CANINE FAECES: THE MICROBIOLOGY OF AN ENVIRONMENTAL HEALTH PROBLEM

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Dedication

I dedicate this thesis to my family, especially to my mother and to my father's soul. To my wonderful wife Maryam Al-Magasbi, who supported me during every step of the way. To my children Azizza, Attia and Mohammed, thank you for patience and understanding. Finally, to all my teachers, my brothers, my sisters and my friends and my neighbours; I respect you all and, I dedicate my modest work to you.

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Abstract

The overall aim of the research work reported in this Thesis was to study a variety of aspects of dog faeces in relation to public health, their fertilizer potential and possibility that such faeces might be remediated using larvae, ultimately to provide a source of biodiesel. The results can be summarized as follows:

1) Dog faeces were shown to be source of pathogenic bacteria, notably *Escherichia. coli* and *Salmonella*. These bacteria were shown to be transferred to the soil of a local playing field by direct, *in situ,* transfer from dog faeces undergoing weathering. *E. coli* and *Salmonella enterica* were isolated from all four sites while no such isolates were obtained from the fifth location which was uncontaminated with dog faeces

2) It was shown here that "common or garden" slugs can transfer potentially pathogenic bacteria from dog faeces to lettuce.

3) The feeding of Black Soldier Fly Larvae on faeces led to a statistically significant increase in the number of bacteria inside the BSFL gut and the same trend was seen in relation to dog faeces fed Fruit Beetle Larvae. This trend of increasing bacterial numbers in larvae fed on dog faeces is particularly worrying in relation to the potential feeding of these larvae to animals- post exposure to faeces.

4) Dog faeces were shown to have potential inherent fertilizer content; the nutrients present being released over a time period mimicking the natural weathering of dog faeces in the environment.

5) As a generalization, the addition of both types of larvae to dog faeces significantly reduced the concentration of indigenous plant nutrients over the entire four week incubation period; exceptions to this were nitrate and phosphate concentrations in BSFL treated faeces, where significant increases were seen at week 4 and 3 respectively and in faeces treated with FBL, where ammonium concentrations were significantly increased at weeks 2-4, and phosphate at week 4. While the addition of both larvae therefore

initially decreased levels of indigenous plant nutrients there was a trend in some of the nutrients to increase the longer the incubation went on. This suggests that perhaps a longer term exposure of dog faeces to the two larvae might have lead to increase in ammonium, nitrate, sulphate and phosphate concentrations. The addition of ammonium, elemental sulphur an insoluble phosphate to dog faeces which had been modified by the two larvae led to significant increases in nitrate, sulphate and plant-available phosphate, results which shows that that dog faeces contains the indigenous microflora required for the transformation of these amendments (which simulate fertilizer addition). The results suggest the possibility that larval modified dog faeces could be used as compost additive fertilizer, or perhaps even be used as an agricultural soil fertilizer.

6) The potential for using fly larvae for the bioremediation of dog faeces was investigated. Black Soldier Fly (BSFL) and Fruit Beetle (FBL) Fly larvae were shown to dramatically improve the physical nature of canine faeces, even after only a short exposure period, giving a bioremediated product which is markedly improved in terms of texture, reduced odour and overall reduced offensiveness. The bioremediated dog faeces product was also found to be suitable as potting compost when "diluted" with proprietary potting compost.

7) The haemolymph and total body extracts of BSFL and FBL were shown to be antibacterial.

8) The potential for using dog faeces and dog faeces which had been treated with BSFL and FB as a source of biodiesel was determined. It was shown that potential biodiesel precursors) (mainly fatty acids) were present both in the raw dog faeces and in faeces which were treated with the two different larvae.

9) The number of bacteria present in dog faeces disposed of in plastic bags dramatically increased over exposure to the UK summer, when temperatures were recorded between 10-27^{0}C.

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Chapter One: Introduction

1.1. The environmental problem of canine faeces

The Dog (*Canis lupus familiaris*) is a member of the Canidae family of the mammalian order "Carnivora. Dogs have been adapted for economic usefulness and are loyal and generally protective of humans, being used as guide dogs for the blind and disabled; their keen sense of smell is also used to detect bombs or drugs (Kim, 2008; Murray, 2007). In contrast, dogs are a source of danger to humans through their bites, and because they transmit zoonotic diseases such as rabies, toxoplasmosis, echinococcosis, trypanosomiasis, filariasis, spirocerosis, hydatidosis, larva migrans etc. (WHO, 1959; Oduyemi and Olayemi, 1977; Hill *et al.*, 1985) resulting in death. Canine waste which is not removed from the local environment due to the irresponsible behaviour of dog owners may represent a source of potential pathogens. Pathogenic bacteria can survive in canine faeces for a long period and can be spread by wind and vehicular traffic. Faeces can also be carried inside dwellings via contaminated shoes (Tarsitano *et al*., 2010). The interactions between plants and other animals including invertebrates in the field may also be an important means of transmission of pathogenic bacteria to crops.

Contamination of the urban environment by dog faeces continues to be a growing problem around the world. While in the UK societal changes in thinking about the dog dirt problem has led more people to pick up the waste products of their pets, our streets continue to be polluted with dog faeces.

1.2. Microbiology of dog faeces

Dog faeces present two major problems. Firstly they smell and are generally offensive, secondly, and more importantly, they spread microbial and parasitic diseases. Since dog faeces often contaminate parks and playing fields, children are likely to be

frequently exposed to the likelihood of catching such damaging and potentially fatal infections. The disease spread to humans by dog faeces include:

Campylobacteriosis and Yersiniosis, dogs which eat raw pork are infected with *Yersinia enterocolitica which can cause* enteritis in dogs and cats; the organism is also shed in the faeces for several weeks after infection, even in the absence of clear symptoms (Fredriksson-Ahomaa, *et al*., 2001), Dogs may therefore be an obvious source of human infection and this has shown to be high amongst children under six years of age, so dog faeces may provide a transmission link for pathogenic bacteria between pigs and young children. Campylobacteriosis is a bacterial infection causing diarrhoea in humans. Wright *et al*. (1982) found that for dog faeces collected in urban parks *Campylobacter* were isolated from 260 collected samples, while *Salmonella* species were found in only three. Most of the *Campylobacter* were isolated during the warm months of June and July, i.e. they were present when children are most likely to be at play outside.

Salmonellosis, represent a very large group of rod-shaped, gram negative bacteria including more than 2000 known serotypes which belong to the family of *Enterobacteriaceae*. All these serotypes are human pathogens and can cause various symptoms from mild gastroenteritis to severe illness or death. In 1890 more than 30 people out of every 100,000 in the United State died of typhoid caused by *S. typhi. Salmonella* can cause food poisoning from eggs, pork, chicken and beef (Jacquelyn, 1999; Blancou *et al.*, 2005) and is the most common bacterial infection transmitted to humans, symptoms include, fever, muscle aches and vomiting and diarrhoea.

Escherichia coli is a gram-negative rod of the family *Enterobacteriaceae* and is found in the gastrointestinal of all warm-blooded animals. All strains of *E. coli* are spread by the faecal-oral transmission route. Many strains can cause gastroenteritis; among these

are the enterotoxgenic (ETEC), enteropathogenic (EPEC), enteroinvasive (EIEL), or enterohemorragic (EHEC) *E. coli.* (Maier *et al.*, 2009).

Shigella flexneri is an intestinal bacterium responsible for severe diarrhoea in people and non-human primates (monkeys); it is also found in dogs (Wang *et al.* 1996). About 25,000 cases are reported in people in the United States every year. Many monkeys carry this bacterium without symptoms, while people are also commonly carriers. Small children at child care facilities and people who handle monkeys are most at risk. The diarrhoea produced in people exposed to human or monkey waste is never life threatening but during the two to three weeks the diarrhoea lasts, the victim is quite ill. The chief danger from this disease is dehydration.

Streptococcus* and *Staphylococci these bacteria are found on all animals that typically associate with humans. In the great majority of cases they cause no disease in the animal. However, in pets, eye infections are occasionally associated with Streptococci and skin infections with Staphylococci. Both bacteria can spread from pets to humans on contaminated hands and objects. Infections are generally limited to the skin and eyes.

Faecal coliforms cause dysentery herpes, typhoid fever and ear infections in humans. Not surprisingly these bacteria are well represented in dog and other animal faeces (Whitlock *et al*., 2002).

1.3. Parasites and diseases associated with canine faeces

1.3.1. Toxoicariasis

Toxoicariasis is the main medical problem relating to dog faeces. It is an infection of the round worm *Toxocara canis* (Gillespie, 1988) and is a zoonotic disease spread via unwashed vegetables and dog faeces (Karadam *et al.*, 2008); young people are particularly at risk due to their weaker immune systems and because of their likely increased exposure by ingesting the eggs (Thompson *et al.*, 1986). Puppies, which are a

major source of environmental contamination, can pass up to 15,000 eggs per gram of faeces. Each *T. canis* female can lay up to 700 eggs a day and these are excreted when the dog defecates; they can survive in soil for as long as three years. After two to three weeks of warm weather the eggs develop into an embryo state, containing larvae which are infective to dogs and people. The larvae attempt to migrate throughout the human body like they would do in a dog's, but the human body regards them as foreign and reacts leading to tissue damage (Overgaauw and Nederland, 1997). Two types of toxocariasis exist, namely: visceral larva migrans (VLM) and ocular larva migrans (OLM). In VLM, the larvae reach the liver, causing inflammation and symptoms including abdominal pain and pyrexia; most people however, recover spontaneously. OLM occurs when a migrating larva reaches the eye where it causes the formation of a granuloma on the retina which leads to significant visual impairment and in severe cases, blindness; around 12 new cases of OLM are diagnosed annually in the UK (Despommier, 2003).

1.3.2. Toxoplasma contamination of dog faeces

Toxoplasma gondii is an obligate intracellular protozoan with worldwide distribution (Frenkel 1990, Wallace, 1973), where it can cause blindness in humans. It is found in the Americas, including southern Mexico, Central America, South America, and the West Indies. Infections with *Toxoplasma* are very common in Panama, although most infections are asymptomatic. Antibodies titres to *Toxoplasma* are high in children, notably where cats and dogs are numerous, sanitation is bad and there is high shade and high humidity. Cats are the main hosts of *Toxoplasma*, but the parasite is also found in dogs (Barutzki and Schaper, 2003). Pets become infected by consuming rodents and birds, which are intermediate hosts that contain cysts (bradyzoites) that help to continue the chronic infection. When pets eat infected animals, the bradyzoites develop into the

enteroepithelial stages and the shedding of oocysts in the cat or dog faeces. The oocysts then sporulate in the soil and then lie dormant from up to weeks or months, especially when in moist, shaded areas. Sporozoites within the oocysts are then infectious to humans and other mammals after being passed by hand to mouth, after which tachyzoites and bradyzoites (multiplying asexual stage) continue to induce active infection. Dogs have only recently been considered a factor in the spread of toxoplasmosis (Frenkel, 1996). Interestingly, by eating or rolling in cat faeces, dogs probably play an important role in the mechanical transmission of *Toxoplasma* oocysts (Lindsay *et al*., 1997) and it is likely that dogs excrete oocysts near human habitats, promoting *Toxoplasma* transmission to humans.

1.3.3. Other protozoan livestock parasite disease related to dog faeces

There is a growing link between two specific protozoan diseases in livestock and the fact that faeces, from infected dogs, is increasingly found on grazing land (Dubey and Lindsay, 2006). The two main diseases in question are:

1.3.3.1. Neosporosis

This disease caused by the parasite *Neospora caninum* is responsible for the highest rate of all cattle abortions reported in the UK. Once this disease occurs in cattle it can remain in the herd as a result of vertical transmission of the parasite between cows and their calves. *Neospora* eggs are produced by infected dogs and then excreted into their faeces. Cattle then become infected when they eat food or drink water contaminated with the eggs. Infection in cattle is common and generally ill effects are not clear, either for the cow or the calf (Williams *et al.,* 2000) The disease becomes obvious when *Neospora* multiplies in the cells of the developing calf and its placenta and causes damage sufficient to bring about abortion or stillbirth. Control of *Neospora* abortion is difficult and there are no drugs available at present to control this disease in cattle or to

cure the infected animals. Similarly, no vaccine is currently licensed in the UK to prevent cattle-neosporosis. Fortunately, current evidence shows that *Neospora* is not a major problem for humans . (Anderson *et al.*, 1995)

The vertical transmission of neosporosis is a main cause of long standing infection within a herd, although spread of the disease between females which are not related only occurs where a dog acts as host to the parasite. The parasite can be picked up by dogs through the consumption of contaminated livestock material, including placentas from newly calved cows, or by being fed contaminated raw meat, faeces from infected dogs then contaminate pasture and also cattle feed, water or bedding (Anderson *et al.*, 1997); (Davison *et al.*, 1999).

Only a small number of infected dogs develop the disease, which produces progressive lameness and paralysis in pups younger than 6 months of age. Infected bitches can pass the parasite to their young during pregnancy by transplacental infection. If dogs do develop symptoms, then the results are usually fatal or lead to euthanasia (Reichel *et al.*, 2007). This disease is very important since it impacts farm economics due to infected cows being more likely to abort and the occurrence of premature culling and reduced milk yields. Since there is no way to prevent (through vaccination), or an effective treatment of neosporosis, a farmer's main defence against the disease is to take action against any likely *Neospora* contamination (Dubey, 2003).

1.3.3.2. Sarcocystosis

This is a disease which is also caused by a parasite, in this case *Sarcocystis* spp, which employs a number of intermediate hosts, including dogs. Sarcocystis eggs are produced by infected carnivores and are excreted in their faeces, and sheep become infected when they eat food, or drink water contaminated with *Sarcocystis* eggs. In many cases, infected livestock show no disease symptoms (Traub *et al.*, 2002). The

disease can be transmitted from ewe to lamb during pregnancy, but vertical transmission is not believed to be important. Dogs can pick up the parasite through the ingestion of contaminated material from carcasses, or by consuming contaminated raw sheep meat. Faeces from infected dogs can also contaminate pasture as well as animal feed, water or bedding. In contrast to neosporosis, no transmission of the *Sarcocystis* parasite occurs between bitch and puppy. The link between infected dogs and sarcocystosis in sheep is compelling, but the disease is generally regarded as less of a problem than neosporosis. No vaccine is available against sarcocystosis in sheep and although there are some treatments available, the high cost and practicality of administration of these prevents their spread use. As with neosporosis, the most feasible option for the farmer is to reduce infection risks. In addition to, round worms, giardiosis, tuberculosis, gastroenteritis and cryptosporidiosis and Cystercercosis, is a human disease involving larval tapeworms. Although parasitic infections are important in relation to dog faeces, no attempt was made here in this thesis to study these infectious agents . (Dubey and Williams, 1980)

1.4. Chemical composition of dog faeces

Dog faeces (and those of cats) contains about 0.7% nitrogen, 0.25% phosphate and 0.02% potasium. As a result, dog faeces are not a particularly good plant fertilizer; they are offensive and often oderous and contain pathogens, as well as *Toxoplasma*. In it its unweathered state therefore dog faeces are not a useful, let alone, ideal organic fertilizer.

Currently most dog waste is allowed to breakdown naturally in the environment where it is deposited, and where collected it is usually incinerated. Dog faeces are not ideal additives to composting plants and as a result, their addition to municipal composters is generally avoided. Similarly, because of their low nutrient and high

pathogen content they are not regarded as ideal or safe fertilizers for agricultural, garden or allotment use.

The problems relating to dog faeces and the environmental pollution they cause has been largely overlooked and surprisingly little research work has been published in this area.

Here are some facts about dog ownership and the resultant waste problem relating to the UK: There are around 24 million UK households and, in 2002, the number of households owning dogs was 4.8 million. Some 21% of households with dogs have more than one. There are around 6.8 million dogs in the UK, the highest levels of dog ownership being among the 45 to 54 year-old age group - around 30%. It has been calculated that the UK dog population produces some 900 tonnes of faeces every day and over a ten-year lifetime, a dog can produce up to half a ton of faeces.

There are an estimated 41 million and 60.7 million dogs in Europe and the USA respectively. Australia has one of the highest rates of pet ownership in the world, with almost 36% of Australian households having a dog (Australian Companion Animal Council 2010).

In the UK, the legal position relating to dog fouling is covered by the following statutes:

The Dog (Fouling of Land) Act 1996 in England and Wales. The Dog Fouling (Scotland) Act 2003. The Litter (Animal Droppings) Order 1991 - Made under section 84 (14) of the Environmental Protection Act 1990 and Statutory Instruments Numbers 2762 and 2763.DoE Circular No 18/96 (Welsh Office No 54/96).

These Acts require that the owner should immediately clean up after his or her dog, should it foul what is termed 'designated land'. Designated areas are usually defined as places where dog faeces have the potential to cause a health hazard to people, including children's play parks, public greens and parks, residential areas, cycle paths and

walkways etc. Individual local authorities can use these Acts as a basis to create bylaws (which allow for instant fixed-penalty fines) and nominate the designated areas in the appropriate borough. The fines begin at around £40, rising to a maximum of £1,000. Dog wardens may be employed to patrol these areas and catch those irresponsible dog owners who fail to remove their dog's faeces.

The Government recommends that the dog faeces-related disease problem can be reduced by the following actions:

a) Poop scooping on each occasion your dog makes a mess.

b) Dogs should be wormed regularly-every three to six months, using a wormer recommended by a veterinary surgeon.

c) Dogs should be exercised in dedicated areas of parks where available.

d) Dogs should be discouraged from parks having children's playgrounds.

e) Pregnant women and individuals who suffer from impaired immunity should use additional extra precautions when cleaning up the faeces, for example by wearing disposable gloves.

1.5. Transmission of pathogenic bacteria from dog faeces to human food

Because of immunization and the use of litter boxes and flea treatments, the transfer of pet diseases to humans has greatly been reduced over the years, but still some dog related diseases infect people; most troubling of all when people are infected by their pet, they usually are unaware of it. Besides the risk of bites, scratches and allergies, several infections can be transmitted to the human as zoonosis. These pathogenics have an oral-faecal transmission cycle and humans can be infected either by faecal contamination of food, water or the environment (gardens, sandpits and playgrounds) or by direct contact (Overgaauw *et al.*, 2009). Outbreaks of diseases caused by infective bacteria as well as parasites have been documented to occur as a result of consumption

of contaminated salad and vegetables. The studies described in this Thesis relating to this problem focus in the potential consumption of contaminated lettuce. Sources of pre-harvest contamination of produce include manure, from livestock operations, and domestic animals (Beuchat, 2006).

Diseases caused by pathogenic bacteria, such as Salmonellosis and Campylobacteriosis are the most frequently reported zoonotic diseases transmitted from animals to humans via food (Norrung and Buncic, 2007). Infections with verotoxin-producing *E. coli* O157 are comparably less frequent but of considerable public health concern as they are associated with life-threatening human diseases such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Roldgaard *et al.*, 2004). Although a variety of foods may serve as vectors of food borne illness, the farm is the original source of all of these pathogens and there is strong association between prevalence in food production and other animals and post-harvest prevalence on carcasses (Elder *et al*., 2000).

1.6. Slugs and snails as intermediate host-vectors of pathogenic bacteria

Slugs (Mollusca: Gastropoda) are a major pest of fruits and vegetables (Godan, 1983; South, 1992), with the Grey Garden Slug *Deroceras reticulatum* (Stylommatophora Agrioimacidae) being the most widespread and most serious pest (Wilson *et al*., 1993). Slugs, by serving as intermediate hosts for many bacterial diseases mainly found in animals faeces (South, 1992), present a risk to plants, animals and humans. Slugs ingest soil bacteria during feeding and as a result, they become hosts of many bacteria including pathogens like such as *E. coli* O157. These can be picked up from dog faeces or from animal faeces which have been spread in open fields (Figure 1.1) (Walker *et al*., 1999).

Emma *et al*. (2006) conducted research on the Yellow Slug (*Limax flavus*) and the Great Gray Slug (*Limax maximus*), and confirmed that both species can carry *E. coli*

O157, both on the surface and internally. The persistent slug species *Deroceras reticulatum* can carry *E. coli* on its external surface for 14 days (Emma *et al*., 2006). Slugs which ingest *E. coli* also pass viable bacteria to their faeces and *E. coli* was found to persist for more than 3 weeks in excreted slug faeces (Hogan, 1985). In a similar study Emma *et al.* (2006) showed that *E. coli*, through contact and/or ingestion can survive for many days both internally and externally.

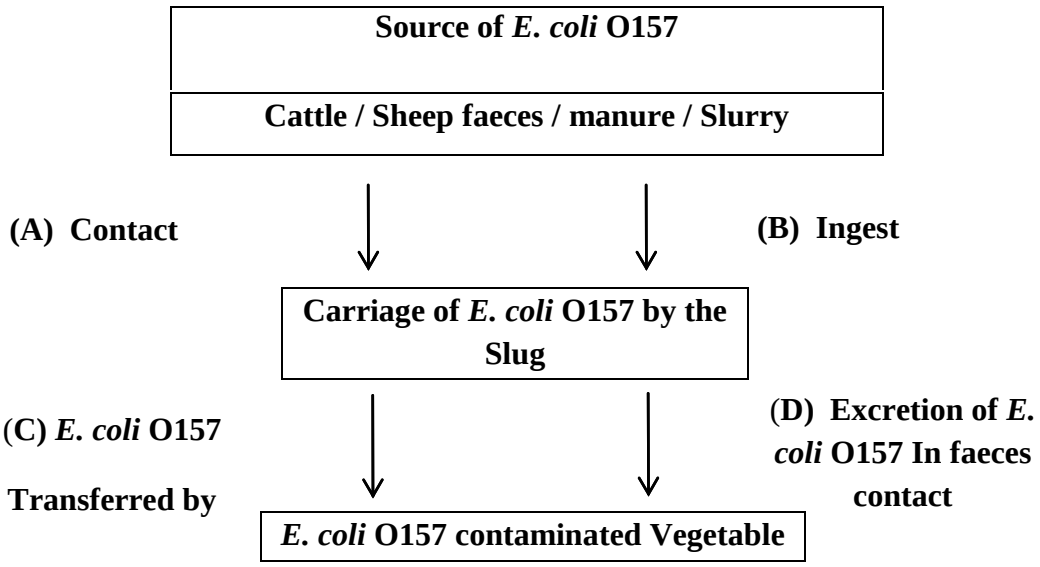


Fig 1. 1: Anticipated transfer pathways of *E. coli* O157 by slugs from an environmental source to vegetable crops.

1.7. Waste composting

Increases in the human population and the expansion of large cities have lead to marked increase in the volume of all kinds of waste. The search for modern approaches to waste management, notably composting has recently gained momentum (Pascual *et al*. 1997; Bhattacharyya *et al*. 2001a and b; Smith and Hughes 2004). Composting is now often a preferred approach to waste management (Lee *et al*. 2004; Sharholy *et al*. 2008) and is defined as the *biological oxidative decomposition of organic matter*

(Stoffella and Kahn 2001) based on the catalytic activity of environmental organisms which are responsible for organic matter decomposition. Under optimum conditions, three stages of traditional composting have been identified: 1) mesophilic, or moderate temperature phase; 2) thermophilic, or high temperature phase and 3) cooling or maturing phase (Kostov *et al*. 1996; Trautmann and Olynciw 2000). The duration of these phases relates to the type of organic matter under compost and its efficiency, which is largely determined by aeration and humidity (McKinley and Vestal, 1985; Strom 1985 , Strom *et al*. 1983; Butler *et al*. 2001).

1.8. Composting and recycling of dogs waste

The risk of canine waste accumulation in the urban environment and agro-ecosystem is an increasing problem. Recycling is a sustainable approach for disposing of waste, and composting can be an important component of recycling approaches. The microbes involved oxidize carbon as an energy source for growth and take in nitrogen for protein synthesis (Taylor, 2004). The correct carbon to nitrogen ratio in composting systems is required for the efficient decomposition of wet dog waste contains 0.7% nitrogen (N), 0.25% phosphate, compared to wet cattle manure, dog waste which contains 40% more nitrogen, the same amount of phosphate, and a twentieth of the amount of potash (Hall and Schulte 1979). Dog waste composting reduces the amount of waste being sent to landfills (Sequi, 1996) and also reduces the amount of methane being released into the atmosphere (Peigne and Girardin 2004, Albaladejo *et al.* 2000). Because of the high nitrogen content of dog manure, a rich source of carbon is required for composting, such as wood chips, shavings or sawdust (C:N ratio of 560-641:1) (Rynk 1992, Miller 1996).

1.9. Bio-conversion of putrescent waste using BSFL

After seven years of research, a patented bioconversion process that effects a 95% reduction in the weight and volume of food waste within a matter of just a few hours has been developed using the Black Soldier Fly. This unique approach to bioconversion requires no energy, no electricity, no chemicals, not even the addition of water. It is totally self-contained and does not produce effluent and, while it produces a small amount of carbon dioxide, it does not produce any other greenhouse gases, such as methane (Craig Sheppard *et al.*, 2002).

The Black Soldier Fly BSF (*Hermetia illucens*) is a tropical fly (Craig Sheppard *et al.*, 2002) indigenous to the whole of the Americas, from the south of Argentina to Boston and Seattle, and in World War II, the fly also spread into Europe, India, Asia as well as Australia.

As a result, the currently discussed bioconversion process does not require the introduction of a foreign or exotic species, involving as it does an organism which is indigenous to the Americas, and now Europe. It is also not associated with the transmission of disease. The BSF has the ability to thrive in the presence of salts, alcohols, ammonia and a various food-based toxins and can process food waste as well as swine, human and poultry waste. Upon reaching maturity, the larvae of the BSF migrate out of the bioprocessing unit into a collection bucket without any human or mechanical intervention and thereby provide a self-harvested grub which is rich in nutrients and is food source which rivals in commercial value the finest fish meal. Unlike many other flies, BSF adults do not enter dwellings and since they do not have functional mouth parts, they do not eat waste and cannot (like the House Fly) regurgitate on human food, and therefore are not involved with disease transmission. Black Soldier Fly adults do not, in any way, bite, bother or annoy humans.

1.9.1. The Black Soldier Fly life cycle

Soldier fly adults come together in small numbers near an isolated bush or tree in order to find and choose a mate. After mating, the female finds an ideal place to lay her eggs, and lays about 900 eggs in 5 to 8 days lifespan. Housefly adults, by contrast, often live for 30 days, and during this long period, they eat, and as result actively spread disease.

Male BSF do not go near wastes since they do not lay eggs. The females in fact, prefer to lay their eggs not upon the waste, but either above or to the side of it, thereby allowing the eggs a far better chance of survival. The eggs are relatively slow in hatching (102 to 105 hours). The newly hatched larvae then crawl or fall onto the waste and begin to eat it with unbelievable rapidity. It takes about two weeks for the larvae to become mature a period which may extend to 6 months if the temperature is not right, or if there is not enough food. This ability of BSF larvae to extend its life cycle under conditions of stress is very useful when using it waste bioprocessing.

Black Soldier Fly larvae pass through 5 stages or instars. When mature, pre-pupal larvae are about 25mm long, 6mm in diameter, and weigh around 0.2 grams. These larvae are extremely tough and robust and can survive under conditions of extreme oxygen starvation. They can be also subjected to several 1000 gs of centrifugation without being harmed (Lord *et al.*, 1994).

1.9.2. Bioconversion of dog faeces using Black Soldier Fly larvae (BSFL)

Black Soldier Fly larvae have been used to dramatically reduce food waste and manure, and convert the nutrients from food waste and manure into insect larval biomass containing over 40% protein and over 30% crude fat (Newton *et al*. 2005). As a result, BSF larvae are potentially excellent source of protein and therefore as a high energy meal for chicken, fish and other domestic animals. As an added benefit,

BSF larvae also aerate and dry manure, thereby increasing rates of breakdown and reducing odours. BSF larvae add value to soil composition as a result of digesting organic material and the release of larval faeces into the soil, which promotes plant growth (Jeon *et al.*, 2011).

1.9.3. The Texas Experimental BSF Composting Research Programme

In an experiment conducted in Texas over a period of one year, it was found that BSF larvae are able to digest over 15 kilograms per day of restaurant food waste per square meter of feeding surface area, or roughly 3 lbs per square foot per day, leading to a 95% reduction in the weight and volume of such waste. As a result, for every 100 lbs of restaurant food waste placed into a bio-processing unit, only 5 lbs of a black, friable residue remain. Over 100,000 active larvae can be found in a typical waste disposal unit, and they can eat and digest just about any type of putrescent waste, including meat and dairy products. The instant waste is deposited into the unit, the larvae begin to secrete enzymes into the waste long before it begins to rot and smell. Since thermophilic and anaerobic bacteria play no role in this process, the larvae can conserve and recycle the majority of the nutrients and energy within the waste (Alvarez, 2012).

1.9.4. Rates of bioconversion

Over a period of one year, approximately 20% by weight of the fresh food waste is converted into fresh larvae. This food waste had an average dry matter content of 37%, and the pre-pupae has an average dry matter content of 44%, i.e., on a dry matter basis, the bioconversion of food waste situates at almost 24%. An input of 100 kg of food waste per day can be handled by three 6-foot bioconversion units.

The BSF pre-pupa is composed of:

42.1% crude protein, 34.8% ether extract (lipids), 7.0% crude fibre, 7.9% moisture, 1.4% nitrogen free extract (NFE),14.6% ash, 5.0% calcium and 1.5% phosphorus.

Live BSF pre-pupae have been successfully fed to bull frogs, tropical fish, reptiles, snakes and many other creatures that have a strong preference for living food, and the value of fresh BSF larvae ranges from $4 to $20 /lb. Chickens are especially fond of the live larvae (Amatya, 2009).

1.10. Does winter pose a problem to composting?

In winter, bioprocessing using BSF can be maintained by simply placing a styrofoam sheet on top of the larval residue to retain the heat generated by larval movement. If this heat is not allowed to escape, the temperature on the surface of the residue easily exceeds 35 degrees C. During summer, the conversion rate of fresh food waste into fresh larvae runs as high as 20%, but during winter, this conversion drops to less than 5%, in spite of the fact that the larvae digest roughly the same daily quantity of food waste per unit surface area. Under the right ideal summer conditions, it takes about two weeks for newly hatched larvae to reach the mature pre-pupal form, but during the cold of autumn and winter, this two-week period may last as long as six months. Well-insulated, BSF technology units can be introduced into some of the coldest regions of Earth. During the hot summer months, overcrowding can often occur, a process which leads to relatively high temperatures within the unit; so as to cool the unit down, some actively feeding larvae are forced to exit the unit (Craig Sheppard *et al.*, 2002).

1.11. Slugs as agents of disease transmission

Slugs (Mollusca: Gastropoda) are the major pests of large varieties of vegetables and fruits in fields, home gardens, landscapes, greenhouses, (Godan, 1983; South, 1992). Slugs can also present a risk to plants, animals and humans because they serve as intermediate hosts for many bacterial diseases which predominate in animal faeces (South, 1992). Slugs usually ingest bacteria in soils as an important source of food. Consequently, they become hosts of many bacterial strains, such as *E. coli* O157, either

via direct contact or being contaminated with animal faeces spread in open fields (Walker *et al*., 1999). Emma *et al*. (2006) showed that both the Yellow Slug (*Limax flavus*) and the Great Gray Slug (*Limax maximus*) carry *E. coli* O157 both on the surface and internally, and that slugs are contaminated with *E. coli* through contact and/or ingestion. Elliot (1969) also suggested that slugs possibly carry *E. coli* O157, and Dawkins *et al.* (1986) showed that four slug species transmit the agent of bacterial soft rot of potatoes (*Erwinia carotovora*) although this was considered to be accidentally rather than obligatory.

1.12. Transfer of bacterial species via slugs and snails to lettuce.

The enteric tract of dogs, like mammals, possesses a complex microbial ecosystem, including several bacteria such as Streptococci, *Bifidbacteria, Lactobacilli, Bacteroides* and *Clostridium* (Drasar and Hill, 1974; Drasar and Barrow, 1985). *Acinetobater baumannii* isolated from a number of dogs and cats, is spread (Thierry *et al*., 2008) nosocomially, a fact which explains the occurrence of several strains *of A. baumannii* in the veterinary hospital environment among dogs.

Some invertebrates may be responsible in transmission of the *E. coli* and other bacteria to field crops either by direct contact or contamination with animal faeces. The greatest concerns to human pathogens on fresh vegetables and fruits is represented by the enteric pathogens (e.g. *E. coli* O157:H7 and *Salmonellae*), which can grow before being eaten.

1.13. Detection and confirmation of Mycoplasma in dog faeces

The first documented occurrence of *Mycoplasma* in dogs was in 1934. During the last 70 years, 15 known species of *Mycoplasma* have been recognised and several have been isolated from or detected in dogs (Chalker, 2005). The isolation of a *Mycoplasma* from a human was first reported in 1937 (Taylor-Robinson, 1996; Kudva *et al*., 1998).

Four species, *M. hominis, M. orale, M. pneumoniae, M. salivarium* have been isolated in humans as well as dogs (Colaizy *et al*., 2003).

Culture techniques remain the most commonly used approaches to the detection of the presence of *Mycoplasma* in environmental samples such as canine samples. However, there are now a wide variety of indirect test methods available for *Mycoplasma* detection, including PCR- based kit, DNA fluorochrome staining, autoradiography, ELISA (McGarrity *et al*., 1985; Lincoln and Gabridge, 1998; Rottem and Barile, 1993)

The most widely used recommended indirect test is DNA fluorochrome staining (McGarrity *et al*., 1983), an easy and relatively fast procedure which stains DNA using a fluorescent dye. When stained and fixed cells are examined under a UV microscope equipped with the proper filter package, DNA fluoresces brightly and negative control slides should always be used to help interpret staining results. These positive and negative *Mycoplasma* control slides are commercially available. The best overall testing approach is a combination of both methods: direct culture can be provided very high sensitivity while DNA fluorochrome staining can detect any fastidious *Mycoplasma*.

1.14. Nitrification

1.14.1. The Nitrogen Cycle

Nitrogen is essential for life, it is the main component of amino acids which are the building blocks of peptides and protein, and is found in important biological components such as chitin and mucopeptides; it is also an integral part of the genetic material of cells, the nucleic acids. Plant growth in soils throughout the world is often restricted by the supply of available N and, as a result, it is nitrogen supply, more than any other soil nutrient which limits UK and world crop production. Because of this large amounts of nitrogen are applied globally as fertiliser in order to increase crop productivity (Lam *et al.*, 1996)

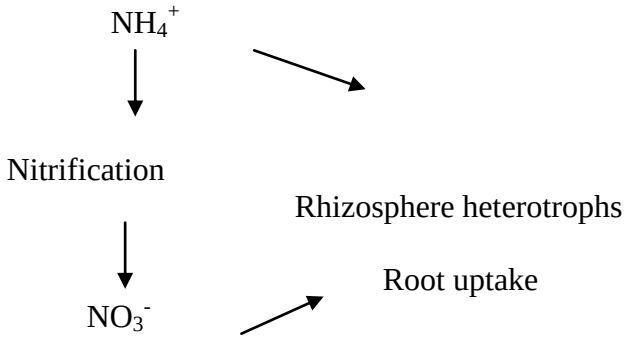
. In agricultural systems, the need to understand the nitrogen cycle is of extreme importance if maximum crop yields are to be achieved. In natural ecosystems, no additional, fertiliser nitrogen is applied, but the need to understand the soil N-cycle is just as critical.

1.14.2. Ammonification

The great bulk (95-99%) of the soil nitrogen is in organic compounds which are largely unavailable to higher plants (Pate, 1973). When soil microorganisms degrade these compounds, simple amino compounds (R-NH2-) are formed. Many soil microorganisms are able to deaminate amino acids (e.g. bacteria, actinomycetes and fungi) with the resultant release of ammonia. Any NH_4-N that accumulates in soil represents the quantity of substrate nitrogen in excess of microbial requirements (Richards, 1987). Ammonium production is referred to as ammonification and the fate of the ion varies, depending upon conditions in the soil. Ammonia as a gas is volatile and leaves the soil; however, if dissolved in soil water the ion-NH_4 is formed. Ammonium can be accumulated and utilized by plants and microorganisms and under favourable conditions can be oxidized to nitrate.

The flow of nitrogen in the soil is intimately tied to flow of carbon and the processes involved in the nitrogen cycle bring about changes to the soil environment that also have an influence on other soil processes and cycles

Recently, the definition of nitrification has extended to refer to the biological oxidation of any reduced of nitrogen to a more oxidised form (Killham, 1994).



1.14.3. Autotrophic nitrification in soil.

It is generally accepted that the major type of nitrification in most agricultural soils is chemoautotrophic, largely carried out by the Gram-negative bacteria *Nitrosomonas* and *Nitrobacter*. (Killham,1994). The reactions carried out are summarised below

$$NH_4^+ + O_2 + 2e^- \xrightarrow{\textit{Nitrosomonas}} NH_2OH + H_2O$$

Ammonium hydroxylamine

$$NH_2OH + H_2O \longrightarrow NO_2^- + 5H + 4e$$

Hydroxylamine nitrite

$$NO_2^- + 5H + 4e \xrightarrow{\textit{Nitrobacter}} H_2O.NO_2^- \longrightarrow NO_3^- + 2H$$

nitrite nitrate

In the case of *Nitrosomonas*, the oxidation state of nitrogen is changed from $^-3$ to $^+3$, and in the cases of *Nitrobacte*r from $^-3$ to $^+5$. The energy yields to the chemoautotrophs are approximately 65 kcal (or 8.8 ATP molecules) per mole for *Nitrosomonas* and l8 kcal (or 2.5 ATP molecules) per mole for *Nitrobacter*, energy yields which are somewhat low compared with heterotrophic metabolism. A mole of glucose can for example optimally give an aerobic microbe 280 kcal (or 38 ATP molecules). This partly explains why autotrophic nitrifiers grow relatively slow in the soil and even in

laboratory culture where conditions for growth are optimised. Natural generation times for nitrifying bacteria of the order of 20-40 h, together with their low numbers in most soils however, gives a highly misleading impression of their vital contribution to nitrogen cycling and to soil ecology in general (Killham,1994).

1.14.4. Nitrate reduction

Once nitrate is formed in soil, it is subjected to the following fates;

1- It can undergo microbial denitrification to gaseous oxides of nitrogen to dinitrogen-N_2.

2- It can be utilized as a source of N for plants and microorganisms. Assimilation of NO_3 is followed by its reduction to NH_4^+, which is then utilized (i.e. assimilatory reduction) (Alexander, 1977, Paul and Clark, 1989).

3- In the absence of O_2, nitrate can be used by microorganisms as an electron acceptor and as a result be reduced to NH_4^+ (dissimilatory reduction) (Paul and Clark, 1989).

4- Being a negatively charged ion, NO_3^- is easily leached through soil and into ground water and soil nitrate leaching has several consequences. When nitrate is leached, it reduces the base saturation of a soil and increases exchangeable acidity. High concentrations of nitrate in surface waters can also lead to eutrophication and fatal diseases such as gastric cancers and methaemoglobinaemia (Alexander 1977).

1.14.5. Nitrogen losses from soil

Of all the nutrients required for plant growth, N is by far the most mobile and subject to greatest loss by physical, chemical, and/or biological processes from the soil-plant system (Knowles 1981, 1982). Even under the best circumstances, no more than two thirds of the fertilizer-N is accounted for by crop removal or recovered in the soil at the end of the growing season and nearly one-half of the applied amount can be lost. Five main processes for N loss occur, including microbial denitrification, chemo-

denitrification, NH_3 volatilization, leaching, and erosion. There are two biological processes for reduction of oxidised N forms (e.g., NO_3^- and NO_2^-). One of these, assimilatory NO_3^- reduction, the other process is dissimilatory NO_3^- reduction , more commonly known as denitrification (Alexander 1977, Cooper, and Smith, 1963, Lynch 1983, Payne 1981).

1.14.6. Assimilatory nitrate reduction

In assimilatory nitrate reduction N is incorporated into cell biomass of plants, bacteria, cyanobacteria, and fungi all of which reduce NO_3^- to NH_4. in the biosynthesis of amino acids and proteins (Atlas and Bartha 1993). The process needs energy and several enzyme systems, including nitrate and nitrite reductases to form ammonia, which is subsequently converted into amino acids. As a result, this process is regulated by the availability of nitrogen, and nitrate utilization occurs when energy exceeds the concentration of ammonium or organic-nitrogen compounds (Atlas and Bartha l998).

1.14.7. Dissimilatory nitrate reduction

Dissimilatory nitrate reduction is a process, in which the N is not utilized and in the absence of O_2. Nitrate ions acts as a terminal electron acceptor. The process is also known as nitrate respiration, or dissimilatory nitrate reduction (Atlas and Bartha 1998). Respiratory denitrification is usually the major dissimilatory process occurring in soils which reduces nitrate and under anaerobic conditions nitrate-respiring bacteria reduce nitrate to nitrite. Facultative anaerobes are often involved and many of these can also further reduce nitrite to ammonium:

$NO_3^- + 4H_2 + 2H_4 \rightarrow NH_4^- + 3H_2O.$

The following genera are involved:

Table 1.1: Bacteria that can dissimilate nitrate to ammonium Based on Tiedje (1988)

Genus	**Typical habitat**
Clostridium	Soil, sediment
Desulfovibrio	Sediment
Selenomonas	Rumen
Veillonella	Intestinal tract
Wolinella	Rumen
Facultative anaerobes	
Citrobacter	Soil, wastewater
Enterobacter	Soil, wastewater
Erwinia	Soil
Escherichia	Soil, wastewater
Klebsiella	Soil, wastewater
Photo bacterium	Seawater
Salmonella	Sewage
Serratia	Sediment
Vibrio	sediment
Microaerophiles	
Campylobacter	Oral cavity
Aerobes	
Bacillus	Soil, food
Pseudomonas	Soil, water
Neisseria	Mucous membranes

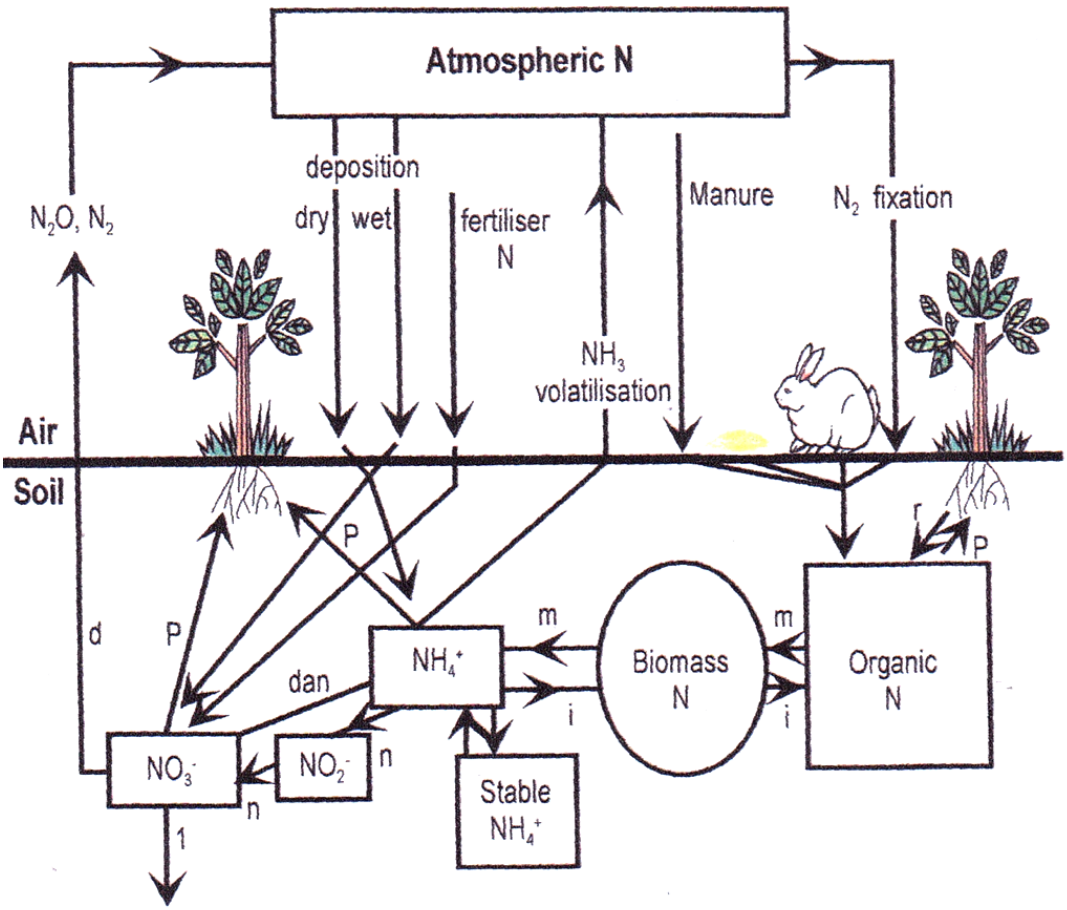


Fig 1. 2: The Nitrogen Cycle

Abbreviations; d, denitrification; dan, dissimilatoryand assimilatory nitrate reduction to ammonium; I, immobilisation; m, mineralisation; n, nitrification and subsequent leaching 1; p, plant; r, root exudation and turnover (Killham , 1994)

1.15. The Sulphur cycle

Sulphur is an essential element which in plants, it is an important component of amino acids methionine and cysteine. Plants contain as much sulphur as phosphorus, and sulphur is as important in the formation of protein. Despite this, sulphur has traditionally been regarded as a plant nutrient of secondary importance to nitrogen, phosphorus and potassium.

1.15.1. Forms of S in soil

It is generally accepted that well over 90% of the sulphur in most non- calcareous, non-tropical, surface soil is in organic forms, about half in the form of sulphate esters and esters with C-O-S linkage (Tisdale, Nelson and Beaton 1985); about 20% of the sulphur directly bonded to carbon such as S-containing amino acids (Biederbeck 1978), and the remainder in a variety of largely inert organic compounds.

1.15.2. Biological and biochemical S-mineralisation

The mineralisation of organic sulphur in soil occurs by two main processes, biological and biochemical. Carbon-bonded sulphur is mineralised biologically during the oxidation of carbon by soil organisms to provide energy, whereas non-carbon bonded organic sulphur is mineralised through enzymatic catalysis outside the cell (Killham, l994). An example of this latter biochemical release of sulphur, the form of sulphates-catalysed by cleavage of sulphate esters, Sulphur is released into the inorganic pool in various oxidation states from sulphide (oxidation state-2) to sulphate (oxidation state in $^{+}6$).

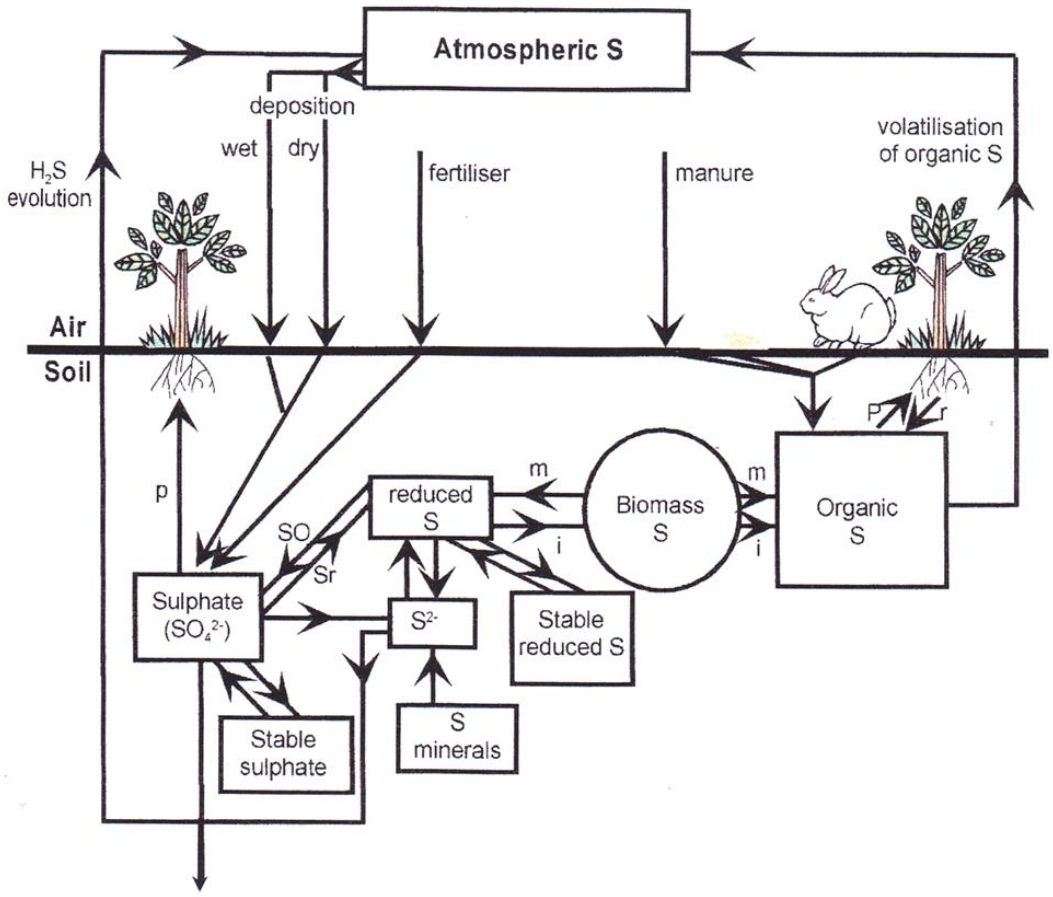


Fig 1. 3: The Sulphur Cycle

Abbreviations: I, immobilisation; m, mineralization; p, plant uptake; r, root exudation and turnover; so, oxidation and subsequent, leaching 1; sr , reduction (Killham, 1994).

1.15.3. Soil ecology and S-mineralization

The rate of S-mineralisation in soil is influenced by similar environmental factors which control N-mineralisation, including water potential, temperature, and pH, the presence of plants, drying/heating cycles and the form and quantity of organic sulphur have the most important influence on rates of soil S-mineralisation.

In most soils, the inorganic S-pool is very small and biological uptake, both as microbial immobilization and plant uptake depends upon an adequate rate of mineralisation. Some soils cannot meet the sulphur demands of all crops in this way,

and this S-deficiency is sometimes not offset by fertilizer, pesticide and atmospheric S-inputs (Hoque and Killham 1987).

The great similarities between the cycling of sulphur and nitrogen (notably with regard to mineralisation from organic matter) suggest a fundamental involvement of soil animals in S-mineralisation. Until further information becomes available, it can be assumed that the quantitative involvement of soil animals in the cycling of sulphur is approximately similar to that for the cycling of N. The atmosphere contains considerable amounts of sulphur released by the burning of fossil fuels and by microbiological sulphate reduction. Plants meet most of their sulphur needs from sulphate, but they may obtain some sulphur directly from the atmosphere. When plants and animals are incorporated into the soil their proteins are hydrolysed to form amino acids, which together with other sulphur containing compounds are further oxidized by microorganisms to form sulphate, while in anaerobic soils H_2S is formed partly from sulphate reduction and partly from the mineralization of organic sulphur. A number of intermediates are produced during S-oxidation, including thiosulphate, polythionates and sulphate. These ions do not generally persist however, and their concentration in nature is as a result, usually low.

1.15.4. Microorganisms involved in the S-Cycle

Microorganisms are responsible for:

(1) The mineralization of organic sulphur to sulphate.

(2) The oxidation of reduced forms of inorganic sulphur to sulphate.

(3) The aerobic reduction of sulphate to sulphides.

(a) The immobilization of sulphate as organic sulphur.

1.15.5. S-Mineralization

Sulphur mineralization is the conversion of organic sulphur into inorganic forms, notably sulphate; it provides a substantial source of soil sulphate, notably in forest ecosystems Johnson (1932). Sulphur mineralization is therefore an important way in which sulphate is mobilised in soils (it also provides a source of H^+ ions) (Tabatabai 1985). Sulphur mineralization increases in the presence of oxygen, temperature (in the mesophilic range, Tabatabai and Al-Khafaji (1980), moisture level and the addition of lime to acid soils (Williams 1967).

1.15. 6. Sulphate reduction

Bacteria of the genus *Desulfuvibrio* are the main microorganisms concerned with the reduction of sulphate. They use sulphate as an electron acceptor for growth. While few microorganisms are able to reduce sulphate to sulphide many bacteria, actinomycetes and fungi can reduce partially reduced inorganic sulphur compounds such as thiosulphate, tetrathionate and sulphite to sulphide (Alexander, 1977).

1.15.7. Sulphur oxidation

The oxidation of reduced sulphur in soil is generally regarded as a microbial process (Wainwright, 1978 Burns 1967), although some non-biological oxidation of the element has been shown to occur in autoclaved soils (Wainwright and Killham 1980, Nor and Tabatabai 1977).

During the microbial decomposition of organic sulphur compounds, sulphides and other incompletely oxidised substances are formed, such as elemental S, thiosulphate, and polythionates; these reduced substances are the available for oxidation. The oxidation of some sulphur compounds, such as sulphites (SO_3^{2-}) and sulphides (S^{2-}) can

occur by strict chemical reactions (Brady and Weil 1974) and such abiotic oxidation can occur to a limited extent in soils, but microbial reactions are clearly dominant:

$S^{o} \longrightarrow S_2O_3^{2-} \longrightarrow S_4O_6^{2-} \longrightarrow SO_4^{2-}$

Elemental Thiosulphate Tetrathionate Sulphate sulphur

The microorganisms involved in S-oxidation can be divided into: chemoautotrophs (lithotrophs), including species of the genus *Thiobacillus*

1- Photoautotrophs, including species of purple and green sulphur bacteria, and

2- Chemoheterotrophs (organotrophs), including a wide range of bacteria and fungi

1.15.8. Heterotrophic sulphur-bacteria

The ability of heterotrophic bacteria to oxidise sulphur was initially studied by Guittoneau (1927) and confirmed by Starkey (1934) who showed that soil heterotrophic bacteria can oxidise thiosulphate to sulphate via tetrathionate. A wide range of heterotrophic bacteria, fungi and actinomycetes (Yagi, 197l) can oxidise a variety of reduced forms of sulphur *in vitro*. Bacterial species of the genera *Arthrobacter, Achromobacter, Bacillus, Beggiatoa, Flavobacterium, Micrococcus, Mycobacterium, Pseudomonas* and *Sphaerotilus* can oxidize sulphur; unlike the S-oxidising autotrophs, heterotrophic bacteria do not seem to obtain energy from the process (Trudinger , 1967, Schook and Berk, 1978).

1.15.9. Chemotrophic sulphur bacteria

The chemotrophic sulphur bacteria vary in both morphology and physiology, ranging from specialist obligate chemolithotrophs through facultative chemolithotrophs which can grow mixotrophically, to specialist heterotrophs, some of which may not benefit directly from the oxidation of reduced sulphur compounds (Kuenen and Beudeket,1982; Table, 1.2); the most widely studied in this group belong to the genus *Thiobacillus*. The

thiobacilli are rod shaped organisms which can obtain energy from oxidizing inorganic sulphur compounds, using oxygen as an electron acceptor, while CO_2 or bicarbonate supplies the carbon for chemoautotrophic growth (London and Rittenberg 1967). Additionally, they can be subdivided into those growing on neutral pH and those, which live at acidic pH. They can also grow both at acidic and alkaline pH values. Vitolins and Swaby (1969) pointed out that that Thiobacilli are important sulphur oxidisers only at pH values below 7, while heterotrophs are the primary sulphur oxidisers in neutral to alkaline soils (pH 6.0-7.5). The reduction in pH resulting from sulphuric acid formation by thiobacilli may also control some diseases of plants including potato scab (Brown, 1982).

Table1. 2: Colourless sulphur oxidising bacteria. (Modified after Kuenen and Beudeker, 1982)

Obligate chemolithotrophic S-bacteria		**Facultative chemolithotrophic S-bacteria**
(a) **Aerobic bacteria**		
Thiobacillus thiooxidans		*T. novellus*
T. neapolianus		*T. intermedius*
T. ferrooxidans		*T. acidophilus*
T. kabobis		*T. organoparus*
T. tepidarius*		*Sulfolobus acidocaldarius*
Thiomicrospira pelophila		*Sulfolobus brierleyi*
(b) **Facultative anaerobic bacteria**		
T. denitrificans		*Thiobacillus A2*
T. thioparus		*Thermothrix thiopara*
Thiomicrospira denitrificans		*Paracoccus denitrificans*
		Thiosphaera pantotropha **
Chemolithoheterotrophs	Heterotrophs	Unclassified
T. permetabolis	*Beggiatoa*	*Thiovulum*
***Pseudomonas* sp**	*Pseudomonas sp*	*Thiophysa*
		Thiothrix
		Thiospira
		Thioploca

1.15.10. The phototrophic sulphur bacteria

The phototrophic sulphur bacteria are found in anaerobic environments, (e.g. in H_2S rich mud, and stagnant waters), which remain exposed to light; since they are not found in most agricultural soils they need not be discussed further here.

1.16. Phosphorus cycling

Phosphorus is second only to nitrogen as an inorganic nutrient required by plants and microbes and is important for the accumulation and release of energy during cell metabolism. Phosphorus is added to the soil as a fertilizer, or is produced during the breakdown of plant residues or animal remains. Microorganisms are involved in a number of transformations of the element including:

(l) Solubilization of inorganic insoluble phosphorus compounds.

(2) Mineralization of organic compounds to form inorganic phosphate.

(3) Immobilization of inorganic phosphate into cell components.

(4) Oxidation and reduction of inorganic phosphorus compounds.

Some 15-85% of phosphorus in soil is organic, notably as minerals existing as insoluble phosphates. These insoluble inorganic compounds are largely unavailable to plants. Microorganisms including fungi and bacteria can solubilize this insoluble phosphate. These phosphate-solubilizing fungi include species of *Penicillium*, *Sclerotium*, *Fusarium* and *Aspergillus* (Alexander 1977). This process involves the production of organic acids and/or chelating agents (Wainwright 1981). The mineralization of organic phosphorus is an important soil process due to the existence of a large reservoir of non-plant available organic phosphorus which is converted to inorganic forms by enzymes collectively known as phosphatases. The microbial immobilization and assimilation of phosphorus may depress crop yields and the resultant phosphorus shortages can be overcome by adding phosphorus fertilizers to the soil. Fungi are also able to oxidize

reduced phosphorus compounds eg. phosphite to phosphate and can use these compounds as sole phosphorus source. There is the possibility of a reductive phosphate pathway occurring in soils with phosphate being reduced to phosphate and hypophosphate; fungi however, have not been implicated in this pathway (Alexander, 1977).

1.16.1. P-Mineralization and immobilization

The maintenance of soluble phosphate in the soil solution depends to some extent on the magnitude of the two opposing processes

$$\text{Organic P} \xleftrightarrow[\text{Immobilization}]{\text{Mineralization}} P_i \text{ (e.g., } H_2PO_4^-. HPO_4^-)$$

Phosphorus mineralization is an enzymatic process. As a group, the enzymes involved, called phosphatases, catalyze a variety of reactions which release phosphate from organic phosphorus compounds.

1.16.2. P-solubilization

Phosphate solubilising microorganisms (fungi and bacteria) include species of *Pseudomonas, Mycobacterium, Microococctts, Bacillus, Flavobacterium, Penicillium, Sclerotium, Fusarium,*and *Aspergillus* (Alexander,1977). Many common microorganisms, including species of *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Streptomyces*, and especially *Aspergillus* and *Arthrobacter*, are able to solubilize insoluble inorganic phosphates in soil. The rhizosphere often has a particularly high proportion of such organisms, for example, having found that 20 - 40% of the bacteria, actinomycetes and fungi isolated from the rhizospheres of many plants are able to dissolve hydroxyapatite, compared with l0 - 14% from non-rhizosphere soil.

1.17. Aims of the Work Reported in this Thesis

The overall aim of the work reported in this Thesis is to attempt to correct this deficiency by providing research data relevant to:

a) The composition of bacterial populations found in dog faeces

b) The distribution of bacteria in playing field soils exposed to dog faeces

c) The fertilizer potential of dog faeces and its potential to be used as additive to potting composts.

d) The question of whether slugs can act as vectors of bacterial disease, transmitting pathogenic bacteria from dog faeces to lettuce.

d) The potential use of Black Soldier Fly and Fruit Beetle larvae to increase the rate of composting of dog faeces.

e) The ability of slugs, snails and earth worms to carry and transfer Mycoplasmas to lettuce from dog faeces.

f) The potential use of dog faeces as a source of biofuels.

Chapter Two: Transfer of Bacteria by Invertebrate Slugs from Canine Faeces to Lettuce

2.1. Introduction

Several bacterial species, by being associated with a variety of invertebrates, can infect humans, plants and animals; either via vectors (such as mosquitoes, the malaria disease carrier) or through infecting the food chain with bacteria, notably vegetables crops and fresh fruits.

Food poisoning outbreaks are frequently associated with cross contamination from insects, such as houseflies, to meat products; however, direct contamination of foods, notably of vegetables and lettuce in the field is also highly. Many field crops are prone to be contaminated with remains of faeces from farm and domestic animals which are in contact with manure contaminated soil or dust, and this is the major source of pre-harvest contamination. Indirect sources of contamination also result from the interaction between vegetables and fruits and phytophagous birds, mammals, and insects.

Pets and farm animals have been shown to be effective vectors of pathogenic bacteria, either in the visceral or on their remains and animal faeces have been shown to contain bacteria such as *Salmonella*, *E. coli* O157, and *Listeria monocytogenes* (Beuchat, 1996).

Dog faeces are likely to be a major a source of bacterial contamination of vegetable and lettuce crops especially where these are produced in allotments, small-holdings, or agriculture land located close to housing estates where dogs are common. The canine enteric tract, like most mammals, has a complex microbial ecosystem, which includes several bacteria such as species of *Streptococci*, *Bifidobacteria*, *Lactobacilli*, *Bacteroides* and *Clostridium* (Drasar and Hill, 1974; Drasar and Barrow, 1985). *Acinetobater baumannii* has also been isolated from a number of dogs and cats, and according to Thierry *et al*. (2008) the molecular typing of samples with limited polymorphisms of ribosomal DNA provided confirmation of nosocomial spread of this

pathogen as well as for the occurrence of several strains of *A. baumannii* in dogs in veterinary hospitals.

Slugs (Mollusca: Gastropoda) are major pests of large varieties of vegetables and fruits in fields, domestic gardens, allotments, landscaped areas and greenhouses, (Godan, 1983; South, 1992). Slugs present a risk to plants, animals and humans because they often serve as intermediate hosts for many bacterial diseases found in animals' faeces (South, 1992). Slugs usually ingest bacteria in soils as an important component of their food. Consequently, they become hosts to many bacteria, such as *E. coli* O157, either via direct contact or by being contaminated with animal faeces spread in open fields (Fig. 1.1), (Walker *et al*., 1999). Emma *et al*. (2006) carried out a study on both yellow slug (*Limax flavus*) and great gray slug (*Limax maximus*), and confirmed that both species can carry *E. coli* O157 both on the surface and internally.

Emma *et al.* (2006) also showed that once a slug has become contaminated with *E. coli* through contact and/or ingestion, the bacterium can survive for many days internally and externally, thereby providing adequate time for successful transmission. Elliot (1969) also suggested that slugs possibly carry *E. coli* O157. However, the laboratory study of Dawkins *et al*. (1986) demonstrated that in four slug species i.e. *Arion hortensis, Deroceras reticulatum, Milax budapestensis* and *Limax maculatus* tested to see if they transmit bacterial soft rot of potatoes *Erwinia carotovora,* the association between the pathogen and its slug vectors was considered to be accidental, rather than obligatory. Invertebrates then may be responsible for the transmission of the *E. coli* O157 and other bacterial strains to fruits and vegetables either by direct contact, or following contamination with animal faeces. Human pathogens present on fresh fruits and vegetables, notably enteric pathogens (e.g. *E. coli* O157:H7 and *Salmonella*), have the ability to grow before being eaten and bacterial pathogens have been isolated from a large number of vegetables and fruits. Many strains of food poisoning bacteria

have also been isolated from soils including *Vibrio cholera, E. coli, Campylobacter jejuni* and *Shigella*. In addition, *Enterococci* have been found in soils, and their distribution is generally associated with animal faeces.

The aim of the work reported in this Chapter was to determine if bacteria can be transferred from canine faeces to vegetable crops (in this case lettuce) by slugs indigenous to the UK.

2.2. Materials and Methods

2.2.1. Slug culturing

One hundred slugs were used in this research. They were divided into small groups; sets of twenty slugs were placed in five sterilised plastic boxes (of size 20cm × 30cm × 10 cm). The grey garden slug *Limax maximus* was obtained from Blades Biological Ltd. In order to maintain the slugs alive, wet tissues were placed in the bottom of the boxes and changed every 3 days. Fresh lettuce was introduced to the slugs twice a week as their only food source. All slug-containing boxes were left at room temperature.

2.2.2. Isolation of bacteria from dog faeces

Samples were collected from fresh dog faeces. Portions (1g) were added to 99 ml of distilled water and were shaken at 70 g for 15 min. Aliquots of the shaken samples were spread onto the surface plates containing Chromagar medium, and left overnight in an incubator at 37^{o}C (Samra *et al.*, 1998).

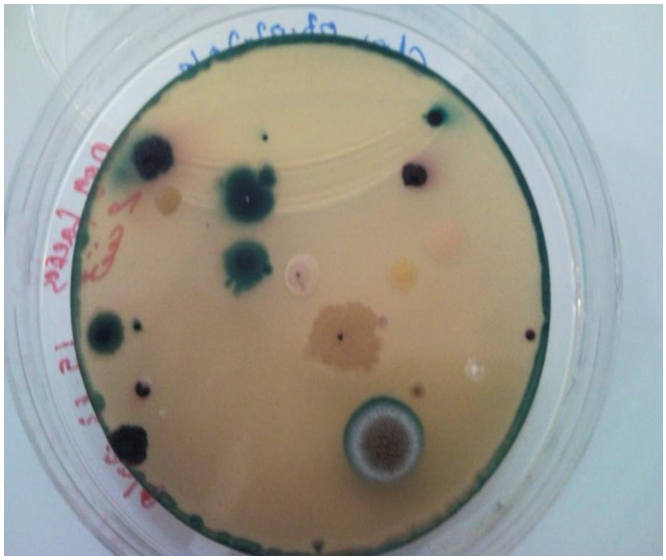


Fig. 2. 1: Bacterial colonies isolated from dog faeces using Chromagar medium

2.2.3. Isolation of bacteria from slugs

The slugs were starved for 2 days and then exposed to fresh dog faeces (which was collected locally) as their sole food source. Bacteria were then isolated from both the outside and inside of the animal.

Samples were collected from inside slugs in sterile deionised water as follows: Slugs were killed using formalin (90% v/v in a Petri-dish), and then dissected. Portions of 2 ml of (dH_2O) were directly injected into the digestive track of the slugs, and the samples were collected using sterile syringes (Fig.2.2). Samples (1 ml) were then added to 9 ml of distilled water and were shaken at 100 g for 15 min, Surface samples were obtained directly from the surface secretion of the slugs. Both collected samples were spread onto the surface of Chromagar media (e.g. Orientation media); and the inoculated plates were incubated at 37^oC for overnight.

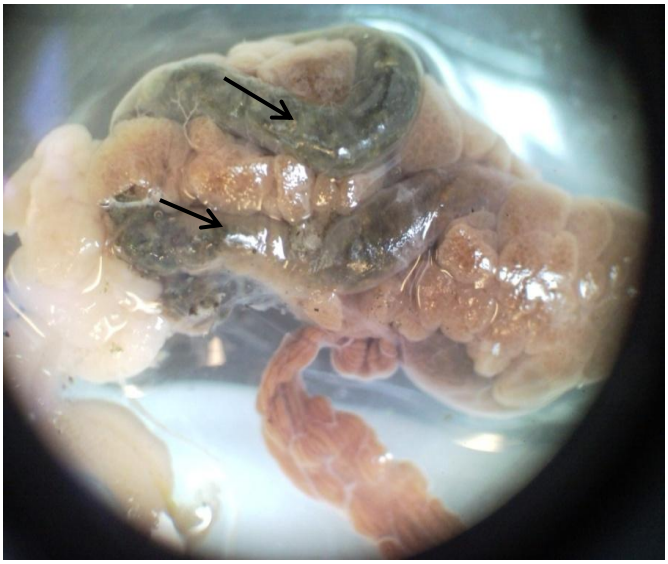


Fig. 2. 2: Section of digestive track of slug from where the samples were taken

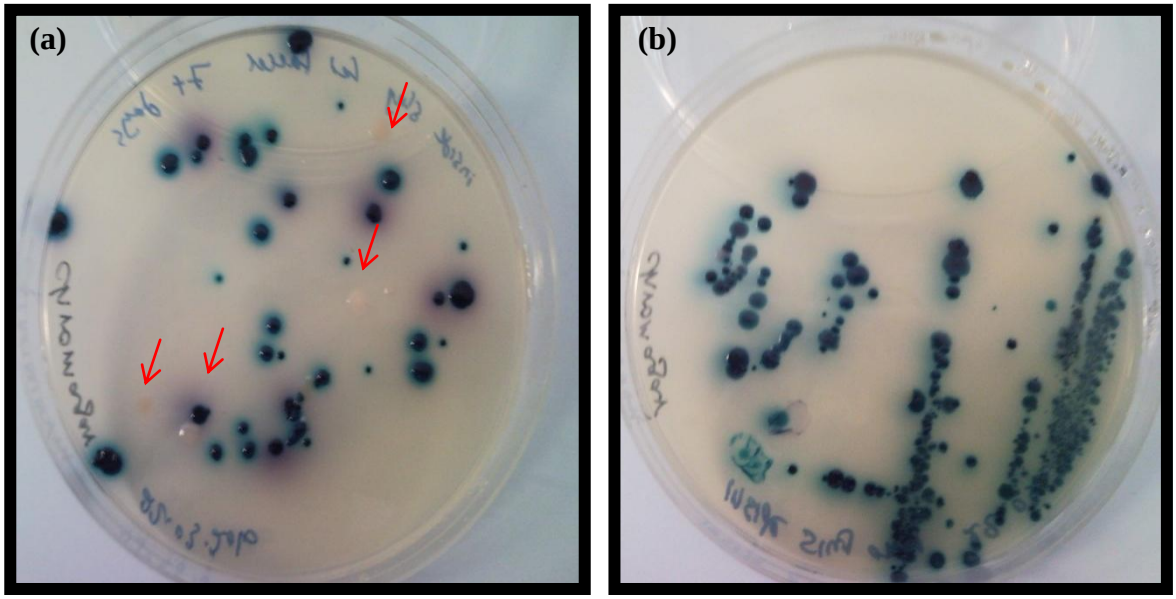


Fig. 2. 3: Isolation of Bacteria from slugs: (a) colonies inside slugs, and (b) colonies from the outside of slugs.

2.2.4 Isolation of bacteria from lettuce

Bacteria were initially isolated from fresh lettuce leaves as controls. Slugs previously fed on dog faeces were then fed lettuce. After two days, the suspension was prepared from both control and contaminated lettuce leaves, by immersing small pieces of lettuce leaves in 50 ml of liquid Nutrient Agar. The samples were incubated overnight at 25^{o}C under continuous shaking (250 g). In order to obtain different isolation batches, four dilution series (e.g. 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} were used in this experiment, followed by spreading the samples (100µl) onto Chromagar plates which were then incubated for 18-24 hours.

2.2.5 Bacterial identification

2.2.5.1 Identification of bacteria using Chromagar medium

Chromagar Orientation medium was prepared as recommended by the Manufacturer's instructions (Chromagar Company, Paris, France). The medium was made up as follows: (per litre) 15 g of agar, 16 g of peptone, (1.3 g of chromogen mix) meat extract, and yeast extract, pH 7. The species of bacteria were presumptively identified on the basis of the colour of the colonies produced when growing on Chromagar. The colour is produced by the reaction of enzymes, produced by a specified genus or species, with certain chromogenic substrates. Chromagar Orientation media contains two chromogenic substrates used for the detection of B-D glucosidase and B-D-glucosidase enzyme activity, together with 1- pyrolidonyl-B-naphtylamide hydrololysis (PYR) which is described for the identification and differentiation of Enterococci from other bacteria (Merlino, 1997). See appendix two (Fig, 2. 2)

2.2.5.2. Extraction of Genomic DNA

Genomic DNA (gDNA) was extracted from the bacterial strains (JS1, JS2, JS3 and JS4) which were grown on LB nutrient solid medium for overnight at 37^{o} C, DNA was isolated using Key Prep-Bacterial DNA Extraction kit by following procedure. 4 ml of culture grown overnight in LB medium was centrifuged at 6,000×g for two minutes at room temperature. The supernatant was decanted completely. The cell pellet was then washed with TE buffer and completely suspended in 100µl of buffer R1 by pipetting it up and down. Then 10µl of lysozyme (50mg/ml) was added to the cell suspension and mixed thoroughly. After 20 minutes incubation at 37°C, the digested cells were collected by centrifugation at 1000×*g* for 3 minutes and the supernatant was decanted immediately. The collected pellet was then re-suspended in 180µl of Buffer R2 and 20µl of Proteinase K, mixed thoroughly and incubated at 65°C for 20 minutes in a shaking water bath. In order to obtain a RNA-free DNA, 20µl of RNase A (DNase-Free, 20mg/ml) was added and followed by incubation at 37°C for 5 minutes. Homogeneous solution was obtained by adding 2volumes of Buffer BG to the previous mixture and mixed thoroughly by inverting tube several times then incubated at 65°C for a further 10 minutes, immediately 200µl of absolute ethanol was added and mixed carefully. The samples were transferred into a column assembled in a clean tube and centrifuged for 1 minute at 10,000×g, supernatants were discarded. The column was then washed with 750µl of the wash buffer and centrifuged twice for 1 minute at 10,000×g in order to remove the residual ethanol, supernatants were also discarded. Finally, the column was placed into a clean microcentrifuge tube and 100µl of preheated Elution Buffer, TE buffer or sterile water was added directly onto column membrane and left for 2 minutes which then centrifuged at 10,000×g for 1 minute to elute DNA. To verify DNA presence and purity, 10µl of each resultant DNA was mixed with 2µl of Blue/Orange 6x

loading dye and electrophoresed using 1% agarose gel. The checked DNA was stored at -20°C

2.2.5.3. Agarose preparation

To separate DNA, RNA or protein. Nucleic acid molecules are separated by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis) where shorter molecules move faster and migrate farther than longer ones due to the sieving effect of the gel. The presence, size and quantity of the DNA were checked and determined by electrophoresis in 1% agarose gel which was prepared as follow: 0.5g of molecular biology grade agarose was dissolved in 1ml of 50x TAE buffer and 40ml distilled water. The agarose was completely dissolved by heating in a microwave oven. The solution was mixed gently and allowed to cool to 55°C, and then 2.5µl ethidium bromide was added. After mixing, the solution was poured into a sealed gel rack and a comb was inserted at one side of the gel and allowed to stand in room temperature for 20 minutes. Then the comb and seal were removed and gel was placed into an electrophoresis tank and submerged in 1x TAE buffer. 10µl of the DNA sample was mixed with 2µl of Blue/Orange 6x loading dye and loaded into the wells. 6µl of Hyper Ladder was loaded as well into one of the wells as a reference. The samples were electrophoresed for 40 minutes at 80V to allow DNA to migrate toward the anode. The DNA fragments were visualised under a UV transilluminator and the images were captured using a connected digital camera

2.2.5.4. PCR amplification

The 16S rRNA gene was amplified with the universal bacterial forward primer (5` CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G 3`) and universal reverse primer (5` CCC GGG ATC CAA GCT TAC GGC TAC CTT GTT ACG ACT T

3`) according to Weisburg e*t al*., (1991). A typical PCR mixture (50 µl in volume) contained the following components: 39µl sterile distilled water, 5µl 10× Buffer , 2.5µl 50 mM $MgCl_2$, 0.5µl forward Primer, 0.5µl Reverse primer, 1.0µl dNTPs, 1ul genomic DNA and 0.5µl Bioline Taq. The PCR reaction mixture, after incubation at 94°C for 3 minutes as an initial denaturation, were cycled 30 times through the following temperature profile: denaturation for 1 minute at 94°C; annealing for 1 minute at 60°C; and elongation for 5 minutes at 72°C with final incubation for 5 minutes at 75°C, after which 10 µl of each PCR amplification mixture was mixed with 2µl of Blue/Orange 6x loading dye and analysed by 1% agarose gel electrophoresis. In addition, 6µl of 1 Kb Hyper ladder I loading was used to confirm the correct sized product (Fig. 2.4). All PCR products were analyzed on agarose gels to check for the successful amplification 16S rRNA gene in the samples

2.2.5.5. Phylogenetic identification of unknown bacteria

All samples sent to Sheffield University Medical School Core Genetics Unit for sequencing. BLAST was used to compare the sequence. Finch TV software was used to correct the sequence then compared it with BLAST to look for the closest matches (Fig. 2.5)

2.2. 6. *Mycoplasma* identification

2.2.6.1. Samples description

A variety of samples were tested here for the presence of *Mycoplasma spp*, namely: a) Earthworms, b) both the internal organs and outer slime of slugs and snails) dog faeces, d) control and contaminated lettuce.

The earthworms were placed in polystyrene boxes together with a sterilized soil. A number of earthworms (10) were then placed in 40 ml of distilled water and then

centrifuged at 5000 g for 10 minutes. A 1 ml of the suspension was then taken to test for *Mycoplasma*. Slugs and snails were collected from the local area.

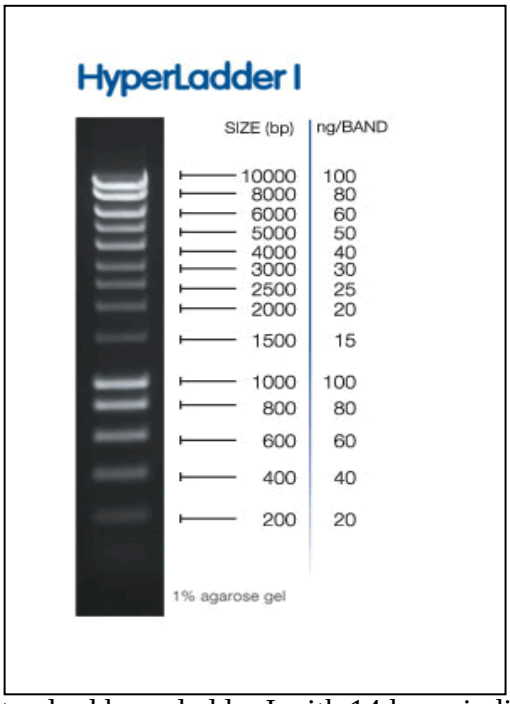


Fig. 2. 4: Standard hyperladder I with 14 lanes indicates higher intensity bands, 1000 and 10,000 and each lane (5µl) provides 720ng of DNA (BIOLINE supplier).

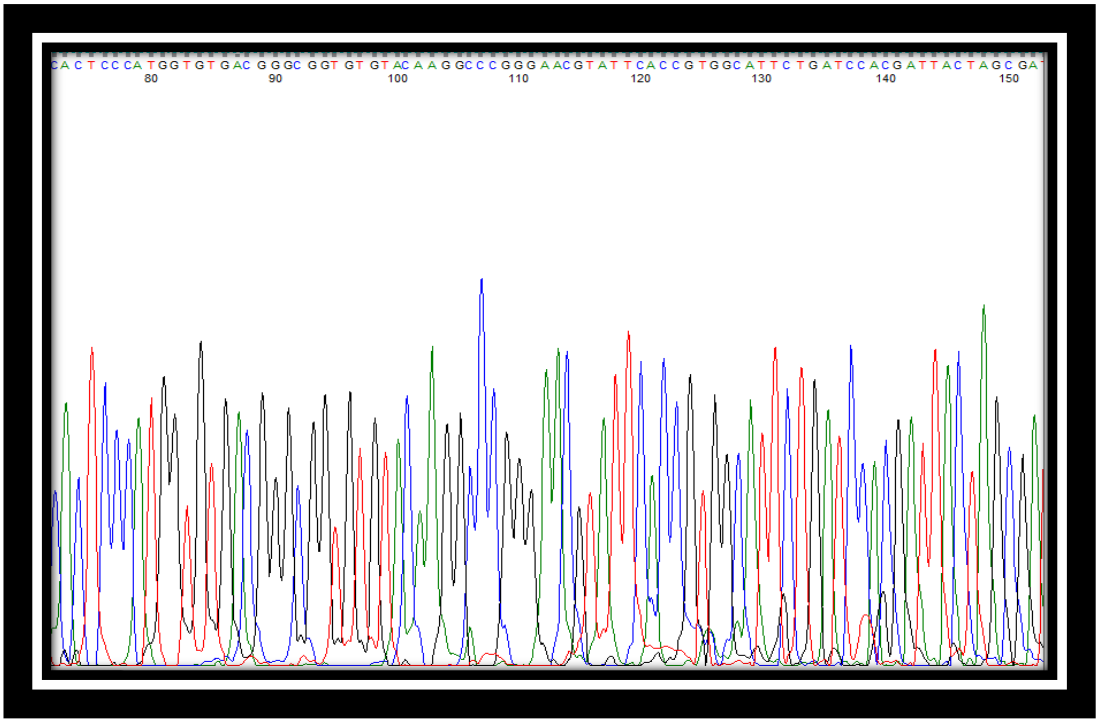


Fig. 2. 5: FinchTV software that manually adjusts errors of consensus sequences before BLASTn NCIMB database

2.2.6.2. Detection of *Mycoplasma*

The followed procedure to detect the presence of *Mycoplasma* or not was using EZ-PCR *Mycoplasma* Test Kit (Gene flow Ltd, Cat No.20-700-20).

2.2.6.3 Test samples preparation for PCR

A quantity of 1.0 ml of sample was transferred to 1.5 ml sterile Eppendoff tube and centrifuged at 10000 g for one 1 minute to sediment the *Mycoplasma.* The supernatant was transferred to a fresh 1.5 ml tube and centrifuged at 15000 g once again for 10 minutes to sediment the *Mycoplasma*. The supernatant was carefully discarded and removed then re-suspended the pellet (not always visible) in 50µl of buffer solution and mixed thoroughly using a micro- pipette samples were heated at 95^{o}C for 3 minutes then stored at -20 ^{o}C.

2.2.6.4 PCR amplification

PCR amplification of *Mycoplasma* DNA was performed as described by the manufacturer. For the test sample, positive and negative control a reaction mixture of the following was prepared (no ice in PCR tube) by addition of the PCR mixture containing sdH_2 O, reaction mix and the test sample (Table 2.1). The tube was placed in a thermal cycle and the *Mycoplasma* programme was run. After PCR amplification, the PCR tube moved from the thermal cycler and 4 µl of 5x loading dye was added to each tube. For the positive control, the DNA template control (270bp) was provided by manufacturer whereas for negative control, the DNA template was replaced with double-distilled water.

Table.2. 1: PCR reaction mixtures for the amplification of *Mycoplasma*-DNA fragment.

Reaction/Component	Volume
Sterile deionized water (sdH_2O)	35 µl
Reaction Mix	10 µl
Test sample Positive control Negative	5 µl

The reaction procedure (Table 2.2.) consisted of the initial denaturation step at 94^{o}C for 30 sec followed by 35 cycles of denaturation at 94^{o}C for 30 sec., primer annealing at 60^{o}C for 120 sec, and extension at 72^{o}C for 60 sec, and was ended by 1 cycle of denaturation at 94^{o}C for 30 sec., primer annealing at 60sec for 120 sec., and extension at 72^{o}C for 5 min.

Table.2. 2: PCR amplification protocol of Mycoplasma DNA fragment

Step	Time	Temperature	Number of cycle
Initial denaturation	30 sec	94 ^{o}C	1
Denauration	30 sec	94 ^{o}C	
Annealing	2 min	60 ^{o}C	35
Extensions/ Elongation	1 min	72 ^{o}C	
Final denaturation	30 sec	94 ^{o}C	
Final annealing	2 min	60 ^{o}C	1
Final extension	5 min	72 ^{o}C	
Hold		4 ^{o}C	

2.2.6.5. Agarose gel electrophoresis

The amplified producers (10µl), including positive and negative control, (2µl) 5x loading dye were separated in a 2% agarose gel in TAE for 45 minutes at a constant voltage of 80 V. Amplified produce by UV transillumination with ethidium bromide staining. A 200 bp Ladder (Bioline, UK) was used as a molecular size standard.

2.2.7. Detection and identification of fungi

2.2.7.1. Samples isolation on PDA and Czapek-Dox media

To determine fungal species in dog faeces samples (0.1 g) of fresh dogs faeces were inoculated onto PDA (Potato Dextrose Agar), while 0.l g inoculated into CDA (Czapek Dox Agar) then incubated at 25 ^{o}C for 3 to 5 days. (Figs, 2.6 a, 2.6 b). Identification of fungi was achieved by extraction of genomic DNA and using 18S rRNA primers samples were 0.5- 1 ml of an overnight Potato Dextrose Broth culture.

2.2.7.2. Extraction of gDNA from pure cultures of fungi for PCR using 18S rRNA

Fungi /Yeast Genomic DNA Isolation Kit, Model 27300 from Sigma was used to extract genomic DNA from fungal isolates using the following procedure:

1**. Lysate preparation**; 5 ml of pure culture grown 3 days on PDA broth medium was centrifuged at 14,000 (g) for 1 minute to pellet the cell. The supernatant was decanted then 500µl of Lysis Solution added to the cell pellet. The suspended pellet was mixed with gentle vortexing , RNase 20µl was added to the cell suspension then the mixture transferred to a provided Bead Tube and vortexing again for 5 minutes at maximum speed. The bead Tube was incubated with lyaste at 65C^{o} for 10 minutes, the lysate mixed 2 times during incubation by inverting the tube. The lyaste including cell debris was transferred to DNase-free microcentrifuge tube by pipetting, and the tube centrifuged for 2 minutes at 14,000 ×g. The supernatant transferred carefully to a new

DNase-free microcentrifuge tube without disturbing the pellet. An equal volume of 96% -100% ethanol was added to the lysate collected above (1µl of ethanol: 1µl of lysate) then vortexe to mix, 300µl of binding solution was added briefly to the suspension which was mixed by vortexing.

2. Binding of nucleic acid. Lysate **(**650 µl) was applied with ethanol to the column and centrifuged for 1 minute at 8,000 ×g, the spin column was reassembled with the collection tube, while the flow was discarded. This step was repeated with remaining lysate.

3. Column wash. 500µl of wash solution was next added to the column and centrifuged for 1 minute at 8,000 ×g.

4. Nucleic Acid elution. The column was transferred into a fresh 1.7 ml Elution tube which is provided with the kit and 100µl of elution buffer was added to the column and centrifuged for 2 minutes at 8,000 ×g; again the column was spin at 14,000 ×g for 1 minute.

5. Storage of DNA; the purified nucleic acid was stored -20^{0}C for a few days, while it is recommended that samples be placed at -70^{o}C for long term storage.

2.2.7.3. PCR amplification of fungal gene

The 18Sr RNA gene was amplified with the ITS1 fungi forward primer (5` TCCGT AGGTGAACCTGCGG 3`) and ITS4 reverse primer (5` TCCTCCGCTTATTGAT ATGC3`) (see appendix). A representative PCR mixture 50 µl included the following reagents: 35 µl sterile distilled water, 5 µl of PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 Mm), 2.5µl of $MgCl_2$, , 1µl forward Primer (ITS1), 1 µl Reverse primer (ITS4), 1.0 µl dNTPs, 4µl genomic DNA and 0.5 µl of Taq polymerase . The following thermal cycling conditions were performed in a thermal cycler BIO Rad 57BR0200; an initial denaturation step at 95^{o}C was conducted for 2 minutes and a

thermocycle for 30 cycle, where each cycle consisted of 30 second at 95^{o}C followed by 30 second at 58^{o}C for annealing , and 30 second at 72^{o}C elongation; and a final extension cycle of 7 minutes at 72^{o}C. The amplified product (10µl) was added to 2 µl 6× loading dye visualized on a 2% (w/v) agarose gel made up in Tris-Borate- EDTA buffer (0.09 M Tris base, 0.09 M boric acid, 2 mM EDTA pH 8.0) containing 0.5 mg ethidium bromide ml^{-1} to confirm the presence of amplified target DNA in the PCR mixture. In addition, 6µl of Hyper Ladder was loaded into one of the wells as a reference. Samples were run at a constant voltage of 80 V for 45 minutes. Twenty microlitres of the remaining PCR product was used for the electrochemical assay (Muir et al., 2011)

2.2.8. Identification of unknown fungi

All samples were sent to the Medical School Core Genetics Unit for sequencing. BLAST was used to compare the sequences and Finch TV software was used to correct the sequence with BLAST and look for the closest matches.

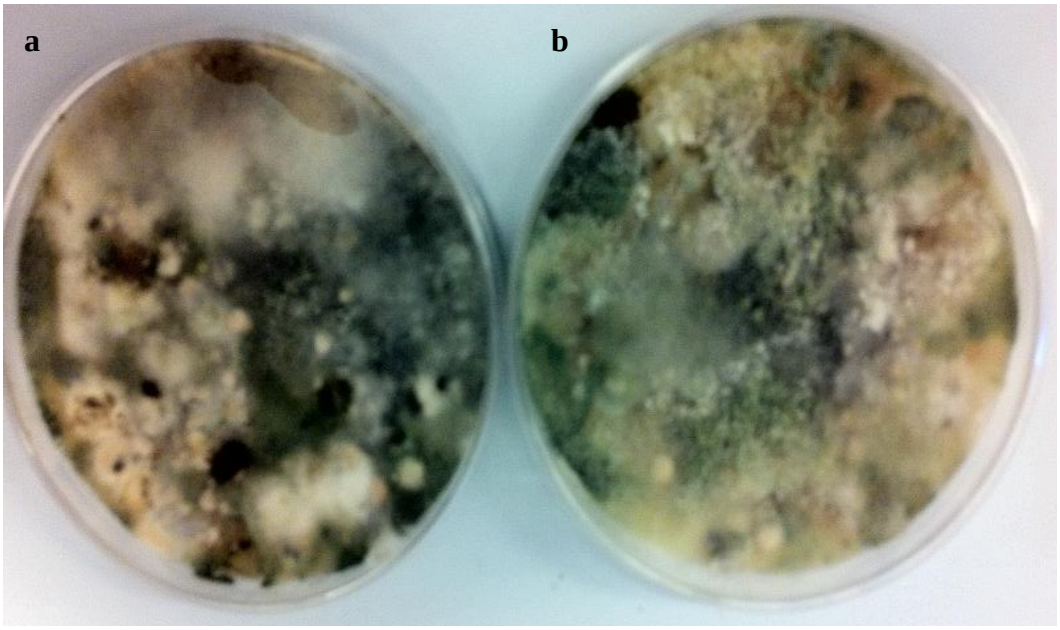


Fig. 2. 6: Fungi isolated from dog faeces grown on a) PDA medium b)

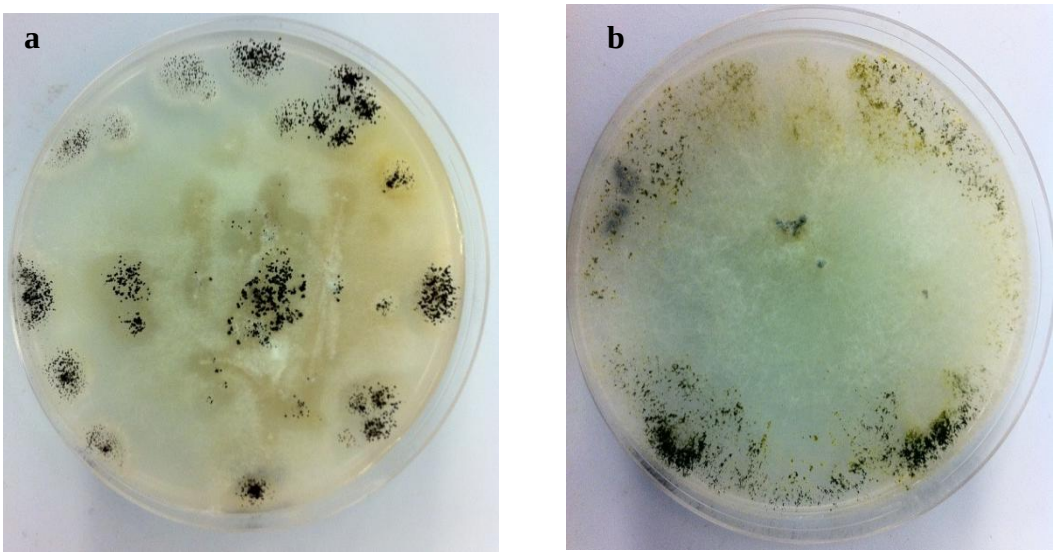


Fig. 2. 7: Purification of fungi species; a) green mould; b) black mould on PDA medium

2.3. Results and Discussion

2.3.1. Transfer of bacteria by slugs to lettuce

All isolated bacteria were grown on Chromagar (Orientation) media and incubated at 37^{o}C for overnight. Cultures shown in (Fig. 2.1) showed different colonies grown on Chromagar media. Chromagar information sheets (reference) were used to identify genera and species of the grown bacterial colonies. There was a consistent colour reaction observed for some species or genus, according to colony colour. For instance, *Enterococcus sp,* from all isolations, appeared as blue (turquoise) colonies, while, other colonies from different isolates showed a variety of colours as shown in Table 2.3. Previous studies on slugs have shown no relationship between *E. coli* transfer and animals faeces (Elliot. 1969) or have failed to detect the presence of pathogens (Shrewsbury and Barson, 1947). On the other hand, field studies by Sproston *et al*. (2006) provided evidence that *E. coli* can be transferred to vegetables from sheep faeces via contaminated slugs.

2.3.2. Bacteria isolated from dog faeces

A number of colonies developing colours on Chromagar media were isolated from dog faeces based on colony-colour blue turquoise, beige, metallic blue, pink-red, cream is shown in Table (2.3) Based on the colour identification coding catalogues for Chromagar (Orientation) media, the genera and species identification of the isolates are: *Entercoccus sp, Salmonella sp, Staphylococcus lentus, E. coli, Proteus sp* and *Acinetobacter sp*.

2.3.3. Bacteria isolated from slugs

2.3.3.1. Bacteria isolated from control slugs

Blue bacterial colonies grew from cultures of both the internal and external parts of slugs; these were identified as an *Enterococcus sp*. Bacterial colonies of a metallic blue colour were found to grow from the outside secretion of slugs and these were identified as *Enterobacter amnigenes*, a finding which was confirmed using 16S rRNA sequence gene anlaysis (i.e.. 99% confirmed).

Table.2. 3: **Analysis of coloured bacterial strains which require further 16S rRNA tests for their identification confirmation to the species level.**

	Colonies colour on Chromagar medium	Presence of bacteria species in different Samples						
		Dogs faeces	Slugs				Vegetable (Lettuce)	
			Control		Fed on faeces		Control	Contaminated
			In	Ex	In	Ex		
Enterococcus spp	**Blue Turquoise**	+	+	+	+	+	+	+
Enterobacter amnigenus	**Metallic blue**	-	-	+	-	-	-	-
Staphylococcus lentus	**Metallic blue**	+	-	-	+	-	-	+
Acinetobacter sp	**Beige**	+	-	-	+	+	-	-
Comamonas sp	**Blue**	-	-	-	-	+	-	-
Delftia sp	**Beige**	+	-	-	+		-	+
Proteus sp	**Beige with brown Halo**	+	-	-	+	-	-	-
E. coli	**Pink-red**	+	-	-	+	-	-	+
Salmonella sp	**Creamy**	+				+	-	-

In = Internal samples Ex= External samples

Grey shading indicates bacteria that were transferred to lettuce

2.3.3.2. Bacteria isolated from slugs fed on faeces

The results showed that five species of bacteria were grown from the inside/ outside of slugs fed on dogs faeces. All five species were isolated from the internal secretions and two out of five species grown from the surface of slugs. *Staphylococcus lentus* exhibited a metallic blue colony colour, while *Acinetobacter sp* was beige and identify confirmed (100%) using 16SrRNA analysis. *Salmonella sp* and *Proteus sp* appeared as cream and beige with a brown halo, respectively, and *E. coli* as a light red colour. Externally isolated species were identified as *Acinetobacter sp* (99%) using 16SrRNA (Table 2.3) and *Comamonas sp* showing a blue colony was also confirmed by 16S rRNA analysis is (91%).

2.3.3.4. Fungi isolated from dog faeces

Several species of fungi and yeasts were isolated from dogs faeces using PDA and Czapek Dox media (Fig 2.5), some of colonies were purified on a number of occasions (Fig 2.6). Results of g DNA and PCR-18SrRNA analysis are respectively shown in gel Fig.2.12 and Fig. 2.13. One fungal isolate was identified using 18SrRNA analysis, namely *Trichoderma asperellum* (99% similarity).

2.3.4. Transmission of bacteria from dog faeces to lettuce

Based on the colour of the developed colonies on Chromagar media, three groups of bacteria dominated the contaminated lettuce leaves, i.e. blue turquoise, metallic blue and beige, whereas only one bacterial species (i.e. *Enterococcus*) was grown from the control lettuce leaves. *Enterococcus* Spp. bacteria were regularly isolated using Chromagar from the control lettuce leaves. Contaminated lettuce samples gave two colonies which were coloured differently from *Enterococcus,*. The colonies were initially identified using colony-colour references as *E. coli* and *Salmonella sp* (Table

2.3); these were not however, confirmed by 16SrRNA analysis. The results of genomic DNA and PCR amplification (Figs 2.8 and 2.9) showed that *Enterobacter amnigenus.* *Acinetobacter sp, Comamonas sp* and *Acinetobacter sp* were isolated from contaminated lettuce (samples, JS1, JS2, JS3 and JS4 respectively (Table 2.4) in addition to *Delftia sp, Staphylococcus lentus* and *E. coli* (samples MS1, MS2 and MS3) were also genomic DNA and PCR amplification achieved (Figs 2.10 and 2.11).

Table.2. 4: **Bacterial identification using 16SrRNA**

S/No	S/ source	Species	S %
JS1	**From outside control slugs (C)**	***Enterobacter amnigenus***	**99%**
JS2	**From inside slugs fed on faeces (C)**	***Acinetobacter sp***	**99%**
JS3	**From outside slugs fed on faeces (B)**	***Comamonas sp***	**91%**
JS4	**From outside slugs fed on faeces (C.)**	***Acinetobacter sp***	**100%**
MS1	**From contaminated lettuce (B)**	***Delftia sp***	**99%**
MS2	**From inside slugs fed on faeces (MB)**	***Staphylococcus lentus***	**100%**
MS3	**From inside slugs fed on faeces (P)**	***E. coli***	**100%**

C = Cream or Beige colony, B= Blue colony, MB= Metallic blue colony, P= Pink-red colony

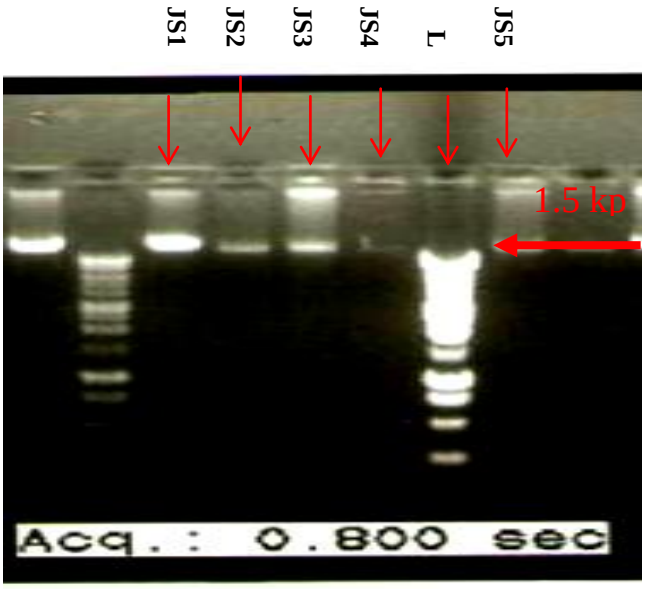


Fig.2.8:.Extraction of genomic DNA of isolated bacterial species; (lane JS1) *Enterobacter amnigenus*; (lane JS 2) *Acinetobacter sp*; (lane JS3) *Comamonas sp*; (lane JS 4) *Acinetobacter sp*(lane L); hyper ladder and(lane JS5) *Delftia sp*

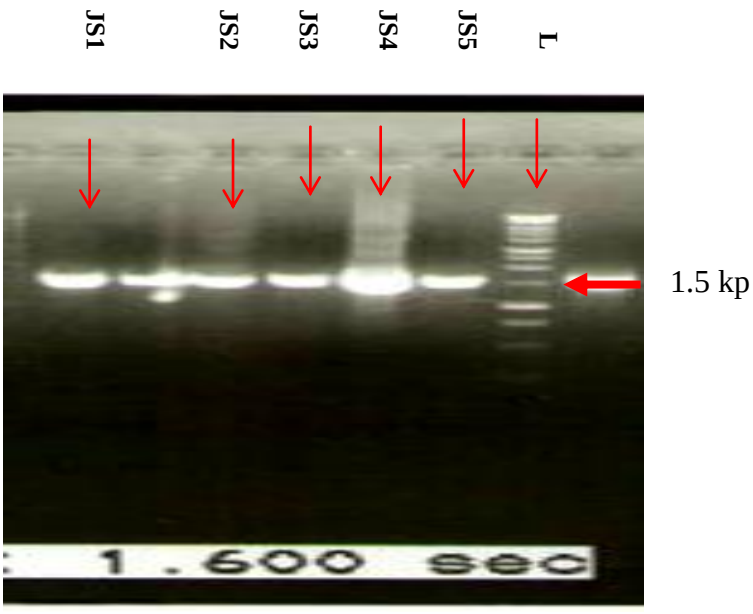


Fig. 2. 9: PCR- 16Sr RNA, amplification products of four isolated bacterial species analyzed by electrophoresis in agarose gel lanes represent; (lane L); hyper ladder;(lane JS1) outside control slugs (Metallic blue); (lane JS 2) inside slugs fed on faeces (B); (lane JS3) outside slugs fed on faeces(B); (lane JS 4) outside slugs fed on faeces (C); (lane 5) contaminated lettuce(Pink-red).

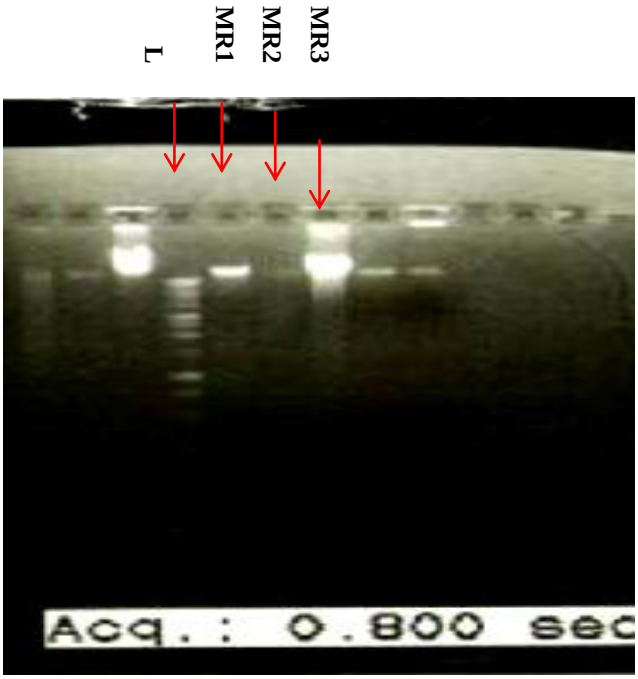


Fig. 2. 10: Extraction of genomic DNA of isolated bacterial species; (lane L); hyper ladder and (lane MR1) *Delftia sp* ; (lane MR 2) *Staphylococcus lentus*; (lane MR3) *E. coli*

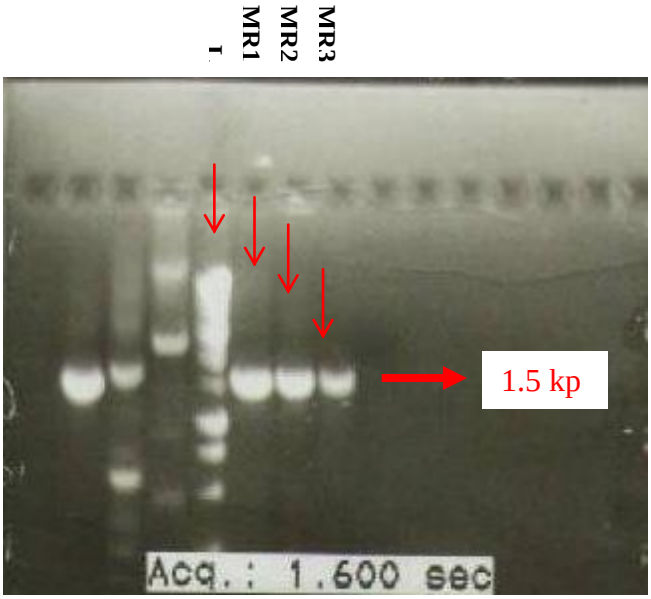


Fig. 2. 11: PCR-16Sr RNA, amplification products of isolated bacterial species analyzed by electrophoresis in agarose gel lanes represent; (lane L); hyper ladder;(lane MR1), contaminated lettuce (Blue); (lane MR 2) Inside slugs fed on faeces (Metallic blue); (lane MR3), Inside slugs fed on faeces (Pink-.red)

2.3.5. *Mycoplasma* detection

2.3.5.1. Attempts to isolate *Mycoplasma* from various samples

Samples were examined for the presence of *Mycoplasma* contamination using the EZ- Polymerase Chain Reaction (PCR) assay. The samples were obtained from the inside and outside of slugs and snails, whole body of earthworm, dog faeces samples and lettuce control and following exposure to slugs fed on dog faeces (Table 2.5).

Table.2. 5: Presence of *Mycoplasma* in various samples

Samples	Symbol	Reaction
Outside slug	SR1	Positive
Inside slugs	SR2	Negative
Earthworm	SR3	Positive
Control lettuce	AZ1	Negative
Contaminated lettuce	AZ2	Positive
Control snail	AZ3	Negative
Outside contaminated slug	AZ4	Negative
Inside contaminated slug	AZ5	Positive
Inside contaminated snail	AZ6	Positive
Outside contaminated snail	AZ7	Negative
Dog faeces	AZ8	Positive

The PCR technique (Figs 2. 14, 2.15 and 2.16) was successful at detecting *Mycoplasma* species from dog faeces and from slugs and earthworm collected from local areas of Sheffield. The main finding of the work reported here is that *Mycoplasma* was transmitted from dog faeces to slugs or snails which they, ingested or carried, and then in turn transferred to lettuce. Earthworms were also shown to carry *Mycoplasma* from soil on or in their bodies, a fact which presumably reflects contamination from animal faeces, mainly dogs.

Fig. 2. 12: Extraction of genomic DNA fungi species ; (lane L); hyper ladder and (lane 6 and 7) fungi isolated from dog faeces

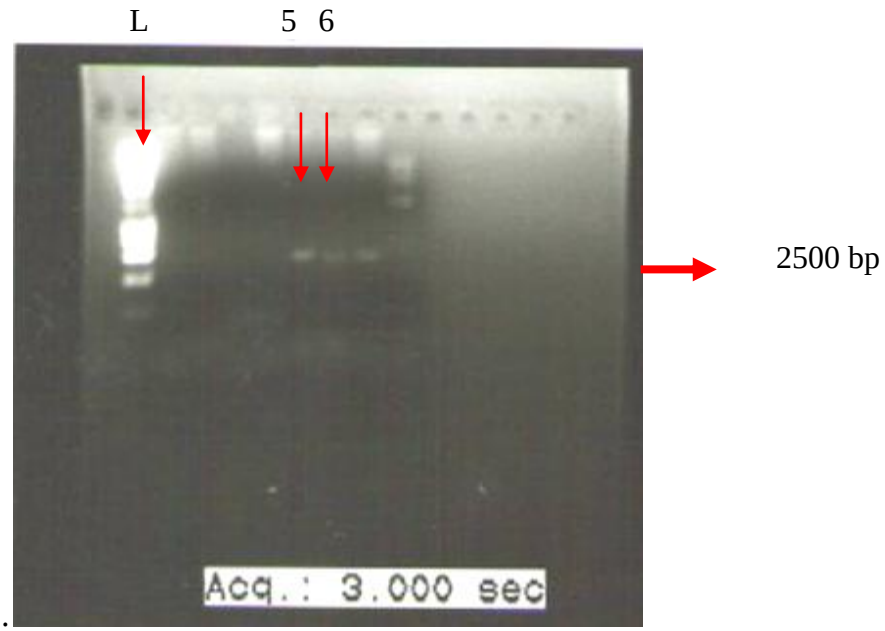


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Fig. 2. 13: PCR-18SrRNA, amplification products of fungal species analyzed by electrophoresis in agarose gel lanes represent; (lane L); hyper ladder; (lane5) *Trichoderma asperellum* (lane 6) no result.

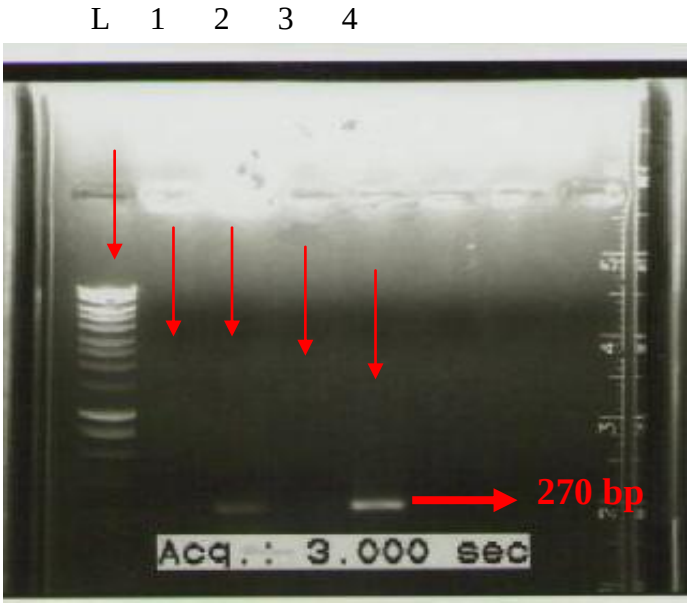

Fig. 2. 14: Polymerase chain reaction (PCR) detection of *Mycoplasma* species in Earth worm samples, EZ-PCR-*Mycoplasma* test analyzed by electrophoresis in 2% agarose gel; the lanes represent (lane M); 1kb hyper ladder, (lane 1); negative control(distilled water). ; (lane 2); earth worm sample; (lane 3); negative result; (lane 4) positive control

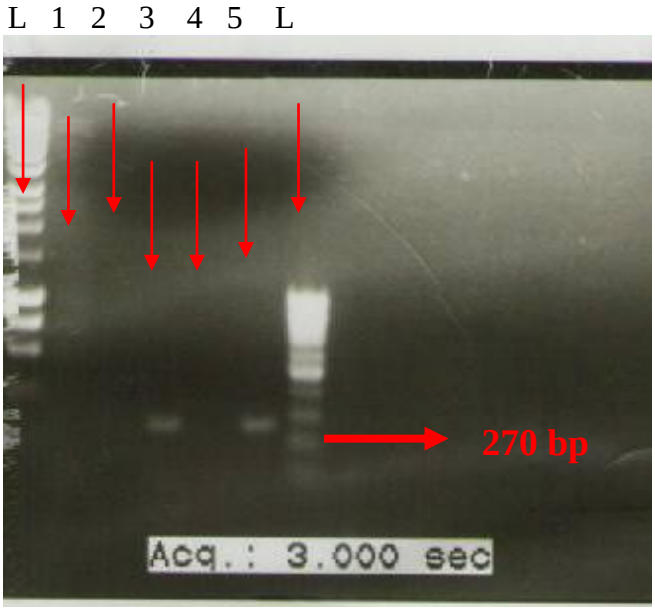

Fig. 2. 15: Polymerase chain reaction (PCR) detection of *Mycoplasma* species in Dog faeces and lettuce samples, EZ-PCR- *Mycoplasma* test analyzed by electrophoresis in 2% agarose gel; the lanes represent (lane L); 1kb hyper ladder, (lane 1); negative control (distilled water).; (lane 2); negative result; (lane 3); dog faeces samples ; (lane 4) control lettuce; (lane 5); contaminated lettuce

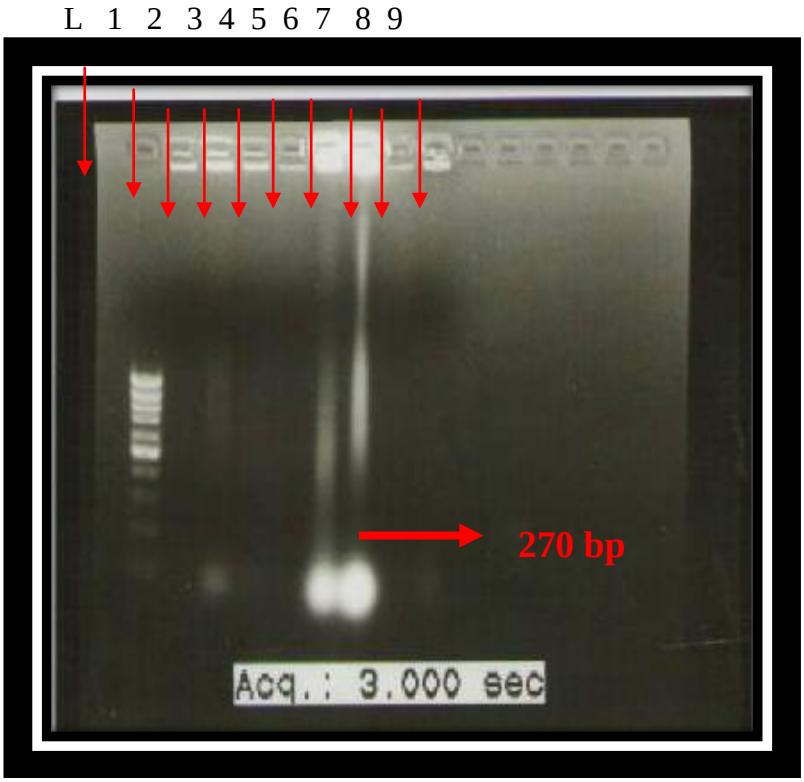

Fig. 2. 16: Polymerase chain reaction (PCR) detection of *Mycoplasma* species in Dog faeces and lettuce samples, EZ-PCR- *Mycoplasma* test analyzed by electrophoresis in 2% agarose gel; the lanes represent (lane L); 1kb hyper ladder, (lane 1); Outside control slug (negative result) (lane 2); Outside contaminated slug; (lane 3); Outside control snails (lane 4) Outside contaminated snails (lane 5); Inside control slugs; (lane 6); Inside contaminated slugs; (lane 7); inside control snails (lane 8); Inside contaminated snails (lane 9) negative control (distilled water)

2.3.5.2. Comments on the detection of *Mycoplasma* using PCR procedure

In this study the EZ-PCR technique was used to detect culturable-independent *Mycoplasma* DNA. This kit can detect a range of *Mycoplasma* species such as (*M. fermentans, M. hyorhinism, M. arginini, M. orale, M. salivarium, M, hominis, M, pulmonis, M, arthitidis, M. bovis, M, pneamoniae, M. pirum and M. carpricolum*) in addition to *Acholeplasma* and *Spiroplasma,* all with a high degree of sensitivity and specificity. The main aspect of this method is that rRNA gene sequences of prokaryotic, including *Mycoplasma*, are well conserved, whereas, the sequences and length of the spacer region in the rRNA operon (e.g. the region between 16S and 23S gene) differ from species to other. Two primers were used to amplify the conserved 16S ribosomal DNA (rDNA) coding region. The primer sequences allow for the detection of *Mycoplasma* DNA but not any other bacterial DNA that may contaminate sample preparations or solutions used for the PCR. Therefore, amplification of the gene sequence with PCR using this primer set enhances not only the sensitivity, but also the specificity of detection. Amplified products are then detected by agarose gel electrophoresis. Although our study was focused on domestic dogs, several species of *Mycoplasma* found in dogs have also been found in humans and other animals which means the potential of transition several species of pathogenic *Mycoplasma* from canine and amongst animal species (e.g. *M. arginini*) has been isolated from a range of hosts which are associated with canines, including goats, sheep, cattle, healthy cats (Tan *et al.*, 1974 and 1977), and camels suffering from pneumonia.

Chapter Three: Reduction in Bacterial Numbers in Dog Faeces Using Black Soldier Fly and Fruit Beetle Larvae

3.1 Introduction

Animal faeces is known to contain pathogens such as *Escherichia coli* and *Salmonella spp,* recent research has shown that waste production from animals is increasing annually as a result of population growth and human activities (Mawdsley *et al.*,1995; Pell,1997). Around a million tons of hen waste is produced annually worldwide (Turnell *et al.,* 2007), while approaching up to one billion tons of cow wastes is produced annually in the United State alone (Islam *et al.,* 2005). This type of waste leads to the pollution of water sources, soils, crops and the air by animal wastes. Factors such as temperature, moisture, aeration and pH value, in addition to manure composition and animal health together influence the population of pathogens present prior to, and during manure storage (Erickson *et al.,* 2004). *Escherichia coli* normally grow over the range of pH 5 to 9 but under laboratory condition can survive several hours at pH 2 to 3 (Small *et al.,* 1994). In order to manage animal wastes a variety of experimental treatments have been attempted, e.g. the mixing of animals manure with carbon sources in order to accelerate the biological oxidation of wastes (Kashmanian and Rynk, 1996). Such a treatment makes the resultant manure more effective for use in agricultural and also kills pathogenic bacteria by the production of high temperature (40-55^{0}C) during the primary stages of composting (Jiang *et al.,* 2003). However, failure to maintain this heat for long periods may allow bacteria to survive (Prysor Williams *et al.,* 2006; Nemiroff and Patterson, 2007). Consequently, the possibility exists that insufficiently treated manure will lead to bacterial contamination of soil and agricultural crops (Cekmecelioglu *et al.,* 2005). As well as biological treatments, chemical approaches can successfully reduce pathogens in manure, examples include: gassing with ammonia and the application of sodium carbonate (Himathongkham, 1999; Park and Diez-Gonzalez, 2003).

Invertebrate larvae have also been evaluated in the treatment of animal wastes and manures; saprophagous or polyphagous larvae can take up as important elements during benefaction of animal waste and increase their protein and carbohydrate contents as a result; in this way, not only is the manure improved for fertilizer use, but the nutrient content of the larvae is increased, making them ideal as food sources for, for example poultry chicken and egg production (Erickson *et al*., 2004). Black Soldier Fly (BSF) larvae, *Hermetia illucens* L. (Diptera:Startiomyidae) have been widely evaluated for this purpose. The life cycle of BSF has four stages; egg, larva, pupa and adult. The larvae range in size from (3– 9 mm) and usually have five larval instars. Black Soldier Fly larvae have been used to bioremediate livestock manure in studies by Sheppard *et al*, (1994) and Sheppard and Newton, (2001) and a number of studies have shown that BSF larvae can reduce the pathogenic bacteria in waste, e.g. *E coli* and *Salmonella Spp* in chicken manure (Erickson *et al.*, 2004; Jeon *et al.*, 2011). Black Soldier Fly larvae can also dry and aerate manure in addition to reducing odours by eating and ingesting of bacterial and fungicidal compound, as a results BSF larvae can modify the microflora of manure and reducing the number of harmful species (Jeon *et al.*, 2011).

The studies reported here were aimed at using BSF larvae and Fruit Beetle grubs (Sun Beetle larvae, FB) *Pachnoda marginata peregrina* (Coleoptera: Scarabaeidae) to reduce the bacterial population of dog waste and also to improve texture and fertilizer nutrient content in the hope that treated dog faeces might be converted from an offensive waste into a useful compost material.

3.2. Materials and Methods

3.2.1 Methods for bacterial isolation and identification

Several broth and agar-based media and selective media based on colony coloration were used:

HiCrome *E. coli* (TM) Agar is a selective medium obtained from Fluka-Sigma-Aldrich Company (see media in Appendix). The medium was used to isolate and enumerate *E. coli* as recommended by Hansen and Yourassowsky, (1984). Most of the *Escherichia coli* strains can be differentiated from other coliforms by presence of the enzyme glucuronidase which is highly specific for *E. coli* (see medium composition in Appendix). The chromogenic agent X-glucuronide used in this medium detects glucuronide activity. *Escherichia coli* cells absorb X-glucuronide and the intracellular glucuronidase splits the bond between the chromophore and the glucuronide. The released chromophore then gives a blue colouration to the colonies (Fig. 3.6 a) of *E. coli*. Brilliance *E. coli*/Coliform agar from Oxoid, on the other hand, gave a purple colour with *E. coli* (Fig.; 3. 6 b).

A highly selective XLT-4 Agar CM 1061 (Xylose Lactose Tergitol™ 4) agar medium obtained from Oxoid was used to isolate and identify *Salmonella* sp., as recommended by Mallinson *et al.*, (2000). Differentiation on this medium is facilitated by the fermentation of xylose, lactose and sucrose as well as the decarboxylation of lysine. *Salmonella* appear as black or red colonies with a black centre (Fig. 3. 7), due to their ability to reduce thiosulphate to hydrogen sulphide, which then causes the colony to blacken (as there are ferric ions present in the medium). The makers instruction for the preparation of this medium are as follows: Suspend 59g of XLT-4 Agar Base in 1 litre of distilled water, then 4.6ml of XLT-4 SR0237 selective supplement was added to the medium and brought to the boil. The medium was not autoclaved. After cooling

to approximately 50°C, the medium was poured into sterile Petri-dishes (Tate *et al.*, 1990).

A range of media including Luria-Bertani (L.B) , Plate Count Agar (Fig. 3.8) and Mueller Hinton (M.H) and Nutrient Broth or Agar were also prepared for routine isolation and growth studies. Bacteria were also identified using 16s rRNA analysis; DNA was extracted according to the instructions of the Anachem Key prep-Bacterial DNA Extraction kit, and then bacterial 16S rRNA gene was amplified and sequenced using PCR. After checking, the purified PCR products were sent to the Medical School, (University of Sheffield) to be sequenced. 16SrRNA gene sequences were adapted using the Finch TV software and then exported into The Basic Local Alignment Search Tool (BLAST), available from the website of National Centre for Biotechnology Information (NCBI)http://www.ncbi.nlm.nih.gov, to identify matches with existing characterized reference sequences

3.2.2. Dog faeces and insects larvae collection

Dog faeces (from an Alsatian) were collected with the cooperation of a dog owner in Sheffield. The fresh dog faeces samples were distributing onto two small boxes (30×20×15 cm) and placed at laboratory condition whereas the first box treated with Black Soldier Fly larvae (Fig.3.1). One hundred larvae per 100g dog faeces approximately were used, as suggested by Li *et al.*, (2011). The second box included 100g of dog faeces (without larvae) as the control. Fruit Beetle larvae were treated in the same manner, except that 10 FB larvae (Fig.3.3) were added to 100 g of dog faeces; controls were also set up. The larvae were purchased online from Ricks LiveFood. Black Soldier flies were reared to the adult stage (Fig.3.2) and Fruit Beetle stages from larvae (Fig. 3.3) pupa (Fig. 3.4) and adults (Fig. 3.5) to the next generation.

3.2.3. Bacterial isolation from dog faeces

Bacteria were isolated at weekly intervals over a 28 day incubation period. Samples 1g were diluted in 9 ml of sterilized water then a serial dilution from 0.1 to 10^6 was performed; the 0.1 ml of each diluted samples were then spread on *E. coli* agar , XLT4, L.B and count plate media, and incubated between 18 and 24 h at 37^0C. In this case, both the control and treatment dog faeces were sampled from control and treatment (with larvae) boxes in triplicate at each sampling day (0, 7, 14, 21or 28)

3.2.4. Bacterial isolation from the larval gut

Bacteria were isolated from BSF and FB larvae gut and surface after being fed on faeces. For internal isolation, the outer surface was rinsed three times in sterilized water. The larvae were then anaesthetized using a piece of cotton wool wetted with chloroform (100%), placed with the larvae in Petri-dishes for few minutes. The anterior end, near the cephalopharyngeal skeleton, was opened and the abdominal contents were squeezed gently to collect 1g of the gut contents. A serial dilution was then prepared for BSF and FB larvae to determine the number and type of bacteria, and 0.1 ml of each diluted samples was taken for both control and treatment larval gut and spread onto the *E. coli* agar , XLT4, and count plate media and incubated between 18 and 24 h at 37^oC; triplicates were used throughout.

3.2.5. Determination of pH

The pH of the dog faeces was determined as follows; 5 g of dog faeces were dissolved in 50 ml deionised water (Erickson *et al.*, 2004); suspension were shaken for 15 min on a reciprocal shaker at 100 g until the suspension became homogenous, The pH of the final solution was measured weekly using a calibrated Jenway 3310 pH meter (Bogdanov *et al.*, 2002).

3. 2. 6. Statistical analyses

All observations are presented as Mean ±SD (Standard deviation). Bacterial populations were converted to log CFU/g before statistical analysis. Statistical analyses were performed on experimental datasets using the t-Test and three way ANOVA at >0.05 probability level compared significance of means of three replication. Results were analysed using Sigma Plot 11.0© software.

Fig. 3. 1: Black Soldier Fly larvae, 1st instar

Fig. 3. 2: A Black Soldier Fly adult

Fig. 3. 3: Fruit Beetle larvae 3rd instar

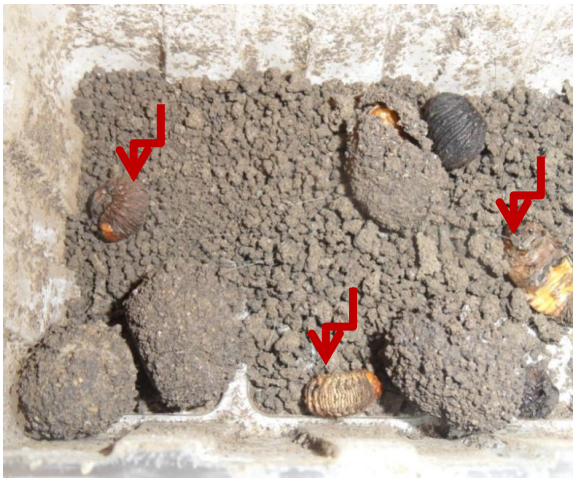

Fig. 3. 4:.Fruit Beetle pupae

Fig. 3. 5: Fruit Beetle adults

3.3. Results and Discussion

3.3.1. Identification of bacteria associated with dog faeces and larvae

Several species of bacteria were isolated from dog faeces and intestinal tract (gut) of both the Black Soldier Fly and the Fruit Beetle FB larvae using the chromogenic media, Hicrome *E. coli* and Brilliance *E. coli*/ Coliform agars. XLT-4 agar was used to isolate and identify *Salmonella Spp*. The results (Table 3.1) show that *E. coli* produced a blue or pink/purple colony (Fig. 3.6), while *Salmonella enterica* produced a black colony on these media (Fig. 3.7). Bacteria growing on Plate Count Agar were not identified (Fig. 3.8). Scanning Electron Microscope (SEM) confirmed that the morphology of bacteria isolated from dog faeces and gut of larvae agreed with *E, coli* and *Salmonella enterica* (Fig. 3.9 a) while some of bacteria species were cocci, e.g. MRSA(Fig. 3.9 b). Molecular identification of gDNA using 16S rRNA results confirmed the species isolated as *Escherichia coli* and *Salmonella enterica*. The samples were sent to Medical School Core Genetics Unit for sequencing, and then we used (BLAST) to compare the sequence. Finch TV software was used and compared with BLAST to look for the closest matches. The results show the species isolated from dog faeces were: *E. coli* and *Salmonella enteric* 100% and 99% respectively see appendix 2 bacteria sequences

Table. 3. 1: Media used to detect and identify *E. coli* and *S. enterica* from dog faeces, BSF and FB larvae gut

Sample	Bacterial species	Appearance of the colony	SEM / Cell morphology	Media name	Gram nature	DNA Similar
Dog faeces	***E. coli***	**Blue or purple**	***Bacillus***	**HiCrome or Brilliance**	**Gram negative**	**100%**
BSFL gut	***E. coli***	**Blue or purple**	***Bacillus***	**HiCrome or Brilliance**	**Gram negative**	**100%**
FBL gut	***E. coli***	**Blue or purple**	***Bacillus***	**HiCrome or Brilliance**	**Gram negative**	**100%**
Dog faeces	***S. enterica***	**Black**	***Bacillus***	**XLT-4**	**Gram negative**	**99%**
BBSFL gut	***S. enterica***	**Black**	***Bacillus***	**XLT-4**	**Gram negative**	**99%**
FBL gut	***S. enterica***	**Black**	**Bacillus**	**XLT-4**	**Gram negative**	**99%**

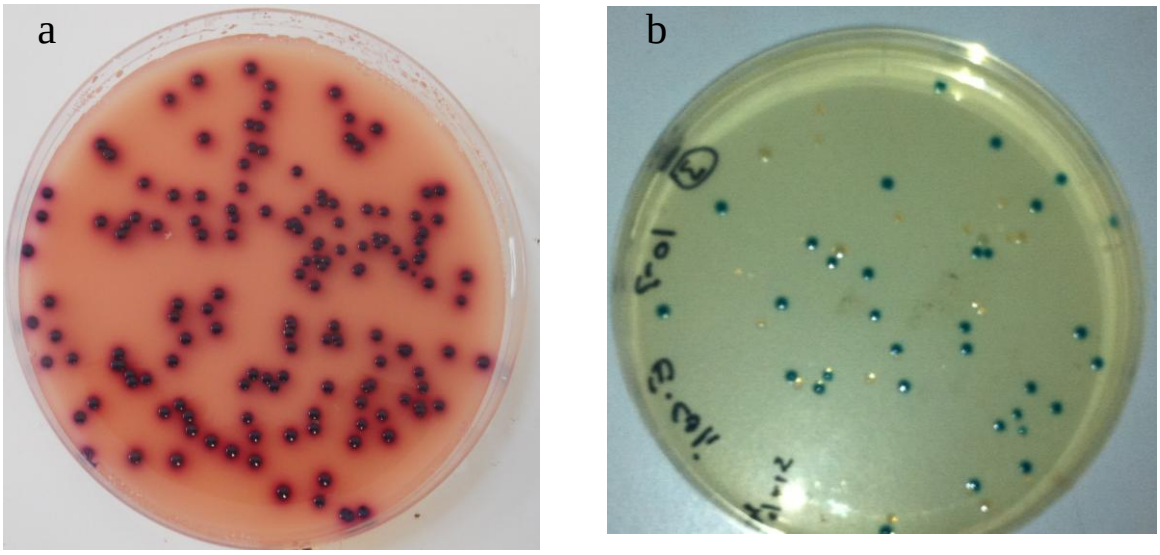

Fig. 3. 6: *Escherichia coli* grown on selective chromogenic agar *E. coli* media isolated from dog faeces and larvae fed on dog faeces; a) Brilliance *E. coli* /Coliform agar showing blue colonies, b) HiCrome *E. coli* agar showing purple/pink coloured colonies

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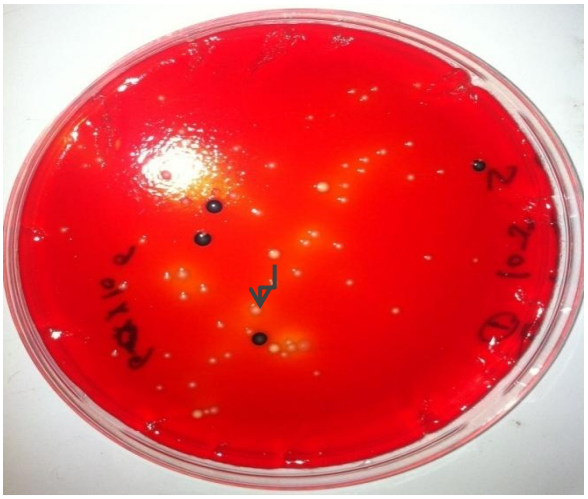


Fig. 3. 7: *Salmonella Spp* grown on XLT-4 selective *Salmonella* agar medium

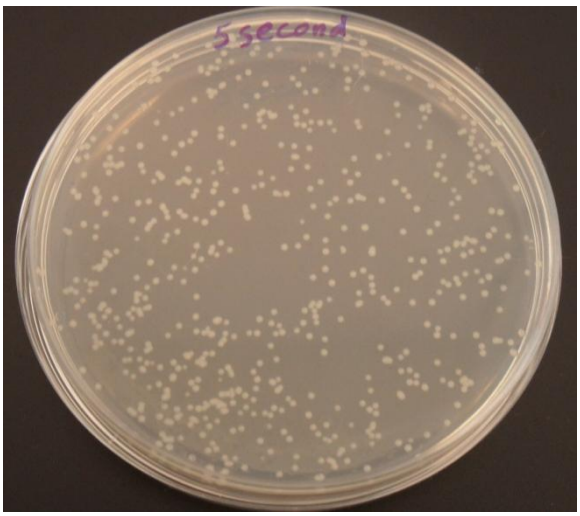


Fig. 3. 8: Colonies of bacteria grown on plate count agar medium

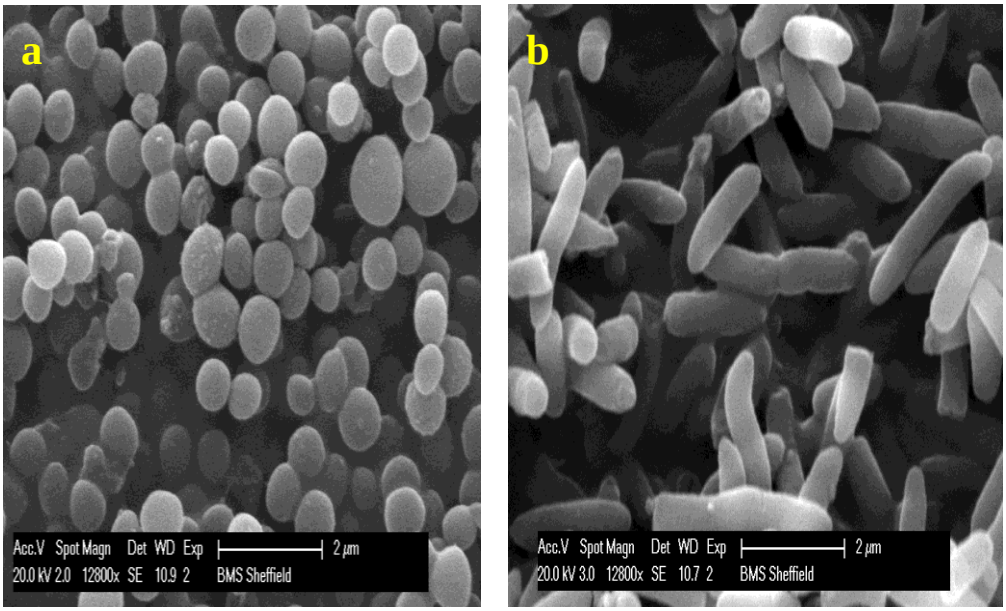


Fig. 3. 9: Two bacterial isolates showing different external morphology; a) cocci, b) bacillus, observed by SEM.

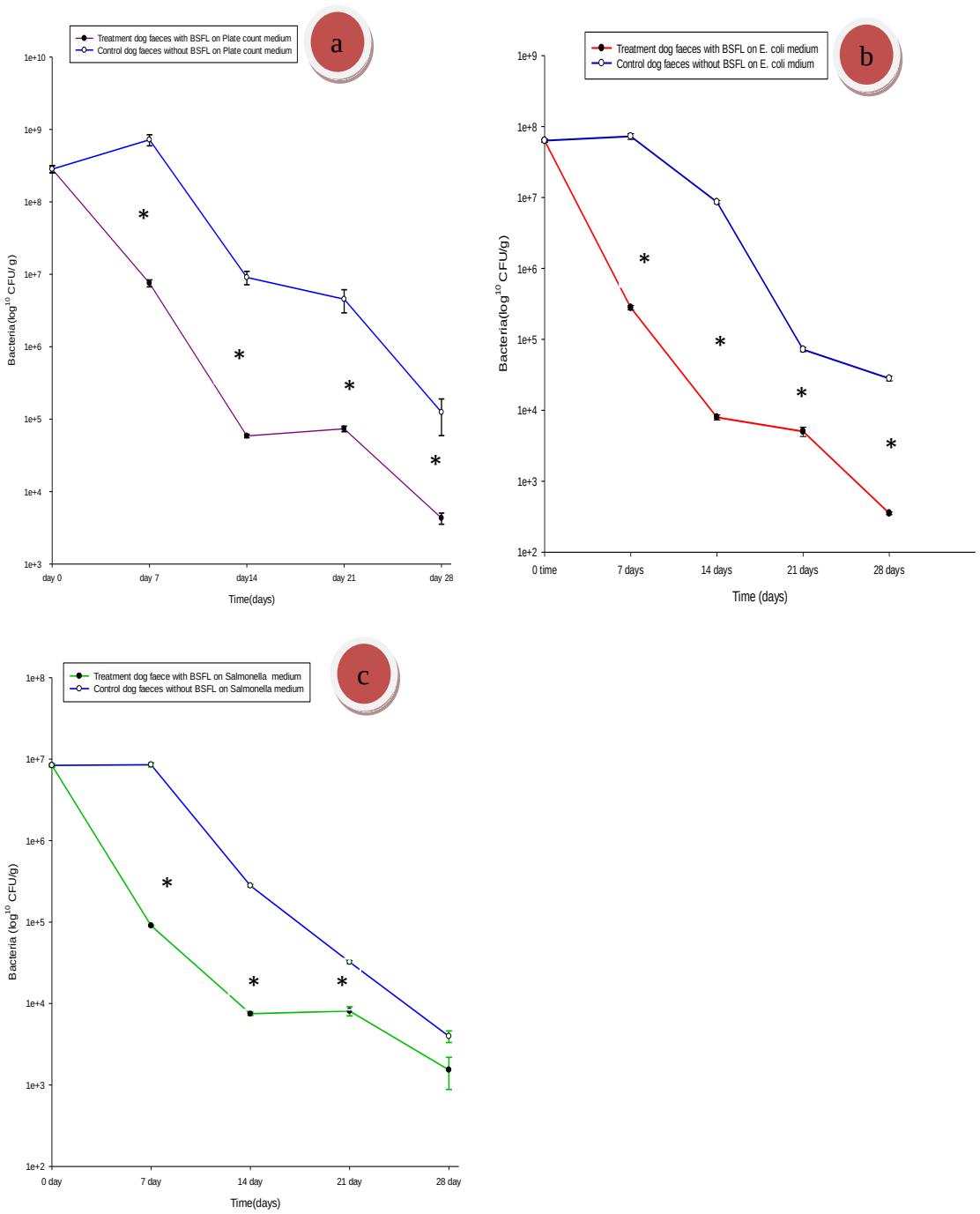


Fig. 3. 10: Bacterial numbers in dog faeces grown on; a) plate count medium, b)HiCore *E. coli* in and c) *Salmonella* medium after treatment with Black Soldier Fly larvae

(Means of triplicate (±) SD log CFU/g (*significantly different from control)

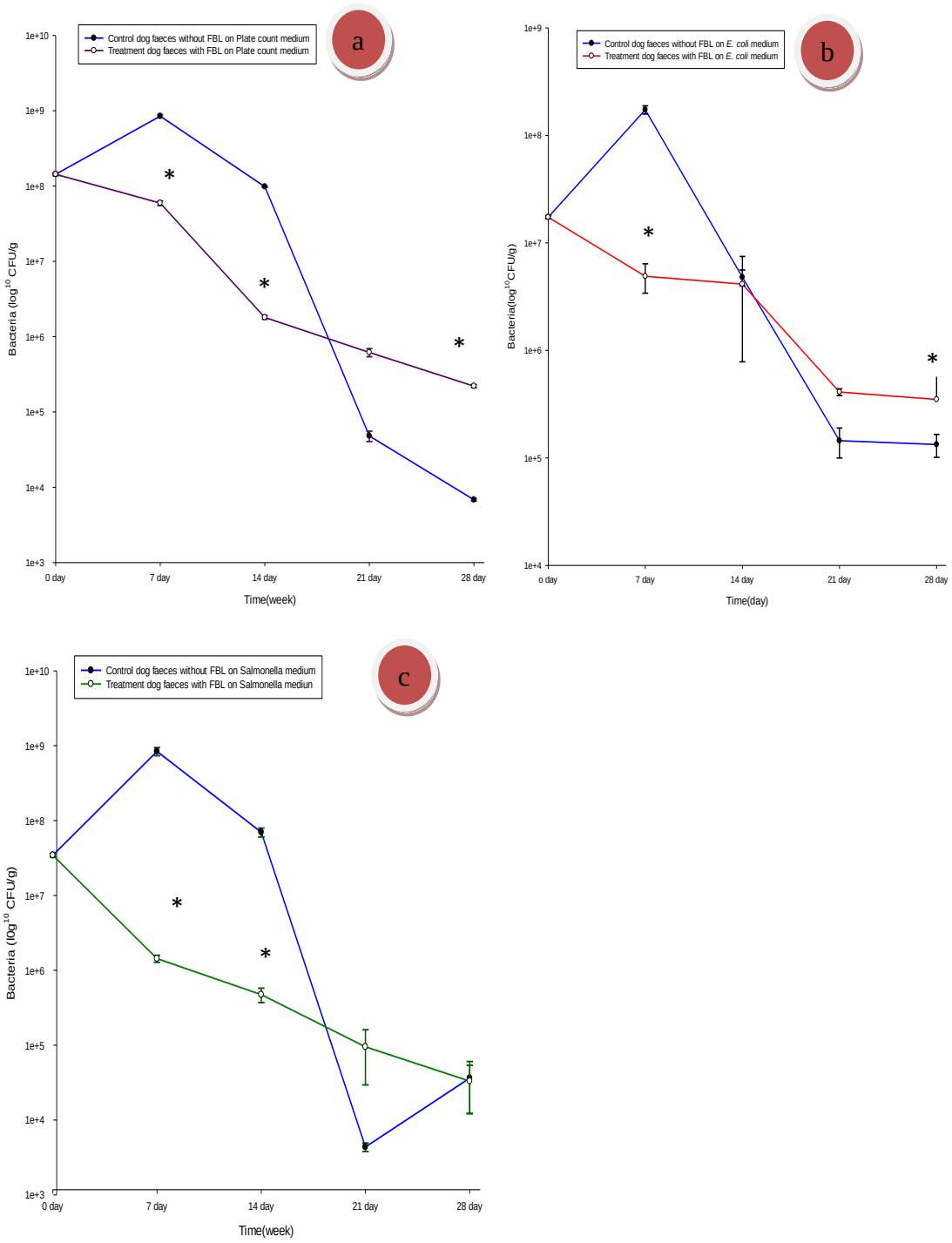


Fig. 3. 11: Bacterial numbers from dog faeces, bacteria grown on; a) plate count medium, b) HiCore *E. coli* medium and c) *Salmonella* medium by Fruit Beetle Larvae.

(*Significantly different from control)

Figure 3.10a shows that the "total heterotrophic bacterial count" in the faeces treated with BSFL were markedly and statistically reduced. The same trend was found in relation to numbers of *E.coli* (Fig.3.10b) and *Salmonella* (Fig.3.10c). Figure 3.11a shows that the total number of heterotrophic bacteria in faeces treated with FBL, as was the case with BSFL declined compared to the control, at least for the first 14 days; the numbers in the control then declined rapidly so that numbers in the treated faeces then exceeded the control at days 21 and 28. This marked decline in bacterial numbers following BSF treatment was also seen in relation to treatment with *E.coli* and *Salmonella* (Figs.3.11b,c).

The overall trend following BSF larval treatment was a reduction in numbers for the first 14 days, followed by an increase over the control for days 21 and 28. The increase in numbers after 14 days may however, have been anomalous due to a rapid and unexplained fall in numbers in the control. The results show that BSFL can be used to reduce the numbers of bacteria, including some important pathogens in dog faeces. The findings relating to the use of FBL are in agreement with this trend up to day 14 but increases in bacterial population then occur which, if the control's rapid decline is not anomalous is worrying in relation to the potential use of FBL as a compost additive or fertilizer.

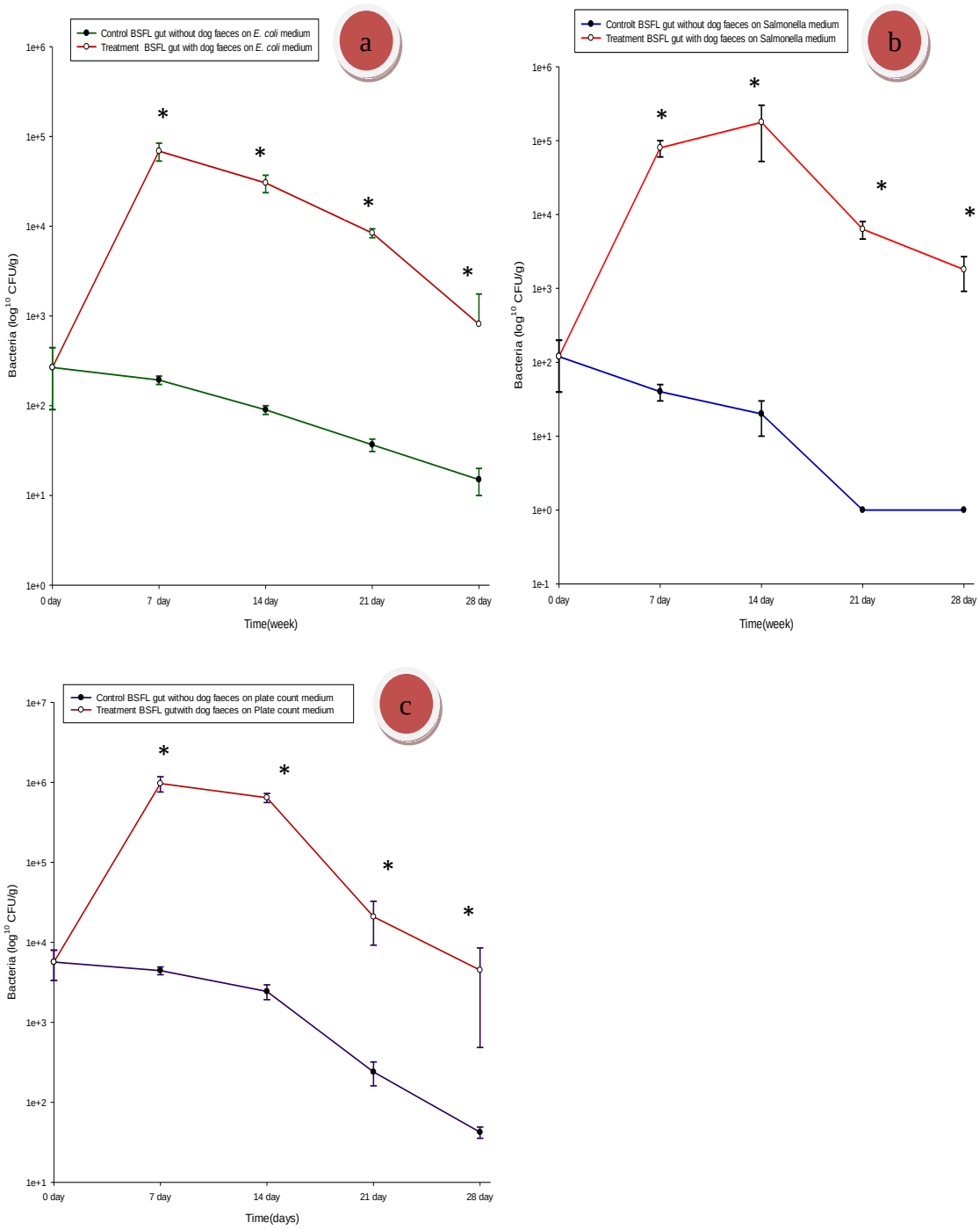


Fig. 3. 12: Numbers of bacteria; a) *E. coli*, b) *Salmonella enterica* and bacterial species grown on plate count medium isolated from gut of BSF larvae

Means of triplicate (±) SD log CFU/g (*Significantly different from control).

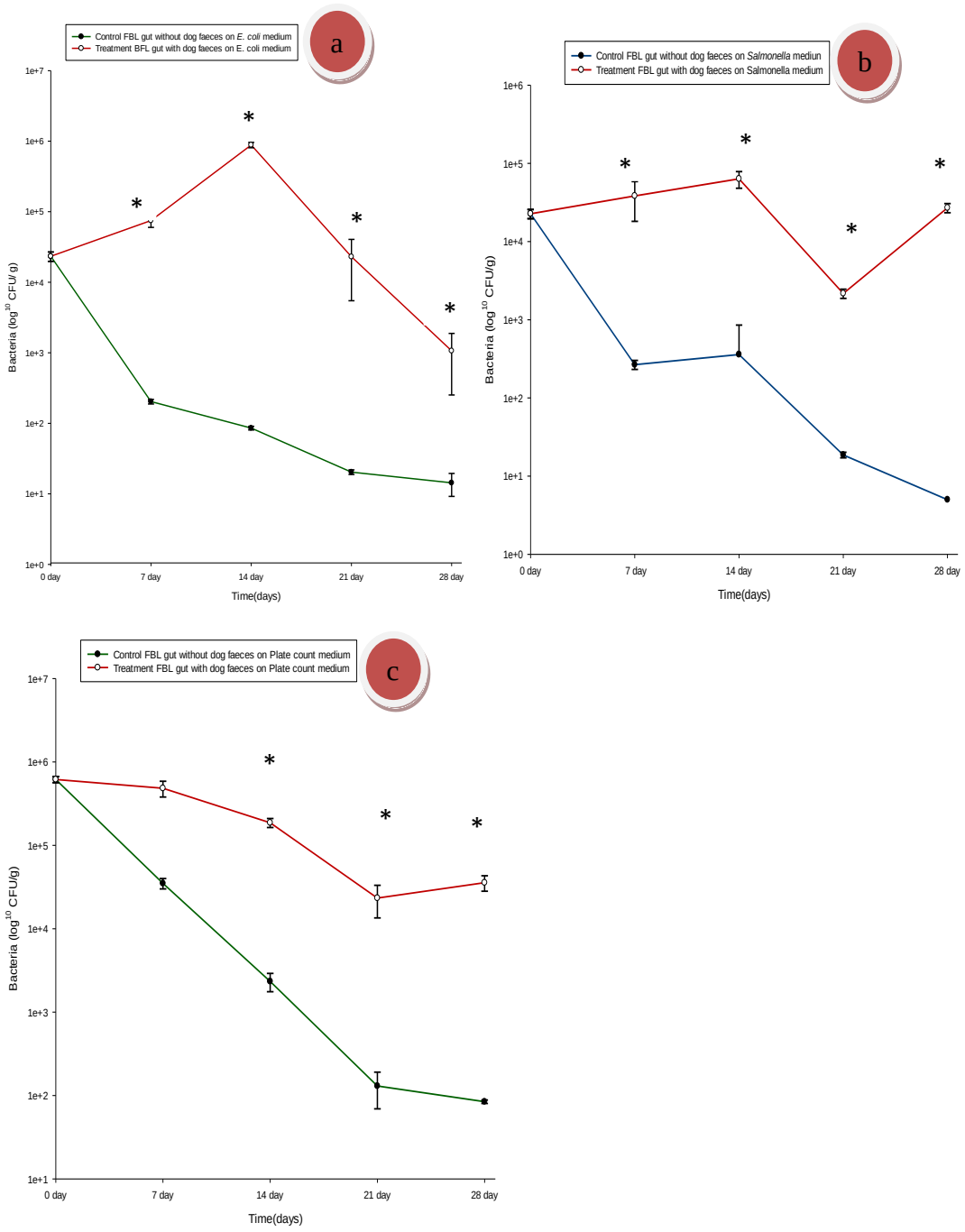


Fig. 3. 13: Numbers of total "heterotrophic bacteria" in gut of FB larvae; a) *E. coli* b) *Salmonella enterica* and c) bacterial species grown on plate count medium

Means of triplicate (±) SD log CFU/g (*Significantly different from control).

Figure 3.12 shows the number of bacteria, including pathogens inside the gut of BSFL following feeding on dog faeces. The overall trend is obvious, namely feeding leads to a statistically significant increase in the number of bacteria inside the BSFL gut. The same trend is seen in relation to dog faeces fed FBL (Fig 3.13). This trend of increasing bacterial numbers in larvae fed on dog faeces is particularly worrying in relation to the potential feeding of these larvae to animals-post exposure to faeces. Black soldier Fly larvae in particular are currently being produced for use as animal feed (mainly to chickens) following feeding on restaurant food waste. It is unlikely then, considering the high pathogen content of larvae fed on dog faeces that these could be safely used as animal feed following feeding on dog faeces. The only reference seemingly available on the effect of feeding BSFL on animal faeces was reported by Erickson *et al.* (2004),who found that they reduced the pathogenic bacteria population, including *E. coli* and *Salmonella* in chicken and hogs manure.

Chapter Four: Modification of Canine Faeces by Black Soldier Fly and Fruit Beetle Larvae in relation to Plant Nutrient Release and Potential Use of Modified Faeces as a Compost Additive

4.1 Introduction

The digestive tract of animals contains a wide range of microorganisms and as a result, their faeces may also contain these organisms which include a variety of pathogenic and saprophytic faecal bacteria (Mawdsley *et al.*, 1995; Pell, 1997).

The accumulation of dog faeces in the urban environment poses a major threat to animals, plants, human health and water sources (Rippy *et al.*1997). In the United States alone, dog faeces contamination is estimated to be around ten million tons annually (Brinton and Storms 2004) and the disposal of dog faeces is a major problem in regions of high-density dog populations. Dog populations vary worldwide; in the city of Quebec for example, approximately 25% of residents own at least one dog (Marketing, 2002). The estimated dog population of Quebec is 742,728, with 432,000 being found in Montreal alone (Institute de la statistique Quebec 2005a). A European survey showed that 44,000,000 domestic animals, including 6,900,000 dogs, were living in Italy in 2002. In 2007 a random study in the UK revealed that dogs were owned by 26% of households, while the 2006 dog population was around 10.5 million. The average daily faecal production of a dog is approximately 100 grams and open spaces, public gardens, pathways, arcades, pedestrian precincts and roadways are the sites with the highest pollution (Tarsitano *et al.*, 2010). Canine faeces, are often not removed from the urban environment, due to the bad habits of the owners and they clearly represent a source of potential pathogens in addition to being an unsightly inconvenience (Nemiroff and Patterson, 2007).

In the past, series of treatments have been used to reduce the pathogenic bacteria content of animals manures; the mixing of animals manure with various carbon sources being the most common method used to activate the biological oxidation process. (Kashmanian and Rynk, 1996). In this treatment method, pathogenic bacteria are killed by the high temperature (40-550C) produced in the primary stages of the composting

process (Jiang *et al.*, 2003). However, the failure to maintain such high temperatures for long periods may allow pathogens to survive (Prysor-Williams *et al.*, 2006, Nemiroff and Patterson, 2007) Consequently, the possibility for bacterial contamination of soil and agricultural crops resulting from the of inefficiently composted manure is a concern (Cekmecelioglu *et al*., 2005). In addition, chemical treatments that have been used with success to reduce pathogens in manure (including gassing with ammonia and the application of sodium carbonate (Himathongkham, 1999) may introduce further environmental risks.

Many insect species depend completely on animals manure as organic materials as their main food source, so that a number of insects (Saprophagous) can be used to help recycle and improve the fertilizer quality of animal manures (Erickson *et al.*, 2004).

Recently, a range of studies on animal waste benefaction have been conducted in China, United States, Mexico, Eastern Europe, Australia and Central and South America, aimed at treating animals faeces with insects to produce manure and animal feed in the form of larvae; insect larvae were found to reduce the nutrient concentration and mass of the manure residue, thereby reducing the pollution potential of the waste by 50-60% or more. (Newton *et al.*, 2005)

A novel approach involves the use of black Soldier Fly (BSF) larvae *Hermetia illucens* L. (Diptera: Startiomyidae). The life cycle of BSF has four stages; egg, larva, pupa and adult. The larvae range in size from (3– 9 mm) and usually have five larval instars. Female adults do not need to feed and lay around 900 eggs in their short life of 5 to 8 days; adults surviving on the large fat body stored from the larval stage. The black Soldier Fly is not recognized as a pest since the adult Fly is not attracted to human habitation or foods, nor does it bite or sting (Newton *et al.*, 2005)

The larva of the black Soldier Fly is an common sight is some countries in decaying organic matter including kitchen waste, spoiled feed and the larval stage of BSF and its

use has been suggested as a means of reducing livestock manures (Craig Sheppard *et al.*, 1994; Sheppard and Newton, 2001) a number of studies have demonstrated the ability of BSF larvae to recycle animals manure and reduce the number of pathogenic bacteria which they contain; *E coli* and *Salmonella spp* populations have, for example, been reduced in chicken manure (Erickson *et al.*, 2004). Previously, several studies have also reported on the possibility of recycling the dog faeces using physical and chemical approaches whereas some have utilized BSF larvae to reduce the bacterial population from animals manure such as hogs, cows and poultry. The work discussed in this Thesis focuses on using the BSF larvae to treat dog faeces. In addition, Fruit Beetle larvae FBL *Cotinis mutabilis* (Scaraaeidae: Coleoptera) were investigated and compared with BSFL in relation to their ability to reduce populations of *E. coli* and *Salmonella Spp*.

The aim of this study therefore was also to investigate changes in the concentration of ammonium and nitrate, and sulphur oxidation and phosphate solubilisation during larval treatment of dog faeces with of BSF and FB larvae in the hope of converting the biomass of these wastes dog faeces from a toxic, unsightly waste-product, into a safe and useable fertilizer and compost additive.

4.2. Materials and Methods

4.2.1. Sample collection

4.2.1.1. Samples collection from canine waste

Samples of fresh dog faeces were collected from a single dog (Alsatian) owned by a local family. The collected samples were distributed into eight plastic boxes (15x 20× 10 cm), each box containing 100 g of dog faeces with or without larvae. Three replicates were used and the results compared using T-Test at 0.05 probability level and ANOVA to determine levels of significance using Sigma Plot 11.0.

4.2.1.2. Samples collected from treated dog faeces

After 28 days, the treated dog faeces (now compost) were collected, weighed and then divided into control and treatment without added larvae. Samples (100g) of the treated and control faeces for each element were then placed in polythene bags. Treated samples were amended by addition ammonium, nitrate, sulphur and insoluble phosphate regents while the control no elements regents were added to the samples . Sample were taken a weekly to measure the elements.

4.2.1.3. Effect of larvae on the nutrient content of dog faeces.

A number of first instar Black Soldier Fly (BSF) and Fruit Beetle (FB) larvae were bought from a commercial company (Ricks Live-Food) in UK. Dog faeces (100)were were placed in the plastic boxes and 100 larvae of BSF and 10 larvae of FBL were added; a control with no larvae was also set up. The treatments were left for 28days under laboratory conditions and sampled at 7 day intervals for the following plant-fertilizer ions: ammonium, nitrate, sulphate and phosphate.

4.2.3. Nutrient transformations in modified dog faeces amended with ammonium nitrate, sulphur and insoluble phosphate

Samples of dog faeces which had been modified by treatment with either BSFL or FBL and the larvae were removed. The two types of modified samples (100g) were then amended, in triplicate (in polythene bags) with ammonium (5ml of a 100ug per ml solution of $NH_4SO_{4)}$ and ammonium and nitrate was measured; elemental sulphur (1g) and sulphate was measured; insoluble calcium phosphate (Ca_3O_8 P_2,1g) and soluble phosphate was measured. Controls lacking amendment were also set up. Then 20 ml of water was added to each bag, which were closed to allow a small hole to allow for gas exchange; the samples were then incubated at 25^o C for 7, 14. 21 and 28 days elements determined as follows.

4.2.3.1. Determination of ammonium–N (NH_4^+-N) in dog faeces

Ammonium was extracted from both control(without larvae) and treatment (with larvae) dog faeces as well as to the modified control and treatment dog faeces (dog's compost) by adding solution of (15% w/v) of KCL in the ratio;(1g) of samples : (10 ml) KCL. The dog faeces were shaken for 30 min at 70 g then, in order to determine the concentration of ammonium N(NH_4^+-N) 2 ml of filtrate was mixed with (1ml) of EDTA (6% w/v), then (7ml) of distilled water, (5ml)of phenolate reagent (see appendix), and (3ml) of sodium hypochlorite solution (10%v/v). The mixture was then incubated at $25C^0$ for 20 min in the dark. The volume was made up to 50 ml and mixed then the concentration of the indophenol-blue ammonium complex was measured at 630 nm (Wainwright and Pugh, 1973), the concentration of ammonium intensity was determined by reference to a standard curve(10-100µg NH_4^+-N) prepared from a standard solution of ammonium sulphate (see Appendix). Urea (0.5g) was added to the treatment samples (amended) dog faeces then while control was set-up lacking urea.

4.2.3.2. Determination of Nitrate – N ($NO3^-$-N)

Samples were extracted (1g) of each and shaken for 15 min with 20ml of water then the samples were filtrated through Whatman No 1 filter paper. Nitrate was determined using the method of (Sims and Jackson, 1971). Chromotropic acid: 7ml was mixed with 3ml of the filtrate then incubated at $40C^0$ in water bath for 45 min; the producer yellow colour was measured at 410 nm and the concentration of nitrate was determinate by reference to a standard curve of nitrate concentration.

4.2.3.3. Determination of Sulphate-SO_4^{2-}- S

Samples (1g) were shaken with 10 ml of water then shaken at 70 g using an orbital shaker for 15 min the samples were filtrated through Whatman No.1 filter paper. Sulphate-S was determined using turbidimetric method to analysis the sulphur element (Hesse, 1971) as follows: Filtrate (10 ml) was transferred into a 250 ml volumetric flask, 1g of barium chloride $BaCl_2$, and 2 ml of gum acacia (0.25% w/v) were added and mixed well then the volume increased up to 25 ml of water. The white suspension resulting from precipitation of $BaCl_2$ was measured at 470 nm using a spectrophotometer and the concentration if of $^{SO42-}$-^{S} was determined by reference to a standard curve (0- 100µg sulphate ml) prepared from a standard solution of Na_2SO_4. (see Appendix).

4.2.3.4. Determination of phosphate solubilisation PO_4^{3-}-P

Samples (10g) were placed into screw capped glass bottle containing 100 ml of 0.5 N $NaHCO_3$ and all the bottles were shaken for 30 min at 70 g, using an orbital shaker, the contents were then filtered through Whatman No. 1 filter paper. Phosphate ions were determined as described by Falih, (1995) as follows: Filter (3 ml) was mixed with 7 ml of work solution (see Appendix) then the mixture incubated at $37C^0$ for 1 hour. A

serial dilution were taken up to log 10^{-3} for the blue colour reaction then was measured at 820 nm using a spectrophotometer and the amount of PO_4-P were determined by reference to a calibration curve(0- 8 µg PO_4-P ml^{-1}.

4.2.4.Measurement of the pH of dog faeces samples

The pH of the dog faeces was determined each from zero time until 28 days, using a 1:1 distilled water suspension , shaken for 15 min on a reciprocal shaker (100 revolution min^{-1}), and a pH meter fitted with a glass electrode.

4.2.5. Determination of the effects of modified dog faeces as a compost additive on plant seed germination and growth

The two types of modified dog faeces (BSFL and FBL) mixed with a local grassland soil, which had not recently been fertilized, in the ratio 25%, 50% and 75%. Controls consisted of modified and unmodified dog faeces. Samples (100g) of each mixed proportions were planted with turnip or lettuce. The treatments were set up in triplicate and watered twice a week and left under laboratory condition and plant growth was observed.

4.3. Results and Discussion

4.3.1. Effect of larvae on the texture of dog faeces

Untreated dog faeces were greasy and tightly packed (Fig.4.1) and possessed an offensive odour. Treatment of fresh dog faeces with Black Soldier Fly and Fruits Beetle larvae reduced two 100g sample to 38g and 43g of modified dog's faeces within 4 weeks (62% and 57% reduction using BSF and FB larvae respectively. The modified dog faeces were more friable and loosely textured and had lost their faecal structure and their offensive odour (Fig.4.2).

Fig. 4. 1: Untreated dog faeces after 28 days incubation

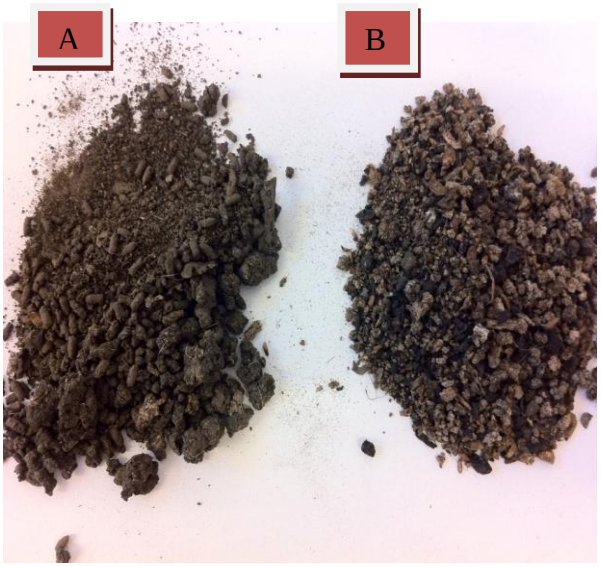


Fig. 4. 2: Modified faeces: a) treated with FBL;
b) treated with BSFL after 28 days

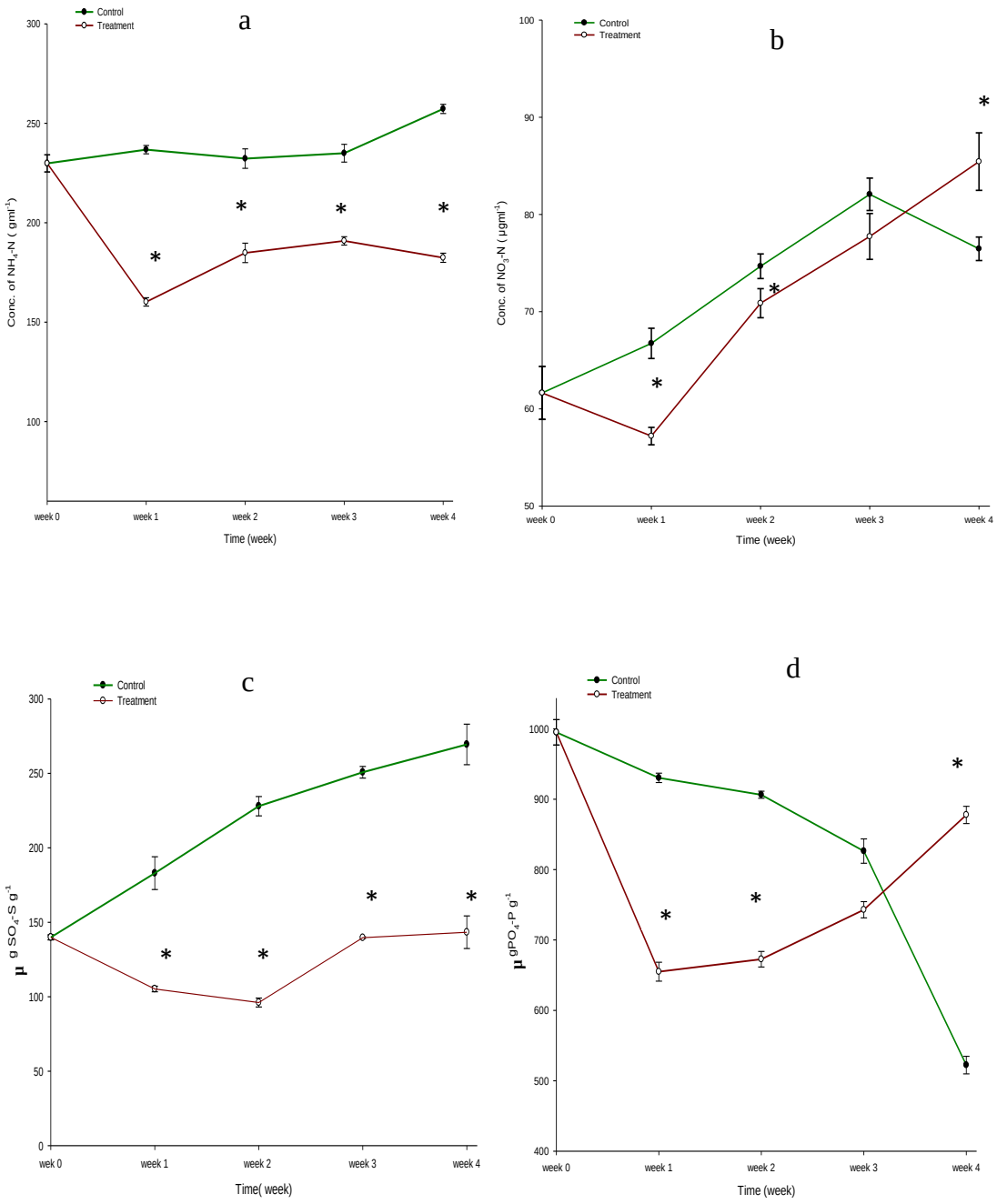


Fig. 4. 3: The effect of BSFL activity on indigenous; a) ammonium, b) nitrate, c) sulphate and d) phosphate concentration in dog faeces(* Significantly different from control, p=0.05).

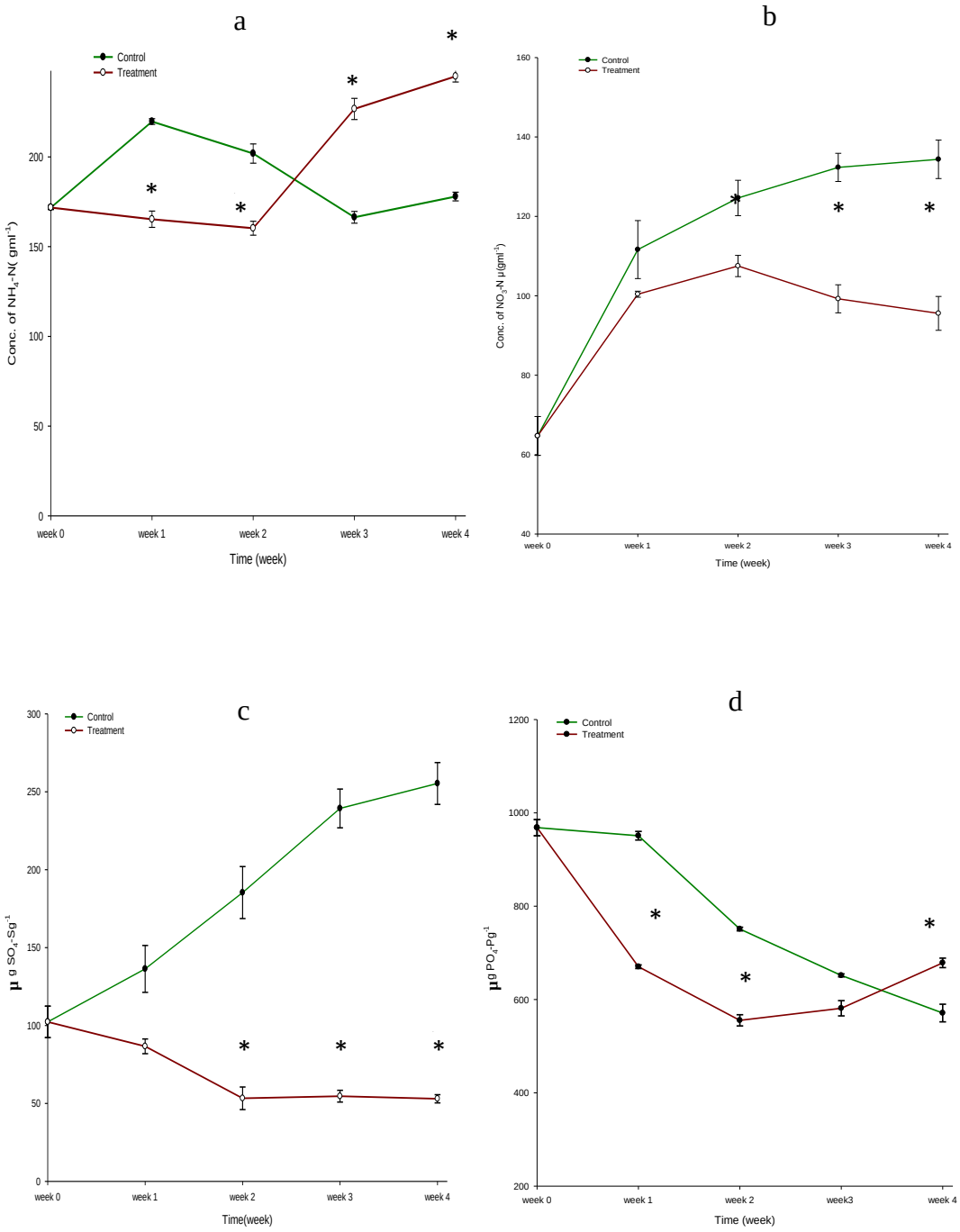


Fig. 4. 4: The effect of FBL activity on a indigenous a) ammonium, b) nitrate, c) sulphate and d) phosphate concentration in dog faeces (* Significantly different from control, p=0.05)

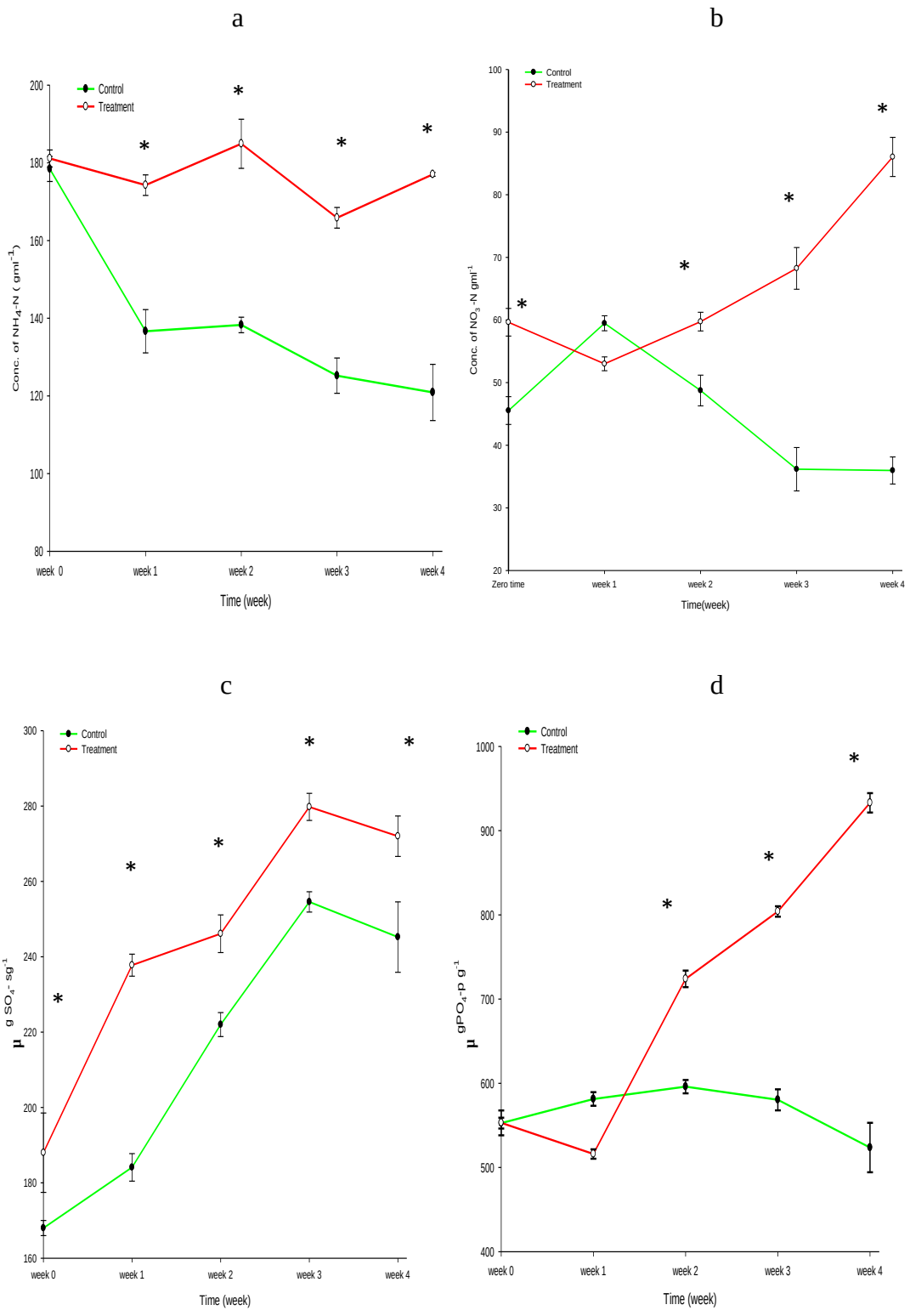


Fig. 4. 5: The effect of length of incubation on a) ammonium concentration following addition of (10µg/ $(NH_4)_2SO_4$-N g) , b) nitrate concentration following the addition of (10µg/ $(NH_4)_2SO_4$-N g), c) S^0 oxidation following the addition of (1% w/w) S^O and d) phosphate solubilisation following the addition of (1% w/w)calcium phosphate to dog faeces modified with BSFL(* Significantly different from control, p=0.05)

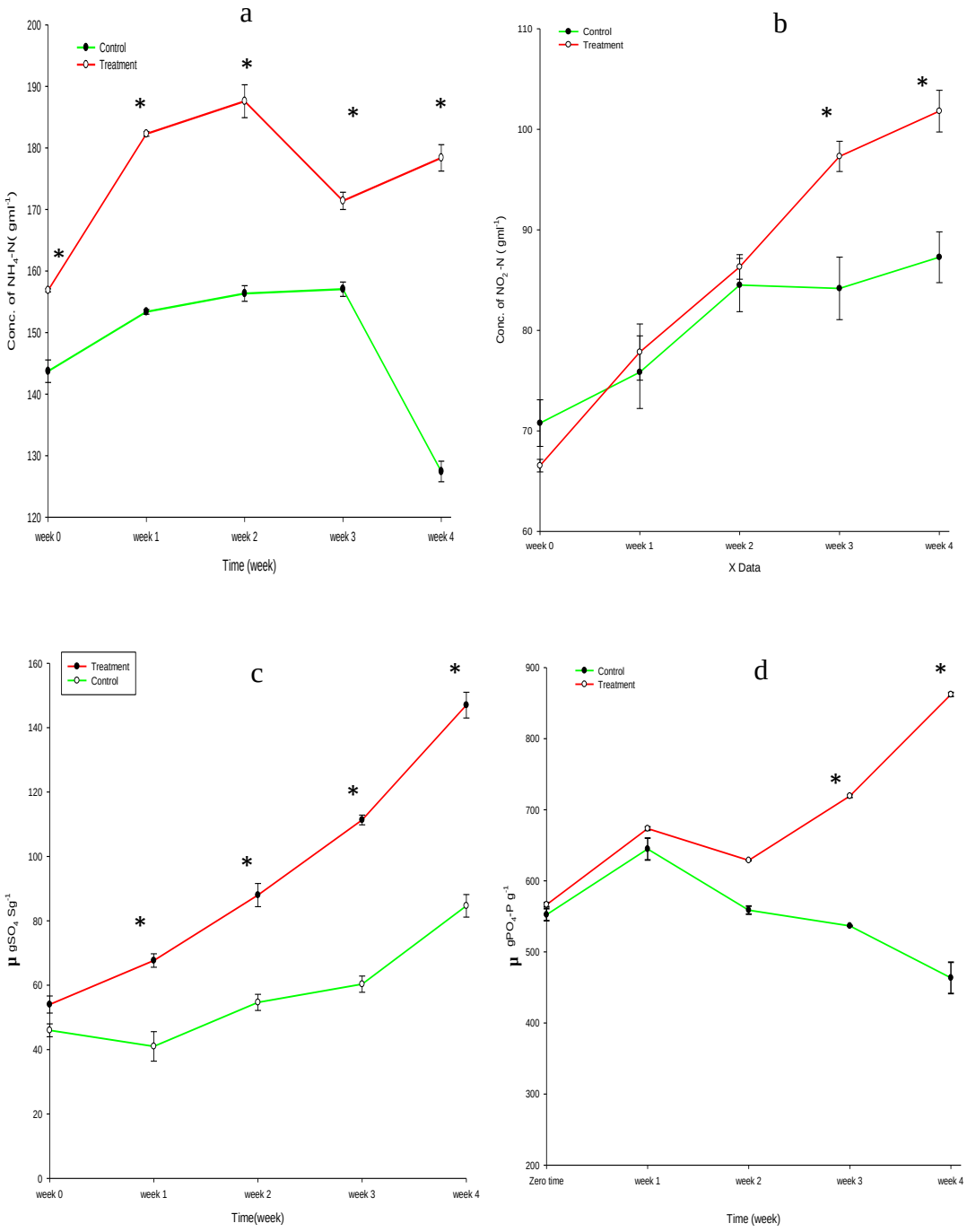


Fig. 4. 6: The effect of length of incubation on a) ammonium concentration following the addition of (10µg/ $(NH_4)_2SO_4$-N g), b) nitrate concentration following the addition of (10µg/ $(NH_4)_2SO_4$-N g) , c) S^0 oxidation following the addition of (1% w/w) S^O , d) phosphate solubilisation following the addition of (1% w/w) of calcium phosphate to dog faeces modified with FBL (* Significantly different from control, p=0.05)

Fig.4.3 shows the effect of BSFL activity on the concentration of the indigenous dog faeces-related plant nutrients: ammonium, nitrate, sulphate and phosphate. Fig (4.3a) shows that there was a significant reduction in ammonium concentration ever the entire incubation period, while (Fig.4.3b) shows a similar decrease in nitrate concentration up to week three, followed by a significant increase at week 4. Sulphate concentration in the dog faeces also decreased following BSFL treatment over the entire incubation period (Fig.4.3c), while phosphate concentrations were significantly reduced up to week three and then significantly increased at week 4 (Fig.4.3d)

Fig.4.4 shows the effect of FBL on the indigenous plant nutrients in the dog faeces. Ammonium concentrations were reduced to week 2, but significantly increased at weeks 3 and 4 (Fig.4.4a). Nitrate concentrations, in contrast were significantly reduced over the entire incubation period (Fig.4.4b), as were sulphate concentrations; phosphate concentrations were significantly reduced up to week 3, but significantly increased at week 4 (Fig4.4d).

The effect of amendment with BSFL modified dog faeces with a source of plant nutrients (ammonium, sulphate and insoluble phosphate) is shown in Fig.4.5. The concentration of all of the released nutrients (ammonium, nitrate, sulphate and phosphate) was generally significantly increased over the control, over the entire incubation period. The same trend was seen in nutrient-amended faeces modified by the addition of FBL (Fig.4.6).

As a generalization, the addition of both types of larvae to dog faeces significantly reduced the concentration of indigenous plant nutrients over the entire four week incubation period; exceptions to this were nitrate and phosphate concentrations in BSFL treated faeces, where significant increases were seen at week 4 and 3 respectively and in faeces treated with FBL, where ammonium concentrations were significantly increased at weeks 2-4, and phosphate at week 4. While the addition of both larvae therefore

initially decreased levels of indigenous plant nutrients there was a trend in some of the nutrients to increase the longer the incubation went on. This suggests that perhaps a longer term exposure of dog faeces to the two larvae might have lead to increase in ammonium, nitrate, sulphate and phosphate concentrations. While this would generally be advantageous to the fertility of soils surrounding dog faeces, the potential long term increase in nitrate could at first sight be considered detrimental as nitrate can be readily leached to ground waters (where, when present in drinking water, it can cause blue baby disease and gastric cancer in humans). However, the relatively small amounts of dog faeces which are present, spread over large areas of soil, would likely make such increased nitrate contributions to drinking water relatively insignificant.

The addition of ammonium, elemental sulphur and insoluble phosphate to dog faeces which had been modified by the two larvae led to significant increases in nitrate, sulphate and plant-available phosphate, results which shows that that dog faeces contains the indigenous microflora required for the transformation of these amendments (which simulate fertilizer addition). The increased friability and therefore increased aeration of the dog faeces following larval modification is also like to result in enhanced rates of nitrification, S-oxidation and phosphate solubilisation. Clearly the microflora of dog faeces can act to modify added fertilizers and convert them to the plant available form.

4.3.5. Determination of the effects of modified dog faeces as a compost additive on plant seed germination and growth

Table 4. 1 shows that none of the seeds used germinated or grew in raw, untreated dog faeces (Control DF), but all grew in untreated compost (control compost). Of the seeds used only turnip germinated/grew in potting compost: modified dog faeces (FBL) at a ratio of 25:75. At a ratio of 50:50 lettuce seed failed to grow in BSFL modified faeces, but 80% of turnip seeds grew in this mix; 20 % of lettuce seeds grew in 50:50 FBL: compost and 40% of turnip seeds. At a ratio of 25:75 BSFL modified dog faeces: compost 20% of lettuce seeds grew and 100 % of turnip seeds grew. The growth figures for FBL modified faeces were relatively, lettuce 40%, and turnip, 80%. The results given in Table 4.1 are shown visually in Figs. 4.7 and 4.8

Table. 4. 1: The percentage seed germination and growth planted in different ratios of in modified dog faeces (treated with BSF and FB larvae) and potting compost

Planted seeds	**Control Modifies DF**	**75% DF**	**50% DF**	**25% DF**	**Control compost**
Lettuce seeds planted in dog faeces treated with BSFL	0%	0%	0%	20%	80%
Turnip seeds planted in dog faeces treated with BSFL	0%	0%	80%	100%	60%
Lettuce seeds planted in dog faeces treated with FBL	0%	0%	20%	40%	60%
Turnip seeds planted in dog faeces treated with FBL	0%	40%	40%	80%	80%

DF= Dog faeces

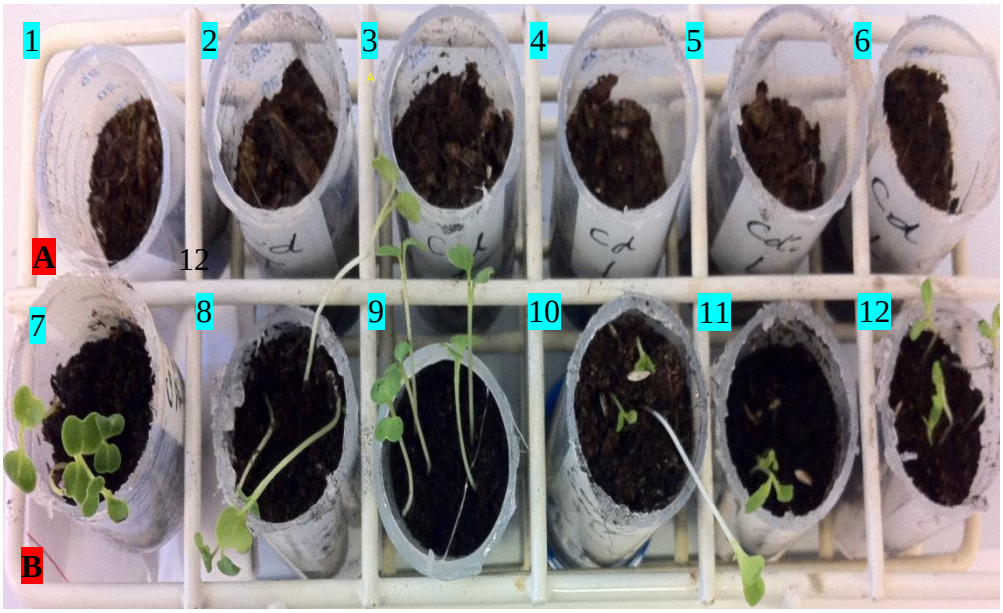


Fig. 4. 7: Shows a) the seeds planted in larval treated dog faeces; from 1 to 3 - turnip seeds, from 4 to 6 is lettuce seeds; b) the seeds planted in compost ; 7 to 9 - turnip seeds, from 10 to 12 is lettuce seeds.

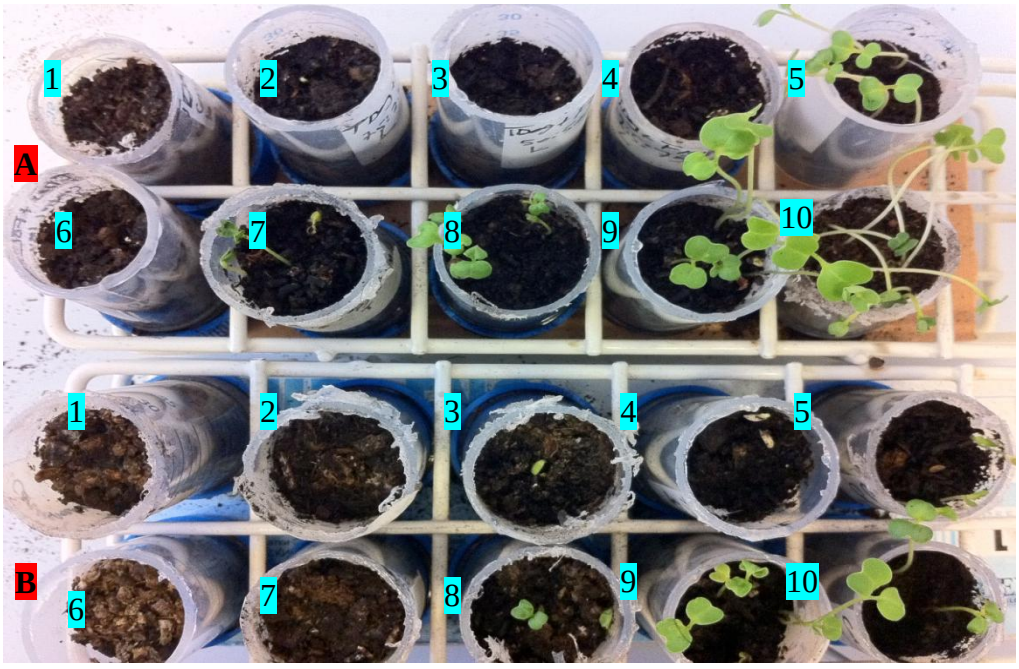


Fig. 4. 8: Shows; a) the lettuce seeds planted in different concentration of larval treated dog faeces with BSFL No; 1, control dog faeces 2, 3 and 4 are 75, 50 and 25% of dog faeces concentration and 5 is a control compost. From 6 to 10 are the turnip seeds planted at the same concentration b) the lettuce seeds planted in different concentration of treated dog faeces with FBL No; 1, control dog faeces 2, 3 and 4 were 75, 50 and 25% of dog faeces and 5 is a control compost. From 6 to 10 showing turnip seeds planted at the same concentration

Clearly, by increasing the ratio of compost to modified faeces the inhibitory effect of raw dog faeces on plant growth was “diluted out”, thereby suggesting the possibility that larval modified dog faeces could be used as a compost additive fertilizer, or perhaps even be used as an agricultural soil fertilizer. These possibilities are however, limited by the fact that dog faeces are widely separated in the environment, so that it would be difficult to economically collect large amounts, making unviable it industrial use. Domestic dog pounds and dog racing kennels might on the other hand provide a sufficiently large source of faeces to make such use economic.

Chapter Five: The Antibacterial Activity of the Haemolymph and Whole Body Extracts from BSFL and FBL against Four Species of Bacteria

5. 1 Introduction

Like many other organisms, insects exhibit an efficient immunity response allowing them to survive in environment and it is known that insects have various levels of internal defence against microbial invasion (Cociancich *et al.,* 1994), including the ability, in some species, to externally secrete antibacterial compounds. Internal components of Green Blow Fly maggots for example, have been used with success, since 1930 (Simmons, 1935) to treat wounds infected with bacteria (Thomas *et al.*, 1999). In medicine, larvae of the Green Blow Fly (*Lucilia sericata* Meigen) are well-known for having the ability to reduce and eradicate bacteria in human wounds (Thomas *et al.*, 1996); they can both ingest and digest such bacteria or may produce active antimicrobial substances. A study by Murry and Hinckley (1992) showed that earthworms (*Eisenia foetida*) can also reduce the population of *Salmonella enterica* in horse manure.

The published work above shows a variety of results relating to the antibacterial activity of larval extracts against several species of bacteria. Bexfield *et al*., (2004) established that *Lucilia sericata* excretions/secretion inhibit growth of *E. coli*. Similarly, they showed that *Lucilia sericata* extracts inhibit the growth of *P. aeruginosa* over a 24-hour period ,whereas Jaklič *et al.*, (2008) reported a prolonged lag phase of more than 5-hours, and Thomas *et al.*, (1999) found only limited inhibition.

More recently, Mumcuoglu *et al.*, (2001) found green fluorescent protein (GFP)–producing *E. coli* in the alimentary canal of Fly larvae and found a decreasing intensity of this marker during passage through the digestive tract. A battery of defence proteins synthesized by insects in response to bacterial challenge may be responsible for this decrease. Cecropins, and lytic proteins for example, have been isolated from larvae of blow flies (*Calliphora vicina*) (Crowley and Houck, 2002). Another antibacterial substance, *p*-hydroxycinnamaldehyde, isolated from the larvae of the Saw Fly,

Acantholyda parki S., has also been found to have a broad antibacterial spectrum against both gram-negative and gram-positive bacteria (Leem *et al.*, 1999).

The Black Soldier Fly, *Hermetia illucens*, is a beneficial insect because its larvae feed on organic material, including the remains of plants, animals and humans. Such larvae can degrade large amounts of waste quickly and efficiently than any other known species of fly due to their potent mouthparts and digestive enzymes (Tomberlin *et al.*, 2002), The effect of larval excretion/ secretion (ES) against some common species of bacteria found in dog faeces was studied in relation to determining how larvae can resist bacterial invasion. Based on these findings, similar antibacterial substances might be present in BSF and FB larvae.

The investigations reported in this Thesis were aimed at determining the ability of Black Soldier Fly and Fruit Beetle larvae to reduce the number of pathogenic bacteria, such as *E. coli*, *Salmonella enterica* and *Staphylococcus aureus* MRSA, in dog faeces. Few studies have reported the ability of Black Soldier Fly or Fruit Beetles to demonstrate immunity or the ability of their internal and external secretion, as antibacterial agents.

The aims of the work reported in this Chapter was to investigate the presence of antibacterial activity in the body extracts of BSF and FB larvae and their inhibitory effects against four species of bacteria.

5.2. Materials and Methods

5.2.1. Culture of larvae

The two species of insects (larvae of Soldier Fly and Fruit Beetle) used in this study were obtained from a commercial company (Ricks, Livefood.Co.UK), the third instar of BSF larvae was purchased, while large sized FB larvae were used.

5.2.2. Antibacterial properties of BSF and FB larvae

5.2.2.1. Collection of excretion/secretion (ES) from larval whole-body and haemolymph

Samples were placed separately in small boxes (10×15x30 cm) under laboratory conditions. In order to collect the whole body and haemolymph extraction, samples 20g of each larval species (approximately 200 larvae of BSF and 10 larvae of FB) were left unfed for 4 days until they were free from gut residues. For whole body extracts and larval secretions, larvae were washed with sterile deionised water (dH2O) and placed in Petri-dish at -4^{o}C for a few days. The larvae were then ground manually, using a mortar and pestle, until they became homogenous. Haemolymph was collected from larvae by clipping the anterior end near the cephalopharyngeal skeleton. The abdomen was then squeezed gently to force the haemolymph to flow from the wound. The resultant suspensions were then filtered (0.2µm) to remove any large particles and bacteria. The excretion/secretion ES from each group of insect larvae were dissolved in 0.1 µ g/ml protein in 0.1% trifluoroacetic acid (TFA) 1: 10 v/v. The suspension was placed on ice for 6 hours and centrifuged four times at 14000 g for 30 min. The supernatant was then harvested and separated into 15 ml centrifuge tubes; samples were used fresh or kept frozen at -80°C for a few days until required (Sahalan *et al.*, 2006; Huberman *et al.*, 2007).

5.2.2.2. Antibacterial assay

Antibacterial activity was determined as follows: Viable counts: the effect of BSF and FB extract on the viability of *E.* coli, *Salmonella enteric, Serratia marcescens and S. aureus* MRSA cells were monitored by inoculating 1ml of an overnight 5.0 x10^{6} cfu/ ml , into 9 ml nutrient agar with and without both extracts and incubating with shaking at37°C (250g). Samples (100 µl) were removed at intervals, diluted serially and 100µl

of the diluted samples were placed on nutrient agar plates and incubated at 37°C for 24 hours. The colonies were then counted after overnight incubation and the results expressed as colony forming units (CFU/ml). Turbidimetric analysis: bacterial cells were incubated the extracted haemolymph and whole body; the final optical density of the treated and untreated cultures were then measured at 600 nm.

5.2.3. Time killing curves for testing the bacterial activity

The time kill assays were prepared by inoculating one or two colonies of overnight test bacteria into 9 ml of Mueller–Hinton broth and incubating for 18-24 hours at 37°C with shaking, adjusting to a 0.5 McFarland 5 x 10^6 CFU ml^{-1}. Haemolymph and whole body were prepared separately by adding 4ml of haemolymph to 16 of ml of Mueller–Hinton broth in 100 ml flasks, solutions lacking haemolymph acted as controls. Treatments and control were incubated with shaking at 37°C and samples were transferred to nutrient agar at 0, 2, 4, 6 and 8 hours. Viable counts were performed by serially diluting each sample 10-fold in PBS and spreading 100 μml volumes from the appropriate dilutions onto nutrient agar. After incubation at 37°C, for 20–24 h, viable counts (CFU ml^{-1}) were determined to provide a quantitative determination of bacterial growth colony forming units. To confirm the sterility of samples of ES prior to bacterial inoculation, a loop (10 μl) of haemolymph and whole body secretions from each larvae, TSB, and sterile dH2O were spread separately onto nutrient agar and incubated at 37°C for 24 h .This procedure was repeated at four, eight and 24 h to ensure the media used to prepare dilutions for viable counts was contamination-free.

5.2.4. Bacterial culture

Four species of bacteria were used in this study. Three species were Gram negatives (*Escherichia coli, Salmonella enterica* and *Serratia marcescens*) were obtained from previous works, whereas the forth, *Staphylococcus aureus* was a Gram positive. The organisms were obtained from the Departmental Culture Collection.

Bacteria were maintained on nutrient agar at 4^{o}C. New subcultures were prepared as required (Alexander and Strete, 2001). In order to test that the colonies were viable, the plates were incubated overnight at 37^{o}C to allow growth.

5.2.5. Transmission Electron Microscopy of Bacteria treated with haemolymph or whole body secretions of the two larvae

For transmission E/M, harvested cells were fixed in 3% glutaraldehyde and 0.1M phosphate buffer overnight at 4°C, and then washed twice times with 30 min intervals at 4°C in 0.1M phosphate buffer. Cells were subjected to a secondary fixation with 2% osmium tetroxide aqueous for 2 hour at room temperature and the previous wash step was repeated. After this, cells at room temperature were serially dehydrated with 75%, 95%, and three times using 100% ethanol, the last one was dried over anhydrous copper sulphate for 15 min. The cells were then placed in an intermediate solvent, propylene oxide, for two changes of 15 min duration. Infiltration was accomplished by placing the cells in a 50/50 mixture of propylene oxide/Araldite resin. The cells were then left in this 50/50 mixture overnight at room temperature. Once this incubation was finished the cells were transferred into full strength Araldite resin and left for 6-8 hours at room temperature after which time they were embedded in fresh Araldite resin for 48-72 hours at 60°C. Semi-thin sections approximately 0.5 μm thick were cut on a Reichert Ultracut E ultramicrotome and stained with 1% toluidine blue in 1% borax. Ultrathin sections, approximately 70-90nm thick, were then cut using a Reichert Ultracut E

ultramicrotome and stained for 25 mins with 3% uranyl acetate in 50% ethanol followed by staining with Reynold's lead citrate for 25 min. The sections were examined using a FEI Tecnai Transmission Electron Microscope at an accelerating voltage of 80Kv.

5.2.6. Statistical analysis

All data were presented as means ± SD. (Standard deviation). The data was analyzed by SigmaPlot© 11.0. $P<0.05$ was considered as significant.

5.3. Results and Discussion

Figures 5.1 and 5.2 show the effects of the haemolymph and whole body extract of the BSFL and FBL respectively on the growth of *E. coli*. Both the BSFL extracts had only a minor effect on the growth of *E. coli* from 2-8h (Fig 5.1). After 8h however, BSFL haemolymp had a marked effect on *E. coli*. In contrast the addition of the haemolymph and whole body extracts of the FBL produced an increase in numbers of *E. coli* compared to the control, from hour 2-8 (Fig.5.2).

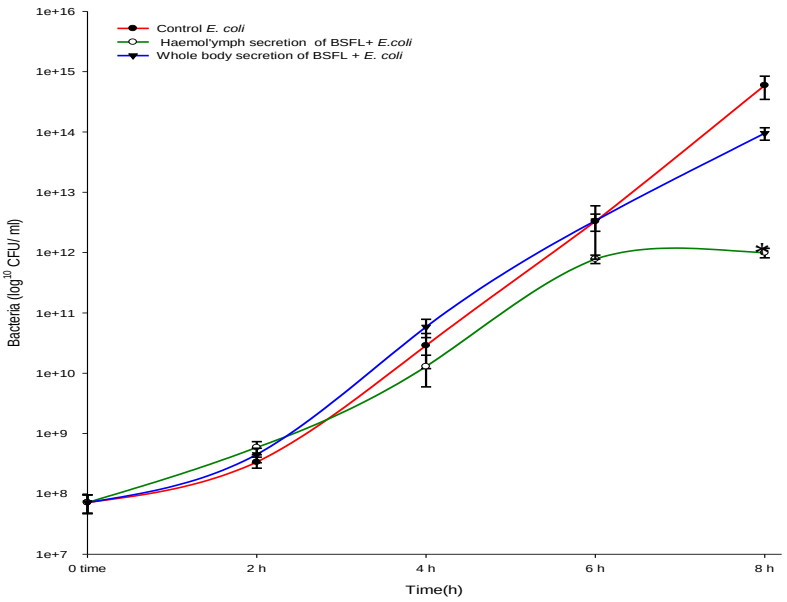


Fig. 5. 1: The effect of BSFL haemolymph and whole body extract on *E. coli*, growth curve over 8 h.

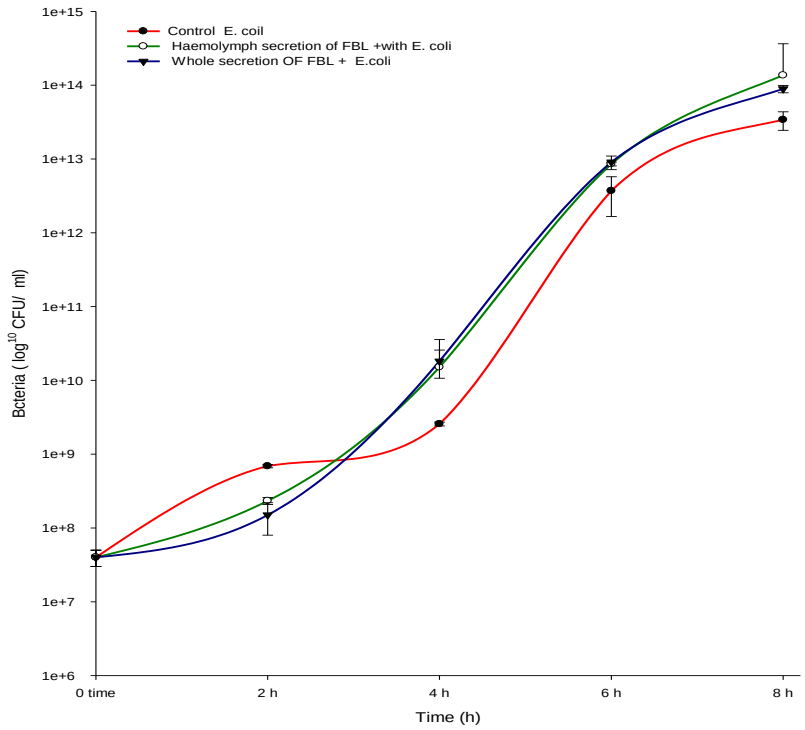


Fig. 5. 2: The effect of FBL larvae haemolymph and whole body extract *E.coli* , growth curve over 8 h.

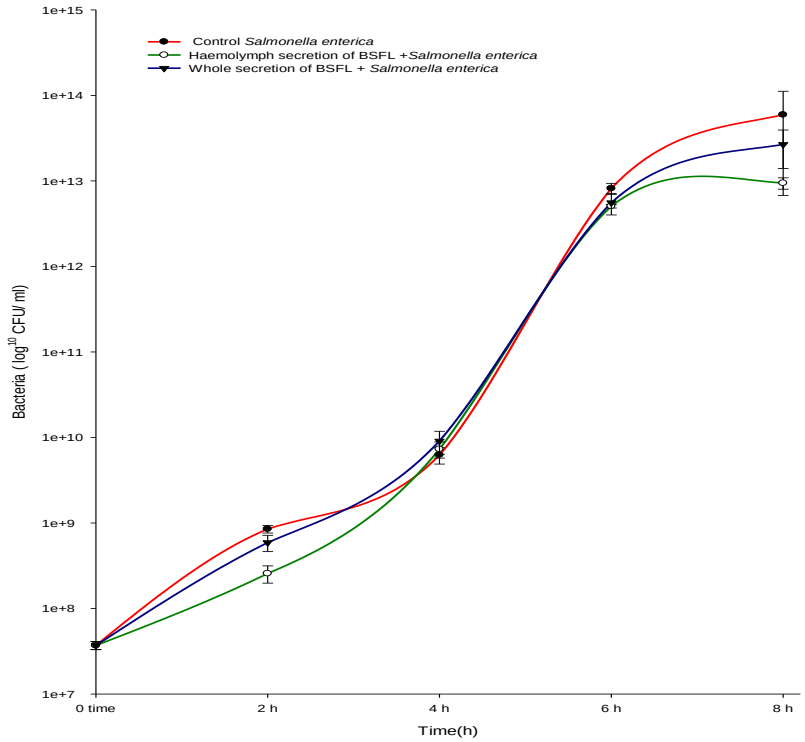


Fig. 5. 3: The effect of BSFL haemolymph and whole body extract on *S.enterica* growth curve over 8 h.

Both the haemolymph and whole body extract of the BSFL inhibited the growth of *S. enterica* at hour 2 of the incubation period, although by hour 4, there was little impact of both extracts on bacterial numbers; the inhibition trend for both bacteria then resumed from hours 6-8 (Fig5.3).

Haemolymph of the FBL had a general slight stimulatory impact on numbers of *Salmonella enterica* over the entire incubation period, with the effect being at its lowest in the middle of the 8 hours. The whole body secretion in contrast, reduced bacterial numbers over the entire incubation period (Fig.5.4).

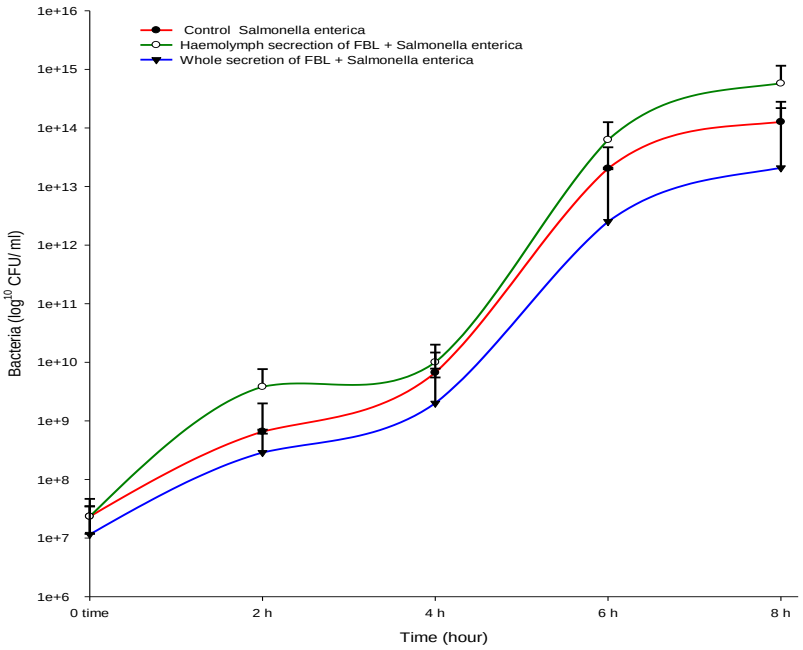


Fig. 5. 4: The effect of FBL haemolymph and whole body extract on *S.enterica* growth curve over 8 h.

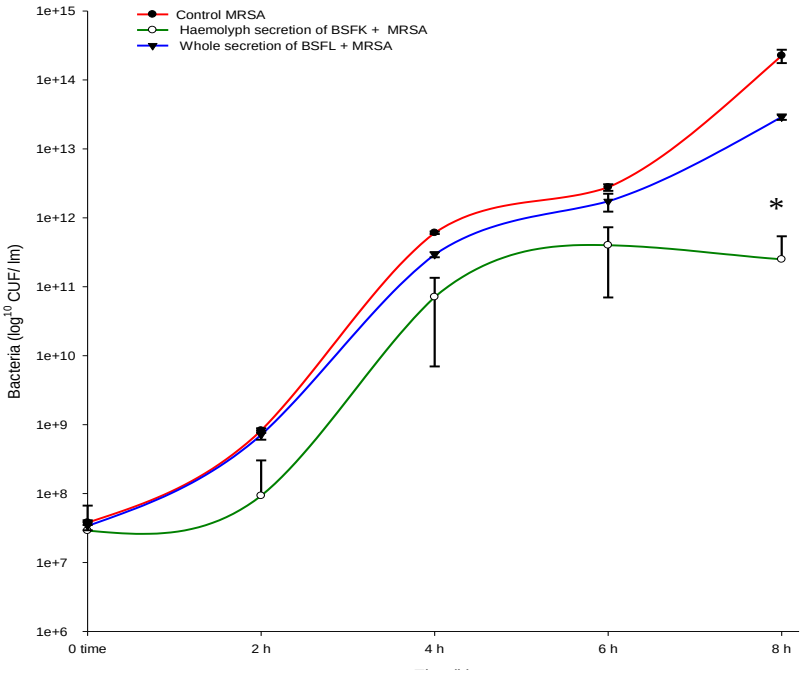


Fig. 5. 5: The effect of BSFL haemolymph and whole body extract on *S.aureus* MRSA, growth curve over 8 h.

Both of the extracts from the BSFL had a general inhibitory effect on the growth of *S. aureus*, the effect being most marked with the haemolymph, which inhibited growth over the entire incubation period (Fig.5.5). The haemolymph extract from the FBL inhibited the growth of MRSA between 4 and 8 hours (Fig.5.6), while the whole body extract increased numbers at 6hours.

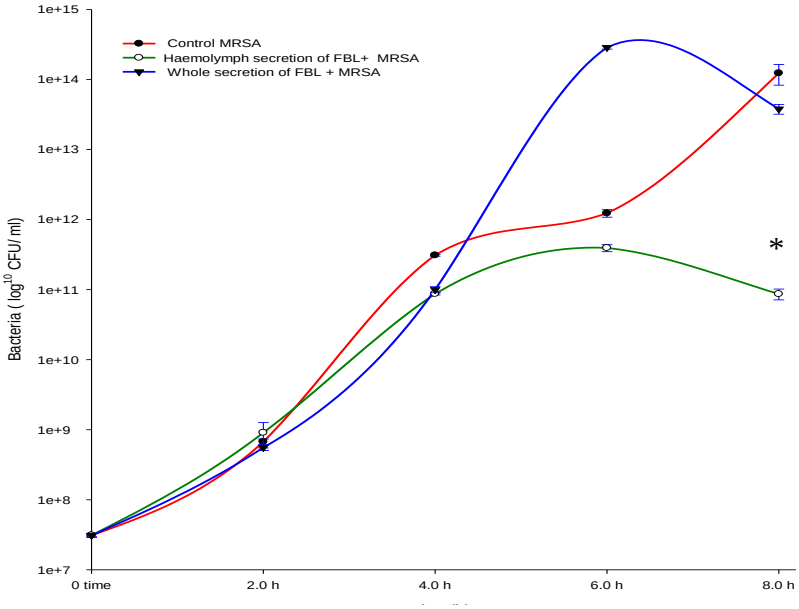


Fig. 5. 6: The effect of FBL haemolymph and whole body on *Staphylococcus aureus* MRSA, growth curve over 8 h.

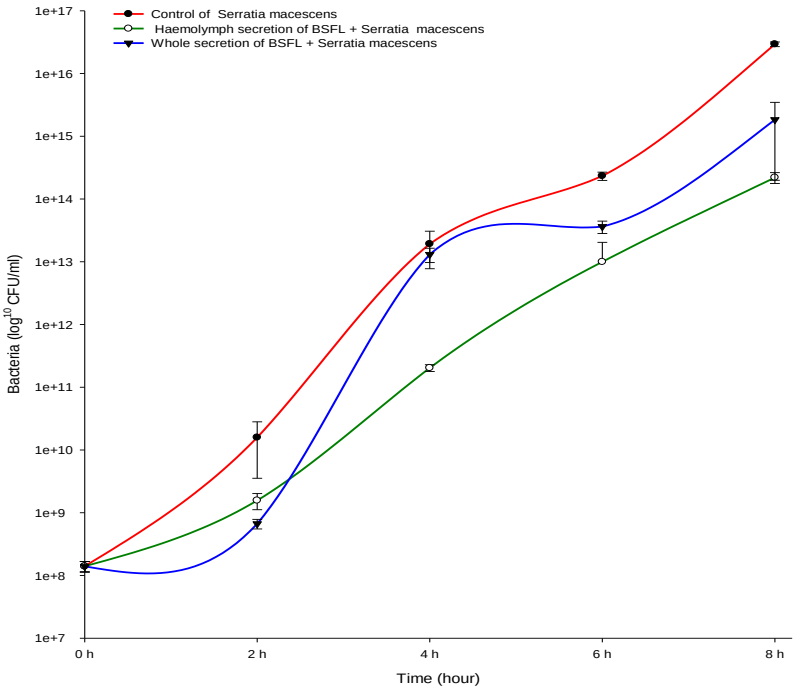


Fig. 5. 7: The effect of BSFL haemolymph and whole body on *Serratia marcescens*, growth curve over 8 h.

Both the haemolymph and whole body extract of BSFL inhibited the growth of *S .marcescens* over the entire incubation period, the effect of the haemolymph extract being most marked at the end of the growth period ((Fig.5.7). The whole body extract of the FBL had less effect on the growth of *S. marcescens* over the entire incubation period, while the haemolymph had a slight, and very transient stimulatory effect, at the 6h point only; the haemolymph increased numbers at the end of the incubation period (Fig.5.8).

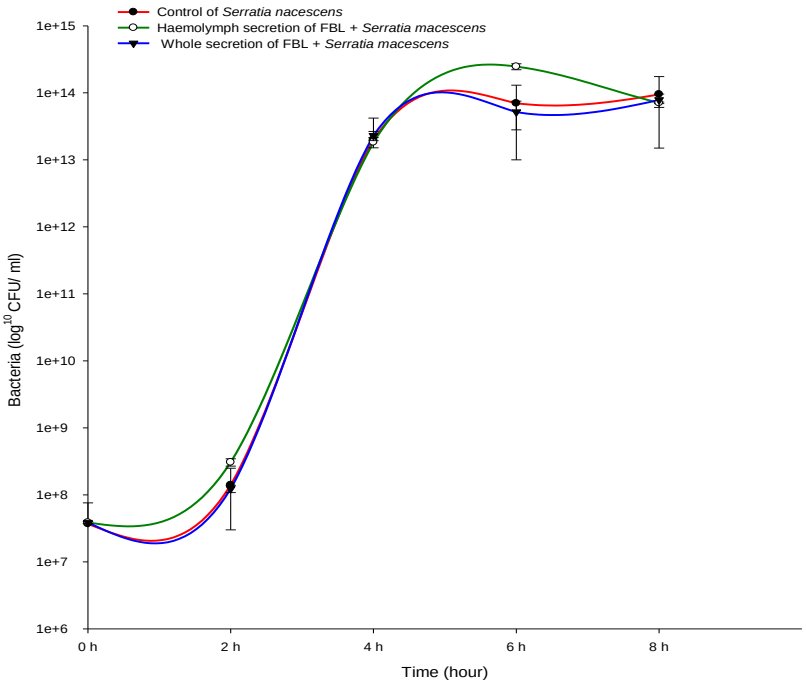


Fig. 5. 8: The effect of FBL haemolymph and whole body extract on *Serratia marcescens,* growth curve over 8 h.

Table. 5. 1:The effect of the two species of larval haemolymph and whole body secretions on the four species of bacteria.

Bacterial strains	Gram strain	BSF larvae		FBL larvae	
		Haemolymph	Whole body	Haemolymph	Whole body
E. coli	Gram -ve	+	+	-	-
Salmonella enterica	Gram-ve	-	-	-	+
S. aureus MRSA	Gram +ve	+	-	+	-
Serratia marcescens	Gram -ve	+	+	-	-

Table. 5.1 shows that the effect of the extracts on bacteria varied with different species of larva and with different types of extracts used. Clearly, no overall generalization can be made about the effects of BSFL and FBL extracts on bacteria can be observed.

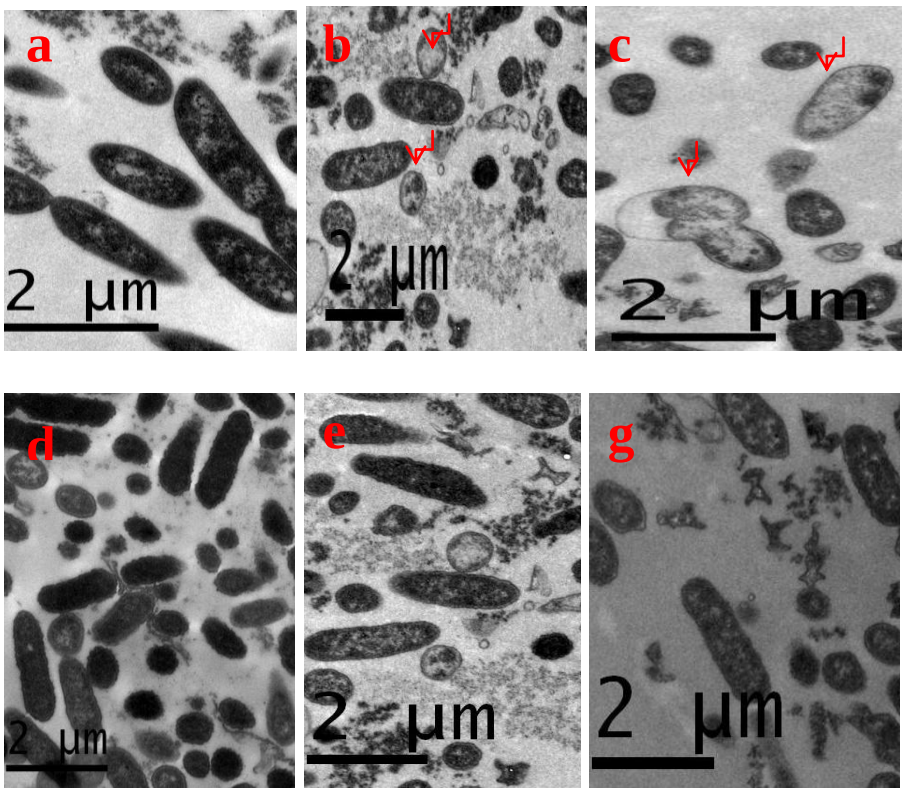


Fig. 5. 9: The effect of larval components on the morphology of *E. coli* observed by TEM, a) control for BSFL, b) whole body extract of BSFL, c), haemolymph of BSFL; d) control for FBL, e) whole body extracts for FBL and g) haemolymph of FBL.

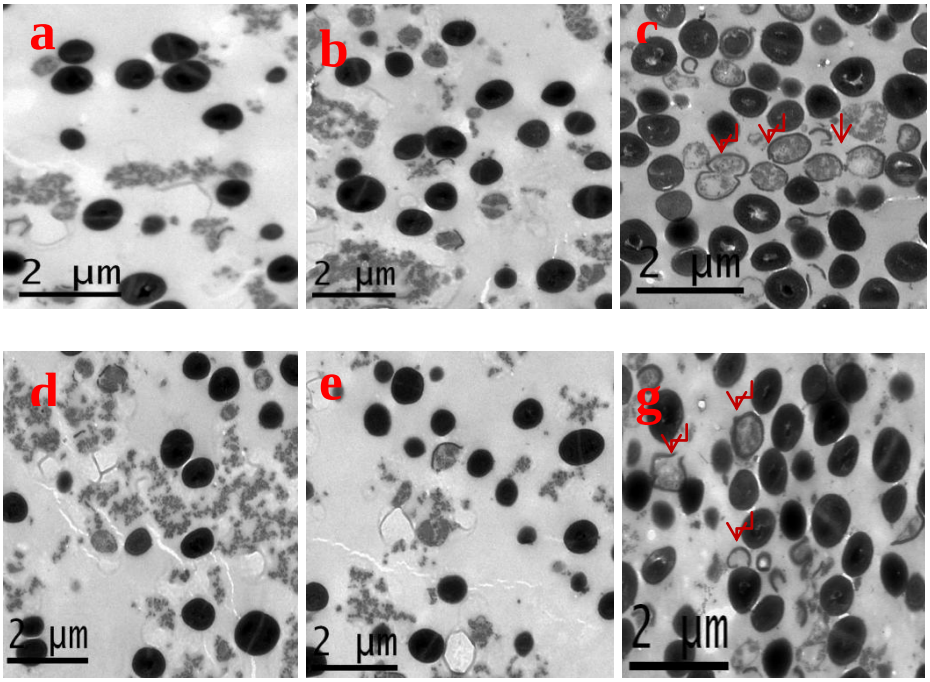


Fig. 5. 10: The effect of larval components on the morphology of *S.aureus* observed by TEM, a) control for BSFL, b) whole body extract of BSFL, c), haemolymph of BSFL; d) control for FBL, e) whole body extracts for FBL and g) haemolymph of FBL.

The effects of the two larval extracts on *E.coli* and *S. aureus* as observed under the transmission electron microscope are respectively shown in Figs. 5.9 and 5.10. The haemolymph extract of BSF is the only one which has a readily observed, and marked, effect on the morphology of *E.coli* (Fig.5.9c); note that the cells are seen to lose some of their electron dense structure, but that cell lysis is not evident. In the same way, the haemolymph of BSFL is the only fly body extract which has an effect on the cell structure of *S. aureus*; again there is no obvious evidence of cell lysis (Fig.5.10c).

Interest in the possible antibacterial activity of the body extracts of the two flies used here was based on two possibilities. Firstly that an antibacterial agent, possibly a protein might be obtained from either or both of the extracts and secondly that BSFL or FBL might be used in infected wounds in the same way that larvae of the Green Blow Fly (*Lucillia sericata*) are used in maggot therapy (Boxfield *et al*.,2004). The latter possibility is perhaps unlikely based on the large size of the two fly larvae compared with maggots, a fact which would restrict their application to open wounds. Maggot therapy is extremely effective, so it is unlikely that the two larvae studied here would replace this approach to wound treatment. Similarly while extracts from the two larvae have been shown to inhibit the growth of bacteria, their effectiveness is unlikely to be comparable to antibiotics like penicillin.

Conclusion: In conclusion, whole body extracts and the haemolymph of the two fly species used here do have antibacterial effects, but such inhibitory effects are not consistent, both in relation to the individual fly species, or in relation to the type of bacterium investigated. The addition of the fly larvae to faeces is likely however, to lead to reduction in overall bacterial numbers. It seems unlikely that BSFL or FB larvae could be usefully applied to the control of bacterial infections in human or animal wounds.

Chapter Six: The Number of Bacteria in Grassed Soil of Public Parks Contaminated with Dog Faeces and Effect of Faeces-Volatiles on Plant Growth.

6. 1. Introduction

The number of dogs in the UK has increased dramatically in recent times especially in cities, and their waste represents a considerable source of pollution in relation to humans and the environment (Carvalho *et al.*, 2009). Dog faeces are known to carry a large and various microbial and parasite population which can cause diseases and public-health problems (Baxter and Leck, 1984). Dog waste is linked with more than 60 zoonotic diseases and provides a serious public-health problem (Rinaldi *et al.*, 2006). Irresponsibility on the part of dog owners in relation to the disposal the dog waste represents a source of potential pathogens in public areas such as playgrounds, parks, gardens, public squares and sandpits (Schantz, 1994; Rubel and Wisnivesky, 2005); the risks to young children being particularly obvious. Children may, by accident consume contaminated soil or grass, or touch their mouths or eyes with hands contaminated with dog faeces or otherwise handle bags left hanging outdoors on trees branches and in parks (Fig.6.1) ; people using hand activated wheelchairs and active sports players may also be at risk (Jackson, 1995). Viable pathogens present in dried canine faeces can also be spread by the wind and are carried into dwellings and workplaces on soiled shoes.

The aim of the work reported in this Chapter was to study faecal contamination accumulation in a local public park, a problem which is likely to be a risk to the health of young children and parks visitors. In addition, the work focuses on the extremely undesirable habit of some dog owners to remove dog faeces from the environment and leave them in a plastic bag on the parks, a habit which contributes to a potentially bigger problem than does exposed faeces. The effect of dog faeces volatiles on plant growth will also be touched upon.

6.2. Materials and methods

6.2.1. Samples collection from public parks

The following protocol was used to determine the presence of *E. coli* and *Salmonella enterica* in addition to other heterotrophic bacteria (as determined using Plate Count Agar) in soil samples collected from a public park in Sheffield UK (a grassed area of playing field behind the former Lodge Moor Hospital). Four samples of soil were taken (0 - 3 cm) from under surface grassed soil contaminated with dog faeces (samples were taken directly from soil which had long exposure to faeces and not from under fresh material); the last sample was taken from an area not contaminated with faeces, i.e. an uncontaminated control. Samples (1 g) were suspended in (9 ml) autoclave-sterilised water in sterile 15 ml tubes and shaken at 70 g for 30 min. A serial dilution was performed, then dilutions from 10^3 to 10^6 were taken and (100 ml) of this suspension was then spread onto the surface of selective media for *E. coli* and *Salmonella* Spp. XLT4 and Plate Count Agar; the plates were then incubated in triplicate at 37^oC overnight. Presumptive isolation was based on the use of selective media such as *E. coli* and XLT4 *Salmonella* Spp. agar media, followed by use of the Gram stain. In addition, the 16s rRNA gene dependant technique was used; DNA being extracted according to the Anachem Key prep-Bacterial DNA extraction kit; bacterial 16SrRNA gene was then amplified and sequenced.

6.2.2. Effect of incubation of dog faeces in plastic bags left under ambient outside conditions

A variety of fresh dog faeces were collected from dog bins from Weston Park. Samples were placed in black plastic bags and left exposed to ambient weather conditions on the roof of the Firth Court building from the middle of May until the middle of June, 2012. Samples (1 g) of the faeces were diluted in (9 ml) in sterilised

water. Then a serial dilution from 10^3 to 10^7 was performed and (100 µl) of the final suspension was then spread onto plates contained the selective media: HiCrome *E. coli* agar and *Salmonella Spp* XLT4, as well as Plate Count Agar. Plates were incubated in triplicate at 37^{o}C for 18-24 h.

Fig. 6. 1: Plastic bags containing dog faeces bags obtained from Sheffield parks

6.2.3 The effect of gasses (odour) from dog faeces on plant growth

Two plastic containers were prepared, one contained compost soil divided to two parts each one include 200 g of compost into which was planted lettuce seeds, and the other turnip; controls not receiving faeces-gasses were also set up. The gasses were transferred, using a pump, from a large Erlenmeyer flask containing 200g dog faeces. The gasses then passed through holes in the bottom of the plant growth container and passed over the growing seedlings (Fig. 6.2). Seeds (50 each of lettuce and turnip) were planted and watered twice a week and left at room temperature in the light. The experiment was continued for two weeks when the growth was visually assessed, i.e. for four weeks. The samples were aerated and suspended with unoccupied space under natural conditions.

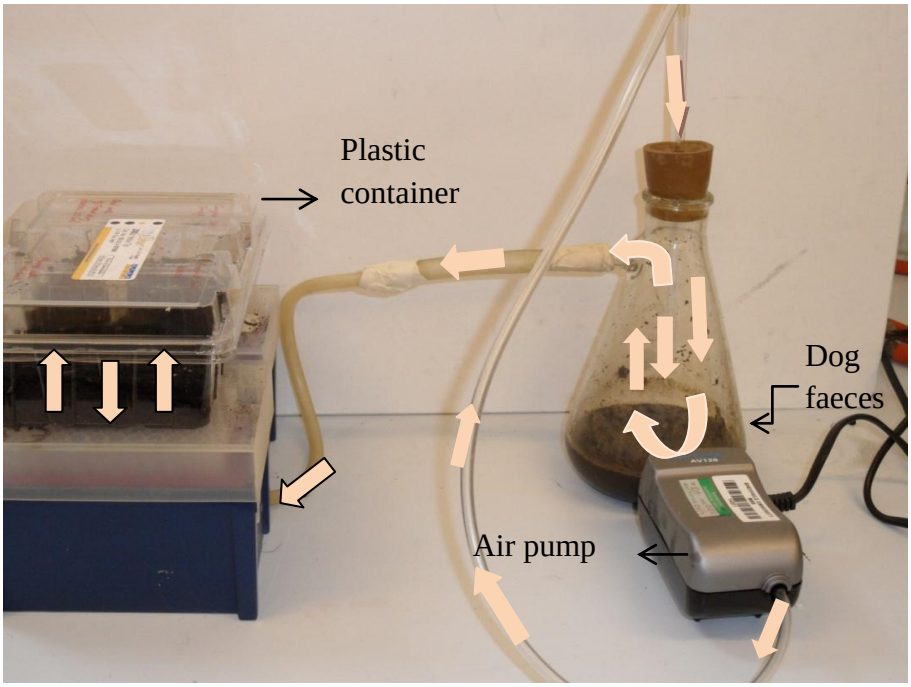


Fig. 6. 2: Airtight container contains 200 g of fresh dog faeces, the dog faeces gases were pumped via the tube into the plastic box containing compost planted with lettuce or turnip seeds.

6.3 Results and Discussion

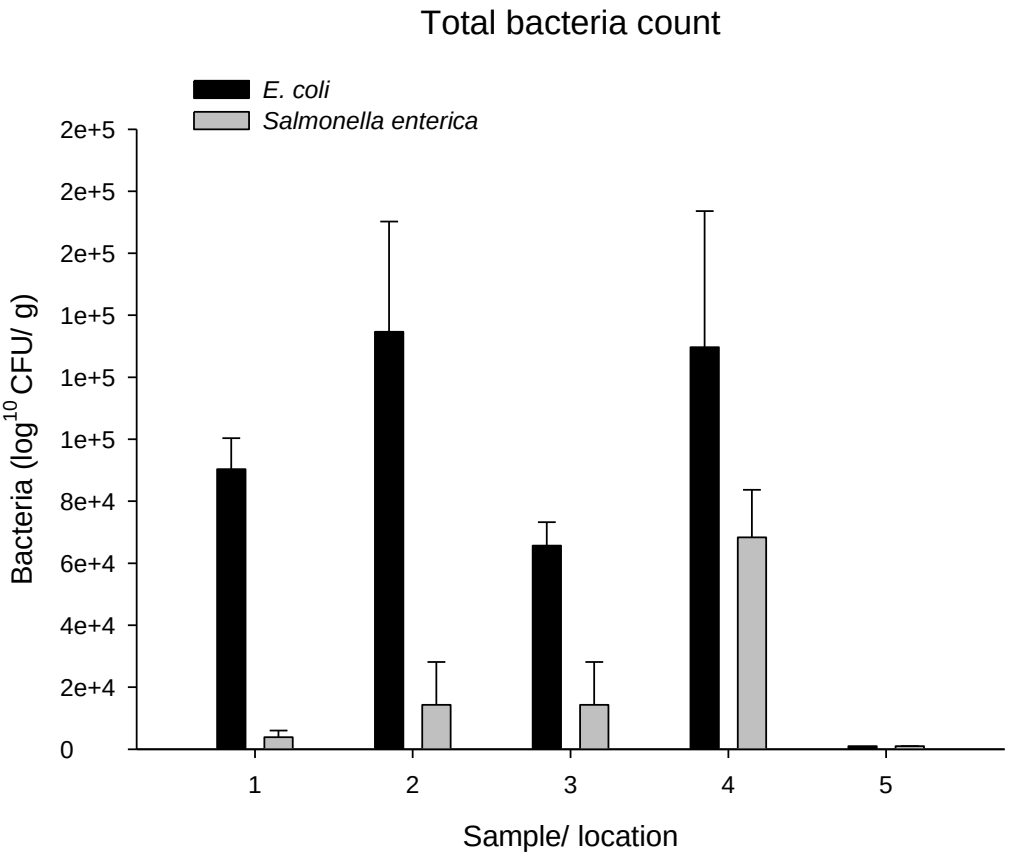


Fig. 6. 3: The presence and population density of *E. coli* and *Salmonella sp* in five locations in public areas of Sheffield (location 5 is the uncontaminated grass sample).

6.3.1. Occurrence of *E. coli* and *S. enterica* in dog faeces contaminated grassed-soil

Escherichia coli and *Salmonella enterica* were isolated from all four sites (Fig. 6.3), while no isolates were obtained from the fifth location which was uncontaminated with dog faeces. The number of *E. coli* varied from site to site (from 10^4 to 10^5 CFU/g); *E. coli* was isolated in highest numbers. The isolation of these pathogenic bacteria from playing fields frequented by children is obviously a major public health concern since

pathogenic bacteria could be picked up from these soils, either on shoes, or by transfer from contaminated skin to the ears, eyes or mouths of infected children (and of course, people in general) from where they could cause disease.

Several studies have concluded that there is a high potential risk to human health of contaminated soil and animal faeces, and direct or indirect contact with dog faeces has been implicated in several human infections with *E. coli* and *Salmonella enterica* (Kudva *et al.*, 1998). The risk of the transfer pathogenic bacteria to young children from dog faeces in public areas-notably gardens, parks and playing-field probably is critical because people do not always know that they have been exposed to contamination and can touch their eyes or mouth with faeces without realising it. Contaminated garden and park soils could possibly influence the health of large number of adults and children and it is therefore important that research and development is carried out on risk assessment and also to develop measures to mitigate such contamination (Alloway, 2004).

6.3.2 Effect of leaving dog faeces in plastic bags on bacterial numbers

An undesirable and unhygienic habit has recently developed amongst some dog owners in Sheffield (and presumably other parts of the country) of picking up their dog waste, transferring it to polythene bags and then leaving the filled bags in the environment as litter, rather than depositing them in dedicated dog-waste containers.

A wide range of potentially pathogenic bacteria were isolated from dog faeces left at ambient conditions in plastic bags (Fig.6.4). The results presented in Fig.6.5 show that numbers of *E.coli* and *S. enterica* increased in the dog faeces, left in sealed plastic bags, over the 28 day incubation period; as did the number of total heterotrophic bacteria.

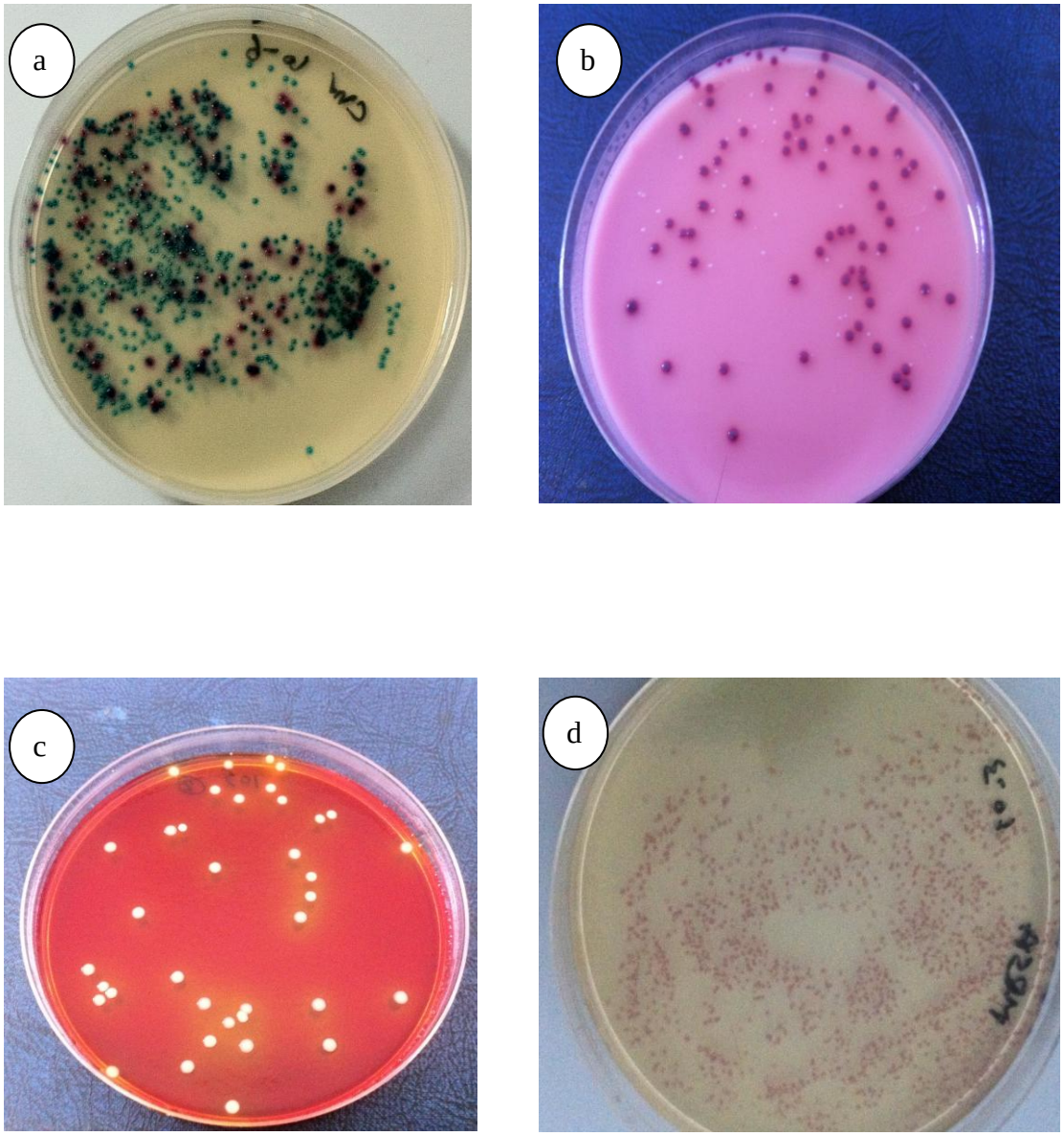


Fig. 6. 4: Bacterial species isolated from dog faeces left in polythene bags; a) red colonies of *E. coli*, and blue *Enterococcus Spp*, b) *E. coli*, c) *Salmonella enterica*, d) *Staphylococcus aureus.*

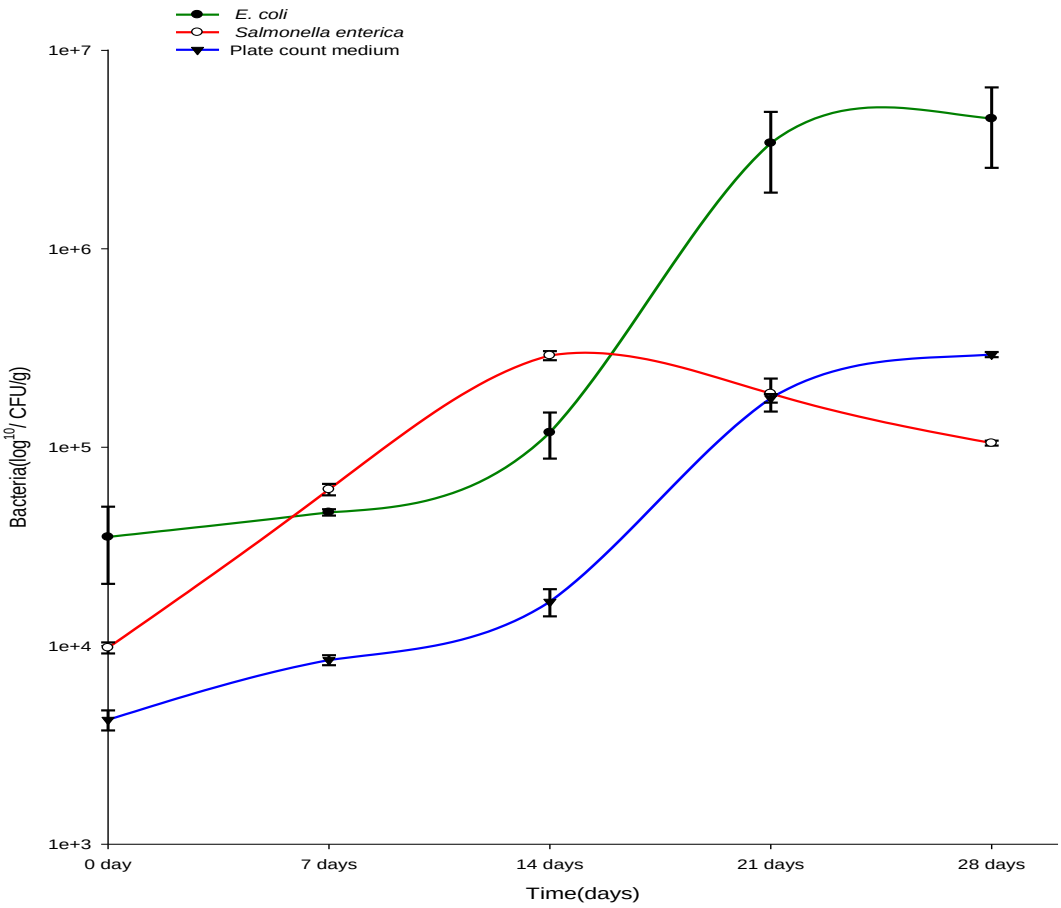


Fig. 6. 5: Numbers of *E. coli*, *Salmonella enterica* and total heterotrophic bacteria (grown on plate count media) isolated from dog faeces left in plastic bags.

This study shows that the numbers of "total bacteria", as well as those of two important human bacterial pathogens increase in dog faeces left under ambient conditions in sealed plastic bags. It would have been interesting to continue the exposure period for longer than the one month used here , since numbers of pathogenic bacteria are likely to have increased even further under such circumstances.

6.3.3 The effect of the gasses (odour) released from dog faeces on the plant growth

Fig.6.6 shows that gasses from dog faeces have a marked detrimental effect on the growth of lettuce and turnip from seed, with seedling growth obviously visually reduced in treated samples when compared to the controls. The question could be asked- why

was the effect of dog faeces volatiles on plant growth determined, and not the direct effect of the faeces themselves? The answer is that it would have been difficult the separate the likely toxic effects of dog faeces, on plant growth, from the positive fertilizer effect resulting from the fact that the faeces are rich in plant nutrients like ammonium and nitrate. Although the nature of the dog faeces gases and volatiles was not determined, the observed inhibitory effects presumably result from the inhibition of seed germination or shoot growth, following the production of a cocktail of toxic gases, including ammonia in high concentration (Van der Eerden, 1982). Several studies have discussed emissions from animals and researchers have obtained varying results; clearly research is needed to discover the impact of gases, emitted from animal manures (such as ammonium) on plants (Kebreab *et al.*, 2006). The effect of ammonia on vegetation has been noticed since 1896, when König observed injuries to the surrounding vegetation near a soda factory where ammonia was accidentally released. Garber (1935) appears to have been the first to expose plants to NH_3 and to prove its phytotoxic effects (Fangmeier *et al.*, 1994). Ammonia can cause various types of injury, including necrosis, growth reduction, growth stimulation and increased frost sensitivity. Several plant species have been assessed for sensitivity to ammonia. Some conifer species were relatively sensitive to low concentrations in the long term; some cultivars of cauliflower and tomato were relatively sensitive to somewhat higher concentrations for a short term. Special attention has been paid to plant injury around intensively managed livestock. The emission from these sources consists of a large number of compounds, ammonia proving to be the main toxic component (Van der Eerden, 1982).

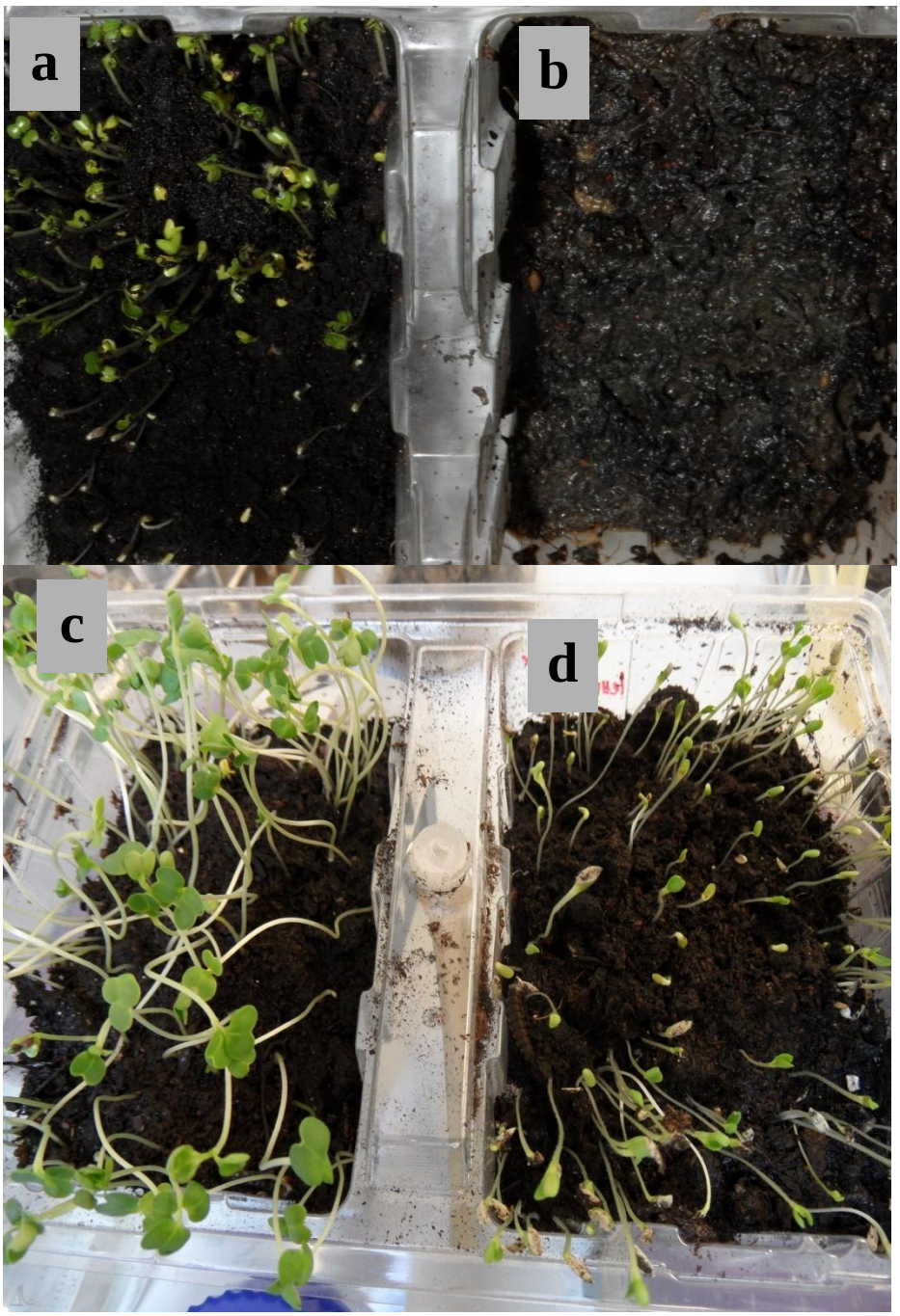


Fig. 6. 6: The effect of gas (odour) emission from dog faeces on vegetable growth; a) turnip treated with dog faeces gasses and, b) lettuce treated with dog faeces gasses c) control turnip seeds d) control lettuce seeds.

Dog faeces represent a potential source for spreading pathogenic bacteria such as *E. coli* and *Salmonella enterica*, bacteria which represent an obvious risk human health. Bacterial pathogens from dog faeces could infect young children and others playing, or using, parks etc (including of course dog owners). Dog faeces have also been shown

here to produce gasses which are injurious to plant germination and/ or growth, and are also likely to be directly inhibitory to plant growth.

There has been a welcomed trend of late for dog owners to act responsibly by picking up waste from their dogs, placing this in plastic bags and than putting these bags into special local council run dog-waste containers, from where they are transported and incinerated (Fig.6.7).

Fig. 6. 7: A typical dog waste bin used in public parks in the UK (author's photograph).

Unfortunately there is another increasing trend, namely the discarding of such bags filled with dog faeces into the local environment. The results presented here show that this practise potentially increases the problem of dog faeces because the sealed polythene bags provide an environment where pathogenic bacteria (and presumably other pathogens, like viruses) can rapidly increase. Such an increase in pathogen numbers was seen during a relatively cool UK summer and could be much greater where summer temperatures are higher. In addition, the possibility exists that the environment inside the bags could become anaerobic and select for, or otherwise encourage the growth of anaerobic, pathogenic bacteria, such as species of *Clostridium*. Clearly this habit should be discouraged. In fact it would be better to encourage dog

owners to leave dog faeces in the open air, where they can break down naturally (with pathogens being outcompeted by saprophytic microbes) than to allow them to discard the faeces in sealed bags which, when broken (by for example a child sliding on them) could release an increased, and potentially more dangerous, pathogen load.

The survival of bacteria in animals waste under environmental condition has been studied by, amongst others, LaGoy, (1987), who showed that *E. coli* in cattle manure survived for 42 to 49 days at 37°C, for 49 to 56 days at 22°C, and for 63 to 70 days at 5°C (LaGoy, 1987) Another study of *E. coli* in manure commutation revealed that the pathogen may survive for up to 47 days, 4 months, and 21 months in bovine, aerated ovine, and non-aerated ovine manure, respectively (Kudva *et al.*, 1998). Himathongkham *et al*, (1999) based on their research findings, recommended that cow manure should be survive for 105 days at 4°C or 45 days at 37°C to achieve a 5-log10 reduction of both *E. coli* and *Salmonella enterica* serovar (Himathongkham *et al.*, 1999). These results indicate that *E. coli* can continue in bovine faeces for an extended period of time and that bovine faeces are a potential vehicle for transmitting the pathogen to cattle, food, and the environment (Jiang *et al*., 2003). However, pathogenic bacteria such as *E. coli* and *Salmonella Spp.* can survive in animals manure or soil up to 70 days under 14^0F (McLaughlin, 2002). A study by Kudva *et al*, (1998) reported that *E. coli* survived in the manure for 21 months, and the numbers of bacteria recovered ranged from $<10^2$ to 10^6 CFU/g at different times over the course of the experiment.

Conclusion: In conclusion dog faeces represent an important means of transfer of bacterial pathogens from dogs to humans via the soil. The reprehensible recent habit amongst some dog owners of leaving faeces in sealed plastic bags is likely to greatly exacerbate this problem.

Chapter Seven: Production of Biodiesel from Dog Waste by BSF and FB Larvae- A Potential Solution To The Dog Faeces Problem

7.1. Introduction

Biodiesel is receiving increased attention as a renewable source of fuel. Biofuels can be made from a variety of feedstocks including starch, vegetable oil, or animal fats as an fuel alternative to help reduce consumption of petroleum. Alternatively cheap waste, such as dairy manure can be used to produce bioethanol (Liao *et al.*, 2008; Predojevic 2008) and is hoped that, as well as producing biofuels, a reduction can be made in the amount of waste which has to be disposed of to the environment (Ann *et al*., 2002). In addition to biodiesel, other fuels can be obtained from wastes as is discussed below.

7.1.1. Fuels from wastes

Many fuels can be derived from waste refuse can be combusted directly in an incinerator to provide power and chicken litter can (Ferrer et al., 2005) , like dog faeces, be dried and directly combusted (Li *et at*., 2011).

7.1.2. Anaerobic digestion to methane

The anaerobic fermentation of animal fats, vegetable oil, starch and sugar can be achieved using conventional technology and results in the production of methane, i.e. biogas (Demirbas, 2008). Digestate, a solid by-product which can be used as a fertilizer is also produced. Dog faeces can fuel an anaerobic digester and produced methane. This might be done in a large centralized facility, but it is more likely that it will be done on a more local scale. Methane is of course a more potent green house gas than carbon dioxide and when burnt its releases CO_2; any treatment will however solve the direct problem of dog waste removal. In the US, and increasingly world-wide, dog waste is being turned into a local energy source (Demirel *et al*,. 2010). Dog owners throw the waste into a feeding tube, turn on a hand crank, so that the anaerobic biogas digester can decompose the faeces and make it into a burnable methane gas. The gas is then used to

power the local street. i.e. the so-called “Park Spark Project”. A criticism of this approach is that during methane production, pathogens are not removed content completely a potentially harmful product will remain after digestion; such residues can however, be treated in the conventional sewage treatment system (Demirel *et al*,. 2010).

Biofuel or agrofuel is obtained from biomass and may be produced in a solid, liquid or gaseous form. The utilization of waste biomass as a source of energy can decrease problems relating to waste management , pollution, greenhouse gaseous emissions and, as a result, the burning of fossil fuels. Some 19 million tons of oil equivalents could be derived from biomass, with some 46% being obtained from bio-wastes like farm waste, agricultural waste, municipal solid waste and other biodegradable waste streams (Demirbas., 2008)

7.1.3. Landfill methane

Landfill waste undergoes anaerobic digestion to landfill gases (LFG). Such gases can be burned and looked up to as a source of renewable energy (Themelis and Ulloa., 2007). The LFG is made of around 50% methane and since it is equivalent to natural gas can be used to generate electricity for public consumption or alternatively it can be burned for heat. If LFG is not used properly, it would be released to the atmosphere. and since methane is a greenhouse gas, it has a global warming potential of 23 times carbon dioxide, i.e. one ton of methane results in the greenhouse gas equivalent of 23 tons of carbon dioxide. Harvesting and burning LFG reduces the global warming potential by a factor of 23 and energy is provided for heat and power. A typical landfill power plant can supply power to 1900 homes and avoid 6000 tons of methane per year from entering the atmosphere. As well as eliminating 18,000 tons per year of carbon dioxide by fossil fuel replacement (De Montfon, 2012)

7.1.4. Biodiesel from waste cooking oils

Each week the UK population uses in excess of 1,500 tonnes of cooking oil. Large scale producers of waste oil like restraints, chip shops and industrial food producers have their waste oil removed by licensed collectors, and around 100,000 tonnes of recoverable waste oil produced each year within the UK (Phan and Phan., 2008). Currently most of this recovered oil is treated and added to animal feed. The EU however, its use for pig feed due to several food scares related to vegetable oil and a similar ban may soon apply to cattle also. Such oilcan however, be converted into biodiesel (Silvis, 2006). The waste vegetable oil currently used to produce UK biodiesel is mostly rapeseed oil, whose trans-esterification produces RME (Rape Methyl Ester), or more properly, FAME (Fatty Acid Methyl Ester) which is the correct chemical term for biodiesel produced from any oil or fat using methanol as the alcohol for the production process (Zhang *et al*., 2003).

7.1.5. Methane from anaerobic digestion

Anaerobic digestion (or gasification) is the use of bacteria to decompose organic matter in the absence of oxygen to produce a gas containing 60% methane, 40% carbon dioxide together with a mix of solid and liquid fertiliser, and since anaerobic digestion does not produce anymore carbon dioxide than would be produced by the natural decomposition of the waste, it regarded as being 'carbon neutral' In this process, waste is pumped once a day into the digester, and remains there for about 10 to 40 days, when the internal of the digester must reach between 30 and 70 degrees C. Any mix of organic waste mater can be used, including animal and human sewage, crop residues, newspaper, abattoir waste and agricultural and food processing waste. Since the resultant product is a gas this fuel is more applicable to electricity generation, rather

than a transport fuel; although methane could be liquefied for vehicular use (Chynoweth *et al*., 2001).

7.1.6. Oil from tyre pyrolysis

Around 40 million tyres (440,000 tonnes) per year are produced world-wide. Currently the UK recovers some value from 70% of the total used tyres produced, with the rest going to landfill. In 2006 a ban on tyre-landfill was introduced, so tyres are increasingly being treated by pyrolysis, a process of thermal degradation in the absence of oxygen at elevated temperatures (430°C) and pressures(Shulman, 2004). The products of pyrolysis (from organic waste) are gases, small quantities of liquid, and a solid residue containing carbon and ash. The gases produced in the process can then be re-used to provide the heat required to continue the process (Makarov and Drozdovskiĭ., 1991).

7.1.7. Biodiesel production

Currently, biodiesel is generally used as a diesel fuel additive to minimize particulate release and the production of carbon monoxide, and hydrocarbons. The relatively high production cost is the main reason why biodiesel is not used as a primary fuel. Three quarters of the cost of biodiesel is derived from the feedstock, such as soybean oil, rapeseed oil and sunflower oil. In addition, the use of limited food supplies for the production of biodiesel is not feasible for developing countries like China. As a result, non-food feedstocks such as *Jatropha curcas*, Chinese tallow and microalgae are being developed as sources of biofuels; the use of these sources however, is certainly not without problems. On the other hand, organic wastes such as animal wastes, residential wastes (e.g., household), commercial and institutional wastes are generated in large quantities in developing countries and present a cheap from of organic wastes which would otherwise present a environmental pollution problem and so can act as a useful resource for biodiesel production (Ma and Hanna., 1999)

7.1.8. Biodiesel production by BSFL

Animal faeces waste can be used for biodiesel production, with the resultant biodiesel being comparable to rapeseed based biodiesel, and met the European biodiesel standard, EN14214. Among the organic wastes evaluated, chicken manure appears to be the best one for achieving high rates of BSFL biomass production. About 30% of the waste-grown BSFL biomass can be extracted as crude fat, which can be used for the production of biodiesel; the resultant fuel properties of the BSFL fat-based biodiesel, including density (885 kg/m3), viscosity (5.8 mm2/s), and cetane number (53) were found to be similar to those of rapeseed oil based biodiesel (Li *et al*., 2011a). To date there appear to be no reports of the use of dogs faeces to produce biodiesel either alone or in conjunction with BSFL or FBL. Among the organic wastes evaluated chicken manure was found to be the best for maximal BSFL growth (327.6 g), which gave 8.5 g crude fat production after petroleum ether extraction. An optimized two step conversion process was performed, yielding 91.4 g of biodiesel with a biodiesel yield of 93% from the crude fat contents (Li *et al*., 2011c). Cattle, pig and chicken manure can also act as a food source for the production of high fat, nutrient–rich larvae. Fat-rich BSFL can be used to degrade organic waste and produce biodiesel as well as providing foods from animals notably chickens(Li *et al*., 2011b).

The results of recent studies have shown that BSFL-produced fatty acids could be a valuable feedstock for biodiesel production having two advantages over crop oil-based biodiesel, namely:

1) It does not compete with food resources or land use

2) It uses ‘‘waste nutrients’’ for insect growth.

The aim of the work reported in this Chapter was to determine if the larvae used here can convert dog faeces into a potentially viable source of biodiesel

7.2. Materials and Methods

7.2.1. Samples collection

Black soldier fly and fruit beetle larvae were obtained from LiveFood Co., while dog faeces samples were collected from local dog waste bins. The faeces were distributed in two boxes, each containing 100g of dog faeces. Ten BSFL were then added to the waste (1000 larvae per kg dog waste) according to Li *et al.*, (2011) and ten FB larvae were similarly added to the dog faeces. The treated faeces were incubated under laboratory conditions for 28 days; samples were obtained in triplicate at weekly intervals.

7. 2.2. Extraction of lipids

In order to extract lipids from dog faeces and larvae, the larval whole body were ground and then weekly samples (1 g) of all samples were added to Eppendorf tubes 1.5 ml and frozen at -80^{o}C overnight, then freeze dried (lypophilized) for 24-48 hours. To estimate the weight of biomass the Eppendorf tubes were re-weighed. Samples were transferred to centrifuge tubes (50 ml), 20 ml of methanol/ chloroform (2:1 v/v) were added; the contents were then sonicated for 1 min on ice. Samples were centrifuged at 5000 g for 5 mins and transferred to a centrifuge tubes in order to determine the volume of supernatant. Then 2:1 methanol: chloroform, and chloroform and 1% NaCl (1 g NaCl in 100 ml) was added to give 2:2:1 methanol: chloroform:1%NaCl. (13.33ml methanol:13.33 chloroform:6.7 1%NaCl) (Fig 7.1). Samples were then centrifuged for 2 mins at 5000 g. The centrifuge tubes were labelled and weighed then transferred to a chloroform phase into pre-weighed sample were left in fume cupboard with tops open to evaporate until dry. The centrifuge tubes were then re-weighed and the weight of lipids calculated. (Lu *et al*. 2008; Chiu *et al*. 2009)

7.2.3.Detection and measurement the fatty acid using a combined mass spectrometer and gas chromatographic GC/MS

The harvested lipids (10mg/ml) of dog faeces and BSF and FB larvae before and after being fed on dog faeces were dissolved in chloroform. The samples were then subjected to gas chromatography-mass spectrometry GC/MS. The GC/MS analyses were performed using a Perkin Elmer Turbo mass spectrometer detector (software version 5.4. Perkin Elmer). Injector and oven temperatures were set as above with a transfer line temperature, 260°C. The injection volume was 1.0µl of dog faeces and larvae and was injected in a capillary column (Zebran ZB-S, 30 m length x 0.25 mm diameter x 0.25µm phase thickness) (see appendix 4). The oven temperature was programmed from 60°C and increased at the rate of 10°C/min to a final temperature of 260°C, which was held at 260°C for 10 min. High purity helium was used as carrier gas at 1ml/min, split ratio and 1:25; Electron ionization (EI) spectra were obtained at 70 eV; the scan range was 50-450 m/z at 30 min. The identity of each compound was determined by comparison of its retention index (RI) as well as of its total ion chromatogram with the NIST mass spectral library version 5.4.2 (Albano *et al.*, 2011; Sivasamy *et al.*, 2011).

7.3. Results and Discussion

7.3.1. Extraction of biodiesel from larvae

A visual representation of the fact that lipids could be extracted from the faeces on which larvae were grown and from the larvae themselves is shown in Fig.7.1. Table 7.1 shows the percentage of lipids produced in the faeces fed on the larvae and in the larvae themselves

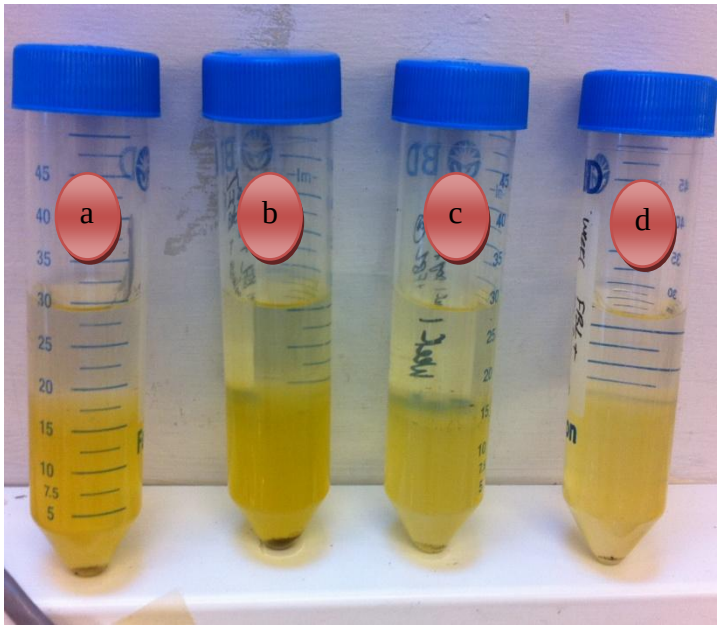


Fig. 7. 1: Fatty acids extracted from; a, BSFL, b) FBL, c) dog faeces with BSFL, d) Dog faeces with FBL

Table. 7. 1: The percentage of lipids in dry weight of 1g samples of faeces and larvae

Time/ week	Dry weight/ g SD±	Total lipids/ g SD±	% of lipids in dry weight
Zero time			
Untreated dog faeces (without larvae)	0.21 ± 0.01	0.15 ± 0.02	71%
BSFL before fed on dog faeces	0.26 ± 0.01	0.22 ± 0.02	85%
FBL before fed on dog faeces	0.20 ± 0.01	0.10 ± 0.02	50%
Week 1			
Dog faeces with BSFL	0.70 ± 0.05	0.10 ± 0.00	14%
Dog faeces with FBL	0.44 ± 0.04	0.12 ± 0.01	27%
BSFL fed on dog faeces	0.28 ± 0.01	0.23 ± 0.01	82%
FBL fed on dog faeces	0.25 ± 0.02	0.14 ± 0.03	56%
Week 2			
Dog faeces with BSFL	0.90 ± 0.02	0.10 ± 0.02	11%
Dog faeces with FBL	0.80 ± 0.02	0.14 ± 0.01	18%
BSFL fed on dog faeces	0.50 ± 0.02	0.15 ± 0.02	30%
FBL fed on dog faeces	0.60± 0.01	0.14 ± 0.04	23%
Week 3			
Dog faeces with BSFL	0.90± 0.03	0.23± 0.03	26%
Dog faeces with FBL	0.80± 0.02	0.22± 0.02	28%
BSFL fed on dog faeces	0.50± 0.02	0.16± 0.04	32%
FBL fed on dog faeces	0.70± 0.02	0.13± 0.01	19%
Week 4			
Dog faeces with BSFL	0.80 ± 0.01	0.23± 0.01	29%
Dog faeces with FBL	0.84± 0.02	0.20 ± 0.02	24%
BSFL fed on dog faeces	0.62± 0.04	0.16 ± 0.02	26%
FBL fed on dog faeces	0.82± 0.02	0.14 ± 0.01	17%

Untreated dog faeces (without larvae) possessed a lipid content of 71% Table 7.1). The unfed larvae, BSFL and FBL, contained 85and 50% dry weight of lipids. This suggests that both types of dog faeces and the two larvae could be used independently as a source of lipids for the production of biofuels. The next step was to determine if feeding the larvae on dog faeces would increase, over time, their lipid content and the lipid content of the faeces on which they were feeding.

The results of the week 1 analysis shows that the lipid content of the dog faeces treated with BSFL and FBL fell from 71 to 14 and 27% respectively, while the lipid content of the two larvae remained broadly the same as before feeding on faeces. By week 2, the lipid content of the dog faeces treated with BSFL and FBL declined, as did the concentration of lipids in both types of larvae (Table 7.1);this trend was also seen at weeks 3 and 4 (Table 7.1).

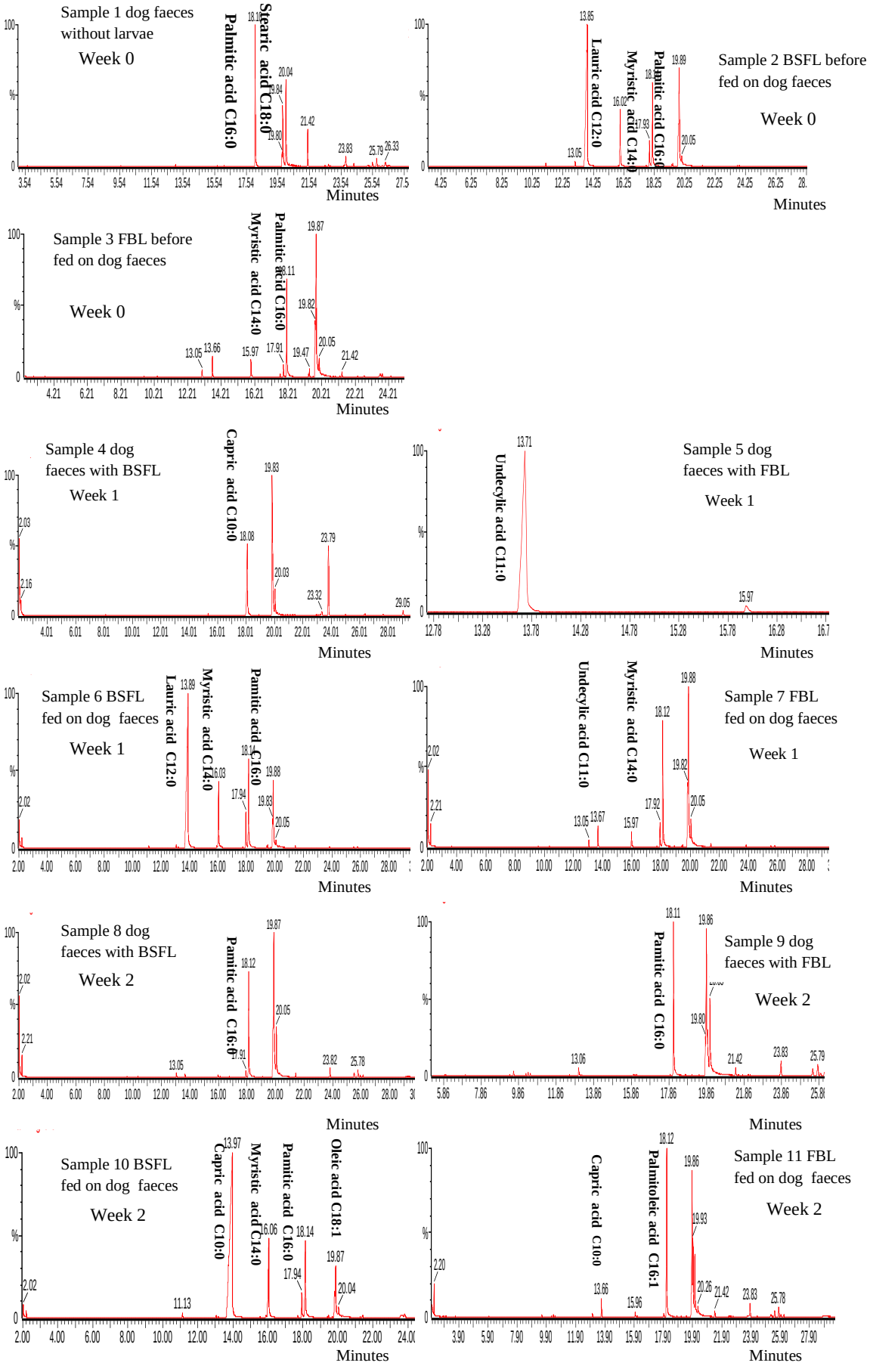
These results show that the decline in dog faeces lipid content was not mirrored by an increase in the lipid content of the two larvae. Presumably, the faeces-lipids were, over time, metabolized by the larvae, ultimately to carbon dioxide, i.e. the larvae were using the faecal lipids as a food source. The results show that no advantage can be gained in relation to total lipid production by treating dog faeces with BSFL and FBL. However, Fig.7.2 shows that the type and concentration of fatty acids (as seen by referring to peak retention times and peak height respectively of the exhibited chromatograms)varied markedly depended on time of exposure of the larvae to dog faeces and the type of larvae used. This is shown more clearly in Table 7.3. Dog faeces without larvae and unfed FBL contained only palmitic and stearic acids, while unfed BSFL contained these fatty acid together with lauric acid.

The type of fatty acid produced in the larvae and in the dog faeces on which the larvae fed was then seen to vary over time. Oleic acid, for example was only produced in the BSFL fed on dog faeces and then only at week 2 (Table 7.2). Clearly, the type of

fatty acid varied with the larvae used and in the faeces treated with the different larvae, all of which varied over time. This finding suggests that BSFL and FBL might be used to produce individual fatty acids, either within their bodies, when fed on dog faeces, or in the dog faeces itself. In this way a single, or desired mixture of fatty acids, might be produced. Thus, if oleic acid alone was needed as a biofuel feedstock, or for some other biotechnological purpose then feeding BSF on dog faeces for 2 weeks and the harvesting the larvae and extracting the fatty acid may provide a source of this individual fatty acid.

Table. 7. 2: The GC/MS analysis chain composition of a number of fatty acids from dog faeces and larval fats

Fatty acid	Lipids Number	Time																		
		Week 0			Week 1				Week 2				Week 3				Week 4			
		Dog faeces no larvae	Control BSFL	Control FBL	Dog faeces+BSFL	Dog faeces+FBL	BSFL fed on faeces	FBL fed on faeces	Dog faeces +BSFL	Dog faeces+FBL	BSFL fed on faeces	FBL fed on faeces	Dog faeces +BSFL	Dog faeces+FBL	BSFL fed on faeces	FBL fed on faeces	Dog faeces +BSFL	Dog faeces+FBL	BSFL fed on faeces	FBL fed on faeces
Capric acid	C10:0				+						+	+								
Undecylic acid	C11:0					+		+												
Lauric acid	C12:0		+				+								+				+	
Myristic acid	C14:0		+	+			+	+			+				+					
Palmitic acid	C16:0	+	+	+			+		+	+	+		+	+	+	+	+	+	+	+
Stearic acid	C18:0	+											+					+	+	
Palmitoleic acid	C16:1																			
Oleic acid	C18:1										+									


Sample 1 dog faeces without larvae
Week 0
Palmitic acid C16:0
Stearic acid C18:0
Minutes
Sample 2 BSFL before fed on dog faeces
Week 0
Lauric acid C12:0
Myristic acid C14:0
Palmitic acid C16:0
Minutes
Sample 3 FBL before fed on dog faeces
Week 0
Myristic acid C14:0
Palmitic acid C16:0
Minutes
Sample 4 dog faeces with BSFL
Week 1
Capric acid C10:0
Minutes
Sample 5 dog faeces with FBL
Week 1
Undecylic acid C11:0
Minutes
Sample 6 BSFL fed on dog faeces
Week 1
Lauric acid C12:0
Myristic acid C14:0
Palmitic acid C16:0
Minutes
Sample 7 FBL fed on dog faeces
Week 1
Undecylic acid C11:0
Myristic acid C14:0
Minutes
Sample 8 dog faeces with BSFL
Week 2
Palmitic acid C16:0
Minutes
Sample 9 dog faeces with FBL
Week 2
Palmitic acid C16:0
Minutes
Sample 10 BSFL fed on dog faeces
Week 2
Capric acid C10:0
Myristic acid C14:0
Palmitic acid C16:0
Oleic acid C18:1
Minutes
Sample 11 FBL fed on dog faeces
Week 2
Capric acid C10:0
Palmitoleic acid C16:1
Minutes

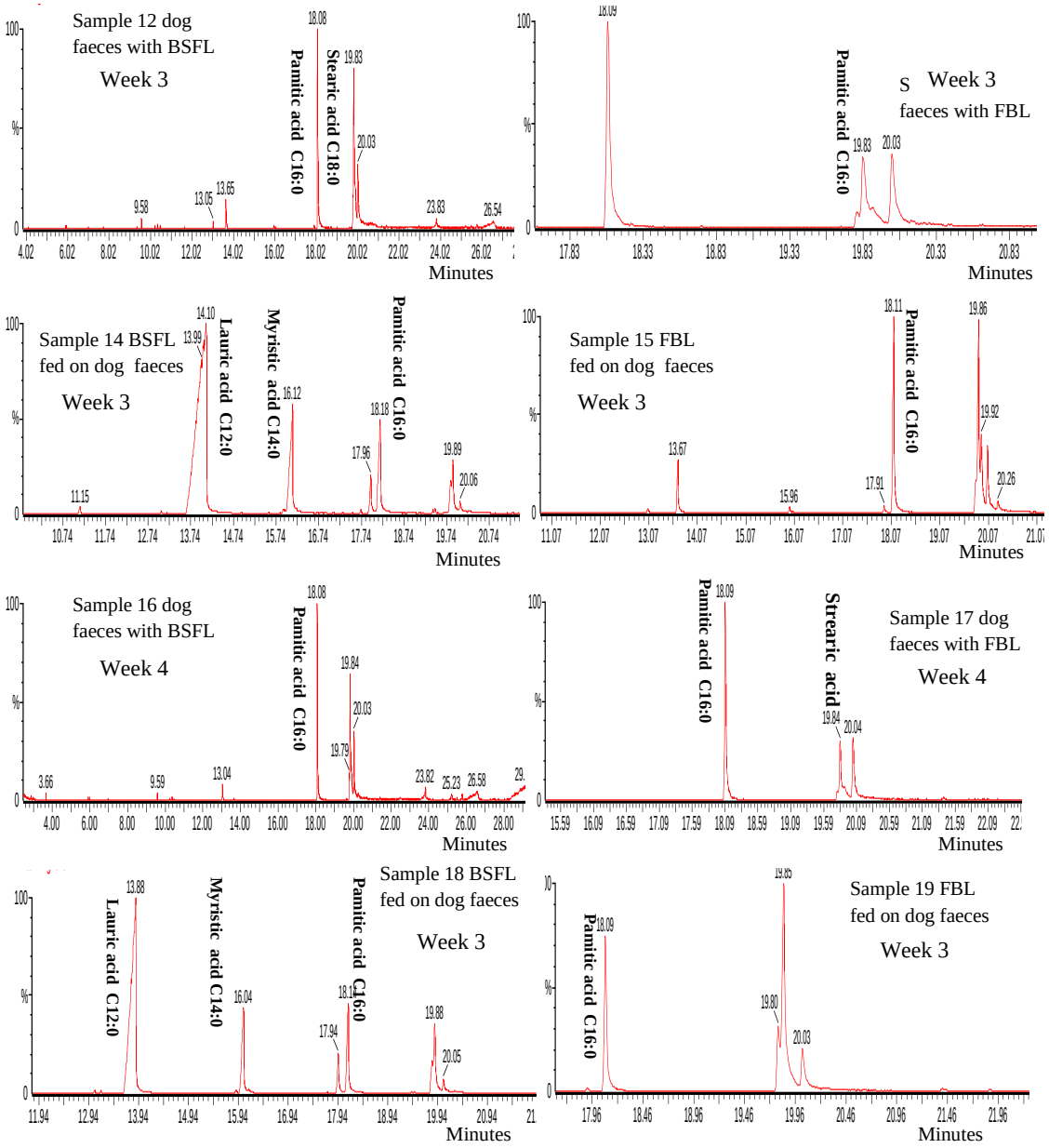


Fig. 7. 2: GC–MS chromatogram of a reference mixture of fatty acid representative the total of samples ion taken from dog faeces and BSF and FB larvae. The chromatogram for this figure and all subsequent figures were obtained with Gas Chromatograph using Zebron ZB-S capillary column

The use of BSF to treat domestic waste has been pioneered by the New York-based Ecosystem Corporation which is asking the US Department of Energy for $1.75 million to prove the concept. This Company is also seeking a matching grant from the Dog's Biomass Research and Development Initiative to build a $3.5 million demonstration project that would use 24,000 tons of food waste from an Ohio waste transfer station to feed a "bioreactor" full of flies (Otis et al., 1980).The pilot plant could produce between 150,000 to 195,000 gallons of oil per year; some 26 million tons of food scraps are dumped by Americans into landfills each year, and only about 3 percent of that is being recovered today. Ecosystem claim that with only one-quarter of all those food scraps it could make 100 million gallons of oil every year; add in livestock manure and other agriculture wastes and that amount could increase – particularly if the company can be paid to take it off waste handlers' hands, a possibility which forms an important part of many waste-to-biofuel business plans (Vaughn, 2009)

Restaurant wastes are also an ideal foodstock for BSFL biodiesel production. The major methyl ester components of the biodiesel derived from BSFL were oleinic acid methyl ester (27.1%), lauric acid methyl ester (23.4%), and palmitic acid methyl ester (18.2%) (Table 7.3, 7.4). Table 7.3 also shows that BSFL biodiesel contains a far richer mix of fatty acid methyl ester compounds than does biodiesel obtained from rapeseed. Certainly the BSFL product provides a better source of fatty acids for other possible industrial/food-based products than does rapeseed oil biodiesel.

Table. 7. 3: Comparison of fatty acid methyl ester composition of BSFL fat-based biodiesel and rapeseed oil-based biodiesel.

Composition	BSFL fat-based biodiesel[a] (%)	Rapeseed oil-based biodiesel[b] (%)
Capric acid methyl ester	3.1	n/a
Lauric acid methyl ester	35.6	n/a
Myristic acid methyl ester	7.6	n/a
Palmitoleic acid methyl ester	3.8	n/a
Palmitic acid methyl ester	14.8	3.5
Oleinic acid methyl ester	23.6	64.4
Linoleic acid methyl ester	5.8	22.3
Linolenic acid methyl ester	Nd	8.2
Stearic acid methyl ester	3.6	0.8
Noadecanic acid methyl ester	1.4	n/a

(See EN 14214 in Refs. Nd stands for not detected).

Most of the properties of this biodiesel met the specifications of the standard EN 14214, including density (860 kg/m^3), viscosity (4.9 mm^2/s), flash point (128 °C), cetane number (58) and ester contents (96.9%) (Table 7.4).

Table. 7. 4: Comparison of fuel properties of BSFL fat-based biodiesel, rapeseed oil-based biodiesel, and the standard EN14214.

Properties	EN14214	BSFL biodiesel[a]	Rapeseed biodiesel[b]
Density (kg/m^3)	860–900	885	880
Viscosity at 40 °C (mm^2/s)	1.9–6.0	5.8	6.35
Sulfur content (wt.%)	0.05	Nd	<0.01
Ester content (%)	96.5	97.2	n/a
Water content (mg/kg)	<0.03	0.03	0.03
Flash point (°C)	120	123	n/a
Cetane index	48–60	53	45
Acid number (mg KOH/g)	<0.8	1.1	0.3
Methanol or ethanol (m/m)	0.2%	0.3%	n/a
Distillation (°C)	n/a	360	352

nd stands for not determined

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(See EN 14214 in Refs. Nd stands for not detected).

Results such as these show that BSFL obtained from larvae grown on fat wastes could potentially be used as a non-food feedstock for biodiesel production, as well as significantly reducing the large quantity of a solid waste.

It has also been shown in the literature (REF) that larval biomass of BSFL fed on animal manure can efficiently produced biodiesel. Approximately15.8 g biodiesel was produced from about 1200 black soldier fly larvae when fed on dairy manure over a 21 days period.

Conclusion

The results reported in this Thesis show that dog faeces and BSFL and FBL fed on such faeces can provide a source of fatty acids which could form the basis of biodiesel production. Again however, the main limitation of using dog faeces for any process, be it agricultural or industrial soon becomes apparent and relates to the economics of collecting sufficient quantities of dog waste. When one compares the vast world-wide production of rapeseed oil with the large, but widely dispersed sources of dog faeces it soon becomes obvious that the former has such a huge competitive advantaged which makes it impossible that bulk dog faeces-biodiesel could ever compete with other sources of this product.

Chapter Eight: Final Discussion

8.1 Final Discussion

It is perhaps surprising, considering the vast amounts of dog faeces which reach the environment that relatively few scientific studies have been made on the bioremediation of this potentially toxic waste which is major source of disease-causing microorganisms in the urban environment. The obvious distasteful nature of dog faeces doubtless contributes to the lack of interest amongst researchers in this waste product. This lack of interest means that there exists relatively few publications available which are directly relevant to the scientific study of dog faeces.

The broad aim of this work reported in this Thesis was to study a variety of aspects of dog faeces in relation to public health, their fertilizer potential and the possibility of them being remediated using larvae, ultimately to provide a source of biodiesel.

Dog faeces were shown to be a source of pathogenic bacteria, notably *E.coli* and *Salmonella*. These bacteria were shown to be transferred to the soil of a local playing field by direct, *in situ*, transfer from dog faeces undergoing weathering. The potential danger to public health is obvious, especially since children play in parks like these, and are therefore at risk of picking up pathogens from contaminated soil, or more directly from the faeces themselves. Dog faeces are known to transmit bacterial pathogens and, perhaps more worryingly, potentially deadly parasites.

It was shown here that "common or garden" slugs can transfer potentially pathogenic bacteria from dog faeces to lettuce. Again this is potentially damaging to public health, especially since slugs frequent agricultural areas. The following genera and species of bacteria were isolated from slugs: *Enterococcus sp, Salmonella sp, Staphylococcus lentus, E. coli, Proteus sp* and *Acinetobacter sp*. *Enterobacter amnigenes* was isolated from the outside secretion of slugs and their identification was confirmed using 16S rRNA sequence gene analysis (i.e.. 99% confirmed). Externally isolated species were identified to *Acinetobacter sp* (99%) using 16SrRNA and *Comamonas sp* was also

confirmed by 16S rRNA analysis is (91%), *Enterobacter amnigenus, Acinetobacter sp, Comamonas sp* and *Acinetobacter sp* were isolated from lettuce contaminated with slugs fed on dog faeces. *Mycoplasma* was transmitted from dog faeces to slugs or snails which they, ingested or carried, and then in turn transferred to lettuce. Earthworms were also shown to carry *Mycoplasma* from soil on/or within their bodies, a fact which presumably reflects contamination from animal faeces, mainly dogs. Perhaps the greatest potential risk relates to gardens and allotments where slugs may be common, and not successfully controlled. The consumption of pathogen contaminated lettuces, or other salad vegetables, is an obvious potential problem, which would presumably only be solved by vigorous washing of locally grown produce. There is no doubt more research effort has been devoted to the potential risk to human and animal of parasites in dog faeces, rather than bacterial and virus pathogens. Viruses have been isolated from dog faeces (Carmichael and Binn, 1981)

Dog faeces were shown to have potential inherent fertilizer content, the nutrients present being released over a time period mimicking the natural weathering of dog faeces in the environment. The fertilizer potential of dog faeces is not however, great and their offensive nature and potentially rich pathogen content means that un-weathered, or non-composted canine faeces are rarely used as an agricultural fertilizer. Dog faeces were also shown to contain an indigenous microflora capable of mineralizing organic nitrogen to ammonium and oxidizing added ammonium to nitrate; elemental sulphur to sulphate and were able to solubilise insoluble phosphate to plant available, soluble phosphate. As a generalization, the addition of both types of larvae to dog faeces significantly reduced the concentration of indigenous plant nutrients over the entire four week incubation period; exceptions to this were nitrate and phosphate concentrations in BSFL treated faeces, where significant increases were seen at week 4 and 3 respectively and in faeces treated with FBL, where ammonium concentrations

were significantly increased at weeks 2-4, and phosphate at week 4. While the addition of both larvae therefore initially decreased levels of indigenous plant nutrients there was a trend in some of the nutrients to increase the longer the incubation went on. This suggests that perhaps a longer term exposure of dog faeces to the two larvae might have lead to increase in ammonium, nitrate, sulphate and phosphate concentrations. While this would generally be advantageous to the fertility of soils surrounding dog faeces, the potential long term increase in nitrate could at first sight be considered detrimental as nitrate can be readily leached to ground waters (where, when present in drinking water, it can cause blue baby disease and gastric cancer in humans). However, the relatively small amounts of dog faeces which are present, spread over large areas of soil, would likely make such increased nitrate contributions to drinking water relatively insignificant.

The addition of ammonium, elemental sulphur and insoluble phosphate to dog faeces which had been modified by the two larvae led to significant increases in nitrate, sulphate and plant-available phosphate, results which shows that that dog faeces contains the indigenous microflora required for the transformation of these amendments (which simulate fertilizer addition). The increased friability and therefore increased aeration of the dog faeces following larval modification is also like to result in enhanced rates of nitrification, S-oxidation and phosphate solubilisation. Clearly, the microflora of dog faeces could act to modify added fertilizers and convert them to the plant available form. Dog faeces represent a potential source for spreading pathogenic bacteria such as *E. coli* and *Salmonella enterica*, bacteria which represent an obvious risk human health. Bacterial pathogens from dog faeces could infect young children and others playing, or using, parks etc (including of course dog owners). Dog faeces have also been shown here to produce gasses which are injurious to plant germination and/ or growth, and are also likely to be directly inhibitory to plant growth.

The potential for using fly larvae for the bioremediation of dog faeces was investigated. Black Soldier Fly (BSFL) and Fruit Beetle (FBL) Fly larvae were shown to dramatically improve the physical nature of canine faeces, even after only a short exposure period, giving a bioremediated product which is markedly improved in terms of texture, reduced odour and overall reduced offensiveness.

The feeding of BSFL on faeces led to a statistically significant increase in the number of bacteria inside the BSFL gut and the same trend was seen in relation to dog faeces fed FBL. This trend of increasing bacterial numbers in larvae fed on dog faeces is particularly worrying in relation to the potential feeding of these larvae to animals- post exposure to faeces. Black soldier Fly larvae in particular are currently being produced for use as animal feed (mainly to chickens) following feeding on restaurant food waste. It is unlikely then, considering the high pathogen content of larvae fed on dog faeces that these could be safely used as animal feed following feeding on dog faeces. The bioremediated dog faeces produced was also found to be suitable as potting compost when "diluted" with proprietary potting compost.

The haemolymph and total body extracts of BSFL and FBL were shown to be antibacterial.

The potential for using dog faeces and dog faeces which had been treated with BSFL and FB was determined. It was shown that potential biodiesel precursors (mainly fatty acids) were present both in the raw dog faeces and in faeces which were treated with the two different larvae. Whether or not dog faeces, treated or otherwise, could be used economically as a source of biodiesel remains to be seen. Although vast amounts of dog faeces are produced annually all over the world problems relating to such collection are obvious. The increasing social trend towards dog owners collecting dog faeces and placing them in council collection containers means that an increasing amount of this potential bioresource is being collected together, at least in the urban environment. The

quantities collected will never however, approach that of other animal faeces, such as cattle feedlot waste. As a result, it might be better to use the relatively small amounts of dog faeces which are collected in any one location for localised methane generation, rather than biodiesel production.

The number of bacteria, including pathogens declined inside the gut of BSFL following feeding on dog faeces. The feeding of BSFL on faeces led to a statistically significant increase in the number of bacteria inside the BSFL gut and the same trend was seen in relation to dog faeces fed FBL. This trend of increasing bacterial numbers in larvae fed on dog faeces is particularly worrying in relation to the potential feeding of these larvae to animals- post exposure to faeces. Black soldier Fly larvae in particular are currently being produced for use as animal feed (mainly to chickens) following feeding on restaurant food waste. It is unlikely then, considering the high pathogen content of larvae fed on dog faeces that these could be safely used as animal feed following feeding on dog faeces. The bioremediated dog faeces produced was also found to be suitable as potting compost when "diluted" with proprietary potting compost. Increasing the ratio of compost to modified faeces "diluted out" the inhibitory effect of raw dog faeces on plant growth suggesting the possibility that larval modified dog faeces could be used as compost additive fertilizer, or perhaps even be used as an agricultural soil fertilizer.

Escherichia coli and *Salmonella enterica* were isolated from all four sites (Fig. 6.3nb) while no such isolates were obtained from the fifth location which was uncontaminated with dog faeces. The number of *E. coli* varied from site to site (from 10^4 to 10^5 CFU/g); *E. coli* was isolated in highest numbers. The isolation of these pathogenic bacteria from playing fields frequented by children is obviously a major public health concern since pathogenic bacteria could be picked up from these soils, either on shoes, or by transfer from contaminated skin to the ears, eyes or mouths of

infected children (and of course, people in general) from where they could cause disease. The number of bacteria present in dog faeces disposed of in plastic bags dramatically increased over exposure to the UK summer, when temperatures were recorded between 10-27^{o}C. The following bacteria were isolated from the enclosed faeces (Fig. 6.4) a), *Enterococcus spp* and *E. coli* in Chromoagar media b), *E. coli* in HiCrome agar media c), *Salmonella* enterica in XLT-4 media while d) was *Staphylococcus aureus* with MRSA selective media respectively. However, the faecal sample counts were:(range 3.5×10^5 to 4.6×10^6, 9.7×10^3 to 1.0×10^5 and 4.2×10^3 to 2.9×10^5) for *E. coli*, *Salmonella enterica* and plate count respectively. This study has demonstrated that the numbers and the diversity of bacteria can increase when dog faeces are enclosed over a month in plastic bags.

There has been a welcome trend of late for dog owners to act responsibly by picking up waste from their dogs, placing this in plastic bags and than putting these bags into special local council run dog-waste containers, from where they are transported and disposed off.

8.2 Future work

The lack of published work on all aspects of the environmental and agricultural impact of dog faeces means that there is considerable potential to continue and extend the work reported in this Thesis. This Thesis reports preliminary work on all of the individual research questions investigated and all of the separate chapters could be extended to form the basis of separate theses.

1) The work described here on biofuels could be extended, particularly in relation to the type of lipids produced by fly larvae growing on faeces. However, since there exist more readily available and technically more readily useable biofuel substrates available

it would seem economically pointless to devote to much attention to this area of green technology.

2) No work was done here on viruses, both in relation to their presence in faeces and transmission to salads and vegetable. This is clearly an opportunity for workers with the experience and methodology in relation to working with viruses.

3) Considerable interest is currently being devoted to the use of BSFL, produced from waste vegetables and other waste organic matter, as a source of poultry feed. It would be of interest to develop this line of research. Problems related to pathogen transfer mean that it is unlikely however, that such animal feeds could be safely produced from dog faeces.

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Appendices

Appendix One: Preparation of chemical solutions and reagents

1.1 Solutions used in analysis of inorganic N-ions:

1.1.1 Indophenol blue method for the determination of ammonium-N (NH4+-N).

(a) Standard ammonium solution..

Standard ammonium solution was prepared by dissolving 0.4717 g ammonium sulphate in 1 litre distilled water for (100 pg N ml-^{1}). (Wainwright and Pugh, 1973)

(A) Ethylenediamineteraaactic acid (EDTA)

EDTA (60g) was dissolved in (900ml) distilled water then diluted to 1 litre

(b) Phenol solution:

Phenol solution was prepared by dissolving phenol (62.5 g) in ethanol (25 ml) and adding acetone (1S.5 ml) to give a total of 100 ml. The phenol solution was stored in the dark at 4^{o} C.

(c) Phenolate reagent:

Phenolate reagent was prepared by mixing 20 ml of phenol solution with 20 ml caustic solution (27%NaOH w/v) and diluting to 100 ml. The reagent was prepared fresh daily.

1.1.2 Chromotropic acid method for nitrate-N determination.

(a) Standard nitrate solution:

Potassium nitrate (KNO3), 0J229 was dissolved in distilled water and made up to 1 litre volumetrically, for 100 NO3-N ml$^{-1.}$

(b) Chromotropic acid reagent (C_{1o} $H_6O_8S_2$ Na_2):

A0.l% (v/v) stock solution of chromotropic acid in concentrated sulphuric acid (H_2SO_4) was prepa.red by dissolving 1.84 g chromotropic acid in litre H_2SO+ (1:l). This solution was stored in an amber bottle in refrigerator at 4^{o}C for several months.

(c) Working chromotropic acid solution (CTA):

A working CTA-solution (0.01% v/v) was prepared by diluting 100 ml of stock solution to 990 ml with concentrated sulphuric acid (H_2SO_4) then adding 10 ml concentrated HCl. This solution was stored at 4^oC for several weeks only

1.1.3 Analysis of inorganic S-ions.

(a) Standard sulphate-S solution:

Sodium sulphate ($Na_2SO+.10H_2O$), 0.443 g was dissolved in 1 litre distilled water, which gives the concentration 100 µg SO_4^{2-}- S ml^{-l}.

b) Gum acacia solution (0.250/" w/v):

Gum acacia0.25 g was dissolved in 100 ml distilled water.

1.3.4Analysis of phosphate

(a) Chemical solution

1- Ascorbic acid solution(10%) was prepared by mixing 10 ml of ascorbic acid with 90 ml of distilled water

2- Ammonium molybdate (0.42Vo) was prepared by dissolved 42gof ammonium molybdate with 100 ml of in (1N) H_2SO_4

(b) Working solution

Working solution was prepared by mixed 1 volume of ascorbic acid solution (10%) and 6 volumes of ammonium molybdate (0.42%)

2. Elements standard curves

2.1 Standard ammonium solution (Wainwright and Pugh, 1973)

3.66 g of $(NH_4)_2SO_4$ and were dissolved it in litre then diluted the solution 10 times (10 ml of ammonium solution with 90ml distal water) = 100µg/NH_4^+-N $ml^{-1.}$ 2ml of previous solution was added to 1ml of EDTA (6%w/v) 7ml of distilled water, 5ml of phenolate reagent and 3ml of sodium hypochlorite solution (10%v/v). The reaction mixture was mixed thoroughly and incubated at 25^oC for 20min in the dark. The volume

was made up to 50ml and mixed and the concentration of the indophenols-blue complex was measured at 630 nm.

2.2 Standard Nitrate solution (Sims and Jackson, 1971)

Weight 0.137g of NaN_{O3} and were dissolved to 100ml of distilled water. Resulting solution is 1mg nitrate ($NaNO_3$) per ml. Solution was pipette to 6 tubes following volumes of the standard solution: (1µl=1µg nitrate) (0µl, 10µl, 20µl, 40µl, 80µl, 100µl). 3ml of filtrate was mixed to 7ml chromotropic acid (work solution)then incubated in water bath for 45 min in 40^{o}C. The yellow colour formed was measured at 41 nm using spectrophotometer and the concentration of nitrate was determined by reference to a standard curve of nitrate concentration.(Fig 1 2)

2.3 Standard sulphate-s solution (Hesse, 1971)

1.47 g of (Na_2SO_4) and were dissolved in 1 litre of distilled water, which give the concentration **1000µg/ SO_4^{-2}-S ml^{-1}** then diluted the solution 10 times (10 ml of sodium sulphate solution with 90 ml of distal water) =**100µ g/ SO_4^{-2}-S ml^{-1}.**

5ml of previous solution were added to 1g barium chloride $BaCl_2$ and 2ml of gum acacia (0.25%w/v) mixed well then the volume was made up to 25ml with distilled water. The white suspension resulting from precipitation of sulphate was measured at 470 nm. (Fig 13)

2.4 Standard phosphate solution (Hesse, 1971)

Weight 0.4393g of potassium dihydrogen (KH_2PO_4) into a litre volumetric flask. This solution was diluted with distilled water to produce 10µg , 20 µg, 40 µg, 50 µg, 60 µg, 70 µg, 80 µg, 90 µg and 100 µg. The control was distilled water without KH_2PO_4.

Mix 1 vol. of ascorbic acid mixed with 6 vol. of ammonium molybdate. The add 0.7 ml of working solution to 0.3 ml of sample and incubate at 37oC for 1 hour and read with blue colour at 820 nm. (Fig 1 4)

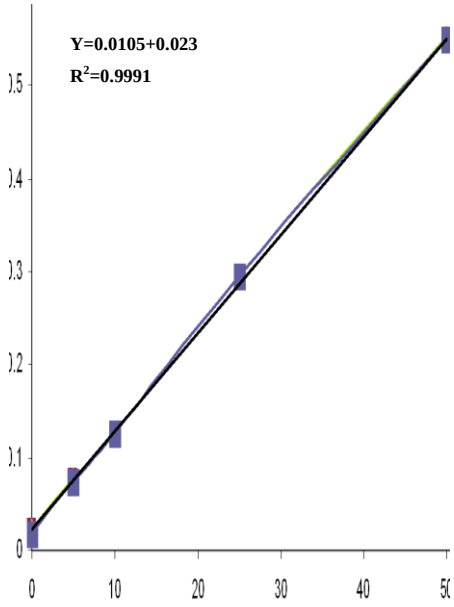


Fig1. 1Ammonium standard curve

Fig1 2: Nitrate standard curve

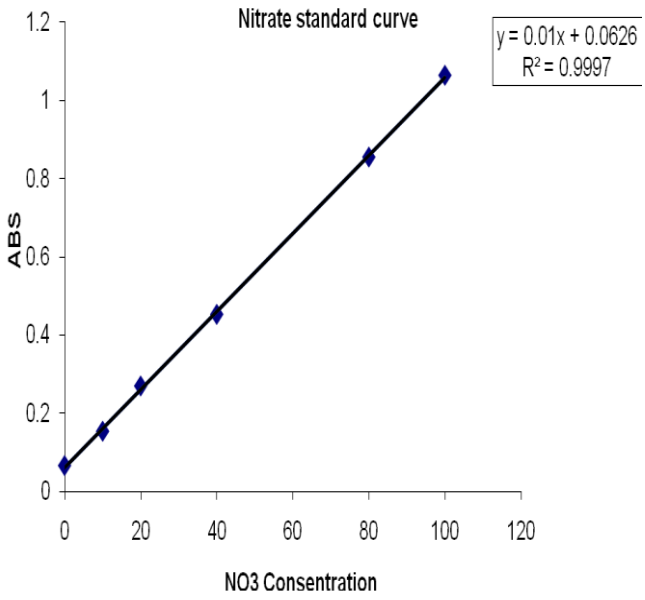


Fig1 2: Nitrate standard curve

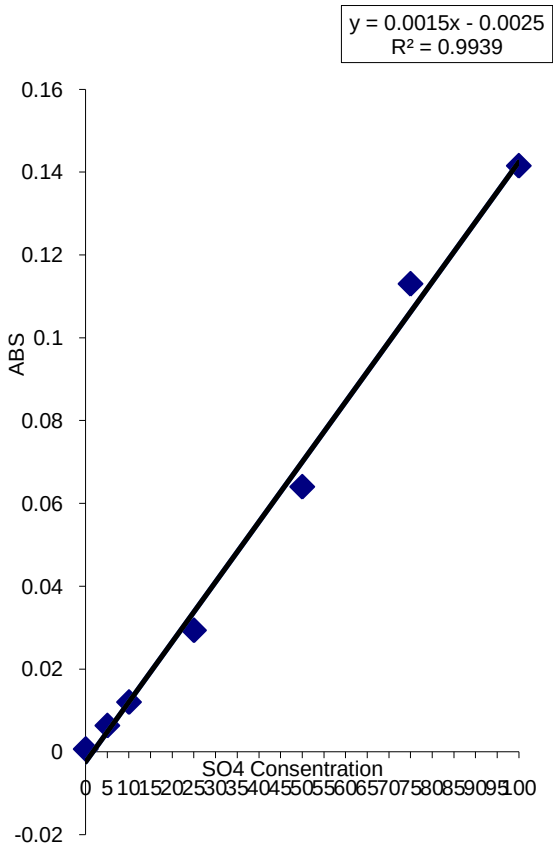


Fig 1 3: Sulphate standard curve

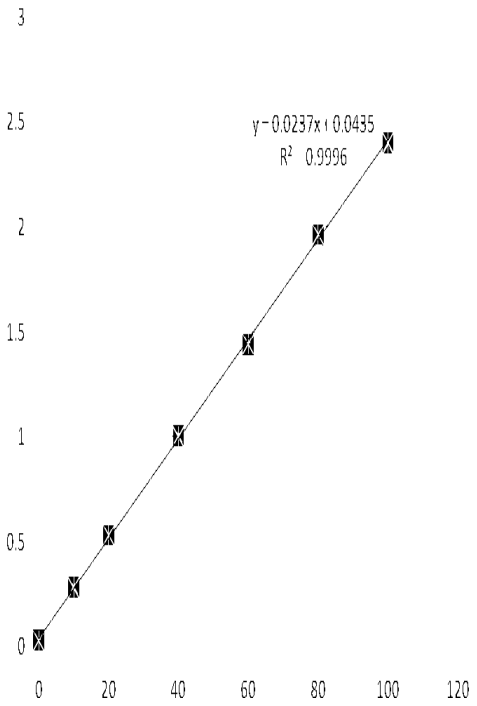


Fig 1 4: Phosphate standard curve

Appendix Two: Identification of bacteria

1. Solid media

1.1 BRILLIANCEUTI CLARITY AGAR

Brillianceuti Clarity Agar (formerly Chromogenic UTI Clarity Agar) is for differentiation and presumptive identification of common urinary tract infection isolates.

Table 2 1 Composition of Brilliant clarity agar medium

Typical Formula*……	Gm/ litre
Peptone……..	**9.00**
Chromogenic Mix…….	**17.00**
Tryptophan…….	**1.00**
Agar……	**10.00**

pH 7.0 ± 0.2 @ 25ºC

Interpretation

The expected colour reactions are laid out in Table 2 2

Table 2 2 Typical colour reactions on *Brilliance*UTI Clarity Agar

Organism	β-galactosidase	β-glucosidase	TDA	Colony colour
E. coli	+			Pink
enterococci		+		Blue / Turquoise
coliforms	+	+		Dark Blue / Purple
Proteus/Morganella			+	Brown halo
pseudomonads				Green/ Brown
staphylococci				White / Cream
S. saprophyticus				Pale Pink / White
streptococci				White

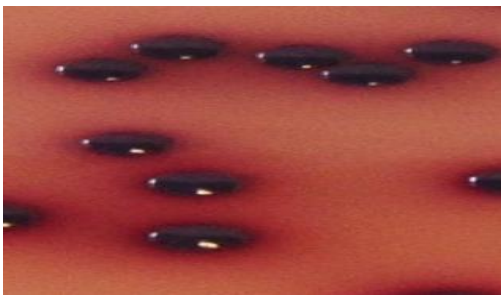
. **Appearance**

Dehydrated *Brilliance*UTI Clarity Agar is a free-flowing straw coloured powder. The prepared medium is a straw-coloured, transparent agar.

Table 2 1 Quality control

Positive controls	Expected results
Escherichia coli ATCC®25922*	Good growth; pink colonies
Enterobacter aerogenes ATCC®13048*	Good growth; purple colonies
Enterococ	
Proteus mirabilis NCTC10975 or *Proteus mirabilis* ATCC®29906*	Good growth; brown colony and halo
Staphylococcus aureus ATCC®25923*	Good growth; typical appearance
Negative control	
Uninoculated medium	

Fig 2 1: *E. coli* on BrillIant agar / Author's photo



***E. coli* on** Brillianceuti Clarity Agar by (Authors)

1.2 CHROMagar Orientation

The principle of this medium is the use of Chromogenic substrates revealing metabolic enzymes. Dehydrated powder was provided by the CHROMagar Company, Paris, France.

The medium is composed of 169 each of peptone, meat, and yeast extracts and 15g of agar per Litre and a special chromogenic mixture. The powder was introduced into an Automatic pippeter, and sterilization process was performed at 120^{o}C for 15 min. Samples were Streaked onto plate and incubate at 37°C for 18-24 hours.

Table 2 4: Composition of Chromagar Orientation medium

Typical Formula……	gm/litre
Agar……	**15.0 g**
Peptone and yeast extract……	**17.0**
Chromogenic mix……	**1.0 g**
Total……	**33 g/L**

pH 7.0 ± 0.2 @ 25°C

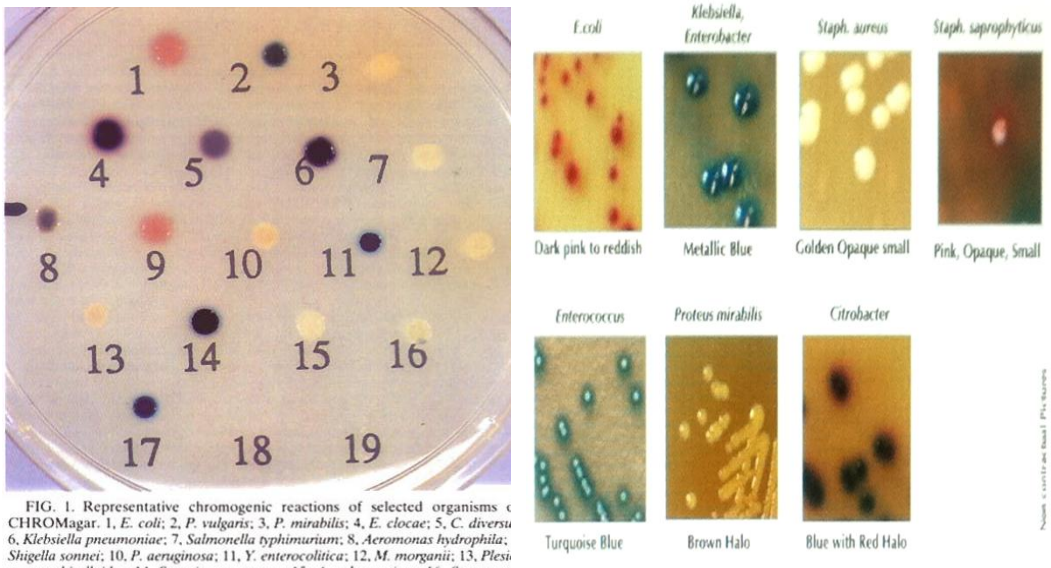


FIG. 1. Representative chromogenic reactions of selected organisms c CHROMagar. 1, *E. coli*; 2, *P. vulgaris*; 3, *P. mirabilis*; 4, *E. clocae*; 5, *C. diversu* 6, *Klebsiella pneumoniae*; 7, *Salmonella typhimurium*; 8, *Aeromonas hydrophila*; *Shigella sonnei*; 10, *P. aeruginosa*; 11, *Y. enterocolitica*; 12, *M. morganii*; 13, *Plesi monas shigelloides*; 14, *Serratia marcescens*; 15, *A. calcoaceticus*; 16, *Stenotrop omonas maltophilia*; 17, *Enterococcus faecalis*.

(Merlino *et al*., 1996)

Fig, 2 2: The colour of colonies of CHROMagar Orientation media reference

1.3 CHROMagar MRSA

BBL CHROMagar MRSA is a selective and differential medium, which incorporates cefoxitin, for the detection of MRSA from specimens.

For pre-weighed dose of medium of CHROMagaTMRSA powder, add dry powder to the corresponding volume of purified water. Alternatively, suspend the powder slowly in water by rotating for swelling of the agar. Heat and bring to boiling (100"C) while swirling or stirring regularly. If using an autoclave, do so without pressure. Do not heat to more than100^{o}C. the mixture may also be brought to a boil in a microwave oven: after initial boiling, remove from oven, stir gently, then return to oven for short repeated155

Table 2 5: Composition of Chromagar MRSA

Typical Formula…….	gm/litre
Chromopeptone ……	**40.0 g**
Sodium Chloride ……...	**25.0 g**
Chromogenic mix……	**0.5 g**
Inhibitory Agents ……	**0.07 g**
Cefoxitin…… …….	**6.0mg**
Agar…… …….	**14.0 g**

pH 7.0 ± 0.2 @ 25°C

Table 2 6: **Quality control**

Positive control:	**Expected result**
Staphylococcus aureus ATCC® 29213	Inhibition (partial to complete); N/A
**Staphylococcus aureus* ATCC®25923	Inhibition (partial to complete); N/A
**Staphylococcus aureus* ATCC®43300	Growth; Mauve
Staphylococcus aureus ATCC ®3359	Growth; Mauve
Enterococcus faecalis ATCC ®29212	Growth; blue

(Garner, 1996 ; Clinical and Laboratory Standards Institute. 2008)

1.4 **Czapek Dox agar**

Code: CM0097 / **Oxoid**

Semi-synthetic solid medium, containing sucrose as C-source and nitrate as the sole source of nitrogen, useful for the general cultivation of fungi, yeasts and soil bacteria. Recommended by Czapek (1902-1903) and Dox (1910). Table 2 1 1

Table 2.7: Composition of Czapek Dox agar medium

Typical Formula	gm/litre
Na NO…….	**2.00 g**
KCI……..	**0.50 g**
Magnesium glycerophosphate……	**0.50 g**
Fe SO_4 ($7H_2O$)……	**0..01 g**
K2 SO_4……...	**0.35 g**
Sucrose……	**30.0 g**
Agar (Oxoid No. 3)…….....	**12.0 g**

pH 6.8 ± 0.2 @ 25°C

Directions

A proprietary formulation (Oxoid) of Czapek Dox Agar was used for fungal growth. It was prepared by suspending 45.4 g of the powder in a litre of distilled water. The medium Was dissolved, and the pH adjusted to 6.8, and sterilised by autoclaving at l2l^{o} C for 15 min.

Appearance

Dehydrated medium: White coloured, free-flowing powder

Prepared medium: Off-white coloured gel

Table 2 8: Cultural characteristics after 24-48 hours at 35°C.

Organisms (ATCC)	Growth
Aspergillus braseliensis (16404)	+++
Saccharomyces cerevisiae (9763)	+++
Candida albicans (10231)	++
Bacillus subtilis (6633)	++
Staphylococcus aureus (25923)	-

1.5 HiCrome (TM) E. coli Agar A

Code: 70722/ Fluka/ Sigma

HiCrome *E.coli* Agar B is recommended for the detection and enumeration of Escherichia coli in foods without further confirmation on membrane filter or by indole reagent.

Table 2 9: Composition of HiCrome (TM) E. coli Agar A

Formula	**gm/litre**
……	

Casein enzymic hydrolysate……..	**14.0 g**
Peptone especial……	**5.0 g**
Bile salts mixture…….	**1.50 g**
Disodium hydrogen……...	**1.0 g**
Sodium dihydrogen phosphate……..	**0.6 g**
Sodium chloride……	**2.4 g**
Glucuronide……...	**0.075 g**
Agar ……	**12.0 g**

pH 7.2 ± 0.2 @ 25°C

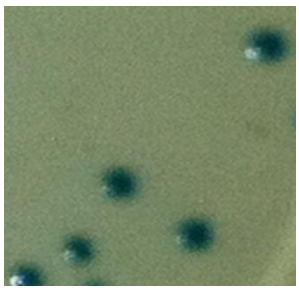
Directions :

Suspend 36,6g in 1 litre distilled water. Sterilize by autoclaving at 121°C for 15 minutes. Cool to 50°C and pour into sterile petri plates

Table 2 10: Culture characterisation after 18-24 hours at 44°C

Positive control:	Expected results
Escherichia coli ATCC® 25922 *	Luxuriant; blue
Klebsiella pneumonia ATCC® 13883	luxuriant colourless, mucoid
Salmonella enteritidis ATCC® 13076	luxuriant colourless
Staphylococcus aureus ATCC® **25923**	inhibited

Anderson and Baird-Parker., 1975; Hansen and Yourassawsky



E. coli

Fig 2 3: *E. coli* on **HiCrome** agar / Author's photo

1.6 Nutrient Agar (Oxoid)

Code: CM0003 (Powder)

A general purpose medium which may be enriched with up to 10% blood or other biological fluid.

Table 2 11: Composition of Nutrient Agar

Typical Formula…….	gm/litre
'Lab-Lemco' powder (Oxoid)……..	**1.0 g**
Yeast extract……....	**2.0 e**
Peptone……	**5.0 g**
Sodium chloride……..	**5.0 g**
Agar…….	**15.0 g**

pH 7.4 ± 0.2 @ 25°C

Directions

The medium was prepared by suspending 28g in litre of distilled water, boiled dissolve completely. Sterilised by autoclaving at l2l^{o}C for 15 minutes.

Table 2 2 12: Quality control

Positive controls:	Expected results
Staphylococcus aureus ATCC® 25923 *	Good growth; straw/white colonies
Escherichia coli ATCC® 25922 *	Good growth; straw colonies
Negative control:	
Uninoculated medium	No change

(Lapage *et al*. 1970)

1.7 Plate Count Agar

The medium was prepared by suspending 17.5g in litre of distilled water, boiled to dissolve completely, pH 7.0, sterilised by autoclaving at 121^{o}C for 15 minutes' Silica gel medium (Parkinson et a1.,1989)

Table 2 13: Composition Plate Count Agar (Oxoid)

Typical Formula…….	gm/litre
Tryptone……..	**1.0 g**
Yeast extract……....	**2.5 e**
Glucose……	**1.0 g**
Agar…….	**9.0 g**

pH 7.0 ± 0.2 @ 25°C

Directions

Add 17.5g to 1 litre of distilled water. Dissolve by bringing to the boil with frequent stirring, mix and distribute into final containers. Sterilise by autoclaving at 121°C for 15 minutes.

Appearance

Dehydrated medium: Straw coloured, free-flowing powder
Prepared medium: Straw coloured gel

Table 2 14: Quality control

Positive control:	Expected results
Escherichia coli ATCC® 25922 *	Good growth; straw coloured colonies
Negative control:	
Uninoculated plate	No change

(PHLS, 1999)

1.8 POTATO DEXTROSE AGAR (EP/USP/JP/BP)

Code: CM0139 (Oxoid)

For the detection and enumeration of yeasts and moulds in butter and other dairy and food products. Also for the preparation of *Aspergillus niger* for the Harmonised Microbial Limit Tests from EP/USP/JP (enumeration test)

Table 2 15: Composition of PAD medium

Formula	gm/litre
Potato tract……..	**4.0***
Glucose ……...	**20.0**
Agar ……	**15.0**

pH 5.6 ± 0.2 @ 25°C

Directions

Suspend 39g in 1 litre of water (purified as requested). Bring to the boil to dissolve completely. Sterilise by autoclaving at 121°C for 15 minutes. Mix well before pouring.

Appearance

Dehydrated medium: Off-white, free-flowing powder

Prepared medium: Light straw coloured gel

Table 2 16: Quality Control

Positive control:	**Expected result**
Aspergillus fumigatus ATCC® 9197 *****	White mycelium, blue green spores
Negative controls:	
Uninoculated medium	No change
At pH 3.5 *Bacillus subtilis* ATCC® 6633 *	No growth

American Public Health Association. (1992)

1.10 XLT-4 AGAR

Code: CM1061 from / **Oxoid**

A highly selective medium for isolation and identification of Salmonellae from clinical, environmental and food samples.

Table 17; Composition of XLT-4 Agar

Typical Formula*	gm/litre
Proteose Peptone …….	**1.6**
Yeast extract…….	**3.0**
Lysine…….	**5.0**
Xylose……	**3.75**
Lactose……..	**7.5**
Sucrose……...	**7.5**
Ferric ammonium citrate……	**0.8**
Sodium thiosulphate……..	**6.8**
Sodium chloride…….	**5.0**
Phenol Red…….	**0.08**
Agar……	**18.0**

pH 7.4 ± 0.2 @ 25°C

Directions

Suspend 59g of XLT-4 Agar Base in 1 litre of distilled water, add 4.6ml of XLT-4 Selective Supplement and bring the medium to the boil. **Do not overheat, do not autoclave.** Cool to approximately 50°C and pour into sterile Petri dishes. It is not advised to hold the medium at 50°C for longer than 1 hour as this may cause the medium to precipitate.

Description

XLT-4 (Xylose Lactose Tergitol™ 4) Agar is a highly selective plating medium used for isolation and identification of salmonellae from clinical, environmental and food samples according to Miller

b) XLT-4 SELECTIVE SUPPLEMENT

Code: SR0237/ Oxoid

Table 2 18: Composition of XLT-4 selective supplement

Supplement available in 100 ml (SR0237C)	**per litre**
Tergitol™ 4…….	**4.6ml**

Appearance:

Dehydrated medium: straw coloured, free-flowing powder
Prepared medium: clear red gel

Table 9: Quality control

Positive control:	Expected results
Salmonella enteritidis ATCC®13076 *	Good growth: black or, red with black centre
Negative controls:	
Escherichia coli ATCC®25922 *	Reduced growth, yellow
Enterococcus feacalis ATCC®29212 *	

(Miller and Tate, 1990; Dusch and Altwegg, 1995)

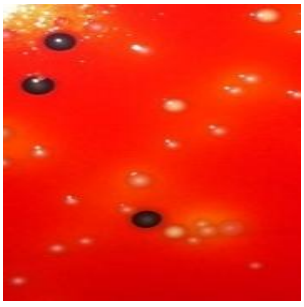


Fig 2 4: *S. enterica* on XLT-4 agar / Author's photo

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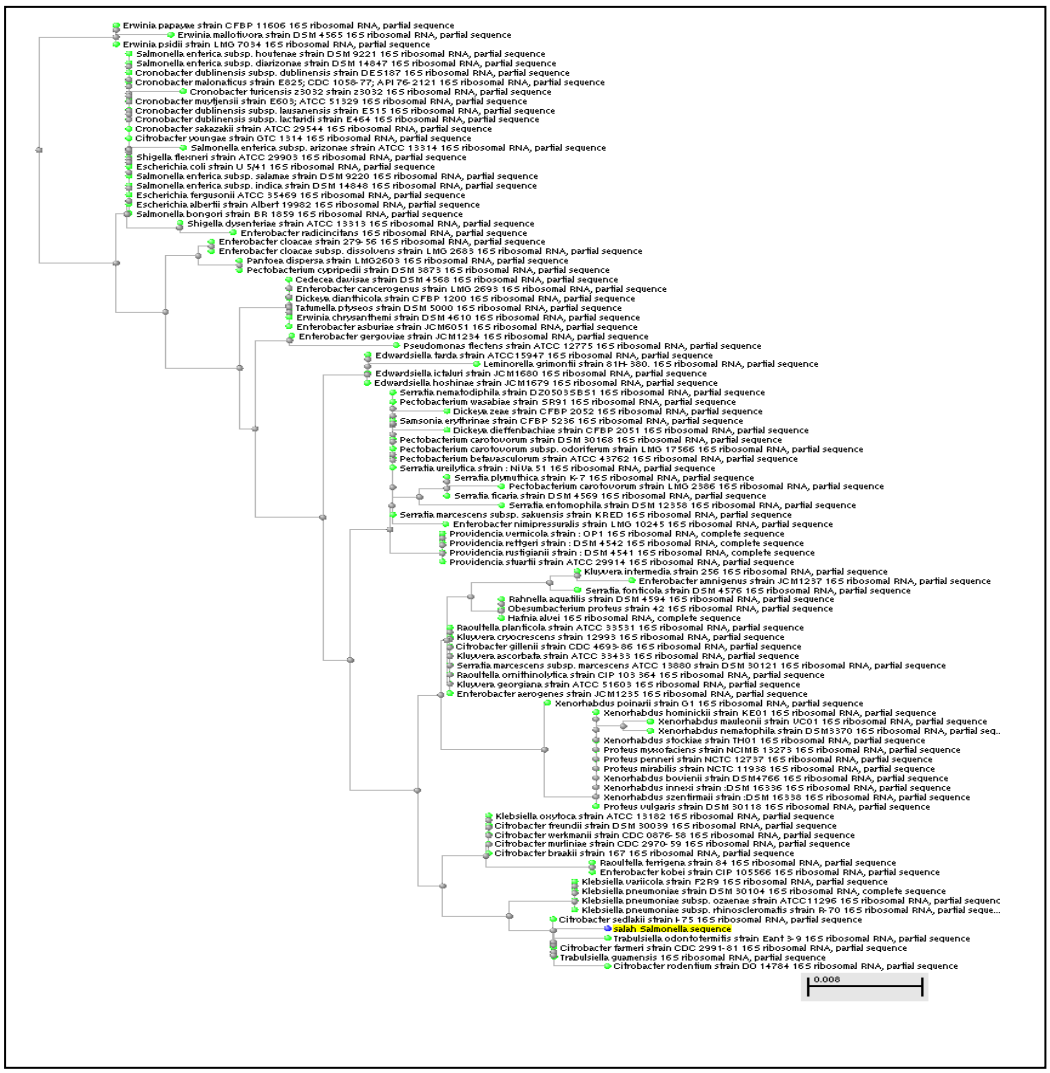

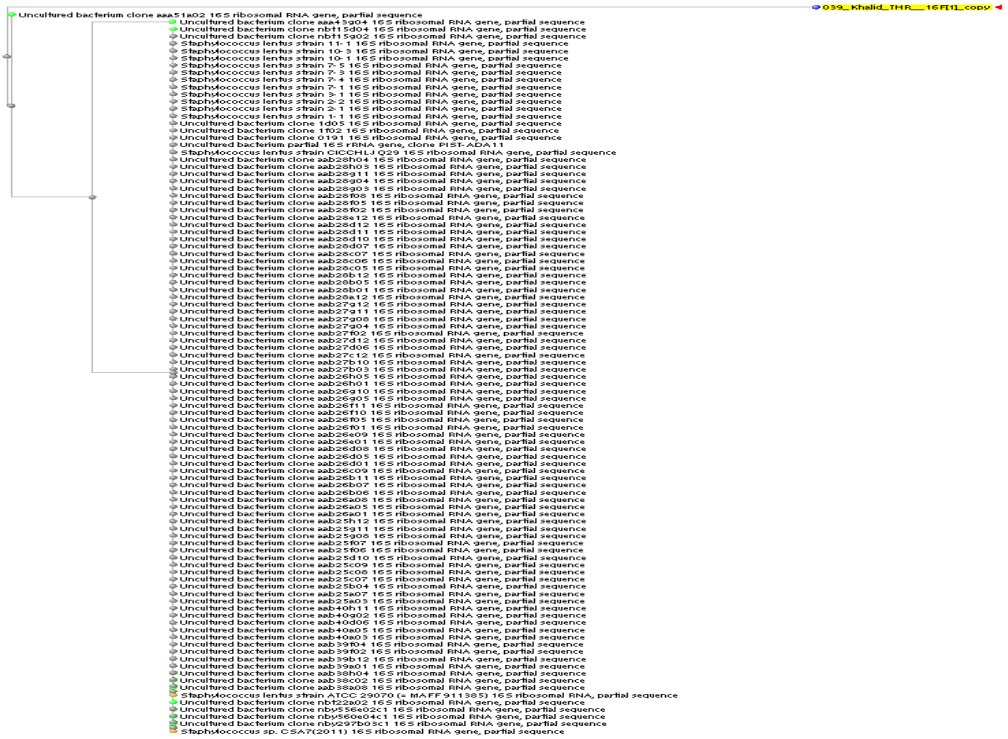
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Uncultured bacterium clone 0191 16S ribosomal RNA gene, partial sequence
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0.0008

L 1 2

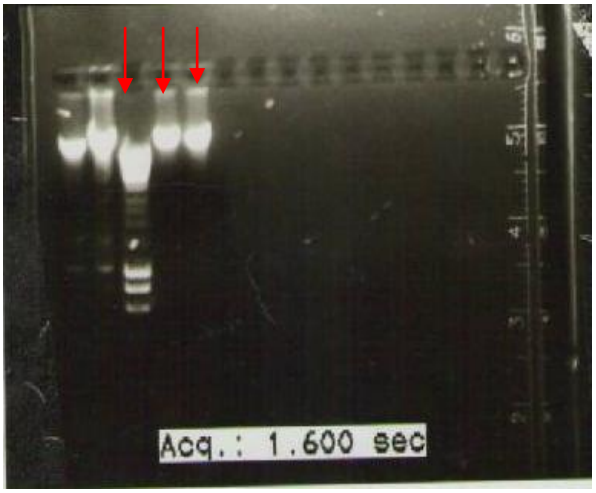

Fig 2 5: g DNA of isolated bacterial species; (Lane 1) *Salmonell*a and (Lane 2) *E. coli*, (lane L); hyper ladder

L 1 2 3

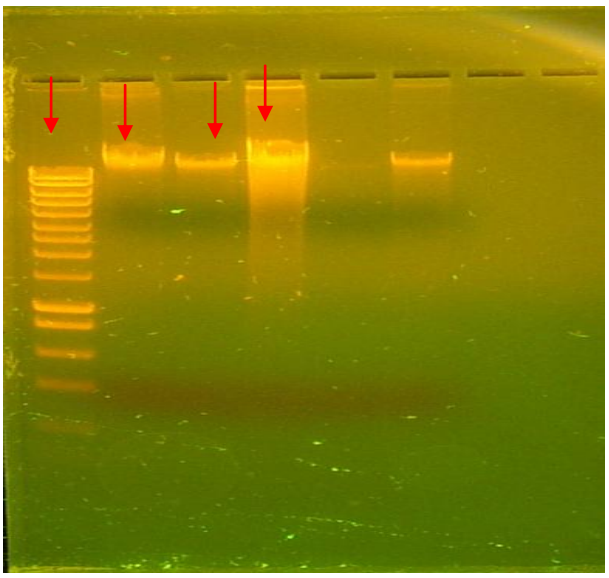

Fig 2 6: PCR- 16Sr RNA, amplification products of *Salmonella enteric* species analyzed by electrophoresis in agarose gel lanes represent; (lane L); hyper ladder;(lane lane 1); *S. enterica* R1, (lane 2); *S. enerica* R2 and (lane 3) positive control.

L 1 2

Fig 2 7: PCR- 16Sr RNA, amplification products of *Salmonella enteric* species analyzed by electrophoresis in agarose gel lanes represent; (lane L); hyper ladder;(lane lane 1); *S. enterica* R1, (lane 2); *S. enerica* R2 negative result l.

L 1

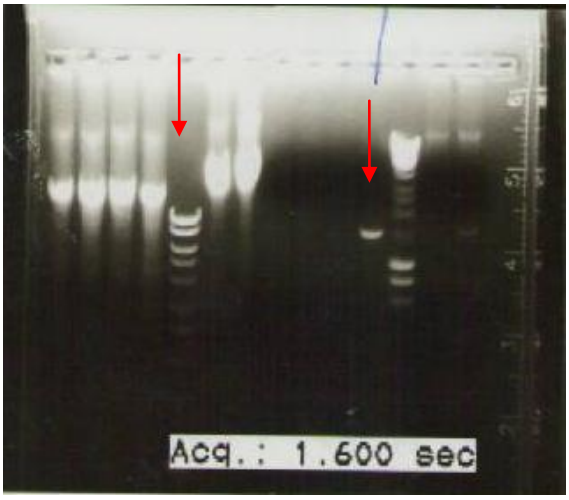

Fig 2 8: PCR- 16Sr RNA, amplification products of *E. coli* analyzed by electrophoresis in agarose gel lanes represent; (lane L); hyper ladder;(lane lane 1); *E.coli* .

Appendix Three: ANOVA tables

Three Way Analysis of Variance Saturday, March 17, 2012, 14:06:28

Data source: E. coli ANOVA of BSFL in BSFL AONVA E. coli

Balanced Design (No Interactions)

Dependent Variable: Y

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
T	1	1.974E+015	1.974E+015	431.898	<0.001
R	4	1.914E+016	4.784E+015	1046.919	<0.001
C	2	2.713E+013	1.357E+013	2.969	0.109
Residual	8	3.656E+013	4.570E+012		
Total	29	2.724E+016	9.394E+014		

The difference in the mean values among the different levels of T are greater than would be expected by chance after allowing for the effects of differences in R and C. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of R are greater than would be expected by chance after allowing for the effects of differences in T and C. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of C are not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in T and R. There is not a statistically significant difference (P = 0.109).

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **T**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	16221816.000	20.782	<0.001	0.050	Yes

Comparisons for factor: **R**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
1.000 vs. 5.000	63319106.667	51.305	<0.001	0.005	Yes

1.000 vs. 4.000	63293650.000	51.284	<0.001	0.006	Yes
1.000 vs. 3.000	59012683.333	47.815	<0.001	0.006	Yes
2.000 vs. 5.000	36459106.667	29.541	<0.001	0.007	Yes
2.000 vs. 4.000	36433650.000	29.521	<0.001	0.009	Yes
2.000 vs. 3.000	32152683.333	26.052	<0.001	0.010	Yes
1.000 vs. 2.000	26860000.000	21.763	<0.001	0.013	Yes
3.000 vs. 5.000	4306423.333	3.489	0.008	0.017	Yes
3.000 vs. 4.000	4280966.667	3.469	0.008	0.025	Yes
4.000 vs. 5.000	25456.667	0.0206	0.984	0.050	No

Comparisons for factor: **C**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
1.000 vs. 3.000	2284562.000	2.390	0.044	0.017	No
2.000 vs. 3.000	1536809.000	1.608	0.147	0.025	No
1.000 vs. 2.000	747753.000	0.782	0.457	0.050	No

Power of performed test with alpha = 0.0500: for T : 1.000
Power of performed test with alpha = 0.0500: for R : 1.000
Power of performed test with alpha = 0.0500: for C : 0.288

Least square means for T :

Group	**Mean**
1.000	12725337.333
2.000	28947153.333

Std Err of LS Mean = 551942.360

Least square means for R :

Group	**Mean**
1.000	63333333.333
2.000	36473333.333
3.000	4320650.000
4.000	39683.333
5.000	14226.667

Std Err of LS Mean = 872697.497

Least square means for C :

Group	**Mean**
1.000	21847017.000
2.000	21099264.000
3.000	19562455.000

Std Err of LS Mean = 675988.575

Three Way Analysis of Variance Saturday, March 17, 2012, 14:54:58

Data source: ANOVA OF BSFL Salmonella in BSFL ANOVA Salmonella

Balanced Design (No Interactions)

Dependent Variable: Y

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
T	1	2.293E+013	2.293E+013	955.133	<0.001
R	4	3.360E+014	8.400E+013	3499.420	<0.001
C	2	62712566000.000	31356283000.0		
Residual	8	192037221999.938	24004652749.992		
Total	29	4.441E+014	1.531E+013		

The difference in the mean values among the different levels of T are greater than would be expected by chance after allowing for the effects of differences in R and C. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of R are greater than would be expected by chance after allowing for the effects of differences in T and C. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of C are not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in T and R. There is not a statistically significant difference (P = 0.323).

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **T**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	1748433.333	30.905	<0.001	0.050	Yes

Comparisons for factor: **R**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
1.000 vs. 5.000	8397250.000	93.875	<0.001	0.005	Yes
1.000 vs. 4.000	8379783.333	93.680	<0.001	0.006	Yes

1.000 vs. 3.000	8256250.000	92.299	<0.001	0.006	Yes
2.000 vs. 5.000	4309083.333	48.172	<0.001	0.007	Yes
2.000 vs. 4.000	4291616.667	47.977	<0.001	0.009	Yes
2.000 vs. 3.000	4168083.333	46.596	<0.001	0.010	Yes
1.000 vs. 2.000	4088166.667	45.703	<0.001	0.013	Yes
3.000 vs. 5.000	141000.000	1.576	0.154	0.017	No
3.000 vs. 4.000	123533.333	1.381	0.205	0.025	No
4.000 vs. 5.000	17466.667	0.195	0.850	0.050	No

Comparisons for factor: **C**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
1.000 vs. 3.000	111920.000	1.615	0.145	0.017	No
1.000 vs. 2.000	59470.000	0.858	0.416	0.025	No
2.000 vs. 3.000	52450.000	0.757	0.471	0.050	No

Power of performed test with alpha = 0.0500: for T : 1.000
Power of performed test with alpha = 0.0500: for R : 1.000
Power of performed test with alpha = 0.0500: for C : 0.0826

Least square means for T :

Group	**Mean**
1.000	1701493.333
2.000	3449926.667

Std Err of LS Mean = 40003.877

Least square means for R :

Group	**Mean**
1.000	8400000.000
2.000	4311833.333
3.000	143750.000
4.000	20216.667
5.000	2750.000

Std Err of LS Mean = 63251.683

Least square means for C :

Group	**Mean**
1.000	2632840.000
2.000	2573370.000
3.000	2520920.000

Std Err of LS Mean = 48994.543

Three Way Analysis of Variance Saturday, March 17, 2012, 13:00:40

Data source: BSFL AONVA Plate count media in Plate count media ANOVA

Balanced Design (No Interactions)

Dependent Variable: Y

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
T	1	1.581E+017	1.581E+017	102.181	<0.001
R	4	7.626E+017	1.906E+017	123.201	<0.001
C	2	7.304E+015	3.652E+015	2.360	0.156
Residual	8	1.238E+016	1.547E+015		
Total	29	1.560E+018	5.378E+016		

The difference in the mean values among the different levels of T are greater than would be expected by chance after allowing for the effects of differences in R and C. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of R are greater than would be expected by chance after allowing for the effects of differences in T and C. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of C are not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in T and R. There is not a statistically significant difference (P = 0.156).

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **T**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	145196940.000	10.108	<0.001	0.050	Yes

Comparisons for factor: **R**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 5.000	363737183.333	16.016	<0.001	0.005	Yes
2.000 vs. 4.000	361496500.000	15.917	<0.001	0.006	Yes

2.000 vs. 3.000	359237333.333	15.818	<0.001	0.006	Yes
1.000 vs. 5.000	283270516.667	12.473	<0.001	0.007	Yes
1.000 vs. 4.000	281029833.333	12.374	<0.001	0.009	Yes
1.000 vs. 3.000	278770666.667	12.275	<0.001	0.010	Yes
2.000 vs. 1.000	80466666.667	3.543	0.008	0.013	Yes
3.000 vs. 5.000	4499850.000	0.198	0.848	0.017	No
3.000 vs. 4.000	2259166.667	0.0995	0.923	0.025	No
4.000 vs. 5.000	2240683.333	0.0987	0.924	0.050	No

Comparisons for factor: **C**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
1.000 vs. 2.000	37619750.000	2.138	0.065	0.017	No
1.000 vs. 3.000	24658710.000	1.402	0.199	0.025	No
3.000 vs. 2.000	12961040.000	0.737	0.482	0.050	No

Power of performed test with alpha = 0.0500: for T : 1.000
Power of performed test with alpha = 0.0500: for R : 1.000
Power of performed test with alpha = 0.0500: for C : 0.209

Least square means for T :

Group	**Mean**
1.000	58213993.333
2.000	203410933.333

Std Err of LS Mean = 10156812.630

Least square means for R :

Group	**Mean**
1.000	283333333.333
2.000	363800000.000
3.000	4562666.667
4.000	2303500.000
5.000	62816.667

Std Err of LS Mean = 16059330.839

Least square means for C :

Group	**Mean**
1.000	151571950.000
2.000	113952200.000
3.000	126913240.000

Std Err of LS Mean = 12439504.178

Three Way Analysis of Variance Saturday, March 17, 2012, 12:27:59

Data source: E. coli ANOVA in FBL E. coli ANOVA

Balanced Design (No Interactions)

Dependent Variable: Y

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
R	1	8.554E+015	8.554E+015	455.393	<0.001
T	4	3.470E+016	8.676E+015	461.905	<0.001
C	2	4.231E+013	2.115E+013	1.126	0.371
Residual	8	1.503E+014	1.878E+013		
Total	29	7.776E+016	2.681E+015		

The difference in the mean values among the different levels of R are greater than would be expected by chance after allowing for the effects of differences in T and C. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of T are greater than would be expected by chance after allowing for the effects of differences in R and C. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of C are not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in R and T. There is not a statistically significant difference (P = 0.371).

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **R**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	33771066.667	21.340	<0.001	0.050	Yes

Comparisons for factor: **T**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 5.000	88992000.000	35.566	<0.001	0.005	Yes
2.000 vs. 4.000	88841000.000	35.505	<0.001	0.006	Yes

2.000 vs. 3.000	84650000.000	33.830	<0.001	0.006	Yes
2.000 vs. 1.000	71783333.333	28.688	<0.001	0.007	Yes
1.000 vs. 5.000	17208666.667	6.877	<0.001	0.009	Yes
1.000 vs. 4.000	17057666.667	6.817	<0.001	0.010	Yes
1.000 vs. 3.000	12866666.667	5.142	<0.001	0.013	Yes
3.000 vs. 5.000	4342000.000	1.735	0.121	0.017	No
3.000 vs. 4.000	4191000.000	1.675	0.132	0.025	No
4.000 vs. 5.000	151000.000	0.0603	0.953	0.050	No

Comparisons for factor: **C**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
1.000 vs. 3.000	2812600.000	1.451	0.185	0.017	No
1.000 vs. 2.000	2049200.000	1.057	0.321	0.025	No
2.000 vs. 3.000	763400.000	0.394	0.704	0.050	No

Power of performed test with alpha = 0.0500: for R : 1.000
Power of performed test with alpha = 0.0500: for T : 1.000
Power of performed test with alpha = 0.0500: for C : 0.0630

Least square means for R :

Group	**Mean**
1.000	5377866.667
2.000	39148933.333

Std Err of LS Mean = 1119016.803

Least square means for T :

Group	**Mean**
1.000	17333333.333
2.000	89116666.667
3.000	4466666.667
4.000	275666.667
5.000	124666.667

Std Err of LS Mean = 1769320.918

Least square means for C :

Group	**Mean**
1.000	23884000.000
2.000	21834800.000
3.000	21071400.000

Std Err of LS Mean = 1370510.090

Three Way Analysis of Variance Tuesday, January 17, 2012, 21:49:21

Data source: FBL Salmonella ANOVA in FBL Salmonella ANOVA

Balanced Design (No Interactions)

Dependent Variable: Y

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
R	1	2.473E+017	2.473E+017	237.919	<0.001
T	4	7.879E+017	1.970E+017	189.502	<0.001
C	2	2.672E+015	1.336E+015	1.285	0.328
Residual	8	8.315E+015	1.039E+015		
Total	29	1.872E+018	6.454E+016		

The difference in the mean values among the different levels of R are greater than would be expected by chance after allowing for the effects of differences in T and C. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of T are greater than would be expected by chance after allowing for the effects of differences in R and C. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of C are not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in R and T. There is not a statistically significant difference (P = 0.328).

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **R**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	181583786.667	15.425	<0.001	0.050	Yes

Comparisons for factor: **T**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 5.000	420681950.000	22.601	<0.001	0.005	Yes
2.000 vs. 4.000	420623483.333	22.598	<0.001	0.006	Yes

2.000 vs. 1.000	386316666.667	20.754	<0.001	0.006	Yes
2.000 vs. 3.000	385480000.000	20.709	<0.001	0.007	Yes
3.000 vs. 5.000	35201950.000	1.891	0.095	0.009	No
3.000 vs. 4.000	35143483.333	1.888	0.096	0.010	No
1.000 vs. 5.000	34365283.333	1.846	0.102	0.013	No
1.000 vs. 4.000	34306816.667	1.843	0.103	0.017	No
3.000 vs. 1.000	836666.667	0.0449	0.965	0.025	No
4.000 vs. 5.000	58466.667	0.00314	0.998	0.050	No

Comparisons for factor: **C**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
1.000 vs. 3.000	20346370.000	1.411	0.196	0.017	No
2.000 vs. 3.000	19675210.000	1.365	0.210	0.025	No
1.000 vs. 2.000	671160.000	0.0465	0.964	0.050	No

Power of performed test with alpha = 0.0500: for R : 1.000
Power of performed test with alpha = 0.0500: for T : 1.000
Power of performed test with alpha = 0.0500: for C : 0.0802

Least square means for R :

Group	**Mean**
1.000	7304353.333
2.000	188888140.000

Std Err of LS Mean = 8324291.397

Least square means for T :

Group	**Mean**
1.000	34400000.000
2.000	420716666.667
3.000	35236666.667
4.000	93183.333
5.000	34716.667

Std Err of LS Mean = 13161860.360

Least square means for C :

Group	**Mean**
1.000	105102090.000
2.000	104430930.000
3.000	84755720.000

Std Err of LS Mean = 10195133.196

Three Way Analysis of Variance Saturday, March 17, 2012, 20:02:03

Data source: FBL Plate count media ANOVA in FBL Plate count media ANOVA

Balanced Design (No Interactions)

Dependent Variable: Y

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
R	1	2.338E+017	2.338E+017	1114.570	<0.001
T	4	8.676E+017	2.169E+017	1034.149	<0.001
C	2	1.363E+014	6.814E+013	0.325	0.732
Residual	8	1.678E+015	2.097E+014		
Total	29	1.815E+018	6.259E+016		

The difference in the mean values among the different levels of R are greater than would be expected by chance after allowing for the effects of differences in T and C. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of T are greater than would be expected by chance after allowing for the effects of differences in R and C. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of C are not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in R and T. There is not a statistically significant difference (P = 0.732).

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **R**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	176550300.000	33.385	<0.001	0.050	Yes

Comparisons for factor: **T**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 5.000	452886583.333	54.163	<0.001	0.005	Yes
2.000 vs. 4.000	452667666.667	54.137	<0.001	0.006	Yes

2.000 vs. 3.000	403100000.000	48.209	<0.001	0.006	Yes
2.000 vs. 1.000	309666666.667	37.035	<0.001	0.007	Yes
1.000 vs. 5.000	143219916.667	17.128	<0.001	0.009	Yes
1.000 vs. 4.000	143001000.000	17.102	<0.001	0.010	Yes
1.000 vs. 3.000	93433333.333	11.174	<0.001	0.013	Yes
3.000 vs. 5.000	49786583.333	5.954	<0.001	0.017	Yes
3.000 vs. 4.000	49567666.667	5.928	<0.001	0.025	Yes
4.000 vs. 5.000	218916.667	0.0262	0.980	0.050	No

Comparisons for factor: **C**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
3.000 vs. 1.000	5214920.000	0.805	0.444	0.017	No
2.000 vs. 1.000	2822550.000	0.436	0.675	0.025	No
3.000 vs. 2.000	2392370.000	0.369	0.721	0.050	No

Power of performed test with alpha = 0.0500: for R : 1.000
Power of performed test with alpha = 0.0500: for T : 1.000
Power of performed test with alpha = 0.0500: for C : 0.0500

Least square means for R :

Group	**Mean**
1.000	41060666.667
2.000	217610966.667

Std Err of LS Mean = 3739382.136

Least square means for T :

Group	**Mean**
1.000	143333333.333
2.000	453000000.000
3.000	49900000.000
4.000	332333.333
5.000	113416.667

Std Err of LS Mean = 5912482.295

Least square means for C :

Group	**Mean**
1.000	126656660.000
2.000	129479210.000
3.000	131871580.000

Std Err of LS Mean = 4579789.093

Larvae Guts

Three Way Analysis of Variance Monday, March 19, 2012, 14:24:45

Data source: ANOVA BSFL gut *E. coli* in ANOVA BSFL gut *E. coli*

Balanced Design (No Interactions)

Dependent Variable: Col 4

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
T	1	3491104687.500	3491104687.500	141.130	<0.001
C	4	5039706996.667	1259926749.167	50.933	<0.001
R	2	91331151.667	45665575.833	1.846	0.219
Residual	8	197894770.000	24736846.250		
Total	29	14123564784.167	487019475.316		

The difference in the mean values among the different levels of T are greater than would be expected by chance after allowing for the effects of differences in C and R. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of C are greater than would be expected by chance after allowing for the effects of differences in T and R. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of R are not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in T and C. There is not a statistically significant difference (P = 0.219).

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **T**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	21575.000	11.880	<0.001	0.050	Yes

Comparisons for factor: **C**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	34163.333	11.897	<0.001	0.005	Yes
2.000 vs. 5.000	34017.500	11.847	<0.001	0.006	Yes

2.000 vs. 4.000	30211.667	10.521	<0.001	0.006	Yes
2.000 vs. 3.000	19218.333	6.693	<0.001	0.007	Yes
3.000 vs. 1.000	14945.000	5.205	<0.001	0.009	Yes
3.000 vs. 5.000	14799.167	5.154	<0.001	0.010	Yes
3.000 vs. 4.000	10993.333	3.828	0.005	0.013	Yes
4.000 vs. 1.000	3951.667	1.376	0.206	0.017	No
4.000 vs. 5.000	3805.833	1.325	0.222	0.025	No
5.000 vs. 1.000	145.833	0.0508	0.961	0.050	No

Comparisons for factor: **R**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
1.000 vs. 3.000	4167.500	1.874	0.098	0.017	No
2.000 vs. 3.000	2904.500	1.306	0.228	0.025	No
1.000 vs. 2.000	1263.000	0.568	0.586	0.050	No

Power of performed test with alpha = 0.0500: for T : 1.000
Power of performed test with alpha = 0.0500: for C : 1.000
Power of performed test with alpha = 0.0500: for R : 0.145

Least square means for T :

Group	**Mean**
1.000	120.333
2.000	21695.333

Std Err of LS Mean = 1284.182

Least square means for C :

Group	**Mean**
1.000	266.667
2.000	34430.000
3.000	15211.667
4.000	4218.333
5.000	412.500

Std Err of LS Mean = 2030.470

Least square means for R :

Group	**Mean**
1.000	12718.000
2.000	11455.000
3.000	8550.500

Std Err of LS Mean = 1572.795

Three Way Analysis of Variance Monday, March 19, 2012, 14:32:22

Data source: ANOVA BSFL gut *Salmonella* in ANOVA BSFL gut *Salmonella*

Balanced Design (No Interactions)

Dependent Variable: Col 4

Normality Test: Passed (P = 0.097)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
T	1	21075980853.333	21075980853.333	12.305	0.008
C	4	35539650118.133	8884912529.533	5.188	0.023
R	2	2254822678.400	1127411339.200	0.658	0.544
Residual	8	13702023586.667	1712752948.333		
Total	29	124078968596.800	4278585124.028		

The difference in the mean values among the different levels of T are greater than would be expected by chance after allowing for the effects of differences in C and R. There is a statistically significant difference (P = 0.008). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of C are greater than would be expected by chance after allowing for the effects of differences in T and R. There is a statistically significant difference (P = 0.023). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of R are not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in T and C. There is not a statistically significant difference (P = 0.544).

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **T**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	53010.667	3.508	0.008	0.050	Yes

Comparisons for factor: **C**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
3.000 vs. 1.000	88390.667	3.699	0.006	0.005	No
3.000 vs. 5.000	87605.000	3.666	0.006	0.006	No

3.000 vs. 4.000	85338.333	3.572	0.007	0.006	No
3.000 vs. 2.000	48490.000	2.029	0.077	0.007	No
2.000 vs. 1.000	39900.667	1.670	0.133	0.009	No
2.000 vs. 5.000	39115.000	1.637	0.140	0.010	No
2.000 vs. 4.000	36848.333	1.542	0.162	0.013	No
4.000 vs. 1.000	3052.333	0.128	0.902	0.017	No
4.000 vs. 5.000	2266.667	0.0949	0.927	0.025	No
5.000 vs. 1.000	785.667	0.0329	0.975	0.050	No

Comparisons for factor: **R**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
3.000 vs. 1.000	18648.400	1.008	0.343	0.017	No
3.000 vs. 2.000	18122.000	0.979	0.356	0.025	No
2.000 vs. 1.000	526.400	0.0284	0.978	0.050	No

Power of performed test with alpha = 0.0500: for T : 0.847
Power of performed test with alpha = 0.0500: for C : 0.689
Power of performed test with alpha = 0.0500: for R : 0.0500

Least square means for T :

Group	**Mean**
1.000	39.867
2.000	53050.533

Std Err of LS Mean = 10685.669

Least square means for C :

Group	**Mean**
1.000	119.333
2.000	40020.000
3.000	88510.000
4.000	3171.667
5.000	905.000

Std Err of LS Mean = 16895.527

Least square means for R :

Group	**Mean**
1.000	20153.600
2.000	20680.000
3.000	38802.000

Std Err of LS Mean = 13087.219

Three Way Analysis of Variance Monday, March 19, 2012, 16:14:50

Data source: ANOVA BSFL gut Plate count medium in ANOVA BSFL gut Plate count medium

Balanced Design (No Interactions)

Dependent Variable: Col 4

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
Col 1	1	795360395700.300	795360395700.300	179.832	<0.001
Col 2	4	1.220E+012 30	5092657541.967	68.982	<0.001
Col 3	2	15524723293.267	7762361646.633	1.755	0.233
Residual	8	35382363173.067	4422795396.633		
Total	2	3. 327E+012 1	14719680659.151		

The difference in the mean values among the different levels of Col 1 are greater than would be expected by chance after allowing for the effects of differences in Col 2 and Col 3. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Col 2 are greater than would be expected by chance after allowing for the effects of differences in Col 1 and Col 3. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Col 3 are not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Col 1 and Col 2. There is not a statistically significant difference (P = 0.233).
All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **Col 1**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	325650.200	13.410	<0.001	0.050	Yes

Comparisons for factor: **Col 2**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 5.000	483278.833	12.587	<0.001	0.005	Yes

2.000 vs. 1.000	479883.333	12.498	<0.001	0.006	Yes
2.000 vs. 4.000	474980.000	12.371	<0.001	0.006	Yes
3.000 vs. 5.000	320612.167	8.350	<0.001	0.007	Yes
3.000 vs. 1.000	317216.667	8.262	<0.001	0.009	Yes
3.000 vs. 4.000	312313.333	8.134	<0.001	0.010	Yes
2.000 vs. 3.000	162666.667	4.237	0.003	0.013	Yes
4.000 vs. 5.000	8298.833	0.216	0.834	0.017	No
4.000 vs. 1.000	4903.333	0.128	0.902	0.025	No
1.000 vs. 5.000	3395.500	0.0884	0.932	0.050	No

Comparisons for factor: **Col 3**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
1.000 vs. 3.000	55721.700	1.874	0.098	0.017	No
2.000 vs. 3.000	28027.000	0.942	0.374	0.025	No
1.000 vs. 2.000	27694.700	0.931	0.379	0.050	No

Power of performed test with alpha = 0.0500: for Col 1 : 1.000
Power of performed test with alpha = 0.0500: for Col 2 : 1.000
Power of performed test with alpha = 0.0500: for Col 3 : 0.134

Least square means for Col 1 :

Group	**Mean**
1.000	2563.133
2.000	328213.333

Std Err of LS Mean = 17171.285

Least square means for Col 2 :

Group	**Mean**
1.000	5666.667
2.000	485550.000
3.000	322883.333
4.000	10570.000
5.000	2271.167

Std Err of LS Mean = 27150.185

Least square means for Col 3 :

Group	**Mean**
1.000	193193.700
2.000	165499.000
3.000	137472.000

Std Err of LS Mean = 21030.443

Three Way Analysis of Variance Monday, March 19, 2012, 15:50:56

Data source: ANOVA FBL gut *E. coli* in ANOVA FBL gut *E. coli*

Balanced Design (No Interactions)

Dependent Variable: Y

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
T	1	289342177348.033	289342177348.033	549.679	<0.001
C	4	865296991978.467	216324247994.617	410.963	<0.001
R	2	2437231576.467	1218615788.233	2.315	0.161
Residual	8	4211070551.867	526383818.983		
Total	29	2.057E+012	70946674171.551		

The difference in the mean values among the different levels of T are greater than would be expected by chance after allowing for the effects of differences in C and R. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of C are greater than would be expected by chance after allowing for the effects of differences in T and R. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of R are not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in T and C. There is not a statistically significant difference (P = 0.161).

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **T**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	196415.267	23.445	<0.001	0.050	Yes

Comparisons for factor: **C**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
3.000 vs. 5.000	441169.000	33.305	<0.001	0.005	Yes

3.000 vs. 4.000	430199.333	32.477	<0.001	0.006	Yes
3.000 vs. 1.000	418376.167	31.585	<0.001	0.006	Yes
3.000 vs. 2.000	404107.833	30.508	<0.001	0.007	Yes
2.000 vs. 5.000	37061.167	2.798	0.023	0.009	No
2.000 vs. 4.000	26091.500	1.970	0.084	0.010	No
1.000 vs. 5.000	22792.833	1.721	0.124	0.013	No
2.000 vs. 1.000	14268.333	1.077	0.313	0.017	No
1.000 vs. 4.000	11823.167	0.893	0.398	0.025	No
4.000 vs. 5.000	10969.667	0.828	0.432	0.050	No

Comparisons for factor: **R**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
2.000 vs. 1.000	21282.200	2.074	0.072	0.017	No
3.000 vs. 1.000	15728.900	1.533	0.164	0.025	No
2.000 vs. 3.000	5553.300	0.541	0.603	0.050	No

Power of performed test with alpha = 0.0500: for T : 1.000
Power of performed test with alpha = 0.0500: for C : 1.000
Power of performed test with alpha = 0.0500: for R : 0.204

Least square means for T :

Group	**Mean**
1.000	4731.400
2.000	201146.667

Std Err of LS Mean = 5923.872

Least square means for C :

Group	**Mean**
1.000	23333.333
2.000	37601.667
3.000	441709.500
4.000	11510.167
5.000	540.500

Std Err of LS Mean = 9366.463

Least square means for R :

Group	**Mean**
1.000	90602.000
2.000	111884.200
3.000	106330.900

Std Err of LS Mean = 7255.231

Three Way Analysis of Variance Monday, March 19, 2012, 15:59:35

Data source: ANOVA FBL gut *Salmonella* in ANOVA FBL gut *Salmonella*

Balanced Design (No Interactions)

Dependent Variable: Y

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
T	1	5083997648.133	5083997648.133	97.628	<0.001
C	4	3124422187.200	781105546.800	15.000	<0.001
R	2	179414888.467	89707444.233	1.723	0.239
Residual	8	416599594.200	52074949.275		
Total	29	13693679987.867	472195861.651		

The difference in the mean values among the different levels of T are greater than would be expected by chance after allowing for the effects of differences in C and R. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of C are greater than would be expected by chance after allowing for the effects of differences in T and R. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of R are not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in T and C. There is not a statistically significant difference (P = 0.239).

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **T**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	26035.867	9.881	<0.001	0.050	Yes

Comparisons for factor: **C**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
3.000 vs. 4.000	30753.333	7.381	<0.001	0.005	Yes

1.000 vs. 4.000	21574.000	5.178	<0.001	0.006	Yes
3.000 vs. 5.000	18341.000	4.402	0.002	0.006	Yes
2.000 vs. 4.000	18207.333	4.370	0.002	0.007	Yes
3.000 vs. 2.000	12546.000	3.011	0.017	0.009	No
5.000 vs. 4.000	12412.333	2.979	0.018	0.010	No
3.000 vs. 1.000	9179.333	2.203	0.059	0.013	No
1.000 vs. 5.000	9161.667	2.199	0.059	0.017	No
2.000 vs. 5.000	5795.000	1.391	0.202	0.025	No
1.000 vs. 2.000	3366.667	0.808	0.442	0.050	No

Comparisons for factor: **R**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
1.000 vs. 2.000	5439.400	1.685	0.130	0.017	No
1.000 vs. 3.000	4892.700	1.516	0.168	0.025	No
3.000 vs. 2.000	546.700	0.169	0.870	0.050	No

Power of performed test with alpha = 0.0500: for T : 1.000
Power of performed test with alpha = 0.0500: for C : 0.997
Power of performed test with alpha = 0.0500: for R : 0.130

Least square means for T :

Group	**Mean**
1.000	4664.133
2.000	30700.000

Std Err of LS Mean = 1863.240

Least square means for C :

Group	**Mean**
1.000	22666.667
2.000	19300.000
3.000	31846.000
4.000	1092.667
5.000	13505.000

Std Err of LS Mean = 2946.041

Least square means for R :

Group	**Mean**
1.000	21126.100
2.000	15686.700
3.000	16233.400

Std Err of LS Mean = 2281.994

Three Way Analysis of Variance Monday, March 19, 2012, 14:42:25

Data source: ANOVA FBL gut Plate countmedium in ANOVA FBL gut Plate count medium

Balanced Design (No Interactions)

Dependent Variable: Y
Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 1.000)

Source of Variation	DF	SS	MS	F	P
T	1	143431898781.633	143431898	781.6106.291	<0.001
C	4	1.541E+012	385284530525.8	285.517	<0.001
R	2	2612610782.	6001306305391.3	0.968	0.420
Residual	8	10795425067.067	1349428133.383		
Total	29	1.930E+0126	6549719835.197		

The difference in the mean values among the different levels of T are greater than would be expected by chance after allowing for the effects of differences in C and R. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of C are greater than would be expected by chance after allowing for the effects of differences in T and R. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of R are not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in T and C. There is not a statistically significant difference (P = 0.420).

All Pairwise Multiple Comparison Procedures (Holm-Sidak method):
Overall significance level = 0.05

Comparisons for factor: **T**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
2.000 vs. 1.000	138290.467	10.310	<0.001	0.050	Yes

Comparisons for factor: **C**

Comparison	Diff of Means	t	Unadjusted P	Critical Level	Significant?
1.000 vs. 4.000	604935.000	28.523	<0.001	0.005	Yes

1.000 vs. 5.000	598791.167	28.233	<0.001	0.006	Yes
1.000 vs. 3.000	522166.667	24.620	<0.001	0.006	Yes
1.000 vs. 2.000	357500.000	16.856	<0.001	0.007	Yes
2.000 vs. 4.000	247435.000	11.667	<0.001	0.009	Yes
2.000 vs. 5.000	241291.167	11.377	<0.001	0.010	Yes
2.000 vs. 3.000	164666.667	7.764	<0.001	0.013	Yes
3.000 vs. 4.000	82768.333	3.903	0.005	0.017	Yes
3.000 vs. 5.000	76624.500	3.613	0.007	0.025	Yes
5.000 vs. 4.000	6143.833	0.290	0.779	0.050	No

Comparisons for factor: **R**

Comparison	**Diff of Means**	**t**	**Unadjusted P**	**Critical Level**	**Significant?**
1.000 vs. 3.000	22800.700	1.388	0.203	0.017	No
2.000 vs. 3.000	12810.200	0.780	0.458	0.025	No
1.000 vs. 2.000	9990.500	0.608	0.560	0.050	No

Power of performed test with alpha = 0.0500: for T : 1.000
Power of performed test with alpha = 0.0500: for C : 1.000
Power of performed test with alpha = 0.0500: for R : 0.0500

Least square means for T :

Group	**Mean**
1.000	130842.867
2.000	269133.333

Std Err of LS Mean = 9484.823

Least square means for C :

Group	**Mean**
1.000	616666.667
2.000	259166.667
3.000	94500.000
4.000	11731.667
5.000	17875.500

Std Err of LS Mean = 14996.823

Least square means for R :

Group	**Mean**
1.000	210918.500
2.000	200928.000
3.000	188117.800

Std Err of LS Mean = 11616.489

Appendix Four: Turbo mass spectrometer GC/MS

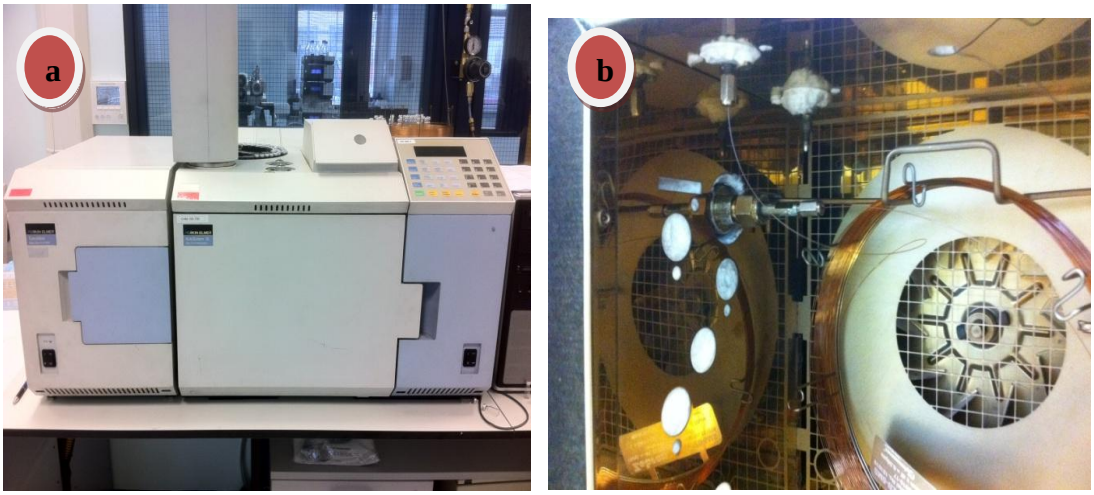

Fig 4 1: Turbo mass spectrometer detector used in GC/MS; a) Outer view b) Inside view

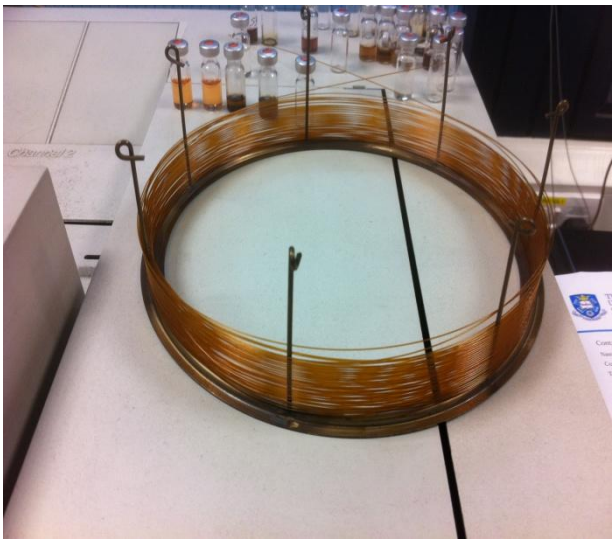

Fig 4 2: Capillary column (Zebran ZB-S, 30 m length x 0.25 mm Diameter x 0.25 µm phase thickness)

Interactions between invertebrates and pathogenic and non-pathogenic bacteria

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Introduction

The risks of canine waste accumulation in urban environment and agro-ecosystem is an ever-growing issue, pathogenic bacteria such as *Escherichia* coli and *Salmonella* Spp. have been isolated from animals faeces including canine faeces. Invertebrates such as slugs and snails play an important role in the food chain and can act as agricultural pests. (Emma *et al*, 2006)observed that the Great Gray Slug, *Limax maximus* and the Yellow Slug, *Limax flavus* can carry *E. coli* O157 from animals manure on their bodies surface and internally as intermediate hosts vectors. Recycling is a sustainable strategy for disposing of animals waste, and composting can be an important part of a recycling programme. The microorganisms involved oxidize carbon as an energy source for growth and ingest nitrogen for protein synthesis (Taylor 2004). Consequently, the right carbon to nitrogen ratio in composting systems is required for efficient decomposition of wet dog manure contains 0.7% nitrogen (N),and 0.25% phosphate (P2O4) (compared to wet cattle manure, dog waste which contains 40% more nitrogen, the same amount of phosphate (Hall and Schulte 1979). Insects such as Black soldier fly larvae have been used in bioremediation to breakdown and recycle animals faeces which lead to a reduction in bacterial number in animal's faeces in may be used for biodiesel production and animal food-stock. This study aims to investigate the problems resulting from dog faeces accumulation in the environment and to find scientific solutions for utilizing and converting dogs waste to a safer material which should be more compatible with environmental aspects,

Results

1. Microorganisms isolated from canine faeces, slugs, snails and lettuce

The results (Figure 1.A) show seven differently coloured bacterial colonies which were isolated from dog faeces grown on CHROMagar media. Six of them were transmitted to the inside (gut) of slugs fed on dog faeces (Figure 1.B); as well, *Staphylococcus lentus* grow in the slime on the surface of slugs. However, in the contaminated lettuce samples, four colonies, different from Enterococci, were isolated. The colonies were initially identified using colour reference as. *S. lentus, Delftia sp, E. coli* and *S. enterica* in addition to *Enterococci spp* which were isolated everywhere (Table. 1)

Further identification of bacterial species using 16S rRNA procedure has been investigated. The isolations JS1, JS2, JS3 , JS4 and JS5 (Table. 2) were subjected to the extraction of genomic DNA (Figure 3.4) which were isolated from; outside control slugs (Metallic blue), Inside slugs fed on faeces (Beige), outside slugs fed on faeces (Beige), outside slugs fed on faeces(Cream) and contaminated salad(Beige) respectively. In addition to three isolations MR1, MR2 and MR3 (Table. 2) which were isolated from the Inside of slugs

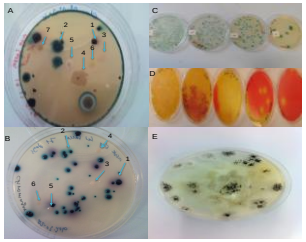


Figure1:A)Bacteria isolated from dog faeces grown on Chromagar medium ,1. *S. letus*; 2. *Enterococcus spp*; 3. *Proteus sp*; 4.*S. entreica*; 5. *E. coli*; 6. *Delftia sp*; 7. *Acinetobacter sp. B)* Bacteria grown on Petri-dish isolated from slugs gut following; 1. *S. letus*; 2. *S. enterica* 3. *Enterococcus spp*; 4. *E.coli*; 5. *Proteus sp*; 6. *Delftia sp*. C) E. coli and D) S. enterica are bacteria grown on selective media isolated from public park and E) *Aspergillus niger* and *A, flavus*

Table 1. Different colours of bacteria species grown on CHROMagar medium

Bacterial strain	Colonies colour on media (Chromagar)	Presence of bacterial species in different Samples						
		Dogs faeces	Slugs				Lettuce	
			Control		Fed on faeces		Control	Contaminated
			1a	1b	1a	1b		
Enterococcus spp	Blue Turquoise	+	+	+	+	+	+	+
Enterobacter aemigenus	Metallic blue	-	-	+	-	-	-	-
Staphylococcus lentus	Metallic blue	+	-	-	+	-	-	+
Acinetobacter sp	Beige	+	-	-	+	+	-	-
Comamonas sp	Blue	-	-	-	-	+	-	-
Delftia sp	Beige	+	-	-	+		-	+
Proteus sp	Beige with brown Halo	+	-	-	+	-	-	-
E. coli	Pink-red	+	-	-	+	-	-	+
Salmonella sp	Cream	+			+	-	-	+

2. Identification of bacteria and fungi transmitted from canine faeces to slugs and snails to lettuce

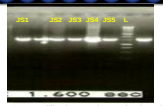


Figure 2. PCR amplification products of four bacterial species analyzed by electrophoresis in agarose gel lanes represent; (lane L); hyper ladder;(lane JS1) outside control slugs (MB); (lane JS 2) Inside slugs fed on faeces (B); (lane JS3) outside slugs fed on faeces(B); (lane JS 4) outside slugs fed on faeces (C); (lane 5) Contaminated salad

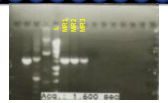


Figure 3. PCR amplification products of bacterial species detecting bacteria test analyzed by electrophoresis in agarose gel lanes represent; (lane L); hyper ladder;(lane MR1) Inside slugs fed on faeces (MB); (lane MR 2) Inside slugs fed on faeces (C); (lane MR3) Contaminated salad

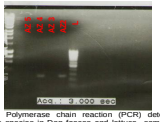


Figure 4. Polymerase chain reaction (PCR) detection of Mycoplasma species in Dog faeces and lettuce samples, EZ-PCR- detecting Mycoplasma test analyzed by electrophoresis in 2% agarose gel; the lanes represent (lane L); 1kb hyper ladder, (lane AZ 2); contaminated slugs (lane AZ 3); control slugs(lane (lane AZ 4) contaminated lettuce; (lane AZ 5); control lettuce

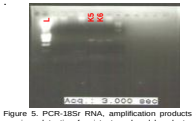


Figure 5. PCR-18Sr RNA, amplification products of fungal species detecting fungi test analyzed by electrophoresis in agarose gel lanes represent; (lane L); hyper ladder; (lane K5 and K6) dog faeces samples.

Table 2. Bacterial identification using gDNA-16SrRNA

S/No	S/ source	Species	S %
JS1	Outside control slugs (MB)	*Enterobacter amnigenus*	99%
JS2	Inside slugs fed on faces (B)	*Acinetobacter sp*	99%
JS3	Outside slugs fed on faces (B)	*Comamonas sp*	91%
JS4	Outside slugs fed on faces (C)	*Acinetobacter sp*	100%
JS5	Contaminated salad (B)	*Delftia sp*	99%
MR1	Inside slugs fed on faces (MB)	*Staphylococcus lentus*	100%
MR2	Inside slugs fed on faces (C)	*Salmonella sp*	99%
MR3	Contaminated salad (F/E)	*E. coli*	100%

Table 3. The presence of Mycoplasma in various samples

Samples	Symbol	Reaction
Earth worm (Whole body)	RM1	Positive
Dog faeces	SR1	Positive
Contaminated lettuce	SR2	Positive
Control lettuce	SR3	Negative
Outside control slug	AZ1	Negative
Outside contaminated slug	AZ2	Positive
Outside control snails	AZ3	Negative
Outside contaminated snails	AZ4	Negative
Inside control slugs	AZ5	Positive
Inside contaminated slugs	AZ6	Positive
inside control snails	AZ7	Negative
Inside contaminated snails	AZ8	Positive

3. Summary of the canine waste accumulation Problems

Dog faeces can carry 7 species of bacteria, mycoplasma and pathogenic fungi

contaminated Slugs and snails can carry 6 species of bacteria and mycoplasma

6 species of bacteria and mycoplasma can be transmitted to lettuce by slugs

Insert BSF and FB larvae as a solution suggested for bioremediation of dog faeces

4. The effect of BSF and FB larvae activity on *E. coli and Salmonella enterica* populations

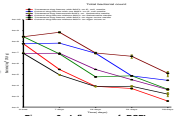


Figure 6. Influence of BSFL on bacterial activity on dog faeces.

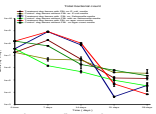


Figure 7. Influence of FBL on bacterial activity on dog faeces.

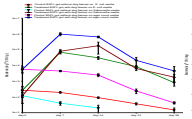


Figure 8. The Influence of BSFL gut on bacterial in dogsfaeces

Figure 9. The Influence of BFBL gut on bacterial in dogs faeces

5. Recycling and composting the dog faeces

Figure 10. The final compound of untreated dog faeces after 28 days

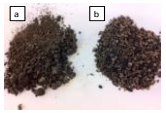


Figure 11. The final compound of treatment dog faces a) treated with FBL; b) treated with BSFL after 28 days

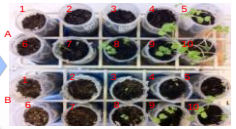
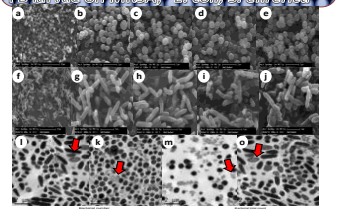


Figure12. Illustrates; a) the lettuce seeds planted in different concentration of treated dog faeces with BSFL No; 1, control dog faeces 2, 3 and 4 are 75, 50 and 25% of dog faeces concentration and 5 is a control compost. From 6 to 10 are the turnip seeds planted at the same concentration b) the lettuce seeds planted in different concentration of treated dog faeces with FBL No; 1, control dog faeces 2, 3 and 4 were 75, 50 and 25% of dog faeces and 5 is a control compost. From 6 to 10 are the turnip seeds planted at the same concentration

6. SEM and TEM studies on the morphological effects of Hemolymph and Whole body secretions of BSF and FB larvae on MRSA, *E.coli, S. enterica*



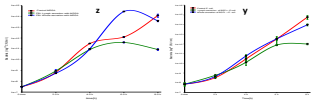


Figure 13. The effect of hemolymph and whole body secretions of BSF and FB Larvae on the morphological of MRSA,(a-e), *E. coli* (f-j), Internal morphology different bacterial species(k-o). Z and y the effect the secretion of larvae on E. coli and MRSA growth

7. Dogs waste from biomass to biodiesel

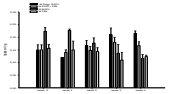
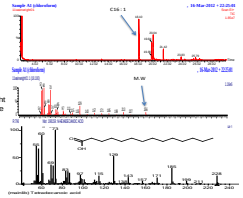


Figure 14. The amount of lipids in dry weight of 1g of dog faeces and BSF and FB larvae



15. Use the GC/MS to identify the extracted fatty acids from dog's faeces, BSF and FB Larvae

8. Conclusion

The results of this study show out that seven species of bacteria were found in dogs' faeces. Six out these seven species were confirmed for being carried by slugs and snails, when obligatory fed on dog's faeces. Slugs transmitted four bacterial species i.e.: *E.coli sp, Salmonella enterica, Staphylococcus lentus* and *Delftia* sp to fresh lettuce. Our study suggests a range of different solutions to convert the dog faeces from a poisonous substance to a less offensive material using insect larvae. Bioremediation of dog faeces was successful in converting dog waste to modified compost for agricultural purpose. Lipids of poetical use as befouls were also extracted from dog faeces treated with larvae.

9. Acknowledgments

I thank my supervisor Prof. M. Wainwright for the valuable guidance and advice . I also thank my advisors Dr. D. J. Gilmour and Dr L. Partridge for their assistance. I am grateful to Mr S. J. Thorpe from chemistry dept for his help in GC/MS. and Mr C.J. Hill in SEM/TEM.

10. References

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Hall, RE., Schulte, E.F. (1979). Using dog and cat manure onhome gardens. University of Wisconsin, Madison, College of Agriculture and Life Sciences.

Taylor, L. (2004). Poop, Scoop and Compost: Proposal to Explore the Feasibility of Composting Dog Waste in Public Parks. Vancouver, Canada: National Coalition for People and Dogs, Handout, pgs. 1-7

Poster Presentation Relating to Some of the Work Described in this Thesis