

Studying novel, natural antimicrobials in food systems

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For the degree of Masters of Research

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Abstract

The purpose of this project was to test a range of novel, natural antimicrobial compounds provided by Phil Stroud Solutions Ltd to determine their effectiveness for use in food products. The idea behind the project was to develop a 'clean label' antimicrobial to have a primary use in meat products. It was hoped that the new antimicrobial would surpass the effectiveness of current antimicrobials being used and be more desirable to a consumer. The antimicrobials were tested against a group of five bacteria including gram positive and gram negative pathogens and spoilage organisms including *Listeria*, *Salmonella*, *E.coli* and *Pseudomonas*. The antimicrobials were first screened to determine their effectiveness using an absorbance measurement method. They were then tested in sausage and mince products by dipping and incorporation. Safety testing was completed in the form of the Ames test. Results showed that compounds D and E had the most effect on all of the five bacteria in screening and in meat trials. In meat trials the antimicrobials were added to the product before being tested over a number of days. In some instances there was a several log reduction in growth. The Ames test also confirmed that the products are safe for use in food. Overall the novel natural antimicrobials tested have scope to be used in food products in the future.

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Chapter 1 Literature Review & Aims

1.1 Literature Review

1.1.1 Introduction

Microbial organisms will always be found within food systems and the composition of food provides nutrients and a suitable environment for these organisms to grow. Hence food products generally have a set amount of time they can be stored before they start to deteriorate, which is referred to as the shelf life of the product. Food producers however, wish to increase the shelf life of their products so that the product can be displayed for longer in the shop and also so the consumer has longer after purchase before it has to be consumed. This both reduces losses to the producers due to spoilage of product before sale and also food waste, which is often due to products not being consumed within use by dates. To achieve this the food industry needs to delay the growth of spoilage organisms to inhibit their role in decomposition or to try to inactivate them entirely.

Globally, the amount of human food waste is a vast problem. From a study by the Food and Agricultural Organisation of the United Nations (FAO) it was estimated that 1.3 billion tonnes of food made for human consumption is wasted each year (Gustavsson et al., 2011). The reasons for wasting food differs depending upon the income level of the country; in lower income countries food wastage occurs early in the production chain. Often harvests can produce crops that are seen as inedible or once harvested products cannot be stored in a manner that will help to preserve them; for example due to a lack of refrigeration (Bräutigam *et al.*, 2014). However, in higher income countries, such as the USA and many countries in Europe, food wastage can occur at any point in the chain. Often waste occurs due to perceived consumer preferences, so that quality control procedures can remove products that would otherwise be edible due to the way they look. For example many UK supermarkets are now making a virtue of selling 'wonky vegetables', allowing them to buy all of a local producers crop that would have previously been rejected and selling the imperfect products at a lower price in an effort to reduce this type of waste (Caplan, 2017). The main issue in higher income countries is that people can afford to waste food (Grethe et al., 2011) and it is estimated that across Europe the average amount of food waste is 173 kg per person per year (Stenmarck et al., 2016). This is set against the fact that in the majority of countries across South America, Sub-Saharan Africa and Asia the average person consumed less than 15 kg per year (FAO, 2017)

Therefore the food industry is working to reduce food waste, and one way in which microbiology can help achieve this is by providing new ways to prolong the shelf life of foods. Spoilage is defined the point where a product becomes unacceptable in appearance, smell or taste to the consumer. Preservation methods are used to prolong the life of food products, and while older methods such as drying, heating and addition of salt are still used, they are employed alongside modern preservation methods which inhibit the growth of spoilage organisms (Gram *et al.*, 2002). The type of spoilage organism that causes this decay depends upon the product; for instance meat products which are normally stored aerobically at refrigeration temperatures will most likely be spoiled by psychrophilic Gram-negative bacteria (Gill and Newton, 1977) such as *Pseudomonas spp.* Other common spoilage organisms in meat and fish products include the Gram-positive *Bacillus spp.* and *Clostridia spp.* as these survive preservation treatments due to their ability to form spores and makes them an issue in vacuum packed meat products (Farber, 1991). Dairy products are another large source of food waste and in the USA it is estimated that 29 % of fluid and fermented milk and 27 % of cheese is wasted each year (Research, 2012). In fluid milk spoilage is largely post pasteurisation, most commonly the spoilage organisms are *Pseudomonas spp.* or *Enterobacteriaceae*. These organisms lead to spoilage in around a week (Trmčić *et al.*, 2015).

Other than spoilage organisms, the other main problem for the food industry is foodborne pathogens and finding ways to prevent them growing to dangerous levels. Not only to they pose a risk to the general population but they produce reputational damage for the manufacturers when they are named as the source of an outbreak, resulting in potentially devastating economic issues for their business. There are several foodborne pathogens which frequently cause illness in the population. Some illnesses, like *Campylobacter spp.*, which is one of the most common bacterial pathogens causing food related illness in the USA, are often the result of consumer error. It is estimated that *Campylobacter spp.* are the cause of 1.3 million gastrointestinal food related illness each year (Scallan *et al.*, 2011) and these illnesses are often related to the consumption of undercooked or raw poultry and therefore could be prevented if correct food handling and preparation were used. *Salmonella spp.* are another foodborne pathogen which causes gastrointestinal illness, but in this case the illness is normally self-limiting and is one of the most common causes of food poisoning, despite a relatively low rate of reporting to health authorities. However, there are cases where *Salmonella* infections can be fatal, especially in the elderly and other immunocompromised individuals. It is estimated that 1.4 million cases of *Salmonella spp.* infection occur in the USA each year with up to 600 cases of fatality (Cummings *et al.*, 2010, Lee *et al.*, 2015). Some foodborne pathogens cause illness as a result of contamination from the environment. For instance *E.coli* O157:H7 is another significant

cause of severe gastrointestinal illness which often fatal or results in long term health problems therefore it is important to limit the risk of infection. In this case contamination of food products from an environmental source is the cause of most outbreaks, so not necessarily to do with consumer actions although infection can also be caused due to consumption of undercooked contaminated minced beef in products such as burgers. Leafy vegetables are one of the most common sources of *E. coli* outbreaks of disease (Heiman et al., 2015). *Listeria spp.* contamination is probably the biggest concern for the food industry. In 2010 there were over 23,000 cases of reported *Listeria spp.* Infection worldwide with over 5000 resulting in fatalities (Rodrigues et al., 2017). *Listeria* is often found in ready-to-eat foods after contamination occurs during production. Therefore when food reaches the consumer infection can easily result, especially in the very young, elderly, immunocompromised or pregnant as no further cooking is required which would destroy the bacteria (Southwick and Purich, 1996).

1.1.2 Listeria

Listeria is a Gram-positive rod which is a facultative anaerobe. It is non-spore forming and its physiology can vary depending upon the growth temperature (Juntilla et al., 1988). For example the development of flagella in *Listeria monocytogenes* is response to temperature and motility is induced at 22 °C due to the production of the flagellin protein however generally above this temperature expression of the flagellin genes to make flagellin does not occur (Peel et al., 1988). This results in the fact that *Listeria* is more motile at lower temperatures, producing peritrichous flagellae which gives these bacteria their characteristic 'tumbling' pattern of motility (Peel et al., 1988).

The type of agar used to identify *Listeria spp.* depends upon the purpose of the research. *Listeria spp.* are relatively fastidious but have been shown to grow on many different nutrient rich, non-selective media including Brain Heart Infusion, Tryptone Soya and nutrient (CURIALE and LEWUS, 1994, Welshimer and Donker-Voet, 1971). Growth on these media is not different for any of the species and colonies are generally small, round and translucent. *Listeria* has been shown to be grown successfully on/in For identification from food samples *Listeria* can be grown on a number of different selective media but Palcam is often used in research for the isolation of *Listeria spp.* *Listeria spp.* colonies on Palcam are greenish, doughnut-shaped colonies with a black halo. (El Marrakchi et al., 2005) found that *Listeria* selective medias such as Palcam and *Listeria* selective agar were successful at isolating *Listeria spp.* however they did not allow differentiation between the species. In contrast it has been shown that the newer chromogenic agar can be used to differentiate between *Listeria* species. For instance using CHROMagar *Listeria* showed that

L. monocytogenes and *L. ivanovii*, the two *Listeria* species which are pathogenic, grew with a distinct colony morphology. Colonies of these two species are pale blue in colour with a white halo whereas colonies of other species from within the genus did not have this morphology. Therefore the use of chromogenic agar does allow rapid identification of presumptive pathogenic *Listeria* isolates (Hosseini et al., 2013). However as other types of bacteria can also grow on these chromogenic agars and produce very similar colony morphologies to the pathogenic species (Angelidis, 2015) it is always important to confirm the isolation of pathogenic *Listeria* species using further tests.

The main physiological characteristic which singles out *Listeria* as the main problem for the food industry is the fact that it is psychrotrophic and therefore has the ability to grow over a wide range of temperatures, including refrigeration temperatures. Therefore growth rates for *Listeria spp.* alter depending on the temperature. Although growth is optimal at 37 °C, studies have shown *Listeria spp.* can generally grow between as low 2 °C and as high as 45 °C however *Listeria spp.* have been seen to grow at -0.4 °C if the growth media does not freeze, albeit this growth is very slow (Rocourt and Cossart, 1997). Below 4 °C, the doubling time for *L. monocytogenes* is generally greater than 50 hours. However at temperatures between 7°C and 10 °C the doubling time has been shown to reduce to around 10 hours and the lag phase taking less than a week instead of several (Lu et al., 2005, Chan and Wiedmann, 2008).

At low temperatures *L. monocytogenes* undergo alterations which allows the cells to adapt quickly to a decrease in temperature. For instance there is a change in lipid composition in the membrane of *L. monocytogenes* which allows enzyme activity and transport processes to continue (Annous et al., 1997). Specifically there are changes in the fatty acid composition of the membrane so that at growth temperatures around 20 °C the levels of the C_{17:0} chain outnumber those of the C_{15:0} chain, however when *Listeria* is grown at refrigeration temperatures the composition is such that the relative levels of these two fatty acids is reversed (Gandhi and Chikindas, 2007). *Listeria spp.* make other adaptations which allow them to maintain growth at lowered temperatures including the production of cold shock proteins which helps the process of gene transcription and the induction of alternative sigma factors in response to the shock experienced to a number of different cell components following a change in temperature. In *L. monocytogenes* the stress sigma factor, σ^B , coordinates the cells response to a decrease in temperature. (Becker et al., 1998) reported that that this includes the accumulation of cryoprotectants which also support membrane structure and function and help the bacterium to continue growth at lower temperatures.

Listeriosis is the disease that results from infection by pathogenic species of *Listeria*. In humans the most common species to cause infections is *L. monocytogenes* however there have been rare cases of *L. ivanovii*, which normally causes infection in animals (Rocourt and Seeliger, 1985), causing infections in humans (Snapir et al., 2006, Guillet et al., 2010, Beye et al., 2016). Although *Listeria spp.* are very common in the environment in water and soil, contaminated food is the most likely source of *L. monocytogenes* that result in infection (Lecuit, 2007) and *L. monocytogenes* infections can occur after eating just one heavily contaminated food product. Human *L. monocytogenes* infections are normally the result of ingesting contaminated food, but patients remain clinically silent for 10-70 days which is the normal incubation range before clinical signs of infection become apparent (Goulet and Marchetti, 1996). As listeriosis is the result of ingestion of contaminated food, access to the body is via the gastrointestinal tract. As can be seen in Figure 1-1 the bacteria crosses the intestinal barrier into the main lymph nodes before being disseminated to other organs through the circulating blood stream and lymphatic system. To begin with symptoms are either 'flu like', such as muscle aches and chills, or can present as gastroenteritis. However these infections are often self-limiting and therefore are not diagnosed.

If, however, infection becomes more serious, it can develop into septicaemia, meningitis or encephalitis which can in many cases results in death (Vazquez-Boland et al., 2001). The more serious symptoms of the infection are caused by the ability of the bacteria to cross the blood-brain barrier. Once in the brain infection is likely to be fatal unless high level antibiotic treatment can be administered successfully (Lecuit, 2007). Hence mortality rates in *L. monocytogenes* infections can be very high and are on average as high as 30%, which is much higher than other food borne pathogens (Altekruse et al., 1997). Fully developed listeriosis most commonly effects children, the elderly, the immunocompromised and pregnant women (Southwick and Purich, 1996) because of the intracellular location of the bacterium. *Listeria* has the ability to induce phagocytosis and can enter many different cell types, essentially hiding within these cells and avoiding interaction with circulating antibodies as shown in Figure 1-2 (Southwick and Purich, 1996). Hence to clear infection patients need a good cell-mediated immune response system and therefore any immunocompromised individuals are more likely to suffer severe infections.

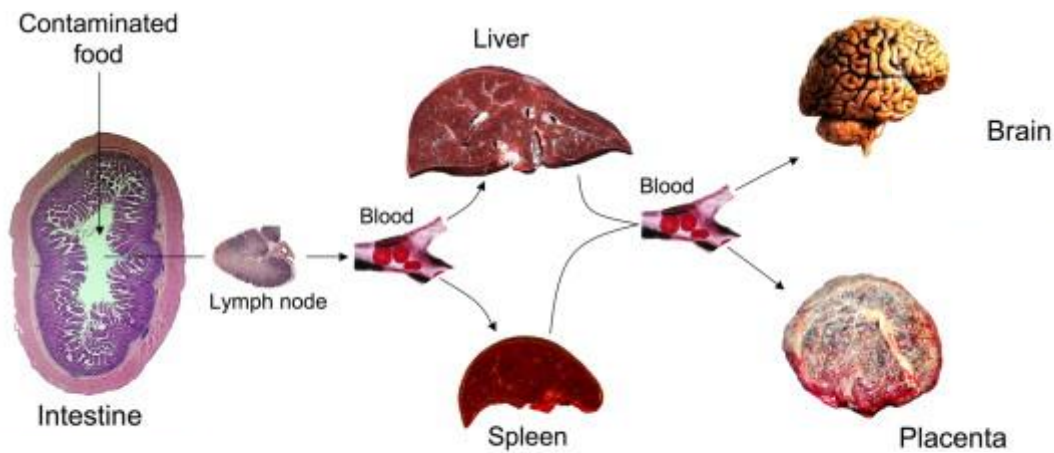


Figure 1-1 - **Infection by *L.monocytogenes*.** *L.monocytogenes* is ingested and enters the intestine through the gastrointestinal tract. Once in the intestine it crosses the intestinal barrier to the mesenteric lymph nodes. This allows the bacteria to spread through the blood to the liver and spleen. The immune system should then be able to detect *L.monocytogenes* and fight the infection, if not the infection becomes prolonged which causes more serious consequences. It is when the bacteria is spread in the blood once more that it reaches privileged sites such as the brain and the placenta and infection becomes uncontrolled. Image adapted from (Lecuit, 2007).

L. monocytogenes infection is also a significant problem for pregnant women due to its ability to cross the placental barrier. This type of infection causes spontaneous abortion of the foetus thus ending the pregnancy. In other cases the neonate can be born suffering from symptoms of the infection such as sepsis and meningitis (Prevention, 2013).

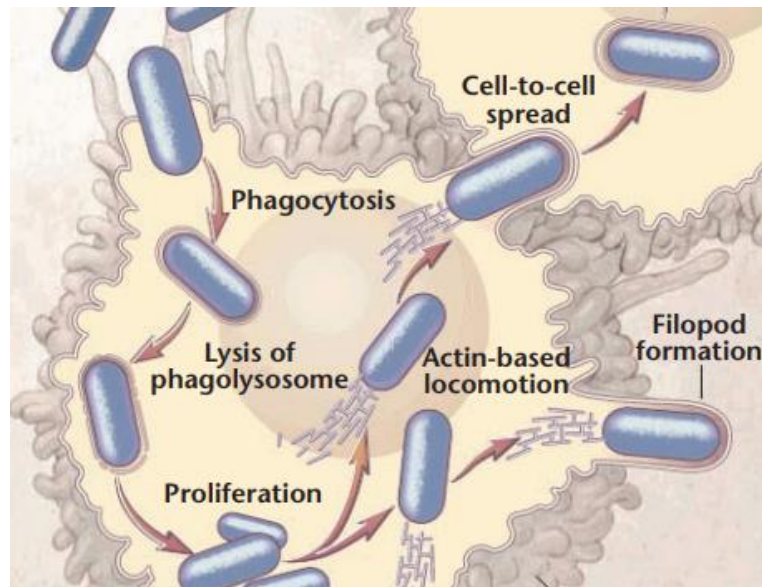


Figure 1-2 – **Cellular invasion by *L.monocytogenes*.** Once phagocytosed by a host cell, listeriolysin O allows access to the cytoplasm. This is where the bacteria can proliferate normally doubling every hour. Once complete *Listeria* will target another host cell; using actin-based locomotion and polarising one end of the host cell the bacteria can form a filopod as an extension of the host cell. This allows the bacteria to transfer from one cell to the next without immune system detection. The process then repeats with another host cell (Southwick and Purich, 1996, Janakiraman, 2008). Image adapted from Janakiraman (2008).

Listeriosis is often hard to diagnose as symptoms can often not present themselves for a number of weeks following infection (Goulet and Marchetti, 1996). When serious symptoms do occur medical professionals often look at different options as to a diagnosis. Listeriosis can be diagnosed relatively easily once presumption of the infection has been established. Diagnosis is complete by culturing a site which should otherwise be sterile and getting a positive culture for *L.monocytogenes*. Common sites which are cultured are blood or cerebrospinal fluid (CSF) however in babies this can be amniotic fluid or ear swabs (Shetty et al., 2009). Whichever site is tested the sample is plated generally onto blood agar and incubated overnight. As culture is not a quick way of diagnosis newer Polymerase chain reaction (PCR) based tests have become available (Allerberger and Wagner, 2010). PCR based diagnosis can take hours instead of days for a culture, the accuracy of the identification is also much higher. As the bacterium is being identified from a specific gene instead of colony morphology there is more confidence in the results. This approach is also

being adopted in the food industry to quickly identify the organism which is contaminating the food. (O' Grady et al., 2008) showed that targeting the *ssrA* gene which is present in all *Listeria* species could be used with FRET hybridization and real time PCR to diagnose *Listeria* presence. Although in medical terms the gene target may be slightly different to make it specific to *L.monocytogenes* the principles are the same.

As with most bacterial infections, listeriosis can be treated using antibiotics. *L.monocytogenes* is generally still susceptible to most common antibiotics with a few exceptions however some rare strains have been isolated which have developed resistance (Heger et al., 1997). Treatment however, will vary depending upon what part of the body the infection has reached and is therefore dependent upon whether it is related to pregnancy, gastrointestinal or neurological infection. In *L. monocytogenes* infection the standard treatment regime is ampicillin or penicillin with the option of these being combined with gentamicin. As these treatments are only bacteriostatic, success of the treatment relies upon the immune system being able to assist the antibiotics in removing all infection (Hof, 2003) and therefore the immunocompromised are less likely to respond to this therapy. If infection occurs during pregnancy then antibiotics are used to treat the placenta and foetus where possible, in these cases the standard two week course is increased to 3-4 weeks to ensure all infection has been eradicated (Temple and Nahata, 2000). The treatment in the case of neuroinvasive *L. monocytogenes* infection is at least 6 weeks to make sure all infection is removed from the brain or brain stem, in this case more powerful antibiotics are used, such as combining ampicillin with acyclovir (Kayaaslan et al., 2009, Jubelt et al., 2011).

Listeria spp. can be found associated with many different food types, contamination has been seen in meat, dairy, fruit and vegetable products and infection by *L. monocytogenes* is often associated with 'ready-to-eat' (RTE) foods as cooking at high temperature will destroy the bacterium. Due to the high fatality rate the food industry takes the issue of potential for *Listeria* contamination very seriously with some countries such as the USA enforcing a zero tolerance policy for detection of *L. monocytogenes* in food (Shank et al., 1996). The UK falls under the European guidelines which, instead of operating zero tolerance where no RTE food can be found to contain any *L. monocytogenes*, these products must be shown to contain less than 100 *L. monocytogenes* cells per 25 g of product. This however is only the case in products which will not support *Listeria* growth (Beaufort et al., 2014), in which case producers must demonstrate absence of the bacterium from food that is to be consumed without cooking.

Recent outbreaks of *L.monocytogenes* have occurred across the globe and have been associated with many different contaminated food stuffs. An outbreak starting in 2015 in Europe was traced in early 2018 to contaminated frozen sweetcorn and mixed vegetables. Five European countries, including the UK, reported cases linked to these products and overall there were 47 confirmed cases with a 19 % mortality rate (European Food Safety Authority, 2018a). Traceback investigations found that the likely source of the outbreak was two production plants, one in Hungary and the other in France. *Listeria* strains isolated from environmental samples taken from these plants were compared to the particular *L. monocytogenes* serovar found in the patient samples to finally identify the source of the outbreak. As a result many products that had been produced from 2016 onwards were recalled due to risk of contamination.

Between 2017 and 2018 South Africa suffered from one of largest *Listeria* outbreaks recorded. As of early 2018 there were 943 confirmed cases with a 19 % mortality rate (Manganye et al., 2018). Isolates from patients and polony sausage product which was confirmed as the source of the outbreak were taken and whole genome sequence analysis revealed that the majority were found to contain *L. monocytogenes* sequence type 6 (ST6) (Allam et al., 2018). It is thought that the contamination of the polony sausage may have occurred during slicing, possibly due to the fact that the oil in the slicing machinery may have become contaminated resulting in the RTE meat product becoming contaminated with *L. monocytogenes*.

Dairy products are another food group susceptible to *L. monocytogenes* contamination, and one particular product which has been the source of many outbreaks is soft cheese. As listeriosis is not a notifiable disease in many countries some outbreaks can go unnoticed. For instance an outbreak in Northern Italy between 2009 and 2011 was traced back to soft cheese but only after 306 samples were tested and *L. monocytogenes* ST38 was found to be the most prominent. A cheese was then confirmed as the source of the outbreak when clinical samples were matched to those taken from cheese still present in patients fridge's (Amato et al., 2017). However, as mentioned above, this type of traceback analysis is often difficult due to the long time it takes for clinical symptoms to appear.

1.1.3 Salmonella

Infection by *Salmonella spp.* is one of the most common food borne infections in the world. There are two types of *Salmonella spp.* which cause illness in humans; typhoid or non-typhoid. Non-typhoid infections are much more common especially in more developed

countries. *Salmonella enterica* (*S. enterica*) is the leading cause of salmonellosis disease in humans. The bacterium is a Gram-negative, rod-shaped, flagellated bacterium which is a facultative anaerobe (Ochman and Groisman, 1994). Like *L. monocytogenes*, the virulence of *S. enterica* is due to its ability to invade human cells. *Salmonella* Pathogenicity Islands (SPI's) encode a type III secretion system which induce uptake of the bacterial cells by phagocytosis (Coburn et al., 2007). The activation of the secretion system allows the bacteria to inject effectors into the host cells cytoplasm which modifies the host cell's cytoskeleton allowing the bacteria to be engulfed (Takaya et al., 2003). This intracellular phase allows the bacteria to replicate and spread and further infect the host (Eng et al., 2015).

Salmonella spp. can be cultured using both selective and non-selective media. In the case of non-selective media, such as nutrient agar, *Salmonella* colonies are white/yellow in colour, relatively large and round. The choice of selective media can depend upon the type of *Salmonella spp.* that is being isolated however a common choice is Xylose Lysine Desoxycholate (XLD) agar (Park et al., 2012). On XLD *Salmonella spp.* grow as round black colonies, and some strains also exhibit a pink halo. One issue with XLD, as well as other selective agars for *Salmonella spp.*, is that colonies are often indistinguishable from other bacteria such as *Proteus spp.* (Cooke et al., 1999). The development of chromogenic agar has been developed to resolve this issue, however they have also been shown to have problems due to colonies forming with morphologies similar to those of normal commensal bacteria. Although chromogenic agar have been shown to have a higher specificity, they are more expensive than other selective medias therefore they are not used in high throughput experiments and tests (Perry et al., 2002).

Generally *Salmonella spp.* are grown at 35-37°C, in which case after 24 hours growth will be seen on agar plates. It has been shown that *Salmonella* can grow at temperatures as low as 3.5 °C however this is only on agar where all nutrients are provided as uptake and metabolism are affected (Matches and Liston, 1968). *Salmonella spp.* will however grow at 'fridge abuse' temperatures, of around 7-10°C, which has resulted in a number of product contamination issues. For example sliced fresh tomatoes were found to support growth at lower temperatures leading to unacceptable levels of the bacteria being present after storage (Pan and Schaffner, 2010).

Food poisoning as a result of *Salmonella spp.* infection can often be self-limiting and treatment can be managed by the patient. In this case recommended treatment is fluid and possibly anti diarrhoeal drugs (Sirinavin and Garner, 1999). *Salmonella* infection causes gastrointestinal illness normally between 6 and 48 hours after infection, but this short timeframe makes traceback investigations more likely to identify a food source.

Symptoms include vomiting, diarrhoea, cramps and fever. However infections often go undiagnosed as symptoms will often resolve within a few days so patients do not present themselves to the healthcare system which then makes identifying an outbreak more difficult (Switaj et al., 2015). Indeed diagnosis of *Salmonella* infection is often not possible as the bacteria have left the body before tests are carried out. The standard diagnosis for *Salmonella* infection is bacterial culture of the patient's faeces (Narayanappa et al., 2010). PCR diagnostic tests can be carried out along with serological antibody studies, however these are only carried out if there are complications as in standard cases they are often carried out too late and the bacteria is no longer present (Kuhn et al., 2012). In more severe cases rehydration via fluids in hospital may be required especially in the case of an elderly or immunocompromised patient. In a minority of cases treatment with antibiotics would be necessary, this is only in the case of invasive disease. Antibiotics primarily used would be amoxicillin and cephalosporin however their use is not common given the number of cases of *Salmonella* infection each year (Cianflone, 2008).

Outbreaks of *S. enterica* can be associated with a number of different food products. Fresh fruit and vegetables are common sources of outbreaks, and in 2011 the USA saw a *Salmonella* outbreak caused by papayas. The source of this outbreak was thought to be the farm where the fruit was grown and contamination of the product could have occurred during storage or washing due to contaminated water (Mba-Jonas et al., 2018). After the outbreak the US Center for Disease Control (CDC) issued a reminder of good practice for the preparation of fruit and vegetables to try and educate the public to avoid further outbreaks (Prevention, 2018). The UK has suffered an outbreak of the same strain of *S. enterica* between 2017-2018 relating to RTE foods containing cucumber. Although microbial evidence suggested that the source of outbreak was cucumbers originating from one area, insufficient epidemiological evidence was available to be able to back this up because, as described above, the self-limiting nature of many *S. enterica* infections means that data is often missing as those infected do not present to medical professionals (European Food Safety Authority, 2018b).

1.1.4 *Escherichia coli*

Escherichia coli (*E. coli*) is a Gram negative, rod-shaped bacterium and is a facultative anaerobe. *E. coli* is generally classed as a commensal bacteria found in the gastrointestinal tract of humans and animals (Kaper et al., 2004). However some *E. coli* strains, including *E. coli* belonging to the O157:H7 surface antigen group, are pathogenic and are a common cause of food borne illness (Lim et al., 2010). *E. coli* O157:H7 is responsible for most

recorded cases of *E. coli* food borne disease. As for *Salmonella*, the illness it causes often presents as a self-limiting gastroenteritis and for most patients any symptoms will resolve themselves within a week and only require common diarrhoeal treatments. However infections can also result in fatality depending upon the health of the individual and the seriousness of the infection. Initially the infection presents with symptoms of abdominal cramps and diarrhoea, it is from this point that symptoms either improve or the condition of the individual deteriorates (Slutsker et al., 1997). In many cases patients can suffer from bloody diarrhoea which often presents 1-2 days after initial symptoms, this symptoms occurs in 70% of cases associated with *E. coli* O157:H7 (MacDonald et al., 1996). In *E.coli* O157:H7 infection vomiting and fever are less common, the main symptom which occurs is diarrhoea. However, for a small percentage of patients haemolytic uremic syndrome (HUS) develops (Mead and Griffin, 1998). HUS is a serious condition which can be fatal and is more common in the under-fives (Bell et al., 1997). The main symptoms are haemolytic anaemia, thrombocytopenia and renal failure or injury (Banatvala et al., 2001). Figure 1-3 shows the various outcomes of HUS along with the average percentage of patients which follow each pathway.

E. coli O157 has several different virulence factors including the cytotoxic Shiga toxins which are able to help the bacteria to infect host by disrupting cell function (Jacewicz et al., 1999). There are two types of shiga toxin involved in *E. coli* O157:H7 pathogenesis, and Shiga toxins 2 (Stx2) is generally more toxic than Stx1. Hence more cases of *E.coli* O157:H7 infection resulting in HUS have been shown to encode the Stx2 cytotoxins (Boerlin et al., 1999). *E. coli* O157:H7 also carries the locus of enterocyte effacement (LEE) in a pathogenicity island in its genome which is required to help it attach tightly to the surface of the human cells as part of the infection process (Caprioli et al., 2005). Another virulence factor is the plasmid pO157 which is a non-conjugative F type plasmid and could be responsible for production of haemolysin and serine proteases that cause damage to the host cells. (Burland et al., 1998); (Law, 2000) but the role of pO157 in virulence is still not entirely clear (Lim et al., 2010).

When culturing in the laboratory *E.coli* are generally grown at 37 °C and growth will be seen within 24 hours. It has been shown the *E. coli* O157:H7 can grow in temperature as low as 4 °C although this is not the normal situation (Palumbo et al., 1995). However growth of *E. coli* O157:H7 at refrigeration abuse temperatures is possible and relatively common and this has accounted for some outbreaks of infections associated with dairy products (McIngvale et al., 2000). As for *Listeria* and *Salmonella*, *E. coli* can be cultured on both selective and non-selective media. On non-selective agars *E. coli* appear as round, white colonies of reasonable size. However the choice of selective agar depends upon the

species of *E. coli* that are being targeted. Generally *E.coli spp.* can be selectively cultured using XLD agar as with *Salmonella spp.*, however in this case the *E. coli* colonies are round and straw yellow in colour. More commonly MacConkey agar is used to select for *E. coli* species (Wright, 1984). However it is important to be able to differentiate between commensal *E. coli* strains and *E. coli O157:H7*, and to do this another selective media is required which takes advantage of the fact that *E. coli O157:H7 cannot* produce acid from sorbitol whereas other *E. coli spp.* can. Therefore Sorbitol MacConkey agar is a variant of MacConkey agar is used for the culture of *E.coli O157:H7* and to distinguish between the two types. Commensal *E. coli* strains grow as pink/red colonies whereas *E. coli O157:H7* grows as clear/grey colonies giving a good distinction between the two (Kang and Fung, 1999).

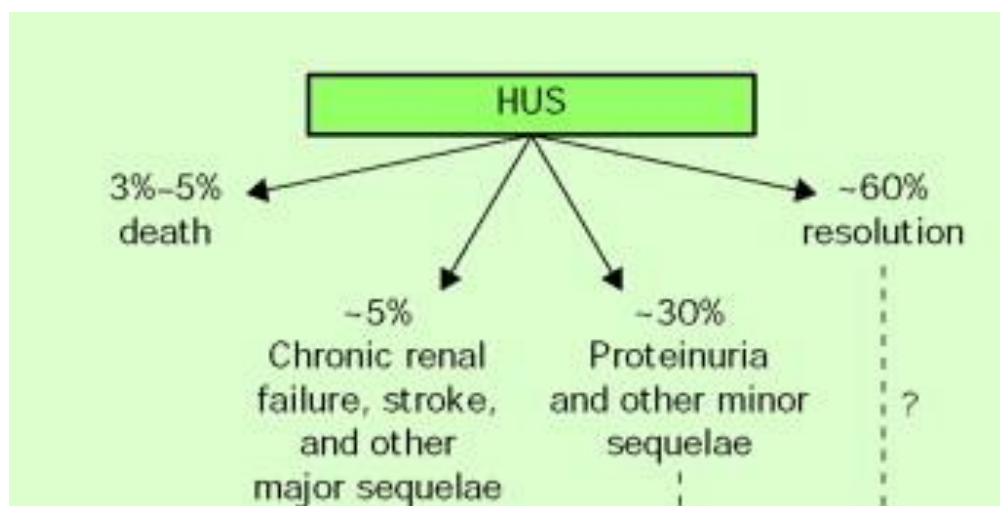


Figure 1-3 – **HUS pathway**. HUS although a serious condition in many situations can be manageable. The 3-5% of illness which ends in fatality is acute fatality. Other chronic issues can develop such as chronic renal failure which can ultimately result in fatality later on. Most cases are treated and resolve and no further complications arise nevertheless some cases result in further late complications which can include chronic pancreatitis, diabetes and glucose intolerance (Mead and Griffin, 1998). Image adapted from Mead and Griffin (1998).

Diagnosis of *E.coli O157:H7* infection is relatively simple, based on the fact that anyone who presents to medical professionals with bloody diarrhoea is suspected of having an *E. coli O157:H7* infection and a stool sample is taken. In the case of an outbreak, other patients may be tested for infection if they have had known contact with the source of the outbreak (Advisory Committee on the Microbiological Safety of Food, 1995). The stool sample is plated on Sorbitol MacConkey agar and incubated overnight to check for the presence of *E.coli O157:H7* (Mead and Griffin, 1998). If colonies which are thought to be *E. coli O157:H7* are present on the plate they are further tested for the O157:H7 antigen to confirm the identification (Nataro and Kaper, 1998).

Despite the potential severity of the disease, treatment for *E. coli* O157:H7 infection is often not necessary and in cases where symptoms are self-limiting the infection can be left to resolve itself. In cases where patients are more susceptible to illness they will be more closely monitored for the development of HUS (Tarr, 1995). There is debate as to whether treatment with antibiotics increases the risk of a patient developing HUS, since antibiotic therapy can induce the production of the Shiga toxins and (Wong et al., 2000) found that treatment with antibiotics in children under ten did increase their chance of developing HUS. Hence HUS treatment involves monitoring patients to check for signs of deterioration while balancing rehydration and electrolytes. It is also necessary to treat any anaemias or other physiological conditions caused as a result of the HUS to support the patient's recovery (Siegler, 1995). In cases of chronic renal failure caused by HUS, dialysis is required with the possibility of the patient ultimately requiring a kidney transplant (Siegler, 1995, Mead and Griffin, 1998).

The majority of *E. coli* O157:H7 outbreaks are food borne. Outbreaks of common in ground beef as cattle are a vehicle for *E. coli* O157:H7 transmission because they can be colonised by this bacterium. Contamination occurs when the surface of the meat comes into contact with faeces and is further spread through the beef when it is ground (Erickson and Doyle, 2007). In 1999 the USA saw an outbreak of *E. coli* O157:H7 which was caused by ground beef in a popular fast food restaurant. It was found that all patients had eaten there in the week before the onset of illness. The outbreak was discovered to be over many states following epidemiological studies by the US public health authority (CDC) (Jay et al., 2004). Vegetables which are generally eaten raw are another common source of outbreaks. They are often contaminated by the water used to wash them before packaging. Consumers then do not wash the product as it is sold as a prewashed product resulting in infection when the contaminated product is consumed. In this respect, the USA saw an *E. coli* O157:H7 outbreak in 2006, and the source was found to be prepacked fresh spinach. There were 191 confirmed cases with a 2 % mortality rate. As a result all spinach products were removed from sale (Sharapov et al., 2016).

1.1.5 The Problem

There are many issues which arise in the food industry which can lead to microbial contamination of a food product. The product can become contaminated during manufacture or by misuse by the customer. Outbreaks of any microbial organism are of serious concern to a manufacturer as it can reflect badly on them and their product.

L. monocytogenes is known for its ability to form biofilms and adhere to a large number of surfaces. There is evidence that *L. monocytogenes* is able to persist in the processing

environment in hard to clean areas and there is a debate whether the biofilms formed in these areas are more resistant to biocides used in the food industry due to regular exposure to low level biocide residues (Wang et al., 2015). In a case in Denmark in 2000 a turkey meat plant was identified as the source of an outbreak. The turkeys that arrived at the plant were tested for the bacteria and none were found and therefore it was concluded that the source of contamination had to be at some point along the production line. *L. monocytogenes* was found in every area of the processing plant and of 99 samples taken in the plant, 41.4 % tested positive for *L. monocytogenes* indicating that the bacterium had colonised the whole factory environment, After disinfection and cleaning 25.9% still tested positive showing its ability to remain at low levels in these hard to clean areas (Ojeniyi et al., 2000).

Hence *L. monocytogenes* is known to be a risk for contamination of RTE foods, and therefore consumer abuse of the product is generally not the reason for outbreaks of disease. However in the case of the 2017-2018 UK outbreak involving frozen sweetcorn and mixed vegetable products, it was consumers using the product in an unexpected way which caused issues in that the producers assume that frozen vegetables are generally cooked before consumption which would destroy the bacteria, but in this case consumers were eating the product without cooking highlighting the risk in countries where a zero tolerance policy is not applied to the whole food chain. However, in this case the amount of *L. monocytogenes* in the product was still above the UK acceptable limit and therefore the source of such high levels of contamination needed to be identified.

E. coli O157:H7 contamination normally comes from the environment as although a hardy organism it does not form biofilms in factories like *L. monocytogenes* so does not have the ability to remain in food manufacturing systems. *E.coli* O157:H7 can often contaminate water used to produce food in agricultural areas where infected cattle shed the bacteria in manure, and this may then lead to contamination of local water sources. Water is used to both irrigate and then wash fresh fruit and vegetables and if contaminated water is used then the bacteria contaminate the products. (Solomon et al., 2002) investigated the use of water and manure to assist the growth of lettuce. They found that when they contaminated the soil at the base of the plant, avoiding the edible part of the lettuce, that within a few days the edible part of the plant became contaminated. This proved that there is a risk of infection during growth of the plant from contaminated irrigation water via the root system, as well as from post-harvest contamination during washing. Hence guidance has been introduced that manure should be composted in line with specific regulations (US Food and Drug Administration, 1998).

Unlike these other two bacteria, *S. enterica* is one of the most likely bacteria to cause infection due to consumer mishandling or cooking of products. The producer of these products expects them to be cooked therefore the presence of *Salmonella spp.* is not a cause for concern. For instance in countries that do not vaccinate their flocks, it is prominent in raw chicken and eggs. Some cases of infection are likely to be caused by consumers undercooking these products and thus be infected with *Salmonella spp.* that should have been inactivated by the cooking process (Garbutt, 1997). Another consumer misuse problem which can cause illness is refrigeration temperature abuse. The growth of *Salmonella spp.* is inhibited between 4 and 6 °C. However when products are left between 7 and 10 °C due to inadequate chill storage, *Salmonella spp.* will begin to grow (Garbutt, 1997). This means that there is a higher risk that when the product is either handled or consumed that the levels of bacteria present will result in infection.

1.1.6 Preserving Food

To avoid these problems the food industry has designed ways to either avoid contamination or prevent the growth of bacteria in the product. This does not only assist in reducing outbreaks of disease, it also helps to prolong the shelf life on food and thus reduces food waste. There are various ways of preserving food, including treatments such as pasteurisation and heat treatment or adding a preservative agent to the product to prevent bacterial growth.

Reducing water activity in food products is one way the food industry reduces microbial growth and increases the shelf life of the products they produce. Common ways of reducing water activity are dehydrating, smoking and using salts or sugars. Salts or sugars are used depending upon the product and what works best to maintain flavour for the consumer. Dehydrating removes water from a product thus reducing growth. Microbes present in food need to maintain an internal osmotic balance which is affected by their external environment. When water activity in a food product is reduced below an acceptable threshold, the bacterial cells can't maintain their internal osmotic balance in a range that will allow them to carry on growing (Erkmen and Bozoglu, 2016).

The way food is packed can also contribute to how the product is preserved. Often products are packaged to that they are in a sealed environment which prevents contamination when displayed and when stored but can also prevent microbial growth. For example when products are vacuumed packed, oxygen is removed therefore preventing the growth of obligate aerobes and slowing the growth of facultative anaerobes who have to switch to the less efficient anaerobic respiration. *Pseudomonas spp.* are obligate aerobic, Gram-negative bacteria that include many common spoilage organisms therefore vacuum

packing reduces spoilage giving products a longer shelf life. Similarly increasing levels of CO₂ in packaging can also reduce microbial growth. These methods are commonly used for chilled meats, such as steak and fish, and is method which allows the aesthetic of the product to remain unchanged as there is minimal addition or treatment of the products.

Pasteurisation is a process commonly used to help preserve products such as milk and eggs from microbial contamination. The pasteurisation process uses heat or gamma irradiation to kill and bacteria in the product. Most mesophilic, non-spore forming bacteria cannot withstand temperatures above 40-45 °C therefore by heating them to in excess of 60-70 °C ensures the inactivation of the bacteria that may be contaminating the product. The time and temperature to which the product is heated to will depend upon the product. Juice for cartons and similar is heated to a higher temperature for a shorter period of time, often less than 30 s. This is because the flavours can be maintained and it allows the product to be stored out of the fridge until opened. If meat is treated this way it is heated to a lower temperature for up to 30 min. This is because if the short treatment at high temperature is used the meat loses its flavouring and appearance that the consumer is looking for. Milk can go through either process but the high temperature treatment with a shorter timescale affects the organoleptic properties of the product less so that is the preferred option. After heating, the products are quickly cooled and stored at the recommended temperature for that product, but generally this is refrigeration temperatures between 4-6 °C.

Chemical preservatives can be used to prolong the shelf life of food products. Preservatives such as benzoic acid, sorbic acid and nitrites can be found in a variety of food products. They have been proven to prolong shelf life therefore they are of benefit to the food industry. However, these non-natural preservatives are given E numbers which are regarded by some consumers as unwanted additions and therefore they can be wary of buying products that contain them. As consumers move towards wanting more natural and organic products, these inorganic acids are not seen as a clean and healthy additions to food products.

1.1.7 Natural Preservatives

Consumers are now looking towards healthier, more natural alternatives to preservatives and additives which were previously chemical based. The food industry is increasingly using more natural products when manufacturing food due to these changes in consumer attitude. However to maintain the quality of the product the natural component must be as effective as an alternative to the original preservative.

'Clean label food' is something that consumers are increasingly looking towards. Although the definition of what this includes is not very well defined. In a review by (Asioli et al., 2017) they described four different definitions for the term. These ranged from 'clean label' being defined as something with no more than five ingredients, to products with labelling that stated they were organic or allergen free as well as a product being free from additives. The level of confusion is clear when differences between the front and back of food packaging are considered. The front of the packaging can display easy to interpret labels that say a product is, for example, organic whereas the back of the packaging generally displays the list of ingredients, including any additives (Draper et al., 2013). Preservatives will always be present in food, this is what allows the food to have a longer shelf life and avoid contamination. The consumers view of the ingredients is what shows whether they are seen as natural or not (Moskowitz et al., 2012).

There are products that can be used as natural preservatives in many different food types. Popular choices are plant and essential oils and herbs. To the consumer these are seen to be readily available in the environment therefore natural. It is the components of the essential oils which make them effective and it is thought that it is a combination of the different organic components rather than any one element which make them effective. Some researchers believe that the phenolic compounds which are found in essential oils are able to alter membrane permeability thus being able to damage the cytoplasmic membrane leading to cell death (Min et al., 2011). In an experiment conducted to see the efficacy of plant essential oils in soft cheese, some effect was seen against *S. enterica* and *L. monocytogenes*. Clove, cinnamon and thyme were used in full and low fat soft cheese and the results showed that the oil of clove seemed successful in slowing the growth of both types of bacteria (Smith-Palmer et al., 2001). In meat essentials oils are sometimes used, however mostly they are used to enhance preservative measures already in place (Mathenjwa et al., 2012). In a study by (da Silveira et al., 2014) it was reported that bay leaf was a successful antimicrobial when added to Tuscan sausage and increased the shelf life of the product. However one of the issues which can arise with the use of preservatives is how it alters the appearance of the product, and in this case bay leaf did alter the appearance of the sausage but not to enough of an extent to affect consumer preferences. (Calo et al., 2015) reviewed this area and reported that a lot of research has been conducted on using essential oils as antimicrobials and they have been shown to have effect on most main food borne pathogens including *L. monocytogenes*, *S. enterica* and *E.coli* O157:H7.

Organic acids are another form of natural antimicrobial if they are sourced from naturally produced products. They are produced during the fermentation of lactic acid and have

been approved for food use by several governing bodies (Mani-López et al., 2012). Their efficacy as antimicrobials has been shown in several meat and dairy products with examples of those used including acetic acid, citric acid and several lactates (Hugo and Hugo, 2015). One study assessed the effects of organic acids lactic acid and acetic acid on *L. monocytogenes* in frankfurter sausages. It found that at 10 °C the two acids provided acceptable control of the bacteria. The acids were applied by dipping the sausage ensuring the surface of the sausage was covered (Barmpalia et al., 2004).

Products with an unappealing smell, look or feel will not be bought by consumers therefore the fact that the shelf life is longer will be irrelevant since the appearance of the product after the preservative has been added is important to the consumer. Hence most products will also go through sensory analysis to ensure that they will be acceptable to the consumer (Mani-López et al., 2012) and other products will be used alongside the antimicrobial to counter any such problems. For instance a study of the efficacy of sodium lactate as an antimicrobial against spoilage organisms found that although the sodium lactate was effective against the spoilage bacteria, and significantly increased the shelf life of the product, to maintain an acceptable appearance antioxidant was also used because of the chemical effects of the lactate on the food composition (Crist et al., 2014) .

1.2 Project Aims

From the literature review it has been shown that food borne pathogens and spoilage organisms are of concern to the food industry. To try and prevent contamination and avoid illness preservatives are an important component of food products. This project aims to show that novel, natural antimicrobials based on organic acids can prevent or slow the growth of five common food borne organisms. Firstly by screening the new compounds to determine their efficacy against the range of organisms including both Gram-positive and Gram-negative and to determine if they are bacteriostatic or bactericidal. Then the aim was to test them in food system to study whether they were effective in the presence of other components in food. The food products chosen were Italian mortadella sausage for *L. monocytogenes* challenges and pork minced meat for challenges against *Salmonella*, *E. coli* and *Pseudomonas*. Finally any candidate antimicrobials would be screened for mutagenicity using the Ames test to determine if the antimicrobials safety for taking forward into food applications.

Chapter 2 Methods

2.1 Bacterial Strains

Table 2-1 - Bacterial strains used in this project

Bacteria	Strain number/source
<i>Listeria monocytogenes</i>	10403S
<i>Listeria innocua</i>	Nottingham laboratory collection
<i>Salmonella enterica (serovar Typhimurium)</i>	DB 7516
<i>Escherichia coli</i>	MG1655
<i>Pseudomonas fluorescens</i>	Nottingham Laboratory collection
<i>Salmonella enterica (serovar Typhimurium)</i>	TA98, TA100, TA1537, TAI535, TA102 Nottingham laboratory collection with deletion mutation

2.2 Growth Media

2.2.1 BHI Broth

Brain Heart Infusion (BHI Oxoid) broth was made by dissolving 14.8 g of broth powder in 400 ml of RO water. The solution was then mixed and autoclaved at 121 °C for 15 min.

2.2.2 BHI Agar

Brain Heart Infusion (14.8 g; BHI Oxoid) broth powder and 6 g of agar powder was added to 400 ml of RO water. It was then mixed and autoclaved at 121 °C for 15 min.

The molten agar was then poured aseptically into sterile petri dishes.

2.2.3 Maximum Recovery Diluent

MRD (0.5 g) was added to 50 ml of reverse osmosis (RO) water. This was then mixed and autoclaved at 121 °C for 15 min.

2.2.4 Buffered Peptone Water

BPW (10 g) of was added to 500 ml of RO water and then mixed and autoclaved at 121°C for 15 min to sterilise.

2.2.5 Palcam Agar

Palcam Agar was prepared by adding 34.5 g of Palcam agar base to 500 ml of RO water. This was mixed and autoclaved at 121 °C for 15 min to sterilise. One freeze dried vial of *Listeria* selective supplement was reconstituted in 2 ml sterile water. Once the molten agar had cooled to approximately 55 °C, 2 ml of the reconstituted selective supplement was

aseptically added to the agar and mixed thoroughly. The agar was then poured into sterile petri dishes.

2.2.6 XLD Agar

XLD was prepared by adding 26.5 g of XLD agar base (Oxoid) to 500 ml of RO water. The media solution was then heated until the mixture had gently boiled for approximately 10 min and the media was stirred using a magnetic stirrer during this step. The media was then cooled to 50 °C before being immediately poured into sterile petri dishes.

2.2.7 Pseudomonas Agar

Pseudomonas agar base CM559 (24.2 g; Oxoid) was diluted in 500 ml RO water. To this 5 ml of glycerol (100 %) was then added before autoclaving at 121 °C for 15 min to sterilise. The media was then cooled to approximately 50 °C and 1 vial of pseudomonas C-F-C supplement (Oxoid) reconstituted 2 ml with sterile water was aseptically added. The agar media was then poured in sterile petri dishes.

2.3 Cultivation of Bacteria

Cultures were prepared by taking a single colony and loop inoculating it into 6ml of BHI broth. These were then incubated at 30 °C, with shaking at 150 RPM overnight. All bacterial strains were taken from the University of Nottingham collection and details of strains used are shown in Table 1.

2.4 Measurement of Cell Number

2.4.1 Optical Density

Optical density was used to determine approximate cfu ml⁻¹ in cultures and thus estimate levels of growth. An Eppendorf Biophotometer Plus spectrophotometer was used to measure optical density. To do this a sample (1 ml) of the culture was put into a 1.5 ml cuvette (Sarstedt) and optical density measured at OD_{600nm}. If required the culture was diluted using BHI broth until an OD_{600nm} value in the linear range (0.05 to 0.9) was reached.

2.4.2 Viable Count

A 10 fold serial dilution of cultures was performed using MRD. Samples were then plated onto BHI agar using the Miles-Misra method. Results were recorded as cfu ml⁻¹.

2.4.3 Automated Plate Reader

A Tecan plate reader was set to monitor cell density at 600nm and the number of reads per sample was set at 10. Temperature was set to 20 °C with a range of 18-25 °C. The shaker was set for 20 sec in orbital mode at 200 rpm to occur before a measurement was taken. The number of cycles was set to 36 at intervals of 2400 s. this was designed through the multi labelling kinetic function and the measurement mode set to absorbance. The plates used were Porvair 96 well plates, with samples loaded in wells B2 to G11 to avoid sample drying effects (see section 2.5.2).

2.4.4 Determining Growth Rate

To calculate growth rate, where μ is the rate, the following equation should be used:

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

The specific growth rate is defined as μ . N_0 is the initial number of cells at time 0 (t_0) whilst N_x is the number of cells present at time x (t_x).

2.5 Microbial Challenge Experiments

2.5.1 Preparation of Antimicrobials

Each antimicrobial was prepared to working strength, as shown in Table 2 below, using sterile water. Powdered compounds were reconstituted to working strength and then vortexed to ensure they had mixed according to the instructions provided by the supplier. Antimicrobials were stored at room temperature protected from light in a sterile universal tubes. Preparation was completed as when they were needed.

Table 2-2 – **The working strength of each compound used in this project.**

Compound	Working Strength
A	0.6%
B	1.4%
C	1.4%
D	1.4%
E	1.4%
F	1.0%
G	1.4%
H	0.8%
J	3%
K	0.9%

2.5.2 Automated Plate Reader

Standardly Porvair 96 well plates was used and set up as shown in Figure 2-1 below. Water was added to the wells marked W to help prevent the evaporation of samples. Each test used 5 bacteria types, and each dilution series was performed in duplicate. Therefore each bacteria occupied 10 wells as shown by the orange and blue in Figure 2-1.

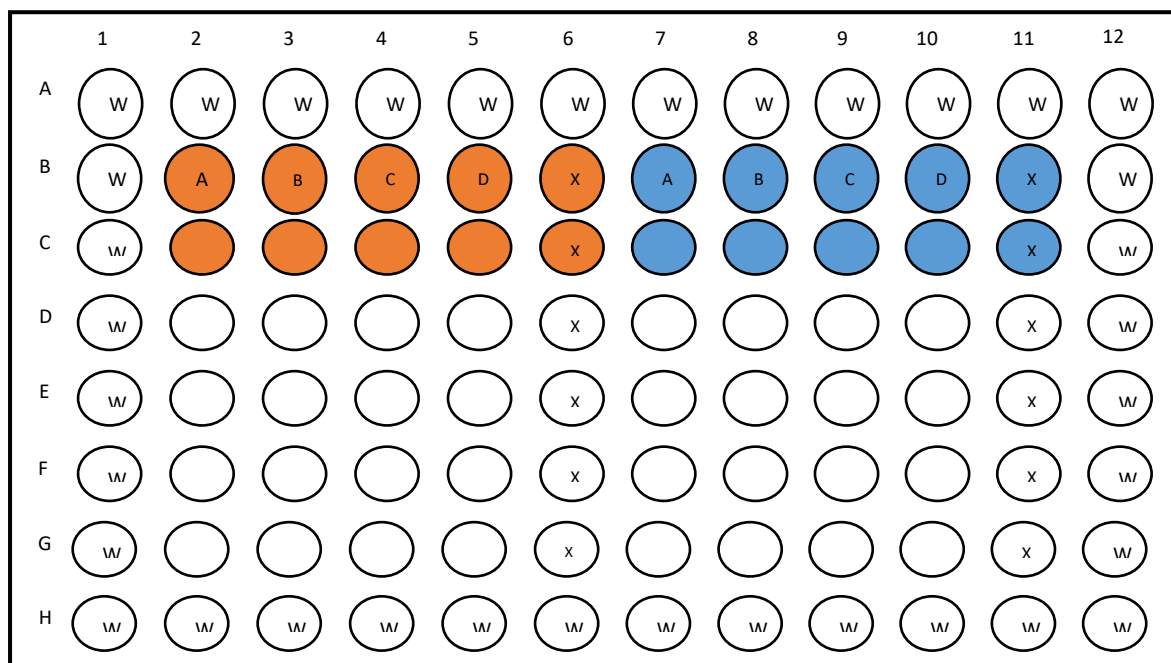


Figure 2-1 - **Layout of 96 well plate for Tecan.** W represents wells containing water whilst X denotes the control. A-D represent wells containing serial dilutions. The orange and blue give an example of the plate layout with orange being the first bacteria and blue the second.

A serial dilution was performed using each antimicrobial compound. The initial concentration of the compounds was 40X. To do this 180 µl BHI broth and 20 µl of the stock solution of the compound was added to the first well, producing a concentration 2X, (well marked A in figure 2-1). From this well, a 100 µl sample was removed and added to the adjacent well (marked B) which already contained 100 µl of BHI broth. This process was repeated until well D was reached at which point 100 µl was discarded thus creating a 2-fold dilution series of the compound. To each well 100 µl of culture at an OD_{600nm} of approximately 0.05 was then added, including the control wells. The plate was then placed into the Tecan where it was sampled every 40 min for 24 h.

2.6 Ames Test

2.6.1 Growth Media

2.6.1.1 Vogel Bonner Salt Solution (50X)

Into a 2 l Erlenmeyer flask containing a magnetic stirrer the following were added to 670 ml of warm RO water: 10 g magnesium sulphate ($MgSO_4 \cdot 7H_2O$), 100 g citric acid monohydrate ($C_6H_8O_7$), 500 g potassium phosphate (K_2HPO_4) and 175 g sodium ammonium phosphate ($NaH_2NH_4(PO_4 \cdot 4H_2O)$). The solution was then adjusted to 1 l before

being aliquoted into 200 ml volumes which were then autoclaved for 20 min at 121 °C to sterilise.

2.6.1.2 Glucose Solution (40 % [w/v])

of D-glucose (400 g) was added to 700 ml of RO water in a 2 l Erlenmeyer flask, and the solution was stirred using a magnetic stirrer until the glucose had dissolved, then the volume adjusted to 1 l. The solution was then split into 200 ml aliquots before being autoclaved at 110 °C for 10 min to sterilise (lower temperature/time profile was used to avoid caramelisation reactions).

2.6.1.3 Limited Histidine and Biotin Solution (0.5mM)

D-biotin (31 mg; F.W. 247.3) was added to 250 ml of RO water. The solution was sterilised by filtration using a 0.22 µm filter (Swinex).

2.6.1.4 Minimal Glucose Agar

Agar No. 2 (7.5 g; Oxoid) was added to 465 ml of RO water and this was then mixed to distribute the solids before it was autoclaved at 121 °C for 20 min. The molten agar was allowed to cool until it was approximately 55 °C then 10 ml of VB salt solution (50X; section 2.6.1.1) and 25 ml of glucose solution (40 %; section 2.6.1.2) were added to this aseptically. Dependent upon the Ames strain to be used (see Table 3), the media was supplemented with either ampicillin alone (24 µg ml⁻¹) or ampicillin and tetracycline (2 µg ml⁻¹) were added to the agar before it was poured. After all of the required components had been added to the agar, it was thoroughly mixed and 25 ml of agar was transferred into sterile petri dishes using a 25 ml pipette.

2.6.1.5 Top Agar

To prepare this 3 g of agar No. 2 (Oxoid) and 3 g of NaCl were added to 450 ml of RO water and thoroughly mixed. To this 50 ml of limited histidine and biotin solution (0.5 Mm) was then added and the mixture was split into 50 ml aliquots before it was autoclaved at 121 °C for 20 min to sterilise.

2.6.1.6 Antibiotics

Table 2-3 – Antibiotics used in Ames test

Antibiotic	Stock Concentration	Working Concentration
Ampicillin	8 mg ml ⁻¹	24 µg ml ⁻¹
Tetracycline	8 mg ml ⁻¹	2 µg ml ⁻¹

2.6.2 Testing Mutagenicity using Ames

Overnight cultures of each of the Ames strains were prepared using 5 ml Nutrient broth, and were grown at 37 °C, with shaking at 200 rpm. The optical density of cultures were

measured at OD_{540nm} to ensure they had reached an optical density of 0.1 and then 0.1 ml of the culture was added to 2 ml molten top agar. The antimicrobial compound being tested was also added to the molten top agar solution at concentration 1X. This was done using a mini incubator set to approximately 45°C to allow the agar to remain molten yet cool enough not to kill the bacteria being added. Once added to the molten top agar the mixture was then poured onto set minimal glucose agar plates and spread to ensure the whole of the plate was covered. The plates were then left for a couple of minutes to set. The solution was then mixed and poured onto a minimal glucose agar plate, which also contained the antibiotics required for selection of strains if required. For the positive control a mutagenic compound was used by pipetting 10 µl of sodium azide (0.15 mg ml⁻¹) on to a sterile filter disc (6 mm, Whatman) which was placed in the centre of the plate. After allowing the agar to set, the plates were inverted and covered in foil before being incubated at 37 °C for 48 h and each test was performed in triplicate. The number of colonies on the plates were counted after 48 h.

2.7 Testing of compound in Sausage as a model food system

2.7.1 Slicing of commercial products

The type of sausage used was Italian mortadella-style sausage supplied by Phil Stroud Solutions Ltd. The plastic covering was removed and the meat sliced using a circular meat slicer. Each slice was cut to be 3 mm in thickness. Smaller slices for antimicrobial analysis were then cut from the larger meat slices using a cookie cutter to give a slice of 75 mm diameter.

2.7.2 Preparation of sausage meat slurry

A slurry was formed by mixing chopped pieces of mortadella sausage using a Thermomixer in the University of Nottingham food hall using hygienic procedures required for commercial food handling. The sausage was mixed for 5 min at 500 rpm until a thick paste was formed.

2.7.3 Inoculation of sausage Slices

Overnight cultures of *L. monocytogenes* were prepared using BHI broth and incubated at 37 °C, grown with shaking at 200 rpm overnight. These cultures were then diluted to approximately 10⁶ cfu ml⁻¹ in BPW either with or without the compound to be tested at 1X concentration. Each slice was dipped into the solution for 30 s and then were left in a

Class II Microbiological Safety Cabinet for 20 min to drain and remove any excess liquid from the slices.

2.7.4 Inoculation of Slurry

Overnight cultures of *L. monocytogenes* were prepared at 10°C, 150 rpm shaking. The slurry prepared as described in section 2.7.2 was placed into a stomacher bag containing 100 ml BPW, which was supplemented, when relevant, with the compound at a 1x concentration (section 2.5,1) and was inoculated with cells at final density of 10^6 cfu ml⁻¹. The meat slurry and material suspended in the BPW were mixed in a stomacher bag (Seward) to ensure the slurry was coated in the solution. Aliquots of this mixture (20 g) were transferred into petri dishes and were incubated

2.7.5 Sampling

At each testing point slices or slurry samples were homogenised using a stomacher machine (Seward 400 circulator). To do this 100 ml BPW was added to a stomacher bag containing the meat sample and was stomached for 1 min at 300 rpm. Samples (1 ml) of the resulting suspension were taken and a 10 fold serial dilution performed using MRD. These were plated on either selective (enumeration of *Listeria* spp.) or non-selective (viable count) agars. At day 0 only, the diluted samples were plated using Miles and Misra onto Palcam agar plates. At other time points duplicate spread plates of three dilution points were used. The Palcam plates were then incubated at 37°C for 48 h. *L.monocytogene* colonies were then counted and the results recorded.

2.8 Mince

2.8.1 Inoculation Method One

The minced pork was sourced from a local food retailer (Morrisons Ltd) and was specified to be 100% pork mince, <5% fat . Overnight cultures of relevant gram negative bacteria were prepared at 30°C, 200 rpm shaking. Mince samples were placed in a stomacher bag containing a 1:1 ratio BPW to mince. This was then accompanied by compound at a 1x concentration (section 2.5,1) where needed and was inoculated with cells at final density of 10^6 cfu ml⁻¹. The mince was mixed in the stomacher bag (Seward) to coat the mince. Aliquots (20 g) of mince were taken and placed in petri dishes and allowed to dry for approximately 10 min.

2.8.2 Sampling

For testing, a 20 g minced pork sample was placed in a stomacher bag with 200 ml of BPW and were stomached for 1 min at 300 rpm in a Seward 400 stomacher. Samples (1 ml) of the resulting suspension were removed and serial dilutions performed using MRD

before samples were plated out onto the appropriate type of agar. Plates were then incubated at 37°C overnight and colonies counted and results recorded.

Chapter 3 Results

3.1 Screening Test

3.1.2 Introduction

The overall aim was to determine whether any these compounds would prevent the growth of food borne pathogens such as *L. monocytogenes*, *Salmonella spp.* and *E. coli spp.* in a food product to improve food safety. As well as this, by preventing the growth of spoilage organisms the shelf life of a product can be increased allowing the consumer to keep food longer and reduce food waste. The compounds being tested were naturally produced by fermentation or were extracted from plants. This allows them to be used and food be given a 'clean' food label which is a current consumer trend.

The antimicrobials being tested were provided by Phil Stroud Solutions Ltd. Each one is a variation of one another with them all being based on naturally occurring organic acids. They are identified by lettering throughout this write up with compounds A-F being the originally provided six, compound G being another variation added later and compound H being a known industry product used in meat.

Each compound was given a working strength by the providers, these were given as percentages and can be seen in section 2.5.1, table 2. The antimicrobials were made up to the working strength using sterile water, this was identified as 1X. Throughout this write up 1X refers to the working strength, with 2X being double this and 0.5X and 0.25X half and a quarter of the working strength respectively. This was done as the exact make up of each compound was chemically different therefore this provided an appropriate way to make each compound comparable.

3.1.3 Aim

The aim of this experiment was to determine whether the antimicrobial compounds provided had any bacteriostatic or bactericidal effects against any of the five bacteria species using rapid, microtitre plate assay format. These would be tested alongside a standard product used in the food industry. The bacteria that were chosen for this assay were *L. monocytogenes*, a well-known food borne pathogen of concern to the food industry, and *Listeria innocua* which is an environmental contaminant common in the food environment. In addition to these two Gram-positive bacteria, three Gram-negative bacteria were included in the test panel. These were *Salmonella enterica* serovar Typhimurium, *E. coli* and *Pseudomonas fluorescens* as representative pathogens, indicator organisms and spoilage organisms from the Gram-negative group, respectively.

The aim of using this screening test was to show whether the different compounds were having any effect on the growth of the bacteria during a 24 h incubation period.

3.1.4 Experimental Design

To carry out these experiments, cultures of each of the bacteria were grown overnight in BHI broth and diluted using fresh BHI media to an OD₆₀₀ of 0.05 to ensure that at the start of the experiment each of the bacterial cultures started the experiment at approximately the same cell density (cfu ml⁻¹). To allow different concentrations of the compounds to be tested, a serial dilution of each compound was performed in a sterile Tecan microtitre plate (see section 2.5.2; figure 2-1) using BHI broth to dilute the compounds to produce a range of concentrations. Initially the compounds were diluted in BHI broth in the well to a concentration of 2X the normal working strength. A serial dilution was then performed to dilute the compounds in the other wells to concentration of 1X, 0.5X and 0.25X working strength and also a control well that contained no compound (0X; control). These concentrations were chosen as they represent the ideal (1X) and also minimum (and therefore most economic) concentrations for industry as well as concentrations which should be high enough to definitely show some effect (2X).

Once the serial dilution of the compounds had been prepared, 100 µl of the bacterial culture was added to each well. The plate was then incubated in a Tecan plate reader run for 24 h set to measure absorbance (A = 600 nm) taken every 40 min section 2.4.3). It was originally intended that the temperature to be maintained between 18°C and 21°C. This temperature range was chosen as it represented a temperature where *Salmonella*, *E. coli* and *Pseudomonas* would still easily grow in 24 h and the *Listeria spp.* being tested would have adapted to its physiological state when it grows in the environment rather than when it grows inside an animal host (Horn and Bhunia, 2018). However, there was some difficulty with this as the temperature of the Tecan depended upon the ambient temperature in the laboratory due to the unusual high temperatures that the UK experienced this year. Therefore the decision was taken to alter the temperature range to between 18°C and 25°C as this should not affect the growth of the bacteria and is still below the environmental adaptation temperature for *Listeria* when it induces motility only seen during environmental growth (Peel et al., 1988).

The results gained were plotted as growth curves and are shown in Figures 3-1 through 3-5. Each graph shows the effect of each concentration of the compound tested in a challenge experiment using one of the five bacteria. Each sample was tested in duplicate and then the average of the two absorbance's taken to generate the results shown on the graph. After the initial screening, some compounds that showed most effect were tested

more than once (compound D) to confirm that the method was successful and that the results were reproducible. The other compound tested more than once was compound H which was the industry standard and was used to compare the effect of novel compounds being tested.

3.1.5 Results

Due to the volume of data generated from this experiment only a selection of graphs are shown. These are those which are helpful in interpreting the data which justify decisions made later in this project. For Compound A, graphs for all five bacteria are shown there on after only the graphs which are of interest and discussed are shown. All other graphs with the data for this experiment can be seen in Appendix A.

Error bars of the standard deviation can be seen on the first graph of compound A. They have not been included past this as the error bars make the graphs difficult to interpret. The ranges of results and standard deviations are small therefore it has been determined that the data is better represented without error bars present.

3.1.5.1 Compound A

The results for Compound A are shown in Figures 3-1 to 3-5. As seen in figure 3-1, although at 2X concentration there is initially evidence of lowered growth, there is very little effect of this compound on the growth of *L. monocytogenes* and therefore there is no evidence of an antimicrobial effect on these types of cells. This was confirmed by the results shown in figure 3-2 since the growth of *L. innocua* was also not significantly inhibited by compound A and as members of the same species it would be expected that there would be a similar outcome for both types of bacteria. Compound A also did not seem to have a significant effect on the growth of the Gram-negative bacteria tested. As shown in the graph in figures 3-3 and 3-4 the difference in growth between 0X (control) and 2X concentration was minimal for *Salmonella* and *E. coli* apart from a small reduction in growth for 2X and 1X concentrations. In panel E compound A showed some effect against the *Pseudomonas spp.* as the growth was reduced in the highest three concentrations tested and the lag phase extended to around 200 min and the final cell density reached was also lower and therefore compound A seemed to have some effect on the growth of *P. fluorescens*. Overall compound A did not have any significant effect on the growth of the bacteria tested apart from *P. fluorescens* and so this combination of organic acids does not seem a suitable option for preventing food borne pathogens, however this combination may be of use to control the growth of spoilage organisms.

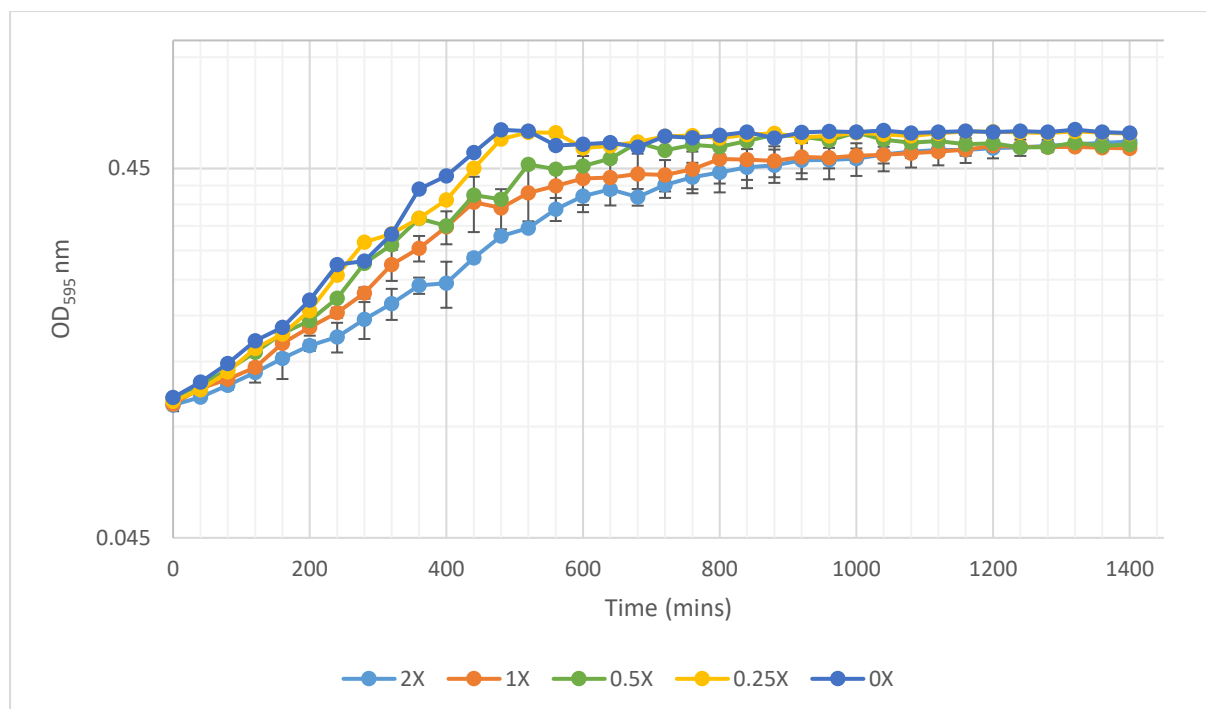


Figure 3-1 - The effects of Compound A on growth of *L. monocytogenes* at five different concentrations: Cultures of *L. monocytogenes* [10403S] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2). Error bars represent $\pm$ SD.

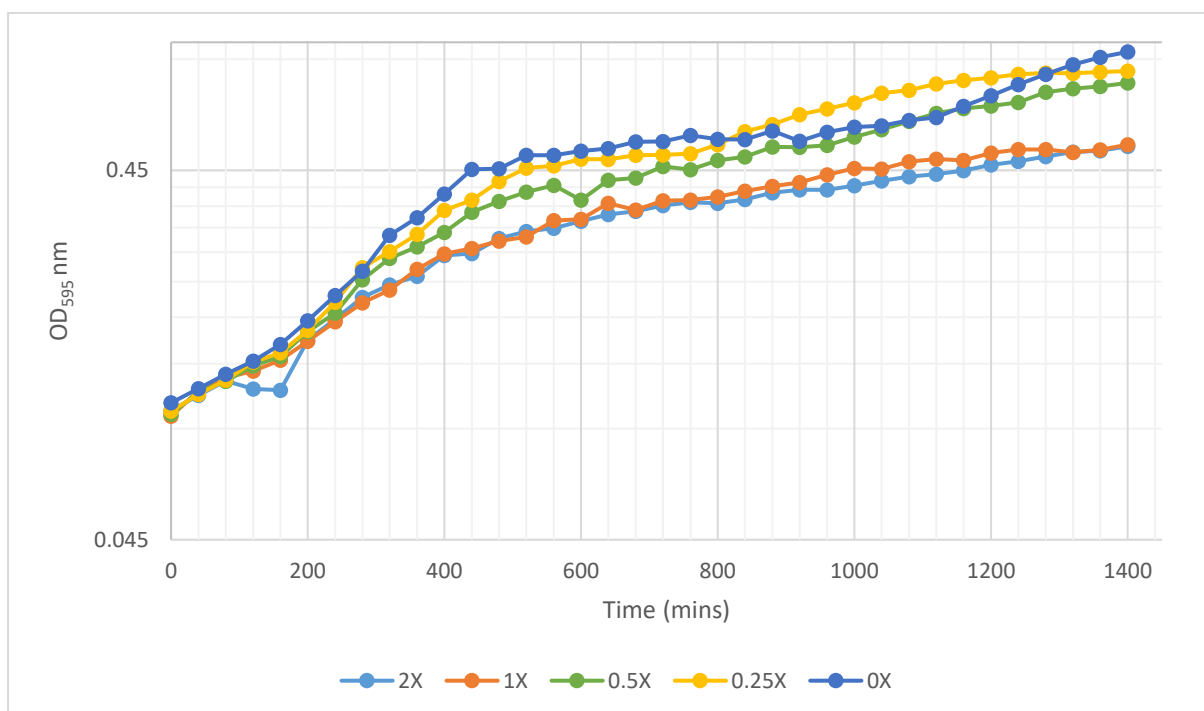


Figure 3-2 - The effects of Compound A on growth of *L. innocua* at five different concentrations: Cultures of *L. innocua* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

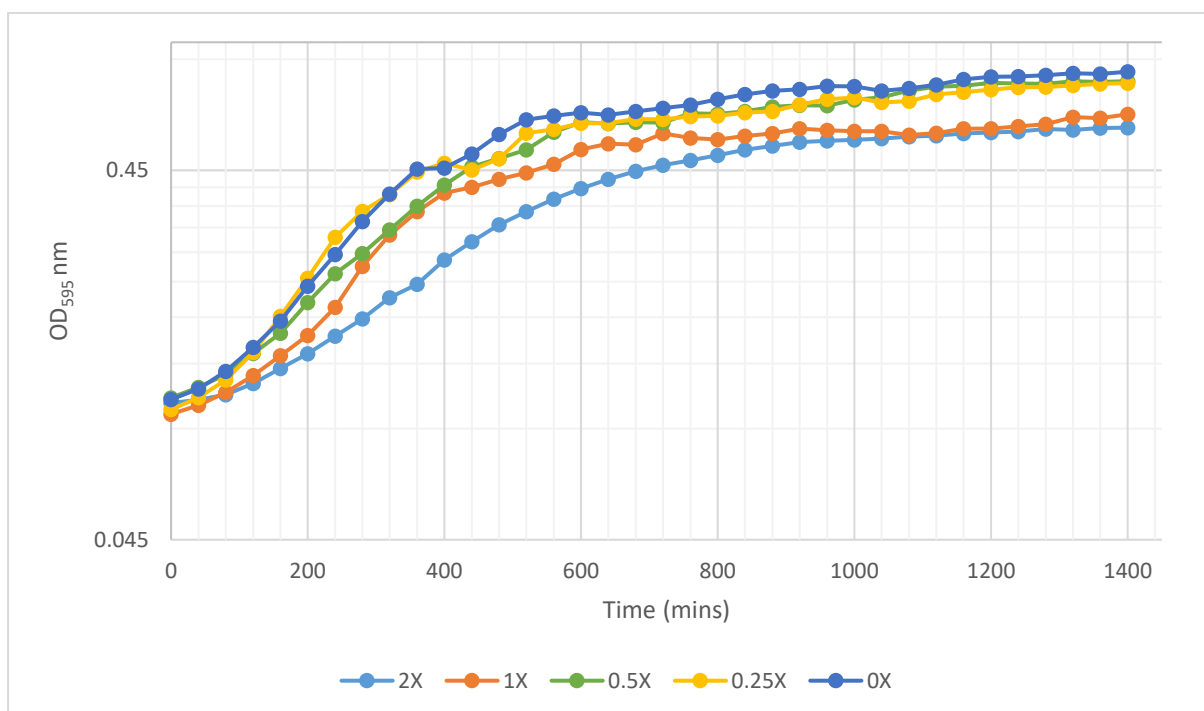


Figure 3-3 - The effects of Compound A on growth of *S. Typhimurium* at five different concentrations: Cultures of *S. Typhimurium* [DB7516] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

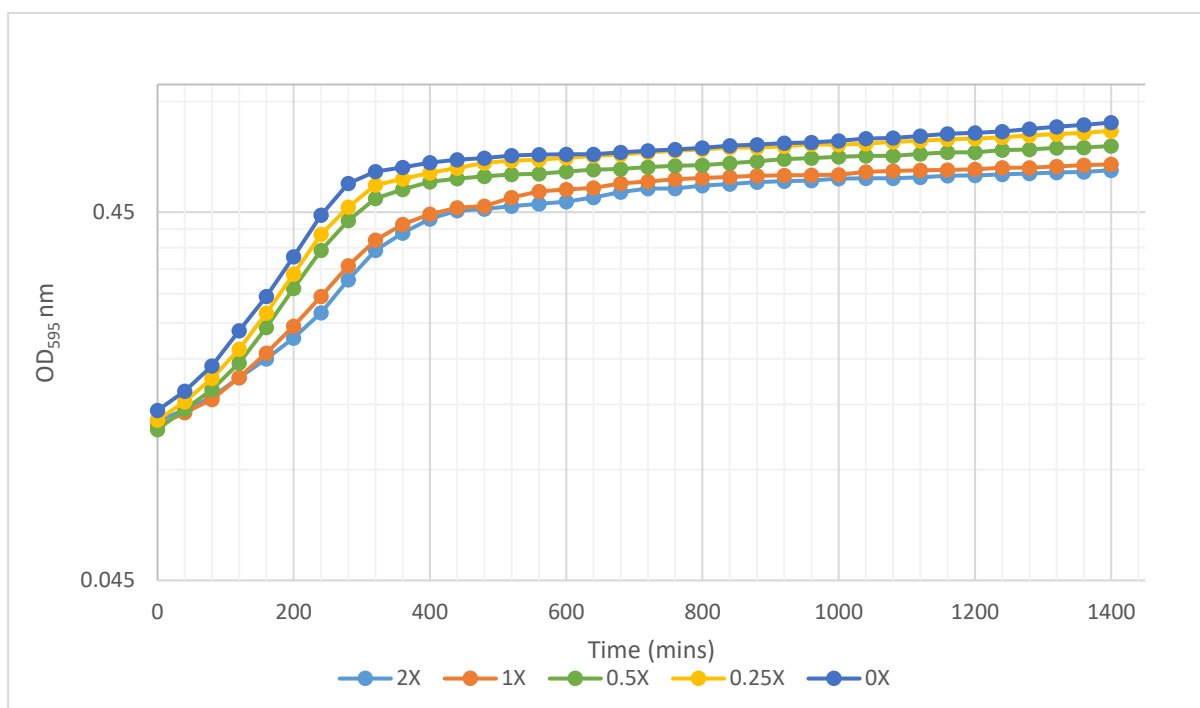


Figure 3-4 - The effects of Compound A on growth of *E. coli* at five different concentrations: Cultures of *E. coli* [MG1655] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

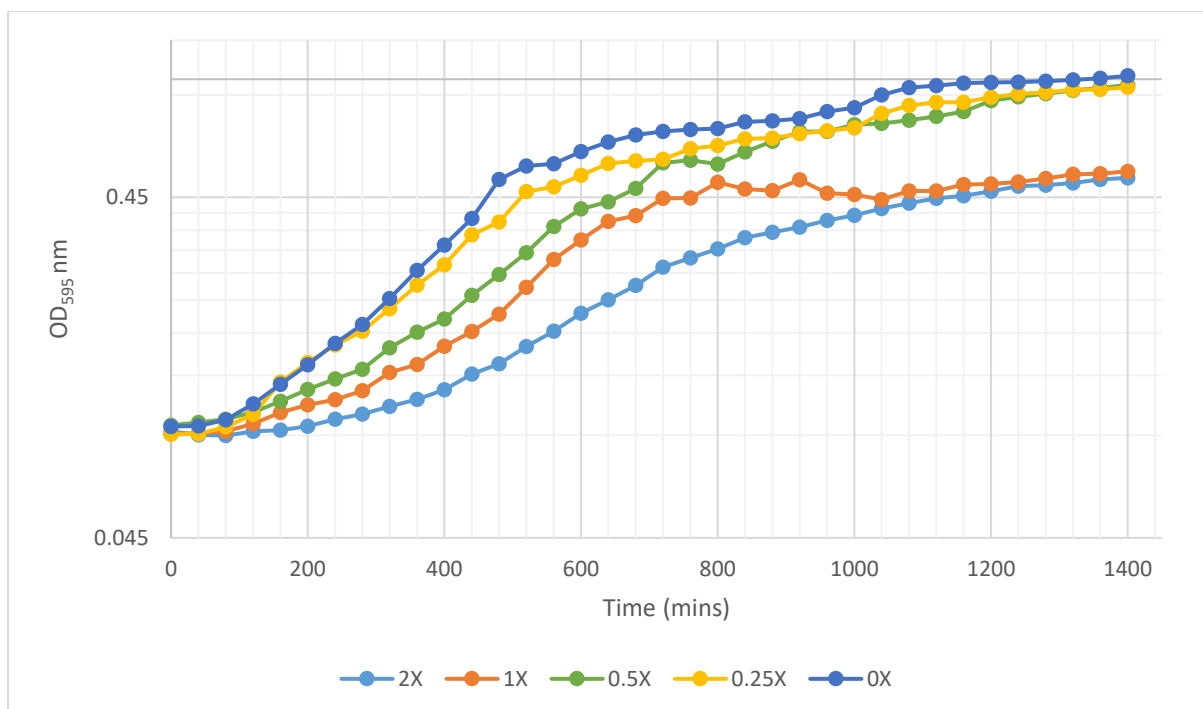


Figure 3-5 - **The effects of Compound A on growth of *P. fluorescens* at five different concentrations:** Cultures of *P. fluorescens* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

3.1.5.2 Compound B

In figure 3-6 the results are shown for compound B, only one graph is shown the others can be seen in appendix A. The effect at 2X on the growth of *L.monocytogenes* is pronounced, the growth is inhibited to an extent that there is no real increase in absorbance until 400 min. When the growth rate does start to increase it is at a significantly slower rate to the other concentrations. At other the concentrations there is no significant difference between them and the control other than perhaps slightly slower growth initially at 1X concentration. These results are confirmed with *L.innocua* where the same trends are seen. The gram negative bacteria show the same effects to compound B as the gram positives. The difference here is that when the growth plateaus it is at the same level as the control. An exception to this is *P.fluorescens* as seen in figure 3-6 below; at 2X concentration it does not show any growth therefore compound B at this concentration has completely inhibited growth. Growth at 1X has also slowed with an extended lag phase until 400 min. 0.5X and 0.25X show reduced growth in comparison to the control.

Overall compound B has a good effect slowing the growth of all five bacteria species at concentration 2X. There was also some slowing of growth at concentration 1X but not as

a significant reduction. As with compound A, compound B was most effective against *Pseudomonas* with decreased growth at all concentrations. Overall the screening has shown that compound B has potential to be used as an antimicrobial at concentration 1X or 2X.

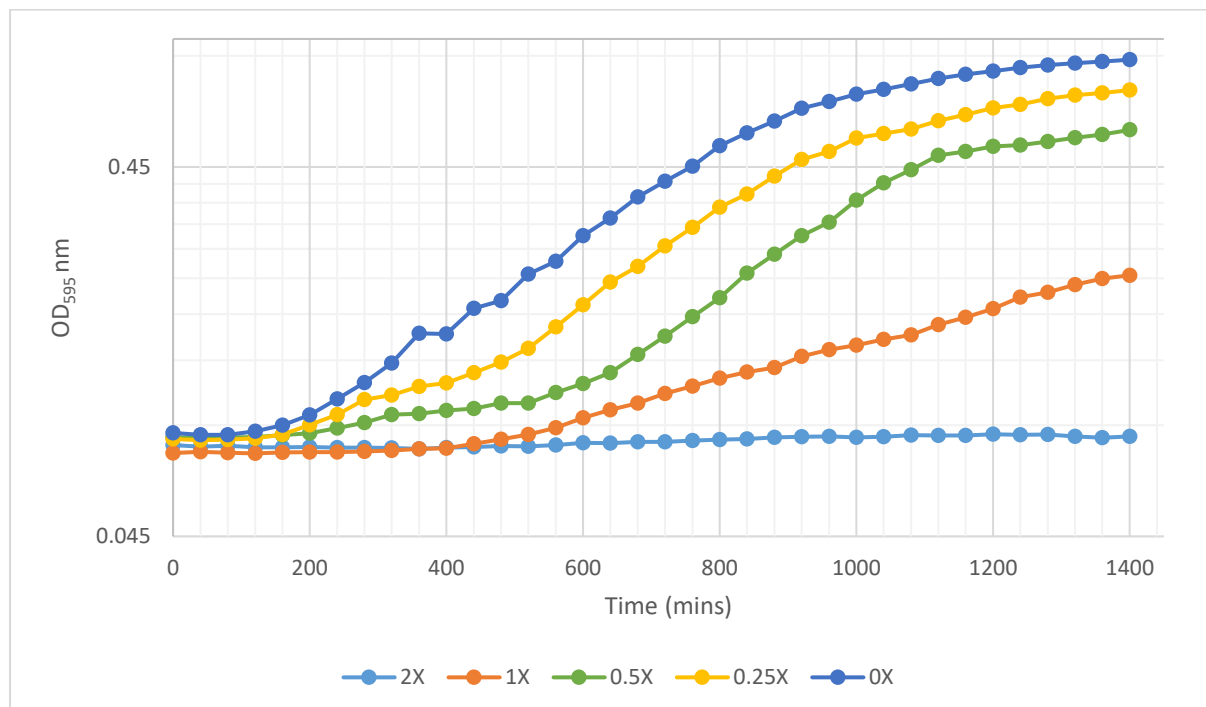


Figure 3-6 - **The effects of Compound B on *P. fluorescens* at five different concentrations:** Cultures of *P. fluorescens* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

3.1.5.3 Compound C

Compound C at concentration 2X shows reduced and slowed growth of *L.monocytogenes* with the increase in growth only starting after approximately 300 min. At 1X growth appears to be slowed in comparison to the control however it continues to increase and reaches the same point after 24 h. There is nothing to compare between the control and 0.5X and 0.25X. The same effect can be seen with *L.innocua*. Compound C's effect on *S. Typhimurium* shows reduced growth at 2X concentration with increased growth occurring after an extended lag phase of approximately 400 min. Concentration 1X also shows slower growth in comparison with the control, again with a lag phase of 400 min. At 0.5X there is slightly reduced growth comparably to the control however this is not particularly significant. The effects of compound C on *E.coli* can be seen in Figure 3-7 below. Concentrations 2X and 1X show reduced growth. At concentration 2X growth appears to be inhibited through an extended lag phase for the first 500 min with no real increase in growth rate occurring. 1X concentration has a much steadier growth rate however it is still reduced compared to the control. At 0.5X and 0.25X there is slightly

reduced growth compared to the control. *P. fluorescens* shows an effect similar to the effect on the other gram negative bacteria. There is the same effect at concentrations 2X and 1X. 0.5X concentration shows a slightly slower growth rate compared to 0X.

Compound C has shown effect against all five bacteria species at concentration 2X. At this concentration it would have potential as an antimicrobial from screening test results. 1X concentration has an effect also on all five bacteria however the reduction is growth is smaller. In comparison to compound A and compound B, compound C has performed similarly against the gram positive and gram negative bacteria therefore from this screening it could be said that compound C has more potential as an all-round antimicrobial for spoilage and pathogenic bacteria if used at concentration 2X.

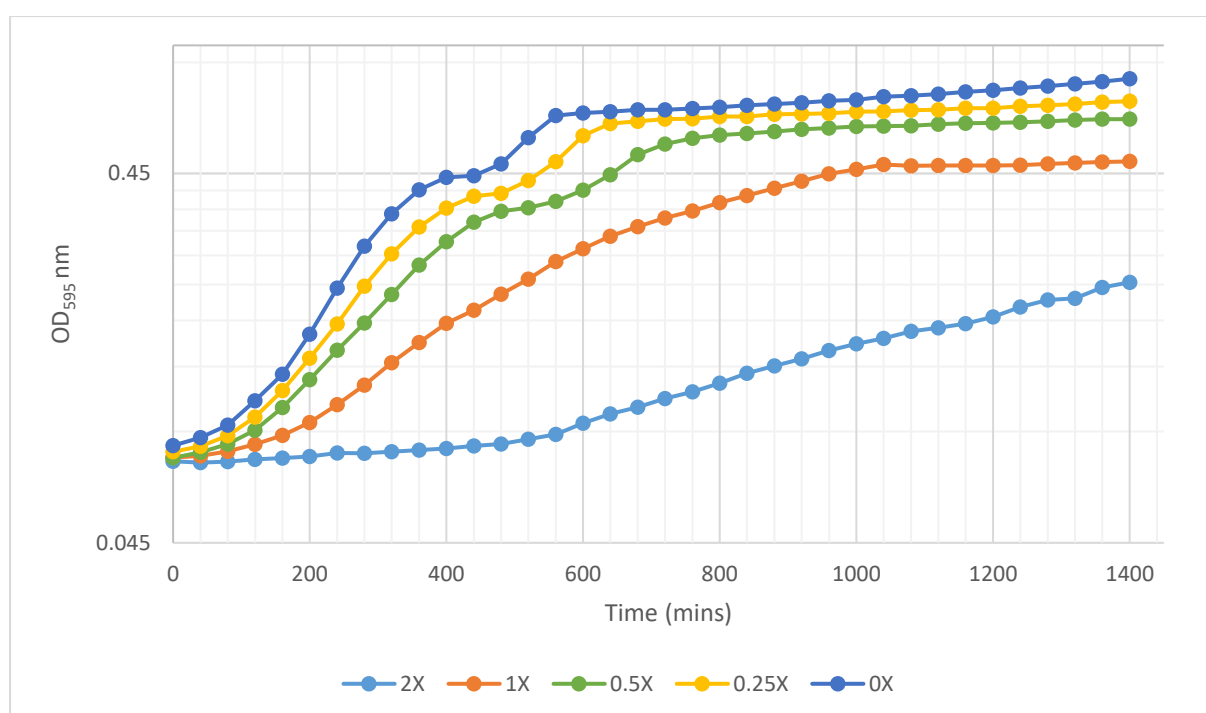


Figure 3-7 - **The effects of Compound C on *E. coli* at five different concentrations:** Cultures of *E. coli* [MG1655] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

3.1.5.4 Compound D

Two runs of compound D were completed, as this was the first compound which was tested using the Tecan method. The two runs were to ensure that comparable results were generated on each run. After this initial compound only one run per compound was completed as there was confidence in the method after this point.

Each run has shown similar results of compound D against *L.monocytogenes*. Concentration 2X has shown reduced growth over the full 24 h, whereas 1X shows reduced growth initially however after 1000-1200 min it has reached the same maximum growth as the control. 0.5X is shown to probably have a slower growth rate over the first 800 min before growth begins to exceed that of 0X, the control. With *L.innocua* which can be seen in Figure 3-8 below concentration 2X shows reduced growth as expected having seen the effects on *L.monocytogenes*. 1X concentration also shows a probable slower growth rate than the control. At 0.5X concentration there is a smaller difference between the control and 0.5X than with *L.monocytogenes* however there is still less growth. Using compound D against *S.typhimurium* has shown effect at 2X, 1X and 0.5X. The growth is significantly less than that of the control. In the second run 1X had a greater effect than 2X however this is probably due to an anomaly in the data. This still shows however that there is an effect from this concentration. There is some effect at all of the concentrations used against *E.coli*. As can be seen each concentration line follows a similar trend but at a slightly lower absorbance each time. The lower absorbance represents decreased growth as more light is passing through the sample. 2X concentration has the greatest effect with there being an extended lag phase until 200 min approximately, however every other concentration begins to increase after 0 min there is a plateau beginning to start after 400 min. The effect on *P.fluorescens* is similar to that on *E.coli*. The two repeats do show slight differences in growth patterns however the trends are similar. It appears that in the first run the lag is longer in the second run does not occur. There is a reduction in growth using all concentrations with growth plateauing at a lower level to that of the control.

Compound D appears to be a good overall antimicrobial from the results shown in this screening test. There is also more effect at lower concentrations, 0.5X for gram positives and 0.5X and 0.25X for gram negatives. Working at lower concentrations is beneficial to industry as less of the product is required to gain the same effect. Therefore there is potential for this compound to be used against all five bacteria species at, at least 0.5X concentration making it more attractive to industry.

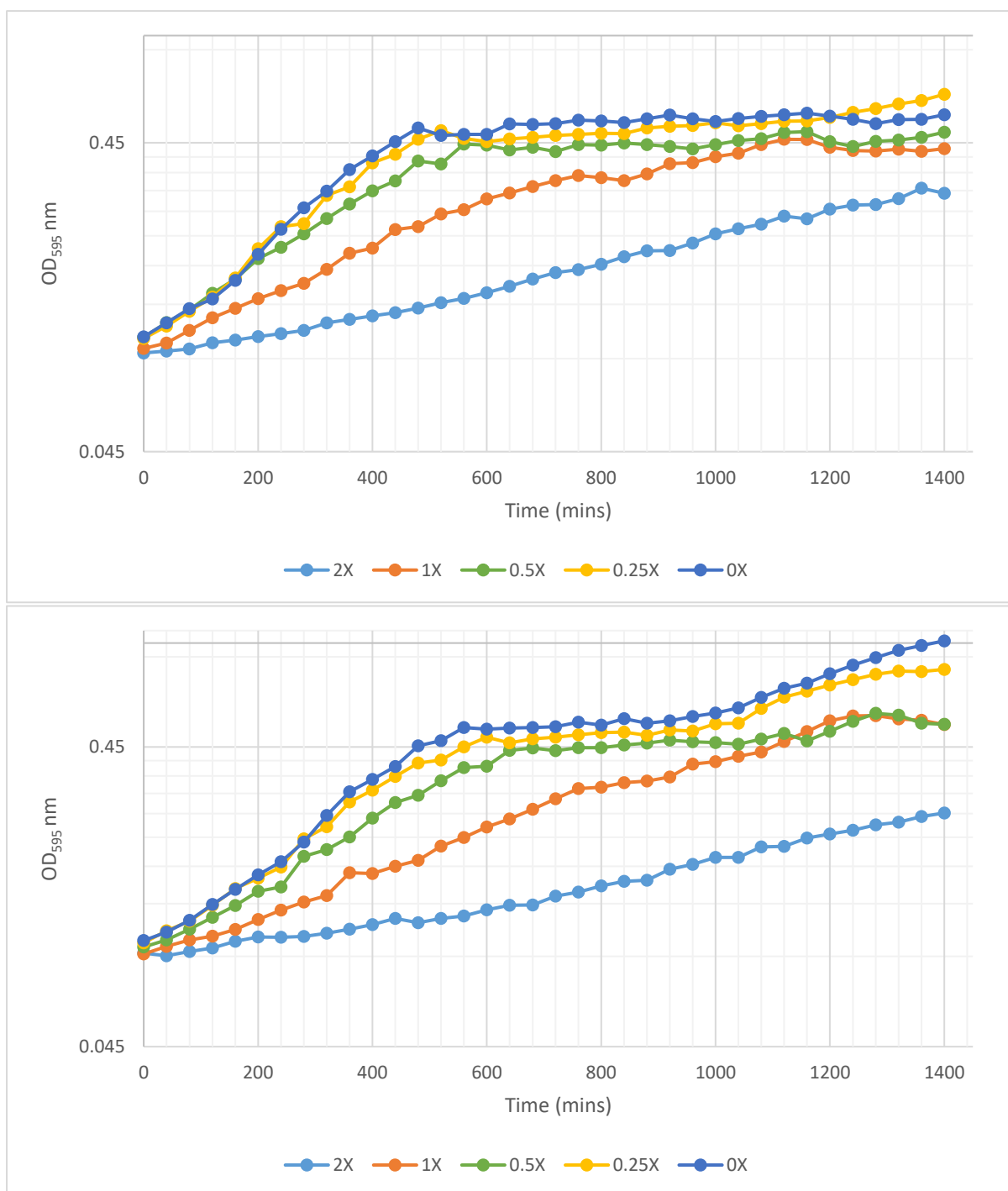


Figure 3-8 - The effects of Compound D on *L. innocua* at five different concentrations (top graph is run 1, bottom is run 2): Cultures of *L. innocua* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

3.1.5.5 Compound E

Compound E's effect on *L.monocytogenes* can be seen Figure 3-9 below. Growth is slowed at 2X concentration with the lag phase extending to approximately 400 min. The lag phase for concentration 1X is extended slightly from that of the control by 100-150 min. Generally growth is reduced also at concentration 0.5X however there is a sharp increase in growth rate at 1200 min however this could be due to some form of anomaly. 0.25X concentration shows slightly decreased growth compared to the control however the difference is quite small. The same effects are seen with *L.innocua* however at concentration 2X the lag phase is extended further too almost inhibit the bacteria's growth over the 24 hour period. At concentration 0.25X there is the same initial growth rate as 0X, the control, but the compound manages to slow the growth after 600 min. compound E's effect on the gram negative bacteria is very similar to that of the gram positive. At concentrations 2X, 1X and 0.5X growth is reduced compared to the control. The lag phase at concentration 2X and 1X is extended to approximately 300 min. The 0.25X concentration is not having any particularly inhibitory effect. The effect on *E.coli* is similar to that of *S. Typhimurium* with no different effect at any concentration. Compound E at concentration 2X almost inhibited the growth of *P.fluorescens* completely, there is only a possible increase in gradient after 800-900 min. At 1X the lag phase lasts until around 500 min and then there is a slow increase in growth over the rest of the 24 h. 0.5X and 0.25X also have an effect on the growth, there is decreased growth compared to the control however the lag phase is the same as that of the control.

Compound E has potential to be effective against both gram positive and gram negative bacteria. From the results of the screening test it appears that as an antimicrobial it could be effective at, at least 0.5X concentration. The compound has shown almost inhibitory effects at concentration 2X with only slow growth occurring after a long lag phase. This occurred in all of the five bacteria species tested therefore it could be a useful antimicrobial for extending shelf life. Concentration 0.25X showed some reduction in growth in the cases of all the bacteria however it may not be enough compared to the control to provide a robust effect.

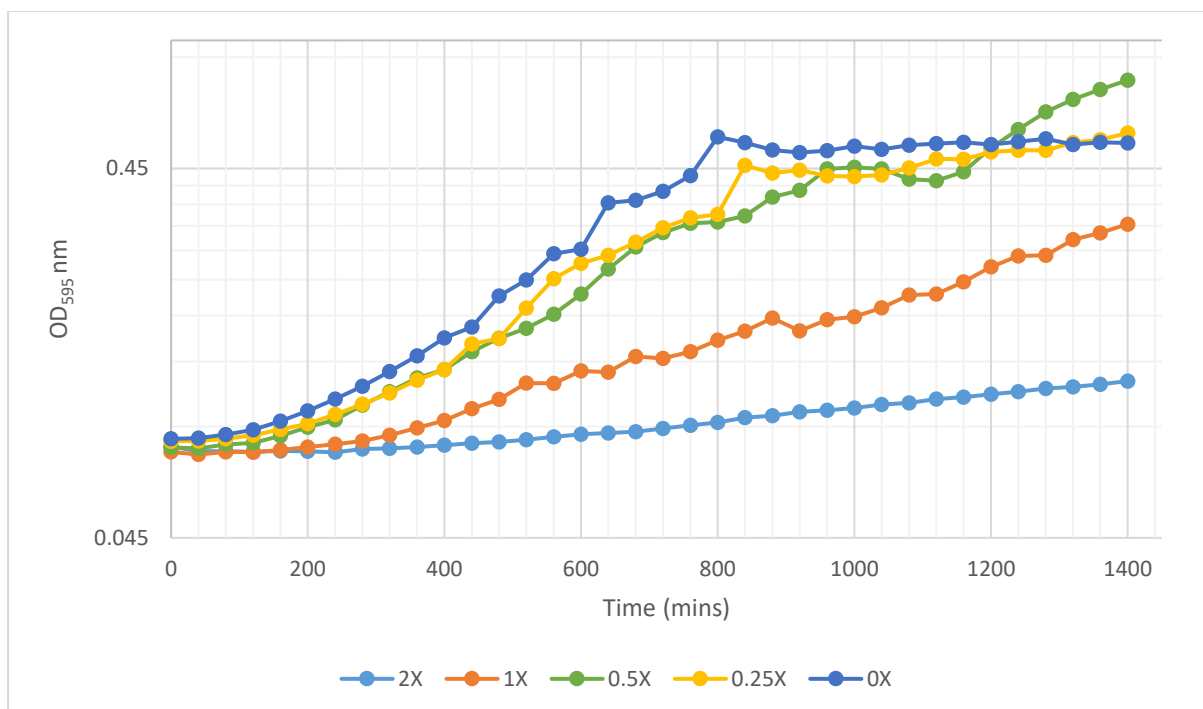


Figure 3-9 - The effects of Compound E on *L. monocytogenes* at five different concentrations: Cultures of *L. monocytogenes* [10403S] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

3.1.5.6 Compound F

Compound F showed little effect on *L. monocytogenes*, the only concentration which is showing any effect on growth is 2X. There is no particular change in lag time however the gradient of the line is smaller therefore growth is slower. None of the other concentrations show any effect compared to the control, 1X growth is slightly slower but not enough to be beneficial as an antimicrobial. The same trends are found with *L. innocua*, the only difference is that concentration 1X shows initial growth to be a bit slower than the control however when the plateau of growth occurs which is after 800 min the growth level is similar to that of 0.25X and 0.5X. Compound F's effect on *S. Typhimurium* is the same as the gram positive bacteria as there is no real effect. Unlike the previous bacteria species, compound F has had an effect on *E. coli* at 2X and 1X. At concentration 2X growth was slowed, the lag phase was the same as the other concentrations yet the maximum growth within the 24 h was less. Concentration 1X shows the same trend but the decreased growth is smaller than that of 2X. The effect of compound F on *P. fluorescens* can be seen in Figure 3-10 below. At concentration 2X growth is inhibited as can be seen by the flat line in the graph. 1X concentration shows an increased lag time of approximately 300 min before growth increases, although the

growth over 24 h is less than the control. At 0.5X concentration the trend is the same as 0X, the control but the growth is slightly reduced. 0.25X concentration follows the same growth as the control.

From the results of the screening test it has been shown that compound F has little effect on the five bacteria in comparison to other compounds tested. There is some effect at 2X concentration but not at any lower concentration with the exception of *E. coli* and *P. fluorescens*. *E. coli* showed some effects in slowing growth at concentration 1X as well as 2X however this wasn't as much of a decrease as seen by other compounds. *P. fluorescens* showed inhibition at 2X and decreased growth at concentrations 1X and 0.5X. However, compound F does not show the overall ability against all five bacteria to effect growth therefore it probably is not as useful as an antimicrobial as some of the other compounds tested.

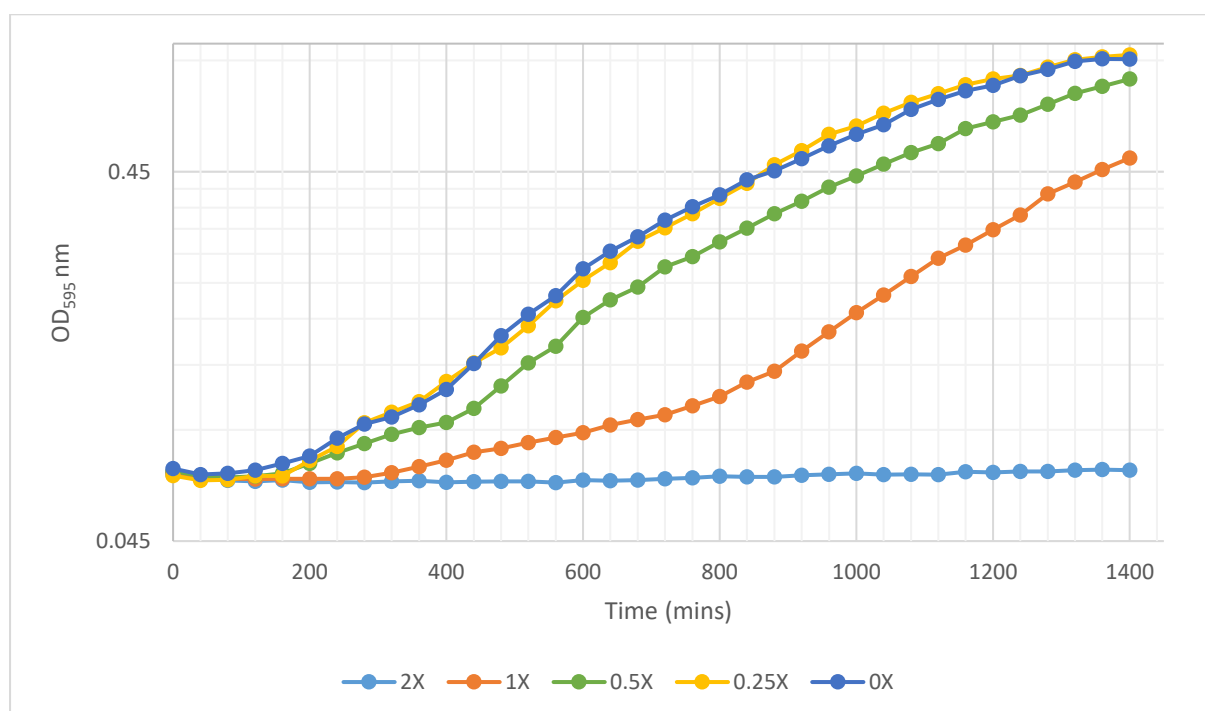


Figure 3-10 - The effects of compound F on *P. fluorescens* at five different concentrations: Cultures of *P. fluorescens* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

3.1.5.7 Compound G

The effect of compound G on *L. monocytogenes* can be seen in Figure 3-11 below. There is effect on the growth of the bacteria at 2X, 1X and 0.5X concentrations. At concentration 2X growth is slowed, the same trend is followed by concentration 1X but at an increased level of growth. Concentration 0.5X follows the same general trend of

the control however initially growth is lower, after 1000 min the growth level reaches the same as the control. 0.25X concentration has no effect as growth is the same as that of the control. Compound F's effect on *L.innocua* is almost the same as that of *L.monocytogenes*. The only difference is that at concentration 2X there is a longer lag phase lasting till approximately 600 min. the effect on the growth of the gram positive bacteria is the same as that of the gram positive. All three bacteria show the same patterns of growth.

Overall compound G appears to have a similar effect against gram positives than gram negative bacteria. From the screening test results it has been shown that when tested against gram positive bacteria compound G has an inhibitory effect at 2X and 1X concentration. When tested against gram negative bacteria there is the same effect against growth at concentration 2X however at concentration 1X there is may be a slightly reduced growth rate compared to the gram positive bacteria. Therefore if compound G was to be used as an antimicrobial, to achieve all round effects against all five bacteria concentration 2X would need to be used.

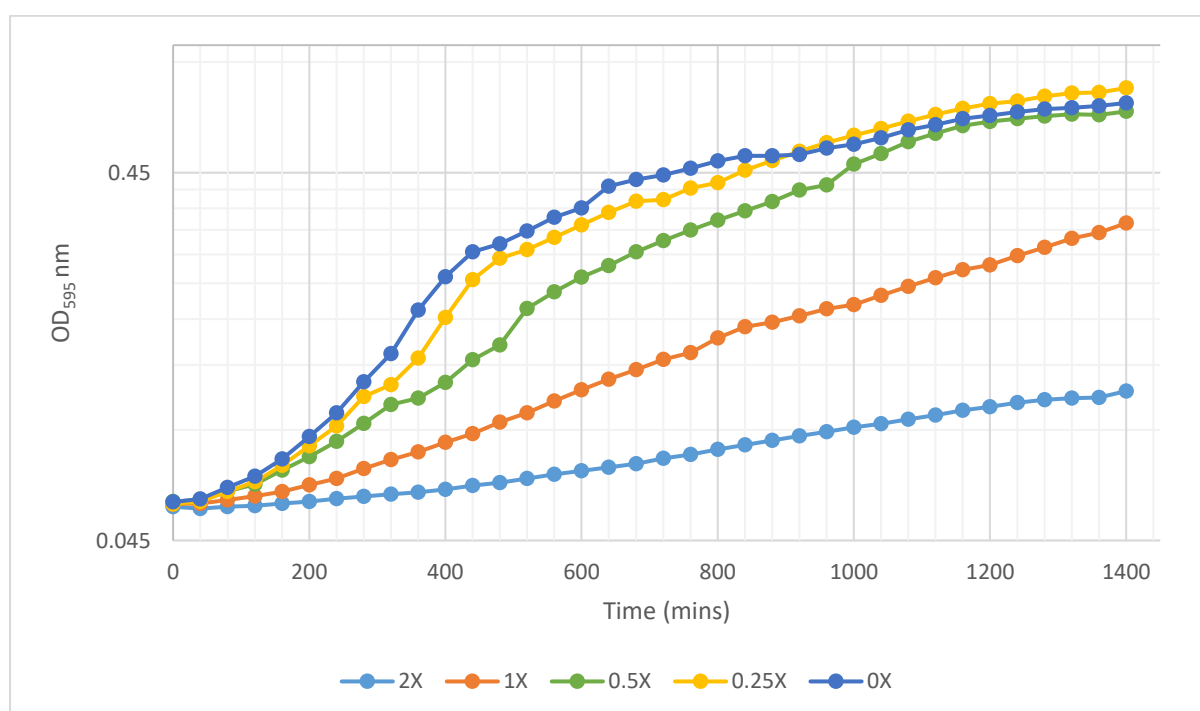


Figure 3-11 - The effects of Compound G on *L. monocytogenes* at five different concentrations: Cultures of *L. monocytogenes* [10403S] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

3.1.5.8 Compound H

Compound H was added to the screening schedule as a current industry standard to be used as a comparison to determine if the novel antimicrobials were having similar or better effects on the five bacteria being tested. Three runs were completed due to an issue with the second run. Due to unusually warm weather the ambient temperature in the laboratory had risen meaning the Tecan was unable to maintain its temperature range of 18-25°C. It was expected that this issue had resulted in the two repeats giving marginally different results. Therefore a third run was completed, the results of this run agreed with the results of the first agreeing that the second run was anomalous probably due to temperature. Run one and three are shown here, run two can be found in appendix A.

The effect of compound H on *L.monocytogenes* in both runs all concentrations apart from concentration 2X have had little or no effect on the growth of the bacteria. They have followed the same trend and reached the same if not increased maximum growth points for each concentration. With *L.innocua* all concentrations apart from 2X show no effect when compared to the control. The results for *S. Typhimurium* can be seen in figure 3-12 below. Other than a slightly slower initial growth at concentration 2X which can be seen by the gentler gradient of the line, however after approximately 700 min the level of growth begins to equal that of the control. All other concentrations follow the same trend as the control. As with *S. Typhimurium* the effect on *E.coli* is minimal with concentration 2X having some effect however the difference in the maximum growth reached within 24 h is not comparably different. With *P.fluorescens* run one and run three show slightly different results. Run one shows concentration 2X having slowed growth and an extended lag phase to 400 min. Concentrations 1X and 0.5X have slower initial growth however then increase quickly to match the maximum growth of the control. In Run three, concentrations 2X and 1X have decreased growth with 1X slowing after approximately 600 min. Overall both graphs show the same trends, that concentration 2X is having some effect along with concentration 1X within the first 1000-1200 min whereas 0.5X and 0.25X have no effect compared to the control.

Compound H is a currently used industry antimicrobial. In these screening test it shows that the effects on the five bacteria tested are limited. At concentration 2X there are some small reductions in growth however the other concentration do not show any real effect in inhibiting the growth of the bacteria. There are the same trends with gram positive and gram negative bacteria therefore there is no specific use against one of these groups. Overall the screening test shows that compound H would not be a potentially useful antimicrobial unless possibly used in higher concentrations.

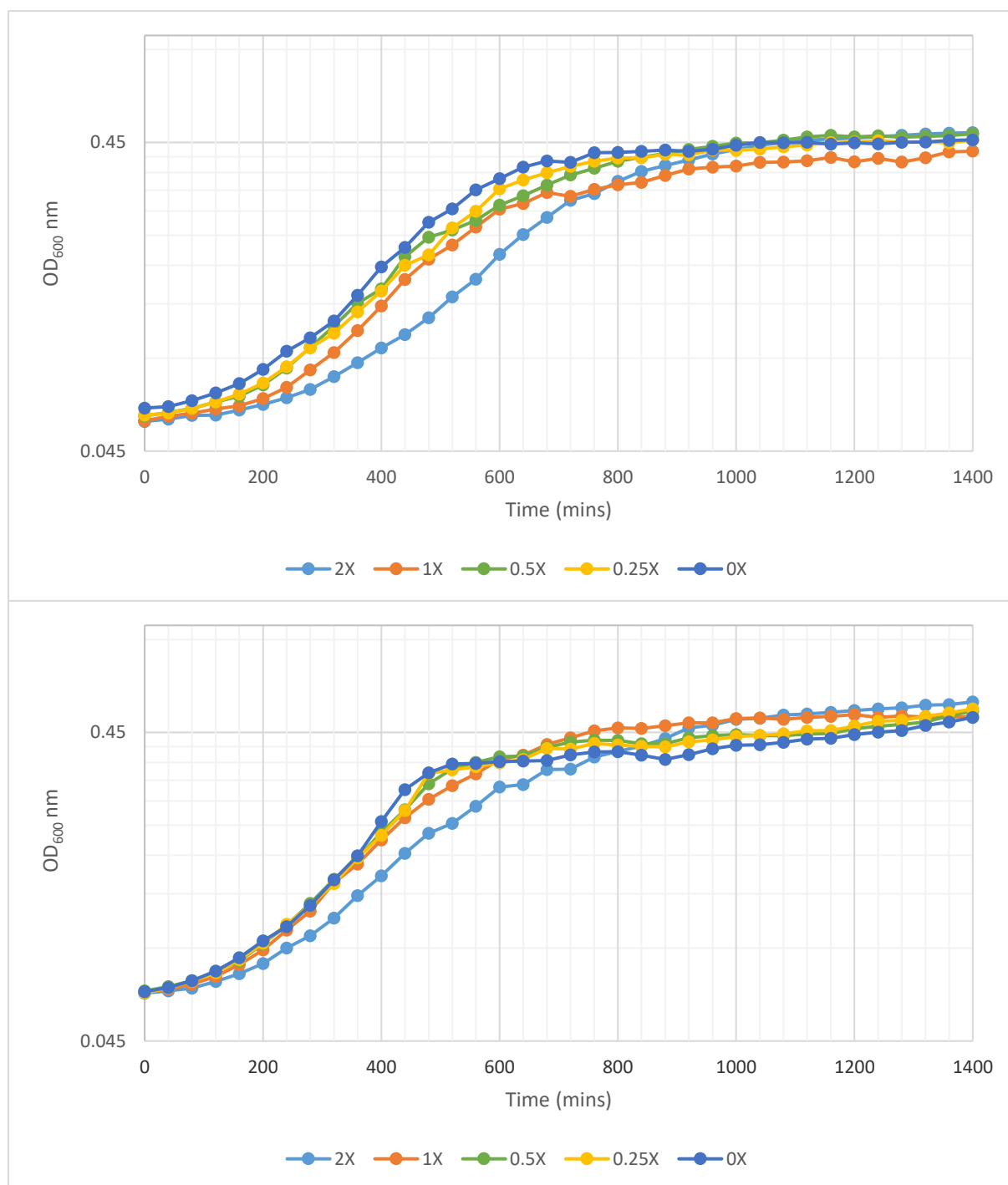


Figure 3-12 - The effects of Compound H on *S. Typhimurium* at five different concentrations: Cultures of *S. Typhimurium* [DB7516] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

3.1.6 Discussion

The screening test was devised as a quick way to determine whether an antimicrobial compound showed potential against any or all of the five bacteria species being used in the test. It was thought that by doing this the number of compounds taken into further testing would be reduced. As each compound is a variation of each other on a sliding scale it was used to see which combination was expected to work better in a food system. The bacteria used were chosen because they represented common pathogenic and spoilage bacteria which are of concern to the food industry.

Screening of compound H was undertaken to give a comparison of what is being used in the food industry currently. It was thought that if the novel antimicrobials performed to an equal or better standard then they definitely showed potential to be sold to industry. The other benefit to the novel antimicrobials is that they are natural therefore could come under the 'clean label' brand which is currently searched for by consumers (Asioli et al., 2017). Therefore to determine which compounds would be progressed forward each one was compared to the results of compound H.

Compound H did not perform very well in the screening test with all the other compounds outperformed it apart from compound A which also had no effect on the bacteria tested. Compound F only showed effect at concentration 2X, all other concentrations showed no effect against any of the bacteria therefore the potential as an antimicrobial is limited. Compounds B and C performed very similarly with them having an effect at concentrations 2X and 1X. Although, the effect at 1X was pointedly smaller than that of 2X. Compound G had a greater effect on gram positives than gram negatives which could make it useful as a more targeted antimicrobial. With the gram positive bacteria tested the compound showed effect at concentrations 2X, 1X and 0.5X, this makes it useful to industry as it is effective at lower concentrations. The compounds which showed the most potential as antimicrobials were compounds D and E. Each was effective against all five bacteria showing efficacy against gram positive and gram negative bacteria. Both compounds were also able to show reduced growth of the bacteria at the lower concentrations of 0.5X and 0.25X as well as 1X and 2X. Using compound E at concentration 2X resulting in inhibition of growth of all the bacterial species tested. By inhibiting growth or slowing it to such an extent is of real benefit to the food industry.

Four different concentrations were used in the screening process. It was expected that 2X would show at least a small effect on growth using any other compound which was

generally what was found. In some cases for example compound E, concentration 2X was able to inhibit the growth or considerably increase the time of the lag phase. It was then expected that the other concentrations would then have a lesser effect and be closer to the growth levels of the control. For the novel antimicrobials to be cost effective to produce they needed to show effect at 0.5X or 0.25X. It was hoped that the lower concentrations could show enough of an effect to exceed the abilities of the industry product so that the product could be economical. From the screening results compounds D and E have the ability to reduce growth of all the bacteria species tested at 0.5X and to a lesser extent 0.25X. Although the effect is not as extreme as the almost inhibition of growth at concentration 2X it is enough to slow the growth in comparison to the control. Therefore as an antimicrobial it would be useful in extending the shelf life of products and slowing the growth of pathogenic bacteria meaning they may not reach infectious levels.

Increasing shelf life is important in the food industry as it will allow for the reduction of food waste (Gustavsson et al., 2011). By an antimicrobial showing decreased growth or an increased lag phase time there is the potential to increase the shelf life of the product it is used in. The increased lag time shows that the antimicrobial is inhibiting the growth of the bacteria for a short time before the exponential phase begins. The reduced growth in the exponential phase shows that the antimicrobial is preventing the replication and growth of the bacteria. The food industry aim to use an antimicrobial at the lowest possible concentration as this using less of the product. If a product is able to be effective at a lower concentration it makes it more economical when used in manufacturing a food product. In this case the desired concentrations were 0.5X and 0.25X. In the screening tests compounds D and E were the only two compounds to show any real effect at this level, therefore these were the compounds which were progressed into further testing.

3.1.7 Next Steps

The progression of experiments after screening was to test the selected compounds in a food product. As Phil Stroud Solutions were interested in the antimicrobials actions in meat products it was decided that a sausage product would be used. The sausage was sourced by industry from Italy and a dipping method was used. This was to determine whether the antimicrobial compounds could inhibit growth in a more complex food system. *L.monocytogenes* was tested using the sausage product whereas the gram negative bacteria were tested using a pork mince product. These decisions were made based on the likelihood of a particular bacteria growing in that food product. *Listeria spp.*

are mainly found in ready to eat foods whereas gram negatives such as *Salmonella spp.* are found in products such as fresh meat.

Another step taken as a result of the screening data was to calculate the growth rates of each bacteria in the presence of each compound. This was to determine whether the growth rate data corresponded with the interpretation of the initial screening data.

3.2 Quantification of effect of antimicrobials on growth

3.2.1 Aim

After all of the compounds to be tested had been screened, and before the compounds were tested in meat, the level of growth inhibition needed to be quantified. There are two features of the effect of growth that can be measured to indicate how an antimicrobial is likely to influence the final growth levels of the test bacterial. These are (a) the length of the lag phase and (b) the growth rate achieved during the exponential phase of growth. Also calculating the growth rates of each bacteria challenged with different concentrations of antimicrobial compound the relative effect of dilution of each of the antimicrobial compounds can be seen.

3.2.2 Approach used to define the exponential phase of growth

For any bacterial growth curve, the growth rates are normally calculated using the gradient of the line when the growth reaches the linear phase when plotted on an exponential scale. Although bacterial growth is a doubling function, data is normally plotted on a $\log_{10}$ scale to linearise the data and then constants in the equation convert the slope of the curve into a value appropriate for a $\log_2$ function. In this case the growth rate equation shown in section 2.4.4 was used.

To identify the exponential phase of growth for each of the graphs shown in section 3.1 (and Appendix A), a line of best fit was used to identify the linear portion of the curve each bacteria. Two points were then taken from this line and then inputted into the equation shown above. The calculated growth rates were then compared for each bacteria and for each concentration of antimicrobial tested. In each case the level of growth inhibition is seen as the difference between the growth rate in the control sample (0x) and the growth rate measured in the presence of the antimicrobial.

The lag phase was also determined from each graph, this established whether the presence of the compounds was delaying bacterial growth. Data for each lag phase can be found in the tables in Appendix B.

3.2.2.1 Results for *L. monocytogenes* and *L. innocua*

These results can be seen in figure 3-13. In the case of *L. monocytogenes*, at the working concentration for all these antimicrobials (1X), compounds B, C and G caused the largest growth inhibition resulting in the lowest growth rates. Interestingly the industry standard (compound H) had the least effect on growth, in particular when the 2X concentration was used, although compound A also showed little effect on the growth of *L. monocytogenes*. In all cases, an increase in growth inhibition was seen as the

concentration of the antimicrobial increased, but again compound H seemed to have the smallest maximal effect (change in growth rate between 0X and 2X = 0.0007) whereas compounds D, E and G showed much larger effects (change in growth rate between 0X and 2X = 0.0025, 0.0027 and 0.0032, respectively). Although compound B showed the largest difference in growth rate between 0X and 2X, the difference at 1X was in the same range as these two compounds (0.0029). It was also interesting to note that all of the compounds tested, apart from F and G showed some effect on growth rate even at the lowest dilution suggesting that sub-inhibitory concentrations are still retaining some antimicrobial activity.

The pattern of results for *L. innocua* were, as expected, similar to those seen for *L. monocytogenes* with compounds B, C and D showing the most effects on growth rate and confirmed again that the industry standard compound had little effect on the growth of this species. However in this case the results for compound E needed to be treated with caution as the growth rate at 0.25X is higher than that at 0X which suggests there may have been an experimental error that affected this set of results.

Compound E has the most effect on extending the lag phase of *Listeria spp.* At concentration 2X the lag phase is extended to more than 500 min in *L. monocytogenes* and over 900 min with *L. innocua*. The results of the lag phase calculations can be seen in appendix B. At concentration 1X compound E is still able to extend the lag phase for 360 min whereas other compounds can generally only extend it for around 120 min which is the same as the control.

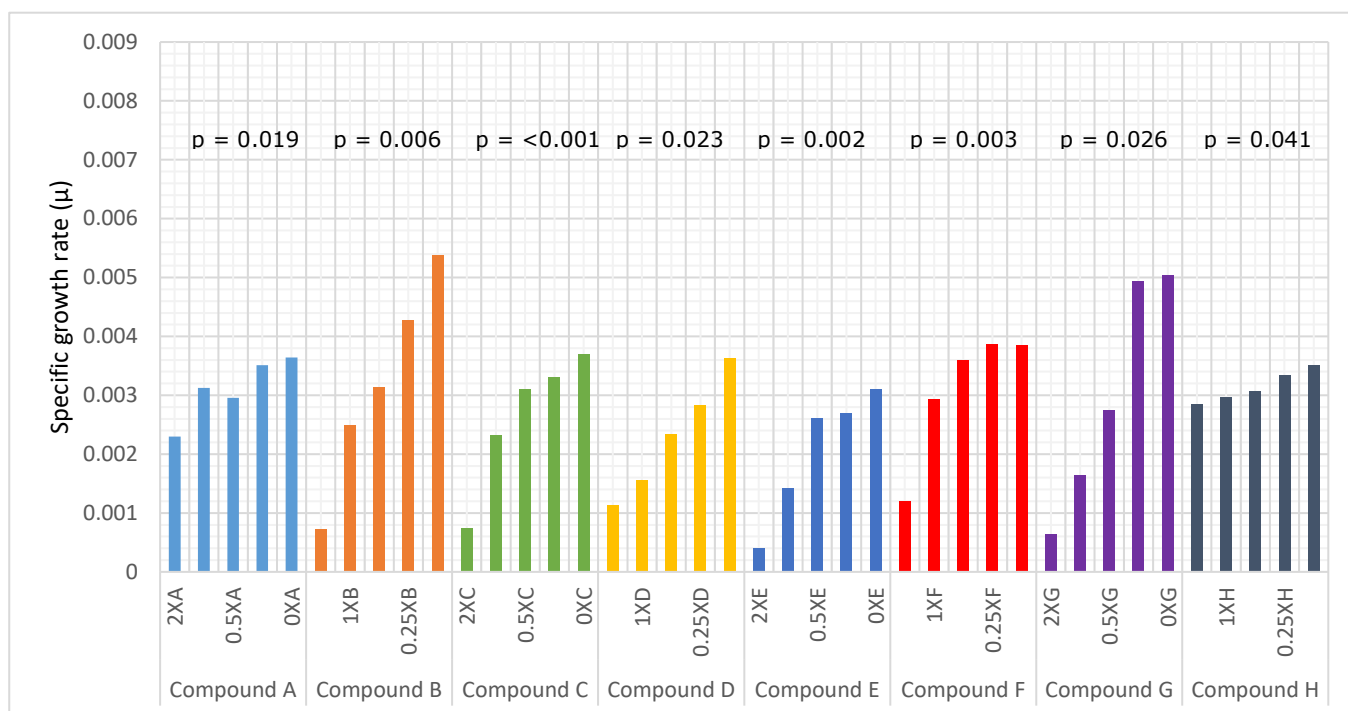
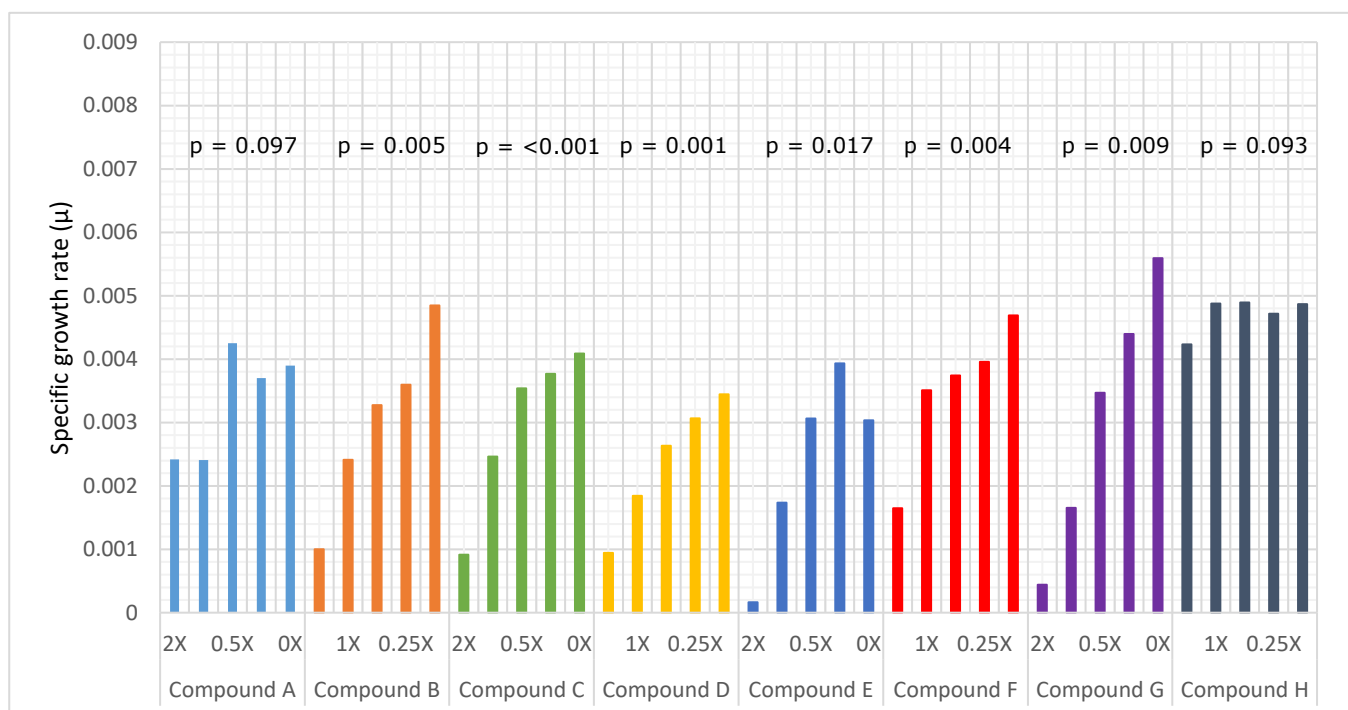
A**B**

Figure 3-13 - **Growth rates of *Listeria* species in the presence of 8 different antimicrobial compounds.** Data are calculated mean ($n=3$) growth rates of Gram negative bacteria: A, *L. monocytogenes*; B, *L. innocua*. Growth rates calculated using specific growth rate equation with data from screening test graphs. There was no significant compound effect (ANOVA, A: $p=0.697$, B: $p=0.128$ compound as a single factor). p values above each compound are values derived from regression.

3.2.2.2 Results for *Salmonella* and *E. coli*

When the results for *Salmonella* are compared (Figure 3-14), only compound E showed significant effect on growth rate at the 1X concentration, although compounds B, C, G and E all showed good levels of inhibition at the 2X concentration. Compound E showed the greatest level of inhibition of concentration 2X (0.0008) and still showed the highest level of inhibition at concentration 1X (0.0017). Compound H shows some inhibition of growth however the difference between the rates of each concentration is still small, the difference in effect between 2X and 0X is 0.00036. This is a very small amount especially for a product which is currently used against these bacteria in the food industry.

Compound G shows the largest difference in growth rate (0.0045) although it is not the most effective at the working concentration 1X. Compound B was the only compound which really showed any effect at the lowest concentrations.

The *E. coli* the rate of growth in the absence of the compounds was generally higher than that of the previous three bacteria tested. The pattern of results was however, similar to that of *S. Typhimurium* as expected. In this case compounds B, C, D and E all showed good levels of growth inhibition at a 2X concentration. Compound H showed poor inhibition of growth at all levels tested and compounds A and F show no real effect on the growth rate of *E. coli* as seen with the previous three bacteria tested.

Again compound E shows the longest extension of the lag phase with values for *S. Typhimurium* and *E. coli* as 440 min and 680 min respectively at concentration 2X. Compound B was also able to extend the lag phase however not to the extent of compound E. Compounds A and H had the shortest lag time but this is as expected when compared to the growth rates.

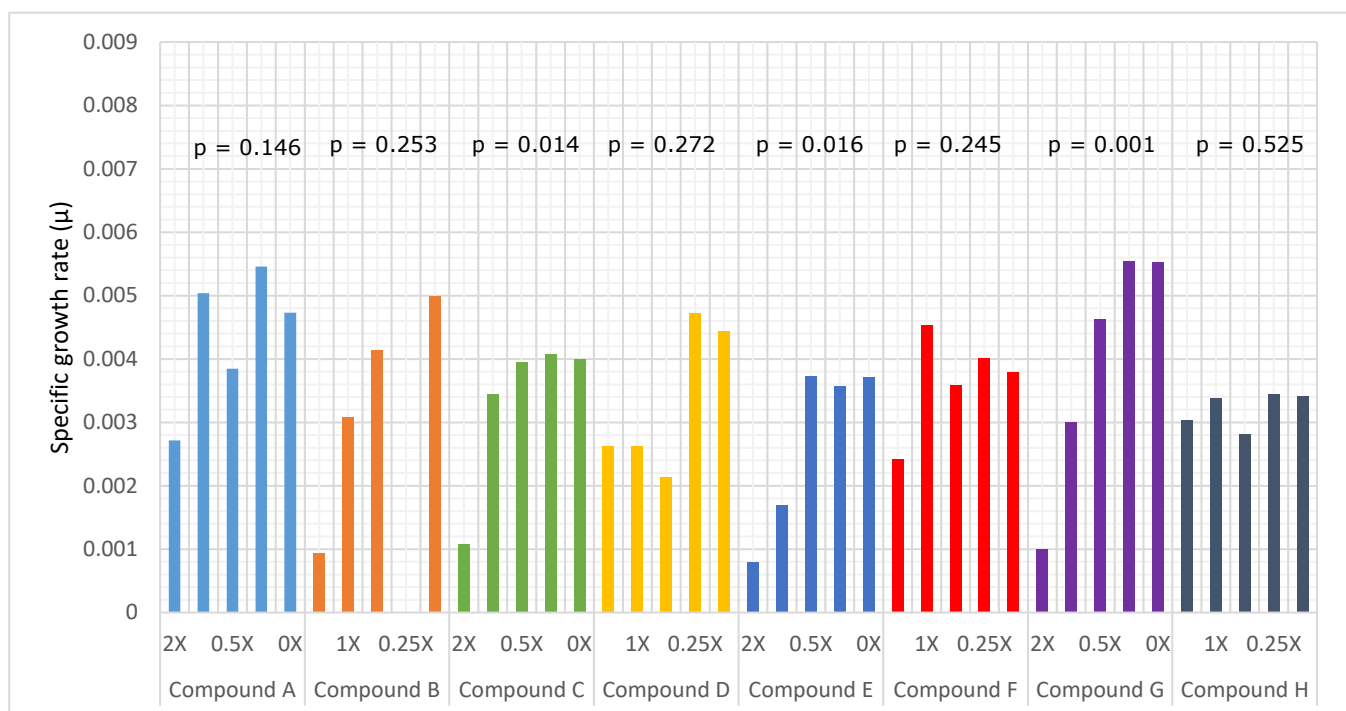
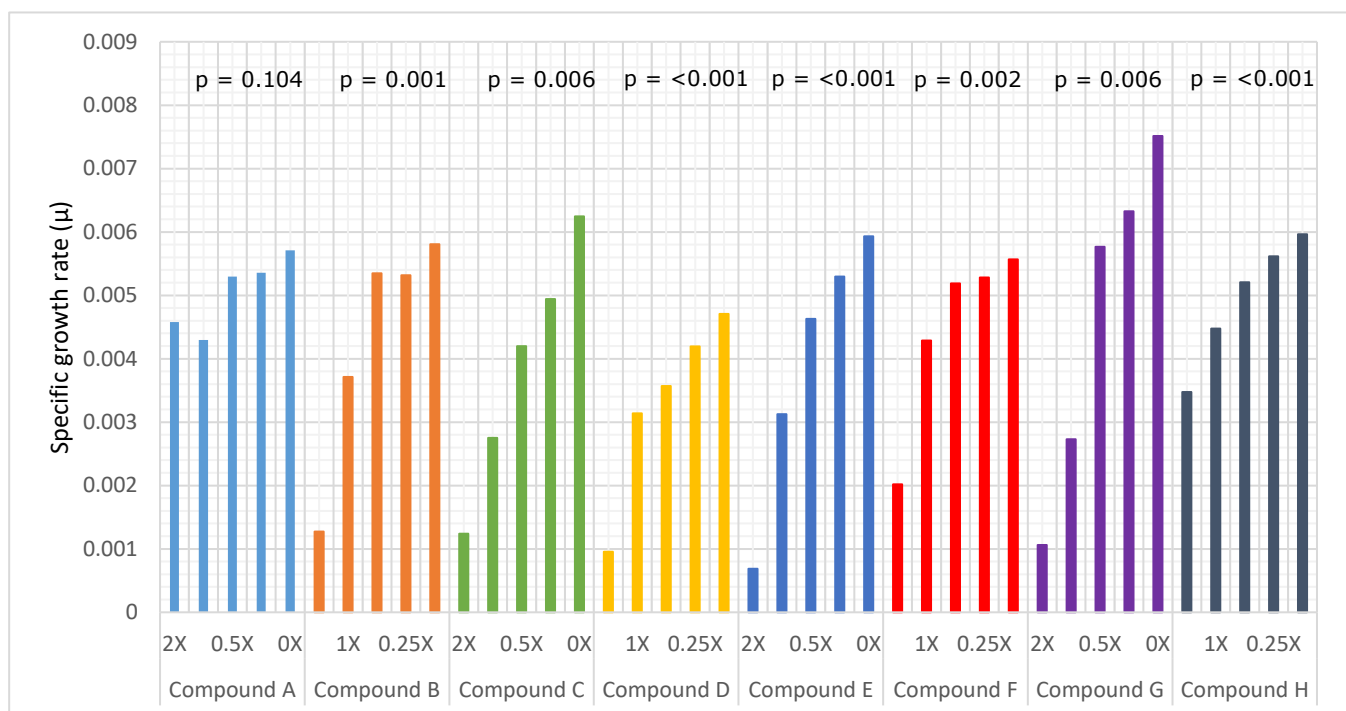
A**B**

Figure 3-14 - Growth rates of Gram negative bacteria in the presence of 8 different antimicrobial compounds. Data are calculated mean ($n=3$) growth rates of Gram negative bacteria: A, *S. Typhimurium*; B, *E. coli*. Growth rates calculated using specific growth rate equation with data from screening test graphs. The missing data point for compound B, concentration 0.25X was removed due to experimental error. There was no significant compound effect (ANOVA, A: $p=0.736$, B: $p=0.712$ compound as a single factor). p values above each compound are values derived from regression.

3.2.2.3 Results for *Pseudomonas*

In these experiments the growth levels at 0x were again generally higher, and more similar to those seen for *E. coli* but as this bacteria is a non-pathogenic mesophile with a lower optimum growth temperature that would be expected. The results can be seen in figure 3-15. Compounds B and E show the greatest inhibition of growth at concentration 1X with growth rates of 0.0011 and 0.0012 respectively. All compounds apart from compound A are effective against *P. fluorescens* at concentration 2X however the effect is quickly diluted as the concentrations increase. Compound shows no real effect with a small growth rate range of 0.0015. The greatest maximal effect on the growth is by compound G which also has the widest range of growth rates (0.007).

At concentration 2X *P. fluorescens* showed some interestingly long lag phases.

Compounds B, E and F were able to extend the lag phase for the full 24 h and little growth occurred. However at the working concentration 1X this effect was reduced with compound B and compound E having lag times of 440 min and 520 min respectively.

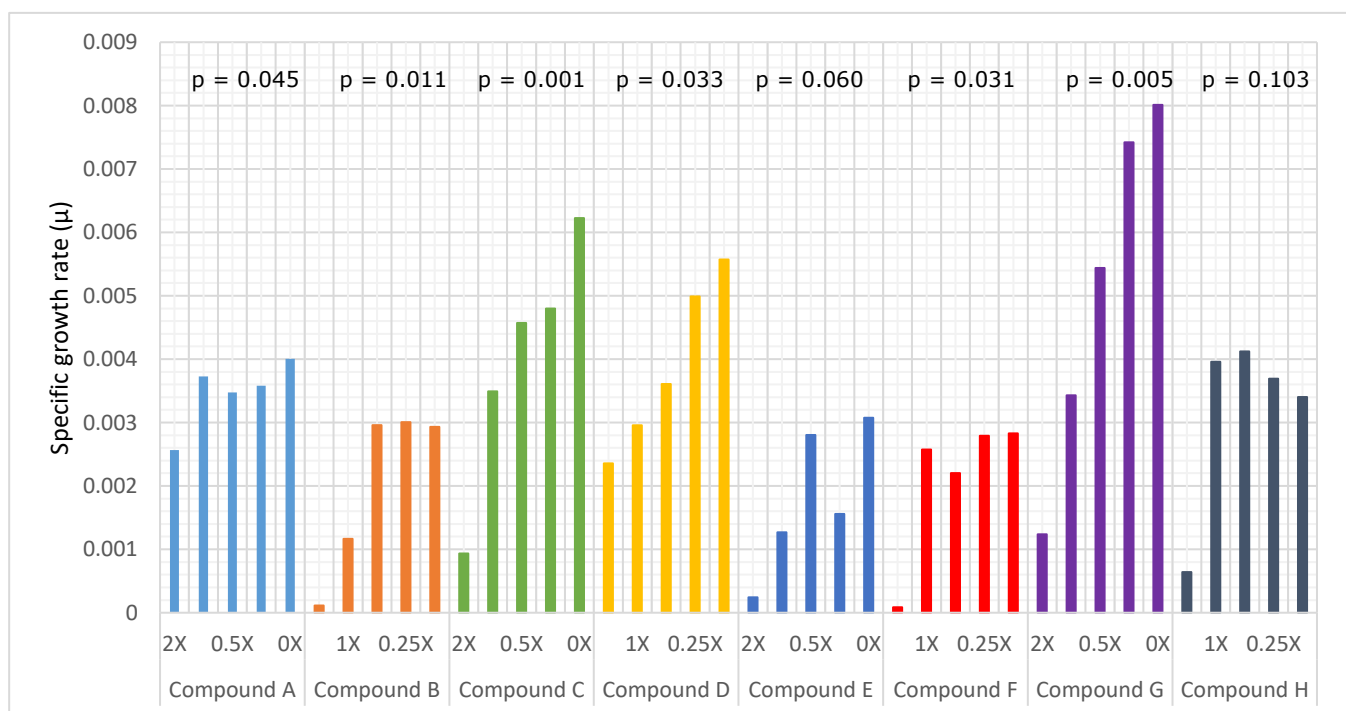


Figure 3-15 - **Growth rates of *Pseudomonas* species in the presence of 8 different antimicrobial compounds.** Data are calculated mean (n=3) growth rates of *Pseudomonas* bacteria. Growth rates calculated using specific growth rate equation with data from screening test graphs. There was no significant compound effect (ANOVA, p=0.148, compound as a single factor). p values above each compound are values derived from regression.

Overall the purpose of these calculations was to assist with the interpretation of the screening data as well as choose the most suitable all round antimicrobials to take forward into the in food tests. Compounds A, F and H were easy to discount as they did not perform particularly well with any of the five bacteria. Apart from the effect of F and H on *P. fluorescens* where there was some effect at 2X concentration. From the remaining

compounds, D and E were chosen to move forward with into the next phase of testing. This was done in discussion with Phil Stroud Solutions Ltd who made the antimicrobial compounds. Overall both of these compounds showed good results with them both having an effect at inhibiting growth at the working concentration 1X. Another factor was how the compounds were made and Phil Stroud Solutions Ltd felt as if these compounds provided a good balance of their ingredients and had the ability to be economically viable in the future.

3.3 Investigation of antimicrobial mechanism

3.3.1 Aim

In the previous sections, it was clear that the antimicrobials being tested were reducing the growth of the bacteria, but it was not clear whether the antimicrobials provided by Phil Stroud Solutions Ltd were acting in a bacteriostatic or bactericidal manner against the bacteria they were being tested against. Alongside the results of the screening tests showing that growth was inhibited, understanding this would provide a better idea of how the novel antimicrobials would behave in further 'in food' tests.

3.3.2 Experimental Design

Rather than monitoring a change in growth rate, these experiments were designed to determine whether an exposure of 24 h resulted in a loss of viability of the cells exposed to the antimicrobial compound. To carry out the experiment overnight cultures of *L. monocytogenes*, *L. innocua*, *S. Typhimurium*, *E. coli* and *P. fluorescens* were prepared by incubating them at 30 °C, with shaking at 200 rpm in BHI broth. A temperature of 30°C was chosen as it allowed all five bacteria to be grown under the same conditions as *Pseudomonas spp.* do not grow at higher temperatures. The viable count of these cultures was then determined using the Miles and Misra method with samples being plated onto BHI agar plates. After this the test antimicrobial compound was added to each of the cultures to a final concentration of 1X and then the samples were incubated overnight under the same conditions. The viable count of the cultures was then again determined using the Miles and Misra method, and it was noted that the dilutions to reduce the cell number to the countable range were sufficient to dilute out the antimicrobial agents to below their active concentration. To detect any bacteriocidal effects, the ratio of viable count (expressed as cfu ml⁻¹) before and after treatment was calculated by dividing the initial viable count by the viable count recorded after treatment.

3.3.3 Results

The results of these experiments are shown in Tables 4.1-4.8 there are two controls as these results were gained over two experiments, the first testing compounds A, B and C and the second D, E, and F. This was done to ensure each experiment was of a manageable size.

The control samples for compound A, B and C produced a ratio range of 2.50 Icfu ml⁻¹ / Fcfu ml⁻¹ to 22.86 Icfu ml⁻¹ / Fcfu ml⁻¹. The lowest ratio was the growth of *L. monocytogenes* whereas the highest was *L. innocua*. In the control sample for

compounds D, E and F the ratio range was 11.58 Icfu ml⁻¹ / Fcfu ml⁻¹ to 42.50 Icfu ml⁻¹ / Fcfu ml⁻¹. The lowest ratio being *L.monocytogenes* and the highest *S. Typhimurium*.

The results for the viable count using compound A can be seen in table 4.3. In this case all values are higher than 1 but less than or within the range of the control. Therefore compound A has a bacteriostatic action. Compound B results can be seen in table 4.4; all of the results apart from *L.innocua* have a ratio value of less than 1 therefore growth is inhibited and there is evidence that exposure at concentration 1X is leading to cell death. The results for compound C can be seen in table 4.5. In this case all ratios are less than 1 therefore there is again evidence that the compound is not only inhibiting growth but also leading to cell death.

Compound D results can be found in table 4.6. There is evidence that this compound at concentration 1X may lead to cell death of gram negative bacteria due to the ratio values of less than 1. In gram positives there appears to be a bacteriostatic action as values are over 1 but less than the control. The effects on compound E can be seen in table 4.7, here there are values of less than 1 for all bacteria apart from *L.monocytogenes*. Therefore in these bacteria there is evidence suggesting cell death as well as inhibition of growth. The ratio for *L.monocytogenes* is greater than one but less than the control range therefore there is still an effect on growth yet no evidence to suggest cell death. The ratio values for compound F can be seen in table 4.8. Apart from *P.fluorescens* all values fall within the range of the control therefore suggesting a bacteriostatic effect. The value of 0.79 Icfu ml⁻¹ / Fcfu ml⁻¹ for *P.fluorescens* suggests there may be evidence of cell death alongside inhibition of growth.

Table 3-1 - **The effects of a control on a viable count of five different bacteria (A,B,C).**

Bacterial suspensions were grown overnight in BHI broth at 30°C. Each suspension was plated onto BHI broth and incubated at 30°C overnight, colonies were counted and recorded (Icfu ml⁻¹). In control samples no antimicrobial was added and suspensions grew for a further overnight period. Samples were plated and grown overnight before colonies were counted (Fcfu ml⁻¹).

Bacteria	Initial viable count (Icfu ml ⁻¹)	Final Viable count (Fcfu ml ⁻¹)	Ratio (Icfu ml ⁻¹ / Fcfu ml ⁻¹)
<i>L. monocytogenes</i>	3.20E+08	8.00E+08	2.50
<i>L. innocua</i>	1.40E+08	3.20E+09	22.86
<i>S. Typhimurium</i>	1.80E+08	3.70E+09	20.56
<i>E. coli</i>	2.40E+08	2.80E+09	11.67
<i>P. fluorescens</i>	1.60E+08	2.40E+09	15.00

Table 3-2 - **The effects of a control on a viable count of five different bacteria (D,E,F).** Bacterial suspensions were grown overnight in BHI broth at 30°C. Each suspension was plated onto BHI broth and incubated at 30°C overnight, colonies were counted and recorded (Icfu ml⁻¹). In control samples no antimicrobial was added and suspensions grew for a further overnight period. Samples were plated and grown overnight before colonies were counted (Fcfu ml⁻¹).

Bacteria	Initial viable count (Icfu ml ⁻¹)	Final Viable count (Fcfu ml ⁻¹)	Ratio (Icfu ml ⁻¹ / Fcfu ml ⁻¹)
<i>L. monocytogenes</i>	9.50E+07	1.10E+09	11.58
<i>L. innocua</i>	7.20E+07	1.20E+09	16.67
<i>S. Typhimurium</i>	4.00E+07	1.70E+09	42.50
<i>E. coli</i>	1.40E+08	3.50E+09	25.00
<i>P. fluorescens</i>	3.40E+07	1.30E+09	38.24

Table 3-3 - **The effects of Compound A on a viable count of five different bacteria.** Bacterial suspensions were grown overnight in BHI broth at 30°C. Each suspension was plated onto BHI broth and incubated at 30°C overnight, colonies were counted and recorded (Icfu ml⁻¹). Compound A was added at a concentration 1X and suspensions grew for a further overnight period. Samples were plated and grown overnight before colonies were counted (Fcfu ml⁻¹).

Bacteria	Initial viable count (Icfu ml ⁻¹)	Final Viable count (Fcfu ml ⁻¹)	Ratio (Icfu ml ⁻¹ / Fcfu ml ⁻¹)
<i>L. monocytogenes</i>	3.70E+08	6.00E+08	1.62
<i>L. innocua</i>	2.00E+08	6.50E+08	3.25
<i>S. Typhimurium</i>	1.20E+08	2.80E+08	2.33
<i>E. coli</i>	2.10E+08	4.00E+08	1.90
<i>P. fluorescens</i>	1.40E+08	3.20E+08	2.29

Table 3-4 - **The effects of Compound B on a viable count of five different bacteria.** Bacterial suspensions were grown overnight in BHI broth at 30°C. Each suspension was plated onto BHI broth and incubated at 30°C overnight, colonies were counted and recorded (Icfu ml⁻¹). Compound B was added at a concentration 1X and suspensions grew for a further overnight period. Samples were plated and grown overnight before colonies were counted (Fcfu ml⁻¹).

Bacteria	Initial viable count (Icfu ml⁻¹)	Final Viable count (Fcfu ml⁻¹)	Ratio (Icfu ml⁻¹ / Fcfu ml⁻¹)
<i>L. monocytogenes</i>	4.20E+08	2.40E+08	0.57
<i>L. innocua</i>	1.70E+08	3.10E+08	1.82
<i>S. Typhimurium</i>	2.30E+08	5.00E+07	0.22
<i>E. coli</i>	2.60E+08	1.10E+08	0.42
<i>P. fluorescens</i>	1.10E+08	2.80E+07	0.25

Table 3-5 - **The effects of Compound C on a viable count of five different bacteria.** Bacterial suspensions were grown overnight in BHI broth at 30°C. Each suspension was plated onto BHI broth and incubated at 30°C overnight, colonies were counted and recorded (Icfu ml⁻¹). Compound C was added at a concentration 1X and suspensions grew for a further overnight period. Samples were plated and grown overnight before colonies were counted (Fcfu ml⁻¹).

Bacteria	Initial viable count (Icfu ml⁻¹)	Final Viable count (Fcfu ml⁻¹)	Ratio (Icfu ml⁻¹ / Fcfu ml⁻¹)
<i>L. monocytogenes</i>	2.30E+08	2.00E+08	0.87
<i>L. innocua</i>	2.70E+08	2.60E+08	0.96
<i>S. Typhimurium</i>	1.30E+08	6.00E+07	0.46
<i>E. coli</i>	1.70E+08	1.20E+08	0.71
<i>P. fluorescens</i>	1.20E+08	4.80E+07	0.40

Table 3-6 - The effects of Compound D on a viable count of five different bacteria. Bacterial suspensions were grown overnight in BHI broth at 30°C. Each suspension was plated onto BHI broth and incubated at 30°C overnight, colonies were counted and recorded (Icfu ml⁻¹). Compound D was added at a concentration 1X and suspensions grew for a further overnight period. Samples were plated and grown overnight before colonies were counted (Fcfu ml⁻¹).

Bacteria	Initial viable count (Icfu ml⁻¹)	Final Viable count (Fcfu ml⁻¹)	Ratio (Icfu ml⁻¹ / Fcfu ml⁻¹)
<i>L. monocytogenes</i>	9.50E+07	1.20E+08	1.26
<i>L. innocua</i>	7.20E+07	8.00E+07	1.11
<i>S. Typhimurium</i>	4.00E+07	3.30E+07	0.83
<i>E. coli</i>	1.40E+08	3.20E+07	0.23
<i>P. fluorescens</i>	3.40E+07	1.70E+07	0.50

Table 3-7 - The effects of Compound E on a viable count of five different bacteria. Bacterial suspensions were grown overnight in BHI broth at 30°C. Each suspension was plated onto BHI broth and incubated at 30°C overnight, colonies were counted and recorded (Icfu ml⁻¹). Compound E was added at a concentration 1X and suspensions grew for a further overnight period. Samples were plated and grown overnight before colonies were counted (Fcfu ml⁻¹).

Bacteria	Initial viable count (Icfu ml⁻¹)	Final Viable count (Fcfu ml⁻¹)	Ratio (Icfu ml⁻¹ / Fcfu ml⁻¹)
<i>L. monocytogenes</i>	9.50E+07	1.20E+08	1.26
<i>L. innocua</i>	7.20E+07	4.80E+07	0.67
<i>S. Typhimurium</i>	4.00E+07	2.70E+07	0.68
<i>E. coli</i>	1.40E+08	3.20E+07	0.23
<i>P. fluorescens</i>	3.40E+07	2.00E+07	0.59

Table 3-8 - **The effects of Compound F on a viable count of five different bacteria.** Bacterial suspensions were grown overnight in BHI broth at 30°C. Each suspension was plated onto BHI broth and incubated at 30°C overnight, colonies were counted and recorded (Icfu ml⁻¹). Compound F was added at a concentration 1X and suspensions grew for a further overnight period. Samples were plated and grown overnight before colonies were counted (Fcfu ml⁻¹).

Bacteria	Initial viable count (Icfu ml⁻¹)	Final Viable count (Fcfu ml⁻¹)	Ratio (Icfu ml⁻¹ / Fcfu ml⁻¹)
<i>L. monocytogenes</i>	9.50E+07	8.00E+08	8.42
<i>L. innocua</i>	7.20E+07	3.00E+08	4.17
<i>S. Typhimurium</i>	4.00E+07	4.10E+07	1.03
<i>E. coli</i>	1.40E+08	2.00E+08	1.43
<i>P. fluorescens</i>	3.40E+07	2.70E+07	0.79

3.3.4 Discussion

The purpose of these viable count experiments was to determine whether the novel antimicrobials being tested in this project had bactericidal or bacteriostatic actions. Some antimicrobial compounds showed ratio values within the range of the control therefore in these cases the action of the antimicrobial at concentration 1X is bacteriostatic. This was the case for compounds A and F. However, for many of the antimicrobial compounds the viable count ratio was less than one in one or all bacteria tested. This means that is some evidence of cell death at concentration 1X of this compound. Compound B showed this for all five bacteria whereas compounds C, D and E had ratios of less than one in some of five bacteria. The value of less than one represents a decrease in cell number and thus growth this is what suggests there might be cell death and therefore a bacteriocidal effect. Due to this suggestion that some compounds may be resulting in cell death at concentration 1X a safety test was needed to ensure the compounds were safe for human consumption. It was decided that the Ames test would be performed to ensure that this effect would not cause problems to the food industry.

Overall the same pattern of results was seen in this viable count data as was seen in the screening results. Therefore this gives confidence in the results being produced.

3.4 Ames Test

3.4.1 Aim

Having established that compounds D and E had the most promising in vitro activity, the next question to consider was whether these mixtures of chemicals were safe to use in the real food products for human consumption, in particular whether there was any evidence that the material had the potential to cause any mutagenicity. One of the simplest screening tests that can be used to determine this is the Ames test which detects different types of DNA damage in cells of *Salmonella* after they are exposed to the test chemicals (Mortelmans and Zeiger, 2000).

3.4.2 Experimental Design

To complete the Ames test five strains of *S. Typhimurium* were used which were TA98, TA100, TA102, TA1535 and TA1537. These bacterial strains each contain different types of mutation in the histidine biosynthesis genes which means they require histidine to grow. This allows antimicrobial compounds to be tested to determine whether they have any mutagenic potential by detecting second mutations that occur in these genes which then allow the strains to grow in the absence of histidine (Florin et al., 1980). Within the five strains used two main mutations were present, the action of some strains was also effected by the presence of a plasmid. Of the *S. Typhimurium* strains used TA100, TA102, TA1535 and TA1537 all have the *rfa* mutation present. This mutation shortens LPS molecules on the outside of the cell. TA102 unlike the other strains does not have a deletion mutation, this is because it requires the chromosome region that would be deleted for DNA repair, this strains is also not biotin dependent unlike the other strains. The plasmid pKM101 is present in strains TA98 and TA100. This plasmid can relate to ampicillin resistance however in this instance there is enhance UV and chemical mutagenesis due to overexpression. These strains were chosen as they represent a number of different mutation strains which the antimicrobial compounds could be tested against. The mutation strains can be seen in Table 5 below.

Table 5 – ***S. Typhimurium* strains used in this test.** Table adapted from Mortelmans and Ziegler (2000).

Mutation (strain)	Histidine operon mutations	LPS defect	Plasmid
TA98 hisC3076	Deletion mutation	<i>rfa</i>	pKM101
TA100 hisD3052	Deletion mutation	<i>rfa</i>	pKM101
TA102	Wild type	<i>rfa</i>	pKM101, pAQ1
TA1535 hisG46	Deletion mutation	<i>rfa</i>	No plasmid
TA1537 hisG428	Deletion mutation	<i>rfa</i>	No plasmid

The five bacterial strains were cultured overnight at 37 °C to a density of 10^9 cfu ml⁻¹, which is required to ensure that enough bacteria can be added to the test to allow detection of mutations which will occur naturally at about a frequency of 1 in 10^6 colonies. Hence a low level of colony growth is expected even if a substance being tested is not mutagenic. To carry out the challenge tests, the bacterial culture and the compound being tested were added to the top agar. Four sets of plates were prepared per bacterial strain, and in addition a positive control was prepared by placing a filter disc with a known mutagen on the surface of an agar plate. For plates testing the antimicrobial compound this was added to the top agar, the negative control agar contained sterile water.

Once the top agar had been spread onto the plates and had set they were wrapped in tin foil. This was to avoid any UV-induced reactions occurring which might lead to the production of mutagens and would invalidate the results gained. The plates were incubated at 37°C overnight and were then photographed to record the results.

3.4.3 Results of the Ames tests

The results for *S. Typhimurium* strain TA98 can be seen in figure 3-16. The positive control shows growth of 6 colonies with no focussed growth surround the sodium azide disc. The negative control also shows a small number of colonies along with a few smaller revertant colonies. The same results can be seen on the plates containing compounds D and E.

On the plates growing *S. Typhimurium* strain TA100, shown in figure 3-17, the positive control plate shows a collection of colonies surrounding the paper disc, this shows the colonies which have been mutated due to the presence of sodium azide. This allows them to grow in close proximity to the mutagen, these are smaller colonies compared to those on the outside of the plate. The negative control plate shows fewer colonies, some are revertants, these are the smaller colonies present. The plates for compounds D and E show similar growth to the negative control however in these cases there are less of the smaller revertant colonies present.

Figure 3-18 shows the results for *S. Typhimurium* strain TA102. The positive control plate shows no concentrated growth surrounding the filter disc however there is high colony count and colonies of varying sizes are spread across the plate. These are both larger colonies and smaller revertant colonies. In the negative control there is a similar pattern of growth to that found on the positive control plate. The growth covers the plate with both larger and smaller revertant colonies. The same pattern is seen on the plates containing compound D and E.

The results for *S. Typhimurium* strain TA1535 can be seen in figure 3-19. The positive control plate shows the typical appearance for this test. There is a focussing of colony

growth around the filter paper containing sodium azide. Surrounding this are a mixture of smaller revertant colonies and larger colonies. The negative control shows little growth with a colony count of just ten. The same pattern of growth can be seen on the plates containing compounds D and E.

In figure 3-20 the results of *S. Typhimurium* strain TA1537 are shown. There is little to no growth on any of the plates. All plates including the positive control only have between one and four colonies growing. Only larger colonies are generally present, in this case there are no smaller revertant colonies.

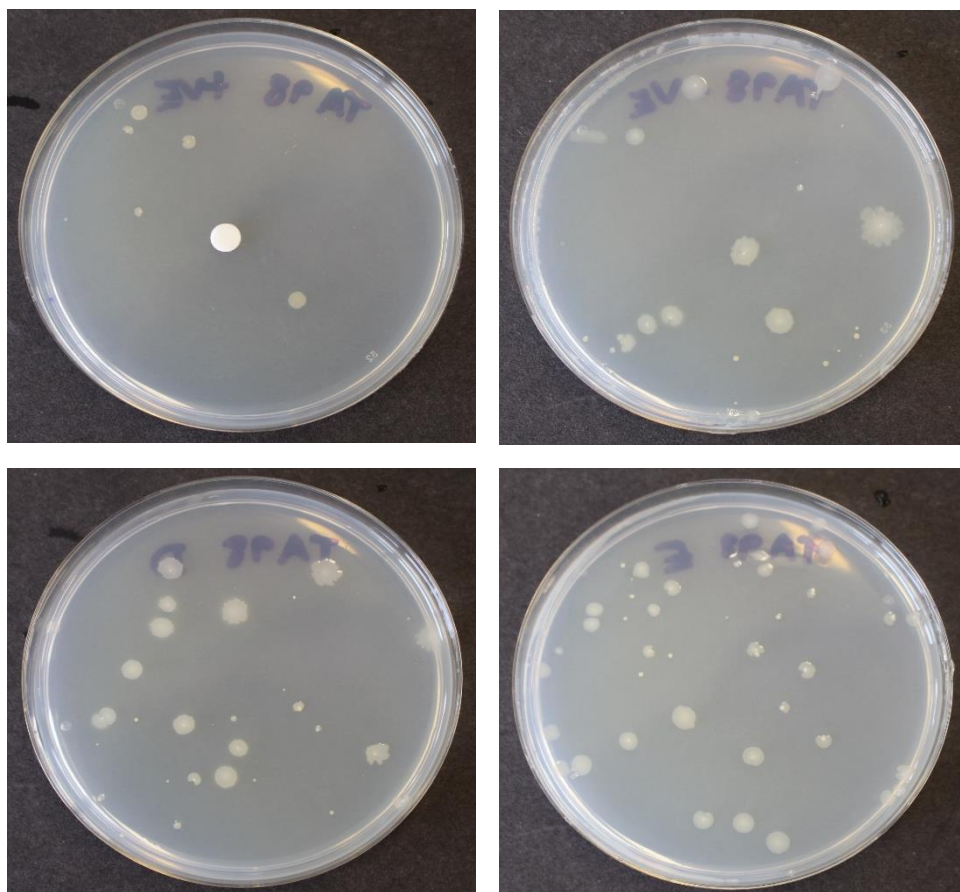


Figure 3-16 - **Ames test for compounds D and E using *S. Typhimurium* strain TA98.** The 5 *Salmonella* strains were grown in suspension overnight at 37°C to give approximately 10^9 cfu ml⁻¹. The bacterial suspension was added to molten top agar along with either compound D (bottom left), compound E (bottom right) or sterile water; negative control (top right). For the positive control (top left) a disc containing a known mutagen was placed onto the top agar. The top agar was poured onto minimal glucose agar plates. The plates were wrapped in tin foil and incubated at 37°C overnight. Photos were taken as a record of results.

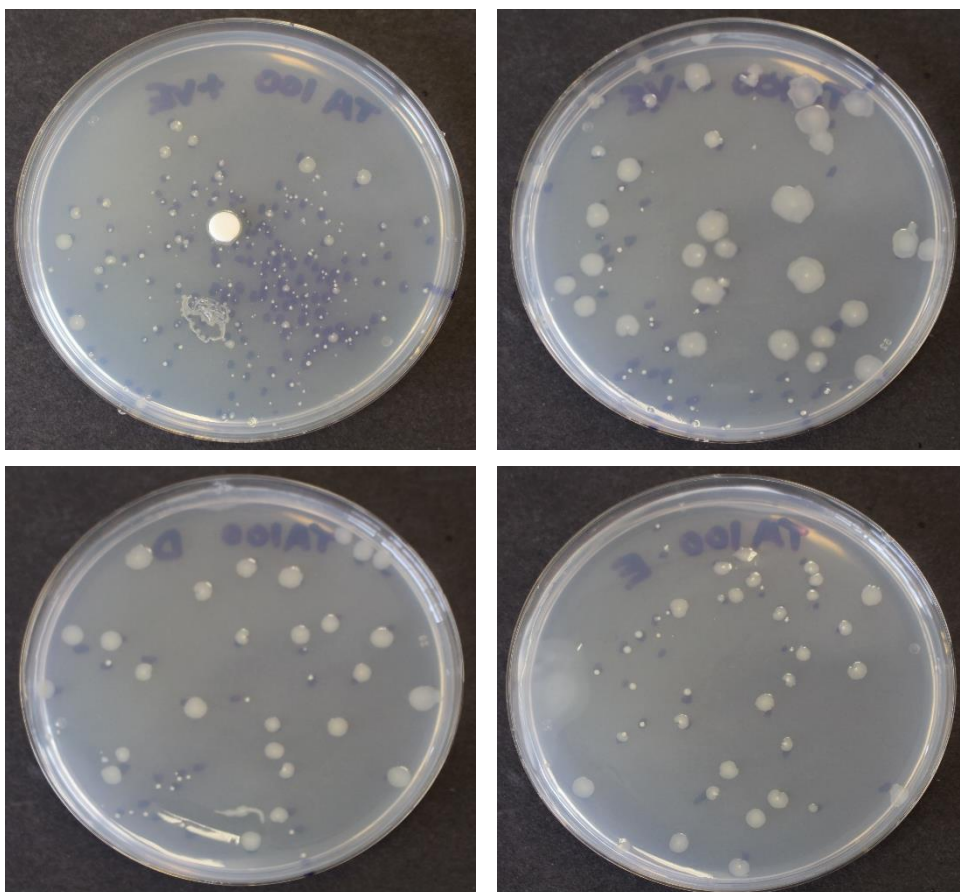


Figure 3-17 - **Ames test for compounds D and E using *S. Typhimurium* strain TA100.** The 5 *Salmonella* strains were grown in suspension overnight at 37°C to give approximately 10^9 cfu ml⁻¹. The bacterial suspension was added to molten top agar along with either compound D (bottom left), compound E (bottom right) or sterile water; negative control (top right). For the positive control (top left) a disc containing a known mutagen was placed onto the top agar. The top agar was poured onto minimal glucose agar plates. The plates were wrapped in tin foil and incubated at 37°C overnight. Photos were taken as a record of results.

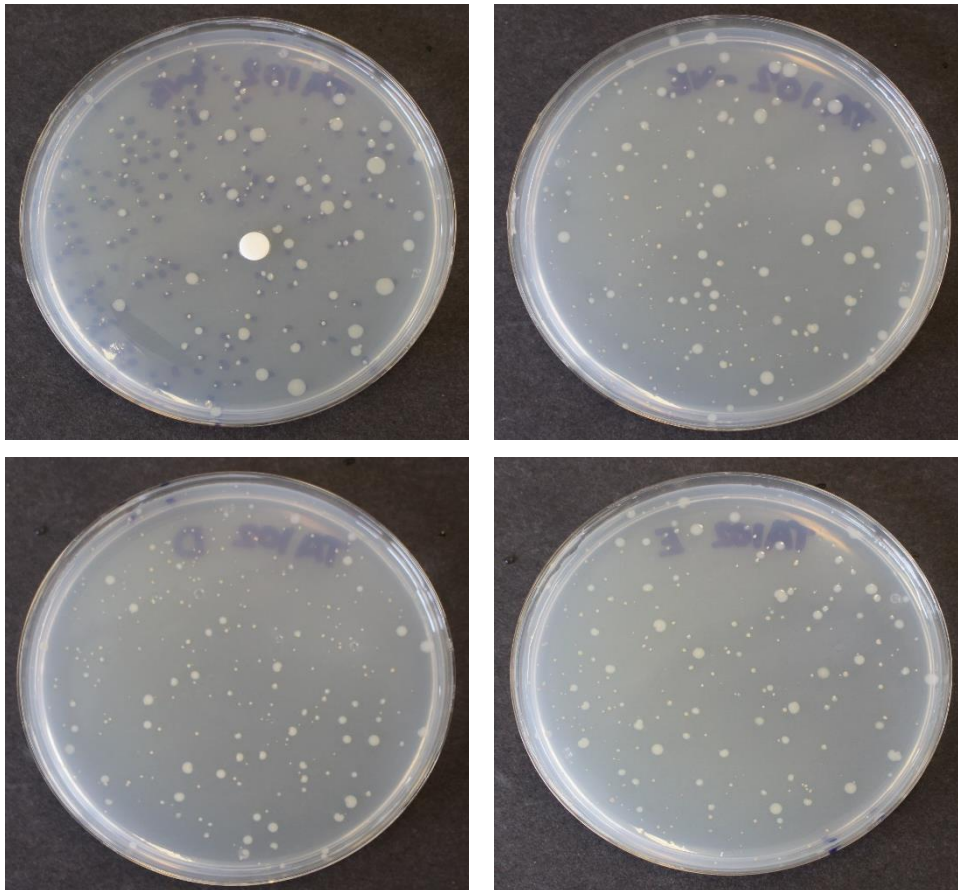


Figure 3-18 - **Ames test for compounds D and E using *S. Typhimurium* strain TA102.** The 5 *Salmonella* strains were grown in suspension overnight at 37°C to give approximately 10^9 cfu ml⁻¹. The bacterial suspension was added to molten top agar along with either compound D (bottom left), compound E (bottom right) or sterile water; negative control (top right). For the positive control (top left) a disc containing a known mutagen was placed onto the top agar. The top agar was poured onto minimal glucose agar plates. The plates were wrapped in tin foil and incubated at 37°C overnight. Photos were taken as a record of results.

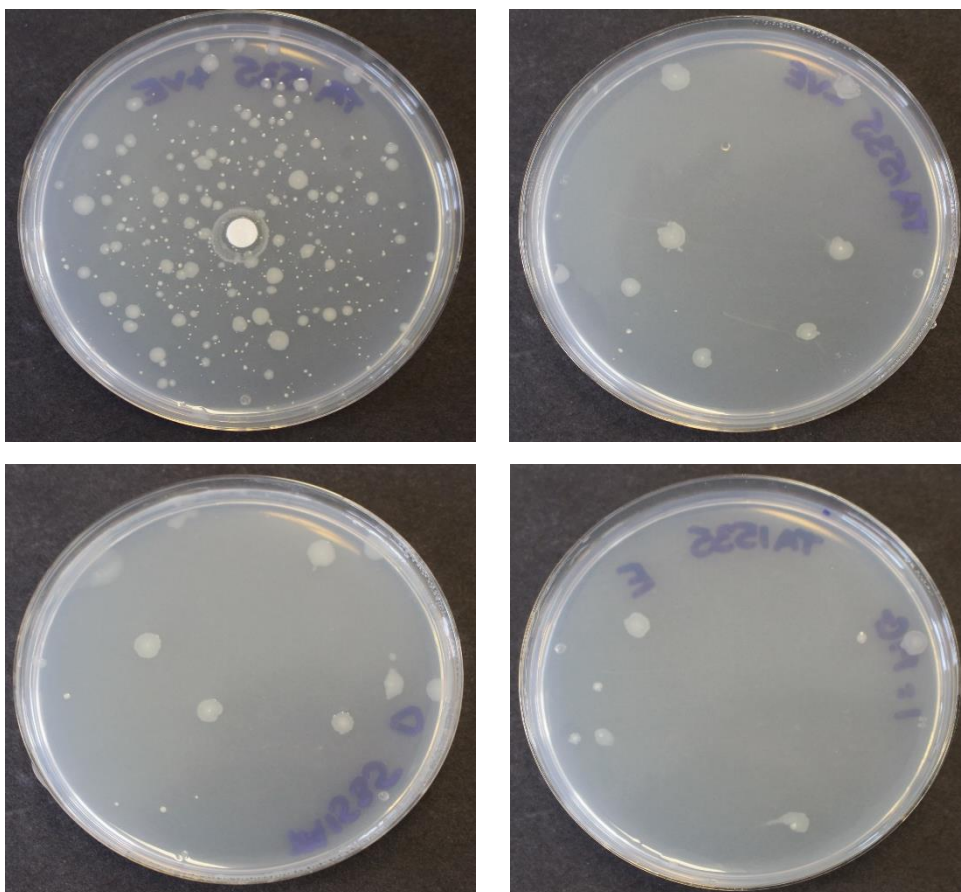


Figure 3-19 - **Ames test for compounds D and E using *S. Typhimurium* strain TA1535.** The 5 *Salmonella* strains were grown in suspension overnight at 37°C to give approximately 10^9 cfu ml⁻¹. The bacterial suspension was added to molten top agar along with either compound D (bottom left), compound E (bottom right) or sterile water; negative control (top right). For the positive control (top left) a disc containing a known mutagen was placed onto the top agar. The top agar was poured onto minimal glucose agar plates. The plates were wrapped in tin foil and incubated at 37°C overnight. Photos were taken as a record of results.

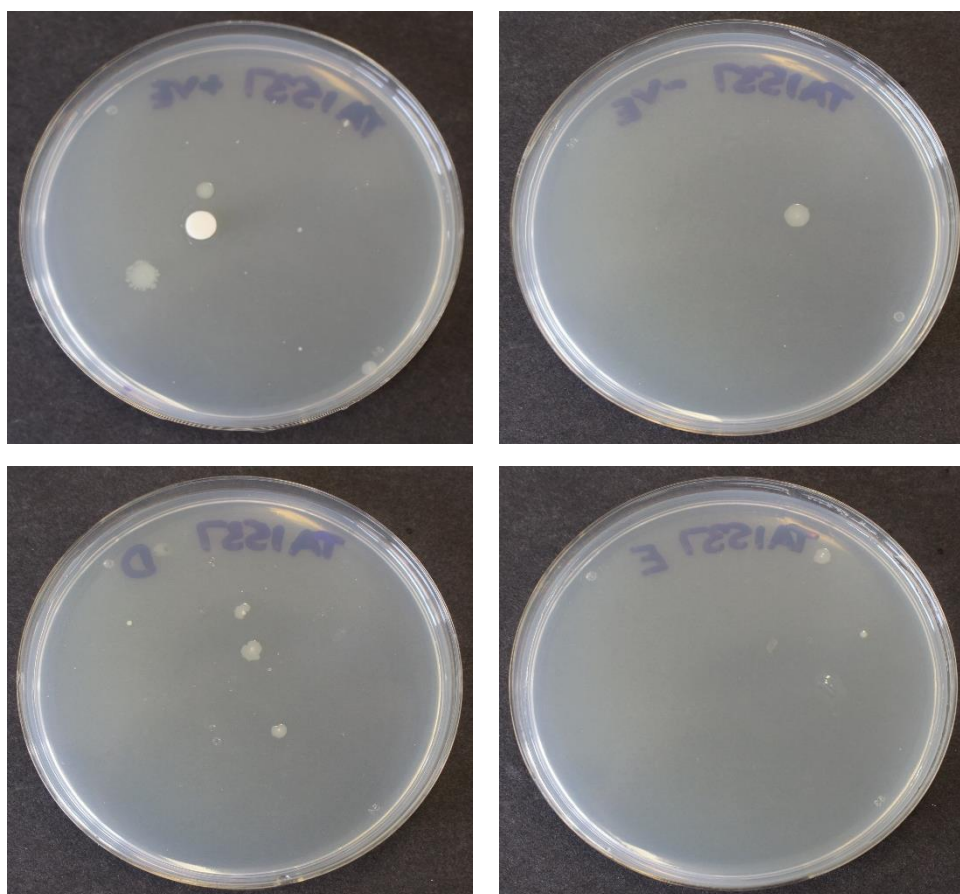


Figure 3-20 - **Ames test for compounds D and E using *S. Typhimurium* strain TA1537.** The 5 *Salmonella* strains were grown in suspension overnight at 37°C to give approximately 10^9 cfu ml⁻¹. The bacterial suspension was added to molten top agar along with either compound D (bottom left), compound E (bottom right) or sterile water; negative control (top right). For the positive control (top left) a disc containing a known mutagen was placed onto the top agar. The top agar was poured onto minimal glucose agar plates. The plates were wrapped in tin foil and incubated at 37°C overnight. Photos were taken as a record of results.

3.4.4 Discussion

Overall some of the *S. typhimurium* strains have worked successfully and have shown that the antimicrobial compounds tested do not show any mutagenicity. This is the case with strains TA100 and TA1535. From these strains it can be presumed that compounds D and E show no evidence of causing the types of mutations required to reverse the mutations in these strains therefore safe to use in food products. There was some colony growth on the plates however this is to be expected as random mutations will always occur. In the case of the other three strains of *S. Typhimurium* there was little or no growth on the positive control. This was the same pattern which occurred on the negative control and plates containing antimicrobial compound. Although there was little growth the conclusion can still be drawn that there is no evidence of compounds D and E causing the mutations needed for the compounds to be an issue to the food industry. Although the correct strains of *S. Typhimurium* were used there is a chance that sodium

azide was not the correct positive control to use. However, due to the lack of growth of smaller revertant colonies this still shows that the compounds tested are safe.

From the results gained it was decided that antimicrobial compounds D and E did not have any mutagenicity therefore were safe to use in food product testing. This was the expected result as the compounds were prepared using various organic acids and plant extracts, many of which are already found as antimicrobials in the food industry. However all novel food additives must be tested to confirm safety.

3.5 Sausage

3.5.1 Aim

After the screening tests determined which compounds had the most potential as antimicrobials, the chosen compounds were tested within a food system. When put into food antimicrobials need to work within a product which often contains a mix of ingredients such as salts and fats. This provides more challenges that the antimicrobial needs to overcome to still inhibit the growth of bacteria. It is important that antimicrobials are tested within the product they are intended for us in because of this. In a screening test the antimicrobial is in a simple environment, the bacteria are growing within broth therefore there are no other components to overcome. The purpose of this experiment was to use a simple method to challenge the antimicrobial with bacteria within a meat product. By dipping the sausage into a solution containing both bacteria and antimicrobial there was a simple solution of incorporating this onto the product. Therefore there was the ability to test the novel compounds efficacy within a food system without having to create a product with the antimicrobials incorporated in it.

3.5.2 Experimental Design

The basis of this sausage dipping experiment was adapted from the method in a paper by (Lara-Lledo et al., 2012). The sausage was sliced into 3mm thick slices using a circular meat slicer before each slice was cut using a cookie cutter, as described in section 2.7.1. This was because the sausage used, a mortadella sausage from Italy, was very large and when initially sliced the slice diameter was still too large for the purposes of the experiment. Once sliced the sausage was stored in a fridge until needed for the experiment, the maximum time of storage was 12 hours.

The solution the sausage slices were dipped in was composed of buffered peptone water (BPW), bacterial culture and antimicrobial compound at concentration 1X. The method for preparation can be seen in section 2.7.3. The amount of bacterial culture added depended upon the level of overnight growth. The aim was to add the culture to the BPW solutions at around 10^6 cfu ml⁻¹ so that the concentration on the sausage slices was approximately 10^4 cfu ml⁻¹. When on the sausage slices at this concentration there is a good level of growth present which will allow for continued growth without a long lag phase. This allows to see in a short space of time the level of growth which can occur and how effective the antimicrobial compounds are at inhibiting this growth. For the initial slice experiments a antimicrobial concentration of 1X was added to the solution however concentrations 10X, 5X, 0.5X and 0.25X were also used depending upon the experiment. Initially concentration 1X was tested with two compounds to determine efficacy then more concentrations were added to explore the effects.

The slices were dipped into the solution for 30 seconds before being removed and dried in a safety cabinet for approximately 10 minutes. The 30 second timing was thought to be enough time for the sausage slice to absorb as much of the liquid as possible.

However, a test was done to prove this. Sausage slices were weighed prior to being dipped into a BPW solution. They were then dipped for 30 seconds and weighed before being dipped for a further 4.5 minutes before being weighed once more. The results of this test can be seen in table 6 below:

Table 3-9 - **The results of the test to see whether the sausage slices absorbed the maximum amount of liquid within 30 seconds.**

Slice Number	Weight T=0 (g)	Weight at T=30 sec (g)	Weight at T=5 min (g)
1	13.4	14.0	14.2
2	13.9	14.6	14.6
3	13.5	14.1	14.2
4	12.6	13.2	13.3
5	13.5	14.4	14.4

As there is little or no difference in the weight of the slices between 30 seconds and 5 minutes it can be concluded that the slice absorbs the maximum amount of liquid within 30 seconds. Once the slices had been allowed to dry meaning excess liquid was removed the slices were placed in sealed petri dishes and incubated at 20°C until required for testing.

The days the slices were tested on depended upon the length of the experiment. Over longer experiments they were tested every five days whereas for shorter experiments this was every 2-3 days. All experiments included a testing point on day 0. On a testing day samples were removed from the incubator in triplicate. By testing each sample in triplicate it allowed for necessary repeats to be conducted without having to repeat the experiment several times. This was necessary as the minimum length of an experiment was 1 week. Each sample was added to 100 ml of BPW in a stomacher bag and stomached for 1 minute, see section 2.7.5. The stomaching process allowed for the sausage slice to be mixed into the BPW giving a solution which could then be diluted and spread onto agar plates. Once stomached each sample was diluted using a 10 fold serial dilution, to the desired concentration. On day 0 this is 10^4 although this increased to 10^6 as the experiment progressed. On day 0 four dilutions were plated using the Miles Misra method onto Palcam agar plates. After this at the other testing points three dilutions were spread plated, generally 10^4 , 10^5 and 10^6 , in duplicate onto Palcam agar plates. The plates were then incubated at 37°C for approximately 36 hours.

Upon removal from the incubator the colonies were counted and results recorded. The results were then formatted using Excel before being turned into a graph. The results of

each triplicate sample were averaged to give one result per time point, any anomalous results were at this point removed from consideration.

3.5.3 Description of Results

The first experiment conducted was to establish the effect compounds D and E had on *L.monocytogenes* growth in sausage slices over 30 day period. Compounds D and E were used as they had shown the most promise in the screening tests. The compounds were added at 10X concentration as it was concluded that this would show a definite effect. The sausage slices were incubated at 20°C and tested every five days. The main purpose of this experiment was to establish whether these compounds which had performed well in the screening test would perform equally as well in a food product. The results are displayed in the graph below.

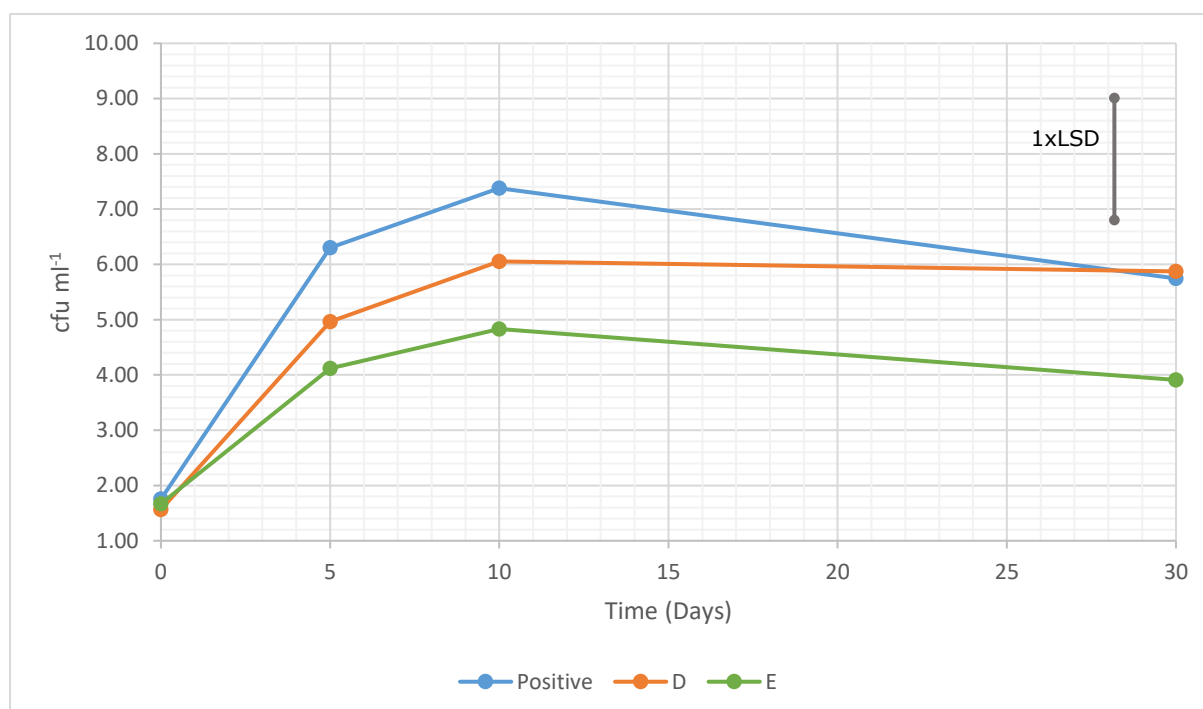


Figure 3-21 - **The effects of two antimicrobial compounds against *L.monocytogenes* in Italian mortadella sausage over a 30 day period:** Slices of mortadella were dipped into a suspension of *L. monocytogenes* [10403S] containing approximately 10^6 cfu ml⁻¹ (control; ●) Separate suspensions were also prepared that contained compounds D (●) or E (●) at a concentration of 10X. Replicate mortadella slices were prepared using each of the antimicrobial compounds, and after excess liquid was removed these were placed into petri dishes. One set of samples (n=3 for each treatment) were sampled on the day of preparation (day 0) and the remainder of the samples were incubated at 20 °C for 30 d. Samples were removed for testing on day 5, day 10 and day 30. At each sampling point, bacterial cells were separated from the meat sample by stomaching and samples were spread plated onto Palcam agar for identification and enumeration of *Listeria*. Data points represent the average results obtained from testing 3 meat slices. Error bar derived from LSD. There was no significant treatment effect as a single factor however there was a significant time effect (ANOVA, Treatment: $p=0.018$, Time: $p<0.001$).

Although there is an increase in growth over the first 10 days there is a substantial reduction in growth in the slices containing the antimicrobial compounds. Over the second five days there is a slowing in growth from all three sets however the slices

containing compounds D and E slow further than the control. After 10 days there is a plateau or reduction in growth from all three sets of data. Any decrease in growth is thought to be for an experimental design reason rather than an actual decrease in growth. It is believed that growth probably plateaued from the maximum level after 10 days. It was thought any decrease could be due to the slices drying up whilst in the incubator thus changing the growth conditions for the *L.monocytogenes*. As the *L.monocytogenes* in the presence of compounds D and E had shown a significant decrease in growth with several log reductions compared to the control it was thought that the products were working very well.

The next experiment which was conducted involved testing one compound under the same circumstances but at a number of lower, more realistic in product concentrations. It was decided that compound E would be used as it had performed more effectively in the previous test. The concentrations used were similar to those in the screening test however 2X was not used. This was because compound E at 1X concentration had been effective in the screening tests. The other concentrations used were 0.5X and 0.25X which were the ideal concentrations for industry partners. The results are shown in the graph below.

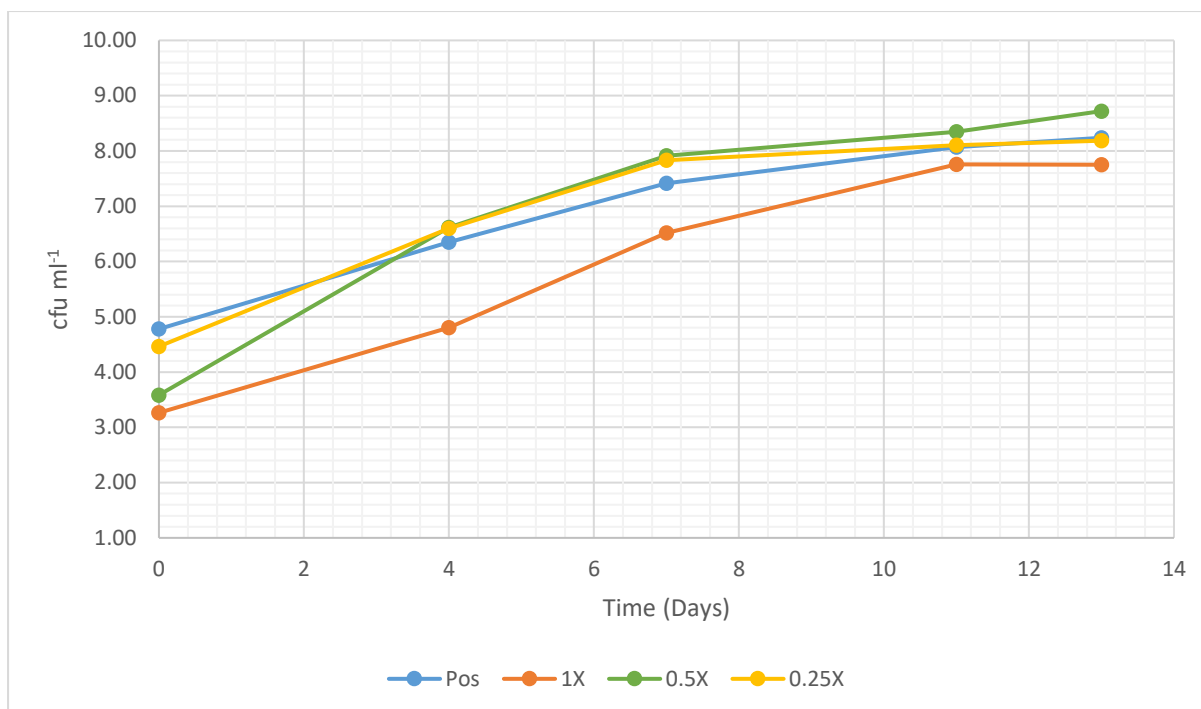


Figure 3-22 - **The effects of compound E at three different concentrations against *L.monocytogenes* in Italian mortadella sausage over a 13 day period:** Mortadella sausage was sliced before being dipped into an *L.monocytogenes* [10403S] suspension. The control (●) solution contained approximately 106 cfu ml⁻¹ of bacterial suspension. Whereas three other suspensions were prepared containing compound E at different concentrations; 1X (●), 0.5X (●) and 0.25X (●). Triplicate slices were prepared for each time point and antimicrobial compound concentration. Excess liquid was removed before slices were placed into petri dishes. One set of samples (n=3 for each treatment) were sampled on the same day as preparation whilst all other samples were stored at 20°C for 13 days. Slices were tested on days 4, 7, 11 and 13. Samples were tested by separating bacterial cells from the meat by stomaching before being spread onto Palcam agar plates. *Listeria* colonies were then identified and counted. Data points represent the average results obtained from testing 3 meat slices. There was no significant treatment effect but there was a significant time effect (ANOVA, Treatment: p=0.166, Time: p=0.012).

It was expected that the effect on the growth of *L.monocytogenes* would not be as great at 1X concentration as it was at 10X concentration. There is still some reduction in growth however there are no whole log reductions. Concentration 1X has slower growth than the control which can be seen by the less steep gradient of the line. Concentrations 0.5X and 0.25X have no effect in comparison to the control. In the screening test some reduction in growth was shown at these concentrations however the more complex challenges in the sausage slice have rendered them ineffective. It was thought that concentration 1X would have more effect in reducing the growth of *L.monocytogenes* however the reduction in growth shown could still be useful to the food industry. By slowing the growth of *L.monocytogenes* the infectious dose may not be reached within the use by date therefore still preventing illness for the consumer.

The next experiment undertaken was to compare the growth at 10X concentration to the growth at 1X concentration. This was done as a replication of the first and second experiments combined to confirm the reliability of the results. Compound E was used at concentrations 10X, 5X and 1X, to see the difference in effect on growth compared to

the control. The experiment length was shortened to 8 days to allow it to fit the four testing points into 1 week. It had been shown in the previous experiment that most growth had occurred by 8 days and after the growth was beginning to slow. The results are displayed in the graph below.

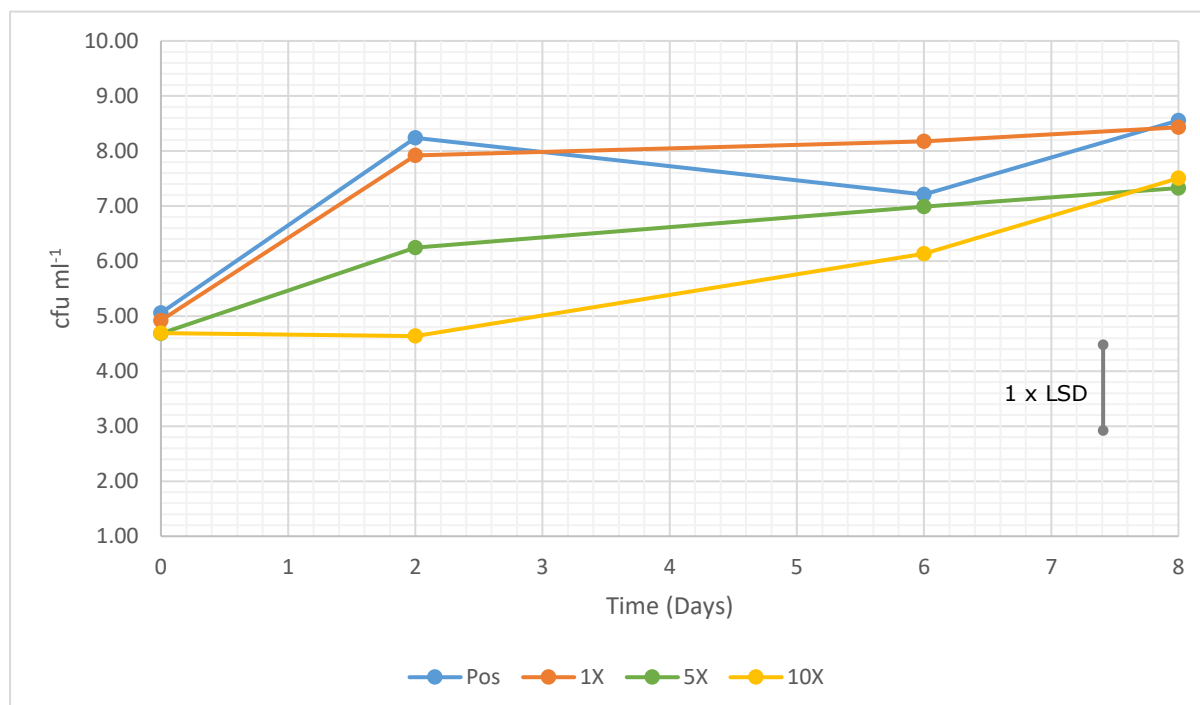


Figure 3-23 - The effects of compound E at three different concentrations against *L. monocytogenes* in Italian mortadella sausage over an 8 day period: Into a suspension of *L. monocytogenes* [10403S], slices of mortadella sausage were dipped. The control (●) suspension contained approximately 10^6 cfu ml⁻¹ and three other suspensions contained compound E at 1X (●), 5X (●) and 10X (●). Replicate sample were prepared for each time point and concentration of antimicrobial. Slices were placed in petri dishes once excess liquid was removed. Samples were tested at day 0, 2, 6 and 8; one set of samples (n=3 for each treatment) for each time point. All but the day 0 samples were stored at 20°C over the 8 days. Bacterial cells and meat were separated by stomaching and bacteria spread onto Palcam agar plates and *Listeria* colonies counted. Data points represent the average results obtained from testing 3 meat slices. Error bar derived from LSD. There was both a significant treatment and time effect (ANOVA, Treatment: $p < 0.001$, Time: $p < 0.001$).

Concentration 1X shows slightly reduced growth compared to the control. The reduction in growth is not as great as in the previous experiment however the difference is not that great so is generally comparable. At 10X concentration there is greater reduction of growth with this concentration showing an inhibition of growth over the first 2 days. Growth then begins to increase but there is a log reduction in growth compared to the control. Concentration 5X, as expected shows growth in between 1X and 10X, however the maximum growth after 8 days shows the same reduction compared to the control as concentration 10X. The control shows a decrease in growth on day 6, this is most probably an anomalous result. It is unlikely that there would be a sudden decrease in growth at that point therefore something probably went wrong during the testing process.

The next experiment involved testing *L.monocytogenes* ability to grow at low temperatures, especially fridge and fridge abuse temperatures. As all previous experiments were run at 20°C which is an unlikely temperature for RTE meat products to be left at unless left out of the fridge an experiment was run at 10°C. although it is unlikely for a consumer to leave a meat product at room temperature it is not uncommon for these products to be left at fridge abuse temperatures, between 8-10°C therefore the antimicrobial was tested at this temperature to determine when the effects on growth changed during cold temperature growth.

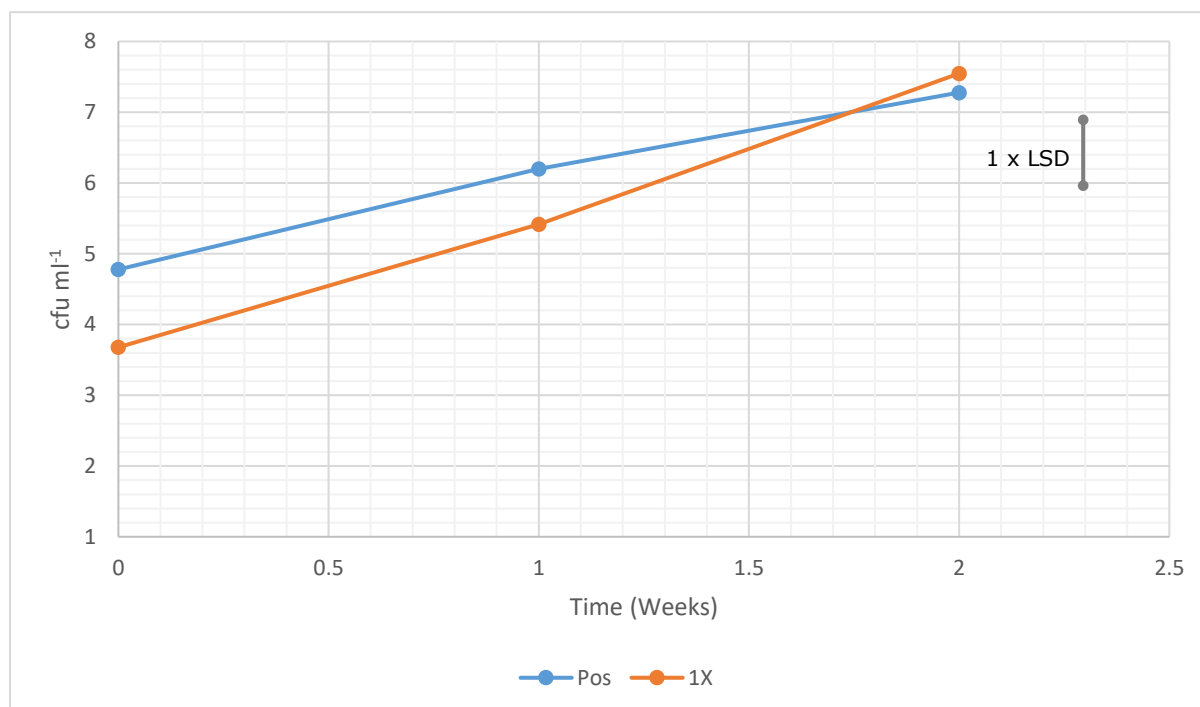


Figure 3-24 - **The effects of compound E against *L.monocytogenes* in Italian mortadella sausage over a 2 week period at refrigeration temperature.** A suspension of *L. monocytogenes* was prepared containing approximately 10^6 cfu ml⁻¹. Slices of mortadella sausage were dipped into this suspension, the control (●) and a suspension also containing compound E at concentration 1X (●). For each time point replicate samples were prepared; slices were air dried and placed into petri dishes. Testing took place after 1 and 2 weeks as well on the same day as preparation (week 0), samples were stored at 10°C over the 2 weeks. Bacterial cells were separated from the meat slices by stomaching and cells spread onto Palcam agar plates (n=3 for each treatment). *Listeria* colonies were then identified and enumerated. Data points represent the average results obtained from testing 3 meat slices. Error bar derived from LSD. There was no significant treatment effect but there was a significant time effect (ANOVA, Treatment: $p=0.503$, Time: $p=0.015$).

As this experiment was conducted at 10°C it took place over 2 weeks and testing was done every week. This is due to the slow replication rate of *L.monocytogenes* at this temperature. There was reduced growth at concentration 1X which can be seen in the graph above. When compared to the control there is just under one log reduction in growth after 1 week. However, after 2 weeks the growth of *L.monocytogenes* at concentration 1X increases to exceed that of the control. From the gradient of the lines on the graph it can be seen that concentration 1X is starting to increase at a higher rate than the control. This was not as expected and could have been due to a number of

reasons. This particular experiment had a lot of issues with mould growth something which didn't inhibit the growth of *L.monocytogenes* could have resulted in this effect. A final experiment was conducted at 20°C to compare the growth of *L.monocytogenes* in the presence of a number of different compounds. The experiment was set up using a control alongside compounds D, E, H, J and K. D and E being novel compounds and H, J and K being industry products, this allowed for the comparison in efficacy between the compounds being developed and those already being used in industry. The experiment was set up in the same way as the previous sausage slice experiments. At day 0 normal results were obtained however when tested on day 3 no *L.monocytogene* colonies were present on the Palcam agar plates. The same results were recorded at day 6 and day 8. As it has already been proven that the compounds are not bactericidal and the bacteria did not grow on the control plates, further investigation was required to establish why the *L.monocytogenes* were not growing.

Two small experiments were set up to establish why *L.monocytogenes* had not grown in the previous experiment. It was suspected that the formulation of the meat had changed resulting in a preservative being added which caused the inhibition of *L.monocytogenes* growth. The first experiment involved placing small slices of the sausage onto a BHI agar plate and leaving them at 4°C for approximately 48 hours. The slices were then removed and *L.monocytogenes* culture spread across the plate which was subsequently incubated at 37°C overnight. In this case if the product within the sausage which was causing the inhibition of growth had been high enough in concentration to contact kill, the bacteria would not grow where the sausage slices had been. This was not the case as *L.monocytogenes* grew across the plate. The second test was to spread bacterial culture across the plate and then add the small sausage slices to the surface. If the sausage contained a preservative or something with the ability to inhibit *L.monocytogene* growth the results would be a halo around the sausage slice.

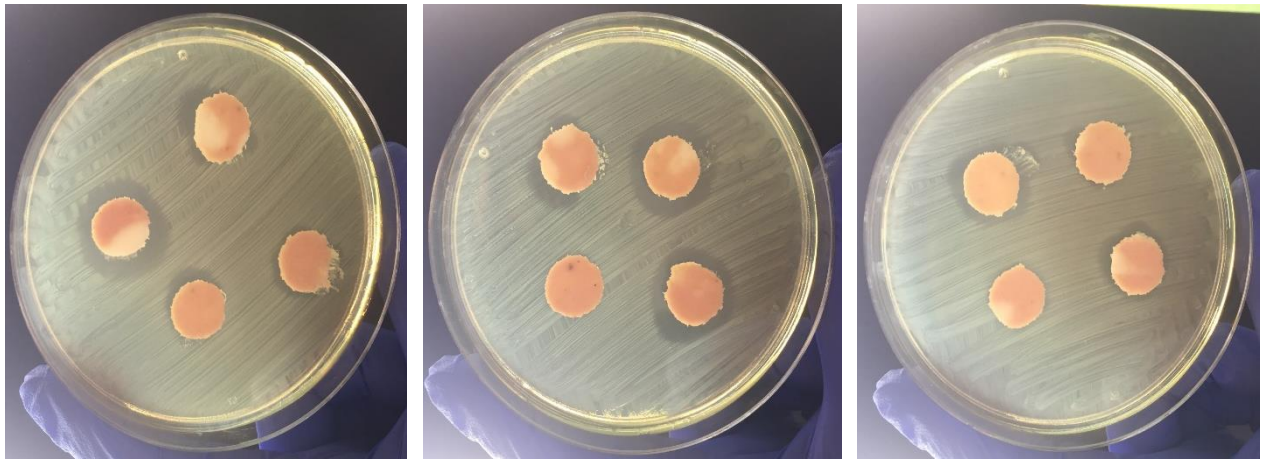


Figure 3-25 - **Images showing the inhibition of growth of *L.monocytogenes* using Italian mortadella sausage slices.** A suspension of *L.monocytogenes* was grown overnight at 30°C in BHI broth. The suspension was spread onto a BHI agar plate using a cotton swab before four sausage slices were placed onto the surface of the agar. Plates were incubated at 20°C overnight. Zones of inhibition surrounding the sausage were analysed. Three plates were prepared to give replicate samples.

As can be seen in the pictures above there is a halo of no growth around most of the sausage slices. Although this isn't present around all of the slices and some zones of inhibition are larger than others this results proves that there is something within the sausage product inhibiting the growth of *L.monocytogenes*. The product is then diffusing out of the slice into the agar thus inhibiting growth in the immediate area surrounding the slice. This creates the zone of inhibition seen. It is thought that the product inhibiting growth must need to gradually diffuse over time which was why in the previous experiment no inhibition was seen. As the products in the sausage had not changed since the initial experiments were conducted where *L.monocytogenes* had grown there is a possibility that lactic acid bacteria are present and producing nicin. Nicin is proven to effectively inhibit the growth of *L.monocytogenes*.

The concern with this discovery is that the results in the other experiments have been effected by this issue. If this is the case then the effects of the products have possibly been decreased due to the other inhibitory product being present. Therefore there is a possibility that the novel compounds have more effect than the previous experiments have shown.

Due to the slices being dipped into the compound and bacterial culture solution it was not certain how much was getting into the product. Although the test of weight proved that the maximum amount of liquid was absorbed in the 30 seconds dip time there was no certainty that the compound and bacteria were spread through the sausage slice. To attempt to incorporate the bacteria and antimicrobial compound into the sausage product a slurry was made. This could then have the bacteria and antimicrobial mixed in before it was formed into patties. This also gave a use for the excess meat generated from the smaller slices being cut out.

The excess meat was placed into a thermomixer where it was mixed until the sausage had the consistency of a smooth paste, see section 2.7.2 for method. The *L.monocytogenes* culture and antimicrobial were then mixed into the slurry before it was aliquoted into 20g aliquots and placed in petri dishes for incubation at 20°C. After this the slurry was treated the same as the slices in terms of sampling and recording of results. The first slurry experiment was conducted at 20°C and involved different concentrations of compound E being tested against *L.monocytogenes*. The concentrations used were 1X, 0.5X and 0.25X and the experiment was run for 13 days alongside the sausage slice experiment of the same length. The results can be seen in the graph below.

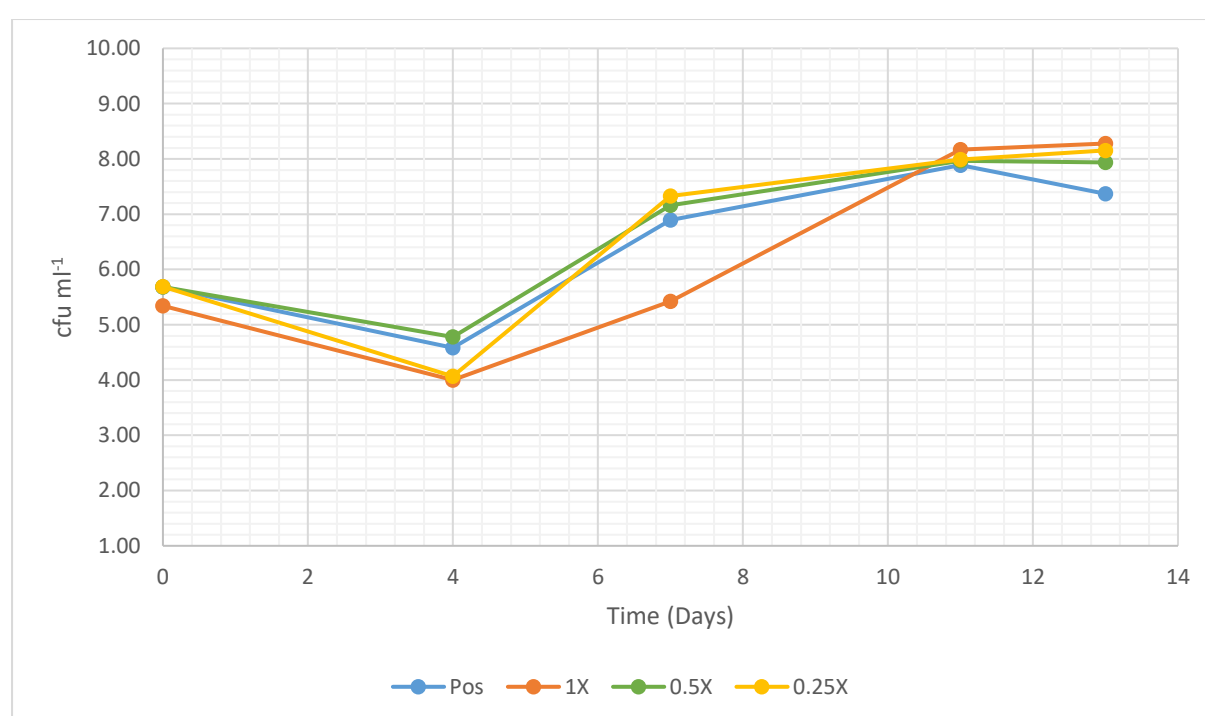


Figure 3-26 - The effects of compound E at three different concentrations against *L.monocytogenes* in Italian mortadella sausage slurry over a 13 day period. Mortadella sausage slurry was mixed with a suspension of *L. monocytogenes* [10403S] at around 10^6 cfu ml⁻¹ (control; ●). Separate suspensions containing compound E at concentrations 1X (●), 0.5X (●) and 0.25X (●) were also prepared. Slurry was then removed and 20g aliquots placed in petri dishes, replicate aliquots were prepared for each concentration of compound E. Testing was carried out at day 0, 4, 7, 11 and 13, day 0 being the day of preparation. Samples (n=3 for each treatment) were incubated at 20°C over the 13 days. On sampling days bacterial cells were separated from the slurry by stomaching and samples spread plated onto Palcam agar. *Listeria* were then identified and counted. . Data points represent the average results obtained from testing 3 meat slices. There was no significant treatment effect as a single factor however there was a significant time effect (ANOVA, Treatment: $p=0.0914$, Time: $p<0.001$).

The results show an unusual trend where there is an initial decrease in growth after 4 days. This is unusual as once added to the meat *L.monocytogenes* is normally able to easily adapt and grow however in this case something is inhibiting the initial growth. This is likely to be due to the incorporation of the antimicrobial into the sausage or the

environment became more unsuitable to the bacteria when the sausage was broken down in the mixer. After 4 days growth begins to increase again, 1X concentration has initially slower growth after this point. However, after 11 days the same maximum point as the control is reached. 0.5X and 0.25X concentration have no effect on the growth of *L.monocytogenes* compared to the control. Overall in this slurry model, compound E does not have a particularly significant effect on the growth compared to the control. Even when compared to the same experiment conducted with slices compound E has less effect. The slurry was more effected by mould than the slices it was also quicker to go rancid which could have contributed to the overall results. The mould was proven not affect the growth of *L.monocytogenes* however it may have contributed to how quickly the slurry 'went off' compared to the slices. The rancidity of the product after approximately 8 days meant that the antimicrobial compounds had more challenges therefore is chanced to have worked as effectively.

The same experiment was conducted at 10°C over a 2 week period. As with the slices experiment this was to see how the antimicrobials worked when challenged with *L.monocytogenes* in its colder temperature morphology. The same method as the slurry experiment at 20°C was used however only concentration 1X of compound E was tested. The results can be seen below.

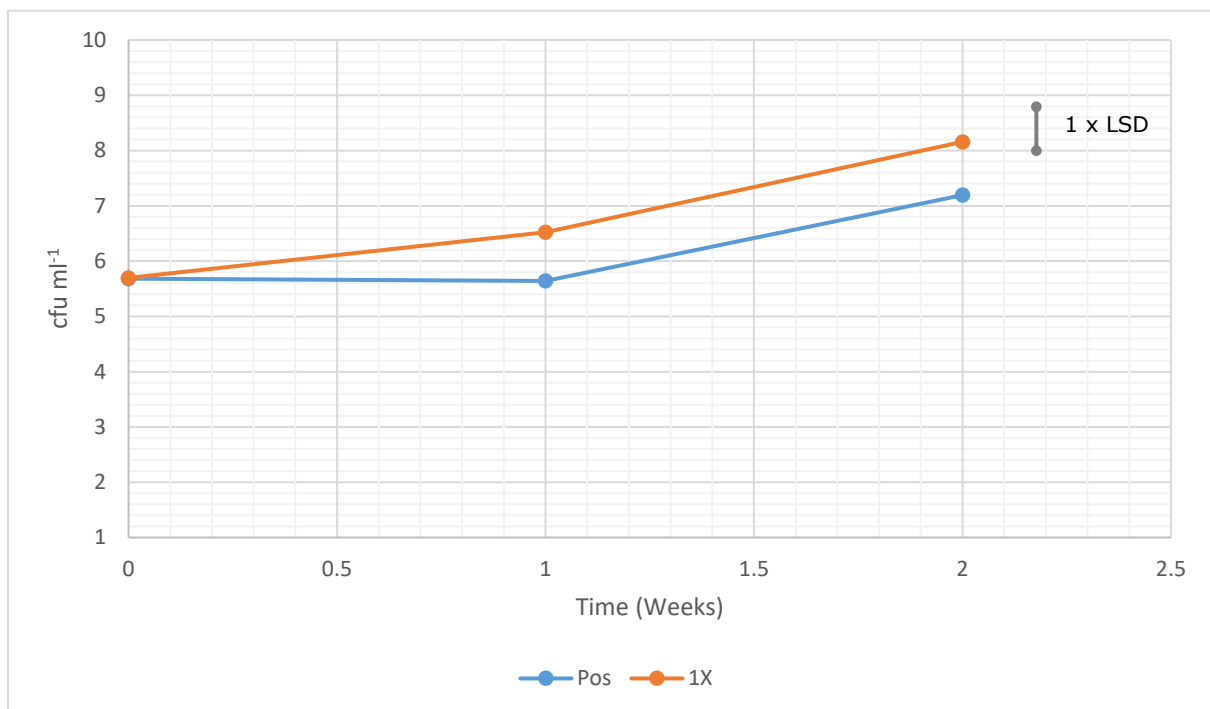


Figure 3-27 - **The effects of compound E against *L.monocytogenes* in Italian mortadella sausage slurry over a 2 week period at refrigeration temperature.** A suspension of *L. monocytogenes* at approximately 10^6 cfu ml⁻¹ and added to mortadella sausage slurry in a stomacher bag (control; ●). Another stomacher bag was prepared also containing compound E at a working concentration 1X (●). Slurry was placed in petri dishes in 20g aliquots and stored at 10°C for 2 weeks. Samples (n=3 for each treatment) were removed for testing on week 0 (day of preparation), week 1 and week 2. Sampling was by separating the meat and bacterial cells by stomaching and spread plating onto Palcam agar. *Listeria* were identified and enumerated. Data points represent the average results obtained from testing 3 meat slices. Error bar derived from LSD. There was both a significant treatment and time effect (ANOVA, Treatment: $p=0.003$, Time: $p<0.001$).

The growth of *L.monocytogenes* was monitored over a 2 week period using a control and a sample with antimicrobial compound E at concentration 1X. This experiment gave some interesting results as the sample containing compound E grew at increased level to the control. This was not to be expected as compound E should be able to slow the growth of *L.monocytogenes* as had been seen in the previous experiments. In the control growth is inhibited for the first week until it starts to increase over the second week. A similar growth pattern is seen in the concentration 1X however the inhibition of growth in the first week is not present. Instead there is a slow increase in growth. There are a few reasons the growth of the sample containing the antimicrobial exceeded the growth of the control. There were mould in these samples therefore this could have affected the growth of *L.monocytogenes*. Compound E may have reduced the growth of the mould therefore allowing more bacterial growth however this is unlikely as tests to check if the mould present effected the growth of *L.monocytogenes* gave negative results. The other explanation would be an anomalous set of results where the bacteria

have grown differently on the occasion. Overall the results of this experiment were not as expected therefore the results cannot be relied upon to form any conclusions.

3.5.4 Discussion

These experiments were designed to test the most promising antimicrobials from the screening test in a basic food system. The bacterial target in these experiments was *L.monocytogenes*, this was because this is the main problem for the food industry in ready to eat foods. As the Italian mortadella sausage being used in this food system is a ready to eat product this was the most relevant bacteria to test it against.

Two forms of experiments were conducted, slices and slurry. The slurry was less successful than the slices, the results gained do not show anything to suggest the antimicrobial used is having any substantial effect. Designed to use up excess meat created from the slice experiment it was hoped that it would allow for a more integrated approach to the in food system testing. As the compound and bacteria would be mixed into the slurry instead of just being placed on the surface it was thought that the distribution would be more equal. However, the method used was possibly not the best as it involved introducing a lot of liquid to the meat. This meant that the water content of each sample was probably very different. The result of this is results that were not particularly reliable. When looking at the literature there was nothing on the subject of sausage slurry. Most papers focused on integrating the product fully into the product and then testing whilst some adopted the quicker dipping method. As it seemed this particular type of experiment had not been conducted before there was no data to compare these results to, however, given the results it could probably be said that overall this method was not a success. As such when further experiments were conducted on sausage slices it was decided that no further slurry experiments would be conducted.

When compound E was tested on the sausage slices there were some encouraging results gained. There was some effect in either slowing or inhibiting the growth of *L.monocytogenes* at every concentration with the higher concentrations having a greater effect on growth. There are several instances in the literature which have tested common organic acid preservatives against *L.monocytogenes* at various different temperatures and packaging types. In an experiment by (Samelis et al., 2001) the results describe a 4-5 log increase in the controls growth whereas in the presence of an antimicrobial this reduces to 2 and 5 log increase depending upon the concentration of antimicrobial used. This is comparable to the results that have been seen in this experiment. By gaining comparable results this shows that these novel antimicrobials might give better results than the standard antimicrobials currently being used. similar results again were gained by (Barmpalia et al., 2004) who showed similar reductions of *L.monocytogenes* in frankfurter sausages. Although these experiments were conducted

at lower temperatures they were left for considerably more time. To reduce the time of experiments due to the time restrictions of this project it was decided that the experiments would be conducted at 20°C. Although the morphology of *L.monocytogenes* should be similar at 20°C as it is a colder temperatures, however the higher temperature allowed for experiments to be completed and results collected in weeks rather than months. Overall the results from these experiments showed that there was scope for the antimicrobial to be tested further as it had given encouraging results in both the screening and slices tests.

The main problem with the results of these experiments is the validity of the results. As the Italian mortadella used in the tests was able to inhibit the growth of *L.monocytogenes* as seen in figure 3-25 it is not known whether the inhibition of growth in the experiments was due to the antimicrobial or the product in the sausage. There are two scenarios which are the most likely to be what will happen. One is that the effect on the growth of *L.monocytogenes* is due to the antimicrobial added to the sausage during dipping. In this case the results may not give an accurate representation of the products abilities. This could be possible as the results from the screening test show compound E reducing the growth of *L.monocytogenes* substantially more than in the slices test. This would mean that compound E has a greater effect on the growth of *L.monocytogenes* thus as an antimicrobial it would perform better. Therefore the antimicrobial compounds ability would probably exceed that of organic acid products currently used in industry. The other option would be that the antimicrobial compound added to the sausage slice was not inhibiting or reducing the growth of *L.monocytogenes*. This would mean that the reduction in growth seen in the results was due to the Italian mortadella sausage product and not the antimicrobial being tested. Although this was only tested after the final unsuccessful experiment there is a chance that it was present in the sausage product throughout the experiments. The most likely cause of the inhibition is nisin. Nisin has been shown to effectively reduce the growth of *L.monocytogenes* in many food products, it is one of the most effective inhibitors of this bacteria (Benkerroum and Sandine, 1988). In the case of the sausage nisin is likely to have been a by-product of lactic acid bacteria contamination. If these bacteria had contaminated the Italian mortadella sausage which is possible due to methods used to make the product. When completed the product is only heated to a low temperature meaning many bacteria can still exist in the product; as a ready to eat product this is an interesting method. By the lactic acid bacteria being present and nisin being produced the growth of *L.monocytogenes* is reduced with or without compound E being present.

3.5.5 Next Steps

The first next step would be to establish whether the antimicrobials are having the effect in food that was initially thought. To do this the antimicrobial needs to added to a

controlled product and incorporated during the manufacturing process. This will allow for results establishing whether the novel antimicrobial compound has an effect in food or maybe an enhanced effect on what has been seen in these results. Or whether the effect shown in this set of results is from a product such as nisin in the sausage which has been used. A controlled product would need to be made and used to allow this experiment to work. By incorporated the antimicrobial instead of dipping this would allow for the same process as a manufacturer in industry would use therefore this would give an idea of effectiveness for manufacturers and the food industry.

If the novel antimicrobial does provide good results in the controlled meat product then the next step would be test using this method, different concentrations of the antimicrobial. This would establish which concentration would be the best balance between effectiveness and economic advantage. Once these results had been gained experiment would need to be conducted in conditions similar to that expected of the product. Therefore tests would need to be done at fridge temperatures and over a timescales of several weeks or months. This would then replicate what would happen to a product purchased by a consumer.

3.6 Mince

3.6.1 Aim

In ready to eat foods *L.monocytogenes* is a bacteria which is of main concern, however in fresh meat products this is not the case. *Salmonella typhimurium* and *E.coli* can cause either spoilage or illness and are prone to grow in fresh meat products at fridge abuse temperatures. At fridge temperature these bacteria do not grow however if fridges reach 7°C, which is commonly found, these bacteria can start to grow. Therefore the aim of testing the antimicrobial compounds in mince products allows for the determination of efficacy against these bacteria in a food system. The effects have been seen in the screening tests against these two bacteria so experiments were carried out to see if they could be used in fresh meat products.

3.6.2 Experimental Design

The method for these experiments were designed from those used in the sausage experiments, adaptations were made so that they suited the product being used in testing. In the first experiment mince was provided by industry, the mince was 100% pork mince which arrived from source. In further experiments including viable count mince was purchased from the supermarket Morrisons. The product bought was 100% pork mince with a use by date for at least a week after the product was bought. Two methods were used in these experiments the second being developed from the first at a later point to try and improve the experimental design.

The mince needed for the samples was mixed with BPW, the antimicrobial compound at concentration 1X and *S.Typhimurium* and *E.coli* culture. This was done in a Seward stomacher bag and the mince mixed thoroughly by hand to ensure it was completely coated. It was decided to mix by hand as using a stomacher machine would have destroyed the mince therefore the product would not be in the state found by a consumer. The amount of BPW depended upon the amount of mince needed, in this case 200 ml of BPW was used alongside antimicrobial at concentration 1X and culture of both bacteria at around 10^6 cfu ml⁻¹ so that the concentration on the mince was approximately 10^4 cfu ml⁻¹. Instead of different concentrations, a number of different compounds was used in this experiment. Compounds D and E were used due to the good effect they had shown inhibiting the growth of the bacteria in the screening tests. Compound H was introduced as an industry comparison, this product is currently used as an antimicrobial in meat products however is not natural.

Once the mince had been mixed in the solution 20 g aliquots were taken and placed in petri dishes. The amount was chosen as it provided enough mince to form a 'patty' type shape yet wasn't too large to fit into a standard petri dish. The mince was then incubated at 20°C for the six day experiment period being tested at three points; day

zero, three and six. The temperature used was to allow for the growth of *S. Typhimurium* and *E.coli* over a week timescale without spoiling the mince product. At lower temperatures the bacteria growth would be inhibited or very slow meaning the experiment time would be weeks rather than days.

On each testing day, triplicate samples were removed from the incubator for testing. Each sample was added to 200 ml of BPW in a stomacher bag and stomached using a Seward stomacher at 300 rpm for 1 min. 200 ml of BPW was used as this was ten times the weight of the mince and allowed the method to be in line with the method used for the sausage slices. Once stomached 1 ml of the resulting solution was removed and a 10 fold serial dilution performed. At day zero, Miles and Misra was used to plate dilutions up to 10^4 onto XLD agar to select for *S. Typhimurium* and *E.coli*. *S. Typhimurium* grows as a round black colony whereas *E.coli* is a round straw yellow colony. This allowed for them to be plated onto the same plate and counted separately. On all other testing days spread plates were spread in duplicate. Three dilutions were plated the dilutions used were between 10^4 and 10^7 depending upon the time point of the experiment. The plates were then incubated at 37°C for 24 hours. After this colonies were counted and the results recorded for further analysis.

In the case of method two a similar process was followed however the BPW was removed to prevent the mince from being so wet. This meant only the antimicrobial compound at concentration 1X and bacterial culture were added to the mince. In this experiment *S. Typhimurium*, *E.coli* and *P.fluorescens* were added to the mince. To add the antimicrobial compound it was diluted in sterile water to give a larger volume to allow the mince to be coated, however the concentration was maintained at 1X. The same principals were applied to the culture however overall the amount of liquid added was around a tenth of the weight of the mince. The same sampling method was used and the solution was still plated onto XLD agar. Due to the addition of *P.fluorescens* the stomached solution was also plated onto Pseudomonas agar so that *P.fluorescens* colonies could be selected and counted.

3.6.3 Description of Results

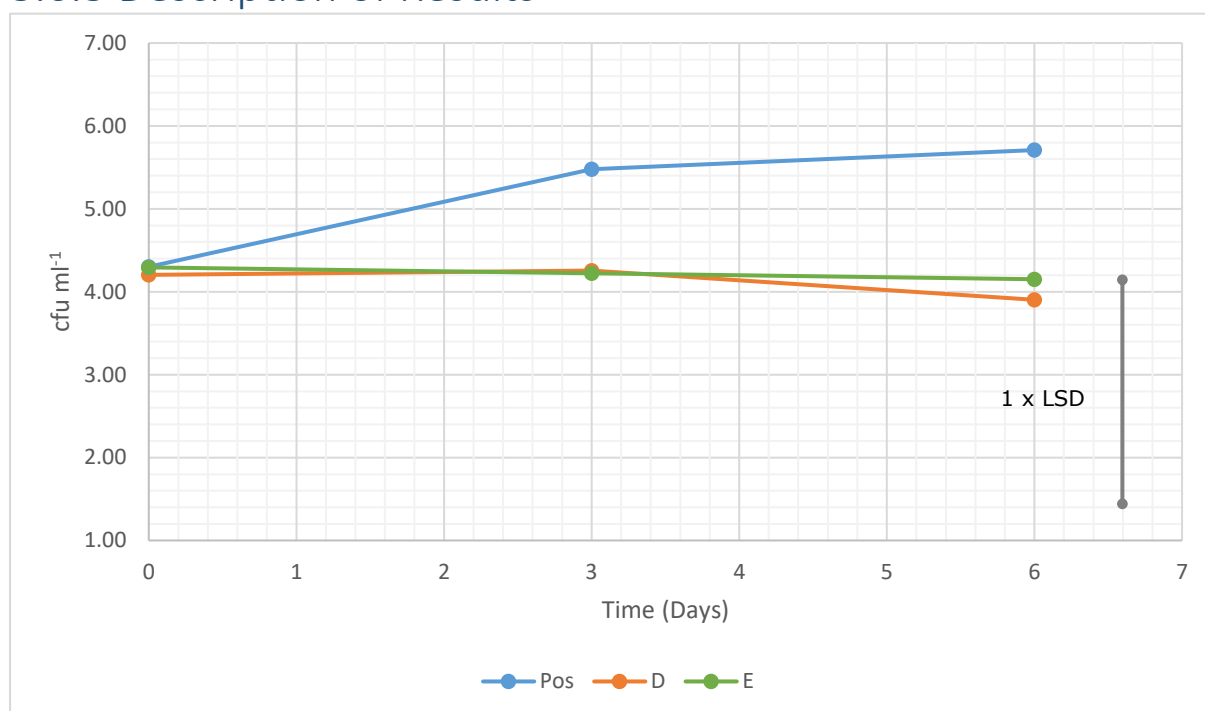


Figure 3-28 - **The effects of two antimicrobial compounds against *S. Typhimurium* in 100% pork mince over a 6 day period using method 1.** A suspension of *S. Typhimurium* [DB7516] at around 10^6 cfu ml⁻¹ was added to pork mince (control; ●). Separate suspensions were also prepared that contained compounds D (●) or E (●) at concentration 1X. Mince was aliquoted into petri dishes with 20g per dish, replicate samples were prepared for each compound. Samples (n=3 for each treatment) were removed for testing on day 0 (preparation day) whilst samples for testing on days 3 and 6 were incubated at 20°C. Bacterial cells were removed from the mince by stomaching and samples were spread onto XLD agar plates. *S. Typhimurium* colonies were identified and counted. Data points represent the average results obtained from testing 3 meat slices. Error bar derived from LSD. There was no significant treatment effect but there was a significant time effect (ANOVA, Treatment: $p=0.383$, Time: $p=0.035$).

In the first experiments conducted using method 1 both compounds D and E had a substantial effect on the growth of *S. Typhimurium*. In comparison to the control there is an estimated two log reduction in growth. The decrease in growth after day 3 for compound D is likely to be an issue with the experimental design and this is most probably not representative of an actual decrease in growth. Therefore for both compounds D and E there is inhibition of growth of the bacteria. There is no bactericidal effect however this is not expected as the compounds have been proven to be bacteriostatic. As the graph shows the growth remains at the starting level throughout whereas the control shows steady increased growth over the 6 days.

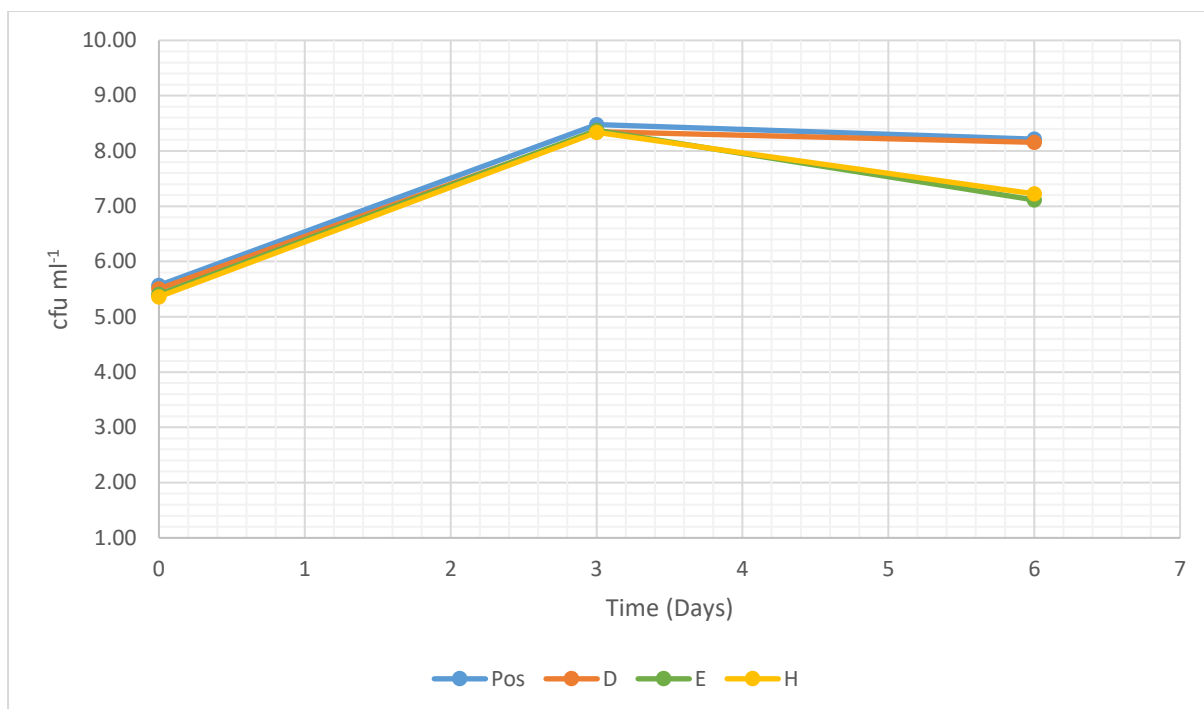


Figure 3-29 - **The effects of three antimicrobial compounds against *S. Typhimurium* in 100% pork mince over a 6 day period using method 1.** A suspension of *S. Typhimurium* [DB7516] at around 10^6 cfu ml⁻¹ was added to pork mince (control; ●). Separate suspensions were also prepared that contained compounds D (●) or E (●) and H (●) at concentration 1X. Mince was aliquoted into petri dishes with 20g per dish, replicate samples were prepared for each compound. Samples (n=3 for each treatment) were removed for testing on day 0 (preparation day) whilst samples for testing on days 3 and 6 were incubated at 20°C. Bacterial cells were removed from the mince by stomaching and samples were spread onto XLD agar plates. *S. Typhimurium* colonies were identified and counted. Data points represent the average results obtained from testing 3 meat slices.

A repeat of the initial experiment was conducted several months later. In this experiment *S. Typhimurium* grew at a quicker rate than had been seen previously and therefore than expected. Due to this the dilutions which were plated at day 3 were not high enough to produce countable colonies. This resulted in the day 3 results being given an estimated colony count value of the same amount meaning the growth between day 0 and day 3 does not give any useful interpretable results. From the results at day 6 it can be seen that compounds E and H had an effect on *S. Typhimurium* showing a log decrease in growth from the control. Compound D shows no effect having the same maximum growth level as the control.

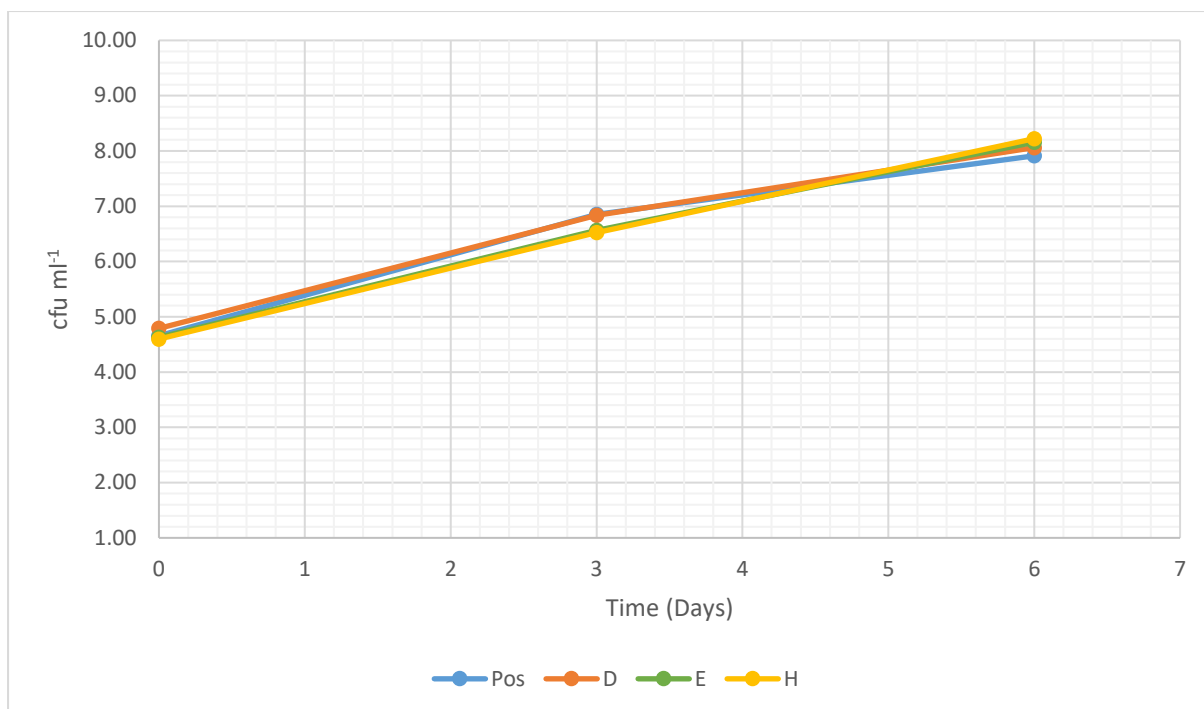


Figure 3-30 - **The effects of three antimicrobial compounds against *S. Typhimurium* in 100% pork mince over a 6 day period using method 2.** Pork mince was mixed with a *S. Typhimurium* [DB7516] suspension. The control (●) solution contained approximately 10^6 cfu ml⁻¹ of bacterial suspension. Whereas three other suspensions were prepared containing compound, D (●), E (●) and H (●) at concentration 1X. Mince was aliquoted into petri dishes with 20g per dish, replicate samples were prepared for each compound. One set of samples (n=3 for each treatment) were sampled on the same day as preparation whilst all other samples were stored at 20°C for 6 days. Slices were tested on days 3 and 6. Samples were tested by separating bacterial cells from the meat by stomaching before being spread onto XLD agar plates. *Salmonella* colonies were then identified and counted. Data points represent the average results obtained from testing 3 mince aliquots.

Due to excess liquid in method 1 the same experiment was repeated using method 2. There was still increased growth at day 3 however higher dilutions were plated on this occasion to get an accurate colony count. Compared to this control there is no difference in either growth rate or maximum growth from any of the 3 compounds being tested. Although compounds E and H have slightly slower growth over the first 3 days it is not enough to be considered of any value.

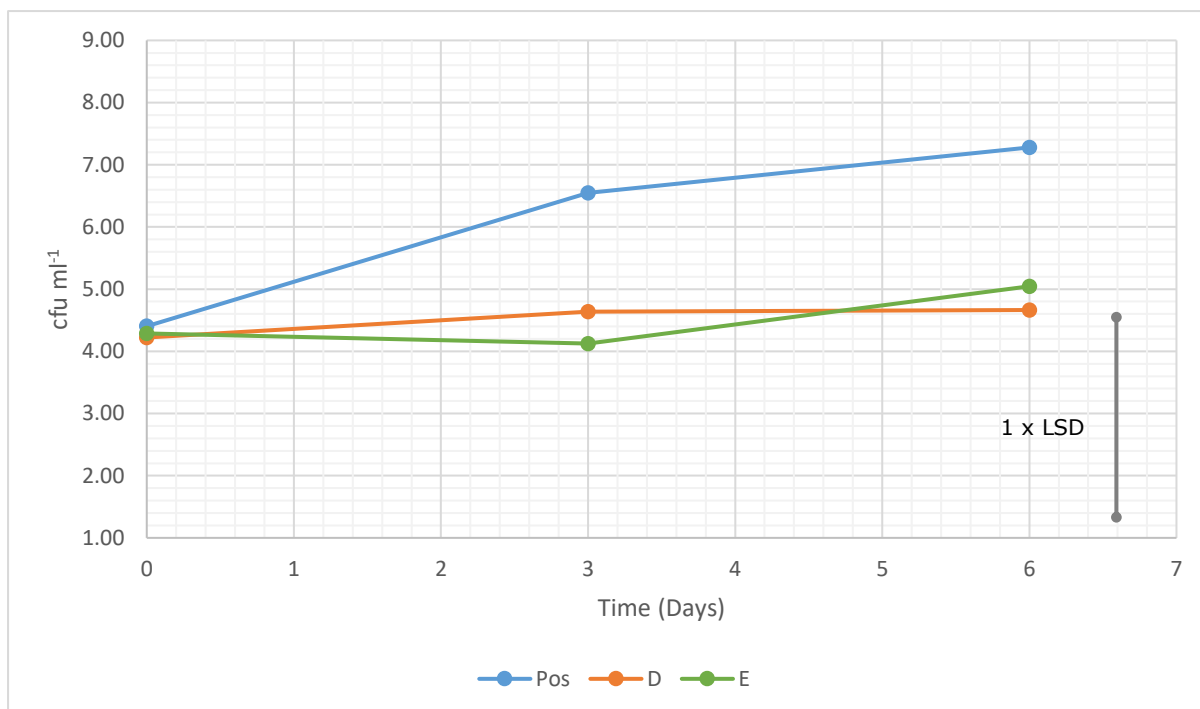


Figure 3-31 - **The effects of two antimicrobial compounds against *E.coli* in 100% pork mince over a 6 day period using method 1.** A suspension of *E. coli* [MG1655] at around 10^6 cfu ml⁻¹ was added to pork mince (control; ●). Separate suspensions were also prepared that contained compounds D (●) or E (●) at concentration 1X. Mince was aliquoted into petri dishes with 20g per dish, replicate samples were prepared for each compound. Samples (n=3 for each treatment) were removed for testing on day 0 (preparation day) whilst samples for testing on days 3 and 6 were incubated at 20°C. Bacterial cells were removed from the mince by stomaching and samples were spread onto XLD agar plates. *E. coli* colonies were identified and counted. Data points represent the average results obtained from testing 3 meat slices. Error bar derived from LSD. There was a significant treatment effect but not a significant time effect (ANOVA, Treatment: $p=0.006$, Time: $p=0.636$).

In this first experiment with *E.coli* the bacteria's growth was effected by compounds D and E. there was a 2.5 to 3 log reduction in growth compared to the control at the end of the experiment on day 6. Compound D shows that there is a small increase in growth between day 0 and day 3 however after this growth plateaus. Whereas with compound E the growth of *E.coli* decreases initially before increasing after day 3 to result in a higher maximum growth compared to compound D. however, both compound D and E show a decreased growth compared to the control.

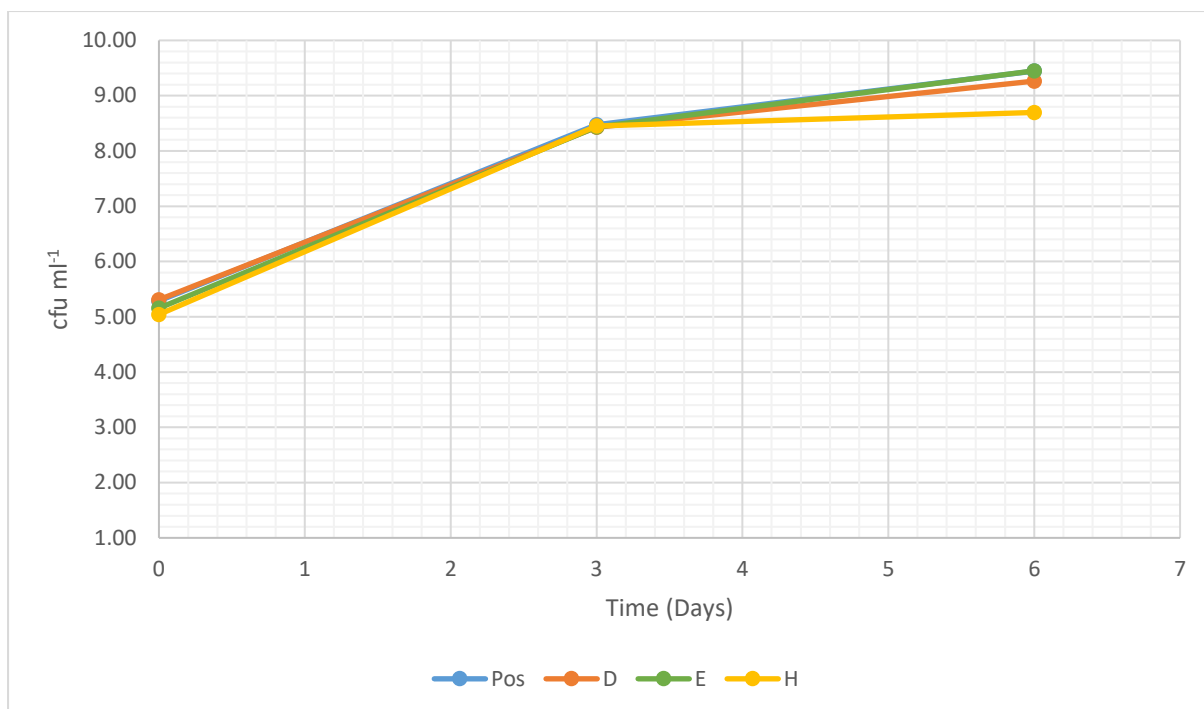


Figure 3-32 - **The effects of three antimicrobial compounds against *E.coli* in 100% pork mince over a 6 day period using method 1.** A suspension of *E. coli* [MG1655] at around 10^6 cfu ml⁻¹ was added to pork mince (control; ●). Separate suspensions were also prepared that contained compounds D (●) or E (●) and H (●) at concentration 1X. Mince was aliquoted into petri dishes with 20g per dish, replicate samples were prepared for each compound. Samples (n=3 for each treatment) were removed for testing on day 0 (preparation day) whilst samples for testing on days 3 and 6 were incubated at 20°C. Bacterial cells were removed from the mince by stomaching and samples were spread onto XLD agar plates. *E. coli* colonies were identified and counted. Data points represent the average results obtained from testing 3 meat slices.

In the second repeat the same issue with the colony counting for day 3 occurred as described relating to Figure 3-32. From day 3 there is no real reduction in growth of *E.coli* compared to the control. Compound E has the same maximum growth as the control and can be seen to grow at exactly the same rate. Compound D shows a small reduction in growth and compound H and slightly bigger decrease. Compared to the control no compound shows a major decrease in the growth of *E.coli* compared to the control.

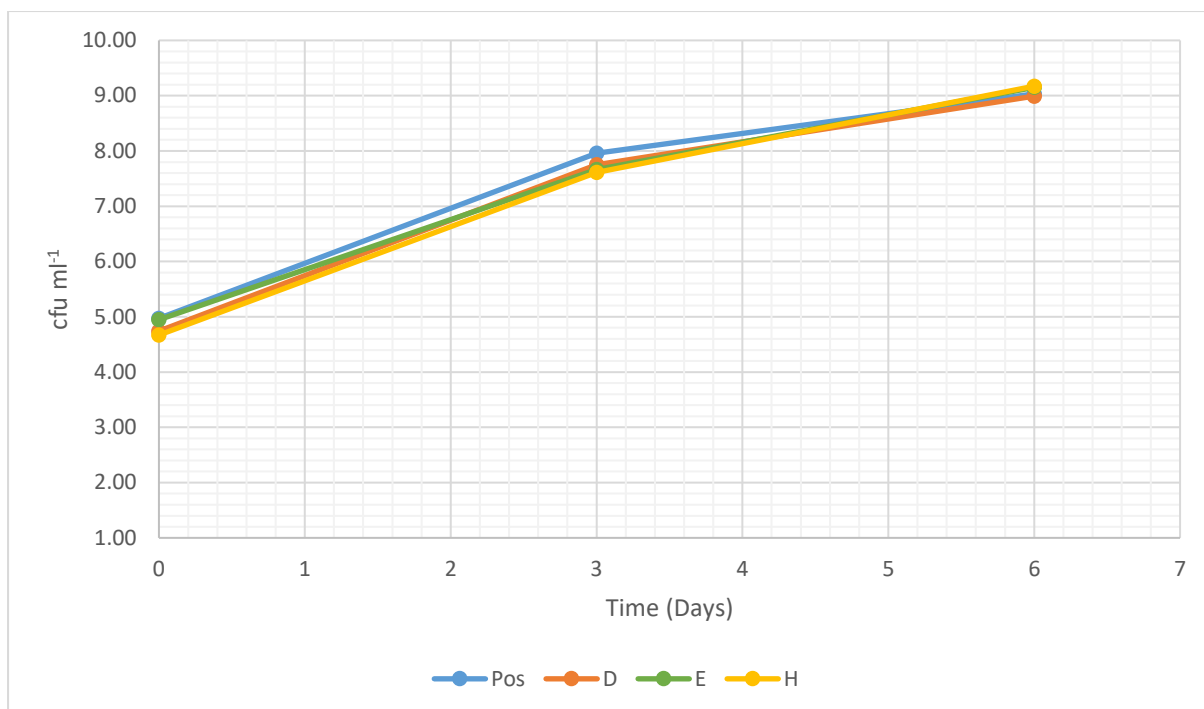


Figure 3-33 - **The effects of three antimicrobial compounds against *E.coli* in 100% pork mince over a 6 day period using method 2.** Pork mince was mixed with a *E. coli* [MG1655] suspension. The control (●) solution contained approximately 10^6 cfu ml⁻¹ of bacterial suspension. Whereas three other suspensions were prepared containing compound, D (●), E (●) and H (●) at concentration 1X. Mince was aliquoted into petri dishes with 20g per dish, replicate samples were prepared for each compound. One set of samples (n=3 for each treatment) were sampled on the same day as preparation whilst all other samples were stored at 20°C for 6 days. Slices were tested on days 3 and 6. Samples were tested by separating bacterial cells from the meat by stomaching before being spread onto XLD agar plates. *E. coli* colonies were then identified and counted. Data points represent the average results obtained from testing 3 mince aliquots.

Using method 2 the antimicrobial effects on *E.coli* appeared to reduce. Compared to the control none of compounds D, E or H have had any effect on the growth of *E.coli*. There is no difference in the growth maximum growth with each compound with some having a slightly higher maximum growth than the control. The growth rate of the control is marginally increased up to day 4 however when reaching day 6 the cfu ml⁻¹ is the same as the growth where compounds are present.

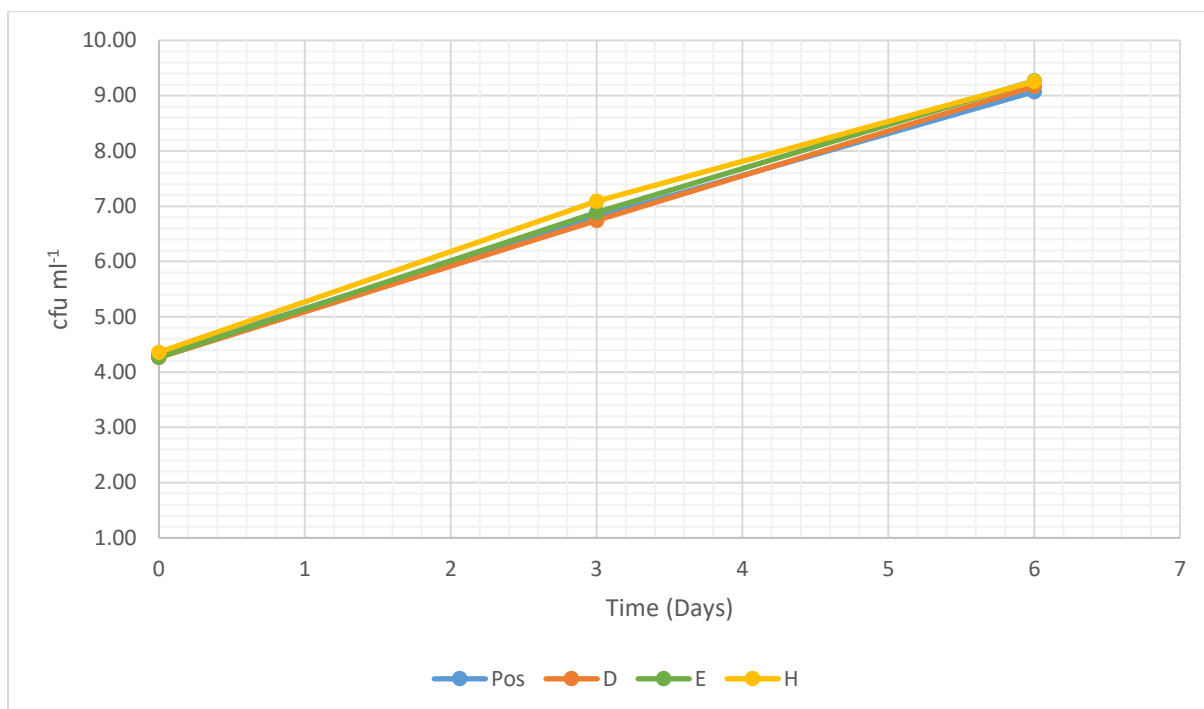


Figure 3-34 - **The effects of three antimicrobial compounds against *P.fluorescens* in 100% pork mince over a 6 day period using method 2.** A suspension of *P. fluorescens* at around 10^6 cfu ml⁻¹ was added to pork mince (control; ●). Separate suspensions were also prepared that contained compounds D (●) or E (●) and H (●) at concentration 1X. Mince was aliquoted into petri dishes with 20g per dish, replicate samples were prepared for each compound. Samples (n=3 for each treatment) were removed for testing on day 0 (preparation day) whilst samples for testing on days 3 and 6 were incubated at 20°C. Bacterial cells were removed from the mince by stomaching and samples were spread onto *Pseudomonas* agar plates. *Pseudomonas* colonies were identified and counted. Data points represent the average results obtained from testing 3 meat slices.

In the final run of this experiment *Pseudomonas fluorescens* was included to represent a spoilage organisms in the fresh meat product. As can be seen in the graph above none of the three compounds had an effect on the growth of *P.fluorescens* when compared to the control. The growth rate and maximum growth point of all three compounds is the same as the control therefore there is no inhibitory effect displayed by compound D, E, or H.

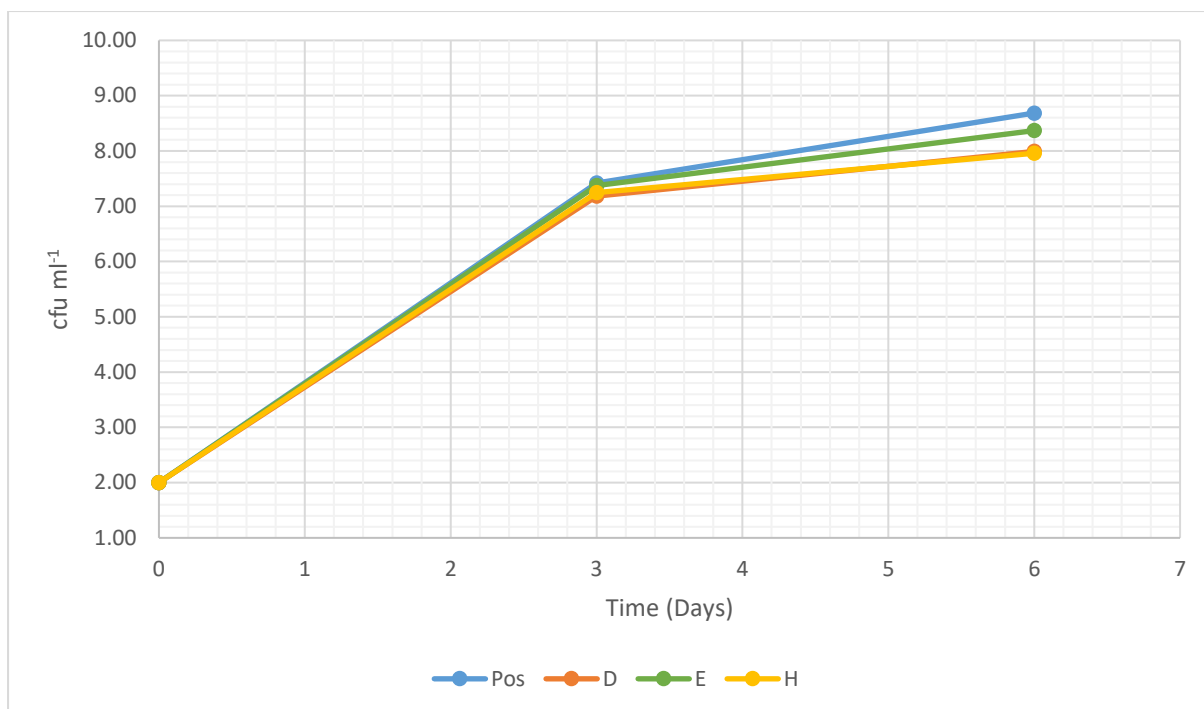


Figure 3-35 - **The effects of three antimicrobial compounds on a viable count in 100% pork mince over 6 days.** Pork mince was added to three different compounds at concentration 1X; D (●), E (●) and H (●). Mince was aliquoted into petri dishes with 20g per dish, replicate samples were prepared for each compound along with a control (●). Samples (n=3 for each treatment) were removed for testing on day 0 (preparation day) whilst samples for testing on days 3 and 6 were incubated at 20°C. Bacterial cells were removed from the mince by stomaching and samples were spread onto Nutrient agar plates. Colonies were counted to determine viable count. Data points represent the average results obtained from testing 3 aliquots.

A viable count was completed to establish the extent of background flora in the pork mince. Over the first three days there was a substantial increase in growth, this was over a seven log increase. From day three to six there was a slower increase in growth and the samples containing one of the antimicrobials had a slightly lower level of growth compared to the control. Overall however, the effect on the growth of the bacteria is not effected greatly by the presence of the antimicrobial which can be seen compared to the control.

3.6.4 Discussion

The aim of these experiments was to test the antimicrobial compounds in a different food system against different bacteria. As the bacteria commonly found varies from food to food it was important to test the novel antimicrobials in different food systems.

Whereas *L.monocytogenes* is currently found in ready to eat foods, in fresh meat it is more commonly *Salmonella spp.*, *E.coli spp.* and *Pseudomonas spp.* which can cause spoilage as well as holding the potential to be pathogenic.

Two methods were used, one being developed from the other. The first method showed mixed results with the first run giving very successful results whilst the second runs

results did not show the same promise. In the first run of method one both compounds D and E were shown to reduce and almost inhibit the growth of *S. Typhimurium* and *E.coli*. In each case there was a two log reduction in growth compared to the control which shows that the antimicrobial is having a good effect reducing the growth of the bacteria. However, when a second run of the experiment was conducted there was very little effect against the growth of either of the two bacteria. It was from these results that a second method was developed. The reasons for this were due to the liquid content of the mince samples. It was thought that because the mince contained more liquid there may have been some quickening of the spoilage process resulting in higher colony counts. The difference between the first and second run was thought to be the temperature difference between spring and an unusually warm summer which may have been causing differences due to the mince having to spend some time outside of a temperature controlled environment during the experimental preparation.

The second method used less liquid in the samples therefore it was hoped that this would help reduce excess growth of the bacteria. Therefore allowing the antimicrobials chance to show their full effects. *Pseudomonas spp.* was also introduced to the experiment to be able to see the effects of antimicrobials against another spoilage organism. In this experiment there was no effect on the growth of bacteria from any of the compounds, all grew at the same rate and to the same point as the control.

Compound H which is an industry standard did not produce any effect which is unusual as it is commonly used in the food industry to reduce the growth of these bacteria. The fact there was still no replication of the effect seen in the first set of experiments indicated that there must have been some other difference between the experiments other than ambient temperature.

To determine the level of background flora in the mince product a viable count experiment was conducted using method two. The experiment was under the same conditions and for the same length of time as the previous experiments. The results of this experiment were unusual and not as expected. The initial growth of all samples containing antimicrobial and the control increased by approximately seven logs over the first three days. Although the test was conducted at 20°C this is an unusually high rate of growth in a food product. There was continued increase over the following three days up to day six, here there was a slight split in growth levels between the control and compounds D, E and H. The second and third set of experiments along with the viable count were performed using 100% pork mince from Morrison's supermarket whereas the first set the mince used was straight from the source and made on site before being delivered directly for this experiment. Due to the viable count having such a high growth rate and growth levels it was suspected that the reason for this may have been the mince product. As supermarket mince has to be produced and then go through a

packaging process and transport before it reaches the consumer who purchases it the process is much longer. Therefore there is more opportunity for bacteria, pathogenic or spoilage to grow in the meat, this can be seen in the results of the viable count. The results from the first experiment show possibly what may occur if the mince did not go through this process and the antimicrobial was added earlier in the mince production. This would mean that the antimicrobials could have a substantial effect on the growth of these bacteria.

An example of each situation can be seen in the literature. Gniewosz and Stobnicka (2018) used fresh pork mince produced for the experiment and have shown reduced growth of the same target bacteria using cranberry pomace extracts as an antimicrobial. Whereas Moroney et al. (2013) found their experiments using brown seaweed gave inconclusive results and it was not proven whether this was due to the seaweeds lack of antimicrobial properties or the mince used in the experiment.

3.6.5 Next Steps

The next steps for this mince set of experiments would be to repeat them using a controlled pork mince product as was used in the first set of tests. To do this a product would need to be sourced from a butcher who can provide details such as the kill date and date of mince production to allow this to be taken into account when the experiment was set up. One of the first experiments would be a viable count so that a comparison can be made between the controlled mince and supermarket mince. Another advantage to doing this would be that an initial check could be done to see if the antimicrobials are working as expected in the product. If they were further challenge tests could be conducted. Method two would also need to be developed again to ensure that the optimum method was used, this may involve standardising the amount of liquid used especially as further more detailed experiments are performed.

If further challenge tests were successful and one or some antimicrobials were outperforming others then further experiments using different antimicrobial concentrations could be conducted. This would allow the optimum concentration of antimicrobial needed to prevent the growth of the three target bacteria. If this was found there would also be scope to test other fresh meat products to see if the same effect could be seen. The other change to the experiments would be the time taken and the temperature they were conducted at. For fresh meat products the temperature would need to be lowered to fridge or fridge abuse temperature, i.e. 4-6°C or 7-10°C. By lowering the temperature the experiments would need to be conducted over a longer period of time, this would mean several weeks rather than several days.

Chapter 4 Conclusion

With a number of outbreaks of food borne illness still increasing, and the amounts of food waste becoming more of an issue as Food security is recognised as an increasing problem, finding an antimicrobial which can inhibit or slow the growth of pathogenic and spoilage bacteria is of growing importance. This year saw the largest reported outbreak of *L. monocytogenes* infections in South Africa, including 943 cases 19% of which resulted in death. This was traced back to a type of Bologna-style sausage, which is a known potential high risk product for *Listeria*, produced by one of the largest food manufacturers in the country (Manganye et al., 2018). Therefore, for both consumer safety and to prevent reputational damage of food companies, it is important to develop new antimicrobials which may be more effective at preventing bacteria from growing in the food or slowing their growth to allow more time for safe consumption.

The antimicrobials used in this project were both novel and sourced from natural ingredients. There has been clear shift in consumer preference for food products without artificial additives and ingredients (Gustavsson et al., 2011). Therefore there is need for new and effective antimicrobials in the industry. This project began with a panel of six antimicrobials that had been designed and developed by our industry partners, Phil Stroud Solutions. Although the exact nature of the ingredients needs to remain confidential to the company because of potential Intellectual Property issues, the company did inform us that they were made from organic acids and plant extracts and therefore qualified as 'green label' preservatives, i.e. the components are also accepted to be natural Food ingredients and therefore do not need to be declared as food additives on the label. This approach to making food products more acceptable for the change in consumer preferences has become more and more common and is now an accepted approach when developing new food products.

The first tests performed were screening tests which were used to quickly establish whether the antimicrobial compounds showed any effect on the bacteria being tested. If there was no effect at this stage there would not have been any point in continuing to test the compounds as they would never be able to be used in food products. The desired antimicrobial spectrum of activity was the ability to control the growth of all five bacteria in the test panel, including both Gram-positive and Gram-negative bacteria and also included pathogenic and spoilage organisms. From the screening tests a few of the compounds showed promise to meet the criteria set out by slowing or inhibiting the growth of the five bacteria, and from these two were chosen for further tests. The chosen compounds, compounds D and E were able to show an effect of slowing growth rate at each of the concentrations tested therefore they were considered good candidates for further testing as they had a good dynamic range of activity. This is

important as it is hard to ensure that the concentration of a Food preservative is uniform through out a complex food product, such as processed meats, and therefore preservatives that quickly lose activity on dilution may not be fully functional in a real food system.

When moving into 'in food' system testing the chosen two compounds were challenged in a variety of ways to test their effectiveness in slowing the growth of bacteria in a food product. Ideally more compounds would have been tested in this way, as even from all of the screening test results, it is hard to predict how new products will perform in application. However the time constraints of this project meant that it was only feasible to take the most promising candidates forward. This does mean that the other compounds tested should not be disregarded entirely as there may be food systems where they have a good antimicrobial activity.

The sausage dipping method was based on a previously published method and had previously been successfully used by another research student in the laboratory. The experiments provided a quick and easy way to gain an initial evaluation of how the compounds would act in a more complex system. Although the product was not incorporated within the food, the results gained did give some insight into what might happen if meat products were prepared that did contain a more even distribution of the compounds. Using this system, some promising results were found with compound E at concentration 1X when it was found to substantially slow the growth of *L. monocytogenes*. However when further experiments were planned using a new source of commercially prepared mortadella sausage unexpected results were gained. Further tests revealed that the sausage contained antimicrobial agents, despite the fact that the product specification did not mention any. Enquiries revealed that a change in ingredients had occurred which would explain the results seen. This highlights the issue when using commercially produced food samples for such tests, as the new ingredients used were green label and were therefore not declared. Hence the work was affected because of exactly the same current trend of moving towards green label products. This problem meant it was not possible to complete the planned experiments to compare the efficacy of the novel compounds with those already used in industry to give a direct comparison to see if the compounds being developed performed equally well or better. Therefore this would be one of the first experiments to take place if further work was completed, but perhaps now planning to produce a meat product in house so consistency of ingredients could be confirmed. If the novel compounds could perform on par or better than the ones currently used in industry this would be an excellent result as many current industry antimicrobials are not natural but chemical based. Therefore for the manufacturer these compounds would provide the same protection against bacteria and

yet also make the products more appealing to the consumer due to their natural ingredients. In fact many manufacturers sell such products at a premium as the consumer is prepared to pay a higher price for items sold as natural and green label. If compound E continues to perform as well as it has done throughout this project there is scope for it to be successfully introduced into the food industry. In screening tests it already has done well when compared to compound H, which was a standard industry product as it provided the same ability to control the growth of *L. monocytogenes* in RTE sausage products.

The same chosen compounds were used in the experiments to assess the antimicrobials effectiveness against the growth of the gram negative bacteria being used in this project. As gram negatives are not commonly found in RTE foods fresh pork mince was used in these experiments. They provided a mixture of results with some showing the compounds to give a several log reduction in growth whereas in others there was no significant difference between the samples containing compounds and the control. From analysing the data and the experimental design it was thought that the first experiment results were the most reliable. Having assessed a viable count on the supermarket mince used in the subsequent experiments it was found that the level of bacteria in the mince was very high therefore this was probably effecting the action of the antimicrobials. When looked at in mince which was bought straight from production the effects on the growth of the gram negative bacteria are much better. This then shows both compound D and E having a near inhibitory effect on growth. This means overall both compounds D and E look promising for use in fresh meat products.

The other aspect of this product was to check the safety of the antimicrobials to be used in food products. The Ames test was carried out to check for mutagenicity in the products. It was important to test for this as if the results were positive for mutagenicity the antimicrobial compounds would not be suitable for use in food. Upon testing the two chosen compounds with the Ames strains of *S. Typhimurium* it was found that there is no mutagenicity. This means that from this test it can be established that compounds D and E are safe for use in food products. The ideal scenario would have included testing all of the compounds using the Ames test however due to time constraints this was not possible. To ensure that the antimicrobials that have been developed are definitely safe for use in food further safety tests would need to be carried out, beyond what has been done in this project. This would most probably include cell toxicity testing.

Overall there is definitely scope for these novel natural antimicrobials to be used in food products. The performance of compounds D and E across all the tests has shown real promise that they could become successful as antimicrobials against a number of different bacteria both gram positive and gram negative. Once furthered with tests incorporating the antimicrobial compounds into the food product the results should

hopefully back up what has been found here and there will be scope to move the testing to a larger more realistic scale.

Chapter 5 References

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Chapter 6 Appendix

6.1 Appendix A

As seen in section 3.1 screening tests were carried out on all compounds to determine their efficacy against the five test bacteria. Over 24 hours, OD was measured every 40 minutes to give an idea of the growth of each bacteria in the presence of each compound. Due to volume of data produced from this experiment and because a lot of the tests produced similar results only those results of interest were featured in section 3.1. The rest of these results can be seen here.

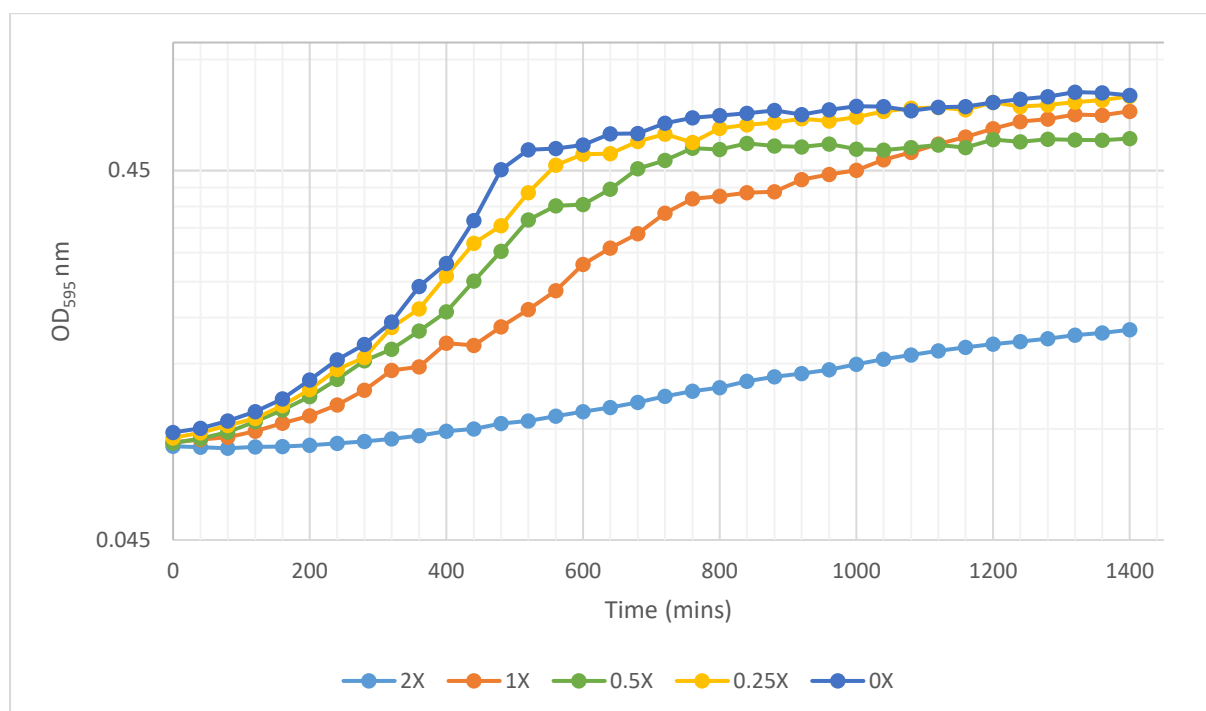


Figure 6-1 - The effects of Compound B on *L. monocytogenes* at five different concentrations: Cultures of *L. monocytogenes* [10403S] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

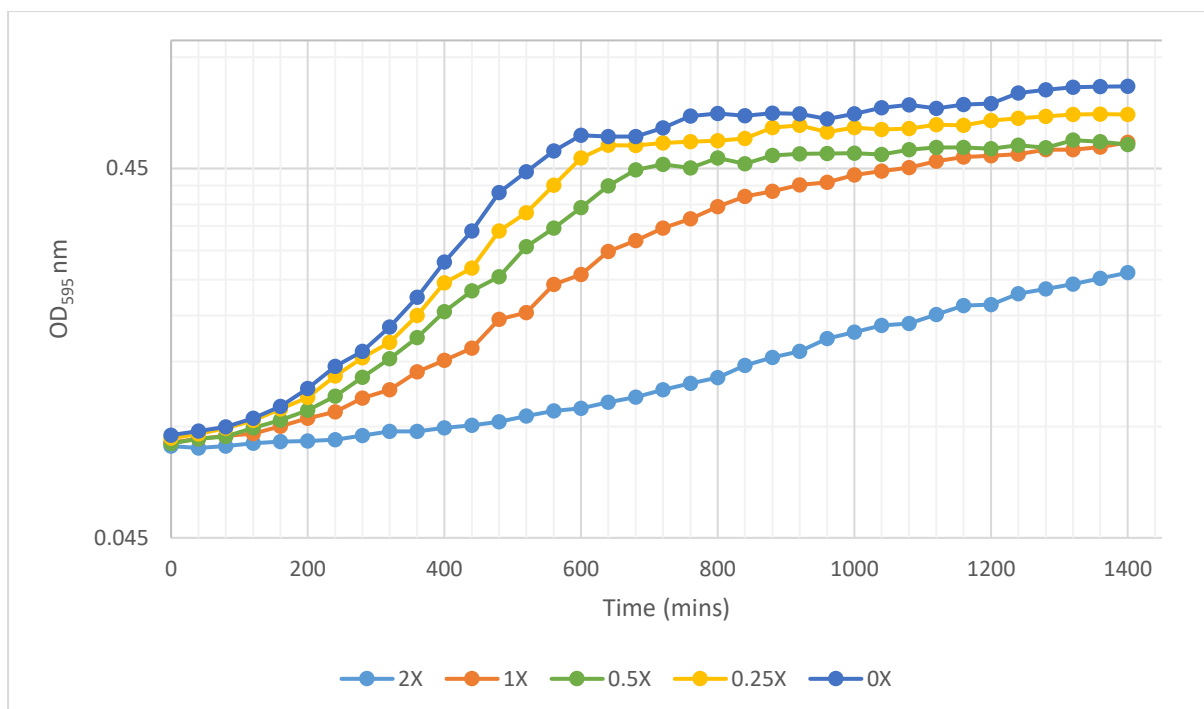


Figure 6-2 - The effects of Compound B on *L. innocua* at five different concentrations: Cultures of *L. innocua* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

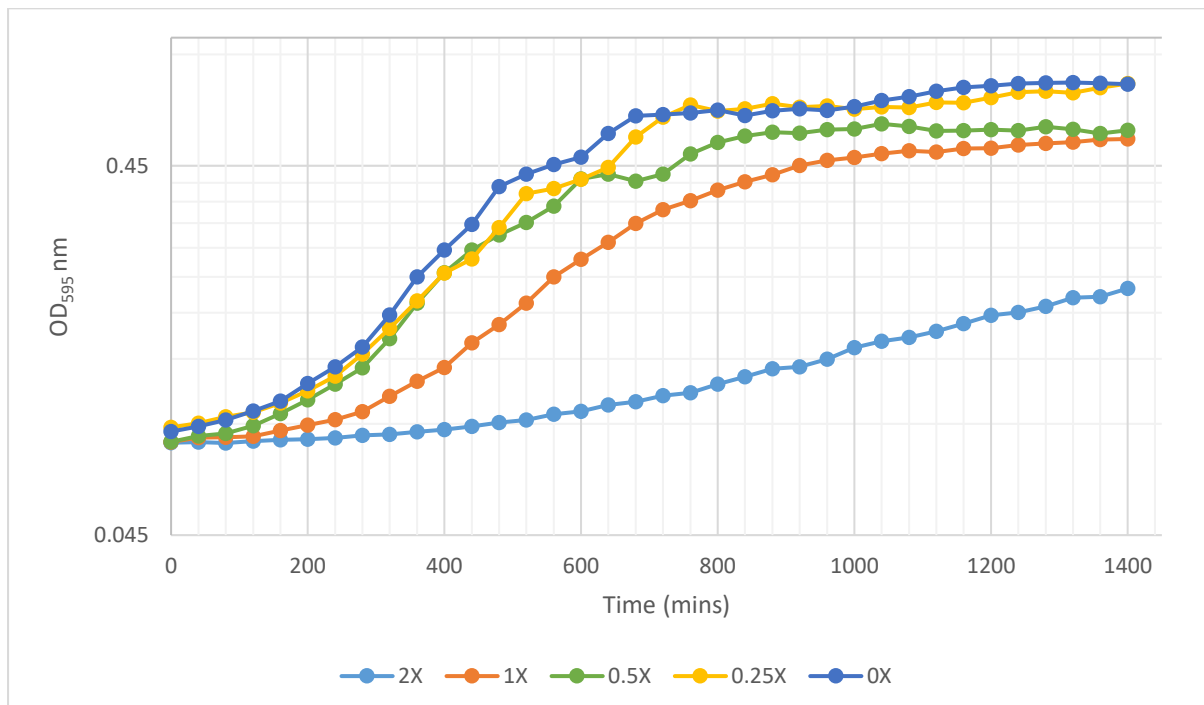


Figure 6-3 - The effects of Compound B on *S. Typhimurium* at five different concentrations: Cultures of *S. Typhimurium* [DB7516] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

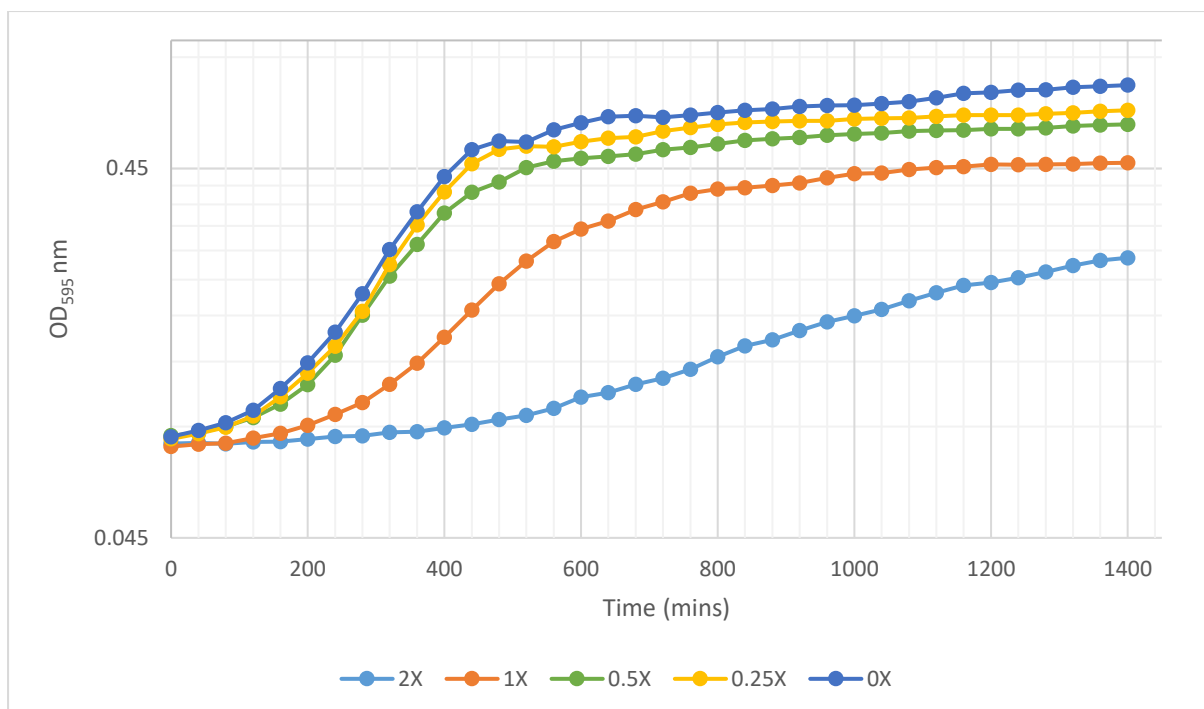


Figure 6-4 - **The effects of Compound B on *E.coli* at five different concentrations:** Cultures of *E.coli* [MG1655] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

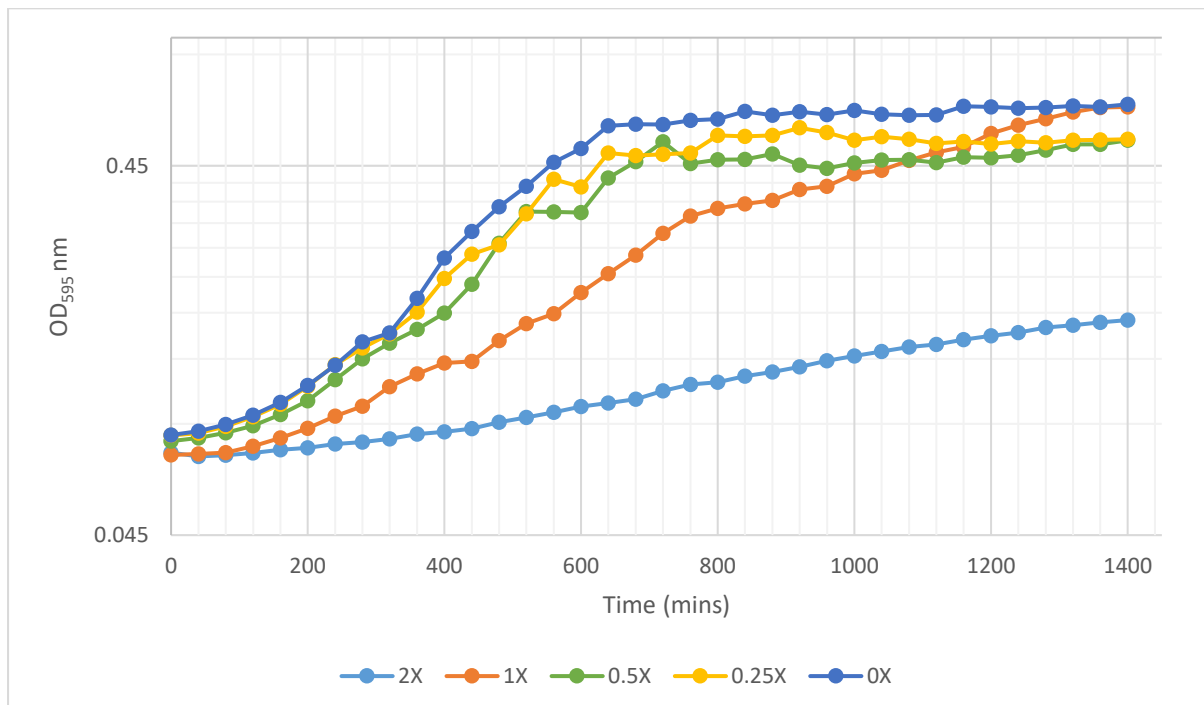


Figure 6-5 - **The effects of Compound C on *L.monocytogenes* at five different concentrations:** Cultures of *L. monocytogenes* [10403S] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

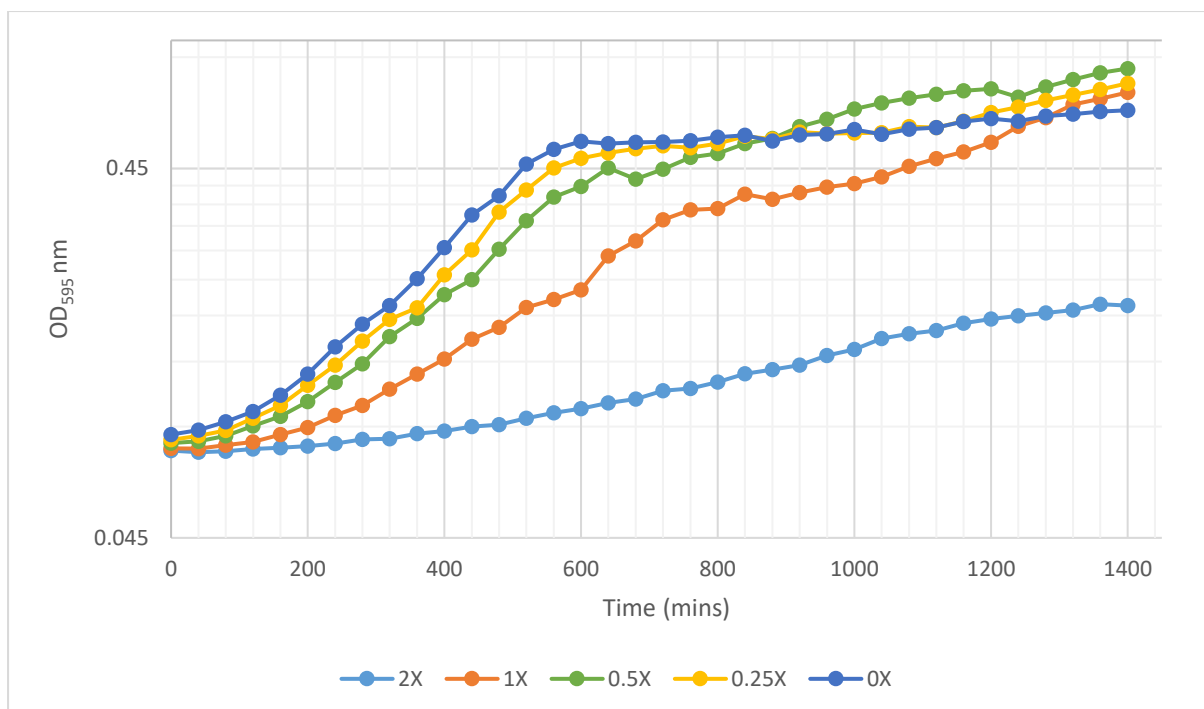


Figure 6-6 - The effects of Compound C on *L. innocua* at five different concentrations:

Cultures of *L. innocua* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

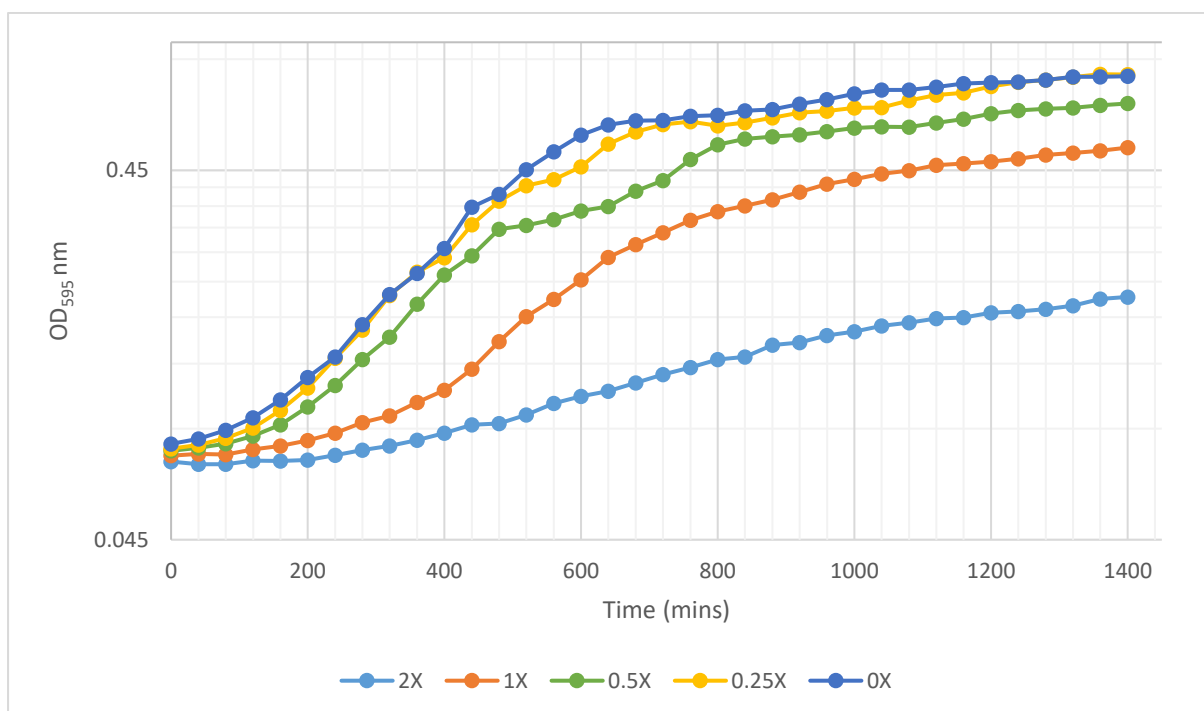


Figure 6-7 - The effects of Compound C on *S. Typhimurium* at five different concentrations: Cultures of *S. Typhimurium* [DB7516] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

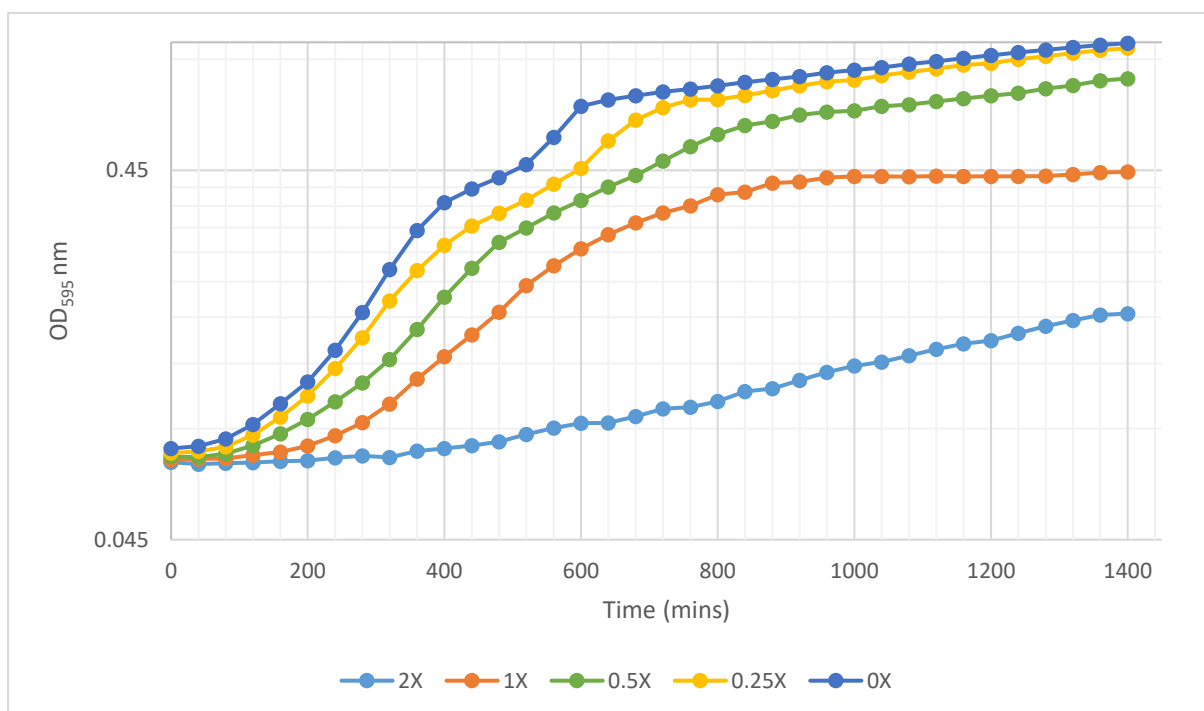


Figure 6-8 - The effects of Compound C on *P. fluorescens* at five different concentrations: Cultures of *P. fluorescens* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

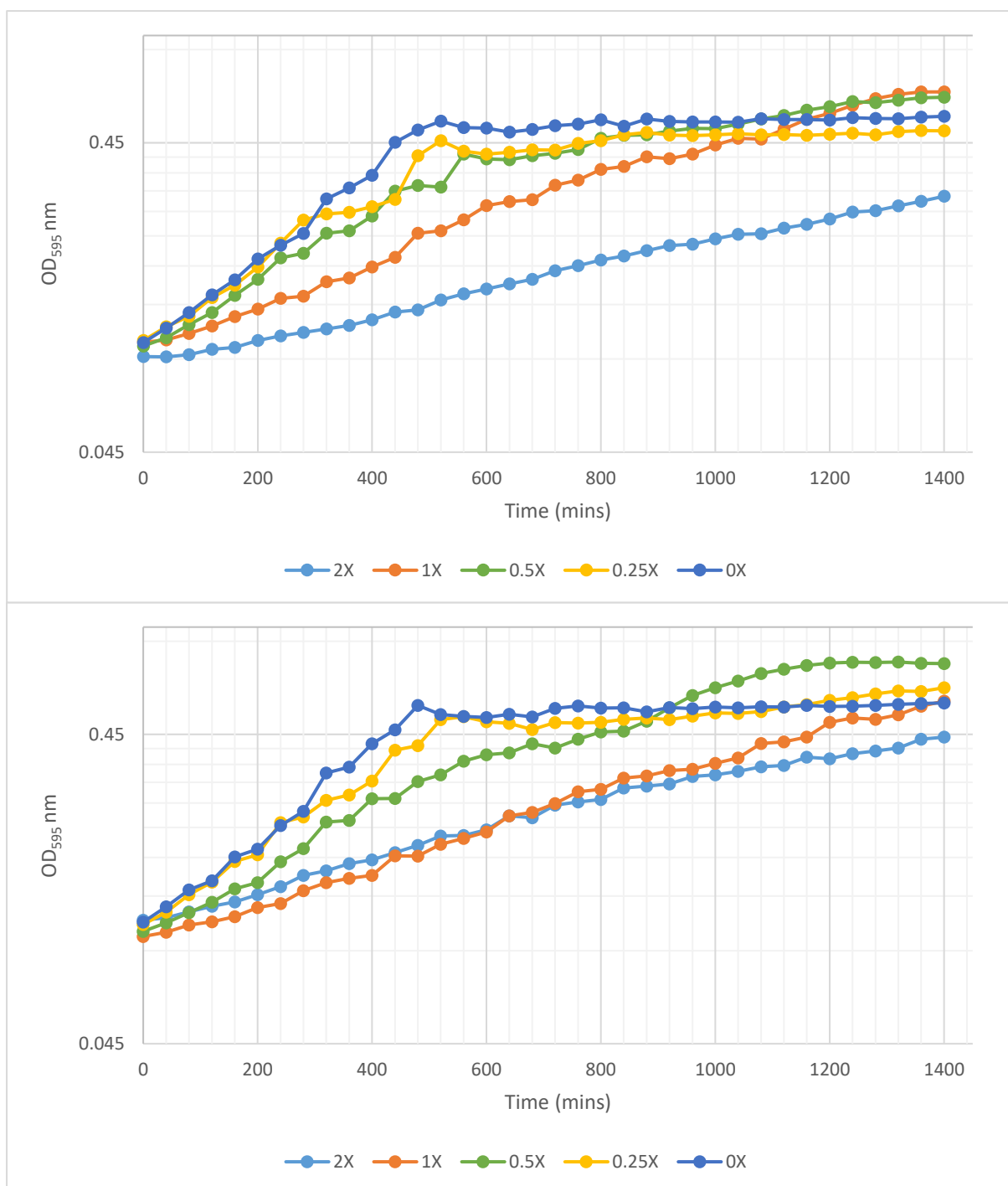


Figure 6-9 - The effects of Compound D on *L.monocytogenes* at five different concentrations: Cultures of *L. monocytogenes* [10403S] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

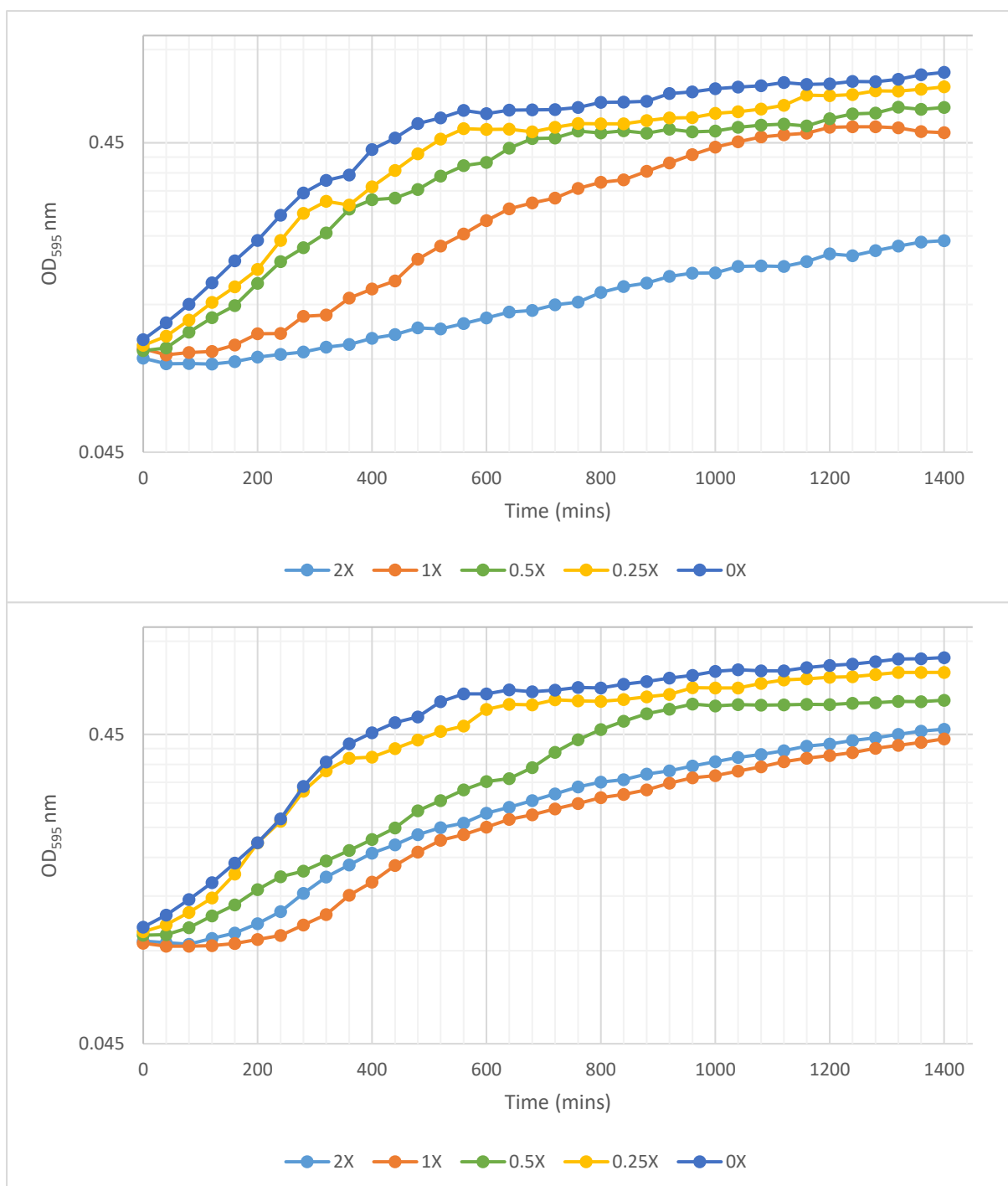


Figure 6-10 - The effects of Compound D on *S. Typhimurium* at five different concentrations: Cultures of *S. Typhimurium* [DB7516] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

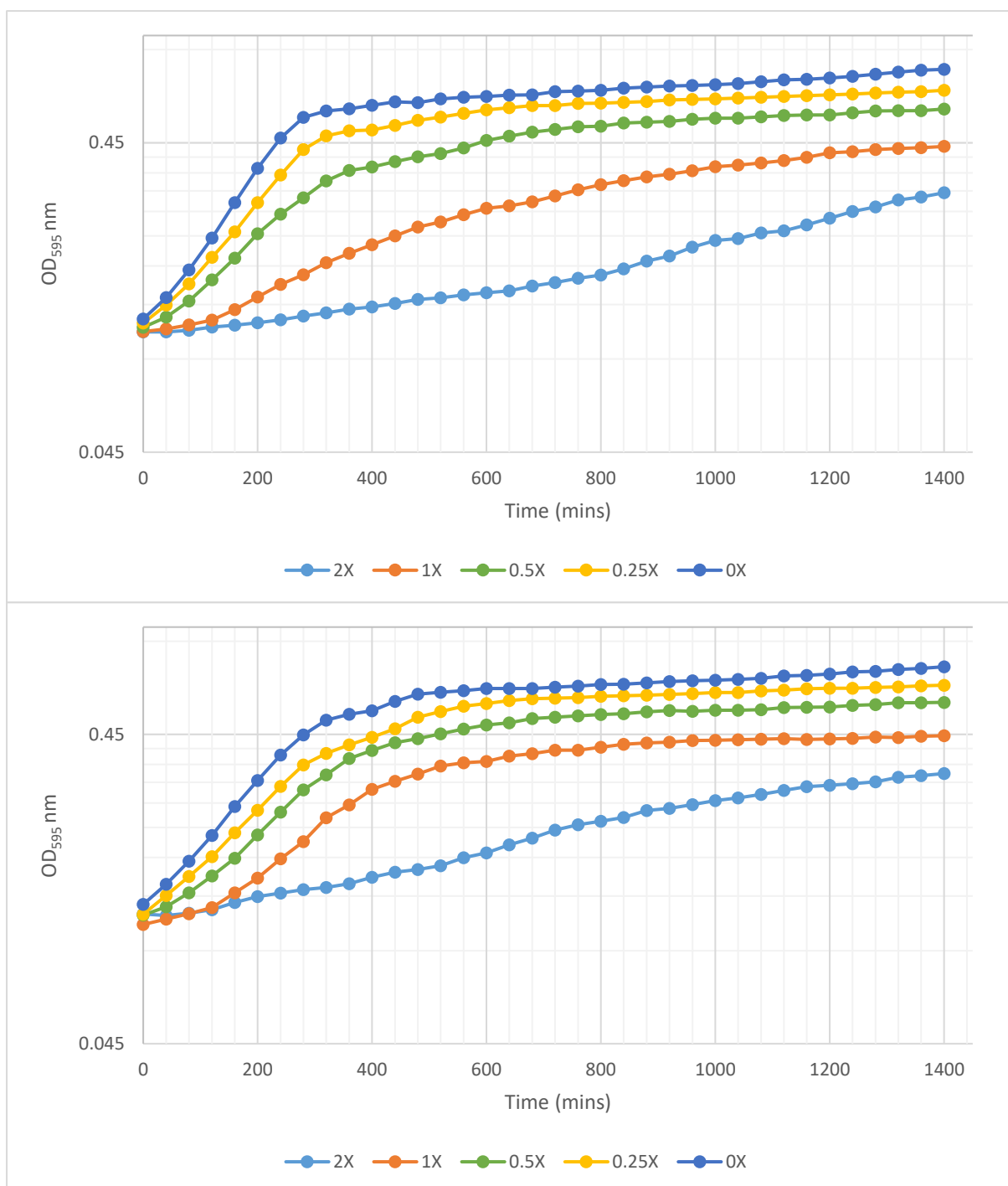


Figure 6-11 - **The effects of Compound D on *E.coli* at five different concentrations:** Cultures of *E.coli* [MG1655] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

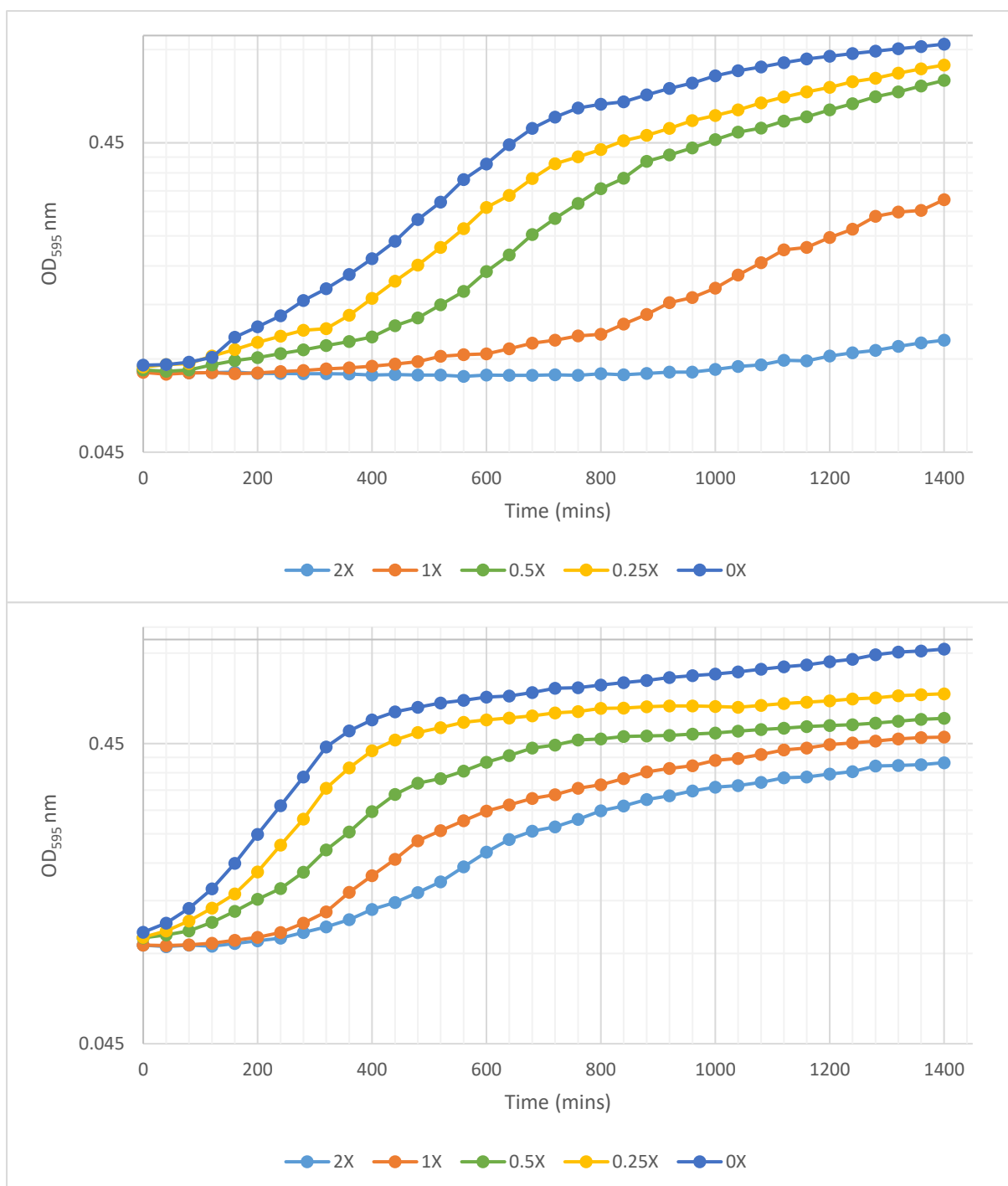


Figure 6-12 - The effects of Compound D on *P. fluorescens* at five different concentrations: Cultures of *P. fluorescens* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

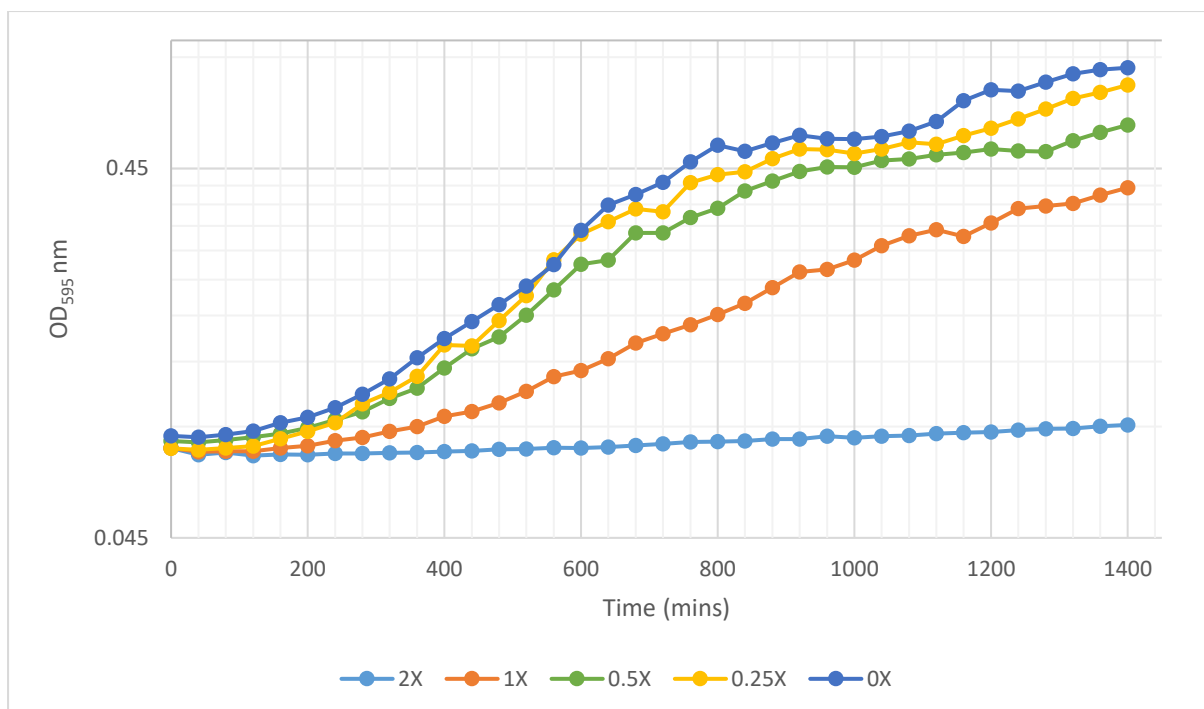


Figure 6-13 - The effects of Compound E on *L. innocua* at five different concentrations: Cultures of *L. innocua* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

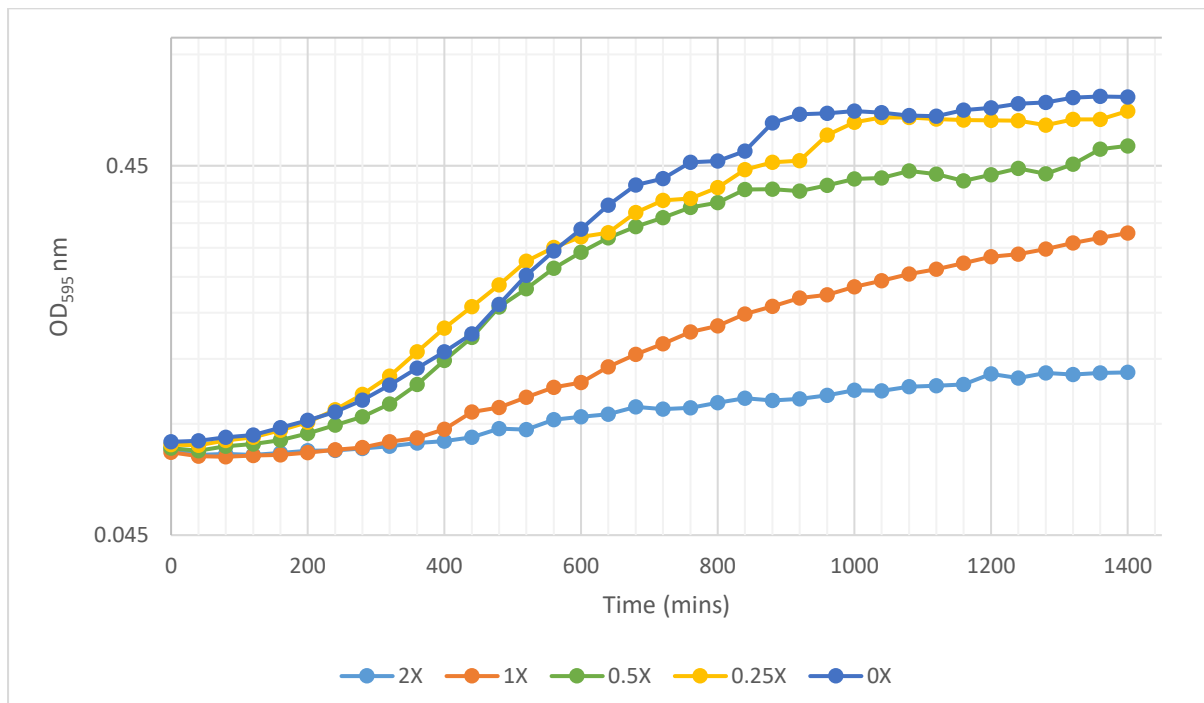


Figure 6-14 - The effects of Compound E on *S. Typhimurium* at five different concentrations: Cultures of *S. Typhimurium* [DB7516] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

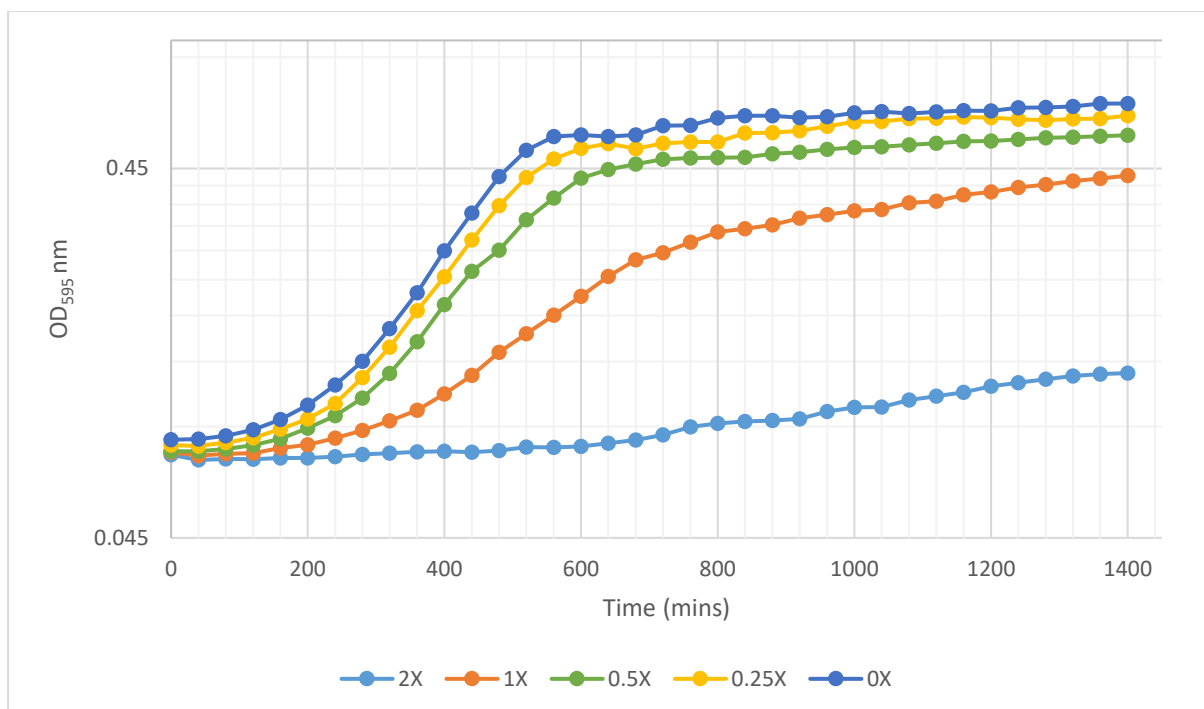


Figure 6-15 - **The effects of Compound E on *E.coli* at five different concentrations:** Cultures of *E.coli* [MG1655] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

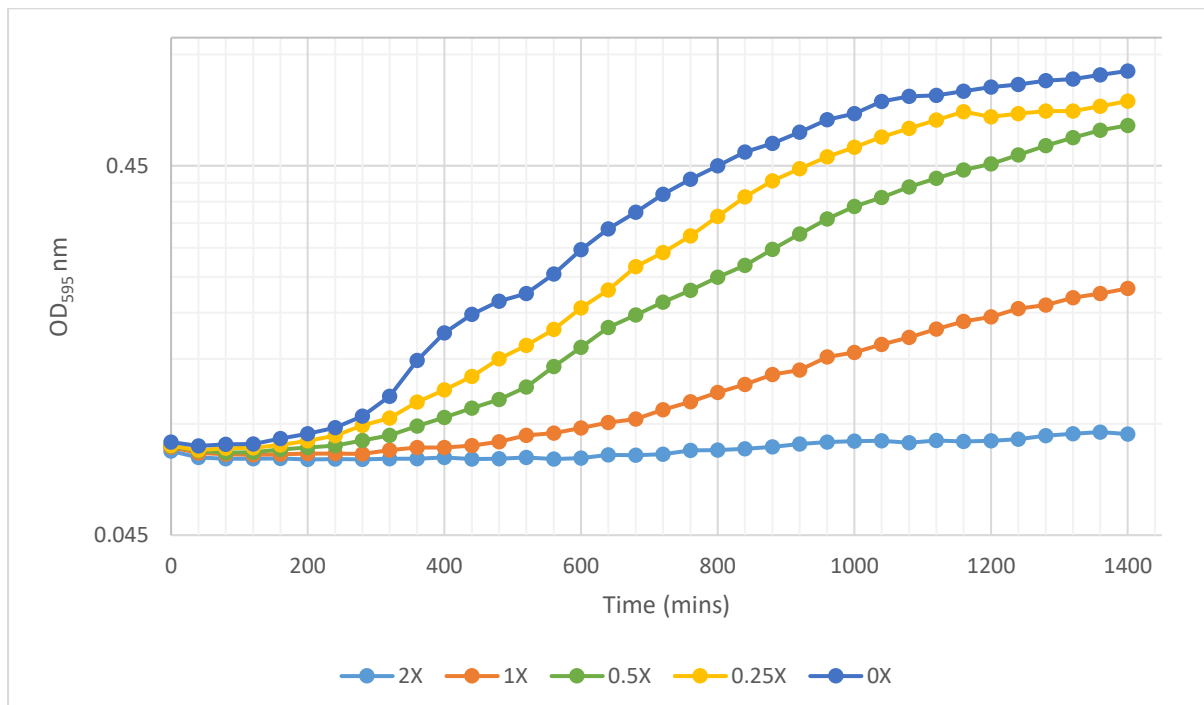


Figure 6-16 - **The effects of Compound E on *P.fluorescens* at five different concentrations:** Cultures of *P.fluorescens* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

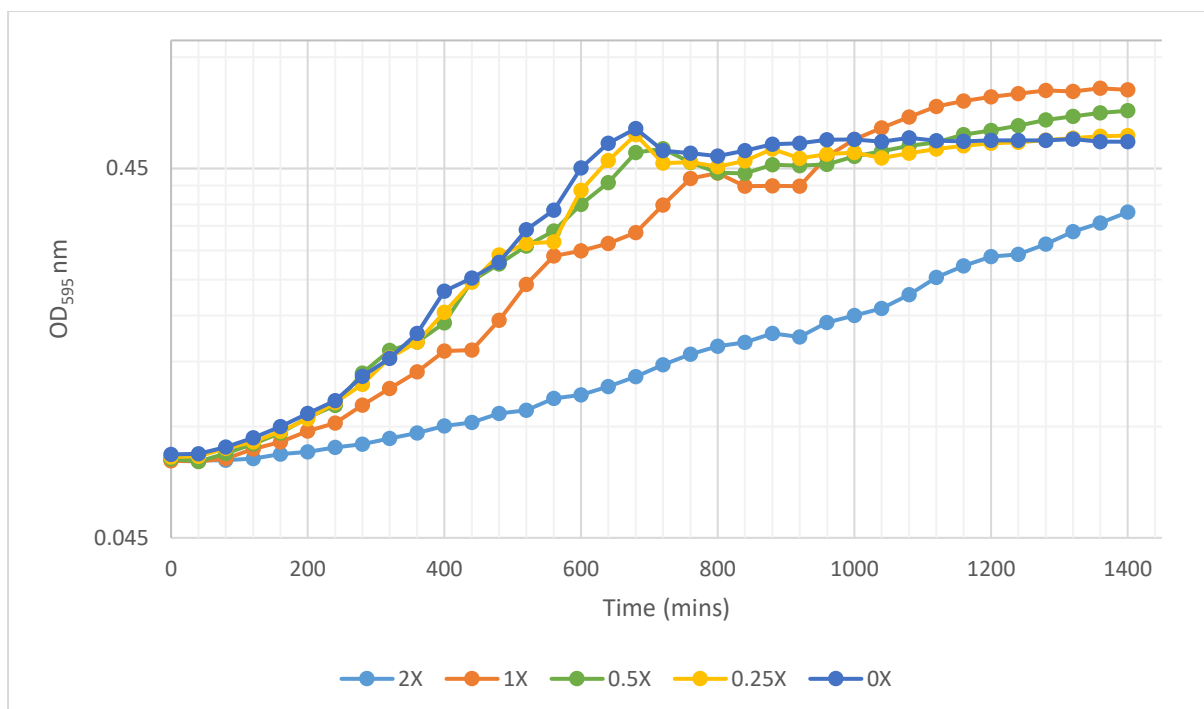


Figure 6-17 - The effects of Compound F on *L.monocytogenes* at five different concentrations: Cultures of *L. monocytogenes* [10403S] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

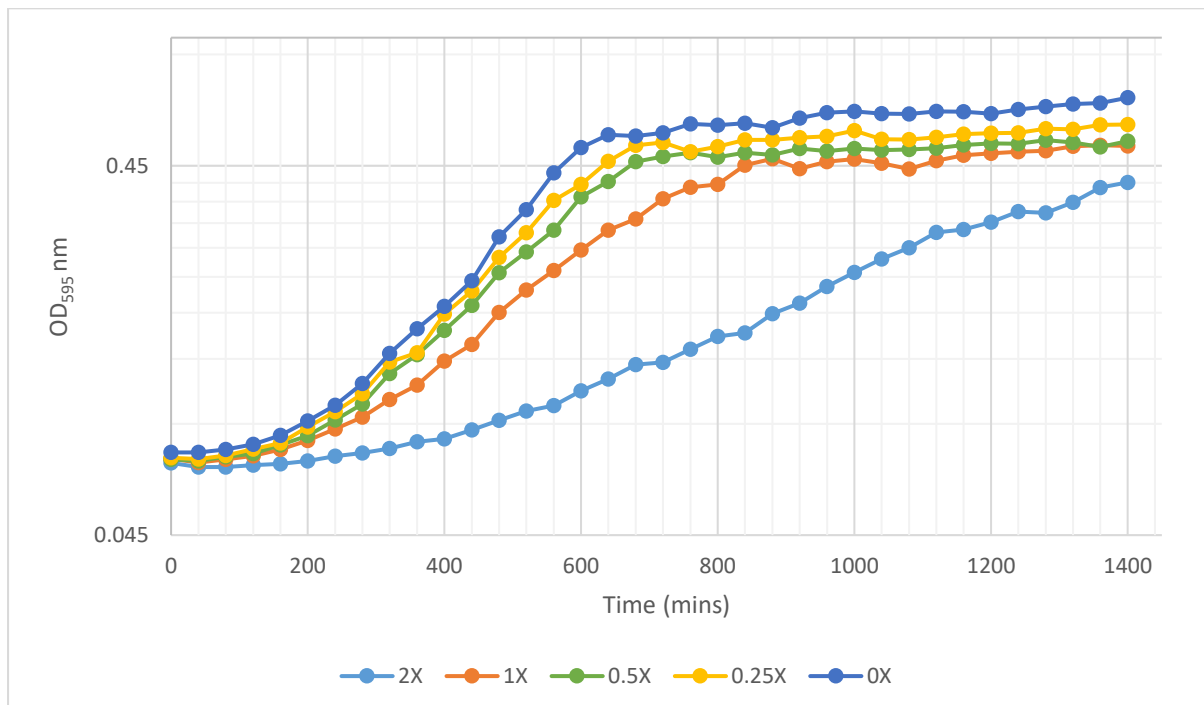


Figure 6-18 - The effects of Compound F on *L.innocua* at five different concentrations: Cultures of *L. innocua* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

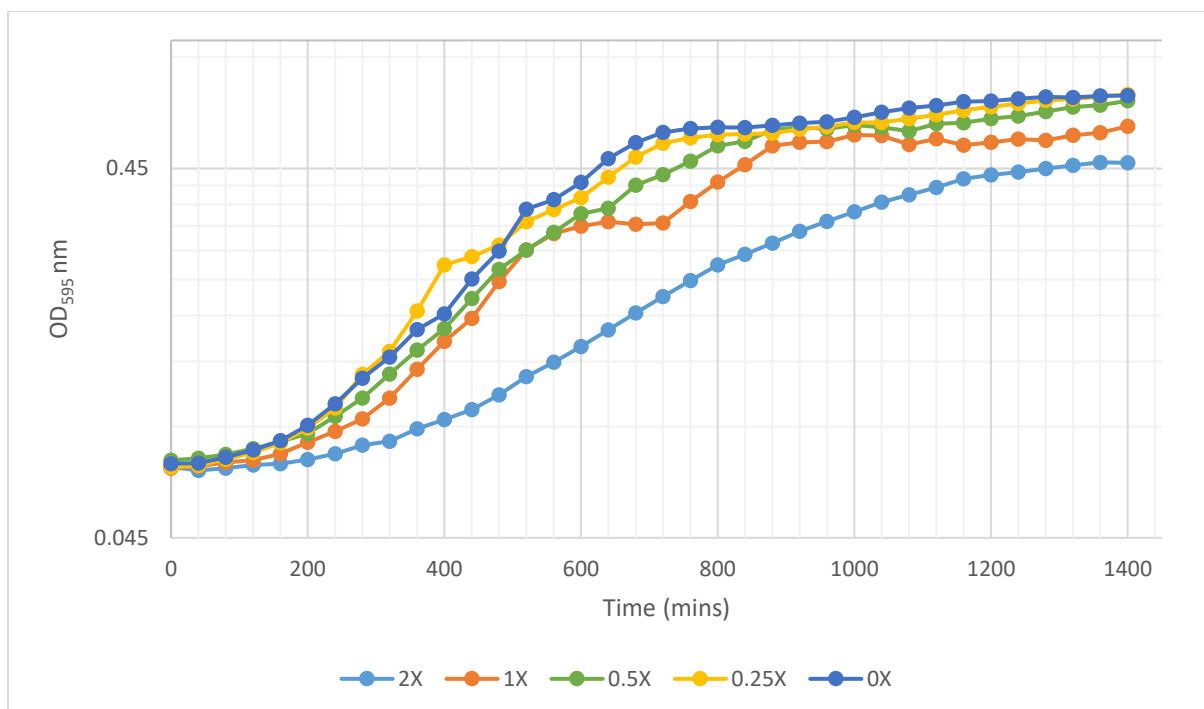


Figure 6-19 - The effects of Compound F on *S. Typhimurium* at five different concentrations: Cultures of *S. Typhimurium* [DB7516] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

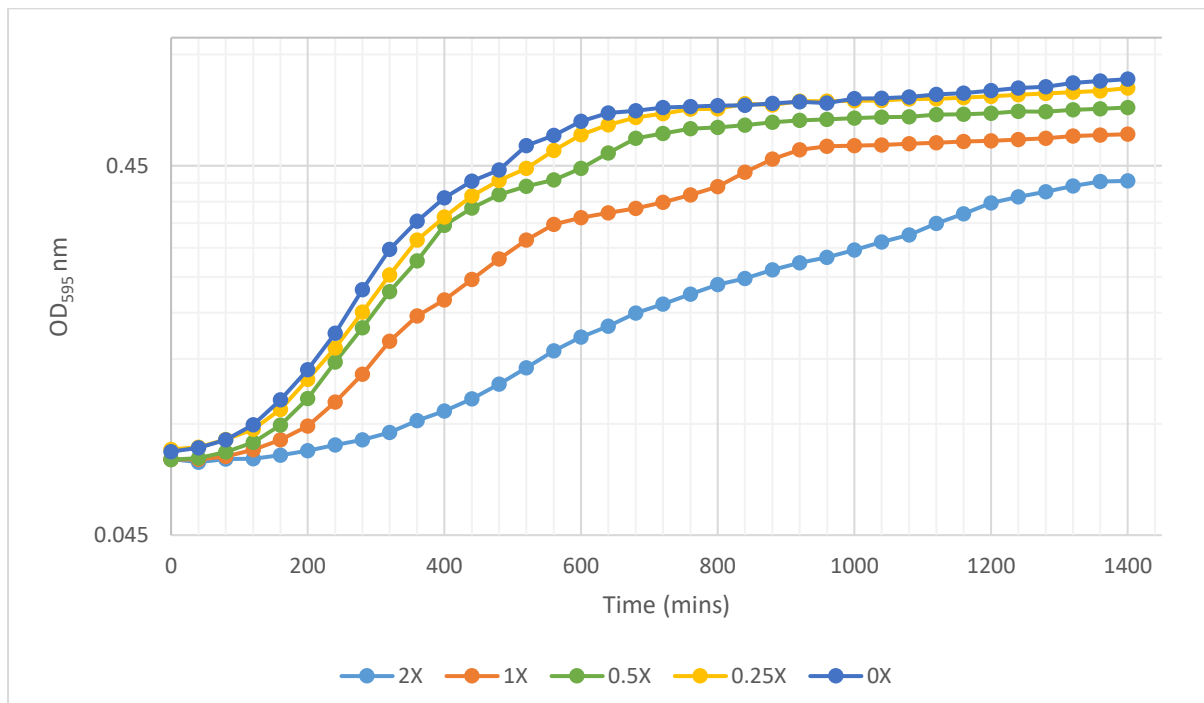


Figure 6-20 - The effects of Compound F on *E. coli* at five different concentrations: Cultures of *E. coli* [MG1655] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

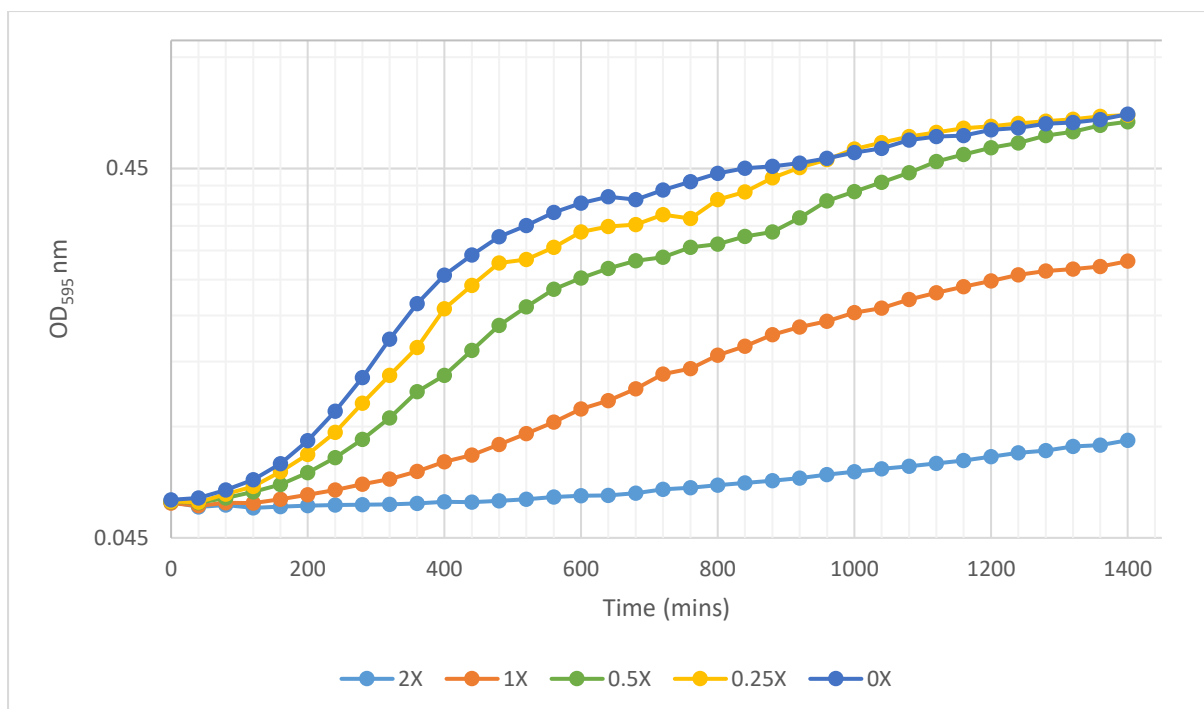


Figure 6-21 - The effects of Compound G on *L. innocua* at five different concentrations: Cultures of *L. innocua* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

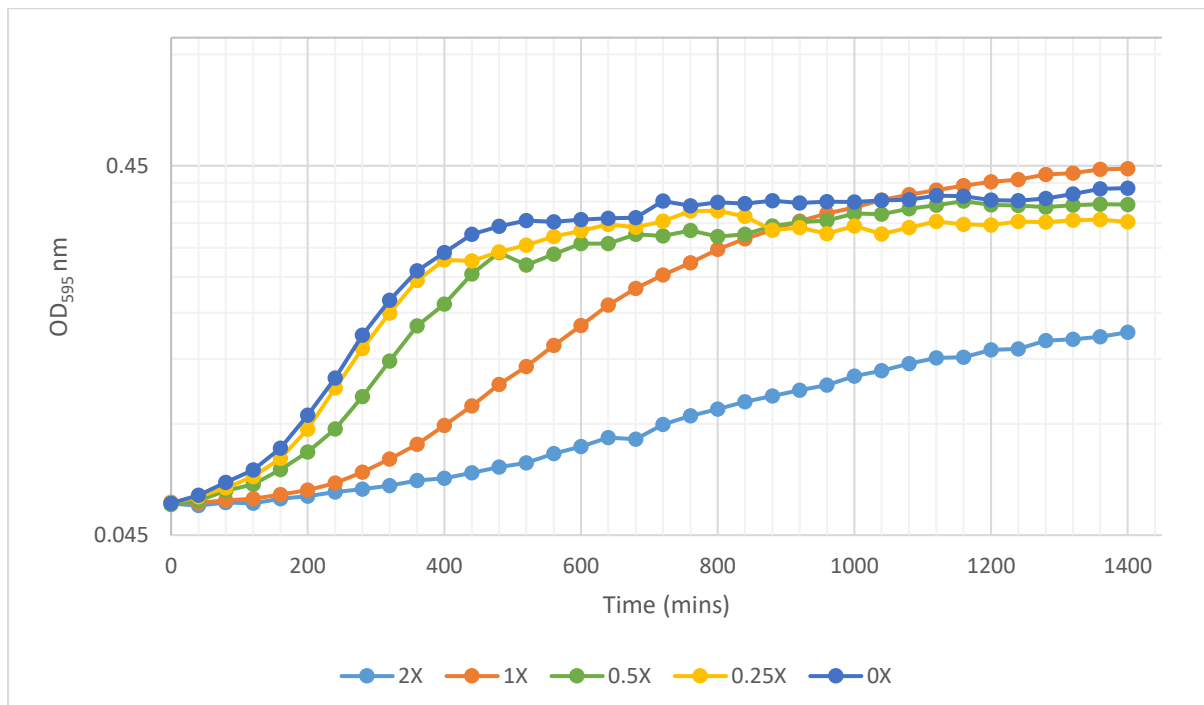


Figure 6-22 - The effects of Compound G on *S. Typhimurium* at five different concentrations: Cultures of *S. Typhimurium* [DB7516] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

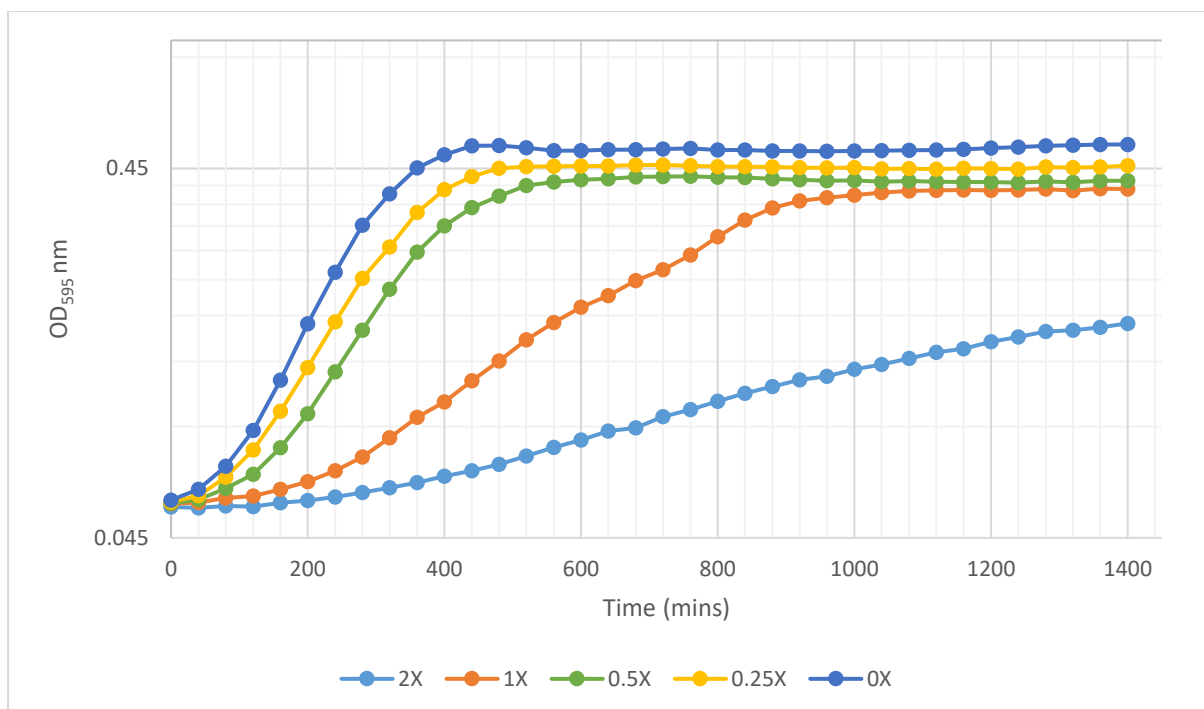


Figure 6-23 - **The effects of Compound G on *E.coli* at five different concentrations:** Cultures of *E.coli* [MG1655] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

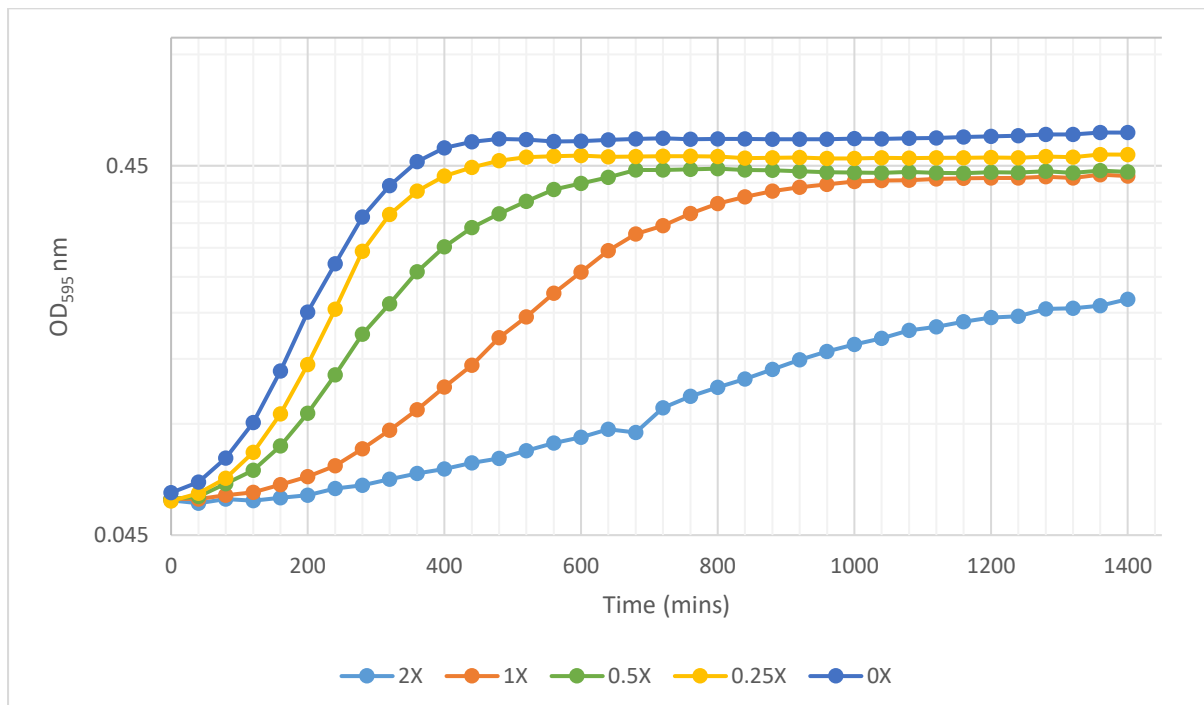


Figure 6-24 - **The effects of Compound G on *P.fluorescens* at five different concentrations:** Cultures of *P.fluorescens* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

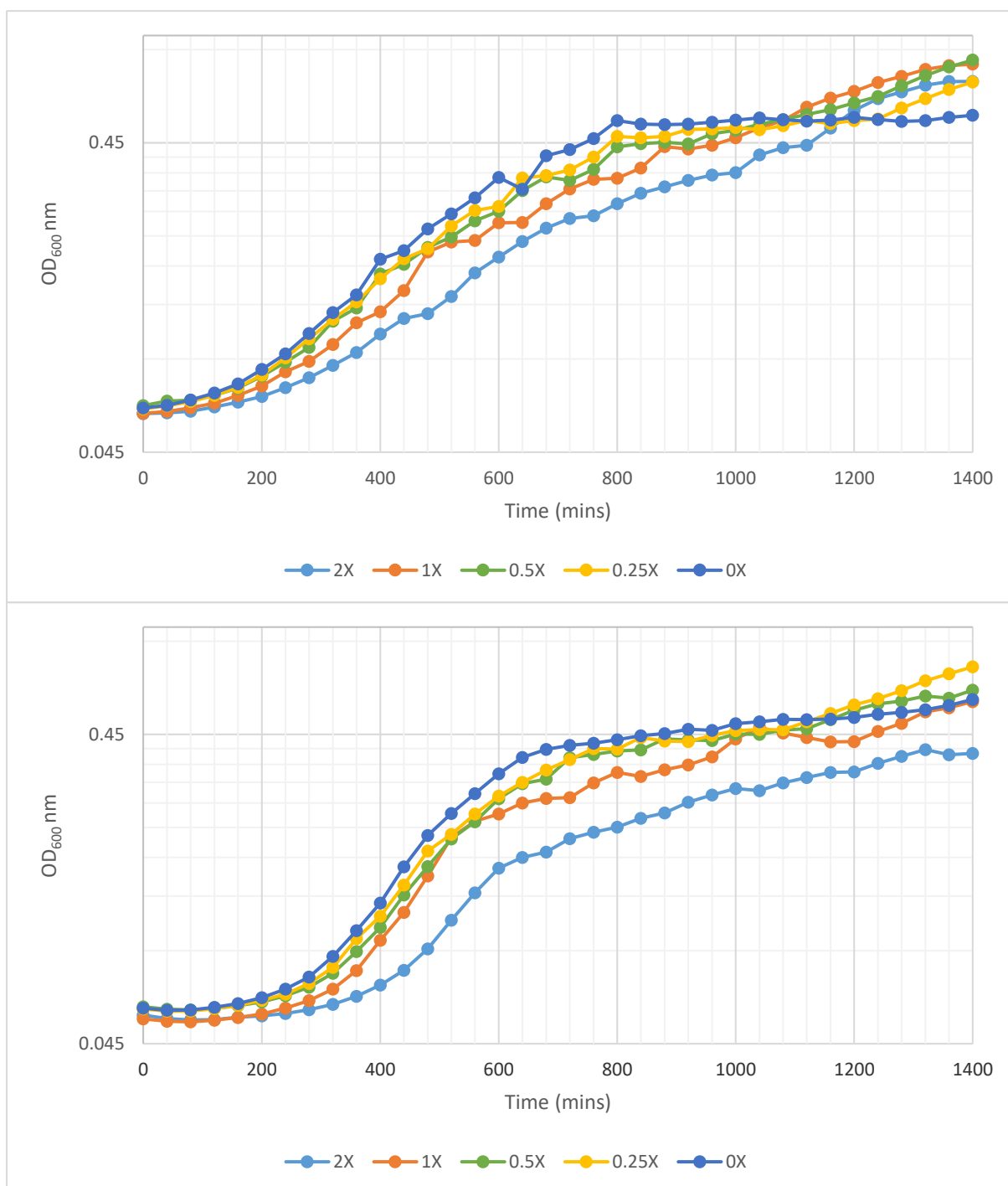


Figure 6-25 - The effects of Compound H on *L. monocytogenes* at five different concentrations: Cultures of *L. monocytogenes* [10403S] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

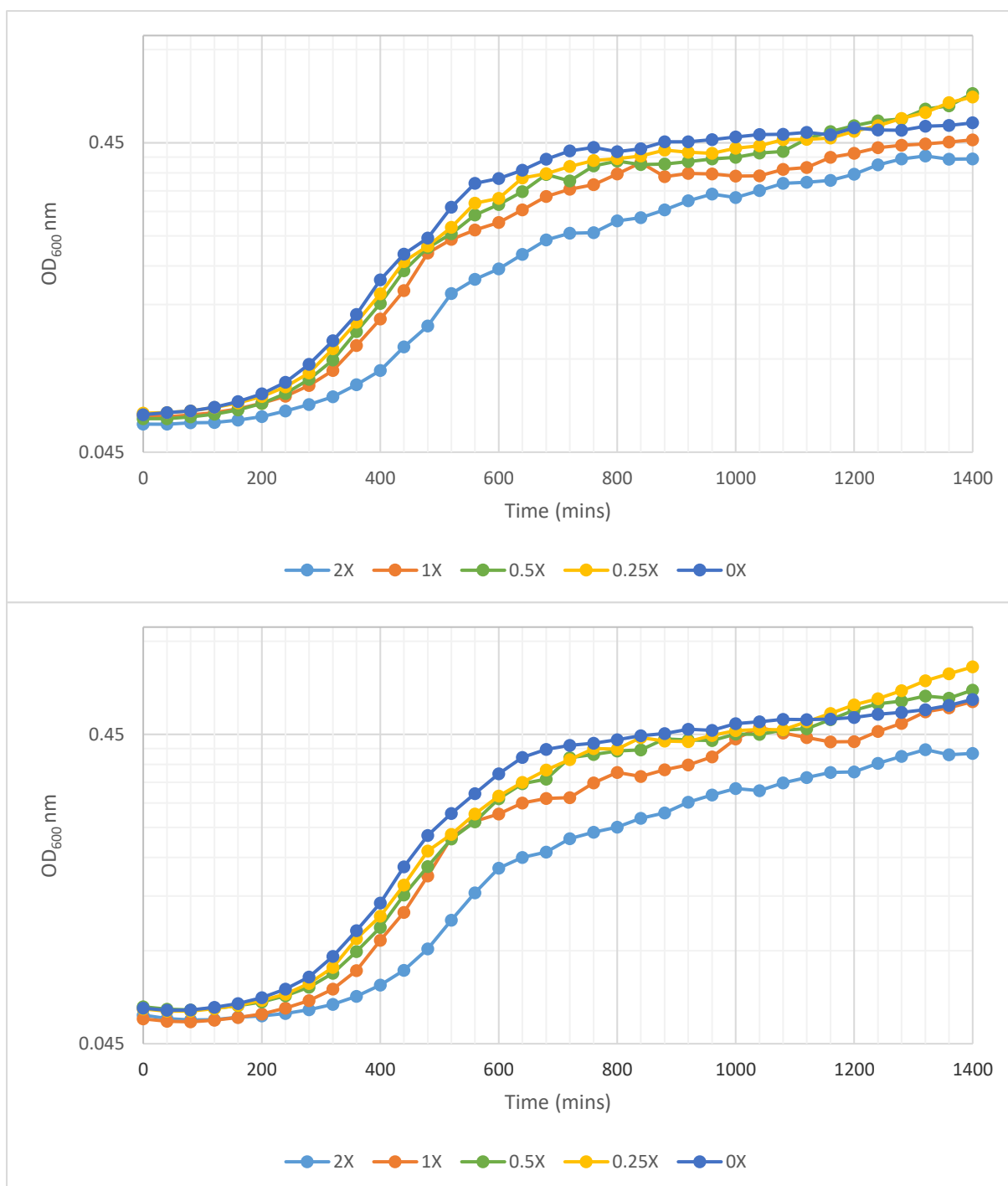


Figure 6-26 - The effects of Compound H on *L.innocua* at five different concentrations:

Cultures of *L. innocua* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

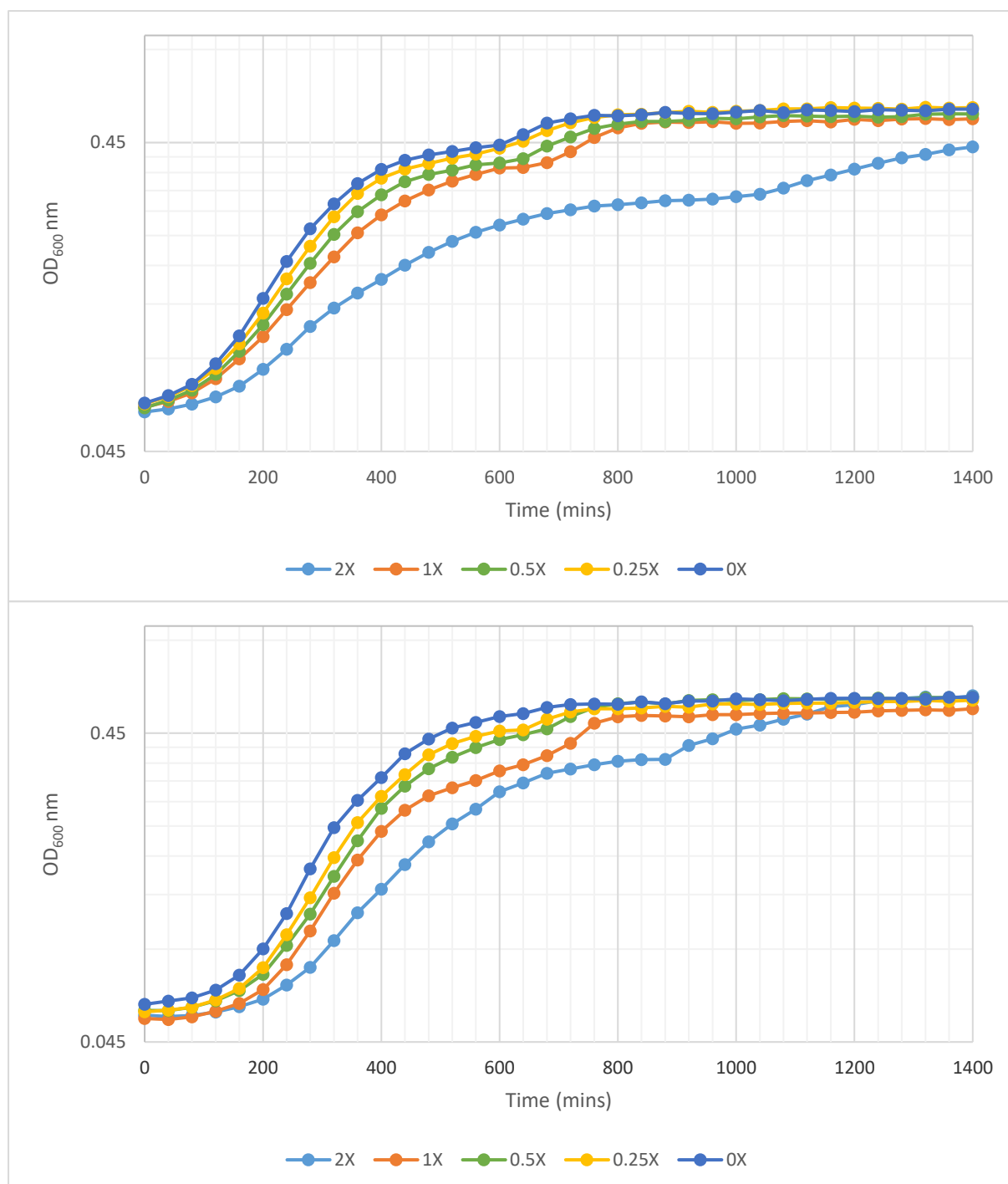


Figure 6-27 - **The effects of Compound H on *E.coli* at five different concentrations:** Cultures of *E.coli* [MG1655] were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

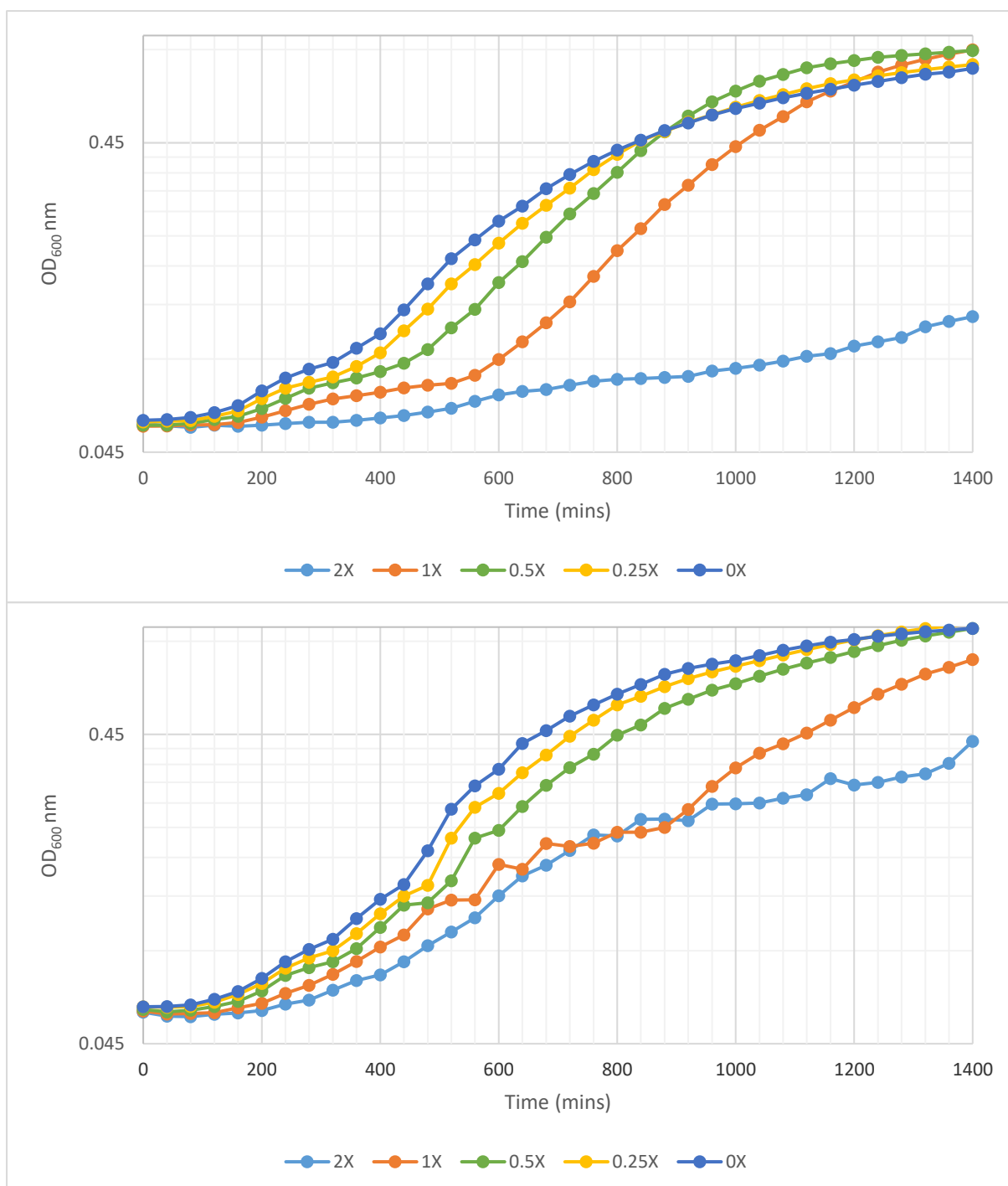


Figure 6-28 - The effects of Compound H on *P. fluorescens* at five different concentrations: Cultures of *P. fluorescens* were inoculated into BHI broth to an OD of ~0.05 in the wells of a microtitre plate. The antimicrobial compound was added to the wells at a range of working concentrations; 2X (●), 1X (●), 0.5X (●) and 0.25X (●). A control sample was also prepared that contained broth only (●). Bacteria were grown at 20 °C and sampled every 40 min. Growth was monitored by measuring optical density of cultures at 595 nm over a 24 h period. Data points represent the averages of two independent samples (n=2).

6.2 Appendix B

When calculating growth rates in section 3.2 the lag phase time was also determined. Lag phase was established as this helped to analyse how each compound slowed the initial growth of each bacteria in the presence of each compound. This data is discussed alongside the specific growth rate data in the results section however the raw data is displayed here.

Table 6-1 - Lag phase times in min for *L.monocytogenes* for the 8 antimicrobials tested.

Concentration	Compound A	Compound B	Compound C	Compound D	Compound E	Compound F	Compound G	Compound H
2X	40	360	320	120	560	280	400	120
1X	0	160	120	160	360	160	200	120
0.5X	0	40	80	40	160	160	120	120
0.25X	0	40	40	40	160	160	120	120
0X (Control)	0	40	40	40	120	160	120	120

Table 6-2 - Lag phase times in min for *L.innocua* for the 8 antimicrobials tested.

Concentration	Compound A	Compound B	Compound C	Compound D	Compound E	Compound F	Compound G	Compound H
2X	0	400	360	360	920	320	720	280
1X	0	120	160	160	360	160	320	200
0.5X	0	40	80	80	240	160	160	160
0.25X	0	40	80	80	120	160	120	160
0X (Control)	0	40	40	80	120	160	120	160

Table 6-3 - Lag phase times in min for *S. Typhimurium* for the 8 antimicrobials tested.

Concentration	Compound A	Compound B	Compound C	Compound D	Compound E	Compound F	Compound G	Compound H
2X	80	440	240	160	440	280	360	40
1X	40	200	200	240	360	160	240	40
0.5X	40	80	120	80	200	160	80	40
0.25X	40	80	80	40	200	160	80	40
0X (Control)	40	80	80	40	200	160	80	40

Table 6-4 - Lag phase times in min for *E.coli* for the 8 antimicrobials tested.

Concentration	Compound A	Compound B	Compound C	Compound D	Compound E	Compound F	Compound G	Compound H
2X	40	480	480	360	680	240	360	160
1X	40	160	120	120	240	160	200	120
0.5X	0	80	80	0	160	120	40	80
0.25X	0	80	80	0	160	40	40	80
0X (Control)	0	80	80	0	160	40	40	80

Table 6-5 - Lag phase times in min for *P.fluorescens* for the 8 antimicrobials tested.

Concentration	Compound A	Compound B	Compound C	Compound D	Compound E	Compound F	Compound G	Compound H
2X	240	1400	480	280	1400	1400	280	240
1X	120	440	240	200	520	320	200	160
0.5X	80	360	120	80	280	160	40	1200
0.25X	80	160	80	40	240	160	40	120
0X (Control)	80	160	80	40	200	80	40	120