Evaluation of a Liquorice Extract as a Novel Antimicrobial

BY

Madiha El Awamie

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PH.D. RESEARCH STUDENT
DIVISION OF FOOD SCIENCE
SCHOOL OF BIOSCIENCES

SUPERVISORS: DR. CATHERINE E. D. REES

ABSTRACT

The foodborne disease listeriosis is rare but has a high mortality rate therefore it is a major concern for the food industry. It is caused by the Gram-positive bacterium *Listeria monocytogenes* which can survive mild food preservation conditions, (e.g., high salt and low pH), and its psychrotrophic nature means that it can grow at refrigeration temperatures. Hence listeriosis is associated with the consumption of a broad range of ready-to-eat (RTE) foods, including sliced deli meats. Liquorice extract is common a food flavouring that is known to have antimicrobial activity and this project was designed to investigate the antimicrobial activity of a waste product produced during the production of liquorice flavourings for the food industry.

The antimicrobial efficacy of the liquorice extract was used to challenge different types of bacteria to determine whether this food by-product had antimicrobial activity. A range of both Gram-positive and Gram-negative bacteria were tested and it was found that the extract was only effective against Gram-positive bacteria suggesting that the extract had a similar mode of action in each case with a concentration of 50 μg ml^{-1} resulting in total growth inhibition, therefore this was defined as the MIC. The use of bacterial strains containing plasmids expressing bioluminescence revealed that at sub-MIC concentrations (12.5 μg ml^{-1}) the metabolic state of the cells was reduced, indicated that membrane integrity was affected. The mechanism of action of the liquorice extract was further investigated using a fluorescent LIVE/DEAD® stain. Exposure to the MIC for this extract (50 μg ml^{-1}) resulted in total growth inhibition and the presence of non-viable cells was detected using fluorescence microscopy. In addition, fluorescent microscopy revealed the formation of long filaments consisting of cells that had not completed the cell division process, which indicates that cell wall synthesis was also affected. In contrast no antimicrobial effect or

toxic effect was detected when the extract was used to treat Gram-negative bacteria or the yeast *Saccharomyces cerevisiae*.

When applied to the surface of sliced deli meat, inhibition of *Listeria* growth was seen for up to 10 days, hence the extract has the potential as a natural food preservative and could be of use to improve the safety of this type of RTE product. To confirm that the extract was safe to use as a food preservative, the mutagenic potential of the extract was assessed using the Ames test for detecting carcinogens and mutagens by using different strains of *Salmonella* Typhimurium including TA100, TA102 and TA1535, no adverse results were gained. In addition *in vitro* cytotoxicity was measured using Caco-2 cells and the MTT cell viability assay. At the MIC concentration, some cytotoxicity was detected, and this requires further investigation but these results could indicate the potential for use of liquorice extract an anti-cancer agent. Overall the findings of this research suggest that the liquorice extract has a specific effect on Gram-positive bacteria and further work could lead to its use as an antimicrobial agent to control the growth of these bacteria either in food systems or in other settings.

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LIST OF ABBREVIATIONS

Amp	Ampicillin
ATP	adenosine triphosphate
BHI	Brain Heart Infusion
BPW	Buffered Peptone Water
Caco-2	Human colonic adenocarcinoma cells
ºC	Degrees Celsius
CFU	Colony Forming Unit
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
FMNH	flavin mononucleotide
Ery	Erythromycin
F.W	Formula weight
FBS	Foetal Bovine Serum
g	gram
GFP	Green Fluorescent Protein
GM	Glucose Minimal Agar
h	hours
KDa	kilodalton
l	Litre
LM	*Listeria monocytogenes*
log	Logarithm
Lux	Bacteria Luciferase Gene
*lux*AB	Bacterial Luciferase
μ	Specific Growth Rate
MBC	Minimum bactericidal Concentration
MDR	Multidrug Resistant
μg	microgram
mg	milligram

MIC	Minimum Inhibitory Concentration
min	minute
mm	micromiter
µl	microliter
ml	mililiters
MRD	Maximum Recovery Diluent
MTT	*3*-(*4*,*5*-dimethylthiazol-*2*-yl)-*2*,*5*-diphenyltetrazolium bromide
NaCl	Sodium Chloride
OD_{600}	Optical Density at a wavelength of 600 nanometers
PBS	Phosphate Buffered Saline
pSB	Plasmid Sutton Bonington
% (v/v)	percentage volume per volume
% (w/v)	percentage weight per volume
PI	Propidium Iodide
RLU	Relative Light Units
RO water	Reverse Osmosis
rpm	Revolutions Per Minute
TSB	Tryptic Soy Broth
RTE	Ready-to-Eat
SDS	Sodium Dodecyl Sulphate
Tet	Tetracycline
TMTC	Too much to count
x *g*	Relative Centrifugal Force
⌀	Diameter
MSC	Biological Safety Cabinet
wt	Wild type

LIST OF ORIGINAL PUBLICATIONS

1-EL AWAMIE, M. & REES, C. 2016. Identification of the Antimicrobial Effect of Liquorice Extracts on Gram-Positive Bacteria: Determination of Minimum Inhibitory Concentration and Mechanism of Action Using a *luxABCDE* Reporter Strain. *World Academy of Science, Engineering and Technology, Int. J. Med., Health, Biomed. Bioeng. Pharmaceut. Eng.*, 10, 294-302.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

According to The World Health Organization (2011) approximately 55 million people died worldwide, and one-third of these deaths were due to infectious diseases. In the developing world, infectious diseases remain the main cause of morbidity and mortality (Nabavi *et al*., 2015). The definition of foodborne illnesses by The World Health Organization (WHO), these are diseases caused by agents that enter the body through the ingestion of food which are either infectious or toxic in nature (WHO, 2015). Serious foodborne disease and poisoning are a widespread and growing public health problem and considered a major contributor to human illnesses, hospitalizations, and deaths each year. According to data from the Centers for Disease Control and Prevention (CDC) it is estimated that about 48 million cases of foodborne diseases occur each year in the United States (about 1 in 6 Americans) resulting in 128,000 hospitalizations and 3,000 deaths annually (Baraketi *et al*., 2018; CDC 2011). Losses due to medical expenditure and loss of productivity associated with foodborne illness are estimated to be approximately $152 billion in the United States per year. Although different countries have made significant progress in decreasing the incidence of foodborne disease. Hence for society and the food industry, outbreaks of human disease have a considerable effect on the economic situation (Ivanek *et al*., 2005) and therefore the problem of food borne disease has been given more attention by both Governments and food industry (Le Loir *et al*., 2003; Choffnes *et al*., 2012).

Over the last few decades, several new microbiological problems in food safety have arisen due to the changes in life style, such as an increase in the consumption of ready-to-cook and ready-to-eat products (Kotzekidou, 2013), meaning that foodborne pathogens have become an increasing concern due to contamination of such processed products. This phenomenon is not restricted to one product type, and an increase in infections associated with meat, fresh fruits and vegetables and dairy products has been seen, caused by bacteria such as

Listeria monocytogenes, *Campylobacter jejuni* and *Escherichia coli* (Harris *et al*., 2013). This is not a localised problem and health hazards posed by microbial pathogens in food are of major concern world-wide because circulation of contaminated food between countries increases the chance of outbreaks of foodborne disease caused by imported products as well as outbreaks associated with more locally produced foods. According to International trade statistics produced by the World Trade Organization (WTO, 2015), food represents 42 % of total world exports, and levels have increased 6 % per year since 2010, and so food is a substantial and increasing area of trade and therefore represents and ever increasing risk to human health. Although generally in developed countries the safety of food has dramatically improved overall, foodborne outbreaks from microbial contamination are still prevalent in many countries, and failure to discover the presence of pathogenic microorganisms especially in the food industry may lead to serious effects on human health (McLinden *et al*., 2014). In addition to the risk of infection, microbes that cause food spoilage can secrete toxins which also impacts on the safety of food. Therefore antimicrobials that can eliminate or delay microbial growth are important to maintain the quality and safety of food products (Davidson *et al*., 2013).

Foodborne diseases have an impact on economic as well as public health. For instance approximately 1.8 million deaths every year caused by diarrhoeal disease mostly in developing countries, and a large proportion of these cases can be attributed to contamination of food and drinking water (WHO, 2004). However, levels of foodborne disease are generally under-reported as most are self-limiting and not reported to the health care system, therefore it is difficult to measure the impact of this accurately (Van de Venter, 2000). In addition to the impact on the consumers, the food industry also faces large problems when bacterial contamination causes outbreaks of food borne infection since these can lead to

loss of consumer confidence, and loss of income. In addition there is also an increasing chance of litigation if an outbreak of disease occurs.

To address these problems, there is an increased emphasis in the food industry to produce safe food through the application of food preservation techniques. Regulatory agencies and consumers are generally more concerned about the microbial safety of foods and the control of foodborne pathogens, but there is also a drive to reduce food spoilage. Some bacteria may bring about desirable changes in food, such as during the production of fermented foods, but there is also a relationship between spoilage of food and microbial activity in terms of the production of metabolic compounds that lead to changes in the taste and colour that are not acceptable for the consumer, resulting in an in economic loss to the industry. This is also a world economic issue since the Food and Agriculture Organization (FAO) of the United Nations reports that one-third of food produced for human consumption is lost or wasted globally, amounting to approximately 1.3 billion tons of food lost per year. This represents a waste of the resources used for the production of the food, including land, water, energy and inputs, and increasing greenhouse emissions without producing any benefit (FAO, 2011).

For the control of microbial spoilage many of methods have been used to reduce contamination levels in food products, such as fermentation, pasteurization or the addition of antimicrobials. The use of heat, chemical compounds, and UV radiation to minimize microbial load may expose food to changes in a taste which are important to consumer acceptability (Lau *et al*., 2014). However the problem of food spoilage is still a challenge leading to high levels of food waste and a solution has not yet been found (Raybaudi-Massilia *et al*., 2009). To make this task more difficult, consumers are demanding products which are less processed and more natural or fresh (Roman *et al*., 2017; da Silva Dannenberg *et al*., 2016) and so the food industry is looking for alternative ways to produce safe food. Hence the use lower amounts of additives in foods has been investigated by the food

industries but it is important that these changes do not affect the quality of food, since preservatives such as salt, sugar and organic acids also contribute to the taste perception of the food, and this is considered to be the most important concern of consumer.

Natural preservatives have been used as alternatives to traditional chemical preservatives, however a limited number have been commercially developed and many remain to be investigated as sources of safer and effective antimicrobials. Therefore, alternative sources of safe, effective and acceptable natural preservatives need to be explored (Lau *et al*., 2014; Negi, 2012). Many plant-derived antimicrobial compounds have been found to have activity against foodborne bacteria and thus, they could be used as natural preservatives in foods (Cho *et al*., 2008). The ability of some Gram-positive bacteria to produce spores poses a particular problem, as their ability to resist tolerate extreme environmental conditions, such as heat and chemical treatments, make them more difficult to eliminate than vegetative cells of the same organisms. Therefore, in addition to targeting the vegetative cells present in a food, agents to prevent the germination the spores into vegetative cells under appropriate condition is an area of concern since outgrowth of these spores can lead to food spoilage and foodborne diseases (Fernández-No *et al*., 2011). Therefore, many natural products such as plant extracts, essential oils and organic acids to ensure food safety and control of bacterial spores have been investigated as alternative sources of antimicrobials (Burt, 2004; Chouhan *et al*., 2017).

In different commercial sectors, including medicinal, the use the plant products growing dramatically due to the variety in phytochemical compounds that have been identified in different species of plants (Al-Terehi *et al*., 2015a). It is estimated the plant species present on Earth number 250,000 to 500,000 and only a small proportion have been explored as sources of useful natural products until now (Negi, 2012; Srujana *et al*., 2012). Plant products have been traditionally

used in folk medicine as a medicinal plant in different countries of the world to treat many diseases including bacterial infections. Geetha and Roy (2012) indicates that the same plants were used by people in different regions of the world to treatment of many ailments for many centuries. As a result of the development of resistance to antibiotics by some types of bacteria caused by the misuse of antibiotics (Li and Webster, 2018), this has also led researchers to try and discover new antimicrobials from novel sources, such as plants. Consequently, researchers have established that herbal remedies such as clove, oregano, thyme, cinnamon, and cumin are effective against a wide range of microorganisms and can be used to treat infectious diseases or protect food because they were shown to possess antimicrobial activities against pathogenic and spoilage fungi and bacteria (Chouhan *et al*., 2017). However it is important to know the chemical constituents of the plants and to screen them *in vitro* for any unwanted toxicity before they are used as therapeutics (Geetha and Roy, 2012) since, in addition to many antimicrobial substances, plants are also known to produce many of the most toxic compounds known to man (Patel *et al*., 2013).

What makes these plant extracts particularly good candidates as sources of food preservatives is that they are commonly used as a flavourings in food products and also as natural food preservatives in many countries and, therefore, the use of plant extracts with known antimicrobial properties can be of important significance in food preservation. The impact of plant extracts on microorganisms is known to be due to the presence of a wide range of chemical ingredients including polyphenols, flavonoids, tannins, alkaloids, terpenoids, lectins, polypeptides which produce inhibitory effects on the growth a range of bacteria, moulds and yeast (Edeoga *et al*., 2005; Cowan, 1999). Hence many studies have been carried out on plant products in different countries worldwide to examine the performance and efficacy these products against many types of microorganisms (Negi, 2012).

1.2 *Listeria* spp. and *Listeria monocytogenes*

Until recently the genus of *Listeria* included 17 species (Orsi and Wiedmann, 2016). However, recently a new species (*Listeria costaricensis*; Núñez-Montero *et al*., 2018) was identified and therefore the genus now contains 18 species. Within the different species of *Listeria*, *L. monocytogenes* and *L. ivanovii* are the only ones considered to be pathogenic. *L. monocytogenes* is an important human foodborne pathogen and is considered to be the third major cause of foodborne deaths in the USA due to contamination of food with this microbe (Scallan *et al*., 2011). Although all strains of *L. monocytogenes* have the ability to cause serious diseases in humans and a broad variety of animals (Drevets and Bronze, 2008). Doumith *et al*. (2004) and Zhang and Knabel (2005) reported that *L. monocytogenes* serotypes 1/2a, 1/2b, and 4b are responsible of the vast majority of human listeriosis. In contrast, *L. ivanovii* can rarely cause infections in humans and is primarily regarded as an animal pathogen, mainly causing disease in sheep and cattle, but their pathogenic potential means that these two species are the best studied members of the group (Graves *et al*., 2010; Leclercq *et al*., 2010). According to European laws regarding Microbiological Criteria for food products (Commission Regulation EC 2073 / 2005), *L. monocytogenes* is the pathogenic bacterium that particular attention should be paid to because many challenges exist for achieving control of *L. monocytogenes* in the food chain such as able to survive and grow in harsh conditions including low temperatures, dry environments and over a wide range of pH values (Bertrand *et al*., 2016).

All members of the genus are small rod-shaped, Gram-positive bacteria that are prevalent everywhere in the environment including soil, decaying plants, sewage, dust, and water. Due to this extensive distribution, *L. monocytogenes* and other *Listeria* spp. regularly contaminate food processing plants, with high risk areas being recognised including receiving, handling, and processing areas (Ryser and Marth, 2007; Weller *et al*., 2015). Although low temperature is normally used

to prevent the growth of bacteria in such environments, *Listeria* spp. are psychrotrophs and therefore have the unusual ability to adapt to low-temperature conditions. It can grow over a wide temperature range between 3 and 45 °C which also means it can grow to dangerous levels in refrigerated foods. The physical state of the lipids in the membrane of the bacterial cells in the form of crystalline liquid is considered important for the preservation of the membranes structure and their function to all enzymes activity and transport of solutes across the membrane (Beales, 2004). To allow growth to continue when the temperature is reduced below 7 °C changes need to occur in the to maintain the liquidity of the membrane. In *L. monocytogenes* one of the main changes seen is an increase in the proportion of C15:0 (Pentadecanoic is a saturated fatty acid) lipid at the expense of C17:0 (Margaric is a saturated fatty acid), as well an increase in the degree of unsaturated fatty acids, which helps enhance the liquidity of the membrane and allow growth to continue at low temperatures (Hsu *et al*., 2011). Another adaptation seen is that when grown at temperatures between 20−25 °C, *Listeria* spp. are actively motile by peritrichous flagella and the production of flagella help the cells attach to food surfaces (Vatanyoopaisarn *et al*., 2000). When grown at temperatures between 30-37 °C, *Listeria* strains are non-motile because the organism does not have the ability to synthesize flagella due to lack the expression of the flagellin proteins at this temperature (Way *et al*., 2004). This is another example of the adaptive processes that *Listeria* spp. undergo when they transition between being an environmental organism and a bacterium adapted to growth in animal hosts.

As a facultative anaerobe, *Listeria* has the ability to multiply rapidly in aerobic or microaerophilic conditions and it can also tolerate and grow over a wide range of pH levels, with growth reported to occur from pH 4.1 to 9.6 (Lungu *et al*., 2009). It is also desiccation-resistant and can tolerate low moisture levels and can form biofilms making it resistant to disinfection. All these features

demonstrate that it has a great adaptability to different environments, which explains why the bacterium can colonize food production equipment and environments. Consequently, these bacteria have been shown to persist for months to years in food-processing plants, acting as a possible source of cross-contamination of product. Indeed, contamination of food products with *Listeria* has been identified at many different stages of food production including slicing, packaging and chill-storage. Thus, control and elimination of this type of bacteria from food products is the best strategy to ensure food safety (Vázquez-Boland *et al*., 2001).

1.2.1 Incidence of listeriosis in humans

Amongst foodborne bacterial pathogens, infections caused by *L. monocytogenes* have the highest hospitalization and mortality rates (Scallan *et al*., 2011). Infection by this bacterium results in the disease listeriosis that can have a number of different serious consequences including meningitis, meningo-encephalitis and septicaemia and is distinguished by a high mortality rate of up to 30 % and represents a significant public health threat (Weller *et al.,* 2015; Painter and Slutsker, 2007). Infection is generally seen in people with weakened immune systems, including pregnant women, infants, and the elderly. With rising consumption of RTE foods or frozen foods which require minimal heat treatment before consumption, the risk of listeriosis has increased due to food processing plants often using conditions that can be tolerated by *Listeria,* such as refrigeration, low pH, or high salt concentration to preserve foods. Moreover, *L. monocytogenes* is known to persist by adhering to food contact surfaces, leading to the post-process contamination of final food products (Ferreira *et al*., 2014). The severity of the diseases caused by this bacterium means that there is a high economic cost associated with infection and therefore it is a major concern for the food industry (Raghu, 2013; Olszewska *et al*., 2015). In the United States *L.*

monocytogenes is considered to be of one of the most important causes of diseases transmitted through foods, where it listed among the top five most important pathogens (Gialamas *et al*., 2010; Theinsathid *et al*., 2012), and the health costs associated with listeriosis was estimated at $2.6 billion per year (Hoffmann *et al*., 2012). Similarly in Canada the costs associated with the cases (including medical and nonmedical costs and productivity losses) were estimated to be nearly $242 million Canadian dollars (Thomas *et al*., 2015).

The incidence of listeriosis has increased in both Europe and North America over the last few decades with at least 13 illness outbreaks associated with RTE meat products and dairy products between 1987 and 2008 where *L. monocytogenes* was identified as the leading cause of infection (Lara-Lledó *et al*., 2012). For instance, during an outbreak in Canada in 2008 related to the consumption of contaminated delicatessen meat, 57 cases of listeriosis were reported, resulting in 24 deaths, and was one of the biggest outbreaks of listeriosis in Canadian food history (Thomas *et al*., 2015). Despite the lessons learned from this case, the difficulty faced by food companies ensuring this bacterium does not contaminate products continues to be an issue. For instance *L. monocytogenes* (LM P93) was found to be responsible for 38 infections, resulting in the death of 5 people, in a food poisoning listeriosis outbreak in cheese made from pasteurized milk in Canada in 2012 (Gaulin *et al*., 2012). Other recent notable *Listeria* outbreaks have been associated with sliced melons in Texas in 2010 (146 cases and 31 deaths; Gaul *et al*., 2012) and in Australia another outbreak associated with melons resulted in 20 cases and 7 deaths (WHO, 2018). Recently in January 2017, South Africa has been affected by the largest outbreak of *L. monocytogenes* infections recorded, which caused a death of at least 200 people and further confirmed infections of 1024 people since the outbreak was discovered (WHO, 2018). Hence *Listeria* is regarded by the food industry as a major cause for concern

(Montañez-Izquierdo *et al*., 2012), and therefore it was chosen in this study as a key organism for testing the efficacy of a novel antimicrobial.

According to the data published by the Public Health England in 2017, 135 cases of listeriosis were reported in England and Wales, representing a 17.7 % decline compared to the previous 6 year period (2010- 2016; Figure 1.1; PHE, 2018). An outbreak of listeriosis has been identified across Europe between August 2016 and June 2018 with cases identified in a number of different countries, including Austria, Denmark, Finland, Sweden, and the United Kingdom. This outbreak was linked with the consumption of frozen corn and other frozen mixed vegetables that had not been cooked before being consumed. As of 15 June 2018 it was reported that there were a total of 47 cases of listeriosis associated with this outbreak which caused nine deaths, 23 cases were revealed in Finland, 11 in United Kingdom, 7 in Sweden, 14 in Denmark and 2 in Austria (Authority *et al*., 2018).

Figure 1.1. Human cases of *L. monocytogenes* in England and Wales 2008-2017

	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017
Incidence rate per 100 000 population	0.33	0.38	0.29	0.26	0.30	0.28	0.29	0.29	0.31	0.23
Number of reported cases	180	209	159	147	168	160	169	167	179	135

2008 - 2017

Data from the Public Health England showing human listeriosis caused by *L. monocytogenes* in England and Wales from 2008 to 2017. Data shows that a decrease occurred in 2017 compared to that recorded in the previous 6 years between 2010-2016 (adapted from PHE, 2018).

1.2.2 *Listeria monocytogenes* as a food pathogen

L. monocytogenes is a foodborne pathogen that is almost exclusively transmitted to humans through consumption of contaminated food, although zoonotic and person-to-person (mother-to-child) infections also occur (Rees *et al*. 2017). As it is a non-spore forming bacterium, the utilization of high temperature treatment of products will eliminate the pathogen, therefore the major concern is contamination of the final product with *Listeria* before packaging, and the psychrotrophic nature of the bacterium means that levels of contamination can increase to high levels over weeks or months of refrigerated storage (Nørrung *et al*., 2009; Sofos and Geornaras, 2010). Therefore many different techniques are

employed to inhibit the spread and survival of *L. monocytogenes* in food products. Its unusual biology – being able to grow equally well in soil or in the GI tract of animals or within food production facilities – means that it is associated with a broad range of ready-to-eat (RTE) foods. *L. monocytogenes* have been discovered in a broad variety of food types including raw and processed foods. For instance in open markets in China it has been reported that *L. monocytogenes* was routinely isolated from raw pork (Luo *et al*., 2017). Fresh produce including pepper, radishes, cabbage, seafood and fish products, milk and dairy products, meats and meat products such as beef and sausages, have also all been associated with *Listeria* contamination (Lianou and Sofos, 2007). As mentioned above, *L. monocytogenes* has the ability to survive in the food-processing and produce-packing environment and equipment. Hence, cross-contamination of product by *Listeria* is a significant concern for the food industry (Montañez-Izquierdo *et al*., 2012), especially because of their ability to survive and grow in a wide range of environmental conditions (Zhu *et al*., 2017), as reviewed above, which amplifies the problem.

1.2.3 Contamination by *L. monocytogenes* in the food industry

As illustrated by the two outbreaks described above, ready-to-eat meat products pose the most serious threat to the public and deli meats are one of the RTE foods which represent a high rate of risk of infection and death caused by *L. monocytogenes* (Gallagher *et al*., 2003). This is due to the high risk of contamination after the heat treatment due to the high number of processing steps after cooking (slicing and packing) and also the fact that these products are not cooked before being consumed which would inactivate any *Listeria* contamination (Barmpalia *et al*., 2005). Although thermal treatments are used in the production of meat products such as bologna-style sausage implicated in the South African outbreak mentioned earlier, contamination of these products by *L. monocytogenes*

can occur from bacteria that have become resident in the food processing environment during slicing and packaging (Swaminathan and Gerner-Smidt, 2007; Espitia *et al*., 2013) as *L. monocytogenes* has the ability to attach to several food-contact surfaces including stainless steel, polystyrene and glass (Di Bonaventura *et al*., 2008). As *L. monocytogenes* can survive well in the food environment, it can also colonise slicing machines which can then lead to large scale contamination of product by spreading this organism on the food contact surface (Beresford *et al*., 2001; Frye *et al*., 2002; Gormley *et al*., 2010).

Since contamination of RTE meat products is very difficult to control, to prevent the growth of organisms in order to ensure the safety and extend the shelf-life of these products, several techniques have been used such as packaging in a controlled atmosphere, packaging using activated films, non-thermal pasteurisation treatments and irradiation are employed (Davidson *et al*., 2013). However, although all of these treatments could help to protect food from the growth of pathogenic *Listeria*, on the other hand they will also have an impact on the product in terms of quality by causing changes in the organoleptic characteristics of foods, such as loss of colour or flavour and which reduces consumer acceptability especially as consumers are now demanding products that are less processed. Therefore chemical food preservatives are required, but even these when used at levels acceptable to the consumer cannot entirely eliminate food pathogens such as *L. monocytogenes* or entirely delay microbial spoilage (Gutierrez *et al*., 2009).

1.3 The use of plant extracts as antimicrobials

Herbal remedies have been used for many centuries for the treatment of a range of illnesses, particularly in developing countries but has also been favoured in some developed countries such as Germany, France, Italy and the United States in order to help maintain health (Murray and Pizzorno Jr, 2000). The increase in

medicines derived from medicinal plants is estimated at about 25 % over the past decade. Today herbal drugs are used by 75-80 % of the world's population, especially in developing countries, where there is significant use of medicinal plants in the healing of ailments, and there is also still a dependency on traditional medicines and folk treatments for their healthcare needs (Kamboj, 2000). Medicinal plants grow in the wild and are wide spread all over the world, and since they are easily obtainable and inexpensive they are considered an important source of income for native and rural populations (Robinson *et al*., 2011).

While scientific research plays a major role in the validation of conventional treatments, there has been an emphasis on identifying the biological activity of plant extracts and the active ingredients used in traditional medicines (Tanaka *et al*., 2009). Many plants with biological and anti-microbiological properties have been studied since there has been a relevant increase in the incidence of antibiotic overuse and misuse and the emergence of new resistant strains of bacteria (Saxena, 2005). Thus there has been a resurgence of interest in the search for compounds which are derived from natural sources and used against bacteria (Sedighinia and Afshar, 2012; Hatano *et al*., 2000; Irani *et al*., 2010). Moreover, about 60 % of the drugs available on the market, such as anti-tumour and anti-microbial drugs are derived mainly from natural products for medicinal plants (Bhatnagar and Kim, 2010; Lv *et al*., 2011).

Primary metabolites produced by plants are those known to have an essential role in plant metabolism, whereas secondary metabolites are those where no role has yet been found in growth, photosynthesis, reproduction, or other "primary" functions. Although they may have essential functions for their producers that have not yet been identified, they could also just be waste products of primary metabolism. Plants produce a high diversity of bioactive secondary metabolites, a great number of which serves to protect themselves against microbial, insect and animal attacks (Wink, 2018; Tiwari *et al*., 2009), and include

antioxidants and antimicrobial agents which enable the plants to survive different sorts of biological challenges. The bioactive ingredients which are considered to have the most significant role in these medicinal plants include alkaloids, tannins, flavonoids and phenolic compounds (Nirmala and Selvaraj, 2011). Thus, food poisoning microorganisms and spoilage microorganisms whose metabolic end products or enzymes cause off-odours and off-flavours, as well as texture problems and discoloration, are considered the main targets for antimicrobials derived from plants that can be employed as food preservatives, such as essential oils extracted from different plants including garlic, onion, thyme, marjoram, rosemary and oregano (Swamy *et al*., 2016).

1.3.1 Use of natural antimicrobials for food preservation

The global issues of foodborne disease and food spoilage mean that it is necessary to find a variety of antimicrobial agents to control the growth of food pathogens (Mastromatteo *et al*., 2010; Hereu *et al*., 2012; Holck *et al*., 2011). Although many chemicals are used as preservatives to control foodborne pathogens as well as to extend the shelf-life by inhibiting spoilage organisms, there is increasing concern about adverse effects of such compounds on human health and so there has been a growing trend towards replacing synthetic preservatives by natural ones. As a result of the potential health risks associated with synthetic additives, and increase awareness of consumers, this has led to increased interest in use natural alternatives (Ekor, 2014).

Plants that have a relatively high level of antimicrobial action may be sources of compounds that inhibit the growth of foodborne pathogens (Ibrahim *et al*., 2006). For instance it is known that essential oils and plant extracts have been used for thousands of years for many purposes such as food preservatives as well as being used as medical and pharmaceutical products to prevent diseases. Zhang *et al*. (2009) studied the antibacterial properties of essential oils from different

plants, including liquorice, and tested them against four common meat spoilage and pathogenic bacteria (*L. monocytogenes, Escherichia coli, Pseudomonas fluorescens* and *Lactobacillus sake*) using modified atmosphere packaged, fresh pork and vacuum packaged ham slices. Their results showed that individual extracts of various plants, including liquorice, contained strong antibacterial activity, but the mixture of rosemary and liquorice extracts (1:1, v/v) was found to be the best inhibitor against all four types of microbes when compared with other spices. Since these herb extracts are widely used in the food industry and are generally regarded as safe, consequently, they may be considered as natural preservatives tolerable by the food industry. Thus, the use of natural antimicrobials such as essential oils and plant extracts could be an excellent alternative to guarantee food safety (Appendini and Hotchkiss, 2002; Lucera *et al*., 2012; Del Nobile *et al*., 2012). Accordingly there has been an increase in research focussing on natural antimicrobials to apply to food products to extend shelf-life by inhibiting microbial growth (Lanciotti *et al*.,2004; Fattouch *et al*., 2007), either focussing on inhibiting/controlling the growth of pathogenic organisms (food safety) or on eliminating the growth of spoilage microorganisms (food preservation) (Tajkarimi *et al*., 2010).

1.4 Effect of natural compounds on bacterial cells

Many natural compounds are not yet utilized commercially and are still under investigation. The antimicrobial compounds in plant materials can be located in different parts of plant, such as essential oil commonly found in leaves (rosemary, sage, basil, oregano, thyme, and marjoram), seeds (caraway, fennel and parsley), flowers or buds (clove), bulbs (garlic and onion), rhizomes (liquorice) or other parts of plants (Gutierrez *et al*., 2008; Burt and Reinders, 2003; Douglas *et al*., 2004).

Some antimicrobial compounds, such as phenols, flavones, and flavonoids, tannins, alkaloids, aldehydes, isoflavonoids and terpenoids which have been shown to possess potent antibacterial activity are known to delay or inhibit the growth of bacteria, yeast, and molds, through affecting the structure of the bacterial cell, which is considered a target for action by these components of natural products. Although, the action of natural antimicrobials against both Gram-positive and Gram-negative bacteria may signal the existence of broad-spectrum antibiotic components or metabolic toxins, generally plant extracts are found to have a much more significant inhibition effect against Gram-positive than Gram-negative bacteria (Srinivasan *et al*., 2001; Tegos *et al*., 2002). For instance (Fiamegos *et al*., 2011) discovered that plant derived compounds had a better effect against Gram-positive bacteria such as *Staphylococcus aureus, Enterococcus faecalis* and *Bacillus cereus* than it did on Gram-negative bacteria, and it has been suggested that Gram-negative bacterial may have natural multidrug resistance to many antimicrobial compounds due to the presence of efflux pumps which confer antibiotic resistance on these types of cells (Piddock, 2006).

Consequently, the mechanisms of action of bacterial agents depends on the type of microorganisms which may be linked to their cell wall structure as well as the presence of an outer membrane. It is known that the cell membrane acts as a selective barrier which allows some small molecules to pass through and excludes others, although the bacterial cell can also control the access of these molecules to the cell. Therefore increased membrane permeability is a critical factor in determining the antimicrobial action of many compounds, although antimicrobial agents may not have to cross the membrane to reach their target site, but rather cause membrane damage, resulting in loss of membrane integrity and eventually leading to cell death (Wu *et al*., 2016).

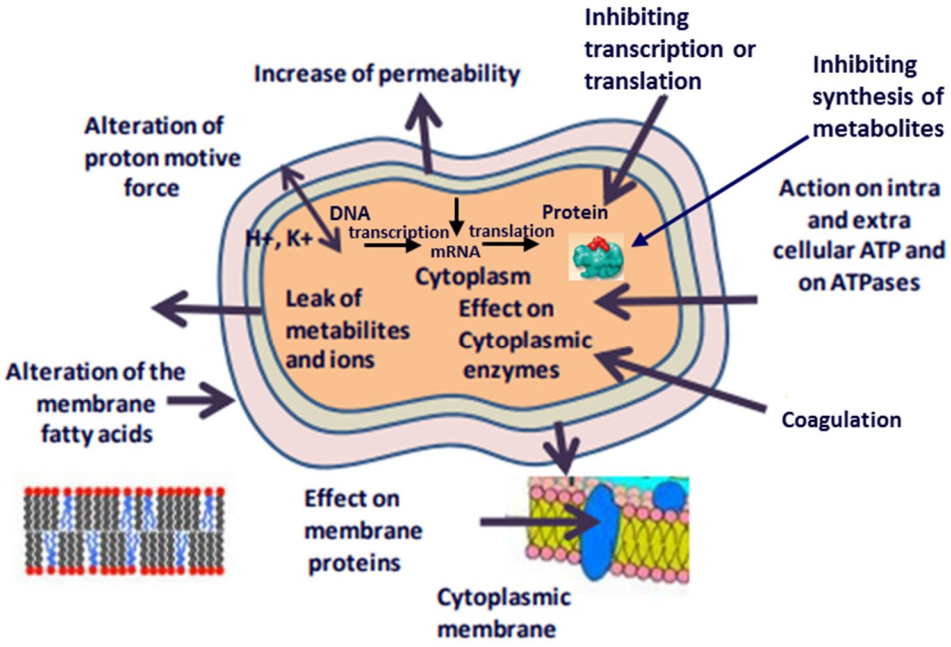
Thus, the difference in cell structure and membrane arrangement between Gram-positive and Gram-negative bacteria may account for differences in sensitivity to different antimicrobials (Bechinger and Gorr, 2017; Wu *et al*., 2016). In general, due to the lipopolysaccharide layer of the outer membrane, Gram-negative bacteria are less sensitive to the antimicrobial agents due to the ability of the outer membrane to limit the diffusion of the hydrophobic compounds. Hence the action of bactericidal agents that interfere with biosynthetic or metabolic functions of cells will be affected by the physical and chemical properties of the chemicals and whether or not they can cross the membrane to reach their target sites to either kill or inhibit the cells. However, it does not mean that Gram-positive bacteria are always more susceptible (Burt, 2004). For instance it has been found that essential oils were more effective against Gram-positive than Gram-negative bacteria (Ceylan and Fung, 2004). The same is true also for many spices and herbs (Shan *et al*., 2007). Another study using the disc-diffusion method confirmed that compounds with phenolic groups, such as oils of clove, oregano, rosemary, thyme, sage, and peppermint, are effective antimicrobials, and were more inhibitory against Gram-positive than Gram-negative bacteria (Kozłowska *et al*., 2015).

Plant antimicrobial compounds have been found to cause disruption of a number of different target sites including the cytoplasmic membrane, by disruption of the phospholipid bilayer. Phytochemical constituents of several extracts have been shown to have antimicrobial activity, which is probably due to the combined effects of adsorption of antibacterial agents with bacterial membranes which cause membrane disruption and dissipation of the proton motive force (PMF) leading to a non-energised membrane which subsequently leads to leakage of cellular contents (Farha *et al*., 2013). Another target are the enzymes of the electron transport system and active transport systems, again resulting in cells with reduced energy levels. Cota *et al*. (2013) reported that the various biologically active compounds found in plants diffuse across the cytoplasmic membrane and

compete for the active sites of particular enzymes inside the cell, thereby prevent cell growth. Inhibition of the ATPase syntheses which lead directly to induce the alteration in the energy state of the bacterial cells and leads to cell death, is caused by plant-derived natural product such as phenolics, flavonoids, quinines, tannins, coumarins, alkaloids, terpenoids and polypeptides (Gill and Holley, 2006a; Gill and Holley, 2006b; Upadhyay *et al*., 2014).

Alternatively they may cause denaturation of cellular components resulting in coagulation of cytoplasmic contents (Figure 1.2; Burt, 2004) or they may compromise the genetic material of bacteria leading to mutations and cell death (Burt *et al*., 2007; Lanciotti *et al*., 2004; Arqués *et al*., 2008; Proestos *et al*., 2008). Cell wall synthesis, metabolic pathways, protein synthesis, nucleic acid synthesis, can all be inhibited by the mechanisms of action of antimicrobial agents such as essential oils (Kherallah, 2002). However some bacteria have mechanisms which help to pump out harmful substances to protect themselves against these antimicrobial compounds. Bacteria can also gain plasmids which encode genes that confer resistance to certain antibacterial compounds or encode additional efflux pumps. Interestingly many active constituents derived from plants act as efflux pump inhibitors (EPIs) and specifically block this resistance mechanism particularly for Gram-positive bacteria (Chérigo *et al*., 2008; Kumar *et al*., 2008; Prasch and Bucar, 2015).

Figure 1.2. The mechanism of action of antibacterial agents on the structure of bacteria at different locations.



Mechanisms and sites of action of antibacterial agents to destroy the microorganisms and disrupt or damage to this structure which lead to leakage of important solutes (adapted from Nazzaro *et al*., 2013). Schematic represents a Gram-positive cell type with a thick layer of peptidoglycan which contains teichoic acids; in Gram-negative bacteria an outer membrane and thin layer of peptidoglycan replaces the thicker layer of peptidoglycan forming the cell wall.

1.4.1 Measuring the susceptibility of bacteria by using different methods

Different laboratory techniques can be applied to examine or screen the antimicrobial activity of an extract or a pure compound. When carrying out the evaluation of antimicrobial agents, researchers routinely use the agar dilution, broth microdilution or disk diffusion assays to measure the *in vitro* susceptibility of bacteria. These are the standard methods recommended by the Clinical and Laboratories Standards Institute (Baraketi *et al*., 2018).

The broth microdilution and agar dilution methods are used to gain quantitative results such as minimum inhibitory concentration and provide qualitative estimations of the sensitivity of different cells types which result in classifications of susceptible, intermediate resistance or resistant. However, the disk diffusion method is not convenient for testing non-polar samples or samples that do not easily diffuse into agar but it is often used as a first screen despite the fact it is unable to accurately determine minimal inhibitory concentrations (MIC; defined as the lowest concentrations of the test compounds that prevented visible bacterial growth) and is not quantitative, so, it is impossible to quantify the amount of the antimicrobial agent diffused into the agar medium. The broth microdilution method can be routinely used due to it being easier to perform and is less time-consuming, whereas the agar dilution method is more time consuming, and is unsuitable to be carried out in most of clinical microbiology laboratories (Kobayashi *et al*., 2004; Cos *et al*., 2006). There are different ways to define the end-point in broth dilution studies, but the most utilized methods are optical density (OD) (turbidity) measurements or the viable count. The first method has the advantage of being automatable using instruments such as plate readers (Stevenson *et al*., 2016) whereas viable counts are more accurate but are more labour intensive (Biesta-Peters *et al*., 2010).

Additional in depth studies about the effect of an antimicrobial on the growth of pathogenic microorganisms can be achieved through the use of a number of different techniques including the time-kill test and flow cytofluorometric methods which give real information on the nature of the inhibitory effect whether bactericidal or bacteriostatic as well as determining whether its action is dependent on the time or concentration and also shows if there is any damage inflicted on the microorganism during treatment with the antimicrobial (Balouiri *et al*., 2016).

1.5 The liquorice plant

Liquorice is derived from the dried roots and rhizomes of *Glycyrrhiza* species (Leguminosae family). The Leguminosae family, also known as Fabaceae, is one of the most widely utilized families for medicinal purposes and an important natural sweetening agent; the Fabaceae family represents a patrimony of high biodiversity known for their abundance of secondary metabolites, which are of especial importance for human health (Wink, 2013). This family includes about 727 genera and 19327 species and contains many genus, including *Glycyrrhiza* distributed over on the tropical and moderate areas which have been shown to be effective against different strains of bacteria (Lewis, 2005). The plant is found to be distributed in the subtropical and hot districts in the world such as Asia, the Mediterranean and regions of southern Europe (Saxena, 2005).

In medical treatments liquorice plant is known as a one of the major plants that has a long history of use in both the medical and culinary spheres due to the fact that it contains flavonoid and phenolic compounds as well as coumarins, volatile oil, saponine and minerals. Extracts are used as spices and flavours for various food products as well as effective drugs for many applications in folk medicine, especially in Africa and Asia (Yamamura *et al*., 1992; Al-Terehi *et al*., 2015b). Several diseases ranging from simple infections to serious diseases such as skin diseases, asthma, malaria and other infections use several plant species including liquorice plant as treatments (Roshan *et al*., 2012; Aggarwal *et al*., 2015). The first documented medicinal use of liquorice can be traced back 4000 years to ancient Assyrian, Egyptian, Chinese and Indian cultures, so it is considered one of the most significant traditional medicinal plants for its therapeutic qualities (Fukai *et al*., 2002a). The medicinal use of liquorice plant was also documented in the written form by the ancient Greeks, and has been well used in Europe since these times (Fiore *et al*., 2005).

Not only has the liquorice plant been used in folk medicine as a medicinal plant in different countries of the world to treat many diseases including bacterial infections, it is also commonly used as a flavouring in food products and also as a natural food preservative in many countries (Škrinjar and Nemet, 2009). Different species of liquorice include *G. glabra, G. uralensis* (Figure 1.3), *G. inflata, G. eurycarpa, G. aspera*, and *G. korshinskyi* (Zhang and Ye, 2009; Yang *et al*., 2014) are all rich in chemical constituents such as flavonoids which are used as drugs for treatment of different kinds of disease such as gastritis, gastric ulcer, stomachic, bronchial diseases, gastrointestinal diseases, cough, liver and bile diseases, duodenal ulcers and gastrointestinal in the Western countries. In Japan it is used as an over-the-counter medicine and may be purchased without a doctor's prescription (Fukai *et al*., 2002b). The extract of different species of liquorice plants consists of many known medically active chemical ingredients, such the flavonoids glabridin, glabrene, licochalcone A, licoricidin and licoisoflavone which have been shown to have inhibitory activity against the growth of *Helicobacter pylori*, which is known to be a major cause of gastric ulcers, *in vitro* (Fukai *et al*., 2002a). A study conducted by Asha *et al*. (2013) reported that Glabridin is the major flavonoid present in GutGard® which exhibited good anti-*Helicobacter pylori* activity in both agar dilution and microbroth dilution methods.

Figure 1.3. Images of *G. uralensis* plants and roots

G. uralensis plant *G. uralensis* root

Glycyrrhiza uralensis is described as a short shrub with oval leaflets, flower clusters with a white and purplish colour shown in panel A and the roots of liquorice shown in panel B (Adapted from Zhang and Ye, 2009; Yang *et al*., 2014).

Recent chemical analyses have found a wide variety of bioactive compounds present in liquorice. So far, more than 400 compounds have been isolated from *Glycyrrhiza* species. The active constituents such as triterpene saponins and flavonoids in liquorice contribute to the bioactivity of liquorice and are considered to be responsible for these beneficial effects seen in the treatment of diseases (Zhang and Ye, 2009). The content of these bioactive in *Glycyrrhiza* vary considerably with the different plant species, due to plant age, seasonal differences, geographical changes and time of harvest and process collect plant, all of these diversity affect their biological activity and thus affect the therapeutic effects of liquorice (Fiore *et al*., 2008). Statti *et al*. (2004) collected *G. glabra* L. from different regions of Calabria, Italy, and confirmed an inhibitory effect on bacteria and fungi, and found that the biological activity varied due to differences in the chemical composition of this plant obtained from different sites. In addition the chemical constituents are often influenced by genetic, environmental and processing factors (Isbrucker and Burdock, 2006).

As described by He *et al*. (2006) bioactive ingredients such as essential oils, flavonoids, triterpenoids, alkaloids, phenols, polyamines, and polysaccharides produced by liquorice have all been evaluated by many research groups for potential use as new drugs. The presence of flavonoids including liquiritin, isoliquiritin (a chalcone) in the liquorice plant gives them a yellow colour (Nazari *et al*., 2017). Most of the pharmacological studies of liquorice saponins indicate that glycyrrhizin and its constituents appear to possess activity against a variety of cancers. In addition to the antioxidant activity due to the presence of isoflavones glabridin and hispaglabridins A and B (Vibha *et al*., 2009). The activity of glycyrrhizin is due to the presence of aglycone and 18β-glycyrrhetinic acid, and these two compounds exhibit extensive biological activities and a large number of biological activities have been reported for liquorice plants such as anti-inflammatory activity (Kwon *et al*., 2013), antiviral, antimicrobial, antioxidative and anticancer activities (Shibata, 2000), and immunomodulatory and cardioprotective effects which are believed to explain the extended spectrum of activities associated with the use of these plants. However, many of these are preliminary studies and further research is needed to enhance the application of these potential impacts (Asl and Hosseinzadeh, 2008).

In addition to the biological activity of Glycyrrhizin, it is also responsible for its sweet taste (Saxena, 2005) and is fifty times sweeter than sugar which is a saponin glycoside (Nazari *et al*., 2017). Saponins are also used as flavouring agents, which are generally considered to be bitter, but, can be sweet (Heng, 2005). Thus, the presence of glycyrrhizin in liquorice extracts had led to its uses in pharmaceutical and sweetmeats industries.

1.5.1 The antimicrobial activity of liquorice extracts

The antimicrobial activities of liquorice extract have been demonstrated against both foodborne Gram-positive and Gram-negative bacteria by many researchers and the extract exhibits potent anti-bacterial activity. For instance Shirazi *et al*. (2007) investigated the antibacterial activity of *G. glabra* extract *in vitro* against Gram-negative bacteria, including *Salmonella* Typhimurium, *Salmonella paratyphi* B, *Shigella sonnei*, *Shigella flexneri* and Enterotoxigenic *Escherichia coli* (ETEC) using both agar-well and disc diffusion methods with different concentrations of liquorice extract (5, 7.5, 10 and 15 %). All of the bacterial strains examined showed sensitivity to high concentrations of liquorice, however, *Salmonella paratyphi* B showed no sensitivity to concentrations less than 7.5 % (Shirazi *et al*., 2007) which is far higher than the allowed levels for food. Compounds derived from the root of *Glycyrrhiza uralensis* have also been evaluated for activity against oral pathogenic bacteria that cause dental caries namely, *Streptococcus mutans* (He *et al*., 2006). Biological activity of the ethanolic *Glycyrrhiza glabra* roots extract was identified by Meghashri (2009) and shown to be antimicrobial against six bacteria and two fungal strains using both the well diffusion method and microdilution method. A significant antimicrobial activity was exhibited by the extract against all tested microorganisms, with MICs ranging from 0.8 to 200 mg ml^{-1} for the fungal strains and MICs ranging from 0.2 to 1.2 mg ml^{-1} for bacterial strains including *S. aureus*, *L. monocytogenes* and *E. coli*. An ethanolic extract of glycyrrhizol B and gancaonin G was also shown to be effective against Gram-positive bacteria with MICs of 1 and 2 µg ml^{-1}, respectively, and it was suggested that it was the flavonoids present in the extract that led to inhibition of growth. A similar study conducted by Ajagannanavar *et al*. (2014) further confirmed the inhibitory effects of liquorice on the growth of pathogenic bacteria by using aqueous and alcoholic liquorice root extract (0.2 %) against Gram-positive bacteria (*Streptococcus mutans* and *Lactobacillus acidophilus*), with results being compared to the activity of the antimicrobial chemical Chlorhexidine.

In this study it found that the alcoholic liquorice root extract showed a higher inhibition of *S. mutans* and *L. acidophilus* compared with both the aqueous form and Chlorhexidine. Rodino *et al*. (2015) tested ethanolic extracts of liquorice (*Glycyrrhiza glabra*) against Gram-positive bacteria (*Staphylococcus aureus, Bacillus cereus* and *Enterococcus faecalis*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas fluorescens*). The inhibition of growth was observed against all bacteria tested with maximum inhibition seen for *E. coli*, *E. faecalis* and *B. cereus*, while *P. fluorescens* showed the lowest level of inhibition.

Chabuck *et al*. (2018) investigated the activity of some herbal extracts including *Glycyrrhiza glabra in vitro* against Gram-positive and Gram-negative bacteria using well-diffusion method, by measuring the diameters of inhibition zones and the results showed that *Glycyrrhiza glabra* exhibited good activity against all bacteria tested with inhibition zones ranging from 18-25 mm, the largest inhibition was against *E.coli* (25 mm) followed by *P. aeruginosa* and *S. saprophyticus* (24 and 23 mm respectively), whereas the lowest inhibition was 8 mm against both *k. pneumoniae* and *E. aerogenes*. Another study showed that flavonoids isolated from *Glycyrrhiza glabra* had a strong inhibitory effect against both *S. aureus* and *E. faecalis* whereas they showed less inhibitory effects against *E. coli* and *Pseudomonas aeruginosa* (Rashid *et al*., 2013). Hence, depending on the extract used, different patterns of sensitivity were seen with both Gram-positive and Gram-negative bacteria being affected. Similar results were reported by Soulef *et al*. (2014) who tested the *in vitro* effect of the glycoside extracted from the liquorice plant *G. glabra* L. against the Gram-positive bacterium *S. aureus* and the Gram-negative bacteria *E. coli and P. aeruginosa* and showed that the extract has an inhibitory effect on *S. aureus* and *P. aeruginosa*, but no effect on *E. coli* suggesting that cell structure alone does not account for the pattern of results seen.

When specific components of liquorice were examined, a more consistent pattern was seen. Fukai *et al*. (2000b) who investigated the antimicrobial activity of nineteen flavonoids commonly found in three species of liquorice which included glabridin and glabrene isolated from *Glycyrrhiza glabra*, licochalcones A and B isolated from *G. inflata* and liquiritigenin, liquiritin, licoisoflavone B Formononetin, licoricidin, glycyrol, isoglycyrol, 3-O-methylglycyrol, (3R)-vestitol, licoricone, glycyrin, isolicoflavonol, gancaonol B, glyasperin D and gancaonin I isolated from *G. uralensis*. They tested these flavonoids against three Gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus* and *Micrococcus luteus*) and three Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and found that these compounds showed significant antibacterial activity against *S. aureus, B. subtilis* and *M. luteus* but did not have an affect against the Gram-negative bacteria. This study concluded that the methanol extract showed activity against Gram-positive rather than Gram-negative bacteria suggesting that a common difference in cell structure was responsible for the difference seen, and may reflect the fact that the compound could not penetrate the outer membrane of the Gram-negative bacteria since the lipopolysaccharide layer in the outer membrane of Gram-negative bacteria which is known to serve as an active permeability barrier to many environmental substances. Irani *et al*. (2010) also reported that extracts of *G. glabra* L. using both paper disc agar diffusion and serial dilution methods and detected a potent activity against Gram-positive bacteria *S. aureus*, *B. subtilis* and *E. faecalis.* Irani *et al*. (2010) and Badr *et al*. (2011) also found that liquorice extract had a potent bactericidal effect against *E. faecalis*. Hatano *et al*. (2000) reported that the main antimicrobial activities of liquorice extract against both methicillin-sensitive and methicillin-resistant of *S. aureus* were due to the phenolic compounds and reported MICs of between 3 and 16 μg ml^{-1} depending on the strain tested. However, again none of the tested compounds showed antibacterial effects on *E. coli* and *P. aeruginosa*. Another group that investigated the antimicrobial effect of β-Glycyrrhetinic acid

isolate from *G. glabra* on both bacteria and fungi found a strong antimicrobial effect against *B. subtilis* and *S. epidermidis* with an MIC of 7.6 and 12.5 µg ml^{-1} respectively, while there was no affect against *E. coli, P. vulgaris* and various fungi tested (Kim *et al*., 2002). These studies are considered as evidence that the effect of liquorice extract is mostly due to flavonoid compounds present in plants, and also that generally the extracts affect Gram-positive bacteria more significantly than Gram-negative bacteria.

Set against these findings is that liquorice and glycyrrhizin have been used for a long time in the treatment of peptic ulcers caused by the Gram-negative bacterium *Helicobacter pylori*. A study conducted by Krausse *et al*. (2004) reported that a total of 29 *H. pylori* strains tested (ca. 79.3 %) were inhibited by low concentrations (≤ 50 mg l^{-1}) including clarithromycin-resistant strains, and therefore liquorice could be considered as the basis for an alternative therapeutic agent against these pathogens. This observation corresponds to another study by Fukai *et al*. (2002a, 2002b) investigated the *in vitro* antibacterial activity of chemical constituents, glabridin, glabrene and licochalcone A isolated from liquorice plants (*G. glabra*, *G. inflata* and *G. uralensis*) against *H. pylori*. Similar results were reported by Malek Jafarian and Ghazvini (2010) who showed that all clinical strains of *H. pylori* tested were sensitive to treatment with *Glycyrrhiza* extract.

Mycobacterium tuberculosis a facultative intracellular bacillus that causes human tuberculosis (TB) and it is considered the number one cause of deaths due to infectious diseases in the world. Friis-Møller *et al*. (2002) reported that several species of *Mycobacterium,* including *M. tuberculosis*, *M. bovis, M. kansasii, M. xenophii* and *M. marinum* were inhibited by licochalcone A with an MIC ≤ 20 mg ml^{-1} whereas all non-*M. tuberculosis* complex mycobacteria were resistant suggesting that there is a target site specific to this type of cell structure for this compound. Chandran (2015) studied the antibacterial activity of different solvent

extracts of liquorice roots against the acid-fast bacterium *Mycobacterium smegmatis* using the disc diffusion method and found that chloroform extract recorded the maximum anti-mycobacterial activity with an inhibitory zone of 22 mm in hot extract process and 24 mm in cold extract. Another study demonstrated the anti-tubercular activity of an acetone extract of *G. glabra* against *M. tuberculosis* H37Rv using Resazurin Microtiter Plate Assay (REMA) and the extract showed significant activity against *Mycobacterium tuberculosis* H37Rv with an MIC of 0.97–1.95 µg ml^{-1} (Nair *et al*., 2015). Similarly Gupta *et al*. (2008) demonstrated antimicrobial activity of extracts of *G. glabra* against of *M. tuberculosis* at a concentration of 500 µg ml^{-1} using BACTEC assay and showed that ethanolic extract from the roots of *Glycyrrhiza glabra* showed antibacterial activity at a concentration of 29.16 µg ml^{-1} against two strains of *M. tuberculosis* H37Ra and H37Rv and subsequent phytochemical analysis identified the active component to be glabridin which was more active against Gram-positive bacteria than Gram-negative. This group also found that an ethyl acetate extract containing glabridin at a concentration of 100–250 µg ml^{-1} showed good activity against these acid fast bacteria that are notoriously resistant to antimicrobial compounds due to their thick, waxy cell walls that contain high levels of mycolic acid (Gupta *et al.,* 2008).

Geetha and Roy (2012) investigated the antimicrobial activities of both ethanol and aqueous liquorice (*G. glabra*) root extracts against *Staphylococcus aureus* (MRSA) using the agar well diffusion method at a concentration of 500, 1000 and 2000 µg ml^{-1}. The ethanolic extract showed a zone of inhibition of 24 mm diameter at concentration of 2000 µg ml^{-1} against *staph. aureus*. Another study focussing on bacterial pathogens which cause oral infections and dental caries by Sedighinia and Afshar (2012) tested the antibacterial activities extracts of liquorice (*G. glabra*) *in vitro* using the agar dilution method and again showed effective inhibitory activity against the microorganisms tested, with an MIC of 12.5

mg ml^{-1} for *S. mutans, Actinomyces viscosus* and *E. faecalis*, no effect seen on *E. coli* which exhibited the most resistance to *G. glabra* (Sedighinia and Afshar, 2012).

The most problematic Gram-positive bacteria in public health and healthcare-related infections is *S. aureus*, not only due to the fact it is widespread but also because it has become more resistant to most of antibiotics available (Chambers, 2001). Especially in hospitals and the communities the Methicillin-resistant *S. aureus* (MRSA) has become a principal source of infection (Long *et al*., 2013). The increase in antibiotic resistance in *S. aureus* strains has led to a search for other therapies to treat it. Consequently, a wide variety of bioactive phenolic constituents have been isolated from liquorice and the antimicrobial activity of liquorice flavonoids have been confirmed against methicillin-resistant *S. aureus* (Fukai *et al*., 2002b; Shibata, 2000). The antimicrobial activities of 70 % ethanolic extracts among of 19 plant were tested against 59 methicillin-resistant *S. aureus* (MRSA) using the agar well diffusion method, and liquorice (*Glycyrrhiza Glabra*) ethanolic extract exhibited the highest antimicrobial effect against all MRSA isolates with MICs of 8 µg ml^{-1} (Bassyouni *et al*., 2012). Hatano *et al*. (2000) also reported that two phenolic compounds, Glicophenone and Glicoisoflavanone present in *G. glabra* had a strong effect against both methicillin resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* with an MIC of 16 µg ml^{-1}.

Licochalcone A extracted from *G. glabra* had potent activity against different types of Gram-positive bacteria and the effective MICs of Licochalcone A was 5 mg ml^{-1} for *Lactobacillus acidophilus* and *Lactobacillus plantarum*, 6 mg ml^{-1} for *Enterococcus faecalis* and *Enterococcus faecium,* as well as 8 and 5 mg ml^{-1}, respectively, for *Streptococcus lactis* and *Staphylococcus mutans* (Nowakowska, 2007). Hao *et al*. (2013) also reported that Licochalcone A isolated from the roots and rhizomes of *Glycyrrhiza inflate*, inhibited the biofilm formation of *Streptococcus suis* with MICs of 4 mg ml^{-1} for *S. suis* serotype 2 strains and 8 mg

ml^{-1} for *S. suis* serotype 7 strains using the microtiter plate assay. Sultana *et al*. (2010) also found that methanolic extracts of *G. glabra* showed activity against both Gram-positive (S*. aureus, Bacillus megaterium* and *B. subtilis*) and Gram-negative (*E. coli, P. aeruginosa* and *Salmonella paratyphi*) bacteria using the disc diffusion method. Most organisms were found to be sensitive to the extract except *Pseudomonas aeruginosa*. However it was most effective against *S. aureus* which produced the largest zones of inhibition of 22 mm in a disc diffusion assay. Activity against these microorganisms was due to the presence of glabrene, licoisoflavone B, isolicoflavonol, and gancaonin I.

The overall conclusion of this research is that active ingredients are present in liquorice, and that essential oils, triterpenoids, alkaloids, phenols, polyamines and polysaccharides have potent antibacterial activities. Generally more effect is seen against Gram-positive bacteria, presumably reflecting the difference in cell wall structure of these two groups of bacteria. However activity against Gram-negative bacteria has been seen, and Glabridin isolated from liquorice has been documented to shown numerous of pharmacological activities shown to equally affect antimicrobial activity against methicillin-resistant *S. aureus* (Fukai *et al*., 2002b; Hatano *et al*., 2000) and *H. pylori* (Fukai *et al*., 2002a) suggesting that there are multiple mechanisms by which the different bioactive compounds present in liquorice affect bacterial cells.

1.5.2 Toxicity of liquorice plant

The most important factor during the search for new antimicrobial agents from plants is a consideration of the toxicity of plant extracts and plant components toward cells, especially mammalian cells. One way to ensure the function of the microbial agents is safe to use as an antibacterial is through inclusion of a parallel evaluation on host cell lines (cytotoxicity evaluation) and other microbial screens on bacteria, fungi, parasites and viruses (Cos *et al*., 2006).

Although the liquorice plant is in general considered safe, hypertension is one of the most usually recorded side effects associated with liquorice consumption. Accordingly, based on data collected between 2006 and 2010 for the European and Brazilian poison centres, liquorice was reported as being one of the ten food plants most frequently reported to have caused harmful side effects (Lüde *et al*., 2016). These adverse effects are probably due to the effect that liquorice has on the renin-angiotensin-aldosterone system, causing salt retention and hypokalaemic hypertension associated with overconsumption of liquorice saponins (White, 2001). Thus, the overconsumption of these products is associated with raised risk of stroke or heart disease (Johns, 2009) since excessive consumption can produce the symptoms of hypertension due to increasing the absorption of sodium and water in kidneys, however, the symptoms usually disappeared when the dose is reduced (Miettinen *et al*., 2010).

According to Renaissance physician Paracelsus (1493-1541) the treatment dose is responsible for the safety of substances, where the right dose distinguishes between a poison and a therapy which means that just the dose determines that if the substance is poisonous or not - otherwise all substances may be considered as poisons (Grandjean, 2016). Therefore, to ensure the efficacy and safety in foods and clinical use, strict control on liquorice is needed (Zhang and Ye, 2009). For instance, liquorice root can be used safely for therapy of duodenal and gastric ulcers, however, deaths from its excessive use have been reported. There is a recommendation that due to reported side effects, an average daily dose should not exceed 5–15 g of dried root, which equates to 200–600 mg glycyrrhizin, for the treatment of gastrointestinal ailments, with treatment course not continuing for more than 4–6 weeks (Isbrucker and Burdock, 2006).

The US FDA, the Council of Europe, and the UK Food Additive and Contaminants Committee recognized liquorice and liquorice extract are safe for use in foods, but it is recommended by the European Scientific Committee on Food

that consumption levels do not exceed 3.5 g/day of liquorice extract which corresponds to about 100 mg/day of glycyrrhizin (Scientific Committee on Food, European Commission, 2003). Thus, the FDA assumes that glycyrrhizin levels in foods do not pose a health hazard, provided that these foods are not consumed in excess or by individuals who are sensitive to low levels of glycyrrhizin (Delbò, 2013). According to The World Health Organisation the consumption of 100 mg/day would not be to cause adverse effects, whereas The Dutch Nutrition Information Bureau advised against daily glycyrrhizin consumption in excess of 200 mg/day (Sontia *et al*., 2008). However, the maximum acceptable amount of liquorice or its extract still unknown. Although severe toxicity of liquorice extract is very rare, and the LD_{50} in mice and rats is greater than 4 g glycyrrhizinate/kg, which is well below the recommended safe use level for foods, however, overconsumption levels of glycyrrhizin may cause pseudoaldosteronism (Isbrucker and Burdock, 2006). Despite this, the major component of liquorice and liquorice derivatives glycyrrhizin or glycyrrhizic acid, has been approved as Generally Recognized as Safe (GRAS) for use in foods by the U.S. FDA.

1.6 Aims and Objectives

The aim of this study was to assess a waste material from the production of commercial liquorice extract for its antimicrobial activity against the Gram-positive bacteria including *L. monocytogenes* strains, *L. innocua*, *B. subtilis*, *Staph. aureus*, *E. faecalis* and *P. aeruginosa*.

Hence the particular aims and objectives of the study were:

1) To investigate the potential of plant extracts as natural antimicrobial compounds. In particular to determine if the compounds had effectiveness against food borne pathogens including *L. monocytogenes*.

2) To determine what the antimicrobial mechanism of action of the extract
3) To determine the MIC for the bacteria that were sensitive to this material and therefore too establish an effective working of concentration of extract.
4) To investigate the physiological effects of the extract on bacterial cells.
5) To evaluate the ability of the extract as a novel food preservative in a model food system.

CHAPTER 2

MATERIALS, METHODS AND STANDARD PROCEDURES

2.1 Chemicals and media

Details for the preparation of solutions and media used in this thesis are presented in Appendix 1 and 2. The suppliers for all chemicals substances and media used in this thesis are listed in Table 2.1.

Table 2.1. Chemical substances and media used during this study

Compound	Supplier
Brain Heart Infusion Broth (CM 1135)	Oxoid, UK Ltd
Agar Bacteriological (Agar No.1) LPOOII	Oxoid, UK Ltd
Nutrient Broth No.2 (IVD) (CM0067)	Oxoid, UK Ltd
Nutrient Broth (CM0001)	Oxoid, UK Ltd
Maximum Recovery Diluent (MRD) (CM0733)	Oxoid, UK Ltd
Palcam agar base (CM087)	Oxoid, UK Ltd
Listeria selective agar Base (CM0856)	Oxoid, UK Ltd
Tryptone soya broth (IVD) (CM0129)	Oxoid, UK Ltd
Tryptone soya agar (IVD) (CM0131)	Oxoid, UK Ltd
phosphate buffered saline	Oxoid, UK Ltd
Listeria Selective Agar Supplement (SR0140)	Oxoid, UK Ltd
Listeria Palcam Selective Supple (VWR), B-3001	Leuven, Belgium
Buffered peptone water (ISO) (F0213W)	VWR,Prolabo chemicals
D-Glucose, anhydrous	Fisher Scientific, UK Ltd
Potassium phosphate, dibasic, anhydrous (K2HP04)	Fisher Scientific, UK Ltd
Sodium ammonium phosphate ($NaHNH_4P0_4.4H_20$)	Fisher Scientific, UK Ltd
Sodium chloride (NaCL)	Fisher Scientific, UK Ltd
Magnesium sulphate ($MgS0_4.7H_20$)	Fisher Scientific, UK Ltd
Glacial acetic Acid	Fisher Scientific, UK Ltd

Table 2.1. Continued

Dimethylformamide	Fisher Scientific, UK Ltd
Erythromycin	ApoLLo Scienctic Limited
Ampicillin Sodium Salt BioChemica	PanReac AppliChem
Tetracycline	Sigma-Aldrich
D-Biotin (F.W.244.3)	Sigma-Aldrich
Citric acid Anhydrous ($C_6H_8O_7$) (C-0759)	Sigma-Aldrich
Sodium dodecyl sulfate, for molecular biology, approx. 99% (L4390)	Sigma-Aldrich
Mitomycin C from Streptomyces. Vial contains 2 mg mitomycin C and 48 mg Nacl (M-0503)	Sigma-Aldrich
Sodium azide (S-2002)	Sigma-Aldrich
Sodium dodecyl sulfate	Sigma-Aldrich
Trypsin – EDTA Solution (T3924)	Sigma-Aldrich
Mem Non-essential amino acid solution (100x) (M7145)	Sigma-Aldrich
L-Glutamine solution (G7513)	Sigma-Aldrich
foetal bovine serum (FBS) (F9665)	Sigma-Aldrich
Dulbecco's Modified Eagle Medium (DMEM) high glucose (D6571)	Sigma-Aldrich
Trypan Blue solution	Sigma-Aldrich
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
L-Histidine. HCL. H_20 (F.W.209.63)	Acros Organics
Isopropanol, 99.5+%, pure	Acros Organics
3-(*4*,*5*-dimethylthiazol-*2*-yl)-*2*,*5*-diphenyltetrazolium bromide (MTT)	Acros Organics

2.2 Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this study are listed in (Tables 2.2 and 2.3), respectively. These strains were obtained from the laboratory stocks of Food Sciences Division, University of Nottingham, United Kingdom. *Listeria* strains were recovered from Microbank^{TM} beads held at -80 °C by inoculating into Brain Heart Infusion (BHI) broth and then plating onto BHI agar with erythromycin (Erm; 5 µg ml^{-1}) to select for the presence of the plasmids when required. Plates were incubated for 24 h at 37 °C and stored at 4 °C until needed. *Staphylococcus aureus* and *Pseudomonas fluourescens* strains chromosomally tagged with a *lux* operon *(luxABCDE*) that was modified to function in both of them.

Salmonella strains for the Ames test carried different mutations in various genes in the histidine operon. When needed, the culture medium was supplemented with antibiotics at the following concentrations; 24 µg ml^{-1} ampicillin for *Salmonella* Typhimurium TA1537, TA100 or 24 µg ml^{-1} ampicillin and 2 µg ml^{-1} tetracycline for *Salmonella* Typhimurium TA102.

In general, the cultures were incubated overnight at optimal temperatures dependent on the type of bacterium. An isolated colony was inoculated in the broth supplements with antibiotic as required. With most organisms in this study, 24 h incubation period is adequate, but an additional time was needed for the slow growing microorganisms.

2.3 Preparation of liquorice extract

3 mg of liquorice powder was weighed into a sterile bijou bottle using scales accurate to 0.1 mg then dissolved in 1 ml of 70 % (v/v) ethanol solvent, and mixed thoroughly before incubating at 37 °C for 30 min to ensure the extract dissolved completely then stored at -20 °C prior to use.

2.4 Preparation of bacterial cultures

2.4.1 *Listeria monocytogenes* strains

Listeria strains from frozen stocks were grown by streaking one bead into brain heart infusion (BHI) agar plates and incubated at 37 °C for 24 h. From these plates one colony of each strain was transferred into 10 ml BHI broth and allowed to grow for 24 h (overnight) at 37 ºC in a rotary shaker at 150 rpm. Growth was routinely measured as optical density at 600 nm (OD_{600nm}). In addition, the number of culturable cells was determined by counting colonies grown on BHI agar plates supplemented with erythromycin (5 µg ml^{-1}), cultures were also always plated onto BHI agar without antibiotics to ensure that the cultures were not contaminated and to ensure marker stability. The inocula were diluted to the appropriate cell densities (OD~0.05) in BHI broth.

2.4.2 *Salmonella* strains/mutants

Strains stored at -80 ºC were streaked into brain heart infusion (BHI) agar to isolate pure isolated colonies and incubated at 37 ºC for 18-24 h subsequent one colony was transferred into 5 ml of nutrient broth then incubated at 37 for 18-24 h in shaking incubator with aeration (150 rpm) to reach approximately 1 x 10^9 cfu ml^{-1}.

2.4.3 *Pseudomonas fluourescens* chromosomally tagged *lux*

P. fluorescens chromosomally tagged *lux* (see Table 2.2 for details) was maintained by streaking the strain on nutrient agar plates which were incubated at 30 °C for 24 h. The grown cultures were stored at 4 °C, one colony was grown in nutrient broth at 30 °C for 18-24 h in shaking incubator with 150 rpm.

Table 2.2. Bacterial strains and yeast used in this study

Bacteria	Strain numbers or source/reference	Gram reaction group
Enterococcus faecalis	NCTC 775	+ve
Staphylococcus aureus	Chicken isolate, Nottingham lab collection	+ve
Bacillus subtilis	var. Niger 168	+ve
Listeria innocua	Factory isolate, Nottingham lab collection	+ve
Listeria innocua	ATCC 11994 (Serotype 6a)	+ve
Listeria monocytogenes	10403S (wild type; 1/2a) (Bishop and Hinrichs, 1987)	+ve
Listeria monocytogenes	EGD (wild type; 1/2a) (Murray *et al.*, 1926)	+ve
Listeria monocytogenes	ATCC 23074 (wild type; 4b)	+ve
Listeria monocytogenes	NCTC 10357 (Δ*prfA*)	+ve
Listeria monocytogenes	NCTC 7973 (*prfA**)	+ve
Staphylococcus aureus	Xen 29 Tn*4001 luxABCDE* (Xiong *et al.,* 2005)	+ve
Pseudomonas fluorescens	Field isolate, Nottingham lab. Collection *mini-Tn5 (gfp:luxABCDE, Tet^r)* Dr P. Hill, University of Nottingham, (unpublished)	-ve
S. enterica serovar Typhimurium TA102	Nottingham lab. Collection Deletion mutation (Levin *et al*., 1982)	-ve
S. enterica serovar Typhimurium TA100	Nottingham lab. Collection Deletion mutation (McCann *et al*., 1975)	-ve
S. enterica serovar Typhimurium TAI535	Nottingham lab. Collection Deletion mutation (Ames *et al*., 1973b)	-ve

Table 2.2. (continued)

Mycobacterium smegmatis	mc^{2}155; ATCC 700084	Acid Fast
Escherichia coli	NCTC 86	-ve
Pseudomonas aeruginosa	Nottingham lab collection	-ve
Saccharomyces cerevisiae	NCYC 363/ Brewing Science	Lab isolate strain
Saccharomyces cerevisiae	S288C/ Brewing Science	Wild type

**ATCC: American Type Culture Collection*

***NCTC: National Collection of Type Cultures*

****NCYC: National Collection of Yeast Cultures*

Table 2.3. Bacteria with plasmids used in this study

Bacteria	**Promoter and Insert**	**Vector**	**Antibiotic selection [μg ml^{-1}]**
L. innocua	BS10, *luxABCDE, gfp*	pUNK1	Erm [5 μg ml^{-1}]
L. monocytogenes	BS10 *luxABCDE, gfp*	pUNK1	Erm [5 μg ml^{-1}]
L. monocytogenes NCTC 10357	*xylA*, *luxABCDE, gfp*	pUNK1	Erm [5 μg ml^{-1}]
S. enterica serovar Typhimurium TA100	*hisG46*	pKM101	Amp [24 μg ml^{-1}]
S. enterica serovar Typhimurium TA1535	*hisG46*	No plasmid	None
S. enterica serovar Typhimurium TA102	*hisG428*	pKM101, pAQ1	Amp [24 μg ml^{-1}] and Tet [2 μg ml^{-1}]
Pseudomonas fluourescens	*mini-Tn5 (gfp:luxABCDE, Tet^r)*	None	Tet [2 μg ml^{-1}]
Staphylococcus aureus Xen 29	Tn*4001 luxABCDE* Perkin Elmer		Kan [5 μg ml^{-1}]

*Amp (Ampicillin), *Tet (Tetracycline) & *Ery (Erythromycin)

Table 2.4. Plasmid used in this thesis

Plasmid	Description	Reference
pSB3007	pUNK1,P*xylA*:*gfp*:*luxABCDE*:*rrnBT1T2*	Perehinec *et al*., 2007
pSB3008	pDEST-pUNK1 containing P_{BS10}::*gfp*3:l*uxABCDE*::*rrnBT1T2*	R. Gaddipati, 2010, PhD Thesis, University of Nottingham

Table 2.5. Human tissues used in this research

Cell line	Description	Source
Caco-2 cells colon	Human Caucasian colon adenocarcinoma	ATCC HTB-37

Table 2.6. Chemicals used as a positive control in the Ames test

Bacterial strain	Chemical
S. Typhimurium TA100	Sodium azide
S. Typhimurium TA102	Mitomycin C
S. Typhimurium TA1535	Sodium azide

The chemical required for every strain is different due to the different mutagenicity in gene operon for all the strains, where sodium azide serves as a positive control for TA100, TA1535 and mitomycin C for TA102.

2.4.4 *Staphylococcus aureus* chromosomally tagged *lux*

Staphylococcus aureus chromosomally tagged with *lux* was maintained by streaking the strain on nutrient agar plates which then were incubated at 37 °C for 18-24 h. Subsequently one colony was grown in nutrient broth at 37 °C for 18-24 h in shaking incubator with 150 rpm.

2.5 Preparation of yeast cultures

Two strains of *Saccharomyces cerevisiae* (S288C, NCYC 363*)* were used in this study. Yeasts were cultured at 30 °C in YPD media, containing (2 % peptone, 1 % yeast extract, 2 % dextrose) for 48 h in shaking incubator with aeration at 230–270 rpm until the OD_{600nm} was in the range of 0.4–0.6.

2.6 Monitoring growth of bacterial cultures

After an adequate period of incubation, culture growth was determined using Optical Density (OD_{600nm}) and the density of each sample was adjusted to a specific level as required. In all tests bacterial cultures were diluted an initial concentration of $\sim 10^7$ cfu ml^{-1} ($OD_{600nm} \simeq 0.05$) whereas, *Salmonella* strains were adjusted to give a final density of $1X10^8$ cfu ml^{-1} (approximately $OD_{600nm} = 0.5$). For the diluted cultures, the samples were used within 30 min to initiate the experiments.

2.7 Frozen stock cultures of bacteria

All strains used in this study were stored long term at -80 ºC by preparing frozen stocks. Cultures were grown on nutrient or BHI agar plates dependent on the type of bacteria (see Section 2.4). The frozen stocks were prepared by using a sterile inoculation loop to pick off enough colonies from a pure overnight culture

into cryovials containing cryopreservative fluids (Microbank®, Pro-Lab Diagnostics U.K). The inoculated vials were tightly closed and inverted 4-5 times to mix the bacteria to allow them to bind to the beads. By using Gilson pipettes the excess cryopreservative was removed from the vials, then the vials were kept at -80 °C. When a fresh culture was prepared, one bead was picked up from the vial by using a sterile tooth then streaked four-way streak plate onto the appropriate agar plates. For short term storage bacteria cultures were kept on BHI agar plates at 4 °C and maintained by sub-culturing on BHI agar plates every 4 weeks.

2.8 Measurement the optical density of bacteria

The bacterial strains used for this study were *E. faecalis*, *S. aureus*, *B. subtilis*, *L. innocua*, *L. monocytogenes* (Gram-positives) and *E. coli* and *P. aeruginosa* (Gram-negatives), one colony inoculated into 10 ml of broth media followed by incubation overnight at 37 °C in 150 rpm shaking incubator then mixed thoroughly using a vortex mixer, cell growth was monitored with a spectrophotometer (Eppendorf Biophotometer Plus - Thermo Fisher Scientific, Germany) at OD_{600nm}, 1 ml of overnight culture was inoculated into sterile 250 ml Erlenmeyer flasks containing 100 ml of BHI broth then 1 ml of samples was transferred into a plastic cuvette for optical density measurement, the inoculum of the broth culture was adjusted until the bacterial suspension reached an OD_{600nm} 0.05 which was equivalent to an approximately $1x10^7$ cfu ml^{-1}.

2.9 Determination of the growth rate of Gram-positive bacterial cultures

To measure the growth rate of bacteria, a single colony was transferred into 10 ml of BHI broth and grown at 37 °C overnight in shaking incubator (150 rpm), then different dilutions were made for overnight cultures (1:10, 1:50 and 1:100) in broth media. Dilution cultures (200 µl) were transferred into wells of a

microtitre plate (at least 4 wells) of sterile 96-well polystyrene microtiter plates. Culture growth was monitored by using optical density at a wavelength of OD_{600nm} with readings taken every 10 min for 24 h, readings were taken at regular periods over the course of the experiment as required. The growth rate of the bacteria was calculated using the equation shown below (Widdel, 2007).

The specific growth rate (μ)

$$\mu = \frac{2.303\ (\log_{10} N_x - \log_{10} N_0)}{(t_x - t_0)}$$

Where: N_0 = initial number of cells at t_0

N_x = number of cells at time t_x

t_o = time at the beginning of log phase

t_x = time at a later time

2.10 Tecan microplate reader use for measuring bacterial growth

To allow multiple growth experiments to be carried out, a microtitre method was developed. This method was used to measure the optical density of cultures as well as luminescence. The sterile polystyrene microtitre plates contained 96-wells (Krystal Microplate, clear bottom or Porvair 96 well black, Porvair Sciences, England), and had a capacity of 200 μl per well. For bioluminescence or fluorescence measurements black plates were used as they reduce the background signal. For optical density measurements clear plates were used.

2.11 Minimum inhibitory concentration determination

The MIC of liquorice extract was determined by a microdilution broth method performed as described by Motyl *et al*. (2006) with a slight modification, using 96 well sterile plates which have a maximum capacity of 200 μl. Overnight cultures were prepared and aliquots inoculated into sterile 250 ml Erlenmeyer

flasks containing 100 ml of BHI broth. The inoculum of the broth culture was adjusted until the bacterial suspension reached an OD_{600nm} 0.05 which was equivalent to an approximately $1x10^7$ cfu ml^{-1}. Three flasks were prepared for each type of bacteria; the first flask (control) contained 1 % (v/v) ethanol, the second flask contained 12.5 µg ml^{-1} of liquorice extract and the third flask contained 50 µg ml^{-1} of liquorice extract.

Samples of the cultures (200 µl) were transferred into the wells of a microtitre plate using at least 12 wells from a sterile 96-well polystyrene microtiter plates for each bacterium. These were tested at two different concentrations (12.5 and 50 µg ml^{-1}) of liquorice extract. Cell suspensions with 1 % (v/v) ethanol and BHI broth without culture were used as a controls. The microtiter plate was then incubated at 37 °C for 24 h in a Tecan Genesis Pro microplate reader. Cell growth was monitored by using OD_{600nm} with readings taken every 10 min for 24 h, readings were taken at regular periods over the course of the experiment as required.

2.12 Miles and Misra technique

The minimum bactericidal concentrations (MBC) were determined using a modified Miles and Misra method (Miles *et al*., 1938) to determine viable counts (cfu ml^{-1}) of a bacterial suspension in Brian Heart infusion (BHI) broth treated with liquorice extract (Section 2.11), 10 µl of treated samples and controls were 10-fold serially diluted into maximum recovery diluent (MRD) with a maximum dilution factor of 10^{-5}. Petri dishes contain BHI agar media were divided into 3 sectors 10^{-3}, 10^{-4} and 10^{-5}, 5 replicate 20 µl samples per dilution (total sample volume = 100 µl) were dispensed onto the surface of BHI agar plate, in triplicate, then allowed to dry into the agar plate next to a Bunsen burner. Once dried, the BHI agar plates were incubated at 37 ºC for 24 h to determine the number of viable bacteria. The colonies were counted after overnight incubation using a

Coulter counter (Digital colony counter, Stuart SC6 Colony counter - Stuart Equipment, protected by BioCote, UK). For counted populations of *L. monocytogenes* counts were made on drops showing between 3 and 30 colonies, data were converted into a log (cfu ml^{-1}) using the following formula:

$$\mathbf{No.\,CFU/ml} = \mathbf{No.\,(In\ countable\ range)} \times \frac{\mathbf{1}}{\mathbf{dilution\ factor}} \times \frac{\mathbf{1}}{\mathbf{volume}}$$

2.13 Gram-staining method

The Gram staining procedure involved placing a loopful of maximum recovery diluent (MRD) on a glass microscope slide. By using a 1 µl inoculation sterile loop, a drop of overnight culture from the test of assessment of cell morphology (Section 3.2.6) was mixed with the MRD and the sample was emulsified. The drop was left to dry for about 30 s and then fixed using a Bunsen flame by passing briefly in the flame 2 to 3 times. The slides were placed on a rack and then immersed in primary stain (methyl violet: purple stain) for 1 min. Excess stain was rinsed off with water, then the slide was placed into mordant (Lugols iodine) for 30 s, and then excess stain was washed off with water and slides were then placed in iodine (mordant) for 30 s and then excess was rinsed off with water. After that the slides were immersed in decolourising agent (alcohol) for 1 min and washed in water. Slides were then placed into secondary stain/counter stain (carbol fuchsin: red stain) for 30 s and excess stain was washed in water. Slides were dried, then cells were imaged using a standard light microscope after Gram-staining using a x100 oil immersion lens. Images were captured using microscope with camera attached to a computer, and also with using electronic microscope (Nikon) connected to a Cannon camera (Canon EOS 700D, Japan).

2.14 Recovery of plasmid containing strains from frozen culture

One colony of *Listeria* strains carrying the *Lux* gene (see Table 2.2 and 2.3 for details) from -80 ºC were streaked onto brain heart infusion (BHI) agar to obtain pure isolated colonies and incubated at 37 ºC for 18-24 h. All colonies arising on agar plates could be visualized both by the *GFP* phenotype (as fluorescent colonies) microscopic and by the luciferase phenotype (as light-emitting colonies) luminometry. The distinction between fluorescent and non-fluorescent colonies was examined using the Biospace lab photon imager (Biospace Lab, France) for Gfp to ensure the present of plasmid.

2.14.1 Confirmation of light emission (luminometry)

The frozen stored *Listeria* strains were reactivated in BHI agar plates and incubated for 24 h at 37 ºC. A colony was transferred into 5 ml of (BHI) broth which contained 5 µg ml^{-1} erythromycin for plasmid selection, as appropriate for 24 h at 37 °C in shaking incubator (150 rpm), the strains were analysed in a Luminometer (Model TD-20e Luminometer, Turner designs) by transferring 3 ml of overnight cultures into luminometer tubes to measure the light emitted. To prepare cultures for monitoring luminescence during growth, 1 ml of the overnight culture was added to 49 ml of fresh BHI broth (1:50) and it was grown in a shaking incubator (approx. 150 rpm) at 37 ºC until mid-exponential phase growth (approx. 3-4 h). In each case strains that did not contain a plasmid were used as a negative control. After 4 h, 3 ml of samples were transferred into tube and again the light was measured in the TD-20e Luminometer.

2.14.2 Confirmation of fluorescence phenotype (GFP)

One colony of *Listeria* strains carrying the *lux* genes from a -80 ºC stock was streaked onto a BHI agar plate than incubated for 24 h at 37 ºC, then the

plates investigated using the Biospace lab photon imager, the colonies that were bright was used in this study.

2.15 Live/Dead stain

Bacterial cell membrane integrity was determined using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes; catalog no. L-7012), for microscopy and quantitative assays. The stock solution of the two stains was prepared according to the protocol issued by Molecular Probes (Mol. Probes, 2004) by adding 2 µl of SYTO 9 and 2 µl of propidium iodide (PI) in equal proportions then mixed and diluted 1:100 in 0.85 % NaCl. To carry out a microscopic assessment of membrane integrity, 60 µl of the sample (Section 3.2.10) was mixed with 18 µl of dyes mixture in an Eppendorf tube. Samples were incubated in the dark for 15 min at room temperature, then 5 µl of the stained samples was placed between a glass slide and a cover slip which was examined after a further 10 min incubation. Fluorescent cells were observed at the excitation and emission wavelengths of 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide by using a fluorescence microscope (Nikon) equipped with an UV lamp and a 100 X magnification objective, visualized by using a digital Canon camera (Canon EOS 700D, Japan) coupled with fluorescence microscope at 100x magnification.

2.16 Evaluating the activity of liquorice extract on bologna-style sausage

Bologna sausages (Morliny Polish Doktorska, 450 g) were purchased from a retail supermarket (ASDA, UK). The inoculation of bologna with *L. monocytogenes* was done according to the method described by Lara-Lledó *et al*. (2012) with some modifications. Bologna-style sausages were sliced (~3 mm) in the laboratory using a food slicer and inoculated by dipping for 30 s in *L. monocytogenes* with and without 50 µg ml^{-1} liquorice extract to achieve an initial

inoculum level of approximately 4 log cfu ml^{-1}. The dipped slices were then allowed to dry for 30 min to remove excess liquid.

The inoculated slices were stored at 6 °C and samples (n = 3) removed for sampling at day 0 (slicing) and thereafter at day 5, 10 and 15. At sampling, slices were placed in a stomacher bag (177 mm x 305 mm) with 0·1 % (w/v) BPW and the blend was mixed for 60 s at 300 RPM speed in a Stomacher Laboratory blender (Stomacher 400, Seward Limited, UK) to distribute the *Listeria* equally. The mixture was serially diluted (1:10) with MRD, and the level of *Listeria* in the samples was determined by selective enumeration by plating appropriate dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) on Palcam Agar to selectively enumerate *Listeria*. This was done by adding 10 µl treatment or control samples to 90 µl maximum recovery diluent (MRD). Then Miles and Misra technique as describe in Section 2.12 was used to determine the viable count.

2.17 Mutagenicity of liquorice extract (Ames test)

The strains used in the experiment were provided from the University of Nottingham Microbiology and Food Saftey culture collection by Dr Jon Hobman. Overnight cultures were grown by transferring a single colony into a Universal tube containing 5 ml of Nutrient broth no. 2 with loose fitting caps. The inoculated tubes were incubated at 37 °C for 16-18 h and cultures were shaken at approximately 200 rpm to insure sufficient aeration and to reach a density of ~1.0 to 2.0 x 10^9 cfu ml^{-1} (OD_{540} between 0.1 and 0.2), 2 ml of molten top agar supplemented with limited histidine and biotin at a temperature between 43 ºC and 48 ºC to avoid killing of the strains was added to 0.1 ml of bacterial suspension with and without 50 µg ml^{-1} of liquorice extract. The contents of the test tubes were then mixed and poured onto the surface of glucose minimal (GM) agar plates then spread over the surface of the agar. The negative control was conducted by replacing the extracts with 1 % (v/v) ethanol solvent.

The antibiotics required for each strain are described in Appendix 2. These were added directly to the GM media after autoclaving and mixed in the agar, for the positive control 0.1 ml inoculum and 2 ml molten top agar with biotin and a trace of histidine were added to a bijou bottle, then spread over the surface of the GM agar. Under aseptic conditions a sterile filter paper disc (6 mmø, Whatman) was placed in the centre of each MG agar plate then 10 µl of the positive control (mutagenic) solution sodium azide or mitomycin C (depending on the strain) was dispensed to the disc (Table 2.6).

When the top agar was set (2-3 min), the plates were inverted and covered with aluminium foil to protect them from the effects of light on photosensitive chemicals and incubated at 37 °C for 48 h.

2.18 Cell culture conditions

2.18.1 Maintenance of cell culture

Human Caucasian colon adenocarcinoma (Caco-2) cells were obtained from the Food Science Department, University of Nottingham, United Kingdom. The stock of Caco-2 cells were kept in liquid nitrogen tank. 1 vial containing 1 ml of the cells was defrosted and maintained in complete medium Dulbecco's modified Eagle's Medium (DMEM) High Glucose (D6571, Sigma), supplemented with 10 % fetal bovine serum (FBS) (F9665, Sigma), 1 % L-Glutamine solution (G7513, Sigma), 2 % non-essential amino acid solution (100x) (M7145, Sigma), cells were grown in T-75 tissue culture flasks (Corning), cells were incubated at 37 ºC in humidified atmosphere of 5 % CO_2 (SANYO CO_2 Incubator) and allowed to adhere for 24 h and the cell lines were maintained by changing medium every 2-3 days. Cell culture was passaged when cells reached to required confluence (approximately 80 % confluence) by removing medium and rinsing with 15 ml of Phosphate Buffer Saline (PBS), then the PBS was removed. The adherent cells were removed by treating with 2 ml of trypsin- EDTA solution and incubating for

2 min at 37 ºC to detach the cells from the surface. Then the cells were examined under the microscope to see separate cells after treatment with trypsin, 8 ml of fresh pre-warmed media was then added to the flask to inactivate trypsin. Cell clumps were disrupted with gentle pipetting up and down on the plate to help the cells to detach to break the cell clumps, then the contents of the flask were transferred into a 50 ml Falcon tube (Sigma-Aldrich), the cells were centrifuged using centrifuge (Thermo Scientific) at 500 x *g* for 5 min and the supernatant was discarded to obtain a cell pellet and cell pellets were re-suspended in 8 ml of fresh medium, again small amount of suspension cells were transferred into a new flask with 8 ml of medium. Cell density were counted using a haemocytometer as a described in Section 2.18.2.

2.18.2 Counting cells with a Haemocytometer

Viable Cells were assessed by trypan blue solution (Sigma, UK), then cells were counted by using a Haemocytometer (Neubauer's chamber). Before each use, the haemocytometer was washed with distilled water and wiped with 70 % (v/v) ethanol. The sample solution was prepared by mixing 50 µl of cell suspension with 50 µl trypan blue into a micro-centrifuge tube, then 50 µl of cells suspension were transferred by a Gilson pipette into haemocytometer. The cells suspension was dropped from the pipette to fill the upper and lower counting chamber. The haemocytometer was then examined under an inverted microscope (PBA Microscopes). Cells were counted in the square surrounded by triple ruled lines in the centre of the haemocytometer which divided into a grid pattern, consisting 25 large squares. Each squares contained 16 sub squares, and cells which touched lines were only counted if on the top or left line of the square and using the contrast to distinguish the cells and count both live and dead cells, the dead cells will appear blue and live cells will appear clear (white). A total of 25 large squares

in the centre were counted in the upper and lower chamber and the average number from these multiplied by 10^4 giving number of cells ml^{-1}.

2.18.3 Determination of cell viability (MTT assay)

Cell viability was determined using the protocol described by Riss *et al*. (2016) with slight modification. Briefly, Caco-2 cells were seeded at a density of 0.3×10^5 cells per well, 5 ml of DMEM with cells was placed at every well in a 6-well plates, cells were treated with two different concentrations of liquorice extract: 12.5 and 50 µg ml^{-1}, 1 % (v/v) ethanol solvent and 4 % vinegar (acetic acid) were used as a negative control and sodium dodecyl sulfate (SDS) (20 %) was used as a positive control. Untreated cells with no extract added were also tested. Triplicate wells were prepared for each individual treatment. Cells were kept in the incubator for 24 h in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 ºC.

After 24 h, culture medium was then aspirated, washed with 5 ml of PBS to remove dead cells and cellular debris. PBS was replaced with fresh DMEM medium containing yellow tetrazolium salt, cells were re-incubated for an additional 3 h at 37 °C. The supernatant was removed, and the residual purple formazan product was solubilized in 2 ml solubilization buffer, then plates were gently shaken and re-incubated for 15-30 min to dissolve the purple formazan dye crystals formed and to stop the reaction. One ml of treated and non-treated cells were transferred into a cuvette and the absorbance was measured by using a spectrophotometer at OD_{570nm} wavelength to measure absorbance of formazan product. The percentage of viable cells was calculated from triplicates using the following formula (1): as a described by (Rezk *et al*., 2015).

$$\textbf{Cell viability}\ (\%) = \frac{\textbf{Absorbance of treated cells}}{\textbf{Absorbance of control cells}} \times \mathbf{100}$$

The percentage of cell viability was calculated by the absorbance readings from the test sample and divided by the control sample then multiplied by 100.

2.19 Cell freezing procedure

To make frozen stocks, Caco-2 cells were harvested using the standard procedure described in Section 2.18.1, after the supernatant was discarded, 90 % DMEM with 10 % Dimethyl sulfoxide (DMSO) were prepared by adding 9 ml of DMEM with 1 ml of DMSO and mixed. The mixture was filter sterilized using 0.22 µm filters, then 6 ml of the solution was added to Falcon tube that contain pellet of cells. The pellet was resuspended in the solution and 1 ml of cell suspension was transferred into a cryogenic vial, and then placed into a container containing 250 ml of isopropanol (99.5+ %, pure). The tubes were marked with name of cells, number of passages and date. Tubes were kept in -80 ºC for one night before permanent storage in the gas phase of a liquid nitrogen tank (at -180 °C) for optimum long-term survival. Frozen cells were recovered by rapid thawing at 37 °C, then washed and maintained in complete medium by mixing directly with 10 to 15 ml of sterile complete medium. Each vial containing 1 ml of frozen cells thawed in a 75 mm tissue culture dish.

2.20 Evaluation of toxicity of liquorice extracts on the yeast

A single colony of yeast strains S288C and NCYC 363 was inoculated in yeast extract -peptone dextrose (YPD) broth medium and incubated at 30 °C for 48 h. Cells were harvested by centrifugation and re-suspended in 20 ml distilled H_2O. This process was repeated twice to ensure all the YPD broth was removed from the culture and then water was added to a final volume of 10 ml. Three dilutions were prepared 10^{-1}, 10^{-2} and 50/50. Two Microcentrifuge Eppendorf tubes were filled with 900 µl water and one was filled with 100 µl methylene blue, 100

µl of culture was added to the first tube mixed thoroughly then 100 µl was transferred into the second Microcentrifuge Eppendorf tube and mixed then 100 µl was transferred into third tube that contain of 100 µl of methylene blue then mixed thoroughly. 10 µl of 50/50 suspension was drawn from the pipette to fill the counting chamber in one side, then cells counted using a Neubaure Haemocytometer. The number of cells was calculated then diluted in YPD broth to a density of $1x10^6$ cfu ml^{-1}. Three 100 ml flasks for each strain were prepared containing a total volume of 20 ml of culture. The first flask (control) contained 1 % (v/v) ethanol, the second flask contained 12.5 µg ml^{-1} of liquorice extract and the third flask contained 50 µg ml^{-1} of liquorice extract. 200 µl of the cultures were transferred into a 96 well plate. The plate was then shaken in a microplate reader (TECAN) at 25 °C for 1800 cycles of orbital (30 min) shaking followed by OD measurements for 48 h.

2.21 STATISTICAL ANALYSIS

Data were converted to log (cfu ml^{-1}) for bacterial counts and parametric statistics (means ± standard errors) were used. All the results were expressed as mean ± SD. Analysis of variance (ANOVA) were used to analysis the data. Each data was compared to one another to show if there is any differences between the means. Statistical analysis was carried out using GraphPad Prism V7 (GraphPad Software, Inc, CA, USA) and Microsoft® Excel 2010/XLSTAT©-Pro (2013.4.03, Addinsoft, Inc., Brooklyn, NY, USA) statistical package at 95 % confidence level. Analysis of variance ANOVA vs Mann Whitney was used. Differences were evaluated by using Tukey's post hoc analysis for multiple comparisons within groups of normally distributed data and the values were considered as statistically significant when p values were less than 0.05 ($p < 0.05$).

CHAPTER 3

EVALUATION OF LIQUORICE EXTRACT AS AN ANTIBACTERIAL

Abstract

In this chapter the antibacterial activity of liquorice extract was determined by screening for growth inhibition using eight species of bacteria of food relevance. The Gram-positive bacteria included *L. monocytogenes, L. innocua, S. aureus, E. faecalis* and *B. subtilis*. The Gram-negative bacteria include *P. aeruginosa, E. coli* and *S.* Typhimurium but none of this latter group were affected by the extract. For all of the Gram-positive bacteria tested, growth was inhibited as monitored using optical density, but the cells were not killed since the cells were still viable when plated out. The Minimum inhibitory concentration [MIC] and Minimum bactericidal concentration [MBC] of the extract was also determined and a concentration of 50 μg ml^{-1} was found to have a strong bacteriostatic effect on Gram-positive bacteria. In addition the growth of *M. smegmatis* was also inhibited, suggesting that a wide spectrum of Gram-positive cell types are sensitive to this extract.

In addition the use of a reporter strain of *Listeria* transformed with the bioluminescence genes *luxABCDE* indicated that cell energy levels were reduced when treated with either 12.5 or 50 μg ml^{-1} of the extract, with the reduction in light output being proportional to the concentration of the extract used and also in this study the mechanism of action of the liquorice extract was further investigated using the LIVE/DEAD® viability stain. Exposure to the MIC for this extract (50 μg ml^{-1}) resulted in total inhibition of bacteria growth and samples imaged using the LIVE/DEAD stain showed that the cells were unable to actively remove the fluorescent dye from the cytoplasm, suggesting that membrane integrity was affected. Exposure to a sub-lethal level of the extract (12.5 μg ml^{-1}) resulted in some growth inhibition and a lower percentage of cells showing evidence of membrane damage.

Evidence that cell division is inhibited was also seen since cells exposed to 50 μg ml^{-1} were generally shorter and did not take up Gram-stain effectively. Together these results indicate that the extract targets a site in the cell wall of

Gram-positive bacteria and it has potential as a natural food preservative either for high risk *Listeria* products or for products where spoilage occurs due to Gram-positive bacteria.

3.1 Introduction

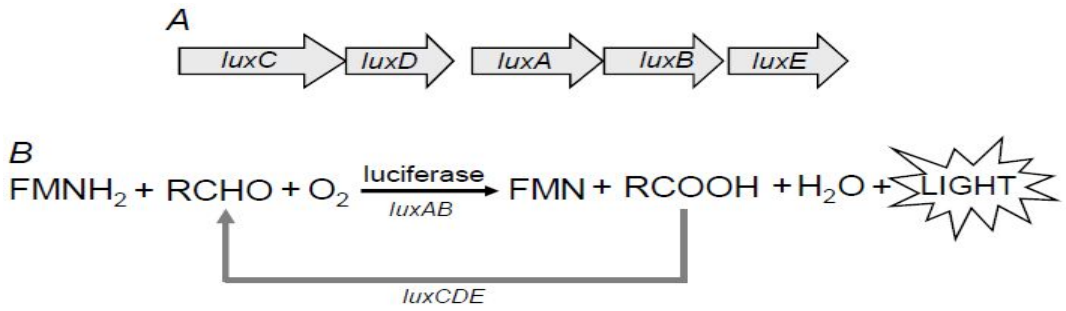
Bacteria such as *Campylobacter*, *Salmonella*, *L. monocytogenes* and *E. coli* are commonly reported to be responsible for foodborne disease and outbreaks (Alocilja and Radke, 2003; Chemburu *et al*., 2005). Generally, all these bacterial pathogens are grouped together, but physiologically they are very different, in particular a major structural difference exists between the different cell types. For instance, *Campylobacter*, *Salmonella* and *E. coli* are all Gram-negative bacteria and have a cell wall structure including two layers of membrane and a thin layer of the structural polysaccharide peptidoglycan (PG). In contrast Gram-positive bacteria, such as *Listeria*, *Staphylococcus*, *Enterococcus* and *Bacillus*, have only one cell membrane encased in a thick layer of PG. Hence when considering the action of new antimicrobial agents, it is important to test them against a range of bacterial cell types.

Generally, when testing antimicrobial agents, cells are challenged with the test chemical and the effect measured by the level of growth inhibition observed. Despite the fact that the definition of viability depends on the capacity of bacteria to multiply and divide, growth of bacteria in a liquid medium, or the formation of colonies on solid agar, is not considered sufficient evidence that bacteria are either alive or dead after treatment since antimicrobial agents may simply be bacteriostatic and prevent growth without causing permanent cell damage. Thus, intermediate situations such as cell injury are hard to identify by plating methods. To address this, other indirect methods have been developed to report on cellular viability, including monitoring enzyme, respiratory activity and measurement of

ATP levels (Riss *et al*., 2011). These parameters can also report on sub-lethal damage to cells, for instance factors that affect membrane integrity or require energy for repair will reduce ATP levels but not result in cell death (Nocker *et al*., 2011; Hill and Stewart, 1994). Indeed many studies have shown the significant effect of microbial stress on cell growth due to the depletion of energy levels and it has been pointed out that the rate of cell growth is related to the stress applied (Khan *et al*., 2010).

Bioluminescence is the process by which visible light is emitted by an organism as a result of a chemical reaction catalysed by a luciferase enzyme and this phenomenon is seen in a variety of organisms, such as insects, fish, squid, shrimp, and jellyfish, but is also seen in bacteria. Bacterial luciferase (Lux) is encoded by the *luxAB* genes which catalyse the reduction of a long-chain fatty aldehyde in the presence of molecular oxygen and flavin mononucleotide ($FMNH_2$) and results in the emission of light (Figure 3.1 B). Cells encoding the full *luxCDABE* operon (Figure 3.1 A) generate a bioluminescence phenotype without the need to add any additional cofactors or exogenous substrates as the accessory genes *luxC*, *luxD* and *luxE* produce the aldehyde within the bacterial cell (Hill and Stewart, 1994; Meighen, 1993). Since $FMNH_2$ production is dependent on a functional electron transport chain, only metabolically active bacteria emit light and therefore it can be used to rapidly monitory antimicrobial activity (Hill and Stewart, 1994). Although many bacteria of interest are not naturally bioluminescent, the *lux* operon can be introduced into a number of different cells on plasmids as long as the genes are engineered to function in that particular cell type (Perehinec *et al*., 2007) and therefore bioluminescence has been widely used to study antimicrobial agents (Hill and Stewart, 1994; Robinson *et al*., 2011).

Figure 3.1. Bacterial bioluminescence



Summary diagram showing the reaction catalyzed by bacterial luciferases: Panel A) Organisation of the *lux* operon found in *Vibrio* and *Photorhabdus* species, including the luciferase genes *luxA* and *luxB* and the *luxCDE* genes which encode a fatty acid reductase complex that regenerates the long chain aldehyde substrate (luciferin). Panel B) The chemical reaction giving rise to light production in bioluminescent bacteria in which the aldehyde is converted to the carboxylic acid in the presence of oxygen reduced flavin mononucleotide ($FMNH_2$); the by-products of this reaction are flavin mononucleotide (FMN) and light in the visible spectrum (bioluminescence) as blue/green light emission ($^{\lambda}MAX$ ~ 490 nm) (Adapted from Gahan, 2012).

Another commonly used marker gene is the green fluorescent protein (Gfp) produced by the jellyfish *Aequorea victoria* which has become a simple and flexible tool used in many applications in the field of molecular biology, medicine and cell biology. The protein is small (27 kDa) and produces green fluorescence at 509 nm when excited with UV light at 398 nm (Lorang *et al*., 2001; Zimmer, 2002). As Gfp protein is extremely stable *in vivo*, it has proved to be useful when studying cells exposed to some sort of stress condition, as the fluorescence signal does not decrease allowing cells that produce this protein to be easily visualised, even if the cells are non-viable (Lowder *et al*., 2000). Again, bacteria do not naturally produce Gfp but this can also be introduced into bacteria either on plasmid vectors or integrated into the chromosome (Qazi *et al*., 2001). It is also possible to introduce multiple marker genes onto a plasmid, so that Gfp can be introduced in conjunction

with the *lux* genes so that cells containing these plasmids are both bioluminescent and fluorescent and as the spectrum of light produced by each marker is different, there is no interference between the two signals (Qazi *et al*., 2001). Overall, these reporter proteins allow the evaluation of bacterial viability in an inexpensive, rapid and easy way. Measurements are also easily automated, which makes luciferase and Gfp-based measurements two of the most efficient ways to investigate the viability of different microorganisms.

The main requirement in different fields of microbiology, including public health, biotechnology, food manufacture and the water and pharmaceutical industries, is the evaluation the viability of bacteria that commonly cause contamination such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, and *Salmonella enterica serovar* Typhimurium (Pyle *et al*., 1999; Nyström, 2001). Because bacterial cell death has long been defined as the failure of a cell to grow to a visible colony on bacteriological media, therefore bacterial death can only be monitored retrospectively using traditional culture methods. However intermediate cases exist, such as cell injury, which are difficult to measure using plating methods (Berney *et al*., 2007). Due to various environmental stresses, such as nutrient starvation, high or low temperature, high pressure or changes in pH, several bacteria have also been shown to enter into a viable but non-culturable (VBNC) state, where they are alive but lose the ability to recover into active growth until suitable conditions for growth are re-established. However there is evidence that ingestion of such VBNC bacteria can lead to infection and therefore this state is a major concern when assessing the effectiveness of antimicrobials (Pienaar *et al*., 2016; Khan *et al*., 2010).

Despite these limitations, the most common method for enumerating *L. monocytogenes* present in food samples is culture, but it is time consuming and depends on cell growth, so that – as mentioned above - injured and non-culturable

cells have no opportunity to be detected (Olszewska *et al*., 2015). In addition, for bacteria which have the ability to form clumps or chains, the viable count is not a good method to accurately measure cell numbers. This is also true for bacteria forming biofilms which is a form of bacterial growth that is widespread in factory environments and makes it difficult to release bacteria from surfaces for accurate determination of viable count by culture methods (Bettencourt *et al*., 2010). Especially in high-throughput experiments, colony counting is a labour-intensive and time-consuming process due to the need to create serial dilutions and plating samples on agar plate, which makes it difficult to use for a large number of samples.

In contrast, traditional direct-count investigations of bacterial viability rely on metabolic characteristics or membrane integrity (Paparella *et al*., 2012). A large number of investigations and processes have been developed in order to investigate the physiological characterization of bacteria at the single-cell level to understand all the processes that occur inside the cell. Most of these methods require long periods of time to complete, in some cases up to 8 hours, such as fluorescence-based methods which involve direct viable count using a microscope or methods combined with nucleic acid staining as well as measurement of membrane integrity (Joux and Lebaron, 1997; Boulos *et al*., 1999; Yokomaku *et al*., 2000). Although bioluminescence measured directly reflects the number of viable cells, all living cells are not necessarily always in the same metabolic state which may limit the effectiveness of bioluminescence as an assay of absolute cell numbers. In contrast, fluorescence can be used to assess bacterial cell number. *GFP* is known as a stable protein and has the capacity to fluoresce in the absence of the need to deliver a substrate, and unlike bioluminescence, *Gfp* fluorescence density is reported to be proportional to total cell number. Thus, Lehtinen *et al*. (2006) reported that the Green fluorescent protein (*Gfp*) might be considered as a flexible tool to study the impact of antimicrobial agents on the growth of bacteria

as a rapid alternative to monitoring cell number using culture-based methods (Lehtinen *et al*., 2006).

However the most commonly used alternative approach to traditional methods of assessing cell viability that has been developed is to use LIVE/DEAD staining kits which can be used for viability assessment for estimating total cell count, including both dead and live cells (Kell *et al*., 1998; Leuko *et al*., 2004). Hence, in different fields of microbiology, the LIVE/DEAD BacLight kit has seen increasing usage by researchers as a rapid method for detection and distinguishing between a live and dead bacteria. This is a fast method frequently used to determine both viable and total counts of bacteria and monitors bacterial cell viability as a function of the membrane integrity. The kits consists of a combination of two nucleic acid stains, the red fluorochrome (propidium iodide) (PI) is a large molecule that can pass through damaged plasma membranes into cells, while the small molecule green fluorochrome (SYTO 9) penetrates all bacterial membranes (Montañez-Izquierdo *et al*., 2012; Alonso *et al*., 2002). A bacterial viability kit which detects the presence of an intact membrane potential based on the differential accumulation of two dyes; component A (SYTO 9 dye, 3.34 mM) and component B (Propidium iodide, 20 mM). The excitation and emission maximum for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for propidium iodide (Probes, 2004). These kits are a rapid and convenient method that are appropriate for routine measurements. When the cell membrane is damaged, PI enters the cell and produces red fluorescence, which causes reduction in an intensity of SYTO 9 fluorescence and labels cells with damaged membranes. Thus, cells with an undamaged membrane will stain green while cells with a disrupted membrane that are considered to be non-viable will stain red and this is taken as evidence of the viable state of the bacteria, but – as discussed above in relation to the VBNC state - there is still some controversy on how to define dead

bacteria (Swaminathan and Gerner-Smidt, 2007; Montañez-Izquierdo *et al*., 2012).

Kits containing propidium iodide and SYTO9 are the most commonly used, and have been used to assess bacterial viability in biofilms, distinguish between pathogenic and non-pathogenic bacteria and to perform a rapid viable count of water-borne bacteria (Yang *et al*., 2009; Hoefel *et al*., 2003). They have also been used to study different bacterial species in pure culture and in various environments and food products (Berney *et al*., 2007; Leuko *et al*., 2004). The technique can be applied rapidly because no extended incubation period is required to allow for colony growth, and quantitative results in some case can be gained within 1 h using a fluorescent microscope or flowcytometer, or can also be used as an alternative methods to monitor membrane integrity (Olszewska *et al*., 2015). The technique is also versatile in that it has been successfully applied to a number of bacterial species, most of which are known pathogens (Berney *et al*., 2007; Stocks, 2004).

Besides all of these indirect methods of measuring cell damage, one important parameter required to understand the mechanism of action of antimicrobial agents on microbes is visualising the morphological structure after treating with antimicrobial agents to determine how these agents could be impacting on microorganisms (Kitajima, 1998).

Objectives: Previous research in this laboratory and in the literature had indicated that liquorice showed a good antimicrobial activity against different types of bacteria as mentioned in Section 1.5.1. So far, liquorice has been extensively studied for its efficacy as an antimicrobial but the antibacterial mechanism of waste material recovered from the end of the production of liquorice has not been specifically reported. Hence the first aim of this Chapter was to investigate the

potential of one particular liquorice extract as natural antimicrobial compound, in particular against Gram-positive bacterial pathogens, including *Listeria monocytogenes* and *Staphylococcus aureus*. At the beginning of this work a product was supplied by a UK company (Phytoquest, UK) that was a waste product of the production of liquorice flavouring and had the potential to be used as a food additive to control these organisms. The first questions to be addressed were (a) what is the range of effectiveness? and (b) what is the minimum inhibitory concentrations required?. The mode of action would also be investigated by determining whether there are any visible effects on the membrane integrity and cell wall morphology.

The second aim was to use a range of techniques to examine the viability of bacteria after liquorice extract treatment. The Live/Dead BacLight™ bacterial viability assay (Live/Dead staining) and viable count were employed to assess the extract treated with *Listeria* strains and *Bacillus*.

The main body of the methods are described in Chapter 2, when necessary some of these details are repeated to make it easier to follow the experiments presented.

3.2 Results

3.2.1 Test solubility of liquorice in ethanol solvent

Before starting the experiments it was important to confirm the percentage of solvent required to solubilise the compound. Phytoquest had provided information that liquorice powder should be dissolved in ethanol, but this is antimicrobial in itself and therefore it was important to determine the lowest concentration of solvent that could be used. Therefore different mixtures of ethanol/water were prepared in order to determine what composition was required to dissolve of liquorice powder completely. Compositions of ethanol/water (30:70, 40:60, 50:50, 60:40, 70:30 v/v) were mixed with different weights of liquorice powder (1.0, 2.0 and 3.0 mg) and the bottles were sealed with Nesco film to avoid

evaporation of the solvent and then the samples were incubated at 37 ºC for less than 30 min. The results indicated that ethanol/water (70:30, v/v) was the optimum concentration solvent as it was able to dissolve 3 mg of liquorice in 1 ml of solvent completely. Thus, in all further experiments in this thesis were carried out as using 70 % (v/v) ethanol to prepare the initial liquorice extracts.

3.2.2 Establishing the growth conditions required for different Gram-positive and Gram-negative bacteria

The aim of this study was to investigate the effect of a different concentrations of liquorice extract on the growth rate of bacteria, these were initially tested by using a broth dilution method. Testing was conducted using organisms that contribute to infectious diseases and food spoilage including *E. faecalis*, *S. aureus*, *B. subtilis*, *L. innocua*, *L. monocytogenes*, *E. coli* and *P. aeruginosa* (see Table 2.2 for details). These organisms were chosen because of their harmful effects on human health and food spoilage including Gram-negative and Gram-positive bacteria, both spore-forming and non-spore forming.

First it was necessary to determine the appropriate method to prepare cultures of the test bacteria to allow convenient growth of different strains of bacteria being used as a part of this study and also to be able to monitor growth using optical density. Overnight cultures of the Gram-negative and Gram-positive bacteria including *E. faecalis*, *S. aureus*, *B. subtilis*, *L. innocua*, *L. monocytogenes*, *E. coli* and *P. aeruginosa* were prepared as described earlier in Section 2.8, then different dilutions were made (1:10, 1:50, 1:100) of these overnight cultures into different broth media (Tryptone Soya Broth, Nutrient Broth, Mueller Hinton Broth and Brain Heart Infusion Broth). Due to the fact that the different types of bacteria in this study had very different growth rates, it was first necessary to determine what dilution of the overnight culture would produce an optimal growth curve using the automated Tecan microtiter plate reader and monitoring optical density at

OD_{600nm}. In particular it was important that the different stages of growth such as lag phase, exponential phase and stationary phase were visible. Samples (200 µl) of the different dilutions of the different cultures were transferred into a at least 4 wells of a sterile 96-well polystyrene microtiter plates which was then incubated at 37 °C for 24 h. Cell growth was monitored by using OD_{600nm} with readings taken every 10 min for 24 h.

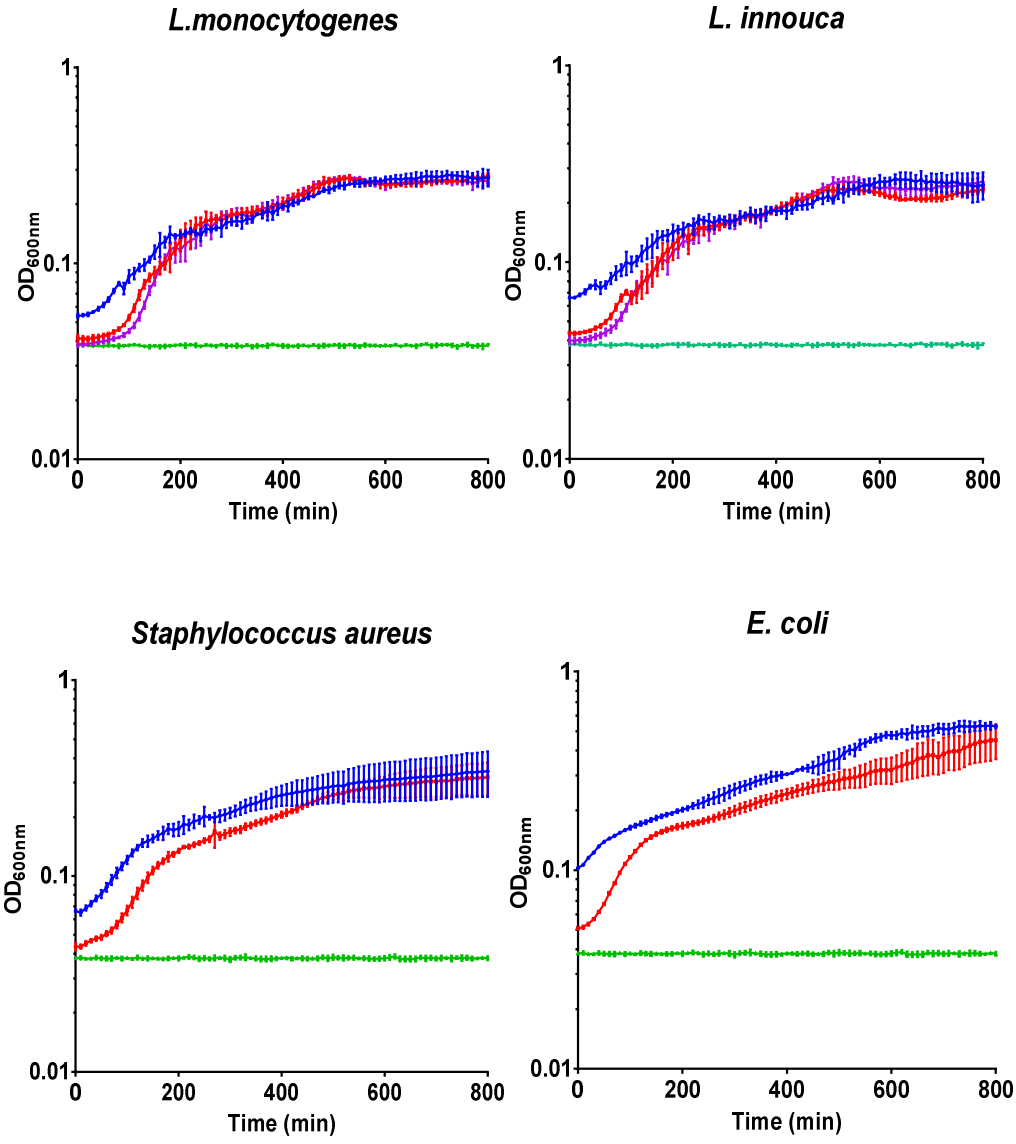
The result showed that all the tested strains grew well in most of the different media and some representative results for two *Listeria* species, *S. aureus* and *E .coli* are shown in Figure 3.2. It was found that using BHI broth good growth was seen, but that all the strains had very different growth rates. Table 3.1 shows a typical result of the initial OD_{600nm} values achieved reached by the different test bacteria after overnight incubation. Hence some of these bacteria required extended incubation times to achieve a high enough cell density to allow the fresh cultures to be prepared to an initial OD_{600nm} of approximately 0.05 which reproducibly allowed the different phases of growth to be identified when samples were incubated in the Tecan plate reader and several test experiments were performed to establish the growth times required to achieve this.

Table 3.1. Growth of bacteria used in this experiment

Type of bacteria	Gram reaction	Optical Density of cultures
Bacillus subtilis	+ve	0.381
Enterococcus faecalis	+ve	0.286
Listeria monocytogenes	+ve	0.101
Listeria innocua	+ve	0.102
Staphylococcus aureus	+ve	1.482
Pseudomonas aeruginosa	-ve	0.585
Escherichia coli	-ve	1.412

Table showing typical results of the OD_{600nm} values for the different types of bacteria after overnight growth. These experiments were performed to establish the growth times required for each organism.

Figure 3.2. Growth curves for different types of Gram-negative and Gram-positive bacteria

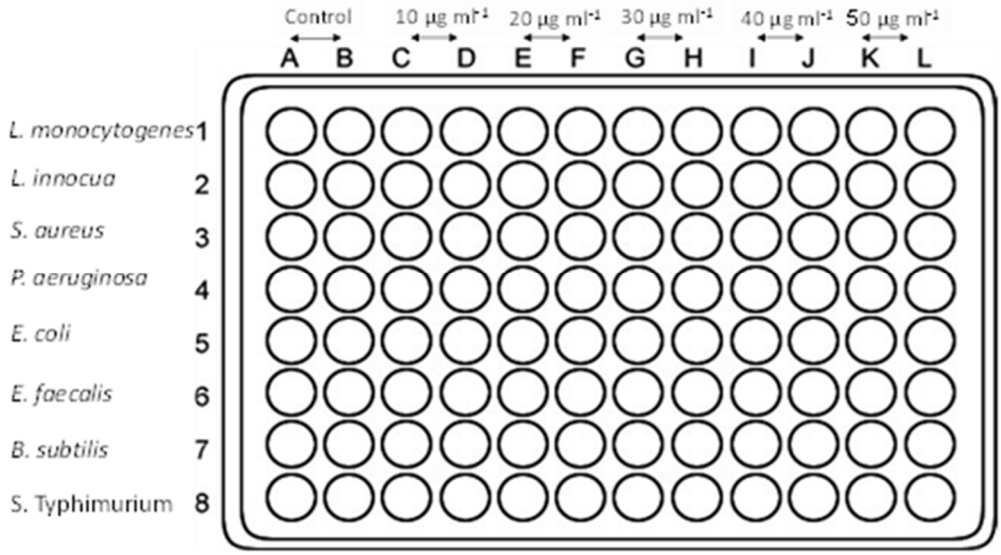


Growth curves of *L. monocytogenes*, *L. innocua*, *S. aureus* and *E. coli*. A colony of each bacteria was used to inoculate 5 ml BHI broth which was incubated at 37 °C overnight (18-24 h). The OD_{600nm} of the cultures was measured. The overnight growth was back-diluted into fresh BHI broth [●] 1:10, [■] 1:50, [▲] 1:100 and [▼] control contain BHI broth. Cultures were incubated at 37 °C in a shaking incubator (150 rpm), and optical density measurements were taken after 24 h to monitor the optical density change for all of bacteria tested.

3.2.3 Bacterial challenge experiments

The first point that needed to be established was whether the extract that had been provided was an active antimicrobial and what range of microbes it was effective against. The second point to be determined was the optimum concentration of the liquorice extract that would inhibit growth of the bacteria. Briefly, overnight cultures of different bacteria were grown in BHI broth, the cultures were adjusted in BHI broth to give a final density of $1X10^8$ cfu ml^{-1} (approximately OD_{600nm} = 0.1), different concentrations of the liquorice extract (10, 20, 30, 40 and 50 µg ml^{-1}) were prepared in BHI broth then 100 µl were transferred into 96 well plate (Figure 3.3). After that 100 µl samples of cultures at optical density 0.1 were transferred into the wells of a 96-well microtitre plate to give a final OD 0.05, two replicates for each concentration were used in addition to control, then the plate was incubated statically at 37 °C for 24 h in Tecan reader and optical density readings were taken throughout the incubation period. As control the bacteria were also grown in BHI broth without liquorice extract. The growth rate for all Gram-positive bacteria was calculated using the formula as was earlier described in Section 2.9.

Figure 3.3. Layout of samples in microtitre plate

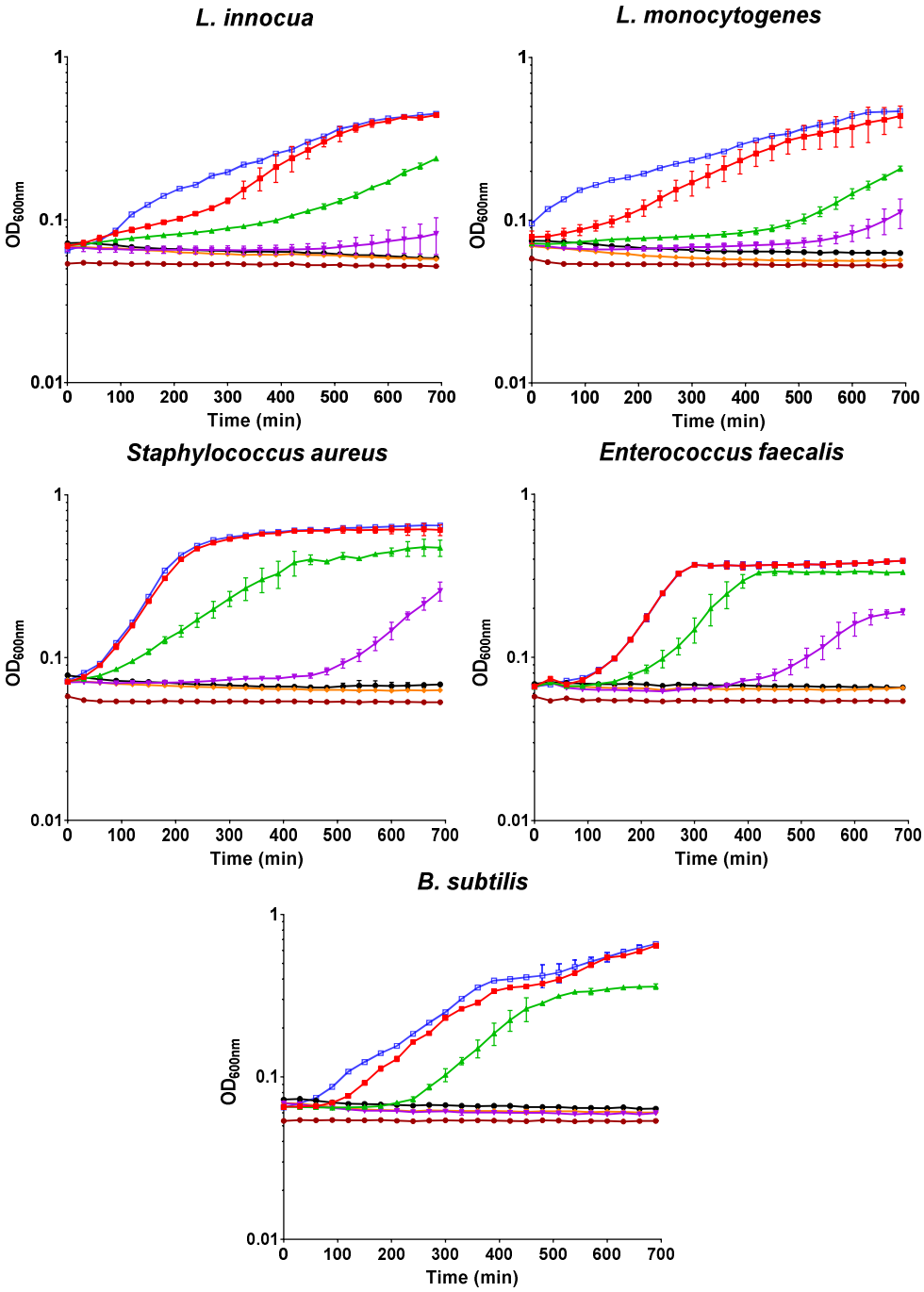


Overnight cultures were prepared with different concentrations of liquorice extract e.g. 10, 20, 30, 40 and 50 µg ml^{-1}, then incubated at 37 ^{o}C for 24 h. Using a microtitre plate to allow more concentrations to be tested, also allowed replicate samples of different bacteria tested.

The results shown in Figure 3.4 indicated that the liquorice extract was highly effective at inhibiting the growth of all the Gram-positive bacteria tested. At a concentration of 40 and 50 µg ml^{-1}, growth of *L. innocua*, *L. monocytogenes*, *S. aureus, E. faecalis* and *B. subtilis* were entirely inhibited. At 30 µg ml^{-1} delayed growth was seen for all strains except *B. subtilis*, while a concentration 20 µg ml^{-1} the growth was low rate for almost strains compared to growth seen in the presence of 10 µg ml^{-1} liquorice extract (Figure 3.4). In contrast growth of none of the tested Gram-negative bacteria was inhibited by the liquorice extract at any of these concentrations (Figure 3.5) and, accordingly there was no significant difference ($p > 0.05$) between the growth rate of these organisms at all concentrations of liquorice extract tested. The specific growth rate (µ) was determined from the slope of the curve and the data expressed per hour. As expected, bacteria grown in BHI alone had the highest growth rate, while bacteria

grown in different concentrations of liquorice extract had variable growth rates, depending on the concentration of the extract used (Figure 3.4). However the growth rates achieved in the presence of 10 μg ml^{-1} of liquorice extract was almost the same in the BHI broth without extract particularly for *Staph aureus*, *E. faecalis* and *B. subtilis* (Table 3.2 and Figure 3.4) and in this case the effect of liquorice extract can be seen as an increase of the lag phase accompanied by a decrease on the specific growth rate (μ) that may result in slower but continual growth, as in for concentrations of 20 and 30 μg ml^{-1} or total growth inhibition as in a concentration of 40, 50 μg ml^{-1} (Figure 3.4). Thus, from the results obtained from this experiment, two concentrations of liquorice extract (12.5 and 50 μg ml^{-1}) were selected to carry out further experiments as representative of concentration that would either give total growth inhibition (50 μg ml^{-1}) or that would be predicted to cause sub-lethal injury (12.5 μg ml^{-1}) to Gram-positive bacteria.

Figure 3.4. The effect of different concentrations of liquorice extract on growth of Gram-positive bacteria



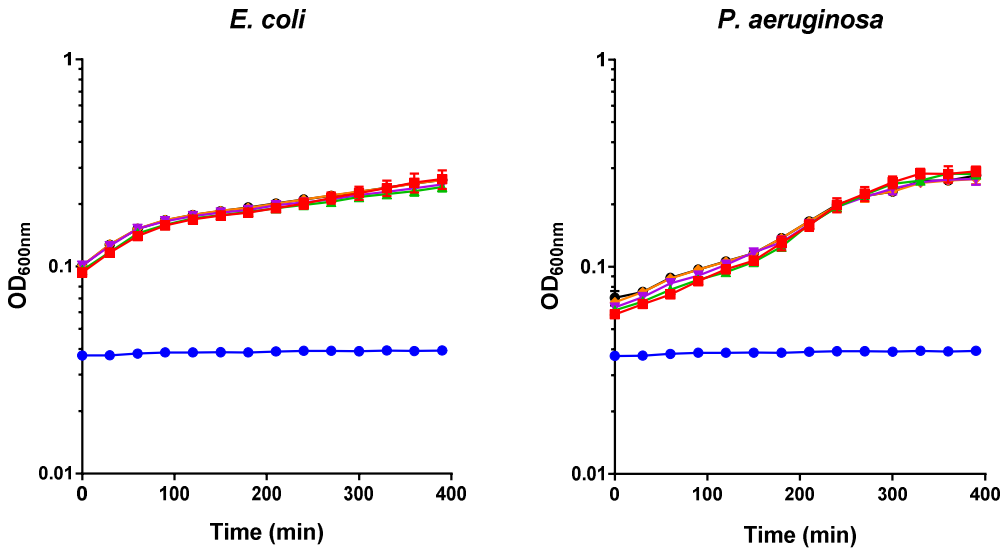
Cultures of *L. innocua, L. monocytogenes, S. aureus, E. faecalis* and *B. subtilis* were grown in BHI broth at 37 °C for 24 h in the presence of different concentrations of liquorice extract (10 [■], 20 [▲], 30 [▼], 40 [◆] and 50 [●] µg ml^{-1}). A control sample [●] containing broth only and control culture [□] was also included. Growth was monitored by optical density measurement at 600nm. Data are given as means ± standard deviations from two independent experiments, each performed in triplicates (n=3).

Table 3.2. The growth rate of Gram-positive bacteria

Bacteria Strains	Growth rate			
	Control	**10 µg ml^{-1}**	**20 µg ml^{-1}**	**30 µg ml^{-1}**
L. innocua	0.34 h^{-1}	0.22 h^{-1}	0.16 h^{-1}	0.08 h^{-1}
L. monocytogenes	0.50 h^{-1}	0.25 h^{-1}	0.24 h^{-1}	0.24 h^{-1}
Staph. aureus	0.50 h^{-1}	0.44 h^{-1}	0.38 h^{-1}	0.40 h^{-1}
E. faecalis	0.60 h^{-1}	0.59 h^{-1}	0.52 h^{-1}	0.38 h^{-1}
B. subtilis	0.48 h^{-1}	0.46 h^{-1}	0.43 h^{-1}	

Table shown that the calculated of growth rate of Gram- positive bacteria which grown for overnight in BHI broth then treated with different concentration of liquorice extract 10, 20, 30, 40, 50 µg ml^{-1}, then incubated at 37 ^{o}C for 24 h.

Figure 3.5. The effect of liquorice extract on the growth of Gram-negative bacteria



Cultures of *E. coli* and *P. aeruginosa* were grown at 37 °C for 24 h in the presence of different concentrations of liquorice extract (10 [■], 20 [▲], 30 [▼], 40 [●] and 50 [●] μg ml^{-1}). A control sample [●] containing broth only was also included. Growth was monitored by optical density measurement at OD_{600nm}. Data are given as means ± standard deviations from two independent experiments, each performed in triplicates (n=3). There is no effect of liquorice extract on the growth of Gram-negative bacteria.

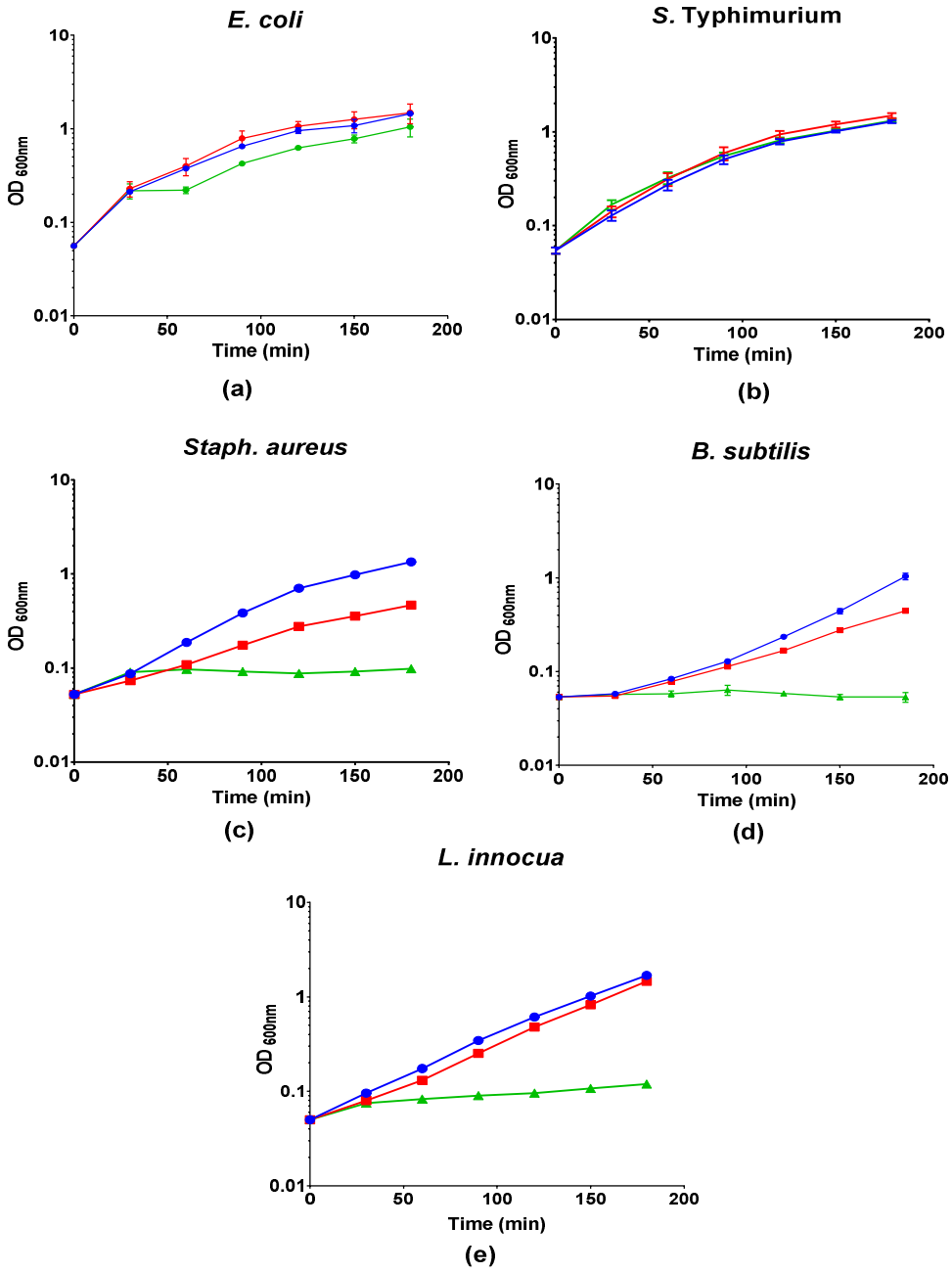
3.2.4 Demonstrating the spectrum of activity of liquorice extract at active concentrations

To confirm the choice from the previous results of the two concentrations of liquorice extract (12.5 and 50 µg ml^{-1}) were correct, these were used to carry out further experiments. In addition to these two test conditions, control experiments were included in this experiment to confirm the inhibition of growth of bacteria was due to liquorice extract rather than solvent. Therefore a control was prepared containing 1 % (v/v) ethanol which was calculated to be the highest concentration of ethanol present in any of the samples (i.e. 50 µg ml^{-1} sample). Biological evaluation of the extract was carried out by screening for antimicrobial activity against a range of bacteria *L. innocua*, *S. aureus*, *B. subtilis*, *E. coli* and *S.* Typhimurium to confirm that the extract had antimicrobial activity. Briefly, overnight broth cultures of Gram-positive and Gram-negative bacteria were adjusted to a population of 10^7 cfu ml^{-1} in fresh brain heart infusion (BHI) broth media then two concentrations of the liquorice extract (12.5 and 50 µg ml^{-1}) were inoculated with cultured bacteria to an OD_{600nm} of 0.05, 200 µl of samples was transferred into microplate and incubated at optimum growth temperature for bacterial strain and growth was monitored over time using optical density at OD_{600nm} every 30 min over 24 hours, using a microplate reader. The MIC was confirmed as the lowest concentration which no growth was revealed.

It was again found that the extract only affected the growth of Gram-positive bacteria since the Gram-negative bacteria *E. coli* and *P. aeruginosa* tested showed normal levels of growth (Figure 3.6 a and b) for any of the test conditions. The extract was found to specifically inhibit only Gram-positive bacteria (Figure 3.6 c, e& f), growth inhibition was seen the growth was completely inhibited at a concentration of 50 µg ml^{-1}, whereas at 12.5 µg ml^{-1} growth rate was reduced, but some growth was still detected. The types of bacteria tested included Gram-positive rods (*L. monocytogene*s (non-spore former) and *B. subtilis* (spore-former) and Gram-positive cocci (*S. aureus* and *E. faecalis*) showing that the extract

inhibited the growth of a wide range of different cell types. The fact that all the different cell types were affected in the same way indicates a shared mode of action rather than a specific effect on one type of cell. However, the important points to note here are that optical density does not report on the viability of the cells detected. Experiments now needed to determine whether at a concentration of 50 μg ml^{-1} cell growth is just inhibited or whether the cells are killed.

Figure 3.6. The effect of liquorice extract on the growth of Gram-negative bacteria (a) & (b) and Gram-positive bacteria (c)-(e)-(f)



Liquorice extract with a two concentration of 12.5 [■] & 50 [▲] µg ml^{-1} was strong effect on Gram-positive bacteria. For *L. innocua* a concentration of 50 µg ml^{-1} inhibited growth, for both *S. aureus* and *B. subtilis*, 12.5 [■] µg ml^{-1} showed a little inhibition, and 50 [▲] µg ml^{-1} inhibited growth, at these concentrations there is no effect on the growth of either *E. coli* or *S.* Typhimurium and the results were the same as before, all bacteria at 1 % [●] ethanol solvent was grown normally, this confirmed that the effect due to the extract.

3.2.5 Effect of liquorice extract on bacterial viability

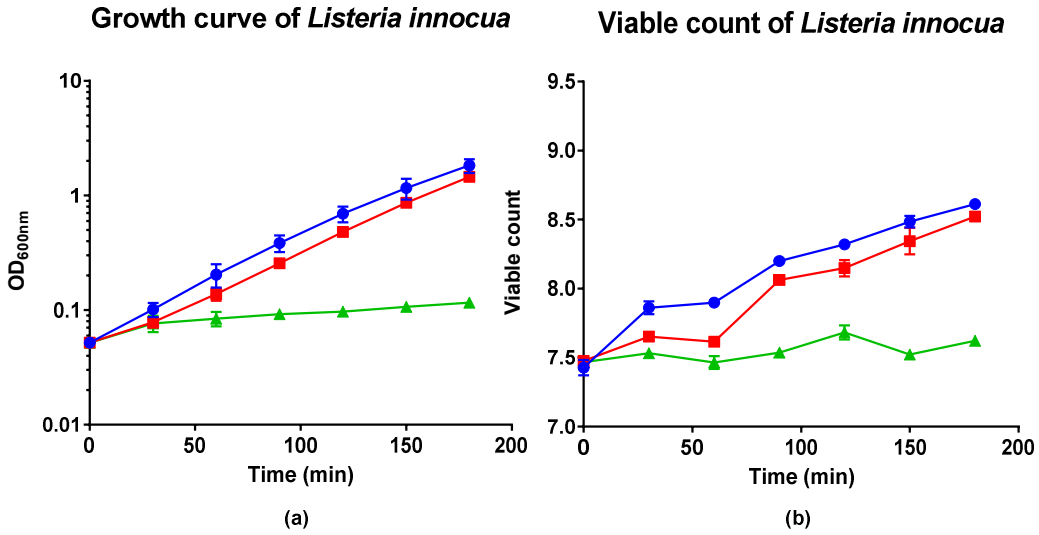
Since bacterial growth inhibition does not necessarily indicate bacterial death, the previous experiment in Section 3.2.4 cannot distinguish between bactericidal and bacteriostatic effects. Thus, the effects on bacterial growth in liquid cultures treated with liquorice extract was studied through both optical density measurements and viable counts in order to determine if the effect of the liquorice extract on the Gram-positive organisms was bactericidal or bacteriostatic. *L. innocua* was chosen as a model organism, being non-pathogenic and non-spore forming. The MIC is interpreted as the lowest concentration that inhibits visible microbial growth and is expressed in terms of μg ml^{-1}, whereas the MBC is interpreted as the lowest concentration that can completely kill the bacteria. To do this the same method was used as previously described but this time both the viable count and optical density were monitored. Briefly, three flasks were prepared as before in Section 2.11 with final concentrations of 12.5 μg ml^{-1} and 50 μg ml^{-1} of liquorice extract and also a control sample only containing the ethanol solvent. These flasks were inoculated with *L. innocua* at an OD_{600nm} of 0.05 (approx. $1x10^7$ cfu ml^{-1}) and were incubated at 37 °C for 30 min in shaking incubator (150 rpm), and samples were taken every 30 min for OD_{600nm} and viable count determination for approximately 3 h. For the viable count, 10-fold dilutions in maximal recovery diluent (MRD) were prepared and plated on the BHI agar plates using Miles and Misra technique as a described in Section 2.12 and the results were recorded after the plates were incubated overnight at 37 °C and the results are shown in Figure 3.7.

The first most obvious result was that the bacteria grew on the agar plates to produce colonies, and therefore although growth was totally inhibited in the sample containing 50 μg ml^{-1} (Figure 3.7 a) there was no effect of the liquorice extract on the *L. innocua* viability (Figure 3.7 b), and the number of viable cells did not decline over time indicating that the effect of the extract was bacteriostatic

rather than bactericidal. Comparing the effects of the difference concentrations used, the results for viable count were the same as those for the optical density with some inhibition occurring when 12.5 μg ml^{-1} of the liquorice extract was added. This was particularly characterised by an increased lag time when viable count was monitored. Again strong inhibition of growth with 50 μg ml^{-1} was seen and confirmed that the MBC of the liquorice extract was at approximately 50 μg ml^{-1}, and this level would taken as the MBC level in future experiments.

Using a Mann-Whitney test it was shown that the inhibition of growth did not decrease significantly ($P > 0.05$) in samples treated with 12.5 μg ml^{-1}, which means that a concentration of 12.5 μg ml^{-1} is not enough to inhibit *L. innocua* growth. In contrast, as seen in Figure 3.7, a concentration of 50 μg ml^{-1} of liquorice extract inhibited the growth of *L. innocua* significantly an effect ($p < 0.0001$). The significant difference in the growth rates determined using the viable count method was confirmed using ANOVA Tukey's test and the results were the same as those gained using optical density measurements, with significantly slower growth occurring when 12.5 μg ml^{-1} of the liquorice extract was added to the cultures.

Figure 3.7. The effect of liquorice extract on the growth (a) and the viable count (b) of *Listeria innocua*



Graphs comparing growth and viable count for *L. innocua* exposed to liquorice extract. Panel (a) shows the optical density and panel (b) shows the results gained using viable count after incubation of *L. innocua* with two concentrations of liquorice extract 12.5 [■] and 50 [▲] µg ml^{-1} for various time intervals. The activity is expressed as log_{10} cfu ml^{-1} observed at each 30 min compared to the control sample that contained 1 % (v/v) [●] ethanol solvent. Data from two independent experiments, each performed in triplicates (n=3). Results are expressed as mean ± SD and the comparison is made by Tukey's post hoc test to calculate the P-values. Significant difference between a concentration 50 µg ml^{-1} and 1 % (v/v) ethanol solvent is indicated as $p < 0.0001$.

3.2.6 Assessment of cell morphology using Gram-staining

As many of the bacteriostatic agents affect cell wall synthesis and cell division, experiments were carried out to monitor the cell morphology after the application of the liquorice. In this case two different Gram-positive bacteria were chosen. *L. innocua* as a representative of the *Listeria* genus which are non-spore forming bacteria and *B. subtilis* which does form spores in case there were differences seen in the effect on these two difference groups. In this case Gram-stain and microscopy were used to detect the effects of the extract on bacterial cell wall integrity. Cultures were prepared as described in Section 2.11 and then three 100 ml flasks for each type of bacteria were prepared containing a total volume of 20 ml of culture. The first flask (control) was supplemented with 1 % (v/v) ethanol to rule out solvent effects, the second flask was supplements with 12.5 μg ml^{-1} of liquorice extract and the third flask was supplemented with 50 μg ml^{-1} of liquorice extract. The flasks were incubated at 37 $^{\circ}C$ for 24 h in shaking incubator (150 rpm), and samples were taken at the beginning and the end of experiment.

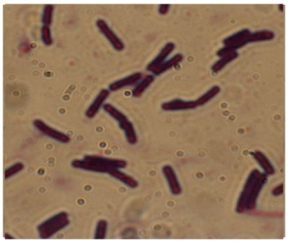
Samples of both *L. innocua* and *B. subtilis* were then transferred onto a microscope slide and distributed by a loop around the centre before the Gram stain procedure was carried out (Section 2.13). Samples were viewed using a light microscope to see if any change in cell morphology could be detected and the results are presented in Figure 3.8.

There was a slight change in cell morphology detected in the treated samples in that the cell shape was shorter and rounder than those seen in the control samples, typical of agents that result in inhibition of cell division. However it was also found that samples treated with the liquorice extract were less able to take up the Crystal Violet stain and therefore appeared paler in colour (Figure 3.8). Measurements were carried on these images to quantify the change in average cell length and the result as shown in Table 3.3 and this confirmed that

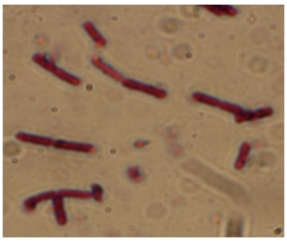
for *Listeria* after 48 h exposure to the 12.5 μg ml^{-1} concentration, the cell length was the same (0.52 μm) as that for the samples treated with 50 μg ml^{-1} (0.51 μm) whereas after 24 h the cell length at a concentration 12.5 μg ml^{-1} was less markedly affected (0.8 μm). The fact that the same pattern was seen for both types of bacteria, and the fact that the growth of all the Gram-positive bacteria tested to date was inhibited, suggest that the primary target of the liquorice extract is something common to Gram-positive bacteria and that it may inhibit cell wall synthesis leading to disruption of the normal synthesis of the peptidoglycan layer.

Figure 3.8. Effect of liquorice extract on cell morphology of Gram-positive bacteria

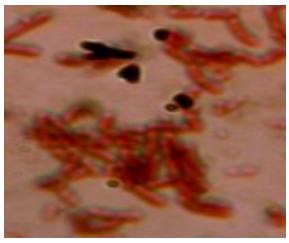
L. innocua



Control 1 % (v/v) ethanol

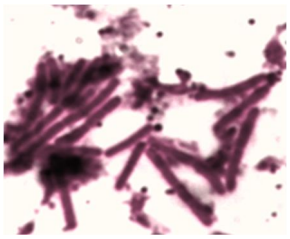


12.5 µg ml^{-1} of extract

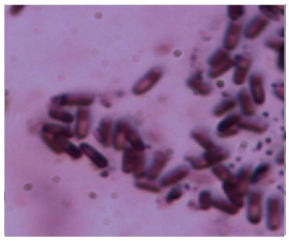


50 µg ml^{-1} of extract

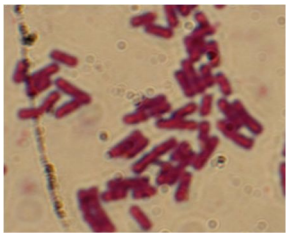
B. subtilis



Control 1 % (v/v) ethanol



12.5 µg ml^{-1} of extract



50 µg ml^{-1} of extract

Gram-stained samples of *L. innocua* and *B. subtilis* were taken after 24 h incubation (x 1000) with two concentration of liquorice extract 12.5 & 50 µg ml^{-1} in addition to control sample contain 1 % (v/v) ethanol solvent. As images were captured using a light microscope (Nikon) connected to a Canon camera (Canon EOS 700D, Japan) after Gram-staining using a x100 oil immersion lens.

Table 3.3. Observed and calculated mean cell dimensions (µm) of *L. innocua* and *B. subtilis*

Time (h)	Treatment with liquorice extract	*L. innocua* Av. Length (µm) n=10	*B. subtilis* Av. Length (µm) n=10
24 h	Control	1 µm	0.99 µm
	12.5 µg ml^{-1}	0.8 µm	0.61 µm
	50 µg ml^{-1}	0.52 µm	0.59 µm
48 h	Control	0.9 µm	
	12.5 µg ml^{-1}	0.52 µm	
	50 µg ml^{-1}	0.51 µm	

Overnight cultures of *L. innocua* and *B. subtilis* treated with a two concentrations of liquorice extract (12.5& 50 µg ml^{-1}) as well as 1 % of ethanol solvent. A Gram stain procedure was carried out on these cells and examine under microscope and images were taken and measured. Images from the light microscope were all produced at the same magnification from the original images to allow differences in cell length to be determined hence cell sizes are given in µm. Target cells were identified by dividing the field into 4 equal sections. The average cell dimensions for 10 individual bacteria per field were measured. Cells showing irregular deformation were excluded from the measurement.

3.2.7 Confirmation of the fluorescent phenotype of *Listeria* strains carrying synthetic *gfp*:*luxABCDE* operon

Fluorescent microscopy is a very useful for studying the effects of different antimicrobials at the cellular level. Green fluorescent protein (GFP) provides an easily detectable phenotype, so it has been used to label many different microorganisms. Many of the strains used in this Chapter which had been transformed to contained the *gfp* gene from the jelly fish *Aquoria victori*a (Chattoraj *et al*., 1996) and therefore the expression of this gene in these bacteria was tested to see if it was sufficient to allow direct visualisation of cells without the need for fluorescent dyes. In addition these strains also carried the full bacterial *lux* operon to allow bioluminescence to be produced. As a range of different strains of *Listeria* that had been transformed to a *lux* phenotype were available, it was also decided to investigate whether any differences would be seen if different species of the same bacteria were used. Hence the effect of strain variation was investigated by using different strains of *L. monocytogenes* in addition to *L. innocua*. Three wild-type strains of *L. monocytogenes* were used, including serovars 1/2a (*L. monocytogenes* EGD*, L. monocytogenes* 10403S) and 4b (*L. monocytogenes* 23074) which represent the major causes of human listeriosis. In addition, two mutant strains which either over express the virulence genes (*L. monocytogenes* NCTC 7973; *prfA** mutant) or cannot express the virulence genes (*L. monocytogenes* NCTC 10357; Δ*prfA*) were used (see Table 2.2 & 2.3 for details).

The cultures were recovered from storage at -80 °C and purified to single colonies on BHI agar. Once pure cultures were obtained the next goal was to confirm that the emission of light could be detected and the level of Gfp protein produced by these strains was sufficient to allow detection of single cells. A complete list of strains and the details of the plasmids they contained are listed in Tables 2.2, 2.3& 2.4.

3.2.7.1 Confirmation of light emission (Luminometry)

First of all to confirm that the *Listeria* strains were emitting light, fresh cultures were prepared (Section 2.4.1) and 3 ml samples of overnight cultures were placed in a Luminometer (Model TD-20e Luminometer, Turner designs) the reading was recorded in (Table 3.4). *L. innocua* without a plasmid was used as a negative control. However, for these stationary phase cultures the light levels were very low, thus to obtain an exponentially growing culture, 1 ml of the overnight culture was transferred into a sterile flask containing 49 ml of BHI broth (1:50) and incubated again for 3-4 h at 37 °C in shaking incubator (150 rpm), samples of cultures (3 ml) were again transferred into the luminometer and the light levels measured. This time the results showed there was a 10-100 fold increase in the light levels detected which is consistent with the fact that the luciferase enzyme requires the cell to be producing reduced $FMNH_2$ through respiration or fermentation and levels of metabolism are much higher in exponentially growing cells than in cells in stationary phase. However a consistent pattern of results was seen in that the same strains still had the highest rate of light production both before and after dilution which were LM 10403S and LM 23074 (Table 3.4). It was interesting to note that *L. innocua* showed the greatest increase in light (approximately 900-fold increase) despite the fact it contained the same plasmid as the majority of the other isolates. In these plasmids (Bs10:Dual:pUNK1) the reporter genes are linked to the ribosomal S10 subunit promoter from *B. subtilis* which is constitutively expressed in *Listeria* (Dr P Hill, University of Nottingham, pers comm.) so it would be expected that light levels would simply reflect levels of metabolism. The strains chosen also included a *prfA** mutant LM 7973 which over expresses the virulence genes (Eiting *et al*., 2005) and a deletion mutant of *prfAΔ* LM 10357 that does not express the virulence genes at all (de las Heras *et al*., 2011). LM 10357 contained a slightly different plasmid that used the XylA promoter which is also constitutively expressed in *Listeria* (Perehinec *et al*., 2007), but the level of induction seen was very similar in these two mutant strains

suggesting that the expression of the virulence genes does not influence the level of light produced. Interestingly in the wild type strains LM 23074 and LM 10403S both produced the highest level of light in stationary phase and the lowest change in light levels in exponential phase (less than 10-fold induction), whereas EGD and *L. innocua* produced the highest changes in light level. This result again suggests that light levels are not affected by the expression of the virulence genes since *L. innocua* is a not a pathogen and does not have these genes.

Table 3.4. Effect of growth phase on light emission by *Listeria*

Strains and plasmids	Light Emitted (RLU)			
	Characteristic	Stationary phase cells	Exponential phase cells	Fold increase
L. innocua (control)	wt	0.088	0.088	0
L. innocua (Bs10:Dual:pUNK1)	wt	2.038	1828	897
LM EGD (Bs10:Dual:pUNK1)	wt serotype 1/2a	1.475	693	470
LM 10403S (Bs10:Dual:pUNK1)	wt serotype 1/2a	318.4	1910	6.0
LM 23074 (Bs10:Dual:pUNK1)	wt serotype 4b	388.2	1472	3.8
LM 7973 (Bs10:Dual:pUNK1)	*PrfA** mutant serotype 4b	8.355	609.5	73
LM 10357 (XylA:Dual:pUNK1)	*PrfAΔ* mutant serotype 4b	1.578	103.6	66

*wt: wild type

Listeria strains were grown at 37 °C in BHI broth that was supplemented with 5 μg ml^{-1} of erythromycin for strains that contained plasmids. After 24 h incubation the light emitted was measured and then the cultures were diluted and incubated at 37 °C for a further for 3 h before the light emitted by these strains was measured again. Light is recorded as Relative Light Units (RLU) per sample. The fold increase was calculated by dividing the light levels at time point 2 by the light levels at time point 1.

3.2.7.2 Confirmation of fluorescence phenotype (GFP)

Listeria strains carrying the plasmids listed in Table 3.4 were then examined to confirm the expression of the Gfp protein from the dual reporter operon (*gfp:luxABCDE*; Perehinec *et al* 2007). To do this individual colonies from a plate were inoculated into samples (200 µl) of BHI broth supplemented with 5 µg ml^{-1} erythromycin in a 96 well plate, using a sterile toothpick. The plate was incubated at 37 ºC for 24 h then fluorescence readings taken using a Tecan plate reader. The results (Table 3.5) showed that all the strains tested had the ability to express Green Fluorescent Protein (GFP) but a range of different levels of fluorescence was detected depending on both the strain and the individual colony used to inoculate the wells. This correlated with the fact that when colonies appeared to fluoresce more than others when imaged under the Biospace lab photon imager in Section 2.14.2. In this case the strain that produced the highest average levels of fluorescence was LM 23074, but given the large standard deviation of the values recorded in each group this is not significantly higher than any of the other strains. In these plasmids the reporter genes are both expressed from the same promoter, so it might be expected that the pattern of expression would be the same for both markers. However, unlike bioluminescence, fluorescence is not linked to the metabolic state of the cell and therefore these results indicated that in all strains the promoter activity is approximately the same, but physiological differences in the cells affect the amount of light produced. The variation in the results for each set of strains also indicates that the level of reporter produced in each individual cell is quite variable.

Samples were transferred into individual Eppendorf tubes and the cells were collected by centrifugation (13,000 rpm for 3 min) and resuspended in 200 µl MRD by vortexing and then transferred into new microtiter plate to measure the fluorescence levels which are shown in (Table 3.6).

Table 3.5. Measurements of green fluorescent protein (Gfp) for all *Listeria* strains

	L. innocua		**LM EGD**		**LM 10403S**		**LM 23074**		**LM 7973**		**LM 10357**	
A	32246	25281	29561	24670	21543	26593	33569	25595	29682	25689	26378	23165
B	31572	24968	17700	22151	26444	26787	42890	27107	20754	21961	23850	20219
C	33827	31478	35698	33372	30210	27895	36051	26815	35504	26877	23332	20937
D	34293	21410	25125	19758	24579	20422	34519	24867	27420	20334	18857	18156
E	34814	23239	34198	26908	34042	28358	34592	25678	33624	21984	20125	17571
F	26204	22220	27070	20536	20539	20048	32258	21363	26956	19779	20026	16441
G	33995	27426	27839	25737	26362	27657	37886	32205	31010	24038	18902	20706
H	36641	25536	34876	21767	35376	24360	37840	24530	38078	25732	32970	24466
Av RFU		21631		26685		26326		31110		26839		29072
SD		±3938		±5466		±4350		±5907		±5363		±4879

Listeria strains carrying plasmids encoding the *gfp* (Table 3.4) were grown in BHI broth supplemented with 5 µg ml^{-1} Erythromycin then incubated at 37 °C for 24 h. The plate was then moved to a Tecan plate reader to measure the fluorescence using an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The gain was set at Optimal (10) and the number of reads taken for each sample was 10 (data in each cell represents the average of 10 readings). Results are reported as Relative Fluorescence Units (RFU) and the average and SD values for each group are shown at the bottom of the table.

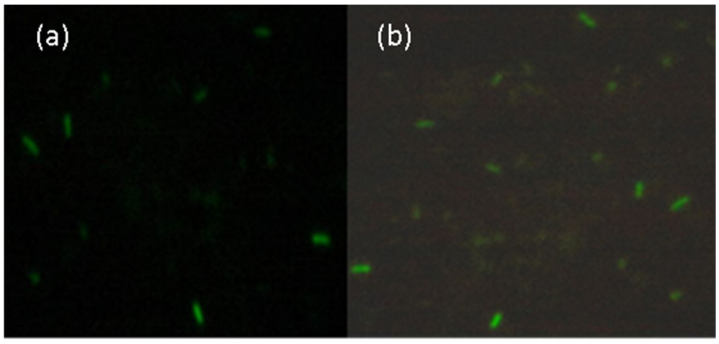
Table 3.6. GFP for *Listeria* strains

Strain	*L. innocua*	LM EGD	LM 10403S	LM 23074	LM 7973	LM 10357
A	7889	14551	17798	4836	8522	6538
B	6484	5728	22176	7486	6111	11522
C	5446	11824	34596	6894	5181	5230
D	6625	6207	24110	5900	5443	7198
E	12733	6299	40321	5490	5625	6432
F	6955	10527	42623	5162	7524	5198
G	6581	12527	38365	6294	5250	9718
H	3904	4024	4120	3762	4147	4112
AV RFU	7077	8961	28014	5728	5975	6994
SD	±2403	±3607	±12450	±1109	±1314	±2322

Cells were grown in BHI broth supplement with 5 μg ml^{-1} of erythromycin and incubated at 37 °C (overnight from the cultures in Table 3.5), the cultures were transferred into Eppendorf tubes and centrifuged and resuspended in MRD and placed in a Tecan microtitre plate. The plate was then moved to a Tecan plate reader to measure the fluorescence using an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The gain was set at Optimal (10) and the number of reads taken for each sample was 10 (data in each cell represents the average of 10 readings). Results are reported as Relative Fluorescence Units (RFU) and the average and SD values for each group are shown at the bottom of the table.

The *Listeria* strains (*L. innocua*, LM EGD, LM 10403S, LM 23074, LM 7973 and LM 10357) that exhibited highest fluorescence from the results shown in Table 3.6 were subcultured on a BHI agar plate supplemented with 5 μg ml^{-1} erythromycin to generate fresh colonies. To determine if the level of fluorescence produced was sufficient to detect individual cells, microscope slides were then prepared by picking up a colony and emulsifying the cells in a drop of PBS (approximately 40 μl) and then covered with cover slip and samples viewed using a fluorescence microscope. The results indicated that all the strains tested produced detectable levels of fluorescence and appeared as individual green cells (Figure 3.9). This experiment confirmed that all strains were producing the Gfp protein before starting treatment with liquorice extract. Thus, the next step was to determine if GFP was still being produce by the cells after treatment with the liquorice extract which was known to be exerting stress on the cells resulting in growth inhibition. One key point was to also demonstrate that when differences in cell shape were detected after prolonged treatment that this was due to changes in cell morphology rather than contamination of the culture with different bacteria that outgrew the test strains. This experiment will be described later in Section 3.2.11 and 3.2.12.

Figure 3.9. Detection of Green fluorescence in individual cells of *Listeria*



Cells were grown in BHI broth contain 5 µg ml^{-1} Ery for 24 h at 37 °C, and were then harvested and resuspended in MRD. The most fluorescent strains were subcultured on agar plates at 37 °C for 24 h, and samples of the fresh colonies used to confirm that the individual cells were visible using fluorescence microscopy. These images representative results for two *Listeria* strains (a) LM 10403S and (b) LM 10357. Samples were imaged using florescence microscope (Nikon).

3.2.8 Use of bioluminescence to investigate the effect of liquorice extract on *Listeria* strains

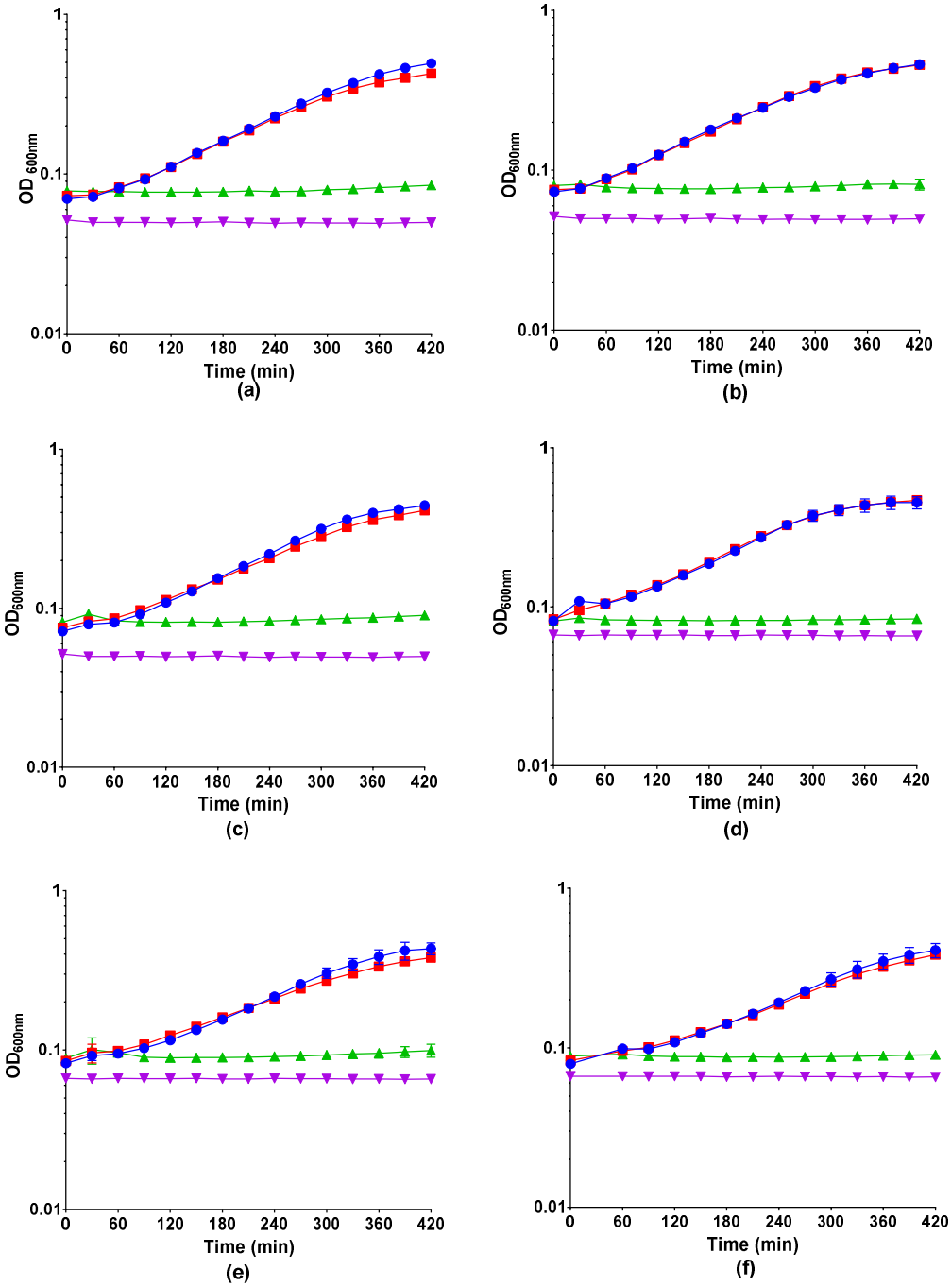
To further investigate of the antimicrobial action of the liquorice extract, the *Listeria* strains containing the bacterial *lux* genes were then treated with the extract. However, these genes are encoded on plasmids and therefore to ensure that all the cells in a population retain the plasmid, antibiotic selection is needed and therefore additional controls will be needed to ensure that loss of light production is not due to plasmid loss. The same set of strains of *Listeria* described in the previous Section were again used (see Table 2.2 & 2.3 for details).

To carry out these experiments, the different strains of *Listeria* were recovered from frozen stocks by growth on BHI agar plates and then a colony was transferred into 5 ml of BHI broth supplemented with Erm (5 µg ml^{-1}) for plasmid selection. These were grown for 24 h at 37 °C in a shaking incubator (150 rpm), before being diluted in fresh BHI broth supplemented with Erm (5 µg ml^{-1}) to an

OD_{600nm} of approximately 0.05. Samples were then supplemented with two concentrations of the liquorice extract (12.5 μg ml^{-1} or 50 μg ml^{-1}) and 1 % (v/v) ethanol was used as a control. Due to the possibility of contamination when carrying out experiments using a microplate reader, an uninoculated control sample was also included (purple line, Figure 3.10) to show that any change in optical density or light recorded was due to the organism inoculated into the broth.

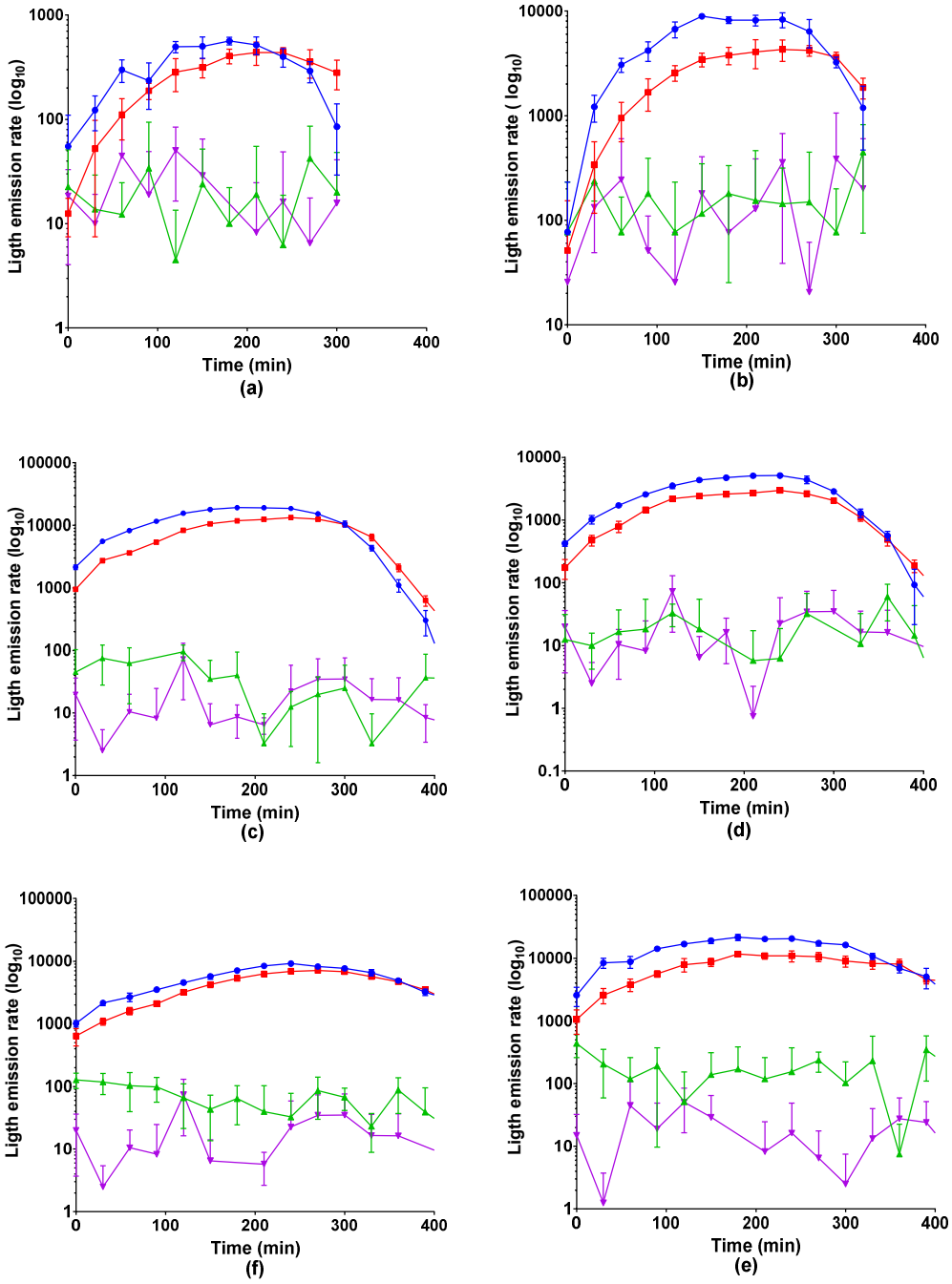
The results from the microtitre plate experiment showed the same pattern as before, in that a concentration of 50 μg ml^{-1} totally inhibited growth whereas at 12.5 μg ml^{-1} very little inhibition of growth was seen in Figure 3.10 confirming that this concentration of the extract is above the MBC. The pattern of results was exactly the same for all strains and species of *Listeria* tested, showing that this effect was the same for both species and all strains of *Listeria* tested. However, when the levels of light produced from each of the strains were examined (Figure 3.11), it was clear that even when growth was not being affected by the addition of the extract at 12.5 μg ml^{-1} (see Figure 3.10), a reduction in the light output could be seen, although this was more pronounced in some strains than others (see Figure 3.11). For comparison, the results are presented on a log scale in the same way as the optical density measurements as they taken during the growth of the cultures when cell mass was increasing. This result indicated that the cells were experiencing some stress at the sub-lethal injury level as the level of light is representative of the overall metabolic state of the cells. It was also noted that the metabolic level of the strains challenged with 50 μg ml^{-1} was very similar to the background control, suggesting that in addition to effects on cell division, the cell membrane may be affected.

Figure 3.10. Growth curve of (a) LM 10357, (b) LM EGD, (c) LM 10403S, (d) LM 7973 and (e) LM 23074 containing plasmid pSB3008 and (f) *L. innocua*(pSB3007)



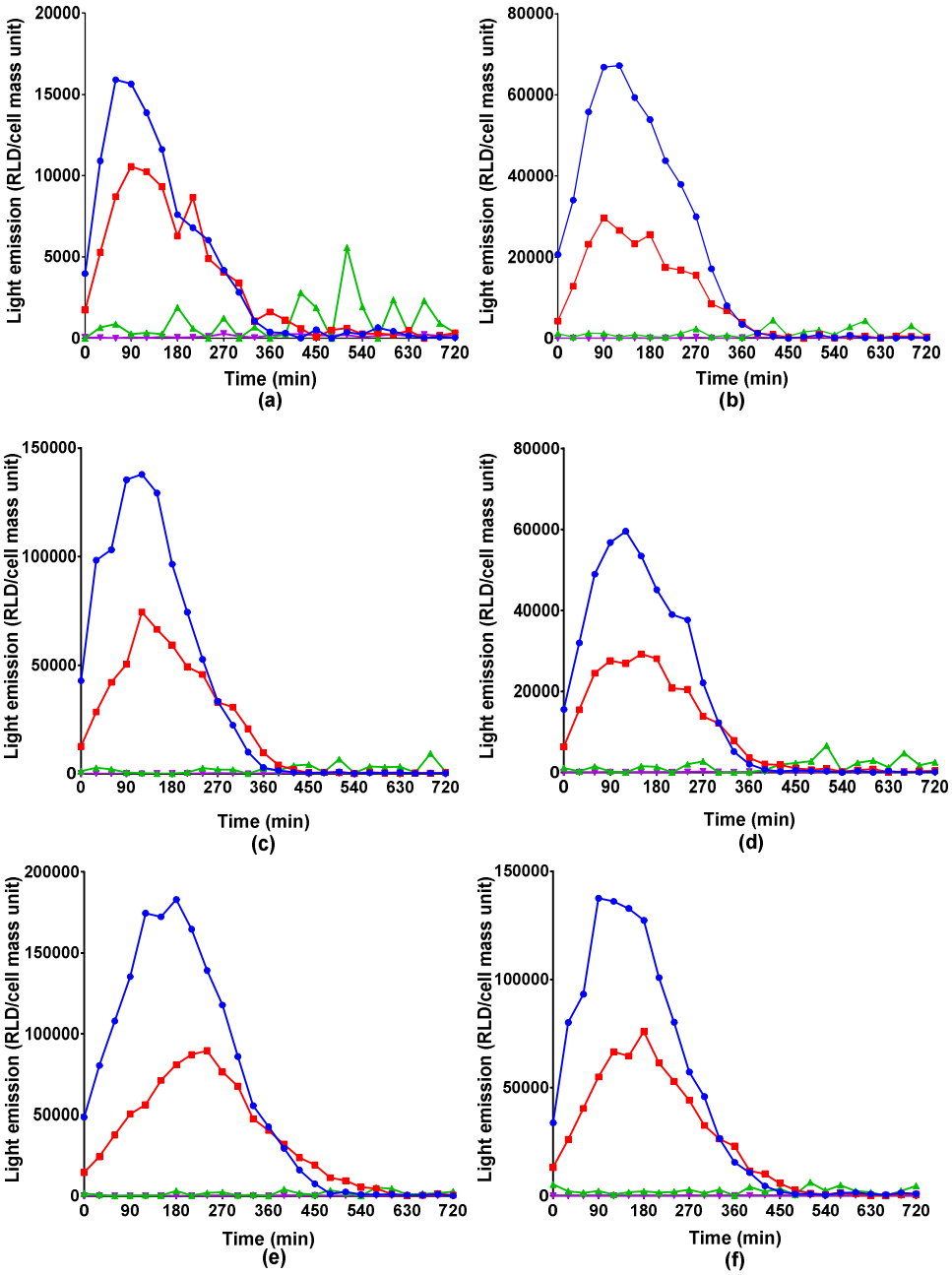
Growth curves of *Listeria* strains carrying *lux* gene were grown at 37 C for 24 h with two concentrations of liquorice extract [■] 12.5, [▲] 50 µg ml^{-1} and 1 % (v/v) [●] ethanol solvent as well as a control sample [▼] containing broth. Data are given as means ± standard deviations from four independent experiments, each performed in 4 replicate (n=4).

Figure 3.11. Effect of liquorice extract on (luminescence) light emitted by (a) LM 10357, (b) LM EGD, (c) LM 10403S, (d) LM 7973 and (e) LM 23074 containing plasmid pSB3008 and (f) *L. innocua*(pSB3007)



Graphs showing light output from the bacterial cells grown in present and absence of liquorice extract 12.5 [■], 50 [▲] µg ml^{-1}, [●] 1 % (v/v) ethanol solvent and [▼] uninoculated broth to confirm sterility. The bioluminescence intensity of the cells shown is the average for the replicate of light output by *Listeria* strains (n= 4 replicates).

Figure 3.12. Effect of liquorice extract on (a) LM 10357, (b) LM EGD, (c) LM 10403S, (d) LM 7973 and (e) LM 23074 containing plasmid pSB3008 and (f) *L. innocua*(pSB3007) reported as RLU per cell mass unit



Metabolic levels were monitored by measuring bioluminescence levels of strains carrying the bacterial *luxABCDE* genes. Light output (RLU) was divided by optical density to normalize light levels produced from samples containing different numbers of cells. Incubation of *Listeria strains* with two concentrations of liquorice extract 12.5 [■], 50 [▲] μg ml^{-1} and 1 % [●] (v/v) of ethanol solvent, a control sample [▼] containing broth only was also included.

Since there were now both variations in growth rate and light output recorded it was important to normalize the bioluminescence data according to the number of cells in the sample (i.e. 10 cells producing 1 unit of light would give the same signal as 1 cell producing 10 units of light). To address this, the bioluminescence data was divided by the optical density data, and the results are presented in Figure 3.12 and as this data now represents the light per cell mass it is plotted on a linear scale. This treatment of the data that adjusts for differences in cell number in each sample more clearly shows that the cells treated with 12.5 μg ml^{-1} of the extract were experiencing sub-lethal injury resulting in overall lower levels of metabolic activity. Since cell division was not affected in these cells as the growth rate was close to that of the control, this suggests that at sub-lethal levels the liquorice extract may be affecting either cell membrane integrity or other enzymes required for the production of $FMNH_2$ within the cells.

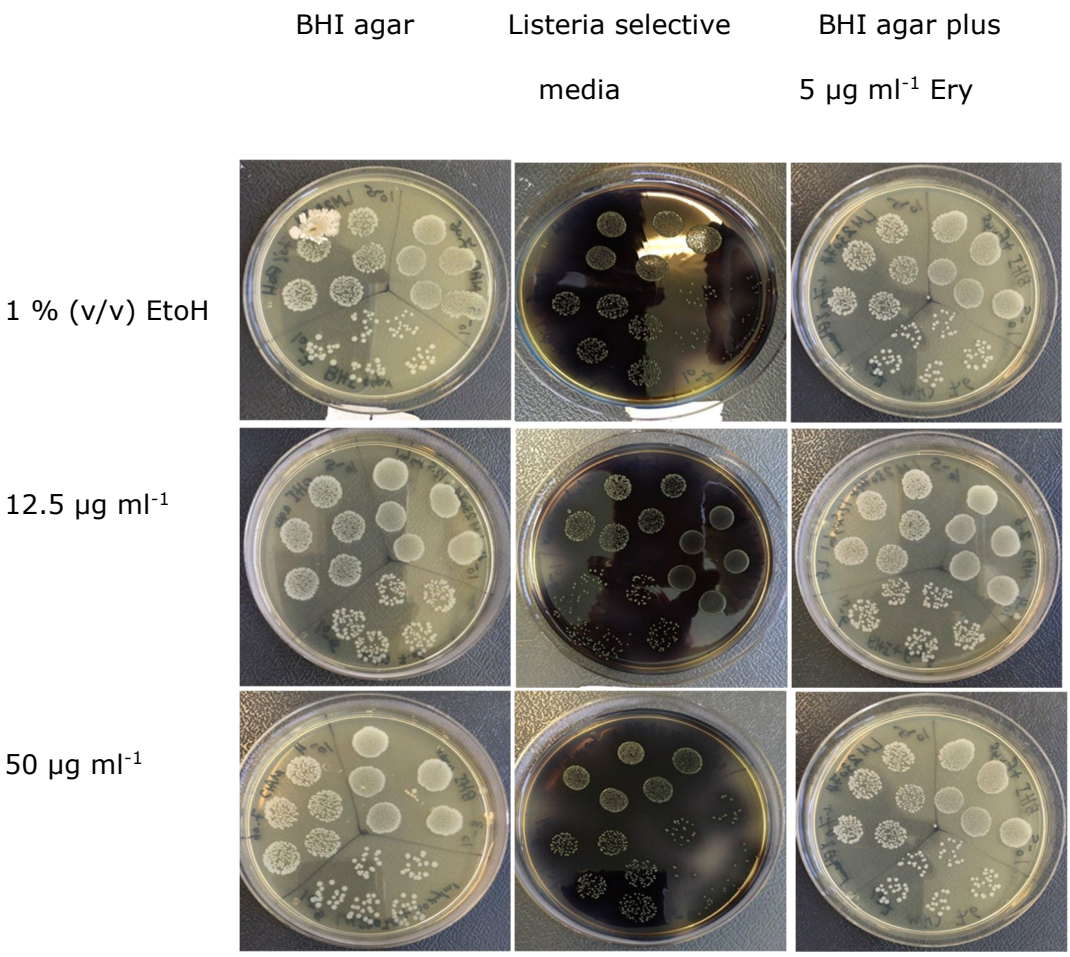
3.2.9 Confirmation of the presence of plasmid after treatment with and without liquorice extract

One question that remained was whether the presence of the antibiotic required for the selection of the plasmids was providing an additional stress on the cells that accounted for the results seen in Section 3.2.8. To determine whether the presence of the antibiotic was required to maintain the *lux* plasmids inside the cells, the stability of the plasmid was determined by growing *L. monocytogenes* strain 10403S(pSB3008) in the presence and absence of the selective antibiotic (Erm 5 μg ml^{-1}). To do this an overnight culture was prepared as a described in Section 2.4.1, and then the inoculum of a fresh broth culture was adjusted until the bacterial suspension reached an OD_{600nm} = 0.05. Culture was prepared, with and without 5 μg ml^{-1} erythromycin then supplemented with 1 % (v/v) ethanol (control), 12.5 μg ml^{-1} liquorice extract or 50 μg ml^{-1} of liquorice extract. Samples of each culture (200 μl) were transferred into a microtitre plate and incubated at

37 °C for 24 h, and rates of plasmid-loss were investigated using viable count as a described in Section 2.12.

The stability of the plasmid was determined by comparing the viable count of samples taken from the experiment and plating on agar with and without the antibiotic Erythromycin (Figure 3.13) and the results are expressed as the percentage of cells retaining the plasmid (viable count on BHI supplemented with Erm) with total cell number being determining by the viable count on BHI agar. When grown in the absence of the antibiotic, the rates of plasmid loss were very low (92-80 %; Table 3.7) indicating that the plasmids themselves were very stable. However plasmid stability decreased with increasing concentration of the liquorice extract, suggesting that it was exerting a metabolic stress on the cells that encouraged plasmid loss. In the presence of the antibiotic in the growth medium, the plasmid was more stable (as expected) but even then plasmid loss was seen when the cells were grown in the presence of the liquorice extract (93-97 %; Table 3.8). Consequently, it was clear that the addition of the antibiotic alone is not causing a major stress on the bacterial cells. Although a concentration of 50 μg ml^{-1} inhibited the growth of bacteria, the bacteria could recover again as demonstrated by the formation of colonies in the viable count experiments after the stress was removed (Figure 3.13).

Figure 3.13. Viable count for *Listeria monocytogenes* 10403S(pSB3008) on different agar plates



Listeria monocytogenes 10403S(pSB3008) treated with 12.5 and 50 µg ml^{-1} liquorice extract and 1 % (v/v) ethanol solvent, then incubated at 37 ºC for 24 h then diluted by serial dilution and plated on BHI agar plate, Listeria selective media and BHI agar plate with 5 µg ml^{-1} Ery using Miles and Misra technique for a viable count to confirm the present of plasmid.

Table 3.7. Plasmid stability in LM 10403S without selection

LM 10403S (BS10*, luxABCDE*, *gfp*) without Erm in the Growth Media	BHI Agar with 5 µg ml^{-1} Erm = Plasmid Present in Cell (cfu ml^{-1})	BHI Agar without Erm = viable count (cfu ml^{-1})	% Bacteria with Plasmid
Control ethanol solvent	$2.7x10^9$	$3.2x10^9$	92 %
12.5 µg ml^{-1} liquorice extract	$2.54x10^9$	$3.06x10^9$	82 %
50 µg ml^{-1} liquorice extract	$1x10^6$	$1x10^6$	80 %

*Erm: erythromycin

Table 3.8. Plasmid stability in LM 10403S with selection (ERM)

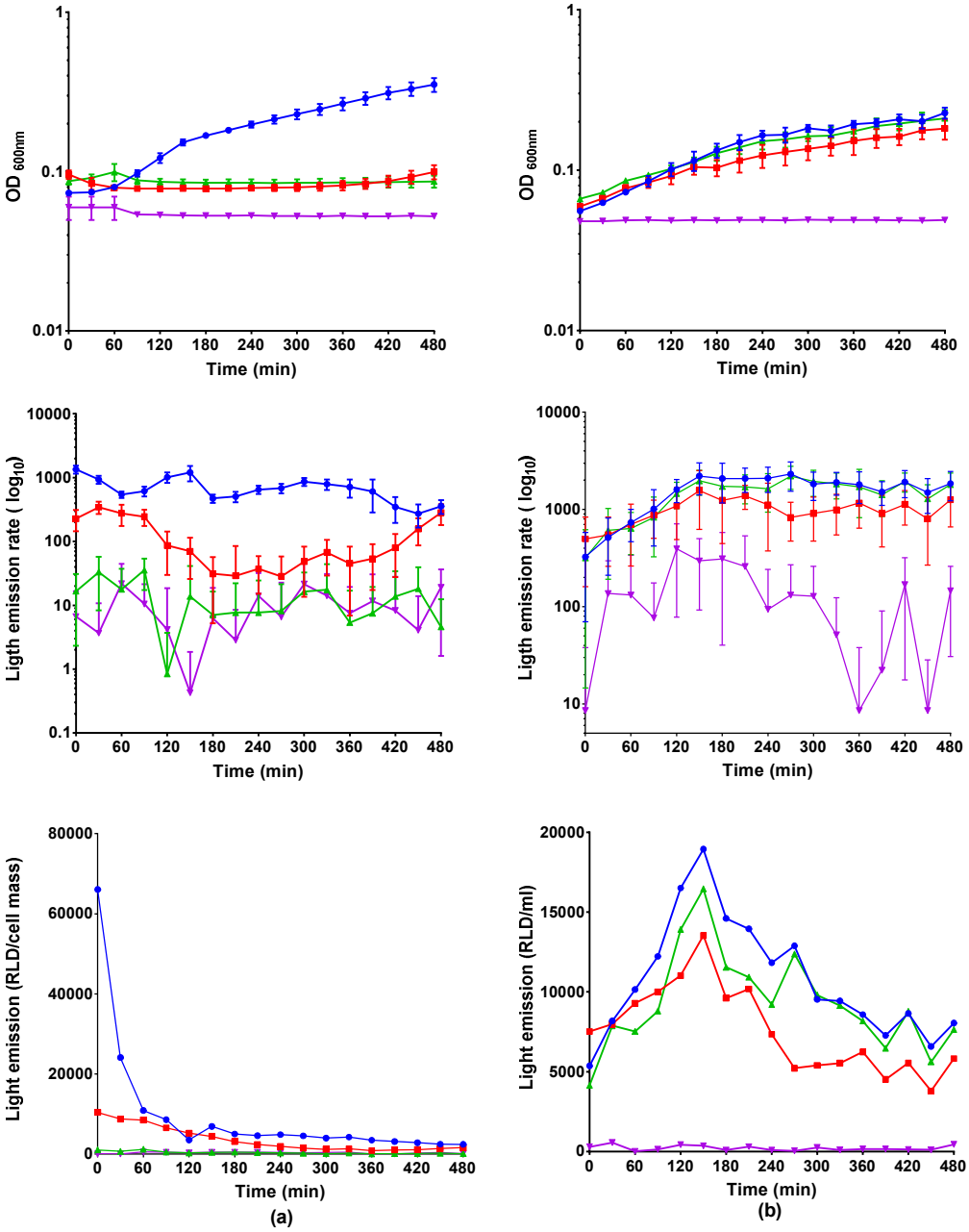
LM 10403S (BS10*, luxABCDE*, *gfp*) with 5 µg ml^{-1} Erm in the Growth Media	BHI Agar with 5 µg ml^{-1} Erm = Plasmid Present in Cell (cfu ml^{-1})	BHI Agar without Erm = viable count (cfu ml^{-1})	% Bacteria with Plasmid
Control ethanol solvent	$1.43x10^{10}$	$1.34x10^{10}$	100 %
12.5 µg ml^{-1} liquorice extract	$2.64x10^9$	$2.83x10^9$	93 %
50 µg ml^{-1} liquorice extract	$9.7x10^7$	$9.9x10^7$	97 %

Overnight culture of LM 10403S was prepared with and without erythromycin in the growth media and incubated for 24 h at 37 ºC in Tecan, then serial dilutions was created and plated on the BHI agar plate with and without 5 µg ml^{-1} Erm to confirm the stability of plasmid.

To see if the extract was also affecting the metabolic state of other bacteria, the effect of the extract on the Gram-positive bacterium *S. aureus* and the Gram-negative spoilage organism *P. fluorescens* bacteria were also tested as bioluminescent derivatives of these organisms were available in the laboratory culture collection. In particular it was important to determine whether, even if there was no evidence of growth inhibition in the Gram-negative bacteria, whether they did experience sub-lethal injury and reduced metabolic levels. For both these strains *S. aureus* and *P. fluorescens*, plasmid stability was not an issue since they were chromosomally tagged with a *lux* operon (*luxABCDE*) that was modified to function in each of them to give a highly bioluminescent phenotype.

The experiment was prepared as described previously in Section 3.2.8 and the growth and bioluminescence of *S. aureus* and *P. fluorescens* were compared by monitoring light and optical density simultaneous and comparing levels of bioluminescence produced (in relative light units [RLU] (Figure 3.14) after exposure to the liquorice extract. The measured light emission was high for *P. fluorescens* for both concentrations of the extract, indicating that there was no significant effect on metabolism. In contrast *S. aureus* showed a dramatic fall in light levels for both concentrations of the extract, and there was no difference between the basal level of light recorded and those measured when a concentration of 50 μg ml^{-1} of liquorice extract was added. There was however some recovery of the light levels during the incubation seen when 12.5 μg ml^{-1} was used, and the increase in light corresponded to the time point where the cells left the lag phase and began to grow. There results confirmed that the extract does not have antimicrobial activity against Gram-negative bacteria, but in addition to affecting the cell wall formation of Gram-positive bacteria also seems to have an effect on cellular metabolism indicative of membrane damage.

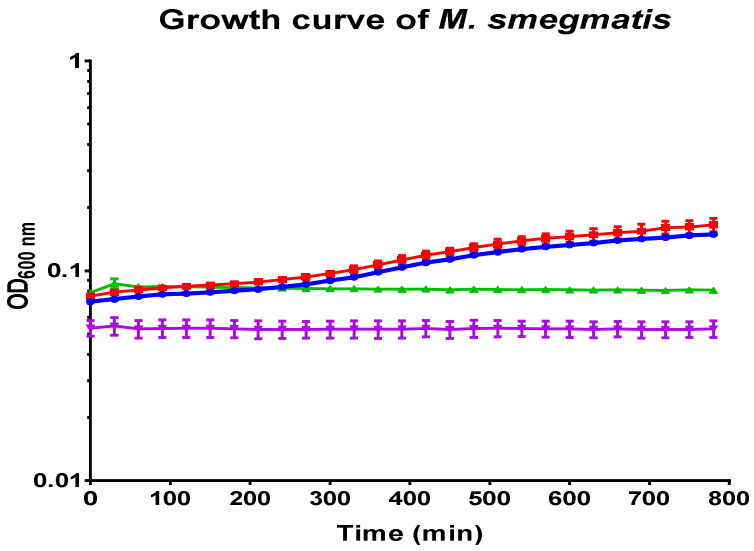
Figure 3.14. Effect of liquorice extract on growth and metabolism of (a) *S. aureus* and (b) *P. fluorescence* chromosomally tagged *lux*



Overnight cultures of *S. aureus* and *P. fluorescens* chromosomally tagged with *lux* were used to inoculate fresh broth to an OD_{600nm} of 0.05 then cultures prepared with two concentrations of liquorice extract 12.5 [■], 50 [▲] μg ml^{-1}, 1 % [●] (v/v) of ethanol. A control sample [▼] containing broth only was also included. Samples incubated at 37 °C and growth monitored by optical density. Bioluminescence was monitored simultaneously and data presented as RLU/OD_{600nm}. Data are given as means ± standard deviations from two independent experiments, each performed in triplicate (n=3).

Because the antimicrobial effect was seen on all Gram-positive bacteria tested, an interesting question to ask is if it also affects acid-fast bacteria which are a part of Gram-positive group but have a very different cell wall structure. Specifically *Mycobacteria* have a complex cell wall structure containing many proteins, lipids, and carbohydrates, many of which are found only in these bacteria and have been identified as excellent targets for further investigations to discover anti-Mycobacterial compounds. So, just to see if the extract would also effect *Mycobacterium*, a growth experiment was carried out and the results are shown in Figure 3.15. As seen for the other Gram-positive bacteria, a concentration of 50 μg ml^{-1} was able to totally inhibit the growth of *Mycobacterium smegmatis*. Although this is a fast growing member of the *Mycobacterium* genus, the structure of the cell wall is very similar to that of the slow growing pathogens such as *M. tuberculosis.* Hence it is likely that this extract would also be effective against pathogenic mycobacterial species, although this needs to be formally demonstrated. Given the differences in the cell wall structures between these bacteria and other Gram-positive bacteria, the target affect must be a highly conserved element fundamental to these bacterial cell types.

Figure 3.15. The effect of liquorice extract on the growth of *Mycobacterium smegmatis*



Culture of *Mycobacterium smegmatis* was grown at 37 °C for 24 h in the presence of liquorice extract 12.5 [■], 50 [▲] µg ml^{-1} and [●] 1 % (v/v) ethanol solvent. A control sample [▼] containing broth only was also included. Growth was monitored by optical density measurement at 600nm. Data are given as means ± standard deviations from two independent experiments, each performed in duplicate (n=3). Significant difference between a concentration 50 µg ml^{-1} and 1 % (v/v) ethanol solvent is indicated as $p < 0.0001$.

3.2.10 Microscopic assay of membrane integrity after exposure to liquorice extract

In Section 3.2.8, the liquorice extract was shown to have an effect on metabolic activity, and one reason for this could be that it was having an effect on membrane integrity. Therefore the purpose of this study was to confirm whether there was any effect of liquorice extract on membrane integrity of Gram-positive bacteria.

Effects on membrane integrity were monitored using a LIVE/DEAD® viability stain and fluorescence microscopy according to the method given by Molecular Probes (2004) with slight modifications. Briefly, overnight cultures of *L. monocytogenes*, *L. innocua* and *B. subtilis* were prepared and aliquots inoculated into sterile 250 ml Erlenmeyer flasks containing 100 ml of BHI broth. The inoculum of the broth culture was adjusted until the bacterial suspension reached an OD_{600nm} equal to 0.05. Three individual flasks were inoculated with each strain; the first flask (control) contained 1 % (v/v) ethanol, the second flask contained 12.5 µg ml^{-1} of liquorice extract and the third flask contained 50 µg ml^{-1} of liquorice extract. To allow sufficient cell mass to be recovered to allow the cells to be detected by microscopy, samples of the cultures (6 ml) were transferred into a sterile disposable centrifuge tube using a 10 ml pipette. Cells were harvested by centrifugation (10,000 x *g* for 15 min), the supernatant was discarded and the pellet was re-suspended in 60 µl of the recommended resuspension buffer (0.85 % NaCl) before mixing with LIVE/DEAD® BacLight™ Bacterial Viability stain. Samples were taken immediately after the flasks were prepared (time = 0) and the flasks containing the remaining cultures were incubated at 37 °C in a shaking incubator (150 rpm) and further samples were taken after 3, 6 and 24 h of incubation. At each time point the cells were concentrated before staining with the LIVE/DEAD® BacLight™ Bacterial Viability Kit as described in Section 2.15.

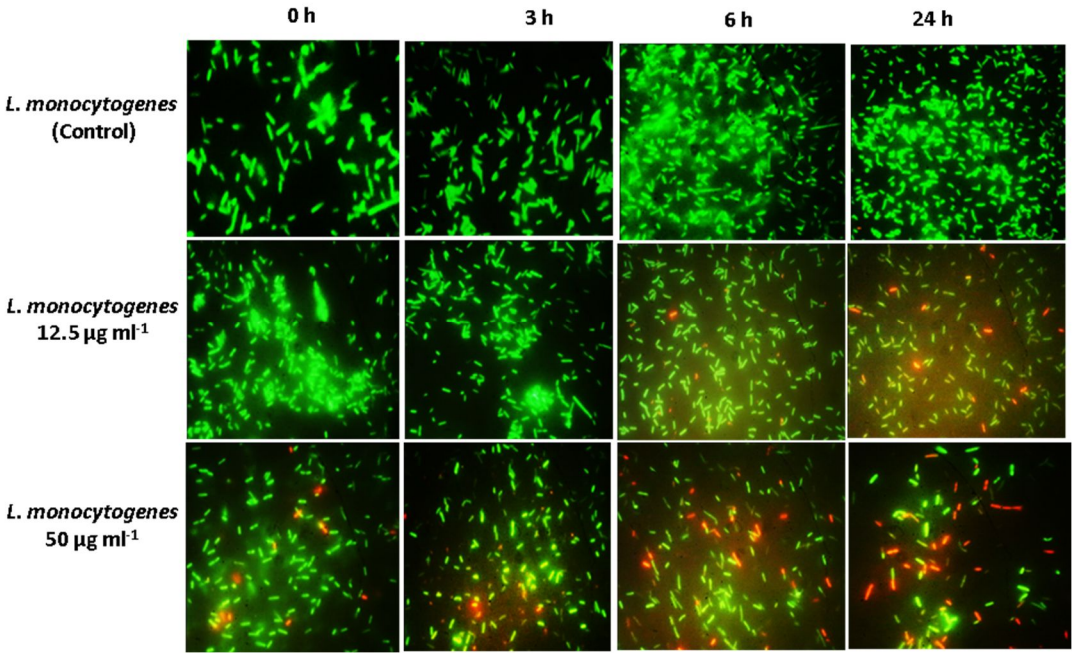
The fluorescence microscope images (Figure 3.16) showed that for all control cultures over time there was an increase in cell number which would be expected in growing cultures and most of the cells taken at all time points showed predominantly green fluorescence (stained with SYTO9 dye), indicating that even though the cultures had reached stationary phase after 24 h incubation the cells were still metabolically active. In the *Bacillus* culture there was some evidence of some orange/red cells present after 24 h incubation, which may be due to cells that have initiated sporulation when the cells entered stationary phase. However from these results with the control there was no evidence of any effect of the ethanol on membrane integrity.

In contrast the cells treated with the extract at 50 μg ml^{-1} showed a large number of cells that were unable to remove the propidium iodide dye, as they were stained red or orange, a characteristic of cells with membrane damage. This was evident even at the first sampling time point but the number of red/orange cells increased over time (Figure 3.16). In addition the intensity of the red stain also appeared to increase with time so, three sub-populations (dead, viable and injured cells) could be distinguished using this viability kit.

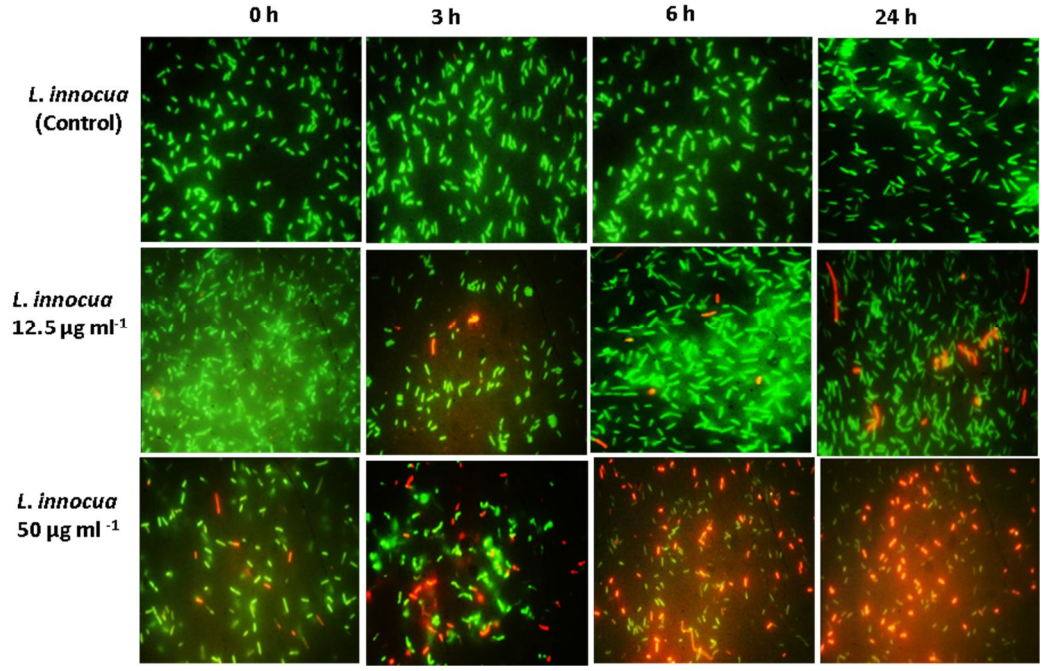
In the samples containing the 12.5 μg ml^{-1} of extract, the number of red cells accumulating was lower in each of the samples, and for both *Listeria* species tested, did not seem to increase over time suggesting that the cells may have been able to adapt to this challenge. These results are consistent with the pattern of growth seen in Section 3.2.8 when strains were challenged with 12.5 μg ml^{-1} of the extract, where there seemed to growth at near normal rates after an extended period. In contrast for *Bacillus* treated with 12.5 μg ml^{-1} of extract, the number of red/orange cells increased over time perhaps indicating that for this sporulating organism the cells responded in a different way to the initial challenge and initiated sporulation rather than using energy to adapt to the antimicrobial challenge.

Figure 3.16. The effect of liquorice extract on membrane integrity after applying LIVE/DEAD® BacLight™ Kit

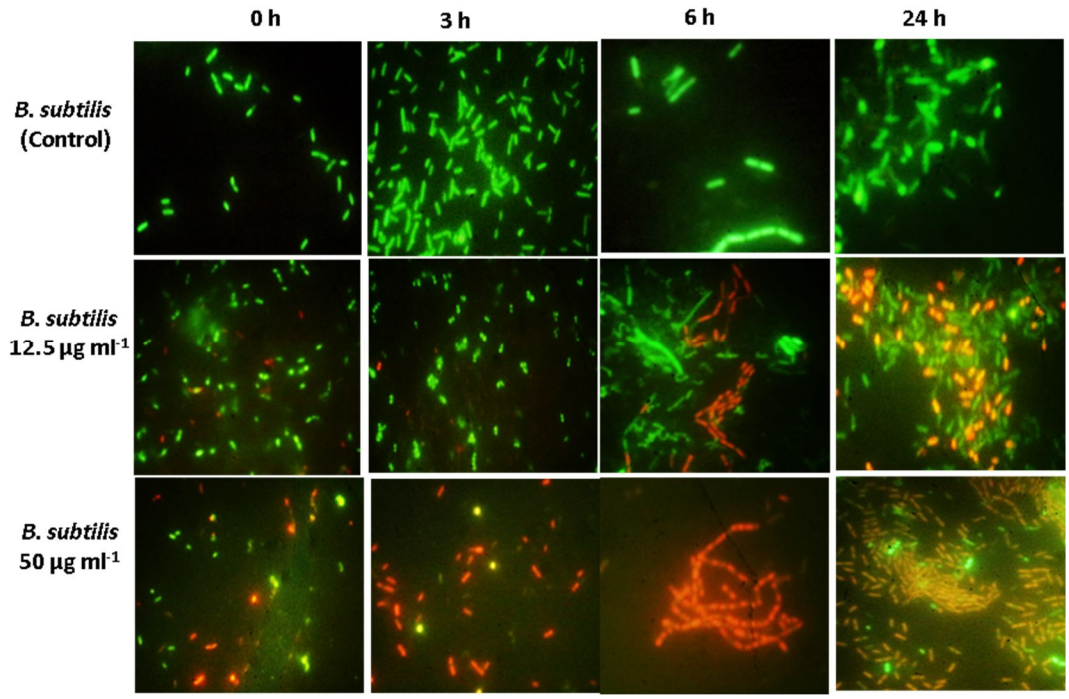
(a) *Listeria monocytogenes*



(b) *Listeria innocua*



(c) *Bacillus subtilis*

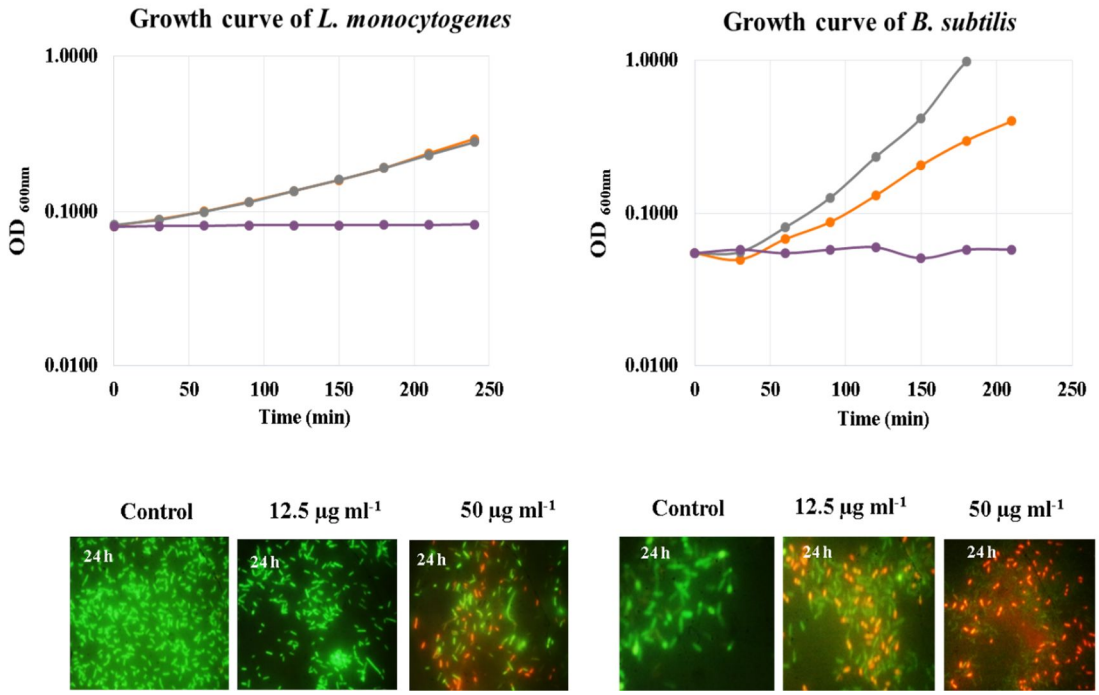


LIVE/DEAD® BacLight™ staining kit applied to three bacterial samples. Culture of *L. monocytogenes*, *L. innocua* and *B. subtilis* were grown in BHI broth overnight at 37 °C to an OD_{660nm} of 0.05. The cells were incubated with liquorice extract (12.5 & 50 µg ml^{-1}) and 1 % (v/v) ethanol solvent for 24 h at 37 °C. The samples were then stained with the LIVE/DEAD BacLight Bacterial Kit-L-7012. 5 µl aliquot was examined by fluorescence microscopy. Control cells (incubated without extract) appear green whereas the cells incubated with extract were fewer in number and appeared red at the 24-h point. In general, it shows that 3–6 h exposure to extract seems to be a susceptible time period after which the rate of cell damage seems rapid in positive bacterial strains tested in this study.

These results were consistent with the results in the previous Section 3.2.8 (Figure 3.12) where a decrease in metabolic activity was detected using bioluminescent strains of bacteria, and provides more evidence that after treatment with liquorice extract and there is inhibition of growth rather than killing of the bacteria. It also suggests that the effect on the metabolic activity for these organisms may be due to an effect on the membrane integrity. However the results in Section 3.2.8 also showed that cell growth was inhibited and that cell structure was changed and therefore there could also be effects on cell division.

Comparing these results with the growth curves of these strains (see Figure 3.17), it is seen that 50 μg ml^{-1} of liquorice extract totally inhibited the growth of *Listeria* and *Bacillus* and therefore we expect a high number of damaged cells to appear. However, as shown in Chapter 3, the treatment is bacteriostatic rather than bactericidal and therefore it would not be expected that all of the cells in the population were non-viable. However at a concentration of 12.5 μg ml^{-1} the growth of *Bacillus* was lower than the control sample whereas at this concentration slightly inhibited the growth of *Listeria* compared to the control. Hence the pattern of growth appears to correspond well with the fluorescent images since at this concentration the *Listeria* cells were more green and *Bacillus* samples contained more orange/red cells. This result confirmed the earlier suggestion that, as reported for Gfp, fluorescence intensity can be used as an indirect measure of viable cell number as fluorescence levels per cell are relatively constant, unlike bioluminescence levels (Lehtinen *et al*., 2006)

Figure 3.17. Comparison of effects of liquorice on growth and viability staining



This figure represented the growth curve of *Listeria* and *Bacillus* strains which was grown in BHI broth with two concentrations of liquorice extract [●] 12.5 µg ml^{-1} and [●] 50 µg ml^{-1} and BHI broth as a control [●], the X axis is indicate to the time and the Y axis is indicate to the optical density, the images were taken after treatment of cells with and without liquorice extract and stained with Live/Dead stain, again showed that the *Listeria* was grown more than the *Bacillus* as see the growth curve matched with the images taken after dye of cells with Live/Dead stain.

3.2.11 Use of *gpf*$^{+}$ marked strains to confirm *Listeria* identification

In Section 3.2.6, changes in cell wall structure and cell shape were detected using the Gram stain method. However these cultures were incubated overnight before the cells were visualized, and Gram-staining requires fixing and treating the cells with dyes which may lead to artefacts if the cells are sub-lethally damaged and then treated with further chemicals. In addition it is difficult to prove that the change in cell staining is not due to contamination of the culture by cells that out compete the strain of interest. Both of these possibilities could be addressed using the strains that had been transformed to a fluorescent phenotype.

The experiment described in Section 3.2.7 confirmed that the Gfp was present in all *Listeria* strains before starting treatment with liquorice extract, thus to confirm that the changes in cell morphology were occurring in the cells being treated rather than apparent changes due to the presence of a contaminating microbe, in this Section it was decided to confirm that *GFP* still remained inside the cells after application of liquorice extract as proof that the changes in cell morphology were occurring in *Listeria* cells and were not due to contamination. To use fluorescence to monitor the effect of the liquorice extract, *L. monocytogenes* 23074(pSB3008) was cultivated in 10 ml of brain heart infusion broth, supplemented with Erythromycin (5 µg ml^{-1}) to select for the plasmid, in a shaking incubator (150 rpm) at 37 °C. The overnight culture of bacteria was adjusted to approximately 1 X 10^{7} cells ml^{-1} (OD ~ 0.05) and then two concentrations of liquorice extract (12.5 and 50 µg ml^{-1}) were added into the culture broth and mixed well. As before 1 % (v/v) ethanol was used as a control and a control sample containing uninoculated BHI broth only was also included. Eight replicate samples (200 µl) for each treatment were transferred into a 96-well microtiter plate which was incubated in a Tecan microplate reader for 24 h at 37 °C.

After incubation, to confirm that the Gfp protein was being expressed at detectable levels, the first stage of the Gram staining procedure was used to

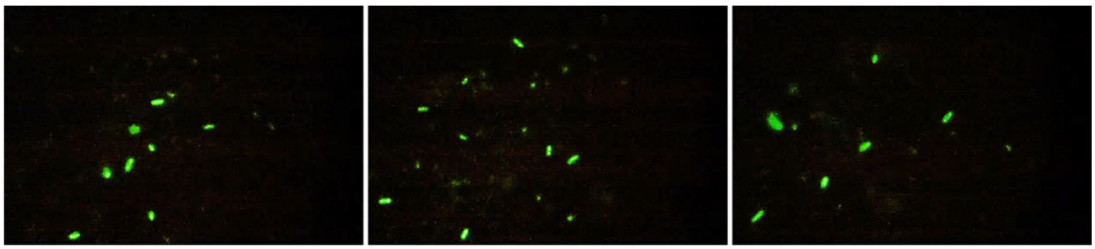
prepare samples for microscopy. Specifically the samples from the microtitre wells were transferred into an Eppendorf tube then centrifuged at 13,000 x *g* for 2 min, then the broth was removed and Phosphate Buffered Saline (PBS w/v) was added into the pellet. The samples were mixed thoroughly by using a vortex mixer, then a loop was used to transfer a small amount of culture on to a slide which was left until dry. PBS solution (20 µl) was added to permeablise the unfixed cells. The sample was then covered with a coverslip and imaged using the x1000 oil immersion lens under the fluorescence microscope to detect Gfp fluorescence.

The results showed that even though the cells were grown with antibiotics to ensure that they contained the plasmid, however, not all cells had the ability to make enough GFP to detect fluorescence, as only a small proportion of *Listeria* cells in the population were seen to fluoresce when examined by fluorescence microscope (Figure 3.18). This is consistent with the results seen in Section 3.2.7 when the fluorescent phenotype of the strains carrying the dual operon were characterised. In this case when individual colonies were grown in a microtitre plate to assess the variability on fluorescence levels, although there was not much difference in the average fluorescence of the replicates, there was a large variation in the fluorescent output of the individual samples. Since they were all incubated until they reached stationary phase, this suggests that the different samples contained a variable number of cells that did not give a fluorescent phenotype.

Despite this limitation it was still important to confirm some of the cells detected with altered morphology had the ability to fluorescence, so samples were viewed using the fluorescence microscope after treatment with 12.5 and 50 µg ml^{-1} of liquorice extract to confirm if any of the cells were detectably green. Only a small proportion of *Listeria* strain population was seen to fluoresce when examined by fluorescence microscope (Figure 3.18), but the presence of the *gfp* gene protein confirmed that cells with altered cell walls are not contaminants. However it was not clear whether the cells that gave a fluorescent signal were a specific sub-

population of the culture or were just a random selection of cells that had retained sufficient Gfp protein to be detectable. There was some evidence that this was the case since none of the fluorescent cells seen in the sample treated with 50 μg ml^{-1} shown in Figure 3.18 were elongated in any way compared to those in the control sample. So perhaps the cells that retained the Gfp phenotype were those cells in the population that were green when detected using the BacLight™ stain (see Figure 3.16).

Figure 3.18. Detection of fluorescence in *L. monocytogenes* 23074(pSB3008) culture after incubation with and without liquorice extract

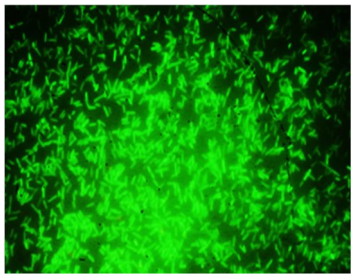


Fluorescence of *L. monocytogenes* 23074(pSB3008) culture after incubation for 24 h at 37 °C with a two concentrations (12.5 and 50 μg ml^{-1}) of liquorice extract as well as 1 % (v/v) ethanol solvent, the green cells were seen under the fluorescence microscope at all treatments to detect Gfp fluorescence.

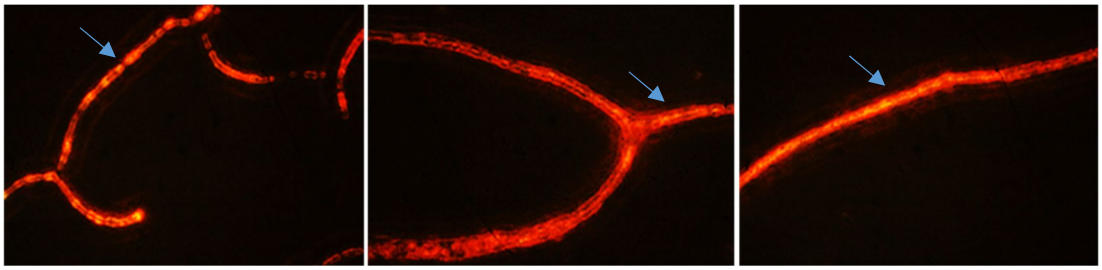
3.2.12 Morphology changes and cell elongation

As the fluorescent marker was not stable enough to allow the cultures of *Listeria* to be directly visualised, it was decided to investigate morphological changes and cell elongation in *L. monocytogenes* caused by liquorice extract when the cells exposed to stress using the BacLight™ stain as all cells should be visible. Overnight culture of *Listeria monocytogenes* 23074 was diluted to 0.05 of optical density then two flasks were prepared with 12.5 and 50 μg ml^{-1} of liquorice extract as well as 1 % (v/v) ethanol solvent. Sample (200 ml) was transferred into microtiter plate. The plate was subsequently incubated for 24 h at 37 °C. Finally, the cells were harvested and exposed to a dye LIVE/DEAD® BacLight™ as a described in Section 3.2.10. In order to visualize the potential existence of septa in elongated cells, a loop of culture was transferred into slide and the changes in the morphology of cells (cell elongation) were observed under fluorescent microscopes, the images of *Listeria* that had been treated with 50 μg ml^{-1} of liquorice extract, after 24 h of incubation are presented in Figure 3.19. Cells treated with liquorice extract for 24 h showed various morphological alteration and damage to the outer surface of the cells as well as clumping of the cells. Numerous cells appeared longer compared to control.

Figure 3.19. Effect confirmed on the elongation of *Listeria monocytogenes* 23074(pSB3008) after 24 h treatment with 50 µg ml^{-1}



Control



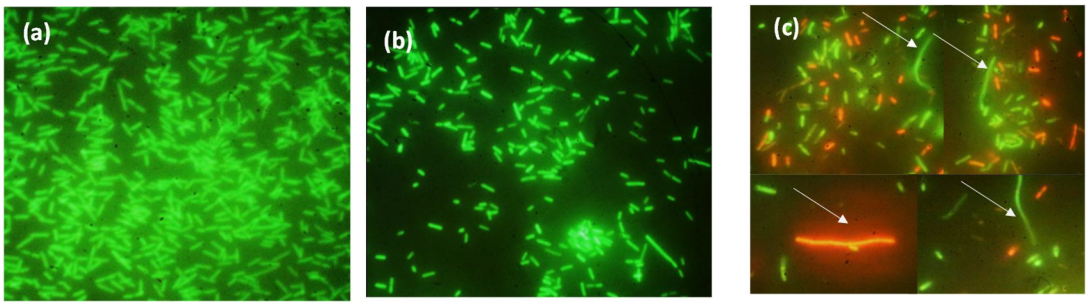
50 µg ml^{-1} liquorice extract

Listeria monocytogenes 23074(pSB3008) was exposed to 50 µg ml^{-1} of liquorice extract and after 24 h of incubation time, the cells were harvested and stained with Live/Dead stain. Under the fluorescence microscope the presence of elongated cells was very evident and the presence of structures that resembled septa (examples indicated with arrow) were evidence between normal size cells, while the untreated sample showed just normal cell structures.

In this study, long filament formations were observed when *Listeria* exposed to liquorice extract that indicate changes in cell length. When bacteria are exposed to stress conditions, the can induce the formation of filaments (Liu *et al*., 2014). In the literature there are many reports that *Listeria monocytogenes* does induce filament formation when the environment became more challenging, in particular when food preservatives are applied that inhibit cell growth (Isom *et al*., 1995; Zarei *et al*., 2012; Hazeleger *et al*., 2006).

Filaments were apparent at 50 µg ml^{-1}, the most predominant filaments occurred after 12 h treatment (Figure 3.20 c). Along with the presence of filaments, normal coccoid rods were observed under all conditions of the treatment with 12.5 µg ml^{-1} (Figure 3.20 b). There were no morphological changes observed in BHI media with 1 % (v/v) ethanol solvent (Figure 3.20 a). Hence changes in bacterial morphology were associated with *L. monocytogenes* cells being treated with liquorice and it was found that the extract induced alterations in the morphology of bacterial cells proportionate to concentration of the extract and time of exposure.

Figure 3.20. Filament formation in *L. monocytogenes* cells grown in BHI broth with and without liquorice extract



1 % (v/v) ethanol solvent 12.5 µg ml^{-1} 50 µg ml^{-1}

Images showing morphology of treated culture of *Listeria monocytogenes* when treated with 1 % (v/v) ethanol solvent and a two concentration (12.5 and 50 µg ml^{-1}) of liquorice extracts. Samples shown in panel (a) untreated culture no morphological changes (b) 12.5 µg ml^{-1} over 24 h showed the cells were green with normal coccoid rods (c) with 50 µg ml^{-1} of liquorice extract the dead cells stained red and the live cells stained green and cells appeared longer compared to control. Images were taken by a fluorescence microscope at 100x total magnification with camera (Canon).

3.3 Discussion

The desire of the public for more natural food preservatives has driven an interest in novel antimicrobial agents derived from plants. As is well known, plants synthesize different groups of secondary metabolites (phytochemicals) which contribute to defence mechanisms, as well as it is recognized that some of these molecules have useful effects such as acting as antimicrobial agents. The current research has produced a more comprehensive picture of the effect of one such natural compound produce by plants on the growth of Gram-positive bacteria revealing an effect on the cell wall, indicated by changes in cell shape as well as the inability to retain the dyes present in Gram-stain reagents.

All Gram-negative bacteria tested in this study were comparably resistant to liquorice extract. In contrast the liquorice extract had a specific effect against Gram-positive bacteria (Figure 3.6 c, e& f). These results are not totally consistent with studies performed by other researchers which described antimicrobial activity of liquorice extract against both Gram-positive and Gram-negative bacteria. For instance Karami *et al*. (2013) showed ethanol extracts of *Glycyrrhiza glabra* root collected at different times of the year exhibited potent antimicrobial activity against *S. enterditis*, *E. coli* and *S. aureus* with the MICs of 0.8 mg ml^{-1} and MBCs of 0.9 mg ml^{-1} whereas *B. cereus* was the most resistant species with MICs of 1.0 mg ml^{-1} and MBCs of 3.0 mg ml^{-1}. Gupta *et al*. (2013) also studied the antimicrobial activity of a methanolic extract of *G. glabra* against *Escherichia coli*, *Vibrio cholerae*, *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis* strains using well-agar diffusion method and they also found that the extract had antibacterial activity against all the bacteria tested. In contrast these results agreed with the report by Tsukiyama *et al*. (2002) in which the extract of roots of liquorice (*G. inflata*), licochalcone A showed effects against all Gram-positive bacteria tested with MICs of 2 to 15 µg ml^{-1}, and the extract especially was effective against all *Bacillus* spp., with MICs of 2 to 3 µg ml^{-1}, but was not effective against Gram-

negative bacteria or eukaryotes. These results, and the results gained in this study, show that Gram-negative bacteria are more resistant to the extracts than the tested Gram-positive bacteria, and this probably could most likely be explained by the differences in the cell wall structure, since the lipopolysaccharide layer in the outer membrane of Gram-negative bacteria is known to serve as an active permeability barrier to many environmental substances, specifically acting as a protective barrier against hydrophobic compounds (Puupponen-Pimiä *et al*., 2001).

The differences in the results gained in these different studies might also be attributable to the fact that the content of these components in *Glycyrrhiza* vary depending on many factors including different plant species, plant age, seasonal differences, geographical changes and time of harvest and collect plant, and all of these divers factors can affect their biological activity (Fiore *et al*., 2008). Known phytochemical groups with antimicrobial activity from liquorice extracts can include alkaloids, saponins, flavonoids, tannins, glycosides and phenols (Meghashri, 2009). Thus, the effect of liquorice extracts against Gram-positive and Gram-negative bacteria may be due to the presence or absence of certain effective biological components or a difference in the quantity of active compounds responsible for bio- efficiency. However the extract provided by Phytoquest Ltd for these experiments was not a direct extract of liquorice but was prepared from the waste material left over after liquorice flavourings were prepared for food products and therefore this extract may contain a different set of bioactive compounds. According to the analysis provided by Phytoquest Ltd, the active constituents in the waste material were Licoricidin and Licorisoflavan A.

Natural preservatives have been used as alternatives to traditional chemical preservatives; however, a limited number have been commercially developed and many remain to be investigated as sources of safer and effective antimicrobials. In this study, we have been investigating the antimicrobial activity

of an extract of *Glycyrrhiza uralensis* (liquorice) that was provided as waste material from the production of liquorice flavourings for the food industry, and to investigate if this retained the expected antimicrobial activity so it could be used as a natural preservative. Testing different concentrations of extract is important when evaluating antimicrobial activity and in this case it revealed that sub-inhibitory concentrations of the extract still had an effect on growth rate and cell metabolism (Figure 3.12). Since food preservatives can be used in combination at this sub-inhibitory levels to produce an overall inhibitory effect hurdle technology; see (Singh and Shalini, 2016), then this information may be useful if this extract is applied in a real food application.

L. monocytogenes is quite fastidious and requires a range of different amino acids and vitamins that it cannot synthesize by itself, so generally it is grown in rich media containing these compounds (Jarvis, 2016). Thus BHI is the most commonly used non-selective media for cultivation of *Listeria* species which is considered rich culture medium that provides all of these growth factors (Jones and D'Orazio, 2013). Therefore in these experiments all bacteria were grown in this medium but while it was found that using brain heart infusion broth most of the bacteria exhibited good growth, the growth rates achieved were very different (see Table 3.1) which then required extended incubation times to achieve a high enough cell density to allow all of the cultures to be prepared the same starting concentration. Since the growth optimal of the bacteria tested were all very similar, these differences in growth rate indicate that not all of the strains being compared are in the same physiological state and this needs to be factored in when assessing antimicrobial effects as the additional stress may make the cells more sensitive to the extract.

Several techniques using different bacterial physiological indicators such as MIC, MBC and metabolism have been used in this study in order to evaluate the extract as antimicrobial agent. Traditionally determining the MBC is performed

using viable count. However, performing these experiments using shake flasks is very laborious and therefore developing a new method to rapidly screen bacteria utilizing a microtitre culture dish and recording cell density automatically using a plate reader allowed a number of concentrations of the compound and simultaneous testing against a number of different bacterial types to be carried out and allow more data to be rapidly generated. However, a limitation of this approach is that when growth inhibition occurs the question remains of whether the effect of the antimicrobial is bacteriostatic or bactericidal. This signifies the need for further tests to further interpret these results. In this case parallel experiments using viable count showed that the effect of the extract was bacteriostatic rather than bactericidal and that the effective concentration is 50 μg ml^{-1} (see Figure 3.7). This is a very low concentration of material, and therefore it may be practical to use it to inhibit Gram-positive bacteria in some foods. According to Kim *et al*. (2002), in agreement with our study, the MIC values for glycyrrhizin, α-glycyrrhetinic acid and β-Glycyrrhetinic acid isolated from *Glycyrrhiza glabra* had antibacterial activity at 7.6 and 12.5 μg ml^{-1} against *Bacillus subtilis* and *Staphylococcus epidermidis*, yet it was not effective against Gram-negative bacteria *Escherichia coli*, *Proteus vulgaris* and fungi.

The emergence of resistance of bacterial strains to antibiotics may be due to the excessive usage of antibiotics and transmission of resistance within and between individuals, where the genes causing resistance can be transferred between different strains of microorganism, then recipient organisms will also become resistant (Cetin-Karaca, 2011). Therefore, there is an urgent need to find solutions to overcome bacterial resistance. Hence, natural alternatives have been also sought for use as new antimicrobial agents as alternatives to antibiotics. Antimicrobial phytochemicals have mechanisms of action similar to antibiotic action in terms of impact and includes inhibition of nucleic acid synthesis (Simões *et al*., 2012), cell wall synthesis, cell membrane damage and inhibition of

respiration (Yap *et al*., 2014; Mc Dermott *et al*., 2003) and it's important to understand the antimicrobial properties of plant species, that could help to contribute to the development of drugs against microorganisms of medical interest.

A Gram stain performed on *L. monocytogenes*, *L. innocua* and *B. subtilis* demonstrated the altered on cell morphology as compared to control group (Figure 3.8), and indicated that Gram-positive bacteria may be more susceptible to the liquorice extract than Gram-negative bacteria due an effect on cell wall synthesis. The fact that a range of different cell types were tested (Gram-positive rods, Gram-positive cocci and spore-forming organisms), and all showed equal sensitivity, suggests that there is a common target in all these different types of Gram-positive bacteria. Using *L. monocytogenes* strains and *L. innocua* as model organisms, the nature of the growth inhibition seen was further investigated, and it was clear that cell growth, including cell division, was affected. At sub-lethal concentrations, even when the growth rate was not particularly affected (Figure 3.10), there was evidence of sub-lethal injury and cell stress (Figure 3.12). Thus, it has been demonstrated that liquorice extract is able to inhibit the growth of Gram-positive bacteria such as *L. monocytogenes*, *L. innocua*, *S. aureus, E. faecalis* and *B. subtilis* (Figure 3.4). In addition an effect on the growth of *Mycobacterium smegmatis* (Figure 3.15), which is considered genetically to be a member of the Gram-positive group was seen, even though the mycobacteria do not give a typical Gram stain result because of the high lipid content of its cell wall (Fair and Tor, 2014).

The fact that all types of Gram-positive bacteria tested to date were inhibited, suggests that the primary target of the liquorice extract is something common to Gram-positive bacteria and that it may inhibit cell wall synthesis leading to disruption of the normal synthesis of the peptidoglycan layer. This raises a question of whether the effect on the cell wall may also there is effect on the

membrane integrity through the effect on the metabolic activity, so, strains carrying *lux* genes were chosen to confirm the effect on the membrane integrity.

Monitoring the bioluminescence levels of the genetically engineered *lux* derivatives of these bacteria, it was clear that even when growth was not affected , a reduction in the light output could be seen. The bacterial *lux* genes are used to monitor cell injury due to the fact that light levels produced by cultures reflects their metabolic state, and agents that inhibit cell metabolism or affect membrane permeability result in decrease levels of bioluminescence. Since it has been shown that light levels produced by cultures reflects their metabolic state, agents that inhibit cell metabolism or affect membrane permeability result in reduced levels of bioluminescence (Robinson *et al*., 2011). This was confirmed by study reported by (Gregor *et al*., 2018; Unge *et al*., 1999), the bioluminescence emitted is directly related to the number of viable cells, since $FMNH_2$ is only produced by metabolically active cells thus allowing an estimation of the sub-lethal effects of antimicrobial agents on the viability of target bacteria to be monitored. Although this approach has been used before by many workers, some caution must be taken to ensure that reduction in light levels are not simply due to the loss of the plasmid from the strains. In this case, we found that the stress caused by treating the bacteria with the liquorice extract did exert some additional metabolic pressure on the cells, leading to plasmid loss, unless antibiotic was added to the media, but there was no evidence of a difference in results gained with and without antibiotic to select for the plasmid (Table 3.7 and Table 3.8) and therefore in this case we can be confident that the loss of light was due to sub-lethal injury.

Treatment with 50 µg ml^{-1} of liquorice extract completely inhibited the growth of all of the Gram-positive bacteria tested, but when plated on to media without the extract the treated cells were able to grow normally indicating that effect was bacteriostatic rather than bactericidal. It is interesting to note that without the use of the bioluminescent reporter genes, the separate effect on cell

metabolism would not have been evident, and therefore this shows that using these tools is a powerful way to better identify stresses that have an effect on metabolic activity through damaging the membrane integrity or affecting enzymes associated with energy generation. So, more studies are needed to confirm that the extract may cause damage to the cell membrane.

Another area of caution is that strains of bacteria such as *Listeria* that have been cultured for long periods of time in the laboratory may have lost some of their fitness. To address this, we tested a range of different isolates, including examples of the two serovars that are responsible for the majority of human infections. Again no difference was seen between the sensitivity of different strains indicating that the results gained were very reproducible and not dependent on the test strain used. In addition, we tested two well characterised mutants of *L. monocytogenes*, one which over expressed the virulence genes (NCTC 7973 prfA*) and another than is incapable of expressing the virulence genes due to a deletion of the virulence gene regulator (NCTC 10357; ΔpfrA). The results gained with these strains were also identical to the results gained with wild-type *L. monocytogenes* and *L. innocua*, which has a complete absence of any of the virulence genes found in *Listeria*. Again these results suggest that the target site is found in all cell types and is not associated with the virulence traits of these bacteria.

The mechanism of action of the liquorice extract was further investigated using the LIVE/DEAD® BacLight™ Bacterial Viability Kit for microscopy and quantitative evaluation of treatment. Bacteria was treated with an inhibitory (50 μg ml^{-1}) or sub-inhibitory (12.5 μg ml^{-1}) concentration of liquorice extract. Exposure to the MIC for this extract (50 μg ml^{-1}) resulted in total inhibition of bacteria growth and samples imaged using the LIVE/DEAD stain showed that the cells were unable to actively remove the fluorescent dye from the cytoplasm, suggesting that membrane integrity was affected. Exposure to a sub-lethal level

of the extract (12.5 µg ml^{-1}) resulted in some growth inhibition and a lower percentage of cells showing evidence of membrane damage. However it must be noted that the cells require a membrane potential to exclude the PI from the cell, and if their metabolism is affected it is also possible that they are not able to efficiently remove the PI from the cell rather than there being a direct damaging effect on the membrane. Consequently, disrupting the membrane lead to cell death, if the cell is not able to repair the damage

The staining of bacterial cells detected three various cell populations: living, compromised, and dead cells as a result separation of the cell occur according to the different viability stages when dealing with two types of stain. These changes in fluorescence intensity are associated with the different viability stages of *Listeria* and *Bacillus* cells when were revealed by using a sub-inhibitory concentrations of liquorice. On this occasion *Listeria* grow more than the *Bacillus* which means *Bacillus* was more affected than the *Listeria*, as illustrated in Figure 3.17. However previously it was seen that a concentration of 12.5 µg ml^{-1} of the extract did cause growth inhibition of *Listeria* and resulted in lower levels of metabolism. This suggests that either there is some variability in the level of active ingredient in the liquorice extract and different solutions prepared on different days had different potency or that this level of the extract is very close to the threshold where no observable effect is seen so small variations in the concentration during preparation of the samples means that it is easy to drop below that threshold. However, at a concentration of 50 µg ml^{-1} the growth of Gram-positive bacteria was consistently inhibited and cell injury was evident even immediately after treatment (appearance of red cells in 0 time point) (Figure 3.16 a, b& c), although the number of orange cells detected over the time of exposure increased suggesting that the cells were being slowly killed by the extract. Thus, the remaining population of *L. monocytogenes* after liquorice extract treatment could be described by the decline of metabolic activity of cells, especially those treated

with 50 µg ml^{-1} of extract. The fact that an increasing proportion of red-stained cells were detected over time confirmed that the membrane was being damaged but for both *Listeria* and *Bacillus* samples treated with the extract, there was also an increase in cell length and the appearance of chains of cells were seen during the 24 h of exposure, suggesting that cell division was also being affected.

Moreover, there was evidence of septa in the elongated cells in *L. monocytogenes* after 24 h of the stress being applied (Figure 3.19) and the length of these filaments continuously increases with time and, progressively, filaments with 4 or more cells are formed (Figures 3.16 and 3.20). Filament formation is considered to be caused by problems in cell division as well as changes in gene and protein expression (Bereksi *et al*., 2002; Kieboom *et al*., 2002; Margolin, 2000). These filamentous cells are longer than single cells but have approximately the same width (Giotis *et al*., 2007). Hence cells that become elongated increase their surface-to-volume ratio which can occur in response to environmental changes or stress (Harris and Theriot, 2016). These results directly confirmed that *L. monocytogenes* undergoes significant changes in cell morphology under the stress. We have found that a concentration of 50 µg ml^{-1} has shown inhibition of growth for *Listeria* and *Bacillus*, as shown by results from experiments after 24 h, cells elongate and filaments are formed. One point to note about the dyes that are typically employed to assess membrane integrity, is that they may be ineffective against cells with a hardy membrane or cell wall, such as spores (Luna *et al*., 2002; Bosshard *et al*., 2002). Although the *Bacillus* has the ability to form spores that make it very resistant to different stress in the environment such as antibiotics, temperature and dyes than other species of bacteria non-spore forming bacteria (Piktel *et al*., 2017; Roszak and Colwell, 1987), in this study we found that the vegetative cells were susceptible to liquorice extract and this limitation was not relevant for this study.

Several adverse environmental conditions, such as exposure to acids, high CO_2 concentration, high osmolality, growth at non-optimum temperatures, low pH and antimicrobial agents, are known to lead to morphological changes in bacterial cells as reaction of these stresses. Thus, for several foodborne pathogens including *Salmonella*, *Escherichia coli*, *Bacillus*, *Clostridium* and *L. monocytogenes*, it has been reported that stress conditions may cause elongation of cells (Gill *et al*., 2007; Hazeleger *et al*., 2006; Everis and Betts, 2001; Jones *et al*., 2013). Specifically for *Listeria* the formation of elongated (filamentous) cells has been reported on exposure to low pH 5.0 at 37 °C (Bereksi *et al*., 2002; Brzin, 1973; Brzin, 1975; Isom *et al*., 1995; Jørgensen *et al*., 1995), osmotic stress (Jørgensen *et al*., 1995), above-optimum growth temperature, (i.e. 42.5 °C; Rowan and Anderson, 1998), and the presence of antimicrobial agents such as trimethoprim and co-trimoxazole (Minkowski *et al*., 2001). Isom *et al*. (1995) observed that filament formation by *L. monocytogenes* in cultures containing high levels of NaCl as environmental challenge increased, the length of the filaments increased. In addition, cells of *L. monocytogenes* grown in the presence of 9 % NaCl were became more thermotolerant than cells were grown in low-salt medium which exposure to the same concentration of NaCl for up to 4 h (Jørgensen *et al*., 1995). These findings are in line with the results seen when the *Listeria* cells were treated with liquorice extract, indicating that the cells are experiencing cell a kind of stress. In particular the fact that staining treated samples with the BacLight™ kit revealed the presence of septa within the filaments in *L. monocytogenes,* consistent with observations that during cell filamentation, it is the cell separation event that is inhibited. Consistent with this idea on the images of the stained *Listeria* cells the cytoplasmic material did seem to be separated into individual cells suggesting that the filaments actually consisted of several normal size cells that have not completed the cell division event.

Several reports indicate that, in some of these cases, removal of such stresses results in the slow return to the normal cell forms in around 24 h, further supporting the idea that the filamentation of *L. monocytogenes* may be an adaptive response to adverse conditions (Brzin, 1973; Isom *et al*., 1995). However, the mechanisms involved in these changes are unclear (Gardan *et al*., 2003). Since the cells plated out from these experiments were able to form normal colonies, this suggest that of the filamented *L. monocytogenes* cells have the ability to revert back to the normal form within one day of incubation at 37 °C when the stress was removed. This is in agreement with the findings by (Brzin, 1973; Brzin, 1975), which showed that *L. monocytogenes* had the ability to form filamentous only as long as the bacteria was grown on media containing high concentrations of NaCl. Following subculture to media without the appropriate high concentrations of NaCl, cell division resumed and normal rods were produced. These findings support the hypothesis that morphological filamentation is a direct result of bacterial adaptation to adverse environmental conditions. Another study conducted by Giotis *et al*. (2007) reported that *L. monocytogenes* 10403S was exposed to sublethal alkaline stress (pH 9.5) led to formed filaments or elongated chains, the cell length was increased over the time, during 1-3 h the cells showed quick develop in filaments/chains. However, these changes were not observed in control samples. When alkali stressed cells were moved into neutral medium, filamentous/chain forms returned to normal cellular morphology. Our results seem to support the observations of Kusumaningrum (2003) that these elongated cells are actually on the verge of division and, when transferred to more favourable conditions, will divide rapidly in single cells and start growing. If this happens in practice, it could have a significant impact on food safety, for instance when elongated pathogens are present. If that food is subsequently kept at conditions where growth is possible, the filaments will split up rapidly into many cells, resulting in a highly contaminated food product.

3.4 Conclusion

The antibacterial activity of liquorice extract was screened for evidence of growth inhibition against eight species of Gram-negative and Gram-positive bacteria, including *L. monocytogenes*, *L. innocua*, *S. aureus*, *E. faecalis* and *B. subtilis*. The Gram-negative bacteria tested include *P. aeruginosa*, *E. coli* and *S.* Typhimurium but none of these were affected by the extract. In contrast, for all of the Gram-positive bacteria tested, growth was inhibited as monitored using optical density. However parallel studies using viable count indicated that the cells were not killed meaning that the extract was bacteriostatic rather than bactericidal. The MIC and MBC of the extract were also determined, and a concentration of 50 μg ml^{-1} was found to have a strong bacteriostatic effect on Gram-positive bacteria. Microscopic analysis indicated that there were changes in cell shape suggesting the cell wall was affected. In addition, the use of a reporter strain of *Listeria* transformed with the bioluminescence genes *luxABCDE* indicated that cell energy levels were reduced when treated with either 12.5 or 50 μg ml^{-1} of the extract, with the reduction in light output being proportional to the concentration of the extract used. Together these results suggest that the extract is inhibiting the growth of Gram-positive bacteria only by damaging the cell wall and/or membrane, all measurements were performed the optical density, bioluminescent and green fluorescence protein (*GFP*) as a quick and reliable techniques for assessing the bacteriostatic, bactericidal or an effects on the metabolic activity during damaging the membrane integrity by using the liquorice extract as the antimicrobial against Gram-positive bacteria. This study confirms that potential of plant-derived molecules could use as a sustainable source of new broad-spectrum antimicrobial products.

Together these results indicate that the liquorice extract targets a site in the cell wall of Gram-positive bacteria and it has potential as a natural food preservative either for high risk *Listeria* products or for products where spoilage occurs due to Gram-positive bacteria. This study contributes to an increasing

number of reports on the occurrence of high numbers of filamentous bacteria under stress conditions and indicates that such morphological changes might play a role in bacterial defense and/or virulence. The next question to be asked was whether this extract could be used to control microbial growth in real food system and whether there was evidence that it was safe for human consumption.

CHAPTER 4

INVESTIGATION OF MECHANISMS OF ACTION OF LIQUORICE EXTRACT AS A FOOD PRESERVATIVE

Abstract

Recently consumers have expressed a preference for more natural foods, but still expect high levels of food safety. This has led to a search for natural antimicrobials that can be used to control the growth of food-borne pathogens in foods. Currently, plant products are being considered as a significant alternative source for novel antimicrobial drugs against antibiotic-resistant microorganism and as preservatives of food.

It was shown in the last Chapter that a liquorice plant extract, prepared from a waste product from the preparation of edible liquorice, is able to inhibit the growth of *Listeria*, resulting in lower levels of metabolic activity. In addition we showed that it was able to inhibit the growth of some other Gram-positive pathogens. In this Chapter an investigation of the effect of liquorice extract as a natural antimicrobial agent on the growth of *L. monocytogenes* 10403S cells applied to the surface of sliced deli meat was carried out. Two initial inoculum concentrations were used in this experiment, 10^6 and 10^8 cfu ml^{-1}. Liquorice exhibited potent activity against *L. monocytogenes*, but the observed effects were dependent on the size of inoculum in the culture medium, and at low inoculum, inhibition of *Listeria* growth was seen for up to 10 days. Although, growth inhibition was seen with a high inoculum, out growth was seen after extended incubation. Hence the liquorice extract has potential as a natural food preservative and could be of use to improve the safety of RTE product, in particular could be of use to improve the safety of sliced deli meats.

4.1 Introduction

In the previous Chapters, it was found that the liquorice extract has antimicrobial activity against the Gram-positive bacterium *Listeria*. *L. monocytogenes* contamination is a particular problem associated with sliced deli meats, but other Gram-positive bacteria, such as *Bacillus*, *Brochothrix* and Lactic

Acid Bacteria commonly contribute to the spoilage of cooked meats (Møretrø and Langsrud, 2017) and therefore if the growth of all of these bacteria were inhibited in product it would be of benefit to the food industry and consumer. Having established that the effect of the liquorice extract was bacteriostatic, and that it caused damage to the cell wall and cell membrane, further studies were now required to determine if this extract could be used in a practical sense in a real food system as a preservative. There are several techniques utilized to investigate the antimicrobial activity of food preservatives, but they are very variable depending on the type of product used. In this case, to further investigate the mechanism of action of the liquorice extract, and towards validating its application as a food preservative, a sliced meat model was chosen.

Microbial Food safety is a major concern for consumers throughout the world, as well as regulatory agencies and food industries. This is due to foodborne disease, as well as spoilage, where growth of microbes cause unwanted changes in food that leads to the alteration of food quality and causes sensory quality deterioration. For this reason there is a growing consumer demand for natural preservatives instead of chemical compounds. Thus there is growing attention by researchers to the discovery of natural antimicrobials to use in food products to extend shelf life as well as to control or inhibit microbial growth (Goni *et al*., 2009; Fattouch *et al*., 200).

L. monocytogenes is widely distributed in meat processing plants (Hof, 2003), and many listeriosis outbreaks have been reported due to the consumption of ready-to-eat (RTE) meat products (Barmpalia *et al*., 2005). Within the factory environment, *L. monocytogenes* is considered as a potential cause of contaminated food products, in particular during the slicing of meats, if proper controls are not in place (Swaminathan and Gerner-Smidt, 2007). Although, pathogenic organisms can be destroyed by the application of high temperature to food products, products can be contaminated by exposure to the environment

after the processing during peeling, slicing and repackaging processes (Sofos and Geornaras, 2010). Thus, food industries have increased attention in developing methods that prevent the growth of, or can kill, this pathogen in RTE meats. In RTE meat products where this bacterium cannot grow, for instance in salami, a limit of 2 log cfu g^{-1} of *L. monocytogenes* is accepted in both the European Commission (Commission, 2005) and Health Canada (Luber *et al*., 2011). However, the United States Department of Agriculture (USDA) has adopted a policy of zero-tolerance for pathogens in all RTE meats irrespective of the growth potential.

Not taking the precautions necessary to prevent contamination during the cutting and packing of meat is one of the reasons that processed products become contaminated with microbes, especially with *L. monocytogenes* which has the ability to grow and survive in the food factory environment and therefore there is a greater risk that this bacterium being present at levels likely to result in cross-contamination events if sufficient control measures are not used (Swaminathan and Gerner-Smidt, 2007). In particular, growth of the organism within slicing machines can lead to high levels of contamination of product by directly distributing *L. monocytogenes* on to the surface directly prior to packaging when there are no further Controls at Critical Points (CCPs) used to inactivate this organism (Beresford *et al*., 2001; Frye *et al*., 2002; Gormley *et al*., 2010) and it can then grow during storage even in the presence of high salt and low pH (Gandhi and Chikindas, 2007). In addition, sliced meat products are stored in the refrigerator and as a psychotroph *L. monocytogenes* has the ability to adapt to cold temperatures and carry on growing.

Although outbreaks of listeriosis have been associated with many different types of sliced meats, bologna-style sausage has been recognized as a particular risk for transmission of *Listeria* since there is nothing in the formulation that prevents growth of the organism if it gets into product (Rodrigues *et al*., 2017).

This has been demonstrated recently by the implication of a bologna-style sausage in the largest recorded outbreak of listeriosis in the world which occurred in South Africa in 2017-early 2018 (WHO, 2018). Therefore when choosing a model system to determine if the liquorice extract can specifically inhibit the growth of *L. monocytogenes* in a real food system, bologna-style sausages were chosen.

For the reasons outlined above, all sectors of the food industry tend to control the growth of organisms through the addition of chemical preservatives. The use of natural antimicrobials as an alternative to traditional, chemical food preservatives has become increasingly popular. Viuda-Martos *et al* (2010) reported that the addition of orange dietary fiber (1 %), rosemary essential oil (0.02 %) and thyme essential oil (0.02 %), combined with specific storage conditions, exhibit very desired effects to preserve the oxidative stability of mortadella, a bologna-type sausage by reducing microbial growth

The ingredients of bologna recipes (Polish Doktorska) used in this study inlude various meat and non-meat ingredients including mechanically separated turkey (44 %), water, turkey skin, soya protein, pork fat (3 %), potato starch, salt, acidity regulator: sodium citrates, stabiliser. triphosphates, antioxidant: sodium erythorbate, spices (contains mustard), spice extracts, flavouring, thickener. carrageenan, flavoure enhancer: monosodium glutamate, preservatives: sodium nitrite.

Objective of this study: The purpose of the research presented in this Chapter was to further investigate the potential of the liquorice extract as a natural alternatives to control *L. monocytogenes* growth in a real food system, using bologna-style sausage as a model to investigate whether it has potential as a food preservative for sliced meats.

4.2 Results

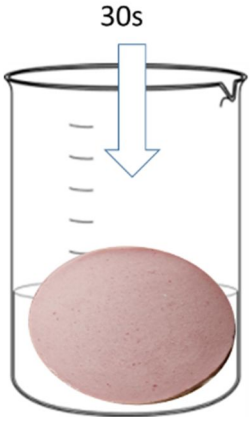
4.2.1 Evaluating liquorice extract activity on bacteria inoculated on to sausage

The aim of this of experiments was to assess the effect of liquorice extract on the growth of *L. monocytogenes* in a model food system. The inoculation of bologna style sausage with *L. monocytogenes* was done using the dipping of sliced sausage samples method according to method described by Lara-Lledó *et al* (2012) with some modifications. Specifically in this study the effect of packaging was not being examined and so the slices were not stored under different conditions after inoculation. For these experiments retail samples of whole bologna style sausage was purchased from a local supermarket (see Section 2.16)

To prepare the samples *L. monocytogenes* 10403S was grown in BHI broth and incubated at 37 °C in shaking incubator 150 rpm for 18 h. The cells were then collected by centrifugation (2,500 x *g* for 10 min at room temperature) and re-suspended in 0.1 % (w/v) BPW to a density of 1 x 10^9 cfu ml^{-1} and then diluted further diluted to the required inoculum level. BPW was used as a neutral buffer that would not support the growth of the bacteria but would allow them to recover from any damage incurred during the preparation of the inoculum.

Initially a high inoculum of *L. monocytogenes* was used so the cells were further diluted 1:10 in BPW to reach 10^8 cfu ml^{-1}. The bologna-style sausage was sliced (~3 mm) and inoculated by dipping for 30 s in to samples of the inoculum that had been supplemented with either 50 µg ml^{-1} liquorice extract or 1 % (v/v) ethanol (Figure 4.1) and then the slices were allowed to dry on a petri dish lid in a Class II microbiological safety cabinet (MSC) for 30 min at room temperature to remove excess liquid for inoculum attachment. It was expected that this method would achieve an inoculation of approximately 10^6 cells onto the surface of the sausage slices.

Figure 4.1. Method used to inoculate *L. monocytogenes* onto slices of bologna-style sausage

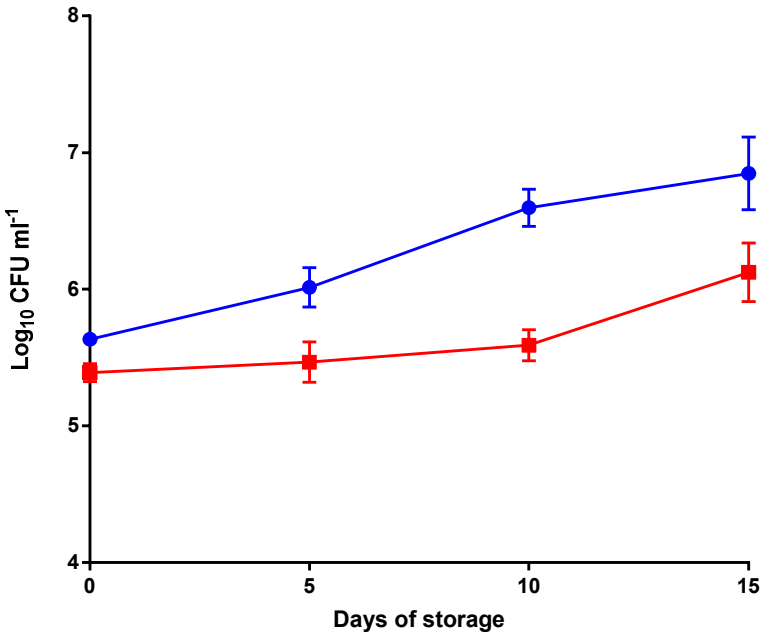


A schematic of the method used to apply liquorice extract and *L. monocytogenes* to the bologna sausages slices to study the effect of the extract on the growth during storage. The cells were diluted into BPW at the appropriate concentration and then the suspension supplemented with either ethanol as the solvent control or the liquorice extract dissolved in ethanol.

To determine if the extract could inhibit the growth of *Listeria* on the surface of the sausage, the inoculated slices were stored at 6 °C and samples (n = 3) were removed for sampling at day 0 (day slices were prepared) and thereafter at day 5, 10 and 15 of incubation. To sample the slices, each one was transferred into an individual sterile stomacher bags (177 mm x 305 mm) and mixed with 180 ml 0·1 % (w/v) BPW and stomached for 60 s at 300 RPM to release the *Listeria* into the liquid phase for sampling. This process was repeated on each day of sampling. After stomaching, appropriate serial dilutions were prepared with MRD and then the level of *Listeria* in the samples was determined by plating appropriate dilutions on PALCAM agar plates using Miles and Misra technique as a described in (Section 2.12) using 5 replicate 20 µl samples per dilution (total sample volume = 100 µl). Selective enumeration of *L. monocytogenes* was required since the

product being used was a commercial sausage product and was not sterile, therefore selective agar was used to specifically enumerate this organism. After allowing the plates to dry, colonies were enumerated after incubation at 37 ºC for 24 h (Section 2.12). The experiment performed in triplicate and the values were expressed as a mean value with standard deviations (M ± SD).

Figure 4.2. Bologna-style sausage inoculated with high numbers of *Listeria monocytogenes*



Cell suspensions (1 ml) of *L. monocytogenes* was diluted 1: 10 in buffered peptone water (BPW) in two flasks, one flask contains a concentration of 50 µg ml^{-1} liquorice extract and second flask contains a 1 % (v/v) ethanol solvent served as a control, slices inoculated by dipping in both solutions, then the slices storage at 6 ºC at different time (0, 5, 10 and 15 days). Three individual samples on each sampling day and for each treatment were used. Data are given as means ± standard deviations, each performed in triplicates (n=3).

Since outgrowth of the cells was seen after a period of incubation (Figure 4.2), it was hypothesized that the high cell number used in this experiment might result in breakdown of the inhibitory substances and allowing a delayed growth of cells after some time. This would reflect the findings reported by other authors that inoculum size is important when evaluating antimicrobials. For instance Silva-Angulo *et al*. (2015) showed that the lag phase of *L. monocytogenes* exposed to citral was shortened when high cell inoculum was used. Therefore, as some inhibition of growth was seen using the high cell inoculum, the experiment was redesigned using a lower cell concentration (10^6 cfu ml^{-1}) in the inoculum.

In this case the initial number of cells detected on the slices of sausage was lower at approximately 10^4 (see Table 4.1). In the absence of the liquorice extract, an increase in *L. monocytogenes* of 1 $\log_{10}$ was seen in the control samples during incubation indicating that *Listeria* was able to grow under the experimental conditions used. After day 5, differences in *L. monocytogenes* levels were observed between the control and the sample treated with 50 µg ml^{-1} liquorice extract (Figure 4.3). From day 5 to day 15 the control exhibited the highest level of growth ($p < 0.0001$) compared to the treatment. By day 10 and 15 bacterial populations in the sample treated with 50 µg ml^{-1} liquorice were lower than in control by 0.96 $\log_{10}$ cfu ml^{-1} and 1.1 $\log_{10}$ cfu ml^{-1}, respectively (Table 4.1, Figure 4.3).

Table 4.1. The number of bacteria emerging on the slices of sausage inoculated with lower numbers of *L. monocytogenes*, counted after 24 h incubation, and converted to log_{10} cfu ml^{-1}

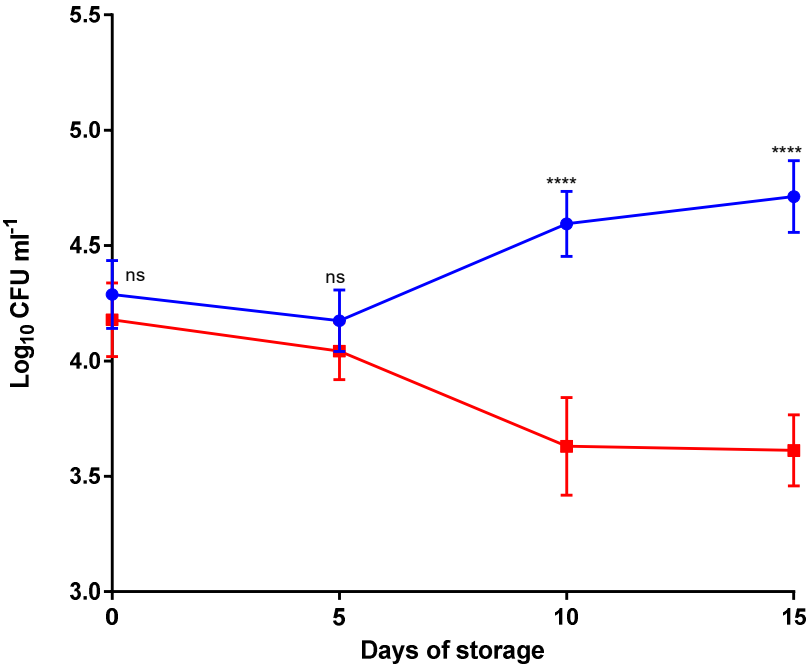
Time (Day)	cfu ml^{-1} (mean ± SD)	
	***L. monocytogenes* with 1 % (v/v) Ethanol solvent**	***L. monocytogenes* with 50 µg ml^{-1} of liquorice extract**
0	4.29 ± 0.15	4.18 ± 0.16
5	4.17 ± 0.13	4.21 ± 0.12
10	4.59 ± 0.14	3.63 ± 0.12
15	4.71 ± 0.16	3.61 ± 0.23

Cell suspensions (1 ml) of *L. monocytogenes* were diluted 1: 100 in buffered peptone water (BPW) in two flasks, one flask contains a concentration of 50 µg ml^{-1} liquorice extract and second flask contains a 1 % (v/v) ethanol solvent to yield 10^6 cfu ml^{-1}, then slices of bologna inoculated by dipping in both solutions, one served as a control 1 % (v/v) and other as treatment with liquorice extract, then the slices storage at 6 ºC at different time (0, 5, 10 and 15 days). Three individual samples on each sampling day and for each treatment were used. Data are given as means ± standard deviations, each performed in triplicates (n=3).

Figure 4.3 illustrates the growth of *L. monocytogenes* and the results showed that the extract was able to completely inhibit the growth of *Listeria* throughout the cold storage, whereas an increase in 1 log_{10} was seen in the control samples. By adding liquorice extract the number of *L. monocytogenes* on bologna slices initially surface inoculated to contain 10^4 *L. monocytogenes* cfu ml^{-1} decreased 0.6 log_{10} after 15 day of storage in the presence of 50 µg ml^{-1} of liquorice extract, compared with a 0.42 log_{10} increase for the slices without liquorice extract (Table 4.1). Analysis of the results indicated that by day 10 of inoculation there was a clear statistical difference ($p < 0.0001$) between the

number of cells detected in the two samples (see Figure 4.3). Interestingly this result in a model food system differed from the early results as there was a reduction in viable count whereas on long term exposure in liquid culture experiments (see Section 3.2.5) the extract was found to be bacteriostatic rather than bactericidal.

Figure 4.3. Growth of *Listeria monocytogenes* on bologna-style sausage at 6°C



Bologna style-sausage was inoculated with *L. monocytogenes* with 50 µg ml^{-1} liquorice extract prepared in 0.1 % BPW [■] and with *L. monocytogenes* with 1 % (v/v) ethanol (solvent control) prepared in 0.1 % BPW [●]. Two way-ANOVA was used to evaluate the treatment by using Sidak's multiple comparisons test to determine the significant differences among treatments by day with the level of significance (P value) set at $p < 0.0001$. Growth of *L. monocytogenes* on bologna incubated for 15 day at 6 ºC, **** indicates the time when growth was significantly higher than inoculation levels.

The results gained here showed that in addition to being effective at controlling the growth of *Listeria* in liquid cultures, it could also be used to control growth in a real food system. It was interesting to note that when the bacterial cells were exposed to the extract in this system, some loss of viability was seen over time. As this was a commercial product, it is possible that the antimicrobials used to produce the product were creating an additional stress on the *Listeria* cells. Bologna-style sausages are non-fermented emulsions and to extend the shelf life of these low acid food products, preservatives are often added. In particular potassium and sodium nitrite are added which, are popular in this type of product because, in addition to being antimicrobial nitrites (NO_2^-) combine with myoglobins present in the meat product to form nitrosylmyoglobins, which are converted to nitrosylhemochromes during cooking and provides the characteristic pink color in these ready-to-eat meat products.

4.3 Discussion

We have previously shown that a liquorice plant extract, prepared from a waste product from the preparation of edible liquorice was able to inhibit the growth of some Gram-positive pathogens and there was evidence that the mode of action of liquorice extract is based on its ability to both disrupt cell wall formation and effect membrane integrity, leading to inhibition of growth for those pathogenic bacteria including *Listeria* strains and resulting in lower levels of metabolic activity.

In vitro, the extract was applied on bologna sausage as a model real food to show whether it could be used to control the growth of *Listeria*. In the initial experiments using a high cell density cells, although was seen inhibition, after extended incubation outgrowth was seen (Figure 4.2). This is consistent with results reported by others using organic food preservatives where the substance is bacteriostatic and can potentially be metabolised by the cells in the culture. It

is interesting to note that even at high cell number (approximately 10^7 cfu ml^{-1}) in the liquid cultures, even after extended incubation, there was no evidence of metabolism and outgrowth. This suggests that in a real food system the extract may be less available due to partitioning into the different food components, such as fats, which would effectively reduce the concentration of the extract and allow outgrowth, as seen with the lower concentration of the extract used in the *in vitro* experiments.

To try and carry out a more realistic evaluation of the potential application of the extract, the method was modified to reduce the initial inoculum but still provide enough cells to allow the *Listeria* to be enumerated without the need for enrichment procedures. When a low inoculum (10^6) was used, good inhibition of *Listeria* which confirmed the hypothesis that the high cell number was leading to metabolism of the extract, resulting in outgrowth of the cells after extended incubation at low temperature, and according to a study by Cos *et al*. (2006), a high inoculum size (e.g. 10^7 cfu ml^{-1}) will increase the chances for false-negatives, while a too low inoculum size (e.g. 10^2 cfu ml^{-1}) will create many false-positives as cell numbers will fall below the limit of detection of plating methods. Another study conducted in *E. coli* by Somolinos *et al* (2010) demonstrated that a large initial inoculum resulted in a smaller amount of inactivation by citral, however it must be noted that such very high levels of *L. monocytogenes* are very unlikely to present in retail RTE foods and therefore the outgrowth seen at very high inoculum levels is less of a concern in real food systems.

In the previous Chapters the effect of the extract was shown to be bacteriostatic, and once the compound was removed the bacteria started to grow again. However in the low dose experiment the number of *Listeria* cells declined overtime. As known in the food industry, salt and nitrates are commonly used as a food preservatives and an antimicrobial agent to inhibit the growth of bacteria in ready-to-eat (RTE) bologna styles sausages contributing to an extended shelf

life of these meat products (Desmond, 2006; Ruusunen and Puolanne, 2005). Salt affects the bacteria by causing damage on the maintenance of osmotic balance between the cytoplasmic and intracellular environments (Burgess *et al*., 2016), and this creates stress on the cell membrane and cell metabolism. Hence during exposure to the extract in the real food system this may have contributed to the increase in the rate of cell death seen when tested in the bologna-style sausages.

The efficacy of naturally derived antimicrobials on the growth of *L. monocytogenes* has been reported in several other studies, such as that as reported by Lara-Lledó *et al*. (2012) the inhibition of *L. monocytogenes* on bologna sausages coated by an antimicrobial film containing mustard extract or sinigrin and stored at 4 °C for 72 day, and showed that on meat packed with pure sinigrin film, *L. monocytogenes* was not inhibited. In contrast, at 17 d storage, yellow mustard extract film caused a significant delay ($p \leq 0.05$) in the growth of *L. monocytogenes* and the viability of *L. monocytogenes* reduced in bologna sausages during the storage period. In addition, no viable *L. monocytogenes* was recovered after enrichment at 52 and 70 d, it is highly likely that the inhibitory effects of oriental mustard observed were bactericidal.

In study conducted by Naas *et al*. (2013) reported that the effect of nisin (produce by *Lactococcus lactis* subsp. *lactis* during fermentation) when combined with different types of packaging used for turkey bologna slices was enhanced and reducing the initial population of *Listeria monocytogenes* by 1.5 to 2 log. Thus, when extra hurdles are applied to control growth of *L. monocytogenes* in RTE meat a synergistic effect is seen that helps to improve the safety in RTE meats during refrigerated storage. Thus, the use of this kind of natural compounds can have effective antimicrobial activity and may eliminate *L. monocytogenes* from food products if used as a food preservative in sliced meats.

L. monocytogenes can be present in a wide variety of fresh produce, thus it is important to minimize the risk of the growth of this pathogen in order to

promote food safety and consumer health protection. Previous studies of chemical compounds isolated from liquorice plants had revealed antitumor, antibacterial, immunomodulatory and antioxidant activities (Karahan *et al*., 2016). Now having established that the liquorice extract could be used in a real food system. However, before using the extract as food preservative we need to confirm the safety and toxicity of the extract as it is known that not all compounds produced by plants are safe. Therefore, the next step required was to check the safety of the product by carrying out mutagenicity and toxicity tests.

4.4 Conclusion

In this research, it was found that liquorice extract inhibited the growth of food-borne pathogenic bacteria, such as *L. monocytogenes*. Therefore this novel antimicrobial extract could be of value to inhibit the growth of *L. monocytogenes* and other Gram-positive spoilage organisms when applied to the surface of sliced deli meats and hence could be of use to improve the safety of sliced deli meats. Thus, application of liquorice extract at 50 μg ml^{-1} was confirmed to be an effective way to control *L. monocytogenes* growth on the slices of bologna sausages.

CHAPTER 5

EVALUATION OF THE SAFETY AND TOXICITY OF LIQUORICE EXTRACT

Abstract

To confirm that the liquorice extract is safe for application as a food preservative, the potential cytotoxicity and mutagenicity of the liquorice extract was studied *in vitro* using Ames and MTT tests. In the Ames test, any mutagenic effects were determined using a concentration of 50 μg ml^{-1} of liquorice extract and the ability of extract to induce reverse mutations was evaluated. Using *Salmonella* Typhimurium strains TA100, TA102 and TA1535, the results showed that there were no increases in the number of revertant colonies at this concentration indicating that no mutagenic activity was detected. In contrast, the results showed that using positive controls there were a 6-7-63 fold increase in the number of revertant colonies detected for TA100, TA102 and TA1535, respectively, and therefore this indicates that there is no evidence that the extract is mutagenic.

A second set of experiments was carried out to investigate whether the liquorice extract had any cytotoxic effects. Using yeast cells as a simple model eukaryotic cell type, it was found that yeast growth was not affected by liquorice extract. Moving on to a human cell line model, MTT (3-(4, 5 dimetylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assays were used to detect any cytotoxicity. In this case it was found that the liquorice extract did have a cytotoxic effect on Caco-2 cell lines after 24 h exposure to liquorice extract at a concentration of 50 μg ml^{-1}. This was in contrast to the control sample as well as samples treated with 12.5 μg ml^{-1} of liquorice extract which had no cytotoxic activity on the Caco-2 cells. However the level of cytotoxicity detected was minimal and was considered unlikely to cause damage to human gut cells if consumed by humans rather than being directly applied to colonic cancer cell culture samples. Therefore these preliminary safety studies indicated that this extract could be taken forward as a candidate novel food preservative, and there is also some evidence that the material could be used as a therapeutic agent.

5.1 Introduction

As reviewed in Chapter 1, not all compounds-derived from plants are safe, and several studies indicated that there are some compounds in plants that have toxic and carcinogenic effects which has led to an emphasis on studying the genetic risks of plant compounds (Boada *et al*., 2016). Accordingly, the use of medicinal plants, crude plant extracts and isolated compounds for food preservation and therapeutic purposes require an evaluation of their cytotoxic and mutagenic potential (Cavalcanti *et al*., 2006). Plant extracts exhibiting explicit mutagenic properties are considered as unsafe since compounds which exhibit a mutagenic activity could possess carcinogenic properties and thus require further investigation before continuing to be used for use in human products (Verschaeve and Van Staden, 2008). Accordingly *in vitro* toxicity testing has acquired significant role as a tool for the assessment of the safety of different drugs and chemicals. In this respect, it is important to note that many researchers have reported that various natural compounds isolated from different species of the liquorice plant (*Glycyrrhiza spp*.) belonging to the *Fabaceae* family possess cytotoxic activities against multiple cancer cell lines (Fukuchi *et al*., 2016; Lee *et al*., 2013; Sheela *et al*., 2006; Bassyouni *et al*., 2012). Moreover, liquorice root has been identified by the National Cancer Institute within a group of herbs which possess cancer-preventive properties (Caragay, 1992). This family represents a group known to have high biodiversity and also for the production of an abundance of in secondary metabolites (Wink, 2013), and because of this has been considered especially important for human health in the form of dietary supplements or as therapeutic treatments (Dixon and Sumner, 2003). However the investigation of liquorice extracts for their potential applications to contain other bioactivities is still important.

Mutagenicity bioassays have been confirmed as a significant tool in environmental monitoring and assessment of pollution, with more than 200 short-

term bioassays were utilized through using micro-organisms, insects or plants which have been developed and applied to assist in the identification of factors that pose genotoxic risks (Ansari and Malik, 2009). Among them, the Ames test was specifically designed to detect if a chemical is a mutagen by detecting its ability to introduce changes into bacterial DNA. This test is able to detect different mutagens which affect single nucleic acid bases by either altering or deleting them or those chemicals that cause insertion of bases into a DNA sequence, and any such changes in eukaryotic cells may result in the formation of cancer cells (Ames *et al*., 1975). Ames employs particular strains of the *Salmonella* Typhimurium were developed by Bruce Ames and form the basis of the "reverse" mutation assays as tools to reveal specific types of mutation (Mortelmans and Zeiger, 2000; Ames, 1971). The Ames strains carry defined mutations in the histidine biosynthetic operon, thus they need histidine for growth and the strains will not normally grow unless the histidine is supplied in growth medium. Each of the different mutations is designed to be reacting to mutagens that act via different mechanisms, and therefore they are susceptible to a broad variety of substances. The basis of the reverse mutation assay is that in the presence of a test product containing a mutagenic chemical compound, a second mutation will be caused in the faulty genes that will convert it back to the functional state. This allows the bacteria to growing in a medium that contains limiting amounts of histidine to form colonies, with the number of colonies formed being proportional to the mutagenic potential of the chemical (Mortelmans and Zeiger, 2000). Over time, the Ames test has become recognized by many governmental and non-governmental organizations as a standard screening test (it has been adopted as an ISO test; ISO, 2012) and is widely used as an initial screen since the Ames test is an inexpensive and flexible method.

In addition to determining whether the compounds are mutagenic, it is also important to determine whether chemicals have any cytotoxic effects on human

cells since bacterial cells have a different structure and cell composition and chemicals may interact with different target sites eukaryotic cells that do not exist in bacterial cells. Under physiological conditions intestinal cells have a high exposure to dietary compounds and for this reason Caco-2 cells are often chosen as a model system to assess cytotoxicity. This cell line was derived from a human colon carcinoma and when grown in culture, it subjects enterocytic differentiation in order to form a monolayer and its functional and structure much like to the human small intestine epithelium, it is known that the responses of live cells are complicated by the membrane permeability (Asano *et al*., 2003; Baker *et al*., 1995). In addition a study conducted by Asano *et al*. (2003) has shown that Caco-2 cells show good absorption of some flavonoids from liquorice and therefore this is an appropriate test system to use for assessing this new liquorice extract.

The overall aim of this study was to carry out a preliminary safety assessment of the liquorice extract using *in vitro* tests to confirm that it is safe for subsequent application as a food preservative. The specific objectives were to assess the cytotoxicity and mutagenicity effects of liquorice extract *in vitro* to confirm that it is safe for subsequent application using the Ames test (Section 2.17) and the MTT assay (Section 2.18.3).

5.2 Results

5.2.1 Evaluating the mutagenicity and toxicity of liquorice extract

The safety of liquorice extract *in vitro* was investigated using the Ames test as the indication of the mutagenicity of the extract. The *Salmonella* Typhimurium strains used were TA100, TA102 and TA1535 (Table 5.1). The *rfa* mutation present in all of these strains shortens the LPS molecules on the outside of the cell to help facilitate the uptake of the larger test compounds by the cells. A deletion mutation in the region of the chromosome between *uvrB* and *bio* genes is also present in all strains except TA102 and this region includes the *uvrB* gene which is required for

the accurate excision DNA repair mechanism. This mutation then allows more DNA lesions to be repaired via the error-prone DNA repair pathway and the deletion in this region also make the cells dependent on the presence of biotin in the media (see review by Tejs, 2008). The presence of the plasmid pKM101 in TA100 and TA98 results in enhanced chemical and UV-induced mutagenesis due to the over expression of the recombination DNA repair pathway that is encoded by this plasmid and also confers ampicillin resistance on the cells. Plasmid pAQ1 which contains the mutation *hisG*428 was introduced into strain TA102 to increase the number of copies of the target mutation, and reversion of any one of these will lead to a reversion to wild type, hence increasing the sensitivity to mutagenic agents. Finally this strain also retains the *uvrB* gene which makes it more sensitive to DNA cross-linking agents as it is able to use the excision DNA repair mechanism induced by these types of chemicals.

Given the phenotypes described above, in the case where the extract is mutagenic, the strains will undergo mutations that revert the mutations in the histidine biosynthetic operon to wild-type (histidine-independence) when exposed in the presence of chemical. If there is no evidence of mutagenic potential this is the first evidence that the extract was safe and could be used as a food preservative. However before using these strains it is also important to carry out tests to confirm that the isolate still retains the expected plasmids which can be easily lost from these strains.

Table 5.1. Genotype of the most commonly used *Salmonella* test strains*

Mutation (strain)	Histidine operon mutations (*bio chlD uvrB gal)*	LPS defect	Plasmid
TA1535 *hisG46*	Deletion mutation	*rfa*	No plasmid
TA100 *hisG46*	Deletion mutation	*rfa*	pKM101
TA98 *hisD3052*	Deletion mutation	*rfa*	pKM101
TA1537 *hisC3076*	Deletion mutation	*rfa*	No plasmid
TA102 *hisG428*	Wild type	*rfa*	pKM101, pAQ1

(*): This table reproduced from Mortelmans and Zeiger (2000)

5.2.1.1 Confirmation of the presence of plasmids in *Salmonella* strains

The presence of plasmid pKM101 was tested by streaking a loop-full of culture across a BHI agar plate supplemented with 24 µg ml^{-1} ampicillin and incubating the samples for 24 h at 37 °C. The result (growth) confirmed that the strains that were expected to contain these plasmids were able to grow and form colonies on these plates and this indicative of plasmid presence (Table 5.2). Similarly the presence of plasmid pAQ1 was tested by streaking a loop-full of culture across a BHI agar plate supplemented with 24 µg ml^{-1} ampicillin and 2 µg ml^{-1} of tetracycline. Plates were incubated for 24 h at 37 °C. The results confirmed that the strain that were expected to contain both plasmids was able to grow and form colonies on these plates and this indicative of plasmid presence in (Table 5.2).

Table 5.2. The genotype of the *Salmonella* Typhimurium TA100 &TA102

Characteristics	Type of genetic change	Result
Tetracycline resistance	Presence of plasmid pAQ1	Growth in the presence of tetracycline
Ampicillin resistance	Presence of plasmid pKM101	Growth in the presence of ampicillin

Salmonella strains (TA100 &TA102) were grown in the plates supplemented with antibiotics to confirm the presence of plasmid

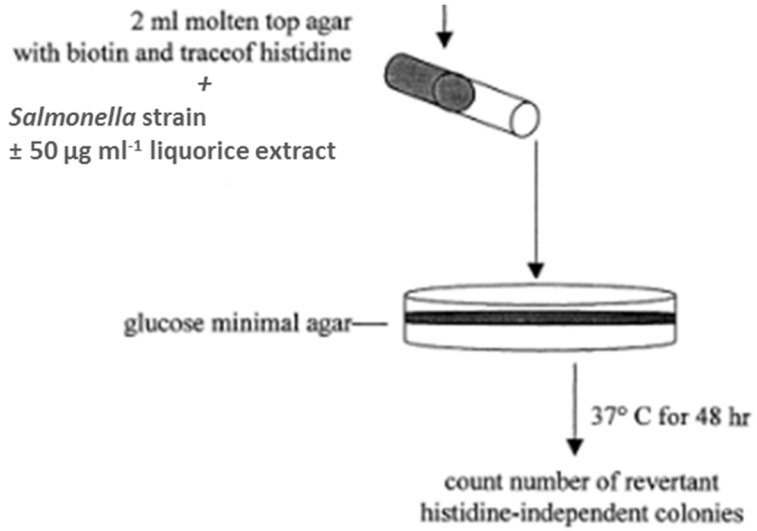
5.2.1.2 Ames test

For the current study, to assess the safety of liquorice extract, a preliminary mutagenic screen was carried out using the Ames test using the *Salmonella* Typhimurium strains TA102, TA100 and TA1535 (see Tables 2.2, 2.3 and 5.1 for details). In these experiments trace amounts of histidine and biotin were added to a molten agar to allow all the *Salmonella* strains grow on the plate of minimum agar medium for a defined number of divisions. Thus, in case there is no chemical compound cause mutagenicity, the *Salmonella* will stop dividing once the histidine and biotin are exhausted. Otherwise, the *Salmonella* will continue division which indicates a DNA mutation had occurred to reverse the histine-dependant growth phenotype. Therefore, after exposure to the chemicals, the number of cells that are able to grown into colonies are counted, and this number reflects the number of *S*. Typhimurium bacteria that undergo a reverse mutation from His^- to His^+.

Cultures of the strains were prepared as described in Section 2.4.2 and then 0.1 ml of bacterial suspension was added to 2 ml of top agar in a bijou bottle, along with the test substances. The liquorice extract was added to a concentration

of 50 μg ml^{-1} and as a control 1 % (v/v) ethanol was added to separate agar samples. The mixture was then poured over the surface of minimal glucose (MG) agar plate (Section 2.17 and Figure 5.1). As a positive control, sterile filter paper disc (6 mm) were placed in the centre of an MG agar plate that had been seeded with the Ames strain using the soft agar method described and then 10 μl of the test mutagenic solution was dispensed onto the surface of the disc (see Table 2.6 for details). The standard mutagens used as positive controls in experiments were sodium azide for TA100 and TA1535, and Mitomycin C for TA102. The plates were incubated at 37 °C for 48 h and the His^+ revertant colonies were counted manually. All experiments were carried out in triplicate. After incubation the colonies were counted on each plate, the average number of colonies on the three plates was recorded and the results expressed as the number of revertant colonies per plate and compared with the negative control plates. Toxicity was detected as a reduction in the number of histidine revertants (His^+) or as an alteration in the auxotrophic background (i.e., background lawn).

Figure 5.1. Method for preparing agar plates for Ames test

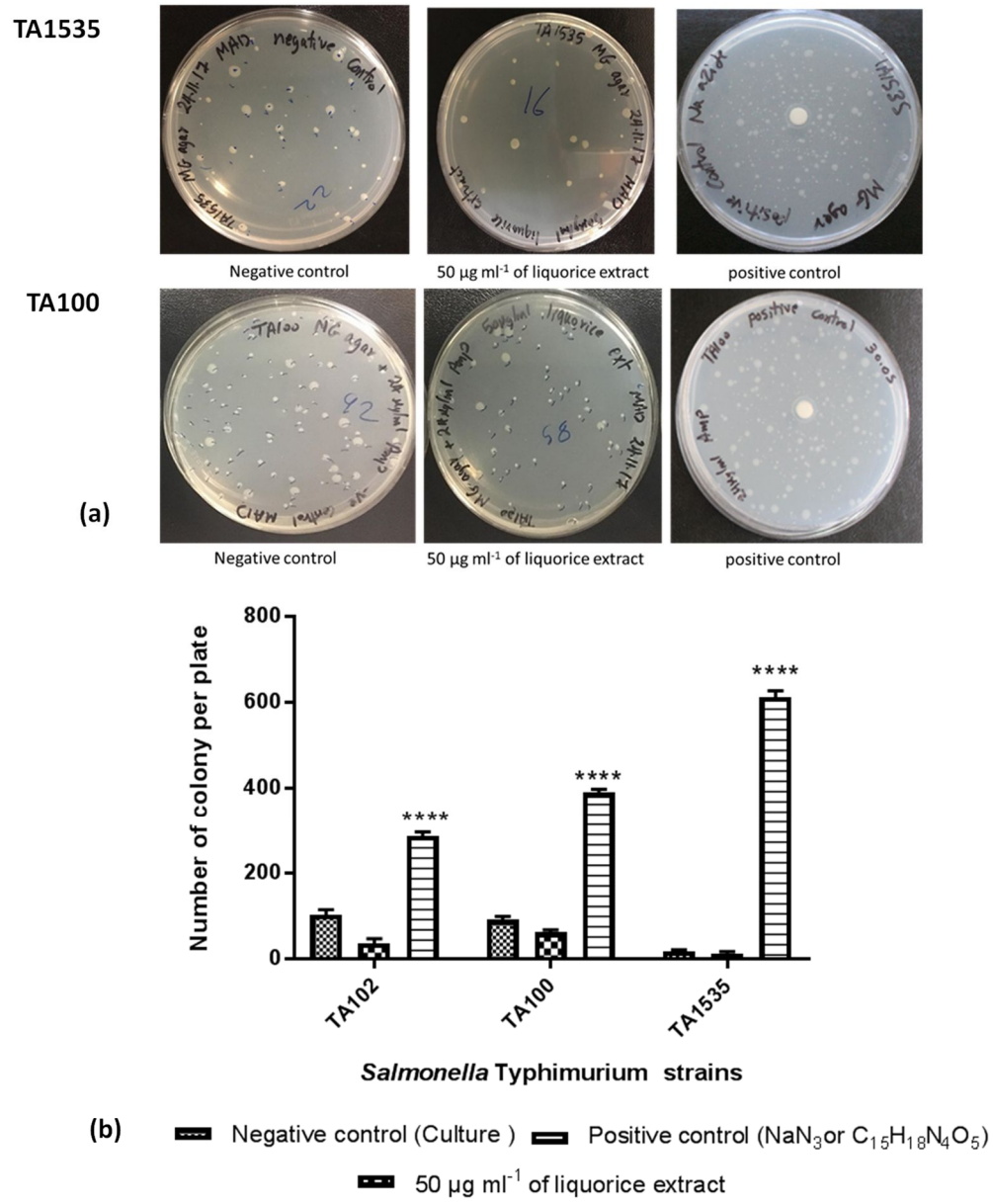


2 ml molten top agar was combined with each of the cultures of bacteria ± 50 µg ml^{-1} of liquorice extract and then poured onto the surface of a minimal glucose (MG) agar plate (Adapted from Mortelmans and Zeiger, 2000).

The results of the reverse mutation test using three bacterial strains are summarized in Figure 5.2. Data showed no significant increase in the number of revertant colonies as a result of liquorice treatment on the three strains of *S*. Typhimurium (TA100, TA102 and TA1535). In contrast the number of revertant colonies in positive controls increased significantly ($p < 0.0001$) compared to the negative control while the number of colonies recorded for the 50 µg ml^{-1} liquorice extract treatment and the negative control were not statistically different ($P > 0.05$) (Figure 5.2). Therefore the treatment with 50 µg ml^{-1} of liquorice extract was not considered to be mutagenic compared to the positive controls of both sodium azide & mitomycin C (Figure 5.2, a & b). Interestingly there was a slightly higher increase in the number of revertants when the TA100 and TA102 cells were exposed to ethanol in the negative control samples. Although generally not considered to be mutagenic in the Ames test (Vedmaurthy *et al*., 2012), it is known

that when converted to acetaldehyde ethanol can cause both DNA cross linking damage and induce transition/transversion mutations in DNA, which are the types of mutations detected by TA102 and TA100, respectively. Although 1 % ethanol was used as a control, this is higher than the level of ethanol present in the sample containing 50 μg ml^{-1} of the extract, since as described in Chapter 3, this was the maximum amount of ethanol present in any of the different concentrations tested and therefore was used as a standard throughout these experiments. The fact that the increase in mutation rate was only 2.7-fold for TA102 and 1.4-fold for TA100 would mean that the dilution of the ethanol in the liquorice extract would take the concentration of this below a threshold where an effect could be detected. Generally only eukaryotic cells are able to convert ethanol to acetaldehyde efficiently through the action of alcohol dehydrogenase, which is believed to be the underlying mechanism for the carcinogenic effect of alcohol consumption in humans (Mizumoto *et al*., 2017), but the pattern of results seen here would be consistent with the presence of low levels of acetaldehyde in these samples due to an unknown source. Despite this anomaly, the results of the reverse mutation test using three bacterial strains proved that dose of 50 μg ml^{-1} of liquorice extract was not mutagenic to *S*. Typhimurium.

Figure 5.2. Effect of treatment of liquorice extract, sodium azide and Mitomycin C treatment on revertant of histidine auxotrophy of the Ames strain S. Typhimurium TA100, TA102 and TA1535

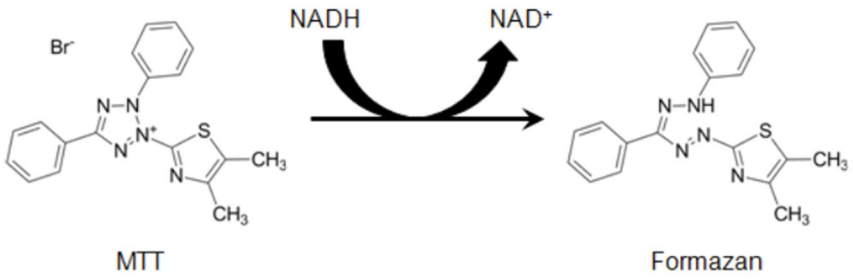


(a) *Salmonella* strains TA102, TA100 and TA1535 were treated with 50 µg ml^{-1} and sodium azide (NaN_3)or Mitomycin C ($C_{15}H_{18}N_4O_5$) as a positive control and 1 % (v/v) ethanol solvent as a negative control, after 48 h incubation time the number of revertant colonies were counted, (b) Values presented are averages of three replicates of each bacterial strain. The results indicated that positive control high significance with ****$p < 0.0001$ compared to negative control and 50 µg ml^{-1} of treatment and the differences were not statistically significant ($P > 0.05$) between 50 µg ml^{-1} of liquorice extract and negative control. Positive control (sodium azide) for TA100, TA1535, positive control (Mitomycin C) for TA102, negative control (1 % (v/v) ethanol solvent with culture).

5.2.2 Evaluating cytotoxicity using a cell viability with MTT assay

In previous Chapters, we confirmed that the extract significantly inhibited the growth of Gram-positive bacteria. *In vitro* experiments were performed using Caco-2 cells for the cytotoxicity to confirm that the liquorice extract has the activity as anticancer through its effect on the viability of Caco-2 cells. The viability of the Caco-2 cells was determined by using the MTT assay, in which the reagent (*3*-(*4*,*5*-dimethylthiazol-*2*-yl)-*2*,*5*-diphenyltetrazolium bromide) tetrazolium is used as a monitor of cell viability. This test is based on the cleavage of a tetrazolium salt by mitochondrial dehydrogenases and this occurs in viable cells (Khairunnisa and Karthik, 2014). In this test soluble MTT bromide is converted by mitochondrial enzyme activity of viable cells into an insoluble coloured formazan dye which can be measured by resolubilize the formazan and the concentration of the dye produced determined spectrophotometrically at 570nm (Figure 5.3).

Figure 5.3. Structures of MTT and coloured formazan product



Viable cells converted MTT bromide into a purple coloured formazan product which is insoluble, the absorption can be measured at 570 nm using a spectrophotometer after cells are solubilized (Adapted from Riss *et al*., 2013).

As previously mentioned Caco-2 cells were selected for this study as they had previously been shown to exhibit an excellent absorption of some flavonoids from liquorice (Asano *et al*., 2003). To carry out these experiments Caco-2 cells were maintained in DMEM medium as a described in Section 2.18.1. The medium was replaced every two days, and the cells were passaged at 80 to 90 % confluence using trypsin-EDTA solution. Cells were stained with trypan blue, and cell number and viability were determined using a haemocytometer as a described in Section 2.18.2.

The MTT assay was then performed using the protocol described by Riss *et al*. (2016) with a slight modification. Briefly, Caco-2 cells were seeded at a density of 0.3×10^5 cells per well by adding 5 ml of DMEM containing the cells to each well in a 6-well plate. The cells were then treated with two different concentrations of liquorice extract (12.5 and 50 µg ml^{-1}) and as negative controls they were also treated with 1 % (v/v) ethanol and 20 % sodium dodecyl sulfate (SDS) was used as a positive control. A second negative control was prepared by treating the cells with 4 % vinegar. It was decided to use vinegar as a negative control since in a study by Bozorgi *et al*. (2017), vinegar was used to extract Squill [Drimia maritima (L.) Stearn] and the results did not show toxicity against cancer cells including HT-29 and Caco-2 . In addition controls were prepared by adding cells to the wells (*i.e.* the well contained medium only with cells). Triplicate wells were prepared for each individual treatment and samples were kept in the incubator for 24 h at a humidified atmosphere of 95 % air and 5 % CO_2 at 37 ºC.

After 24 h, culture medium was removed by aspiration and the cells washed with 5 ml of PBS to remove dead cells and cellular debris. The PBS was then replaced with fresh DMEM medium containing yellow tetrazolium salt and samples were re-incubated for an additional 3 h at 37 °C to allow the tetrazolium salt to be converted to the purple formazan dye. Finally the supernatant was removed, and the residual purple formazan product was solubilized in 2 ml solubilization buffer,

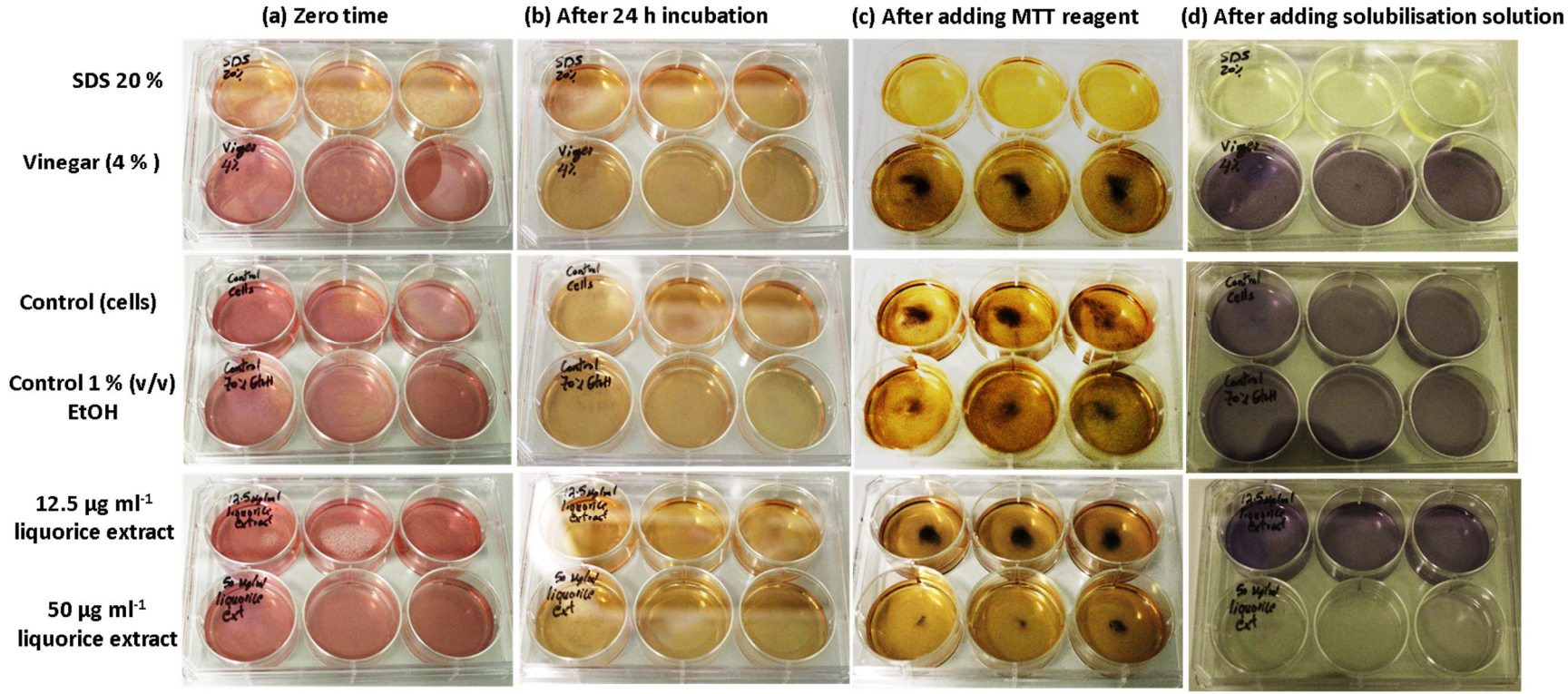
the plate was gently shaken and re-incubated for 15-30 min to dissolve the purple formazan dye crystals formed and to stop the reaction. Finally 1 ml samples were transferred into cuvettes and the absorbance was measured at OD_{570nm}. The percentage cell viability was calculated from triplicate results using the following formula as a described by (Rezk *et al*., 2015). Absorbance values higher than the control indicated that the cells had retained viability, while values lower than the control indicates that cell death or inhibition of respiration had occurred.

$$\textbf{Cell viability}\ (\%) = \frac{\textbf{Absorbance of treated cells}}{\textbf{Absorbance of control cells}} \times \mathbf{100}$$

The experiment was performed in triplicates and repeated at least three times in independent experiments, and the number of cells was expressed by averaging of triplicate readings in each well (Table 5.3; Figure 5.5) and the results gained at each step of the assay are shown in Figure 5.4.

The results showed that the cell control culture contained very similar levels of viable cells to that detected in the presence of 1 % ethanol (96 % viability retained; Table 5.3; Figure 5.5) and that there was no significant difference (p= 0.15) between the results gained using the solvent control and the liquorice extract at a concentration of 12.5 µg ml^{-1} indicating that at this concentration no cytotoxic effects were detected. Interestingly the control sample challenged with 4 % vinegar showed no effect on the viability of the Caco-2 cells – in fact there was a slight increase in the cell viability recorded (111 %; Table 5.3). Acetic acid is commonly used as a component of the solvent for the insoluble formazan dye (Berridge *et al*., 2005), and therefore it is possible that this apparent increase in cell viability just represented a better solubilisation of the dye produced by the cells in these wells rather than a stimulation of cell growth or enzyme activity.

Figure 5.4. Caco-2 cell line with different concentration of liquorice extract and positive and negative control



Cell viability was measured by detecting purple formazan that was metabolized from MTT by mitochondrial dehydrogenases, which are active only in live cells. Panel (a) samples taken at zero time, (b) samples after 24 h incubation, (c) samples after fresh DMEM medium containing yellow tetrazolium salt was added and re-incubated for an additional 3 h at 37 °C and (d) samples after added solubilization solution and incubated for 20 to 30 min at 37 ºC.

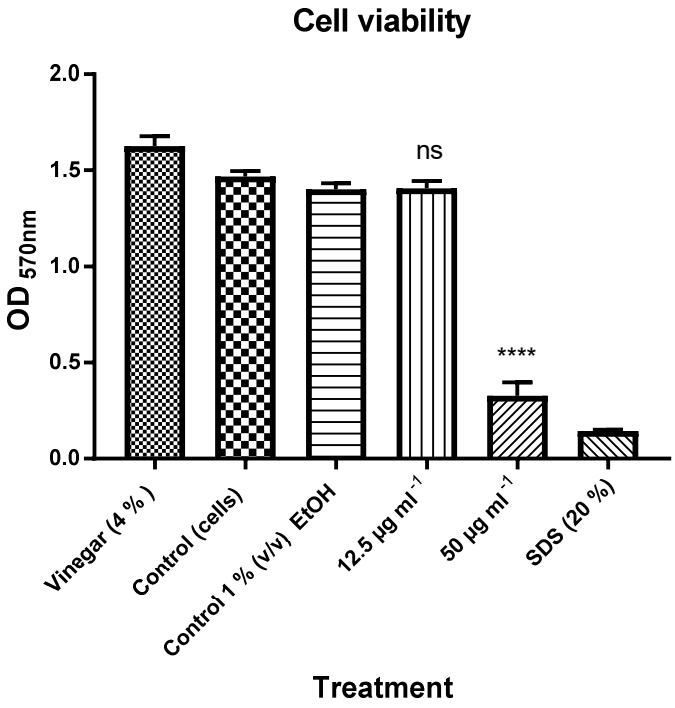
However when the cells were treated with the liquorice extract at a concentration of 50 µg ml^{-1} the cell viability recorded was only 2-fold higher than that for the SDS positive control sample. This result was significantly lower than the negative control samples ($p < 0.0001$) indicated that there was damage to the Caco-2 cells caused by exposure to this concentration of the extract.

Table 5.3. The cell viability MTT Assay

Treatment	Average	% Cell Viability
Control (cells)	1.47	100
Control 1 % (v/v) EtOH	1.40	96
12.5 µg ml^{-1} Extract	1.41	96
50 µg ml^{-1} Extract	0.33	22
Vinegar (4 %)	1.63	111
SDS (20 %)	0.14	10

Caco-2 cells treated with and without liquorice extract as well as vinegar and SDS then incubated for 24 h at a humidified atmosphere of 95 % air and 5 % CO_2 at 37 ºC. The experiment was performed in triplicates and repeated at least three times in independent experiments, and the number of cells was expressed by averaging of triplicate readings in each well. Caco-2 cells have a good relationship between absorbance and the number of cells was observed ($r = 0.9971$), and the extract caused more than 50 % growth inhibition which considered as a cytotoxic agent. The findings showed that the reduction in cell viability caused by the liquorice extract was statistically significant ($P < 0.0001$).

Figure 5.5. Histogram showing results of MTT assays



The cytotoxicity effect of liquorice extract on Caco-2 tumour cells. Cells were treated with and without the extract at two concentrations (12.5 and 50 µg ml^{-1}), then readings were taken after 24 h using spectrophotometer at OD_{570nm}. Data is given as means ± standard deviations from three independent experiments, each performed in triplicates (n=3). Differences between means were evaluated using one-way ANOVA analysis; and values of $p < 0.0001$ were considered statistically significant.

5.2.3 Evaluation of toxicity of liquorice extracts on yeast

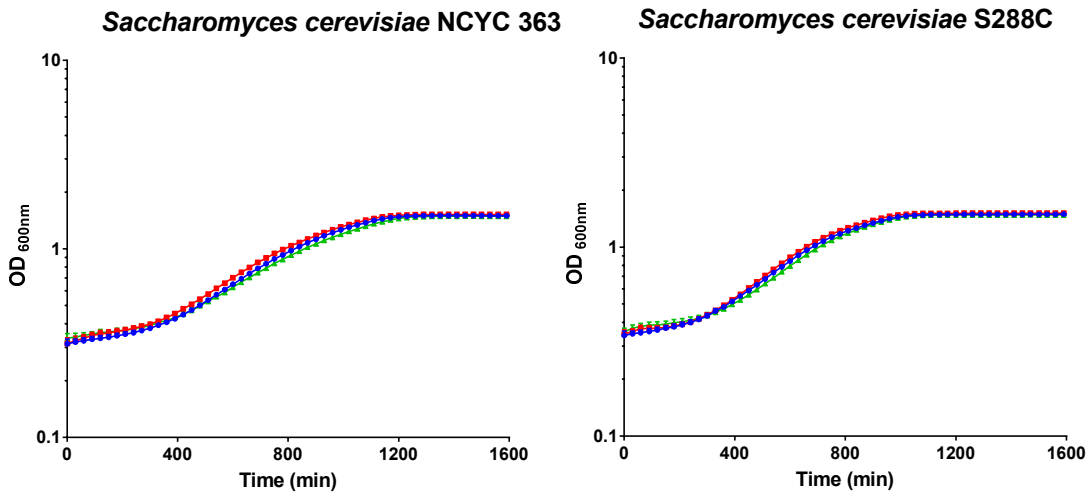
Since the MTT assay had indicated that there was a cytotoxic effect seen when the higher concentration of the liquorice extract was used in the MTT assay, it was decided to test the effects of the cytotoxicity of the liquorice extract against *Saccharomyces cerevisiae* which was chosen as another eukaryotic cell. The cellular structure and functional of *Saccharomyces cerevisiae* much resemblance to cells of higher-level organisms, *S. cerevisiae* is also increasingly utilized in the toxicological evaluation of chemicals (Kasemets *et al*., 2009). To confirm if there is any an effect on growth of yeast, two strains of *S. cerevisiae* were used in this study are listed in (Table 2.2) including a lab isolate strain (S288C) and one wild yeast (NCYC 363).

To carry out these experiments a single colony of yeast strains S288C and NCYC 363 were inoculated in yeast extract peptone dextrose (YPD; Section 2.5) broth and incubated at 30 °C for 48 h. Cells were harvested by centrifugation and re-suspended in 20 ml distilled H_2O, this process was repeated twice to ensure all the YPD broth was removed from the culture and then the cells were finally re-suspended in 10 ml. To determine the number of viable cells present in the sample, the cell suspension was diluted and then stained with methylene blue (Section 2.20) so that the viable count could be determined microscopically using a Neubaure Haemocytometer (Section 2.18.2)

The number of viable cells in the suspension was then calculated and the cells further diluted in YPD broth to a density of $1x10^6$ cfu ml^{-1}. Three flasks were then prepared which had been supplemented with 12.5, 50 µg ml^{-1} liquorice extract or 1 % (v/v) ethanol solvent as a described in Section 2.20. Samples (200 µl) of these different cultures were transferred into plate 96 well which was then incubated in a microplate reader (TECAN) at 25 °C for 1800 cycles of orbital (30 min) shaking followed by OD_{600nm} measurements for 48 h.

The result are shown in Figure 5.6 indicated that the growth of both strains was the same as the control sample containing (1 % v/v) ethanol at both concentrations of the liquorice extract tested (12.5 and 50 µg ml^{-1}). Although ethanol is known as an antimicrobial, the percentage of ethanol used in these experiments is well below the level known to effect on the growth of yeast as ethanol-producing yeast can tolerate levels of ethanol of up to 8 % in the culture media without affecting growth (Kalathenos and Russell, 2003). Hence in this case, the yeast appeared to be resistant to the antimicrobial effects of the extract seen when testing Gram-positive bacteria as there was no significant difference between the control and the samples treated with the liquorice extract. Hence for this eukaryotic cell type, no toxicity was detected.

Figure 5.6. The growth of yeast in the presence of liquorice extract



The growth curves of *S. cerevisiae* of yeast cultures NCYC 363 and S288c grown at 25 °C for 48 h in a culture with and with liquorice extract at concentrations of [■] 12.5 µg ml^{-1} and [▲] 50 µg ml^{-1} and the control sample [●] containing 1 % (v/v) ethanol. Growth was monitored by optical density at 600nm. Data shown are given as means ± standard deviation (SD) for 12 replicates for every concentration; SD values are very small and are very close to the data points and therefore are not visible on the graphs.

5.3 Discussion

Current research is focussing on natural molecules and natural products that are produced by plants as novel food preservatives since they can be sourced relatively easily and many are selected for evaluation based on their medicinal use as traditional medicine for many years (Mussarat *et al*., 2014). However, compounds or extracts with a specific activity at a non-toxic dose need further evaluation to define the toxicity status. Thus, toxicological properties in this study were defined by using three methods, for mutagenicity (Ames test) and toxicity (MTT test and yeast growth inhibition). The Ames test was chosen as a rapid and simple method to perform, and this test is specifically designed to reveal a wide range of chemical substances that can cause genetic damage which turn lead to gene mutation (Mortelmans and Zeiger, 2000). However even in the absence of a mutagen a small number of spontaneous mutations will occur in the high number of bacterial cells present in the test sample leading to the formation of a low number of colonies due to the occurrence of random mutations in the population (Mortelmans and Zeiger, 2000; Wessner *et al*., 2000; Ames *et al*., 1973a; Ames *et al*., 1973b). This was seen in the experiments reported here, but clearly when the strains were exposed to both positive control substances (sodium azide and mitomycin C) the rate of mutations increased dramatically.

The Ames test is considered a convenient tool for quantifying the genotoxicity of composite herbal mixtures that are consumed to improve human health. Although it is generally recognised that identifying the level of genotoxic compounds present in the herbs is dependent on the use of several *Salmonella* strains for the Ames assay (Maron and Ames, 1983). As known, chemicals can cause damage in germ lines leading to fertility problems and causing mutations in future generations, in addition they also are able to induce cancer. Gene mutations can occur with only a single base changes (base-pair substitution mutants), and others contained additions or deletions of one or more bases

(frameshift mutants) (Mortelmans and Zeiger, 2000) as a described in (Table 5.1) and therefore different Ames strains were used to allow these different types of mutations to be detected. However, a positive result does not necessarily indicate that the compound is a carcinogen, the Ames assay just confirms whether or not a compound is mutagenic (Zeiger, 2001).

In the experiments described in this Chapter, no statistically significant difference ($P > 0.05$) was observed between the effects of the liquorice extract and ethanol solvent, and indeed the ethanol alone seemed to have a more mutagenic effect than the extract showing the importance of including controls in these experiments to rule out the possibility that the solvents used are adversely affecting the test cells. Importantly there has been no reported toxicity of liquorice in the previous studies and therefore the results gained are in agreement with the published literature. For instance Moosavi *et al*. (2013) reported that *Abrus precatorius* seeds belonged to the family Fabaceae - and known commonly as Indian liquorice and contains glycyrrhizin and are used for medicinal purposes - were not mutagenic to the TA100 strain of *S.* Typhimurium when tested in the suggested dose range of 0.2-0.5 mg ml^{-1}. However this study would have been more convincing if a range of the different Ames strains were used to cover all of the possible mechanisms of DNA damage that can be detected. Based on the results obtained in this study in Figure 5.2 it is confirmed that liquorice extract is not mutagenic in the *Salmonella* Typhimurium reverse mutation assay (Ames test).

Fruits and vegetables considered as a plant-based diet which contains a large number of molecules that have the chemopreventive potential to fight against cancer evolution. It has been discovered that there is a strong correlation between diet and cancer; where the dietary constituents that inhibit mutagenesis and/or carcinogenesis play an important role in the protection from cancer such

as vitamins. Previous studies have documented the anti-cancer properties of plants due to their high content in polyphenols (Fresco *et al*., 2010). Other compounds including carotenoids, flavanoids, isoflavones and catechins are known to reduce the risk from several forms of human cancers (Kris-Etherton *et al*., 2002; Gagandeep *et al*., 2005). In a study conducted by Xuesheng *et al*. (2009), it was reported that liquorice could be used to minimize the production of toxic intermediates when included in some mixtures containing poisonous herbs, but this would probably be related to the well-known antioxidant effect of liquorice extracts (Kim *et al*., 2012; Tohma and Gulçin, 2010; Martins *et al*., 2015).

The cytotoxicity of liquorice extract was determined using MTT Assay against Caco-2 cell line. The MTT colorimetric assay has been used for assessing the reduction in the cell culture viability in many studies of plant extracts (Wang *et al*., 2015). Anti-cancer activity for plants has also been evaluated by many researchers using methods such as MTT assay (Carvalho *et al*., 2010; Cheshomi *et al*., 2016). Thus, liquorice extract was tested in this study using cultures of human Caco-2 cells, although the results are often equally interpreted as evidence of anticancer activity. Liquorice has already been studied extensively as a potential anticancer or cancer chemopreventive efficacy.

The results of this study showed that the extract at a concentration of 12.5 μg ml^{-1}, the cells have higher cell viability of more than 90 % indicated that the compound at this concentration was non-toxic to the cells. However, after 24 h exposure, the cell numbers were significantly reduced ($p < 0.0001$) at a concentration of 50 μg ml^{-1} (Table 5.3). Thus, the results of this study showed that the extract exhibited cytotoxic effects toward cancer cells at a concentration of 50 μg ml^{-1} by decreasing cell viability after 24 h incubation time. In liquorice, some of phytochemicals compounds have been reported to possess antineoplastic activities against different cancer cell lines. Several reports have confirmed that

various extracts of liquorice (*Glycyrrhiza* spp.) root exerted anticancer effects. The National Cancer Institute has been identified that liquorice root possesses cancer-preventive properties (Fiore *et al*., 2005; Fiore *et al*., 2008; Wang and Nixon, 2001). It has been used among patients with prostate cancer and it has been proposed that liquorice can be used efficiently for the cancer therapy without causing severe side-effects to the healthy body cells. However the preparation of herbal formulations to fight the cancerous cells without affecting healthy cells of the body requires a thorough understanding of the complicated synergistic interaction that can occur between the different constituents of anticancer herbs (Larkin, 1983; Saxe, 1987).

Licochalcone A, the main chalcone in liquorice, which possesses many potential biological activities including antitumor activities (Fukuchi *et al*., 2016; Lee *et al*., 2013; Fu *et al*., 2004). It was found that chalcones, such as licochalcone E isolated from the roots of *G. inflata*, exhibited the highest potent cytotoxic effect compared with the known antitumor agents such as licochalcone A and isoliquiritigenin (Yoon *et al*., 2005). Cytotoxicity of the active constituents isolated from *Glycyrrhiza uralensis* was evaluated against several human cancer cell lines including HepG2 (liver cancer), SW480 (colorectal cancer), A549 (lung cancer), and MCF7 (breast cancer) cells. Licoricidin was the most potent compound among the isoflavans tested, and some compounds showed apparent selectivity among different cell lines, and the liquorice ethyl acetate extract showed more cytotoxic effects between 76−99 % against all cell lines at a concentration of 25 μg ml^{-1} (Ji *et al*., 2016). The results of these studies did not definitively conclude whether or not the liquorice can be used as a therapeutic plant, especially for cancer protective.

When considering cytotoxic effects, our results are in accordance with those reported by Badr *et al*. (2013) where the crude methanolic extract of liquorice has

shown cytotoxic activities against intestinal carcinoma cell line (Caco-2) and prostate carcinoma cell line (PC-3) with different concentration of liquorice extract (0, 12.5, 25, 50 and 100 µg ml^{-1}). In agreement with our results earlier studies conducted by Rao *et al*. (2014) reported that among of different plant extracts liquorice (*Glycyrrhiza glabra*) extract were evaluated for their cytotoxicity activity, different concentrations of plant extracts (50, 100 and 150 µg ml^{-1}) were added to cultured HepG2 cells and incubated for 48 h, it was observed that all plant extracts were cytotoxic to the liver cancer cell line at all the tested concentrations by using MTT assay, it was noticed that the percentage of viable cells decreased as the concentration of extracts increased. On the other hand, when the same extracts were added to normal lymphocytes and examined after 48 h for cell viability by MTT assay, it was observed that the percentage viability was approaching to 100 in most of all the treatments and this is proof that liquorice is entirely safe for human consumption. Therefore although some evidence of cytotoxicity was detected at the higher concentration, and this may be undesirable in a food preservative, it could also be taken as yet more evidence of the presence of anticancer agents in these liquorice extracts. Hence in general, and in agreement with our results, the results of other published studies have concluded that extracts from *Glycyrrhiza* species are not toxic or genotoxic *in vitro* but did exhibited cytotoxicity of the against different cancer cell lines.

5.4 Conclusion

The fact that liquorice extract did not affect yeast cells and the Ames test revealed no mutagenic potential is very significant from a food safety standpoint should these antimicrobials be leveraged as bio-preservatives.

According to the results presented in this study and also in comparison with similar studies, it can be concluded that the liquorice extract has anticancer effects

for colon cancer treatment. However, further research is needed to explore effective compounds that present in the waste material of liquorice extract.

Set against this is the cytotoxicity observed when high concentrations were used, and this is in line with previous reports that liquorice plant helps to reduce the proliferation of tumour cells, such as the Caco-2 cell line. Studying the effects of these extracts on normal cell line is necessary to ascertain selectivity of cytotoxic activity to cancer cells and to determine if this extract could be safely used as a novel antimicrobial.

CHAPTER 6

GENERAL DISCUSSION AND FUTURE WORK

6.1 General discussion

Currently, the research on derivatives produced by plants has being gaining interest in the food industry due to the increasing demand by consumers for natural derivatives produced by plants instead of artificial additives that are considered unsafe and have adverse effects on the health of consumers (Essawi and Srour, 2000). These active constituents derived from plants have a long history of use in traditional medicine for the prevention and treating of illness and infections and immensely acceptable by individuals in order to understanding that they are secure (Guarrera, 2005; Kurek *et al*., 2010). There are two interesting points to mention here. First – as mentioned in the introduction – plant products are also sources of many toxic compounds and medicinal herbs, including liquorice, are known to have adverse effects when consumed above a safe threshold. Therefore it is somewhat counter intuitive that consumers would see these as safer alternatives to well characterised and purified preservatives. However may be the fact that these new ingredients have less of an impact on the organoleptic properties of the food that make then attractive to the consumer. The second point to mention is that many of the common food preservatives include organic acids, such as acetic and propionic acids. These weak acids are naturally formed by many fermentation processes and therefore would be present in fermented foods, which remain acceptable to the consumer on the grounds that they are traditional. Hence this extensive attention in these plant derived compounds as replacements for microbial control is being driven in part as a marketing tool to allow food producers to make their products more attractive rather than because the existing antimicrobials are not performing well. In a recent review of "European consumer healthiness evaluation of 'Free-from' labelled food" (Hartmann *et al*., 2018); a major finding of the research was that consumers who were interested in 'free-from' labelled foods had a preference for naturalness as a feature of the product and were prepared to pay a price premium for products labelled as 'free-from'. Hence developing these products also makes good

economic sense for food producers. Nonetheless by developing these novel products the food industry also aims to protect producers protect human health through minimize the usage of chemical preservatives and also produce food with less acidic, with a lower salt content without compromising product quality in line with consumer demands (Naidu, 2000).

However, this work is not entirely driven by marketing since there is a real need for novel antimicrobials. There have been serious attempts by academia, industry and government agencies to control *Listeria* in foods and in food processing facilities which have so far not managed to find an answer to the problem posed to the food industry by this organism. Therefore new and improved methods to prevent the survival and growth of *Listeria* are still required. Different preservation techniques have been used in many studies in order to try and control of *Listeria* in foods, with a focus on achieving food safety without an effect on the sensory and nutritional qualities of foods (Gandhi and Chikindas, 2007). In line with this, the aim of this study was to evaluate the antimicrobial potential of liquorice extract that was provided as waste material from the production of liquorice flavourings for the food industry, with a specific idea of demonstrating that it was able to control the growth of this important food borne pathogen. The presented results in this study confirmed the fact that this extract did have significant antibacterial activity, specifically targeting Gram-positive bacteria and therefore is a good candidate to be tested as a strong antimicrobial agent to control both pathogens and Gram-positive spoilage organisms.

6.2 Inhibition of Gram-positive bacteria by liquorice extract

This study was first conducted *in vitro* to determine the antibacterial activity of liquorice extract of *Glycyrrhiza glabra* and confirm the promising role of a natural plant source as an antimicrobial that may help to reduce the current

problem of the resistance of many pathogens to chemicals traditionally used to preserve foods. To determine whether the extract had antimicrobial activity, a broad range of organisms consisting of Gram-positive and Gram-negative bacteria were selected to test the ability of liquorice extract to inhibit their growth. It was found that the extract only affected the growth of Gram-positive bacteria and at these concentrations all the Gram-negative bacteria tested showed normal levels of growth. Several previous studies have also found that the most bioactive compounds extracted from the plants were more active against Gram-positive bacteria than Gram-negative bacteria (Karahan *et al*., 2016). In this study growth inhibition was noted for a range of different types of Gram-positive bacteria, including Gram-positive rod-shaped bacteria (*L. monocytogene*s; non-spore former and *B. subtilis*; spore-former) and Gram-positive cocci (*S. aureus* and *E. faecalis*), which might suggest that the extract exerted a similar mode of action on all of the bacteria tested. However the fact that the growth of the acid fast *M. smegmatis* was also inhibited was unexpected and suggests that while most types of Gram-positive bacteria were sensitive to this extract, it is unlikely that they all possess identical target sites that are affected by the same compound. This is also supported by the fact that as multiple effects were seen on the treated cells (membrane integrity and cell division). Perhaps the fact that the extract contains a mixture of bioactive compounds better explains the broad spectrum effects seen against Gram-positive bacteria and explains why some of the more purified compounds described in the literature did not have such a broad spectrum of activity.

Initial antimicrobial studies used different concentrations a range between 10-50 μg ml^{-1} to determine the antimicrobial activity of extract. An important point to note is that using optical density measurements alone, it was not possible to determine if the extract was having a bactericidal or bacteriostatic effect on the cells when growth was inhibited. Hence experiments were carried out using viable count to determine whether cell growth is just inhibited or whether the cells are

killed, and it was clear that the growth inhibition of the Gram-positive bacteria was reversible indicating that the extract was bacteriostatic. This suggests that the compound is not irreversibly binding to a target, and that the cell is possibly able to repair sub-lethal injury that is occurring, but the processes required to carry out this repair results in inhibition of bacterial growth. This idea is supported by the fact that it was found that a concentration of 50 μg ml^{-1} of liquorice extract was sufficient to inhibit the growth of Gram-positive bacteria, whereas at a lower concentration (12.5 μg ml^{-1}) the growth of some of the bacteria tested was reduced but growth was still detected, and in some cases this was very close to the levels seen in the control sample indicating that this concentration was close to the break point (NOEL; No Observable Effect Level (Esdaile, 1995) for the extract at which no observable effect is seen. The pharmaceutical industry is very familiar with the concept of NOEL, in that it is accepted that cells or organisms treated with a drug will tolerate or overcome any adverse effects at concentrations below this threshold. In general food companies need to use preservatives at well above this level due to the heterogeneous nature of food products which can interact with the antimicrobials and affect their activity (Rai and Bai, 2014). Therefore these results suggest that 50 μg ml^{-1} is the minimum concentration that should be used as the in food application level.

It is interesting to note that other studies have identified specific extracts prepared from liquorice that produced a similar pattern of results to those seen in this study. Fukai *et al*. (2002b) investigated the antimicrobial activity of nineteen flavonoids commonly found in three species of liquorice namely, *G. glabra, G. inflata and G. uralensis* and found that they were active against the three Gram-positive bacteria tested (*B. subtilis, S. aureus* and *M. luteus*) with MIC values in a similar range to the unpurified extract used in this study (MIC = 1.56–25 μg ml^{-1}), but had not effect on the Gram negative bacteria tested (*E. coli*, *K. pneumonia* and *P. aeruginosa*). Similarly Kim *et al*. (2002) reported that the MIC of

glycyrrhizin, α-glycyrrhetinic acid and β-glycyrrhetinic acid isolated from *Glycyrrhiza glabra* was in the range of between 7.6 to 12.5 μg ml^{-1} for Gram-positive bacteria such as *B. subtilis* and *S. epidermidis* but had no activity against *E. coli* and fungi. This group also used confocal microscopy to show that β-glycyrrhetinic acid penetrated into the bacteria but did not cause membrane disruption suggesting that the mechanisms of action these compounds was not through disruption of the membrane potential or loss of the semi-permeable nature of the membrane itself. Since these different purified extracts had MIC values very close to those recorded in this study it suggests that the waste material used here contains a significant level of bioactive compounds, and the fact that multiple effects were seen on the cells suggests that there are different bioactive molecules which target different aspects of the Gram-positive bacterial cells. This makes the extract quite attractive for the food industry as it is a waste product that therefore could now be used as a food preservative and if it targets multiple sites in the bacterial cells they are less likely to be able to overcome inhibition of growth, eventually leading to cell death as occurs when Hurdle Technology is applied to food preservation. In fact there was evidence of this seen when the extract was applied to bologna style sausage, which created more stress conditions for the cells and the population of cells declined following a long period of exposure to the extract.

In contrast to the results gained using Gram-positive bacteria, no effect was detected on any Gram-negative bacteria tested. One suggestion is that the outer membrane of the Gram-negative bacteria helps prevent the passage of hydrophobic compounds into the cells (Zhang *et al*., 2013). However the fact that other bioactive compounds prepared from liquorice can affect Gram-negative bacteria, like *H. pylori* as discussed in Chapter 1, suggests that this is not a full explanation. The fact that the highly hydrophobic *M. smegmatis* was also inhibited also argues against a model where the molecules are simply excluded from Gram-

negative cells because they have a hydrophobic surface layer. However cell structure and composition does play an important role in the sensitivity to antimicrobial agents (Hayrapetyan *et al*., 2012). The outer membrane is impermeable and does not allow the passage of large molecules but does allow limited diffusion of hydrophobic substances through its lipopolysaccharide covered surface (Zhang *et al*., 2013). Whereas the cytoplasmic membrane acts to protect the internal contents of cell from leaking out by controlling the exit and entry of the substances into and out of the cell, the presence of the outer membrane in Gram-negative bacteria also provides an extra layer (the periplasmic space) which contains enzymes (including antibiotic degrading enzymes) and efflux pumps which contribute to the resistance of microorganisms to antibiotics by rapidly removing them from the cell (Levy, 2002; Poole, 2004) and to help the organism protect itself against antimicrobial challenge without affecting the exchange of material required for sustaining life (Delcour, 2009). As evidence was seen when very high levels of *Listeria* were added to the bologna style sausage that the bioactive molecules in the extract could be metabolized leading to outgrowth of the cells after prolonged incubation, perhaps the location of enzymes in the periplasmic space that can degrade the inhibitory compounds before they reach the target site might also help explain the difference in sensitivity seen.

However, although the basis of this result is not known, the finding is in line with the results of many other studies reported in the literature which showed that generally unpurified liquorice extracts affect Gram-positive bacteria more significantly than Gram-negative bacteria (Lin *et al*., 1999; Parekh *et al*., 2006; Parekh and Chanda, 2007). In general, plants have been shown to have much greater inhibition impact against Gram-positive than Gram-negative bacteria. The activity may be signal of the existence of broad-spectrum antimicrobial agents or metabolic toxins cause an inhibitory effect on both Gram-positive and Gram-negative bacteria (Srinivasan *et al*., 2001). Over the last decades, bacterial

infectious disease becomes a significant problem and spread considerably among patients worldwide due to the high increase in antibiotic resistance (Dagan, 2003). Despite great progress in other areas of human medicine, infectious diseases caused by pathogenic bacteria are still a major threat to public health, in particular in developing countries due to the shortage of medicines and the emergence of widespread drug resistance (Okeke *et al*., 2005; Sosa *et al*., 2010). The emergence of antibiotic-resistant microorganisms has been attributed to the overuse or inappropriate use of antibiotics leading to serious problems in the treatment of infectious diseases (Nimmo *et al*., 2003). So increasingly research is being carried out to identify new antimicrobial substances derived from natural products which may play an important role in healthcare in the future (Shakya, 2016). Hence the fact that the extract had a broad spectrum of activity and is a food grade material could be of great significance. One suggestion is that it could be used to inhibit the growth of *Clostridium difficile* in the human gut in patients suffering with hospital acquired infections. This difficult to treat Gram-positive bacterium causes a major problem, particularly in patients recovering from extensive antibiotic therapy. A formulation of this extract given as a drink could help target these bacteria and allow normal gut flora to re-establish without the need for further extensive antibiotic therapy, and an extract that did not affect the Gram-negative bacteria in the gut may produce less problems of dysbiosis commonly associated with aggressive antibiotic treatments (Francino, 2016).

6.3 Evaluation of safety of liquorice extract as a food preservatives

The belief that natural products and herbs are safe and have no side effects and the possibility of using them in the long term is a misguided belief. This assumption is due to the lack of sufficient data on these herbs which confirm prove to be potentially hazardous, and therefore requires further studies to investigate their side effects and toxicity (Alves *et al*., 2009). Thus, when plant compounds

are used for long term treatment, it is important to evaluate the toxic, cytotoxic and genotoxic damage caused by these plant compounds in order to reduce the possible risks of these agents (Rodeiro *et al*., 2006). Although natural products will continue to be extremely important as sources of medicinal agents, the literature also describes many plants containing mutagenic compounds, such as furocoumarins, tannins, anthraquinones, alkaloids, and flavonoids (Rietjens *et al*., 2005; Nesslany *et al*., 2009; da Rosa Guterres *et al.,* 2013; Mininel *et al*., 2014) and therefore caution is required before these are introduced into human foods at significant levels. This evidence draws attention to the importance of studying the genetic risks of plant compounds, since the presence of mutagens in medicines can be dangerous to human health. In this study, Ames test and MTT assay were used to confirm if there are any side effects or mutagenicity for liquorice extract and the result obtained confirmed that there is no effect of liquorice on the cell line and there is no mutagenicity, so, this extract may play an important role toward the basic health requirements and may offer a new drugs use as antimicrobial and anticancer agents with significant activity against Gram-positive bacteria.

Although many of active constituents derived from plants may be safe and could be used as herbal therapies, other herbal products may be unsafe to use as the can be toxic to humans in high doses. This study investigated of the potential cytotoxicity of liquorice extract against Human Caucasian colon adenocarcinoma (Caco-2) cells using the MTT assay and the cytotoxicity activity of this extract was found to vary depending on concentration of liquorice as already found when determining its antimicrobial activity. Cytotoxicity was observed on Caco-2 cells treated with extract at a concentration of 50 µg ml^{-1} after 24 h incubation. However in this study a dose of 12.5 µg ml^{-1} showed no effect on the Caco-2 cells. Encouragingly Rao *et al*. (2014) found that liquorice (*Glycyrrhiza glabra*) extract had no effect on healthy cells when cells were exposed to different concentrations of the extract (50, 100 and 150 µg ml^{-1}) but did see a toxicity against different

types of cancer cells. Therefore it will be important to evaluate the cytotoxicity of the extract used in this study against non-cancer cell lines, since a concentration of 12.5 µg ml^{-1} seems to be close to the NOEL it would be difficult to use this effectively as an antimicrobial if a general effect on human cells was detected. Several herbs have been described as unsafe by The Food and Drug Administration, even in small doses, and for this reason they should not be utilized in either foods or beverages (Ekor, 2014). Thus, further research is required to investigate and understand the compounds responsible for cytotoxicity activity seen and their potential mechanisms.

However Glycyrrhizin and other liquorice compounds have been shown to have anti-carcinogenic properties and our results using a cancer cell line are in accordance with those reported by Badr *et al*. (2013) where the extract of liquorice has shown cytotoxic activities against intestinal carcinoma cell line (Caco-2) and prostate carcinoma cell line (PC-3) with different concentration of liquorice extract (0, 12.5, 25, 50 and 100 µg ml^{-1}). In *in vitro* the anticancer activity was also observed against cancer cell lines, including HT-29 and MCF-7 cells treated with extract of *G. glabra* at different concentration (20, 200, and 2000 µg ml^{-1}), after 24, 48 and 72 h incubation with a significant reduction observed in the viability of the treated cells (Nazmi *et al*., 2018). Similar to the previous study, (Khazraei-Moradian *et al*. (2017) reported that treating CT25, HT29, and Hek293 cells with different concentrations of liquorice extract (0, 20, 50, 100, and 200 µg ml^{-1}) for 24 h showed decreases in the viability of cancer cell line significantly more than non-cancerous cell line and once the dose is increased, cell viability was reduced. In other similar studies it has been reported that liquorice and its main constituents decrease cell viability in different types of cancer cells such as gastrointestinal, leukemia, breast and prostate cancer cell lines (Hibasami *et al.,* 2006; Lee *et al*., 2013; Shen *et al*., 2015). Although the exact mechanisms are still under investigation research has confirmed they inhibit tumor formation and growth of breast, skin and liver cancers (Vibha *et al*., 2009).

6.4 Conclusion

In conclusion, our study has demonstrated that liquorice extract exhibits antibacterial activity against all Gram-positive bacteria tested by using several techniques. A range of methods was used to investigate the mechanisms of action where Gram-stain was used to provide evidence that the extract affects the bacterial cell wall, shape change indicates cell division affected, bioluminescence showed that levels of metabolism were lower due to membrane damage. So, this work has found that a waste product from the food industry has potential as a novel antimicrobial product which could be used as a food additive to control bacteria either in food products.

Although liquorice extract at a concentration of 50 μg ml^{-1} displayed *in vitro* cytotoxicity it was not mutagenic during Ames test. Hence, this plant species can be an important source of biologically active compounds and aid in the search for new effective and safe agents that act against spoilage bacteria and as food preservatives.

The antibacterial mechanism of liquorice extract in Gram-positive bacteria was investigated by examining morphology changed, membrane integrity, evaluating on bologna sausage and MTT assay. Based on the data obtained, it could be concluded that the antimicrobial effect of the extract against bacteria is attributed to its action of liquorice extract to Gram-positive bacteria cell membrane, which consequently increases bacteria cell membrane permeability and destroys the membrane integrity.

The results obtained in this thesis confirm the hypothesis that the herbal plants which traditionally are used in various forms in folk medicine since ancient times, due to the presence of antimicrobial compounds could be a potential source of antimicrobial compounds as well as a natural agents for food preservation.

6.5 Future study

This work has found that a waste product from the food industry has potential as a novel antimicrobial product which could be used as a food additive to control bacteria either in food products. In order to use this extract as a preservative to avoid contamination by *Listeria*, further studies are needed to determine an inhibitory dose and the qualitative aspects of the final product. These tests provides evidence that liquorice extract may have antimicrobial property at 50 µg ml^{-1} concentration but more research on their mechanism of action are needed to understand the practicality of using them as an antimicrobial agent. In addition studies would be required to establish whether there would be any organoleptic changes to the foods if used at this level, as it has been noted that natural food preservatives can affect the acceptability of the food product to the consumer (Hintz *et al*., 2015). In addition it would be necessary to see how different food products interacted with the product to determine what type of foods it could be successfully applied to. Non-fermented meat products such as bologna style sausage, typically have high fat and protein content and have a pH between 6-7. In particular it would be interesting to see whether a change in pH of the product affected the ability of the extract to inhibit the growth of *Listeria* since many commonly used antimicrobials are only active in low pH foods (Boziaris and Nychas, 2006). In addition to its potential as a food preservation, an other area that could be investigated would be antioxidant activity which can also help to keep food in a palatable state. Sultana *et al*. (2010) and Li *et al*. (2016) have reported such studies when looking at the identification of the phytochemical properties of liquorice, and identifying how these could be applied to foods. Hence another area of study could be investigating these properties of the liquorice extract to prevent chemical food spoilage.

Currently in the UK, a "Novel Food" is defined as food that had not been consumed to a significant degree by humans in the EU before 15 May 1997, (Regulation (EU) 2015/2283). Before such foods can be marketed, pre-market

safety assessments are evaluated by the regulatory authorities, which in the UK is the Advisory Committee on Novel Foods and Processes (ACNFP) before a decision is made on EU-wide authorisation. To do this the producer must prepare a dossier on the new novel food, providing the information needed so the European Food Standards Agency can carry out a safety assessment. In this study evaluating the safety and toxicity of the extract was carried out *in vitro* using cultured human cell lines and also using a bacterial mutagenicity test (Ames test). If the extract is to be used for food preservation or medicinal purposes, it is essential that more safety studies are carried out *in vivo* using animal models to identify any toxicological effects of the liquorice extract as these would be needed before the extract could be used in any commercial food products.

Several reviews have reported that the efficacy of conventional antimicrobials can be improved by use in combination with plant extracts and there are some reports in the literature about the effect of liquorice plant extracts being used in combination with other plant extracts and showing that their action may be improved by synergistic interactions with other compounds. Haroun and Al-Kayali (2016) have been looking at how antibiotics interact with other natural plant extracts, however no published studies have investigated the effect of combination of liquorice extract with antibiotics against growth of bacteria. Since the results in this study have shown that this extract inhibits Gram-positive bacterial growth at concentrations similar to the MIC of antibiotics, it would be interesting to see if an additive effect can be seen if using combinations of antibiotics and extract. Although there have been a number of papers that have focused on activity of antimicrobial studies of liquorice plant against Gram-positive and Gram-negative bacteria (Gupta *et al*., 2013; Soulef *et al*., 2014), this has been noted in other studies where the components isolated from liquorice plant were found to have good activities against *Mycobacterium tuberculosis* (Gupta *et al*., 2008), however, very little research has been conducted on the effect of extract on Mycobacteria. This will be interesting to follow in terms of understanding the mode of action as

Mycobacteria have a very different cell wall structure to the other Gram-positive bacteria. Understanding what the active site is – or determining whether it is the same as that in Gram-positive bacteria - would be an interesting area of research to take forward.

In addition to the food applications, there are many examples in the literature where these types of extracts from plants have been shown to have potential anti-cancer activities. Therefore another area of study on the liquorice extract could be as a good source of alternative medicines for treating cancer. If work is performed to determine the active compounds in the liquorice extract, it may be possible to predict whether such activity is likely, based on the structure of active molecules that have previously been studies, and therefore, there is the possibility of using this material as a source of the active compounds that confer anticancer activities on liquorice extract. In addition, the long history of use of liquorice plant as a medicinal means there are also reports that suggest it contains many active constituents that have been reported to have antispasmodic, antitussive, anti-ulcer and anti-inflammatory effects. For instance a study conducted by Tanemoto *et al*. (2015) reported that active constituents isolated from *G. uralensis* and examined its anti-inflammatory effects. However, reports comparing the anti-inflammatory effects of these constituents are very few. Thus, isolation and purification of components to determine the active compounds in liquorice extract could be another interesting study to carry out.

Hence there are many possible applications of this extract that could be studied in the future, and the results presented in this thesis now provide a basis for future research that may reveal its potential uses in a number of different areas.

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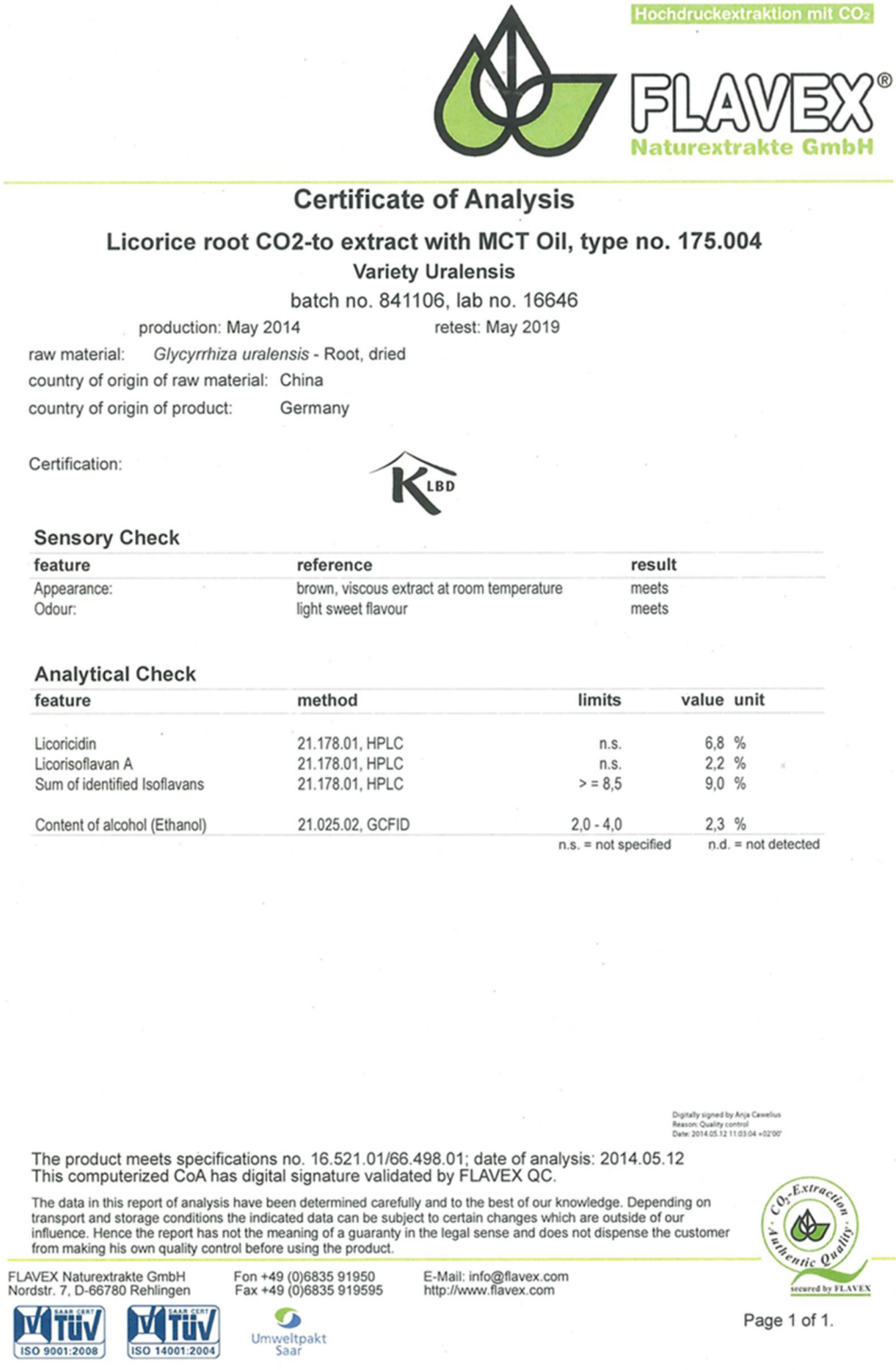
APPENDICES

APPENDIX 1: Certificate of Analysis of the liquorice extract

1.1 Analysis of the liquorice extract

According to the analysis provided by the company who produced the material (Phytoquest Ltd), the active constituent in the waste material was generated from *Glycyrrhiza uralensis* Root during the production of liquorice extracts for food production contains Licoricidin and Licorisoflavan A (see Figure 1.1 and 1.2). While this had been evaluated as a potential antioxidant material, it had not been assessed as an antimicrobial.

Figure 1.1 Certificate of Analysis of the liquorice extract



Hochdruckextraktion mit CO_2

FLAVEX®
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Certificate of Analysis

Licorice root CO2-to extract with MCT Oil, type no. 175.004

Variety Uralensis

batch no. 841106, lab no. 16646

production: May 2014 retest: May 2019

raw material: *Glycyrrhiza uralensis* - Root, dried

country of origin of raw material: China

country of origin of product: Germany

Certification: KLBD

Sensory Check

feature	reference	result
Appearance:	brown, viscous extract at room temperature	meets
Odour:	light sweet flavour	meets

Analytical Check

feature	method	limits	value	unit
Licoricidin	21.178.01, HPLC	n.s.	6,8	%
Licorisoflavan A	21.178.01, HPLC	n.s.	2,2	%
Sum of identified Isoflavans	21.178.01, HPLC	> = 8,5	9,0	%
Content of alcohol (Ethanol)	21.025.02, GCFID	2,0 - 4,0	2,3	%

n.s. = not specified n.d. = not detected

Digitally signed by Anja Cawelius
Reason: Quality control
Date: 2014.05.12 11:03:04 +02'00'

The product meets specifications no. 16.521.01/66.498.01; date of analysis: 2014.05.12
This computerized CoA has digital signature validated by FLAVEX QC.

The data in this report of analysis have been determined carefully and to the best of our knowledge. Depending on transport and storage conditions the indicated data can be subject to certain changes which are outside of our influence. Hence the report has not the meaning of a guaranty in the legal sense and does not dispense the customer from making his own quality control before using the product.

CO_2-Extraction Authentic Quality secured by FLAVEX

FLAVEX Naturextrakte GmbH
Nordstr. 7, D-66780 Rehlingen
Fon +49 (0)6835 91950
Fax +49 (0)6835 919595
E-Mail: info@flavex.com
http://www.flavex.com

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Page 1 of 1.

Figure 1.2 Certificate of Analysis of the liquorice extract

Licorice root CO2-to extract with MCT Oil
Variety Uralensis, Type no. 175.004

Raw material:	*Glycyrrhiza uralensis* - Root, dried
Production:	By supercritical fluid extraction with natural carbon dioxide and a small amount of ethanol as entrainer, no inorganic salts, no heavy metals, no reproducible microorganisms [1]. The extract is standardised with MCT oil to a content of NLT 8,5 % isoflavanes.
Description:	Contains all CO2-soluble lipohilic and semipolar components. Brown, viscous extract at room temperature with light sweet flavour
Declaration:	NCI-Name (CTFA): Glycyrrhiza Uralensis (Licorice) Root Extract, CAS-No. 94349-91-4, EINECS-No. 305-209-3 and Caprylic/Capric Triglyceride, CAS-No. 73398-61-5, EINECS-No. 277-452-2
Halal - Status:	not certified
Kosher - Status:	Certified by London Beth Din Kashrut Division (KLBD). The certification is marked with the Kosher logo on the product.
Transport:	No dangerous good in the sense of the transport regulations.
Ingredients:	The extract contains NLT 8,5 % isoflavanes , e.g. licoricidin and licorisoflavan A and a small amount of essential oil, but no glycyrrhizic acid, 2 - 4 % ethanol.
Application:	Due to the ingredients the extract has antimicrobial, antioxidant and anti-inflammatory properties. It is used in cosmetics and supplements.
Naturalness:	The product is manufactured from the named raw material. It contains apart from the MCT oil no additives and no other technical adjuncts. The product is 100 % natural and corresponds to the EC Flavouring Regulation No. 1334/2008 for flavouring preparations.
Stability:	Unopened container under cool and dry storage conditions and exclusion of light at least 5 years.

References: [1] Manninen P., Haivala E., Sarimo S., Kallio H. : Z. Lebens Unters Forsch A (1997) 204: 202-205

FLAVEX Naturextrakte

FLAVEX Naturextrakte GmbH Nordstraße 7 D-66780 Rehlingen
Tel. +49 - (0) 68 35 - 91 95-0 Fax +49 - (0) 68 35 - 91 95-95
Internet www.flavex.com E-Mail info@flavex.com

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APPENDIX 2: PREPARATION OF MEDIA

2.1 Media preparation

The suppliers for all chemicals substances and media used in this thesis are listed in Table 2.1. All media used in this thesis were prepared using reverse osmosis (RO) water followed by sterilization by autoclaving at 121 °C for 15 min or, if the components were heat labile, were filter sterilised using a 0.22 or 0.45 µm filter prior to use. The supplements and selective antibiotics were added to the molten agar after sterilizing, once it had cooled to approximately 50 °C. The media was poured into sterile Petri dishes (Sarstedt, Leicester, UK) in a laminar flow cabinet. Liquid media was generally stored at room temperature (unless specified) and prepared agar plates was stored at 4 °C.

2.1.1 PALCAM agar media

A vial of PALCAM Supplement (VWR, B-3001, Belgium) 5 ml was brought to room temperature prior to using and 5 ml of sterile RO water was added to it and mixed well. PALCAM agar Base (CM0877, Oxoid) was prepared by adding 34.5 g into 500 ml of RO water and sterilized by autoclaving then was cooled to 45 to 50 ºC. Aseptically, the 5 ml of PALCAM Supplement was transferred into the medium then mixed well before dispensing into sterile Petri dishes.

2.1.2 *Listeria* Selective agar Base

27.75 g of Listeria Selective agar Base (CM0856, Oxoid) was suspended into 500 ml of RO water. This was sterilised by autoclaving then cooled to 50 °C before adding the contents of one vial of Listeria Selective Supplement (SR0140, Oxoid) dissolved in 5 ml of 70 % (v/v) ethanol then mixed well and poured into sterile Petri dishes.

2.1.3 Yeast Extract -Peptone Dextrose broth medium (YPD)

YPD powder (50 g) was suspend in 1 l of distilled water and autoclaved for 15 min at 121 ºC. YPD broth was stored at room temperature for a several weeks.

2.1.4 Dulbecco's Modified Eagle medium (DMEM)

Dulbecco's Modified Eagle Medium (DMEM, 500 ml) high glucose was supplemented with 50 ml of foetal bovine serum (FBS), 5 ml of L-Glutamine solution and 10 ml of MEM Non-essential Amino Acid Solution (100x). Then 10 ml aliquots of the media were dispensed in tissue culture flasks and incubated at 37 °C in a humidified incubator gassed with CO_2 5 % (v/v) for 48 h to ensure no contamination of the media had occurred during preparation. DMEM was stored at 4 ºC.

2.1.5 Mueller Hinton agar

15.2 g Mueller Hinton agar (CM0337, Oxoid) was dissolved in 400 ml of RO water, sterilized by autoclaving at 121 °C for 15 min cooled the agar and poured into Petri dishes which were stored at 4 ºC.

2.1.6 Nutrient broth No. 2

Nutrient Broth No.2 (CM0067, Oxoid) was prepared by adding 5 g of Nutrient broth No. 2 to 200 ml of RO water. After autoclaving the broth was stored at room temperature for several weeks.

2.1.7 Brain Heart Infusion (BHI) broth

BHI broth (CM1135, Oxoid) was prepared by dissolving 7.4 g of BHI broth in 200 ml of RO water and autoclaved for 15 min at 121 °C. After autoclaving, BHI broth was stored at room temperature for a several weeks.

2.1.8 Brain Heart Infusion (BHI) agar

BHI agar (Lp00II, Oxoid) was prepared by adding 14.8 g of BHI broth and 6 g of agar to 400 ml of RO water and autoclaved at 121 °C for 15 min. After cooling to 45 °C the media was poured into sterile Petri dishes. For use with the Miles & Misra technique (Section 2.12) the agar plates were left in a laminar flow cabinet at room temperature overnight to ensure the plate surface was completely dry before use.

2.1.9 Nutrient broth

Nutrient broth (CM0001, Oxoid) was prepared by dissolving 2.6 g of nutrient broth powder in 200 ml of RO water and autoclaved at 121 °C for 15 min and then was stored at room temperature.

2.1.10 Nutrient agar

Nutrient agar (CM0003, Oxoid) was prepared by dissolved 11.8 g of nutrient agar powder in 400 ml of RO water. The medium was autoclave-sterilized at 121 °C for 15 min and the cooled agar was poured into Petri dishes which were stored at 4 ºC.

2.1.11 Top agar supplemented with limited histidine and biotin

Top agar was prepared by adding 3 g agar and 3 g sodium chloride to a flask containing 450 ml of RO water and dissolved by heating on a magnetic stirrer in order to melt the agar. Then, 50 ml of limited histidine and biotin solution (0.5 mM) were added to the molten agar and mixed. The mixture was dispensed into 50 ml in 100 ml Duran bottle then autoclaved at 121 °C for 20 min.

2.1.12 Minimum Glucose (MG) agar plates

MG agar media was prepared by adding of 7.5 g of agar to 465 ml of RO water in 500 ml Duran bottle. The mixture was autoclaved for 20 min at 121 °C. Once the agar had been cooled to approximately 55 °C, 10 ml of VB salt solution (50x) and 25 ml of 40 % (w/v) glucose solution were added aseptically then mixed thoroughly then ampicillin or ampicillin and tetracycline were added to the medium as required per each strain, the amount was added as indicated in (Table 2.4 and 2.5). Thorough mixing was applied after the addition of each of these ingredients to the sterilized agar. The agar medium was distributed into a petri dishes. The plates were stored at 4 °C after the agar was solidified for several weeks.

APPENDIX 3: PREPARATION OF CHEMICAL SOLUTIONS

3.1 Antibiotic solution preparation

Selected antibiotics were prepared in appropriate solvent or water according to manufacturer's instructions. The appropriate amount was added to the media to give final concentrations recommended for each strain to the molten agar media just before pouring of medium into Petri dish. Stock concentrations for antibiotics used in this thesis are listed in (Table 3.1) and antibiotic required for *Salmonella* and *Listeria* strains are listed in (Table 3.2).

3.1.1 Erythromycin 5 µg ml^{-1}

Stock solution of erythromycin 5 µg ml^{-1} was prepared by dissolving 50 mg of erythromycin in 10 ml of 70 % (v/v) ethanol and stored at -20 ºC.

3.1.2 Ampicillin solution (0.8 %, W/V)

Ampicillin sodium salt solution was prepared by dissolving 200 mg of ampicillin in 25 ml of warm RO water. Once the ampicillin was dissolved the solution was sterilized using a 0.45 µm membrane filter and stored at -20 °C.

3.1.3 Tetracycline solution (0.8 %, W/V)

Tetracycline solution was prepared by dissolving 200 mg of tetracycline powder in 25 ml of 70 % (v/v) ethanol, once the tetracycline was dissolved the solution was sterilized using a 0.45 µm membrane filter then aliquots of 5 ml were dispensed and stored at 4 °C in the dark to protect against light (tetracycline is light sensitive).

Table 3.1. Antibiotics used in this thesis

Antibiotic	Solvent	Stock concentration	Working Concentration
Erythromycin	70 % Ethanol	5 mg ml^{-1}	5 µg ml^{-1}
Ampicillin sodium salt	Warm RO water	8 mg ml^{-1}	24 µg ml^{-1}
Tetracycline	70 % Ethanol	8 mg ml^{-1}	2 µg ml^{-1}

Table 3.2. Antibiotics required for *Salmonella* and *Listeria* strains

Strains	Strain numbers	Antibiotics Required for plasmid selection
S. Typhimurium	TA98 and TA100	Amp (24 µg ml^{-1})
S. Typhimurium	TA102	Amp (24 µg ml^{-1}) and Tet (2 µg ml^{-1})
L. innocua	ATCC 11994 (Serotype 6a)	Ery (5 µg ml^{-1})
L. monocytogenes	10403S (wild type; 1/2a) EGD (wild type; 1/2a) ATCC 23074 (wild type; 4b) NCTC 10357 (Δ*prfA*) NCTC 7973 (*prfA**)	Ery (5 µg ml^{-1})

*Amp (Ampicillin), *Tet (Tetracycline) and Ery (Erythromycin)

3.2 Mutagenic solutions (positive controls for Ames strains)

3.2.1 Sodium azide

A stock solution of sodium azide (S-2002- Sigma) (12.5 mg ml^{-1}) was made by dissolving 12.5 mg of sodium azide powder in 1 ml of RO water. From the stock solutions, 60 µl was transferred into a 30-ml Universal tube (Sterilin, UK Ltd) containing 5 ml of RO water in order to prepare a working solution of 0.15 mg ml^{-1} concentration. Both solutions were stored in the dark at 4 °C for several weeks.

3.2.2 Mitomycin C

A stock solution of mitomycin C (M-0503- Sigma) (2 mg ml^{-1}) was prepared by transferring 1 ml of RO water into a vial containing 2 mg of mitomycin powder.

A working solution of 0.05 µg ml^{-1} concentration was made by adding 50 µl of mitomycin C stock solution into a bijou bottle containing 2 ml of RO water then mixed well, then stored in the dark at 4 °C.

3.3 Buffers and solutions

3.3.1 Maximum recovery diluent (MRD)

MRD was prepared by suspending 1.9 g of maximum recovery diluent powder (CM0733, Oxoid) in 200 ml RO water and autoclaving for 15 min at 121 °C. After autoclaving, MRD was stored at room temperature for a several weeks.

3.3.2 Phosphate buffered saline (PBS)

PBS was prepared by dissolving one phosphate buffered saline tablet (Oxoid) in 200 ml of RO water. After autoclaving, PBS buffer was stored at room temperature.

3.3.3 Buffered peptone water (BPW)

BPW (F0213W, VWR, Prolabo Chemical, ISO) was prepared by suspending 0.5 g in 500 ml RO water and autoclaved for 15 min at 121 ºC. After autoclaving, BPW was stored at room temperature for a several weeks.

3.4 Other chemical solutions

3.4.1 Sodium chloride 0.85 % (w/v)

0.85 g of NaCl (Fisher Scientific) was added into 100 ml of RO water. Nacl was stored at room temperature for a several weeks.

3.4.2 Vogel-Bonner medium E (50X)

The salts (10 g of magnesium sulphate, 100 g of citric acid monohydrate, 500 g of potassium phosphate, dibasic (anhydrous) and 175 g of sodium ammonium phosphate) were transferred in the order indicated into 670 ml of warm RO water in a 2 l Erlenmeyer flask with magnetic stirrer, every salt was completely dissolved before adding next salt. The volume was adjusted to 1 l, the solution was distributed into 200 ml and autoclaved for 20 min at 121 ºC. The bottles caps were tightened after cooled and stored at room temperature in dark for several weeks.

3.4.3 Glucose solution 40 % (w/v)

Glucose solution (40 %) (Fisher Scientific) was prepared by adding 400 g of D-glucose to 700 ml of RO water in a 2-l Erlenmeyer flask. The glucose was completely dissolved by stirring. The volume was adjusted to 1 l using RO water. Divided into 200 ml then autoclave sterilized at 110 °C for 10 min. The bottles caps were tightened after cooled and stored at 4 °C.

3.4.4 Limited histidine/ biotin solution (0.5mM)

31 mg of D-biotin (Sigma-Aldrich) and 26.3 mg of L- Histidine. HCL (Acros Organics) compounds were added into 200 ml of RO (about boiling) in a 250-ml volumetric flask. Each salt was thoroughly dissolved by stirring on a magnetic stirrer when the mixture became very clear the next salt was added. The volume was adjusted to 250 ml using OR water. The solution was filter-sterilized by using a 0.45 µm membrane filter. The mixture was divided into 20 ml into glass 30 ml universal tubes and stored at 4 °C for several weeks.

2.23.3 Solubilization solution

Solubilization solution was prepared by adding 2 ml of Glacial acetic acid (Fisher Scientific) to 40 ml of RO water in a beaker then 40 ml of Dimethylformamide (DMF) was added. The mixture was mixed before adding 16 g of sodium dodecyl sulfate (SDS) (L4390, Sigma) thorough mixing was applied after the addition of each of these compounds then the solution was incubated at 37 °C to dissolve. The volume was adjusted to 100 ml using RO water.

2.23.4 MTT Assay reagent

A stock solution of MTT (*3*-(*4*,*5*-dimethylthiazol-*2*-yl)-*2*,*5*-diphenyltetrazolium bromide, 5 mg ml^{-1}) (Acros Organics) was made by adding 10 ml of phosphate buffered saline solutions (BPS, Oxoid) into a vial containing 50 mg of MTT powder. MTT solution was stored at 4 ºC in a bottle covered by aluminium foil to protect from light due to the MTT is light-sensitive and MTT stock was stored for up to 18 months.

PUBLICATIONS

Posters

SfAM Early Careers Autumn Meeting; Oct 2014	Inhibition of Gram-positive bacteria by liquorice extracts; identification of a potential new food grade antimicrobial.
Global Food Microbiology Conference; Aug 2016	Identification of the Antimicrobial Effect of Liquorice Extracts on Gram- Positive Bacteria: Determination of Minimum Inhibitory Concentration and Mechanism of Action Using a *luxABCDE* Reporter Strain.
International Association for Food Protection; Mar 2017	Characterisation of the antimicrobial effects of liquorice extract: selective inhibition of a broad range of Gram-positive bacteria.

Paper

El AWAMIE, M. & REES, C. 2016. Identification of the Antimicrobial Effect of Liquorice Extracts on Gram-Positive Bacteria: Determination of Minimum Inhibitory Concentration and Mechanism of Action Using a *Luxabcde* Reporter Strain. *World Academy of Science, Engineering and Technology, International Journal of Medical, Health, Biomedical, Bioengineering and Pharmaceutical Engineering*, 10, 294-302.

Identification of the Antimicrobial Effect of Liquorice Extracts on Gram-Positive Bacteria: Determination of Minimum Inhibitory Concentration and Mechanism of Action Using a *luxABCDE* Reporter Strain.

Madiha El Awamie and Catherine Rees

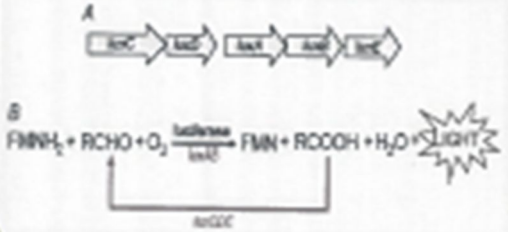
Division of Food Sciences, School of Biosciences, The University of Nottingham, Sutton Bonington Campus, LE12 5RD, United Kingdom
Email: [redacted]

The University of Nottingham
UNITED KINGDOM · CHINA · MALAYSIA

Introduction

The public is demanding foods that are more natural and less processed; this has led to a search for new antimicrobial substances from other sources including plants. Liquorice plant is used as a traditional medicine in different countries around the world to treat many diseases including bacterial infections and also commonly used as flavouring in food products. Studies of antimicrobial activity are normally carried out using challenge tests to determine both the activity range and MIC (minimum inhibitory concentration) of a substance. However it is also important to understand the mechanism of action of preservatives. Bacterial bioluminescence has been used in many studies as an in direct reporter of cellular viability. When transformed with the bacterial lux operon (Fig. 1), bacteria produce light and the level of light produced is representative of the metabolic activity of the cells [1].

Figure 1: Bacterial bioluminescence



In this study we have used both traditional microbiological techniques and also bacterial bioluminescence to investigative the antimicrobial activity of an extract of *Glycyrrhiza glabra* (liquorice) that was provided as food industry waste material as a potential natural preservative [2].

Materials & Methods

- Growth was monitored using optical density (600nm) or by viable count.
- All strains were cultured at 37 °C in BHI broth.
- Microscopic observation were made using a x100 oil immersion jens using light microscopic and images were captured using microscope with camera attached to a computer following Gram staining.
- A Tecan microplate reader was used to simultaneously monitory optical density (600nm) and to measure light output from cultures (relative light units; RLU). For experiments in the Tecan samples of the cultures (200 µl) transferred into a microtitre plate and incubated at 37 °C in a Tecan microplate reader. Cell growth was monitored by using OD_{600nm} with readings taken every 10 min for 24 h. To normalise light measurements against cell number the RLU values were divided by the OD values and the data plotted on a linear scale (Fig. 6).

Results

To initially determine whether the extract had antimicrobial activity, bacteria were grown in the presence of liquorice extract at a range of concentrations and growth monitored over time by optical density. It was found that the extract only affected the growth of Gram positive bacteria (all the Gram-negative bacteria tested showed normal levels of growth; see Fig. 2). For Gram- positive bacteria, the MIC was found to be 50 µg ml^{-1} with sub-lethal inhibition of growth still occurring at a concentration of 12.5 µg ml^{-1} (Fig. 3).

To determine if the effects on Gram-positive bacteria were bacteriocidal or bacteriostatic, viable count was also determined and it was found that for the 50 µg ml^{-1} sample where growth with totally inhibited, the number of viable cells did not decline over time indicating that the effect of the extract was bacteriostatic rather than bacteriocidal (Fig. 4).

In addition changes in cell morphology were detected following Gram-staining of samples (Fig. 5). It was found that samples treated with the liquorice extract were less able to take up the Crystal Violet stain and therefore appeared paler in colour and also the cell shapes were less regular than those seen in the control samples.

Monitoring the bioluminescence levels of the genetically engineered *lux* derivatives of these bacteria, it was clear that even when growth was not affected, a reduction is the light output could be seen. It was also noted that the metabolic level of the strains challenged with 50 µg ml^{-1} were very similar to the background control, suggesting that in addition to effects on cell division, the cell membrane may be affected.

Figure 2: Effect of extract on growth of *Salmonella* Typhimurium

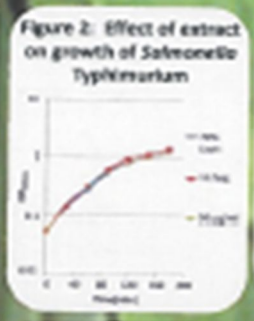


Figure 3: Effect of extract on growth of Gram-positive bacteria

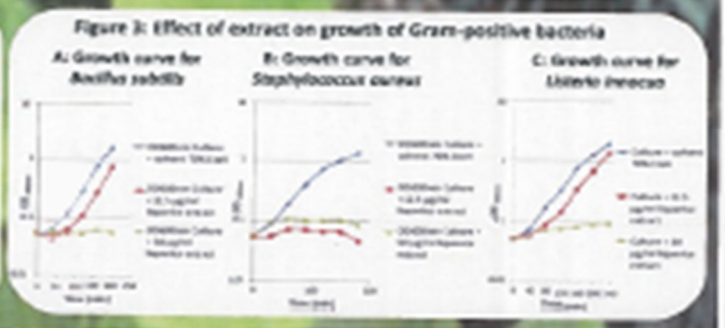


Figure 4: Effect on *Listeria innocua* viable count

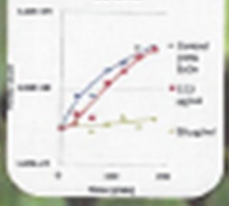
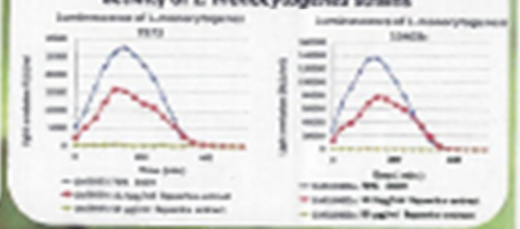


Figure 5: Effect of extract on cell morphology

Figure 6: Effect of liquorice extract on metabolic activity of *L. monocytogenes* strains



Conclusions

- The extract was found to specifically inhibit only Gram-positive bacteria.
- The fact that all the different cell types were affected in the same way indicates a shared mode of action rather than a specific effect on one type of cell.
- Cells were able to grow normally when liquorice removed indicating that the extract was bacteriostatic.
- The extract was active at 50 µg ml^{-1} and this is a level that could be applied in a practical way.
- Microscopic analysis indicated that there were changes in cell shape that suggests an effect on cell wall synthesis.
- Bioluminescence used to quickly evaluate the effect of an antimicrobial on bacterial metabolism.

References

1. HILL, P. J. & Stewart, G. S. 1994. Use of *lux* genes in applied biochemistry. *Journal of Bioluminescence and Chemiluminescence*, 9, 211-215.

2. EL AWAMIE, M. & REES, C. 2016. Identification of the Antimicrobial Effect of Liquorice Extracts on Gram-Positive Bacteria: Determination of Minimum Inhibitory Concentration and Mechanism of Action Using a *Luxabcde* Reporter Strain. *World Academy of Science, Engineering and Technology International Journal of Medical, Health, Biomedical, Bioengineering and Pharmaceutical Engineering*, 10, 294-302.

Characterisation of the Antimicrobial Effects of Liquorice Extract: Selective Inhibition of a Broad Range of Gram-Positive Bacteria

Madiha El Awamie and Catherine Rees

Division of Food Sciences, School of Biosciences, The University of Nottingham, Sutton Bonington Campus, LE12 5RD, United Kingdom

Email: [redacted]

The University of Nottingham

UNITED KINGDOM · CHINA · MALAYSIA

Introduction

Liquorice extract is commonly used as a food flavouring and we have previously shown that a waste material from the production of liquorice extract has antimicrobial activity against the Gram-positive bacterium *Listeria*[1]. We also had evidence that the extract was able to inhibit the growth of other Gram-positive bacteria. Since *Bacillus, Brochthrix* and Lactic Acid Bacteria (all Gram-positive) commonly contribute to the spoilage of cooked meats and *L. monocytogenes* contamination is a particular problem associated with sliced deli meats and therefore this extract could be of use to improve the safety of sliced deli meats. The purpose of this study was to further investigate the mechanism of action of the liquorice extract and investigate whether it has potential as a food preservative for sliced meats.

Results

Bioluminescent *luxABCDE* derivatives of *Listeria* were used to monitor cell viability after treatment with different concentrations of the liquorice extract (Fig. 1). It was clear that even at sub-inhibitory concentrations, when growth was not affected, there was a reduction is the light output indicated that the cells were experiencing sub-lethal injury. The metabolic levels of the strains challenged with 50 µg ml^{-1} were very similar to the background control, suggesting that the integrity of the cell membrane may be affected.

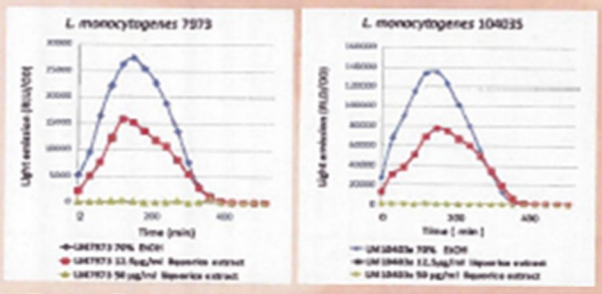


Figure 1: Effect of liquorice extract on metabolic activity of *Listeria*

Metabolic levels were monitored by measuring bioluminescence levels of strains carrying the bacterial *luxABCDE* genes. Light output (RLU) was divided by optical density (600nm) to normalize light levels produced from samples containing different numbers of cells

Evaluating activity on bologna-style sausage

To carry out these experiments *L. monocytogenes* cultures were centrifuged and cells re-suspended in buffered peptone water to a density of 1 x 10^6 CFU ml^{-1}. Bologna-style sausages were sliced (~3 mm) and inoculated by dipping for 30 s in *L. monocytogenes* ± 50 µg ml^{-1} liquorice extract and then allowed to dry for 30 min to remove excess liquid. The inoculated slices were stored at 6°C and samples (n = 3) removed for sampling at day 0 (slicing) and thereafter at day 5, 10 and 15. At sampling, slices were placed in a stomacher bag with 0-1% (w/v) BPW and stomached for 60 s (300 RPM). The level of *Listeria* in the samples was determined by selectively enumeration by plating appropriate dilutions on Palcam Agar to selectively enumerate *Listeria*. The results showed that the extract was able to completely inhibit the growth of *Listeria* throughout the cold storage (Fig. 3) whereas an increase in 1 $\log_{10}$ was seen in the control samples.

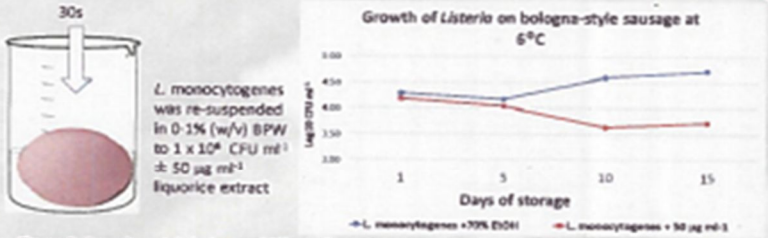


Figure 3: Experimental design and Effect of extract on growth of *Listeria monocytogenes*

Total numbers of bacteria on slices were detected. Control samples contained 1% (v/v) ethanol which was the solvent used for the liquorice extract.

The growth of both *Bacillus subtilis* and *S. aureus* are also inhibited by this compound (Fig. 2). A LIVE/DEAD® stain was used to investigate the effect of the extract using both *L. monocytogenes and Bacillus subtilis*. Cells were exposed to either 12.5 or 50 µg ml^{-1} of the extract and then imaged using fluorescent microscopy (Fig. 3). Cell injury was evident even immediately after treatment (appearance of red cells in 0 time point). An increasing proportion of red-stained cells were detected over time confirming that the membrane was being damaged. For both *Listeria* and *Bacillus* samples treated with the extract, an increase in cell length and the appearance of chains of cells were seen during the 24 h of exposure, suggesting that cell division was also being affected

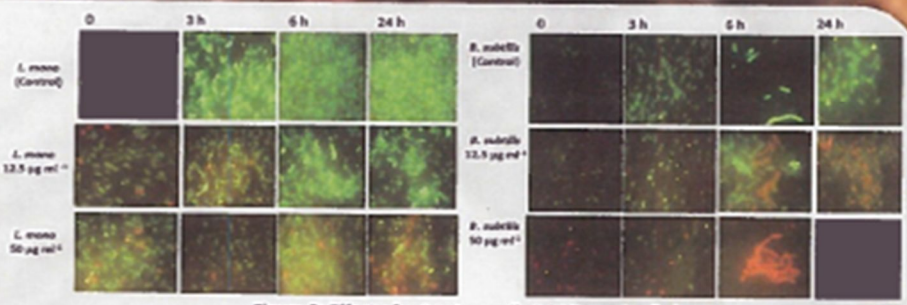


Figure 3: Effect of extract on cell viability and cell shape

Overnight culture of *L. monocytogenes* and *B. subtilis* were prepared with liquorice extract at 12.5 µg ml^{-1} & 50 µg ml^{-1} and a control sample contained 1% (v/v) ethanol (solvent for the extract). Samples were taken at 0 time then incubated at 37 °C with aeration. Samples were taken at 3, 6 and 24 h. Cells were harvested by centrifugation (15 min at 10,000 x g) and then diluted into 0.85 % (w/v) NaCl before mixing with LIVE/DEAD® BacLight™ Bacterial Viability stain. Samples were incubated for 15 min in a dark, then 5 µl of the stained samples examined using a fluorescent microscope.

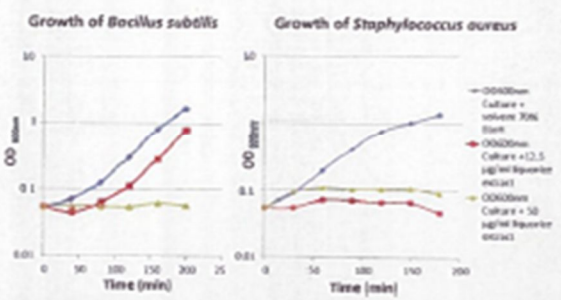


Figure 2: Effect on growth of other Gram-positive bacteria

Conclusions/Significance

- The extract was found to specifically inhibit only Gram-positive bacteria at low concentrations (50 µg ml^{-1}) but effects were reversible and therefore it has a bacteriostatic action.
- All the different Gram-positive cell types tested are affected in the same way, indicating a common mode of action.
- Microscopic analysis indicated that there were effects both on cell membrane integrity and cell division.
- This novel antimicrobial extract can inhibit the growth of *L. monocytogenes* and other Gram-positive spoilage organisms when applied to the surface of sliced deli meats.

Reference

1. El Awamie, M., and Rees C (2016) Identification of the Antimicrobial Effect of Liquorice Extracts on Gram Positive Bacteria : Determination of Minimum Inhibitory Concentration and Mechanism of Action Using a *luxABCDE* Reporter Strain. Int. J. of Medical, Health, Biomedical, Bioengineering and Pharmaceutical Engineering 10 [6] 294-302

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Identification of the Antimicrobial Effect of Liquorice Extracts on Gram-Positive Bacteria: Determination of Minimum Inhibitory Concentration and Mechanism of Action Using a *luxABCDE* Reporter Strain

Madiha El Awamie, Catherine Rees

***Abstract*—**Natural preservatives have been used as alternatives to traditional chemical preservatives; however, a limited number have been commercially developed and many remain to be investigated as sources of safer and effective antimicrobials. In this study, we have been investigating the antimicrobial activity of an extract of *Glycyrrhiza glabra* (liquorice) that was provided as a waste material from the production of liquorice flavourings for the food industry, and to investigate if this retained the expected antimicrobial activity so it could be used as a natural preservative. Antibacterial activity of liquorice extract was screened for evidence of growth inhibition against eight species of Gram-negative and Gram-positive bacteria, including *Listeria monocytogenes*, *Listeria innocua*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus subtilis*. The Gram-negative bacteria tested include *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhimurium* but none of these were affected by the extract. In contrast, for all of the Gram-positive bacteria tested, growth was inhibited as monitored using optical density. However parallel studies using viable count indicated that the cells were not killed meaning that the extract was bacteriostatic rather than bacteriocidal. The Minimum Inhibitory Concentration [MIC] and Minimum Bactericidal Concentration [MBC] of the extract was also determined and a concentration of 50 μg ml^{-1} was found to have a strong bacteriostatic effect on Gram-positive bacteria. Microscopic analysis indicated that there were changes in cell shape suggesting the cell wall was affected. In addition, the use of a reporter strain of *Listeria* transformed with the bioluminescence genes *luxABCDE* indicated that cell energy levels were reduced when treated with either 12.5 or 50 μg ml^{-1} of the extract, with the reduction in light output being proportional to the concentration of the extract used. Together these results suggest that the extract is inhibiting the growth of Gram-positive bacteria only by damaging the cell wall and/or membrane.

***Keywords*—**Antibacterial activity, bioluminescence, *Glycyrrhiza glabra*, natural preservative.

I. Introduction

OVER the last decade, the foodborne pathogens became an increasing concern through food contamination of products, including meat, fresh fruits and vegetables, caused by bacteria such as *Listeria monocytogenes*, *Campylobacter jejuni* and *Escherichia coli* [1]. Health hazards posed by microbial pathogens in food are of major concern to all governments because of circulation of contaminated food between countries increases the chance of outbreaks of foodborne disease. According to International trade statistics (2015) by World Trade Organization (WTO), food represents 42% of total world exports, and levels have increased 6% per year since 2010, and so food is a substantial and increasing area of trade [2]. According to the definition of foodborne illnesses by The World Health Organization (WHO), these are diseases caused by agents that enter the body through the ingestion of food which either infectious or toxic in nature [3]. Approximately 1.8 million deaths were registered in 2005 caused by diarrhoeal disease, and a large proportion of these cases can be attributed to contamination of food and drinking water. However, levels of foodborne disease are generally under-reported and are difficult to measure accurately [4]. Although generally the safety of food has dramatically improved overall, foodborne outbreaks from microbial contamination are still prevalent in many countries, and failure to discover the presence of pathogenic microorganisms especially in the food industry may lead to serious effects on human health [5], [6].

Bacteria such as *Campylobacter*, *Salmonella*, *L. monocytogenes* and *E. coli* are commonly reported to be responsible for foodborne disease and outbreaks [7], [8]. Generally, all these bacterial pathogens are grouped together, but physiologically they are very different, in particular a major structural difference occurring in the different cell types. For instance, *Campylobacter*, *Salmonella* and *E. coli* are all Gram-negative bacteria and have a cells wall structure including two layers of membrane and a thin layer of the structural polysaccharide peptidoglycan (PG). In contrast Gram-positive bacteria, such as *Listeria*, *Staphylococcus*, *Enterococcus* and *Bacillus*, have only one cell membrane encased in a thick layer of PG. Hence when considering the action of new antimicrobial agents, it is important to test them against a range of bacterial cell types.

L. monocytogenes is a foodborne pathogen that is almost exclusively transmitted to human through consumption of contaminated food. Its unusual biology – being able to grow

Madiha El Awamie is with the Microbiology and Food Safety, The University of Nottingham, Nottingham, United Kingdom (e-mail: stxma46@nottingham.ac.uk)

equally well in soil or in the GI tract of animals and within food production facilities – means that it is associated with a broad range of ready-to-eat (RTE) foods, including dairy products, fresh vegetables, processed meats and shellfish. Infection by this bacterium results in the disease listeriosis that can have a number of different serious consequences including meningitis, meningo-encephalitis and septicaemia and is distinguished by a high mortality rate of up to 30%. The severity of the diseases caused by this bacterium means that the economic costs associated with an infection is high. For instance, during an outbreak in Canada in 2008 related to consumption of contaminated delicatessen meat, 57 cases of listeriosis were reported, resulting in 24 deaths. The costs associated with the cases (including medical and nonmedical costs and productivity losses) were estimated to be nearly $242 million Canadian dollars [9]. Infection is generally seen in people with weakened immune systems, including pregnant women, infants, and the elderly and although listeriosis occurs infrequently, it is a major concern for the food industry [10], [11].

There are many challenges exist for the control of *L. monocytogenes* in the food chain, including its ability to survive and grow at low temperatures. Although low temperature is normally used to prevent the growth of bacteria, *L. monocytogenes* has the unusual ability to adapt to low temperature conditions and grow in refrigerated foods. It can also tolerate and grow over a wide range of pH and moisture levels and can form biofilms making it resistant to disinfection. The bacterium can colonize in food production equipment and environments and, consequently, these bacteria have been shown to persist for months to years in food-processing plants, acting as a possible source of cross-contamination of product. Indeed, contamination of food products with *Listeria* has been identified at many different stages of food production including slicing, packaging and chill-storage [12], and therefore many different techniques are employed to inhibit, spread and survival of *L. monocytogenes* in food products. So, the control and elimination of this type of bacteria from food products is the best strategy to ensure food safety [13].

Generally, when testing antimicrobial agents, cells are challenged with the test chemical and the effect measured by the level of growth inhibition observed. Despite the fact that the definition of viability depends on the capacity of bacteria to multiply and divide, growth of bacteria in a liquid medium, or the formation of colonies on solid agar, is not considered sufficient evidence that bacteria are either alive or dead after treatment since antimicrobial agents may simply be bacteriostatic and prevent growth without causing permanent cell damage. To address this, other methods have been developed to report on cellular viability, including enzyme and respiratory activity and measurement of ATP levels. These parameters can also report on sub-lethal damage to cells, factors that affect membrane integrity or require energy for repair will reduce ATP levels but not result in cell death [14], [15].

Bioluminescence is the process by which visible light is emitted by an organism as a result of a chemical reaction catalysed by a luciferase enzyme and this phenomenon is seen in a variety of organisms, such as insects, fish, squid, shrimp, and jellyfish, but is also seen in bacteria. Bacterial luciferase (Lux) is encoded by the *luxAB* genes which catalyse the reduction of a long-chain fatty aldehyde in the presence of molecular oxygen and $FMNH_2$ and results in the emission of light (Fig. 1 B). Cells encoding the full *lux* operon (Fig. 1 A) generate a bioluminescence phenotype without the need to add any additional cofactors or exogenous substrates [15], [16]. Since $FMNH_2$ production is dependent on a functional electron transport chain, only metabolically active bacteria emit light and therefore it can be used to rapidly monitory antimicrobial activity [15]. Although many bacteria of interest are not naturally bioluminescent, the *lux* operon can be introduced into a number of different cells on plasmids as long as the genes are engineered to function in that particular cell type [17] and therefore bioluminescence has been widely used to study antimicrobial agents [15], [18].

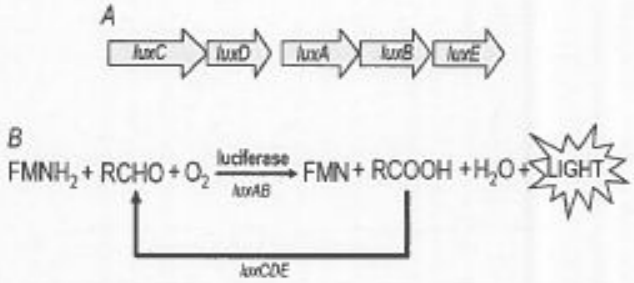


Fig. 1 A) Organisation of the *lux* operon in *Vibrio* and *Photorhabdus* species including the luciferase genes *luxA* and *luxB* and the *luxCDE* genes which encode a fatty acid reductase complex that regenerates the long chain aldehyde substrate (luciferin) B) The chemical reaction giving rise to light production in bioluminescent bacteria in which the aldehyde is converted to the carboxylic acid in the presence of oxygen reduced flavin mononucleotide ($FMNH_2$); The by-products of this reaction are flavin mononucleotide (FMN) and light in the visible spectrum (bioluminescence)

Another commonly used marker gene is the green fluorescent protein (GFP) produced by the jellyfish *Aequorea victoria* which has become a simple and flexible tool used in many applications in field of molecular biology, medicine and cell biology. The protein is small (27-kDa) and produces green fluorescence at 509 nm when excited with UV light at 398 nm [19], [20]. As GFP protein is extremely stable *in vivo*, it has proved to be useful when studying cells exposed to some sort of stress condition, as the fluorescence signal does not decrease allowing cells to be easily visualised, even if non-viable [21]. Again, bacteria do not naturally produce *Gfp* but this can also be introduced into bacteria on plasmids [22] and can also be introduced onto plasmid in conjunction with the *lux* genes so that cells containing these plasmids are both bioluminescent and fluorescent [23].

II. Material and Methods

A. Bacterial Strains, Plasmids, and Growth Conditions

Bacterial strains and plasmids used in this study are listed in Tables I and II, respectively. These strains were transformed with plasmids carrying the bacterial *luxABCDE* and *gfp* genes modified for expression in each of the different bacteria genera used. Strains were recovered Microbank™ beads held at -80 °C by inoculating into Brain Heart Infusion (BHI) broth and then plating onto BHI agar with erythromycin (Erm; 5 μg ml^{-1}) to select for the presence of the plasmids. Plates were incubated for 24 h at 37 °C and stored at 4 °C. For each experiment, an isolated colony was inoculated in BHI broth supplements with Erm and incubated at 37 °C for 24 h.

TABLE I
Bacterial Strains Used in This Study

Bacteria	Strain numbers or source	Gram reaction group
Salmonella enterica serovar Typhimurium	Nottingham lab collection	-ve
Escherichia coli	NCTC 86	-ve
Staphylococcus aureus	Chicken isolate	+ve
Bacillus subtilis	var. Niger 168	+ve
Listeria innocua	Nottingham lab collection	+ve
Listeria monocytogenes	10403S (wild type, 1/2a) EGD (wild type, 1/2a) ATCC 23074(wild type, 4b) NCTC 10357 (*ΔprfA*) NCTC 7973 (*prfA**)	+ve

TABLE II
Bacteria with Plasmid Used in This Study

Bacteria	Promoter & Insert	Vector	Antibiotic selection [μg ml^{-1}]
L. innocua	BS10, *luxABCDE, gfp*	pUNK18	Erm [5]
L. monocytogenes	BS10 *luxABCDE, gfp*	pUNK18	Erm[5]
L. monocytogenes NCTC 10357	*xylA, luxABCDE, gfp*	pUNK18	Erm [5]

B. Liquorice Extract

Liquorice extract (3 mg) was weighed into a sterile bijoux bottle then dissolved in 1 ml of 70% (v/v) by incubating at 37 °C 10 min and was then stored at -20 °C prior to use.

C. Determining Antibacterial Activity of Liquorice Extract

Overnight cultures were prepared and aliquots inoculated into sterile 250 ml Erlenmeyer flasks containing 100 ml of BHI broth. The inoculum of the broth culture was adjusted until the bacterial suspension reached an OD_{600nm} 0.05 which was equivalent to an approximately $1x10^7$ CFU ml^{-1}. Three flasks were prepared for each type of bacteria; the first flask (control) contained 1% (v/v) ethanol, the second flask contained 12.5 μg ml^{-1} of liquorice extract and the third flask contained 50 μg ml^{-1} of liquorice extract. Ethanol was used as the solvent to dissolve the liquorice extract and therefore ethanol alone (which is known to have antimicrobial activity at high concentration) was used as a control. The concentration of ethanol used was 1% (v/v) as this was the maximum level of ethanol present in either of the test samples following addition of the liquorice extract. Samples were incubated at 37 °C for 30 min with aeration (shaking at 150 rpm), and samples collected for OD measurement at 30 min intervals for approximately 3 h. In addition, the viable count of the culture was determined for each of the time points using standard dilution and plating methods.

D. Gram Stain

Cells were imaged using a standard light microscope after Gram-staining using a x100 oil immersion lens. Images were captured using microscope with camera attached to a computer, and also with using electronic microscope connected to a Cannon camera.

E. Growth in Tecan Microplate Reader

Overnight cultures of bacteria were used to inoculate fresh broth to an OD_{600nm} of 0.05. Samples of the cultures (200 μl) were transferred into the wells of a microtitre plate (Krystal Microplate, clear bottom or Porvair 96 well black, Porvair Sciences, England), and samples incubated at 37 °C in a Tecan Genesis Pro microplate reader. Cell growth was monitored by using OD_{600nm} with readings taken every 10 min for 24 h. Fluorescence (RFU; Excitation wavelength = 485 nm, Emission wavelength = 535 nm) and bioluminescence levels (RLU) readings were taken at regular periods over the course of the experiment as required.

III. Results and Discussion

To determine whether the extract had antimicrobial activity, bacteria were grown in the presence of liquorice extract at range of concentrations and it was found that 50 μg ml^{-1} was sufficient to inhibit the growth of bacteria. For all further experiments two different concentrations were used (12.5 μg ml^{-1} and 50 μg ml^{-1}) and growth was monitored over time using optical density. In addition to these two test conditions, a control was prepared containing 1% (v/v) ethanol to rule out effects of the solvent used to prepare the liquorice extract. It was found that the extract only affected the growth of Gram-positive bacteria and at these concentrations all the Gram-negative bacteria tested showed normal levels of growth (see Fig. 2). For the Gram-positive bacteria, at 50 μg ml^{-1} growth was completely inhibited, whereas at 12.5 μg ml^{-1} growth rate was reduced but some growth was still detected (Fig. 2; panels C-E).

To determine if the effect of the liquorice extract on the Gram-positive organisms was bacteriocidal or bacteriostatic, *Listeria innocua* was chosen as a model organism being non-pathogenic and non-sporeforming. The experiment was repeated, this time monitoring both viable count and optical density. When 50 μg ml^{-1} was used, it was found that although growth was totally inhibited, the number of viable cells did not decline over time indicating that the effect of the extract was bacteriostatic rather than bacteriocidal (Fig. 3).

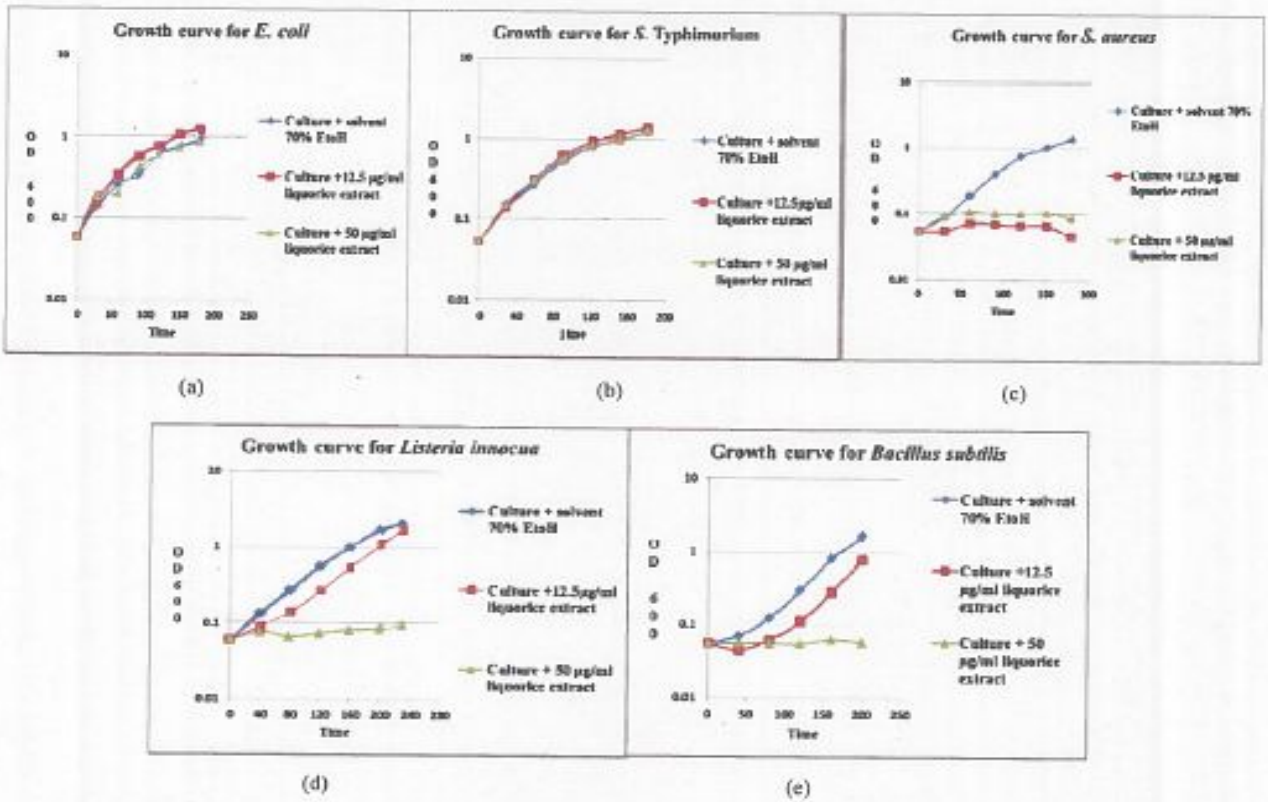


Fig. 2 The effect of liquorice extract on the growth of Gram-negative bacteria (a) & (b) and Gram-positive bacteria (c)-(e)

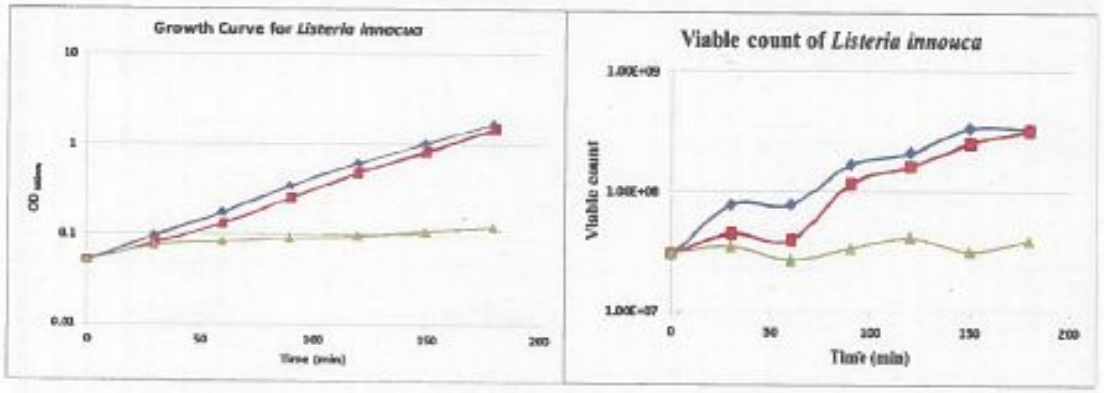
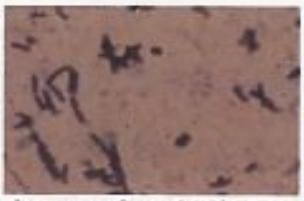


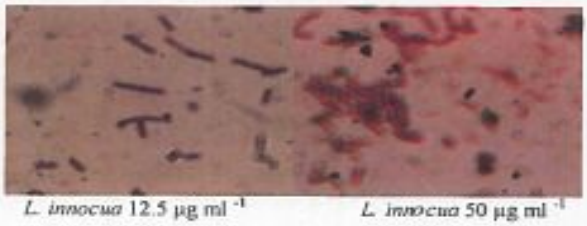
Fig. 3 The effect of liquorice extract on the growth (a) and the viable count (b) of *Listeria innocua*. Blue lines, 1% EtOH (control); Red lines, 12.5 µg ml^{-1}; Green lines, 50 µg ml^{-1}

As many of the bacteriostatic agents affect cell wall synthesis, experiments were carried out to monitor the cell morphology of *L. innocua* after the application of the liquorice was examined. Cultures were prepared as described above and then samples were taken at the begging and the end of experiment, assessment of cell morphology using Gram-staining of samples. It was found that samples treated with the liquorice extract were less able to take up the Crystal Violet stain and therefore appeared paler in colour and also the cell shapes were shorter and rounder than those seen in the control samples (Fig. 4), typical of agents that result in inhibition of cell division. Therefore, the results suggested that the primary

target of the liquorice extract is something common to the Gram-positive bacteria and may inhibit cell wall synthesis.



L. innocua Control (1% EtOH)



L. innocua 12.5 μg ml $^{-1}$ *L. innocua* 50 μg ml $^{-1}$

Fig. 4 Effect of extract on cell morphology

To try and further investigate the action of the liquorice extract, genetically engineered derivatives were used that contain the bacterial *lux* genes. However, these genes are encoded on plasmids and therefore to ensure that the cells all retain the plasmid, antibiotic selection is needed. In addition, we wanted to investigate whether any differences would be seen if different strains of the same bacteria were used. Hence the effect of strain variation was investigated by using different strains of *L. monocytogenes* in addition to *L. innocua*. Three wild type strains of *L. monocytogenes* were used, including serovars 1/2a and 4b which represent the major causes of human listeriosis (Table I). In addition, two mutant strains which either overexpress the virulence genes (NCTC 7973) or cannot express the virulence genes (NCTC 10537) were used. To carry out these experiments, different strains of bacteria (Table I) were grown in BHI broth supplemented with Erm (5 μg ml^{-1}). These cultured were then diluted into fresh BHI + Erm to a standardized OD_{600}nm of 0.05. To allow multiple samples to be monitored, and to allow simultaneous measurement of OD and light, cultures were transferred into a microtiter plate and incubated at 37 °C over a 24 h period. Due to the possibility of contamination using a microplate reader an uninoculated control sample was included (purple line, Fig 5) to show that any change in optical density or light was due to the organism inoculated into the broth.

The results gained using the microtitre plate showed the same pattern as before, in that a concentration of 50 μg ml^{-1} totally inhibited growth, but at 12.5 μg ml^{-1} very little inhibition of growth was seen (Fig. 5).

The pattern of results gained was exactly the same for all strains and species of *Listeria* tested, showing that this was a very reproducible effect. However, when the levels of light produced from each of the strains was examined (Fig. 6) it was clear that even when growth was not affected, a reduction is the light output could be seen, although this was more pronounced in some strains that others. This result indicated that the cells were experiencing some stress at the sub-lethal injury level as the level of light is representative of the overall metabolic state of the cells. It was also noted that the metabolic level of the strains challenged with 50 μg ml^{-1} were very similar to the background control, suggesting that in addition to effects on cell division, the cell membrane may be affected.

Since there were now both variations in growth rate and light output recorded it was important to normalize the bioluminescence data according to the number of cells in the sample (i.e. 10 cells producing 1 unit of light would give the same signal as 1 cell producing 10 units of light). To address this, the bioluminescence data was divided by the optical density data and the results are presented in Fig. 7. This treatment of the data clearly revealed that the cells treated with 12.5 μg ml^{-1} were experiencing sub-lethal injury resulting in overall lower levels of metabolic activity. Since cell division was not affected in these cells as the growth rate was close to that of the control, this suggests that at sub-lethal levels the liquorice extract may be affecting either cell membrane integrity or other enzymes required for the production of $FMNH_2$ within the cells.

One question that remained was whether the presence of the antibiotic required for the selection of the plasmids was providing an additional stress on the cells that accounted for the results seen in the last experiment. To determine whether the presence of the antibiotic was required to maintain the *lux* plasmids inside the cells, the stability of the plasmid was determined by growing *L. monocytogenes* 10403S containing the plasmid in the presence and absence of the selective antibiotic (Erm 5μg ml^{-1}), and rates of plasmid-loss were calculated (Tables III & IV). These experiments were also performed with and without the additional stress caused by the presence of the liquorice extract. The stability of the plasmid was determined by comparing the viable count of samples taken from the experiment and plating on agar with and without Erm and is expressed as the 100% of cells (viable count on BHI) retaining the plasmid (viable count on BHI Erm).

When grown in the absence of the antibiotic, the rates of plasmid loss were very low (92-80%; Table III) indicating that the plasmids themselves were very stable, but that plasmid stability varied with the concentration of the liquorice extract, suggesting that it was exerting a metabolic stress on the cells. In the presence of the antibiotic in the growth medium, the plasmid was more stable (as expected) but even then plasmid loss was seen when the cells were grown in the presence of the liquorice extract (93-97%; Table IV). This result validated the data shown in Fig. 7, since it is clear that the addition of the antibiotic alone is not causing a major stress on the bacterial cells.

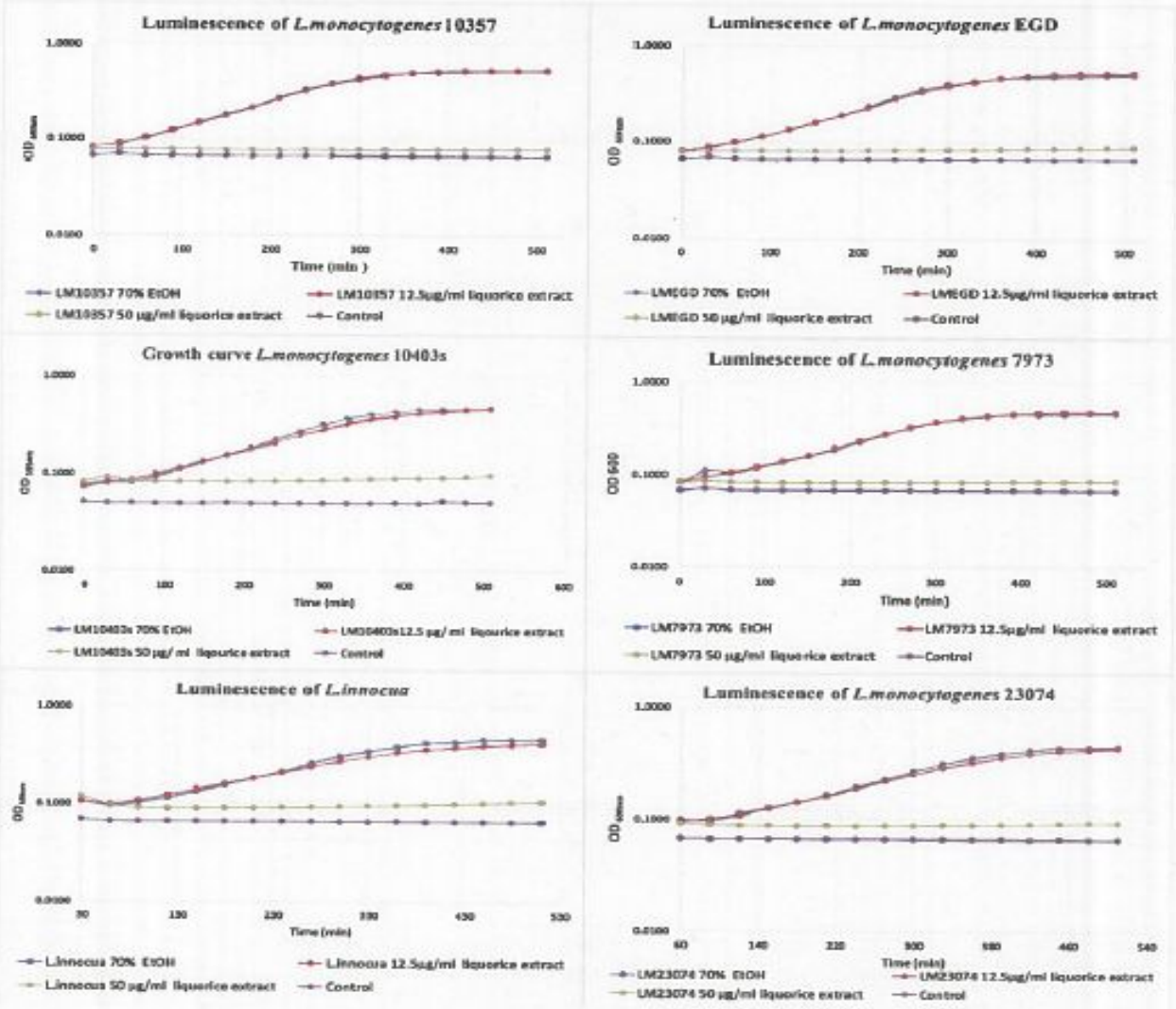


Fig. 5 Growth curve of six strains of *Listeria monocytogenes*

TABLE III
PLASMID STABILITY IN LM10403S WITHOUT SELECTION

LM 10403s (BS10, *lisABCDE, gfp*) without Erm in the Growth Media	BHI Agar with 5 µg ml^{-1} Erm = Plasmid Present in Cell (cfu ml^{-1})	BHI Agar without Erm = viable count (cfu ml^{-1})	% Bacteria with Plasmid
EtOH Control	$2.7x10^{9}$	$3.2x10^{9}$	92%
12.5 µg/ml liquorice extract	$2.54x10^{9}$	$3.06x10^{9}$	82%
50 µg/ml liquorice extract	$1x10^{6}$	$1x10^{6}$	80%

TABLE IV
PLASMID STABILITY IN LM10403S WITH SELECTION (ERM)

LM 10403s (BS10, *lisABCDE, gfp*) with 5 µg ml^{-1} Erm in the Growth Media	BHI Agar with 5 µg ml^{-1} Erm = Plasmid Present in Cell (cfu ml^{-1})	BHI Agar without Erm = viable count (cfu ml^{-1})	% Bacteria with Plasmid
EtOH Control	$1.43x10^{10}$	$1.34x10^{10}$	100%
12.5 µg/ml liquorice extract	$2.64x10^{10}$	$2.83x10^{10}$	93%
50 µg/ml liquorice extract	$9.7x10^{7}$	$9.9x10^{7}$	97%

*Erm: erythromycin

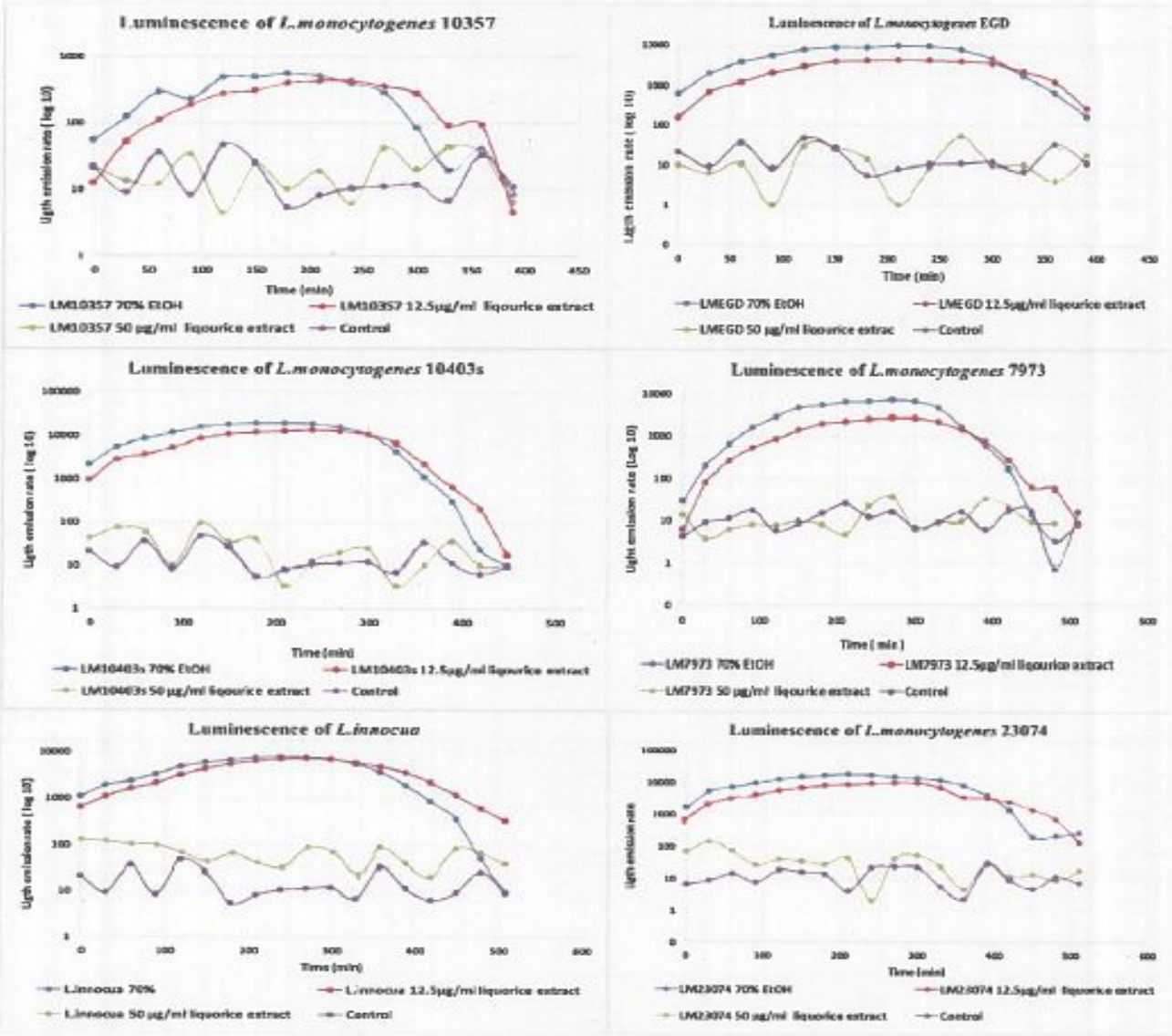


Fig. 6 Effect of liquorice extract on light emitted by *Listeria* strains

IV. Conclusion

In conclusion, the results of this work show that Gram-positive bacteria are more susceptible to the liquorice extract than Gram-negative bacteria. A range of different cell types were tested (Gram-positive rods, Gram positive cocci and spore forming organisms) and all showed equal sensitivity suggesting there is a common target in all these different types of Gram-positive bacteria. Using *L. monocytogenes* strains and *L. innocua* as model organisms, the nature of the growth inhibition seen was further investigated and it was clear that cell growth, including cell division, was affected. At sub-lethal concentrations, even when growth rate was not particularly affected, there was evidence of sub-lethal injury and cell stress.

In this study, we have used bioluminescence to quickly evaluate the effect of an antimicrobial on bacterial metabolism. This approach has been used before by many workers but some caution must be taken to ensure that the results gained are not simply due to the presence of the plasmid in the strains. In this case we found that the stress caused by treating the bacteria with the liquorice extract did exert some additional metabolic pressure on the cells, leading

to plasmid loss unless antibiotic was added to the media but there was no evidence of a significant difference in results gained with and without antibiotic.

Another area of caution is that strains of bacteria such as *Listeria* that have been cultured for long periods of time in the laboratory may have lost some of their fitness. To address this, we tested a range of different isolates, including examples of the two serovars that are responsible for the majority of human infections. Again no different was seen between the sensitivity of different strains indicating that the results gained were very reproducible and not dependent on the test strain used. In addition, we tested two well characterised mutants of *L. monocytogenes*, one which over expressed the virulence genes (NCTC 7973 *prfA**) and another than is incapable of expressing the virulence genes due to a deletion of the virulence gene regulator (NCTC 10357; Δ*prfA*). The results gained with these strains were also identical the results gained with wildtype *L. monocytogenes* and *L. innocua*, which has a complete absence of any of the virulence genes found in *Listeria*. Again these results suggest that the target site is found in all cell types and is not associated with the virulence traits of these bacteria.

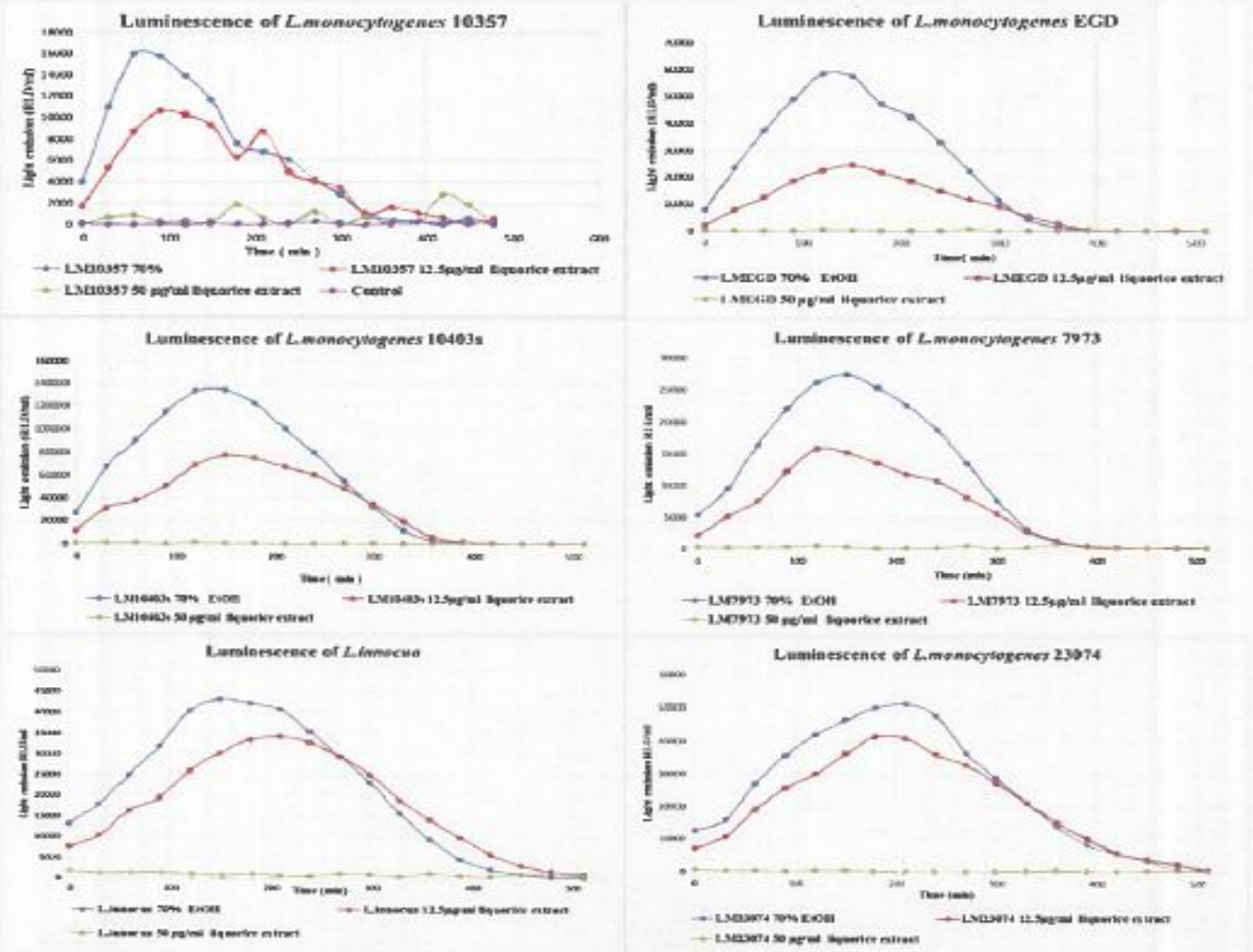


Fig. 7 Effect of liquorice extract on light emitted by *Listeria* strains, light emission was normalized by dividing RLU/OD

Treatment with 50 μg ml^{-1} of liquorice extract completely inhibited the growth of all of the Gram-positive bacteria tested, but when plated on to media without the extract they were able to grow normally indicating that stress is bacteriostatic rather than bactericidal. It is interesting to note that without the use of the bioluminescent reporter genes, the separate effect on cell metabolism would not have been evident and therefore this shows that using these tools is a powerful way to better identify stresses that have an effect on metabolic activity through damaging the membrane integrity or affecting enzymes associated with energy generation.

Since this extract was produced as a waste product from the food industry, there is the potential that it could be applied as a novel food preservative. The extract was active at 50 μg ml^{-1} and this is a level that could be used for such a practical application. However, more work would be required to establish whether there would be any organoleptic changes to the foods if used at this level. Another area of interest is if it could be used to inhibit the growth of *Clostridium difficile* in the human gut in patients suffering with hospital acquired infections. This difficult to treat Gram-positive bacterium causes a major problem, particularly in patients recovering from extensive antibiotic therapy. A formulation of this extract given as a drink could help target these bacteria and allow normal gut flora to re-establish without the need for further extensive antibiotic therapy.

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