School of Public Health

Antimicrobial Efficacy of Biodegradable Films and Coatings on Beef

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Declaration of Originality

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Signature: 

Date: 30.08.2016

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Abstract

Contamination of chilled fresh meat and processed meat product surfaces with pathogenic and spoilage bacteria are of concern to the meat industry with respect to both consumer safety and economic loss. The impact of these bacteria in meat spoilage and safety is associated with many intrinsic and extrinsic factors such as packaging, preservation, temperature and storage conditions. New approaches to effectively control bacteria on meat are constantly being sort. Antimicrobial film and coating represent one method which may be effective in ensuring the safety of fresh and processed meat products. Kafirin and zein films or coatings, which are from the same Panicoideae grasses subfamily, have received attention and been widely investigated. Studies have shown that spoilage on meat can be controlled by films and coatings, natural antimicrobial agent or combinations of both. This research in this thesis is focused on the potential of kafirin as an antimicrobial film or coating to improve meat safety and extend shelf life of meat during chilled storage. There is limited work reported on kafirin (K) films or coatings containing essential oils such as oregano (OEO) or thyme (TEO) alone, or a combination of both (OTEO), or antimicrobial peptides such as nisin.

The first part of this study was a preliminary screening of the characteristics of biodegradable zein (Z) and K films with OEO incorporated with respect to drying temperature; the residual hexane in both films without essential oil and; the antimicrobial activities of both films containing OEO, TEO and OTEO against spoilage and pathogenic bacteria *in vitro*. The results indicated that films that were dried in a forced draft oven were uniform in thickness and had a smoother surface than films dried at room temperature. There was a low content (<4 mg/kg) of residual hexane in both films. In this preliminary study, antimicrobial activity results showed that zein films did not produce any inhibition zones against six bacteria regardless of the type or concentration of essential oils added (0.1%, 0.5%, 1.0% and 1.5%). Kafirin films containing OEO inhibited the growth of *B. thermosphacta* and *P. aeruginosa* at 1.5% (0.54 mm^2 inhibition zone) and 0.5% (1.22 mm^2 inhibition zone), respectively. Films of K+OTEO at 0.5%, 1.0%, and 1.5% inhibited *B. thermosphacta* with inhibition zones of 0.52 mm^2, 0.46 mm^2 and 0.85 mm^2, respectively. Kafirin

films were deemed suitable for further work on their potential for application on meat.

The second part of this study examined the antimicrobial effects of biodegradable K films containing OEO, TEO and OTEO at different concentrations against 6 bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Brochothrix thermosphacta*, *Listeria monocytogenes*, *Lactobacillus sakei* and *Pseudomonas aeruginosa*) were evaluated. In addition, the most effective K films without or 5% OTEO were examined for physical, mechanical, barrier and optical properties. Results showed that the antimicrobial activity of OTEO was found to be the most effective antimicrobial agent followed by OEO and TEO. This indicated the potential that there were additive or synergistic effects of these compounds. Kafirin films loaded with OEO were effective at concentrations of more than 4% against all bacteria tested except *P. aeruginosa*. A particularly strong inhibitory effect against *B. thermosphacta* ($p < 0.05$) was apparent. Kafirin films containing OTEO were effective ($p < 0.05$) against all bacteria tested. Kafirin films loaded with TEO and OTEO were also ineffective against *P. aeruginosa*. Zein and K control films did not display any antimicrobial effects against the six bacterial strains tested. Incorporation of 5% OTEO into kafirin films was found to: increase film thickness (0.17 ± 0.01 mm); not significantly affect moisture content values; increase film water solubility significantly ($p < 0.05$) (41.06 ± 0.66%); improve mechanical strength (reduced tensile strength (TS), ($p < 0.05$) and higher % elongation at break (%EAB), ($p > 0.05$)); improve barrier and optical, relative to control films. It was also established that different batches of K may influence some film characteristics such as colour.

The third part of this study investigated the effectiveness of using different antimicrobial coating treatments on the physicochemical characteristics and surface microflora of vacuum packaged fresh beef stored at 2 ± 0.5°C. The results showed that the *Enterobacteriaceae* counts of all antimicrobial coating treatments remained below the limit of detection at the end of the storage period. The total viable count (TVC) was significantly ($p < 0.05$) lower in all the antimicrobial coating treatments as compared to the control group. A slightly lower lactic acid bacteria count (as compared to TVC count) was apparent in all the antimicrobial coating treatments as compared to the control group ($p < 0.05$), but no significant effect ($p > 0.05$) was

observed over the storage periods. This part of the study showed that kafirin coating treatments combined with vacuum packaging affect pH and redness colour of fresh beef. It can be concluded that kafirin coating based, with and without essential oils, and vacuum packaging have potential for improving the shelf life of beef meat during the storage.

The final part of this study was carried out to evaluate the antimicrobial effect of K films with nisin incorporated in them against *L. monocytogenes in vitro*. In addition the effects of antimicrobial kafirin coatings containing nisin for controlling *L. monocytogenes* inoculated onto the surface of processed beef meat stored at 4°C for up to 12 days, as well as on naturally occurring bacteria, were also studied. A nisin concentration of greater than 3% showed an antimicrobial effect against *L. monocytogenes* ($p < 0.05$) and produced clear inhibition zones underneath the film discs (contact area) *in vitro*. Kafirin coatings with nisin at 10% on processed meat resulted in the highest reduction of *L. monocytogenes*, followed by coating with 1% nisin and control films, but none of the treatments were effective in reducing TVC during storage at 4°C. This part of the study demonstrated nisin can be effectively incorporated in K films and act to control *L. monocytogenes* on processed meat.

Overall, the findings of this study indicate that the kafirin films and coatings containing oregano essential oil, thyme essential oil (or a combination of both) and nisin can be used to inhibit meat spoilage and pathogenic bacteria with the potential of extending shelf life and safety of chilled fresh meat and processed meat products.

Keywords: Antimicrobial; biodegradable films; biodegradable coatings; fresh meat; kafirin; meat spoilage bacteria; nisin; oregano essential oils; pathogenic bacteria; processed meat; thyme essential oils

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List of Abbreviations

ANOVA	Analysis of variance
ASTM	American society for testing and materials
ATCC	American type culture collection
BHIA	Brain Heart Infusion Agar
BPS	Phosphate buffered saline
BPW	Buffered peptone water
CDC	The centers for disease control and prevention
CFU	Colony forming unit
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EAB	elongation at break
EU	European union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FSO	Food safety objectives
GHP	Good hygiene practices
GMP	General manufacturing practice
GRAS	Generally Recognized As Safe
HACCP	Hazard analysis and critical control points
HCI	Hydrochloric acid
LAB	Lactic acid bacteria

LSD	Least significant difference
MHA	Mueller-Hinton Agar
MLA	Meat & Livestock Australia
MRD	Maximum recovery diluent solutions
MRS	De man, rogosa, sharpe agar
MSA	Meat Standards Australia
NaOH	Sodium hydroxide
OEO	Oregano essential oil
OP	Oxygen permeability
OTEO	Oregano and thyme essential oil
PBS	Phosphate buffered saline
PEG	polyethylene glycol
PCA	Plate count agar
RH	Relative humidity
RNA	Ribonucleic acid
SD	Standard deviation
TEO	Thyme essential oil
TS	Tensile strength
TVC	Total viable counts
TPC	Total psychrotrophic counts
US	United States

USDA	United States Department of Agriculture
UV	Ultraviolet
VRBG	Violet red bile glucose agar
WHO	World Health Organization
WPI	Whey protein isolate
WTO	World Trade Organization
WVP	Water vapour permeability
WVTR	Water vapour transmission rate

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Chapter 1: Introduction and Literature Review

1.1 Introduction

The control and elimination of spoilage bacteria and foodborne pathogens remains an important challenge at all stages of meat production - from the producer to the consumer. Common meat spoilage bacteria and pathogens found on fresh and processed meat products during chilled storage and packaging include *Pseudomonas* spp., *Enterobacteriaceae*, lactic acid bacteria (LAB), *Brochothrix thermosphacta*, *Listeria monocytogenes* and *Staphylococcus aureus*. Meats are highly vulnerable to spoilage during slaughtering, processing, preparation, storage, packaging and distribution (Lucera, Costa, Conte & Del Nobile, 2012). In addition, microbial contamination can accelerate physicochemical deterioration. The application of a protein film or coating in combination with other methods to preserve fresh meat and processed meat products can reduce spoilage as a consequence of bacteria. Sometimes fresh meat or processed meat products are packaged using vacuum packaging to extend shelf life. However, although these methods assist in preserving perishable foods, they cannot guarantee the elimination of undesirable meat spoilage and pathogenic bacteria. Hence, the addition of natural antimicrobial agents (such as essential oils or antimicrobial peptides) is promising (Jayasena & Jo, 2013). There are a limited number of studies on the application of biodegradable kafirin as a film or coating material, and specifically its combination with antimicrobials to prevent meat spoilage and the growth of pathogenic bacteria. Similarly, there has been little investigation into the effectiveness of using film or coating treatments on the microbiological and physicochemical characteristics of meat in combination with vacuum packaging. The objective of this thesis is to investigate and determine the effectiveness of antimicrobial films or coatings containing essential oils or nisin in controlling spoilage or pathogenic bacteria on meat.

1.2 The Consumption of Fresh Meat and Processed Meat Products

Meat is the first preferred source of animal protein for most people around the globe (Dave & Ghaly, 2011). According to the World Health Organization (WHO), the predicted world annual meat consumption per capita is expected to reach 45.3 kg by 2030 (Kouvari, Tyrovolas & Panagiotakos, 2016). In the United States the average

annual beef consumed per capita reached 54.5 pounds in 2012 (USDA, 2015). The beef meat industry continues to maintain a sizable market share (Belk et al. 2014). For instance, Australia is among the world's top three exporters and efficient producers of beef. From 2013 to 2014, the Australian beef and veal industries generated more than $7.7 billion (Jie, Parton & Mustafid, 2015). Australian consumers alone spent approximately $154.26 million on beef during June, 2011 (MLA, 2012).

1.3 The Consequences of Unsafe Meat

Fresh beef meat and processed meat products are well known to harbour bacteria that are capable of causing foodborne illnesses and may threaten human health. Foodborne disease, or food poisoning, is frequently related to the consumption of contaminated fresh meat and its processed products. Since meat is nutritious, has a high moisture and water content (a_w > 0.99), has a moderate pH that supports microbial growth (5.5–6.5) and is considered highly perishable, it supports the growth and survival of a diverse population of bacteria and the potential for chemical deterioration if not properly preserved and stored (Doulgeraki, Ercolini, Villani & Nychas, 2012; Jayasena & Jo, 2014). Meat-related product recalls can seriously affect both industry and the general economy by resulting in reduced sales at a national or international level (Sofos, 2008). Numerous examples of outbreaks or individual cases of disease associated with meat, and their associated economic losses, have been reported. For example, a total of 115,929 cases and 64 deaths of foodborne disease in England and Wales with beef as a vector were reported from 1996 to 2000 (Adak, Meakins, Yip, Lopman & O'Brien, 2005). The Centers for Disease Control and Prevention (CDC) state that in 2014 58% of sick individuals were hospitalised because of an outbreak associated with ground beef contaminated with *E. coli* O157: H7 produced by the Wolverine Packing Company. In addition 1.8 million pounds of ground beef were recalled in the United States. The economic cost of foodborne illness in the United States was reported to be $77.7 billion (Scharff, 2012).

1.4 Meat Definitions

The beef meat industry has been influenced by consumer demand for variation and convenience of preparation due to a busy lifestyle, which has led to the creation of processed beef meat products, rather than fresh beef or primal cuts (Resurreccion, 2004). The term 'fresh beef' refers to slaughtered or processed meat products supplied as vacuum-packaged meat or presented in any antimicrobial packaging that has not had treatment applied to it other than chilling to improve preservation (Zhou, Xu & Liu, 2010). Many processed beef meat products are marinated or smoked, include salt and other ingredients and are prepared in small portions such as slices, small pieces and steaks. Beef meat is also sold using various packaging and temperatures (from chilled to frozen) and is delivered by different methods of transportation. The term 'chilled beef' denotes beef meat that has undergone cold temperature treatment to maintain meat safety and shelf life and has been cooled and maintained at a temperature of or below 7ºC, but not under –2ºC, during storage and distribution (Fernandes, 2009).

Generally, spoilage is the process by which chilled meat deteriorates to a point at which it becomes unsuitable for human consumption (Gram et al., 2002). Chilled beef meat is susceptible to microbiological contamination as previously discussed and this creates an excellent environment for the surface growth of meat spoilage bacteria and meat borne pathogens (Aymerich, Picouet & Monfort, 2008; Ercolini et al., 2011; Nowak et al., 2012; Turgis et al., 2012). At chilled temperatures, the initial microbial population on fresh meat and processed meat products is within the range of $10^2 – 10^4$ CFU/g (Holzapfel, 1998; Sun & Holley, 2012).

1.5 Overview of Meat Spoilage Bacteria and Pathogenic Bacteria

There are a variety of common spoilage bacteria and pathogenic bacteria found on chilled fresh meat and processed meat product surfaces during different storage conditions (see Table 1). *Pseudomonas spp*., *Enterobacteriaceae*, LAB and *Brochothrix thermosphacta* represent bacteria that have been recognised as constituting a large proportion of spoilage organisms on chilled beef meat, whether fresh or processed (Borch, Kant-Muermans & Blixt, 1996; Dainty & Mackey, 1992; Doulgeraki, Paramithiotis, Kagkli & Nychas, 2010; Labadie, 1999; Lucera et al., 2012; Nychas, Skandamis, Tassou & Koutsoumanis, 2008; Shahbazi, Shavisi & Mohebi, 2016). The growth of meat spoilage bacteria is usually influenced by temperature, oxygen, type and number of spoilage bacteria present (Huis in't Veld, 1996). It is also well established that pathogenic bacteria, such as *Listeria monocytogenes, Salmonella enteridis, Escherichia coli* and *Staphylococcus aureus* are able to survive and/or grow on meat (Coma, 2008; Oussalah et al., 2007). Hence, this study was focused on several spoilage and pathogenic bacteria that are responsible for the contamination of meat, specifically, *Brochothrix thermosphacta, Lactobacillus sakei, Pseudomonas aeruginosa, Listeria monocytogenes, Escherichia coli* and *Staphylococcus aureus*. Fresh and processed meat products can be contaminated during both slaughter and processing (Doulgeraki, Ercolini, Villani, & Nychas, 2012; Loiko et al., 2016).

Meat spoilage and pathogenic bacteria are responsible for a high proportion of deterioration of fresh and processed meat quality and safety issues. Spoilage of fresh meat and processed meat products can be ascertained by changes in pH levels and colour. Growth of bacteria to unacceptable levels, depending on the species of spoilage organism, can affect the appearance of entire meat surface, formation of slime, unpleasant odour and degradation of protein (Ercolini, Russo, Torrieri, Masi, & Villani, 2006; Sánchez-Ortega et al., 2014). Unacceptable levels are defined as microbial loads greater than 10^7 to 10^9 CFU cm^{-2} and contribute to 'off' odours, such as cheesy, buttery or fruity smells (Ercolini et al., 2006). The numbers of bacteria present can vary on fresh meat and processed meat products based on their storage conditions, packaging and temperature (Casaburi, Piombino, Nychas, Villani &

Ercolini, 2015; Pennacchia, Ercolini & Villani, 2011; Sun & Holley, 2012). These factors may result in physicochemical and organoleptic changes at various stages of the chilled beef meat storage and distribution process.

Table 1
Common Spoilage and Pathogenic Bacteria Present on Fresh and Processed Meats.

Bacteria	Gram reaction	Fresh	Processed
Achromobacter	-	X*	
Acinetobacter	-	XX*	X
Aeromonas	-	XX	X
Alcaligenes	-	X	
Alteromonas	-	X	X
Arthrobacter	±	X	X
Bacillus	+	X	X
Brochothrix	+	X	X
Campylobacter	-	X	
Carnobacterium	+	X	
Chromobacterium	-	X	
Citrobacter	-	X	
Clostridium	+	X	
Corynebactenum	+	X	X
Enterobacter	-	X	X
Enterococcus	+	XX	X
Escherichia	-	X	
Flavobacterium	-	X	
Hafnia	-	X	X
Janthinobacterium	-		X
Klebsiella	-	X	
Kluyvera	-	X	
Kocuria	+	X	X
Kurthia	+	X	
Lactobacillus	+	X	XX
Lactococcus	+	X	
Leuconostoc	+	X	X
Listeria	+	X	X
Microbacterium	+	X	X
Micrococcus	+	X	X
Moraxella	-	XX	
Paenibacillus	+	X	X
Pantoea	-	X	
Proteus	-	X	
Providencia	-	X	X
Pseudomonas	-	XX	X
Salmonella	-	X	X
Shewanella	-	X	X
Staphylococcus	+	X	X
Streptococcus	+	X	X
Vibrio	-	X	
Weissella	+	X	X
Yersinia	-	X	

Based on Nychas et al. (2007); Coma (2008); *X = known to occur, XX = most frequently isolated.

Chilled meat spoilage bacteria and pathogenic bacteria have the capacity to grow on fresh meat or processed meat when stored under air, vacuum or antimicrobial packaging (see Table 2). Typically, their spoilage activities are likely to depend on oxygen availability and the production of many volatile organic compounds (Penacchia et al., 2011). The use of packaging at chill temperature and antimicrobials could influence the succession and metabolic activities of specific spoilage microorganisms (SSOs), which usually dominate the process of spoilage (La Storia et al., 2012; Sun & Holley, 2012). This review focuses on common chilled beef meat spoilage associated with bacteria microorganisms and pathogenic bacteria. It examines the sources of such microbial contamination on fresh meat and processed meat products and the factors influencing microbial growth and shelf life.

Table 2

Spoilage and Pathogenic Bacteria on Fresh Meat and Processed Meat under Different Storage Conditions

Bacteria	**Storage conditions**		
	Air	**Vacuum packaging**	**Antimicrobial packaging***
Brochothrix thermosphacta	x	x	x
Escherichia coli	x	x	x
Lactobacillus sakei	x	x	x
Pseudomonas aeruginosa	x	x	x
Staphylococcus aureus	x	x	x
Listeria monocytogenes	x	x	x

Based on Doulgeraki et al. (2012); Shahbazi et al. (2016); Pavelková et al., (2014); Casaburi et al. (2015).

x indicates the ability to grow microorganism in the storage condition.

*The antimicrobial packaging refers to essential oils or nisin in the film or coating.

1.5.1 Important spoilage and pathogenic bacteria

1.5.1.1 *Pseudomonas spp.*

Pseudomonas are a Gram-negative bacterial genus of which several species, such as *P. aeruginosa*, *P. fragi* and *P. fluorescens,* are responsible for meat spoilage and which grow under aerobic storage at different temperatures (-1 to 25°C) (Nychas et al., 2008). Studies have shown that *Pseudomonas* spp. can also occur in meat stored in air, vacuum packaging and antimicrobial packaging (Casaburi, Piombino, Nychas, Villani & Ercolini, 2015; Jayasena & Jo, 2013). Vacuum packaging is widely used commercially for export or long distance distribution of chilled beef (Hernández-Macedo, Barancelli& Contreras-Castillo, 2011). The storage shelf life of vacuum packaged beef is expected to be a minimum of 90 days, and for some countries storage is at a temperatures below zero (Youssef et al., 2014). However, if the temperature of storage shelf life is at 0ºC–4ºC, chilled beef can be maintained for only approximately 40 days (Youssef et al., 2014). The desired shelf life also depended on vacuum packaging films, with films with an oxygen permeability below than 300 cc/m^2/24h/atm stored for 11 weeks at 0°C (Lee & Yoon, 2001). Extended shelf life of vacuum packaged chilled beef can be of concern as it may support the growth and survival of facultative anaerobic psychotropic pathogens (Dykes et al., 2001; Jiang et al., 2011) and the possibility of resulting in deterioration in colour and odour quality that is observed after the packaging is removed (Lee & Yoon, 2001). *Pseudomonas* spp. compete with other bacteria better at lower temperatures, particularly those between 0°C to 2°C (Labadie, 1999). Members of this genus metabolise glucose in the meat and utilise it until they begin to consume proteins and amino acids. As a result, ammonia, amines and sulphides are produced, which lead to the formation of ‘off’ odours and ‘off’ flavours (Koutsoumanis et al., 2005). *P. aeruginosa* is a common and resilient species and therefore has the highest spoilage potential in this genus across almost all meats under different storage conditions, particularly vacuum packaging, air and antimicrobial packaging (Doulgeraki et al., 2012; Emiroğlu, Yemiş, Coşkun & Candoğan, 2010).

1.5.1.2 *Enterobacteriaceae*

Enterobacteriaceae can contribute to spoilage of fresh meat or processed meats at chilled temperatures (Coma, 2008; Doulgeraki et al., 2012). They are Gram-negative, rod-shaped and facultative anaerobic bacteria. Vacuum packaging can efficiently inhibit and control the growth of the *Enterobacteriaceae* family (Chen et al., 2012). *E. coli* is a member of the *Enterobacteriaceae* family and can be used as a hygiene indicator of microbial contamination for the food industry during preparation and processing. This is due to the fact that *E. coli* can be found in animal and human gastrointestinal tracts and it can also survive during processing environment, storage and packaging. Yang et al. (2012) reported that the numbers of *E. coli* on beef products and conveyor belts were higher than the numbers on carcasses and this may have contributed to the contamination of beef during carcass dismemberment and preparation. *E. coli* may also be present in vacuum packaging and antimicrobial packaging during storage. A large number of *E. coli* strains can cause human illnesses and symptoms such as diarrhoea, abdominal cramping, fever, chills, nausea, headache, loss of appetite and haemorrhagic colitis (Estrada-Garcia, Hodges, Hecht & Tarr, 2013).

1.5.1.3 *Lactic acid bacteria (LAB)*

LAB are Gram-positive organisms and a heterogeneous group that are often associated with fresh beef meat and processed meat. The dominant species of psychrotrophic LAB include *Lactobacillus sakei*, *Lactobacillus curvatus*, *Lactobacillus fuchuensis* and *Lactobacillus algidus*, each of which can reach high populations in chilled storage vacuum-packaged meat (Chen et al., 2012; Doulgeraki et al., 2010; Sakala et al., 2002). In particular, *L. sakei* contributes to beef meat spoilage when stored in vacuum packaging at 4°C. Under these conditions the concentration of oxygen is decreased and the concentration of carbon dioxide is increased (Doulgeraki et al., 2012; Doulgeraki, Paramithiotis, Kagkli & Nychas, 2010; Pennacchia et al., 2011; Pothakos, Devlieghere, Villani, Björkroth & Ercolini, 2015). Processed meat products, such as marinated and value-added products, are more susceptible to contamination by LAB owing to ingredients such as sugar on/in

meat tissue (Sofos & Geornaras, 2010). However, LAB also may act as protective cultures in meat products since they are able to produce antimicrobial metabolites such as organic acids, acetoin, diacetyl, hydrogen peroxide and bacteriocins (Katikou, Ambrosiadis, Georgantelis, Koidis & Georgakis, 2005). The presence of a high LAB population does not essentially effect quality of a product (Pothakos et al., 2015).

1.5.1.4 *Brochothrix thermosphacta*

The species *B. thermosphacta* is important in the spoilage of fresh and processed meat products as it can grow at chilled temperatures (Gribble & Brightwell, 2013). This bacterial species is Gram-positive, rod-shaped, non-spore forming and facultatively anaerobic. It has an ability to tolerate high salt concentrations (around 10%), even at low pH (5.5–6.5). *B. thermosphacta* has also been isolated in slaughterhouse, processing and chilling facilities (Labadie, 1999). It is among the dominant bacteria associated with a refrigeration temperature of 4°C (Gribble & Brightwell, 2013). *B. thermosphacta* spoils beef stored in vacuum packaging and in air (Ercolini et al., 2011). The signs of meat quality deterioration due to *B. thermosphacta* include discoloration, 'off' flavours and gas production (Casaburi, de Filippis, Villani & Ercolini, 2014). Inhibition of this species growth has also been reported under refrigeration and vacuum packaging conditions (Holley & McKellar, 1996).

1.5.1.5 *Staphylococcus aureus*

S. aureus is a Gram-positive and non-spore forming and grows at a temperature of 7°C (Koutsoumanis & Taoukis, 2005). It contributes to a broad range of diseases from mild skin infections to severe illnesses, particularly food poisoning, septicaemia, toxic shock and pneumonia (Dhup, Kearns, Pichon & Foster, 2015). Food poisoning from *S. aureus* occurs because of the ingestion of staphylococcal enterotoxins that are heat stable and stimulate nausea, with or without diarrhoea, abdominal cramping and vomiting (Argudín, Mendoza & Rodicio 2010; Balaban &

Rasooly, 2000). Fresh beef and processed meat products provide suitable surfaces for the growth of *S. aureus* (Millette, Le Tien, Smoragiewicz & Lacroix, 2007). *S. aureus* can be found throughout the natural environment such as in air, water and soil, as well as in the human nose and skin. As *S. aureus* is a facultative anaerobe, it can potentially grow in meat stored aerobically, anaerobically or in antimicrobial packaging (Djenane et al., 2012; Emiroğlu et al., 2010; Millette et al., 2007). The presence of this bacteria in or on meat may be attributed to inadequate hygienic handling and cross-contamination of raw meats or products during slaughtering, processing and storage, thereby posing a potential risk to the community (El Bayomi et al., 2016; Hennekinne, de Buyser & Dragacci, 2012; Hennekinne, Herbin, Firmesse & Auvray, 2015). Many types of foods (such as roast beef, chicken, hamburgers and turkey) are sources of *Staphylococcus* food poisoning and cause outbreaks in restaurants, schools, flights, cruise ships, universities and entire countries (Hennekinne et al., 2012; Kérouanton et al., 2007). *S. aureus* has been reported to be resistant to methicillin with resistant *S. aureus* (MRSA) a current public health concern (Wendlandt, Schwarz & Silley, 2013).

1.5.1.6 *Listeria monocytogenes*

L. monocytogenes is a Gram-positive, non-spore forming, facultative anaerobic bacteria that can grow actively at refrigeration or chilled temperatures and is known for its ubiquitous distribution and association with livestock (Quinto, Marín & Schaffner, 2016). This versatile foodborne pathogen can growth at temperatures from 2°C to 4°C (Lungu & Johnson, 2005). *L. monocytogenes* can adapt to grow and survive in a various situations and environmental conditions (Gandhi & Chikindas, 2007). Vacuum packaging and refrigeration are not able to efficiently inhibit or control this strain (Chen et al., 2012). *L. monocytogenes* is a dangerous causative agent of foodborne disease (Mead et al., 1999) and a serious potential contaminator of fresh meat and processed meat products after processing, slicing, during repackaging and other procedures (Marcos, Aymerich, Monfort & Garriga, 2008; Meloni et al., 2013; Muhterem-Uyar et al., 2015). *L. monocytogenes* are able to form biofilm, allowing it to contaminate food processing surfaces and equipment (Møller et al., 2016). In Europe, the notification rate for listeriosis cases was from 0.33 to

0.44 people per 100,000 of the population (Bolocan et al., 2016). In general, listeriosis infections can cause meningitis and septicaemia in vulnerable people and miscarriage in pregnant women (Allen et al., 2016; Farber & Peterkin, 1991).

Although many studies have been conducted on *Pseudomonas spp.*, LAB, *Enterobacteriaceae*, *Brochothrix thermosphacta*, *Listeria monocytogenes* and *Staphylococcus aureus*, these spoilage bacteria continue to provide new challenges to food safety, particularly as a consequence of the evolution of new microorganism populations resulting from different packaging and antimicrobial use. There is therefore a continuing need for research into, and greater knowledge of, meat spoilage bacteria to maintain meat safety.

1.5.2 Temperature effect

Temperature is an important factor influencing fresh meat and processed meat spoilage rates and bacteria growth during storage and distribution (Nychas et al., 2008). Low temperatures have been demonstrated to inhibit the growth of several species of spoilage and pathogenic bacteria and extend shelf life performance (Pothakos, Samapundo & Devlieghere, 2012). Chilling is a refrigeration preservation method used successfully with meat to protect its shelf life and safety (Zhou et al., 2010). During the process of cooling beef carcasses from slaughter, the refrigeration temperature should be 7°C and this is referred to as the primary chilling process (Sun & Holley, 2012). Secondary chilling occurs when further processing of primal and subprimal beef carcass cuts are maintained at 4°C and then fresh beef subprimal cuts are packed under vacuum. A chilled storage temperature of 4ºC usually retards the mesophilic (cold-intolerant) growth species. It also facilitates spoilage flora, which are psychrotrophic (cold-tolerant) bacteria originally present in a small population on the meat (Sun & Holley, 2012). In general, fresh meat and processed meat products are chilled from -1 to 5° C for a certain period. Fresh meat and processed meat product surfaces stored in packaging (films or coatings) and with antimicrobial agents (such as essential oil), could be exposed to unintended contamination with spoilage bacteria, undesirable physical changes (texture and colour) and biochemical reactions associated with these storage conditions (Ercolini et al., 2011). Gill and

Newton (1978) indicated that the concept of chilling is related to dry meat surfaces that have reduced water activity and may possibly inhibit and slow the growth rate of the microbial but do not affect yeast and fungi. Further, an initial bacteria presence of around 10% can grow and spoil meat during chilled storage (Borch et al., 1996; Sun & Holley, 2012).

1.5.3 Meat pH effect

Another factor that influences the spoilage of fresh meat and processed meat products bacteria is pH. Meat spoilage bacteria and pathogenic bacteria well grow between pH 5.5 to 7.0 (Jayasena & Jo, 2013). It is well-known that meat muscle pH during slaughter is around 7.0 afterward decreasing about 5.3-5.8 (Reid et al., 2017). While stored beef in chilled condition showed chilled beef stabilized pH at approximately 5.8 due to lactic acid is produced from glycogen in the meat muscle (EFSA, 2014). During aerobic storage, spoilage bacteria were detected in meat with a normal pH from 5.4 to 5.8. In fresh meat with a high pH value of greater than 6.0, the colour is darker than standard and the meat is usually firm and dry (Sun & Holley, 2012). If vacuum packaging is poor or film packaging cannot prevent oxygen from existing, several facultative anaerobes, such as *B. thermosphacta*, may grow especially at pH 5.8 (Bell, 2001). Under anaerobic situation, it has been observed that the beef primal surfaces pH when vacuum packed were within 5.65 to 5.99 for the 6 weeks storage (Reid et al., 2017). If meat pH is high indicates rapid spoilage process because of bacteria growth and used most nutrients (Lulietto et al., 2015). Despite many research on meat pH, there is still very limited research on the meat pH particularly during storage and even less based on its type of packaging or antimicrobial use.

1.5.4 Influence on the colour of meat

Interaction with oxygen influences the colour of fresh meat and processed products because spontaneous oxidation of myoglobin can develop metmyoglobin, resulting in a brownish colour (Bonilla et al., 2012). A similar effect can be observed when meat

is exposed to light, especially in the ultraviolet range, which can lead to the formation of metmyoglobin. Lipid oxidation processes can trigger the deterioration of meat proteins. Lipid and pigment oxidation can cause the colour of meat to change, to develop an 'off' taste and to possibly produce toxic compounds that can limit shelf life (Bonilla et al., 2012; Sun & Holley, 2012). Vacuum packaging can change meat colour to purple when there is no oxygen present (McMillin, 2008). Thus, packaging can play a big role in the colour of fresh and processed meat products.

1.5.5 Sources of meat spoilage contamination

The bacteria present in the contamination of fresh meat also depend on the physiological status of the animal during slaughter, processing and storage (Casaburi et al., 2015; Huis in't Veld, 1996). The sources of various bacteria contaminating meat can be found in the abattoir environment, where bacterial or pathogens are manifest in the raw beef meat itself (i.e. natural microflora from beef carcasses). Alternatively these bacteria may be present during slaughter and the production of processed meat as a consequence of cross-contamination arising from poor hygiene (hands and skin) and handling practices among workers. Although fresh meat is sterile in the animal's body before slaughter, particularly in healthy animals, bacteria from the gastrointestinal tract or hide can contaminate the meat. Spoilage can only begin when the fresh meat surface comes into direct contact with slaughter equipment (such as knives) in the abattoir environment through contaminants such as soil, water, animal hides and faeces that may possibly transfer bacteria and pathogenic bacteria to the processing facility or plant (Appendini & Hotchkiss, 2002; Gill & Newton, 1978; Nychas et al., 2008). Implementation of the hazard analysis and critical control points (HACCP) system, along with food safety objectives (FSOs), general manufacturing practices (GMPs) and good hygiene practice (GHP) control, can prevent and reduce the risk of microbial growth and assist in ensuring the safety of chilled beef meat during storage (Chen et al., 2012).

1.6 Overview of Packaging for Meat

1.6.1 Definitions

Food packaging has historically continuously developed to cater to consumer demands and markets. Packaging can be defined as a method that enables the distribution of goods in excellent condition intended for consumer use (Lockhart, 1997). It has also been referred to as a 'coordinated system that begins with goods preparation for storage, distribution, retailing and shipping at the optimum cost with excellent conditions for the product' (Ahmed & Alam, 2012, p. 1237). Packaging for food can be described as protection created for food through applying wrap and coating (Ahmed & Alam, 2012, p. 1237).

1.6.2 Role of food packaging in beef meat supply chain

Food packaging is principally designed to contain and protect food against physical, chemical and biological hazards during storage and distribution (Marsh & Bugusu, 2007; Tihminlioglu, Atik & Özen, 2010). The roles of packaging include convenience of use and the ability to communicate efficiently to the consumer through providing clear product information and indirectly promoting the food. An important trend in food packaging materials is the change from synthetic to biodegradable materials as an advantage for secure disposal (Marsh & Bugusu, 2007). The application of biodegradable packaging has significantly contributed to resolving long-term environmental health problems through decreasing solid waste, reducing space for landfill and incineration, preventing environmental pollution and posing less risk to human health (North & Halden, 2013). It has been demonstrated that types of packaging have the capacity to influence the entire ecological system.

The basic functions of beef packaging are similar to those of conventional food packaging. The primary role of beef packaging is to maintain beef safety and security as well as to increase its shelf life by improving beef quality (Coma, 2012; Han, 2014; Kerry, O'Grady & Hogan, 2006). In the storage and distribution of beef, all three hazard elements (i.e., biological, physical and chemical contaminants) may affect beef if it is not covered with suitable packaging that can secure it from extrinsic environmental influences (Robertson, 2013, p. 445). Although the packaging industry has grown tremendously, it is necessary for all packaging to meet

basic criteria. Films and coatings provide fundamental physical and mechanical protection from dust, vibrations, pressure and other physical and mechanical factors that arise when wrapping or coating beef (Bell, 2001; P. Dawson, Cooksey & Mangalassary, 2012; Sánchez-González, Vargas, González-Martínez, Chiralt & Cháfer, 2011). They serve to preserve fresh meat and processed meat products from deterioration and contamination agents, protecting them from spoilage caused by the growth of bacterial and pathogenic bacteria and preventing water loss (Bell, 2001).

Antimicrobial packaging performs better than conventional packaging because microbial growth and chemical changes on the beef's surface are effectively controlled and stabilised by incorporating antimicrobial agents (Han, 2014; Quintavalla & Vicini, 2002). The material used in antimicrobial films or coatings for beef meat are specifically designed to meet strategies for preventing meat from spoilage and foodborne pathogens or bioterrorism, and for prolonging meat shelf life and maintaining its quality (Coma, 2012; Han, 2014; Kerry et al., 2006). For instance, spoilage and pathogenic bacteria may occur in or on the beef because of mishandling (Cannarsi, Baiano, Marino, Sinigaglia & Del Nobile, 2005). Several applications of antimicrobial film packaging have demonstrated that it promotes fresh, tasty beef cuts as an end product and reduces the rate of rancidity arising from lipid oxidation, allowing the monitoring of mass transfers to prevent moisture loss and the development of unwanted aromas (Camo, Lorés, Djenane, Beltrán & Roncalés, 2011; Fernandez & Vieira, 2012; Kerry et al., 2006). In considering the sensory aspect of beef meat consumption for the consumer, these films or coatings may provide an organoleptic improvement of the colour of the beef meat. Therefore, by ensuring beef safety and extending its shelf life, antimicrobial packaging can be considered a vital addition to the storage period.

1.6.3 Modern food packaging

Cellulose plastic packaging was first created in 1856 (Miller & Krochta, 1997). The food packaging industry has since developed from simple preservation techniques to create new materials with advanced preservation methods to protect food from microbial and physical contamination while maintaining excellent food quality.

After World War II, plastic from synthetic materials was introduced into food packaging (Risch, 2009). Rigid and flexible packaging was invented after and the use of polyethylene and other polymer materials was initiated. These materials were selected for packaging because they were safe for use, inexpensive, consumed less energy and were flexible and versatile (Cutter, 2006). However, these properties contributed little to resolving issues such as recyclability and degradability of these materials. Another problem with polymer food packaging materials is that the cost of petroleum is constantly increasing and this limits the supply of resources, as oil sources are depleted (Gillgren & Stading, 2008). Up until now, the majority of food packaging materials have been petroleum-based and from non-renewable sources. This situation has placed economic stress on the food-packaging industry.

For the above reasons, food packaging technology is growing and evolving in an attempt to discover natural renewable material sources as alternatives to non-renewable packaging materials. The development of natural renewable materials is a consequence of the need for materials to be biodegradable, edible and meet biocompatibility criteria so that packaging materials are able to deal with environmental and sustainability issues (Fabra et al., 2013; Ruiz-Navajas, Viuda-Martos, Sendra, Perez-Alvarez & Fernández-López, 2013; Shi, Yu, Lakshmana Rao & Lee, 2012; Taylor et al., 2005a).

Today, the food industry mostly implements principles of sustainability in designing food packaging. The concept of sustainability dictates that raw materials must come from renewable sources, indirectly increase energy efficiency in their production processes and allow the reuse or recycling of lighter packaging material to ensure smaller quantities of solid waste (Fernández-Pan, Royo & Maté, 2012). Although the issue of recycling is important in reducing solid waste, using recycled packaging for beef meat produces potential problems when packaging that has already been used may have microbiological and chemical contamination as demonstrated by the food-contact theory (Dawson et al., 2012, p. 458). However, for several decades until the present, biodegradable film and coating packaging has been the preferred material for beef meat packaging because it is cost effective and easy to produce in compact, lightweight form (Cutter, 2006; Sánchez-Ortega et al., 2014).

1.7 Biodegradable Films or Coatings from Biopolymers

Biodegradable materials for films and coatings are typically derived from agricultural resources and suitable for food packaging applications (Cuq, Gontard & Guilbert, 1998; Zhang & Mittal, 2010). There are three types of agriculture-based materials that fall under the category of biopolymer packaging materials: proteins, lipids and polysaccharides. The most successful materials used for the commercial application of biopolymer are protein based (Falguera, Quintero, Jiménez, Muñoz & Ibarz, 2011). Biopolymer films and coatings are packaging materials that have been developed from natural, sustainable and renewable resources (Cha & Chinnan, 2004; Di Maio et al., 2010; Wihodo & Moraru, 2013). Despite the fact that biopolymers may cost more and their physicochemical properties are not always as great as those of synthetic film packaging, they are have environmentally friendly properties such as their biodegradability and edibility (Petersson, 2007). Biopolymer material is naturally non-toxic and recyclable. Biodegradable film and coating materials are capable of being naturally decomposed by bacteria and can be used as fertilisers and soil conditioners (Siracusa et al., 2008). These properties generate great advantages for countries whose waste disposal systems still rely principally on landfill. The term biodegradable film or coating means packaging material that will degrade more naturally than other polymeric packaging materials.

1.7.1 Biodegradable films and coating: Edible or non-edible

Biodegradable films have two forms: edible and non-edible. Biodegradable edible films refers to free-standing film material used to wrap or cover food (Hanani et al., 2014; Hernandez-Izquierdo & Krochta, 2008). Their film forming solution has received significant attention with respect to their food grade status or the degree of its edibility. In contrast, edible coatings are materials in solution which can be applied directly to food surfaces by dipping or spraying (Hanani, Roos & Kerry, 2014). Critics of biodegradable edible ingredients have been concerned about ensuring human safety during consumption. There is also uncertainty as to whether biodegradable edible films can be directly consumed or whether the film packaging must be removed before swallowing. However, the most important consideration for the purposes of this thesis is the advantages of biodegradable films or coatings for disposal, in that they naturally degrade and decay even if they are not eaten

(Bourtoom, 2008). The decision of whether the film should be edible or non-edible depends on its purpose (Joerger, 2007), that is, whether it is used for consumption or as a food preservative. There is current interest in the development of antimicrobial packaging for food from biodegradable materials, particularly fresh meat and processed meat products (Marcos, Aymerich, Monfort & Garriga, 2010; Sánchez-Ortega et al., 2014).

1.8 Antimicrobial Packaging as a Control Strategy

Various methods are available to control meat spoilage and extend the shelf life of fresh meat and processed meat. Packaging is one way to overcome spoilage problems and increase levels of safety and quality. Hence, it is possible that the next era of food packaging will have antimicrobial properties (Hosseinnejad & Jafari, 2016). Antimicrobial packaging from plant proteins is increasingly gaining attention for its potential uses in the food industry (Kuorwel, Cran, Sonneveld, Miltz & Bigger, 2011a). Antimicrobial packaging is an effective approach for delivery of natural antimicrobial agents, such as essential oils and nisin, and can be used with a combination of agents with synergistic or additive effects. In addition it exhibits significant antimicrobial properties against food spoilage and foodborne pathogens, thus increasing meat safety (Irkin & Esmer, 2015). Incorporation of antimicrobial agents (such as essential oils or nisin) into meat film or coatings can prevent moisture loss, discolouration and changes in flavour and texture and delay spoilage bacteria (Sánchez-Ortega et al., 2014).

Antimicrobial packaging can be used to coat the entirety of food surfaces by direct coating (e.g., the dipping method) or indirect coating (e.g., the wrapping method) (Guo, Jin & Yang, 2014a). Antimicrobial films or coatings containing antimicrobial agents have proven more effective to food than directly placed antimicrobial agents. In fact, the antimicrobial agent incorporated into films or coating solutions is mainly designed to release/migrate slowly during periods of food storage. This means the application of adequate concentrations could be used for microbial protection when necessary (Guo, Jin & Yang, 2014b; Ouattara, Simard, Piette, Bégin & Holley, 2000).

1.8.1 Essential oils

The application of essential oils from plants on fresh meat and processed meat products can be considered as natural preservatives (Holley & Patel, 2005; Seow, Yeo, Chung & Yuk, 2014). Extensive studies have focused on using natural essential oils such as oregano, thyme, basil and rosemary both alone, in combination with other essential oils or in combination with other preservation methods to enhance the organoleptic properties and extend the shelf life of fresh meat and processed meat products (Jayasena & Jo, 2014). In response to consumer demand for food safety, high food quality, increased shelf life and minimal use of chemical additives, essential oils from plants provide an interesting alternative to chemical preservatives (Aider, 2010; Cha & Chinnan, 2004; Espitia, Du, Avena-Bustillos, Soares & McHugh, 2014). Chemical preservatives may produce toxicity in the body and can be carcinogenic (Calo et al., 2015).

Natural essential oils comprise volatile (85-99%) and non-volatile components (1-15%). They have also been classified as aromatic liquids usually obtained from herbs and different parts of plants (such as from flowers, seeds, buds, leaves, twigs, bark, fruits, wood and roots) (Sánchez-González et al., 2011). They can be obtained through several methods, for example, through fermentation, expression, steam distillation and extraction (Tongnuanchan & Benjakul, 2014). Among these methods, steam distillation is popular and has been used commercially to obtain essential oils (Burt, 2004; Seydim & Sarikus, 2006). The chemical composition of essential oils can be analysed using gas chromatography, headspace and spectrometry methods (Seow et al., 2014). Essential oils have been widely used as an approach to food preservation (Hyldgaard, Mygind & Meyer, 2012; Tongnuanchan & Benjakul, 2014; Viuda-Martos, Ruiz-Navajas, Fernández-lópez & Perez-Alvarez, 2010). They have a large range of antimicrobial properties (Oussalah, Caillet, Saucier& Lacroix, 2007) and are not only effective for food preservation, but also have antifungal and antiviral properties (Hyldgaard et al., 2012). Natural plant essential oils are classified as ‘generally recognised as safe’ (GRAS) by the US Food and Drug Administration. This recognition has triggered the interest of the food industry in their use (Lucera, Costa, Conte & Del Nobile, 2012).

Essential oil compounds have been frequently documented as antimicrobial agents that can be incorporated into films or coatings for packaging materials because of their strong active components that can prevent and reduce the level of food spoilage bacterial and pathogenic bacteria. Essential oils are able to improve the safety and shelf life of food without compromising consumer acceptability (Burt, 2004; Emiroğlu et al., 2010; Fernández-pan, Royo & Maté, 2012; Negi, 2012; Oussalah, Caillet, Saucier & Lacroix, 2006; Sánchez-González et al., 2011), food flavouring (Campos et al., 2011; Chorianopoulos, Tassou, Skandamis & Nychas, 2012; Govaris, Solomakos, Pexara & Chatzopoulou, 2010; McBride et al. 2007; Royo, Fernández-Pan & Maté, 2010; Seydim & Sarikus, 2006; Viuda-Martos et al., 2010) and are safer additives for meat (Govaris et al., 2010).

The application of essential oils into antimicrobial film and coating packaging materials may affect film or coating odour and colour as a consequence of the indigenous pigments of the aromatic plants and food itself (Tongnuanchan & Benjakul, 2014). Research has found that the antimicrobial activity of essential oils is due to chemical compositions or bioactive compounds that serve to inhibit certain types of spoilage bacterial or pathogenic bacteria and they have also been found to function as antioxidants (Jayasena & Jo, 2014). Adding essential oils to film or coating packaging may improve water-vapour barriers because of the hydrophobicity of essential oils (Tongnuanchan & Benjakul, 2014; Vergis, Gokulakrishnan, Agarwal & Kumar, 2013).

1.8.1.1 Active chemical composition: oregano and/or thyme essential oils

Oregano and thyme are among the most effective natural essential oils. These have been frequently used in films or coatings packaging materials (Del Nobile, Conte, Incoronato & Panza, 2008; Emiroğlu et al., 2010; Karabagias, Badeka & Kontominas, 2011; Lin, Wang & Weng, 2011; Matiacevich, Acevedo & López, 2015; Nowak, Kalemba, Krala, Piotrowska & Czyzowska, 2012) and are common in food (Nabavi et al., 2015). However, limited data exist about the antimicrobial activities of oregano and thyme in antimicrobial kafirin film.

Both oregano and thyme are in the *Lamiaceae* family. Oregano (*Origanum vulgare L.*) and thyme (*Thymus vulgaris L.*) are widely used for human consumption. Oregano is a well-known herb in the culinary field. Generally, oregano is predominant in the Mediterranean, Europe and Asia, whereas thyme, which is originally from Europe, is distributed and grows in Australia, the Mediterranean, Asia and South Africa. However, the use of both plants is spreading globally because of their commercial, research and recreational applications. The chemical composition of plant essential oils may vary based on their species, chemotype, harvest season, country and extraction technique (Hyldgaard et al., 2012; Kokkini, Karousou, Dardioti, Krigas & Lanaras, 1997; Nabavi et al., 2015).

Natural essential oils are usually comprised of terpenoids, sesquitepenes, diterpenes and other groups such as alcohol, acids, aliphatic hydrocarbons, aldehydes, acyclic esters or lactones (Tajkarimi, Ibrahim & Cliver, 2010). The major constituents of oregano oil (carvacrol, thymol, γ-terpinene and p-cymene) and thyme oil (p-cymene, γ-terpinene and carvacrol) have all been found to have significant antimicrobial properties (Chorianopoulos et al., 2012; Del Nobile et al., 2008; Karabagias et al., 2011; Kuorwel, Cran, Sonneveld, Miltz & Bigger 2011b; Pranoto, Salokhe & Rakshit, 2005b). The major active chemical compounds of essential oils from oregano and thyme were extensively reviewed by Teixeira et al. (2013a) and Tongnuanchan and Benjakul (2014). Table 3 presents some of these active chemical compounds. The chemical composition profiles of essential oils from oregano and thyme are different and difficult to compare. Their variations arise from differences in geographic origin, plant parts used, harvesting times and extraction techniques (Kuorwel et al., 2011b).

Table 3

Chemical Compositions of Oregano and Thyme Essential Oils

Essential oil	Bioactive chemical compounds	Reference
Oregano (*Origanum vulgare*)	Apigenin, luteolin, quercetin, caffeic acid, myricetin, p-coumaric acid, carvacrol, thymol, rosmarinic acid	Kaefer & Milner (2008)
Thyme (*Thymus vulgaris*)	Thymol, carvacrol, cineole, α-pinene; apigenin,β-carotene, eugenol, limonene, ursolic acid, luteolin, gallic acid, caffeic acid, rosmarinic acid, carnosic acid, hispidulin, cismaritin	
Oregano (*Origanum vulgare*)	Carvacrol (80%),thymol (64%),γ-terpinene (2-52%), p-cymene (52%)	Burt (2004)
Thyme (*Thymus vulgaris*)	Thymol (10-64%), carvcrol (2-11%),γ-terpinene (2-31%), p-cymene (10-56%)	
Oregano (*Origanum vulgare*)	Carvacrol (30.17%),p-cymene (15.20%) and γ-terpinene (12.44%)	Lv, Liang, Yuan & Li (2011)
Oregano (commercial)	Carvacrol (57.7%),thymol (2.58%),γ-terpinene (6.4%), p-cymene (28.7%)	Petrou, Tsiraki, Giatrakou & Savvaidis (2012)
Thyme (*Thymus vulgaris*) (commercial)	Thymol (57.24%), p-cymene (18.91%),carvacrol (2.82%), linalool (2.03%)	Peng & Li (2014)
Thyme (*Thymus vulgaris*) (commercial)	Thymol (46.42%), p-cymene (22.31%),carvacrol (12.42%)	Jouki, Mortazavi, Yazdi & Koocheki (2014a)
Oregano (*Origanum vulgare*) (commercial)	Carvacrol (68.5%), thymoquinone (12.1%)	Turgis et al. (2012)
Thyme (*Thymus vulgaris*) (commercial)	Linalool (60%), Linalyl acetate (10%)	

1.8.1.2 Effectiveness of antimicrobial film and coating packaging containing essential oils from oregano and thyme

Antimicrobial film or coating packaging material incorporating essential oils can assure the reduction and control of the rate of microbial population growth, as well as inhibiting target bacteria through antimicrobial activity. A significant amount of research has demonstrated that film enhanced with oregano and/or thyme essential oils possesses antimicrobial activity characteristics for a broad range of target bacteria (Aguirre, Borneo & León, 2013; Govaris et al., 2010; Jouki et al., 2014a, 2014b; McBride et al., 2007; Nowak et al., 2012; Skandamis & Nychas, 2001; Tsigarida, Skandamis & Nychas, 2000).

1.8.1.3 Antimicrobial activity

The antimicrobial activity of oregano (OEO) and thyme essential oils (TEO) is principally attributed to their major compounds: carvacrol, thymol and p-cymene (Kuorwel et al., 2011b). Table 4 presents the significant effects of incorporating OEO or TEO into various film packaging, particularly protein-based polymers. It has also been demonstrated that a variety of concentrations of essential oils influence the effects of clearing inhibition zone areas on various food spoiling and pathogenic bacteria, including Gram-negative or Gram-positive species, moulds and yeasts. The antimicrobial mode of action of essential oils is influenced by their chemical structure and the concentration applied. For instance, the presence of hydrophilic functional groups or the lipophilicity of essential oils.

There are several ways that essential oils are able to inhibit bacteria: through the disruption of the cytoplasmic membrane, through cell content coagulation, by the leakage of intracellular constituents, through protein-synthesis inhibition, by the connection of enzymes with cell wall synthesis, with deoxyribonucleic acid (DNA) or with ribonucleic acid (RNA) synthesis and through the destruction of the osmotic integrity of the cell membrane (Kuorwel et al., 2011b).

The majority of antimicrobial films containing OEO and/or TEO have the capability to produce antimicrobial activity against a wide range of target microorganisms with

a minimum effective concentration of 1% (see Table 4). Positive results also occurred when film polymer was successfully added with OEO and TEO.

Table 4

Antimicrobial Activity of Oregano and Thyme Plant Essential Oils Produced by Film Polymer

Essential oil	**Country**	**Film packaging**	**Method**	**Target microorganisms**	**Effective concentration and microorganisms**	**Reference**
Thyme (0%,1,1.5,2.0%)	Iran	Quince seed mucilage (QSM)	Agar disk diffusion assay	*E. coli, Yersinia enterolitica, S. aureus, Bacillus cereus, Pseudomonas aeruginosa, Lactobacillus plantarum, S. thyphimurium, E.coli 0157:H7, L. monocytogenes, V. cholera, S. putrefaciens*	Minimum effective concentration is 1.0% against all microorganisms	Jouki et al. (2014a)
Oregano (*Origanum vulgare*) (0%,0.5%,1.0% and 1.5%)	Chile	Alginate	Agar diffusion	*E. coli, S. aureus, L. monocytogenes, S. enteritis*	Minimum effective concentration is 1.0% against all microorganisms	Benavides, Villalobos-Carvajal, & Reyes (2012)
Oregano (*Origanumminutiflorum*) (1.0%,2.0,3.0,4.0%) wt/vol	Turkey	Whey protein	Agar disk diffusion assay	*Lactobacillus plantarum, E. coli 0157:H7, L. monocytogenes, S. enteriti, S. aureus*	1% was not effective against any microorganisms. Minimum effective concentration is 2% against all microorganisms	Seydim, Acton, Hall & Dawson (2006)
Oregano (*Origanum vulgare*) (Commercial) (1.0-10.0%)	Spain	Polypropylene (PP) and polyethylene/ ethylene vinyl alcohol copolymer (PE/EVOH)	Agar diffusion	Gram-negative: *Escherichia coli, Yersinia enterocolitica*, *Pseudomonas aeruginosa*, and *Salmonella choleraesuis*; Gram-positive: *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Enterococcus faecalis*; Moulds: *Penicilliumislandicum*, *Penicilliumroqueforti*, *Penicilliumnalgiovense*, *Eurotiumrepens*, *Aspergillusflavus;* Yeasts: *Candida albicans*, *Debaryomyceshansenii* and *Zigosaccharomycesrouxii*.	Minimum effective concentration is 4.0% against all fungi, whereas all gram-positive bacteria is10% and there is no gram-negative bacteria	López, Sánchez, Batlle & Nerín (2007)
Thyme (*Thymus vulgaris*)(1-5%)	Turkey	Soy protein	Agar disk diffusion	*Escherichia coli, E. coli O157:H7, Staphylococcus aureus, Pseudomonas aeruginosa and Lactobacillus plantarum*	Minimum effective concentration is 1% against all microorganisms	Emiroğlu et al. (2010)

1.8.2 Nisin

Nisin is a peptide bacteriocin produced by strains of *Lactococcus lactis*. It is classified as GRAS internationally by the FDA and is thus suitable for the food industry and can applied directly to food as a preservative (Delves-Broughton, 2005; Gálvez, Abriouel, López & Omar, 2007; Ndoti-Nembe, Vu, Doucet & Lacroix, 2015). Nisin has a diverse spectrum of antimicrobial effects on Gram-positive bacteria, and is particularly active against foodborne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus* or *Bacillus cereus* (Cao-Hoang, Grégoire, Chaine & Waché, 2010; Chan, Lee, An, Park & Sun, 2003; Chen et al., 2016; Delves-Broughton, 1996; Ercolini et al., 2010). In some cases, the application of nisin in foods has resulted in several species of Gram-positive bacteria developing resistance to it (H. Zhou, Fang, Tian & Lu, 2014). Nisin has amphipathic properties that allow it to bind to bacterial cell walls, resulting in the leakage and death of the bacteria (Shiroodi et al., 2016).

The addition of nisin into various types of film and coating materials, such as zein, soy protein, whey protein isolate (WPI), cellulose and chitosan, has greatly enhanced its antimicrobial activity against spoilage and pathogenic bacteria and extended the storage life of meat products (Deegan, Cotter, Hill & Ross, 2006; Ercolini et al., 2010; Gadang, Hettiarachchy, Johnson & Owens, 2008; Jiang, Neetoo & Chen, 2011; Ku & Song, 2007; Nattress, Yost & Baker, 2001; Nguyen, Gidley & Dykes, 2008; Pattanayaiying, Kittikun & Cutter, 2015; Siragusa, Cutter & Willett, 1999; Theivendran, Hettiarachchy & Johnson, 2006; Zhou et al., 2010).

Nisin, used alone or combined with other antimicrobial agents or preservation methods, has demonstrated promising results in the effort to control or reduce meat spoilage or pathogenic bacteria. The antimicrobial activity of nisin in cellulose films against *Listeria monocytogenes* on meat has been demonstrated (Nguyen, Gidley & Dykes, 2008). Casting films including nisin generally showed more antimicrobials than heat-pressed films (Dawson, Hirt, Rieck, Acton & Sotthibandhu, 2003). Lu et al. (2010) evaluated the effect of cinnamon and nisin in alginate-calcium coatings for fresh northern snakehead fish fillets stored at 4±1°C. These treatments could inhibit microbial growth during storage in terms of total viable counts (TVCs) and total psychrotrophic counts (TPC) as well as reduce lipid oxidation. The effectiveness of

nisin in film or coating material when applied to food products depends on its diffusion throughout the food matrix. However, its reaction depends on the type of food (its composition and physicochemical properties), packaging and storage temperature (Cao-Hoang et al., 2010). For example, biodegradable films with nisin stored at a temperature of 4°C showed a reduction in bacteria eight hours after inoculation, but those stored at a temperature of 22°C showed a reaction after only four hours (Shiroodi et al., 2016). Kafirin films or coatings containing nisin still lack adequate research.

1.8.3 Methods to determine antimicrobial activity

Antimicrobial compounds or properties can be investigated *in vitro* to ascertain their effectiveness against food spoilage bacteria and pathogens (Burt, 2004; Irkin & Esmer, 2015; Seow, Yeo, Chung & Yuk, 2014). There are a number of antimicrobial activity test methods, including diffusion (agar diffusion), dilution and biautographic methods (broth and agar dilution) (Burt, 2004; Kuorwel et al., 2011b). These tests are also known as 'screening tests' and usually provide preliminary assessment data for antimicrobial performance and the effectiveness of essential oils or nisin (Kuorwel et al., 2011b). Agar diffusion has been most commonly used for the antimicrobial activity test. This method uses a film disk (containing the antimicrobial agent), or a paper disk impregnated with the antimicrobial agent, placed on top of an inoculated agar plate or media (Sun & Holley, 2012). Agar diffusion has been noted to be useful for the selection of essential oil and nisin and for determining the minimum or optimum concentration of an essential oil or nisin for targeting spoilage bacteria or pathogenic bacteria. Sometimes problems occur at low concentrations of antimicrobial agents (such as essential oils or nisin) when added into antimicrobial films because of a failure to produce a clear inhibition zone. However, high concentrations essential oils in film display very effective and clear inhibition zones. These results may be because of the hydrophobic and lipophilic characteristics of essential oils. It is frequently mentioned that such tests cannot mimic or match the original conditions found in food as a consequence of the variable conditions that exist for food items (Kunicka-Styczynska, 2012).

1.8.4 Antimicrobial plant protein material: films and coatings

Plant-protein films and coatings are the most actively used and are potentially the most useful when used in applications for perishable food, particularly beef (Dawson, Cooksey & Mangalassary, 2012; Miller & Krochta, 1997; Sánchez-González et al., 2011). Most plants have their own protein to support their structure and biological activity (Krochta, 2002).

Antimicrobial films or coating packaging material can also be made from a variety of plant protein-based biopolymer sources such as corn zein, sorghum kafirin, soy protein and wheat gluten (Hernandez-Izquierdo & Krochta, 2008; Zhang & Mittal, 2010). Zein and kafirin films or coatings, which are from the same Panicoideae grasses subfamily, have received attention and been widely investigated (Belton, Delgadillo, Halford & Shewry, 2006; Buchner et al., 2011; Emmambux, Stading & Taylor, 2004; Petersson et al., 2007; Taylor et al., 2005a). Kafirin and zein have been classified as similar in their degree of prominent homology (amino-acid composition), solubility and structure (Buchner et al., 2011; Derose et al., 1989; Shull, Watterson & Kirleis, 1991). Both are categorised into four classes: α-grain, β-grain, γ-grain, and δ-grain prolamin (Belton et al., 2006). Prolamins can be soluble or dissolved in aqueous ethanol mixtures. Zein has been used to compare or provide frameworks and support for kafirin in many research projects (Belton et al., 2006; de Mesa-Stonestreet et al., 2010; Gillgren & Stading, 2008). Zein is the most widely used plant polymer in antimicrobial film and coating packaging. However, kafirin is currently gaining interest but with very few studies conducted with regards to incorporating antimicrobial agents into kafirin films and coatings.

1.8.5 Antimicrobial plant proteins: Kafirin and zein

Kafirin grain prolamin is derived from sorghum seed. Sorghum (*Sorghum bicolor L. Moench*) originates from Africa and Asia and is also cultivated in the US, Mexico and Argentina. In the 1950s, sorghum gained attention and experienced increased production as an important cereal (Rooney & Serna-Saldivar, 2000). There are many benefits of sorghum grains and they are used in foods such as bread, porridge, cake, cookies, pasta, snacks and noodles, as well as for alcoholic beverages and as forage

for livestock (Espinosa-Ramírez & Serna-Saldívar, 2016; Oom, Pettersson, Taylor & Stading, 2008; Taylor, Schober & Bean, 2006). This protein plant is able to survive in much hotter environments than maize and is resistant to fungi and mycotoxin. A high level of temperature adaptability may enable sorghum to become a possible alternative to maize under conditions of climate change. Currently, sorghum can be found worldwide in countries and regions including the US, Australia, Africa and Asia.

In Australia, sorghum is grown in Queensland and northern New South Wales. It is an important export market for the pet food industry rather than being used for human consumption (Department of Agriculture and Fisheries, 2012). Australia is one of the top six sorghum producers in the world after Nigeria, the US, India, Argentina and Ethiopia (FAO, 2015). Similar to maize, sorghum is recognised as safe for people suffering from celiac disease and it is non-allergenic (de Mesa-Stonestreet, Alavi & Bean, 2010; Schober, Bean, Tilley, Smith & Ioerger, 2011).

Sorghum is the fifth ranking cereal or grain crop in the world. According to the FAO (2015), the global trade in maize was slightly lower in 2014 and 2015 than in previous years, however, sorghum is now in high demand internationally. Consequently, sorghum kafirin is economically important in several regions of the world and there is great potential for its application in new markets.

Zein comes from maize or corn (*Zea mays L.*) grain prolamin. The US has been the top global exporter of zein since the twentieth century (FAO, 2015; Lawton, 2002; US Grains Council, 2015). Zein can be acquired from corn gluten, is created through the process of wet milling and is a coproduct of the bioethanol industry (Shukla & Cheryan, 2001; Singh, Saengerlaub, Ali Abas & Horst-Christian, 2012).

1.8.6 Kafirin films and coatings

Kafirin has great potential in terms of its biodegradability and antimicrobial properties against meat spoilage and pathogens, in addition to its physical and optical properties for use in food packaging films and coatings (Atarés & Chiralt, 2016; Buchner, Kinnear, Crouch, Taylor & Minnaar, 2011; Giteru et al., 2015; Kuorwel,

Cran, Sonneveld, Miltz & Bigger, 2011a; Petersson, Hagström, Nilsson & Stading, 2007; Sun, Wang, Kadouh & Zhou, 2014; Taylor, Taylor, Belton & Minnaar 2009a, 2009b). Its broad range of applications that appear to have superior functional properties for film and coating packaging have made it the subject of growing research interest and demand (Schober, Bean, Tilley, Smith & Ioerger, 2011; Taylor, Taylor, Belton & Minnaar, 2009a; Taylor, Anyango & Taylor, 2013; Xiao et al., 2015). Kafirin has biodegradable, non-toxic and biocompatible properties that make it a suitable candidate for use in food antimicrobial film or coating material (Buchner et al., 2011; de Mesa-Stonestreet, Alavi & Bean, 2010; Gao et al., 2006; Gillgren & Stading, 2008; Giteru et al., 2015; Marcos et al., 2010; Petersson et al., 2007; Taylor et al., 2016). Fundamentally, kafirin is highly hydrophobic and has low solubility in water as well as excellent solubility in ethanol or acetic acid (Taylor, Taylor, Dutton & de Kock, 2005b).

Recently, the application of kafirin as a packaging material in active films or coatings has received considerable attention and its potential use as alternative packaging needs to be further explored. Kafirin film formulations with essential oil citral and polyphenol quercetin were found to be effective against *Campylobacter jejuni*, *Listeria monocytogenes* and *Pseudomonas fluorescens* (Gram-negative and Gram-positive bacteria) (Giteru et al., 2015). Kafirin also has good film-forming abilities, is a flexible film, is highly compatible with other antimicrobial agents and is an excellent oxygen barrier (Gillgren & Stading, 2008; Petersson et al., 2007).

1.8.7 Antimicrobial film and coating preparation

1.8.7.1 Extraction methods

The extraction methods for kafirin and zein begin with wet milling. The wet-milling process involves the separation of the grain components (i.e., starch, protein, germ and bran). The process of wet milling zein takes a short time as compared to sorghum (de Mesa-Stonestreet et al., 2010). However, the flow process of both types of wet milling is quite similar. Anderson and Lamsal (2011) and de Mesa-Stonestreet et al. (2010) have comprehensively reviewed the extraction methods and chemicals used for kafirin and zein.

Several extraction methods are used for zein. Shukla and Cheryan (2001) reviewed zein extraction methods particularly on an industrial scale. Some of these methods have patents in the US (Lawton, 2002). Zein is insoluble in water and, thus, during the zein extraction process, two types of solvents are employed, an aqueous ethanol solution or isopropanol (as a polar solvent) at an elevated temperature (65ºC) and, subsequently, a solvent hexane (non-polar solvent) to remove lipids and pigments (Singh et al., 2012). As a result, commercial zein has been extensively selected and used by researchers for the majority of zein film research. Commercial zein was effectively soluble in 50% to 90% aqueous ethanol (Shukla & Cheryan, 2001). Zein protein content varies from 6 -12% on the basis of dryness and depending on corn types (Shukla & Cheryan, 2001). The extraction method is important because it may change the properties of zein and can sometimes create inter-batch variation (Schober et al., 2011). While the kafirin extraction method is not marketable, the wet-milling process can be commercialised (de Mesa-Stonestreet et al., 2010). Kafirin protein content ranges from 6 - 18% (de Mesa-Stonestreet et al., 2010), kafirin and zein consist of different classes of prolamin and their compositions differ depending on the extraction methods used.

1.8.7.2 Antimicrobial film and coating preparation methods

To obtain biodegradable films, many studies have used casting methods rather than extrusion or high-pressure techniques (Campos, Gerschenson & Flores, 2011). As an initial step, the biodegradable material must be dissolved or dispersed into a solvent such as ethanol, water or a diluted acid solution. The addition of a plasticiser is usually necessary to equip the films with better physical and mechanical properties, particularly flexibility. The most commonly used film or coating plasticiser is glycerol as a result of its stability and compatibility with biopolymer. Depending on the film or coating solution, materials used may need to be heated or have their pH levels adjusted to dissolve the macromolecules properly. Finally, antimicrobial agents can be added to the mixture to make the desired film or coating through homogenisation and subsequent drying (Atarés & Chiralt, 2016).

Preparation methods for antimicrobial film and coating packaging are similar to those applied in synthetic polymer packaging. These preparation methods use dry and wet techniques. The dry techniques usually involve the extrusion or compression moulding of the biopolymer to convert it to thermosplastic, whereas wet techniques involve casting with solvent. This solvent casting method is the most commonly used in antimicrobial kafirin or zein film preparation in the laboratory and for research (see Table 5). The casting technique involves spreading a prepared film-forming solution onto a flat, non-stick surface where it is subsequently dried. A suitable solvent for kafirin and zein is ethanol. Warm aqueous ethanol (65% - 85%) is considered as convenient; it is frequently applied to food and has been broadly used as a suitable solvent for plant-protein film because it is safe (Buffo & Han, 2005; Kim & Xu, 2008; Zhong & Ikeda, 2012).

As noted earlier, another important element of antimicrobial film or coating packaging is the plasticiser. Antimicrobial kafirin and zein films or coatings have the potential to be brittle and rigid in most practical applications as a consequence of the strong forces of plant-protein intermolecules. This means that kafirin and zein have limited capacities to function as free-standing, flexible films. Therefore, a plasticiser is frequently needed to improve the flexibility and mechanical properties of antimicrobial kafirin and zein films. Important characteristics of plasticisers are their low levels of volatility and low molecule weight. Plasticiser is generally added to polymer material to improve its three-dimensional material network, decrease the interaction between intermolecular forces and boost the volume and chain mobility of the polymer material (Hernandez-Izquierdo & Krochta, 2008; Nur Hanani, Roos & Kerry, 2012; Zhang & Mittal, 2010). A plasticiser's role is usually to modify film properties for elasticity, extensibility, rigidity, flexibility and for influencing mechanical or physical properties (Arcan & Yemenicioğlu, 2013; Campos et al., 2011). Glycerol is widely used for developing antimicrobial kafirin and zein films without affecting the film drying period (see Table 5) and frequently added to protein biopolymers (Hernandez-Izquierdo & Krochta, 2008). It assists antimicrobial film or coating packaging material to become softer and more flexible; it prevents pores or cracks and enhances film stability compared to sugars, sorbitol and polyethylene glycol (PEG) (Campos et al., 2011; Gao et al., 2006). However, incorporating plasticiser into antimicrobial films can significantly change the film

properties causing increased moisture, gases and aroma compounds (Espitia et al., 2014). With respect to these issues, the quantity and type of plasticisers used are critical for developing good antimicrobial films or coatings because the efficiency of antimicrobial films or coatings may deteriorate in terms of mechanical, physical and barrier performance if the correct plasticiser is not used.

1.8.8 Factors affecting antimicrobial film or coating efficiency

Prior to achieving better performance in developing and designing antimicrobial films or coatings, several factors need to be considered: the pH of the antimicrobial film or coating, the homogenisation methods, physical treatments involved and the application of temperature during casting.

Antimicrobial film or coating forming solutions can be affected by high pH levels during preparation. As a result, the efficacy of film or coating antimicrobial properties may decrease. In the casting method, antimicrobial kafirin film-forming solutions are usually adjusted to pH5 (Taylor et al., 2005a) because of the sensitisation of proteins that have zwitterionic characteristics influencing the viscosity and homogeneity of the final film (Gällstedt, Hedenqvist & Ture, 2011). Nisin is also effective at lower pH levels (Mauriello, Luca, Storia, Villani & Ercolini, 2005).

Homogenisation is part of the preparation method for antimicrobial kafirin and zein films used to mix the film-forming solution material between the plasticiser and the antimicrobial agent (see Table 5). In addition, this method has been indirectly reported to be able to enhance antimicrobial activities and improve the interaction between oils and polymer, as well as to enhance the appearance of antimicrobial films by removing identifiable oil droplets (Atarés & Chiralt, 2016).

During the casting or drying period, antimicrobial film or coating packaging is well known to be unable to withstand high temperatures, owing to the potential loss of residual volatile antimicrobial agents and subsequent chemical compound stability (Han, 2000; Han & Floros, 1997). In addition, when the drying temperature is high, the mean relative humidity is low and, consequently, the film can crack, shrink and

sometimes develop bubbles if solvent evaporation occurs too quickly. For these reasons, oven and incubator equipment are suitable for the developing of antimicrobial film packaging in the laboratory (Gällstedt et al., 2011).

At some stage after film formation, antimicrobial film packaging must undergo physical treatments such as ultraviolet (UV) irradiation (Zhang & Mittal, 2010). The application of UV light on antimicrobial films reportedly eradicates any bacteria on antimicrobial films before contact with food.

In recent studies, antimicrobial kafirin and zein film or coating packaging have been developed using the approaches presented in Table 5. Currently, many different film names are being considered for antimicrobial applications. The approach presented seems to provide evidence for the importance of the methods (e.g., the solvent used, the type of plasticiser, the homogenising method and the conditions needed) for producing antimicrobial kafirin and zein films or coatings. Most studies revealed that antimicrobial films, whether in small or large batches, need to dry or be cast for more than 12 hours. However, the time taken to dry and may affect the efficacy or effectiveness of residual antimicrobial agents. A normal ambient temperature is used for drying because of the requirement for a low temperature to maintain essential oil encapsulation (Burt, 2004). In the coating method, the period of drying is determined by the food type. However, none of these studies note the difficulty of peeling the film, which suggests that antimicrobial film packaging may not be affected by the small amount of plasticiser used when several types of plasticisers and only a small amount of essential oil are applied. It must be remembered that, if all mixture ingredients and antimicrobial agents used for producing antimicrobial kafirin and zein films or coatings are compatible, they may only minimally affect the physical, mechanical and chemical properties of the film or coating (Han & Floros, 1997; Kuorwel et al., 2011a; Sánchez-González et al., 2011).

Table 5

Antimicrobial Kafirin and Zein Film Methods Used by Others

Name of film or coating type	Application	Antimicrobial agent	Solvent	Plasticiser	Homogenise (yes/no)	Drying method	Period/place/ temp. before peeling/dried	Difficulty of peel?	Author and year
Active zein film	Antimicrobial efficacy	Thymol	Ethanol (96%)	Glycerol (1 g)	Yes, Magnetic stirrer (10 min)	Casting	48 h/ambient temperature	Not mentioned	Del Nobile et al. (2008)
Antimicrobial zein edible film	Antimicrobial activity	*Zataria multiflora* Boiss'	Ethanol	Glycerol (1.5 ml)	No	Casting	Overnight/ laboratory temperature	Not mentioned	Ghasemi, Javadi, Moradi, Oromiehie & Khosravi-Darani (2012)
Flexible zein film	Developed flexible film	Lysozyme	Ethanol (96%)	Glycerol (0.4mL) and catechin	Yes,10,000 rpm (4 min)	Casting	19±2h/incubator 25ºC	Not mentioned	Arcan & Yemenicioğlu (2013)
Flexible antimicrobial zein	Food application	Gallic acid	Ethanol (97%)	Glycerol (8.1 mL)	Yes,10,000 rpm, (4 min)	Casting,	19h/incubator 25ºC	Not mentioned	Alkan et al. (2011)
Edible zein film	Ground beef patties	Lysozyme, Na2EDTA	Ethanol (97%)		Yes, 8000rpm, (2 min)	Casting	22±1ºC	Not mentioned	Ünalan et al. (2011)
Antimicrobial zein	Freshkashar cheese	Lysozyme, catechin, gallic acid	Ethanol (96%)	Glycerol (0.4 mL)	Yes,10,000 rpm, (4 min)	Casting	19±2h/incubator 25ºC	Not mentioned	Ünalan, Arcan, Korel & Yemenicioğlu (2013)
Bioactive kafirin film	-	Citral, quercetin	Ethanol (96%)	PEG 400, lactic acid, glycerol - 12.64g(1:1:1 w/w)	Yes, 6,000 rpm, (2 min)	Casting	24 h/oven (force draft) 25±2ºC	Not mentioned	Giteru et al. (2015)
Antimicrobial zein coating	Maintain quality fish ball	Nisin, nisin + EDTA*	Ethanol (95%)	Propylene glycol (5 ml)	-	-	1 hr/room temperature		Lin et al. (2011)
Zein ethanol film coating	Ready to eat chicken	Nisin and/or calcium propionate	Ethanol (95%)	Glycerine, citric acid	-	-	20 mins		Janes et al. (2002)

* EDTA = ethylenediaminetetraacetic acid

1.8.9 Effect of essential oil or nisin on physical, mechanical and barrier properties

The incorporation of antimicrobial agents into films or coatings can affect physical, mechanical and barrier properties when the antimicrobial agents added are not compatible with the polymer.

In some cases, the application of essential oils in films or coatings can cause discontinuities (essential oil droplets) that make the film or coating structure thicker and more open (Atarés & Chiralt, 2016), in turn, causing an increase in film thickness. Adding essential oils to films can therefore sometimes cause surface roughness or irregularities as a result of the migration of droplets or aggregates on top of the film during drying (Sánchez-González et al., 2011). Similarly, the effect of kafirin films with citral (essential oil) alone or in combination with quercetin (polyphenol) revealed a significant increase in terms of thickness (Giteru et al., 2015). Further, the addition of essential oils rarely made films slightly oily unless the concentration level of incorporated essential oils was high. For example, when garlic oil was added at 6% w/w to LDPE/EVA polymers, films became slightly oily (Sung, Sin, Tee, Bee & Rahmat, 2013a). Jin et al. (2009), studying the effect of nisin included in pectin/PLA films showed a rough morphology when observed by confocal reflection microscopy. Nonetheless, these films were homogenous, flexible and transparent. Guo et al. (2014a) reported that chitosan films containing nisin had a cloudy appearance compared to control films without nisin.

It has been observed that protein films (kafirin, triticale and WPI) containing essential oils did not have their moisture contents significantly affected (Aguirre, Borneo & León, 2013; Giteru et al., 2015; Zinoviadou, Koutsoumanis & Biliaderis, 2009). Nevertheless, the moisture content of the film may be influenced by humidity (Zinoviadou et al., 2009).

In addition to the analysis of film solubility in water, biodegradable films are analysed for their properties of water resistance and integrity (Rhim, Gennadios, Handa, Weller & Hanna, 2000). Film with moderate to high solubility have potential for the design and development of biodegradable film or coating packaging material intended for easy solubility or coating. This type of film or coating application provides an advantage as it may not require any removal before the consumption or

cooking of the food product (Ahmad, Benjakul, Prodpran & Agustini, 2012; Ghasemlou, Khodaiyan & Oromiehie, 2011). The solubility of kafirin films was not influenced by the addition of citral and quercetin (Giteru et al., 2015).

Several studies have investigated film packaging mechanical properties when antimicrobial agents are added, especially essential oil. Tensile strength (TS) measures film strength and the percentage of elongation at break (EAB%) to ascertain film stretchability at breakage. Both factors are evaluated using tensile tests ASTM D882, which extend the film at a certain rate upon breakage and are based on parameters such as strength versus time or distance. Protein film tensile properties are dependent on film constituents, preparation conditions and the amount or type of antimicrobial agents. However, TS does not decrease if small amounts of antimicrobial agents are applied (Sung et al., 2013b). Further, the effect of essential oil incorporated into films could provide more extensibility as a result of essential oil characteristics and the formation of oil droplets in the film matrix (Fabra, Talens & Chiralt, 2008). Pranoto, Rakshit and Salokhe (2005a) observed the effect of different concentrations of essential oils, nisin and potassium sorbate in chitosan film, finding reduced TS but increased elongation properties when using nisin compared to essential oils. For kafirin films, the addition of citral essential oil displayed lower TS and higher EAB% than the combination of both antimicrobial agents (Giteru et al., 2015). Zinoviadou et al. (2009) reported that the incorporation of oregano essential oil (OEO) into WPI increased EAB when essential oil concentration was greater.

Another important element that needs to be taken into consideration is water vapour permeability, especially when films or coatings are applied to moist products. The water vapour permeability (WVP) of biodegradable films can be quantified using the gravimetric method ASTM E96-95 (Giteru et al., 2015). The water vapour transmission rate (WVTR) can be defined as the rate at which moisture penetrates and passes through a material. According to Giteru et al. (2015), kafirin film, including citral and polyphenol, did not show any difference in terms of WVP, whereas, for WVTR films, the addition of essential oils (citral) demonstrated lower rates than other film formulations. Benavides et al. (2012) conducted a WVP test on alginate films containing OEO, finding that the inclusion of OEO leads to reduced WVP values and even more so when the essential oil levels are increased. Further,

Ku and Song (2007) reported that the WVP values for corn zein and gelatin films were reduced with the increase of nisin concentration. Antimicrobial film or coating packaging material depends on the hydrophilic-hydrophobic ratio of the film constituents (Benavides, Villalobos-Carvajal & Reyes, 2012). It is desirable that low WVP values be obtained to minimise weight loss in coated products and, at the same time, influence product appearance and firmness (Sánchez-González et al., 2011). Normally antimicrobial coating films provide lower water vapour transmission because they may prevent some moisture from passing through packaging as a consequence of the addition of antimicrobial agents (Sung et al., 2013b).

The oxygen permeability (OP) of films and coatings is another essential consideration for maintaining meat product integrity. Films with added essential oil seem to possess good barriers against gases, but information about this is limited (Sánchez-González et al., 2011). Kafirin films with added citral essential oil showed significantly less OP values than control films (Giteru et al., 2015). Conversely, OP values increased when higher amounts of OEO were added to quince seed mucilage films (Jouki, Yazdi, Mortazavi & Koocheki, 2014b). It has been suggested that the addition of plasticisers to film-forming solutions can increase the OP, as glycerol, lactic acid and PEG 400 can modify the basic structure of the kafirin film network. Moreover, films incorporating essential oils could contribute to poor oxygen barriers, as more oxygen is dissolved in the non-polar oil phase increasing the transfer rate of the oxygen molecules into the plasticised polymer matrix (Jouki et al., 2014b). Nonetheless, the excellent oxygen barrier properties of film or coating packaging generally depend on the film matrix as well. For example, when films have a porous structure, gas is encouraged to cross it (Sánchez-González et al., 2011).

The interaction between antimicrobial packaging material (films or coatings) and antimicrobial agents such as essential oils and nisin is significant with respect to the alteration of colour. The addition of essential oil into film or coating directly influences the colour based on the type and concentration applied. Giteru et al. (2015) showed that the addition of citral essential oil or/and quercetin (polyphenol) changed the kafirin film to yellowish and brown colours. This could suggest the presence of natural pigments or polyphenols in sorghum that may have been co-extracted with the kafirin (Da Silva & Taylor, 2005; Shukla & Cheryan, 2001). In

addition, Da Silva and Taylor (2005) reported that there was variation in colour depending on different kafirin films. Murillo-Martínez et al. (2013) reported that WPI films incorporating only nisin were whitish, those incorporating WPI + glycerol did not change colour, while those with WPI + glucose oxidase became yellowish and those with WPI + nisin + glucose oxidase became slightly yellowish and pale. This study indicated that nisin could change the film colour property based on the ingredients and the material of the films. However, there is no significant effect on alginate film colours when adding garlic essential oil (Pranoto, Salokhe & Rakshit, 2005b).

Another characteristic that needs to be evaluated is light transmission and film transparency with the main purpose of acceptable product appearance for consumers and indications of good quality (Imran et al., 2012). Lighting without UV radiation can delay meat spoilage by changing the surface colour (a*) and causing the development of metmyoglobin, which leads to brown colouration (Djenane, Sánchez-Escalante, Beltrán & Roncalés, 2001). Indeed, the majority of consumers desire and prefer food products with a transparent film or coating so as to see foods and evaluate their quality. The light transmission barrier of films can be measured against UV and visible light at 200–900 nm (Imran et al., 2012). The transparency value is calculated by dividing the absorbance at 600nm by the film thickness (Han & Floros, 1997). It was observed that essential oil may induce the reduction of film transparency and light transmission (Atarés & Chiralt, 2016). Ahmad et al. (2012) listed several factors that influence light transmission or the transparency of films that incorporate essential oils, including essential oil concentration and the extent of the distribution of essential oils throughout the film matrix. It was found that the addition of plasticiser to films could improve light transmission and good transparency (Imran et al., 2012). Imran et al. (2012) revealed that film containing glycerol had lower transmission but more reduction when nisin was incorporated to hydroxypropyl methylcellulose film (HPMC).

1.8.10 Antimicrobial packaging to extend shelf life

In the context of this thesis, shelf life refers to the period of time that fresh meat and processed meat products retain acceptable microbiological quality during storage. Although shelf life properties include microbial growth, lipid oxidation, appearance, texture, colour, flavour and nutritional value, these tend to be limiting factors in the shelf life of meat (Lorenzo & Gomez, 2012). Fresh meat and processed meat products can be controlled by using vacuum packaging and chilled storage, which are effective for prolonging shelf life. TVC of *B. thermosphacta, Pseudomonas* spp. and *Enterobacteriaea* was reduced with the application of vacuum packaging; however, LAB was not affected after 20 days of at 4°C (Pennacchia et al., 2011).

Many studies have investigated the efficiency of different antimicrobial film and coating materials containing various antimicrobial agents (such as essential oils and nisin), combining them with other methods to extend the shelf life of fresh and processed meat. Several studies have demonstrated that the combined use of natural essential oils and vacuum packaging can improve the shelf life of food (Lucera, Costa, Conte & Del Nobile, 2012; Ouattara et al., 2000; Sánchez-González et al., 2011; Skandamis & Nychas, 2002; Viuda-Martos, Ruiz-Navajas, Fernández-lópez & Perez-Alvarez, 2010). Another study showed that the addition of 0.8% oregano oil to vacuum-packaged beef decreased by 2 to 3 log CFU/g in its TVC value (Tsigarida, Skandamis & Nychas, 2000). An extension of shelf life for four days was obtained after applying oregano oil to minced beef stored aerobically under refrigeration (Skandamis & Nychas, 2001).

Lin et al. (2011) investigated the microbiological quality of zein coating containing nisin alone and added EDTA to fish balls during storage at 4°C; this revealed better reduction during the initial TVC population and for a further 15 days when zein was coated with both antimicrobial agents (Nisin + EDTA) compared to nisin alone. Meanwhile, Emiroğlu et al. (2010) observed that fresh ground beef patties wrapped with soy edible films containing oregano, thyme and a combination of both essential oils did not have significant effects on TVC, LAB and *Staphylococcus* spp., but reduced the initial counts of *Pseudomonas* spp. as well as the coliforms population throughout the storage period (12 days) at 5°C. Populations of LAB, *Enterobacteriaceae*, TVC and *Pseudomonas aeruginosa* were reduced in a chicken

breast when stored under vacuum packaging and dipped in different groups, oregano and thyme essential oils; this extended the shelf life at least eight to nine days, which was longer than for the control samples at 4°C (Pavelková et al., 2014).

Theivendran et al. (2006) reported that the addition of nisin to soy protein coating on turkey frankfurters significantly lowered the population of *L. monocytogenes* when stored at 4°C by reducing 2.1 log CFU/g compared to control coating. Nguyen et al. (2008) also showed that increasing nisin (2500 IU/ml) in cellulose film sharply decreased the *L. monocytogenes* counts on frankfurters after two days of storage at 4°C. Further, Gadang et al. (2008) indicated the effectiveness of WPI coating containing nisin (6000 IU/g) combined with malic acid and grape seed extract, which successfully reduced the cell population by 3.2 log CFU/g after 28 days stored at 4°C.

The expected shelf life of refrigerated fresh and processed meat products is also influenced by environmental conditions, such as temperature, pH levels and storage conditions. The relationship of different packaging to microbial growth is presented in Table 6.

1.8.10.1 pH

It appears that antimicrobial soy edible films containing OEO and/or TEO increase the initial pH of meat (5.87) after day one and then it decreased slowly until day twelve. Sakala et al. (2002) reported the initial pH values of vacuum-packaged fresh beef cuts stored at 2°C to be 5.62 ± 0.04 while Karabagias et al. (2011) reported a pH value of 6.6 on day nine for lamb meat samples containing 0.1% TEO. According to Mancini & Hunt (2005), pH levels ranged from 4.0 to 5.0, which likely decrease the lightness of meat. In addition, at low pH, the hydrophobicity of an essential oil is increased, allowing the film to more easily dissolve in the lipids of the cell membrane of the target bacteria (Burt, 2004; Juven, Kanner, Schved & Weisslowicz, 1994). Antimicrobial films or coatings containing nisin demonstrated a better response at lower pH levels (Murillo-Martínez et al., 2013).

1.8.10.2 Other aspects

There are several aspects that can influence the shelf life of fresh meat and processed meat. Food components, especially fat in meat, can affect antimicrobial film and coating performance (Sánchez-González et al., 2011; Seow et al., 2014). Further, the stability of essential oils or nisin in film or coating packaging material during heating or storage at high temperatures can also affect their efficacy (Dawson et al., 2003; Sánchez-González et al., 2011).

Table 6

Expected Shelf Life of Refrigerated Fresh Meat and Processed Meat Products

Meat	Storage	Expected shelf life	Growth[a]			
			***Pseudomonas* spp.**	***Enterobacteriaceae***	**LAB**	**B. *thermosphacta***
Meat, normal pH	Air	Days	+++	++	++	++/+++
	Vacuum	Weeks–months	+	+/++	+++	++/+++
Meat, high pH	Vacuum	Days	+	++/+++	+++	++/+++
Meat products	Air	Days	+/++	+	++	+++
	Vacuum	Weeks	+	+	+++	++/+++

[a] +++: dominant part of the microflora; ++: intermediate part of the microflora; +: minor part of the microflora.

Source: Borch et al. (1996), Sun and Holley (2012)

1.9 Objectives of the Research

There have been limited studies undertaken into kafirin films or coatings as antimicrobial packaging particularly for fresh meat or processed meat products. Most research to date by Giteru et al. (2015) has suggested that kafirin films incorporating citral essential oil have antimicrobial properties. Conversely, Buchner et al. (2011) and Taylor et al. (2016) demonstrated the potential to use kafirin film as coating material, but suggested that further research may be required. Therefore, the objectives of this study were to:

1. Screening biodegradable zein and kafirin films with respect to the effect of the drying temperatures on the antimicrobial films and their antimicrobial effect against food spoilage and pathogenic bacteria *in vitro*. Determining the amount of hexane present in each film (see Chapter 2a).

2. Investigating the antimicrobial activity of biodegradable zein films including oregano (OEO) or thyme (TEO) essential oil alone, and the combination of both essential oils (OTEO), against common meat spoilage and pathogenic bacteria. Determine the effect of essential oils on zein film thickness and colour (see Chapter 2b).

3. Evaluate the antimicrobial activities of biodegradable kafirin films containing OEO or TEO alone and in combination (OTEO) at different concentrations against common meat spoilage and pathogenic bacteria. Investigate the most effective concentrations of OEO and TEO for maintaining the physical, mechanical, barrier and optical properties of kafirin films (see Chapter 3).

4. Investigate the effectiveness of using different antimicrobial coating treatments on the reduction of surface microflora on vacuum packaged fresh beef stored at 2 ± 0.5°C and determine its microbiological and physicochemical characteristics (see Chapter 4).

5. Evaluate the antimicrobial properties of kafirin films containing nisin against *L. monocytogenes in vitro*. In addition, investigate the efficacy of antimicrobial kafirin coatings containing nisin for controlling *L.*

monocytogenes and other bacteria on the surface of processed beef meat stored at 4°C (see Chapter 5).

Chapter 2: Preliminary Investigation into Biodegradable Antimicrobial Films Containing Essential Oils

This chapter describes a range of preliminary studies were conducted prior to the development of antimicrobial zein (Z) and kafirin (K) films containing essential oils. Initial tests involved the investigation of both film types with respect to key standard film criteria: their ability to peel from casting plates and hexane residuals (which should be within the limits prescribed by standard regulation around the world). In addition, both films were evaluated and screened for antimicrobial activity using a range of essential oils concentrations. These results from this chapter informed further investigations. The study is divided into two parts: (a) screening of the ability of biodegradable zein and kafirin films with essential oils incorporated to inhibit food spoilage and pathogenic bacteria and; (b) preliminary investigation into the antimicrobial properties of zein films, containing oregano or thyme essential oils, alone or in combination, against spoilage and pathogenic bacteria.

Chapter 2a Screening the ability of biodegradable zein and kafirin films with essential oils incorporated to inhibit food spoilage and pathogenic bacteria

2a.1 Introduction

Protein film packaging made from biodegradable materials are increasingly used in the food industries (Kuorwel, Cran, Sonneveld, Miltz, & Bigger, 2011a). The preparation of biodegradable protein films can be accomplished by using a wet process (casting method) that requires solubilisation of protein followed by a drying step to remove the solvent (Andreuccetti et al., 2012; Kuorwel et al., 2011a). Zein and kafirin are similar with respect to their degree of homology (amino-acid composition), solubility and structure (Derose et al., 1989; Shull, Watterson, & Kirleis, 1991), and non-toxicity (N. Singh, Georget, Belton, & Barker, 2010). Zein has been used as a comparator or to provide a framework for kafirin film in many studies (Belton et al., 2006; de Mesa-Stonestreet et al., 2010; Gillgren & Stading, 2008). Kafirin is more hydrophobic as compared to zein (Belton et al., 2006). The films that are in contact with food should have levels of residual chemicals that are

necessary to satisfy food safety regulations, which are different in each country (Lee, 2005, p. 118).

The addition of natural essential oils into biodegradable films could improve their antimicrobial properties, which is an attractive prospect for the food and packaging industry (Atarés & Chiralt, 2016). However, essential oils are sensitive to film processing conditions. The processing technique used during film formation are critical, as different factors such as the temperature applied during processing or drying may affect antimicrobial film packaging (Kuorwel et al., 2011a; Singh, Singh, Kaur, & Bakshi, n.d.). There is, at present, a lack of research with respect to the potential use of kafirin and zein as antimicrobial packaging incorporating essential oils to improve food microbial safety. This study reports an initial comparative screening of zein and kafirin films with respect to the effects of drying temperature on the antimicrobial films, determination of the residual hexane in each film and the antimicrobial effects of these films against food spoilage and pathogenic bacteria.

2a.2 Materials and methods

2a.2.1 Bacterial cultures and growth conditions

A range of common meat spoilage and foodborne pathogens associated with beef meat were used in this study. *Brochothrix thermosphacta* (DSM 20171) and *Lactobacillus sakei* (DSM 20017) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Germany). *Pseudomonas aeruginosa (*ATCC 9721), *Staphylococcus aureus* (NCTC 6571), *Listeria monocytogenes* (NCTC 11994) and *Escherichia coli* (NCTC 10418) were provided by the School of Biomedical Sciences, Curtin University (Perth, Australia). *P. aeruginosa*, *E. coli* and *S. aureus* were grown on Mueller-Hinton Agar (MHA, PathWest Laboratory WA, Perth, Australia) at 37°C, while *B. thermosphacta, L. monocytogenes* and *L. sakei* were maintained at 30°C on Brain Heart Infusion Agar (BHIA, PathWest Laboratory WA, Perth, Australia).

2a.2.2 Preparation of antimicrobial films

2a.2.2.1 Extraction

Kafirin was extracted from white sorghum that was provided by the Queensland Department of Agriculture, Fisheries and Forestry (Brisbane, Australia), as described by Emmambux and Taylor (2003) and Giteru et al. (2015) with the following modifications: kafirin (500 g) was dispersed in 70% ethanol (w/w) in deionized water (1750 ml) containing sodium metabisulphite (12.5 g) and sodium hydroxide (8.75 g). The mixture was then heated at 70°C with constant stirring. The kafirin mixture was centrifuged at 3500 rpm (1,200 g) for 5 min (Centrifuge 5810R, Hamburg, Germany), the supernatant was discarded and the remaining suspension placed overnight in a laminar flow cupboard to remove the solvent. The kafirin suspension was adjusted to pH 5.0 and collected by vacuum filtration. The wet kafirin was freeze-dried for 21 h (Christ Alpha 1-2/LD plus, John Morris Scientific, Sydney, Australia) and ground to a fine powder using a coffee grinder.

2a.2.2.2 Defatting

The defatted zein (Z3625, Sigma-Aldrich Chemical Co., Australia) and kafirin were obtained by stirring with three times *n*-hexane (protein to solvent ratio of 1:10 w/w) for 3 h at ambient temperature after which the residuals were decanted (Lin, Wang, & Weng, 2011; Schober, Bean, Tilley, Smith, & Ioerger, 2011). The remaining *n*-hexane (95%, Thermo Fisher Scientific, Australia) was removed by evaporation in a laminar flow cupboard overnight. The isolated defatted zein and kafirin had protein contents of 89.92 ± 4.85% and 86.31 ± 4.58% (dry basis), respectively, as determined by the Kjeldahl method of AOAC 925.10 (AOAC, 2005) using a nitrogen to protein conversion factor of N x 6.25.

2a.2.3 Antimicrobial films

Antimicrobial zein and kafirin films were prepared with the same plasticizer mixture and amount used in the previous studies (Gillgren & Stading, 2008; Giteru et al., 2015). Briefly, defatted kafirin (1.77 g) or zein (1.70 g) powder was dissolved in a mixture of plasticizers (0.66 g) (a 1:1:1 w/w of glycerol, lactic acid and polyethylene glycol 400) in 97% ethanol (9.0 g), and stirred continuously and heated until boiling using a magnetic stirrer on a hotplates. The volume of ethanol lost during evaporation was replaced and the mixture was reheated for 5 mins. The film solutions for kafirin were filtered with cheesecloth and cooled to room temperature. Zein and kafirin solutions (4 g) were weighed and transferred to small beakers and 1% (w/w) oregano essential oil was added. The solution was then homogenized (Ultra-Turrax, IKA®T18, Germany) at 9500 rpm for 1 min. Finally, the mixture was degassed through vacuum to remove air bubbles.

2a.2.3.1 Effects of drying temperature on zein and kafirin films containing essential oils

To ensure the antimicrobial films produced were easy to peel from the petri dish after the drying process, three drying techniques were investigated: room temperature (22.5 ± 0.5°C, 46.3 ± 5% RH); forced draft oven (Panasonic Biomedical, Leicestershire, UK) (32 ± 2°C, 34 ± 2% RH) and; a humidity oven (Memmert D91126, Germany) (27.7 ± 1.67°C, 55 ± 8% RH). All drying was overnight. TinytagUltra data loggers were used to monitor the temperature. Duplicate samples were measured for each film and temperature used.

2a.2.4 Effects of residual hexane in zein and kafirin films

The determination of residual hexane in zein and kafirin films without essential oils were analysed at the ChemCentre (Western Australia, Australia), using gas chromatography equipped with a mass spectrometer (Agilent 5973 MSD, USA). Both samples were extracted with methanol and allowed to settle. A 1 mL aliquot of

each film solution was transferred to a 40 mL headspace vial containing 10 mL of Evian water and was analysed by purge and trap (Atomx Teledyne Tekmar, USA). The purge and trap autosampler was set up to extract and concentrate volatile organic compounds (VOCs) by purging the extract solution with helium and collecting the liberated VOCs on a sorbent trap. The trap was then desorbed and the VOCs transferred to the GC inlet (operating in split mode at 200 °C) for analysis. The GC oven contained a DB-VRX column (60 m x 0.25 mm x 1.4 µm film fused silica column) and was temperature programmed at 40 °C for 5 min and heated at 8 °C/min to 190 °C, followed by a stage from 6 °C/min to 240 °C for 1 min, giving a total run time of 35.08 mins. The mass spectrometer had a solvent delay of 8 minutes and monitored m/z = 86, 71, 57, 56 for the duration of the analysis. Data were analysed using Chemstation software.

2a.2.5 Antimicrobial film screening

Antimicrobial zein and kafirin films containing the commercial essential oils (Range Products Pty Ltd Company, Perth, Australia) oregano (OEO) and thyme (TEO) alone, and the combination of oregano and thyme essential oils (OTEO), at low concentrations were produced as described in Section 2a.2.3, but in larger quantities. Zein and kafirin film solutions were prepared was follows: zein (51 g) or kafirin (53.1 g) were added into a 500 mL Erlenmeyer flask, 97% ethanol (270 g) was added, followed by plasticizers (19.8 g) (1:1:1 w/w mixture of glycerol: polyethylene glycol 400: lactic acid). The solution was stirred and heated until boiled using a magnetic stirrer on a hot plate (Buffo, Weller, & Gennadios, 1997). The amount of ethanol (97%) lost during evaporation was replaced and re-heated for 5 min. Film solutions were cooled to room temperature, and then OEO, TEO and OTEO were added to each zein and kafirin solution at 0% (control), 0.1%, 0.5%,1.0% and 1.5% w/w for Z+OEO, Z+TEO, K+OEO and K+TEO, respectively. Films with the combination of both essential oils (Z+OTEO and K+OTEO) were added with a similar percentage of each essential oil per film. Each film solution was homogenized (Ultra-Turrax, IKA®T18, Germany) at 9500 rpm for 60s and degassed under vacuum to remove bubbles. Zein (4 g) and kafirin (4 g) solutions were poured into each glass petri dish and placed in a forced draft oven (Panasonic Biomedical,

Leicestershire, UK) at 32 ± 2°C and 34 ± 2% RH for 19 h. The dried antimicrobial films were peeled carefully and used in antimicrobial activity test.

2a.2.5.1 Agar diffusion method

All antimicrobial films were initially screened against the bacteria detailed in section 2a.2.1 using the zone of inhibition or agar disc diffusion method (Bell, Pham, Newton, & Nguyen, 2013; Weerakkody, Caffin, Dykes, & Turner, 2011). Stock bacterial cultures were diluted in phosphate buffered saline (BPS) (pH 7.2) to approximately 10^8 CFU/ml standardized using the 0.5 McFarland (Giteru et al., 2015). Bacterial cultures were flooded inoculated (1000 µL) onto appropriate media and the plates dried in a biosafety cabinet to remove extra moisture on the plate surface and achieve the desired surface attached tested bacteria.

The films were cut into disks (6 mm diameter) using a sterile punch, and all films were sterilized using UV light for 2 min (Kuorwel et al., 2011a). They were then placed on the inoculated agar plates of the different indicator bacteria. Antimicrobial effectiveness of films and the clearing inhibition zones around each film were measured after 24 h and 36 h incubation at 37°C and 30°C using a digital calliper (AccuRemote, USA) with a precision of 0.001 mm.

2a.3 Results and discussion

2a.3.1 Effects of temperature on the antimicrobial films

The drying temperature affected the visual appearance of the antimicrobial film. Film dried in the forced draft oven were uniform in thickness and had a smoother surface than films dried at room temperature. It is possible that room temperature did not properly dry the film, and film thickness was not uniform and the surface of antimicrobial films from this method was also not smooth. This could be due to insufficient heat at ambient temperature not effectively drying films containing plasticizers and antimicrobial agents. Alkan et al. (2011) reported that antimicrobial zein-based films containing gallic acid were very sticky and hard to peel from glass

plates after being dried at 25°C for 19 h in an incubator. Arcan et al. (2011) also demonstrated that zein films containing different phenolic compounds (catechin, gallic acid, ferulic acid, flavone, quercetin or hydroxy benzoic acid), when added at more than 3 mg/cm^2 became extremely sticky and were not easy to handle or peel from glass templates after being dried for 19 h at 25°C in incubator. However, chitosan films containing cinnamon essential oil at a concentration of 1.5% easily peeled from the glass plates at 25°C for 30 h (Ojagh, Rezaei, Razavi, & Hosseini, 2010).

2a.3.2 Residual content of hexane in zein and kafirin films

The likelihood of the potential migration of residual hexane is of considerable concern in food packaging. The results of the hexane residue analysis from zein and kafirin films without essential oils showed <4 mg/kg, indicating that residuals of hexane concentration in zein and kafirin films after drying were low. Food Standards Australia New Zealand (2013) (http://www.foodstandards.gov.au/) state that the maximum permitted level for hexane in food is 20 mg/kg (Provision 13). Furthermore, the EU rules on extraction solvents for use in foodstuffs under the Directive 2009/32/EC of the European Parliament and of the council (2016)(http://ec.europa.eu/food/safety/food_improvement_agents/extraction-solvents/index_en.htm) indicate that extraction with hexane for the preparation of defatted protein products and defatted flours should have maximum residue limits of 10mg/kg.

2a.3.3 Antimicrobial activities of films containing essential oils

The findings of the screening for antimicrobial activity of kafirin based films against six meat spoilage and pathogenic bacteria are shown in Table 2a.1. Zein films did not produce any zones of inhibition regardless of the type or amount of essential oil added. Arcan et al. (2013) reported that zein control films did not form any inhibition zones against *L. innocua*. Khalil et al. (2014) indicated that zein film loaded with succinic anhydride, succinic acid, citric acid, or eugenol had no

antimicrobial activity towards *L. innocua*, *L. monocytogenes*, and *C. sporogenes*. Pagno et al. (2016) reported that quinoa flour-based films containing 0.5% OEO or TEO exhibited small inhibition zones 16.5 ± 0.9 mm^2 and 13.1 ± 0.8 mm^2, respectively against *S. aureus.* Films containing 1% and 2% of either essential oils (OEO or TEO) also inhibited *E.coli*.

Kafirin films without essential oil did not inhibit the growth of any of the bacteria tested. Similarly, kafirin film with TEO added did not inhibit any of the bacteria, regardless of the concentration of the TEO. K+OEO inhibited the growth of *B. thermosphacta* and *P. aeruginosa* at 1.5% (in radius= 0.54 mm^2) and 0.5% (1.22 mm^2), respectively. Addition of OTEO into kafirin films at 0.5%, 1.0%, and 1.5% inhibited *B. thermosphacta* with inhibition zones of 0.52 mm^2, 0.46 mm^2 and 0.85 mm^2, respectively. K+OEO inhibited the growth of *B. thermosphacta* and *P. aeruginosa* at 1.5% (in radius= 0.54 mm^2) and 0.5% (1.22 mm^2), respectively. Addition of OTEO into kafirin films at 0.5%, 1.0%, and 1.5% inhibited *B. thermosphacta* with inhibition zones of 0.52 mm^2, 0.46 mm^2 and 0.85 mm^2, respectively. Similar results were observed in antimicrobial activity tests conducted by Giteru et al. (2015), which reported that kafirin films containing citral (1.5%) and quercetin (1%) produced slight inhibition zones for *L. monocytogenes* (0.52 ± 0.10 mm^2), *P. fluorescens* (0.50 ± 0.01 mm^2), and the largest inhibition zone was for *C. jejuni* (6.37 ± 4.20 mm^2). However, there is a lack of literature in antimicrobial activity of zein-based films or kafirin-based films containing the same antimicrobial agent. These differences made it hard to compare the effectiveness of OEO, TEO and OTEO concentrations in our study with other studies.

2a.4 Conclusion

Both zein and kafirin antimicrobial film solutions were suitable to be formed by the casting method in a forced draft oven. The residual content of hexane in both control films was significantly lower than the maximum allowable level, suggesting they are safe for food contact application. No antimicrobial activity of the zein-based films with OEO, TEO and OTEO incorporated was detected against meat spoilage and pathogenic bacteria. However, the addition of OEO and OTEO into kafirin-based

films at lower concentrations resulted in smaller inhibition zones towards *B. thermosphacta* and *P. aeruginosa*.

Table 2a.1

Inhibition zone (in radius, mm^2) observation and measurements for kafirin-based film discs containing different essential oils.

Film treatments	Essential oil concentrations (%)	*E.c*	*S.a*	*B.t*	*P.a*	*L.s*	*L.m*
K+TEO	0	No	No	No	No	No	No
	0.1	No	No	No	No	No	No
	0.5	No	No	No	No	No	No
	1.0	No	No	No	No	No	No
	1.5	No	No	No	No	No	No
K+OEO	0	No	No	No	Not clear	No	No
	0.1	No	No	No	Not clear	No	No
	0.5	No	No	No	Yes =1.22	No	No
	1.0	No	No	No	Not clear	No	No
	1.5	No	No	Yes = 0.54	Not clear	No	No
K+OTEO	0	No	No	No	No	No	No
	0.1	No	No	No	No	No	No
	0.5	No	No	Yes = 0.52	No	No	No
	1.0	Not clear	No	Yes = 0.46	No	No	No
	1.5	No	No	Yes = 0.85	No	No	No

* Not clear inhibition zone

Film treatments: Kafirin containing: thyme essential oil (K+TEO), oregano essential oil (K+OEO), and oregano and thyme essential oils (K+OTEO)

Bacteria: *E. coli (E.c), S. aureus (S.a), B. thermosphacta (B.t), P. aeruginosa (P.a), L. sakei (L.s) and L. monocytogenes (L.m).*

Chapter 2b : Preliminary Study of Antimicrobial Zein-Based Films, Containing Oregano or Thyme Essential Oils, Alone or In Combination against Spoilage and Pathogenic Bacteria

2b.1 Introduction

The control and elimination of foodborne pathogenic and spoilage bacteria remain important for the meat industry. Fresh meat and processed meat products are considered the main source of contamination from common meat spoilage bacteria, such as *Brochothrix thermosphacta*, lactic acid bacteria (LAB) and *Pseudomonas aeruginosa*, that can lead to nutrients loss, colour change and off-flavour (Ercolini et al., 2011; Radha krishnan, 2015; Nychas, Skandamis, Tassou, & Koutsoumanis, 2008; Pothakos, Devlieghere, Villani, Björkroth, & Ercolini, 2015). *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* are categorised as foodborne pathogens that are responsible for many illnesses, disease or food poisoning outbreak and even deaths (Hsi, Ebel, Williams, Golden, & Schlosser, 2015; Jacob, Mathiasen, & Powell, 2010; Oussalah, Caillet, Saucier, & Lacroix, 2007; Sohaib, Anjum, Arshad, & Rahman, 2015). There are various traditional ways to reduce microbial contamination rates such as the hazard analysis and critical control points (HACCP) system at meat processing plants or retail, or through the use of UV light, heat and other techniques, however contamination cannot be eliminated using these techniques. The application of novel antimicrobial food packaging is therefore still important to protect food safety, quality and extend product shelf life (Quintavalla & Vicini, 2002; Sung et al., 2013b).

Biopolymer materials, including protein materials such as zein, are biodegradable and biocompatible which make them suitable for the formulation of antimicrobial film packaging (Chen, Ye, & Liu, 2014; Padgett, Han, & Dawson, 1998a). Zein is a major storage protein in corn, and is hydrophobic in nature, containing high amounts of non-polar amino acids such as alanine, leucine and glutamic acid (Ghanbarzadeh & Oromiehi, 2008; Shukla & Cheryan, 2001). The use of zein films for packaging food products is well-developed (Lin et al., 2011; Ünalan, Korel, & Yemenicioğlu,

2011). Films made from zein are strong, have excellent flexibility and film forming properties, and also have unique antimicrobial properties (Del Nobile, Conte, Incoronato, & Panza, 2008; Ghasemi, Javadi, Moradi, Oromiehie, & Khosravi-Darani, 2012; Marcos, Aymerich, Monfort, & Garriga, 2010; Xiao, Davidson, & Zhong, 2011a). Other compounds can be added to zein because of the diffusion characteristics of the film. Several studies have investigated the efficacy of zein films containing different concentrations of antimicrobial agents using controlled release systems (Arcan & Yemenicioğlu, 2013; Del Nobile et al., 2008).

Antimicrobial films with essential oils, such as oregano and thyme, incorporated have antimicrobial activities against many foodborne pathogenic and spoilage bacteria (Abdollahzadeh, Rezaei, & Hosseini, 2014; Boskovic et al., 2015; Calo, Crandall, O'Bryan, & Ricke, 2015; Pesavento et al., 2015; Reyes-Jurado, López-Malo, & Palou, 2016). Previous studies have revealed that the effectiveness of the antimicrobial activity may differ depending on the type of bacteria tested, experimental conditions and the antimicrobial compounds used (Reyes-Jurado et al., 2016). However, there is a lack of literature dealing with zein films containing essential oils (Del Nobile et al., 2008). The objective of this study was to investigate the antimicrobial activity of biodegradable zein-based films incorporating oregano (OEO) or thyme (TEO) essential oil alone, and the combination of both essential oils (OTEO), against common meat spoilage and pathogenic bacteria. In addition, film thickness and colour analysis were performed to determine the essential oil effects on zein-based films.

2b.2 Materials and methods

2b.2.1 Film preparation

The antimicrobial films were prepared by defatting zein powder according to the method used in section 2a.2.2.2.

2b.2.2 Antimicrobial film preparation

Zein films were prepared as described in Padgett, Han, and Dawson (1998) with modifications. In this study, a different volume of film forming solution was made, namely, zein powder (296.55 g) and plasticizers (114.12 g, a 1:1:1 w/w mixture of glycerol: polyethylene glycol 400: lactic acid) were dissolved in 97% ethanol (1,314.06 g). The formulation for one petri dish and method used is similar to that described in section 2a.2.3.

After the film forming solution was cooled to room temperature and transferred (~4 g) to a small beaker, different amounts of essential oils (oregano and thyme alone, and in combination of both essential oils) were added as follows: oregano essential oil (OEO) or thyme essential oil (TEO) was added to film solutions at varying concentrations (1% - 7%), and the combination of both essential oils (OTEO) at 1% to 4%. The final essential oil concentration (%) for the combination of each essential oils (OTEO) in the zein solution was similar to the percentage of each essential oil (for example, the final combination of essential oil at 1%, was calculated based on a ratio of 1%:1% of each essential oil from ~4 g (film forming solution). The solution was homogenized (Ultra-Turrax, IKA®T18, Germany) at 9500 rpm for 60s and vacuum degassed to remove air bubbles. The final film-forming solution was poured in a glass petri dish and dried in the forced draft oven (Panasonic Biomedical, Leicestershire, UK) for 19 h at 34 ± 2% RH. The dry films were peeled off and immediately investigated for its antimicrobial activity. Films without essential oil were designated as film 0%, which was used as a control (ZC).

2b.2.3 Film thickness and colour measurement

Film thickness was measured using a digital micrometer (AccuRemote, USA) with an accuracy of ± 0.001 mm in five random locations for each film. Results were presented as the average. All zein-based films surface colours were evaluated using a portable Minolta Hunter LAB colorimeter (CM-508i, Konica Minolta, Tokyo, Japan) to measure L*, a* and b*. Films were placed on the surface of a standard white

background. The mean value of three measurements was recorded for each colour attribute.

2b.2.4 Antimicrobial activity of biodegradable zein films

Six different meat spoilage and pathogenic bacteria, namely *E. coli*, *S. aureus*, *P. aeruginosa*, *B. thermosphacta*, *L. monocytogenes* and *L. sakei* were used in this study. Briefly, the isolates were inoculated on agar and incubated at a suitable temperature as described previously in section 2a.2.1. The agar disc diffusion method was prepared as explained in section 2a.2.5.1 to assess films' antimicrobial activity against tested bacteria. Each sample was made in triplicate and experiments were repeated three times on different days. The zone of inhibition was measured based on the whole zone of inhibition area and then the diameter of the film disk subtracted.

2b.2.5 Statistical analysis

One-way analysis of variance (ANOVA) was conducted to analyse the data from each experiment using SPSS software version 22 (IBM Corp, NY, USA). Differences between the mean values were determined using Duncan's and least significant difference (LSD) tests for multiple comparisons. The significance of the difference was assessed at a 95% confidence level ($p = 0.05$).

2b.3 Results and discussion

2b.3.1 Appearance, thickness and colour of the films

Both control films and films with essential oils (1% to 7%) were visually homogeneous, flexible, without cracking, oily, sticky or bubbles and were easily to peel after casting. All of these elements suggest that the films are stable. By contrast, Altiok et al. (2010) reported that addition of TEO into chitosan films resulted in roughness, cloudiness and change in the whiteness of the film. In addition, Benavides et al. (2012) reported that alginate colour changed to yellowish due to the presence of

OEO. On the other hand, cassava starch-chitosan films containing OEO were homogenous, easy to handle, and had smooth surfaces without cracks and bubbles (Pelissari, Grossmann, Yamashita, & Pineda, 2009).

The addition of OEO or TEO into zein films at the maximum concentration of 7% affected the films became slightly oily and sticky. Films with the combination of essential oils (OTEO) were slightly oily and sticky when 4% concentration was applied. In this case, the maximum concentration of essential oil that can be used in zein films can be seen to depend on film matrix and the amount of essential oil used.

The effects of the addition of OEO, TEO and OTEO on the thickness and colour properties of the antimicrobial zein films are displayed in Table 2b.1. Film thickness varied from 0.145 to 0.189 mm. The thickness of films containing TEO (1-7%) was significantly greater than control films ($p < 0.05$). The increase could be attributed to the entrapment of TEO microdroplets in the zein film matrix. Similar increases in film thickness were found by Jouki et al. for quince seed mucilage (QSM) films containing TEO (Jouki, Mortazavi, Yazdi, & Koocheki, 2014a). However, no significant difference in thickness was observed between film treatments containing 4 -7% OEO and control films ($p > 0.05$). This finding suggests that the matrix of the zein film could not form the compact film network in the presence of OEO. When OEO was incorporated into alginate films they showed an increase in thickness with increasing OEO concentration (Benavides et al., 2012). The thickness of zein films with 1% OTEO was significantly greater than the control film ($p < 0.05$), however, there was no difference at the higher concentrations of OTEO. This may be because greater levels of essential oils cause weakening of the film matrix and directly increase the film thickness (Han & Krochta, 1999; Jouki et al., 2014b).

Film colour is one of the main elements for consumer acceptance. The effects of essential oils at different concentrations on film colour values are shown in Table 2b.1. The colour of the films was affected by the type and concentrations of essential oils added. Zein films including TEO was lighter than Z+OEO and Z+OTEO, with L* values from 84.71 to 89.54. The* and b* values slightly decreased at some concentrations. The Z+OTEO and Z+OEO film treatments ranged between 83.15 and 88.80 and 84.82 and 88.52, respectively. There was no obvious difference in L* values between all film treatments and control ($p > 0.05$).

It was established that the addition of OEO, TEO and OTEO at various concentrations to zein-based films resulted in slight changes in a* values in the direction of green. For the b* (yellow-blue) values, zein films incorporating essential oils were slightly lower than zein control film (46.75), except for Z+TEO at 6% and Z+OTEO at 2% and 3%, which gave values of 46.94, 53.51 and 51.01, respectively. These results indicated that OEO, TEO and OTEO incorporated into zein-based films influenced film colour, but the changes depend on the type of essential oil.

Table 2b.1

Thickness and colour parameters of zein-based films containing essential oils.

Films	Essential oil concentration (%)	Thickness (mm)	L*	a*	b*
ZC	0	0.153 ± 0.003^{aA}	86.26 ± 1.57^{bB}	4.84 ± 0.67^{bA}	46.75 ± 7.42^{ecB}
Z+TEO	1	0.165 ± 0.006^{b}	87.93 ± 0.39^{c}	5.50 ± 0.05^{c}	39.07 ± 2.12^{b}
	2	0.178 ± 0.007^{c}	87.43 ± 1.06^{bc}	4.26 ± 0.45^{b}	34.28 ± 5.03^{b}
	3	0.184 ± 0.016^{c}	87.17 ± 1.11^{bc}	4.94 ± 0.33^{b}	44.75 ± 6.94^{d}
	4	0.186 ± 0.005^{c}	86.15 ± 1.52^{b}	4.17 ± 0.12^{b}	39.57 ± 8.74^{bc}
	5	0.165 ± 0.008^{b}	84.71 ± 1.80^{a}	3.07 ± 1.79^{a}	43.27 ± 5.08^{d}
	6	0.176 ± 0.007^{bc}	86.33 ± 0.31^{b}	5.19 ± 0.36^{c}	46.94 ± 0.50^{e}
	7	0.180 ± 0.006^{c}	89.54 ± 0.29^{d}	3.81 ± 0.47^{ab}	22.99 ± 2.57^{a}
Z+OEO	1	0.184 ± 0.010^{b}	86.35 ± 0.20^{b}	4.74 ± 0.19^{b}	42.57 ± 0.97^{b}
	2	0.183 ± 0.022^{b}	86.41 ± 1.24^{b}	4.78 ± 0.50^{b}	43.38 ± 5.19^{b}
	3	0.181 ± 0.004^{b}	85.94 ± 2.12^{b}	4.41 ± 0.81^{b}	41.05 ± 6.53^{b}
	4	0.156 ± 0.008^{a}	84.82 ± 1.06^{a}	2.66 ± 0.86^{a}	42.91 ± 4.09^{b}
	5	0.149 ± 0.005^{a}	87.99 ± 1.02^{c}	4.44 ± 0.32^{b}	32.25 ± 5.99^{a}
	6	0.145 ± 0.004^{a}	88.52 ± 0.61^{c}	4.95 ± 0.14^{b}	31.61 ± 3.49^{a}
	7	0.154 ± 0.004^{a}	87.72 ± 0.32^{c}	5.04 ± 0.15^{b}	35.55 ± 1.80^{a}
Z+OTEO	1	0.189 ± 0.014^{B}	86.46 ± 0.45^{B}	4.83 ± 0.55^{A}	42.84 ± 1.29^{B}
	2	0.148 ± 0.005^{A}	83.15 ± 0.43^{A}	2.88 ± 0.43^{A}	53.51 ± 3.06^{C}
	3	0.151 ± 0.002^{A}	83.87 ± 1.95^{A}	3.10 ± 2.05^{A}	51.01 ± 1.33^{C}
	4	0.150 ± 0.002^{A}	88.80 ± 1.10^{C}	3.59 ± 0.51^{A}	26.39 ± 4.01^{A}

Values are given as mean ± standard deviation (thickness, $n = 5$; colour, $n = 3$)
Different letters or different capital letters in the same column within the film containing the same essential oil together with the control indicate significant differences (Duncan's test, $p < 0.05$). Film samples: Zein containing: thyme essential oil (Z+TEO), oregano essential oil (Z+OEO), and oregano and thyme essential oils (Z+OTEO).

2b.3.2 Antimicrobial properties

The antimicrobial activity of zein-based films containing TEO, OEO and OTEO at different concentrations against *E. coli*, *S. aureus*, *B. thermosphacta*, *L. monocytogenes*, *L. sakei* and *P. aeruginosa* are presented in Table 2b.2. The zein

control film without essential oil did not display any antimicrobial effect against the bacterial strains tested. Similar findings were reported by Khalil et al. (2014) that pure zein film did not inhibit any bacteria. No concentration of essential oil tested was effective against *L. sakei* or *P. aeruginosa*. However, Del Nobile et al. (2008) have found zein films loaded with thymol at concentration of 10%, 20% and 35% are inhibited pseudomonads microorganisms.

The Z+TEO film at the highest TEO concentrations (6% and 7%) slightly inhibited *E. coli*, *S. aureus*, and *L. monocytogenes*, producing clear inhibition zones between 0.56 and 3.24 mm^2 in diameter. Whereas, quince seed mucilage (QSM) films loaded with thyme essential oil at lower concentrations (1%, 1.5% and 2%) exhibited higher inhibition zones against *E. coli*, *S. aureus*, and *L. monocytogenes* (Jouki et al., 2014a). In contrast for *B. thermosphacta* antimicrobial activity was observed at the minimum concentration of zein with 1% TEO incorporated. At the minimum concentration of TEO, the films did not affect any bacteria except *B. thermosphacta*. This may be because thyme essential oil were trapped within the films thereby limiting its diffusion and activity (Arcan & Yemenicioğlu, 2011).

The ranking of antimicrobial activity of Z+OEO films against the different bacteria was *B. thermosphacta* > *S. aureus* > *E. coli* > *L. monocytogenes*. The largest diameter of the zone of inhibition of zein films included OEO against *B. thermosphacta* ranging between 3.65 mm^2 and 329.92 mm^2. This indicates that the inhibition zones increased significantly ($p < 0.05$) with increasing essential oil concentration for *B. thermosphacta*. Zein films at concentration of 5% demonstrated inhibited four bacterial strains with inhibition zone diameters 0.30 mm^2, 3.30 mm^2, 256.62 mm^2 and 3.42 mm^2 for *E. coli*, *S. aureus*, *B. thermosphacta* and *L. monocytogenes.* These results are not in agreement with Jouki et al. (2014b) who is reported that QMS films incorporated with oregano essential oil at various concentrations (1%, 1.5% and 2%) produced greater inhibition zones.

The addition of OTEO at 1% in zein films exhibited obvious inhibition zones for *B. thermosphacta*. The Z+OTEO films at 2% demonstrated antimicrobial activities on both *E. coli* and *S. aureus.* The essential oils in zein film were least effective against *L. monocytogenes*. The antimicrobial efficacy of essential oils has been mainly attributed to the oregano or thyme terpenoid fraction that includes carvacrol, thymol

and p-cymene (Corrales Fernandez & Han, 2014; Hosseini, Rezaei, Zandi, & Farahmandghavi, 2015; Soković, Glamočlija, Marin, Brkić, & van Griensven, 2010). The major and minor constituents of thyme essential oil (p-cymene and ɣ-terpenine) may also play a major role in inhibiting bacteria (Bagamboula, Uyttendaele, & Debevere, 2004).

The antibacterial properties of essential oils in zein-based films is greater against the Gram-positive bacteria *B. thermosphacta*, *S. aureus* and *L. monocytogenes*, compared to the Gram-negative bacteria *E. coli*. The action of essential oils against food spoilage and pathogenic bacteria is recognised as more effective against Gram-positive than Gram-negative bacteria (Ahmad, Benjakul, Prodpran, & Agustini, 2012). In this study, all antimicrobial zein films containing TEO, OEO or OTEO were not effective against *L. sakei* and *P. aeruginosa* (Mann, Cox, & Markham, 2000; Teixeira et al., 2013). Some LAB bacteria are more resistance towards essential oil components (Holley & Patel, 2005). In general, the effectiveness of Z+TEO films was lower against all bacteria tested than for Z+OEO and Z+OTEO. Films of Z+OTEO had moderate antimicrobial activity, while the Z+OEO film displayed the greatest film against the bacteria tested.

2b.4 Conclusion

The incorporation of oregano (OEO) or thyme (TEO) alone, and the combination of both essential oils (OTEO), into zein-based films was successful. However, their effectiveness depended on the essential oil concentrations used in zein film. Furthermore, increased essential oil concentrations in zein could lead to the film becoming sticky and oily. OEO, TEO and OTEO can affect the film differently owing to the different modes of action of chemical compounds in these essential oils. Films containing OEO exhibited more antimicrobial activity than OTEO and TEO against four bacteria tested. All films were more effective against Gram-positive (*B. thermosphacta*, *S. aureus*, *L. monocytogenes*) than Gram-negative bacteria (*E. coli*), but could not inhibit *P. aeruginosa* and *L. sakei*. The antimicrobial properties of zein-based films can be modified by selected essential oils.

Table 2b.2

Antimicrobial activity of zein-based films containing thyme (TEO) or oregano (OEO) alone, and the combination of both essential oils (OTEO), against selected meat spoilage and pathogenic bacteria

Film	Essential oil concentration (%)	Inhibition zones in annular radius (mm^2)					
		E. coli	***S. aureus***	***B. thermosphacta***	***L. monocytogenes***	***L. sakei***	***P. aeruginosa***
Z+TEO	1	ND	ND	6.94 ± 1.92^{a}	ND	ND	ND
	2	ND	ND	49.98 ± 6.93^{b}	ND	ND	ND
	3	ND	ND	42.74 ± 11.08^{b}	ND	ND	ND
	4	ND	ND	111.85 ± 26.67^{c}	ND	ND	ND
	5	ND	ND	178.87 ± 38.99^{cd}	ND	ND	ND
	6	0.90 ± 0.24^{a}	0.88 ± 0.23^{a}	209.57 ± 45.45^{d}	2.87 ± 0.52^{a}	ND	ND
	7	0.56 ± 0.17^{a}	1.45 ± 0.64^{a}	148.94 ± 24.52^{c}	3.24 ± 1.86^{a}	ND	ND
Z+OEO	1	ND	ND	3.65 ± 0.95^{a}	ND	ND	ND
	2	ND	ND	27.06 ± 6.69^{b}	ND	ND	ND
	3	ND	0.91 ± 0.24^{a}	95.01 ± 12.94^{c}	ND	ND	ND
	4	0.71 ± 0.48^{a}	1.96 ± 1.28^{ab}	193.09 ± 32.98^{c}	ND	ND	ND
	5	0.30 ± 0.15^{a}	3.36 ± 1.05^{b}	256.62 ± 46.07^{cd}	3.42 ± 2.03^{b}	ND	ND
	6	1.44 ± 0.71^{b}	2.71 ± 0.64^{b}	302.27 ± 34.83^{d}	0.84 ± 0.41^{a}	ND	ND
	7	7.89 ± 2.66^{c}	15.62 ± 4.46^{c}	329.92 ± 33.05^{d}	2.98 ± 0.88^{b}	ND	ND
Z+OTEO	1	ND	ND	81.58 ± 37.99^{a}	ND	ND	ND
	2	0.22 ± 0.08^{a}	1.02 ± 0.65^{b}	133.86 ± 36.80^{ab}	ND	ND	ND
	3	0.56 ± 0.19^{a}	0.66 ± 0.13^{a}	197.75 ± 34.80^{b}	0.35 ± 0.29^{a}	ND	ND
	4	1.27 ± 0.48^{a}	3.94 ± 0.73^{c}	129.43 ± 15.58^{a}	1.06 ± 0.45^{a}	ND	ND

Values are given as mean ± SEM (n = 9). Diameter of inhibition zones excluded disc film (6 mm).

* ND - Antimicrobial activity not detected.

For a film test, means within a column (between concentrations) with different letters differ significantly ($p < 0.05$).

Film samples: Zein containing; thyme essential oil (Z+TEO), oregano essential oil (Z+OEO), and oregano and thyme essential oils (Z+OTEO).

Chapter 3 : The Antimicrobial Activity, Physical, Mechanical and Barrier Properties of Biodegradable Kafirin Films Containing Oregano or Thyme Essential Oils

3.1 Introduction

Food packaging material with better physical characteristics, mechanical strength, barrier properties and biodegradablity, as well as antimicrobial activity is clearly desirable for perishable foods. The development of biodegradable bioplymer packaging materials is a current focus as an alternative to traditional petroleum-based packaging materials (Ruiz-Navajas, Viuda-Martos, Sendra, Perez-Alvarez, & Fernández-López, 2013).

Kafirin is a biopolymer protein from sorghum grain (*Sorghum bicolor L. Moench*). Kafirin is biodegradable and environmentally friendly and has been successfully used as antimicrobial packaging materials for food preservation (Buchner, Kinnear, Crouch, Taylor, & Minnaar, 2011; Giteru et al., 2015; Taylor, Muller, & Minnaar, 2016). Kafirin films and coatings have a promising potential impact on food shelf life extension during storage, due to their nature as an effective barrier to gas, but poor water barrier and moisture properties limits their uses (Buchner, Kinnear, Crouch, Taylor, & Minnaar, 2011). There are few studies investigating the antimicrobial activity of kafirin films with essential oils added to them, and their physical, mechanical and barrier properties. The addition of essential oils to film packaging could improve the water vapour barrier and increase oxygen permeability due to its hydrophobic character (Atarés & Chiralt, 2016; Tongnuanchan & Benjakul, 2014; Vergis, Gokulakrishnan, Agarwal, & Kumar, 2013). While there is no research on the antimicrobial activity of kafirin films containing oregano and thyme essential oils, but citral essential oil and quercetin have been effectively incorporated into them. Films containing these compounds displayed antimicrobial activity against *Campylobacter jejuni*, *L. monocytogenes* and *Pseudomonas fluorescen* (Giteru et al., 2015).

The aim of the work in this chapter was to evaluate the antimicrobial activities of biodegradable kafirin films containing oregano or thyme essential oil alone, and the combination of both essential oils, at different concentrations, against common meat spoilage and pathogenic bacteria. The physical, mechanical, barrier and optical properties of the most effective kafirin films containing oregano and thyme essential

oils at a concentration of 5% were also examined to asses their potential applications as food antimicrobial packaging material.

3.2 Materials and Methods

3.2.1 Materials

3.2.1.1 Sorghum flour

Two batches of white sorghum grain were used in this study. They were obtained from Queensland Department of Agriculture, Fisheries and Forestry (Brisbane, Australia) and Mirfak Pty. Ltd. (Victoria, Australia).

3.1.1.2 Bacterial strains

Bacterial strains used were as described in the previous chapter 2a (section 2.1).

3.2.2 Kafirin extraction

White sorghum kafirin was extracted following the similar method described in Chapter 2a (section 2.2.1 and 2.2.2) with the only difference being ingredient quantities: White sorghum flour (800 g) was extracted with 2800 ml aqueous ethanol (70% v/v) in deionized water, containing 20.0 g sodium metabisulphite and 14.0 g sodium hydroxide. The isolated defatted kafirin protein contents from two different batches were 86.30 ± 4.58% and 90.23 ± 5.24% protein (db) as determined by the Kjeldahl method of AOAC 925.10 (AOAC, 2005) using nitrogen to protein conversion factor of N x 6.25.

3.2.3 Preparation of antimicrobial kafirin films

Kafirin film was developed as described in the previous method with the only difference being ingredient quantities (Chapter 2a section 2.3). In this study, two different batches and formulations (based on casting material size) of kafirin were prepared as follows. In study one, kafirin flour, ethanol (97%), and the amount of plasticizers used were the same (Chapter 2a section 2.3). For the second study, a large batch of kafirin solution, kafirin flour (23.40g) was dissolved in 125 g and 9.18 g plasticizers were used. The commercial essential oils oregano (OEO) or thyme (TEO) alone or in combination (OTEO) (Range Products Pty Ltd Company, Perth, Australia) were added to the kafirin film solutions to reach a final concentration of 1%-10% w/w for kafirin-based films (K+OEO and K+TEO) and 1%-6% w/w for K+OTEO films, measured as essential oil concentration per film. Films with the combination of both essential oils (K+OTEO) had a similar percentage of each essential oil (oregano or thyme; at the percentage of 5%:5%) to the film solution. The film solutions were homogenized (Ultra-Turrax, IKA®T18, Germany) at 9500 rpm for 60s and degassed under vacuum to remove bubbles and cast as described below.

Casting films

In study one, about 4 g of mixture was cast onto glass petri dishes (9 cm diameter) for antimicrobial activities and in the second around ~56g was cast on square glass templates (32 cm x 28 cm; L x W) usually for physical, mechanical, barrier and optical properties. Fims were then dried for 19 h at 32 ± 2°C and 34 ± 2% RH using forced draft oven (Panasonic Biomedical, Leicestershire, UK). Dried films were peeled and directly evaluate for antimicrobial activities at several concentrations were investigated. Films for physical, mechanical, barrier and optical properties determinations were stored in a desiccator at 23.7 ± 2°C and 50.7 ± 1% RH until evaluation. Kafirin film without essential oils was prepared and used as a control. Films were produced in batches, according to their type of essential oils and concentrations, respectively. All antimicrobial films were prepared in triplicate.

3.2.4 Essential oil composition analysis

Commercial OEO and TEO were analysed by GC/MS to identify their principle components. The ChemCentre (Western Australia, Australia) conducted these analyses using the following method. GC/MS was performed using an Agilent 5975C GC/MSD system. Both essential oil samples were diluted with dichloromethane. The system was set up to inject the diluted samples (1μL) into a split/splitless inlet held at 280°C operating in pulsed splitless mode. The oven contained a HP-5MS column (30 m × 0.25 mm; film thickness 0.25 μm), and the temperature was programmed at 40°C for 3 min, and then raised to 320°C at the 15°C / min, then held for 5 min. The total run time was 26.6 mins. The mass spectrometer had a solvent delay of 6 min and scanned from 40 to 500 amu (atomic mass units) for the duration of the analysis. The acquisition data and instrument control were achieved by the MSDChemstation software (G1701CA; version C.00.00; Agilent Technologies, Santa Clara, CA, USA). The relative amounts (RA) of individual components were expressed as percentages of the peak area relative to the total peak area.

3.2.5 Antimicrobial activity of kafirin film containing essential oils

Antimicrobial activity was determined using the same method described in 2a.2.5.

3.2.6 Properties of films

3.2.6.1 Film thickness

Film thickness was determined as described in section 2b.2.4. The mean thickness values were used to calculate the mechanical and permeability properties of the films.

3.2.6.2 Film moisture content

The moisture content of the film (1cm x 3cm) was determined by measuring the weight loss of the film before and after drying in an oven at 105°C until a constant dry weight reached. Three replications of each film treatment were used. The percentage of film moisture content was calculated according to the equation MC (%) = (W_i – W_d/ W_i) x 100, where W_i is the initial sample weight (g) and W_d is the dry sample weight (g).

3.2.6.3 Water solubility

The solubility in water of the different kafirin films was determined by a method described by Shojaee-Aliabadi et al. (2013). A square piece (4.0cm^2) was cut from each film and weighed to determine the dry film mass. The dried films were placed into glass beakers with 100mL distilled water. The sample was immersed for 6 h under constant agitation at 25ºC. Subsequently, the film was filtered and dried in an oven at 105ºC until constant weight (Ghasemlou et al., 2013). The film solubility percentage was calculated based on this equation WS (%) = ((W_O - W_F)/W_O) x 100, where W_O is the initial weight of the film (dry matter) and W_F is the final weight of dried film after filtered.

3.2.6.4 Mechanical properties

All films were cut into rectangular strips 10mm wide and 60mm long, after conditioning at 50 ± 1% relative humidity (RH) for 24h. Tensile strength and elongation at break of the kafirin films were determined using a texture analyser TexVol, model TVT-300XP/XPH (Perten, HÄGERSTEN, Sweden) according to ASTM standard method D882-12 (ASTM, 2012). The films were fixed with grip separation (40 mm) and stretched at a crosshead speed of 0.6 mm/s, pre-test and post-test speeds were 1.0 mm/s and 8.0 mm/s, respectively, the distance was 100mm and a 5 kg load cell was used. The maximum force and distance at the break point were determined automatically by a software texture analyser. The tensile strength

(MPa) and elongation at break (ELB) (%) were measured according to Hosseini et al. (2009). Film thicknesses were measured in eight places using a digital micrometer (AccuRemote, USA). Fourteen replicates were measured for each film and the average values were reported.

3.2.6.5 Water vapour permeability (WVP)

The WVP of the films was performed by the ASTM E96-95 gravimetric method with slight modifications proposed by Taylor et al. (2005a) and Giteru et al. (2015). The films were conditioned for 24 h and 50 ± 1%RH. Circular samples and aluminium samples (as a control) (42 mm) were cut and eight random thickness measurements were taken for each film. Film and control samples were placed on the top of Schott bottles (100 mL) containing 90 mL of distilled water with petroleum jelly, followed by parafilm and silicon tape, to ensure that the water migration occurred only through film and maintained sealed. All samples were weighed (± 0.001 g) and placed in an oven with forced-air circulation at 34.2 ± 1 ºC and the 37.2%RH. The weight loss from the Schott bottles was measured every 4 h intervals for 8 days were plotted in a scatter plot (R^2>0.97). Film water vapor transmission rate (WVTR) was determined from the slope of weight over time, Eq. (1) and water vapor permeability (WVP) was determined using Eq. (2) (Giteru et al., 2015)

$$\text{WVTR (g/h m}^2\text{)} = (G/t)/A \quad (1)$$

$$\text{WVP (g mm/m}^2\text{ h kPa)} = G \cdot X/A \cdot \Delta P \quad (2)$$

where, G is the weight change from the straight line (g), t is the time during which G occurred (h), G/t is the slope of the straight line (g/h), A is the test area (cup mouth area, m2). X is the film thickness (mm), ΔP is the differential water vapour partial pressure across the film, and ΔP = (Po (kPa) x (RH1- RH2)/100: Po at 30ºC = 4.76 kPa. RH1 is the relative humidity inside the bottle and RH2 is the relative humidity outside the bottle. An assumption was made that the relative humidity inside the bottle (RH1) was 100%. The temperature and relative humidity outside bottle were measured using a TinyTag Ultra2 data logger.

3.2.6.6 Oxygen permeability (OP)

OP was determined by measuring the amount of oxygen diffusing through the film over time (Imran et al., 2012; Khwaldia, Banon, Desobry, & Hardy, 2004). Briefly, the film is placed between two Teflon rings (attached to a permeation cell). Film samples were pre-conditioned (23.7 ± 2°C and 50 ± 1% RH) before analysis. In the lower channel O_2 was supplied at a controlled flow rate to maintain a constant pressure. Nitrogen gas was blown into the other chamber through one channel at a controlled flow. In this case, nitrogen functioned as a carrier for the O_2. At suitable time intervals, oxygen gas samples were withdrawn from the lower channel and analysed using a gas chromatographic system (Hewlett-Packard 5700, Kyoto, Japan). Oxygen gas concentrations were collected with a gas sampling syringe and injected into a gas chromatograph equipped with a thermal conductivity detector (TCD). The flow rate of argon carrier gas was 25 mL min^{-1} and a column molecular sieve 5A 80/100, at 110ºC. Oxygen content is reported as percent of detected peaks (O_2 and N_2). Eq. (3) was used to determine the oxygen permeability (Imran et al., 2012). The results were analysed in triplicate.

$$OP = \frac{a \times x \times V}{S \times 60 \times \Delta P} \qquad (3)$$

where OP = oxygen permeability (m^3 m/m^2 s kPa), a is the coefficient of the slope indicating the percentage of oxygen, x is the thickness of film (m), V is the volume of the permeation cell (m^3), surface area of film (m^2) and ΔP (kPa) is the pressure difference.

3.2.6.7 Film colour parameters

Kafirin films surface colours were evaluated as described in section 2b.2.4. The mean value results of five measurements from different locations were recorded.

3.2.6.8 Light transmission and film transparency

The barrier properties of kafirin films (1 cm x 3 cm) against ultraviolet (UV) and visible light were measured at wavelength range 200 to 900 nm using a UV spectrophotometer (UV-1201, Shimadzu Corp., Kyoto, Japan) according to the Fang et al. (2002) method. The percentage of light transmission (T%) of the film were directly attaching the film to the wall of cuvette test cell. Finally, film transparency was calculated using Eq. (4) (Jung H Han & Floros, 1997):

$$\text{Transparency} = -\log T_{600}/x \qquad (4)$$

Where "T_{600}" is the transmittance at 600 nm and x is the film thickness (mm). Each film was evaluated in triplicate.

3.2.7 Statistical analysis

Statitical analysis was performed using the IBM SPSS software (Version 22, NY, USA). All the experiments were carried out with three or more replicates. Antimicrobial activity experiments were conducted in triplicate on different days. Data was initially evaluated by analysis of variance (ANOVA), and the LSD test (least significant difference) was employed to detect significant ($p < 0.05$) differences among antimicrobial properties of kafirin films. A Duncan's multiple range tests were applied for comparisons of means, and the differences between the control and the kafirin films incorporating essential oils at various concentrations were considered significant at $p < 0.05$ for thickness and colour parameters. A T-test was used to compare mean differences between the control film and kafirin with essential oil incorporated at a concentration of 5% to detect significant ($p < 0.05$) differences for physical, mechanical and barrier properties.

3.3 Results and discussion

3.3.1 Composition of oregano and thyme essential oils

Chemically essential oils are a combination of volatile oils and aromatic compounds, including terpenoids, sesquiterpenes and diterpenes with different groups of aliphatic hydrocarbons, acids, alcohol, aldehydes, ketones, amines and sulfides (Calo, Crandall, O'Bryan, & Ricke, 2015; Fisher & Phillips, 2006). The major active chemical components in commercial OEO and TEO are phenols, terpenes, aldehydes and ketones. The components of OEO and TEO were identified by GC-MS. The dominant components (< 0.1%) of OEO and TEO are listed in Table 3.1 (the content of the compounds exceeding 2% are marked in bold). Sixteen compounds were tentatively identified in TEO representing 99.8% of the total whereas 18 components were identified in OEO representing 99.9% of the total. The main components of TEO were *p*-cymene (37.3%), ɣ-terpinene (36.2%), and D-limonene (6.8%). Similar findings have been reported by other authors who also did not identify carvacrol or thymol compounds in their *T. vulgaris* essential oils (Hyun, Bae, Yoon, & Lee, 2015; Turgis, Vu, Dupont, & Lacroix, 2012). Bagamboula et al. (2004) reported that no major differences were seen between the commercial thyme essential oil and that obtained by steam distillation of finely ground dried thyme leaves. Only linalool and ɣ-terpinene compounds were present in a greater percentage in the commercial TEO. Gavaric et al. (2015) also found a lower percentage of thymol and carvacrol in their *Thymus vulgaris* essential oil when analysed by GC-MS. Our results are similar to studies on oils from the early flowering period for plants as these possess many phenols (*p*-cymene and ɤ-terpinene)(Sáez, 1998). Previous studies have reported thymol and carvacrol as major compounds of thyme essential oil (Jouki, Mortazavi, Yazdi, & Koocheki, 2014a; Nowak, Kalemba, Krala, Piotrowska, & Czyzowska, 2012; Teixeira et al., 2013a), but, these were not detected in our sample. Burt (2004) stated that the dominant components of TEO typically consists of carvacrol, thymol, *p*-cymene (10-56%), ɤ-terpinene and linalool. Variability in chemical compositions in essential oils could be influence by species and its chemotype (Nabavi et al., 2015). Studies have identified *p*-cymene and ɣ-terpinene as the major antimicrobial compounds of genus *Thymus* plants (Nabavi et al., 2015), however, they act differently against Gram-positive or Gram-negative bacteria (Hyldgaard, Mygind, &

Meyer, 2012). It is believed that the terpene group could be effective as antimicrobials when applied with other compounds, with *p*-cymene reported to have a synergistic effect with carvacrol.

Carvacrol (37.7%), followed by *p*-cymene (21.9%) and thymol (14.3%) were identified as the major constituents of OEO. The result of the chemical profile of OEO compares favourably with those of earlier studies (Martucci, Gende, Neira, & Ruseckaite, 2015). Lv, Liang, Yuan & Li (2011) reported different percentages of the main compounds of carvacrol and thymol, representing 30.17% and 8.62% of the total oregano essential oil, while another study reported an even lower percentage was reported for carvacrol (14.5%) and thymol (12.6%) (Teixeira et al., 2013b). According to Aguirre et al. (2013), trans-hydrate sabinene (34.3%) and thymol (17.93%) were the principal components of OEO. Thymol and carvacrol are terpenoids and are the most active antimicrobial compounds against a broad spectrum of food spoilage or pathogenic bacteria (Tajkarimi, Ibrahim, & Cliver, 2010). Both of them can disintegrate the outer membrane of Gram-negative bacteria releasing lipopolysaccharide and increasing the permeability of the cytoplasmic membrane to ATP. It has been suggested that the hydrophobicity of essential oils enables them to accumulate in cell membranes and mitochondria, disturbing the structures and increasing permeability, ultimately leading to leakage of ions and other cell contents (Lambert, Skandamis, Coote, & Nychas, 2001).

Some variance of essential oil compounds was observed between our results and others, these variances could be due to several factors such as different plant material, geographic origin and harvesting time, as well as extraction technique used (Calo et al., 2015; Ličina et al., 2013). These differences may influence antimicrobial properties of the oils. Dorman and Dean (2000) stated that another factor related to the efficiency of essential oil components is the sensitivity of individual bacterial strains. The chemical composition of OEO and TEO in this study indicates that these two essential oils have antimicrobial components and could reasonably be applied in food packaging material.

Table 3.1

Main chemical components of *Origanum vulgare* and *Thymus vulgaris* essential oils.

Components (%)[a]	Thyme (T. vulgaris)	Oregano (O. vulgare)
α-pinene	**2.0**	1.4
Camphene	1.7	1.0
β-myrcene	3.6	**4.2**
α-Phellandrene	0.9	0.9
p-Cymene	**37.3**	**21.9**
D-limonene	**6.8**	**4.4**
Eucalyptol	0.6	
g-terpinene	0.8	**5.9**
4-carene	**4.7**	**4.6**
Borneol	0.7	
p-menth-1-en-8-ol	**3.3**	0.6
Isoterpinolene	0.7	
α-terpinene	**36.2**	
β-caryophyllene	0.5	1.0
β-pinene		0.5
3-carena		0.5
Thymol		**14.3**
Carvacrol		**37.7**
Trans-ocimene		0.5
Cis-Ocimene		0.5

[a]Results were expressed as percentage of chromatographic area.

3.3.2 Antimicrobial properties of kafirin films

The antimicrobial activity of kafirin films containing OEO and TEO alone, and in combination (OTEO), at a range concentrations against strains of *E. coli, S. aureus, B. thermosphacta, L. monocytogenes, L. sakei* and *P. aeruginosa* were tested and the results are presented in Table 3.2.

The control film (KC) showed no antimicrobial activity against any of the six bacteria tested. This suggested that kafirin film (which contained glycerol, lactic acid and polyethylene glycol 400) are unlikely to have not been released into the agar. However, Giteru et al. (2015) reported an inhibition zone (in radius = 0.17 mm^2) for the pure kafirin film against *L. monocytogenes* and weak inhibition effect under the film disk (contact areas on the agar surface). According to Petersson et al. (2007), the release of preservatives from kafirin films containing lactic acid or calcium

propionate can be influenced by the water activity of the food as a slow release was found in the model food with 0.85 water activity, but higher release when water activity of the model food was 0.95. On the other hand, as the sorghum grain used naturally contained phenolic compounds the antimicrobial activity observed by Giteru et al. (2015) could be due to this (Afify, El-Beltagi, Abd El-Salam, & Omran, 2012). In another study, Kil et al. (2009) demonstrated that there was variation in antimicrobial activity due to the different solvents used to extract kafirin from crude sorghum.

The antimicrobial activity increased with increasing concentration of essential oils in the kafirin film matrix. The addition of thyme essential oil in kafirin films at 8% and 4% was effective ($p < 0.05$) against *S. aureus* and *B. thermosphacta*, but no inhibition zones were observed at any concentration of TEO for *E. coli*, *L. monocytogenes*, *L. sakei* and *P. aeruginosa*. For *S. aureus*, the inhibition zones ranged from 0.13 to 0.48 mm^2 with no statistical differences ($p > 0.05$) between concentrations of 9% and 10% TEO. Kafirin films containing TEO produced greater inhibition zones against *B. thermosphacta* with increasing TEO concentration up to 10% ($p < 0.05$), (10.77 to 126.07 mm^2). Emiroğlu et al. (2010) reported that edible soy films containing 1% of TEO had strong antimicrobial activities against *S. aureus*, *E. coli*, *E. coli O157:H7*, *P. aeruginosa* and *L. plantarum*. Thyme essential oils incorporated into films effectively inhibited *E. coli*, *L. monocytogenes* and *P. aeruginosa* (Altiok, Altiok, & Tihminlioglu, 2010; M. H. Hosseini et al., 2009; Jouki et al., 2014a). The differences in this antimicrobial activity may be associated with the chemical compounds presence in TEO, as noted carvacrol or thymol were not present in our TEO components, and the origin of plant, time of harvest, processing and including storage condition might be different.

K+OEO films were effective at concentrations of greater than 4% against five meat of the spoilage and pathogenic bacteria. They were not effective against *P. aeruginosa* and exhibited moderate antimicrobial effects on *E. coli*, *S. aureus*, *L. monocytogenes* and *L. sakei*, with a strong inhibitory effect apparent against *B. thermosphacta* ($p < 0.05$). The most sensitive strain, *B. thermosphacta* showed the greatest inhibition zones with values between 65.82 and 326.52 mm^2 with significant difference between essential oil concentrations ($p < 0.05$). Inhibition of *E. coli*, *S.*

aureus, *L. monocytogenes* and *L. sakei* was seen at 7% OEO in kafirin films with the largest inhibition zones were observed at the highest concentration of 10% OEO. There was a significant difference ($p < 0.05$) between concentrations. The results show that K+OEO films at a concentration of 10% can be ranked in efficiency as against the different bacteria as follows *B. thermosphacta* > *L. monocytogenes* > *S. aureus* > *E. coli* > *L. sakei*. The antimicrobial efficiency of OEO can be influenced by the concentration of essential oil added and the amount of chemical compounds presence. The main terpenoid fraction of oregano comprised of carvacrol, thymol and *p*-cymene (Burt, 2004; Corrales Fernandez & Han, 2014). The dissimilarity in sensitivity of target bacteria is in agreement with earlier reported data for incorporation of OEO into films (Aguirre et al., 2013; Benavides et al., 2012; S. F. Hosseini et al., 2015; Muriel-Galet, Cran, Bigger, Hernández-Muñoz, & Gavara, 2015; Pelissari, Grossmann, Yamashita, & Pineda, 2009; Seydim & Sarikus, 2006).

Kafirin films containing both oregano and thyme essential oil (OTEO) were more effective than K+TEO or K+OEO films in inhibiting five of the bacteria tested. This indicates that the combined effect of these compounds can enhance the antimicrobial activities. K+OTEO films were effective ($p < 0.05$) against all bacteria tested except *P. aeruginosa*. *B. thermosphacta* showed the largest zone of inhibition at all concentrations tested, with values ranging from ~28 to ~392 mm^2. Statistical differences ($p < 0.05$) between K+OTEO 6% and other concentrations except K+OTEO 5% ($p > 0.05$) were apparent. For *S. aureus*, inhibition was observed at concentrations 1% and above 3% and this increased with increasing concentration of OTEO ($p > 0.05$). Films containing OTEO at 5% had antimicrobial activity towards E. *coli*, *L. monocytogenes* and *L. sakei* with significantly greater inhibition zones at 6% ($p < 0.05$).

It can be concluded that the minimum effective concentration of OTEO in kafirin-based films was 5%. These results indicate that the increased antimicrobial activities of K+OTEO at 5% can be related to the combined antimicrobial compounds of these both essential oils. Synergism can be defined as "when two or more essential oils work together to produce an effect greater than the sum of their individual effect" (Seow, Yeo, Chung, & Yuk, 2014). Many studies have demonstrated that oregano and thyme, as well as thyme and orange essential oils, produced a synergistic effect

on antimicrobial activity (Gutierrez & Bourke, 2009; Thanissery & Smith, 2014). The application of thymol and carvacrol in combination can improve the efficacy of essential oils against food pathogenic bacteria (Lambert et al., 2001). For example, synergism between carvacrol and *p*-cymene might facilitate carvacrol's transportation into the cell by better swelling on the *B. cereus* cell wall (Burt, 2004). Giteru et al. (2015) found kafirin films containing citral and quercetin exhibited antimicrobial activity against *C. jejuni*, *L. monocytogenes* and *P. fluorescens* when used at concentrations of 1.25% and 1%. A combination of essential oils could reduce the amount required as antimicrobial preservatives and hence minimise the cost.

None of the kafirin-based films containing TEO, OEO or OTEO could inhibit *P. aeruginosa*. It has been suggested that this foodborne pathogen is resistant to the effect of essential oils and other compounds (Ahmad, Benjakul, Prodpran, & Agustini, 2012; Jouki et al., 2014b). In addition, the external membrane (double layer) surrounding their cell wall restricts diffusion of hydrophobic compounds enter their lipopolysaccharide covering (Burt, 2004; Mann, Cox, & Markham, 2000; Ruiz-Navajas et al., 2013; Teixeira et al., 2013b). Conversely, Aguirre et al. (2013) reported that OEO (2%) was required for triticale films to produce inhibition zones against *P. aeruginosa*. In another study, films containing TEO or OEO exhibited a strong antimicrobial effect against *P. aeruginosa* species with concentration applied between 1% and 1.5% (Emiroğlu et al., 2010; Hosseini, Razavi, & Mousavi, 2009; Jouki et al., 2014a, 2014b).

Table 3.2

Antimicrobial activity of kafirin films incorporated with essential oils against common meat spoilage and pathogenic bacteria

Film samples	Concentrations (%)	Inhibition zones in annular radius (mm^2)					
		E.coli	*S. aureus*	*B. thermosphacta*	*L. monocytogenes*	*L. sakei*	*P. aeruginosa*
K+TEO	1	ND	ND	ND	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND
	4	ND	ND	10.77 ± 5.06^{a}	ND	ND	ND
	5	ND	ND	33.11 ± 13.32^{b}	ND	ND	ND
	6	ND	ND	59.44 ± 18.78^{b}	ND	ND	ND
	7	ND	ND	79.93 ± 30.78^{b}	ND	ND	ND
	8	ND	0.13 ± 0.04^{a}	106.00 ± 29.69^{c}	ND	ND	ND
	9	ND	0.48 ± 0.21^{b}	90.17 ± 21.39^{bc}	ND	ND	ND
	10	ND	0.45 ± 0.10^{b}	126.07 ± 32.62^{c}	ND	ND	ND
K+OEO	1	ND	ND	ND	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND
	3	ND	ND	ND	ND	ND	ND
	4	ND	ND	65.82 ± 12.78^{a}	ND	ND	ND
	5	ND	ND	99.00 ± 10.52^{b}	ND	ND	ND
	6	ND	ND	119.79 ± 15.46^{bc}	ND	ND	ND
	7	0.13 ± 0.04^{a}	0.36 ± 0.06^{a}	201.36 ± 20.84^{c}	1.10 ± 0.47^{a}	0.15 ± 0.04^{a}	ND
	8	0.80 ± 0.19^{b}	1.45 ± 0.38^{b}	225.84 ± 34.05^{cd}	3.19 ± 1.15^{b}	1.05 ± 0.29^{b}	ND
	9	0.98 ± 0.20^{b}	1.58 ± 0.59^{b}	317.14 ± 56.20^{de}	4.02 ± 1.17^{b}	1.24 ± 0.26^{b}	ND
	10	3.50 ± 0.79^{c}	4.11 ± 0.71^{c}	326.52 ± 42.61^{e}	8.18 ± 2.97^{c}	3.05 ± 0.32^{c}	ND
K+OTEO	1	ND	ND	27.75 ± 7.70^{a}	ND	ND	ND
	2	ND	ND	46.65 ± 12.74^{a}	ND	ND	ND
	3	ND	0.07 ± 0.03^{a}	167.76 ± 88.31^{b}	ND	ND	ND
	4	ND	0.14 ± 0.03^{b}	266.62 ± 70.15^{b}	ND	ND	ND
	5	0.78 ± 0.25^{a}	2.58 ± 0.63^{c}	344.04 ± 70.07^{bc}	1.40 ± 0.86^{a}	1.45 ± 0.43^{a}	ND
	6	8.55 ± 3.55^{b}	9.74 ± 2.91^{d}	392.20 ± 70.97^{c}	5.79 ± 1.64^{b}	6.83 ± 2.88^{b}	ND

Values are presented as mean ± SEM ($n = 9$). Diameter of inhibition zones excluded disc film (6 mm). * ND - Antimicrobial activity not detected.

For a film test, means within a column (between concentrations) with different letters differ significantly ($p < 0.05$).

Film samples:Kafirin incorporated with; thyme essential oil (K+TEO), oregano essential oil (K+OEO), and oregano and thyme essential oils (K+OTEO).

3.3.3 Effect of the incorporation of oregano or thyme essential oils, or a combination both, on kafirin film thickness and colour properties

3.3.3.1 Appearance, thickness, visual aspect and colour of kafirin films

The flexibility and manageability of all kafirin films were qualitatively evaluated at the time of peeling from the petri dish. In order to determine the maximum concentration of essential oil that could be added into the kafirin matrix high amounts of the essential oils were incorporated into the film forming solutions (10%). It was observed that kafirin films containing essential oils from 1% to 10% for individual oils and 1% to 6% in combination were not oily, sticky or cracked and were easy to handle and required less care when peeling after cast.

The thickness of K+OEO or K+TEO films alone, and films with a combination of both essential oils (K+OTEO), at various concentrations is shown in Table 3.3. Thickness of kafirin films with TEO (3-10%) incorporated, increased in comparison with the kafirin control film ($p < 0.05$). It ranged from 0.143 to 0.171 mm. There was a significant difference in thickness observed between films with added TEO in the range of concentration 1%, 2%, 3% and 4% ($p < 0.05$). Jouki et al. (2014a) reported that the addition of thyme essential oil to the film-forming solutions increased QSM film thickness. The mean thickness of antimicrobial zein films containing thymol was 230 ± 5 mm (Del Nobile, Conte, Incoronato, & Panza, 2008). However, Altiok et al. (2010) showed that the incorporation of thyme essential oil concentration had no effect on chitosan films thickness.

OEO incorporated into kafirin films had a similar trend in thickness, regardless of OEO concentrations applied ($p < 0.05$). The thickness of these films ranged between 0.142 to 0.179 mm. Incorporation OEO into the alginate film-forming solution increased film thickness (Benavides et al., 2012). On the other hand, Aguirre et al. (2013) reported that the incorporation of OEO was observed to have no effect on the thickness of triticale protein films. For OTEO, the addition of these essential oils into the kafirin film forming solutions led to increasing in film thickness ($p < 0.05$). The thickness of films varied from the thinnest at 0.128 mm to thickest at 0.169 mm.

Similar findings were reported by Giteru et al. (2015), who also found that the incorporation of citral and quercetin essential oils had a significant effect on the thickness of kafirin films. This difference may be due to differences in film formulations and the chemical components present in essential oils (oregano and thyme) (Jouki et al., 2014b). OEO, TEO and OTEO may contribute to the formation of a loose film matrix. Film thickness can be influenced by the solid content of film forming solution with essential oils resulting in a loose matrix and ultimately in increased thickness (Han & Krochta, 1999; Jouki et al., 2014a).

The colour parameters of kafirin films with TEO, OEO and OTEO incorporated at various concentrations are shown in Table 3.3. The values of colour measurement were L* (brightness), a* (red-green) and b* (yellow-blue). The L* parameter values ranged between 72.85 and 82.26 in films incorporated with TEO and which were the brightest among all films. QSM films with thyme essential oil added to them showed decreased in brightness (L*) and increased b* values (Jouki et al., 2014a). Different brightness values for K+OEO (L* = 73.46 – 78.79) and K+OTEO (L* = 68.77 – 76.46) were observed as compared to the control films (L* = 72.18). The control film results were slightly lighter than reported previously (Buffo, Weller, & Gennadios, 1997; Da Silva & Taylor, 2005; Giteru et al., 2015). Kafirin films with citral and quercetin essential oils incorporated were less bright (L* = 61.94 – 69.48). This difference could be due to the differences in film formulations and type of essential oil. However, there was no obvious difference in all L* values for films containing the three essential oils at all essential oils concentrations tested ($p > 0.05$). The kafirin films showed homogeneity, transparency and were glossy regardless of the essential oils concentration (Figure 3.1). When essential oils (TEO, OEO or OTEO) are incorporated into kafirin films, there was a moderately lower in the a* and b* values, which is also lower than that of the control film. This present study demonstrates that incorporating essential oils (TEO, OEO or OTEO) into kafirin films made films slightly lighter than control films. It was also suggested that the incorporation of essential oil had an influence on kafirin film colour, but changes were depending on the type and concentrations of essential oil.

Table 3.3

Thickness and colour parameters of kafirin films (K) incorporated with oregano (OEO) or thyme (TEO) alone and a combination of (OTEO) at various concentrations.

Films	Essential oil concentration (%)	Thickness (mm)	L*	a*	b*
KC	0	0.137 ± 0.004^{aA}	72.18 ± 1.79^{aB}	$16.45 \pm 1.63^{e,cB}$	37.92 ± 2.01^{dD}
K+TEO	1	0.131 ± 0.021^{a}	81.94 ± 1.13^{c}	7.46 ± 0.86^{a}	22.58 ± 2.51^{a}
	2	0.125 ± 0.008^{a}	81.39 ± 2.51^{c}	8.03 ± 2.06^{ab}	24.03 ± 5.00^{a}
	3	0.143 ± 0.008^{bc}	79.18 ± 1.81^{bc}	10.03 ± 1.47^{b}	27.71 ± 2.62^{b}
	4	0.138 ± 0.011^{a}	82.26 ± 0.49^{c}	7.35 ± 0.34^{a}	22.48 ± 1.13^{a}
	5	0.139 ± 0.009^{a}	79.74 ± 1.26^{c}	10.06 ± 1.13^{b}	26.18 ± 2.77^{ab}
	6	0.152 ± 0.006^{c}	75.12 ± 1.08^{a}	12.64 ± 0.88^{cd}	33.65 ± 1.50^{c}
	7	0.157 ± 0.005^{d}	75.63 ± 0.74^{ab}	12.16 ± 0.61^{c}	32.54 ± 0.71^{c}
	8	0.156 ± 0.006^{d}	72.87 ± 0.47^{a}	14.65 ± 0.39^{d}	35.67 ± 0.72^{cd}
	9	0.160 ± 0.007^{de}	79.21 ± 1.64^{bc}	9.49 ± 1.33^{b}	27.20 ± 2.09^{b}
	10	0.171 ± 0.011^{e}	72.85 ± 0.41^{a}	14.42 ± 0.33^{d}	34.11 ± 0.40^{c}
K+OEO	1	0.176 ± 0.008^{e}	73.46 ± 1.38^{a}	14.95 ± 1.44^{c}	36.62 ± 1.96^{d}
	2	0.154 ± 0.004^{c}	78.13 ± 0.52^{d}	10.99 ± 0.49^{a}	30.62 ± 1.18^{a}
	3	0.142 ± 0.003^{ab}	78.36 ± 0.66^{d}	10.86 ± 0.75^{a}	29.17 ± 1.69^{a}
	4	0.150 ± 0.005^{bc}	78.79 ± 1.34^{d}	9.77 ± 1.05^{a}	29.93 ± 2.05^{a}
	5	0.146 ± 0.008^{b}	76.00 ± 0.24^{c}	12.47 ± 0.24^{b}	34.43 ± 0.77^{c}
	6	0.148 ± 0.007^{b}	77.90 ± 0.39^{d}	10.74 ± 0.31^{a}	31.51 ± 0.58^{ab}
	7	0.165 ± 0.013^{d}	75.12 ± 1.84^{bc}	13.12 ± 1.39^{b}	34.84 ± 1.87^{c}
	8	0.151 ± 0.006^{bc}	77.45 ± 1.03^{d}	11.26 ± 1.10^{ab}	32.22 ± 2.02^{b}
	9	0.171 ± 0.004^{de}	76.41 ± 1.37^{cd}	12.12 ± 0.82^{b}	32.55 ± 0.67^{b}
	10	0.179 ± 0.006^{e}	74.39 ± 0.77^{b}	16.09 ± 0.86^{c}	35.05 ± 1.43^{cd}
K+OTEO	1	0.128 ± 0.005^{A}	76.46 ± 1.38^{D}	12.94 ± 1.65^{A}	27.45 ± 1.23^{A}
	2	0.151 ± 0.008^{B}	75.87 ± 1.28^{C}	13.30 ± 0.94^{A}	28.38 ± 2.41^{A}
	3	0.169 ± 0.010^{D}	73.57 ± 1.25^{B}	15.13 ± 0.94^{AB}	31.08 ± 1.02^{A}
	4	0.161 ± 0.023^{C}	75.67 ± 2.53^{C}	13.70 ± 2.42^{A}	31.93 ± 4.24^{B}
	5	0.162 ± 0.009^{CD}	68.77 ± 0.88^{A}	22.12 ± 0.69^{C}	35.73 ± 1.79^{C}
	6	0.150 ± 0.006^{B}	73.01 ± 1.42^{B}	17.21 ± 1.83^{B}	31.18 ± 1.24^{AB}

Values are given as mean ± standard deviation (thickness, $n = 5$; colour, $n = 3$). Different letters or different capital letters in the same column within the film containing the same essential oil together with the control indicate significant differences (Duncan's test, $p < 0.05$).

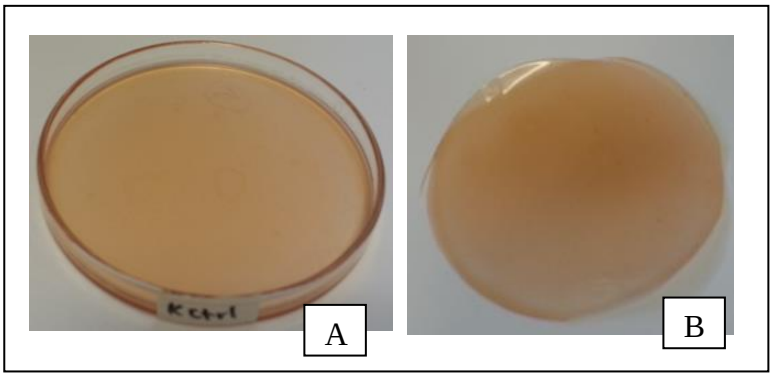

Figure 3.1 Kafirin film colour based on visual observation:

A) KC (control film), B) kafirin film back is glossy.

3.3.4 Effect of kafirin film incorporated with a combination of oregano and thyme essential oils on film properties

Based on the antimicrobial activity of kafirin-based films a concentration of K+OTEO5% was selected to be tested in kafirin film properties. The addition of essential oil leads to modifications of films in terms of physical, mechanical, barrier and optical properties. The interaction between OTEO5% and the kafirin polymer matrix are complex and it is important to take this into consideration when using it as a film or coating material.

3.3.4.1 Thickness and Moisture content

The thickness of kafirin film incorporated with essential oils (K+OTEO5%) (0.17 ± 0.01 mm) increased compare to kafirin control film (KC) (0.14 ± 0.01 mm). No significant difference in thickness was observed between films with essential oils added ($p > 0.05$).

The addition of OTEO into kafirin films did not significantly affect the moisture content values compared to the control films ($p > 0.05$). Increasing the concentration of OTEO at 5% led to a small but non-significant increase in the film's moisture

content (Table 3.4). These results are in agreement with Aguirre et al. (2013) and Zinoviadou et al. (2009) who showed that incorporation with OEO at 0.5% to 2.0% did not markedly affect the water content of triticale protein films and whey protein isolate films. Furthermore, kafirin films with quercetin and citral oils incorporated into them also demonstrated no significant effect on their moisture content (Giteru et al., 2015). This suggests that kafirin films with hydrophobic compounds represent a moderate moisture barrier.

3.3.4.2 Film water solubility

Kafirin films with OTEO showed increased film water solubility compared to control film ($p < 0.05$) (Table 3.4). The differences might be associated with the hydrophobic nature of essential oils and plasticizers (Aguirre et al., 2013). Non-polar components of essential oils may strongly interact with the hydrophobic kafirin film matrix and as a consequence increase the hydrophobicity of the film. However, Giteru et al. (2015) noted that kafirin film water solubility was not affected by the incorporation of citral or quercetin. Our KC result is a little higher than that reported by Giteru et al. (2015) in terms of film solubility and this differences may be due to the amount of plasticizers and essential oils used. According to Gillgren and Stading (2008), increases in plasticizer in kafirin could lead to the hydrophilization of films which may in turn affect product firmness and appearance. Solubility in water is an essential property for biodegradable or edible films (Rhim, Gennadios, Handa, Weller, & Hanna, 2000). In some cases films with moderate to high solubility could have the potential as biodegradable and edible packaging material. This type of film or coating application provides an advantage as it may not require any removal before consumption or cooking of food product (Ahmad et al., 2012; Ghasemlou, Khodaiyan, & Oromiehie, 2011).

3.3.4.3 Mechanical properties

The tensile strength (TS) and elongation at break (EAB) are parameters indicating mechanical strength and extensibility properties for films. They are important elements in maintaining film integrity and allowing it to endure the external stresses that occur during food handling and storage (Table 3.4). They are dependent on film structures. Incorporation of OTEO at a concentration of 5% into kafirin film significantly reduced its TS ($p < 0.05$). However, these essential oils did not act as plasticizers, and therefore caused no statistically significant difference between films in elongation at break ($p > 0.05$). Other studies observed similar trends (Aguirre et al., 2013; Zinoviadou et al., 2009). Buffo et al. (1997) reported low TS (2.1 ± 0.3 MPa) and greater EAB (106.1 ± 9.7) upon addition of essential oils to polymer coatings. The finding of our study suggest that with increased essential oils concentration, reduced TS of films resulted from the development of a heterogenous film structure, featuring discontinuities or irregularities. On the other hand, it had been suggested that the incorporation of essential oil at an appropriate concentration might strengthen the film matrix through enhancing the interaction between protein chains (Ahmad et al., 2012). The kafirin films in the present study demonstrated more expansion than Giteru et al. (2015), suggesting that some interaction occurred between kafirin film proteins and the combination of OTEO in the film forming solution. These may have contributed to reducing the intermolecular forces and improve flexibility and chain mobility. The extensibility of film containing essential oils could be enhanced due to the characteristics of essential oils, which formed oil droplets in the film and, as a consequence, films became easily deformed and improved in its flexibility (Fabra, Talens, & Chiralt, 2008).

Table 3.4

Physical and mechanical properties of kafirin films incorporated with essential oils.

Film	Moisture content (%)	Solubility in water (%)	Tensile Strength, TS (MPa)	Elongation at break, EAB (%)
KC	0.030 ± 0.002^{a}	37.40 ± 0.28^{a}	0.76 ± 0.10^{a}	195.14 ± 40.85^{a}
K+OTEO 5%	0.050 ± 0.009^{a}	41.06 ± 0.66^{b}	0.22 ± 0.12^{b}	201.36 ± 80.51^{a}

Means ± standard deviation in the same column followed by different letter are significantly different ($p < 0.05$).

3.3.4.4 Water vapor permeability (WVP)

Water transfer through packaging from food itself or the external environment may impact food shelf life and quality. The WVP and water vapor transmission rate (WVTR) values are essentials in estimating the water barrier efficiency of kafirin films with or without OTEO.

WVP and WVTR of kafirin film with OTEO incorporated and without essential oils are shown in Table 3.5. Generally, kafirin films with OTEO incorporated lead had a lower WVP value than the control. However, a slightly significant difference in WVP values between kafirin films with and without essential oils was observed ($p < 0.01$). The finding from the films in the present study were more promising barrier properties than those reported by Giteru et al. (2015), who prepared antimicrobial kafirin films embedded essential oils and revealed relatively high WVP values (between 0.65 and 0.74 g mm m^{-2} h kPa). This might be due to differences in the hygroscopic nature of essential oils used, which had a different ability to attract water to the film network (Jouki et al., 2014a). Even higher WVP values (0.43 g mm m^{-2} h kPa) have been recorded for kafirin control films (Taylor et al., 2005b). However, Zinoviadou et al. (2009) showed that oregano essential oil incorporated into whey protein isolate films did not change the WVP. This indicates that kafirin films with added essential oils may minimize food weight losses, firmness as well as appearance by wrapping or coating.

No significant change ($p > 0.05$) in WVTR was seen between control and K+OTEO5%. This result was similar to Giteru et al. (2015) who had WVTR values ranging from 23.7 to 31.5 (g/h m^2). In another study Taylor et al. (2005a) found that the WVTR of kafirin film was lower 10.9 (g/h m^2). Hosseini et al. (2009) indicated that, although the hydrophobic nature of essential oils could affect the hydrophilicity or hydrophobic property of the film, the physical factors had a dominant influence on the water vapor transmission rate through the film. In fact, essential oils incorporated in films may cause micro-pore formation due to the essential oils evaporating during the casting or drying process (Ahmad et al., 2012). This situation might increase and assist the water vapor transmission rate values of the films (Jouki et al., 2014a).

3.3.4.5 Oxygen permeability (OP) of the films

The OP of kafirin films with and without OTEO5% incorporated are presented in Table 3.5. The O_2P of the kafirin control film was the highest, with a value of 6.71± 1.37 x 10^{-7} m^3 m $m^{-2}s^{-1}kPa^{-1}$ while kafirin films with OTEO5% incorporated had a value of 0.0127 ± 0.24 x 10^{-7} m^3 m $m^{-2}s^{-1}kPa^{-1}$. The incorporation of OTEO5% into kafirin film significantly lower OP with respect to control film ($p < 0.05$), probably due to the use and addition of essential oils in film forming solutions that changed the entire kafirin film network and favoured oxygen diffusion (Imran et al., 2012). The OP values of quince seed mucilage films have been shown to increase when higher amounts of oregano essential oil were added to them (Jouki et al., 2014b). The same study on OP of kafirin control films showed a significant difference when any of the essential oil were added into the kafirin-based films (Giteru et al., 2015). It has been suggested that the addition of plasticizers into film forming solutions can increase the OP, as glycerol, lactic acid and polyethylene glycol 400 can modify the basic structure of the kafirin film network.

Generally, the OP of protein-based films demonstrates greater oxygen permeability transference, as biopolymer films are hydrophilic and have a large number of hydrogen bonds, which makes them excellent barriers to non-polar substances, for example, oxygen and aromatic compounds (Miller & Krochta, 1997). However, films with essential oils incorporated could have poor oxygen barrier properties as more oxygen can be dissolved in the non-polar oil phase increasing the transfer rate of the oxygen molecules into the plasticized polymer matrix (Jouki et al., 2014a).

Table 3.5

Barrier properties for kafirin films incorporated with OTEO concentration at 5%.

Film	WVP (g mm/m^2 h kPa)	WVTR (g/h m^2)	O_2P (m^3 m $m^{-2}s^{-1}kPa^{-1}$)
KC	0.017 ± 0.003^a	28.11 ± 4.53^a	$6.71 \pm 1.37 \times 10^{-7b}$
K+OTEO 5%	0.028 ± 0.001^b	29.22 ± 1.48^a	$0.0127 \pm 0.24 \times 10^{-7a}$

Values are given as means ± standard deviation (*n*=3). Different letters in the same column indicate significantly different ($p < 0.05$).

3.3.4.6 Light transmission and film transparency

Transparency is able to directly impact on the consumer acceptance of the food packaging films or coatings product appearance. Indeed, the majority of consumers desire and prefer food products with a transparent film or coating so as to be able to see a food and evaluate its quality. Transmission of UV and visible light wavelength of 200 and 900 nm through kafirin films with and without essential oils are presented in Figure 3.2. Both films showed a very low transmittance values close to 0 (0.0-0.2%) in the wavelength range of 200-300 nm, which indicated that kafirin control film and kafirin with OTEO at 5% concentration could be an excellent barrier to prevent UV radiation. These results suggest that both kafirin films could effectively delay lipid oxidation caused by UV and visible light. Furthermore, it can be suggested that combination of oregano and thyme essential oils was able to block the light transmission through kafirin film possibly by light scattering at the surface of essential oil presented in the film matrix (Tongnuanchan, Benjakul, & Prodpran, 2012).

Light transmission in visible range of 400 nm to 900 nm for control film was in the range of 20.3-40.0%. However, a higher transmission trend was observed when kafirin film had OTEO incorporated (50.6-79.3%). This result suggested that the addition of essential oils into kafirin film, especially at higher essential oil concentrations, most likely produced more light transmission at visible ranges. According to Ahmad et al. (2012), several factors that can influence light transmission or transparency of films when essential oils are incorporated.

Kafirin control films had lower transparency (0.64 ± 0.05) than kafirin with OTEO5% (1.03 ± 0.03). The increase in transparency of kafirin-OTEO5% films may be linked with the decreased in light scattering effect when OTEO5% was added ($p < 0.05$). Generally, when the concentration of an essential oil was higher it resulted in good light scattering intensity and as consequences may decrease the transparency values (Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2009). It has been proposed that the addition of plasticizer to films could improve the light transmission and good transparency (Imran et al., 2012). The result achieved in this study indicated that kafirin films with or without OTEO are clear enough to be used as transparent packaging or coating materials. This finding agreed with Ahmad et al.

(2012) and Kavoosi et al. (2014) who showed that protein-based films are usually recognized to have greater light barrier properties due to the presence of aromatic amino acids which absorb UV light.

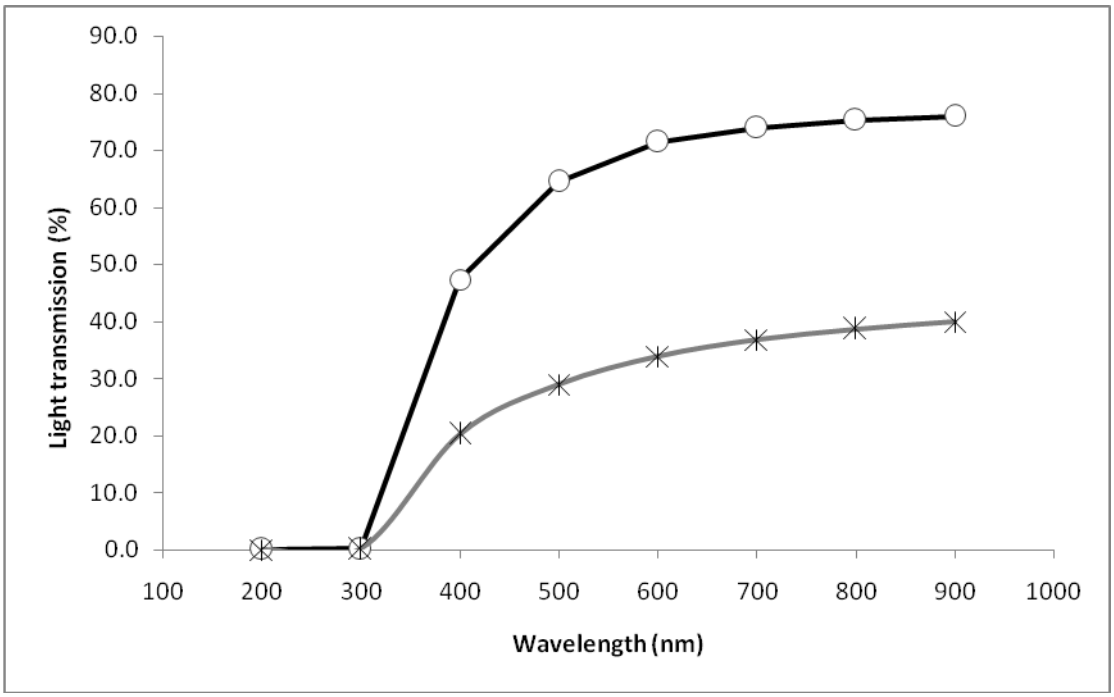

Figure 3.2 Transmission of UV and visible light (%T) through biodegradable kafirin film (○) KOT5% film, (✳) KC film. Mean of triplicate analysis.

3.3.4.7 Film colour

The second batch of kafirin commercial powder used to produce control kafirin films for experiments were lighter (Figure 3.3A,B) than the previous batch. The brightness values (L*) of KC and K+OTEO5% films were 86.34 and 85.52, respectively. The lowest a* values were obtained for KC (0.71± 0.24) and K+OTEO5% (0.81 ± 0.10), while the b* values of KC and K+OTEO5% films were 19.56 ± 1.08 and 19.70 ± 0.92, respectively. However, there was no differences ($p > 0.05$) in a* and b* values between the two films. These results showed that kafirin-based films for the second batch had lower values for a* and b* compared to the first batch films. This variation could be explained by the differences in the white sorghum source and processing procedures. Da Silva et al. (2005) also reported there was variation in colour due to different kafirin sources. The results obtained in the present study suggested that the

colour of both kafirin films with or without essential oils, even at a higher concentration of essential oils are suitable for food film or coating application as acceptable and convenient for consumers.

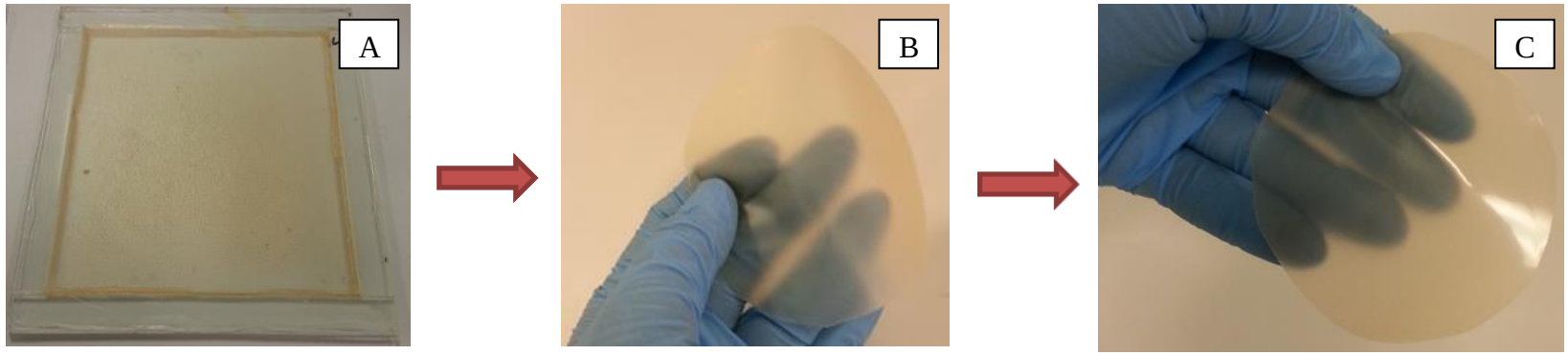

Figure 3.3A. Kafirin films containing OTEO at a concentration of 5% (K+OTEO5%): (A) lighter colour after cast, (B) transparent film, and (C) Glossy (at the back of the film)

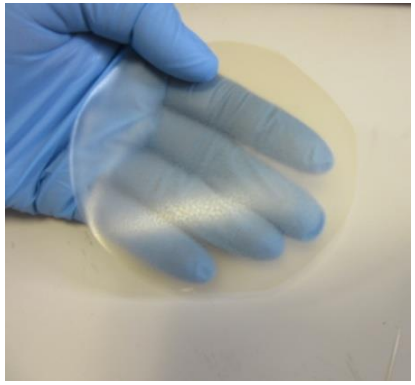

Figure 3.3B. Kafirin film without essential oil

3.4 Conclusion

The commercial oregano and thyme essential oil used in this study had similar chemical components to other studies. Kafirin films containing OEO, TEO and OTEO can migrate and diffuse to inhibit meat spoilage and pathogenic bacteria due to the dominant chemical compounds (such as carvacrol, thymol, p-cymene, ɣ-terpinene etc.) present in these essential oils. Kafirin films containing OEO and OTEO showed antimicrobial activity against five of the six bacteria tested. Films containing both types of essential oils demonstrated the highest inhibition against *B. thermosphacta*, followed by *S. aureus*. None of the essential oils or concentrations used were effective against *P. aeruginosa*. The thickness and colour properties of kafirin films were affected by the addition of OEO, TEO and OTEO. Incorporation of OTEO at 5% influenced physical, mechanical, barrier and optical properties of kafirin-based films. The properties of kafirin films can be modified by selecting the essential oil and concentration used. Overall, the findings suggest a strong potential for kafirin films with oregano (OEO) or a combination of both essential oils (OTEO) incorporated to control food spoilage or pathogenic bacteria when used as an antimicrobial film or coating material.

Chapter 4 : Effects of Antimicrobial Coating Treatments on Microbiological and Physicochemical Characteristics of Vacuum Packaged Fresh Beef Stored at 2°C

4.1 Introduction

Fresh meat and processed meat products are consumed worldwide as a major source of protein and harbour a diverse of variety of bacteria even during chilled storage (Aymerich, Picouet, & Monfort, 2008; Sánchez-Ortega et al., 2014). The microbiological safety of fresh and processed meat is of increasing concern. Specifically, Hennekinne et al. (2015) reported that most food poisoning outbreaks in Europe were associated with meat and meat-based products. Packaging and temperature are the most critical factors influencing the type of bacteria that can grow during meat storage. Meat spoilage occurs when high numbers of bacteria grow and cause biochemical changes during storage (Casaburi, Piombino, Nychas, Villani, & Ercolini, 2015; Doulgeraki, Ercolini, Villani, & Nychas, 2012; Gram et al., 2002).

In order to extend shelf life and quality of fresh and processed meat, control of microbial contamination and growth is critical. Oregano and thyme essential oils are effective natural antimicrobial essential oils (Emiroğlu et al., 2010; Van Haute, Raes, Van der Meeren, & Sampers, 2016). The antimicrobial compounds in oregano and thyme essential oils have been widely investigated (Abdollahzadeh, Rezaei, & Hosseini, 2014; Teixeira et al., 2013a, 2013b). They have also been identified as antimicrobial agents that can be incorporated into coating material to effectively control and prevent microbial growth (Quintavalla & Vicini, 2002).

Kafirin, a sorghum-based biopolymer, can be used to produce biodegradable coatings for food (de Mesa-Stonestreet, Alavi, & Bean, 2010; Taylor et al., 2005a). Kafirin with essential oils incorporated in it demonstrates promising antimicrobial activity against foodborne bacteria (Gillgren & Stading, 2008; Giteru et al., 2015; Petersson et al., 2007). Coatings have been used regularly in the food industry as a barrier to oxygen and moisture, as well as a cost effective, low waste solution to protect the food after the package has been removed (Cha & Chinnan, 2004). Biodegradable coatings, such as kafirin, also have mechanical and barrier properties that may affect the transparency of packaging.

Despite the potential of essential oil as antimicrobials, coating of these compounds on food surfaces usually requires high concentrations to achieve effective

antimicrobial activity (Bonilla, Vargas, Atarés, & Chiralt, 2014; Djenane, Yangüela, Montañés, Djerbal, & Roncalés, 2011; Dorman & Deans, 2000; Seydim & Sarikus, 2006). Many studies demonstrate that combined use of natural essential oil and vacuum packaging can improve the shelf life of food (Lucera, Costa, Conte, & Del Nobile, 2012; Ouattara, Simard, Piette, Bégin, & Holley, 2000; Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2011; Skandamis & Nychas, 2002; Viuda-Martos, Ruiz-Navajas, Fernandez-Lopez, & Perez-Alvarez, 2010). However, vacuum packaging for fresh beef held under chilled storage still cannot eliminate all microbial spoilage (Pennacchia, Ercolini, & Villani, 2011).

To the best of our knowledge, the application of kafirin as a coating material alone or in combination with oregano and thyme essential oils has not been investigated with respect to fresh beef. In Chapter 3 it was established that kafirin films containing oregano and thyme essential oils have good antimicrobial activity against meat spoilage and pathogenic bacteria. The objective of this study was to investigate the effectiveness of using different antimicrobial coating treatments in reducing the surface microflora on vacuum packaged fresh beef stored at 2± 0.5°C. The effects of all treatment groups on the microbiological and physicochemical characteristics of vacuum packaged fresh beef meat during storage were also investigated.

4.2 Materials and methods

4.2.1 Kafirin extraction

Commercial kafirin flour (Maralong Milling, Queensland, Australia) was used in antimicrobial kafirin coating preparation. Kafirin was extracted and defatted as described in chapter 2a (section 2.2.1 and 2.2.2). White sorghum with an isolated defatted kafirin protein content of 90.05 ± 2.73% proteins (dry basis,N x 6.25) was used.

4.2.2 Preparation of antimicrobial coating solutions

The kafirin coating solutions were prepared by dissolving defatted kafirin (106.25g), glycerol (13.83g), lactic acid (13.83g) and polyethylene glycol 400 (13.83g) in 97% aqueous ethanol (566g) with constant stirring and heating until boiling using a magnetic stirrer on a hot plate (Buffo et al., 1997). The amount of aqueous ethanol lost during evaporation was replaced and the mixture heated for 10 mins. Kafirin coating solutions were filtered through a layer of cheesecloth and cooled to room temperature. Kafirin coating solution without essential oil was prepared and used as a control. Kafirin coating solution containing 5% each of oregano and thyme essential oils (OTEO) was prepared by homogenization (Ultra-Turrax, IKA®T18, Germany) at 9500 rpm for one minute and degassing by vacuum to remove air bubbles. All antimicrobial coating treatment solutions were prepared fresh and used within a few hours.

4.2.3 Meat preparation

Vacuum packaged chilled beef meat (whole eye round) was purchased from a local registered establishment (Goodchild Meats, Western Australia, Australia). The beef was from cattle that had been slaughtered, fabricated and vacuum packaged on the same day at the plant. Samples were transported in an insulated box on ice to the testing laboratory. A LogTag was located between adjacent meat samples to record the temperature. Samples were stored under refrigeration (2 ± 0.5°C) until processing. Before opening samples the table, cutting boards and knives were disinfected to maintain aseptic conditions. Whole eye round samples were aseptically cut into smaller pieces and approximately 10 - 20g portions of muscle weighed.

4.2.4 Antimicrobial coating treatment application

Fresh beef pieces were subject to four different antimicrobial coating treatments; 1) vacuum packaged without applying any coating (VP) (control), 2) 97% ethanol (solvent) and vacuum packaged (VPS), 3) kafirin film forming solution without

essential oil (VPK) and 4) kafirin film forming solution with 5% oregano and thyme essential oils (VPKOT5%). The level of essential oil used was based on results of Chapter 3, in which kafirin film with oregano and thyme essential oils incorporated at a minimum concentration of 5 % (K+OTEO5%) showed the most positive effect on antimicrobial activities. The aqueous ethanol (97%) was used as an additional control as it is used in making antimicrobial kafirin-based coating solutions.

The fresh beef pieces were individually dipped into the different treatments and controls for 60s at room temperature to ensure thoroughly covering with each coating solutions. The coatings were dried on sterile stainless steel racks for 15 min at room temperature using fans to allow for attachment of the coating materials. Individual pieces of beef were placed in vacuum bags (175x145mm) (Vital Packaging Pty Ltd, Western Australia, Australia) and vacuum packed using a vacuum machine (WeboMatic, MBL Australia, Australia). The vacuum plastic packages had a water vapour transmission rate 2.6 gm/mq.24h and oxygen permeability 50gm/mq.24h at 23°C and 85% humidity. The vacuum packaged samples were stored in a refrigerator at 2 ± 0.5°C for up to 42 days. The refrigerator temperature was monitored at 30 min intervals using a LogTag (Model HAX0-8, China) to ensure the temperature consistency. All samples coated and uncoated were analysed for microbiological and physicochemical (pH and colour) immediately after coating (day 0) and on days 7, 14, 21, 28, 35 and 42 of storage.

4.2.5 Microbiological analysis

On each sampling day triplicate samples from each group were aseptically opened and transferred using sterile forceps to individual sterile stomacher bags (SARSTEDT, South Australia, Australia). Sterile 90 ml maximum recovery diluent (MRD) was added to each bag and the resulting mixtures were then homogenized for one minute in a stomacher (Colworth, Stomacher 400, London) to make an initial dilution. Serial decimal dilutions of meat samples were prepared using the same diluent. A 1 ml or 0.1 ml aliquots of diluted samples were surfaces plated on appropriate selective agar media to enumerate microbial.

Plate count agar (PCA, PathWest Laboratory WA, Perth, Australia) was used for determination of total viable counts (TVC, PathWest Laboratory WA, Perth, Australia) and incubated at 35°C for 48 h. *Enterobacteriaceae* counts were determined by plating (with overlay) on Violet Red Bile Glucose agar (VRBG, Oxoid, CM048, Thermo Fisher Scientific, Australia) with incubation at 37°C for 48 hours. Lactic acid bacteria (LAB) counts were determined on de Man, Rogosa, Sharpe agar (MRS, PathWest Laboratory WA, Perth, Australia) with incubation at 35°C for 48 h. All counts were performed in triplicate and expressed as $\log_{10}$ CFU/g.

4.2.6 Physicochemical analyses

4.2.6.1 pH

The pH value was recorded using a TPS pH meter (TPS Pty. Ltd., Brisbane, Australia). Beef samples were homogenized (Masticator, Barcelona, Spain) thoroughly using 100 ml of distilled water for 60s. The homogenate of each sample was measured after 1 min by immersing an pH electrode in the stomacher bag. Three readings were reported for each sample and the mean recorded.

4.2.6.2 Surface colour

The sample surface colour was measured using a Minolta Hunter LAB colorimeter (CM-508i, Konica Minolta, Tokyo, Japan) at five different locations on each piece of beef 30 min after opening each package to allow colour stabilization upon air exposure. The average CIE L* (lightness), a* (redness) and b* (yellowness) values were recorded.

4.2.7 Statistical analysis

All experiments were performed independently. Microbial counts obtained from each sample were averaged and transformed to $\log_{10}$ CFU/g. Data were analysed by one-way analysis of variance (ANOVA). Mean values and standard deviations were reported. The LSD tests were used to determine the significant differences between microbial population, pH and colour of antimicrobial treatments and controls at a significance level of $p < 0.05$.

4.3 Results and discussion

4.3.1 Effects of antimicrobial coating treatments on vacuum packaged fresh beef

4.3.1.1 Total viable counts (TVC)

The chilled meat industry uses total viable counts (TVC), *Enterobacteriaceae* counts and LAB counts as spoilage microflora indicators of food safety and quality of meat products (Mcevoy, Sheridan, Blair, & Mcdowell, 2004; Nychas et al., 2008). During storage numbers of bacteria should be within acceptable ranges (Pothakos, Samapundo, & Devlieghere, 2012). Changes in TVC ($\log_{10}$ CFU/ g) on fresh vacuum packaged beef with an antimicrobial coating treatment group and a control group during 42 days of storage at 2 ± 0.5°C are shown in Table 4.1. The TVC was significantly ($p < 0.05$) lower in all the antimicrobial coating treatments (VPS, VPK and VPKOT5%) and some reached the detection limit as compared to the control group (VPB) for 42 days. All treatment groups were not significantly different ($p > 0.05$) from each other during the entire storage period. However, The TVC of samples ranged from 2.00 (below the detection limit) to 4.78 $\log_{10}$ CFU/g. A similar result was reported by Tsigarida et al. (2000) who found a reduction in the initial microflora of beef meat pieces between 2 to 3 $\log_{10}$ CFU/g after treatment with 0.8% oregano essential oil. These results are also in agreement with those of Kanatt et al. (2013), who reported the initial TVC in ready to cook chicken kababs were reduced around 1.6 log CFU/g when coated with chitosan material during chilled storage.

On day zero the TVC for all treatment groups were 3.07 $\log_{10}$ colony forming units (CFU)/g, limit of detection and below the detection limit for VPB, VPS, VPK and VPKOT5%, respectively. It was expected that the initial TVC counts for VPKOT5% were lower than for the other treatments. This was due to the presence of a combination of essential oils which likely begin to act immediately after coating. These counts are indicators of meat quality and reflect hygienic conditions as well as good handling practices employed during slaughtering, processing and packaging of fresh beef meat. In the present study, VPK and VPKOT5% treatment were effective in inhibiting the TVC during the storage. The TVC of the fresh beef samples with all treatments remained well below the value of 7.00 $\log_{10}$ CFU/g after a storage period of 42 days, which is considered as the maximum acceptable limit for fresh beef according to International Commission on Microbiological Specifications for foods (ICMSF) (Dainty & Mackey, 1992).

Many studies suggest that oregano or thyme essential oils are effective against meat spoilage and pathogenic bacteria due to their chemical components (Skandamis & Nychas, 2002). The antimicrobial effect caused by adding both essential oils can be explained by the carvacrol, p-cymene, thymol and ɤ-terpinene as major phenolic components (Burt, 2004). The addition of combined oregano and thyme essential oils or with other preservation methods showed synergistic effects against spoilage and pathogenic bacteria when applied to meat (Gutierrez & Bourke, 2009; Klein, Rüben, & Upmann, 2013; Solomakos, Govaris, Koidis, & Botsoglou, 2008). A synergistic effect in combination with oregano and thyme essential oils was found in this study. As the gradual release of volatile oregano and thyme essential oils from kafirin coating within the vacuum packaging influenced the spoilage association of meat packaged at 2°C can be observed. According to Coma (2008), antimicrobial agent incorporated into coating packaging materials can be released through evaporation (if they are volatile antimicrobial agent) or migrate (if they non-volatile antimicrobial agent) into the food through diffusion onto the food surface. Studies using cinnamon essential oil in alginate-based edible coatings demonstrated that increasing concentrations of antimicrobial compounds resulted in greater inhibition of microbial growth (Lu, Ding, Ye, & Liu, 2010; Oussalah, Caillet, Salmieri, Saucier, & Lacroix, 2006). Han et al. (2014) coated to fresh beef steaks with polypropylene/polyvinyl alcohol incorporated with cinnamon essential oil and rhubarb ethanolic extracts and

found TVC growth was inhibited in 12 days. Cha & Chinnan (2004) stated that antimicrobial agents can be effective against surface growth (by contact area) without migration of the antimicrobial agent into the food. However, samples (fresh ground beef patties) coated with 5% oregano and thyme essential oils added into soy edible films showed no significant differences ($p > 0.05$) in TVC among all groups (Emiroğlu, Yemiş, Coşkun;,& Candoğan, 2010).

4.3.1.2 Lactic acid bacteria (LAB)

Lactic acid bacteria (LAB) are the predominant microflora of refrigerated vacuum packaged fresh beef (Sakala et al., 2002) and it were expected that they would be resistant to antimicrobial treatments (Emiroğlu et al., 2010). The changes in the LAB count of fresh beef samples stored under vacuum packaged at 2 ± 0.5°C are showed in Table 4.1. A similar trend in the changes of LAB counts was observed to that of TVC for all groups. A slightly lower LAB count (as compared to TVC count) was apparent in the fresh beef samples treated with the solvent (VPS), kafirin alone (VPK) and kafirin containing essential oils (VPKOT5%) as compared to the control (VPB) ($p < 0.05$), but no significant effect ($p > 0.05$) was observed over the storage periods. The initial mean log count of LAB on the fresh beef during storage day 0 to 7 were under the detection limit for VPB, VPS, VPK and VPKOT5%, respectively. The same antimicrobial coating trend was found with the oregano and thyme essential oils treatment (VPKOT5%) for LAB as was seen with TVC of the vacuum packaged fresh beef. These results suggested that the antimicrobial agents used, including the combination treatment, reduced the initial LAB count in the fresh beef during storage. At 42 days storage, the total LAB population of the control (VPB) was 5.75 log10 CFU/g. Lee et al. (2001) reported that LAB count of imported vacuum packages beef chuck stored at 0°C for 45 days was 4.57 $\log_{10}$ CFU/cm^2. This result agrees with those of Pennacchia et al. (2011) who found that LAB counts increased in vacuum packaged beef during storage at 4°C. The highest LAB count of 3.09 $\log_{10}$ CFU/g (day 35) and the lowest count (under the detection limit) since day 0 were detected in the VPS samples. The reduction in LAB number seen in the kafirin coated samples can be attributed to the inhibitory effect of kafirin coating solutions on spoilage bacteria. It was observed that kafirin coating in combination

with oregano and thyme essential oils (VPKOT5%) resulted in a constant value of below the detection limit until 21 days, except on day 7 which had a slight increase of around 0.10 $\log_{10}$ CFU/g. After 21 days of storage, the trend of LAB in VPK and VPKOT5% were similar. No significant effect on LAB number were observed throughout the storage period ($p > 0.05$) of the treatment of beef coated with kafirin alone (VPK) or kafirin with 5%OT incorporated essential oils (VPKOT). Emiroğlu et al. (2010) reported that there were no significant differences ($p > 0.05$) in LAB counts in samples of edible soy films with oregano or thyme essential oils added to them as numbers continued to increase over 12 days storage. Compared to all coating treatments, these results suggest that the slightly reduction numbers for VPK and a constant LAB counts over a period of 42 days storage at refrigeration temperature probably due to the antimicrobial effects of kafirin coating solution and essential oils (oregano and thyme). Similarly, Petrou et al. (2012a) reported a strong antimicrobial effect on LAB counts in chicken fillets over the entire storage period when treated with chitosan combined with oregano oil added to it in modified atmosphere packaged. Pavelková et al. (2014) reported 1.34 log CFU/g (day 15) and 1.43 log CFU/g (day 6) reduction of LAB population in chicken breast when stored dipped in oregano oil group, and thyme oil group and stored under vacuum packaging.

4.3.1.3 Enterobacteriaceae

All antimicrobial treatments significantly affected *Enterobacteriaceae* counts (Table 4.1). On the initial day (0) *Enterobacteriaceae* counts of all the treatment groups (VPB, VPK and VPKOT5%) were below the limit of detection, except for the VPS treatment which was at the limit of detection. The *Enterobacteriaceae* counts of all antimicrobial coating treatments remained below the limit of detection until the end of the storage period. Several authors reported similar trends using different coating material, packaging (VP or MAP) and meat (Kanatt et al., 2013; Karabagias, Badeka, & Kontominas, 2011; La Storia et al., 2012; Lorenzo & Gómez, 2012; Michalczyk, Macura, Tesarowicz, & Banas, 2012; Zivanovic, Chi, & Draughon, 2005). Pavelková et al. (2014) reported the similar behaviour of *Enterobacteriaceae* (0 log CFU/g) when oregano and thyme essential oils were coated on chickens and then vacuum packaged. In general, the initial values of *Enterobacteriaceae* were low and then

increased during the storage for vacuum packaging, particularly at the end of storage days. Lee & Yoon (2001) showed the *Enterobacteriaceae* count for imported beef was 5.11 $\log_{10}$ CFU/cm^2 under vacuum packages after 45 days of storage at 0°C.

The results of the present study indicated that kafirin coating was effective against the natural microflora found in fresh beef vacuum packaged when compared to the control group. Kafirin combined with essential oils treatments (VPKOT5%) was the most effective coating with respect to TVC, LAB and *Enterobacteriaceae* counts. The combination of essential oils in kafirin coating and vacuum packaged treatment is capable of affecting metabolic activity and growth of microbial in relation to the surface of fresh beef stored at 2 ± 0.5°C. The least effective treatment was vacuum packaged with solvent (VPS) treatment.

Table 4.1

Microbiological counts (log_{10} CFU/g) of natural microflora on fresh beef with different antimicrobial coating treatments followed by vacuum packaging and storage at 2°C.

Bacterial counts	Treatments	Storage period (days)						
		0	7	14	21	28	35	42
Total viable count (TVC)	VPB	3.07 ± 0.44^{Ab}	3.01 ± 0.21^{Ab}	3.20 ± 0.84^{Ab}	4.46 ± 0.27^{Aa}	3.39 ± 0.57^{Ab}	2.99 ± 0.90^{Ab}	4.78 ± 1.58^{Aa}
	VPS	LD	LD	LD	3.26 ± 0.59^{Ba}	LD	2.86 ± 0.91^{ABa}	2.79 ± 0.99^{Ba}
	VPK	LD	LD	LD	2.57 ± 0.75^{Ba}	LD	LD	LD
	VPKOT5%	< LD	LD	LD	LD	LD	LD	LD
Lactic acid bacteria	VPB	LD	LD	2.86 ± 1.23^{Ac}	5.08 ± 0.70^{Aa}	3.58 ± 1.11^{Ab}	2.84 ± 1.20^{Ac}	5.75 ± 0.14^{Aa}
	VPS	LD	LD	LD	2.72 ± 0.79^{Ba}	LD	3.09 ± 1.89^{Aa}	2.74 ± 1.03^{Ba}
	VPK	LD	LD	< LD	LD	LD	< LD	LD
	VPKOT5%	< LD	LD	< LD	< LD	LD	< LD	LD
Enterobacteriaceae	VPB	< LD	< LD	< LD	LD	< LD	< LD	< LD
	VPS	LD	< LD	< LD	LD	< LD	< LD	< LD
	VPK	< LD	LD	< LD	< LD	< LD	< LD	< LD
	VPKOT5%	< LD	< LD	< LD	< LD	< LD	< LD	< LD

Values are mean ± standard deviation of three replicate experiments.
A-C: Means in a column, within a treatment group (between treatment groups), not having a common capital superscript letter are different ($p < 0.05$).
a-c: Means in a row, within a storage period (between storage days) not having a common lowercase superscript letter are different ($p < 0.05$).
*LD: Limit of detection

4.3.2 Effect of antimicrobial coating treatments on the physicochemical of vacuum packaged fresh beef

4.3.2.1 pH

There was a significant decrease in pH of the meat ($p < 0.05$) over the storage period as well as a significant difference in pH of the meat between treatments (Table 4.2). During refrigerated storage, the initial pH of the fresh beef of different antimicrobial treatment groups VPB, VPS, VPK and VPKOT5% were 5.39, 5.47, 5.05 and 5.03, respectively. Sakala et al. (2002) reported the initial pH values of vacuum packaged fresh beef cuts stored at 2°C was 5.62 ± 0.04. Kafirin coating may affect the pH of the fresh beef as the pH of kafirin solution was adjusted to 5.00 during kafirin extraction. However, a pH of 5.00 for coating treatments can be considered as a low pH and may have an impact on microbial population (Burt, 2004; Seow et al., 2014). Emiroğlu et al. (2010) reported that pH (10.00) of soy-protein based film applied to the surface of beef patties caused an increase in the pH of the samples.

In general, the pH values of all treatment groups decreased during storage although the decrease was smaller for the VPB, VPK and VPKOT5% than for the VPS treatments ($p < 0.05$). All groups had statistically significant differences between them ($p < 0.05$) throughout storage. The pH values of three groups (VPB, VPS and VPK) decreased slightly during the first 7 days and then increased slightly after 14 days. VPKOT5% had a pH value that gradually increased to 5.10 after 2 weeks storage (14 days). At the end of the storage period, the pH values of the treated sample were slightly lower than control samples (VPB and VPS), with the VPK group having the lowest value (4.96). These results suggested an interaction between antimicrobial treatments and bacterial cell surface when coated with kafirin and essential oils. Burt (2004) reported that at low pH the hydrophobicity of essential oils increases, facilitating it dissolving more easily in the lipids of the cell membrane of target bacteria.

Han et al. (2014) observed a drop in the pH of antimicrobial coated films samples (containing cinnamon oil and rhubarb extract) from 5.82 at day 0 to in the range of 5.34 – 5.51 after 12 days of refrigerated storage. By contrast, Karabagias et al. (2011)

reported pH values of 6.6 on day 9 for lamb meat samples containing 0.1% thyme essential oil. Emiroğlu et al. (2010) found that the pH values of ground beef packaged with a combination of oregano and thyme essential oils in soy edible films during refrigerated storage decreased from 5.87 in the beginning to 5.69 over a storage period of 12 days. The decreasing meat pH may also related to the growth of lactic acid bacteria and other aerobic bacteria in most of the packaged meat (Bingol & Ergun, 2011; Han et al., 2014; Jones, 2004; Sakala et al., 2002).

Table 4.2

pH values of vacuum packaged fresh beef for different antimicrobial coating treatment groups during storage at 2°C.

Storage period (days)	VPB	VPS	VPK	VPKOT5%
0	5.39 ± 0.03^{Ab}	5.47 ± 0.09^{Aa}	5.05 ± 0.10^{Ac}	5.03 ± 0.04^{BCc}
7	5.33 ± 0.10^{Aa}	5.34 ± 0.02^{Ba}	4.99 ± 0.05^{BCb}	5.04 ± 0.04^{BCb}
14	5.38 ± 0.08^{Ab}	5.45 ± 0.05^{Aa}	5.02 ± 0.04^{ABd}	5.10 ± 0.05^{Ac}
21	5.33 ± 0.06^{Aa}	5.26 ± 0.07^{Cb}	4.91 ± 0.05^{Dd}	5.01 ± 0.06^{Cc}
28	5.33 ± 0.04^{Aa}	5.34 ± 0.09^{Ba}	4.94 ± 0.04^{CDc}	5.05 ± 0.01^{Bb}
35	5.17 ± 0.02^{Bb}	5.33 ± 0.08^{Ba}	4.96 ± 0.03^{CDd}	5.02 ± 0.02^{BCc}
42	5.24 ± 0.09^{Ca}	5.24 ± 0.03^{Ca}	4.96 ± 0.04^{CDc}	5.02 ± 0.03^{BCb}

*The mean ± standard deviation.
A-D: Means in a column, within a treatment group (between storage days), not having a common capital superscript letter are different ($p < 0.05$).
a-c: Means in a row, within a storage period (between treatment groups) not having a common superscript letter are different ($p < 0.05$).

4.3.2.2 Colour

Meat colour can influence consumer purchasing decisions and is an important element indicating freshness (Mancini & Hunt, 2005). Minimal colour change is therefore desirable when using an antimicrobial coating. The effect of specific treatments on the colour of vacuum packaged fresh beef during refrigerated storage are shown in Table 4.3. Changes in colour parameters L* (lightness), a* (redness) and b* (yellowness) showed significant differences ($p < 0.05$) between treatments, but no significant differences ($p > 0.05$) during the storage period.

The initial day (0) L* values for all three groups (VPB, VPK and VPKOT5%) were higher than those for VPS. The addition of kafirin coating to the fresh beef samples produced a higher L* values ($p < 0.05$) as compared to control (VPB) or VPS throughout the storage period. This may be due to the ability of kafirin to form transparent and bright polymers when it is used as coating material. According to Mancini & Hunt (2005) a pH ranged from 4.0 to 5.0 decreased the lightness of meat.

Values of a* (redness) for all treatments of beef samples varied with significant differences ($p < 0.05$) apparent among the group treatments examined. The initial a* values for VPB, VPS, VPK and VPKOT5% were 13.29, 13.15, 7.73 and 7.67, respectively. These indicate that the redness of fresh beef coated with kafirin alone or with essential oil had changed. With the increase of storage times, the a* values of all groups decreased. The a* values of VPB gradually fell to 12.10 on day 21 and 10 on day 35, indicating a loss of red colour. The redness value of group VPS and VPK increased to 13.46 (on 14 days) and 8.04 (on 7 days), respectively, and by the end of storage period to 10.15 for VPS and 5.64 for VPK. The addition of a combination of oregano and thyme essential oils in kafirin at a concentration of 5% resulted in a significant ($p < 0.05$) decrease in colour loss as compared to the control (VPB) and VPS groups during storage. The loss of redness could be due to the colour of the kafirin solution used to produce the coatings. Furthermore, the decrease in redness may be related to the pH of fresh beef treatments (Mancini & Hunt, 2005). Petrou et al. (2012) indicated that a decrease in a* value was related to a reduced redness of fresh meat owing to oxidation of myoglobin and formation of metmyoglobin. Metmyoglobin is formed when myoglobin, the dominant sarcoplasmic pigment in muscle, is oxidized and the resultant Fe^{3+} results in a brown or grey beef meat colour (P. Singh, Wani, Saengerlaub, & Langowski, 2011). Camo et al. (2011) reported that fresh beef steaks packaged with oregano extract resulted in greater colour loss, but active packaging with 1% to 2% oregano extract delayed metmyoglobin formation during storage of 23 days. Karabagias et al. (2011) reported that the colour of lamb meat added with thyme oil (0.1%) and applied MAP packaged changed with their a* values ranging from 6.61 (on day 1) to 7.39 (on day 25).

The b* value yellowness was significantly different ($p < 0.05$) among all groups (Table 4.3). The initial b* values for VPB, VPS, VPK and VPKOT5% were 16.08, 14.99, 14.22 and 14.50, respectively, but increased in three cases over the first 7 days of storage ($p < 0.05$), except for the VPKOT5% treatment. The VPS b* value appeared the highest at the end of storage compared to other groups ($p < 0.05$). The b* values of VPK and VPKOT5% was slightly lower than other group treatments. Zinoviadou et al. (2009) reported that the addition of oregano oil into WPI films increased the b* values during 2 days of storage of wrapped beef cuts at 5°C compared to the control samples. Petrou et al. (2012) reported that b* values were variable and not significantly different between dipping treatments with chitosan, oregano and their combination using modified atmosphere packaged on chicken breast meat stored at 4°C. The differences in L*, a* and b* values compared to other results reported in the literature, may depend on the type of meat used (chicken, beef, ostrich, etc.).

4.4 Conclusion

All treatments using kafirin coatings reduced the natural microbial population of beef. Kafirin coating based material affected the pH and redness of fresh beef, but successfully maintained fresh beef lightness. This study suggests a potential for kafirin coatings for use as antimicrobial coating and extending the shelf life of vacuum packaged fresh beef.

Table 4.3

Changes in *L**, *a**, *b** parameters of fresh beef with different antimicrobial coating treatments followed by vacuum packaging and storage at 2°C.

	Treatments	Storage period (days)						
		0	7	14	21	28	35	42
L*	VPB	49.79±5.07^{Ba}	48.87±2.04^{Ba}	48.72±1.74^{Ba}	49.49±2.27^{Ba}	46.27±2.32^{Bb}	46.78±1.50^{Cb}	46.16±2.11^{Bb}
	VPS	45.09±4.02^{Ca}	46.15±2.58^{Ca}	45.43±3.36^{Ca}	46.07±2.42^{Ca}	46.36±3.45^{Ba}	46.52±2.35^{Ca}	46.09±2.62^{Ba}
	VPK	54.86±2.89^{Aa}	54.34±3.90^{Aa}	54.09±2.84^{Aa}	53.28±2.53^{Aa}	52.86±1.90^{Ab}	53.40±2.44^{Aa}	51.81±3.54^{Ab}
	VPKOT5%	54.54±3.22^{Aa}	49.54±3.03^{Bb}	50.59±3.00^{Bb}	49.56±3.49^{Bb}	51.49±2.92^{Ab}	51.23±3.66^{Bb}	50.73±2.27^{Ab}
a*	VPB	13.29±1.26^{Aab}	14.26±2.16^{Aa}	13.98±2.44^{Aa}	12.10±2.91^{Ab}	12.35±1.62^{Bb}	10.00±1.56^{Bc}	9.84±1.53^{Ac}
	VPS	13.15±1.32^{Aa}	13.26±2.67^{Aa}	13.46±2.74^{Aa}	12.56±2.28^{Aab}	13.89±2.65^{Aa}	11.03±1.88^{Abc}	10.15±1.86^{Ac}
	VPK	7.73±1.09^{Ba}	8.04±1.43^{Ba}	6.19±0.82^{Bb}	5.79±0.75^{Bb}	5.67±0.59^{Cb}	5.96±0.61^{Cb}	5.64±1.01^{Bb}
	VPKOT5%	7.67±1.59^{Ba}	5.85±1.04^{Cb}	6.55±1.62^{Bb}	6.53±1.67^{Bb}	5.97±1.45^{Cb}	5.89±1.36^{Cb}	5.82±1.30^{Bb}
b*	VPB	16.08±1.21^{Aab}	17.22±1.72^{Aa}	17.06±1.83^{Aa}	16.89±1.76^{Aa}	15.31±1.71^{Abc}	14.71±1.33^{Ac}	14.25±1.77^{Bc}
	VPS	14.99±2.74^{Ba}	15.34±1.78^{Ba}	15.05±1.85^{Ba}	15.69±1.69^{Ba}	16.04±2.00^{Aa}	15.17±1.56^{Aa}	15.52±1.45^{Aa}
	VPK	14.22±1.45^{Ba}	15.14±1.16^{Ba}	13.75±2.24^{Ca}	13.91±0.84^{Ca}	13.91±1.05^{Ba}	14.41±1.03^{Aa}	13.62±1.42^{Ba}
	VPKOT5%	14.50±0.90^{Ba}	12.62±1.94^{Cb}	14.38±1.52^{Ba}	13.89±1.12^{Ca}	13.66±1.17^{Ba}	13.81±1.40^{Aa}	13.77±1.00^{Ba}

Values represent mean ± standard deviation.

A-C: Means in a column, within a treatment group (between treatment groups), not having a common capital superscript letter are different ($p < 0.05$).

a-c: Means in a row, within a storage period (between storage days) not having a common superscript letter are different ($p < 0.05$).

Chapter 5 : The Effects of Antimicrobial Biodegradable Kafirin Films and Coatings with Containing Nisin against *Listeria Monocytogenes*

5.1 Introduction

Processed meat products can be a source of foodborne infections. *Listeria monocytogenes* (*L. monocytogenes*) is a foodborne pathogenic bacterium that may cause a range mild to severe clinical symptoms, including fever, gastroenteritis, meningitis and central nervous system damage (Rhoades, Duffy, & Koutsoumanis, 2009). The transmission of this pathogen via food vehicles is of significant concern to risk categories such as pregnant women, immunocompromised patients and elderly people (Rhoades et al., 2009).

L. monocytogenes is a Gram-positive pathogen which is widely distributed in the environment, can survive at refrigeration and mild heat temperatures, is capable of growing in high salt environments and is therefore of major concern to the meat industry (Solomakos et al., 2008). As *L. monocytogenes* can survive and proliferate for long or short storage periods at low temperatures, a number of risks arise related to consumers' health (Kennedy, O'Rourke, McLay, & Simmonds, 2000; Pal, Labuza, & Diez-Gonzalez, 2008; Theivendran, Hettiarachchy, & Johnson, Michael, 2006). Food is prone to post-process contamination with *L. monocytogenes*, especially during slicing and packaging (Sofos & Georgnaras, 2010). Processed meats are considered to be of high risk for contamination by this pathogen.

Antimicrobial films and coatings containing antimicrobial agents are promising tools for inhibiting *L. monocytogenes* in/on meat products during storage (Abee, Krockel, & Hill, 1995; Cao-Hoang, Grégoire, Chaine, & Waché, 2010). Antimicrobials can be coated onto entire food surfaces by direct coating (e.g., the dipping method) or indirect coating, such as a wrapping method (Guo, Jin, & Yang, 2014a). Antimicrobial films or coatings containing antimicrobial agents have proven more effective than antimicrobial agents placed directly on food.

The addition of nisin into various types of film and coating materials greatly enhanced their antimicrobial activity against spoilage and pathogenic bacteria and extended the storage life of meat products (Deegan, Cotter, Hill, & Ross, 2006; Ercolini et al., 2010; Gadang, Hettiarachchy, Johnson, & Owens, 2008; Jiang, Neetoo, & Chen, 2011; Ku & Song, 2007; Nattress, Yost, & Baker, 2001; Nguyen,

Gidley, & Dykes, 2008; Pattanayaiying, H-Kittikun, & Cutter, 2015; Siragusa, Cutter, & Willett, 1999; Theivendran et al., 2006; Zhou, Xu, & Liu, 2010).

In chapter 4 kafirin coating material with essential oils incorporated in them successfully reduced the microbial population and extended the shelf life of vacuum packaged fresh meat. In this study, kafirin films and coating containing nisin were assessed for their ability to act against *L. monocytogenes* on processed meat. Specifically, the objectives of this study is to: 1) evaluate the antimicrobial properties of kafirin films with nisin against *L. monocytogenes in vitro* 2) and; investigate the efficacy of antimicrobial kafirin coatings containing nisin to control *L. monocytogenes* and other bacteria on the surface of processed beef meat storage at 4°C.

5.2 Materials and methods

5.2.1 Preparation of antimicrobial kafirin films with nisin

Commercial kafirin flour (Maralong Milling, Queensland, Australia) was extracted and defatted as described in chapter 2a (section 2.2.1 and 2.2.2). The isolated defatted kafirin containing 90% protein content (dry basis) was similar to that described in section 4.2.1.

The kafirin polymer films were prepared by the casting method described in 2a.2.3 using the following quantities of components: kafirin powder (8.45g), lactic acid (1.1g), glycerol (1.1g) and polyethylene glycol (PEG 400) (1.1g) in 45 mL aqueous ethanol (97%). Commercially available Nisin-S (N) (2.5% with sodium chloride, Siveele Company, Netherlands) was added to the kafirin film-forming solution (K) at the following concentrations: 0% (as control), 1%, 3%, 5% and 10% w/w. Each mixture was stirred until the nisin was completely dissolved in the kafirin film solution. Kafirin films without nisin were also prepared as controls. The films were prepared by casting 4.0 g on glass petri dishes (90mm) and drying overnight at 32 ± 2°C in a forced draft oven (Panasonic Biomedical, Leicestershire, UK) at 34 ± 2% RH to produce films of uniform thickness. The dried films were then peeled from the casting surface and directly assessed for antimicrobial activity test.

5.2.2 Antimicrobial properties

To determine whether the addition of nisin to kafirin films could improve the antimicrobial properties, the antimicrobial activities of the developed film samples were tested against *L. monocytogenes* using the agar diffusion method as described in 2a.2.5.1. The contact area of the films with agar surfaces were examined (Pranoto, Salokhe, & Rakshit, 2005a). Agar disc diffusion experiments were performed in triplicate.

5.2.3 Preparation of kafirin coating solutions with nisin

To prepare the kafirin coating solutions, defatted kafirin (132.82 g) and plasticiser (13.83 g) (1:1:1 w/w glycerol, lactic acid and polyethylene glycol 400) were dissolved in 97% aqueous ethanol (707.32 g). The mixture was constantly stirred with a magnetic stir bar over a hot plate until it boiled. The amount of aqueous ethanol lost during evaporation was added and the solution was then re-heated for an additional 10 minutes. The kafirin coating solution was filtered through a layer of cheesecloth and cooled at ambient temperature. The kafirin coating solution was divided into three parts: a kafirin coating solution without nisin was used as a control (KC) and two nisin concentrations of KN1% and KN10%. The concentrations used in this study were selected based on the results of antimicrobial properties of kafirin films containing nisin.

5.2.4 Inoculum preparation

The *L. monocytogenes* strain (NCTC 11994) from the culture collection at the School of Biomedical Sciences, Curtin University (Perth, Australia) was streaked for growth on Brain Heart Infusion Agar (BHIA, PathWest Laboratory WA, Perth, Australia) and incubated at 30°C for 24-48 h. For the preparation of inoculum, a single colony of this culture was transferred into a 500 mL Brain Heart Infusion Broth (BHIB, PathWest Laboratory WA, Perth, Australia) and incubated for 48 h at 37°C, to ensure the organisms grew well. The bacterial cells were pelleted by centrifugation

(Allegra® X-12R Centrifuge, Beckman Coulter, BREA, CA, USA) at 3270 x *g* for 15 min at 5°C. Supernatants were discarded and pellets were then resuspended in sterile PBS (pH 7.0) by vortexing. The cells were then washed with the same solution. A 500 mL aliquot of *L. monocytogenes* was used to inoculate the processed beef meat samples.

5.2.5 Evaluation of antimicrobial effect of the kafirin-coated on the growth of *Listeria monocytogenes* on processed beef meat

Further experiments were performed using kafirin-based coating containing nisin at concentrations of 1% and 10% to verify the diffusion of nisin into food systems, particularly processed beef meat. The previous antimicrobial activity tests showed that a nisin concentration of 1% in kafirin films had a minimal antimicrobial effect, based on the 'contact area surface with the agar' result (see results below). Whereas kafirin film containing nisin at concentration of 10% was selected as the maximum concentration of nisin added to the film forming solutions and which demonstrated a clear antimicrobial effect against *L. monocytogenes*.

5.2.5.1 Meat sample preparation

Vacuum packaged corned beef meats were purchased from a local butcher shop. Each 100 g serving consisted of protein (15.2 g), fat (1.4 g), saturated fat (0.6 g), sodium (1335 mg) and potassium (218 mg). The meat also contained salt, mineral salt, sugar, water, antioxidants, hydrolysed vegetable protein, flavour, flavour enhancers, vegetable oil, yeast extract, canola oil, colours, vegetable extract, spice and sodium nitrate. A commercial meat slicer was used to slice the corn beef samples to be approximately 1 cm thick. The samples were then placed on a polystyrene tray at the store before being transported to the laboratory in an insulated box, placed on ice and processed immediately upon arrival. At the laboratory, sterile knives and cutting boards were used to aseptically cut the beef slices into small pieces weighing approximately 10 g each. The samples were then refrigerated until inoculation.

5.2.5.2 Meat inoculation

The samples were randomly divided into three groups. The three groups contained beef pieces were dipped for 2 minutes at room temperature to ensure they were thoroughly covered with *L. monocytogenes* before being placed on disposal aluminium barbeque trays (29 cm x 19 cm x 5 cm) that had been swabbed with 70% ethanol and allowed to air dry for 15 minutes in a biosafety cabinet to allow bacterial attachment.

5.2.5.3 Applied antimicrobial coating treatments on processed beef

The inoculated beef pieces were divided and dipped for 1 min into on of three coating treatments: 1) kafirin coating alone (KC); 2) kafirin coating with 1% nisin (KN1%) and; 3) kafirin coating with 10% nisin (KN10%). All coated samples were placed on sterile disposal aluminium barbeque trays (29 cm x 19 cm x 5 cm) and air dried for about 30 min in a biosafety cabinet to ensure good coat drying. All of the sterile disposal aluminium barbeque trays used to place samples were wrapped twice with cling wrap and stored in a walk-in cooler at 4°C. The beef pieces samples were sampled every three days for 12 days and analysed microbiologically after inoculation (day zero) using the methodologies described below.

5.2.6 Microbiological analysis of inoculated beef samples

On each sampling day, the coated beef samples were aseptically transferred into an individual sterile stomacher bag (SARSTEDT, South Australia, Australia) using flamed forceps. Sterile 0.1% maximum recovery diluent (MRD) solutions (90ml) (pH 7.1) were added and homogenised for 1 min in a stomacher (Colworth, Stomacher 400, London) to make a 1:10 (w/v) dilution. Appropriate serial dilutions of the bacterial suspensions were prepared with 0.1% MRD solution (pH 7.1) and 0.1 ml of each dilution was inoculated onto an appropriate media using the spread plate method. Total viable count populations (TVC) were determined using plate count agar (PCA, PathWest Laboratory WA, Perth, Australia), after incubation for 48 h at

35°C. The *L. monocytogenes* count was determined by plating on *Listeria* selective agar (LSA, PathWest Laboratory WA, Perth, Australia) after incubation at 35°C for 24 h. Small black colonies with black haloes on the plates were counted (Jiang et al., 2011). Three coated beef samples were prepared for each coating treatment per day. The results were expressed as $\log_{10}$ CFU/g.

5.2.7 Statistical analysis

The antimicrobial activities of films (measured as inhibition zones) were reported as the means of three replicates. Six film discs were used for each replicate and a total of 18 measurements were made to determine the area of inhibition zones of the agar diffusion test. Data were analysed using a one-way analysis of variance (ANOVA), followed by a least significant difference (LSD) test. Work on the kafirin coating treatments of inoculated processed beef samples were independently replicated three times. The microbiological analysis results were transformed into log values. The analysis of variance (ANOVA) and the LSD procedures used to compare the means. Significant differences among the treatments were determined at the 95% confidence level ($p < 0.05$).

5.3 Results and discussion

5.3.1 Film characterisation

The kafirin films containing nisin were visually homogeneous with no signs of brittleness or bubbles and could be easily peeled away from the casting surface. However, the addition of nisin to kafirin films may have affected the clearness or transparency of the films, as their surfaces did were not smooth, particularly when higher amounts of nisin were added. These results are similar to those of Guo et al. (2014a), who reported that transparency of chitosan films containing nisin had cloudy appearance compared to control film without nisin. It appears that antimicrobial agent concentration and pH interactions could affect films transparency (Murillo-Martínez, Tello-Solís, García-Sánchez, & Ponce-Alquicira, 2013).

Although the kafirin film solution was produced at pH 5.0, some films still had poor transparencies. Murillo-Martínez et al. (2013) reported that WPI films containing nisin produced high transparent films (~95%).

5.3.2 Antimicrobial properties of films

Table 5.1 shows the antimicrobial activity of kafirin films containing nisin at various concentrations against *L. monocytogenes*. The antimicrobial properties of films were evaluated with respect to the inhibition zones or clear zones around the films and the contact area underneath the film discs following incubation. The inhibition zone differed significantly ($p < 0.05$) depending on the nisin concentration added to the kafirin films (see Table 5.1). As expected, control kafirin films without nisin added to them did not display antimicrobial activity around the film discs (inhibition zone = 0 mm^2). However, observation of the contact area revealed that kafirin films (KC) showed clear inhibition zones underneath film discs that were in direct contact with the *L. monocytogenes* on the agar surfaces. Giteru et al. (2015) evaluated the contact area of kafirin control films and found weak inhibition zones underneath film discs. It is therefore suggested that kafirin films (containing a mixture of plasticisers and aqueous ethanol) had better antimicrobial diffusion into the agar due to the hydrophobic nature of the kafirin protein and the hydrophilic nature of some ingredients used in the mixture (Belton, Delgadillo, Halford, & Shewry, 2006). The antimicrobial compounds most likely did not develop strong interactions with kafirin protein polymer, allowing them to release rapidly. In addition, kafirin film may possess the innate characteristic of antimicrobial activity is able to migrate (Giteru et al., 2015; Petersson et al., 2007). Pranoto et al. (2005a) showed that the addition of nisin into chitosan film inhibited *L. monocytogenes* growth underneath chitosan film discs.

Kafirin films containing 1% and 3% of nisin were not effective against *L. monocytogenes* (inhibition zone = 0 mm^2). The reason for this lack of antimicrobial action could be attributed to the low nisin concentration used in this study. According to Dawson et al. (2003), one of the main factors effecting nisin migration is the temperature during drying or cooling stages that usually varies and can cause

different arrangements and crystallinity of protein molecules in films. However, it has been suggested that the casting method did not affect nisin activity (Dawson et al., 2003). According to Hoffman et al. (1997), nisin can be stably heated up to 100°C. Another reason could be due to the reaction between plasticisers and nisin, or the interaction between nisin and kafirin film protein polymer which could affect and prevent the diffusion of antimicrobial agent in the film to the agar. The opposite occurred in the contact area results, with KN1% film discs demonstrating weak inhibition zones compared to control films. On the other hand, KN3% film discs formed clear inhibition zones underneath the film discs.

The results of the statistical analysis showed that when nisin concentration was increased to higher than 3%, film discs exhibited antimicrobial effect against *L. monocytogenes* ($p < 0.05$). Among them, the KN5% film disc revealed the highest inhibition zone and *L. monocytogenes* was significantly sensitive to it as compared to other film treatments ($p < 0.05$). However, increasing concentrations of nisin at higher concentration (KN10% film treatment) did not increase the antimicrobial activity against the bacteria tested. Even the inhibition zone of the KN10% film disc was considered small and weak when compared to the KN5% film treatment ($p > 0.05$). The pH used for forming kafirin films was 5.0 and should be suitable for stable nisin activity. However, it is recommended to use or produce film forming solutions at low pH (3.5 – 6) to ensure antimicrobial efficacy in the presence of zones of inhibition after casting (Dawson et al., 2003; H.-B. Lee, Noh, & Min, 2012; Mauriello, Luca, Storia, Villani, & Ercolini, 2005; Murillo-Martínez et al., 2013). Furthermore, kafirin film discs containing nisin at 5% and 10% experienced shrinkage. The diameter of the inhibition zone was accurate if it included the diameter of film disc. Although these inhibition zones are considered small, a strong inhibition zone was found at the contact area under film discs on the agar surface when the growth of *L. monocytogenes* was inhibited.

Several studies have shown increased antimicrobial activity against *L. monocytogenes* or *Listeria* spp. by nisin when added to other polymer films such as zein, chitosan, tapioca starch and whey protein isolate films (Ku & Song, 2007; Ming, Weber, Ayres, & Sandine, 1997; Padgett, Han, & Dawson, 1998; Pranoto, Rakshit, & Salokhe, 2005b). The results of this study may be differed due to the

different protein films used. Further investigation is required to verify this phenomenon.

The area of inhibition zones and release of nisin depends on several factors, including the type of polymer used (hydrophobic/hydrophilic), the ability of antimicrobial agents to diffuse and migrate, the temperature applied during storage or process (drying/casting, cooling and incubation) and the low or high pH of the film forming made as discussed above (Karam et al., 2013; Xiao, Davidson, & Zhong, 2011b). In the present study, the antimicrobial activity of kafirin-based films appeared to be proportional to the concentration of the nisin added. Thus, it is important to determine an effective film and nisin concentration that should be applied to real food products.

Table 5.1

Antimicrobial activity of kafirin films formulated with nisin against *L. monocytogenes*

Film treatments	Diameter inhibition zones (mm^2)	Contact area
KC	0.00 ± 0.00^{a}	++
KN1%	0.00 ± 0.00^{a}	+
KN3%	0.00 ± 0.00^{a}	++
KN5%	5.42 ± 0.47^{c}	+++
KN10%	4.18 ± 0.72^{b}	+++

Film treatments: KC (kafirin film alone, the control) and KN (kafirin film with nisin).
Reported values are the mean ± SEM (*n*=18). Zone of inhibition with different lowercase letters in the same column indicate significant differences ($p < 0.05$).
+ Weak inhibition zone underneath film disc; ++ clear inhibition zone; +++ strong inhibition zone present.

5.3.3 *Effect of antimicrobial kafirin-based coating treatments on the growth of bacteria on processed beef meat during storage*

As described in Section 5.2.5, processed beef pieces were inoculated with *L. monocytogenes* and coated with antimicrobial kafirin-based coatings. Bacterial survival was analysed on days 0, 3, 6, 9 and 12 days of storage at 4°C. All antimicrobial coating treatment groups effectively reduced numbers of *L. monocytogenes* on the processed beef meat samples over the storage period of 12 days (Table 5.2). Samples coated with kafirin alone (KC) showed a gradual decrease in the *L. monocytogenes* population with reduction of 2.95 $\log_{10}$ CFU/g by the end of storage days and significant reduction in the bacterial population ($p < 0.05$) between the storage days. A previous study showed that adding ethanol, lactic acid, glycerol and polyethylene glycol 400 into coating solution could improve the antimicrobial performance (Han, 2005). The drop in numbers of this pathogen induced by the KC coating treatment suggested that the coating solution may contain natural active compounds (Afify et al., 2012; Giteru et al., 2015).

The decrease in the KN1% treatment group was more drastic compared to the other coating treatment groups after 3 days of storage, corresponding to 1.42 $\log_{10}$ CFU/g reduction in cell population of *L. monocytogenes*. This was followed by KC and KN10% coating treatment groups with drops of 1.07 $\log_{10}$ CFU/g and 0.95 $\log_{10}$ CFU/g, respectively, detected on day 3 ($p < 0.05$), followed by a slow reduction until day 12. A similar trend was observed in relation to the processed beef meat samples inoculated with *L. monocytogenes* which decreased by 2.67 $\log_{10}$ CFU/g over the 12 days storage period compared to the initial day (day 0) ($p < 0.05$) when coated with KN1%.

Nisin at the maximum concentration of 10% in the kafirin coating formulation (KN10%) resulted in a significant reduction of *L. monocytogenes* populations during the earlier period of the storage (day 0) in comparison to other coating treatments ($p < 0.05$). The mean population of *L. monocytogenes* inoculated on samples decreased significantly from an initial inoculum of 6.23 $\log_{10}$ CFU/g on day 0 to 3.12 $\log_{10}$ CFU/g on day 9 of storage ($p < 0.05$). Nisin may have initially been released at a moderate rate and then diffused efficiently until reaching its maximum on day 9 after which the antimicrobial concentration during may have become less or nearly

depleted. Consequently, the population of *L. monocytogenes* on meat samples increased slightly to 3.21 $\log_{10}$ CFU/g by the end of the storage period. It seems that the KN10% treatment was the most effective coating treatment as compared to the others ($p < 0.05$), as the lowest number of *L. monocytogenes* were observed on meat samples stored at 4°C on day 12. However, there was no significant difference ($p > 0.05$) between storage days 9 and 12 in bacterial counts.

It should also be noted that the effectiveness of antimicrobial kafirin-based coating treatment groups can generally be ranked in the order of KN1% > KC > KN10%. In this study, it was observed that kafirin coating alone and kafirin coating containing different concentrations of nisin were able to diffuse into meat samples tested. The possible explanation for this situation is the interaction between the constituents of the kafirin polymer and the antimicrobials itself. Further, it was shown that the KC and KN1% coating groups showed slowly released and diffused antimicrobial effect with resulting in slower suppressed *L. monocytogenes* growth on processed beef meat samples during storage. It is evident that antimicrobial coating relies on its capability to control the diffusion rate from the coating layer into the food product. It is essential to control or optimise the release rate of the antimicrobial agent to maintain the concentration of antimicrobial agent over the storage period to match the growth kinetics of the target bacteria and maintain the efficiency of antimicrobial packaging (Han, 2005; Jiang et al., 2011; Nguyen, Gidley, & Dykes, 2008). These findings suggest that kafirin coatings may perform in a similar way to kafirin film result (in relation to the result of contact area underneath the film discs and have been mentioned in Section 5.3.2), which can be also arranged from KN1% > KC > KN10%. The behaviour of the *L. monocytogenes* population after being coated with kafirin-based treatments depends on the nisin concentration and temperature during the storage period.

Nisin acts on the cytoplasmic membrane of sensitive strains to cause lesions (Chen et al., 2016; Montville & Chen, 1998). Previous research has shown that nisin can be efficiently added to various packaging (films and coatings) to control foodborne pathogen in different foods, especially meat. In addition, numerous studies have shown that nisin is also effective at 4°C storage. Theivendran et al. (2006) reported the addition of nisin into soy protein coating on turkey frankfurters significantly

lowered the population of *L. monocytogenes* when stored at 4°C by reducing 2.1 log CFU/g when compared to control coating. Similarly, Nguyen et al. (2008) showed that increasing nisin (2500 IU/ml) in cellulose film sharply decreased the L. monocytogenes counts on frankfurters, to approximately 2 log CFU/g after 2 days of storage at 4°C. Gadang et al. (2008) indicate the effectiveness of whey protein isolate (WPI) coating containing nisin (6000 IU/g) combined with malic acid and grape seed extract successfully reduced the cell population by 3.2 log CFU/g after 28 days stored at 4°C. These results imply that antimicrobial kafirin-based coating treatments were able to reduce or control the *L. monocytogenes* population during storage.

Table 5.2

Effect of kafirin-based coatings containing nisin against surface inoculated *L. monocytogenes* on processed beef meat stored at 4°C.

Antimicrobial Coating treatments	Storage period (days) (log_{10} CFU/g)				
	0	3	6	9	12
KC	7.11 ± 0.25^{Aa}	6.04 ± 0.20^{Ab}	5.02 ± 0.12^{Ac}	4.94 ± 0.50^{Ac}	4.16 ± 0.07^{Ad}
KN1%	7.07 ± 0.29^{Aa}	5.65 ± 0.12^{Bb}	4.90 ± 0.36^{Abc}	4.45 ± 0.33^{Ac}	4.40 ± 0.73^{Ac}
KN10%	6.23 ± 0.28^{Ba}	5.28 ± 0.06^{Cb}	5.12 ± 0.17^{Ab}	3.12 ± 0.13^{Bc}	3.21 ± 0.26^{Bc}

Abbreviations of coating treatments: KC (Kafirin coating alone) and KN (Kafirin coating with nisin). Values are the means ± standard deviations across the three replicate experiments.
A-B: Means in a column, within a treatment group (between coating treatment groups), that do not have a common capital superscript letter are different ($p < 0.05$).
a-d: Means in a row, within a storage period (between storage days) that do not have a common lowercase superscript letter are different ($p < 0.05$).

The total viable counts (TVC) were also determined to investigate the potential implication of kafirin-based coatings without and with nisin in extending the shelf life of processed meat. Table 5.3 shows the effect of antimicrobial kafirin coating treatments on the survival and growth of the TVC population on processed beef meat samples throughout storage at 4°C. Kafirin coating treatments without any antimicrobials showed an initial TVC of 7.40 log_{10} CFU/g on day 0 and this increased slightly by 0.76 log_{10} CFU/g after day 3, but then decreased 0.11 log_{10} CFU/g and 0.34 log_{10} CFU/g on days 6 and 9, respectively. However, the coating

treatment (KC) increased the TVC population by 0.53 $\log_{10}$ CFU/g in meat samples by the end of storage period.

The KN1% coating treatment did not demonstrate a change in the initial TVC until day 3, which then increased after 6 days of storage to ~ 0.5 $\log_{10}$ CFU/g. The addition of 1% nisin to the kafirin coating showed no significant difference ($p > 0.05$) over the storage period.

Increasing the nisin concentration to 10% into the kafirin coating did not significantly ($p > 0.05$) reduce the TVC present. KN10% coated meat samples displayed an increase of more than 0.5 $\log_{10}$ CFU/g from the initial inoculation level of day 0 to day 6 of storage. However, the TVC population on processed beef samples with KN10% coating decreased continuously between days 9 and 12 of storage to 0.12 $\log_{10}$ CFU/g and 0.22 $\log_{10}$ CFU/g, respectively. The rate of increase in TVC was higher in the control than for the kafirin coating treatment groups containing nisin ($p < 0.05$), even though final numbers of TVC populations were not different across both coating treatment groups (KN1% and KN10%) ($p > 0.05$).

The TVC remained at levels of >7 $\log_{10}$ CFU/g for the duration of 12 days storage at 4°C. The maximum acceptable level for food is 6-7 $\log_{10}$ CFU/g. Nisin is not effective against Gram-negative bacteria, molds and yeasts (De Arauz, Jozala, Mazzola, & Vessoni Penna, 2009). Generally, Gram-negative bacteria cells are resistant to nisin as to their outer layers are composed of lipopolysaccharidic (LPS) that create a barrier to the nisin action on the cyctoplasmatic cell wall (De Arauz et al., 2009). The results suggest that these three surface coating treatments could not inhibit TVC growth. Three hypotheses may explain this situation: (i) Kafirin coating (KC) surface was hydrated accommodating a suitable environment for the growth of bacteria; (ii) there was low diffusion or release of nisin from the coating into processed beef samples (food); (iii) nisin antimicrobial activity could be lost due to the inactivation of the nisin by food components, especially fat and protein. Aasen et al. (2003) reported that 80% of incorporated nisin could be lost due to the interactions with protein in foods.

Table 5.3

Effect of kafirin-based coating treatments incorporating nisin on the growth of TVC on processed beef meat stored at 4°C.

Antimicrobial Coating treatments	Storage period (days) (log_{10} CFU/g)				
	0	3	6	9	12
KC	7.40 ±0.00^{Ab}	8.16 ±0.17^{Aa}	8.05 ±0.57^{Aa}	7.71 ±0.54^{Aab}	8.24 ±0.10^{Aa}
KN1%	7.40 ±0.00^{Aa}	7.40 ±0.00^{Aa}	7.88 ±0.44^{Aa}	7.65 ±0.44^{Aa}	7.63 ±0.40^{Ba}
KN10%	7.14 ±0.44^{Aa}	7.40 ±2.00^{Aa}	7.74 ±0.59^{Aa}	7.62 ±0.50^{Aa}	7.40 ±0.00^{Ba}

Abbreviations of coating treatments: KC (Kafirin coating alone) and KN (Kafirin coating with nisin). Values are the means ± standard deviations across the three replicate experiments.
A-B: Means in a column, within a treatment group (between coating treatment groups), that do not have a common capital superscript letter are different ($p < 0.05$).
a-b: Means in a row, within a storage period (between storage days) that do not have a common lowercase superscript letters are different ($p < 0.05$).

There have been limited studies investigating the efficacy of antimicrobial kafirin coating treatments with nisin against *L. monocytogenes* and spoilage bacteria on inoculated meat products. Further work is needed to clarify the nisin release from kafirin-based films and coatings, investigate antimicrobial effects on other food-related bacteria and specify the nisin modes of action on the microbial cell. Additionally, variables such as concentration and combinations of other antimicrobials should be evaluated. The results of such studies could provide an effective and promising approach to meat system preservation in the future.

5.4 Conclusion

There is great potential to enhance the antimicrobial properties of kafirin films through the incorporation of nisin. Kafirin films containing nisin at 5% and 10% produced strong inhibition zones against *L. monocytogenes*. Antimicrobial kafirin-based coating treatments with nisin and without nisin significantly reduced *L. monocytogenes*, but did not effectively control TVC, on processed beef meat samples throughout storage periods at 4°C. Overall, antimicrobial kafirin-based films and coatings can be applied to meat products to inhibit or control foodborne pathogens during storage.

Chapter 6 : General Discussion and Conclusions

6.1 Major findings and contribution of this study

The focus of this study was to investigate the potential of kafirin as an antimicrobial film or coating to improve meat safety and prolong the shelf life of beef meat during chilled storage. The main aims of this study were to develop antimicrobial films and coatings from kafirin-based materials containing antimicrobial agents (oregano and thyme essential oil, alone and in combination of both essential oils, and nisin) at various concentrations, and to study their effects on antimicrobial activity and film properties, as well as their efficacy in controlling microbial and foodborne pathogens and also their physicochemical characteristics during storage period.

Based on the results of this study a number of conclusions can be drawn. Preliminary evidence indicating a low content of residual hexane in zein and kafirin films were obtained (Chapter 2a). It can be concluded that both films are suitable to be used for food packaging. A casting method using a force draft oven was successfully developed to produce antimicrobial zein and kafirin films. Zein films containing OEO, TEO and OTEO at low concentrations of 0.1%, 0.5%, 1.0% and 1.5% did not display any antimicrobial activity towards *E. coli*, *B. thermosphacta*, *S. aureus*, *L. monocytogenes*, *L. sakei* and *P. aeruginosa*, but small zone of inhibitions for kafirin films containing oregano (OEO) and a combination of both essential oils (OTEO) were observed against *B. thermosphacta* and *P. aeruginosa* (Chapter 2a). Furthermore, zein films loaded with OEO demonstrated antimicrobial effects against four bacteria (*B. thermosphacta*, *S. aureus*, *L. monocytogenes* and *E. coli*) compared to OTEO and TEO at higher concentrations (Chapter 2b). The addition of essential oil did not improve film structure. Further work should be focused on the amount of plasticizer and essential oil used and further testing against different food spoilage or pathogenic bacteria.

The antimicrobial activity of kafirin films containing OEO, TEO and OTEO were not evaluated previously in any literature. OTEO was found to be the most effective antimicrobial agent followed by OEO and TEO when incorporated into kafirin films (Chapter 3). The additions of OEO and OTEO into kafirin films are able to produce antimicrobial activity against meat spoilage and pathogenic bacteria, *E. coli*, *B. thermosphacta*, *S. aureus*, *L. monocytogenes* and *L. sakei*, depending on the concentration and type of essential oil used (Chapter 3). The presence of

antimicrobial compounds, such as carvacrol, thymol, *p*-cymene, D-limonene and ɤ-terpinene, in oregano or thyme essential oils were positively identified (Chapter 3). The presence of phenolic compounds may be responsible for cell membrane disruption and lead to leakage the cell internal contents (Calo et al., 2015). Zein or kafirin films loaded with OEO, TEO and OTEO were ineffective against *P. aeruginosa*. Similarly zein and kafirin control films did not display any antimicrobial effects against the six bacterial strains tested (Chapter 2a and 3). Based on the findings above, this study indicates kafirin films containing OEO and OTEO are potential alternative antimicrobial films to control or eliminate meat spoilage and pathogenic bacteria. It was also established that the different batches of kafirin may influence some film characteristics such as colour (Chapter 3).

The incorporation of 5% OTEO into kafirin films was found to improve antimicrobial, physical, mechanical and barrier properties (Chapter 3). Films containing different essential oil and concentrations were not investigated in this study. Antimicrobial kafirin coating treatments containing 5% concentration of oregano and thyme essential oils in combination with vacuum packaged (VPKOT5%) were effective in reducing the natural microbial population of fresh beef during storage at 2°C (Chapter 4). This method is a promising preservation technique for fresh beef. However, this study showed that kafirin coating treatments combined with vacuum packaging affect pH and redness colour of fresh beef.

To investigate the potential of coatings and films against foodborne pathogenic bacteria, and particularly *L. monocytogenes*, kafirin film and coating treatments were supplemented with nisin (Chapter 5). This work is the first to demonstrate the activity of kafirin-based films containing nisin against *L. monocytogenes*. It was also established that the antimicrobial effect of kafirin films containing 5% and 10% were relatively weak was measured by diffusion parameters but that strong inhibition zones were found under both film discs. Literature has shown that nisin can enhance the antimicrobial activity of films by effecting the cytoplasmic membranes of Gram-positive bacteria and causing lesions and cell death (Montville & Chen, 1998). Further research to investigate the effect of kafirin-based coatings containing nisin on inoculated processed beef meat samples demonstrated that kafirin-based coatings with nisin significantly reduced *L. monocytogenes*, but not TVC during storage at

4°C for up to 12 days. It can be suggested that kafirin coatings with nisin could be an effective alternative for controlling foodborne pathogens during storage of processed beef meat.

Overall, these findings contribute to the literature on the effect of essential oils and nisin in kafirin-based films with respect to antimicrobial activity, physical, mechanical and barrier properties, and beef meat preservation. Based on the conclusion above it can be suggested that kafirin films and coatings treatments with oregano and thyme essential oil also nisin should be effective against meat spoilage and pathogenic bacteria to some extent on fresh and processed beef meat.

6.2 Future directions

This study provides some understanding of the role of antimicrobial kafirin films and coatings containing essential oil or bacteriocin against meat spoilage and pathogenic bacteria. The ability of antimicrobial kafirin coatings to reduce microbial populations during different storage, antimicrobial agents, beef meat and packaging treatment were also established. Potential research areas for future studies on these films are suggested below.

6.2.1 Study of the effects of kafirin films containing essential oils at lower concentration

The antimicrobial activity of oregano and thyme essential oil alone or in combination in kafirin-based films demonstrated effective at high concentrations (Chapter 4). In future research, the antimicrobial effects of these antimicrobial agents and kafirin-based films should be further explored at lower concentrations particularly if they are to be commercialized.

6.2.2 Identification of the interactions of essential oil with kafirin films

Fourier transforms infrared (FTIR) spectra could be used in the future to verify and investigate the types of interaction between kafirin polymer matrix and essential oils that occur.

6.2.3 Study the effects of nisin in kafirin films with regards to antimicrobial agent concentrations and physical, mechanical and barrier properties

Kafirin films with nisin incorporated showed antimicrobial activity against foodborne pathogens but also displayed film shrinkage at higher nisin concentrations (Chapter 6). Further research is needed to clarify the effect of high concentrations of nisin added to kafirin films. It is also important to investigate kafirin films containing nisin with regards to their mechanical, barrier and optical characteristics in order to apply as food packaging and may enhance film properties.

6.2.4 Usage of wider range of food bacterial and pathogenic strains

Kafirin films with essential oils and nisin were found to be active against a limited range of bacteria in this study reduced (Chapter 6). It is important to investigate the antimicrobial activity of kafirin films against other food spoilage and pathogenic bacterial strains and species to make sure the effects observed are not limited to the bacteria tested.

6.2.5 Study the effects of kafirin-based film pH and during storage

The antimicrobial efficacy of kafirin films could be affected by film solution pH and temperature. As reported by Hosseinnejad and Jafari (2016) the antimicrobial activity could be influenced by film pH as essential oils can efficiently be dissolved in the lipids of cell membrane of bacteria. It is therefore appropriate to investigate the film solution pH and temperature during storage. In addition, the pH and colour of

samples coated with kafirin-based with nisin during storage should be assessed in order to determine the antimicrobial efficacy of these films for a longer period of storage.

6.2.6 Identification of films microstructure properties

Film structures can be influenced by the addition of antimicrobial agents or plasticizers, therefore further confirmation of good dispersion of oregano and thyme essential oils or nisin in antimicrobial films should be determined using techniques such as scanning electron microscope (SEM) (Sun, Wang, Kadouh, & Zhou, 2014).

6.2.7 Sensory evaluation

A knowledge of the effects of essential oil (oregano and thyme essential oils) and nisin on the organoleptic properties of beef samples would be valuable. Essential oil used at high concentration may have an impact on sensory properties of the vacuum packaged fresh beef (Chapter 5). In future research, consumer acceptability and sensory evaluation should be conducted during storage of fresh beef and processed beef meat samples (Chapter 5) in terms of flavour, colour and odour.

6.2.8 Application to food systems

It may be sensible to investigate the effects of antimicrobial agents incorporated into kafirin-based films or coatings against food spoilage and pathogenic bacteria on various foods such as other meat, fresh fruit or vegetables.

6.2.9 Antioxidant effects

The antioxidant effects of kafirin films containing oregano and thyme essential oils could also be further investigated.

6.2.10 Antifungal properties

The antifungal effects of the films containing oregano and thyme essential oils as well nisin could be explored in the future.

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