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Competitive exclusion as a means to reduce *Escherichia coli* regrowth in digested sludge

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Abstract

In recent years, it has been reported that numbers of *Escherichia coli* increase significantly following centrifugation of sludge during the treatment process. *E. coli* is used as an indicator of the microbiological quality of sludge-derived products destined for agricultural recycling and of the efficacy of the sludge treatment processes. The re-growth phenomenon is of concern because of the potential for additional treatment requirement / higher disposal costs and loss of consumer confidence associated with a compliance failure. It is hypothesised that a competitive exclusion treatment could be the solution wherein the digestate be exposed to a 'probiotic' or defined mixture of micro-organisms, to effectively out compete or eliminate any resident *E. coli* remaining following treatment. The competitive exclusion principle as a treatment method has already seen application in various industrial sectors, the most well-known being the poultry industry. In experiments it was determined that an antimicrobial producing organism would be most likely to succeed. From the candidates screened, *Lactobacillus reuteri* proved the most promising. *L. reuteri* is a known producer of reuterin in the presence of glycerol and organic acids as a part of its normal metabolic activity. In sludge derived nutrient broth in the presence of glycerol and low pH, *L. reuteri* addition resulted in a reduction of *E. coli* to undetectable levels. In sludge cake under the same conditions, *L. reuteri* was less successful. However the addition of glycerol and *L. reuteri* to sludge cake restricted *E. coli* growth to a 2 log increase from the initial concentration of *E. coli* recorded following pasteurisation (an average of around $1x10^2$ cfu/gDs), in comparison in the positive control a 4 log increase was recorded. From this result the sludge cake could be defined as conventionally treated. It can be concluded that competitive exclusion and *L. reuteri* show promise as a treatment for reducing *E. coli* re-growth in sludge cake

Acknowledgements

Firstly, I would like to thank my supervisors, Professor Jim Harris and Dr Sean Tyrrel; they have been a great source of guidance throughout my project. I would also like to acknowledge the assistance of Dr Ronald Lambert and the expertise he provided on statistical analysis of data. I also wish to thank all the members of staff within Cranfield University and the laboratory technicians for their assistance and Miss Rachel Whitton for keeping morale up during the long working days in the laboratory. Finally I wish to thank my parents, Julie and Michael Williams for their continued support throughout.

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Section 1

Chapter 1 Introduction and literature review

1.1 Introduction

The recycling of sludge to agriculture is now regarded as the Best Practical Environmental Option in Europe. However, there are many challenges with the practice, with concern about the risk of pathogen transfer to farm produce being a major issue. In recent years, it has been reported that numbers of *Escherichia coli* increase significantly following centrifugation of sludge during the treatment process. *E. coli* is used as an indicator of the microbiological quality of sludge-derived products destined for agricultural recycling and of the efficacy of the sludge treatment processes. The re-growth phenomenon is of concern because of the potential for additional treatment requirement / higher disposal costs and loss of consumer confidence associated with a compliance failure.

The aim of this study is to utilise the “competitive exclusion principle” to control *E. coli* re-growth in digested cake. Competitive exclusion relies on the principle that two or more species competing for the same resources cannot co-exist if other ecological factors are constant. The technique has seen widespread implementation and success in other industries. The overall aim of the research in the short term is to identify organisms with the ability to suppress the growth of *E. coli* either as a sole competitor or in combination in digested sludge. If successful, experimental work will focus on optimizing the competitive exclusion product.

1.2 Introduction into waste treatment processes

Waste water derived from sewage is a combination of liquid carried water products removed from residential, institutional, commercial and industrial establishments, together with ground water, surface water and storm water, as may be present (Metcalf & Eddy *et al*., 1991). In its untreated form wastewater cannot be disposed of for several reasons. First, the biological decomposition of the organic materials in wastewater consumes oxygen and thus reduces the quantity available in the receiving waters for the aquatic life. The decomposition also produces large quantities of malodorous gases. Secondly, the numerous

pathogenic micro-organisms in untreated wastewater pose a considerable health hazard to humans. Third, the toxic compounds, especially heavy metals, contained within waste water can be dangerous to both plants and animals, and finally the presence of phosphates and nitrogen may lead to uncontrolled growth of aquatic life (Tchobanoglous and Burton, 1991). It is therefore necessary to reduce the organic components, nitrogen and phosphorus, toxic compounds, as well as eliminate the indigenous pathogenic microbial community prior to wastewater disposal (Werther and Ogada, 1999).

Whilst the processes used to treat wastewater may have a number of variations, generally they have two main stages: primary and secondary. In primary treatment, a physical operation, usually sedimentation is used to remove a portion of the suspended solids and organic matter from wastewater. This typically removes 60-70% of the total chemical oxygen demand (COD) of the wastewater (COD is commonly used to indirectly measure the amount of organic compounds in water, and therefore is a useful measure of water quality). The solids that are removed in the primary treatment are commonly known as primary sludge. In secondary treatment, biological and chemical processes are used to remove most of the less settleable or soluble organic matter still present. In the majority of municipal plants the activated sludge process is the most common method utilised. This is performed via injecting air into the wastewater to promote the growth of micro-organisms (bacteria and protozoa) which remove and oxidise most of the remaining organic components. Secondary treatment also incorporates a clarification step where settling tanks are employed to remove the micro-organisms (biomass) from the treated water. This biomass is also known as secondary sludge or surplus activated sludge (SAS). To further improve the effluent quality before it is discharged, a tertiary treatment step can also be introduced. This involves the removal of residual suspended solids and other constituents that are not reduced significantly by conventional secondary treatment usually by granular medium filtration or microscreens (Metcalf & Eddy *et al*., 1991). Disinfection and nutrient removal particularly nitrogen and phosphorus is also typically a part of

tertiary treatment. Figure 1.1 shows an overview of the whole treatment process.

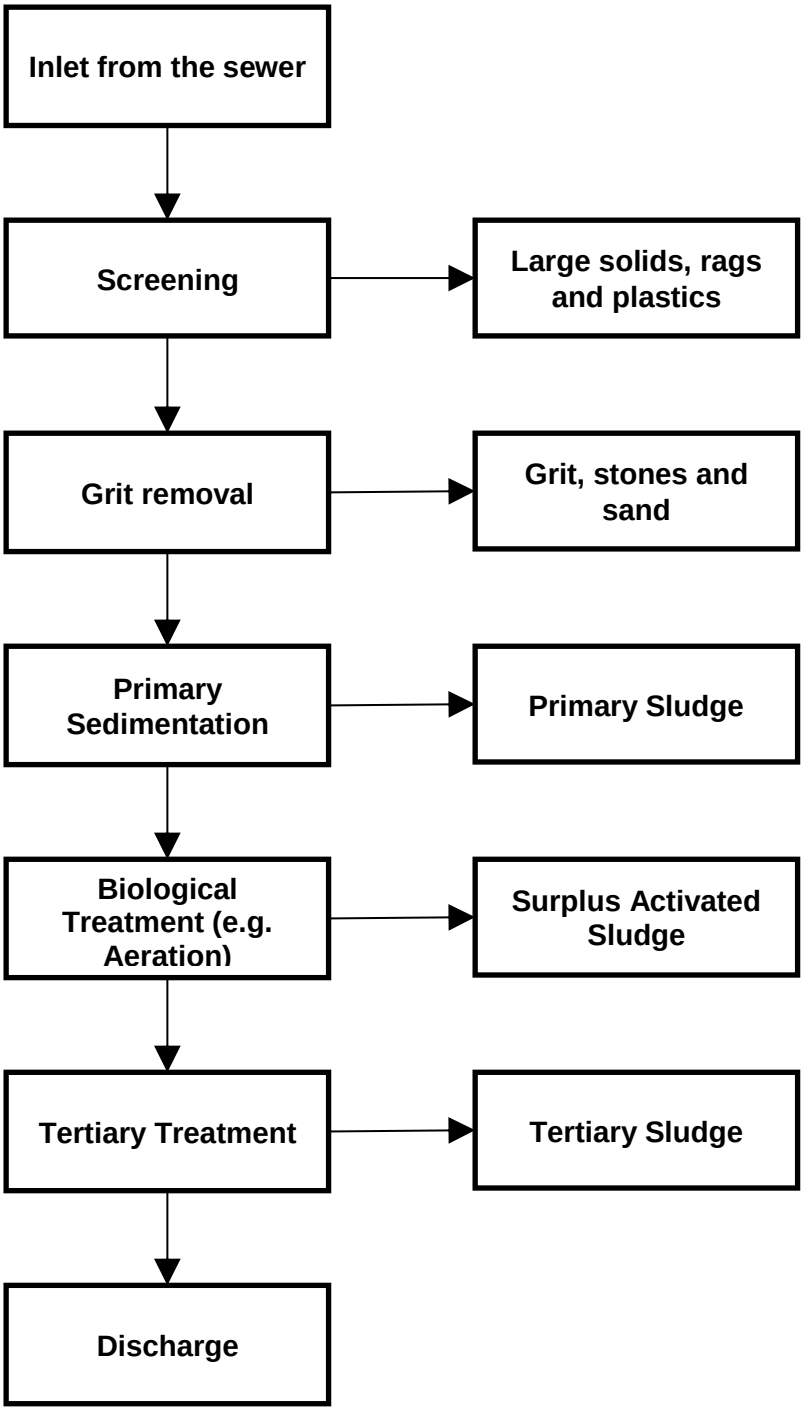

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Figure 1.1 - Flow chart depicting the treatment of wastewater. The tertiary treatment step displayed in this chart is only utilised in cases where high quality effluent is required.

1.3 How and why sludge is treated

Following its removal from waste water, sludge is usually in the form of a very dilute suspension, which typically contains from 0.25 to 12% solids, depending on the operation and treatment process used (Werther and Ogada, 1999).The typical chemical composition and properties of untreated sludge are reported in Table 1.1. Due to the physical and chemical process involved in the treatment of waste water and simply because of what sludge is derived from it tends to contain a very heterogeneous microbiological flora, comprising of a variety of pathogens including *Salmonella* spp., *Shigella* spp., *Yersinia* spp., *Brucella* spp. and *Staphylococcus* spp. as well as enterotoxigenic and enteropathic *Escherichia coli* strains (Dumontet *et al*., 1999). A more complete list of the pathogens found in sludge is shown in Table 1.2. In addition sludge can also contain concentrated levels of heavy metals (e.g. cadmium, chromium, copper, lead, mercury, nickel etc.). These biological and chemical components of sludge can potentially pose a risk to the environment, public health and food safety, especially if sludge is applied to agricultural land; therefore sludge must undergo treatment prior to disposal. Indeed the Sewage Sludge Directive 86/278/EEC prohibits the use of untreated sludge in agriculture (Directive, 1986).

Table 1.1 - Typical chemical composition and properties of untreated sludge (Metcalf & Eddy *et al*., 1991).

Item/Sludge	Untreated Primary		Activated range
	Range	Typical	
Total dry solids (TS) %	2.0-8.0	5.0	0.83-1.16
Volatile solids (% of TS)	60-80	65	59-88
Protein (% of TS)	20-30	25	32-41
Nitrogen (N, % of TS)	1.5-4	2.5	2.4-5.0
Phosphorus (P_2O_5, % of TS)	0.8-2.8	1.6	2.8-11.0
Potash (K_2O, % of TS)	0-1	0.4	0.5-0.7
Cellulose (% of TS)	8.0-15.0	10.0	-
Iron (not as a sulphide)	2.0-4.0	2.5	-
Silica (SiO_2. % of TS)	15.0-20.0	-	-
Energy content	10,000-12,500	11,000	8000-10,000
pH	5.0-8.0	6.0	6.5-8.0

Table 1.2 - A selection of possible pathogens found in sewage sludge (Carrington, 2001; Deportes *et al*., 1998).

Viruses	**Bacteria**	**Fungi**
Polio virus	*Arizona hinshawii*	*Aspergillus fumigatus*
Coxsachivirus	*Aeromonas* spp.	*Candida albicans*
Echovirus	*Bacillus cereus*	*Candida guillermondii*
Influenza	*Bacillus anthracis*	*Candida krusei*
Adenovirus	*Brucella* spp.	*Candida tropicalis*
Astrovirus	*Campylobacter jejuni*	*Cryptococcus neoformans*
Calicivirus	*Citrobacter* spp.	*Epidermophyton* spp.
Coronavirus	*Clostridium botulinum*	*Geotrichum candidum*
Enterovirus	*Clostridium perfringens*	*Microsporum* spp.
Parovirus	*Enterobacteriaceae*	*Phiolophora richardsii*
Rotavirus	*Escherichia coli*	*Trichosporon cutaneum*
Norwalk virus	*Klebsiella* spp.	*Trichophyton* spp.
Hepatitis A virus	*Leptospira ichterohaemorrhagiae*	**Helminths**
Hepatitis E virus	*Listeria monocytogenes*	*Ankylostoma duodenale*
Protozoa	*Mycobacterium tuberculosis*	*Ascaris lumbricoides*
Acanthomoeba	*Pasturella pseudotuberculosis*	*Echinococcus granulosus*
Dientamoeba fragilis	*Proteus* spp.	*Echinococcus multilocularis*
Entamoeba hystolitica	*Providencia* spp.	*Enterobium vermicularis*
Giardia lambila	*Pseudomonas aeuriginosa*	*Hymenolepsis nana*
Giardia intestinalis	*Salmonella* spp.	*Necator americanus*
Isospora belli	*Serratia* spp.	*Strongyloides stercoralis*
Naeglaria fomleri	*Shigella* spp.	*Taenia saginata*
Palantidium coli	*Staphylococcus aureus*	*Taenia solium*
Sarcocystis spp.	*Enterococcus* spp.	*Toxocara cati*
Toxoplasma gondii	*Vibrio parahaemoliticus*	*Toxocara canis*
	Yersinia enterocolitica	*Trichuris trichura*

Treated sludge is defined as having undergone biological, chemical or heat treatment, long term storage or any other appropriate process so as to significantly reduce its fermentability and the health hazards resulting from its use (Directive, 1999). *E. coli* is used as an indicator species to demonstrate the

presence of faecal and pathogenic bacteria and their removal. The use of indicator species is attractive because it reduces the cost and complexity of analyzing biosolids for individual pathogens (Sprigings and Le, 2011). *E. coli* was chosen as an indicator species because it is abundant in raw sewage sludge and there is a strong correlation between the presence/absence of the indicator and faecally derived pathogens (Fenlon *et al*., 2000; Rice *et al*., 1992). On the standard of the degree of *E. coli* elimination from sludge, the safe sludge matrix (SSM) outlines two different forms of treatment – conventional and enhanced (ADAS, 2001). To be defined as conventionally treated, the sludge has to have undergone defined treatment processes and standards that ensure at least 99% of *E. coli* present has been destroyed (ADAS, 2001). Additionally conventionally treated sludge is only permitted to contain an upper limit of 10^5 *E. coli* per gram dry weight of sludge (The Environment Agency, 2003a). In comparison enhanced treatment, originally referred to as 'advanced treatment', refers to processes which are capable of eliminating 99.9999 per cent of *E. coli,* furthermore enhanced treated sludge also must be free of *Salmonella* (ADAS, 2001). Additionally enhanced treated sludge is only permitted to contain an upper limit of 10^3 *E. coli* per gram dry weight of sludge (The Environment Agency, 2003a). There are many technologies used in sludge treatment, Table 1.3 outlines the more common types of sludge treatment employed, of which various combinations are used according to the end product required

Anaerobic digestion is a naturally occurring process of decomposition and decay, by which organic matter is broken down by micro-organisms into its basic chemical components under anaerobic conditions (Monnet, 2003). Anaerobic digestion is considered the most attractive method for the treatment and recycling of sludge since it reduces waste volume, generates an energy-rich gas in the form of methane (CH_4), and yields a nutrient rich final product (Mata-Alvarez *et al*., 2000). During sludge treatment, the digestion process itself takes place in a digester, which can be classified in relation to the temperature and the water content of the feedstock. A general schematic for an anaerobic digester is shown in Figure 1.2.

Table 1.3 - Common methods of sludge treatment (Fytili and Zabaniotou, 2008; Metcalf & Eddy *et al*., 1991)

Sludge Treatment	Description
Lime stablisation	Used when digestion is not available. The lime reacts with the water and produces heat as well as increasing the pH of the sludge, which helps to kill pathogens. Limed sludge is a good soil conditioner in many upland regions with acidic soils.
Thermal drying	Used to convert the sludge to a pelletised or granular form comprising of about 90% solids. The heating involved also destroys any pathogens. Thermally dried sludge is often used in agriculture or for amenity uses.
Pasteurisation	In this process sludge is heated to around 70°C for at least 30 minutes, after which it is cooled. This does not kill all micro-organisms, instead pasteurization aims to reduce the number of viable pathogens but retain the biological quality of the sludge
Dewatering	Centrifugation or filter presses are often used after digestion to separate the solid fraction from the liquid. The solid fraction, commonly known as Biosolids cake, is a compost-like material which can be conveniently transported, stored on farms and spread using standard agricultural equipment.
Composting	Is an aerobic process that involves mixing the sludge with sources of carbon such as sawdust, straw and wood. In the presence of oxygen, bacteria digest both the sludge and the added carbon source and, in doing so, produce a large amount of heat, causing pathogen destruction.

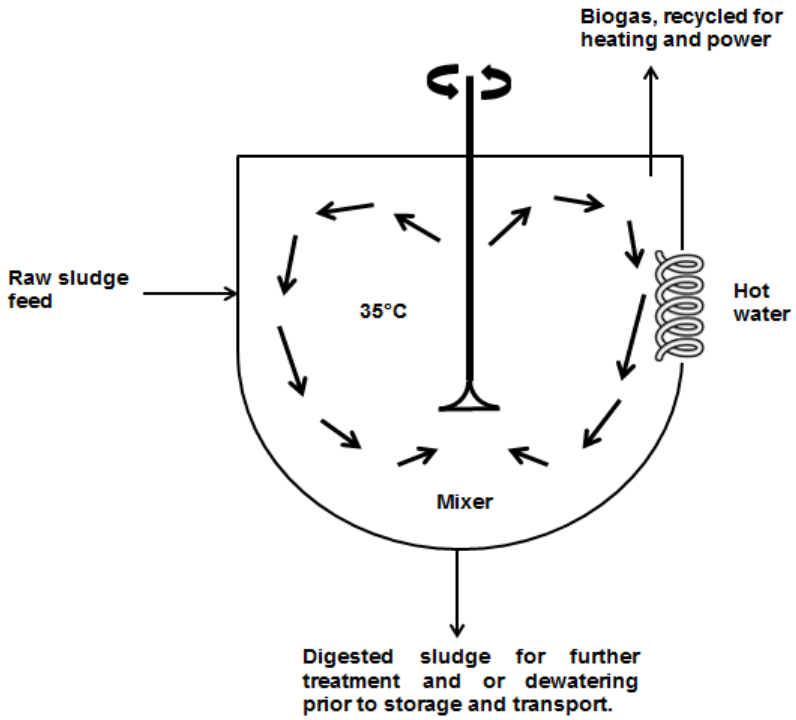


Figure 1.2 - Schematic diagram of an anaerobic digester under mesophilic conditions

Anaerobic digestion can occur at two main temperature ranges. Mesophilic conditions are designated as between 20-45°C, usually 35°C, with a retention time depending on the waste composition of between 15 and 30 days. Thermophilic conditions are however between 50-65°C, usually 55°C with a retention time between 12 and 14 days (Monnet, 2003). The thermophilic anaerobic digestion process is usually characterised by accelerated biochemical reactions, higher growth rate of microorganisms and accelerated interspecies hydrogen transfer resulting in an increased methanogenic potential and therefore a higher gas production rate at lower hydraulic retention times (Zbransk *et al*., 2000). Also, the enhanced hygienisation effect of the thermophilic process (Oropeza *et al*., 2001; Watanabe *et al*., 1997) complies with the safe sludge matrix guidelines for the elimination of pathogens, allowing the sludge produced to be defined as enhanced treated (ADAS, 2001; Lafitte-Trouque and Forster, 2002). Furthermore it has been reported that thermophilic anaerobic digestion of sewage sludge can lead to the production of the U.S. Environmental Protection Agency's class A biosolids, which are essentially pathogen free and are suitable for subsequent land application (Watanabe *et al*., 1997). However, the use of thermophilic anaerobic digestion has been limited, because of some disadvantages like a high energy input requirement, poor supernatant quality and poor process stability related to chronically high propionate concentrations (Kugelman and Guida, 1989). In comparison mesophilic digesters are more stable (Fannin *et al*., 1987), require less energy and have a reduced risk of inhibition by ammonium (Angelidaki and Ahring, 1994; Hansen *et al*., 1998) and long-chain fatty acids. Thus on an industrial scale, mesophilic digesters are the most widely utilised for the treatment of organic waste (Fernández Rodríguez *et al*., 2012). Regardless of which method is used however the performance and stability of anaerobic digestion is highly dependent upon the active microbial groups involved in the process (Shin *et al*., 2010). Therefore, characterisation of the microbial community structure is critical to the efficiency of anaerobic digestion. However, in a study by Laffite-Trouque and Forster (2002) mesophillic digestion was only capable of reducing the faecal concentration of sludge from $1.5x10^6$ g^{-1}DS to $1x10^4$ g^{-1}DS. Berg and

Berman (1980) also recorded a similar reduction in faecal coliform concentration when using mesophilic digestion as a treatment method for sludge. This level of reduction in faecal coliforms however means that sludge treated solely by mesophilic digestion cannot be classified as enhanced treated under safe sludge matrix guidelines. To produce enhanced treated sludge using mesophilic anaerobic digestion, a variety of pre-treatment technologies such as enhanced enzymic hydrolysis and thermal hydrolysis can be utilised (Mller, 2001). Thermophilic digestion however is capable of producing enhanced treated sludge, in the same study by Laffite-Trouque and Forster (2002); all the thermophilic digestions produced extremely low faecal coliform counts in sludge. Following digestion, sludge is then dewatered by centrifugation or belt press to produce a sludge ‘cake’ allowing easier storage and transport.

1.4 Sludge Disposal

During the last few decades there has been a major change in the way that sludge is disposed of. Prior to the banning of sludge disposal at sea in 1998 in accordance with the terms of the North Sea Conference Agreement (Goldsmith, 1994), 30% of sludge produced in the United Kingdom (UK) was disposed of via this method (Werther and Ogada, 1999). The other conventional option, disposal in landfill, has also been effectively eliminated by increased costs and legislation such as EU directive 99/31 (Directive, 1999) which set mandatory targets for the reduction of biodegradable waste to landfill. Due to these restrictions sludge disposal is becoming an increasing problem. The situation is only expected to get worse considering the implementation of further EU directives such as the urban waste water treatment directive 91/271/EEC concerning water purification and enhanced water quality standards expected to increase sludge output drastically in the coming years (Council, 1991). It was estimated in the EU between 1998 and 2005, 9.4 million tonnes (dry weight) of sludge was produced, by 2020 it is expected to exceed 13 million tonnes (Léonard, 2011)

The obvious answer would to increase the role of incineration in the disposal of sludge. As of 2008 in the UK only 16% of sludge produced is incinerated (Water, 2010). Sludge incineration enjoys a combination of several advantages that are not found in other treatment alternatives, including a large reduction of sludge volume to a small stabilized ash, which accounts for only 10% of the volume of mechanically de-watered sludge and thermal destruction of toxic organic constituents (Vesilind and Ramsey, 1996). Further, the calorific value of dry sludge corresponds to that of brown coal, and therefore through incineration this energy content maybe recovered (Römer, 1991). Furthermore in large urban areas where large quantities of wastewater sludge is produced but land available for disposal space and the objections in terms of aesthetics and odor generation of the local population have to be taken into account, incineration of sludge is a promising disposal method. However a number of disadvantages are apparent, firstly incineration does not constitute a complete disposal method since approximately 30% of the solids remain as ash (Malerius and Werther, 2003). This ash is generally landfilled and in certain cases, it is considered as highly toxic because of its heavy metal content. Second, is the general negative public reaction to the burning of waste which makes planning permission for such facilities difficult to obtain (Matthews, 1992). Finally, during the incineration process polynuclear aromatic hydrocarbons and dioxins can be produced and possibly released into the environment; this poses an extreme risk due to the hazardous properties to human health these chemical compounds possess. In addition the amount of greenhouse gases such as carbon dioxide being released into the atmosphere has to be considered, especially with the UK government agreeing that by 2050, it would achieve an 80% reduction in total UK greenhouse-gas emissions from concentrations recorded in 1990, and has further committed to achievement of a 34% reduction in greenhouse gases by 2020 (HM Government, 2009). For these reasons incineration as a principal option of disposal is not ideal.

This leaves the recycling of sludge as biosolid based products or to agriculture. Regarded as the best practicable environmental option, agricultural recycling is

not only economical when compared to other disposal routes such as incineration; it is also the most environmentally sustainable. Furthermore based on the content of nitrogen, phosphorus and potassium in sludge (Poletschny, 1988), it shows good fertilizer properties. Sludge recycling as fertilizer has several advantages which include the return of organic materials into the bio-cycle and improves soil structure and water retention (Wallace *et al*., 2009). Sludge also replaces the need for the application of artificial fertilizers whose production requires a lot of energy, which comes at an expense and in general public opinion of the use of artificial fertilizers on agricultural land is poor (Mara *et al*., 1989). Due to these advantages the sewage sludge directive 86/278/EEC seeks to encourage the use of sewage sludge in agriculture (Directive, 1986). As of 2008, application of sludge in agriculture takes 77% of sludge produced in the UK (Water, 2010). However, despite its advantages agricultural disposal comes under pressure from consumer groups and the public due to faecal aversion, objection to odour and fears regarding the contamination of grazing land and food crops (Le, 2007). Due to this risk of pathogen transfer to farm produce, in the UK, biosolids bound for agriculture must conform to standards stipulated in the safe sludge matrix (ADAS, 2001).

Iranpour *et al*. (2002) indicated that wastewater treatment plant operators that operate thermophilic digesters designed to meet Class A biosolids requirements (ADAS, 2001) have become aware that increases in faecal coliform densities are readily occurring in post-digestion biosolids. Cheung *et al.* (2003) and Qi *et al.* (2004) have also reported significant growth of *Escherichia coli*, with increases as high as 1–2 $\log_{10}$/g dry solids, being measured in digested sludge samples collected immediately after centrifugation. This has potentially important implications for compliance with *E. coli* reduction requirements for conventional and enhanced treated sludge for agricultural recycling (ADAS, 2001). With *E. coli* limits being met after digestion but not after dewatering, uncertainty exists about the true density of *E. coli* and other faecal coliforms in biosolids being applied to land. This potentially limits the use of enhanced

treated biosolids and consequentially leads to higher disposal costs and loss of consumer confidence in the industry.

1.5 Escherichia coli re-growth in digested sludge cake.

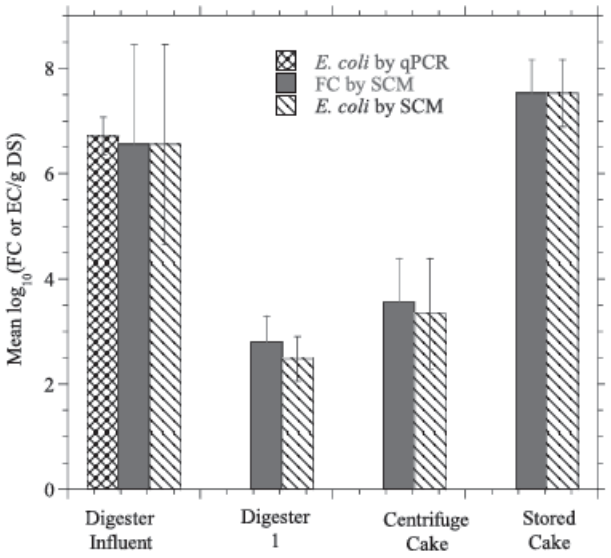


Figure 1.3 – Fecal coliform and *E. coli* density measured using qPCR and standard culturing methods (SCM) in temperature phased anaerobic digestion process with high-solids centrifugation dewatering. *E. coli* densities in stored cake, is after 24 hour storage (Chen *et al*., 2011b).

Several theories have been proposed and investigated to explain the sudden increases in fecal coliforms and in particular *E. coli* density immediately after dewatering and subsequent storage, a classic example of this is shown in Figure 1.3. For example, Qi *et al.* (2004) suggested regrowth of fecal coliforms as a possible cause of the sudden increase. Monteleone *et al*. (2004) theorized that the shear experienced by the solids during high solids centrifugation improved the 'release' of the bacteria from the floc matrix which increased the numbers that could be cultured compared with before dewatering. Iranpour *et al.* (2003) on the other hand reported that contamination of the biosolids with fecal coliforms could explain the high counts measured after dewatering and storage. Supporting this Baddeley *et al.* (2009) identified several routes of contamination that were believed to be the cause of the increases in bacterial density seen in cake; including the use of final effluent as carrier water for polymer dilution in dewatering. Removal of this contamination source resulted in

the reduction of *E. coli* numbers in the immediate cake demonstrating that, at least for some cases, the increase in *E. coli* concentration observed may simply be due to contamination.

However problems do exist with these possible explanations. For example, for regrowth to occur, a significant amount of time would be required to increase the counts to the levels seen in cake immediately following the dewatering process. Since the doubling time under ideal conditions for *E. coli* is around 20 minutes (about the same as the retention time in high solid centrifuges), the large increase in *E. coli* and indeed fecal coliforms cannot be explained by regrowth alone. Secondly it is unlikely the release of *E. coli* in floc during centrifugation is the cause as during conditioning and dewatering, coagulants such as cationic polymer are added, which aggregate the floc and allow for the formation of cake (Higgins *et al*., 2007). In addition during the preparation of samples for *E. coli* enumeration samples are typically diluted with water and homogenized to break up flocs and release bacteria. Finally, even though Baddeley *et al.* (2009) did report that removal of the known contaminant did indeed reduce *E. coli* numbers in the subsequent cake it did not eliminate the bacteria completely and did not prevent the secondary growth in the stored cake, suggesting an alternate cause.

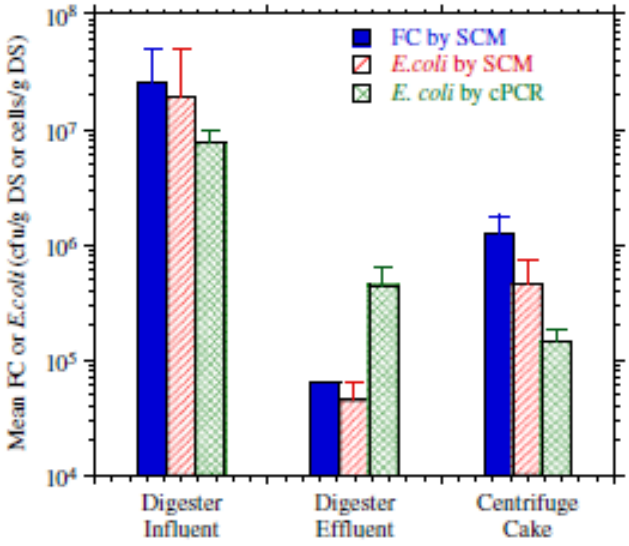


Figure 1.4 - Fecal coliform (FC) and *E. coli* measured by standard culturing method (SCM) and cPCR for mesophillic digestion (Higgins *et al*., 2007).

Higgins *et al*. (2007) proposed an alternate hypothesis in that two separate phenomena, termed reactivation and regrowth were actually responsible for the increases in *E. coli* and fecal coliforms in cake following dewatering and subsequent storage. It was theorized that that the bacteria present during digestion enter a state known as viable but non-culturable (VBNC), meaning the bacteria are non-culturable and therefore impossible to enumerate and detect after digestion using standard culturing methods even though they are still present and effectively viable. It is thought bacteria enter this state following exposure to environmental stress such as nutrient or substrate deprivation, salinity and extreme temperature (Lisle *et al*., 1998; Makino *et al*., 2000; Mizunoe *et al*., 1999). Considering the conditions present during digestion, namely low substrate and nutrient concentrations and for thermophillic digestion, high temperature, it is not unlikely the bacteria would be stressed enough to be induced into a VBNC state. Indeed, Higgins *et al*. (2007) using a specific type of quantitative polymerase chain reaction (qPCR) called competitive PCR (cPCR) to enumerate *E. coli* after digestion/before dewatering and also immediately after dewatering, showed that the concentration based on copies of *E. coli* DNA were not significantly different before and after dewatering, despite the large difference in concentrations shown by the standard culturing method (Figure 1.4). This supports the theory that low counts following digestion are caused by *E. coli* entering a VBNC state, however it has to be noted that cPCR does not distinguish between live and dead cells and as such the concentration of DNA measured cannot be conclusively linked to viable cells. However it has been shown previously in various studies that *E. coli* has the capability to enter a VBNC state (Mizunoe et al., 1999; Reissbrodt *et al*., 2002)

Following on from this Higgins *et al* (2007) hypothesized that during the dewatering process *E. coli* 'reactivates' rendering the cells culturable and therefore enumerable by the standard culturing method again. The mechanism by which reactivation occurs is unclear although it can be speculated that high shear forces caused by the dewatering process (centrifugation) rupture the cell membranes of the digestate flora releasing the nutrient rich cell contents. It is

these nutrients that support the reactivation/resuscitation of the VBNC *E. coli* cells and subsequent proliferation and rapid colonization of the cake following storage. Alternatively the shear forces may result in the release of signaling compounds or growth factors such as autoinducers which resuscitate the bacteria into a culturable state. Reissbrodt *et al.* (2002) has shown that addition of autoinducers to the media for enumeration of VBNC bacteria resulted in a significant increase in the numbers enumerated. Furthermore Higgins *et al.* (2007) showed that the components of centrate (from biosolids displaying bacterial growth patterns associated with the VBNC phenomena) and the addition of a polymer (used for conditioning) could increase fecal coliform enumeration considerably (Figure 1.5). However repeat experiments on different months failed to show the same reactivation suggesting any compounds in the centrate may be unstable or other factors are important. The role of the polymer remains unclear. Either by releasing nutrients or chemical compounds the shear forces created by centrifugation clearly have a role in reactivation since cake produced by alternative dewatering processes such as belt presses which cause minimal shear suffer significantly less re-growth (Chen *et al*., 2011a). Additionally, the basic action of the centrifuge re-oxygenates the digestate. In the majority of cases where anaerobic digestion is used as treatment this rapid shift from an anaerobic to an aerobic environment not only aids rapid proliferation and reactivation but also provides *E. coli* (a facultative anaerobe) with a competitive advantage over the digestate flora which is mostly comprised of obligate anaerobes. This means the flora cannot adapt to the new conditions allowing *E. coli*, despite only being in smaller numbers, to utilize the nutrients with little hindrance, leading to the ‘re-growth’ and increase in *E. coli* concentration. A possible solution to this and to prevent unhindered proliferation of *E. coli* during storage to the point where it exceeds safe sludge matrix guidelines is the utilization of the competitive exclusion

principle.

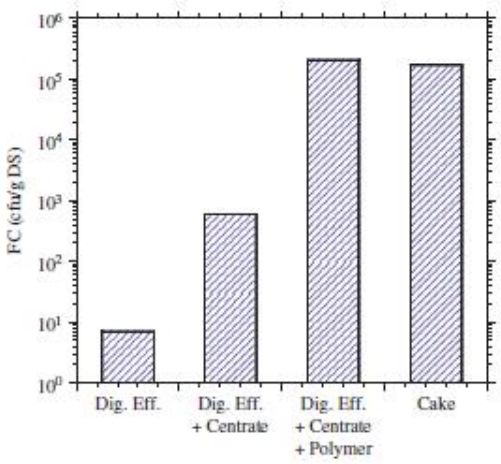


Figure 1.5 - Impact of centrate and polymer on the reactivation of VBNC fecal coliforms (Higgins *et al*., 2007).

1.6 Competitive exclusion

1.6.1 Introduction to the principle

The competitive exclusion principle, sometimes referred to as Gause's law of competitive exclusion was formulated by Russian ecologist Georgii Frantsevich Gause and states two species competing for the same resources cannot coexist if other ecological factors are constant. When one species even has the slightest advantage or edge over another, the one with the advantage will dominate in the long term, leading to either the extinction of its competitor or an evolutionary or behavioral shift towards a different ecological niche. The principle itself was formulated upon competition experiments using two species of *Paramecium* (unicellular protozoa), namely *P. aurelia* and *P. caudatum* (Gause, 2003). It was found that in constant conditions (readily available water and nutrients) that *P. caudatum* dominated, however following a prolonged lag phase *P. aurelia* recovered and out competed *P. caudatum* to the point of extinction (Figure 1.6). This is just one example of competitive exclusion. The principle itself applies to all organisms such as yeasts where *Schizosaccharomyces kefir* was proven to consistently out compete *Saccharomyces cerevisiae* by producing a higher concentration of ethyl alcohol

and thereby causing cessation of its growth (Gause, 1932), to more complex life forms, such as the competitive exclusion of the native red squirrel by the grey squirrel in Great Britain.

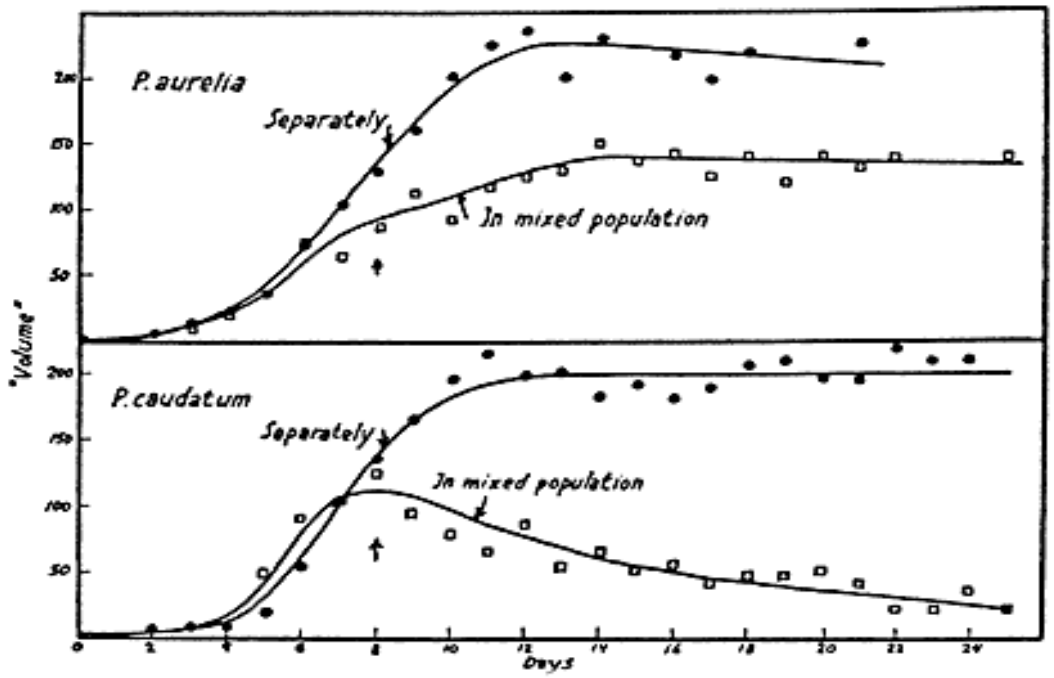


Figure 1.6 - The growth of the "volume" in *Paramecium caudatum* and *Paramecium aurelia* cultivated separately and in mixed population on osterhout medium (Gause, 2003).

1.6.2 Competitive exclusion treatment application and possible mechanisms of action

The competitive exclusion principle as a treatment method has already seen application in various industrial sectors, the most well-known being the poultry industry. In this case chicks are administered prophylactically a ‘probiotic’, which is defined as a live microbial feed supplement which beneficially affects the host animal by improving its microbial balance (Fuller, 1991). Basically, the newly hatched chicks are exposed to either a defined bacterial mixture or undefined caecal culture, in an attempt to instate them with a fully developed intestinal flora, thereby controlling and reducing the colonisation of several enteropathogens such as *Salmonella* spp., *Escherichia coli, Clostridium perfringens, Listeria* spp. and *Campylobacter* spp.

The exact mechanism of this protection is uncertain; though via simply providing the chicks with a non-pathogenic gut microbiota, this effectively could mean there are no receptor sites for pathogenic bacteria to adhere to and thereby colonise and cause disease, or alternatively the addition of the microbiota reduces the nutrients available in the gut, reducing the ability of pathogenic bacteria to colonise. Either way it is well established within the literature that gut microflora is known to play a major role in the protection of the colonized host against invasion by pathogens. For example, dosing newly hatched chicks *per os* with fecal suspension from adult hens prevented the establishment of salmonellae in the gut (Nurmi and Rantala, 1973). Additionally germ free animals have been shown to be more susceptible to disease than their conventional counterparts who carry a complete gut flora. This difference has been shown for infections caused by *Salmonella enteritidis* (Collins and Carter, 1978) and *Clostridium botulinum* (Moberg and Sugiyama, 1979).

Another example of the importance of the gut microflora in preventing pathogen colonisation is in humans where the main cause of noso-comial infectious diarrhoea in hospitals is believed to be due to the antibiotic disruption of the normal intestinal flora, resulting in overgrowth of *Clostridium difficile* (Kyne *et al*., 2002; Naaber *et al*., 1998; Thorens *et al*., 1996). Like with chicks, this is often treated by giving patients a mixed probiotic culture along with the antibiotics. D'Souza *et al.* (2002) reported that live organisms may be effective in preventing antibiotic associated diarrhoea, with in this study lactobacilli and *Saccharomyces boulardii* proving particularly successful. This was further supported by Plummer *et al.* (2010) who reported that only 46% of patients provided with a probiotic consisting of *Lactobacillus* and *Bifidobacterium* along with their antibiotics tested positive for *C. difficle* toxin compared to the 78% that tested positive when provided with a placebo. As the competitive exclusion treatment for the chicks the mechanism of action is unclear, though it is thought competition for receptor sites makes colonisation difficult for pathogens.

However the competition for nutrients and receptor sites may not be solely responsible for the reductions in pathogen colonisation and overall inhibiton of growth. Instead, production of anti-microbial compounds maybe the source of inhibition. There are numerous examples of this within nature and the human body. *Lactobacillus reuteri*, *Lactobacillus plantarum* and *Lactobacillus acidophilus* just to name a few are all known to be able to produce antimicrobial compounds such as reuterin which are effective against pathogens such as *E. coli* (Brashears *et al*., 2003; Cleusix *et al*., 2008; Hamdan and Mikolajcik, 1974; Niku-Paavola *et al*., 1999; Talarico and Dobrogosz, 1989; Barefoot and Klaenhammer, 1984; De Klerk and Coetzee, 1961; Silva *et al*., 1987). *Streptococcus mutans* is known to inhibit the growth of many other oral micro-organisms via producing lactic acid from fermentable carbohydrates present in the host diet (Loesche, 1986). Interestingly, *Streptococcus oligofermentans* has developed the counter-offensive strategy of using the *S. mutans*-produced lactic acid to generate hydrogen peroxide, which is in turn inhibitory to *S. mutans* and many other micro-organisms (Tong *et al*., 2007). It has also been postulated that the production of volatile fatty acids (VFAs) such as propanoic acid could be a source of inhibition. Results from Wolin (1969) strongly suggested that volatile fatty acid fraction of rumen fluid was capable of inhibiting the growth of *E. coli*. This inhibitory effect of VFAs on *E. coli* was also reported by Prohaszka (1980) in the caecal contents of rabbits, further corroborating VFAs possible role in the competitive exclusion of pathogens in particular within the gut.

1.6.3 Possible application of competitive exclusion treatment in sludge cake

It is hypothesised that competitive exclusion as a treatment method could be transferred over to digested sludge cake and that in effect the digestate be exposed to a ‘probiotic’ or defined mixture of micro-organisms, to effectively out compete or eliminate any resident *E. coli* remaining following treatment, thereby preventing or reducing the level of re-growth. Though the use of competitive exclusion as a method of reducing pathogen growth within digested sludge cake has never been properly investigated, there are other examples in the literature where it has been investigated within similar environments, such as in silage.

Silage is fermented, high-moisture stored fodder which can be fed to ruminants or used as a biofuel feedstock for anaerobic digesters. Silage is made either by placing cut green vegetation in a silo, by piling it in a large heap covered with plastic sheet, or by wrapping large bales in plastic film. The production of silage relies on anaerobic digestion and it is prone to aerobic and anaerobic spoilage by micro-organisms. Many of these spoilage organisms such as Clostridia and Listeria not only decrease the nutritional value of the silage, but also have a detrimental effect on animal health and/or milk quality (Driehuis and Elferink, 2000). Therefore it is beneficial for the production of high quality silage to inhibit the growth of these micro-organisms, similar to the need to reduce *E. coli* growth in digested sludge cake. In recent years the addition of bacterial inoculants to silage has become popular within European countries such as the United Kingdom, Germany, Italy and the Netherlands (Wilkinson *et al*., 1996). In the case of silage bacterial inoculants are added in order to stimulate lactic acid fermentation, accelerating the decrease in pH, and thus improving silage preservation via inhibition of the other microflora and more importantly the spoilage micro-organisms. Most available inoculants consist of selected strains of homofermentative lactic acid bacteria, such as *Lactobacillus plantarum*, *Pediococcus*, and *Enterococcus* species (Weinberg and Muck, 1996). Many studies have shown the advantages of such lactic acid bacteria inoculants (Filya *et al*., 2000; Weinberg *et al*., 1988; Lindgren *et al*., 1983). A review by Driehus and Elferink (2000) further concluded that the quality of silage was dependent on the competition between different groups of micro-organisms, with the predominance of lactic acid bacteria a requirement for high quality silage. Furthermore Van Elsas *et al.* (2007) showed that in unfumigated soil, over a 60 day incubation period, the CFU numbers per gram of dry soil of *E. coli* declined by at least six orders of magnitude. In comparison the survival of *E. coli* in all fumigated soils was strongly and significantly enhanced. This result demonstrates that modifying soil's microbial community and removing competing organisms can affect the survival of *E. coli.* Soil and silage are similar growth environments to digested sludge cake in that they all contain a high concentration of highly diverse microflora. These studies essentially show

that using competition exclusion as a treatment method to inhibit the growth of undesirable microflora is possible in an environment similar to that of digested sludge cake Therefore it can be hypothesised that the use of competitive exclusion to reduce *E. coli* re-growth in sludge cake following treatment is feasible.

However applying competitive exclusion treatment to control the growth of *E. coli* in digested sludge cake does pose a challenge for a number of reasons. Firstly digested sludge cake has a fairly high nutrient and moisture content, hence why it is utilised on agricultural land, this along with storage conditions, especially in the summer months, where temperatures can reach up to 30°C within the sludge cake, does mean that growth conditions for *E. coli* are fairly hospitable. Considering that *E. coli* is a fast growing bacterium, capable of doubling in population size every 20 minutes given the optimum conditions, preventing or reducing exponential growth in digested sludge cake to the point where there is no risk of compliance failure will be difficult. Furthermore any inoculant added to sludge cake will not only be competing with *E. coli,* but also the rest of the microflora which is very vast and diverse as shown in Table 1.2. Digested sludge cake is also not a consistent medium, its physical, chemical and biological properties will be subject to change throughout the year and between batches, making any effect a probiotic has in one batch of sludge cake not necessarily consistent with another.

Being able to apply a competitive exclusion treatment to sludge cake would hold its advantages over conventional methods such as chemical addition. Firstly costs, a probiotic purely derived from bacteria found freely within the environment or gut microflora would cost less than antimicrobials or chemicals such as lye especially in the quantities that would be required, even when factoring in the cost of culturing and delivery of the probiotic into the sludge cake. In addition due to the fact biosolids will inevitably go to agricultural land, the question has to be raised with chemicals what would their environmental impact be, while with a probiotic the bacteria utilised could be easily screened to

ensure no potential pathogens would be present. The use of antimicrobials and antibiotics can also result in insufficient exposure for eradicating bacteria such as *E. coli* and potentially create an environment that promotes antibiotic resistance. Misuse of antibiotic therapy has ramifications on health and safety especially when considering the application to agricultural land. In summary probiotics potentially provide a cheaper, safe and possibly effective method of preventing *E. coli* re-growth in stored biosolids.

1.7 Aims and objectives

The principal hypothesis is:

'the regrowth of Escherichia coli in biosolids can be reduced by the application of the competitive exclusion principle; e.g. by competition between E. coli and non-pathogenic bacteria that will be introduced to the dewatered sludge.'

This was achieved via controlled and replicated bench-scale experiments, with the aim of identifying promising candidate organisms with the capability of suppressing *E. coli* growth in digested sludge cake. Experimental work was split into three sections: preliminary, proof of concept and finally application in sludge cake.

1.7.1 Preliminary experiments

The aim of the preliminary experiments was two-fold. First the basic premise was to assess the methodology for accuracy and repeatability. To test this, controlled replicated bench scale experiments were designed in which sludge samples were sterilised via autoclaving and then spiked with a pure culture of *E. coli*. The *E. coli* were then recovered from the sludge cake via membrane filtration and the recovery rate deduced to determine its accuracy. This experiment ensured that the results of every experiment using the methodology would be accurate and valid. Second, it was determined whether it is possible to simulate *E. coli* re-growth in digested sludge cake at laboratory scale. Once again replicated bench scale experiments were designed in which sludge samples were pasteurised and then incubated with *E. coli* growth measured over time. Development of a consistent *E. coli* re-growth methodology was

essential for future competition experiments; it also provided a curve for *E. coli* re-growth in optimal conditions in sludge cake which growth curves from competition experiments were compared against.

1.7.2 Proof of concept

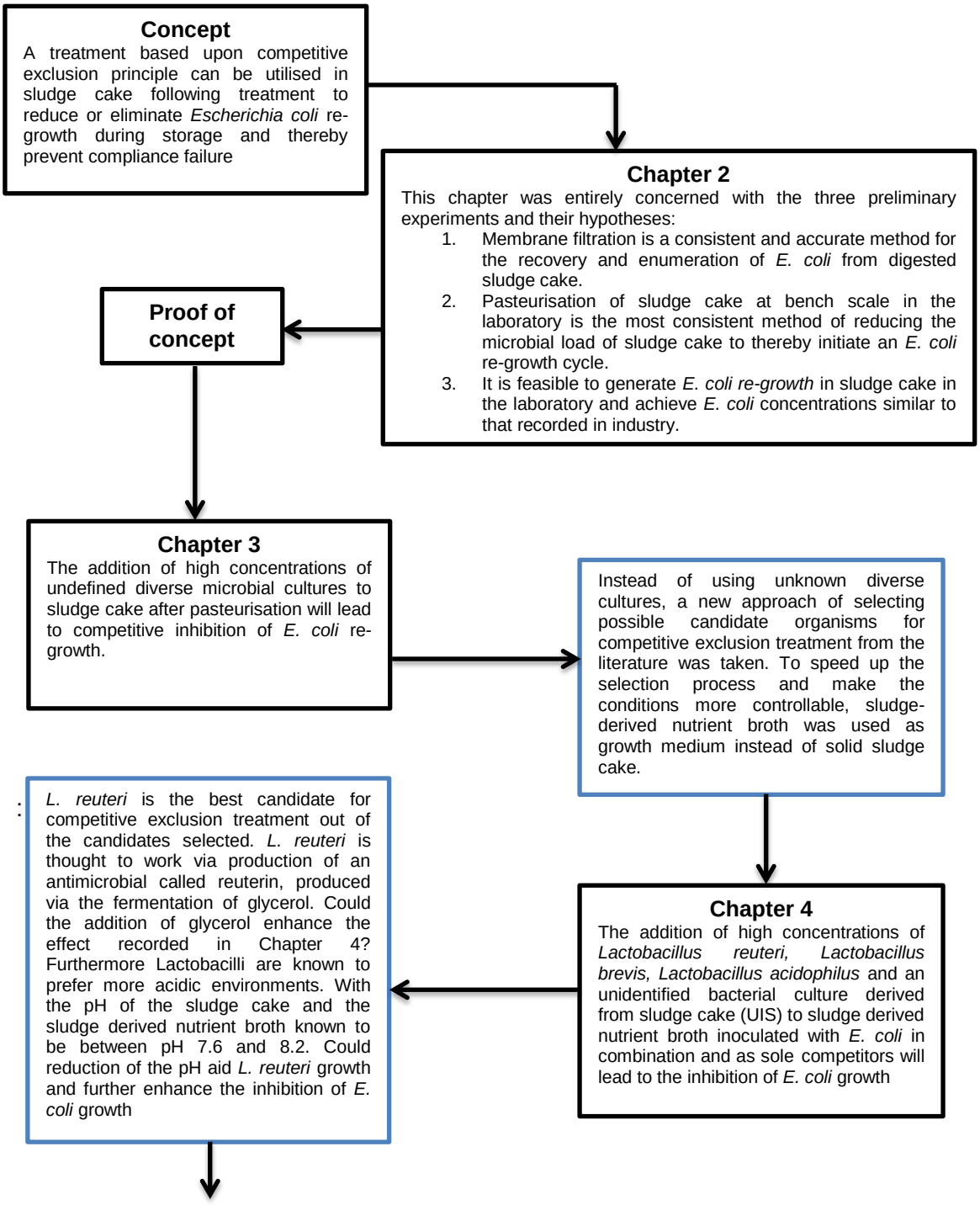
Though the competitive exclusion principle has previously been utilised as a treatment method in a number of industries to combat
whether a competitive exclusion treatment for reducing *E. coli* re-growth pathogens such as *E. coli*, it has never previously been applied to sludge cake. Sludge cake is an extremely complex growth matrix. It is therefore unknown which micro-organisms will be able grow and thrive in sludge cake and competitively inhibit *E. coli* re-growth. Furthermore it is not known which competitive exclusion mechanisms will lead to the highest inhibitory action against *E. coli* in a solid sludge cake environment. Therefore utilising a number of bench scale experiments the ability of a range of bacteria and undefined mixed cultures to inhibit *E. coli* growth in both solid sludge cake and a liquid sludge derived nutrient broth derived from raw sludge were assessed. These experiments provided a rapid screening and optimisation step, with the premise being if a competitor organism or culture cannot compete with *E. coli* in optimal conditions in terms of nutrient availability it is unlikely to be effective in sludge cake.

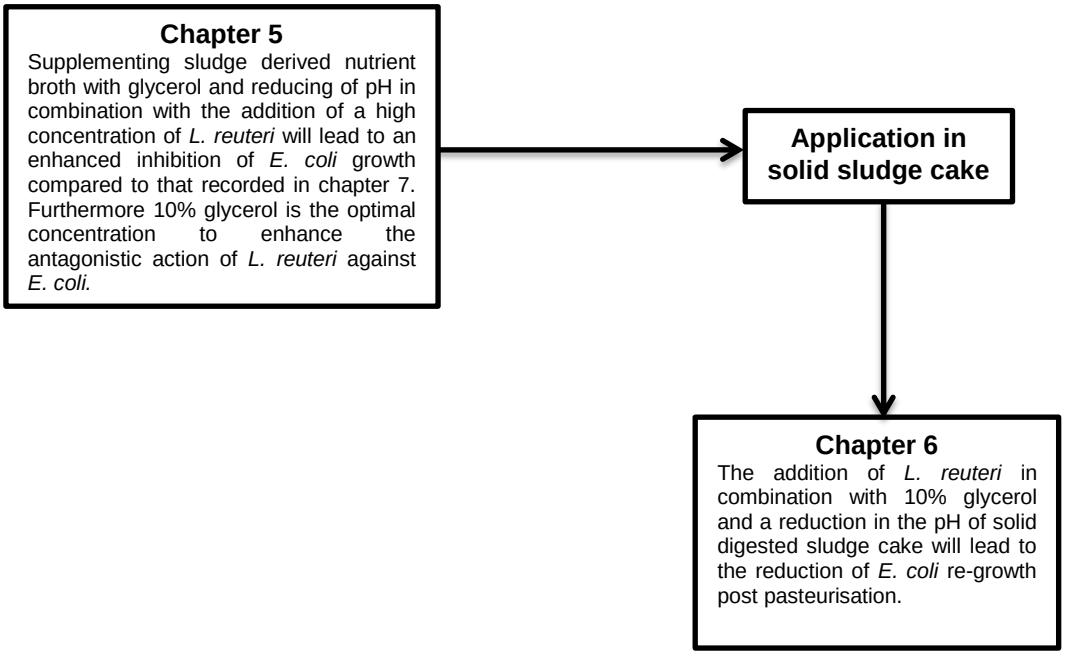
1.7.3 Application in sludge cake

In these experiments the results and conclusions of the previous competition experiments during the screening and optimisation steps were applied in the form of a treatment method to sludge cake post pasteurisation and its effect on *E. coli* re-growth monitored. These experiments determined whether competitive exclusion as a treatment method in sludge cake was feasible. They also highlighted the differences between growth in solid sludge cake and liquid sludge-derived nutrient broth and the effect the growth of the indigenous microbial community contained within cake had on the antagonistic effect of

competitors on *E. coli.* Treatment of sludge cake did pose a challenge in that what can be added and how much liquid was restricted so as to maintain its physical and chemical properties.

A chapter by chapter overview of the experimental hypotheses is displayed below




Chapter 5
Supplementing sludge derived nutrient broth with glycerol and reducing of pH in combination with the addition of a high concentration of L. reuteri will lead to an enhanced inhibition of E. coli growth compared to that recorded in chapter 7. Furthermore 10% glycerol is the optimal concentration to enhance the antagonistic action of L. reuteri against E. coli.
Application in solid sludge cake
Chapter 6
The addition of L. reuteri in combination with 10% glycerol and a reduction in the pH of solid digested sludge cake will lead to the reduction of E. coli re-growth post pasteurisation.

Chapter 2 Preliminary experiments

2.1 Introduction

To investigate the ability of competitor organisms or cultures to compete with *E. coli* and reduce re-growth in digested sludge cake in a laboratory environment at bench scale, two basic methodologies were required. First an accurate and reliable method for the recovery and enumeration of resident *E. coli* in digested sludge cake. Second, a reliable method of generating *E. coli* re-growth in digested sludge cake in the laboratory at bench scale to an extent that the results mirror that which is recorded in industry.

With regards to the recovery and selective isolation of *E. coli* from digested sludge cake there are three main methods used within the wastewater treatment industry (Eccles *et al*., 2004). Two of these methods use membrane filtration techniques, utilising chromogenic *E.coli*/coliform (CEC) media or membrane lactose glucuronide agar (MLGA). The third method however, applies the most probable number (MPN) technique using Colilert in Quantitray 2000 (IDEXX, Westbrook, ME). All these methods are based upon the expression of the enzyme ß-glucuronidase. This enzyme has been reported to be present in over 94% of *E. coli* (Hansen and Yourassowsky, 1984).The methodology for the enumeration and recovery of *E. coli* from sewage sludge cake is reviewed in more detail elsewhere (The Environment Agency, 2003b). In these preliminary experiments only membrane filtration onto MLGA was investigated. The method was selected for its simple procedure and low apparatus and reagent requirements. Furthermore this method is known to provide clear results with the use of selective agar (MLGA) making additional confirmation tests in general unnecessary. To determine the accuracy and repeatability in terms of recovery percentage of *E. coli* from digested sludge cake, controlled replicated bench scale experiments were designed in which sludge samples were sterilised via autoclaving and then spiked with a pure culture of *E. coli*. The *E. coli* were then recovered from the sludge cake via membrane filtration onto MLGA and the recovery rate deduced to determine its

accuracy. These experiments were important in confirming the validity of future data concerning the concentration of *E. coli* recovered from digested sludge cake using membrane filtration onto MLGA as the primary method of recovery.

With regards to establishing a methodology for generating *E. coli* re-growth in digested sludge cake in the laboratory, upon delivery to Cranfield University the *E. coli* concentration of the sludge cake was measured in certain cases as high as 10^5 cfu/gDs. *E. coli* as stated previously (Chapter 1.3) is a good indicator organism for microbial growth in sludge cake. Considering the high concentration of *E. coli* in the sludge cake it was assumed this indicated a high bacterial load. The sludge cake being utilised had already undergone treatment at United Utilities wastewater treatment plant at Ellesmere Port and therefore the concentration of *E. coli* and other micro-organisms would have been expected to be within compliance guidelines (ADAS, 2001). However the sludge cake was not collected immediately following dewatering, and as shown by Figure 2.1, growth of coliforms including *E. coli* in sludge cake at room temperature can occur within hours, leading to as much as a one log increase within the first 8 hours of sampling in that case. It is therefore highly possible that re-growth of the micro-organisms within the cake had begun prior to collection and transport. It is hypothesised that during delivery from Ellesmere Port to Cranfield University re-growth continued leading to the high bacterial load contained within the sludge cake. In these conditions recording an *E. coli* re-growth cycle at bench-scale in the laboratory, never mind the effect of the addition of competing organisms on *E. coli* growth would be improbable. It was hypothesised a process could be applied to significantly reduce the number of all bacteria in the sludge cake including the *E. coli*. By leaving a small number of *E. coli* in the sludge cake with a lot of dead cells it would be expected that *E. coli* numbers would resurge making use of the dead bacteria cytoplasm and any remaining nutrients in the sludge cake. It was hypothesised a pasteurisation process could provide the necessary reduction in bacterial and thereby generate regrowth in the laboratory in a consistent and repeatable way.

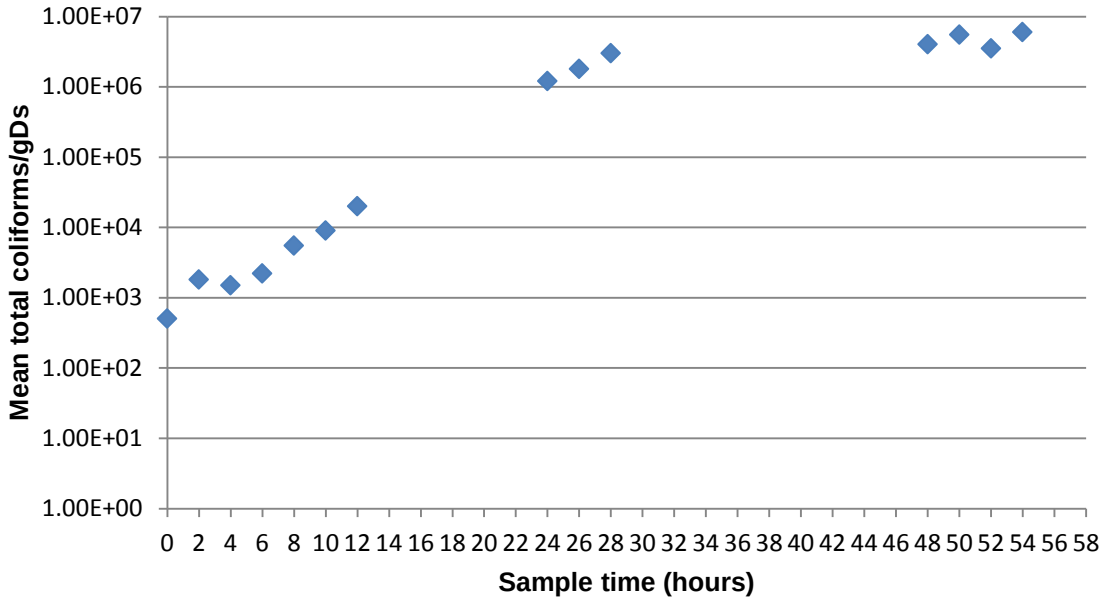


Figure 2.1 – Rate of growth of coliforms in sludge cake at room temperature. (Baddeley, 2010)

Pasteurisation is a process which, unlike sterilisation, is not intended to kill all micro-organisms, but instead aims to reduce the number of viable pathogens, primarily to prevent the transmission of disease. Thermal pasteurisation is the most common method applied and is utilised on occasion for wastewater treatment. In the case of sludge, whilst specific conditions may differ as a result of treatment designs and configurations, generally sludge is heated at a minimum temperature of 70°C for at least 30 minutes or a minimum of 55°C for at least 4 hours (The Environment Agency, 2003a). Appropriate intermediate conditions may also be used. Pasteurisation's application in wastewater treatment is reviewed in more detail at Godfree and Farrell (2005), Hudson and Lowe (1996) and Lundin *et al.* (2004). Though there are a number of alternative methods available to treat the sludge cake in such a way to reduce the bacterial load, in these preliminary experiments only pasteurisation was investigated. It was selected as it is a simple method of reducing the bacterial load of digested sludge cake without affecting the chemical, nutritional and physical properties of the sludge cake. Considering the sludge cake is intended for agricultural use maintaining its properties is an important experimental parameter.

The development of a methodology for achieving *E. coli* re-growth in digested sludge cake in the laboratory at bench scale was two-fold. The principle of the first experiments was to define the ideal conditions for the thermal pasteurisation of sludge cake to effectively reduce its bacterial load to thereby initiate an *E. coli* re-growth cycle and to determine the reliability and consistency of the method. To achieve this, controlled replicated bench scale experiments were performed, in which sludge samples were pasteurised via placement in a dry heat oven set at 62ºC for varying durations of time, with the *E. coli* concentration of the cake measured before and after the process. The temperature of 62ºC was selected as it was found that at temperatures above 70ºC, the *E. coli* within the sludge cake was eliminated in a relatively short duration of time, while at temperatures between 60 and 65ºC the die-off was much more controllable. The principle of the second experiments was to determine whether thermal pasteurisation and reducing the bacterial load of sludge cake would subsequently lead to *E. coli* re-growth. This was achieved via controlled replicated bench scale experiments in which sludge cake was pasteurised using the conditions determined in the first experiment and then subsequently incubated at a set temperature. To re-create conditions the sludge cake would be exposed to in industry, the cake was incubated at 20ºC to simulate the conditions experienced during storage.

It was also hypothesised however, that the initial re-growth of micro-organisms occurring immediately following de-watering may have caused a decrease in the overall nutrient content of the sludge cake. Coupled with the hypothesised reduction in moisture content caused by the pasteurisation process, it was possible any generated re-growth would be restricted, certainly below levels recorded in industry (Figure 2.2). Therefore to increase the nutrients availability, a nutrient broth was added following the pasteurisation process to a designated set of sludge cake samples. So as to maintain consistency in respect to what nutrients the indigenous micro-organisms within the sludge cake would be exposed to, the nutrient broth was derived from raw sludge sourced from the Cranfield wastewater treatment plant. These experiments were important as

they not only proved that generating *E. coli* re-growth in sludge cake was possible at bench scale, they also provided a curve for re-growth in optimum conditions where the only competing organisms were those indigenous to sludge cake.

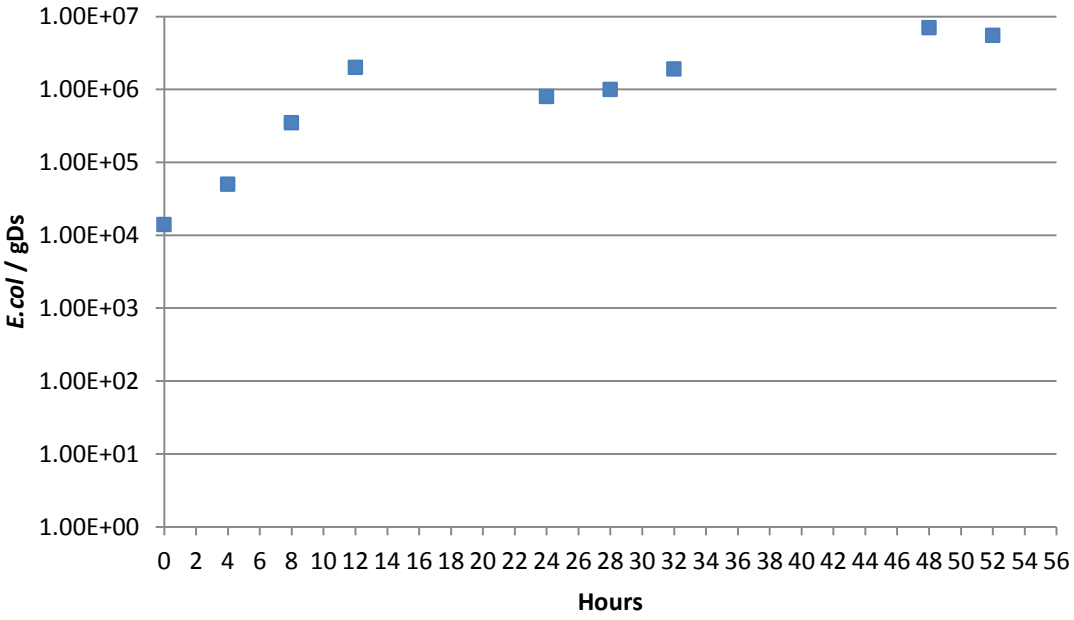


Figure 2.2 – *E. coli* re-growth on site in enhanced treated sludge cake (Sprigings and Le, 2011).

2.2 Materials and methodology

2.2.1 Recovery and enumeration of E. coli from digested sludge cake

2.2.1.1 Strains, culture media and growth conditions

Escherichia coli was recovered and isolated from digested sludge cake provided by United Utilities and sourced from Ellesmere Port and grown and maintained at 37°C on tryptone soya agar (TSA; Oxoid CM131) slopes. TSA was prepared following the manufacturer's instructions and autoclaved at 121°C for 20 minutes prior to use.

2.2.1.2 Collection and storage of digested sludge cake

Digested sludge cake was collected from the outlet of the United Utilities' sludge centrifuge at the Ellesmere Port wastewater treatment plant and dispatched to Cranfield University via courier contained within a refrigerated box and subsequently stored at 5 ºC upon delivery. Though the supplier of the sludge cake and source remained constant throughout the study, deliveries were limited to 5kg batches. This means that the sludge cake was not constant throughout in terms of nutritional, physical and microbial composition, due to the use of different batches in different experiments. The concentration of *E. coli* contained within the sludge cake was recorded upon delivery and 48 hours prior to every experiment.

2.2.1.3 Preparation of inocula

E. coli was grown up overnight in 100ml of tryptone soya broth (TSB; Oxoid CM129) in a Duran bottle and incubated at 37°C under constant shaking at 150rpm. The cells were then harvested by centrifugation at 755g for 10 minutes. The supernatant was removed and the cells subsequently re-suspended in 10ml maximum recovery diluent (MRD; Oxoid CM0733). The optical density (O.D) at 600nm of this suspension was then recorded and standardised at an O.D of 1.7 using sterile MRD. At this optical density from plate counts previously performed it is known that the concentration of the *E. coli* suspension is between 10^7 and 10^8 cfu/ml. Following this the suspensions were 10 fold serially diluted in MRD to a degree of 10^{-8}.

2.2.1.4 Preparation and inoculation of digested sludge cake

Firstly the digested sludge cake was broken down manually to ensure an even particle size was achieved. The sludge cake was then divided into 5g sub-samples (wet weight) and placed in separate 30ml universal bottles (Figure 2.3) and sterilized by autoclaving at 121ºC for 30 minutes. The sludge cake samples were then inoculated with 1ml of the 10^{-4} and 10^{-5} (chosen due to the higher likelihood of providing readable counts) *E. coli* inocula dilutions prepared earlier and vortexed for 1 minute to ensure even distribution of the *E. coli* within the

sludge cake. This was done in triplicate. To determine the concentration of the *E. coli* inoculum, 100µl of dilutions 10^{-6}, 10^{-7} and 10^{-8} of the original *E. coli* suspension were plated onto TSA in triplicate. After incubation at 37ºC for 24 hours, colonies were counted.

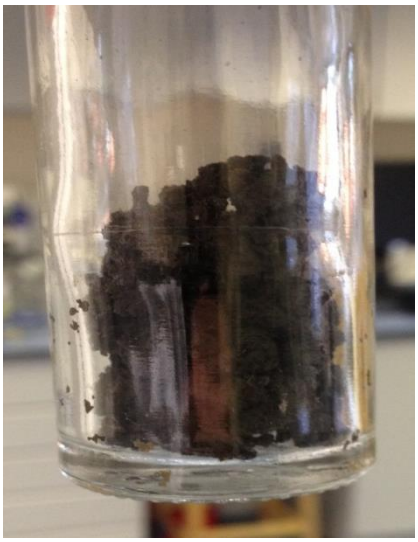


Figure 2.3 – 5g sub sample of sludge cake in a universal bottle following manual break down to ensure even particle size.

2.2.1.5 Recovery of E. coli from digested sludge cake

Immediately following inoculation, 10ml of MRD was added to the universals (Figure 2.4) and vortexed for 1 minute. A negative control was also prepared, in which the digested sludge cake was only autoclaved and not inoculated to ensure the autoclaving process was successful in sterilising the sludge cake.

Figure 2.4 – The three bottles to the left show the 5g samples of sludge cake in a universal bottles following the addition of 10 ml MRD prior to vortexing. The four bottles to the right are preparation for serial dilution.

2.2.1.6 Enumeration of E. coli via membrane filtration

1ml of sample was removed from the universal bottle and 10-fold serially diluted in MRD. 1ml of each dilution was then filtered through a 0.45µm cellulose acetate filter (Eccles et al., 2004; Sartory and Howard, 1992). Due to the volume of the inocula to be filtered being below 10ml, additional MRD was added to the funnel to aid the dispersion of the bacteria over the entire surface of the membrane filter during the filtration process. Following filtration, the filter was placed onto membrane lactose glucuronide agar (MLGA; Oxoid CM1031) and incubated at 30°C for 4 hours and then transferred to 37°C for a further 14 hours. This was performed in triplicate for each time point. The colonies were then enumerated with all green colonies counted and considered as presumptive *E. coli*. Figure 2.5 shows examples of MLGA plates after incubation following the membrane filtration of samples derived from sludge cake.

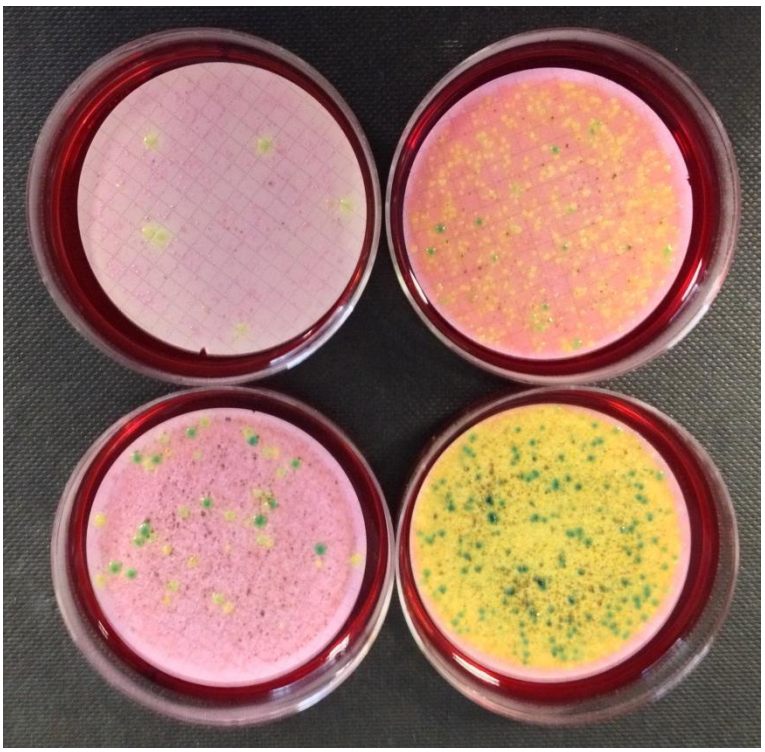


Figure 2.5 - MLGA plates after incubation following the membrane filtration of samples derived from sludge cake. Green colonies represent E. coli, yellow colonies represent faecal coliforms and pinks colonies represent non-lactose fermenters.

2.2.2 Bench scale pasteurisation of digested sludge cake

2.2.2.1 Preparation and pasteurisation of digested sludge cake

Firstly the digested sludge cake was broken down manually to ensure an even particle size was achieved. The sludge cake was then divided into 5g sub-samples and placed in separate 30ml universal bottles, this was done in triplicate so each time point had three samples each to be analysed. The cake was then heated in a dry heat oven at 62ºC.

2.2.2.2 Sampling

Samples were removed from the oven every 15 minutes for one hour, and 10ml of maximum recovery diluent (MRD; Oxoid CM0733) was added to the universals and then vortexed for one minute. The procedure for the enumeration of *E. coli* via membrane filtration reviewed in section 2.2.1.6 was then performed. A sample was also analysed prior to heating (i.e. time 0 hours) to act as a control. The moisture content of the digested sludge cake was established via heating a 5g sample at 121ºC for 24 hours and measuring the percentage weight difference.

2.2.3 Generating Escherichia coli re-growth in digested sludge cake at bench-scale in the laboratory

2.2.3.1 Preparation of sludge derived nutrient broth

Raw sludge from Cranfield University's sewage treatment works was first stirred to break up the biomass and ensure nutrients would be released into suspension and then centrifuged at 84g for one minute to remove the heavier biomass. The supernatant was poured off and then autoclaved at 121ºC for 15 minutes to ensure sterility.

2.2.3.2 Preparation and pasteurisation of digested sludge cake

Firstly the digested sludge cake was broken down manually to ensure an even particle size was achieved. The sludge cake was then divided into 5g sub-

samples and placed in separate 30ml universal bottles, this was done in triplicate so each time point had three samples each to be analysed. Using the conditions determined in the previous experiment regarding pasteurisation the cake was then heated in a dry heat oven at 62ºC for 45 minutes. In the first experiment immediately following pasteurisation the digested sludge cake samples were incubated at 20°C. In the second experiment immediately following pasteurisation 1ml of sludge derived nutrient broth was added to half the samples and 1ml of 50% sludge derived nutrient broth (10ml MRD and 10ml sludge derived nutrient broth) added to the other half, they were both then incubated at 30ºC. The incubation temperature was raised from 20ºC to more accurately mirror the conditions during storage especially during the summer months, as it was discovered that on site storage temperatures of sludge cake can reach up to and above 30ºC (Sprigings and Le, 2011). One set of samples post pasteurisation was not inoculated with sludge derived nutrient broth to act as a negative control and one set was inoculated with 1ml of sterile MRD to determine if any effect was caused by the addition of nutrients or moisture. These were then incubated under the same conditions.

2.2.3.3 Sampling

A universal bottle containing a 5g sample of sludge cake was taken immediately after the pasteurisation process and 10ml of maximum recovery diluent (MRD; Oxoid CM0733) was added to the universal and then vortexed for one minute. The procedure for the enumeration of *E. coli* via membrane filtration presented in section 2.2.1.6 was then performed. This was repeated every 6 hours for 24 hours and then every 12 hours for the subsequent 24 hours. A sample was also analysed prior to pasteurisation to determine how effective the pasteurisation process was. The moisture content of the digested sludge cake was also established via heating a 5g sample at 121ºC for 24 hours and measuring the percentage weight difference.

2.2.3.4 Statistical analysis

All data was input into Microsoft Excel. An F-test was performed to determine variance and then a t-test to determine statistical differences between data sets. A p value below 0.05 resulted in the rejection of the null hypothesis. Graphs were generated by converting the data into log numbers and inputting into JMP statistical software (SAS). An adjusted form of the Baranyi-Roberts equation (Baranyi and Roberts, 1994) devised by Miconnet *et al.* (2005) was utilised to fit curves to the data. Only curves with a root-mean-square error (RMSE) of below 0.5 were accepted. This modified equation was used because it allows for a more accurate representation of the lag phase of growth. Furthermore the Baranyi-Roberts model has proven to be more robust when compared with the alternative, the modified Gompertz equation, when used to fit survival curves, such as those with lag and sigmoidal (Xiong et al., 1999). Additionally when used in conjunction with JMP software the equation provides figures to calculate the growth rate. This equation, however does not allow for the death phase of the bacterial growth curve. Hence, the death phase is not represented in the graphs with a line. All graphs were produced using Microsoft Excel. The mean generation times (g) were calculated using the equations shown in Figure 2.6. N_t was determined from the graphs and the curve of the line and represents the number of *E. coli* at the point where exponential growth ceases. N_0 was also determined from the graphs and the curve of the line and represents the number of *E. coli* at the point in which the lag phase of growth ceases and exponential growth begins. The value of t is dependent upon the incubation time in hours between point N_0 and N_t.

$$g = \frac{(log_{10}N_t - log_{10}N_0)}{log_{10}2}$$

$$\left(\frac{t\ (hours)}{g}\right) \times 60$$

Figure 2.6 – Equations for the calculation of the mean generation times (g) in minutes.

2.3 Results and discussion

2.3.1 Recovery and enumeration of E. coli from digested sludge cake

It was determined from spread plate counts of the original *E. coli* suspensions that 1.14 x 10^4 *E. coli* were added to the sludge cake in the initial experiment, 1.39 x 10^4 in the first repeat and 1.24 x 10^4 in the second repeat (Table 2.1). The average number of *E. coli* recovered in 1ml from the sludge cake was 1.16 x 10^2 in the initial experiment, 1.4 x 10^2 in the first repeat and 1.15 x 10^2 in the second repeat (Table 2.1). This represents a recovery rate of 101.75%, 100.5% and 92.5% in the initial, first repeat and second repeat experiments respectively. The recovery rate was calculated using the equation shown in Figure 2.7. Taking into consideration the overall volume of the sludge cake following the addition of MRD for recovery of *E. coli* was 10ml and the sludge cake/MRD suspension had to be diluted 10 fold (10^{-1}) to allow membrane filtration.

Table 2.1 - Recovery of *E. coli* from digested sludge cake using the membrane filtration technique onto MLGA

Number of *E. coli* added to sludge cake per ml	Number of *E. coli* recovered from sludge cake per ml	Average number of *E. coli* recovered from sludge cake per ml
Initial experiment		
1.14E+04	106	116 ± 11
	113	
	128	
First repeat		
1.39E+04	137	140 ± 14
	156	
	128	
Second repeat		
1.24E+04	108	115 ± 7
	116	
	121	

$$Recovery\ Rate\ (\%) = \left(\frac{\left(\begin{array}{c} Average\ number\ of\ E.coli\ recovered \\ from\ sludge\ cake \\ \times\ Sludge\ cake\ dilution \\ \times\ Volume\ of\ sludge\ cake\ suspension\ (ml) \end{array} \right)}{Number\ of\ E.coli\ added\ to\ sludge\ cake} \right) \times 100$$

Figure 2.7 – Equation for the calculation of the recovery rate of *E. coli* from digested sludge cake using the membrane filtration method.

The high recovery rates calculated in these experiments were unexpected, especially considering only vortexing was used to homogenise and mix the samples when the standard operating procedure states the need for a stomacher (The Environment Agency, 2003b). There are a number of possible causes for the high recovery rates, one of which is contamination. During the first repeat experiment, the negative control tested positive for *E. coli*. This suggests the autoclave process was unsuccessful in sterilising the sludge cake in this case, compromising the validity of the *E. coli* count in this experiment and leading to a high recovery rate. However at no point did any of the negative controls test positive for *E. coli* in the initial and second repeat experiments. This suggests the probability that contamination is the cause of the high recovery rates doubtful. Another possible cause is growth of the *E. coli* following inoculation into the sludge cake. As stated in chapter 1, sludge cake contains numerous nutrients, which is why it is applied to agricultural land; this makes it an ideal growth matrix for micro-organisms. *E. coli* inoculation into sterilised digested sludge cake would undoubtedly lead to unrestricted exponential growth, not only because of the high nutrient availability but also because of the lack of microbial competition, leading to a high *E. coli* recovery. This would also explain the recovery of a higher number of *E. coli* than inoculated into the sludge cake. However the time between inoculation and recovery was restricted to at most a few minutes, making any growth and thereby increases in the recovery of *E. coli* highly unlikely. The most likely cause of the high recovery rates is that the *E. coli* being recovered is not indigenous and therefore ingrained in the cake in such a way that homogenisation is essential for recovery. The sludge cake in these experiments

has been spiked with *E. coli.* Spiking 5g of sterilised sludge cake with a high moisture content (in these experiments consistently around 75%) with 1ml of an *E. coli* suspension, lead to excess inoculum in the universal bottle, due to the cake being saturated. Furthermore the inoculum that was absorbed into the sludge cake will only have been on the surface, these factors combined means the vortexing procedure will have been sufficient to release the majority if not all the *E. coli* inoculated into suspension allowing a high *E. coli* recovery. It would be expected in future experiments, where the sludge cake is not spiked and vortexing is the sole method of homogenisation and mixing, the recovery rate would be significantly lower.

Regardless the results from the initial and second repeat experiments do confirm that membrane filtration onto MLGA is a consistent and accurate means of recovering and enumerating *E. coli* from digested sludge cake, these findings match those of Eccles *et al.* (2004) and Sartory and Howard (1992). With regards to the other methods not tested Eccles *et al.* (2004) found that all three methods gave comparable recoveries and results did not vary by greater than one order of magnitude (1 log). However Eccles *et al.* (2004) found membrane filtration onto MLGA occasionally gave lower counts compared to the other methods. This was attributed to MLGA providing slightly lower presumptive results. Sartory and Howard (1992) reasoned that the green colouration by *E. coli* can be suppressed when the membrane has high numbers of non-target organisms present. This was not investigated during these experiments; however this effect can be nullified by sample dilution to a range of between 20 to 70 target colonies, making accurate dilution key to this technique.

2.3.2 Bench scale pasteurisation of digested sludge cake

For pasteurisation to be deemed an acceptable method for the reduction in the bacterial load of sludge cake, the method had to be capable of consistently reducing the concentration of *E. coli* within the sludge cake below 10^3 cfu/gDs. This was to ensure conditions in the sludge cake in terms of microbial growth were as close to what is recorded in industry as possible. Enhanced treated

sludge cake to be applicable for disposal to land is only permitted to contain an upper limit of 10^3 *E. coli* per gram dry weight of sludge (The Environment Agency, 2003a). Results from the pasteurisation of digested sludge cake (Figure 2.8), show that an exposure time of at least 30 minutes at 62°C, had little to no effect on the viability of the resident *E. coli* within the sludge cake. While an exposure time of 45 minutes at the same temperature caused a near 3 log reduction in the concentration of *E. coli* recovered, reducing it below the 10^3 cfu/gDs threshold previously stated. However 60 minutes eliminated all resident *E. coli* within the sludge cake with no viable count found from any of the samples. Furthermore there was very little variability between the repeats, confirming the pasteurisation method is consistent, regardless of exposure time.

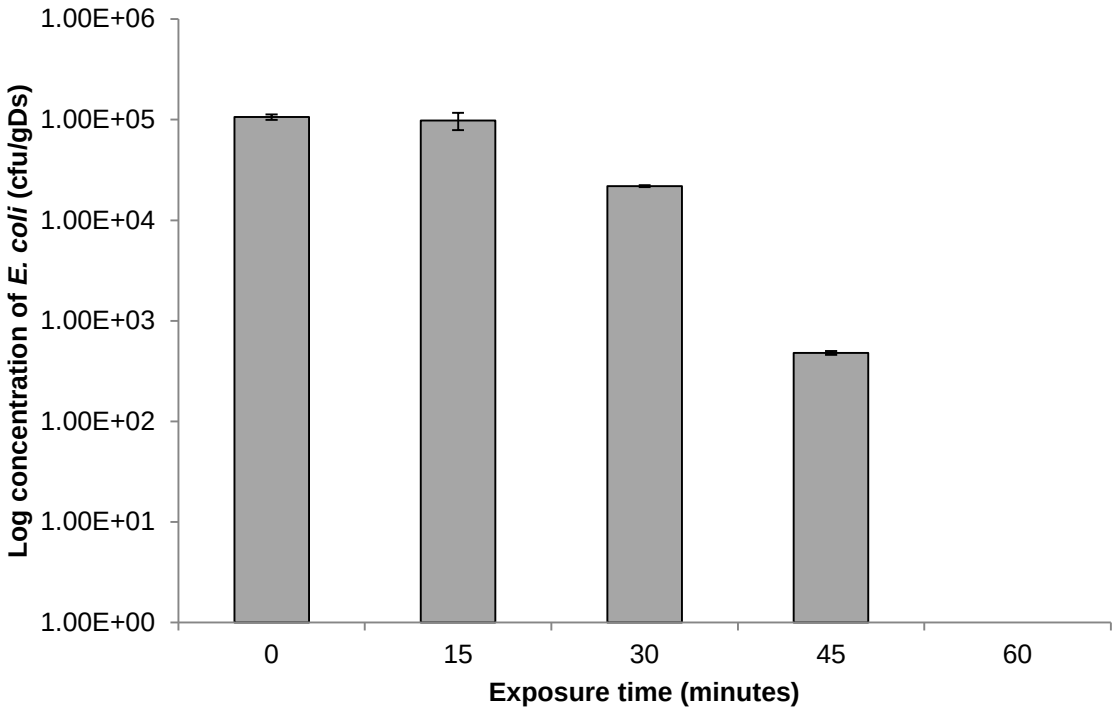


Figure 2.8 - Results for the enumeration of *E. coli* derived from digested sludge cake using the membrane filtration technique following pasteurisation at 62°C for between 0 and 60 minutes. The log concentration of *E. coli* was calculated from the averages of plate counts, the error bars represent the standard deviation.

However in the final two exposure times (45 and 60 minutes) there was a significant drying of the cake, with a between 5 and 30% drop in moisture recorded. As no investigation into growth after pasteurisation was performed at

this stage it is unknown what the impact this reduction in moisture content could have on growth of the indigenous microbial community and in particular *E. coli*. It is also unknown if the heat led to other changes in the physical or nutritional properties of the sludge cake which could also hamper microbial growth. Despite this at this stage only an exposure time of 45 minutes at 62°C showed the capability of consistently reducing the concentration of *E. coli* contained within sludge cake below 10^3 cfu/gDs. Therefore these were the conditions used in future experiments to pasteurise sludge cake to reduce the bacterial load.

2.3.3 Escherichia coli re-growth in digested sludge cake at bench-scale in the laboratory

2.3.3.1 Re-growth with no nutrient addition

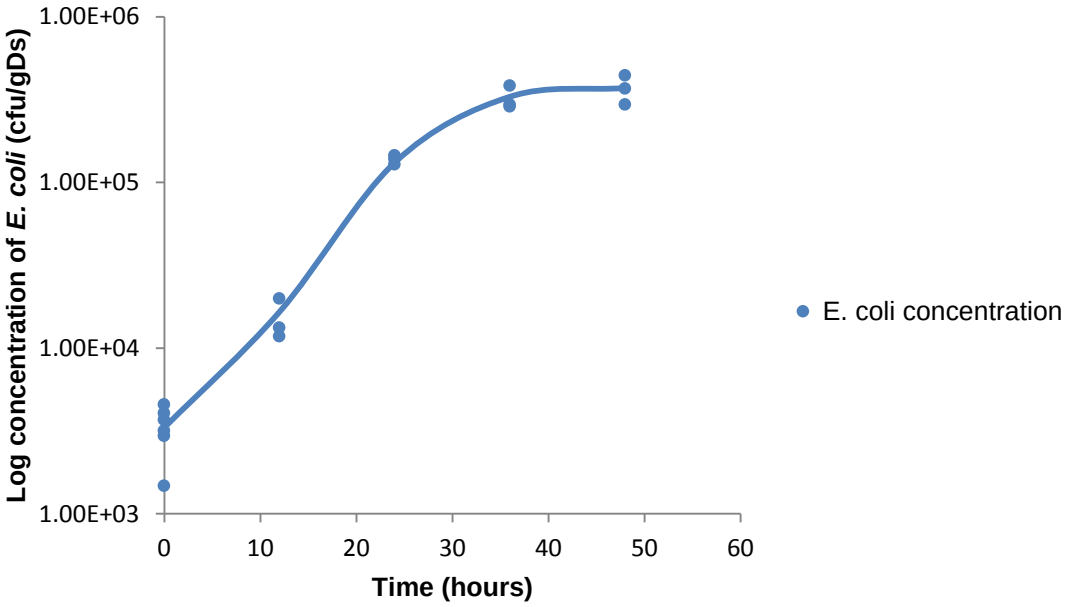


Figure 2.9 - Enumeration of *E. coli* in digested sludge cake incubated at 20°C following pasteurisation at 62°C for 45 minutes using the membrane filtration technique. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

As shown in Figure 2.9 *E. coli* regrowth in cake was generated following pasteurisation in the laboratory, with a 2 log increase recorded. This is a similar result to that reported by Qi *et al.* (2004) in digested sludge samples immediately following centrifugation. However in this experiment, it took *E. coli*

48 hours to achieve this level of growth, and even then the highest concentration of *E. coli* recorded was only $4.41x10^5$ cfu/gDs. Furthermore the mean generation time was calculated to be 326 minutes. Comparing this result to what is documented in industry, Baddeley (2010) recorded a 3 log increase in *E. coli* concentration within 24 hours in class A biosolids following dewatering and storage, with *E. coli* reaching a high of just under $1x10^7$ cfu/gDs after 48 hours. Additionally, Sprigings and Le (2011), recorded a similar level of *E. coli* re-growth on site at a wastewater treatment facility in enhanced treated sludge cake (Figure 2.2), from these results it was estimated the mean generation time to be 101 minutes, 3 times faster than that recorded in Figure 2.9. Although in this case environmental temperatures were much higher than the incubation temperature of 20ºC used in this experiment, therefore it is unsurprising a faster growth rate was recorded. Overall it is clear from these results that not only the growth rate of *E. coli* but also the amount of re-growth generated after 48 hours in this experiment is much reduced when compared to the levels recorded within industry. Therefore the methodology at this stage is unsuitable for future competitive exclusion experiments due to insufficient amount of *E. coli* generated within 48 hours and the slow growth rate

2.3.3.2 Re-growth with nutrient addition

As shown by Figure 2.10, the addition of nutrient to the digested sludge cake post pasteurisation resulted in a reduction in lag times, increase in growth rate and a significant increase ($p \leq 0.05$) in *E. coli* growth when compared to the sludge cake without nutrient addition. The addition of undiluted nutrient lead to an around 3 log increase recorded after 18 hours and a mean generation time of 89 minutes, this is a similar value to that estimated for Figure 2.2. In comparison, with no nutrient addition only an around 1 log increase was documented within the same time frame and the mean generation time was calculated to be 184 minutes. This is a similar to result to that documented in the first experiment (Figure 2.9), in terms of the maximum concentration of *E. coli* recorded, however the growth was significantly reduced by almost a factor

of 2. This is most likely due to the 10ºC increase in incubation temperature between experiments.

There was no significant difference ($p > 0.05$) in *E. coli* growth between sludge cake supplemented with 1ml undiluted nutrient or 1ml of half fold diluted nutrient. This suggests that a higher concentration of nutrient had little to no effect as the *E. coli* within the sludge cake was growing at or near its maximal rate regardless. Although the use of half fold diluted nutrient, did minimally increase the mean generation time to 94 minutes, 5 minutes more than in cake supplemented with undiluted nutrient. Furthermore the addition of a higher nutrient concentration did result in a marginally higher final concentration of *E. coli*; this is most likely due to the sludge cake being able to support more growth due to the excess nutrient availability.

Without nutrient addition a 10°C increase in incubation temperature still resulted in reduced overall growth. This suggests the *E. coli* is at its maximal growth rate in the conditions of the unaltered cake, validating the hypothesis that the sludge cake was nutritionally deficient, most likely as a result of the previous microbial re-growth cycle. This is further supported by the significant increase in *E. coli* growth following nutrient addition. Additionally re-wetting the sludge cake with MRD had no significant effect on growth ($p > 0.05$). This confirms that the reduction in moisture content caused by the pasteurisation process does not hinder *E. coli* growth and is not the source of the reduced growth.

Overall these experiments have shown that it is possible to generate *E. coli* re-growth in digested sludge cake in the laboratory at bench scale; however the supplementation of additional nutrient is required to achieve similar results to that which is recorded in industry.

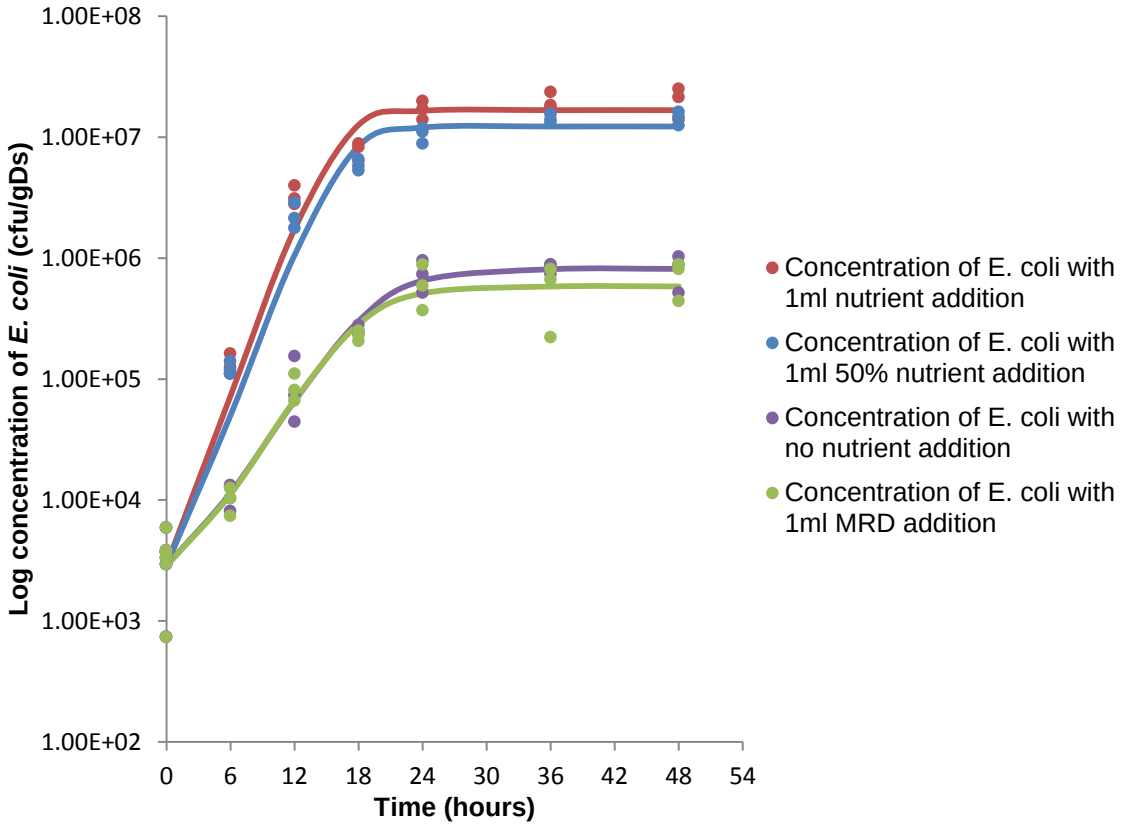


Figure 2.10 - Enumeration of *E. coli* in digested sludge cake incubated at 30°C following pasteurisation at 62°C for 45 minutes and addition of a nutrient broth derived from raw sludge using the membrane filtration technique. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

2.4 Conclusions

- Membrane filtration onto MLGA is a consistent and accurate method of recovering and enumerating *E. coli* from digested sludge cake. Additionally the method has a simple procedure, low apparatus and reagent requirements and provides clear results with the use of selective agar (MLGA) making additional confirmation tests in general unnecessary. Therefore membrane filtration onto MLGA will be the method of choice for the recovery and enumeration of *E. coli* from digested sludge cake in future experiments.

- Reviewing the literature there are no large differences in terms of accuracy of enumeration between the three suggested methods of membrane filtration onto either CEC media or MLGA and utilising the

MPN technique. Furthermore though not tested during these experiments, MLGA can occasionally provide lower counts if in presence of a high concentration of non-target organisms, making proper dilution key to this technique, especially considering the high microbial diversity of sludge cake.

- During the recovery and enumeration of *E. coli,* higher than expected recovery rates were recorded. This is likely attributed to the spiking of sludge cake with a high moisture content with *E. coli* making the recovery simpler as homogenisation and mixing is not as essential in non-spiked sludge cake. It is anticipated in future experiment where vortexing is the sole method of homogenisation and mixing prior to recovery, the rates will be significantly lower.

- Thermal pasteurisation at bench scale using a dry heat oven is an acceptable method for reducing the bacterial load in digested sludge cake. Heating of the digested sludge cake to 62°C for 45 minutes was found to be capable of consistently reducing the *E. coli* concentration below the 10^3 cfu/gDs threshold. However under these conditions the sludge cake experienced a between 5 and 30% reduction in moisture content.

- It is possible to generate *E. coli* re-growth in digested sludge cake in the laboratory at bench scale; however the supplementation of additional nutrient post pasteurisation and incubation at 30ºC is required to achieve similar results to that which is recorded in industry. This was shown in the mean generation times. Using the results from Sprigings and Le (2011) shown in Figure 2.2 it was estimated the mean generation time of *E. coli* in industry in enhanced treated sludge cake on that particular site was 101 minutes. This is a similar result to that recorded for sludge cake supplemented with undiluted nutrient (89 minutes), while sludge cake with no nutrient addition had a much higher mean generation time (184

minutes). Furthermore with the addition of nutrient broth, the *E. coli* concentration within cake was measured at over $1x10^7$ cfu/gDs within 24 hours; this is once again a similar result to what is recorded in industry. In comparison without nutrient broth addition, *E. coli* concentration only ever reached a maximum of around $5x10^5$ cfu/gDs within the same timeframe.

- There was no significant difference between addition of 1ml undiluted nutrient broth and 1ml of half fold diluted nutrient broth, in both cases addition led to an increased level of *E. coli* growth when compared to the sludge cake with no nutrient addition. This suggests that a higher concentration of nutrient had little to no effect as the *E. coli* within the sludge cake was growing at or near its maximal rate regardless.

- Addition of 1ml MRD had no effect on the growth of *E. coli* in sludge cake, confirming that the reduction in moisture content caused by the pasteurisation process does not hinder *E. coli* growth. Furthermore it validates the hypothesis that the increased availability of nutrients and not the addition of moisture is the cause of the increased *E. coli* in sludge cake growth following supplementation with nutrient broth.

- Without nutrient addition a 10°C increase in incubation temperature still resulted in reduced overall *E. coli* growth within the sludge cake, only when nutrients were introduced did it increase. This suggests the *E. coli* is at its maximal growth rate in the conditions of the unaltered cake, validating the hypothesis that the sludge cake was nutritionally deficient.

Section 2

Section 2: Proof of concept

Introduction

In industry it has been shown that *E. coli* in sludge cake is capable of a 2 log increase in a 12 hour period (Figure 2.2), at bench scale a 3 log increase was recorded in the same time frame (Figure 2.10). This rapid growth rate combined with a number of other variables including the nutrient content of and high biodiversity of sludge cake makes the development of a competitive exclusion treatment extremely difficult. Furthermore the experimental parameter of not adversely changing the physical and/or chemical properties of sludge cake due to its land applications restricts what can be added and in what volume. The following chapters detail the experiments setup to develop and optimise such a method for use in digested sludge cake.

Chapters 3 and 4 focus on the initial screening process for candidate organisms and cultures capable of competing with *E. coli,* thereby inhibiting its growth. Initially the emphasis was on utilising undefined mixed cultures such as soil, the hypothesis being that the addition of a high concentration, diverse microbial culture to digested sludge cake would result in the reduction of *E. coli* re-growth via most likely direct nutrient competition. Following on from this the possible use of single candidate organisms capable of producing antimicrobials or alternatively those with a rapid growth rate capable of directly competing with *E.* coli for nutrients were also analysed as individuals and combined in defined mixed cultures. These experiments were important in proving the concept of competitive exclusion as a means to reduce *E. coli* growth in a sludge cake environment, and in highlighting promising candidates for optimisation.

Chapters 5 focuses on the optimisation of the competitive exclusion treatment process using the candidate organisms identified during the screening process. There were two defined methods of optimisation utilised. First it was hypothesised that by changing the sludge cake environment to suit the growth of the competitor organisms, for example by reducing the pH it would increase

the ability of the organisms to compete with *E. coli* and thereby increase any reduction in growth recorded in previous experiments. Second it was hypothesised that the addition of precursor compounds to antimicrobials generated by the competitor organisms would increase their production thereby increasing the inhibition of *E. coli* growth in sludge cake. These experiments were important in maximising any effect the competitor organisms had on *E. coli* growth, and understanding their mechanism of action.

Chapter 3 Competitive exclusion of *Escherichia coli* re-growth in digested sludge cake using high diversity cultures

3.1 Introduction

The competitive exclusion principle also known as Gause's Law (Gause, 2003), contends that to coexist in a stable environment two competing species must differ in their respective ecological niche; without differentiation, one species will eliminate or exclude the other through competition. Throughout nature, there are a large number of well-studied examples of populations which are held in balance, or driven to transition, by competitive forces, a classic example within bacterial populations is within the gastrointestinal tract. The indigenous microflora of the gastrointestinal tract inhibit exogenous pathogenic colonisation by creating a barrier effect and occupying available adhesion sites at the mucosal layer, competing for metabolic substrates and producing regulatory factors such as short-chain fatty acids and bacteriocins (Hao and Lee, 2004).

Competitive exclusion as a treatment method to control the concentration of pathogens has been utilised in a number of industries from the production of silage (Driehuis and Elferink, 2000; Wilkinson *et al*., 1996) to preventing nosocomial infectious diarrhoea in hospitals (D'Souza *et al*., 2002; Plummer *et al*., 2010). In the poultry industry competitive exclusion treatment is used primarily, as a prophylactic measure that is aimed at increasing the resistance of chicks or turkey poults to salmonella infection by the administration of an oral preparation from a pathogen-free adult bird (Mead, 2000; Nurmi and Rantala, 1973).

Currently for poultry several competitive exclusion treatment products are commercially available, including Aviguard (Bayer) and Mucosal Starter Culture (MSC; Wayne Farms LLC, Oakwood, Ga.). Both of these products are mixed cultures derived from the caecal contents and/or wall of domestic fowl (Schneitz, 2005), and have shown to be effective in both laboratory and field trials for controlling *Salmonella* in chickens. For example, Ferreira *et al.* (2003) showed that the administration of Aviguard or MSC, protected chicks from the specific challenge of *Salmonella* Kedougou, as evidenced by the statistically significant reduction ($p < 0.05$) in chicks that tested positive for *Salmonella* (7.3% for MSC and 18.2% for Aviguard) and by the low levels of caecal carriage observed. Competitive exclusion treatments using mixed cultures have also been shown to be effective against other pathogens other than *Salmonella*. Schoeni and Doyle (1992); showed that the addition of a nine-strain mixture of caecal bacteria provided from 41 to 85% protection from *Campylobacter jejuni* colonisation. In another study by Hakkinen and Schneitz (1996) protection against both avian pathogenic *E. coli* and human pathogenic *E. coli* O157:H7 was obtained in chickens by administering Broilact. Broilact like MSC and Aviguard is a competitive exclusion product defined as mixed culture derived from the caecal contents and scrapings of the caecal wall of a healthy adult hen (Schneitz, 2005). The precise mechanism of the protective effect of these products is unknown, and may never be determined because of the complexity of the gut as a habitat for micro-organisms and the variety of host–microbe and microbe–microbe interactions that can occur (Rolfe, 1991). However several theories have been proposed, including competition for (unspecified) receptor sites within the gut (Soerjadi *et al*., 1981; Soerjadi *et al*., 1982), production of antimicrobials such as volatile fatty acids (Barnes *et al*., 1979; Corrier *et al*., 1995a; Corrier *et al*., 1995b; Mead, 2000; Nisbet *et al*., 1993) and finally competition for limiting nutrients (Ha *et al*., 1994).

It was hypothesised that by applying the methodology used within the poultry industry for competitive exclusion treatment, in terms of the addition of a carefully selected mixed culture, a similar protective effect against *E. coli* growth

could be obtained in sludge cake. This hypothesis was tested by separately adding two competitor cultures derived from soil and a commercially available fermented milk product in high concentration to digested sludge cake post pasteurisation. Soil was selected as a source for competitor organisms due to its large microbial biomass. It has been calculated that in temperate grassland soil the bacterial and fungal biomass amounted to 1–2 and 2–5 t ha^{-1}, respectively (Killham, 1994). In addition soil is known to contain a high level of microbial diversity; Torsvik *et al.* (1996) calculated the presence of about 6000 different bacterial genomes per gram of soil by taking the genome size of *E. coli* as a unit. It was hypothesised that this high biodiversity would increase the probability of introducing an organism or group of organisms capable of competing with *E. coli* within sludge cake either via direct nutrient competition or via production of antimicrobials. The fermented milk product was chosen on the other hand due to it being stated to contain a number of lactobacilli including *Lactobacillus casei* in high concentration. In the poultry industry as stated previously a large proportion of mixed cultures utilised in competitive exclusion treatments are sourced from the microflora of the intestinal tract of healthy chickens. It was therefore hypothesised that the highest probability of success was via using cultures of organisms that are normal inhabitants of the intestinal tract, such as lactobacilli. Furthermore lactobacilli are known to possess anti-microbial activity, with the primary antimicrobial effect of lactobacilli exerted via the production of lactic acid and reduction of pH (Daeschel, 1989). In addition lactobacilli can produce various antimicrobial compounds, such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione) and bacteriocins (Ouwehand and Vesterlund, 2004; Piard and Desmazeaud, 1991; Piard and Desmazeaud, 1992). Additionally previous competitive exclusion experiments using lactic acid bacteria have proven successful albeit in cattle (Brashears *et al*., 2003) and turkeys (Milbradt *et al*., 2014). These factors make lactobacilli ideal candidate organisms for competitive exclusion treatment.

3.2 Materials and methodology

3.2.1 Collection and storage of digested sludge cake.

Digested sludge cake was collected from the outlet of the United Utilities' sludge centrifuge at the Ellesmere Port wastewater treatment plant and dispatched to Cranfield University via courier contained within a refrigerated box and subsequently stored at 5 ºC upon delivery. The concentration of *E. coli* contained within the sludge cake was recorded 48 hours prior to the experiment, to ensure there as a sufficient amount (above 10^3 cfu/gDs) for an experiment to be performed.

3.2.2 Preparation of sludge derived nutrient broth

Raw sludge from Cranfield University's sewage treatment works was first stirred to break up the biomass and ensure nutrients would be released into suspension and then centrifuged at 84g for one minute to remove the heavier biomass. The supernatant was poured off and then autoclaved at 121ºC for 15 minutes to ensure sterility.

3.2.3 Preparation of soil and fermented milk product inoculums

In the initial experiment 50g of soil was collected from a location on Cranfield University campus and put through a fine sieve to ensure even particle size. Following this 100ml of maximum recovery diluent (MRD; Oxoid CM0733) was added and the solution vigorously stirred until the soil was in complete suspension. In a previous chapter (Chapter 2.3.3.2) it was concluded the addition of sludge derived nutrient broth to sludge cake was required to generate an acceptable amount of re-growth within 48 hours, due to a hypothesised nutrient deficiency within the sludge cake. Therefore to support the growth of *E. coli* and the competitor cultures, 10ml of the soil suspension and 10ml of a commercially available fermented milk product were removed and separately combined with 10ml of sludge derived nutrient in separate 30ml universal bottles and vortexed for 1 minute. In the second experiment, the inoculum was concentrated so that only the bacterial portion of the fermented

milk product was added to the sludge cake. This was achieved by isolating the cells from the fermented milk product via centrifugation at 755g for 10 minutes. The supernatant was removed and the cells washed via re-suspension in 10ml MRD. This suspension was then centrifuged again under the same conditions. The supernatant was once again removed and the cells re-suspended in 10ml MRD. This was then combined with 10ml of sludge derived nutrient in a 30ml universal bottle and vortexed for 1 minute. The soil inoculum was not used in the second experiment.

3.2.4 Preparation and pasteurisation of digested sludge cake and addition of nutrient and competitor cultures

Firstly the digested sludge cake was broken down manually to ensure an even particle size was achieved. The sludge cake was then divided into 5g sub-samples and placed in separate 30ml universal bottles. The sludge cake was then heated in a dry heat oven at 62ºC for 45 minutes. In the first experiment immediately following pasteurisation 1ml of the soil inoculum was added to half the samples and 1ml of the fermented milk product inoculum was added to the others, they were both then incubated at 30ºC. To determine the effect the competitor cultures had on *E. coli* growth one set of samples post pasteurisation was inoculated with 1ml of a 1:1 dilution of sludge derived nutrient to act as a positive control. To determine the effect of adding 1ml of moisture and to confirm the addition of nutrient was once again required to achieve re-growth as in the previous chapter (Chapter 2.3.3.2) 1ml of sterile MRD was added to another set of samples post pasteurisation to act as a negative control. Both of these controls were then incubated under the same conditions. In the second experiment immediately following pasteurisation 1ml of the bacterial culture derived from the fermented milk product inoculum was added to the sludge cake sample and then incubated at 30ºC. One set of samples post pasteurisation was inoculated with 1ml of sterile MRD and another inoculated with 1ml of a 1:1 dilution of sludge derived nutrient and MRD to act as a controls, these were then incubated under the same conditions. This segment of the methodology is summarised in Figure 3.1.

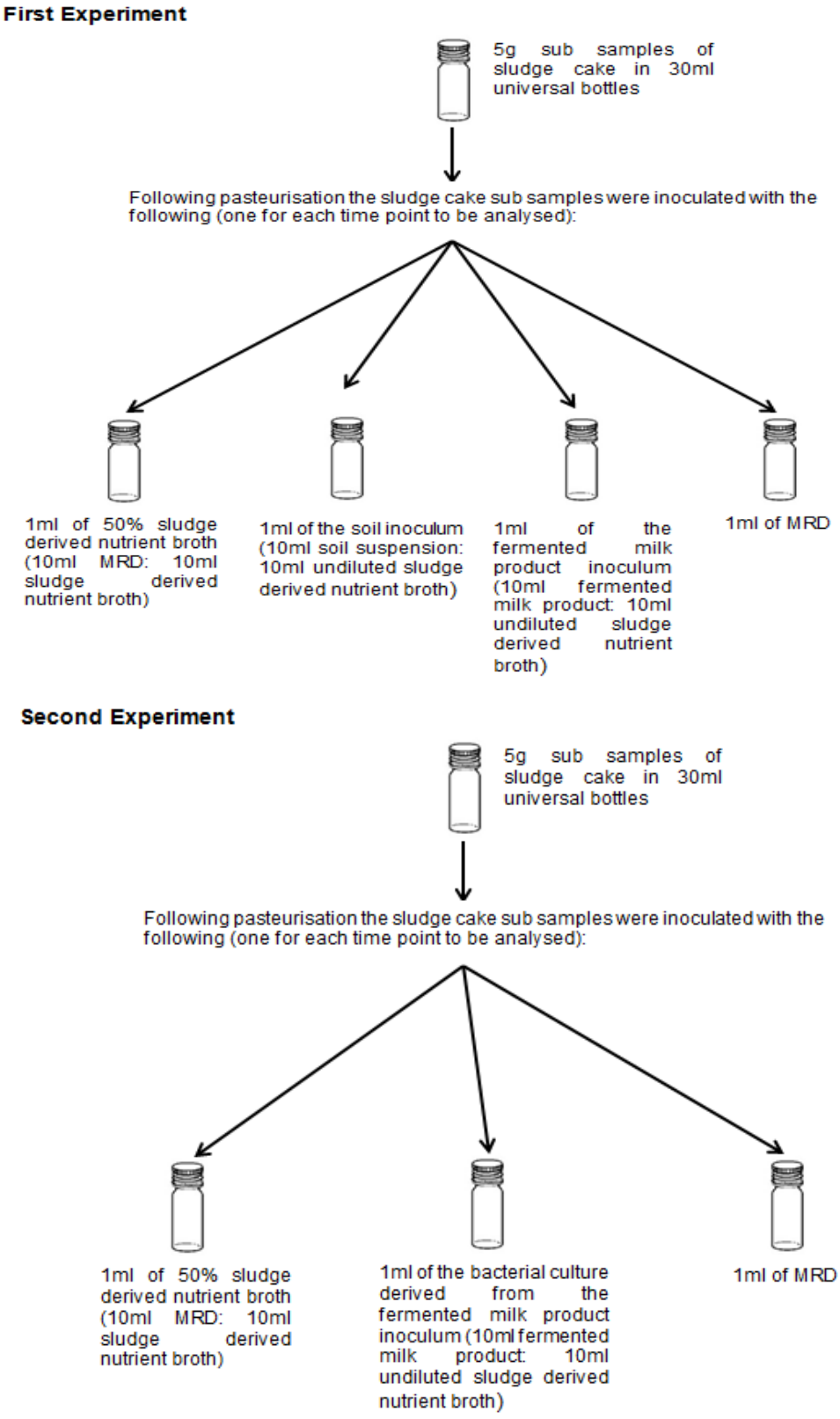


Figure 3.1 – Diagram explaining the inoculation of sludge cake post pasteurisation with competitor cultures and the controls, described in Chapter 3.2.4

3.2.5 Sampling and enumeration of E. coli via membrane filtration

A sample of the sludge cake was taken immediately after the pasteurisation process and 10ml of maximum recovery diluent (MRD; Oxoid CM0733) was added to the universal and then vortexed for one minute. 1ml of the sludge cake/MRD suspension was then removed from the universal bottle and 10-fold serially diluted in MRD. 1ml of each dilution was then filtered through a 0.45µm cellulose acetate filter (Eccles et al., 2004; Sartory and Howard, 1992), this was performed in triplicate for each dilution at each time point. Due to the volume of the inocula to be filtered being below 10ml, additional MRD was added to the funnel to aid the dispersion of the bacteria over the entire surface of the membrane filter during the filtration process. Following filtration, the filter was placed onto membrane lactose glucuronide agar (MLGA; Oxoid CM1031) and incubated at 30°C for 4 hours and then transferred to 37°C for a further 14 hours. The colonies were then enumerated with all green colonies counted and considered as presumptive *E. coli*. This was repeated every 6 hours for 24 hours and then every 12 hours for the subsequent 24 hours. A sample was also analysed prior to pasteurisation to determine how effective the pasteurisation process was. The moisture content of the digested sludge cake was also established via heating a 5g sample at 121ºC for 24 hours and measuring the percentage weight difference.

3.2.6 Statistical analysis

See section 2.2.3.4.

3.3 Results and discussion

In the first experiment as shown by the results of Figure 3.2 there was no statistically significant ($p > 0.05$) effect on *E. coli* re-growth as a result of the addition of either soil or the fermented milk product to sludge cake post pasteurisation. In both cases *E. coli* was able to achieve a near 4 log increase in growth to reach a concentration of around 10^7 cfu/gDs within 24 hours. This result is comparable with that of the positive control, in which only sludge

derived nutrient broth was added. Though it does appear the addition of the fermented milk product caused an increase in the final concentration of *E. coli*, it was confirmed not a statistically significant ($p > 0.05$) increase. The mean generation times of *E. coli* were calculated as 92.5 minutes in the positive control, 93 minutes in the sludge cake in which the soil inoculum was added and finally 94.7 minutes in the sludge cake in which the fermented milk product inoculum was added. The lack of difference in generation times in comparison to the positive control, shows the growth rate of *E. coli* was largely unaffected by the presence of the soil or fermented milk product inoculum. This further proves that neither culture was capable of competing with *E. coli* in sludge cake.

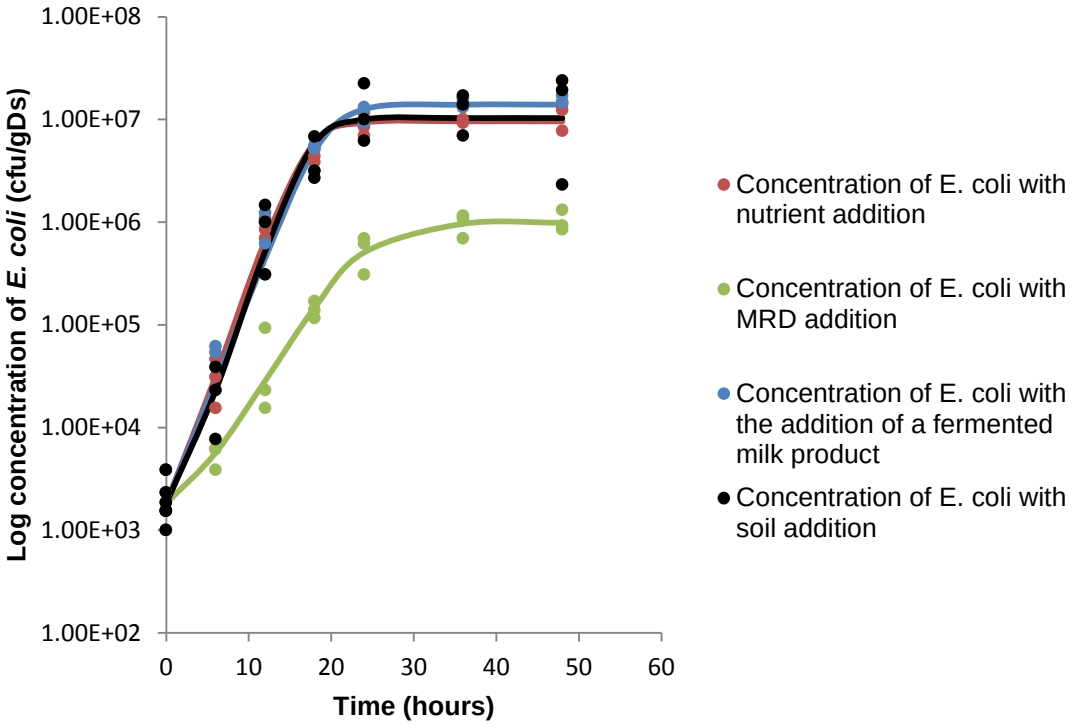


Figure 3.2 - Competitive exclusion of *Escherichia coli* in digested sludge cake using a solution undefined micro-organisms defined from soil and a commercially available fermented milk product stated to contain *L. casei shirota.* The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet et al. 2005 was used to fit curves to the data.

The statistically significant ($p < 0.05$) reduction in *E. coli* growth caused by not adding sludge derived nutrient broth and only adding 1ml MRD to sludge cake, confirms as in the previous chapter (2.3.3.2) that its addition is required to generate sufficient *E. coli* re-growth. This also justifies the addition of sludge derived nutrient broth in combination with the competitor cultures to support their growth and that of *E. coli* in sludge cake.

In the second experiment as shown by the results in Figure 3.3 isolating the micro-organisms contained within the fermented milk product thereby concentrating the number of competitor organisms added to the digested sludge cake had no statistically significant ($p > 0.05$) effect on *E. coli* growth. Even in the presence of the competitor culture, *E. coli* was able to achieve a near 4 log increase in growth to reach a concentration of around 10^7 cfu/gDs within 24 hours. The mean generation time of *E. coli* in this case was calculated to be 97.1 minutes. In comparison in the positive control, in which only sludge derived nutrient broth was added to sludge cake, the mean generation time was calculated to be 97.4 minutes. Furthermore *E. coli* achieved similar increases in growth in the same timeframe. This is a comparable outcome to that shown in Figure 3.2, further proving that the fermented milk product in particular is unsuitable for use in competitive exclusion treatment of sludge cake to reduce *E. coli* re-growth.

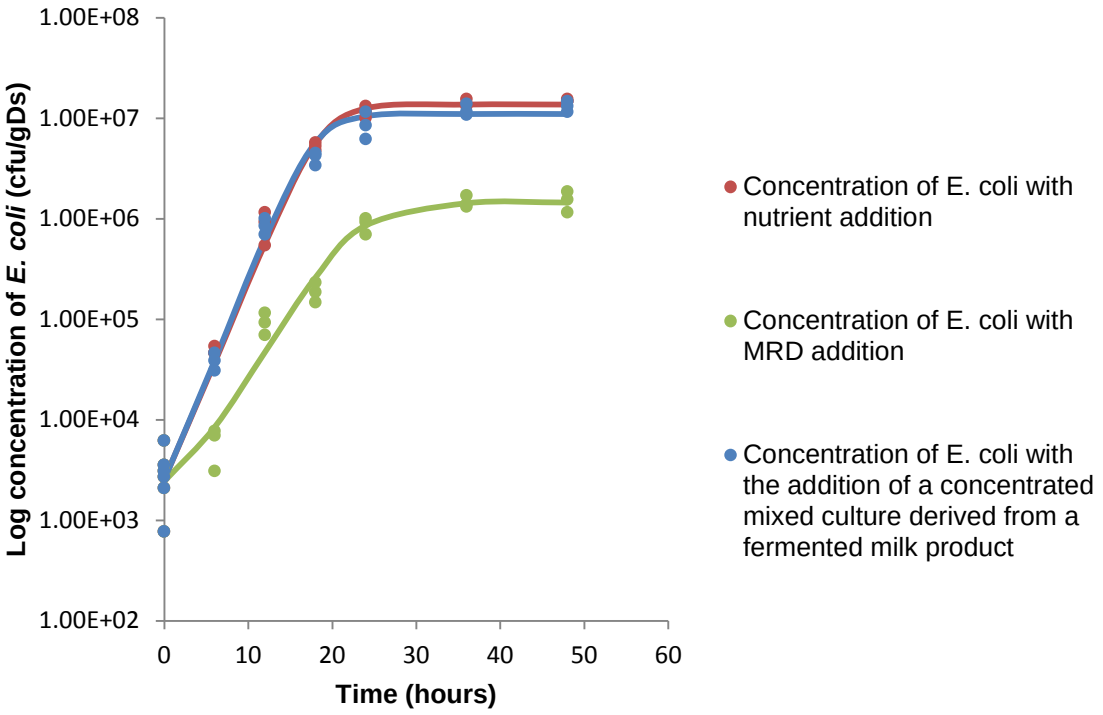


Figure 3.3 - Competitive exclusion of *Escherichia coli* in digested sludge by a commercial fermented milk product stated to contain *L. casei shirota* following centrifugation and re-suspension of cells. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet et al. 2005 was used to fit curves to the data.

There are a number of explanations as to why the addition of neither soil nor a commercially available fermented milk product had any effect on *E. coli* growth.

Firstly, the fermented milk product itself was designed for oral administration and to be effective within the human intestinal tract, which is an anaerobic environment. As well as *L. casei*, the product was stated as containing other bacterial species including a high concentration of bifidobacteria. Bifidobacteria are anaerobic (Lievin *et al*., 2000), therefore due to the aerobic nature of these experiments, the sludge cake would not have supported their growth. Essentially a fraction of the micro-organisms contained within the fermented milk product were not suited for growth in the sludge cake or the environment created by these experiments. This reduces the probability the product as a whole would be capable of competing with *E. coli.* This could explain why even after isolating and concentrating the micro-organisms contained within the fermented milk product, no effect on *E. coli* re-growth was recorded. This also could be true for the soil inoculum. Due to the lack of a screening step it is unknown what micro-organisms were actually present, especially when considering the high biodiversity of soil (Torsvik *et al*., 1996). It therefore can be hypothesised an insufficient number of micro-organisms suited to the growth environment within sludge cake and consequently capable of growing and competing with *E. coli* in said environment and those created for these experiments were present. Due to the advantages that the indigenous *E. coli* has including being already adapted to the sludge cake environment, a rapid growth rate and in the case of these experiments a fairly high initial concentration at around 10^3 cfu/gDs. Any increase in lag times or reduction in ability to produce antimicrobial compounds or compete for nutrients as a result of not being suited to the growth environment would lead to unrestricted growth of *E. coli* as shown in Figure 3.2 and 3.3. It can also be hypothesised that the micro-organisms that could have been effective were too low in concentration and were essentially out competed by *E. coli* and the other micro-organisms indigenous to sludge cake leading to results recorded in Figure 3.2 and 3.3.

Alternatively the mechanism of competing with *E. coli* or the micro-organisms present could have been unsuitable for sludge cake. Some strains of bacteria contained within probiotics like the fermented milk product in question are

selected for their ability to adhere to the epithelial wall of the gut mucosa and thus compete with pathogens for the adhesion receptors (Fuller, 1991). Essentially this method creates a physical barrier preventing colonisation and has been shown to be effective (Soerjadi *et al*., 1981; Soerjadi *et al*., 1982; Stavric *et al*., 1987). However in sludge cake this mechanism of action would be ineffective, not only due to the lack of corresponding receptor sites because of the non-existence of an epithelial cell wall but also because creating a physical barrier to prevent colonisation would be highly improbable in sludge cake. It is also possible that without a screening step, considering that the fermented milk inoculum was derived from a commercial product and the soil inoculum from a random environmental source it is possible that the micro-organisms contained within were in poor condition or non-viable, leading to the unhindered *E. coli* growth recorded.

It is hypothesised that with a proper identification and screening step for an ability to grow in sludge cake and inhibit *E. coli* growth, the use of mixed cultures in competitive exclusion treatments could still prove successful. Despite the results in these experiments soil is still a promising mixed culture, due to the fact it is known to contain numerous micro-organisms capable of producing potent antimicrobials. Nolan and Cross (1988), even go as far to recommend the screening of new soils for micro-organisms able to produce bioactive compounds. Actinomycetes are one of the major communities of the microbial population present in soil, with historically the most commonly isolated of the genera being *Streptomyces* and *Micromonospora*. In a study by Basilio *et al.* (2003) 77% of *Streptomyces* and 49% of other actinomycetes isolated from soil showed antimicrobial activity against a panel of clinically relevant bacteria, yeast and fungi. Furthermore many species and strains of the *Bacillus* genus commonly isolated from soil produce a large number of substances with antimicrobial action. In a study by Todorova and Kozhuharova (2010), *Bacillus subtilis* showed antimicrobial action against a number of fungi and bacteria including *Pseudomonas syringae* and *Alternaria solani*. Foster, Yasouri and Daoud (1992) also reported that 77% of soil myxobacteria had antibacterial

activity against *Micrococcus luteus.* Many of these also showed activity against fungus and gram negative bacteria. If soil was to be utilised again however in competitive exclusion treatments, it would have be as defined mixed cultures. Using undefined mixed cultures of soil as in these experiments in terms of accurately repeating successful experiments using the exact same combination of micro-organisms in the same concentration for every experiment would be problematic. However the production of defined mixed cultures in terms of isolating and identifying these antimicrobial producing bacteria would be very difficult considering the high biodiversity of soil (Torsvik *et al*., 1996). Furthermore this process would also be inevitably time consuming, especially considering the source of the soil with regard to location having to also be carefully selected. It is known that the conditions of the environment have a large impact on the composition of indigenous micro-organisms. Therefore it can be hypothesised that soils in some locations would be better at producing antimicrobials than others and therefore be more suited for utilisation in competitive exclusion treatments. For example Bull *et al.* (1992) stated that Brazilian tropical soils were an extremely rich source of biological compounds. Furthermore in a study by Basilio *et al* (2003) it was concluded that the best group of isolates in terms of production of active secondary metabolites were the ones isolated in saline conditions. For these reasons and the time constraints of this study, soil was not used in further competitive exclusion experiments. This is also true for other undefined mixed cultures, as they also pose the possible problem of containing pathogens due to the lack of a screening step. In the case of digested sludge cake destined for agricultural land this would be highly undesirable. Furthermore the unsuccessful nature of these experiments doesn't provide any significant incentive to proceed with further investigation of their possible application. Therefore an alternative approach was taken in subsequent experiments, with the careful identification of individual organisms and analysis of ability to inhibit *E. coli* in a sludge cake environment the desired method to proceed with.

3.4 Conclusions

- The addition of an inoculum derived from soil or a commercially available fermented milk product to digested sludge cake post pasteurisation had no statistically significant ($p > 0.05$) effect on *E. coli* re-growth.

- It is unknown why either inoculum failed to cause any significant reduction in *E. coli* growth. Although it was hypothesised that the majority of the micro-organisms contained within the mixed cultures were either unsuited for growth within sludge cake or in the experimental conditions. It also thought that the rapid growth rate of *E. coli* and the fact it is adapted to the sludge cake environment alongside the questionable viability of the competitor organisms considering their origin played a role.

- Undefined mixed cultures will not be used in future experiments, due to the problems they can pose. However considering their successful use within the poultry industry and other applications, the promise of the approach cannot be overlooked. There would almost certainly be an organism within soil for example capable of competing and possibly eliminating *E.coli*, however this would entail a substantial and time-consuming screening programme, considering its high biodiversity, which is at present not feasible.

- A targeted review of the literature and a more rapid screening process is required to identify candidate organisms capable of competing with *E. coli* in a sludge cake environment.

Chapter 4 Screening for competitive exclusion candidate organisms

4.1 Introduction

Nicholson (1954) loosely categorized competition for a limiting resource into two broad groups, scramble and contest. Scramble competition (or exploitation competition), involves rapid utilisation of the limiting resource without direct interaction between competitors. Contest competition (or interference competition) involves direct, antagonistic interactions between competitors, with the better adapted organism appropriating the resource. Study into interspecies competitive strategies has revealed that there are many diverse mechanisms by which micro-organisms can coexist with, or dominate, other organisms. A few examples of such mechanisms include biofilm formation, activation of flagellum and the production of adhesins or receptors that bind to specific surface features; these are reviewed in more detail by Hibbings *et al*. (2009). In terms of competitive exclusion strategies, that could prove successful against *E. coli* in digested sludge cake, the focus will be on two mechanisms; nutrient acquisition and production of antimicrobial compounds.

There are a number of methods micro-organisms can employ to sequester nutrients and thereby out-compete other organisms; this includes the restriction or removal of essential nutrients such as carbon, phosphorus or iron from an environment by direct targeted acquisition. An example of this is via the production of iron scavenging molecules called siderophores (Wandersman and Delepelaire, 2004). However the simplest method of direct nutrient competition is a fast growth rate. *E. coli* in particular is known to be a fast growing bacterium. This is shown in previous experiments (Figure 2.10), where a 4 log increase in *E. coli* concentration was recorded within 12 hours in optimal conditions in digested sludge cake. Possessing a fast growth rate allows *E. coli* to utilise a high proportion of the nutrients within an environment, making it less hospitable and thereby limiting the growth of any competitors, while maximising its own growth. Considering this, to compete with *E. coli* directly for nutrients in

digested sludge cake, any competitors will need a similar if not faster growth rate or alternative method of competing.

Some micro-organisms are capable of producing antimicrobials to eliminate or inhibit the competing organisms in their environment. Certain species of the soil bacteria *Streptomyces* have been shown to synthesize multiple antimicrobial compounds, with their genome sequence analysis indicating a potential for the synthesis of even more putative antimicrobial compounds that have yet to be detected under laboratory culture conditions (Challis and Hopwood, 2003). *Staphylococcus epidermidis* is capable of producing a protease to inhibit *Staphylococcus aureus* biofilm formation and nasal colonisation (Iwase *et al*., 2010). It has also been reported by several investigators that lactobacilli are able to produce antimicrobial substances when grown in specific media (Barefoot and Klaenhammer, 1984; Chung *et al*., 1989; De Klerk and Coetzee, 1961; Hamdan and Mikolajcik, 1974; Silva *et al*., 1987). For example, *Lactobacillus salivarus* has specifically been shown to exhibit antagonistic properties against *Listeria* (Barrett *et al*., 2007; O'Shea *et al*., 2011), *Salmonella* (Casey *et al*., 2007; Pascual *et al*., 1999), *Campylobacter* (Robyn *et al*., 2012) and other pathogenic bacteria (Corr *et al*., 2007; Neville and O'Toole, 2010). The ability of *L. salivarus* to inhibit these pathogens has been attributed to production of bacteriocins (Corr *et al*., 2007; O'Shea *et al*., 2011) and the antimicrobial compound salivaricin (Barrett *et al*., 2007) among other attributes. However, to effectively inhibit competitors, antimicrobials must be produced in a sufficient quantity, therefore to inhibit *E. coli* growth in digested sludge cake competitors will need to be capable of growing and thriving in digested sludge cake and as a result be able to produce enough antimicrobials.

In previous experiments (Chapter 3) the use of highly diverse undefined cultures proved unsuccessful. It was hypothesised this was due to the micro-organisms contained within the cultures not being suited to the sludge cake environment and therefore unable to compete with *E. coli* effectively. In response a screening step was put in place using sludge derived nutrient broth to rapidly assess the ability of any candidate organisms to compete with *E. coli*

in a sludge cake environment. This was performed via replicated bench-scale experiments in which the candidate organisms were separately inoculated in high concentration to sludge derived nutrient broth inoculated with a low concentration of *E. coli.* It was hypothesised that organisms that were capable of reducing *E. coli* growth in the nutrient rich environment of sludge derived nutrient broth would be more likely to be successful when applied to digested sludge cake. The nutrient broth was inoculated with a low concentration of *E. coli* to simulate the conditions post pasteurisation, a point in which the concentration of *E. coli* in sludge cake would be at its lowest. While a high concentration of candidate organisms was inoculated to provide a competitive advantage and to off-set the rapid growth rate of *E. coli* and the fact the bacteria is already adapted for growth in a sludge cake environment. Sludge derived nutrient broth was used because unlike standard broth media such as tryptone soya broth (TSB; Oxoid) it is a more realistic representation of the sludge cake environment in terms of nutrients available. Furthermore variables that exist in sludge cake do not in nutrient broth, such as, differing moisture contents, competing indigenous microflora and changing nutrient availability and composition. This makes the results more consistent and representative of how effective the candidate organisms are at inhibiting *E. coli* growth when in optimal conditions.

To select candidate organisms for screening prior to this experiment a targeted review of the literature was performed to identify promising candidates capable of competing with *E. coli* in a sludge cake environment. The criteria by which candidates were selected are shown in Table 4.1 and the candidates identified in Table 4.2. Of the candidate organisms identified *Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus reuteri* and an unidentified bacterial specimen (UIS) isolated and derived from digested sludge cake were selected.

UIS only satisfied two of the essential criteria but was still selected due to the bacterium being an indigenous member of the digested sludge cake microflora; therefore adapted for growth. Due to the mechanism of competitive action of UIS being unknown, this factor at least maximises the possibility of direct

Table 4.1 - Criteria for identifying candidate organisms for competitive exclusion screening experiments

Criteria	**Reason for requirement**
Has the candidate organism previously shown a capability of competing with or inhibiting *E. coli* growth?	Main aim of these experiments is to reduce *E. coli* growth, if a candidate has not shown a previous ability to do so; it is unlikely to be successful in sludge cake.
Has the candidate organism shown an ability to actively grow in sludge cake or a similar environment?	In Chapter 4 one of the hypotheses for the lack of reduction in *E. coli* re-growth was the competitor cultures were unsuited for the environment. Therefore it is essential any candidate can actively grow in a sludge cake environment.
Can the candidate organism be readily handled, maintained and isolated in the laboratory?	If a candidate does not meet this criteria it cannot be utilised in any controlled experiments, making it unsuitable
Does the candidate organism produce any antimicrobial compounds?	Not an essential criteria, however if successful during the screening process it is an ability to exploit during optimisation

nutrient competition with *E. coli. L. acidophilus, L. brevis* and *L. reuteri* however were chosen due to the fact they satisfied all of the essential criteria (Table 4.1). Furthermore isolates of each of these bacteria were readily available, with each being derived from the mammalian digestive tract. It was hypothesised that this factor would make the probability that candidates would be able to readily grow in sludge cake and therefore compete with *E. coli* more likely. All of the selected

lactobacilli have also been shown to possess antimicrobial activity. *L. acidophilus* is known to produce a number of antimicrobial compounds, including lactocidin, a substance active against gram negative bacteria (Vincent *et al*., 1959) and acidolin, which was found to be inhibitory to enteropathogenic and spore forming organisms (Hamdan and Mikolajcik, 1974). *L. brevis* has been reported to produce bacteriocins that have a broad spectrum of inhibition against both pathogenic and food spoilage organisms (Ogunbanwo *et al*., 2003). *L. reuteri* in the presence of glycerol is known to produce reuterin, a broad-spectrum antimicrobial substance active in a wide range of pH values against Gram-positive and Gram-negative bacteria (especially *E. coli*), yeasts, fungi, protozoa and viruses (Cleusix *et al*., 2007; Vollenweider and Lacroix, 2004). Furthermore lactobacilli overall are known to exert an antimicrobial effect via the production of lactic acid and reduction of pH (Daeschel, 1989) and additionally their use has previously proven successful in competitive exclusion treatments (Brashears *et al*., 2003; Milbradt *et al*., 2014; Schneitz, 2005). These factors make lactobacilli ideal candidate organisms for competitive exclusion treatment.

Table 4.2 – Candidate organisms for possible selection to the screening process.

Organism	Reason for selection	Satisfy Criteria?	Paper(s) information was sourced from
Lactobacillus acidophilus	Capable of inhibiting *E. coli* in manure over a 48 hour period suggesting a strong antagonistic action. Furthermore *L. acidophilus* has been reported to produce a number of antimicrobial compounds.	*L. acidophilus* satisfies all criteria	(Barefoot and Klaenhammer, 1984; Brashears *et al*., 2003; De Klerk and Coetzee, 1961; Hamdan and Mikolajcik, 1974)
Lactobacillus brevis	Like *L. acidophilus* showed a capability to competitively inhibit *E. coli* in manure over a 48 hour period. Stated to be a promising candidate as a probiotic supplement in dairy products. *L. brevis* has also been reported to produce antimicrobial compounds.	*L. brevis* satisfies all criteria	(Brashears *et al*., 2003; Ogunbanwo *et al*., 2003; Rönkä *et al*., 2003)
Lactobacillus salivarus	Shown to be capable of significantly inhibiting *E. coli* and *Salmonella* growth. *L. salivarus* is a normal member of the mammalian digestive tract and is known to produce a number of antimicrobial compounds.	*L. salivarus* satisfies all criteria, although strains are expensive.	(Barrett *et al*., 2007; Corr *et al*., 2007; Diaz *et al*., 2013; O'Shea *et al*., 2011)
Enterococcus faecium, Enterococcus faecalis and *Enterococcus hirae*	Intestinal bacteria currently used in probiotics and reported to depress the growth of *E. coli.* However can be sometimes considered pathogens themselves.	*Enterococcus* spp. satisfies all 3 essential criteria.	(AFRC, 1989; Vahjen *et al*., 2007)
Proteus mirabilis	Reduced *E. coli* number in the digestive tract of a number of animals in vitro. Mode of action however reported to be via preventing 0157:H7 attaching to the intestinal cell wall, which would be ineffective in sludge cake.	*P. mirabilis* satisfies all 3 essential criteria, but due to the reported mechanism of inhibition there are doubts over its effectiveness in sludge cake.	(Zhao *et al*., 1998)
Lactobacillus reuteri	Known to produce the broad spectrum antibiotic reuterin. Shown to be effective at inhibiting *E. coli* growth. The MIC of *E. coli* to reuterin was found to be between 7.5 and 15mM. *L. reuteri* is also derived from the mammalian digestive tract.	*L. reuteri* satisfies all criteria	(Cleusix *et al*., 2007; Cleusix *et al*., 2008; Diaz *et al*., 2013; Schneitz, 2005)
Bifidobacterium	Currently used in commercially available probiotics. Claimed in many papers to be similar to *Lactobacillus* in their ability to depress *E. coli* growth and go as far to state antimicrobial activity. No particular strains have been highlighted though. However Bifidobacteria do require anaerobic conditions.	Bifidobacteria satisfy 3 criteria, however due to their requirement for anaerobic conditions they are unsuitable.	(Liévin *et al*., 2000)
Unidentified bacterial specimen (UIS)	Bacterial isolate derived from sludge cake. Indigenous, therefore adapted to growth in sludge cake. It is unknown if it is capable of producing any antimicrobials or indeed compete efficiently with *E. coli* at this stage.	UIS satisfies 2 criteria.	N/A-

4.2 Materials and methodology

4.2.1 Strains, culture media and growth conditions

Escherichia coli and the unidentified bacterial specimen (UIS) were both recovered and isolated from digested sludge cake provided by United Utilities and sourced from Ellesmere Port and grown and maintained at 37°C on tryptone soya agar (TSA; Oxoid CM131) slopes. *Lactobacillus acidophilus* (NCIMB 2663), *Lactobacillus brevis* (NCIMB 11973) and *Lactobacillus reuteri* (NCIMB 11951) were grown and maintained at 37°C on De Man, Rogosa, Sharpe agar (MRSa; Oxoid CM361). All agar was prepared following the manufacturer's instructions and autoclaved at 121°C for 20 minutes prior to use. Stock cultures were stored in a cold room at 5°C, and sub-cultured and analysed for purity every two weeks.

4.2.2 Preparation of sludge derived nutrient broth

Raw sludge from Cranfield University's sewage treatment works was first stirred to break up the biomass and ensure nutrients would be released into suspension and then centrifuged at 84g for one minute to remove the heavier biomass. The supernatant was poured off and then autoclaved at 121ºC for 15 minutes to ensure sterility.

4.2.3 Preparation of inoculum

The *E. coli* and UIS were both grown up overnight in 100ml of tryptone soya broth (TSB; Oxoid CM129) in a Duran bottle and incubated at 37°C under constant shaking at 150rpm. *L. acidophilus*, *L. brevis* and *L. reuteri* were grown up overnight in 100ml of De Man, Rogosa, Sharpe broth (MRSb; Oxoid CM359) in a Duran bottle and incubated at 37°C under constant shaking at 150rpm. For every bacteria the cells were then harvested by centrifugation at 755g for 10 minutes. The supernatant was removed and the cells subsequently re-suspended in 10ml maximum recovery diluent (MRD; Oxoid CM0733). Following this the suspensions were 10 fold serially diluted in MRD and a plate

count performed on TSA for *E. coli* and UIS and MRSa for *L. acidophilus, L. brevis* and *L. reuteri*.

4.2.4 Inoculation of sludge derived nutrient broth

1ml of the UIS inoculum (i.e. dilution 0) and 1ml of the *E. coli* inoculum (i.e. dilution 10^{-8}) were added to 100ml of the sludge-derived nutrient broth and incubated at 30°C in a shaking incubator set at 150rpm. This was repeated for *L. acidophilus, L. brevis* and *L. reuteri*. 100ml of sludge-derived nutrient broth was also inoculated with 1ml of all the inocula. A positive control (only inoculated with *E. coli*) and a negative control (sterile sludge-derived nutrient broth) were also incubated under the same conditions. Growth of *E. coli* was measured every 3 hours for 24 hours with a break after 15 hours, if the stationary phase of growth was not reached after 24 hours, sampling would continue until it was reached.

4.2.5 Enumeration of E. coli via membrane filtration

1ml of sample was removed from the Duran bottles and 10-fold serially diluted in MRD. 1ml of these dilutions was then filtered through a 0.45µm cellulose acetate filter. Due to the volume of the inocula to be filtered being below 10ml, additional MRD was added to the funnel to aid the dispersion of the bacteria over the entire surface of the membrane filter during the filtration process. Following filtration, the filter was placed onto membrane lactose glucuronide agar (MLGA; Oxoid CM1031) and incubated at 30°C for 4 hours and then transferred to 37°C for a further 14 hours. This was performed in triplicate. The colonies were then enumerated with all green colonies counted and considered as presumptive *E. coli*.

4.2.6 Statistical analysis

See section 2.2.3.4.

4.3 Results and discussion

As shown in Figure 4.1 in sludge-derived nutrient broth it was found *E. coli* had a lag time of 1 hour and reached a maximum concentration of around $1x10^9$ cfu/ml in 15 hours, with a mean generation time of 24.4 minutes. The addition of UIS did have a statistically significant effect ($p < 0.05$) on the growth of *E. coli,*. The mean generation time of *E. coli* was increased to 33. 7 minutes and the maximum concentration *E. coli* reached was reduced to around 1.6 $x10^8$ cfu/ml, representing a 1 log reduction. The increase in growth rate and reduction in overall *E. coli* growth does suggest that UIS was capable of growing and competing with *E. coli* in sludge derived nutrient broth, most likely via direct nutrient competition. However the largely unhindered growth of *E. coli* recorded suggests that UIS couldn't sequester enough nutrients or does not have a fast enough growth rate to fully out-compete *E. coli*. This result however may have been compromised by a 2 log increase in the initial *E. coli* concentration. It is unknown what the cause of this increase was, as no contamination was recorded throughout these experiments.

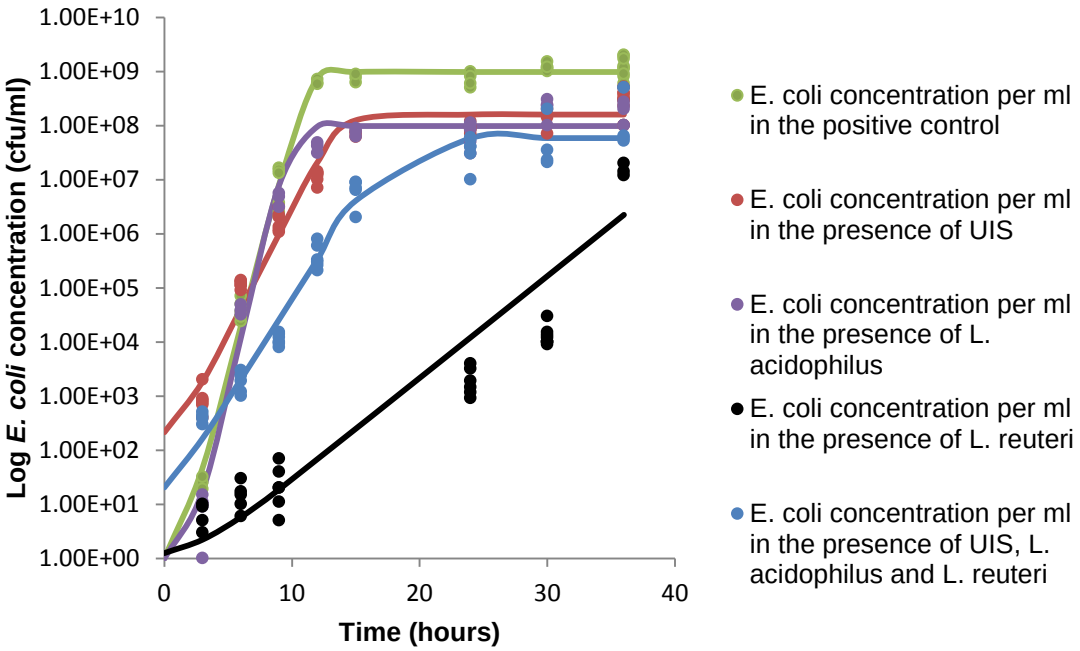


Figure 4.1 - Competitive exclusion of *E. coli* in sludge derived nutrient broth using UIS, *Lactobacillus acidophilus* and *Lactobacillus reuteri* and a combination of all three. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

The addition of *L. acidophilus* led to a very similar result to that recorded for UIS in that the addition of *L. acidophilus* did have an overall significant effect ($p < 0.05$) on *E. coli* growth. The effect on the lag time of *E. coli* was very small when compared with the positive control, extending it by only 0.7 hours and the maximum concentration of *E. coli* was reduced by 1 log to 9.8 $x10^7$ cfu/ml. However the addition of *L. acidophilus* had no effect on the growth rate of *E. coli*, with the mean generation time calculated to be 24.1 minutes. The initial increase in lag time and decrease in final concentration of *E. coli* does suggest *L. acidophilus* was capable of growing and competing with *E. coli* in sludge derived nutrient broth to a least a minor degree. As stated previously *L. acidophilus* is known to be capable of producing antimicrobial compounds (Barefoot and Klaenhammer, 1984; Vincent *et al*., 1959). This lack of inhibition suggests that the *L. acidophilus* strain used in these experiments isn't capable of producing the antimicrobials or simply sludge derived nutrient broth doesn't support sufficient *L. acidophilus* growth, or doesn't contain the precursor compounds for their production, making antimicrobial production not possible or ineffective. It is therefore hypothesised that without the ability to produce antimicrobial compounds *L. acidophilus* is unable to directly compete with *E. coli* for nutrients leading to the unrestricted growth recorded. These results therefore suggest it is unlikely UIS or *L. acidophilus* will find much success in digested sludge cake. It could also suggest that direct competition for nutrients is not a method that will provide much success in reducing *E. coli* numbers in sludge cake following treatment.

As with *L. acidophilus* and UIS, the addition of *L. reuteri* had a significant effect ($p < 0.05$) on *E. coli* growth, however *L. reuteri* had a greater deal of success. Though the lag time of *E. coli* was only extended to 2.9 hours, the growth rate was significantly depressed, with the stationary phase of growth not reached even after 36 hours and the mean generation time of *E. coli* calculated to be 97 minutes, three times longer than that calculated for the positive control. However N_t could not be accurately calculated as the stationary phase was not met during the duration of the experiment due to the presence of contaminant recorded during the 48 hour sampling point. Therefore the last point recorded

on the graph was used, if the experiment had continued to completion, it would be expected that the mean generation time calculated would be higher. As stated previously the *Lactobacillus* metabolism is known to produce antimicrobials as standard such as hydrogen peroxide, lactic acid and other organic acids, which inhibit pathogen growth by chelating essential nutrients or sensitising bacteria to antimicrobial assault (Neville and O'Toole, 2010). Furthermore *L. reuteri* is also a known producer of reuterin (Cleusix *et al*., 2008), an anti-microbial compound, and it is hypothesised that the production of these antimicrobial compounds is the cause of the depressed growth rate and slightly extended lag time as opposed to direct competition for nutrients.

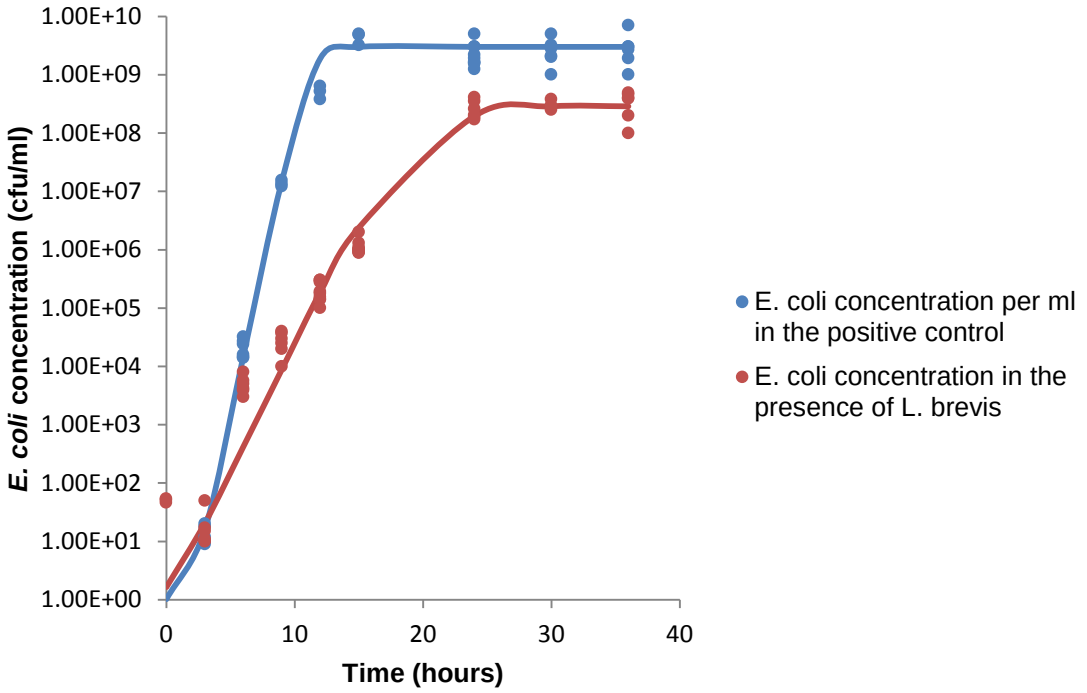


Figure 4.2 - Competitive exclusion of *E. coli* in sludge derived nutrient broth using *Lactobacillus brevis*. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

As shown in Figure 4.2 *L. brevis* had a similar statistically significant effect ($p < 0.05$) on *E. coli* growth as *L. reuteri*, but to a lesser degree. A significant drop in exponential growth was recorded, with the mean generation time of *E. coli* calculated to be 54.4 minutes, nearly 3 times longer than that recorded in the positive control (20.13 minutes. In the case of *L. brevis* addition no extension of the lag time was recorded. Furthermore despite this drop in exponential growth,

E. coli still reached a final concentration of around $1x10^8$ cfu/ml within 30 hours, when compared to the positive control; this represents only a 1 log reduction in the final concentration of *E. coli*. This final result is similar to that recorded in Figure 4.1 for *L. acidophilus* and UIS. As stated previously *L. brevis* is a known producer of the bacteriocin OG1, which has shown the ability to inhibit pathogenic bacteria including *E. coli* (Ogunbanwo *et al*., 2003). While the reasons why *L. brevis* is less effective than *L. reuteri* in supressing *E. coli* growth are unknown it can be speculated that the antimicrobials produced by *L. brevis* are less effective or *L. brevis* growth rate or antimicrobial production is lower than *L. reuteri* in sludge derived nutrient broth. The eventual failure of *L. brevis* and *L. reuteri* to repress *E. coli* growth could be because of a number of reasons. Firstly the antimicrobials may have eliminated all the susceptible *E. coli* leaving those that are only resistant. It is these *E. coli* that following the removal of the competition began to grow, leading to the exponential growth that was recorded. Secondly it is possible that *E. coli* simply adapted to the conditions in the nutrient broth and the antimicrobial compounds present or that in the conditions provided in this experiment *L. brevis* and *L. reuteri* were just unable to produce enough antimicrobial compounds to permanently suppress or eliminate *E. coli*.

In an attempt to maximise any effect UIS, *L. acidophilus* or *L. reuteri* had as sole competitors on *E. coli* growth, they were used in combination. The addition of this mixed culture had a statistically significant effect on *E. coli* growth ($p < 0.05$). A reduction in the growth rate of *E. coli* was recorded with the mean generation time of *E. coli* calculated to be 67.17 minutes; this is significantly longer than that calculated for the positive control (24.4 minutes) and the experiments involving UIS (33.7 minutes) and *L. acidophilus* (24.1 minutes) as sole competitors. However, once again the maximum concentration of *E. coli* was reduced by only 1 log to 5.9×10^7 cfu/ml. Overall the combination of UIS, *L. acidophilus* and *L. reuteri* together was more successful in slowing *E. coli* growth than either UIS or *L. acidophilus* as sole competitors, though it was less successful than *L. reuteri* on its own. Furthermore the reduction in *E. coli* growth is most likely largely due to the presence of *L. reuteri* and not UIS or *L.*

acidophilus. In fact the higher growth rate of *E. coli* seen from these results in comparison with those for *L. reuteri* as the sole competitor is most likely due to UIS and *L. acidophilus* competing with *L. reuteri* and thereby restricting its growth and ability produce enough antimicrobial compound to effectively compete with *E. coli*. *L. brevis* was not used in combination, due to poor growth during the culturing step of the experiment, therefore there were no cells available for harvesting via centrifugation and a *L. brevis* inoculum could not be made.

4.4 Conclusions

- UIS and *L. acidophilus* did have a statistically significant impact ($p < 0.05$) on *E. coli* growth in sludge-derived nutrient broth though it was minimal, with addition leading to a 1 log reduction in the final concentration of *E. coli* recorded in comparison with the positive control. However no significant effect was recorded on the growth rate or lag time of *E. coli*. Therefore it is unlikely that any effect will be recorded in solid digested sludge cake. From these results direct nutrient competition is probably not going to be successful considering that *E. coli* can reach the stationary phase of growth within 15 hours.

- *L. brevis* showed an increased capability of competing with *E. coli* in sludge-derived nutrient broth, possibly due to the production of antimicrobials, however the inhibition wasn't long lasting, with final concentration recorded similar to that detected in the experiments involving UIS and *L. acidophilus.*

- *L. reuteri* showed the most promise in suppressing *E. coli* growth in sludge-derived nutrient broth. It is hypothesised that this organism is capable of competing with *E. coli* due to the production of an antimicrobial compound, probably reuterin.

- Though combining competitors proved to hinder the effect of *L. reuteri* on *E. coli* growth, it will not be ruled out as a possible method in the future to

attain exclusion of *E. coli*. Just organisms that complement each other's effects need to be utilised.

- The successful demonstration of the competitive exclusion principle in sludge-derived nutrient broth is a promising development. The next steps are to repeat the results in solid digested sludge cake and attempt to optimise process in a bid to enhance the inhibition of *E. coli* growth.

Chapter 5 Competitive exclusion using *Lactobacillus reuteri* as a means to reduce *E. coli* growth in sludge-derived nutrient broth

5.1 Introduction

Lactic acid bacteria are a physiologically diverse group of organisms, which can be generally described as Gram-positive, non-sporing cocci or rods with lactic acid as the major product of carbohydrate fermentation (Axelsson, 2004). Lactic acid bacteria comprise four genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (Yang, 2000). Lactic acid bacteria have been used for centuries in the production of a variety of foods such as cheese and fermented meat products. As well as contributing to the flavour and general characteristics of these foods (Fox and Wallace, 1997), it is thought the lactic acid bacteria also exert a strong antagonistic effect against many food borne pathogens as a result of the production of organic acids, hydrogen peroxide, diacetyl, inhibitory enzymes and antimicrobial compounds (Piard and Desmazeaud, 1992). Indeed it has been reported in several studies that lactobacilli are capable of producing antimicrobial substances when grown in specific media (Barefoot and Klaenhammer, 1984; De Klerk and Coetzee, 1961; Hamdan and Mikolajcik, 1974; Tagg *et al*., 1976; Vincent *et al*., 1959). Furthermore Brashears *et al.* (2003) was able to reduce the concentration of *E. coli* 0157:H7 recorded in the manure and rumen fluid of cattle over the space of 48 hours via introduction of lactic acid bacteria as a primary probiotic agent. Brashears *et al*. (2003) speculated the mode of action of these lactic acid bacteria would most likely be due to factors that may include production of bacteriocins, hydrogen peroxide, low-molecular weight metabolites or enzymes. One lactic acid bacteria of particular interest with regards to antimicrobial activity is *Lactobacillus reuteri.*

Despite its well documented antimicrobial activity, *L. reuteri* has not been widely utilised or well researched as a possible probiotic agent, with the exception of its application as a probiotic in dairy products (Rothschild, 1995). Lactobacilli as a group have been used successfully as probiotics, being used to reduce

Salmonella loads and eradicate various pathogens from chickens, pigs and other animals (Avía *et al*., 1998; Corr *et al*., 2007; Pascual *et al*., 1999). It is hypothesised that *L. reuteri* can be utilised as a means of competitive exclusion (Gause, 2003; Hardin, 1960) treatment to control (if not eliminate) *E. coli* growth in digested sludge cake following the final dewatering step of cake manufacture where *E. coli* numbers have been reported to significantly increase (Qi *et al*., 2007; Higgins *et al*., 2007) leading to compliance failure (ADAS, 2001).

L. reuteri in previous experiments (Figure 4.1) was capable of significantly inhibiting *E. coli* growth in sludge derived nutrient broth for a number of hours; however the effect was not sustained. The source of the inhibition is hypothesised to be via the production of antimicrobial compounds. *L. reuteri* is known to produce the antimicrobial compound 3-hydroxypropionaldehyde (3-HPA), also referred to as reuterin (Axelsson *et al*., 1989; Talarico and Dobrogosz, 1989). Reuterin has proven to be a potent antimicrobial agent active against Gram positive and Gram negative bacteria, as well as yeasts, moulds and protozoa (Axelsson *et al*., 1989). Cleusix *et al.* (2007) calculated the minimum inhibitory concentration of reuterin against *E. coli* to be between 7.5-15mM and the minimum bactericidal concentration to be between 15-30mM. The mechanism of action by which reuterin exerts its antimicrobial effects has largely remained unknown. However Schaefer *et al.* (2010) using microarray analysis of cells treated with reuterin discovered that the cells showed signs of oxidative stress and through further analysis concluded that the bioactive component of reuterin is the aldehyde form interacting with thiol groups of small molecules and proteins.

Reuterin is produced as an intermediate step in the conversion of glycerol to 1,3-propanediol, a pathway proposed to regenerate NAD^+ from NADH and to contribute to improved growth yield (Lüthi-Peng et al., 2002). For reasons that are unclear, *L. reuteri* secretes high levels of reuterin when grown or incubated in the presence of excess amounts of glycerol. It was therefore hypothesised that supplementing sludge derived nutrient broth with glycerol would increase the ability of *L. reuteri* to produce reuterin, thereby enhancing and or prolonging

the ability of *L. reuteri* to inhibit *E. coli* growth. This hypothesis was tested by performing replicated bench-scale experiments in which *L. reuteri* was inoculated in high concentration to sludge derived nutrient broth inoculated with a low concentration of *E. coli* and supplemented with varying concentrations of glycerol. The aim of the experiment was to determine whether the addition of glycerol enhanced the ability of *L. reuteri* to inhibit *E. coli* growth via the hypothesised production of reuterin and if so which concentration of glycerol yielded the best results.

Following this the effect of lowering pH along with addition of glycerol on *E. coli* growth in the presence of *L. reuteri* was also tested. Lactobacilli are known to prefer lower pH environments usually around pH 6.0 for optimal growth (De Man *et al*., 1960; Giraud *et al*., 1991). Therefore it was hypothesised that lowering the pH of sludge derived nutrient broth would favour *L. reuteri* growth, leading to reduced lag times, an increased growth rate and hasten the production of antimicrobials. Furthermore the optimal pH for the growth of *E. coli* is around pH 7.0, so lowering the environmental pH, will have the reverse effect, in terms of reducing growth rate, providing *L. reuteri* with a competitive advantage and maximising the inhibition of *E. coli* growth further. This was performed via replicated bench-scale experiments in which *L. reuteri* was inoculated in high concentration to pH adjusted sludge derived nutrient broth inoculated with a low concentration of *E. coli* and supplemented with 10% glycerol. The aim of the experiment was to determine whether changing the environmental conditions to suit *L. reuteri* growth would increase its ability to compete with *E. coli* in sludge derived nutrient broth.

5.2 Materials and methodology

5.2.1 Strains, culture media and growth conditions

Escherichia coli was recovered and isolated from digested sludge cake provided by United Utilities and sourced from Ellesmere Port and grown and maintained at 37°C on tryptone soya agar (TSA; Oxoid CM131) slopes. *Lactobacillus reuteri* (NCIMB 11951) was grown and maintained at 37°C on De

Man, Rogosa, Sharpe agar (MRSa; Oxoid CM361). All agar was prepared following the manufacturer's instructions and autoclaved at 121°C for 20 minutes prior to use. Stock cultures were stored in a cold room at 5°C, and sub-cultured and analysed for purity every two weeks.

5.2.2 Preparation of sludge-derived nutrient broth

Raw sludge from Cranfield University's sewage treatment works was first stirred to break up the biomass and ensure nutrients would be released into suspension and then centrifuged at 84g for one minute to remove the heavier biomass. The supernatant was poured off and then autoclaved at 121ºC for 15 minutes to ensure sterility.

5.2.3 Preparation of inoculum

E. coli was grown overnight in 100ml tryptone soya broth (TSB; Oxoid CM129) in a Duran bottle and incubated and incubated at 37°C under constant shaking at 150rpm. *L. reuteri* was grown overnight in 100ml De Man, Rogosa, Sharpe broth (MRSb; Oxoid CM359) in a Duran bottle and incubated at 37°C under constant shaking at 150rpm. The cells were then harvested by centrifugation at 755 g for 10 minutes. The supernatant was removed and the cells subsequently re-suspended in 10ml maximum recovery diluent (MRD; Oxoid CM0733). Following this the suspensions were 10 fold serially diluted in MRD and a plate count on TSA for *E. coli* and MRSa for *L. reuteri* performed.

5.2.4 Inoculation of sludge derived nutrient broth

A 1ml volume of the *L. reuteri* inoculum (i.e. dilution 0) and 1ml volume of the *E. coli* inoculum (i.e. dilution 10^{-8}) were added to 100ml of the sludge-derived nutrient broth and incubated in a shaking incubator at 30°C and 150rpm. A positive control (inoculated with *E. coli* only) and a negative control (sterile sludge-derived nutrient broth) were also incubated under the same conditions. Growth of *E. coli* was measured every 3 hours for 24 hours with a break after 15 hours. If the stationary phase of growth was not reached after 24 hours, sampling continued until it was reached.

5.2.5 Enumeration of E. coli via membrane filtration

1ml of sample was removed from the Duran bottles and 10-fold serially diluted in MRD. 1ml of these dilutions was then filtered through a 0.45µm cellulose acetate filter. Due to the volume of the inocula to be filtered being below 10ml, additional MRD was added to the funnel to aid the dispersion of the bacteria over the entire surface of the membrane filter during the filtration process. Following filtration, the filter was placed onto membrane lactose glucuronide agar (MLGA; Oxoid CM1031) and incubated at 30°C for 4 hours and then transferred to 37°C for a further 14 hours. This was performed in triplicate. The colonies were then enumerated with all green colonies counted and considered as presumptive *E. coli.*

5.2.6 Effects of the addition of L. reuteri and glycerol

A 100ml volume of sludge-derived nutrient broth was combined with 29ml of sterile distilled water and 15ml sterile glycerol (Fisher scientific, 98.0+ %). This was then inoculated with 5ml of the *L. reuteri* inoculum (i.e. dilution 0) and 1ml of the *E. coli* inoculum (i.e. dilution 10^{-8}), to give a total volume of 150ml and a 10% glycerol solution in sludge-derived nutrient broth. A concentration of 10% glycerol was chosen as it was determined via spread plates to be the highest concentration of glycerol that could be used without adversely affecting *E. coli* growth. This was then incubated at 30°C in a shaking incubator set at 150rpm. A positive control (sludge-derived nutrient broth inoculated with *E. coli* only) and a negative control (sterile sludge-derived nutrient broth with 10% glycerol) were also incubated under the same conditions. Growth of *E. coli* was measured every 3 hours for 24 hours with a break after 15 hours. If the stationary phase of growth was not reached after 24 hours, sampling would continue until it was reached.

5.2.7 Analysing of Lactobacillus reuteri growth in sludge derived nutrient broth with glycerol addition

A 100ml volume of sludge-derived nutrient broth was combined with 34ml of sterile distilled water and 15ml sterile glycerol. This was then inoculated with 1ml of the *L. reuteri* inoculum (i.e. dilution 10^{-7}), to give a total volume of 150ml and a 10% glycerol solution in sludge-derived nutrient broth. This was then incubated in a shaking incubator at 30°C and 150rpm. An additional 100ml volume of sludge-derived nutrient broth was combined with 49ml sterile distilled water and inoculated with 1ml of the *L. reuteri* inoculum (i.e. dilution 10^{-7}), to give a total volume of 150ml. This acted as a control. Growth of *L. reuteri* was measured every 3 hours for 24 hours with a break after 15 hours. If the stationary phase of growth was not reached after 24 hours, sampling would continue until it was reached. To analyse growth a 1ml sample was removed and 10-fold serially diluted in MRD. 100µl of these dilutions was then plated onto De Man, Rogosa, Sharpe agar (MRS; Oxoid CM361) in triplicate and incubated at 37ºC for 48 hours, after which all colonies were counted.

5.2.8 Effects of the addition of L. reuteri and varying concentrations of glycerol

Varying volumes of sterile glycerol (Fisher scientific, 98.0+ %) and sterile distilled water were added to several different Duran bottles containing 100ml of sludge derived nutrient broth. These were then inoculated with 5ml of the *L. reuteri* inoculum (i.e. dilution 0) and 1ml of the *E. coli* inoculum (i.e. dilution 10^{-8}), to give a total volume in each bottle of 150ml and five different glycerol concentrations (1%, 5%, 10%, 15% and 20%). They were then incubated at 30°C in a shaking incubator set at 150rpm. A positive control (sludge-derived nutrient broth inoculated with *E. coli* only) and a negative control (sterile sludge-derived nutrient broth with 10% glycerol) were also incubated under the same conditions. Growth of *E. coli* was measured every 3 hours for 24 hours with a break after 15 hours. If the stationary phase of growth was not reached after 24 hours, sampling would continue until it was reached.

5.2.9 Effects of the addition of L. reuteri and glycerol at reduced pH

Varying volumes of sterile distilled water and 0.1M hydrochloric acid (Fisher Scientific) were added to 100ml volume of sludge-derived nutrient broth supplemented with 15ml sterile glycerol (Fisher scientific, 98.0+ %), to create four batches of sludge derived nutrient broth at pH 6.5, 6, 5.5 and 5. These were then inoculated with 5ml of the *L. reuteri* inoculum (i.e. dilution 0) and 1ml of the *E. coli* inoculum (i.e. dilution 10^{-8}), to give a total volume of 150ml and a 10% glycerol solution in sludge-derived nutrient broth. They were then incubated at 30°C in a shaking incubator set at 150rpm. Two positive controls (sludge-derived nutrient broth inoculated with *E. coli* only with and without pH reduction) and a negative control (sterile sludge-derived nutrient broth with 10% glycerol) were also incubated under the same conditions. Growth of *E. coli* was measured every 3 hours for 24 hours with a break after 15 hours. If the stationary phase of growth was not reached after 24 hours, sampling would continue until it was reached.

5.2.10 Isolating and confirming the production of reuterin

Reuterin was produced as previously described by Vollenweider *et al.* (2003). *L. reuteri* was inoculated at 1% (v/v) in 10 ml MRS broth, incubated overnight in a shaking incubator at 37°C in a shaking incubator set at 150rpm and added to 50 ml MRS medium which was then incubated for 3 h under the same conditions. This culture was then added to 1L of MRS broth supplemented with 20 mM glycerol and incubated overnight at 37°C in a shaking incubator set at 150rpm. The cells were then harvested by centrifugation at 1500g for 10 minutes, washed with potassium phosphate buffer (0.1 M, pH 7.0), re-suspended in 300-ml sterile aqueous solution of glycerol (200 mM) and incubated for 2 h at 37°C in a shaking incubator set at 150rpm. The cells were removed by centrifugation (1500g for 10 minutes). The supernatant was then filter sterilised through a 0.22µm cellulose acetate filter. To confirm the presence of reuterin, the agar disc diffusion method was used. For this 0.1ml of *E. coli* was inoculated and spread evenly across the surface of a TSA plate to create an even bacterial

lawn. Following this, a 0.45µm cellulose acetate filter was cut into circles and saturated with the filter sterilised supernatant. After a brief drying period, two were placed on each inoculated TSA plate. To act as a control another cellulose acetate filter circle was saturated with a sterilised aqueous solution of glycerol (200mM) and also placed on each inoculated TSA plate. The TSA plates were then incubated overnight at 37°C. The zones of inhibition were measure using a standard 30cm ruler.

5.2.11 Statistical analysis

See section 2.2.3.4.

5.3 Results and discussion

5.3.1 Effect of L. reuteri on the growth of E. coli

Results for the competitive exclusion of *E. coli* in sludge-derived nutrient broth using *L. reuteri* proved variable. During the initial screening experiment conducted in Chapter 4 (Figure 4.1) the addition of *L. reuteri* resulted in a statistically significant reduction ($p < 0.05$) in *E. coli* growth with the lag time of *E. coli* calculated to be 2.9 hours and the mean generation time 97 minutes, both 3 times longer than that calculated for the control. Overall the stationary phase of growth for *E. coli* was not reached within 36 hours when *L. reuteri* was present. In the repeat of the screening experiment (Figure 5.1) the addition of *L. reuteri* once again caused a significant reduction ($p < 0.05$) in the growth of *E. coli.* The growth rate of *E. coli* was again restricted with the mean generation time calculated to be 54.2 minutes. The stationary phase of growth was also not reached until after 24 hours, in both cases this over twice as long as that calculated for the control. Furthermore the final concentration of *E. coli* was reduced by around one log. Though *L. reuteri* proved successful in restricting *E. coli* growth the effect was less pronounced than that recorded in the initial experiment (Figure 4.1). The inconsistencies in the ability of *L. reuteri* to inhibit *E. coli* growth are further seen in the second repeat (Figure 5.2), where addition of *L. reuteri* had no significant effect ($p > 0.05$) on *E. coli* growth. Despite the

conditions of the experiments being replicated throughout, the inhibitory effect *L. reuteri* has on *E. coli* is highly variable. These inconsistencies are most likely linked with the ability of *L. reuteri* to produce antimicrobial compounds in the sludge-derived nutrient broth. If this is the case then this variability suggests that without the production of antimicrobials, *L. reuteri* is unable to directly compete with *E. coli.*

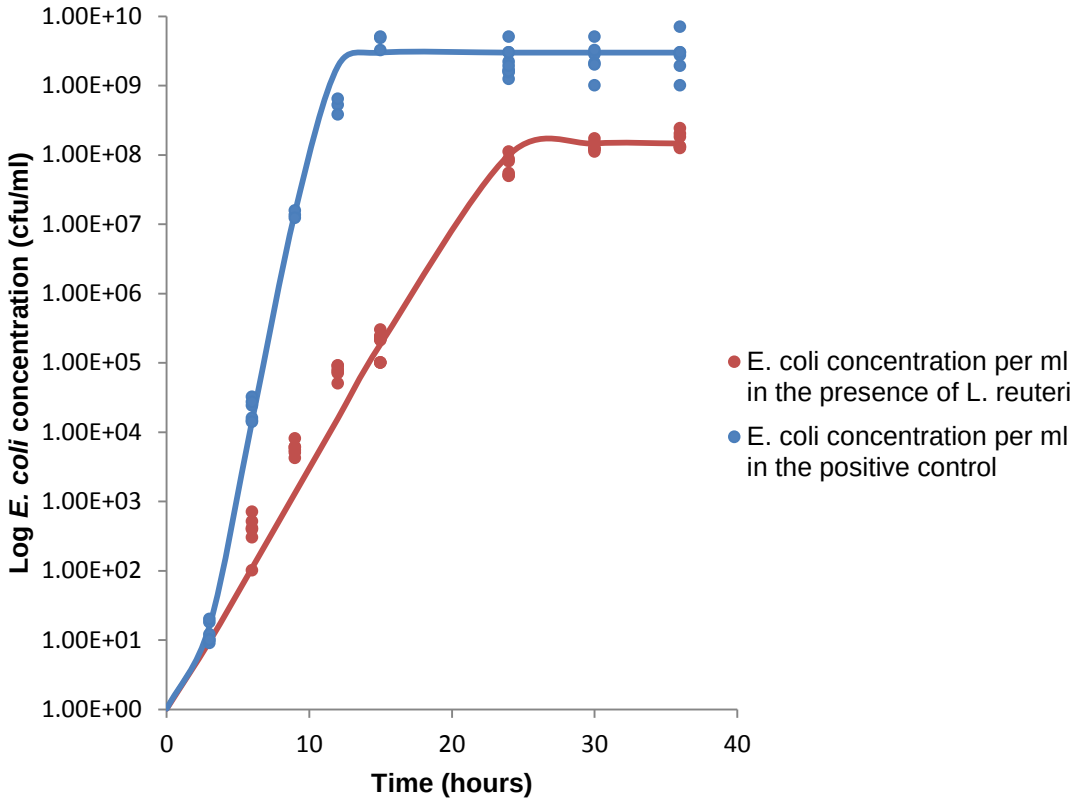


Figure 5.1 - Competitive exclusion of *E. coli* in sludge derived nutrient broth using *Lactobacillus reuteri* in the first repeat. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

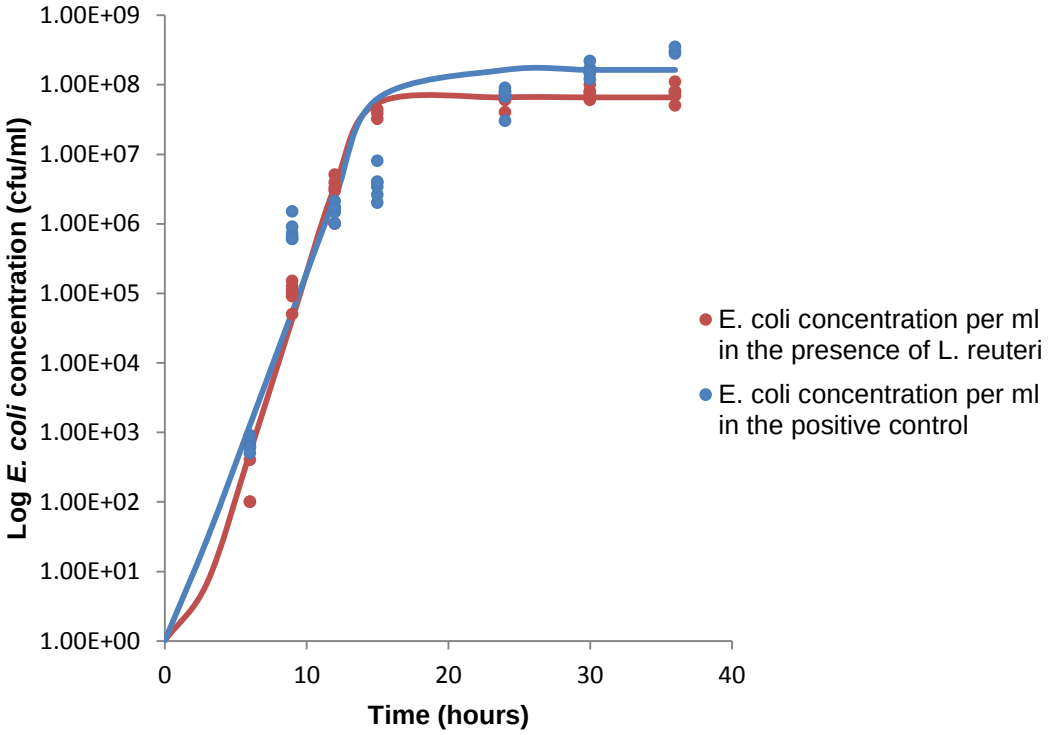


Figure 5.2 - Competitive exclusion of *E. coli* in sludge derived nutrient broth using *Lactobacillus reuteri* in the second repeat. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

5.3.2 The effect of glycerol addition

Despite the inconsistencies between experiments (Figure 4.1, 5.1 and 5.2), *L. reuteri* did show potential as a possible competitor organism. As stated previously it is hypothesised these inconsistencies are due to an inability to produce antimicrobials and in particular reuterin consistently. Considering that the nutrient broth is derived from raw sludge which in turn consists of organic and inorganic components originating from among other things human waste. Glycerol is a precursor of triacylglycerols (a main constituent of vegetable oils and animal fats) and phospholipids (major component of cell membranes) and is a compound found naturally, it is therefore likely that at least a small concentration of glycerol would be present in the sludge-derived nutrient broth. However the exact nutritional composition of sludge-derived nutrient broth and its consistency between experiments is unknown. It is therefore suggested that in the initial experiment (Figure 4.1) *L. reuteri* was exposed to a glycerol concentration sufficient to enable the production of enough reuterin to suppress

growth but not enough to do so permanently, thereby allowing the eventual exponential growth recorded. In contrast, in the two repeat experiments, it is likely that *L. reuteri* was exposed to limited amounts of glycerol and therefore produced little or no reuterin, and as a consequence *E. coli* growth was only marginally affected. Therefore to maximise the ability of *L. reuteri* to produce antimicrobials and in particular reuterin and thereby consistently compete with *E. coli*, glycerol, the precursor compound of reuterin, was added to the sludge-derived nutrient broth.

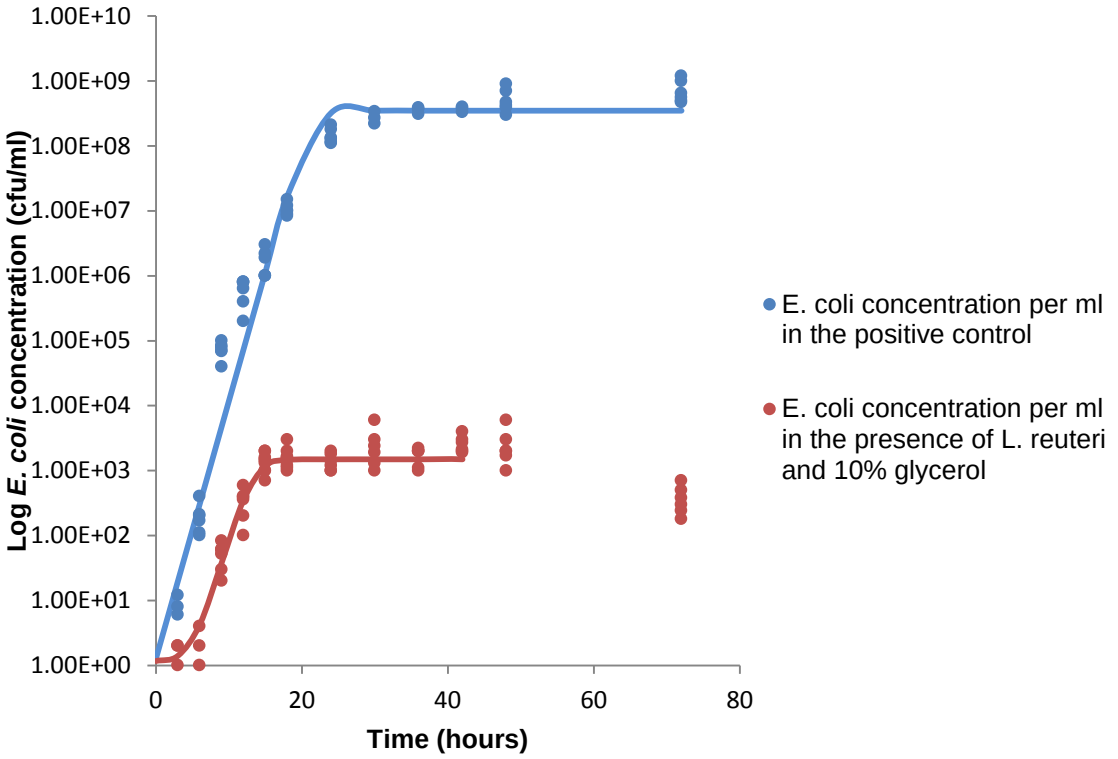


Figure 5.3 - Competitive exclusion of E. coli in sludge derived nutrient broth containing 10% glycerol using *Lactobacillus reuteri* initial experiment. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet et al. 2005 was used to fit curves to the data

The addition of 10% glycerol and *L. reuteri* to cultures had a significant impact ($p < 0.05$) on *E. coli* growth (Figure 5.3). The initial rate of growth of *E. coli* was not too dissimilar to that observed in the positive control, with the mean generation times calculated as 66.6 minutes and 52.6 minutes respectively. This may be attributed to a lag in *L. reuteri* growth and the conversion of glycerol to reuterin and eventual release. However, the final concentration of *E. coli* recorded was significantly reduced in comparison to the positive control to

around 1x10^3 cfu/ml, representing a 5 log reduction. Furthermore it appears that after 72 hours the concentration of *E. coli* was actually in decline. This experiment was repeated and as shown in Figure 5.4 the effect was similar. The addition of *L. reuteri* in combination with 10% glycerol resulted once again in a significant reduction ($p < 0.05$) in the maximum concentration of *E. coli* recorded, but not in the initial growth. However in the case of the repeat experiment *E. coli* was completely eliminated from the sludge-derived nutrient broth within 48 hours. The reason *L. reuteri* was able to completely eliminate *E. coli* in the repeat (Figure 5.4) and not the initial experiment (Figure 5.3) within the duration of the experiment is unknown. However, in the initial experiment *L. reuteri* displayed a longer lag time and sluggish initial growth and this is most likely the cause. Clearly the addition of glycerol not only maximises the effect of *L. reuteri* on *E. coli* growth, but also makes it more consistent.

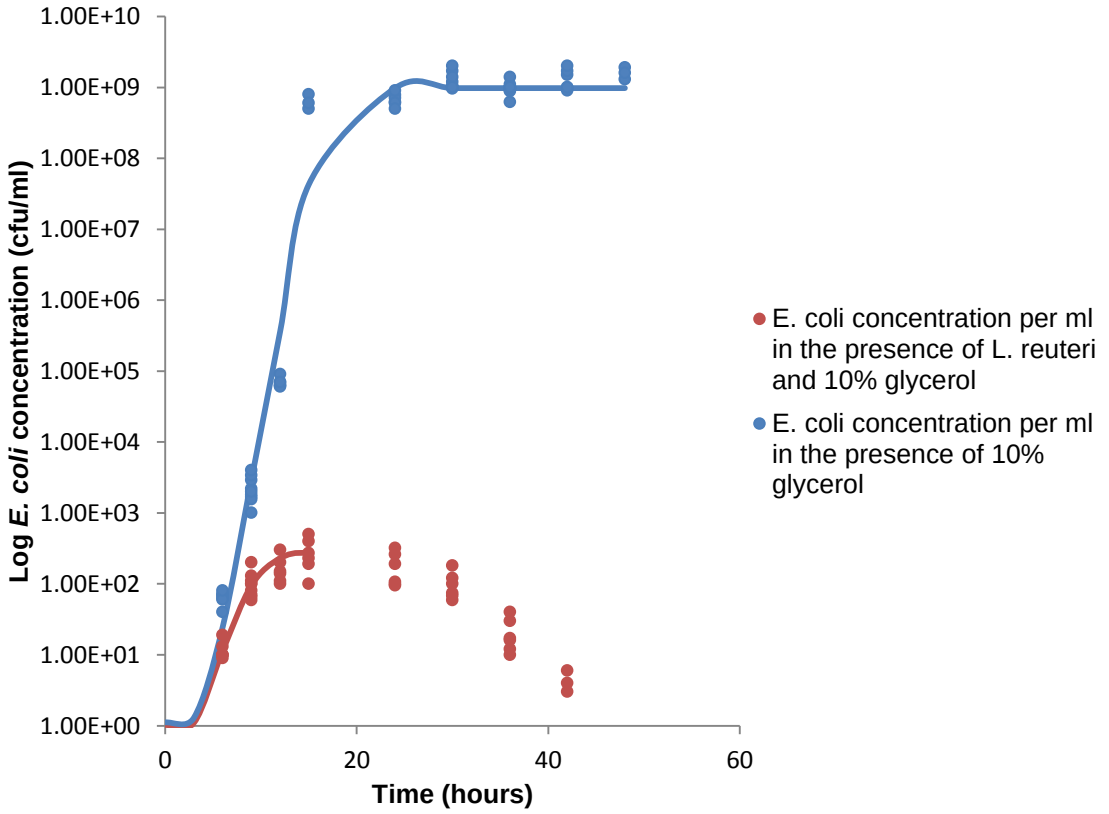


Figure 5.4 - Competitive exclusion of E. coli in sludge derived nutrient broth containing 10% glycerol using *Lactobacillus reuteri* repeat experiment. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet et al. 2005 was used to fit curves to the data

To eliminate the possibility that the effect on *E .coli* was due solely to the addition of glycerol, the experiment was repeated but the sludge-derived nutrient broth of the positive control was also supplemented with 10% glycerol. Comparing the results for the positive control in Figure 5.3 to those for Figure 5.4 it is clear that the addition of 10% glycerol alone to sludge-derived nutrient broth does have a significant effect ($p < 0.05$) on the initial growth of *E. coli*, extending the lag time. This can be largely attributed to the effect that glycerol has on the water activity (A_w). However following this, *E. coli* reached a concentration of around $1x10^9$ cfu/ml within 30 hours, this is a similar result to that recorded in the positive control of the initial experiment. Furthermore the mean generation time in the first repeat was calculated as 42.7 minutes, this is actually a reduction to that calculated for the initial experiment. This suggests that following the initial lag, growth was unhindered by the presence of glycerol. This further supports the hypothesis that *L. reuteri* is the source of inhibition,

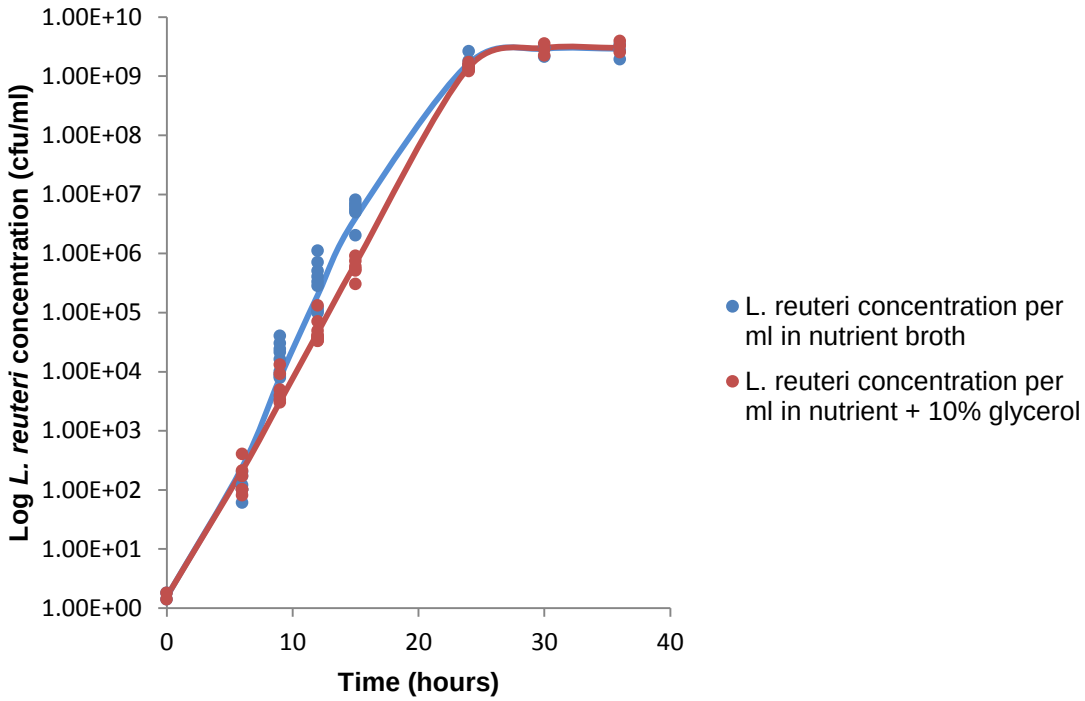


Figure 5.5 - Growth of *Lactobacillus reuteri* in sludge derived nutrient broth and sludge derived nutrient broth supplemented with 10% glycerol. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet et al. 2005 was used to fit curves to the data.

Figure 5.5 shows that *L. reuteri* was capable of growing in sludge-derived nutrient broth and that the addition of 10% glycerol had no significant effect ($p > 0.05$) on growth. With or without glycerol addition *L. reuteri* reached the stationary phase of growth within 24 hours, achieving a final concentration greater than 10^9 cfu/ml. This further confirms the results shown in Figure 5.3 and 5.4 and is consistent with the hypothesis that *L. reuteri* and the subsequent conversion of glycerol to reuterin is responsible for the inhibition and the eventual elimination of *E. coli* in sludge-derived nutrient broth. Furthermore it confirms that the inhibition is not caused by the addition of glycerol or any other variable

5.3.3 The effect of varying glycerol concentration

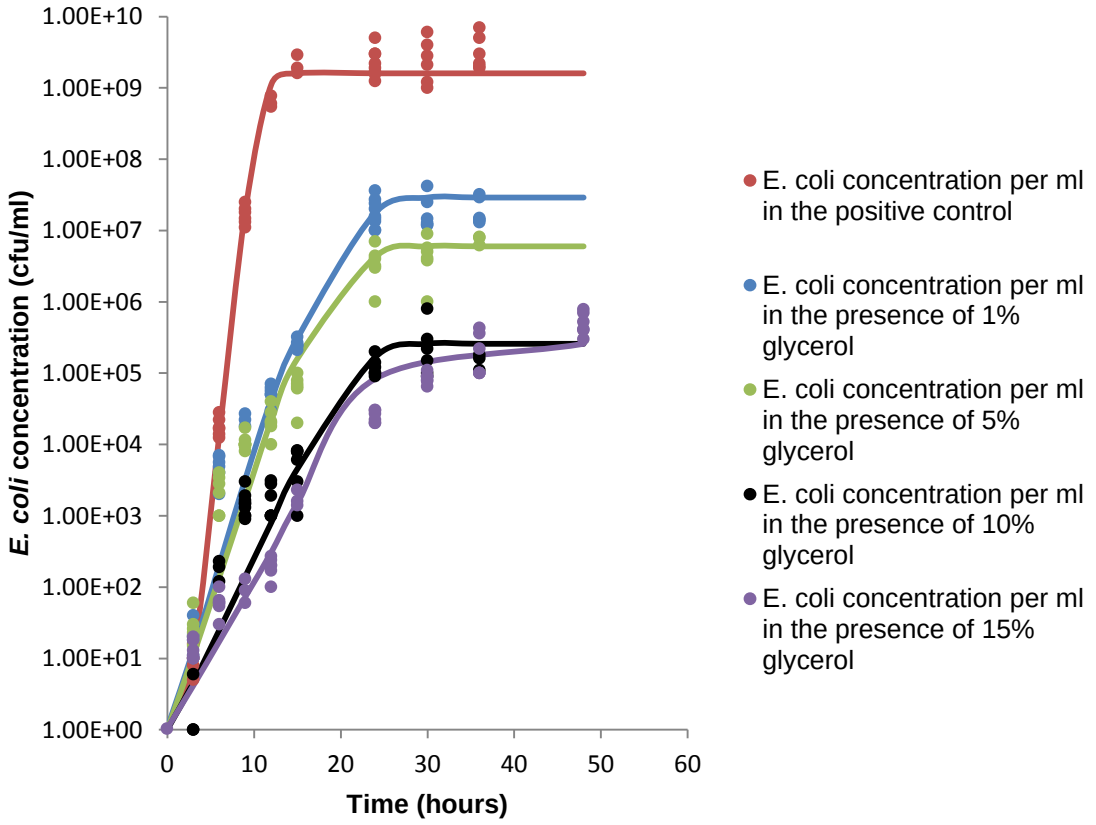


Figure 5.6 - Growth of *E. coli* in sludge derived nutrient broth supplemented with varying concentrations of glycerol in the presence of *Lactobacillus reuteri*. The positive control represents growth of *E. coli* in sludge derived nutrient without glycerol supplementation and without addition of *L. reuteri*. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

From Figure 5.6 it is clear that addition of *L. reuteri* combined with even the lowest concentration of glycerol results in substantial significant effect ($p < 0.05$) on not only *E. coli* growth rate but also the maximum cell yield, in comparison with the positive control. The optimal concentration of glycerol however, seems to be between 10-15%, with there being no significant statistical difference ($p > 0.05$) in *E. coli* growth rate or maximum cell yield between these two concentrations. The most likely cause of this being that *L. reuteri* is producing as much reuterin at this point to suppress *E. coli* as it can regardless of the glycerol that is present, hence the lack of change between the two concentrations. To further confirm *L. reuteri* as the source of the inhibition, from Figure 5.7 it is obvious that the addition of glycerol by itself is not the cause, with in all cases where no *L. reuteri* was added, *E. coli* was capable of reaching a maximal cell yield of $1x10^9$ cfu/ml by at least 36 hours regardless of the concentration of glycerol present. In comparison even with just 1% glycerol supplementation, in the presence of *L. reuteri*, *E. coli* could only manage to reach a maximum cell yield of roughly $5x10^7$ cfu/ml within the same time period (Figure 5.6). That being said glycerol is still hygroscopic in nature and does have an effect on water activity (A_w), as shown in figure 5.7. Glycerol by itself did at concentrations of 10 and 15% initially limit *E. coli* growth and at 20% completely eliminated both *E. coli* and *L. reuteri*. This is a similar result to that recorded in Figure 5.4 where the addition of 10% glycerol to sludge-derived nutrient broth led to an increase *E. coli* lag time. Due to this fact and with future implementation in sludge cake destined for agricultural land in mind, it is important to limit the effect any treatment has on the chemical composition and properties of the sludge cake. Therefore since 10% glycerol supplementation by itself has only a minor effect on *E. coli* growth, while significantly enhancing the inhibitory effect *L. reuteri has on* E. coli, it was chosen as the default concentration of glycerol to be utilised in further competition experiments.

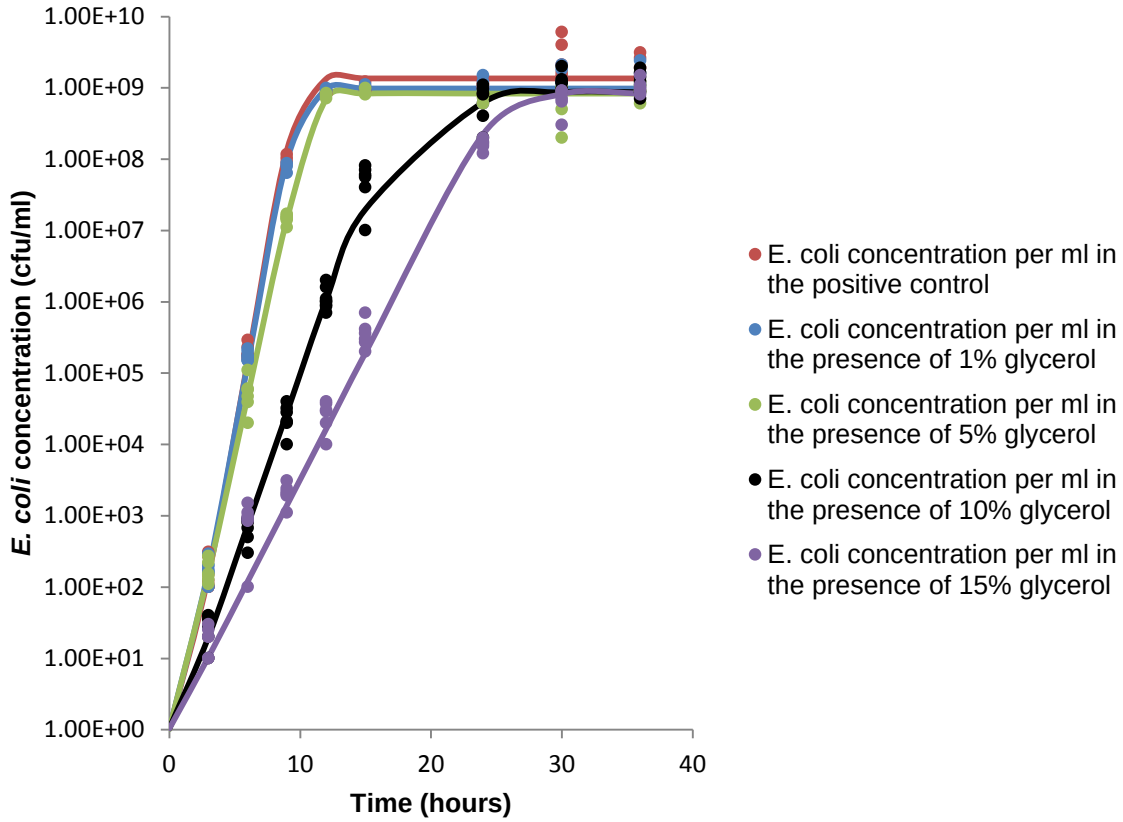


Figure 5.7 - Growth of *E. coli* in sludge derived nutrient broth supplemented with varying concentrations of glycerol. The positive control represents growth of *E. coli* in sludge derived nutrient broth without glycerol supplementation. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

5.3.4 The effect of reduced pH

The results from supplementing sludge derived nutrient with glycerol and introducing *L. reuteri* were highly successful with a 6 log reduction in *E. coli* growth recorded in the case of 10% glycerol addition (Figure 5.4). However it is hypothesised that this inhibition could be enhanced further. Lactobacilli are known to be acid-tolerant, with their optimal growth conditions being below neutral pH, around 6.0 (De Man *et al*., 1960; Giraud *et al*., 1991). When analysed using a pH meter, sludge derived nutrient broth with and without glycerol addition measured consistently between pH 7.6 and 7.9, making growth conditions less than ideal for *L. reuteri*. Furthermore though *E. coli* is also acid tolerant (Conner and Kotrola, 1995), its optimum growth conditions are more neutral, making the sludge derived nutrient more suited for *E. coli* growth. Effectively this means that in sludge derived nutrient without pH reduction the

lag time for *E. coli* will be shorter. However the lag time for *L. reuteri* will be extended as it requires time to adjust to the pH of the environment, probably via the release of organic acids such as lactic acid, which is made naturally during the fermentation of glucose, which would in turn reduce the pH of the environment and thereby allowing exponential growth. However since *E. coli* has a fast growth rate and is capable of reaching a concentration of $1x10^9$ cfu/ml within roughly 9 hours as shown in Figure 5.7, any delay in the ability of *L. reuteri* to achieve exponential growth and produce reuterin and thereby compete with *E. coli*, will ultimately lead to uninhibited *E. coli* growth. Assuming no attempts are made to pre-manufacture reuterin for introduction to sludge cake, this could have severe consequences for the success of any treatment process using *L. reuteri* as its main probiotic

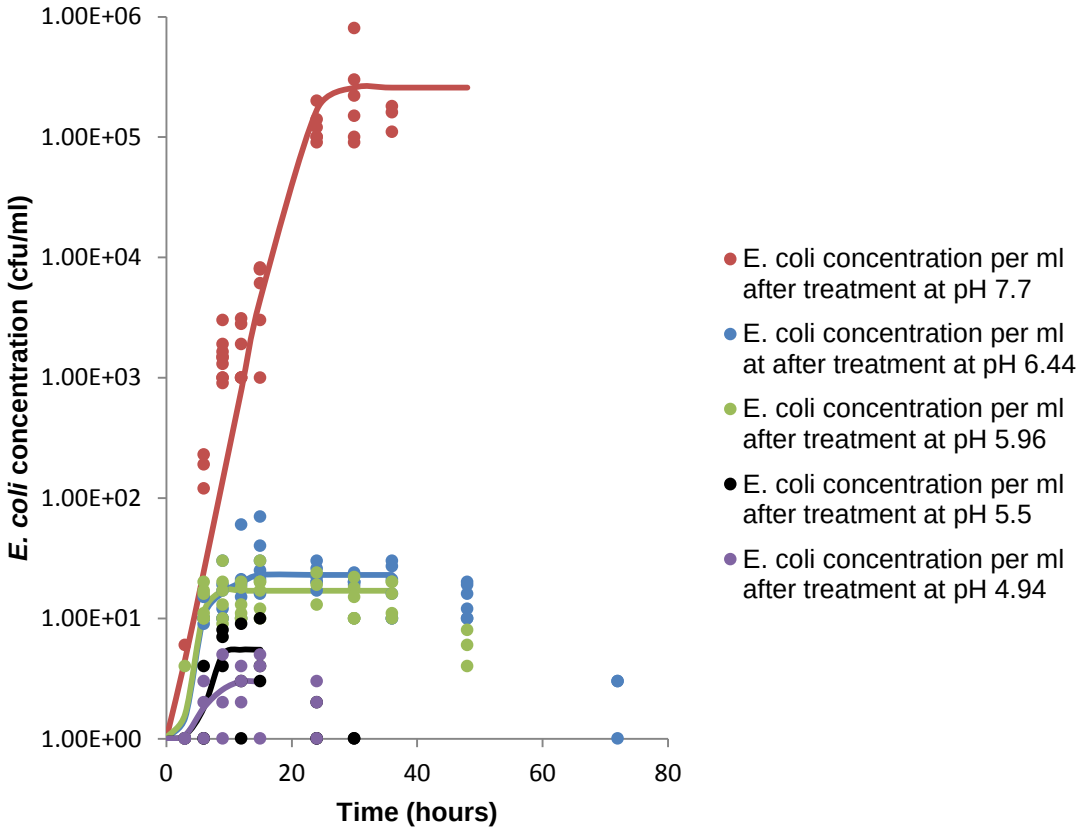


Figure 5.8 - Growth of *E. coli* in sludge derived nutrient broth supplemented with 10% glycerol in the presence of *Lactobacillus reuteri* at a range of different pH values. The positive control represents growth of *E. coli* in sludge derived nutrient at pH 7.7 without glycerol supplementation and without addition of *L. reuteri*. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

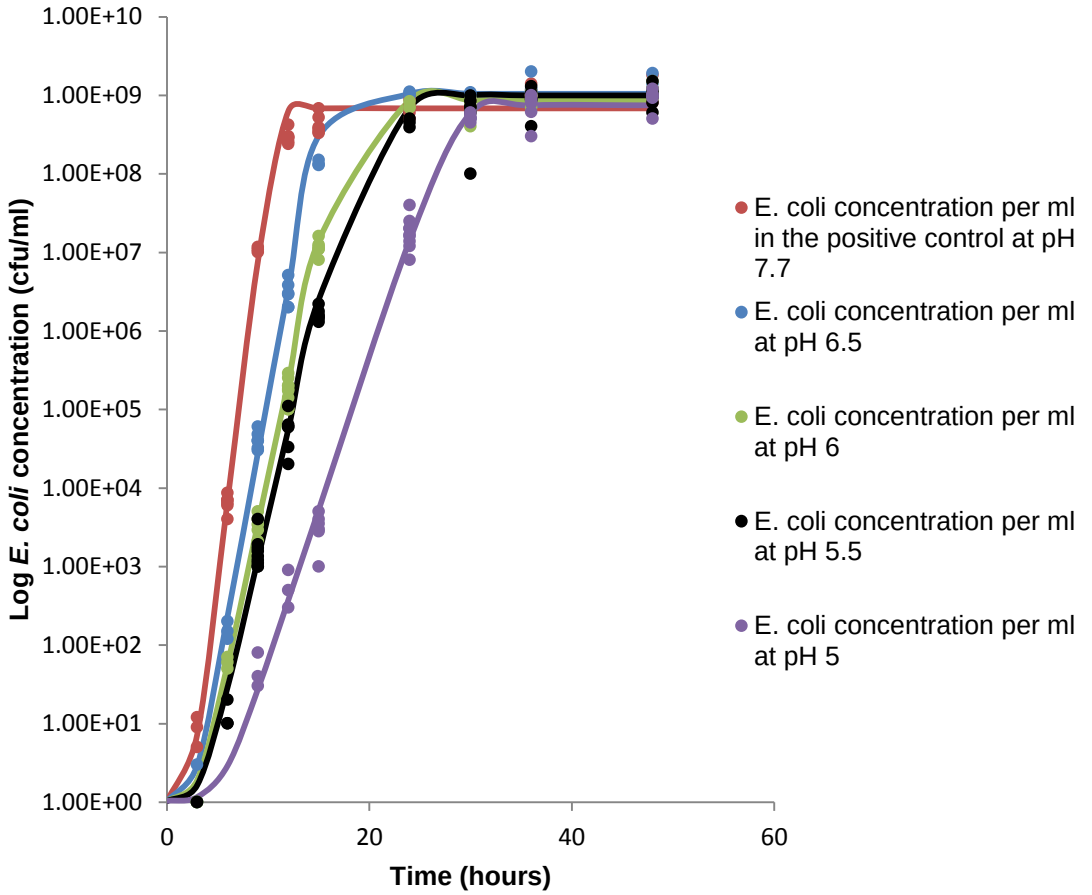


Figure 5.9 - Growth of *E. coli* in sludge derived nutrient broth supplemented 10% glycerol at a range of pH values The positive control represents growth of *E. coli* in sludge derived nutrient broth at pH 7.7 without glycerol supplementation. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

As shown by Figure 5.8, lowering the pH had a significant effect ($p < 0.05$) on the ability of *L. reuteri* to inhibit *E. coli* growth in the presence of 10% glycerol. When the pH of the sludge derived nutrient was unaltered in the presence of 10% glycerol, *L. reuteri* was capable of limiting *E. coli* growth, however in all cases where the pH reduced; *E. coli* was completely eliminated within 75 hours of the experiment. Where the pH was reduced to 5.5 and 4.94, the *E. coli* concentration measured within the sludge derived nutrient never increased above $1x10^1$ cfu/ml and was eliminated within 30 hours. As shown in figure 5.9, solely reducing the pH and adding 10% glycerol does have a statistically significant effect ($p < 0.05$) on *E. coli* growth, however the effect is only seen on the growth rate, as expected the lag time is lengthened, however maximum cell yield is not, with *E. coli* reaching a maximum cell yield regardless of pH and

glycerol concentration of between 1 x10^8 and 10^9 cfu/ml within 36 hours in all cases. This once again proves that *L. reuteri* is the source of the inhibition.

5.3.5 Isolation and confirmation of reuterin production

It is hypothesised the increased inhibition seen at reduced pH is due to *L. reuteri* being in its optimal growth conditions leading to a faster growth rate and therefore an enhanced production of reuterin. As shown in Figure 5.10 and Figure 5.11, the agar disc diffusion method though just a preliminary experiment proved that a soluble substance was being produced that led to an inhibitory effect on *E. coli* growing in ideal conditions. Though it wasn't conclusively proven that the antimicrobial substance is reuterin, it is reasonable to assume so, considering *L. reuteri* is known to produce reuterin in the presence of excess glycerol. Despite the apparent success of this preliminary experiment at most a 1-2mm zone of inhibition was recorded. Cleusix *et al.* (2007) determined the minimum inhibitory concentration (MIC) of *E. coli* to reuterin was between 7.5 and 15mM, therefore it would be expected the zones of inhibition would be larger. However it is possible that the *E. coli* used in this experiment is partially resistant to reuterin or more likely that the reuterin concentration in suspension is at a far too low concentration to be isolated and detected by methods utilised in these experiments. Indeed Talarico and Dobrogosz (1989) for example utilised liquid chromatography mass spectrometry (LC-MS) a far more sensitive method to identify reuterin. However, an alternative mechanism of action could be responsible for the inhibitory action exerted by *L. reuteri* on *E. coli.* Lactobacilli are known to produce organic acids, indeed lactic acid is the major metabolite of lactobacilli fermentation (Yang, 2000). Organic acids are known to possess antimicrobial activity (Bracey *et al*., 1998; Gould, 1991; Podolak *et al*., 1996; Thevelein, 1994; Van Immerseel *et al*., 2006; Van Immerseel *et al*., 2004; York and Vaughn, 1964). However the antimicrobial activity of an organic acid depends on its pKa, or the pH at which it is partially dissociated, which is between pH 3 and 5 (Dibner and Buttin, 2002), this would explain the enhanced *E. coli* inhibition recorded at low pH. The production of organic acids also

explains the decrease in pH recorded in the sludge derived nutrient broth following the inoculation of *L. reuteri.* However the production of organic acids does not explain the enhanced effect *L. reuteri* has on *E. coli* in the presence glycerol especially at a more neutral pH, it is therefore most probable that reuterin and organic acid production play a dual role in inhibiting *E. coli* growth. Furthermore it is hypothesised that reuterin production by *L. reuteri* occurs under anaerobic conditions (Chung *et al*., 1989). It is possible due to the aerobic nature of these experiments that reuterin production is minimalised, hence why without pH reduction and with only reuterin production as a source of inhibition *E. coli* growth is only reduced at neutral pH while at low pH with the additional inhibitory action of organic acids it is eliminated.

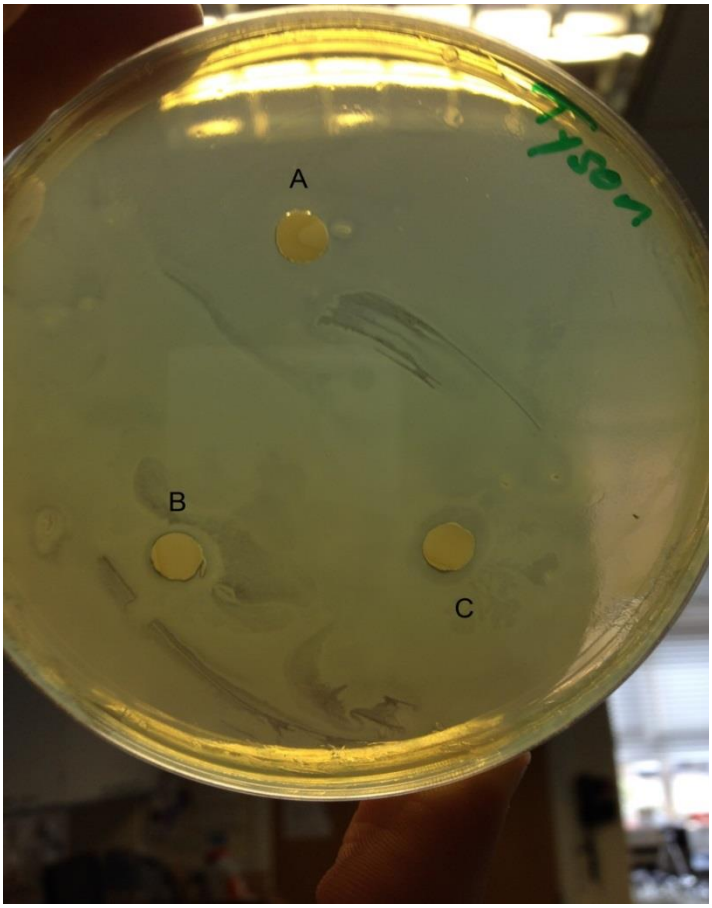


Figure 5.10 – Initial agar disc diffusion analysis on TSA plate inoculated with *E. coli* to confirm the production of reuterin. Disc A is saturated with 200mM glycerol. Disc B and C are saturated with a solution suspected to contain reuterin

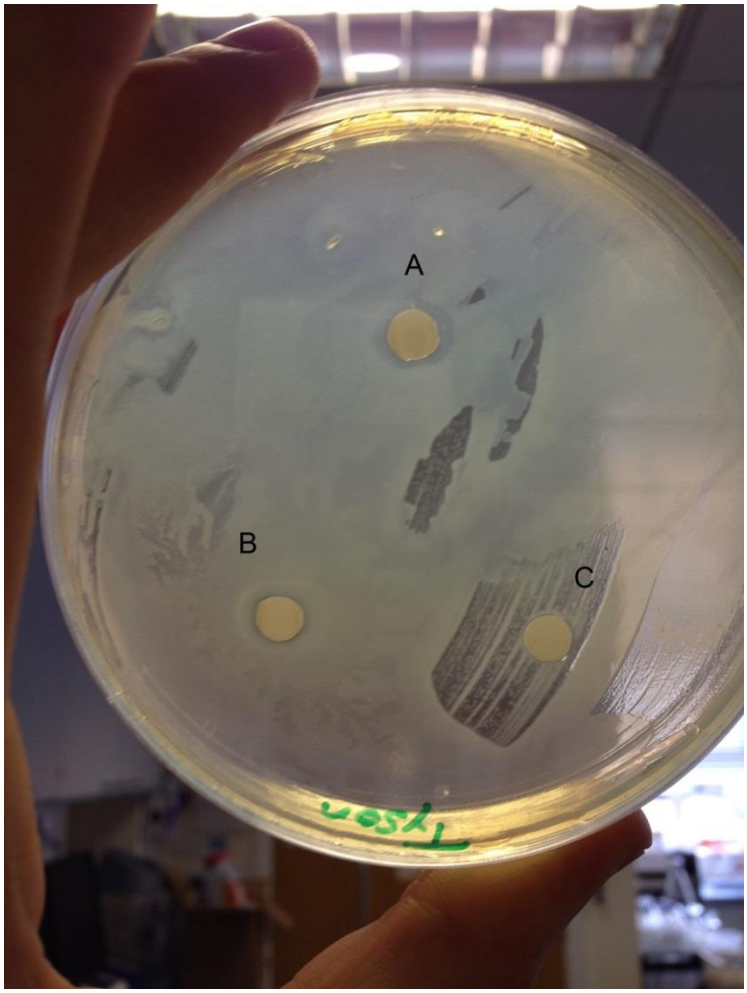


Figure 5.11 – Repeat agar disc diffusion analysis on TSA plate inoculated with *E. coli* to confirm the production of reuterin. Disc C is saturated with 200mM glycerol. Disc A and B are saturated with a solution suspected to contain reuterin

5.4 Conclusions

- *Lactobacillus reuteri* when added as a sole competitor in sludge-derived nutrient broth has an inconsistent effect on E. coli growth

- With the addition of 10% glycerol *L. reuteri* is more consistent and is able to completely eliminate *E. coli* within 48 hours

- The optimal glycerol concentration for *Lactobacillus reuteri* to have the maximum effect on *Escherichia coli* growth in sludge derived nutrient is between 10-15%

- Glycerol as a sole inhibitor does have an effect ($p < 0.05$) on *E. coli* growth at concentration of 10% and over, however at 10% and 15% only the lag time and growth rate were affected, with the maximum cell yield unaffected. This confirms *L. reuteri* is the source of the *E. coli* inhibition.

- Reducing the pH has a significant effect ($p < 0.05$) on the ability *L. reuteri* to compete with *E. coli*, reducing the pH to 6.5 and below results in complete inhibition of *E. coli* within 75 hours.

- Reduced pH and addition of 10% glycerol to sludge derived nutrient does have an effect on *E. coli* growth, but once again only the lag time and growth rate were affected, confirming *L. reuteri* is still the source of the inhibition and not pH or glycerol addition.

- The link between glycerol addition and the enhancement of the inhibitory action of *L. reuteri,* suggest reuterin production is the source. Preliminary experiments using the agar disc diffusion method proved the presence of a soluble antimicrobial substance, though the zones of inhibition were smaller than expected suggesting a low level of production. This combined with the reduction in the pH of sludge derived nutrient broth following *L. reuteri* inoculation and further enhancement of inhibitory action at low pH suggests a role for organic acids, which *L. reuteri* is known to produce as a natural part of its metabolic action.

Section 2 conclusions

The addition of undefined diverse microbial inoculum derived from soil and a fermented milk product to digested sludge cake post pasteurisation had no significant effect ($p > 0.05$) on *E. coli* growth. It is unknown why either inoculum failed to cause any significant reduction in *E. coli* growth. Although it is hypothesised that the micro-organisms contained within the mixed cultures were in majority unsuited for growth within sludge cake or in the experimental conditions. However it also thought that the rapid growth rate of *E. coli* and the fact it is adapted to the sludge cake environment alongside the questionable viability of the competitor organisms considering their origin played a role. It was therefore determined that a rapid screening step was required to screen candidates for a number of criteria (Table 4.1), including essentially an ability to grow in a sludge cake environment and compete with *E. coli.* Soil though not successful as an undefined mixed culture, would certainly contain a number of organisms which fit these essential criteria due to its high biodiversity. However identification and isolation would entail a substantial and time-consuming screening programme which would not be feasible, therefore a targeted review of the literature was performed and a number of candidates identified (Table 4.2).

Of the candidate organisms identified, *L. acidophilus, L. brevis*, *L. reuteri* and UIS were selected. For the targeted screening process sludge derived nutrient broth was used because unlike solid sludge cake certain variables do not exist such as differing moisture contents, competing indigenous microflora and changing nutrient availability and composition. This makes the results more consistent and representative of how effective the candidate organisms are at inhibiting *E. coli* growth when in optimal conditions. During the screening process it was concluded that competition for nutrients was an unfeasible mechanism of action for the inhibition of *E. coli* in digested sludge cake. Both *L. acidophilus* and UIS addition only resulted in a 1 log reduction in the maximum cell yield and didn't have any significant effect on growth rate of *E. coli*. However both *L. reuteri* and *L. brevis* were the complete opposite, with addition

leading to a significant inhibition of initial *E. coli* growth, the mechanism of action of both these organisms was speculated to be via production of antimicrobials. Out of these two bacteria, *L. reuteri* proved to be the most promising candidate. Though *L. brevis also* showed an increased capability of competing with *E. coli* in sludge-derived nutrient broth, the inhibition wasn't long lasting, with the final concentration recorded similar to that detected in the experiments involving UIS and *L. acidophilus* (Chapter 4).

In repeat experiments involving *L. reuteri*, it was the found the effect its addition had on *E. coli* growth proved inconsistent. *L. reuteri* is known to produce an array of antimicrobial compounds including organic acids and reuterin. Reuterin is produced as an intermediate step in the conversion of glycerol to 1,3-propanediol, a pathway proposed to regenerate NAD^+ from NADH and to contribute to improved growth yield. With the addition of 10% glycerol *L. reuteri* proved more consistent in its inhibition of *E. coli* growth, capable of completely eliminating *E. coli* within 48 hours. This result suggests that the production of reuterin by *L. reuteri* is not only the source of inhibition but required for *L. reuteri* to effectively compete with *E. coli.* The optimal glycerol concentration for *L. reuteri* to have the maximum effect on *Escherichia coli* growth in sludge derived nutrient was determined to be between 10-15%. However glycerol as a sole inhibitor was found to have an effect on *E. coli* growth at concentrations of 10% and over, however at 10% and 15% only the lag time and growth rate were affected, with the maximum cell yield unaffected. This confirmed *L. reuteri* as the source of the *E. coli* inhibition. To limit the hygroscopic effect of glycerol addition, but still maintain the elevated antagonistic action of *L. reuteri,* supplementation with 10% glycerol will be used in future competition experiments.

Though the results from supplementing sludge derived nutrient with glycerol and introducing *L. reuteri* were highly successful with a 6 log reduction in *E. coli* growth recorded in the case of 10% glycerol addition. It was hypothesised that the effect could be enhanced further. Lactobacilli are known to be acid-tolerant,

with their optimal growth conditions being below neutral pH. When analysed using a pH meter, sludge derived nutrient with and without glycerol addition measured consistently between pH 7.6 and 7.9, making growth conditions less than ideal for *L. reuteri*. Reducing the pH had a significant effect on the ability *L. reuteri* to compete with *E. coli*, with pH 6.5 and below resulting in complete inhibition of *E. coli* within 75 hours. Reduced pH and addition of 10% glycerol to sludge derived nutrient does have an effect on *E. coli* growth, but once again only the lag time and growth rate were affected, confirming *L. reuteri* is once again still the source of the inhibition and not pH or glycerol addition.

The link between glycerol addition and the enhancement of the inhibitory action of *L. reuteri,* suggest reuterin production is the source. Preliminary experiments using the agar disc diffusion method proved the presence of a soluble antimicrobial substance, though the zones of inhibition were smaller than expected suggesting a low level of production. This combined with the reduction in the pH of sludge derived nutrient broth following *L. reuteri* inoculation and the further enhancement of inhibitory action recorded at low pH suggests a role for organic acids, which *L. reuteri* is known to produce as a natural part of its metabolic action. Organic acid production however does not explain the increased inhibitory action recorded after glycerol supplementation, therefore it is likely reuterin and organic acids work in combination to eliminate *E. coli* at low pH, but at more neutral pH only reuterin is effective leading to only a reduction in *E. coli* growth not elimination.

The next step was to experiment with reducing the pH of sludge cake and the addition of *L. reuteri* and glycerol in solid digested sludge cake. Issues that were investigated include: the effect of lower nutrient availability in cake; the ability of *L. reuteri* to grow in solid digested sludge cake; the effect of additions on cake structural integrity; and, confirmation of the production of reuterin by *L. reuteri*, to determine the optimal conditions required to maximise reuterin production.

Section 3

Section 3: Application in digested sludge cake

Introduction

In the previous chapters it has been shown that competitive exclusion as a treatment method to reduce *E. coli* growth in sludge derived nutrient broth has been successful. Furthermore *Lactobacillus reuteri* has proven to be the most effective candidate at inhibiting *E. coli* especially when used in combination with glycerol and low pH (below pH 6.5). However sludge-derived nutrient broth does not provide an accurate representation of the conditions within sludge cake.

Firstly sludge-derived nutrient broth is likely to have more readily utilisable carbon contained within than digested sludge cake. Digested sludge cake has been through anaerobic digestion, converting readily degradable carbon into methane and more biomass. Sludge derived nutrient broth however is derived from undigested raw sludge, effectively it is a mix of fresh faeces and biomass from the filters at the Cranfield sludge treatment works**.** Therefore it is unknown if *L. reuteri* will be able to grow to the same degree as seen in broth (Figure 5.5). Furthermore previous attempts of using lactobacilli for competitive exclusion treatments in sludge cake were unsuccessful (Chapter 3), though the viability of these cultures was questionable. Second, is the presence of indigenous microflora other than *E. coli.* Sludge derived nutrient broth is sterilised via autoclaving and therefore previously the only competing organism has been *E. coli.* However in sludge cake pasteurisation only reduces the bacterial load, therefore in competition experiments in sludge cake, *E. coli* will not be the only organism *L. reuteri* has to compete with, potentially limiting growth and any inhibition on *E. coli* growth.

Finally, an important parameter of these experiments is that the sludge cake being used must retain its basic physical structure. Consequently the amount of liquid and therefore additives supplemented into sludge cake is

limited. From previous experiments it has been estimated, the limit is around 1-2ml. This is a relatively small amount considering, glycerol, sludge-derived nutrient broth to support growth, a high concentration of *L. reuteri* and acid to reduce pH must be added to replicate conditions used in sludge derived nutrient broth that proved so successful (Chapter 5).. The following chapter details the experiments setup to apply a competitive exclusion treatment methodology in sludge cake using the previous research as a guideline.

Chapters 6 focuses on the use of *L. reuteri* as the primary probiotic agent, with the initial hypothesis being *L .reuteri* can competitively inhibit *E. coli* re-growth following pasteurisation in digested sludge cake. Following on from this the emphasis was on the optimisation of the process with addition of glycerol and reduction in pH being the primary methods, on the basis of their success in sludge derived nutrient broth. These experiments are important in proving competitive exclusion to be a feasible method of pathogen control in sludge cake.

Chapter 6 Competitive exclusion using *Lactobacillus reuteri* as a means to reduce *E. coli* regrowth in solid digested sludge cake

6.1 Introduction

For several years, reports in the literature have documented significant increases in concentrations of pathogen indicator bacteria (*E. coli*/faecal coliforms) in biosolids following centrifugal dewatering (Cooper *et al*., 2010; Higgins *et al*., 2007; Qi *et al*., 2007). The concentration of *E. coli* in particular can often reach levels which exceed both enhanced and conventional treatment standards as defined by the safe sludge matrix (ADAS, 2001), making this resurgence of *E. coli* following treatment a major concern. There are a number of theories behind the cause of this phenomenon (Higgins *et al*., 2007; Iranpour *et al*., 2003; Monteleone *et al*., 2004; Qi *et al*., 2004), though no definitive mechanism has been elucidated.

Nurmi and Rantala (1973) introduced the concept of competitive exclusion treatment as a means to reduce *Salmonella* infections. This was achieved via introducing a probiotic comprised of intestinal flora from adult chicken to young birds as suspensions. A probiotic is defined as a live microbial food supplement that benefits the host by improving its microbial balance (Hentges, 1992). Competitive exclusion treatment and use of probiotics has already been adopted in a number of industries for the purpose of combating unwanted pathogen growth, examples include preventing noso-comial infectious diarrhoea in hospitals (D'Souza *et al*., 2002; Plummer *et al*., 2010) to preventing or controlling paratyphoid salmonella bacteria colonisation of the gastrointestinal tract in poultry (Schneitz, 2005). It is hypothesised that by adding a probiotic organism or culture to sludge following treatment that the *E. coli* re-growth detected following centrifugal dewatering and during sludge cake storage could be inhibited.

During the screening process for possible candidate probiotic organisms *Lactobacillus reuteri* proved to be the most effective in terms of inhibitory action against *E. coli* in sludge-derived nutrient broth. It is theorised the mechanism of action of *L. reuteri* is via the production of antimicrobial compounds. *L. reuteri* is known to produce an array of antimicrobials including reuterin (Axelsson *et al*., 1989; Talarico and Dobrogosz, 1989). Reuterin is a potent antimicrobial agent, produced via the conversion of glycerol by glycerol dehydratase (Talarico *et al*., 1988) and is proven to be active against Gram positive and Gram negative bacteria, as well as yeasts, moulds and protozoa (Axelsson *et al*., 1989). Indeed when combined with glycerol in reduced pH sludge-derived nutrient broth (adjusted to below pH 6.5), *L. reuteri* was capable of completely supressing *E. coli* growth in previous experiments. It is hypothesised that this effect can be transferred to solid digested sludge cake if the conditions of the previous experiments are replicated. It is also hypothesised *L. reuteri* will be able to actively grow and compete in sludge cake, since the bacteria has shown to be previously capable of achieving exponential growth in sludge-derived nutrient

broth. It is also speculated that *L. reuteri* will be capable of producing the antimicrobial agent reuterin in sludge cake based on the data recorded in previous experiments using sludge-derived nutrient broth. This is unsurprising considering the faecal content of sludge cake and that *L. reuteri* naturally resides in the gastrointestinal tract of healthy humans and animals (Axelsson and Lindgren, 1987; Kandler *et al*., 1980). Therefore it is feasible that a treatment method using *L. reuteri* will be successful in inhibiting *E. coli* re-growth in solid digested sludge cake.

In these experiments the ability of *L. reuteri* to inhibit *E. coli* re-growth in solid digested sludge cake following pasteurisation in the laboratory were analysed and the effect of the addition of a precursor molecule, glycerol determined. Following this the effect of lowering the pH of the sludge cake combined with the addition of a set concentration of glycerol and *L. reuteri* was investigated. Though these treatment methods proved highly successful in liquid nutrient broth derived from sludge, solid sludge cake is a highly complex growth matrix and a number of variables which cannot be readily controlled exist, which do not in the liquid based circumstances. Examples include the existence of other organisms in the form of the natural microbial microflora of sludge cake, inconsistences of the nutrient content of sludge cake and the pasteurisation process. It will be determined how these variables affect how successful the treatment method is and the extent of the difference between solid sludge cake and liquid nutrient broth derived sludge as growth media.

6.2 Materials and methodology

6.2.1 Collection and storage of digested sludge cake and strains, culture media and growth conditions

Digested sludge cake was collected from the outlet of the United Utilities' sludge centrifuge at the Ellesmere Port wastewater treatment plant and dispatched to Cranfield University via courier contained within a refrigerated box and subsequently stored at 5 ºC upon delivery. The concentration of *E. coli* contained within the sludge cake was recorded 48 hours prior to the

experiment, to ensure there as a sufficient amount (above 10^3 cfu/gDs) for an experiment to be performed. *Lactobacillus reuteri* (NCIMB 11951) was grown and maintained at 37°C on De Man, Rogosa, Sharpe agar (MRSa; Oxoid CM361). All agar was prepared following the manufacturer's instructions and autoclaved at 121°C for 20 minutes prior to use. Stock cultures were stored in a cold room at 5°C, and sub-cultured and analysed for purity every two weeks.

6.2.2 Preparation of sludge derived nutrient broth

Raw sludge from Cranfield University's sewage treatment works was first stirred to break up the biomass and ensure nutrients would be released into suspension and then centrifuged at 84g for one minute to remove the heavier biomass. The supernatant was poured off and then autoclaved at 121ºC for 15 minutes to ensure sterility.

6.2.3 Preparation of L. reuteri inoculum

L. reuteri was grown overnight in De Man, Rogosa, Sharpe broth (MRSb; Oxoid CM359) at 37°C. The cells were then harvested by centrifugation at 755 g for 10 minutes. The supernatant was removed and the cells subsequently re-suspended in maximum recovery diluent (MRD; Oxoid CM0733). 10ml of the *L. reuteri* suspension was then combined with 10ml of sludge derived nutrient in a 30ml universal bottle and vortexed for 1 minute. The original *L. reuteri* suspension was also 10 fold serially diluted in MRD and a plate count on MRSa performed.

6.2.4 Preparation and pasteurisation of digested sludge cake and addition of L. reuteri inoculum

Firstly the digested sludge cake was broken down manually to ensure an even particle size was achieved. The sludge cake was then divided into 5g sub-samples and placed in separate 30ml universal bottles. The sludge cake was then heated in a dry heat oven at 62ºC for 45 minutes. Immediately following pasteurisation 2ml of the *L. reuteri* inoculum was added to the samples, they

were then incubated at 30ºC. One set of samples post pasteurisation was inoculated with a combination of 1ml of a *L. reuteri* suspension (dilution 0) and 1ml sterile MRD, another inoculated with a combination of 1ml of sludge derived nutrient and 1ml sterile MRD and another was inoculated 2ml of sterile MRD to act as a controls, these were then incubated under the same conditions.

6.2.5 Sampling and enumeration of E. coli via membrane filtration

A sample of the sludge cake was taken immediately after the pasteurisation process and 10ml of maximum recovery diluent (MRD; Oxoid CM0733) was added to the universal and then vortexed for one minute. 1ml of the sludge cake/MRD suspension was then removed from the universal bottle and 10-fold serially diluted in MRD. 1ml of each dilution was then filtered through a 0.45µm cellulose acetate filter (Eccles *et al*., 2004; Sartory and Howard, 1992), this was performed in triplicate for each dilution at each time point. Due to the volume of the inocula to be filtered being below 10ml, additional MRD was added to the funnel to aid the dispersion of the bacteria over the entire surface of the membrane filter during the filtration process. Following filtration, the filter was placed onto membrane lactose glucuronide agar (MLGA; Oxoid CM1031) and incubated at 30°C for 4 hours and then transferred to 37°C for a further 14 hours. The colonies were then enumerated with all green colonies counted and considered as presumptive *E. coli*. This was repeated every 3 hours for 24 hours with a break after 15 hours. If the stationary phase of growth was not reached after 24 hours, sample was taken every 12 hours until reached. A sample was also analysed prior to pasteurisation to determine how effective the pasteurisation process was. The moisture content of the digested sludge cake was also established via heating a 5g sample at 121ºC for 24 hours and measuring the percentage weight difference.

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6.2.6 Effect of the addition of L. reuteri and glycerol

Following pasteurisation a combination of 1ml of the *L. reuteri* inoculum and 1ml of a sterile 60% glycerol solution was added to the samples, they were then

incubated at 30ºC. A solution of 60% glycerol was used to accommodate for diluting effect of the moisture content of sludge cake and the addition of the *L. reuteri* inoculum. It was estimated a 60% glycerol solution would actually be 10-15% in the sludge cake. One set of samples post pasteurisation was inoculated with a combination of 1ml of 50% sludge derived nutrient solution and 1ml a 60% glycerol solution to act as a control; the samples were then incubated under the same conditions. The sample procedure detailed in section 6.2.5 was then repeated

6.2.7 Effect of the addition of L.reuteri, glycerol and reduction in the pH of digested sludge cake

Firstly the digested sludge cake was broken down manually to ensure an even particle size was achieved. 5M hydrochloric acid (Fisher scientific) was added to the sludge cake to adjust the pH of the cake to between pH 4 and 5. The sludge cake was then divided into 5g sub-samples and placed in separate 30ml universal bottles. The cake was then heated in a dry heat oven at 62ºC for 45 minutes to simulate pasteurisation. Following this, a combination of 1ml of the *L. reuteri* inoculum and 1ml of a sterile 60% glycerol solution was added to the samples, they were then incubated at 30ºC. One set of samples post pasteurisation was inoculated with a combination of 1ml of 50% sludge derived nutrient solution and 1ml a 60% glycerol solution to act as a control; the samples were then incubated under the same conditions. The sample procedure detailed in section 6.2.5 was then repeated

6.2.8 Statistical analysis

See section 2.2.3.4.

6.3 Results and discussion

6.3.1 The effect of L. reuteri on E. coli re-growth

The addition of *L. reuteri* as a sole competitor had no statistically significant effect ($p > 0.05$) on *E. coli* re-growth following pasteurisation of the digested sludge cake (Figure 6.1). The mean generation time of *E. coli* in presence of *L. reuteri* with and without nutrient addition was 63.4 and 52.9 minutes respectively. In comparison in the positive controls they were calculated as 47.7 minutes with nutrient addition and 48.6 minutes without nutrient addition. First of all this result shows that nutrient addition had a very little effect in terms of generating *E. coli* re-growth in sludge cake and in fact seems to have proven more useful in supporting *L. reuteri* growth, allowing the bacteria to more actively compete. This result confirms that there is variation in terms of nutrient content between batches of sludge cake, with the cake used in Chapters 2 and 3 requiring nutrient addition to generate high levels of re-growth, however in this chapter this was not the case. This is unsurprising since sludge cake is derived from wastewater and wastewater itself is not consistent in terms of composition and therefore nutrient content and can be influenced by a number of factors including environmental (e.g. seasonal changes) and even which wastewater treatments are used to generate the sludge cake. This natural variation could be problematic in terms of consistency. It is possible a competitive exclusion treatment methodology could be highly successful in one batch of sludge cake but not in another. Apart from this, the results also show the addition of *L. reuteri* did have a minimal effect on the growth rate of *E. coli*, though it was to a much lower degree when compared with the results recorded in similar experiments but in sludge derived nutrient broth (Chapter 4 and 5). In both cases with and without sludge-derived nutrient addition *E. coli* was able to reach a final concentration of around $1x10^7$ cfu/gDs within 24 hours, a similar result as recorded for the positive control.

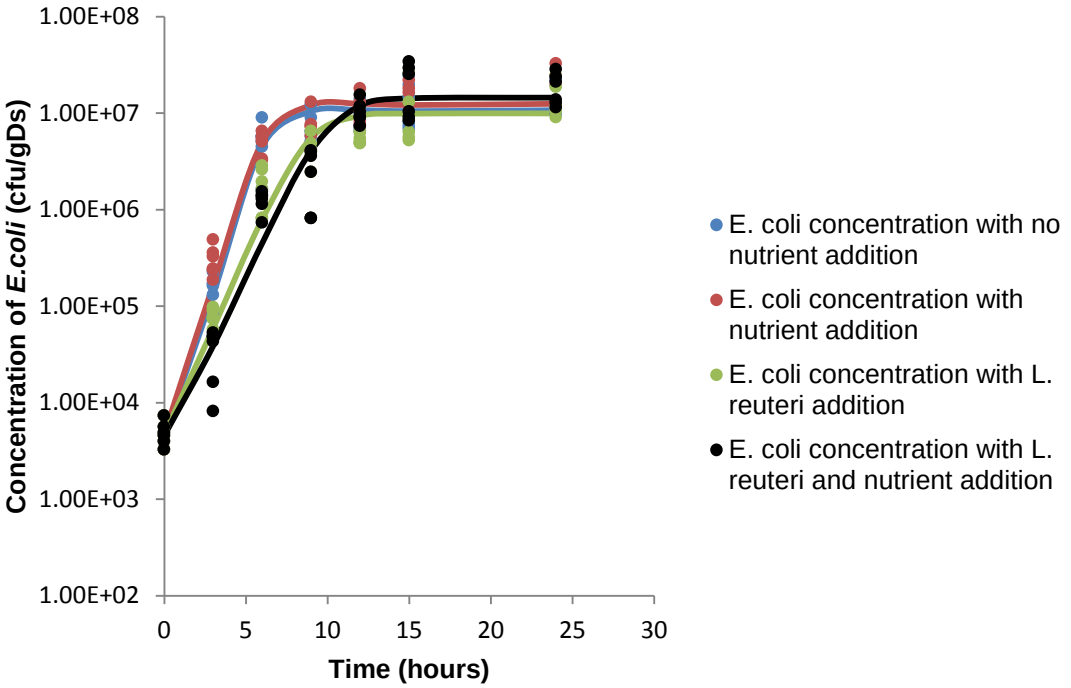

Figure 6.1 - Growth of *E. coli* in digested sludge cake in the presence of *Lactobacillus reuteri* with and without sludger derived nutrient addition. The positive control represents growth of *E. coli* in digested sludge cake with and without nutrient addition. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

There are a number of reasons for the difference between the results in liquid sludge-derived nutrient broth and solid digested cake. First, is that *L. reuteri* is unable or finds it more difficult to utilise or sequester nutrients contained within the solid sludge cake. This is supported by the minor increase in the capability of *L. reuteri* to compete with *E. coli* as a result of the addition of sludge-derived nutrient broth as evidenced by the decrease in *E. coli* growth rate. The ability of *L. reuteri* to grow in sludge cake had never previously been assessed, instead it had been assumed so based upon its ability to achieve exponential growth in sludge-derived nutrient broth. This result does suggest that the sludge derived nutrient broth screening process is flawed in terms of determining whether a candidate organism can actively grow and compete in sludge cake. As stated previously nutrient availability in terms of carbon is higher in the broth due to fact it is derived from undigested raw sludge. It is also possible that *L. reuteri* simply prefers a liquid environment to growth on a solid matrix. Either of these would lead to an increased lag time and reduced growth rate and therefore a reduced production of antimicrobial compounds including reuterin. Any delay in

the competition for nutrients and production of antimicrobial compounds would inevitably lead to the dominance of *E. coli* due to its rapid growth rate. It also has been hypothesised previously based on the variance in effectiveness of *L. reuteri* in inhibiting *E. coli* when glycerol was not readily available (Chapter 5 and Figure 5.2) that *L. reuteri* is unable to inhibit *E. coli* growth if unable to produce antimicrobials compounds. This would explain the result recorded in Figure 6.1, glycerol was not supplemented into sludge cake at this stage of the experiment and no investigation has been performed on the chemical composition of sludge-derived nutrient broth or sludge cake, so it is unknown if glycerol in any concentration is present.

Alternatively competition could be the source of the lack of inhibition. Sludge cake is known to contain a large variety of indigenous micro-organisms (Table 1.2), even following treatment and pasteurisation in these experiments a variety of heat resistant micro-organisms would inevitably survive. In a previous experiment it was shown that the ability of *L. reuteri* to inhibit *E. coli* growth can be reduced by the presence of competing organisms other than *E. coli* (Figure 4.1). In the case of that experiment, the competing organisms *Lactobacillus acidophilus* and an unidentified bacterial specimen derived from sludge were able to significantly reduce the inhibitory effect *L. reuteri* exerted on *E. coli* growth, possibly via direct nutrient competition. Furthermore in sludge cake, the competition for nutrients alone, without factoring in the possibility of antimicrobial production by other organisms would be far greater in terms of concentration and variety of competing organisms. Additionally sludge-derived nutrient broth is not a selective medium and it would have supported and aided in the growth of all the organisms contained within sludge cake, increasing the competitive pressure. Overall an increased level of direct nutrient competition on *L. reuteri* from other sources than *E. coli* would explain the difference in the level of inhibition between the competition experiments using sludge derived nutrient broth as a growth medium (Figure 4.1 and Figure 5.1) and those using solid digested sludge cake (Figure 6.1), especially considering the sludge derived nutrient broth was sterile prior to inoculation with *L. reuteri* and *E. coli.*

However due to time restrictions none of these hypotheses could be validated by performing the necessary experiment, analysing the ability of *L. reuteri* to grow in sludge cake following pasteurisation using selective agar to recover, isolate and enumerate only *L. reuteri*.

6.3.2 Effect of glycerol addition

In previous experiments the addition of glycerol, the precursor compound for reuterin production increased the ability of *L. reuteri* to consistently inhibit *E. coli* growth in sludge derived nutrient broth (Figure 5.3 and 5.4).. As shown in Figure 6.2 and Figure 6.3, the addition of glycerol at a concentration between 10 and 15% and *L. reuteri* in combination had a similar effect in solid digested sludge cake. As shown in Figure 6.2 and Figure 6.3 a significant decrease ($p < 0.05$) in the overall final concentration of *E. coli* was documented, with only $1x10^4$ cfu/gDs recorded in the initial experiment and around $3x10^4$ cfu/gDs in the repeat. This represents in both cases in comparison with the positive control a just over 1 log decrease. Furthermore in the initial and repeat experiments for the positive controls the mean generation times were calculated as 48 and 58.39 minutes respectively. However with the addition of *L. reuteri* and glycerol they were significantly increased to 85.4 and 125.6 minutes in the initial and repeat experiments, this represents a substantial decrease in growth rate. It should also be noted though in the initial experiment a more profound effect upon the lag time was recorded, with it being extended it to around 9 hours, this effect was not evident in the repeat. Overall though in both experiments the results were a significant improvement on those shown in Figure 6.1. Additionally as shown in Figure 6.3 the addition of glycerol alone did have an effect on *E. coli* growth in terms of reducing the overall final concentration of *E. coli* recorded. However in comparison with the result where *L. reuteri* was present, the reduction was minimal and there was no effect on growth rate or lag time, confirming *L. reuteri* as the primary source of inhibition. This is the same conclusion established in previous experiments (Figure 5.4, 5.6 and 5.7)

In both experiments where *L. reuteri* and glycerol were added in combination the concentration of *E. coli* within the sludge cake was only increased by 2 logs from the initial concentration measured post pasteurisation. It is hypothesised that with an enhanced pasteurisation procedure to reduce the initial concentration of *E. coli* to below $1x10^1$ cfu/gDs, the inhibitory effect of *L. reuteri* and glycerol addition to sludge cake recorded in Figure 6.2 would be sufficient to allow the sludge cake to be classified as enhanced treated. The upper limit of *E. coli* permitted for this type of sludge is 10^3 *E. coli* per gram dry weight of sludge (The Environment Agency, 2003a), which the sludge cake at the conclusion of this experiment could have satisfied. Currently with these results the sludge cake could be classified as conventionally treated since the upper limit in that case is 10^5 *E. coli* per gram dry weight of sludge (The Environment Agency, 2003a).

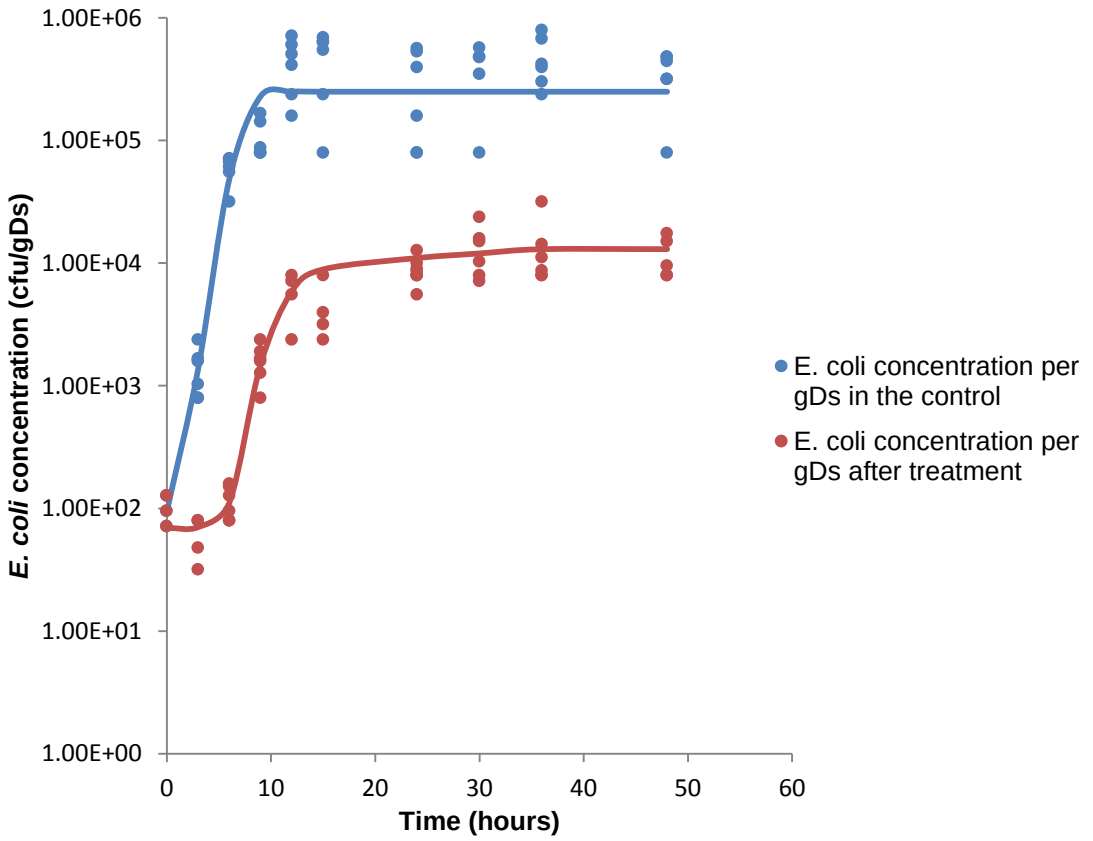

Figure 6.2 – Competitive exclusion treatment of digested sludge cake following pasteurisation, initial experiment Treatment represents the addition of *Lactobacillus reuteri* and a 60% glycerol solution The positive control represents growth of *E. coli* in digested sludge cake with addition of a 25% sludge-derived nutrient broth solution. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

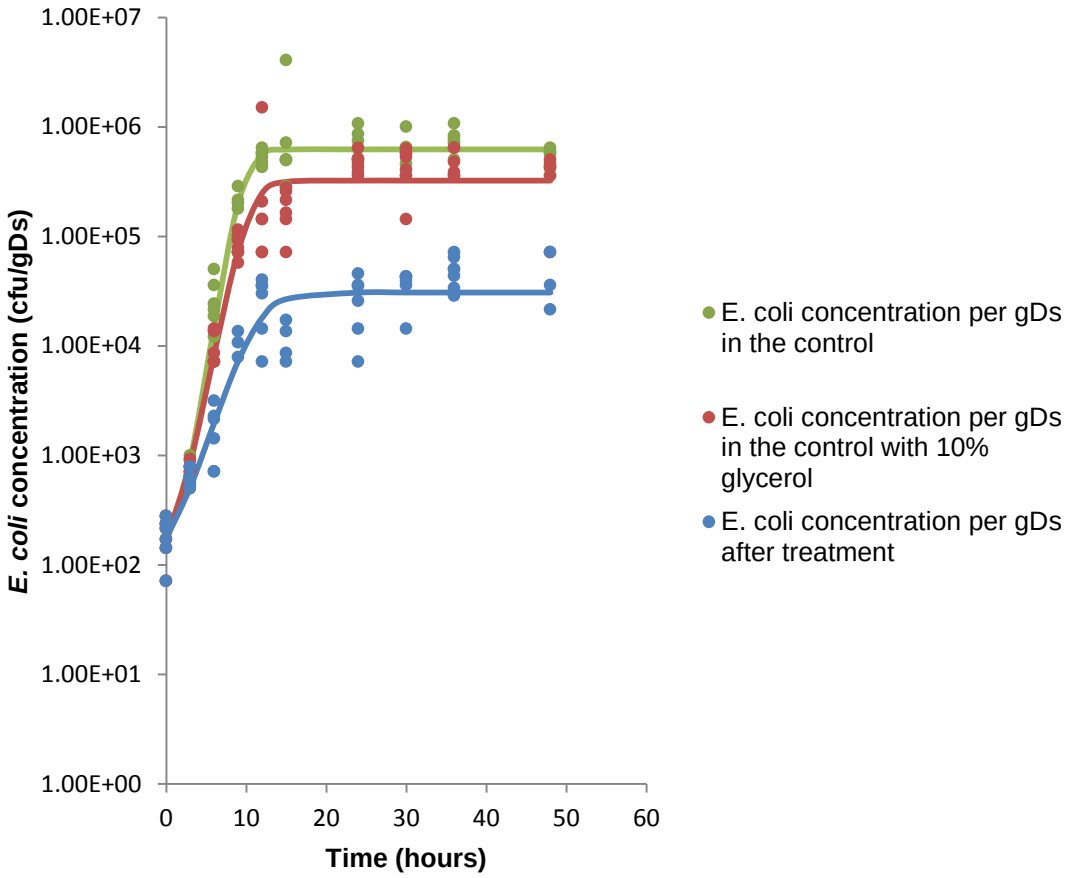

Figure 6.3 - Competitive exclusion treatment of digested sludge cake following pasteurisation, repeat experiment Treatment represents the addition of *Lactobacillus reuteri* and a 60% glycerol solution. The positive controls represent growth of *E. coli* in digested sludge cake with addition of a 25% sludge-derived nutrient broth solution and growth of *E. coli* in digested sludge cake with addition of 1ml of a 50% sludge-derived nutrient broth solution and 1ml of a 60% glycerol solution . The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

In comparison however with the results of the same competition experiment in sludge derived nutrient broth (Figure 5.3 and 5.4) the addition of 10% glycerol had much more substantial impact on the ability of *L. reuteri* to inhibit *E. coli* growth. In the case of these experiments an on average 5 log reduction in the final concentration of *E. coli* was recorded. Additionally in one instance *E. coli* was completely eliminated from the sludge-derived nutrient broth within 48 hours (Figure 5.4), further proving that *L. reuteri* is either most likely more effective in a liquid environment or is itself being competitively excluded by the indigenous micro-organisms in sludge cake. Alternatively the source of the variance in *L. reuteri* effectiveness may lie in the differing mixing procedures in these experiments. In the case of the experiments involving sludge derived

nutrient broth, the nutrient is under constant shaking and therefore *L. reuteri*, glycerol and any antimicrobials including reuterin produced are evenly distributed throughout the liquid. With solid digested sludge cake, there are certain restrictions which do not exist in the liquid medium experiments. Firstly only a maximum 2ml of treatment inoculum can be added per 5g of sludge cake, so it maintains its structural integrity. Secondly the sludge cake cannot undergo constant mixing and is only vortexed after inoculation with the treatment inoculum and prior to sampling. These two factors combined means there is a possibility the treatment inoculum is not evenly distributed and sections of the sludge cake are not exposed to glycerol, *L. reuteri* and any antimicrobials produced. Therefore in sections of the cake *E. coli* may be able to grow unrestricted leading to the higher concentrations recorded in sludge cake.

6.3.3 Effect of pH reduction

In the previous experiment (Figure 6.2 and 6.3) the pH of the sludge cake was measured to be 8.01, this is fairly alkaline. Lactobacilli are known to prefer acidic environments for growth; therefore sludge cake is less than optimal growth environment for *L. reuteri*, reducing its ability to effectively compete with *E. coli*. Reducing the pH of sludge derived nutrient broth below 6.5 and subsequent addition of a high concentration *L. reuteri* inoculum in combination with glycerol in previous experiments (Figure 5.8) led to the complete elimination of *E. coli* within 75 hours of the experiment. Where the pH was reduced to 5.5 and 4.94, the *E. coli* concentration measured within the sludge derived nutrient never increased above $1x10^1$ cfu/ml and was eliminated within 30 hours. In solid sludge cake however, the replication of the conditions of the previous experiments was not as successful.

As shown in Figure 6.4 the reduction of the pH of cake down from 8.05 to 5.85 combined with the addition of an *L. reuteri* and glycerol based treatment inoculum post pasteurisation led to an around 1.5 log reduction in *E.coli* re-growth. In comparison the addition of the same treatment to sludge cake post pasteurisation but without pH reduction, led to a less than 1 log reduction in *E.*

coli re-growth recorded. In the repeat of this experiment (Figure 6.5) the result was very similar. In the case of this set of experiments the reduction of pH increases the effectiveness of *L. reuteri* in its ability to inhibit *E. coli* re-growth. This result is the same as recorded in sludge derived nutrient broth, though at reduced magnitude, suggesting a similar mechanism of inhibitory action. Furthermore as in sludge derived nutrient broth a decrease in pH was recorded in sludge cake inoculated with *L. reuteri* from pH 5.85 to 4.83 over the course of the experiment (Figure 6.3), supporting the hypothesis that organic acids along with reuterin play a role in the inhibition of *E. coli* re-growth.

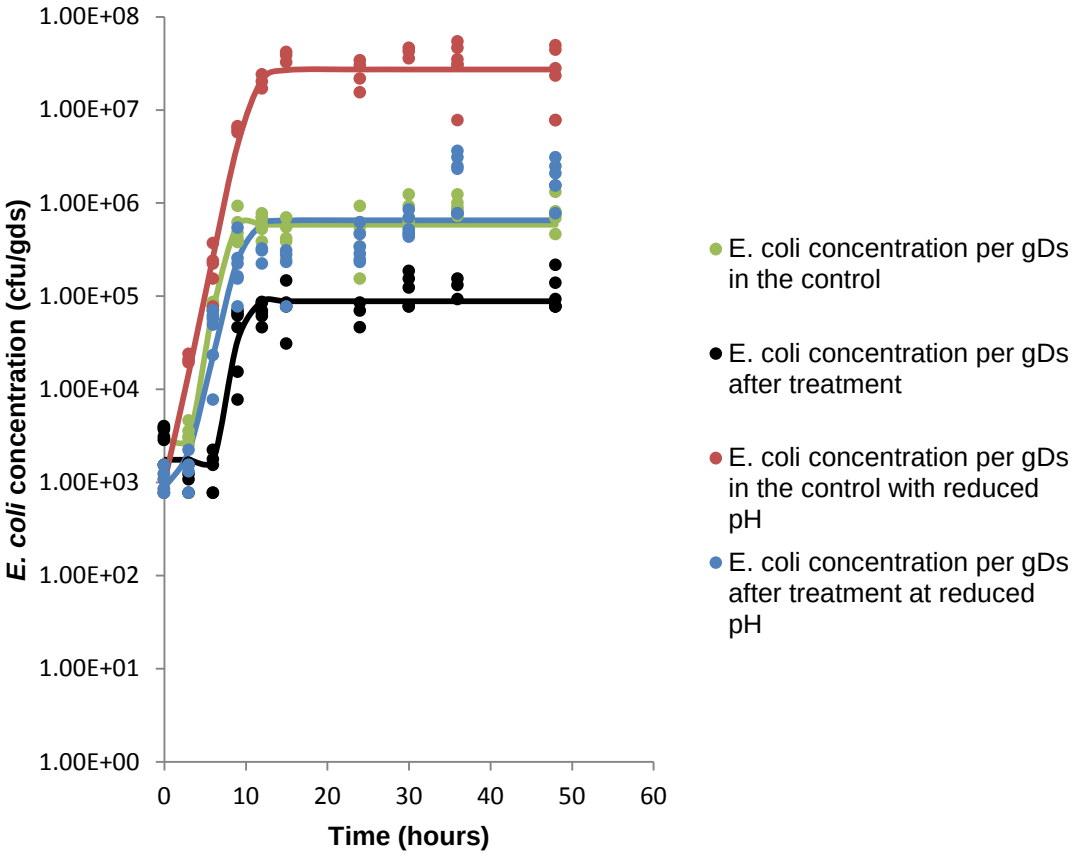

Figure 6.4 - Growth of *E. coli* in pH reduced digested sludge cake (pH 8.05) and pH reduced digested sludge cake (5.85) with the addition of a *Lactobacillus reuteri* and 60% glycerol. The positive control represents growth of *E. coli* in digested sludge cake (pH 8.05) and pH reduced digested sludge cake (5.85) with addition of a 25% sludge-derived nutrient broth solution. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

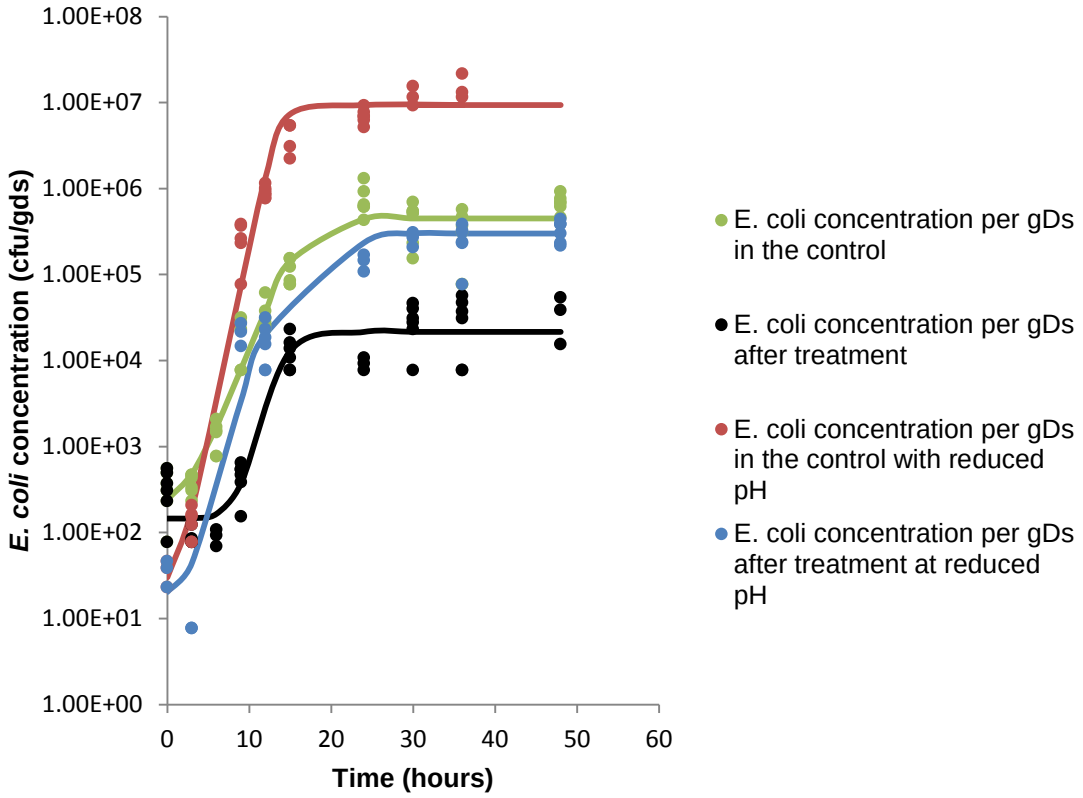

Figure 6.5 – Repeat experiment analysing the growth of *E. coli* in pH reduced digested sludge cake (pH 8.05) and pH reduced digested sludge cake (5.85) with the addition of a *Lactobacillus reuteri* and 60% glycerol. The positive control represents growth of *E. coli* in digested sludge cake (pH 8.05) and pH reduced digested sludge cake (5.85) with addition of a 25% sludge-derived nutrient broth solution. The Baranyi-Roberts model (Baranyi and Roberts, 1994) adjusted by Miconnet *et al.* 2005 was used to fit curves to the data.

However in both the initial and repeat experiments (Figure 6.4 and 6.5) reducing the pH of the sludge cake resulted in an overall 1.5 log increase in the final overall concentration of *E. coli* recorded. The cause of this occurrence is unknown, however due to it happening in both the positive control and samples with treatment inoculum addition, it is hypothesised the cause of the increase most likely lies with the reduction in pH of the sludge cake using hydrochloric acid. There is no documented evidence in the literature of hydrochloric acid addition resulting in elevated *E. coli* concentrations, furthermore in sludge-derived nutrient broth hydrochloric acid addition had very little effect on *E. coli* growth, and actually resulted in growth inhibition at higher concentrations (Figure 5.9). It is possible that the hydrochloric acid interacted with the sludge cake resulting in an elevated release of nutrients, allowing the sludge cake to support an increase level of microbial growth, leading to the higher final

concentration of *E. coli* recorded. This would explain the shorter lag time but similar growth rates recorded throughout and why such increases in *E. coli* growth didn't occur in sludge derived nutrient broth following pH reduction using hydrochloric acid. Unfortunately due to time restrictions this hypothesis could not be validating by repeating the experiment using alternative methods of reducing the pH of sludge cake.

As in the previous experiments in sludge derived nutrient broth (Chapter 5) the addition of *L. reuteri* to sludge cake led to the decrease in the pH over the course of both experiments (with and without pH adjustment). This suggests organic acids were being produced and since organic acid production is a by-product of *L. reuteri* metabolic activity, it can be deemed an indicator of growth. The consistent reduction in pH in only the sludge cake inoculated with *L. reuteri* suggests it is capable of growing and indeed thriving in sludge cake. However without analysing the growth of *L. reuteri* in sludge cake using selective media to recover, isolate and enumerate the bacteria, this cannot be confirmed. Though the increased inhibition in *E. coli* re-growth recorded following the supplementation with glycerol does suggests it is present and capable of producing reuterin. It is suggested that the reduction in inhibitory action exerted by *L. reuteri* in sludge cake when compared to sludge derived nutrient broth is not caused by an inability to grow in sludge cake or produce antimicrobial compounds. This does validate the earlier hypothesis, that a lack of constant mixing during the solid cake experiments is a likely cause of the increased *E. coli* re-growth.

6.4 Conclusions

- *Lactobacillus reuteri* as a sole inhibitor is ineffective in sludge cake in terms of inhibition of *E. coli* re-growth, with and without the addition of sludge derived nutrient broth to support growth. The cause is unknown but it is hypothesised to be due to *L. reuteri* being unable to use or sequester readily the nutrients available within sludge cake, or possibly

due to increased competition due to the presence of indigenous microflora other than *E. coli*

- The addition of glycerol with *L. reuteri*, with a minimal amount of sludge-derived nutrient broth to support early growth resulted in an around 1 log reduction in *E. coli* re-growth in sludge cake following pasteurisation in both the initial and repeat experiments. Furthermore this treatment limited growth to a 2 log increase above the initial concentration of *E. coli* recorded following pasteurisation. With these current results, the sludge cake could be designated as conventionally treated as the upper limit for *E. coli* in that case is 10^5 *E. coli* per gram dry weight of sludge (The Environment Agency, 2003a). It is hypothesised that with an enhanced pasteurisation procedure to reduce the initial concentration of *E. coli* to below $1x10^1$ cfu/gDs, the inhibitory effect of *L. reuteri* and glycerol addition to sludge cake recorded in Figure 6.2 would be sufficient to allow the sludge cake to be classified as enhanced treated. The upper limit for *E. coli* in that case being 10^3 *E. coli* per gram dry weight of sludge (The Environment Agency, 2003a).

- Reducing the pH of sludge cake in combination with the addition of glycerol with *L. reuteri*, with a minimal amount of sludge-derived nutrient broth to support early growth resulted in a minor increase in the ability of *L. reuteri* to inhibit *E. coli* re-growth in sludge cake following pasteurisation.

- In liquid sludge derived nutrient broth *L. reuteri* in combination with glycerol and reducing pH was capable of completing eliminating inhibiting *E. coli,* however this was not the case in solid sludge cake. Indeed throughout *L. reuteri* was less effective in sludge cake. It was hypothesised due to the lack of constant mixing during the solid sludge cake competition experiments.

- Reducing the pH of sludge cake however also resulted in a 2 log increase in the final concentration of *E. coli* recorded in sludge cake. It is hypothesised that the hydrochloric acid used for pH reduction interacted with the sludge cake resulting an increased release of nutrients to support growth.

Chapter 7 Final discussion

7.1 Conclusions

Analysing the experiments performed previously it can be concluded that a treatment method based upon the competitive exclusion principle, where in a probiotic organism or culture would be added to sludge cake to reduce *E. coli* re-growth is feasible. It was determined that as a probiotic agent, a defined mixed culture or sole competitor organism would be preferred. The use of undefined microbial cultures derived from soil and a fermented milk product in competition experiments proved unsuccessful. Furthermore their use as a probiotic presents a number of concerns. First it is unknown whether the inoculum itself may also contain pathogens, which in the case of digested sludge cake destined for agricultural land would be undesirable. Secondly in the event of a reduction in *E. coli* growth following treatment, it would be very difficult to ascertain the cause and which micro-organisms were responsible. However, soil cannot be ruled out as a possible source in the future for candidate organisms for competitive exclusion treatment. This is mainly due to the fact soil is known to contain a number of antimicrobial producing organisms (Basilio *et al*., 2003; Bull *et al*., 1992; Foster *et al*., 1992; Nolan and Cross, 1988; Todorova and Kozhuharova, 2010) that potentially not only have the ability to actively inhibit *E. coli* growth but also grow and thrive in digested sludge cake. It was only due to time constraints that this avenue of research was not pursued, due to the biodiversity of soil (Torsvik *et al*., 1996) and its composition largely relying on environmental factors, any screening process would inevitably be costly in terms of time.

Through screening defined cultures and individual competitor organisms for inhibitory action against *E. coli* in sludge derived nutrient broth it was concluded that the optimal mechanism of inhibition of any organisms utilised in a competitive exclusion treatment would be via the production of antimicrobial compounds. Alternative mechanisms of inhibition such as direct competition were largely unsuccessful, unsurprising considering the rapid growth rate of *E.*

coli and the conditions within sludge cake, in terms of the nutrients available, the storage temperature and pH being fairly optimal for exponential growth.

Lactobacillus reuteri proved to be the most promising candidate organism, fulfilling the criteria in terms of being a known antimicrobial producer. *L. reuteri* is a known to produce reuterin (Cleusix *et al*., 2008; Cleusix *et al*., 2007; Lüthi-Peng *et al*., 2002), a potent antimicrobial agent via the fermentation of glycerol. *L. reuteri* was capable of consistently inhibiting *E. coli* growth in sludge derived nutrient broth. Furthermore the addition of glycerol and reduction of sludge derived nutrient broth pH below 6.5 in combination enhanced the effect of *L. reuteri*, leading to the complete elimination of *E. coli*. Additionally low pH and glycerol as sole inhibitors proved ineffective, confirming *L. reuteri* as the source of the inhibition. However the mechanism of action of *L. reuteri* is suggested to be two-fold. At neutral pH it is thought reuterin production is the source of inhibition due to the link between glycerol addition and enhanced antagonistic action of *L. reuteri*. Furthermore preliminary experiments using the agar disc diffusion method proved the presence of a soluble antimicrobial substance capable of inhibiting *E. coli* growth in optimum growth conditions following incubating *L. reuteri* in broth supplemented with glycerol. However at low pH it is thought the combined effect of reuterin and the production of organic acids are the cause of the complete elimination of *E. coli*. Furthermore organic acids are a natural by-product of lactobacilli metabolic activity (Yang, 2000), the decrease in pH recorded in the sludge derived nutrient broth following the inoculation of *L. reuteri* lends evidence of their production and the fact organic acids are known to exert a higher antimicrobial activity at low pH (Eklund, 1983), validates this hypothesis.

Regardless of the mechanism of action and despite the successes in sludge derived nutrient broth, the application of *L. reuteri* in solid sludge cake post pasteurisation to reduce *E. coli* re-growth was less successful in every experiment performed in terms of magnitude of *E. coli* inhibition recorded. However despite this the addition of glycerol and *L. reuteri* in combination still

inhibited the growth of *E. coli* to a 2 log increase above the initial concentration of *E. coli* recorded following pasteurisation. With this result, the sludge cake could be designated as conventionally treated as the upper limit for *E. coli* in that case is 10^5 *E. coli* per gram dry weight of sludge (The Environment Agency, 2003a). It is hypothesised that with an enhanced pasteurisation procedure to reduce the initial concentration of *E. coli* to below $1x10^1$ cfu/gDs, a designation of enhanced treated could be achievable, with the upper limit for *E. coli* in that case being 10^3 *E. coli* per gram dry weight of sludge (The Environment Agency, 2003a). Following on from this, pH reduction in combination with glycerol and *L. reuteri* addition did slightly increase the inhibition of *E. coli* as was the case in sludge- derived nutrient broth. However the results were compromised by an elevated level of *E. coli* growth possibly caused by the interaction of the hydrochloric acid (HCl) used to adjust the pH interacting with the sludge cake leading to an increased availability of nutrients. An experiment was planned to investigate this in which two sludge cake samples, one acidified with HCl and one not, were to be sterilised and then spiked with *E. coli*, to determine if additional nutrient availability was the cause. Unfortunately this result was obtained at the conclusion of the study and there was not enough time to thoroughly perform this experiment or analyse the sludge cake further.

As for the source of the variance in *L. reuteri* effectiveness between sludge cake and sludge-derived nutrient broth there are a number of hypotheses. Firstly *L. reuteri* is unable to utilise or sequester nutrients contained within the solid sludge cake. This was supported by the minor increase in the ability of *L. reuteri* to compete with *E. coli* as a sole competitor when sludge-derived nutrient broth was also added (Figure 6.1). Prior to that experiment, the growth of *L. reuteri* within solid sludge cake had never been analysed, it had been assumed capable due to its proven ability to achieve exponential growth in sludge-derived nutrient broth (Figure 5.5) and its status as a normal inhabitant or the mammalian digestive tract. However, without an experiment to analyse the growth of *L. reuteri* in sludge cake, these hypotheses cannot be definitively confirmed. Though if this hypothesis is correct it does suggest that using

sludge-derived nutrient broth in the screening process to determine the capability of candidates to grow in sludge cake is flawed and maybe should be limited to proving an ability to compete with *E. coli*, or replaced altogether. A promising alternative would be utilising sterilised sludge cake, wherein sludge cake would be autoclaved to eliminate all micro-organisms. Following this step a competitor organism would be spiked into the sludge cake and the growth monitored. Using sterilised sludge cake would ensure any candidate organisms would be capable of growing and competing in sludge cake and would provide a more realistic screening process. However it is unknown how the autoclaving process would affect the physical and chemical composition of sludge cake and as stated previously there is natural variation in composition of sludge cake making it an inconsistent growth medium. Furthermore sludge-derived nutrient broth though not ideal, does provide a fast and simple screening process, with easily controllable variables.

Another viable hypothesis for the variance in results between sludge derived nutrient broth and sludge cake is competition. In a previous experiment (Figure 4.1), the ability of *L. reuteri* to inhibit *E. coli* growth was reduced by the presence of two other competing organisms, in that case *L. acidophilus* and UIS. In sludge cake this competition would magnified as sludge cake is known to contain a large variety of indigenous micro-organisms (Table 1.2) a number of which will have the capability of antimicrobial production and even following treatment and pasteurisation in these experiments a variety of heat resistant micro-organisms would inevitably survive. A possible solution to this would be to pre-manufacture the reuterin and add it directly to sludge cake. However this does pose the risk that any resistant strains to the antimicrobial would be unaffected by the treatment and therefore free to grow, leading to re-growth regardless.

However the favoured hypothesis is that unlike the sludge derived nutrient broth competition experiments; sludge cake cannot undergo constant mixing and is only vortexed after inoculation with the treatment inoculum and prior to

sampling. These two factors combined means there is a possibility the treatment inoculum is not evenly distributed and sections of the sludge cake are not exposed to glycerol, *L. reuteri* and any antimicrobials thereby produced, leading to unrestricted growth of *E. coli*. This is validated by decrease in sludge cake pH recorded following inoculation with *L. reuteri*. This suggests organic acids were being produced, which due to them being a metabolic by-product can be deemed an indicator of *L .reuteri* growth, suggesting it is capable of growing and indeed thriving in sludge cake. Therefore the reduction in inhibitory action exerted by *L. reuteri* in sludge cake when compared to sludge derived nutrient broth is likely not caused by an inability to grow in sludge cake and therefore production of antimicrobial compounds. This validates the hypothesis that the lack of constant mixing during the solid cake experiments is the most likely cause of the increased *E. coli* re-growth. A possible solution to this is scaling up the experiment. At present a 5g sub-sample in a 30ml universal bottle is difficult to thoroughly mix consistently, however if the process was to be scaled up to a higher quantity of sludge cake in a larger container this process would be simpler. Alternatively *L. reuteri* and glycerol could be added prior to sludge cake manufacture, to ensure even mixing in the liquid digestate.

Overall in conclusion the use of competitive exclusion treatment to reduce or prevent *E. coli* re-growth in digested sludge cake is feasible with the appropriate organism. *L. reuteri* did show promise as a possible candidate and was capable of reducing the re-growth of *E. coli* to below conventionally treated sludge standards (The Environment Agency, 2003a). It is hypothesised that with an enhanced pasteurisation procedure to reduce the initial concentration of *E. coli* to below $1x10^1$ cfu/gDs, enhanced treated sludge standards could have been achieved. With an improved understanding of how reuterin produced and the array of antimicrobials *L. reuteri* is capable of producing including organic acids, any inhibition of *E. coli* recorded in these experiments could be enhanced further. For this reason it is concluded that *L. reuteri* is suitable for scale up experiments. However using the current methodology that eventuality is improbable. If used in the exact same concentrations in terms of glycerol and *L.*

reuteri in particular if used in a higher quantity of sludge cake the amount of reagents and consumables required would be enormous and unfeasible at present. Furthermore considering the hypothesis of a required mixing step, this would be essential, probably to the scale of what is used in an anaerobic digester (Figure 1.2) if any scale up was performed to ensure a constant distribution of glycerol, *L. reuteri* and any antimicrobials produced. Also further research is required on the reduction of pH in combination with *L. reuteri* and glycerol addition, as the effect recorded in sludge-derived nutrient was too promising to discount on the basis of a hypothesised unforeseen reaction to the addition of hydrochloric acid to sludge cake.

The competitive exclusion principle as a treatment method shows great promise. Apart from *L. reuteri* from the literature lactobacilli do seem to be a good starting point for candidate selection, with *L. acidophilus, L. plantarum* and *L. salivarus*, known to produce antimicrobials and in the case of a few are known to be utilised in the control of spoilage microbial growth in silage. This method of treatment also has possible applications in other industries and is worth pursuing in the future.

7.2 Experimental limitations

The primary limitation of these experiments involved the inconsistencies between batches of sludge cake delivered from the United Utilities' sludge centrifuge at the Ellesmere Port waste water treatment plant. Despite being collected from the same location, in the same quantity and delivered to Cranfield University by the same method, there was a constant shift in the nutrient availability of sludge cake batches. This led to poor results or complete experimental failures. In sludge cakes batches with poor nutrient availability, *E. coli* re-growth was sub-standard and not reflective of what is recorded in industry, therefore not suitable for competition experiments and required the addition of a sludge derived nutrient broth to support growth and make it usable. However the addition of sludge derived nutrient broth to sludge cake with high nutrient availability led to a higher rate of *E. coli* re-growth, causing variance in

results. Furthermore there was no method of assessing the nutrient content of sludge cake prior to an experiment. A possible solution to this would have been manufacturing the sludge cake, rather than relying on a supplier. This would have ensured that no extra nutrient would have been required to generate *E. coli* re-growth and *L. reuteri* and glycerol could have been added prior to cake manufacture.

A secondary limitation was that the sludge cake upon delivery had already undergone a cycle of re-growth during transport from Ellesmere Port to Cranfield University, leading to an on average high concentration of resident microbial growth. This level of growth made the cake unsuitable for use in re-growth or competition experiments, therefore to reduce microbial load to allow for re-growth, a pasteurisation step had to be added to the methodology, which was successful.

A third limitation was the inconsistencies in the microbial concentration within solid sludge cake upon delivery. While assessing the *E. coli* concentration using MLGA plates, it was found on occasion that sludge cake had a low *E. coli* concentration but high faecal coliform concentration and sometimes the exact opposite. This led to inconsistencies in the pasteurisation process. The conditions used in pasteurisation for sludge cake with a low resident *E. coli* concentration were often too extreme, leading to the complete elimination of *E. coli* and experimental failure. In sludge cake with a high resident *E. coli* concentration the conditions were not strong enough, leading to a sub-standard pasteurisation and higher starting *E. coli* concentration, which in the case of competition experiments is undesirable. To monitor the inconsistencies, the concentration of sludge cake stored was measured upon delivery and 24 hours prior to an experiment.

A fourth limitation was that the amount of fluid that could be added to sludge cake and therefore the amount of competitor organisms to reduce *E. coli* re-growth was restricted. Due to the high moisture content of sludge cake (70-

75%) the amount of fluid that can be added without the cake losing its structural integrity was limited to 2ml in 5g cake. Maintaining the physical and chemical properties of cake was an important condition of any treatment method. This proved difficult considering especially in the final competition experiments cake the requirement for the addition of sludge derived nutrient broth, glycerol and a high concentration of *L. reuteri.*

7.3 Future research

The work carried out during this thesis suggests substantive possible future research opportunities as follows:

- More research is required on the cause of the increase in *E. coli* growth as a result of acidification of sludge cake using hydrochloric acid. The method when used in combination with *L. reuteri* and glycerol, led the complete elimination of *E. coli* in most cases. If the methodology could be successfully applied in sludge cake and the problems overcome it would be very advantageous.

- The addition of more vigorous and constant mixing step than vortexing to test the hypothesis that a lack of mixing led to the reduction in the ability of *L. reuteri* to inhibit *E. coli* in sludge cake. If a simple procedure such as constant mixing is the only obstacle preventing similar reductions in *E. coli* growth recorded in sludge-derived nutrient broth to be repeated in sludge cake, then it has to be implemented. To consistent mixing procedure with a 5g sample of sludge cake is unfeasible, therefore a scale up would be required for this to be achieved. However a scale up does pose its own challenges in terms of the amount of reagents and consumables required to achieve the same conditions utilise in bench scale experiments. Furthermore the pasteurisation process would have to be intensified due to the increase quantity of sludge cake being used.

- Analysing the possible application of other organisms such as lactobacilli known to produce antimicrobials such as *L. plantarum* and *L. salivarus*. Furthermore look at the possibility of pre-manufacture of antimicrobials for addition to sludge cake. This has the benefit of removing the requirement for the candidate organisms to competitive or indeed be able to grow in sludge cake. However it does pose the risk that resistant micro-organisms would survive and have no competitors to hinder growth leading to re-growth regardless of treatment

- Analyse the possible application of a defined mixed culture of lactobacilli known to produce antimicrobials.

- Analyse the effect of the consistent use of antimicrobial producing bacteria in sludge cake, in the generation of highly resistant strains in particular of *E. coli.* This poses a problem, if resistant strains emerge and the competitive exclusion treatment relies on the production of antimicrobials then the treatment will no longer be feasible.

- Longer term experiments, to analyse the long term survival of *L. reuteri* and *E. coli* in storage conditions and to assess the long term effectiveness of any antimicrobials produced.

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