PET corona treated films coated with GSE/or GSKE

The weight of the circular LDPE and PET corona treated films that covers the interior surface of petri dish used (area =58 cm^2) was 0.252g and 0.113g respectively. Commercial plastic films (LDPE, PET, etc.) have a rather chemically inert and nonporous surface with a low surface tension causing them to be non-receptive to bonding of printing inks, coatings, and adhesives. Corona treatment is a surface modification technique using a low temperature corona discharge plasma to increase the surface energy of plastic films (Zhang *et al*., 1998). A 'corona pen' was used to ensure that the films were corona treated; the corona treated side is colored with this pen while the untreated side remains uncolored.

The corona treated LDPE or PET film was placed in the petri dish with an open cover as shown in (Fig.28b). Solutions of grape seed/or skin extract were prepared in distilled water. The first solution concentration prepared was 0.6 g of Grape seed/or skin extract in 20 ml distilled water in a beaker.The beaker containing a magnetic stirring bar, was placed on a magnetic stirring plate for complete dissolution of the extract at a temperature of 50-60 ^{o} C for 30 minutes (Zam et al., 2012) (Fig. 28a).The solution of GSE/or GSKE was then poured on the surface of the LDPE/or PET corona treated film after cooling down to room temperature. The petri dishes were balanced by a water balance so as to achieve an even distribution of GSE/or GSKE solution on the film surface. After drying under the fume hood, the GSE/GSKE coated LDPE/and or PET films were tested for their antimicrobial activity against both *E.coli* and *Staph. aureus*.



(a) (b)

Figure 28: (a) Solutions of grape seed/or skin extract were prepared in distilled water in a beaker containing a magnetic stirring bar on a stirring magnetic plate (b) Pouring solutions of grape seed/or skin extract on the corona treated LDPE or PET film in petri dish with an open cover.

Petri dishes with nutreint agar were prepared under the laminar hood. Swabs of freshly cultured bacteria; *E.coli* (4.7 x10^7 CFU/ml)and *Staphylococcus aureus* (6.5 x10^7 CFU/ml)were evenly spread over the petri dish surfaces using sterilized plastic disposable loops. The dried coated LDPE or PET films were cut into pieces of ca. 1x1cm. One piece of ca. 1x1cm of prepared film was placed over the fresh bacterial culture (see Fig. 29).The control sample consisted of a piece of uncoated corona treated film of the same dimensions placed over the bacterial culture. A series of GSE/or GSKE solutions of decreasing concentration were prepared to determine the minimum inhibitory concentration (MIC) for each film/microorganism combination. Concentrations were: 0.6, 0.5, 0.3, 0.2, 0.1, and 0.15g of GSE/or GSKE/58 cm^2(area of used petri dish)/film wt.

3.2.4. Preparation of chicken samples wrapped in corona treated LDPE films coated with GSE or GSKE

The corona treated LDPE/or PET films were spread on the interior surface of glass molds (dimensions, ca. 37x19.4 cm) (see Fig. 30)so as to have a good flat surface for pouring the solution of GSE or GSKE, prepared as mentioned before. The glass molds were balanced using a water balance before and after pouring the solutions, as to achieve a uniform distribution of

GSE/or GSKE solutions. The coated films were allowed to dry for 5-6 days at room temperature. After drying, films were cut into strips of ca. 9.25x19.4 cm.

Chicken breasts fillets from freshly slaughtered chickens were packaged in Styrofoam trays, over wrapped in PVC film and directly transferred to the Food Microbiology Lab., University of Ioannina, in an insulated ice box on ice within 20 min. The chicken breasts were ground in a pre-sterilized home type meat grinder and samples of 80±5 g in weight, in the shape of a burger were immediately wrapped in 9.25x19.4 cm film strips covering both sides of the burger with the film. Control samples were prepared using corona treated uncoated (no GSE or GSKE) LDPE films. Given the comparable TPC content of the two extracts (GSE was 315.32g (GAE)/kg, and GSKE was 265.326g (GAE)/kg), two very different concentrations of extracts on the surface of corona treated LDPE films were chosen to investigate if this difference in TPC will show up in the extent of antimicrobial activity of the film in contact with chicken meat. Concentration of GSE coatings on LDPE films was 0.3 g GSE/ca. 37x19.4cm (glass mold dimensions) of LDPE film area; 0.0015g GSE/each ca. 9.25x19.4 cm strip. Respective concentration used of GSKE was 3.7 g/film area that covers the glass mold; 0.0185g GSKE/each ca. 9.25x19.4cm strip.

All samples were placed inside sterile LDPE bags of dimensions 15 x 15 cm which were thermo-sealed using a Boss model, 61352 thermal sealer (Bad Homburg vacuum sealer, Germany) to avoid environmental contamination and stored in the refrigerator at 4+/-1 ^{o} C. Sampling was carried out on days: 0, 2, 4, 6, 8, 10 for test samples, and on days: 0,2,4,6 and 8 for control samples.

Figure 30: The corona treated LDPE films were spread on the interior surface of glass molds (dimensions, 37x19.4 cm) with different concentrations of GSE or GSKE solutions.

3.2.5. Microbiological analyses

On each sampling day, ground chicken samples (10 g) were weighed aseptically, transferred to a stomacher bag with 90ml of 0.1% of sterilized buffer peptone water and homogenized using a stomacher blender for 60 s at room temperature. For microbial enumeration, 0.1 ml samples of serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) of chicken homogenates were spread on the surface of different selective media. Total viable count (TVC) were determined using plate count agar (PCA) (LAB M code LAB149, UK) after incubation for 48 h at 37°C. Pseudomonads were determined on Pseudomonas agar base (Oxoid code CM 0559, supplemented with Pseudomonas "C-N" Selective Supplement (Oxoid Code SR0102, UK) after incubation at 30 °C for 48 h. *Brochothrix thermosphacta* was determined on streptomycin sulphate-thallous acetate cycloheximide (actidione) agar after incubation at 30 °C for 84 h. For members of the Enterobacteriaceae family, a 1.0 ml sample was inoculated into 10 ml of molten (45 °C) violet red bile glucose agar (Oxoid code CM0485, UK). After setting, a 10 ml overlay of molten medium was added and samples incubated at 37 °C for 24 h. The large colonies with purple

haloes were counted. Lactic acid bacteria (LAB) were determined on de Man Rogosa Sharpe medium (Oxoid code CM 0361, UK) (APHA, 1984) after incubation at 37◦C for 3 days.

3.2.6. Colorimetric Determination of Total Phenolic Content of GSE/and or GSKE

For the determination of total phenolic content Folin–Ciocalteu reagent, pure gallic acid as standard (Merck) and 20% sodium carbonate (Merck) were used. A stock solution of gallic acid was prepared in a methanol–water mixture (60:40) at a concentration of 1,000mg l^{-1} and stored at 4 °C. Working solutions were freshly prepared by diluting appropriate amount of stock solution to prepare the different concentrations of gallic acid (10, 20, 40, 55, and 74ppm) (Table 18) to construct a calibration curve as illustrated in Fig. 44. One g of GSE or GSKE was weighed in test tubes and 50ml of (MeOH: H_2O: CH_3COOH) at a ratio of (80:15:5) were added. Tubes were placed on a stirring plate, in the dark for 1h for agitation purposes. Tubes were then centrifuged for 10 min (4000 rpm, at 4°C) (Rockenbach *et al.*, 2011). To 0.2ml of each extract (GSE or GSKE) 2.3ml of distilled water and 0.25ml of Folin- Ciocalteu reagent were added and the tubes were left aside for 3min. Then 0.5ml of a 20 % (w/v) aqueous solution of Na_2CO_3 and water were added to adjust the volume in both tubes to 5 ml. After 2h incubation in the dark, and dilution of 1/100 (0.1ml of each extract was added to 9.9 ml of distilled water) of both colored extracts, a spectrophotometer was used to measure the absorption of both the diluted extracts at a λ=725nm (Fuentes *et al*., 2012).

3.2.7. Statistical Analysis

For the first experiments: initially, the inhibition zone data was explored graphically using the boxplot (Fig.1& Table1, Appendix C).Then a multiple linear regression analysis was conducted to choose the factors that significantly affect the inhibition zone according to this experiment. A

stepwise method was used to choose all significant factors. The adjusted R squared of the chosen model is 53.3%. This implies that the set of these significant factors explains 53% of the variability of the inhibition zone. The factors included in our final model are concentration, bacteria type and substrate. For the second experiment, the aim was to determine whether there is a significant difference between the test and control in the mean number of colonies or not. Data was explored graphically using the boxplot (Fig.2 and Table 2, 3 Appendix C). Both ANOVA[a] and Paired Samples Test were used.

Chapter 4
Results and discussion

4. Results and discussion

4.1. Thermoformed polyethylene sheets

The thermoformed polyethylene sheets containing 10% GSE where tested against both *E.coli* and *Staph. aureus*, after incubation for 24 hours at 37°C. Sheets showed no inhibition zone for both bacteria as shown in (Fig. 25) with *Staph.aureus*. The thermoformed LDPE film with 10%w/w GSE did not influence the bacterial growth of *E.coli* or *Staph.aureus* most probably because the GSE was trapped in the polythelyne mass. Thus, no inhibition zone was observed.

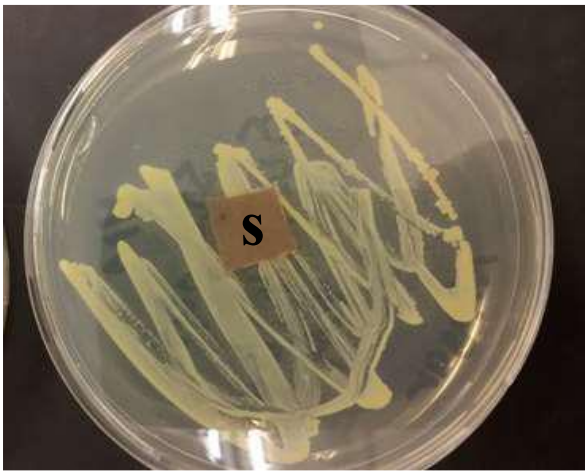


Figure 25: Testing of thermoformed LDPE sheet containing 10% w/w of GSE against *Staph. aureus.* There is no inhibition zone for *Staph. aureus* around the sheet(S).

4.2. GSE Solution

A clear bacterial inhibition zone was observed when 5% GSE solution was added directly, using 100 µl pipette, to a fresh culture of both *E.coli* and *Staph. aureus* as shown in (Fig. 26) for *Staph.aureus*. A positive control plate was also prepared using agar with ampicillin (commercial antibiotic) and tested bacteria (*E.coli*). As shown in (Fig. 27), an area of inhibition (no bacterial growth) was observed around the antibiotic disc. The GSE solution (5% w/v distilled water) caused a great inhibition zone when used directly against *E.coli* and *Staph.aureus* as seen in (Fig. 26); direct contact between the GSE and bacteria showed a strong antibacterial activity of GSE.

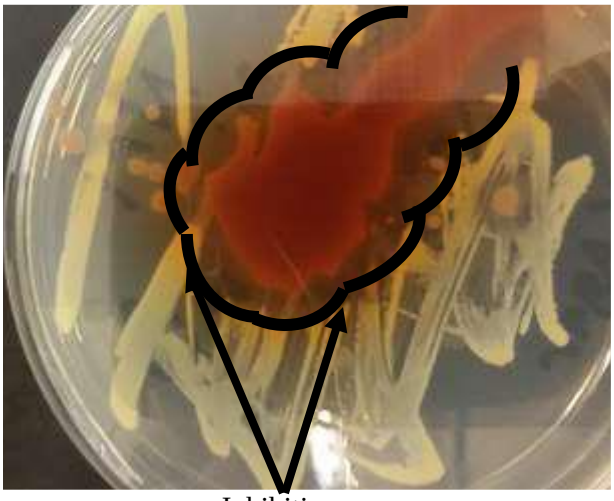


Figure 26: Inhibition zone of GSE solution (5% w/v distilled water) when adding 100 µl of GSE solution to agar plate bearing the *Staph.aureus* culture. Addition of 100 µl of 5 % GSE solution on agar plate showing a large zone (marked by black arcs) of bacterial (*Staph. aureus*) inhibition.

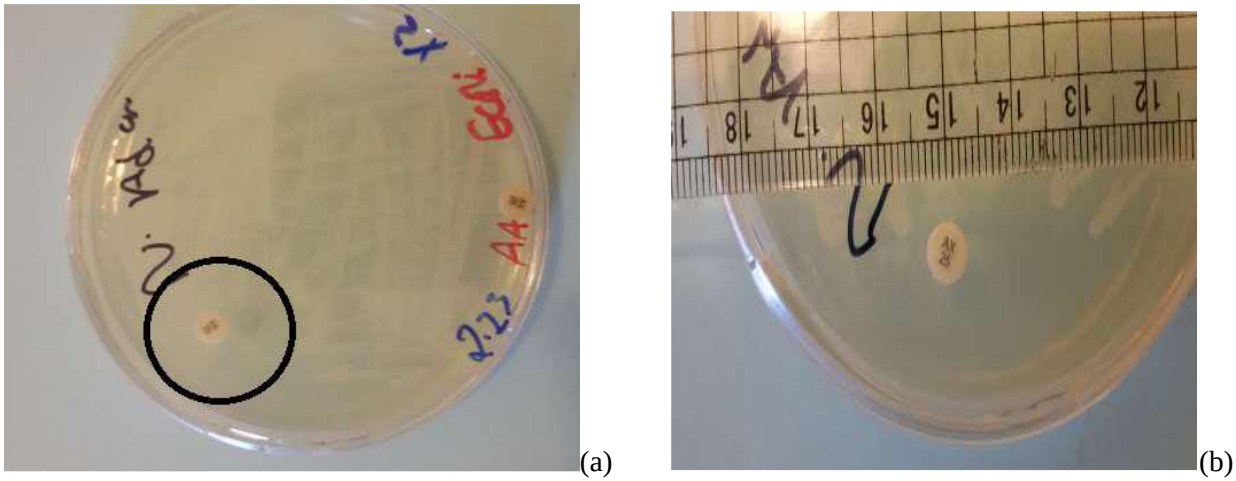


Figure 27: Positive control, (a) Control (agar with ampicillin) showing a zone of inhibition of *E. coli* around antibiotic disc. (b) Higher magnification of antibiotic inhibition.

4.3. LDPE and PET corona treated films coated with Grape seed extract (GSE) or grape Skin extract (GSKE)

The next step was to test the antimicrobial activity of the coated LDPE and PET corona treated films with grape seed or skin extracts.

4.3.1. LDPE films coated with GSE

The uncoated/corona treated side and the uncoated/untreated one of the LDPE were tested against both *E.coli* and *Staph.aureus* as negative controls. Plates were incubated for 24 hours at 37 °C, their photos were taken and then plates were re-incubated for another 24 hours as done with tested samples (LDPE and PET corona treated films coated with GSE). This test was run to see possible differences in bacterial populations between 24 and 48 h. For both *E.coli* and *Staph aureus* there was no inhibition zone formed around the film.

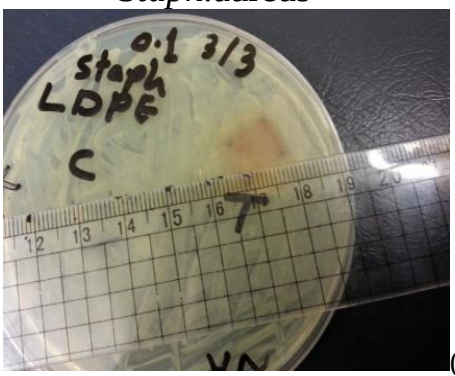
As for the LDPE corona treated coated with GSE, there was an obvious inhibition zone around the 1x1cm film coated with 0.6gm GSE/film wt. An average inhibition zone of 20.5 and 22mm for *E.coli* and *Staph.aureus* respectively was recorded (Table 16). With LDPE film coated with 0.5 gm GSE, there was an average inhibition zone of 25mm and 21mm for *E.coli* and *Staph.aureus* respectively (Table 16).

In the corona treated LDPE film coated with 0.3g/film wt. when tested against *E.coli* and *Staph.aureus* and plates incubated for 24 hours at 37°C there was a zone of inhibition formed around the test film in the case of *E.coli*. After 48 hours incubation for *E.coli* no significant change was observed between 24h to 48 h of incubation. As for *Staph.aureus* a zone of inhibition in bacterial growth was observed around the test film. The average size of inhibition zone for corona treated LDPE film coated with 0.3g of GSE/film wt. against *E.coli* and *Staph. aureus* was 23mm and 21mm respectively(Table 16).

For the corona treated LDPE film with 0.2 GSE/film wt. when tested against *Staph*. *aureus* and *E.coli*; the zone of inhibition for *E.coli* was 19mm and for *Staph.aureus* 16 mm(Table 16). At a GSE concentration of 0.15gm/film wt., the inhibition zone for *E.coli* was 20 mm and for *Staph.aureus* 15mm. For corona treated LDPE film coated with 0.1gm GSE/film wt. for *E.coli* a 16mm zone of inhibition was observed around the test film. For *Staph. aureus*; no inhibition was seen both for the control film and the test film. The inhibition zone for *E.coli* was 16mm as seen in (Fig.29).

E.coli *Staph.*aureus

(a)



(b)

Inhibition zone

Figure 29: Inhibition zone of corona treated (T) LDPE film coated with 0.1gm of GSE/film wt. against *E.coli* and *Staph.aureus vs.* control (C) films. (a)Inhibition zone of 16mm (marked with black arrow) with *E.coli.* (b) No inhibition zone was observed with *Staph.aureu.*

4.3.2. PET Films coated with GSE

The same concentrations that were tested against *E.coli* and *Staph.aureus* with corona treated LDPE film were used with corona treated PET film : 0.6, 0.5, 0.3, 0.2, 0.15 and 0.1 g/PET film wt. Control tests were run using the corona treated and non-treated sides of corona treated PET film, as done in case of LDPE. The GSE did not adhere to the surface of the corona treated PET film in case of 0.6 and 0.5gm/film wt. Moderate zones of inhibition were observed around the test film for *Staph.aureus* and *E.coli* with 0.3gm/film wt. No significant difference was observed between incubation for 24 and 48 hours. No inhibition was seen around the control PET film. The inhibition zone for *Staph. aureus* was 15mm and for *E.coli* 19mm(Table 16).

The corona treated PET film coated with 0.2gm/film wt. when tested against *Staph. aureus* and *E.coli*, gave a zone of inhibition equal to 15 mm for *Staph. aureus* and 17 mm for *E. coli*(Table 16). The corona treated PET film coated with 0.15gm GSE/film wt. when tested against *E.coli* and *Staph.aureus* gave an inhibition zone of 17 mm for *E.coli* and 16.5 mm for *Staph.aureus* (Table 16). Small zones of inhibition were observed around the corona treated PET film coated with 0.1gm GSE/film wt. when tested against both *E.coli* and *Staph.aureus*. Inhibition zone for *E.coli* was 16 mm and for *Staph.aureus* 15.5mm (Table 16).

Cumulative results on the antimicrobial effect of Grape seed extract against *E.coli* and *Staph. aureus* are given in Table 16.

Table 16. Inhibition zones of Corona treated LDPE and PET films coated with GSE.

	GSE Conc. (Gm/film wt.)	%of GSE/film wt.	*E-Coli* inhibition Zone (mm)	*Staph.* inhibition Zone (mm)
LDPE corona treated Films coated with GSE	0.6 gm/0.252gm	238%	20.5mm	22mm
	0.5gm/0.252gm	198%	25mm	21mm
	0.3gm/ 0.252gm	119%	23mm	21mm
	0.2gm/0.252gm	79.4%	19mm	16mm
	0.15gm/0.252gm	59.5%	20mm	15mm
	0.1gm/0.252gm	39.7%	16mm	No inhibition zone
PET corona treated Films coated with GSE	0.3gm/0.113gm	265.5%	19mm	15mm
	0.2gm/0.113gm	179.9%	17mm	15mm
	0.15gm/0.113gm	132.7%	17mm	16.5mm
	0.1gm/0.113gm	89.8%	16mm	15.5mm
Control films(LDPE and PET corona treated films without GSE)		No inhibition zone		

As for the corona treated LDPE and PET films coated with grape seed extract (GSE); there was an obvious inhibition zone the value of which was directly proportional to the concentration of

GSE/film wt. The antibacterial activity of GSE is greater against *E.coli* than against *Staph. aureus*, expressed by the greater inhibition zones with *E.coli* than in case of *Staph. aureus*. Grape seed extract has a notable antibacterial effect on both *E.coli* and *Staph*. aureus. With both tested films (corona treated LDPE and PET films) inhibition zones for *E.coli* were greater than those for *Staph. aureus.* The higher the concentration of the GSE, the greater the inhibition zone. Grape seed extract is an effective, natural antibacterial agent when used at appropriate concentrations coated on corona treated plastic films.

The corona treatment imparts more changes in the surface energy of the LDPE film than it does with PET film ; as a result, GSE adhesion to the corona treated LDPE film is greater (Farghal *et al*., 2017). **For LDPE corona films the minimum inhibitory concentration (MIC)** for *E.coli* and *Staph.aureus* was 0.1and 0.15 gm/0.252 gm (film wt.)/area of used petri dish respectively, or **0.002g/1cm^2**(area of used test film (T)) for *E.coli* and **0.003g/1cm^2** for *Staph.aureus*. While **for corona treated PET films, the MIC** for both *E.coli* and *Staph.aureus* was 0.1gm/0.113 gm (film wt)/ 58cm^2 (area of used petri dish), or **0.002g/1cm^2** (area of pieces of film used).

Inhibition zones of corona treated LDPE and PET films are shown in Table 16, as well as the percentage of GSE to the tested film weight.

The antibacterial effect of GSE in this study is in a partial agreemnt with the results obtained by Baydar *et al*., (2004). These researchers examined the antibacterial properties of GSE extracts at 1%, 2%, 4% and 20% concentrations against *Escherichia coli*, *Staphylococcus aureus* and some pathogenic and spoilage food bacteria. The results showed that GSE at 4% and 20% is an effective antibacterial agent against *E.coli, S.aureus* and all the examined bacteria. Also this study is in partial agreement with the results obtained by Jayaprakasha and Sakariah, (2003). These researchers used the pour plate method to investigate the antibacterial activity of grape

seed extracts against *Escherichia coli*, *Staphylococcus aureus* and other bacteria. The study showed that Gram-positive bacteria (*Staphylococcus aureus)* were totally inhibited at 850-1000 ppm, while *Escherichia coli* and other Gram-negative bacteria were inhibited at 1250–1500 ppm concentration of grape seed extract.

The increase of inhibition zone as the concentration of grape extracts increases was reported by Rodriguez-Vaquero et al., (2007). They confirmed that grape wine inhibited microbial growth especially *Escherichia coli*, and the inhibition increased as the concentration of polyphenol increased. Papadopoulou et al., (2005) suggested that red wines polyphenolic compounds were responsible for the antimicrobial properties exhibited against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans.* Their study concluded that *Staphylococcus aureus* were most sensitive to wine extract, followed by *Escherichia coli*, with the least inhibition occurring with *Candida albicans.* Radovanovic *et al*., (2009) reported similar results with inhibition zones for *Escherichia coli* and *Staphylococcus aureus* of 12–20 mm and 16–22 mm respectively. This result differs from the results of this study where the greater inhibition zone was with *E.coli*; 16-25 mm, while that of *S.aureus* was almost the same as that obtained by Radovanovic *et al*., (2009);15-22mm. Inhibition of *Staphylococcus aureus* and *Escherichia coli* growth by phenolic compounds from defatted grape seed extract was also reported by Rotava *et al*., (2009).

The results of this study are also, comparable with those of Baydar et al., (2006). They examined three grape seed extracts with different phenolic content; (Hasandede) 589.09, (Emir) 506.60 and (Kalecik Karasi) 549.54 mg gallic acid equivalent (GAE) per gram using acetone:water:acetic acid (90:9.5:0.5) as an extraction solvent mixture. The antibacterial activity of these extracts was examined at 1%, 2.5%, 5% and 10% concentrations by the agar diffusion method against *Escherichia coli, Staphylococcus aureus* and other pathogenic and spoilage bacteria. All the

examined bacteria were inhibited at all concentrations of grape seed extracts with the greatest effect showed by (Hasandede) with (TPC) of 589.09 mg (GAE)/g against the tested organisms. Corrales et al. (2009), reported different results compared to this study when investigating the antibacterial properties of grape seed extracts using the cylinder–plate assay method. The growth of Gram-positive food-borne pathogens e.g., *Staphylococcus aureus* was inhibited while Gram-negatives e.g., *Escherichia coli* were not inhibited. This deference may be due to different phenolic content as a result of different grape variety or different extraction method that may influence the potency of the antimicrobial activity of the phenolic compounds.

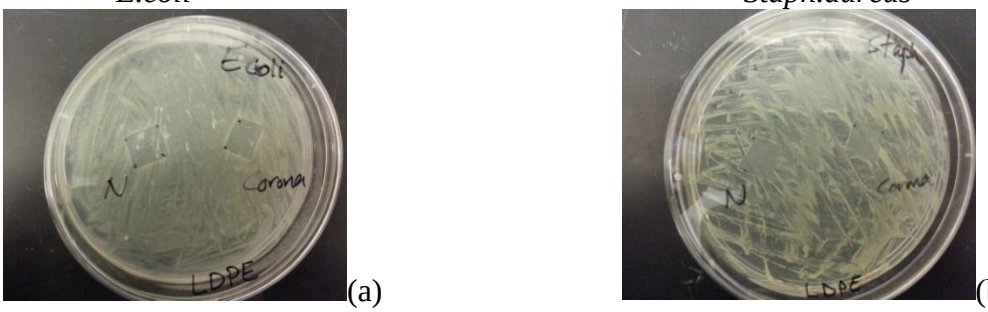
LDPE and PET corona treated films coated with Grape Skin Extract (GSKE)

4.3.3. LDPE films coated with GSKE

The corona treated/coated side of the LDPE film was tested against both *E.coli* and *Staph.aureus* by adding a piece of the film of dimensions 1x1 cm on the petri dish surface. The treated/uncoated side (without GSKE) comprised the negative control sample. Plates were incubated for 48 hours at 37 °C and photos were taken for both test and control samples. In control samples there was no inhibition zone observed around the corona treated (and untreated) films for both *E. coli* and *Staph aureus* as shown in Figure 31.

As for the LDPE corona treated coated with GSKE, there was an obvious inhibition zone around the film coated with 0.6 g GSKE/film wt. The average size of the inhibition zone was 16.3 and 20mm for *E.coli* and *Staph.aureus* respectively (Table 17). Decreasing concentrations of GSKE in water were 0.5, 0.3, 0.2, 0.15, 0.1 and 0.05 g GSKE/film wt. /58cm^2 (area of used petri dish). With LDPE film coated with 0.5 g GSKE, there was an average inhibition zone of 16 mm and 19mm for *E. coli* and *Staph*. *aureus* respectively (Table 16and Fig. 32).

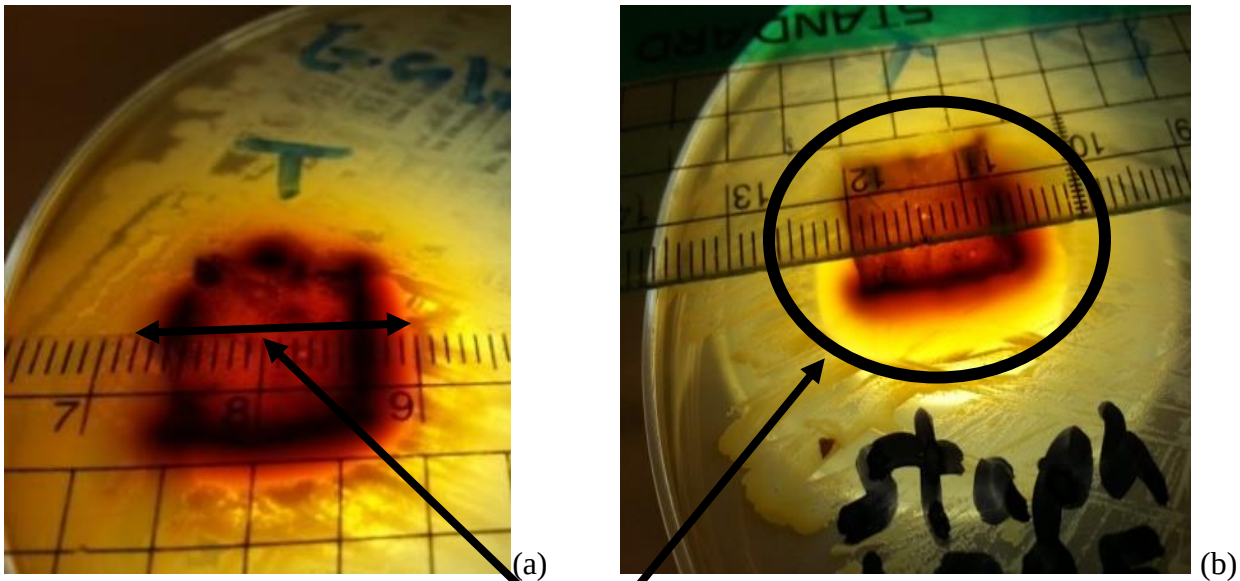
E.coli *Staph.aureus*



(a) (b)

Figure 31: Negative controls; corona treated LDPE side and the untreated side against *E.coli* and *Staph.aureus*. (a) No inhibition zone observed with *E.coli* around both corona treated (corona) and untreated (N) films (no GSKE coating). (b) No inhibition zone observed with *Staph.aureus* around both corona treated and untrated films (no GSKE coating).

E.coli *Staph.aureus*

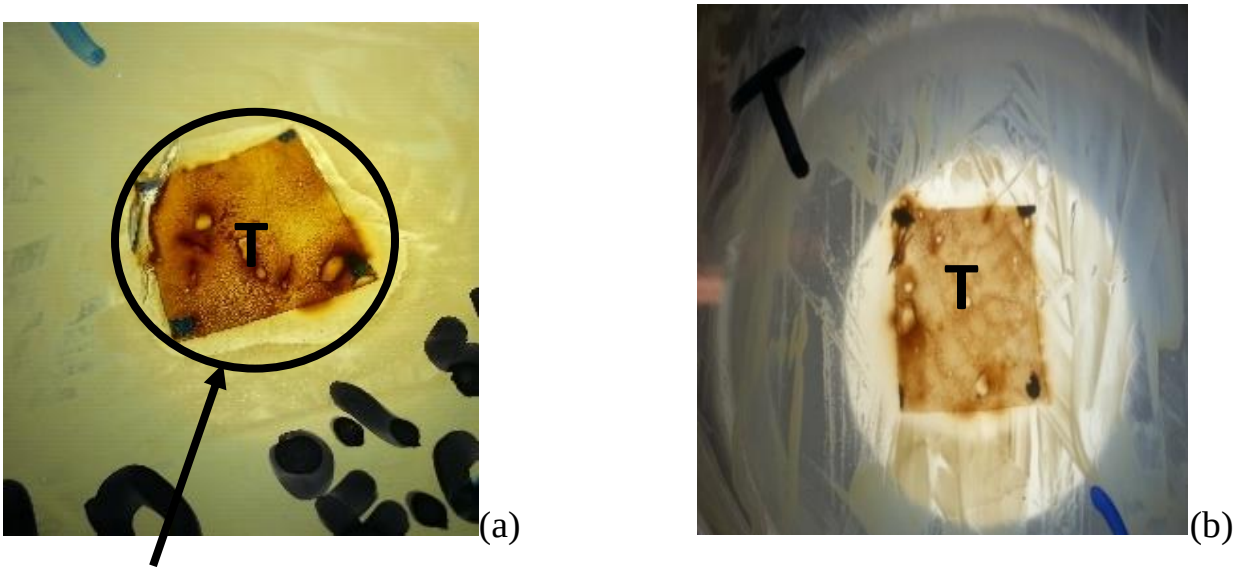


(a) (b)

Figure 32: Corona treated (T) LDPE film coated with 0.5gm GSKE/film wt. against *E.coli* and *Staph.aureus.* (a) An average inhibiton zone of 16 mm(marked by black arrow) *(E.coli)* was observed around the test (T) film but not around the control (C) film. (b) An average inhibition zone of 19 mm(the diameter of the black circle) (*St. aureus*) was observed around the test (T) film but not around the control (C) film.

In the corona treated LDPE film coated with 0.3g GSKE/film wt. the average inhibition zone for corona treated LDPE film coated with 0.3gm of GSKE/film wt. against *E.coli* and *Staph.aureus* was 15mm and 15.7mm respectively (Table 17). Regarding the corona treated LDPE film coated with 0.2 g GSKE/film wt. the inhibition zone for *E.coli* was 14.7 mm and for *Staph.aureus* 15mm (Table 17). As for the corona treated LDPE coated with 0.15 gm GSKE /film wt., the inhibition zone for *E.coli* was 14 mm while for *Staph.aureus*, there was no inhibition zone formed around both the test and control film (Table 17). For corona treated LDPE film coated with 0.1g GSKE/film wt. no inhibition zone was observed for *Staph. aureus while the* inhibition zone for *E. coli* was 13.3mm (Fig. 33).

E.coli *Staph.aureus*



(a) (b)

Moderate inhibition zone No inhibtion zone around the test film(T)

Figure 33: Corona treated (T) LDPE film coated with 0.1g GSKE/film wt. against *E.coli* and*Staph.aureus vs*. control. (a) A moderate inhibition zone of 13.3mm *(E.coli*)(Black circle) was observed around the test (T) film but not around the control (C) film. (b) No inhibition zone *(Staph.aureus)* was observed around both the test (T) film and (C) control film.

Finally, for corona treated LDPE film coated with 0.05g GSKE/film wt. an inhibition zone of 12mm was observed for *E. coli* around the test film but not around the control film . Based on above results, regarding *St. aureus*, the coated film was not tested against this bacterium.

4.3.4. PET films coated with GSKE

The same concentrations that were tested against *E.coli* and *Staph.aureus* with corona treated LDPE film were used with corona treated PET film; 0.6, 0.5, 0.3, 0.2, 0.15, 0.1 and 0.05 g GSKE/PET film wt. /**58cm^2**(area of used petri dish).The control test was run using the corona treated and non-treated sides of PET film, as done in case of LDPE. **The GSKE did not adhere to the corona treated PET film in case of 0.6, 0.5, 0.3, 0.2g/film wt.**

For the corona treated PET film coated with 0.15 g GSKE/film wt. the average inhibition zone against *E.coli* and *Staph.aureus* was 15mm and 12 mm respectively (Table 17). For the corona treated PET film coated with 0.10 g GSKE/film wt. the average inhibition zone against *E.coli* was 13 mm while there was no inhibition of *Staph. aureus* (Table 17). Finally for the corona treated PET film coated with 0.05 g GSKE/film wt. there was no measurable inhibition zone against *E. coli*. Inhibition zones of corona treated LDPE and PET films including GSKE concentrations used are shown in Table 17.

For corona treated LDPE and PETfilms coated with GSKE there was an obvious inhibition zone, the value of which was directly proportional to the concentration of GSkE/film wt., that is, the higher the GSKE/film wt. the greater the inhibiton zone observed.The inhibition observed in case of LDPE/*S. aureus* was greater than that of *E.coli* for the same concentration of GSKE/film wt. This holds for GSKE concentrations down to 0.2 g/film wt. In contrast, inhibition of *E.coli* was greater than that of *S. aureus* at GSKE concentations 0.15-0.05 g/film wt. This second trend also holds for PET GSKE coated films. Grape skin extract demonstrated considerable antibacterial activity against *Staph. aureus* and to lesser extent against *E.coli*. It is

clear that Grape skin extract is an effective, natural antibacterial agent when used at appropriate concentrations coated on corona treated plastic films such as LDPE and PET.

Corona treatment imparts more profound changes to the surface energy of the LDPE compared to PET film. As a result, GSKE adhesion to the corona treated LDPE film is greater (Farghal *et al*., 2017). **For LDPE corona films the minimum inhibitory concentration (MIC)** for *E.coli* and *Staph. aureus* was 0.05 and 0.2 g/0.252gm (film wt.)/58cm^2 respectively, or **0.0009g/1cm^2**(area of piece of film used) for *E.coli* and **0.003g/1cm^2**for *Staph.aureus*. While **for corona treated PET films, the MIC** for *E.coli* and *Staph. aureus* was 0.1 and 0.15g /0.113gm (film wt.) respectively, or **0.002g/1cm^2** for E.coli and **0.003g/1cm^2** for *Staph. aureus.*

Table 17. Inhibition zones of Corona treated LDPE and PET films coated with GSKE.

	GSKE Conc. (G/film wt.)	% of GSKE /film wt.	*E.coli* inhibition zone (mm)	*Staph*. inhibition zone (mm)
LDPE corona treated films coated with GSKE	0.6 g/0.252g	238%	16.3 mm	20 mm
	0.5g/0.252g	198%	16 mm	19 mm
	0.3g/ 0.252g	119%	15 mm	15.7 mm
	0.2g/0.252g	79.4%	14.7 mm	15 mm
	0.15g/0.252g	59.5%	14 mm	No inhibition zone
	0.1g/0.252g	39.7%	13.3 mm	No inhibition zone
	0.05g/0.252g	19.8%	12mm	-
PET corona treated films coated with GSKE				
	0.15g/0.113g	132.7%	15 mm	12 mm
	0.1g/0.113g	89.8%	13 mm	No inhibition zone
	0.05g/0.113g		No measurable inhibition zone	No inhibition
Control films(LDPE and PET corona		No inhibition zone		

treated films without GSKE)		

The obtained results are in partial agreement with the results of Vijayakumar et al., (2016) who tested grape skin aqueous extract against a broad spectrum of bacteria among which *E.coli* was inhibited to the least extent whereas significant inhibition was observed for *S. aureus*. The same holds for the results of Katalinić et al., (2010); in their study the antimicrobial activity of grape skin extracts from 14 grape varieties (seven white and seven red grape varieties) was investigated using broth the microdilution test against Gram-positive bacteria (*Staphylococcus aureus, Bacillus cereus*) and Gram-negative bacteria (*Escherichia coli* O157:H7, *Salmonella Infantis, Campylobacter coli*). The antimicrobial properties of grape skin extracts was confirmed against all examined bacteria with minimum inhibitory concentrations (MICs) in the range of 0.014–0.59 mg of gallic acid equivalents (GAE)/ml. Differences between results of this study and those of the literature is probably due to the fact that grape phenolic compounds' concentration is variety dependent, and affected by environmental and viticultural factors, e.g. cultivar, geographical origin, climate, soil composition, and cultivation practices (Xia *et al*., 2010).

It has been confirmed that the higher the phenolic content the greater the antimicrobial and antioxidant properties of the grape extracts (Katalinić et al., 2010). For example in this study the phenolic content of grape skin extract was 265.326g/kg while in the study by Katalinić et al., (2010) it was about 1000 mg GAE/kg.

GSKE used in this study had much higher phenolic content and consquently more potent antibacterial and antioxidant properties.

The phenolic compounds obtained from different grape drupe parts exhibited different antimicrobial properties. In agreement with other studies, the present work confirmed that the antimicrobial activity of seed extracts was higher than that of other grape parts. Brown *et al*.,

(2009) showed that grape skin had the strongest activity against *Helicobacter pylori*, followed by grape synergy (skin and seed) and seed.

Data analysis

The boxplot (Appendix C) shows that there is a difference in the median between the two types of bacteria, *E.coli and S.aureus* in the GSE group and a slight difference in the median in the GSKE group. This graph suggests that the bacteria type and the substrates are affecting the inhibition zone.

According to the coefficients Table, the concentration has a positive effect on the inhibition zone, while the GSE has a higher average than the GSKE given that all other factors are fixed at the same level. Also, *E.coli* has a higher mean than *S.aureus* bacteria given that all other factors are fixed. The p-value for all the coefficients is below 0.05.

4.4.1. Antimicrobial activity of Grape Seed Extract (GSE) coated onto LDPE films in ground chicken samples

4.4.1.1 Total Viable Count (TVC)

Microbiological analysis for tested samples was done on day 0, 2,4, 6, 8, and10, while for control samples on day 0, 2, 4, 6, and 8 only. Each and every sample, control or test, was duplicated twice with different chicken samples (n=2x2). Total viable counts (TVC) for chicken samples, both test and control, were determined using plate count agar (PCA) (LAB M code LAB149, UK) after incubation for 48 h at 37°C. The TVC count of test chicken samples wrapped with LDPE coated with GSE and control samples is given in Figure 34. On day 0 the TVC of fresh chicken sample was 4.9 log cfu/g, indicative of acceptable good quality of poultry meat (Dawson *et al*., 1995). The TVC for control samples reached 6.85 log cfu/g, closely related to the upper

microbiological limit for fresh poultry meat as suggested by ICMSF (1986); (Karabagias, *et al*., 2011a) on day 6 for control and day 10 for test samples (7.2 log cfu/g). The use of GSE extended the micro-biological shelf life of chicken samples by 2-3days (Fig. 34). The odour of control samples on day 8 was undesirable, while for test samples, it was only on day 10 when a slight unacceptable odour developed. Preservation of fresh poultry using refrigeration is the most common approach for extending the shelf-life of fresh poultry but using it alone resulted in a microbiological shelf life of no more than 4–5 days (Lee *et al*., 1996).

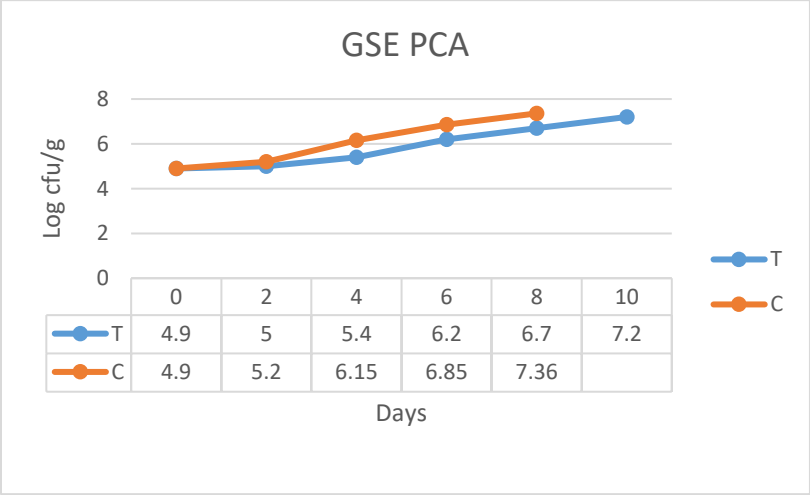


Figure 34: TVC of GSE coated vs. uncoated LDPE films in ground chicken patties

4.4.1.2 Pseudomonads Count

Pseudomonas spp. are one of the main spoilage group of microorganisms in meat and poultry. They are gram negative strictly aerobic bacteria (Jay, 1992). Pseudomonads were determined on *Pseudomonas* agar base (Oxoid code CM 0559, supplemented with *Pseudomonas* "C-N" Selective Supplement (Oxoid Code SR0102, UK) after incubation at 30 °C for 48 h. The *Pseudomonas spp*. count of test chicken samples wrapped with LDPE coated with GSE and control samples is given in Figure 35. The initial pseudomonads count on day 0 was 3.6 log cfu/g. On days 2 and 4 the difference between the control and test samples was very slight ($p>0.05$); 0.3 and 0.4 log cfu/g respectively (Fig. 35). While on days 6 and 8, GSE resulted in a

reduction of *Pseudomonas spp*. of the test samples by 0.95 and 1 log cfu/g respectively (($p < 0.05$). The smell of control samples was unacceptable on day 8 and for test samples, experiments were continued until day 10 when the population of pseudomonads reached 7.1log cfu/g. Reduction of the Pseudomonads population using GSE coated onto LDPE corona treated films is beneficial for the preservation of meat products as this group of bacteria have, the major role in spoilage of both meat and poultry.

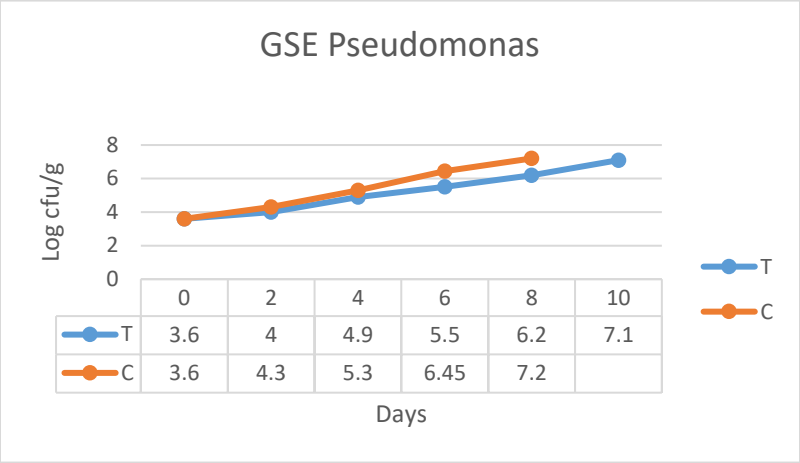


Figure 35: *Pseudomonas spp* count of GSE coated vs. uncoated LDPE films in ground chicken patties

4.4.1.3 Enterobacteriacea Count

For members of the family Enterobacteriaceae, a 1.0 ml sample was inoculated into 10 ml of molten (45 °C) violet red bile glucose agar (Oxoid code CM0485, UK). After setting, a 10 ml overlay of molten medium was added and samples incubated at 37 °C for 24 h. The large colonies with purple haloes were counted (Mossel *et al*., 1979). Enterobacteriaceae are Gram negative bacteria and their count reflects the hygienic status of meat products. The initial Enterobacteriaceae count on day 0 was 1.6 log cfu/g indicative of a good quality poultry product. On days 2 and 6 the difference in Enterobacteriaceae count between control samples and chicken samples wrapped with corona treated LDPE coated with 0.3 g GSE/LDPE film area (dimensions, 20x40 cm) was 1.4 and 1.25 log cfu/g respectively (Fig. 36) ($p < 0.05$). While the

Enterobacteriaceae count of control sample reached 5 log cfu/g on day 8, the test sample reached 5.4 log cfu/g on day 10. GSE resulted in the reduction of Enterobacteriaceae population by approximately 1.5 log cfu/g on day 2.

4.4.1.4 *Brochothrix thermosphacta* Count

After incubation at 30 °C for 2 days, *Brochothrix thermosphacta* was determined on streptomycin sulphate-thallous acetate cycloheximide (actidione) agar.

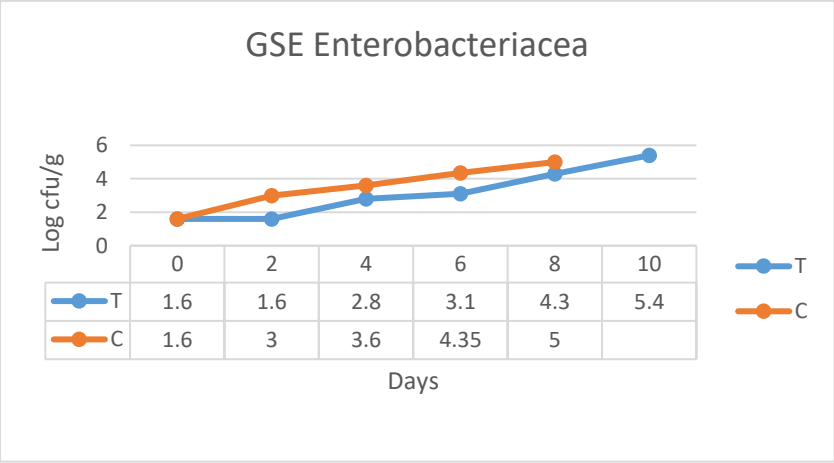


Figure 36: Enterobacteriacea count of GSE coated vs. uncoated LDPE films in ground chicken patties

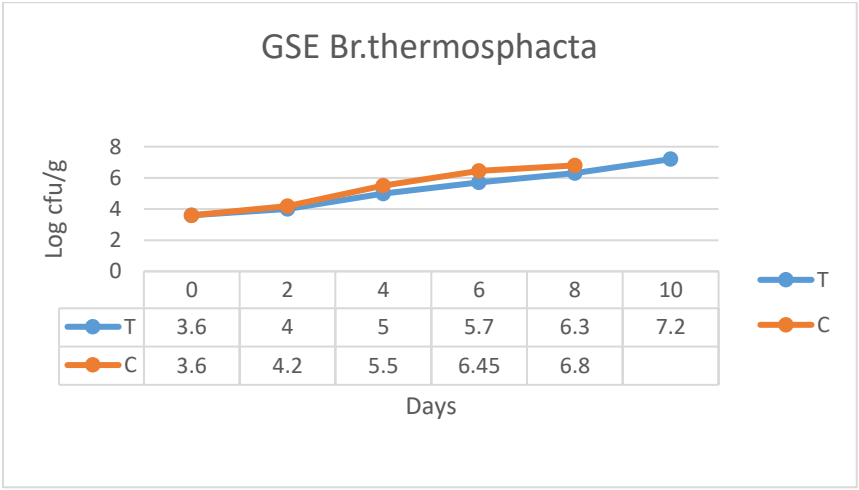


Figure 37: *Br.thermosphacta* count of GSE coated vs. uncoated LDPE films in ground chicken patties

Br.thermosphacta populations (test and control) are given in Figure 37. As a Gram positive facultative anaerobe, *Br.thermosphacta* is a part of fresh meat microflora t packaged aerobically or under MAP (Labadie, 1999). On day 0 the *Br.thermosphacta* count was 3.6 ; the reduction of the *Br.thermosphacta* population in the case of corona treated LDPE coated with GSE was as follows: 0.2, 0.5, 0.75, and 0.5 log cfu/g lower than the control samples on days 2, 4, 6, and 8 respectively (Fig. 37). The reduction of *Br. thermosphacta* in this study is less than that obtained by Corrales et al., (2009). They examined the effect of GSE incorporated in pea starch films on pork loins inoculated with *Brochothrix thermosphacta* in vitro, and reported a reduction in the bacterial growth by 1.3 log cfu/ml after 4 days of storage at 4 ◦C. The explantion of this difference may be due to different phenolic content of the GSE extracts and the different food substrate used in the study. Taguri et al., (2004) investigated the relationship between compound structure and antimicrobial activity of phenolic compounds, and concluded that number of hydroxyl groups in phenolics may be critical for phenolic compounds antimicrobial activity.

4.4.1.5 Lactic acid bacteria Count

After incubation at 37 °C for 3 days, Lactic acid bacteria (LAB) were determined on de Man Rogosa Sharpe medium(Oxoid code CM 0361, UK) (APHA, 1984).

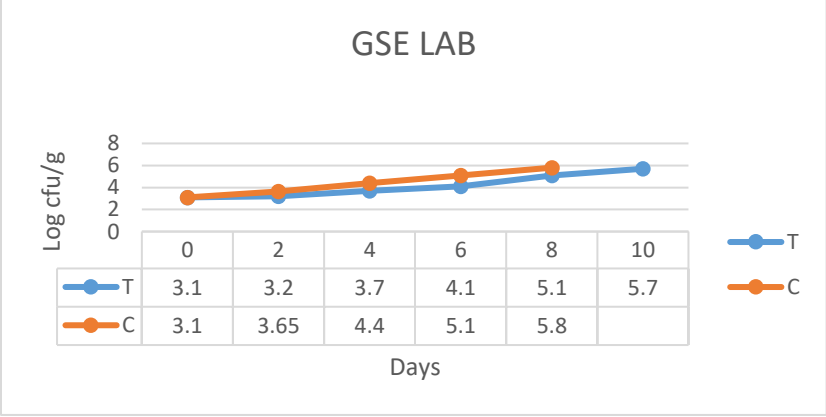


Figure 38: Lactic acid bacteria count of GSE coated vs. uncoated LDPE films in ground chicken patties

The initial LAB population (Fig. 38) was 3.1 log cfu/g.The Lactic acid bacteria count of GSE coated LDPE films in ground chicken patties was slightly lower than that of the control samples throughout storage (Fig. 38). The highest differnce between test and control was 1 log cfu/g reduction in lactic acid count on day 6 in the test sample over the control sample of the same day ($p < 0.05$).

Regarding the small concentration used; 0.3g GSE/LDPE film area (dimensions, 20x40 cm), GSE had a considrable antibacterial effect against TVC and all the examined groups of bacteria($p < 0.05$). Sensory attributes of test samples; color, and odor did not change until day 10 of storage. Based on the microbiological data, the shelf life of ground chicken patties can be extended by 2-3days if wrapped with GSE coated onto corona treated LDPE films.

The results of this study are comparable to those in the literature. Banon et al., (2007) examined the antibacterial properties of GSE for the preservation and shelf life extension of low sulphite raw beef patties. SG (100 SO2 + 300 GSE) (mg per kg of meat) was one of five groups of preservatives tested. The other groups were: Control (with no additives), ST (100 SO2 + 300 GTE (Green Tea Extract), S (100 SO2), and SA (100 SO2 + 400 sodium ascorbate). SG, ST and SA increased the shelf life of the raw sulphite beef patties by 3 days through delaying microbial spoilage, loss of redness and lipid oxidation.

Using GSE as potential natural preservative for raw pork was investigated by Shan et al., (2009). Grape seed extracts were used among other natural extracts and their antimicrobial activity was investigated in order to be used for raw pork preservation. GSE antimicrobial activity was evaluated against *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella enterica* in raw pork at room temperature (~20 ◦C). GSE and other natural extracts showed effective antimicrobial properties against all the tested bacteria. The study concluded that GSE and other

extracts could be used as natural preservatives to reduce pathogenic bacteria populations, change of color and oxidation of lipid in raw pork.

Also, the present study results are in partial agreement with Ahn et al., (2004). They used grape seed extract (ActiVin) for the inhibition of *Escherichia coli* O157:H7, *Salmonella Typhimurium,* and *Listeria monocytogenes* on raw ground beef. GSE (ActiVin 1%) resulted in the reduction of all three pathogen populations by 1-log CFU/g after 9 days of refrigerated storage. It was also reported by Baydar et al. (2006) that grape seed extracts at low concentrations can be used in both food and beverage preservation.

4.4.2. Antimicrobial activity of Grape Skin Extract (GSKE) coated onto LDPE films in ground chicken samples

Microbiological analysis of ground chicken patties wrapped in corona treated LDPE films coated with 3.7g GSKE/film area (dimensions, 20x40 cm), was carried out on day 0, 2,4, 6, 8, and 10, while for control samples on day 0, 2, 4, 6, and 8 only. Each and every sample, control or test, was duplicated twice with different chicken samples; n=2x2.

4.4.2.1 Total Viable Count (TVC)

The initial TVC value (day 0) was 4.5 log cfu/g indicative of acceptable quality poultry meat. Figure 39 gives the TVC for test and control chicken samples. While on day 2 and 4 there was a slight difference between test and control samples' TVC count, on day 6 and 8 test samples' TVC count was lower than that of control by 1.4 log cfu/g.

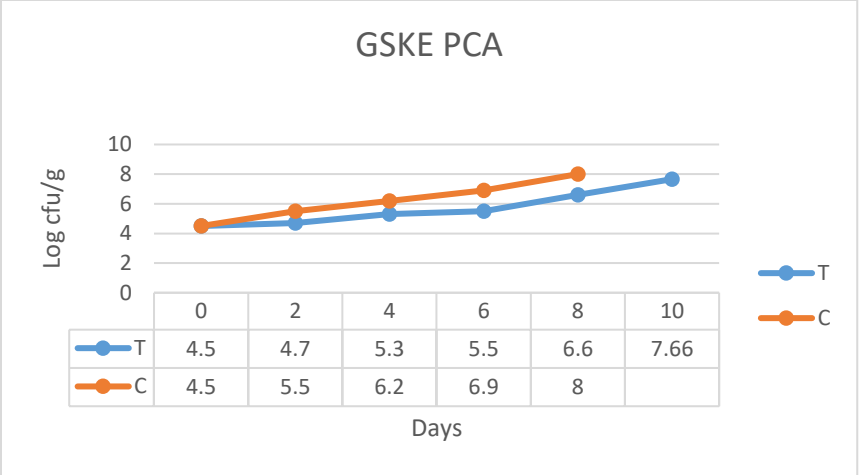


Figure 39: TVC of GSKE coated vs. uncoated LDPE films in ground chicken patties

4.4.2.2 Pseudomonads count

On day 0 Pseudomonads count was 4.5 log cfu/g and it increased slightly in test samples during the storage period of 10 days at 4◦C (Fig. 40). The highest reduction of Pseudomonads count in test samples over the control samples was on day 6 by 1.95 log cfu/g. Figure 40 gives the Pseudomonads count of test samples and control samples as a function of storage time.

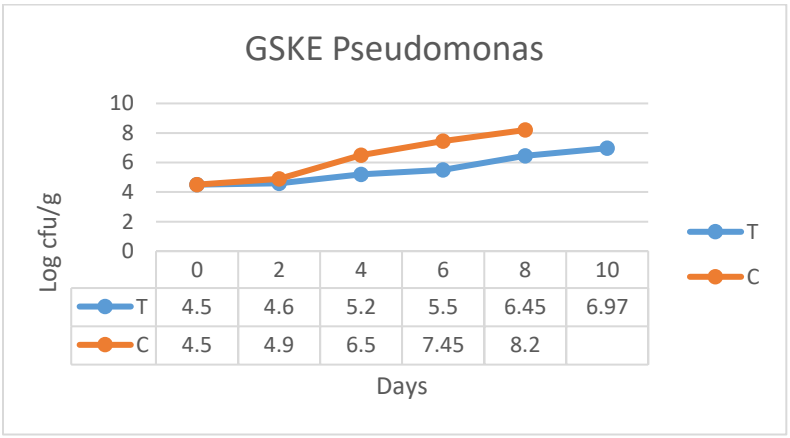


Figure 40: *Pseudomonas spp.*count of GSKE coated vs. uncoated LDPE films in ground chicken patties

4.4.2.3 Enterobacteriacea count

The initial Enterobacteriaceae count on day 0 was 1.5 log cfu/g indicative of good hygienice status of fresh poultry meat. Figure 41 gives the Enterobacteriaceae counts of test and control

samples as a function of storage time (p<0.05). On day 6 and 8 the difference between Enterobacteriaceae counts of test samples and control samples was 1.8 log cfu/g for test samples over the control samples.

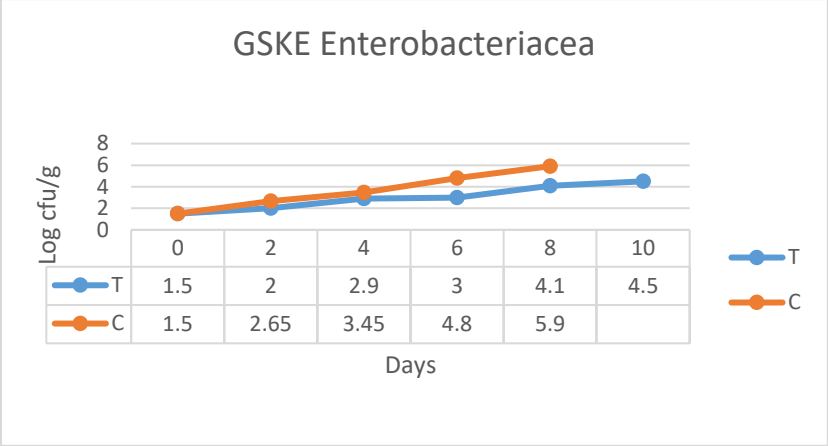


Figure 41: Enterobacteriacea count of GSKE coated vs. uncoated LDPE films in ground chicken patties

4.4.2.4 *Brochothrix thermosphacta* count

The initial *Brochothrix thermosphacta* count was 3.8 log cfu/g (day 0). Following that, its population increased during storage in both the test and control chicken samples (Fig. 42). The reduction in *Brochothrix thermosphacta* count of test samples wrapped with GSKE coated LDPE films as compared to the control samples was as follows: 0.55, 0.7, 0.85, and 1.4 log cfu/g on day 2, 4, 6, and 8 respectively. Figure 42 gives the *Brochothrix thermosphacta* count of test samples and control samples as a function of storage time

4.4.2.5 Lactic acid bacteria count

Figure 43 gives the LAB count of test samples and control samples as a function of storage time. The initial LAB population (Fig. 43) was 2.5 log cfu/g.The Lactic acid bacteria count of GSKE coated LDPE films in ground chicken patties was slightly lower than that of the control samples throughout storage (Fig. 43). The highest differnce between test and control was 1.1 log cfu/g

reduction in lactic acid count on day 4 in the test sample over the control sample of the same day ($p < 0.05$).

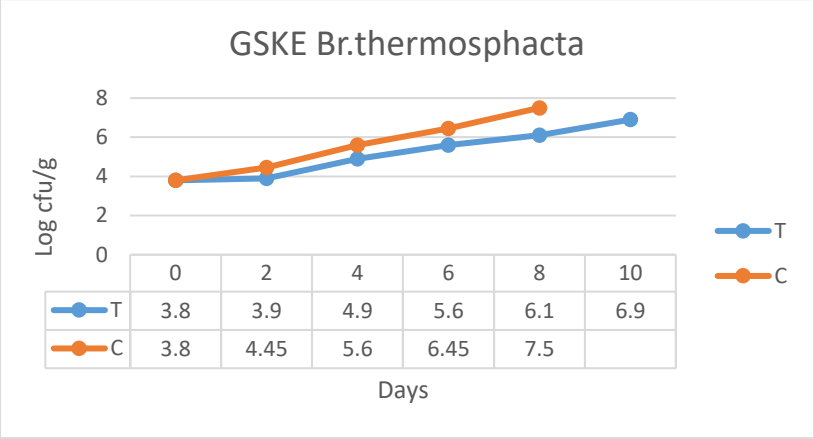


Figure 42: *Br.thermosphacta* count of GSKE coated vs. uncoated LDPE films in ground chicken patties

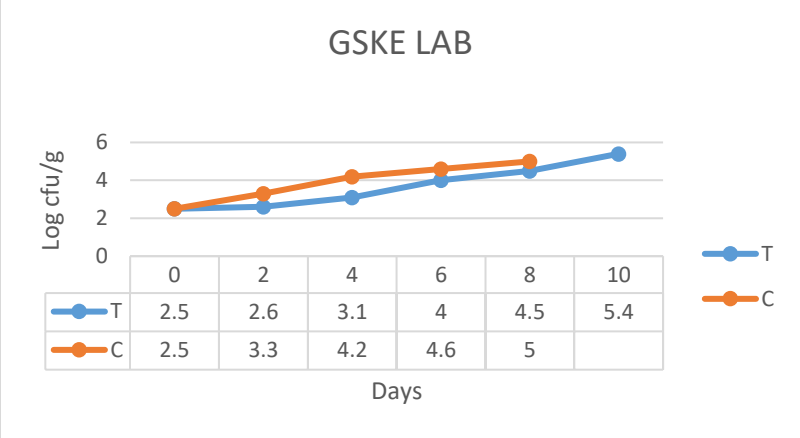


Figure 43: Lactic acid bacteria count of GSKE coated vs. uncoated LDPE films in ground chicken patties

Sensory attributes of test samples; color, and odor did not change until day 10 of storage (official sensory analysis was not carried out). Based on the microbiological data, the microbiological shelf life of ground chicken patties can be extended by 2-3days if wrapped with GSKE coated onto corona treated LDPE films. The results of this study are comparable to those in the literature.

In a comparison study done by Arnous et al.(2008) between grape (*Vitis vinifera L.*) and apple (*Malus domestica*) skins, extraction of the examined samples' skins showed that grape skins had almost fivefold total phenolic content than apple samples' skins. The study concluded that grape skin is a rich source of anthocyanin pigments, especially the 3-glucosides of cyandin and malvidin with antibacterial and antioxidant properties.

The results of the GSKE study are in partial agreement with Katalinić *et al*., (2010). They used the broth microdilution test for the examination of the antimicrobial properties of grape skin extracts of the 14 grape varieties. *Bacillus cereus* and *Staphylococcus aureus* were used as Gram-positive bacteria, while *Campylobacter coli*, *Salmonella infantis* and *Escherichia coli O157:H7* were used as Gram-negative bacteria. The antimicrobial properties of grape skin extracts of the 14 grape varieties were evaluated against all the examined bacteria with a minimum inhibitory concentration range 0.014–0.59 mg of gallic acid equivalents (GAE)/ml.

Also, the present study results partially agree with Smullen *et al*., (2007). These researchers used a standard susceptibility agar dilution technique for the determination of the antibacterial activity of commercially available and 70% aqueous propanone (P70) extracts from different plants that are known for their high polyphenol content on *Streptococcus mutans* and other bacteria. The extracts used were seed and skin extracts of red and white grapes, green tea, cocoa, Pynogenol, and sloe berry. The lowest MIC was that for the P70 extract of red grape skin (0.5 mg ml $^{-1}$). This study also showed that both red and green grape extracts possessed higher activity than other tested fruits.

Also, the results of this study are in partial agreement with Rhodes et al. (2006). They investigated the antibacterial activity of *Vitis vinifera* var. *Ribier* grape commercial juice and of fresh Ribier grape extracts as well as that of seed, skin, and pulp extracts on *Listeria*

monocytogenes. The results of the study showed that commercial grape juice was highly inhibitory to *L. monocytogenes* serotypes 1/2a and 4b, the cause of foodborne listeriosis, and to all tested *Listeria* species. Its effect was so strong that it decreased the number of *L. monocytogenes* colonies from 10^6–10^7 CFU/ml to non-detectable within 10 min. Regarding the fresh extracts of *Ribier* grape skin, seed and pulp, skin and seed extracts had strong antilisterial activity, while the grape pulp had no inhibitory activity at all. Inhibition of tested *Listeria* species by fresh grape skin and seed extracts was similar to the inhibitory effect exhibited by commercial grape juice. Grape skin extract caused reduction in *L. monocytogenes* counts by 1-log, while grape seed extract caused the reduction in *L. monocytogenes* counts by 2-log after a 1 min exposure.

The present study results for GSE and GSKE confirmed that Grape seed (GSE) and skin (GSKE) extracts are rich sources of phenolic compounds considered as natural food preservatives against microbial growth. Xia et al., (2010) reported similar findings including antioxidant properties of the extracts.

Data analysis of the above experiment, using boxplot (Appendix C), showed that there is a difference in the median in the number of colonies between test and control samples for both antimicrobials (GSE and GSKE). A regression analysis was conducted to choose the factors significantly affecting the number of colonies. The output showed that the number of days, the type of bacteria and the type of antimicrobial used significantly affect the number of colonies at 5% significance level. All regression assumptions were valid in our analysis (randomness and normality of errors).

4.5. Colorimetric Determination of Phenolic Content of GSE and GSKE

Phenolic content of GSE and GSKE using the Folin- Ciocalteu methodology in this study was 315.32g (GAE)/kg, and 265.326g (GAE)/kg respectively (Appendix B).

Table 18 gives Gallic acid concentrations (ppm) and the corresponding absorptions (nm). Figure 44 gives the standard calibration curve of Gallic acid concentration (ppm) against the corresponding absorption (nm).

Table 18. Gallic acid concentrations (ppm) and the corresponding absorptions (nm).

Concentration of Gallic Acid (ppm	Absorption (nm)
10	0.049
20	0.09065
40	0.178
55	0.275
74	0.3326

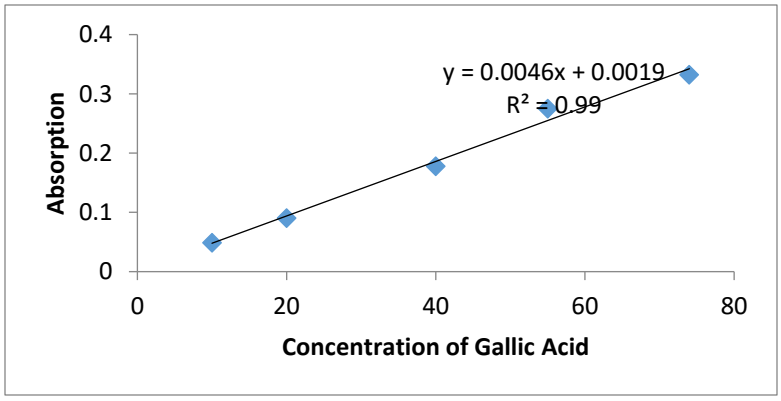


Figure 44: Standard calibration curve of Gallic acid concentration (ppm) against the corresponding absorption (nm)

Present results are in agreement with the literature; grape seed extract phenolic content is higher than that of grape skin extract. Xia et al., (2010) reported that phenolic content of grape seed,

skin, flesh, and leaf was 2178.8, 374.6, 23.8, and 351.6 mg/g GAE (gallic acid equivalent) respectively. Negro et al., (2003) determined the total phenolic content of grape seed extract, the peel and the marc. These researchers showed that grape seed extract's total phenolic content was higher than that of peel and marc, GSE total phenol concentration was equal to 2.86 g/l, while peel and marc total phenol concentrations were 1.11 and 1.40 g/l respectively. Baydar et al., (2004) determined GSE and bagasse extracts' total phenolic content using the extraction solvent mixture; acetone: water: acetic acid (90: 9.5 : 0.5) and reported it to be 627.98 mg and 45.44 mg gallic acid equivalent (GAE)/g respectively. With the solvent mixture; ethyl acetate: methanol: water (60: 30: 10), the TPC of GSE and bagasse extract was 667.87mg GAE/g and 29.55mg GAE/g respectively. Thus, extraction solvents influence the TPC of grape extracts.

Corrales et al., (2009) also determined the total phenolic content of GSE using Folin- Ciocalteau reagent and reported it to be 327.58 ± 7.24 mmol GAE/g extract.

TPC content of both grape seed and skin extracts in this study is higher than that in most literature studies.

5. Conclusion

Corona treated LDPE and PET films were coated with grape seed and skin extracts. Agar plate diffusion test showed that grape seed extract has a notable antibacterial effect on both *E.coli* and *Staph. aureus.* With both tested films (corona treated LDPE and PET films), inhibition zones for *E.coli* were greater than those for *Staph. aureus.* Grape skin extract also demonstrated considerable antibacterial activity against *Staph. aureus* and to a lesser extent against *E.coli*. In general, the higher the concentration of the GSE or GSKE/film wt. the greater the inhibition zone observed. LDPE corona treated films when showed better adhesion than that to PET corona

treated films. The microbiological data indicated that the experimental films coated with both grape seed and skin extracts exhibited antimicrobial activity when used to wrapp ground chicken samples. GSE antimicrobial activity was 10 fold to that of GSKE. Grape phenolic content varies according to the part of grape that it was extracted from; seeds have higher phenolic content than grape skin. The Total Phenolic Content of GSE and GSKE was 315.32g (GAE)/kg, and 265.326g (GAE)/kg respectively.

6. Future Work

Grape extracts as potent antimicrobial agents and as potential natural food preservatives may be applied at optimal concentrations to treated film surfaces for the preservation of a series of muscle foods such as fish and meat. Also, other methods of surface modification should be investigated e.g., chemical modification, plasma treatment, etc. for better adhesion. Commercial trials for the production of active food packaging using GSE/or GSKE will also be useful.

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Appendix A.

Calculations of *E.coli* and *Staph. aureus* populations:

The OD_{600} of *E.coli* and *Staph. aureus* were respectively 1.500 and 2.071. According to Peñuelas-Urquides et al., (2013) the best way to calculate the CFU is to go with an approximate that: 1 OD = 1^9 Cells/ml or 1 OD = 3.13 X 10^7 CFU/ml. Thus,

E.coli population = $1.500X3.13X10^7$ CFU/ml,

Staph. aureus population = $2.071X3.13X10^7$ CFU/ml

Appendix B

Calculations of Total Phenolics Content of GSE and GSKE

Absorption of GSE is: 0.292

& of GSKE is: 0.246

Phenolic Content of GSE

Y= 0.0046X + 0.0019

Abs =0.0046C+0.0019

C= (Abs- 0.0019)/0.0046

C= (0.292-0.0019)/0.0046

C=63.065ppm

Considering the dilution factor; 1/100

CX100 (dilution) =63.065X100=6306.5ppm

6306.5mg/1000ml

X/50ml (the used extract)

X=315.32g/kg

Phenolic Content of GSKE

C= (0.246-0.0019)/0.0046

C= 53.065ppm

Considering the dilution factor; 1/100

CX100=53.065X100= 5306.5ppm

5306.5mg/1000ml

X mg/50ml (the used extract)

X=50ml/1000mlX5306.5mg= 265.326g/kg.

Appendix C.

Data Analysis

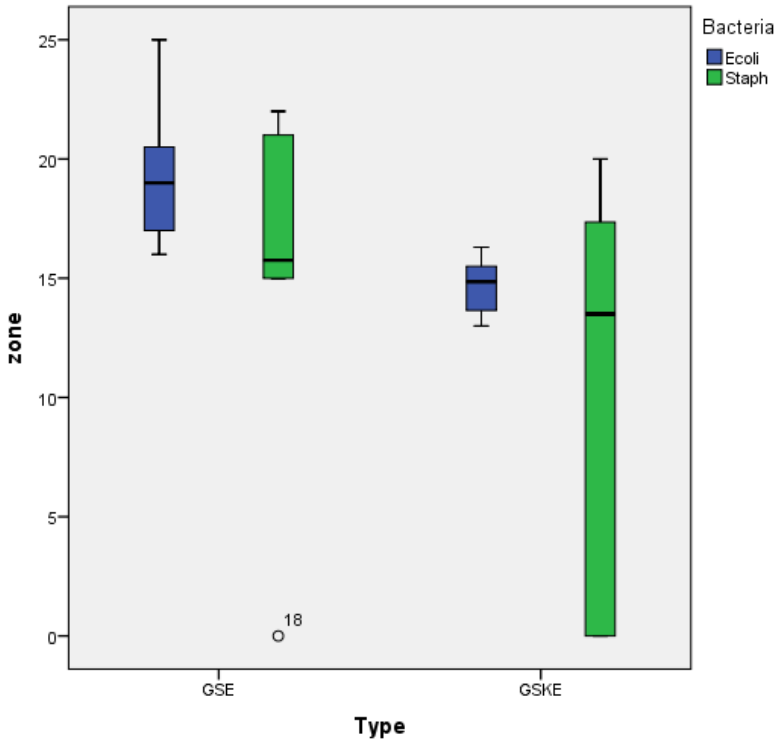

Figure 1: The boxplot shows that there is a difference in the median between the two types of bacteria in the GSE group and a slight difference in the median in the GSKE group. This graph suggests that the bacteria type and the substrates might be affecting the inhibition zone.

Table 1. Inhibition zones of Corona treated LDPE and PET films coated with GSE/ or GSKE

	GSE Conc. (Gm/film wt.)	*E-Coli* inhibition Zone (mm)	*Staph.* inhibition Zone (mm)	**GSKE Conc. (Gm/film wt.)**	*E-Coli* inhibition Zone (mm)	*Staph.* inhibition Zone (mm)
LDPE corona treated Films coated with GSE	0.6 gm	20.5mm	22mm	0.6gm	16.3mm	20mm
	0.5gm	25mm	21mm	0.5gm	16mm	19mm
	0.3gm	23mm	21mm	0.3gm	15mm	15.7mm
	0.2gm	19mm	16mm	0.2gm	14.7mm	15mm
	0.15gm	20mm	15mm	0.15gm	14mm	No inhibiton zone
	0.1gm	16mm	No inhibition zone	0.1gm	13.3mm	No inhibiton zone
PET corona treated Films coated with GSE	0.3gm	19mm	15mm			
	0.2gm	17mm	15mm			
	0.15gm	17mm	16.5mm	0.15gm	15mm	12mm
	0.1gm	16mm	15.5mm	0.1gm	13mm	No inhibiton zone
Control films(LDPE and PET corona treated films without GSKE or GSE	No Inhibition Zones					

Table 2. GSE against different bacteria

Days		0	2	4	6	8	10
GSE TVC	Test	4.9	5	5.4	6.2	6.7	7.2
(PCA)	Control	4.9	5.2	6.15	6.85	7.36	
GSE	Test	3.6	4	4.9	5.5	6.2	7.1
Pseudomonas	Control	3.6	4.3	5.3	6.45	7.2	
GSE Enterobacteriacea	Test	1.6	1.6	2.8	3.1	4.3	4.5
	Control	1.6	3	3.6	4.35	5	
GSE Br.thermosphacta	Test	3.6	4	5	5.7	6.3	7.2
	Control	3.6	4.2	5.5	6.45	6.8	
GSE Lactic acid bacteria	Test	3.1	3.2	3.7	4.1	5.1	5.7
	Control	3.1	3.65	4.4	5.1	5.8	

Table 3. GSKE against different bacteria

Days		**0**	**2**	**4**	**6**	**8**	**10**
GSKE TVC	**Test**	**4.5**	**4.7**	**5.3**	**5.5**	**6.6**	**7.66**
(PCA)	**Control**	**4.5**	**5.5**	**6.2**	**6.9**	**8**	
GSKE	**Test**	**4.5**	**4.6**	**5.2**	**5.5**	**6.45**	**6.97**
Pseudomonas	**Control**	**4.5**	**4.9**	**6.5**	**7.45**	**8.2**	
GSKE Enterobacteriacea	**Test**	**1.5**	**2**	**2.9**	**3**	**4.1**	**4.5**
	Control	**1.5**	**2.65**	**3.45**	**4.8**	**5.9**	
GSKE Br.thermosphacta	**Test**	**3.8**	**3.9**	**4.9**	**5.6**	**6.1**	**6.9**
	Control	**3.8**	**4.45**	**5.6**	**6.45**	**7.5**	
GSKE Lactic acid bacteria	**Test**	**2.5**	**2.6**	**3.1**	**4**	**4.5**	**5.4**
	Control	**2.5**	**3.3**	**4.2**	**4.6**	**5**	

ANOVA[a]

Model		Sum of Squares	df	Mean Square	F	Sig.
3	Regression	772.897	3	257.632	14.413	.000[d]
	Residual	572.006	32	17.875		
	Total	1344.903	35			

a. Dependent Variable: zone

b. Predictors: (Constant), concentration

c. Predictors: (Constant), concentration, Type1

d. Predictors: (Constant), concentration, Type1, Bacteria1

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
3	(Constant)	5.234	1.678		3.119	.004
	concentration	19.920	4.175	.550	4.771	.000
	Type1	5.087	1.418	.414	3.587	.001
	Bacteria1	3.950	1.409	.323	2.803	.009

a. Dependent Variable: zone

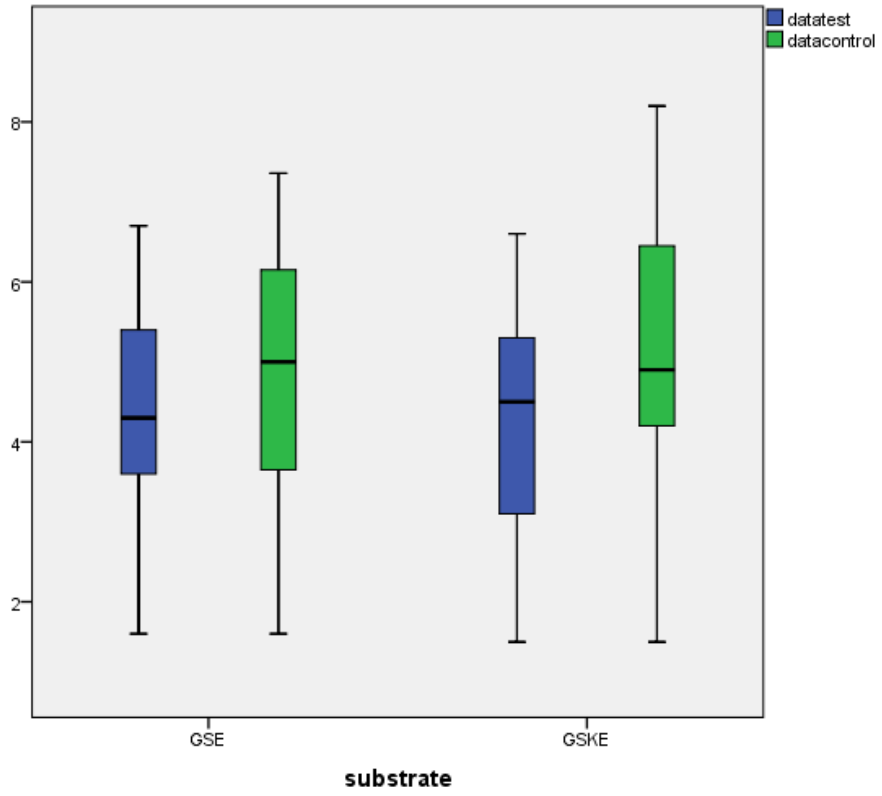

Figure 2: boxplot shows that there is a difference in the median of colonies between test and control for both substrates.

The sample size in our experiments is more than 30, so we are allowed to use parametric tests according to the central limit theorem. The paired t-test is used here to check whether there is a significant difference between the test and control regardless of the substrate and bacteria type. The t-test shows that there is a significant difference in the means of test and control at 5% significance level.

Paired Samples Statistics

		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	datatest	4.34	50	1.366	.193
	datacontrol	5.04	50	1.610	.228

Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	datatest & datacontrol	50	.947	.000

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	datatest - datacontrol	-.697	.539	.076	-.850	-.544	-9.141	49	.000

To investigate the factors affecting the number of colonies we defined 4 dummy variables representing the different types of bacteria and then conducted a regression analysis to choose the factors significantly affecting the number of colonies.

ANOVA[a]

Model		Sum of Squares	Df	Mean Square	F	Sig.
4	Regression	131.978	4	32.994	399.012	.000[e]
	Residual	4.548	55	.083		
	Total	136.526	59			

a. Dependent Variable: datatest

b. Predictors: (Constant), days

c. Predictors: (Constant), days, Entero1

d. Predictors: (Constant), days, Entero1, Lactic

e. Predictors: (Constant), days, Entero1, Lactic, TVC1

The set of chosen factors explain 96.4% of the variability in the number of colonies.

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
4	(Constant)	3.778	.080		47.234	.000
	days	.307	.011	.695	28.246	.000
	Entero1	-2.322	.102	-.616	-22.836	.000
	Lactic	-1.397	.102	-.370	-13.738	.000
	TVC1	.492	.102	.130	4.836	.000

a. Dependent Variable: datatest

Another regression analysis was conducted to check the factors affecting the difference between the test and control data. We created a new variable which is the difference between the number of colonies of the test and control.

The results show that the chosen model is able to explain 54.5% of the variability in the difference between the number of colonies for test and control.

ANOVA[a]

Model		Sum of Squares	Df	Mean Square	F	Sig.
2	Regression	8.026	2	4.013	30.300	.000[c]
	Residual	6.225	47	.132		
	Total	14.251	49			

a. Dependent Variable: Difference

b. Predictors: (Constant), days

c. Predictors: (Constant), days, substrate1

Coefficients[a]

Model		Unstandardized Coefficients		Standardized Coefficients	t	Sig.
		B	Std. Error	Beta		
2	(Constant)	-.311	.103		-3.017	.004
	days	-.132	.018	-.701	-7.273	.000
	substrate1	.286	.103	.267	2.775	.008

a. Dependent Variable: Difference

The coefficients table shows that as the number of days increases the difference between the number of colonies for test and control decreases given that all other factors are fixed.

The substrate also affects the difference significantly. The GSE has a higher difference in the number of colonies than the GSKE at the same number of days. All these coefficients are significant at 5% significance level.

All regression assumptions were valid in our analysis (randomness and normality of errors).

Appendix D

Table 4. Genera of Bacteria Most Frequently Found on Meats and Poultry

Genus	*Gram Reaction*	*Fresh Meats*	*Fresh Livers*	*Poultry*
Acinetobacter	–	XX	X	XX
Aeromonas	–	XX		X
Alcaligenes	–	X	X	X
Arcobacter	–	X		
Bacillus	+	X		X
Brochothrix	+	X	X	X
Campylobacter	–			XX
Carnobacterium	+	X		
Caseobacter	+	X		
Citrobacter	–	X		X
Clostridium	+	X		X
Corynebacterium	+	X	X	XX
Enterobacter	–	X		X
Enterococcus	+	XX	X	X
Erysipelothrix	+	X		X
Escherichia	–	X	X	
Flavobacterium	–	X	X	X
Hafnia	–	X		
Kocuria	+	X	X	X
Kurthia	+	X		
Lactobacillus	+	X		
Lactococcus	+	X		
Leuconostoc	+	X	X	
Listeria	+	X		XX
Microbacterium	+	X		X
Micrococcus	+	X	XX	XX
Moraxella	–	XX	X	X
Paenibacillus	+	X		X
Pantoea	–	X		X
Pediococcus	+	X		
Proteus	–	X		X
Pseudomonas	–	XX		XX
Psychrobacter	–	XX		X
Salmonella	–	X		X
Serratia	–	X		X
Shewanella	–	X		
Staphylococcus	+	X	X	X
Vagococcus	+			XX
Weissella	+	X	X	
Yersinia	–	X		

Note: X = known to occur; XX = most frequently reported.

(From Jay *et al*., 2005)

Table 5. Genera of Yeasts and Molds Most Frequently Found on Meats and Poultry

Genus	*Fresh and Refrigerated Meats*	*Poultry*
Molds		
Alternaria	X	X
Aspergillus	X	X
Aureobasidium	X	
Cladosporium	XX	X
Eurotium	X	
Fusarium	X	
Geotrichum	XX	X
Monascus	X	
Monilia	X	
Mucor	XX	X
Neurospora	X	
Penicillium	X	X
Rhizopus	XX	X
Sporotrichum	XX	
Thamnidium	XX	
Yeasts		
Candida	XX	XX
Cryptococcus	X	X
Debaryomyces	X	XX
Hansenula	X	
Pichia	X	X
Rhodotorula	X	XX
Saccharomyces		X
Torulopsis	XX	X
Trichosporon	X	X
Yarrowia		XX

Note: X = known to occur; XX = most frequently found.

(From Jay *et al*., 2008)

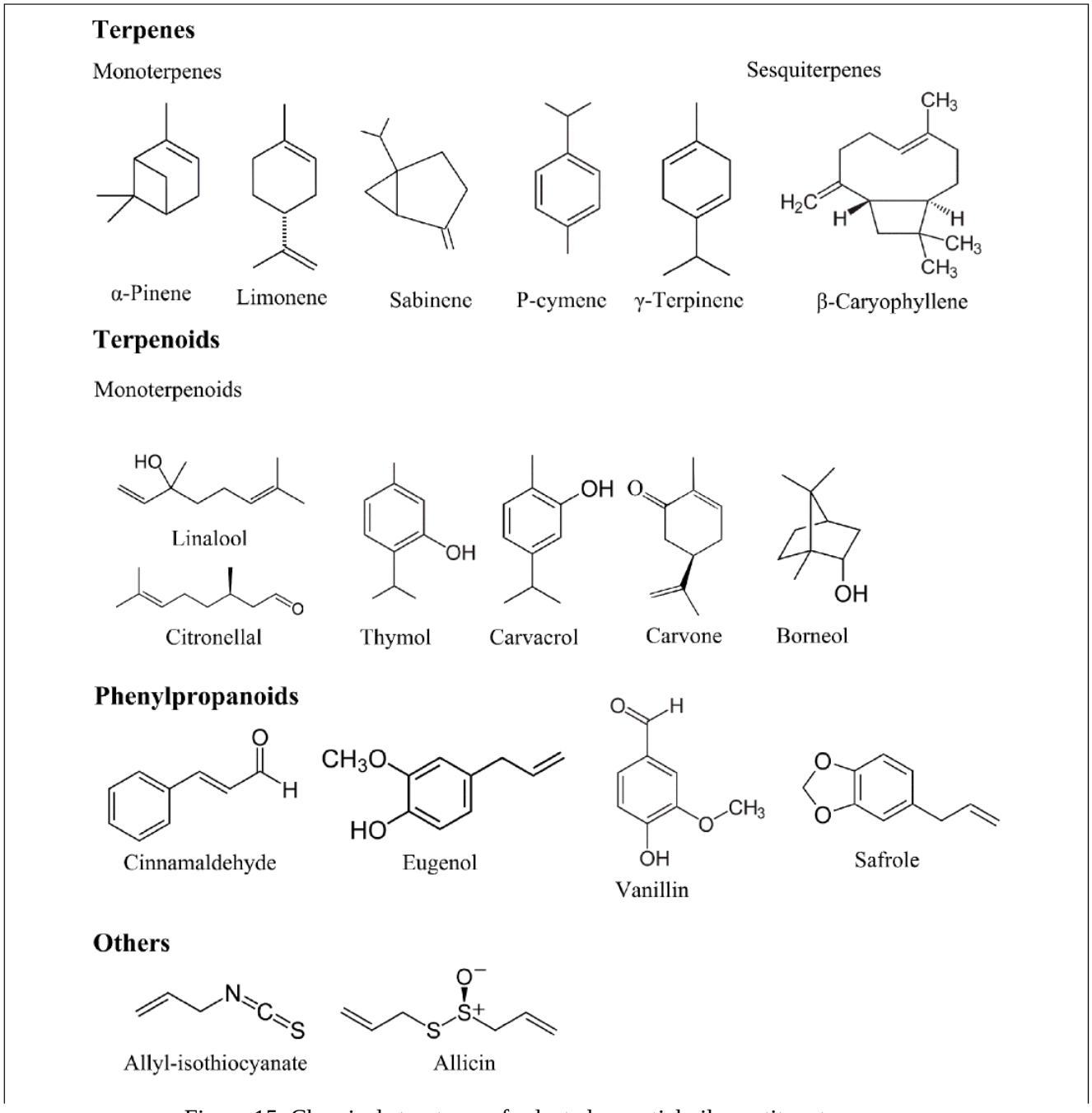

Figure 15: Chemical structures of selected essential oil constituents
(From Hyldgaard *et al*., 2012)

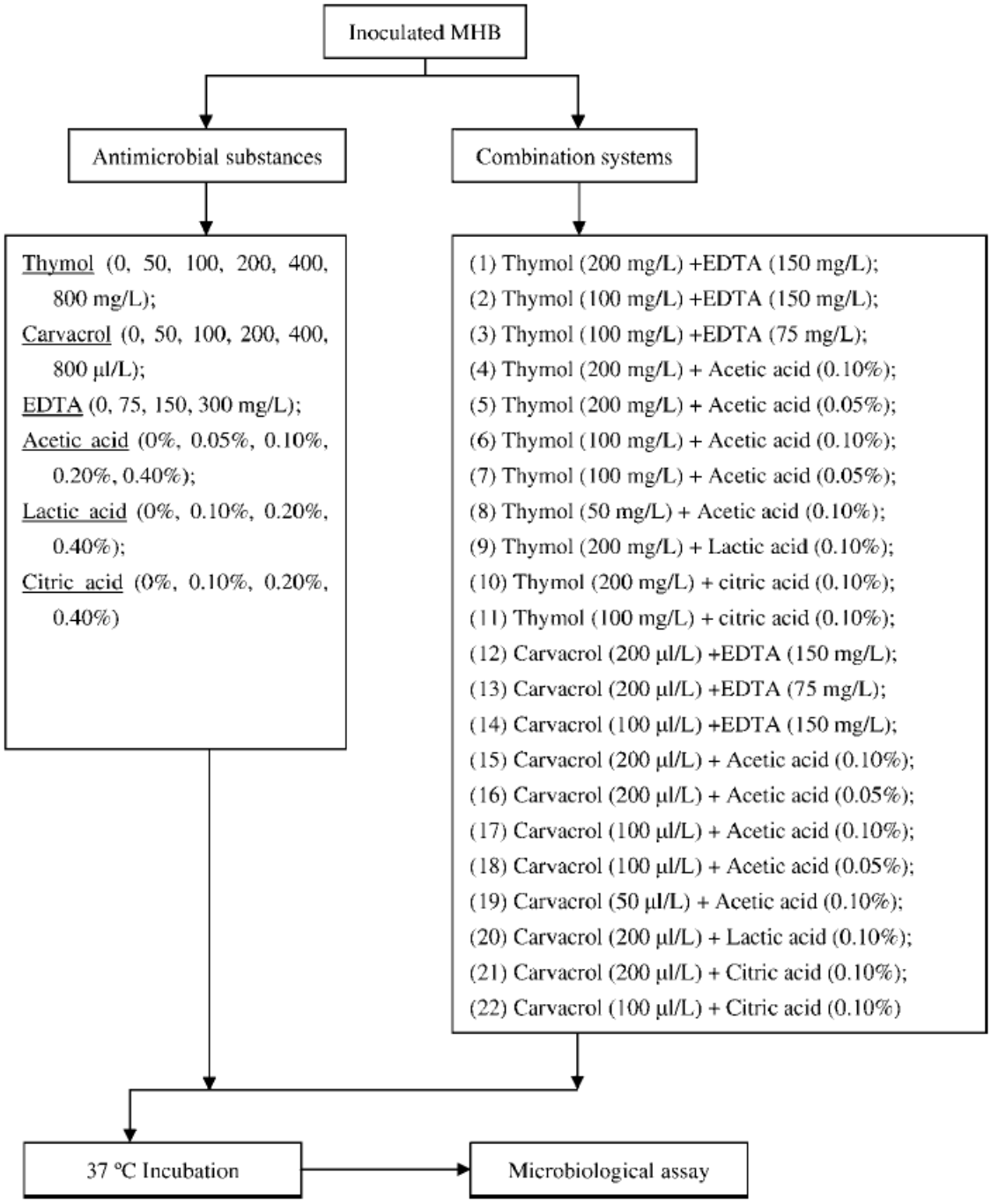

Figure 16: Flow chart and treatment diagram of antimicrobial system application protocols. (From Zhou *et al*., 2007)