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Impact Of Selected Rodenticides On The Acidic And Enzymatic Coagulation Properties Of Milk

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Biographical Sketch

Madhavi Hathurusinghe was born in Kegalle, Sri Lanka, where she attended elementary and secondary schools. Madhavi received her Bachelor's degree in Veterinary Medicine from University of Peradeniya, Sri Lanka in 2000. She joined as a research assistant in the department of Veterinary Public Health and Pharmacology, University of Peradeniya where she was involved in research on antimicrobial residues in food of animal origin. She then earned her Master's degree in food safety from the same University in 2004. Madhavi worked as a senior lecturer in the Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka from 2005 to 2008, where she was involved with teaching and research related to chemical residues in food. Madhavi also worked as a part time veterinarian in small animal practice in Sri Lanka. During her career as a Senior Lecturer, Madhavi was involved in FAO/IAEA project on antimicrobial residues in food and worked with several European laboratories where she presented her findings nationally and internationally.

Madhavi joined North Carolina A&T State University in 2009 to pursue a PhD in Energy and Environmental Systems interdisciplinary program under the thrust of Environmental Food Science. She received several scholarly awards at North Carolina A & T State University. She was given the award for scholarly accomplishment and academic excellence by the International Students and Scholars Office (ISSO) from 2009–2012. She is a recipient of the Wadaran L. Kennedy 4.0 Scholars award from the School of Graduate Studies from 2009–2011. Madhavi is a member of the Sri Lanka Veterinary Association, American Dairy Science Association and Phi Kappa Phi honor Society. While working on her PhD, she presented her findings in a number of national and international conferences and she has authored several scientific papers. After

finishing her Ph.D. degree, Madhavi intends to continue her career in research and teaching in food safety.

Dedication

To my son, Manuka, whose life and innate happiness have given me perspective, motivation, and joy, which can be hard to find in the last stages of writing a dissertation.

To my husband, Melaka, for always reminding me that I can do whatever it is I think I can't. Your unending love, support, and confidence in me have made me a better person, scholar, and mother.

Most importantly, to my parents for their unconditional love, affection, encouragement and who, through both their words and actions, have always made education a priority.

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Nomenclature

^{0}C	Celsius
ml	Milliliters
CFU	Colony Forming Units
CFU/ml	Colony Forming Units/milliliter
DAD	Photodiode Array Detector
ELISA	Enzyme Linked Immunosorbent Assay
FDA	Food and Drug Administration
g	Grams
Gal U/ml	Galactose unit per milliliter
Gal U/mg	Galactose unit per milligram
GRAS	Generally Recognized as Safe
HPLC	High Performance Liquid Chromatography
LA	Lactic Acid
LAB	Lactic Acid Bacteria
mg	Milligram
mins.	Minutes
µl	Microliters
µm	Micrometers
mM	Millimolar
µM	Micromolar
MRS	DeMann Rogosa Sharpe media
MW	Molecular weight

N	Normality
RIA	Radioimmunoassay
rpm	Revolution per minute
Spp.	Species
Subsp	Subspecies
U/mg	Units per milligram
WHO	World Health Organization
w/v	Weight/volume

Abstract

The early detection of chemical contaminants in milk at the farm level is essential to prevent them entering into the food chain. The overall objective of this study was to determine the effect of selected rodenticides on the acidic and enzymatic coagulation properties of milk by using selected strains of lactic acid bacteria (LAB) and to evaluate the suitability of lactic acid bacteria as a biomarker for early detection of toxins in milk. Specific objectives of this study were (a) to determine the optimum growth temperature for lactic acid bacteria in MRS broth for early detection of rodenticides; (b) to determine the effect of selected rodenticides on the growth and activity of different strains of lactic acid bacteria in MRS broth; and (c) to determine the effect of selected rodenticides on the growth and activity of different strains of lactic acid bacteria in milk. Serially diluted rodenticides were added to tubes containing MRS broth/milk. Commercial yogurt culture was then inoculated into the medium and incubated at 42 °C for 6 hours. The pH of the broth was recorded at 2, 4, and 6 hour intervals. The bacterial counts, lactic acid production, α-galactosidase and β-galactosidase activity were also measured at the end of incubation. In the MRS broth, the yogurt cultures YF001 and YI 885 showed highest sensitivity to diphacinone at the level of 0.005 mg/ml. YF001 showed detectable sensitivity to brodifacoum and bromadiolone at the level of 0.02 mg/ml. YG011 showed detectable sensitivity to brodifacoum, bromadiolone and diphacinone at the levels of 0.02, 0.04, and 0.02 mg/ml, respectively. The growth of YI 885 was inhibited by brodfacoum and bromadiolone at the levels of 0.01 and 0.04 mg/ml, respectively. In milk, the yogurt cultures YG011 and YI 885 showed highest sensitivity to diphacinone at the level of 0.04 mg/ml. YF001 showed detectable sensitivity to brodifacoum, bromadiolone and diphacinone at the levels of 0.12, 0.08, and 0.08mg/ml, respectively. The growth of YG011 was inhibited by brodfacoum and bromadiolone

at the levels of 0.08 and 0.16mg/ml, respectively. YI 885 showed detectable sensitivity to brodifacoum and bromadiolone at the levels of 0.08 and 0.16mg/ml, respectively. A significant ($p < 0.05$) growth inhibition of all the yogurt cultures was observed in the presence of all the three rodenticides. There were significant differences ($p < 0.05$) in the bacterial count, pH, lactic acid production, α-galactosidase and β-galactosidase activity in the presence of rodenticides in the medium. The results indicate that yogurt culture could be used as a biomarker for the early detection of rodenticides in milk. With some adjustments, this process could be utilized as a fast and accurate test for the presence of rodenticides in dairy food products.

CHAPTER 1

Introduction

Cow's milk is ubiquitous, and it is an essential part of the human diet. It is the basis for all the dairy products. Milk is a natural source that produces a highly nutritious part of the daily diet. It is an excellent source of high quality proteins that human beings cannot synthesize, and milk is the major source of calcium in the diet. Milk is a good source of all the essential vitamins for humans, especially vitamin A, D, E, and K that are essential for bone health. It also provides riboflavin and vitamin B12 that are necessary for cardiovascular health and energy production. In addition, cow's milk is a very good source of iodine, a mineral essential for thyroid function, and it provides phosphorous, magnesium, potassium, and trace elements such as zinc (A Haug, Høstmark, & Harstad, 2007; B. Roy, 2008).

During production at the farm level, there is a risk of microbiological and chemical contamination of milk. The chemical contamination of milk can be unintentional or intentional. Milk can be contaminated unintentionally with detergents (Hettinga, van Valenberg, & van Hooijdonk, 2008), environmental chemicals such as persistent lipophillic organic contaminants (PCB, PCDD/Fs), pesticides, antibiotics, and heavy metals (Fayed, Zidan, Abou-Arab, & Magdoub, 1995; Jensen, 1995; McLachlan, 1995; Ramos et al., 1997). Poor level of bio-security in a dairy farm may permit uninvited people to enter the farm and engage in intentional contamination of milk with harmful chemicals. This may lead to possible terrorist attacks. Rodenticides and pesticides are possible chemicals that can be used in an intentional contamination of milk. Such undetected contamination from a single farm may contaminate the milk collected in bulk, causing these chemicals to enter the food chain.

Chemical contamination of milk at the point of production may lead to potential human health hazards (M. I. Yamani, L. M. A. Al-Kurdi, M. S. Y. Haddadin, & R. K. Robinson, 1999). Previous researches prove that acute and prolonged exposure to chemicals through food can cause harmful effects to human health (Okolie & Osagie, 1999; Soto-Blanco & Górniak, 2003; Sousa, Soto-Blanco, Guerra, Kimura, & Górniak, 2002). Milk contaminated with chemicals causes problems in the processing industry, mainly in the production of yogurt and cheese (Boor, Brown, Murphy, Kozlowski, & Bandler, 1998). The chemicals in food not only cause harmful effects to the consumer, but also cause severe economic losses to the country.

The milk is distributed from the farm soon after production, and it reaches the consumers around the country within a short period of time. If the milk is contaminated with chemicals at the dairy farm, it may cause potential health risks to the consumer. The early detection of chemical contaminants in milk at the farm level is essential to prevent them entering into the food chain. Chemical residues in milk are analyzed using advanced methods such as High Performance Liquid Chromatography (HPLC) and Gas Chromatography–Mass Spectrometry (GC-MS) (García-Mayor, Garcinuño, Fernández-Hernando, & Durand-Alegría, 2006; Ramírez et al., 2003). These existing methods are expensive and time consuming, and it takes two-three days to obtain results from them.

Bioassay techniques, which use bacteria as a biomarker to detect chemicals in food, are widely used to screen antibiotic residues. Numerous studies have been carried out to establish techniques to detect antibiotic residues in milk (Montero, Althaus, Molina, Berruga, & Molina, 2005; Stead et al., 2008; Zeng, Escobar, & Brown-Crowder, 1996). There is very little research on developing microbiological techniques to detect harmful chemicals in milk. Microbiological assays are easy to perform, cheaper, and give results within a few hours. Simple and robust on-

farm technique, which detect harmful chemicals in milk, will prevent them entering into the food chain.

Microbiological assays are developed using microorganisms as biomarkers. Selecting a microorganism, which is sensitive to a wide range of chemicals, is essential in developing a bioassay method to achieve a broader sensitivity. The microorganism used in the bio-assay system should be nonpathogenic and environment friendly. Lactic acid bacteria (LAB) are nonpathogenic microorganisms possessing beneficial effects on human health. They are widely used in food fermentation, especially in yogurt production. They do not require special laboratory conditions or expensive media for their growth. They do not cause environmental or human health hazards and they are capable of producing lactic acid rapidly and changing the acidity of the medium. The slow reaction in lactic acid production can be used as a triggered response to changes in the milk content due to the presence of foreign substances. These qualities of LAB increase its suitability as an indicator organism in bioassays. Therefore it is important to identify the growth of LAB in the presence of toxins and the effect of toxins in the milk coagulation process in order to determine the suitability of LAB as a biomarker.

1.1 Objectives

The overall objective of this project is to determine the effect of selected rodenticides on the acidic and enzymatic coagulation of milk by using selected strains of lactic acid bacteria and eventually to evaluate the suitability of lactic acid bacteria as a biomarker for early detection of rodenticides in milk.

The specific objectives of this study are:

1. To determine the optimum growth temperature for lactic acid bacteria in MRS broth for early detection of rodenticides.

2. To determine the effect of selected rodenticides on the growth and activity of different strains of lactic acid bacteria in MRS broth.
3. To determine the effect of selected rodenticides on the growth and activity of different strains of lactic acid bacteria in milk.
4. To determine the effect of rodenticides on enzymatic coagulation properties of milk.

1.2 Organization of Dissertation

Chapter 2 the Literature Review, presents the background for the research. It identifies the problem and discusses several approaches other researchers have taken to solve it. Chapter 3 presents the optimum growth temperature for lactic acid bacteria in MRS broth for early detection of rodenticides. Chapter 4 of this dissertation provides the effect of selected rodenticides on the growth and activity of lactic acid bacteria in MRS broth. Chapter 5 elaborates the effect of selected rodenticides on the growth and activity of different strains of lactic acid bacteria in milk. Chapter 6 summarizes the conclusions and recommends future research possibilities.

CHAPTER 2

Literature Review

2.1 Milk as a Food

The health benefits of milk and milk products are well known from ancient times. Consumption of cow's milk gives extensive nutritional values to children, adults, and elderly. It also provides several health benefits. There is a growing evidence that milk has health enhancing effects such as antimicrobial properties, immune-stimulatory effects, cancer prevention, and regulation of obesity related metabolic disorders (Ebringer, Feren ík, & Kraj ovi 2008; A Haug et al., 2007).

Cow's milk is a reserve of lipids, proteins, amino acids, vitamins, and minerals. It contains immunoglobulins, hormones, growth factors, cytokines, nucleotides, peptides, polyamines, enzymes and other bioactive peptides. The lipids in milk are emulsified in globules coated with membranes. The proteins are in colloidal dispersions as micelles. The casein micelles occur as colloidal complexes of protein and salts, primarily calcium. Lactose and most minerals are in solution. Milk composition has a dynamic nature, and the composition varies with stage of lactation, age, breed, nutrition, energy balance and health status of the udder. Colostrum in milk differ considerably; the most significant difference is the concentration of milk protein that may be double in colostrum compared to later in lactation (Liu, Chen, & Lin, 2002). The change in milk composition during the whole lactation period seems to match the changing need of the growing offspring, giving different amounts of components important for nutrient supply, specific and non-specific host defense, growth and development. Specific milk proteins are involved in the early development of immune response, while others take part in the

non-immunological defense (e.g., lactoferrin). Milk consists of different types of fatty acids (Guce et al., 2010). All these components make milk a nutrient rich food item.

The USA is one of the largest producers of cow's milk in the world accounting for 15.0% of the world production. It has produced nearly 200 billion pounds of milk in 2012, an increase of 2.1% when compared to that in 2011 (Table 2-1). The rate per cow, at 21,697 pounds, was 361 pounds above 2011. The cash receipt from marketing of milk during 2012 was $37 billion (NASS, 2013).

Table 2-1

Milk Production and Number of Milk Cows in USA: 2011-2012 (NASS, 2013)

Item	Year	
	2011	2012
Milk Cows (Thousands)	9,194	9,233
Milk Per Cow (Lbs)	21,336	21697
Milk Production (Million Lbs)	196,164	200,324

There is a risk of unintentional or intentional contamination of milk by a wide variety of chemicals. Chemical residues can be introduced unintentionally via treatment of the cow, the feed, the milking environment, and the processing plant. The potential chemical contaminants are: antibiotics, hormones, disinfectants, nitrites, pesticides including rodenticides, polychlorinated biphenyls (PCBs), mycotoxins, toxic metals, and dioxins (Heeschen & Harding, 1995). Numerous studies have revealed that acute and prolonged exposure to chemicals through food can lead to harmful effects to human health including allergies, toxic effects, mutagenic effects, and teratogenic effects (Okolie & Osagie, 1999; Soto-Blanco & Górniak, 2003; Sousa et

al., 2002; Teuber, 2001). Milk contaminated with chemicals create problems in processing industry leading to acute monetary losses to the country (Boor et al., 1998).

2.2 Rodenticides

Rodenticides (rat poison) are category of pesticides intended to kill rodents. It is difficult to kill rodents due to their scavenging behavior in feeding. They eat small quantities of new food and wait for several hours, and if they don't get sick, they continue. Therefore an effective rodenticide must be tasteless and odorless in lethal concentrations, and have a delayed effect. Rodenticides are an important tool for public health, including controlling domestic mice and rats, but the mal practices in marketing and use have been associated with accidental exposures to thousands of children each year (Chua & Friedenberg, 1998; Watt, Proudfoot, Bradberry, & Vale, 2005). These products also pose significant risks to non-target wildlife, including both birds and mammals. The wide commercial availability of these rodenticides has increased accidental and intentional ingestion for both animals and human beings (Erickson & Urban, 2004; Fauconnet, Pouliquen, & Pinault, 1997; Jin, Chen, Ye, & Zhu, 2008; Spurr et al., 2005).

2.2.1 Anticoagulant rodenticides. Anticoagulant rodenticides are used widely in agricultural and urban rodent control. Most rodenticides are anticoagulants and have a single mode of action of blocking the vitamin K cycle causing coagulation impairments (Petterino & Paolo, 2001). Anticoagulant rodenticides are divided into two main groups depending on their chemical structure; Hydroxycoumarins and indandiones. Hydroxycoumarines are subdivided as either first-generation anticoagulants like chlorophacinone, coumatetralyl and warfarin or as second-generation anticoagulants. This second generation group includes 4-hydroxycoumarins brodifacoum, bromadiolone, difenacoum, flocoumafen and the indandione derivatives chlorophacinone and diphacinone (see Figure 2-1; (Ivan Valchev, Binev, Yordanova, Nikolov, &

Pasha, 2008). The emergence of resistant strains of rats for warfarin led to the introduction of a new group of these second generation anticoagulant rodenticides commonly referred to as 'superwarfarins,' 'single dose,' or 'long-acting' (M. Hadler & A. Buckle, 1992; Watt et al., 2005). In addition to their ability to control rodents resistant to traditional warfarins, the second generation products are characterized by their higher overall potency. These are more effective due to the greater affinity to binding sites in the liver, greater accumulation and persistence in the body (M. Hadler & A. Buckle, 1992; Huckle, Hutson, Logan, Morrison, & Warburton, 1989; Watt et al., 2005).

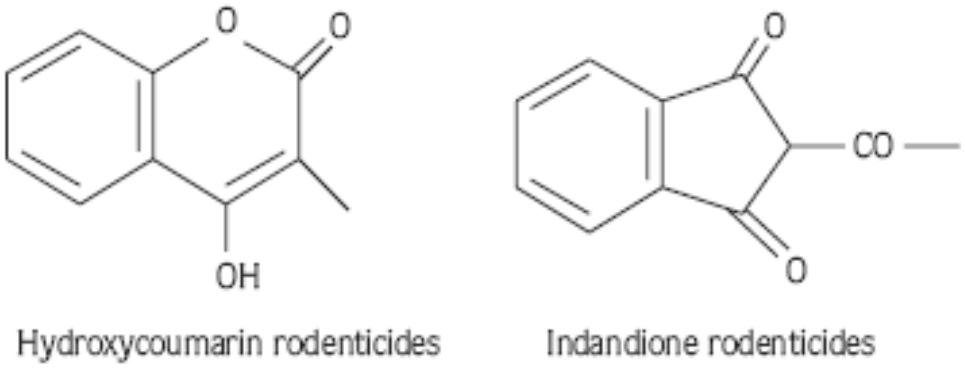
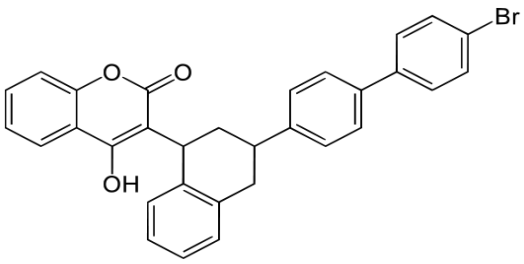


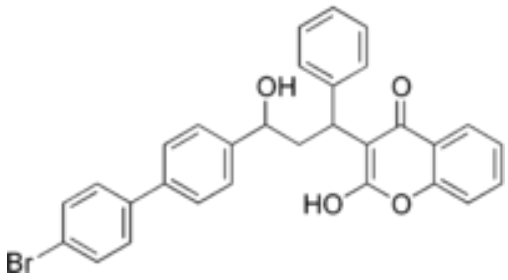
Figure 2-1. Chemical structure of anticoagulant rodenticides (Valchev et al., 2008).

The "first-generation" anticoagulants (warfarin and coumafuryl) require multiple feedings to result in toxicity and they inhibit the clotting factors for 1-10 days. The "intermediate" anticoagulants such as chlorophacinone and diphacinone require fewer feedings than "first-generation" chemicals and depress clotting factors for 3-4 weeks. Therefore they are more toxic to nontarget species (P. Berny, 2007). The "second-generation" anticoagulants (i.e., brodifacoum and bromadiolone; see Figure 2-2) have a greater affinity to binding sites in the liver and are highly toxic to nontarget species after a single feeding. and they stay much longer periods usually more than 100 days in the body (Thijssen, 1995). According to a research

carried out in 2008, brodifacoum, showed a plasma elimination half-life of 91.7 days and the elimination half-life in liver was 307.4 days (Ivan Valchev et al., 2008).



(a)



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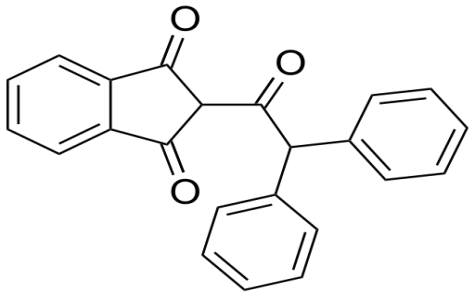


(c)

Figure 2-2. Chemical structure of anticoagulant rodenticides (a) brodifacoum, (b) bromadiolone, and (c) diphacinone (Adapted from http://en.wikipedia.org/wiki).

Superwarfarins are the most commonly used rodenticides in the United States. They were first introduced in the 1970s after the development of resistant rats to the traditional warfarin rodenticide (Juajun et al., 2011). Over 95% of all rodenticides used in United States consist of superwarfarin. Brodifacoum is the commonest rodenticide among them. Marketing

and use malpractices have been associated with accidental exposures to thousands of children each year. (Watson et al., 2005). These products also pose significant risk to non-target species including both birds and mammals. Anticoagulant rodenticide poisoning has significantly affected conservation and management of non-target wildlife. Numerous studies have documented the primary or secondary rodenticide poisoning of endangered and common non-target wildlife. Primary exposure is by direct ingestion of bait and secondary poisoning is through consumption of exposed insects or animals (P. Berny, 2007; Fournier-Chambrillon et al., 2004; Gabriel et al., 2012; Rattner et al., 2012; Ruder et al., 2011; Thomas et al., 2011). The EPA has also developed a risk management decision for ten rodenticides including brodifacoum, bromadiolone, and diphacinone, which the agency has categorized as rodenticides that pose the greatest risk to human health and environment (Erickson & Urban, 2004).

The rodenticides are available to purchase over the counter and they are commonly used to control rodents in agricultural farms and household (J. Spahr, J. Maul, & G. Rodgers, 2007). This could lead to unintentional contamination of milk and other food commodities. Their long acting, fat soluble nature (J. Spahr et al., 2007) could make them remain for longer periods in food producing animals and their products. The tasteless and odorless nature of rodenticides at their lethal concentrations makes impossible to detect them physically as food contaminants. This is one of the major reasons for the extensive incidences of intoxication in humans and animals (Eason, Murphy, Wright, & Spurr, 2001; J. Spahr et al., 2007; Wu et al., 2009). Although the rodenticides are introduced as less toxic to non-target species than to rodents, clinical experience and research has shown they are a significant health threat to other species including human and the environment (Huiæ, Francetiæ, Bakran, Macoliæ-Šariniæ, & Bilušiæ, 2002; Lipton & Klass, 1984; Papin et al., 2007). Since the rodenticides are designed to kill

mammals, their toxicity is very similar to the target rodents and to the humans. See Table 2-2 for classification and toxicological features of anticoagulant rodenticides.

Table 2-2

Classification and Toxicological Features of Anticoagulant Rodenticides (Valchev et al., 2008)

Group	Generic name preparations	Commercial Formula	Chemical	Acute oral toxicity LD_{50} (mg/kg)		
				Albino rats	Dogs	Cats
Hydroxycoumarin						
First generation	Cournachlor	Tomorin	$C_{15}H_{15}ClO_4$	900	-	-
	Cournafuryl	Kumatox, Ratafin	$C_{17}H_{14}O_5$	0.4	6-40	-
	Cournatetralyl	Rodentin	$C_{19}H_{16}O_3$	16.5	-	-
	Wafarin	Warfart, Zoocommarin	$C_{19}H_{15}O_4$	58(11-323)	20-50	1-5
Second generation	Brobifacoum	Folgoart, Kierat,	$C_{31}H_{23}BrO_3$	0.26	0.25-3.56	25 (11-33)
	Bromadiolone	Talon, Rodend	$C_{30}H_{23}BrO_4$	1.125	>10(11-15)	>25
	Difenacoum	Lanirat, Contrac,	$C_{31}H_{24}O_3$	1.8	50	100
	Difethialone	Bromorat, Musal	$C_{31}H_{23}BrO_2S$	0.56	5	>16
	Flocounafen	Matrac, Rastop, Ratak, Silo Frap, Queil Storm	$C_{33}H_{25}F_3O_4$	0.46	0.075-0.25	>10
Indandione	Chlorophacinone	Delta, Patrol	$C_{23}H_{15}ClO_3$	20.5	-	-
	Diphacinone	Ratindan, Ratik	$C_{23}H_{16}O_3$	3	3-7.5	14.7
	Pindone	Pival, Tri-ban	$C_{14}H_{14}O_3$	50	50	-
	Valone		$C_{14}H_{14}O_3$	-	-	-

2.2.2 Human exposure to anticoagulant rodenticides. The incidence of human ingestion of superwarfarin around the world has increased over the past several years (A. T. Nelson, J. D. Hartzell, K. More, & S. J. Durning, 2006; J. E. Spahr, J. S. Maul, & G. M. Rodgers, 2007; S.-L. Zhao, Li, Ji, Zong, & Zhang, 2010). The majority of superwarfarin intoxications occur in the pediatric population. Superwarfarin can be obtained easily over the counter and found in many households and businesses as rodenticides. Superwarfarins have a 100-fold increase in potency compared with warfarin. Therefore if exposed to an amount as little as 1 mg in adults, the patient will show clinical signs of poisoning with coagulopathy and deficiencies of vitamin K-dependent clotting factors II, VII, IX, and X (Mack, 1994). Oral ingestion (whether

intentional or unintentional) and inhalation are the main routes of superwarfarin exposure in humans. However, absorption through the skin can occur, and numerous cases with an unknown origin have been reported (Laposata, Van Cott, & Lev, 2007; J.-H. Lee, Kim, Han, Lee, & Kim, 2009; A. T. Nelson et al., 2006; Pavlu et al., 2005)

Warfarin related compounds are the most commonly ingested rodenticides in the United States, with 13,345 human exposures reported in 1996 (Litovitz et al., 1997). Over 15000 of pediatric cases of superwarfarin poisoning has been reported in 2004 (Watson et al., 2005). Out of the rodenticide exposures of human reported in 2007, there were 11,926 (77.4%) cases of anticoagulant superwarfarin exposures (see Table 2-3) and majority of them (10,220) are children below 6 years of age (Bronstein, Spyker, & Cantilena Jr, 2008). The major clinical signs of humans exposed to superwarfarin are significant coagulopathy or abnormal bleeding, haematemesis, haematuria and cutaneous haemorrhages (Dolin, Baker, & Buck, 2006; Hui, Lie, Lam, & Bourke, 1996; Moery & Pontious, 2009; Watts, Castleberry, & Sadowski, 1990; Wu et al., 2009). Majority of the human cases of superwarfarin poisoning reported have unknown etiology (A. Nelson, J. Hartzell, K. More, & S. Durning, 2006; J. Spahr et al., 2007). Therefore diagnosing a patient with superwarfarin poisoning can be difficult. There may not be a history of superwarfarin exposure, and the initial clinical sign may not be obvious. However, in patients presenting with a coagulopathy of unknown cause, serum superwarfarin (brodifacoum, difenacoum, etc.) tests can confirm superwarfarin intoxication (J.-H. Lee et al., 2009). Furthermore, the documentation of superwarfarin poisoning has been hindered by the lack of readily available assays for these compounds (Watts et al., 1990).

Table 2-3

Human exposures to rodenticides in USA in 2007 (Bronstein et al., 2008)

Rodenticide	Number of cases mentioned	Age		
		< 6	6–19	> 19
Anticoagulant: Long acting superwarfarin	11,926	10,220	350	874
Anticoagulant: Warfarin-type	380	294	13	47
ANTU	5	1	3	0
Bromethalin	533	393	21	72
Cholecalciferol	12	6	0	4
Cyanide	4	0	0	0
Monofluoroacetate	5	3	0	1
Strychnine	89	8	5	48
Vacor	1	0	0	1
Zinc phosphide	123	25	9	50
Other	720	495	64	109
Unknown	1607	977	81	287

2.2.3 Mechanism of toxic activity of anticoagulant rodenticides. Hydroxycoumarins and indandiones depress the hepatic synthesis of vitamin K dependent blood clotting factors and increase permeability of capillaries throughout the body leading to widespread internal hemorrhage (Katona & Wason, 1989). Warfarin and superwarfarin interfere with vitamin K metabolism and inhibit the recycling of vitamin K_1. Vitamin K_1 is an important cofactor for activation of blood clotting factors II, VII, IX and X by the enzyme vitamin K-dependent carboxylase. Superwarfarins inhibit both vitamin K epoxide reductase and vitamin K reductase (see Figure 2-3). Vitamin K epoxide reductase converts vitamin K epoxide (inactive) into vitamin K quinone (active). Vitamin K reductase converts vitamin K quinone (active) into vitamin K1 hydroquinone which involves in activation (carboxylation) of clotting factors II, VII,

IX , and X as well as regulatory proteins S, C and Z (Petterino & Paolo, 2001; Smith, Kraft, Lewis, Melethil, & Freeman, 2000). The inhibition of vitamin K epoxide reductase leads to an accumulation of vitamin K epoxide, resulting in lack of active vitamin K and production of undercarboxylated vitamin K-dependent proteins. They may also cause decreased synthesis of vitamin K-dependent protein in the endoplasmic reticulum (Chua & Friedenberg, 1998; I VALCHEV, BINEV, YORDANOVA, & NIKOLOV, 2008; Ivan Valchev et al., 2008; Wu et al., 2009).

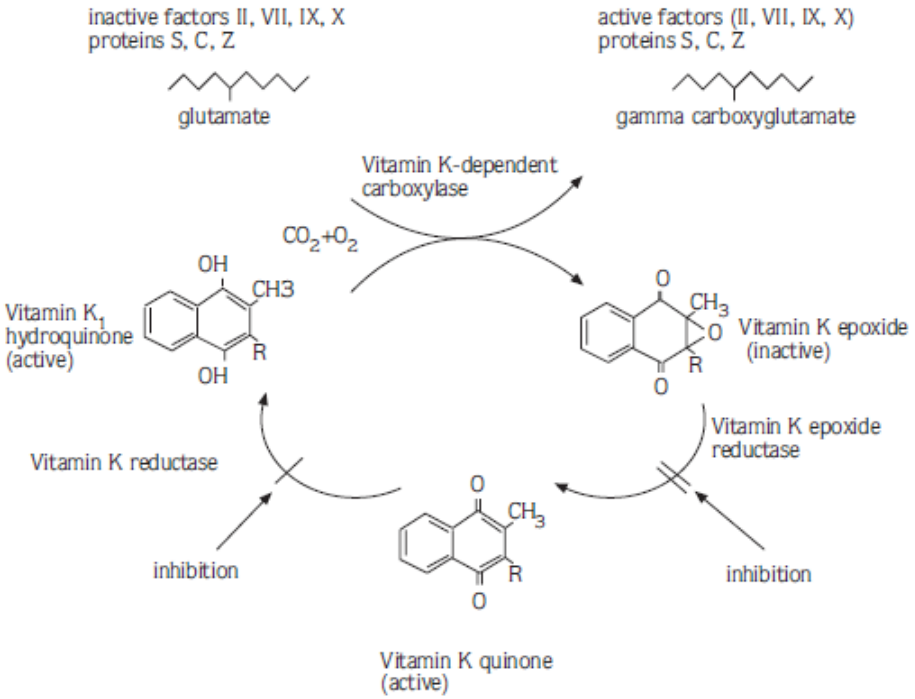


Figure 2-3. Mechanism of toxic effects of anticoagulant rodenticides (Valchev et al., 2008)

Studies have revealed that these rodenticides also can cause birth defects in new born infants due to maternal exposure (Loffredo, Silbergeld, Ferencz, & Zhang, 2001). The modern compounds such as brodifacoum and difenacoum can cause serious poisoning in non-target species including humans. Brodifacoum is much more toxic with a dose as low as 1mg in adult or 0.014mg/kg in children (Mack, 1994). Super warfarins such as brodifacoum and flocoumafen have an elimination half-life from liver greater than 100 days (Huckle, Hutson, et al., 1989;

Katona & Wason, 1989). Due to the long half-lives of these super warfarins it takes a long period of time for elimination from the body after a single exposure.

2.2.4 Strychnine. Strychnine is a very toxic colorless crystalline alkaloid mainly used as a pesticide to control rats, moles, gophers, and coyotes. Strychnine causes muscular convulsions and eventually death through asphyxia or sheer exhaustion. The source of strychnine is seeds of the Indian tree *Strychnos nux vomica*. Strychnine is highly toxic to most domestic animals. Its oral LD_{50} in dogs, cattle, horses, and pigs is between 0.5-1 mg/kg, and in cats is 2 mg/kg. Intentional or unintentional strychnine poisoning occurs mainly in small animals, especially dogs and occasionally cats (Wang, Kruzik, Helsberg, Helsberg, & Rausch, 2007). It is also can be fatal to human (Rosano, Hubbard, Meola, & Swift, 2000). Strychnine is a competitive and reversible inhibitor of the inhibitory neurotransmitter glycine at postsynaptic neuronal sites in the spinal cord and medulla (Martens & Vandevelde, 1993). Strychnine is ionized in an acidic pH and then rapidly and completely absorbed in the small intestine. It is metabolized in the liver by microsomal enzymes. The highest concentrations of strychnine are found in the blood, liver, and kidneys. Strychnine and its metabolites are excreted in the urine. Depending on the quantity ingested and treatment measures taken, most of the toxic dose is eliminated within 24-48 hr (Rosano et al., 2000).

2.3 Techniques Detecting Chemical Residues in Milk

Numerous studies have been carried out to detect chemicals in milk; most of them are to detect antibiotic and pesticide residues. The methods used for detection of antibiotic residues consist of both qualitative and quantitative techniques. Among the quantitative methods, immunoassays, High Performance Liquid Chromatography, and Gas Chromatography methods are common (Ferguson et al., 2005; Knecht et al., 2004; Zacco et al., 2007). Chromatographic

methods are widely used for detection and confirmation of pesticide residues in milk (Bennett, Chung, & Lee, 1997; Pagliuca, Gazzotti, Zironi, & Sticca, 2005; Schenck & Wagner, 1995). According to Cheng, Jandik, and Avdalovic (2005), Ion Chromatographic methods can be used effectively to detect sulfide, cyanide, iodide, thiosulfate, bromide, and thiocyanate in biological samples. Another procedure has been developed by Gordon, Haines, and Wolfe (1961) to detect micro amounts of thiocyanate in milk and animal tissue extracts, using a colorimetric procedure. Several studies have been done to detect rodenticide residues in animal tissues and milk (Huckle, Warburton, Forbes, & Logan, 1989; Hunter, 1985). A reversed phase HPLC has developed to detect anticoagulant rodenticides in animal liver (Fauconnet et al., 1997). A method was developed by Marek and Koskinen (Marek & Koskinen, 2007) for the determination of seven anticoagulant rodenticides, coumafuryl, pindone, warfarin, diphacinone, chlorophacinone, bromadiolone, and brodifacoum, in diverse matrices, including animal feed, cooked beef, and fruit-flavored beverages using high-performance liquid chromatography/electrospray/mass spectrometry. Another rapid procedure was developed by (Palazoglu, Tor, Holstege, & Galey, 1998) for the determination of nine anticoagulant rodenticides in animal serum by high-performance liquid chromatography (HPLC) with a fluorescence and photodiode array detector (DAD). The anticoagulants coumafuryl, pindone, warfarin, coumachlor, diphacinone, chlorphacinone, bromadialone, brodifacoum, and difethialone were extracted and quantitated simultaneously with this method (Palazoglu et al., 1998). A quick and easy method for the analysis of anticoagulant rodenticides in blood or tissue using principles of dispersive solid-phase extraction (dSPE), commonly known as QuEChERS (short for quick, easy, cheap, effective, rugged, and safe), was developed in 2010 by Vudathala, Cummings, and Murphy (2010). Numerous other chromatographic methods have been developed for the simultaneous

determination multiple anticoagulant rodenticides in whole blood, serum, animal plasma and other animal tissues (P. J. Berny, Buronfosse, & Lorgue, 1995; Felice, Chalermchaikit, & Murphy, 1991; Fourel, Hugnet, Goy-Thollot, & Berny, 2010; Guan et al., 1999; Hunter, 1983). Several other methods also have been published for the analysis of indandione anticoagulant rodenticides, such as spectrophotometry (Y. Zhao, 1989) and gas chromatography (GC;(J. Chen, Li, Mai, & Cao, 2007), high-performance liquid chromatography with ultraviolet detector (HPLC–UV;(Fauconnet et al., 1997; Medvedovici, David, & Sandra, 1997), HPLC in combination with mass spectrometry (HPLC–MS; (Guan et al., 1999; Hai-yan, Xiao-hong, & Mi-cong, 2008) and ion chromatography coupled with mass spectrometry(IC–MS; (Cai, Chen, Yan, & Jin, 2009; X. h. Chen, Cai, OuYang, & Jin, 2009).

The existing procedures to detect chemicals and toxins in milk need extensive sample preparation, and they are time consuming. Further, these sophisticated techniques need specific laboratory equipment and trained personnel. Since the milk is distributed among the consumers soon after it is produced, there is a need to screen toxins using a simple on-farm technique, which gives results within few hours, to prevent contaminated milk leaving the farm.

2.4 Immunological Techniques

Antigen and antibody reaction has been used for many years to detect a wide variety of food adulterations and contaminations. There is a specific antigen–antibody interaction which is useful for the detection of residues of chemical and veterinary drugs in animal foods. Enzyme-linked-immunosorbent assay (ELISA) is the commonest technique and the detection system is based on enzyme-labelled reagents. There are different types of ELISA for antigen quantification. In double antibody or sandwich ELISA tests, a primary antibody is bound to the plate well. The antigen of the sample extract added to the well complexes with the bound

antibody and remains bound to the plate after washing. Then, a second antibody labelled with an enzyme such as peroxidase is added to the well followed by a new wash. The quantity of conjugate bound to the plate is detected after incubation with a specific substrate. Colour is developed during incubation and measured with a microplate reader, which is proportional to the amount of analyte in the sample.

Radioimmunoassay (RIA) measures the radioactivity of immunological complex using a counter (Alazzeh, Ibrahim, Song, Shahbazi, & AbuGhazaleh, 2009). Other possibilities include the measure of chemiluminiscence with a luminometer when a chemiluminiscent compound is bound to the antibody or fluorescence with a fluorimeter when a fluorescent compound is used. They allow an enhanced detectability in relation to conventional colorimetry (Ulezlo & Zaprometova, 1981). The advantages of this kit include the capability of analyzing large number of samples per kit, fast to operate and its high specificity and sensitivity in comparison to conventional detection methods. Another advantage is the possibility to use the kit within the food-processing facility without the need to transport the sample to the laboratory. ELISA test kits are widely used for the detection of chemical residues. ELISA kits are available for a large number of substances within each group such as b-agonists, corticoids, steroids, stilbenes, resorcylic acid lactones and several antibiotics. Research continues for the development of new ELISA tests for other substances like sedatives and the b-blocker carazolol (Carrera-Silva et al., 2006).

Development of biosensors is another recent approach to screen animal products for veterinary drugs, to ensure quality and safety of meat and dairy products. These instruments comprise two elements: a biological recognition element, usually an antibody, and a signal transduction element which is in close contact and connected to data acquisition and processing

systems (Gote, Umalkar, Khan, & Khire, 2004). Biosensors are getting expanded applications in food analysis. Biosensors are designed to operate in real time and be able to detect single or multiple veterinary drugs residues in a sample simultaneously.

2.5 Bioassay Techniques

Bioassay techniques, which use bacteria as a biomarker, are widely used qualitative techniques to screen contaminants in food. These techniques are based on the principal of growth inhibition of bacteria in response to chemicals. The bioassay techniques are robust and easy to perform. They detect a wide range of chemicals with satisfactory sensitivity (Popelka et al., 2003; Rault et al., 2004). Numerous studies have been carried out to establish on-farm techniques to detect antibiotic residues in milk (Montero et al., 2005; Seymour, Jones, & McGilliard, 1988; Stead et al., 2008; Zeng et al., 1996). A few researches have been carried out on developing microbiological techniques to detect toxins in milk. *Bacillus stearothermophillus, Bacillus cereus,* and *Bacillus subtilis* have been commonly used as biomarkers in detection of antibiotic residues in food commodities (Nouws et al., 1999; Van Eenennaam et al., 1993). A simple on-farm technique has been developed by Yamani et.al, using yogurt culture to detect antibiotic residues in milk. The yogurt culture they used contains *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub-sp. *bulguricus* (Mohammed I. Yamani et al., 1999). They have shown the sensitivity of LAB to antibiotic residues. LAB have been able to indicate a sufficient growth in 2.5 hours, changing the color of the medium giving satisfactory results. This study shows the suitability of the LAB as a biomarker.

2.6 Lactic Acid Bacteria

Lactic acid bacteria (LAB) comprise a wide range of genera including a considerable number of species. Generally LAB are Gram-positive (Figure 2-4) and usually catalase-negative

non-spore forming bacteria which grow under microaerophillic to strictly anaerobic conditions (Stiles & Holzapfel, 1997). The most important genera of LAB are Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Weissella, Carnobacterium, Tetragenococcus, and Bifidobacterium (Klein, Pack, Bonaparte, & Reuter, 1998).

Figure 2-4. Lactic acid bacteria (source: http://www.biotech-weblog.com).

LAB are found in a variety of habitats where rich carbohydrate-containing substances are available such as the mucosal membranes of humans and animals (oral cavity, intestine and vagina), on plants and material of plant origin, in manure and man-made habitats such as sewage and fermenting or spoiling food. Lactobacilli are omnipresent in the diet and are found in the gastro-intestinal tract in infants soon after birth. In healthy humans, lactobacilli are normally present in the oral cavity (103–104 cfu/g), the ileum (103–107 cfu/g) and the colon (104–108 cfu/g), and they are the dominant microorganism in the vagina (Merk, Borelli, & Korting, 2005; Nagpal et al., 2012).

The group of LAB plays a central role in food fermentation process and has been used from ancient times. It is involved in the production of fermented foods: vegetables, meats and particularly fermented dairy products. Understanding of lactobacilli (e.g., their metabolism and functions) has expanded considerably, over the last decade, opening the way to more reliable

process control in production and an increasing range of industrial dairy applications as starters and adjunct starters/cultures including probiotics; (Leroy & De Vuyst, 2004; Petterino & Paolo, 2001). LAB ferment carbohydrates with lactic acid as a major end product. They also produce acetic acid, ethanol, aroma compounds, bacteriocins, exopolysaccharides, and several enzymes which improve the texture, sensory profile, microbial safety and shelf life of the fermented food. LAB convert the sugar in the medium almost completely into lactic acid via pyruvate to produce energy and equilibriate the redox balance (Leroy & De Vuyst, 2004; Rattanachaikunsopon & Phumkhachorn, 2010). LAB were divided into three sub genera namely "Thermobacterium," Streptobacterium," and Betabacterium according to their growth temperature and their hexose fermentation pathway (Kandler & Weiss, 1986) but modern molecular methods show that these subgroups are inconsistent. Table 2-4 shows the new molecular based grouping along with the classical subgenera.

The LAB used in dairy fermentation industry can be roughly divided into two groups on the basis of their optimum growth. Mesophilic lactic acid bacteria have an optimum growth temperature between 20 °C and 30 °C and the thermophilic have their optimum between 30 °C and 45 °C. Thermophilic lactic acid bacteria are mainly found in the traditional fermented products from sub-tropical countries. The products with mesophilic bacteria originate mainly from Western and Northern European countries (Wouters, Ayad, Hugenholtz, & Smit, 2002). The LAB is also could be divided into two groups as either Homofermenters or Heterofermenters based on the end products formed during the fermentation of glucose. The Homofermenters produce lactic acid as the major product of glucose fermentation whereas Heterofermenters produce carbondioxide, acetic acid and ethanol besides lactic acid during their glucose fermentation. The Homofermenters are able to ferment glucose directly to lactic acid because

they possess the enzyme adolase. Heterofermenters use the alternate pentose monophosphate pathway and convert six carbon sugars (hexoses) to five carbon sugars (pentoses) by enzyme phospoketolaze. They produce both aldehyde and diacetyl substances which enhance the aroma and flavor of the final product (Axelsson, 2004).

Table 2-4

Division of the genus Lactobacillus into subgenera (no longer valid) and into molecular based subgroups (Klein et al., 1998)

Group of lactobacilli	Fermentation pathway	Growth temperature	Representatives used as probiotics
Subgenera:			
Thermobacterium	Homofermentative	15 °C negative 20 °C negative 45 °C positive	*L. acidophilus* group
Streptobacterium	Homofermentative	15 °C positive 45 °C negative	*L.casei* group *L. sake/curvatus*
Betabacterium	Heterofermentative	No general rule	*L. reuteri/L.fermentum*
Molecular based subgroups:			
Group A	Obligatory Homofermentative, no fermentation on pentoses	Not applicable	*L. acidophilus* group
Group B	Facultatively heterofermentative (gas from pentoses)	Not applicable	*L.casei* group, *L. sake/curvatus*
Group C	Obligatory heterofermentative (gas from glucose and pentoses)	Not applicable	*L. reuteri/L. fermentum*

There is a relatively small amount of lactic acid bacteria in raw milk. Therefore most dairy industries today use starter cultures for rapid acidification, because of the slow acidification by existing LAB in milk. Usually the LAB in starter cultures are selected and maintained by sub cultivation in milk which has reduced the number of strains present in the starter culture. These pre-prepared starter cultures are necessary in fermenting pasteurized milk and they are responsible for a certain uniformity of the fermented milk products and cheeses. The main

disadvantage of the introduction of new starter cultures and fermentation techniques is that the danger of raw milk floras being lost. These nonstarter lactic acid bacteria in normal floras are believed to be essential for producing the typical flavors of conventional cheeses (Wouters et al., 2002).

2.6.1 *Streptococcus thermophilus.* *Streptococcus thermophilus* is considered to be the second most important species of commercial LAB after *Lactococcus lactis.* It is used in combination with *Lactobacillus* strains, in starter cultures for the manufacture of fermented milks, yogurt, and various Swiss and Italian cheeses (Delcour, Ferain, & Hols, 2000). Due to the significant increase in the consumption of these milk products during the past two decades, the worldwide use of *S. thermophilus* has enhanced greatly. It is one of the most widely used bacteria in the dairy industry with a market value of approximately 40 billion US dollars. Over 10^{21} of live cells are ingested annually by the human population (Blomqvist, Steinmoen, & Håvarstein, 2006; Mahajan, Desai, & Lele, 2012; Smith et al., 2000). *S. thermophilus* is part of the genus streptococcus, which includes several pathogenic species such as *S. pyogenes* and *S. pneumoniae*. Due to the long history of safe use in food production, it is ‘‘Generally Recognized as Safe’’ (GRAS) in USA and has a Qualified Presumption of Safety (QPS) status in the European Union. Several of the acquired genes found in *S. thermophilus* appear to originate from other dairy species, such as *L. lactis* and *Lactobacillus delbrueckii;* therefore contribute to its adaptation to the milk environment (De Vuyst & Tsakalidou, 2008; J. LeBlanc et al., 2004; Smith et al., 2000). *S. thermophilus* strains have also been isolated from plant sources (J. G. LeBlanc et al., 2008).

2.6.2 Technological and functional characteristics of *S. thermophilus.* *S. thermophilus* is mostly important for the food industry due to its extensive use for the

manufacture of dairy products (Mahajan et al., 2012). *S. thermophilus* is traditionally used in yoghurt in combination with *Lb. delbrueckii subsp. Bulgaricus*. *S. thermophilus* is also used to produce different varieties of cheese, such as Swiss cheese, Brick cheese, Parmesan, Provolone, Mozzarella, and Asiago. It is recently used for the production of Cheddar in combination with mesophilic starters (Delcour et al., 2000; Donkor, Henriksson, Vasiljevic, & Shah, 2007; Ibrahim et al., 2010). One of the main roles of *S. thermophilus* in milk fermentation process is to provide rapid acidification. *S. thermophilus* is the most rapidly acidifying and principal species among LAB throughout the ripening of artisanal cheese (Iyer, Tomar, Uma Maheswari, & Singh, 2010). The rate of acidification is a metabolic characteristic that depends on the strain of *S. thermophilus*. It can be influenced by factors such as lactose–galactose metabolism, proteolytic system and ureolytic activity (Marino, Maifreni, & Rondinini, 2003; Toba, Tomita, Itoh, & Adachi, 1981). Various studies have also documented the prevalence and role of *S. thermophilus* in acid production in dahi samples prepared under household conditions in the Indian subcontinent (Donkor et al., 2007); (Honda, Yajima, & Saito, 2012). It also produces low levels of additional end procucts. Some of these end products includes formate, acetoin, diacetyl, acetaldehyde in addition to lactic acid (Ibrahim et al., 2010; Ott, Germond, & Chaintreau, 2000). Therefore the role of *S. thermophilus* in fermentation of milk is not confined to the production of lactic acid. It has several other important technological properties, such as sugar metabolism, galactose utilization, proteolytic activity, and urease activity. The diversity of phenotypic characteristics that present within the species of *S. thermophilus* is responsible for its diverse technological performance (Iyer et al., 2010).

2.6.3 Lactobacilli. Lactobacilli are widely found in raw milk and dairy products. Many species of lactobacilli are found in fermented milk, including *L. acidophilus, L. rhamnosus, L.*

reuteri, *L. casei*, *L. plantarum*, *L. johnsonii*, *L. crispatus*, *L. paracasei*, and *L. gasseri* (Altman-Hamamdzic et al., 1997). Lactobacilli can be added (*L. delbruecki* subsp. *Bulgaricus)* in yogurt production. Lactobacilli play an important role of the microflora of most of the cheese varieties during ripening. They are also important for flavor of the cheese (Halbmayr et al., 2008; Oliveira, Guimarães, & Domingues, 2011; Wouters et al., 2002). Most frequently isolated species of mesophilic *Lactobacillus* from cheese are *L. casei*, *L. paracasei*, *L. plantarum*, *L. rhamnosus* and *L. curvatus* (Halbmayr et al., 2008). There is a wide biodiversity of lactobacilli in cheeses which has been revealed in previous studies (Macfarlane, Steed, & Macfarlane, 2008). *L. plantarum*, *L. casei* ssp. *casei* and *L. brevis* have been isolated from Spanish goat's milk cheese called Armada cheese (Kim & Rajagopal, 2000). *L. plantarum*, *L. paraplantarum*, *L. paracasei* ssp. *tolerans*, *L. sake*, *L. curvatus*, and *L. pentosus* have been isolated from a traditional Greek cheese made from raw goat's milk named as Batzos (Gänzle, Haase, & Jelen, 2008). Lactobacilli is also added intentionally as starters in the fermentation industry. They play a major role in primary cultures among the various microorganisms involved in cheese making. Its function is to produce lactic acid from lactose. The commonly used *Lactobacillus* species in cheese are *L. delbrueckii* subsp. *bulgaricus*, *L. delbrueckii* subsp. *lactis*, and *L. helveticus*. They have been recovered from Emmental, Parmegiano Reggiano and Italian-type Gorgonzola, Mozzarella, Provolone and Caciocavallo cheeses (Kumar, Ramakrishnan, Teeri, Knowles, & Hartley, 1992). Other species such as *L. casei*, *L. plantarum*, and *L. salivarius* are used to produce many artisanal cheeses from Mediterranean countries (Cortés, Trujillo-Roldán, Ramírez, & Galindo, 2005). Lactobacilli are also used as secondary and adjunct cultures. The species used are all heterofermentative lactobacilli, mainly *L. paracasei* subsp. *paracasei*, *L. rhamnosus*, *L. plantarum*, and *L. curvatus*. Their role is strictly concerned with cheese ripening, and

properties such as proteolysis, lipolytic and antagonistic activity. Probiotic potential of lactobacilli is also used for selecting heterofermentative *Lactobacillus* strains (Greenberg & Mahoney, 1982).

2.6.4 Yogurt culture. The thermophilic LAB *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) is used in association with *S. thermophilus* in the production of yogurts (Weignerová, Simerská, & Kren, 2008). The flavor of yoghurt is mainly determined by the volatile metabolites produced by combination of these two LAB. The mutual benefit between them occurs by producing the amino acids from the milk and organic acids. Therefore they produce more lactic acid and aromatic compounds. Flavor of yoghurt is determined by various compounds, in which lactic acid involved as the major contributor together with other aroma compounds (Routray & Mishra, 2011). *L. bulgaricus* preferentially ferments lactose. It also ferments glucose, fructose and mannose (Garman & Garboczi, 2004). Even though some strains of *L. bulgaricus* are slow fermenters of galactose, it generally does not grow on galactose, (Ghazi, Rooke, & Galbraith, 2003). Most strains of *L. acidophilus* can ferment amygdalin, cellobiose, fructose, galactose, glucose, lactose, maltose, mannose, salicin, sucrose, trehalose and aesculine (Driedonks, Fellinger, Verbakel, Verhue, & Theo Verrips, 1990). *L. acidophilus* utilizes sucrose more effectively than lactose (Shabalin, Kulminskaya, Savel'ev, Shishlyannikov, & Neustroev, 2002) and metabolize citrate releasing CO_2, acetate, diacetyl, acetoin, and 2,3-butanediol (Linden, 1982). *S. thermophilus* and *L. bulgaricus* exhibit mutually favorable interaction in the milk The symbiosis phenomenon was studied by several researchers. It is not characterized by the fact that each bacterium produces one or more substances that stimulate the growth of the other. They have observed a positive effect of the co-culture compared to mono-cultures in growth, acidification, production of flavors and exopolysaccharides, and of

proteolysis (Clarke et al., 2000). Studies on the mutual stimulating factors of these two bacteria has showed that *L. bulgaricus* is stimulated by formic acid and CO_2 produced by the streptococcus. On the other hand *S. thermophilus* is stimulated by the amino acids and small peptides produced from the metabolic activity of *L. bulgaricus* (Schiffman et al., 2001). CO_2 excreted by *S. thermophilus* from the decarboxylation of urea by urease is a stimulating factor of *L. bulgaricus*. Most of the thermophilic LAB (*S. thermophilus, L. bulgaricus* and *L. acidophilus),* preferentially metabolize the glucose moiety of lactose, after its transport and cleavage by β-galactosidase (Mignani & Cagnoli, 2004; Tari, Ustok, & Harsa, 2010).

2.7 Enzyme Activity of LAB

LAB produce functional enzymes that are beneficial for human health. These enzymes has the capability of digesting complex carbohydrates which cannot be metabolized by monogastrics and human beings due to the lack of these enzymes (J. G. LeBlanc et al., 2008; Mahajan et al., 2012). These complex carbohydrates include oligosaccharides (i.e., glycosides, arylglucosides, raffinose, stachyose, cellobiose) and disaccharides such as lactose. When poorly digested, these carbohydrates cause digestive problems in humans resulting in flatulence and abdominal discomfort (J. LeBlanc et al., 2004). LAB can produce number of functional enzymes which include α-glucosidase, α-galactosidase, β glucosidase, β galactosidase and acid phosphatase which can resolve indigestibility problems associated with complex carbohydrates(Donkor et al., 2007; Honda et al., 2012; Ibrahim et al., 2010). The ability LAB to produce functional enzymes and ferment the available carbohydrates varies with the strain, protein source, viable cell count and metal ions in the growth medium (Alazzeh et al., 2009; Donkor et al., 2007; Ibrahim et al., 2010).

2.7.1 α-Galactosidase. *α*-galactosidase (alpha-d-galactoside galactohydrolase; EC 3.2.1.22) is an exoglycosidase (Figure 2-5) that catalyses the hydrolysis of terminal α-1,6-linked D-galactose residues from oligosaccharides (i.e., melibiose, raffinose) and galactopolysaccharides (Carrera-Silva et al., 2006; Ulezlo & Zaprometova, 1981) *α*-galactosidase has been isolated from microorganisms, plants and humans. This enzyme is intracellular, and partially membrane-bound in most microorganisms. It can be produced by microorganisms in culture conditions supplemented with one or more *α*-d-galactopyranosyl groups as carbon sources (Gote et al., 2004). The ability of α-Galactosidase to hydrolyse raffinose and stachyose in leguminous foods and feed, has been used extensively to develop various fermented products using several fermentation approaches. Most of them use LAB strains that ferment soy products and degrade *α*-galactosides, which is economically attractive and also due to many positive effects including the improvement of aroma and other sensory qualities (Kamaly, 1997; Liu et al., 2002).

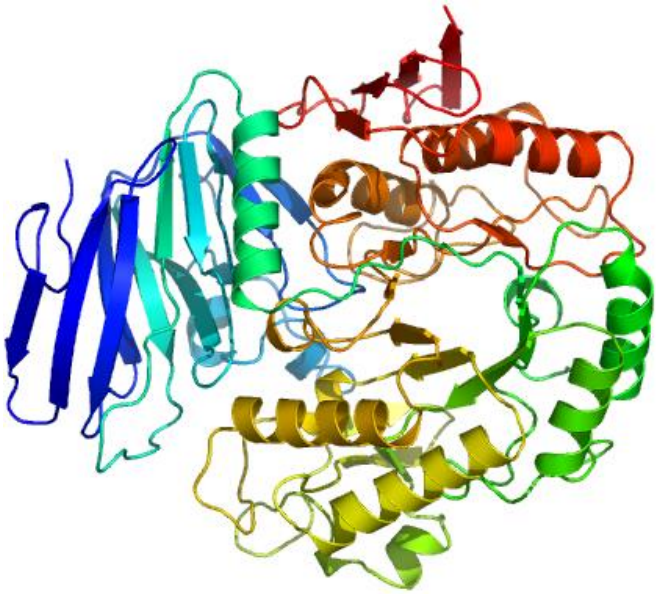


Figure 2-5. The structure of *α*-galactosidase (Source: http://www.jcsg.org/images/stim/1zy9-TM1192.html)

Other than the hydrolyzing of complex sugars in leguminous foods, α-galactosidase has several other food science, industrial and medical applications (Weignerová et al., 2008). α-galactosidase can be used in the pre-treatment of animal feed to hydrolyze non-metabolizable sugars which improves their nutritive value (Ghazi et al., 2003).

Galactose content in guar gum can be reduced using α-galactosidase and improve its properties. Gua gum is a thickening agent used in food products such as jams, drinks and dressings (Driedonks et al., 1990; Shabalin et al., 2002). In the sugar industry α-galactosidase is used to improve the crystallization of sucrose via the hydrolytic cleavage of raffinose (Linden, 1982). Paper and pulp industry uses α-galactosidase to enhance the bleaching effect (Clarke et al., 2000).

Therapeutically α-galactosidase is used in the treatment of Fabry's disease by enzyme replacement therapy (Mignani & Cagnoli, 2004; Schiffman et al., 2001). Defects in human α-galactosidase lead to the development of Fabry disease, a lysosomal storage disorder characterized by the buildup of α-galactosylated substrates in the tissues(Garman & Garboczi, 2004; Guce et al., 2010).

2.7.2 β galactosidase. β galactosidase (β-D-galactoside galactohydrolyse, lactase EC.3.2.1.23) is an enzyme (Figure 2-6) used commercially in the food and pharmaceutical industries (Cortés et al., 2005). β galactosidases catalyze the hydrolysis and transgalactosylation of β-d-galactopyranosides (such as lactose;(Juajun et al., 2011). β-Galactosidases are found widespread in nature. They have been isolated from many different sources including microorganisms (Tari et al., 2010), plants (D. H. Lee, Kang, Suh, & Byun, 2003), and animals (Altman-Hamamdzic et al., 1997). Numerous studies have been carried out on biotechnological

applications of Microbial β-galactosidases (Alazzeh et al., 2009; Halbmayr et al., 2008; Ibrahim et al., 2010; Oliveira et al., 2011; Rajakala & Selvi, 2006).

β-Galactosidases produce by food-grade organisms can be successfully used to solve problems which are related to the milk sugar lactose in the food industry. β-galactosidase hydrolyses lactose into glucose and galactose, which improve digestibility, solubility, and sweetness of dairy products. β-galactosidase also help to digest lactose in lactose-intolerant individuals thereby increase their milk consumption. Lactose maldigestion and intolerance are caused by lactase insufficiency(Kim & Rajagopal, 2000). β-galactosidase also helps to eleminate the problems related to lactose crystallization in frozen concentrated desserts such as ice cream and condensed milk. Crystallization occurs at high lactose concentrations, resulting in sandiness of the products (Gänzle et al., 2008). β-galactosidase also helps to eliminate problems related to disposal of whey which is a lactose-containing by-products in the cheese industry. β-galactosidase converts whey into cheap, renewable, and fermentable sugars after catalyzing the hydrolysis of lactose (Gänzle et al., 2008; Kumar et al., 1992)

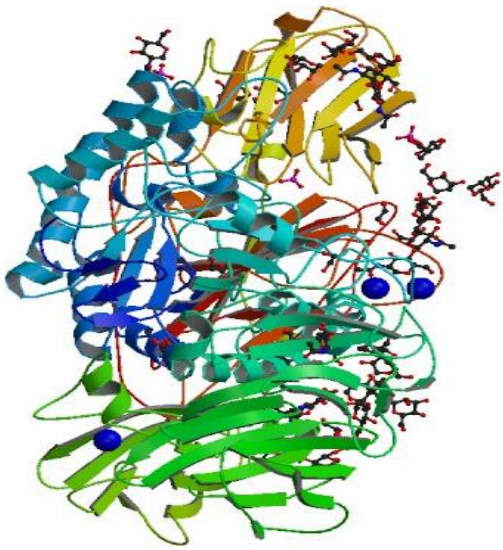


Figure 2-6. The structure of β-galactosidase (Source: http://www.pdb.org/pdb/explore.do?structureId=1TG7).

β-galactosidases also plays an important role in the catalysis of transgalactosylation reaction and production of galacto-oligosaccharides (GOS). GOS are considered as prebiotic food ingredients that can stimulate the growth of beneficial bacteria such as bifidobacteria and lactobacilli which gives beneficial effects to humans (Macfarlane et al., 2008).

S. thermophillus and L. bulgaricus in yogurt culture are promising microorganms for the production of β-galactosidase. The β-galactosidase produced by the yogurt culture is more heat stable and active at neutral pH (Greenberg & Mahoney, 1982). β-galactosidases produced by these thermophillic bacteria is an intracellular enzyme. Therefore they need to be extracted by disruption of the cell membrane using different methods (Bury, Jelen, & Kalab, 2001; Tari et al., 2010).

Safety and security of the milk supply is extremely important to the American dairy industry due to the possibility of intentional contamination of milk. Since the events of Sept. 11, the dairy industry has been working closely with the U.S. Department of Homeland Security, the Food and Drug Administration, the U.S. Department of Agriculture, and other government departments to further safeguard the milk supply from a variety of possible threats. The poor level of bio-security in dairy farms may permit uninvited people, which can lead to intentional contamination of milk with harmful chemicals, leading to possible terrorist attacks. Rodenticides are readily available and widely used pesticides to control rats in the farms and household. They are possible chemicals that can be used in intentional contamination of milk. The milk produced in the farm is distributed around the country; it reaches the consumers within a short period of time. Undetected contamination of milk from a single farm that contains harmful chemicals may contaminate the milk collected in the bulk milk tank. This contaminated milk may distribute rapidly among the consumers, causing severe health hazards to them. Thus, the early detection of chemical contaminants in milk is essential to prevent them entering into the food chain.

CHAPTER 3

Optimum Growth Temperature for Lactic Acid Bacteria in MRS Broth for Early Detection of Rodenticides

3.1 Introduction

Milk is a natural source that produces highly nutritious part of the daily diet. Milk is an excellent source of high quality proteins that human beings cannot synthesize, and milk is the major source of calcium in the diet (A. Haug, Hostmark, & Harstad, 2007; B. D. Roy, 2008). There is a risk of chemical contamination of milk during production at the dairy farm (Martinez, Angulo, Pozo, & Jodral, 1997). The poor level of bio-security in dairy farms, which permits uninvited people, may lead to intentional contamination of milk with harmful chemicals.

Rat poisons are an important tool for pest control, including controlling domestic mice and rats, but the mal practices in marketing and use have been associated with accidental exposures to thousands of children each year (Chua & Friedenberg, 1998; Watt et al., 2005). These products also pose significant risks to non-target wildlife, including both birds and mammals (Fauconnet et al., 1997; Spurr et al., 2005). Rat poisons are chemicals that may be used in intentional contamination of milk. Undetected contamination of milk from a single farm may lead to potential health hazards in human consumers. Acute and prolonged exposure to chemicals through food can cause harmful effects to human health (Hui et al., 1996; Moery & Pontious, 2009; Okolie & Osagie, 1999; Ramirez et al., 2003; Soto-Blanco & Górniak, 2003; Watts et al., 1990; Wu et al., 2009). The low quality milk contaminated with chemicals causes problems in the processing industry, mainly in the production of yogurt and cheese. This type of problem at the processing level, (Boor et al., 1998) can result in severe economic losses.

Rodenticides are available for purchase over the counter as they are commonly used to control rodents in agricultural farms and household (J. Spahr et al., 2007). Warfarin related compounds are the most commonly ingested rodenticides in the United States, with 13,345 human exposures reported in 1996 (Litovitz et al., 1997). Out of the rodenticide exposures of human reported in 2007, there are 11,926 (77.4%) cases of anticoagulant superwarfarin exposures and majority of them (10,220) are children below 6 years of age (Bronstein et al., 2008). Rodenticides are tasteless and odorless at their lethal concentrations. Therefore, it is impossible to detect rodenticides physically when they contaminate food. Rodenticides must be considered as chemicals of choice for intentional adulteration. The existing procedures to detect chemicals and toxins in milk need extensive sample preparation, and these techniques are time consuming (Ler, Lee, & Gopalakrishnakone, 2006). Further, these sophisticated techniques need specific laboratory equipment and trained personnel. Since the milk is distributed among the consumers soon after it is produced, there is a need to screen toxins using a simple on-farm technique, which gives results within few hours, to prevent contaminated milk leaving the farm. Thus, there is an urgent need for developing simple on-farm techniques that can detect toxins in raw milk. Simple and robust on-farm bioassay techniques for detecting harmful toxins in milk before it leaves the farm have both practical and economic benefits.

Bioassay techniques, which use bacteria as a biomarker, are widely used qualitative techniques to screen contaminants in food. These techniques are based on the principal of growth inhibition of bacteria in response to chemicals and detect a wide range of chemicals with satisfactory sensitivity (Popelka et al., 2003; Rault et al., 2004). Selecting a microorganism, which is sensitive to a wide range of chemicals, is essential in developing a bioassay method to

achieve a broader sensitivity. The microorganism used in the bio-assay system should be nonpathogenic and environment friendly.

Lactic acid bacteria (LAB) are nonpathogenic microorganisms possessing beneficial effects on human health. Lactic acid bacteria are widely used in food fermentation, especially in yogurt production. Lactic acid bacteria do not require special laboratory conditions or expensive media for their growth. They do not cause environmental or human health hazards. LAB are capable of producing lactic acid rapidly and changing the acidity of the medium (Leroy & De Vuyst, 2004). The slowed reaction in lactic acid production can be used as a triggered response to changes in the milk content due to the presence of foreign substances. Therefore, it is important to identify the growth of LAB in the presence of toxins in order to determine the suitability of LAB as a biomarker. The objective of this study was to determine the optimum growth temperature for lactic acid bacteria in MRS broth for early detection of rodenticides.

3.2 Materials and Methods

3.2.1 Raw materials and inoculums. The bacterial cultures were obtained from the culture collection of the Food Microbiology and Biotechnology Laboratory at North Carolina A&T State University. Three different commercial yogurt cultures (YF001, YG011 and YI 885) consisting of *Lactobacillus bulgaricus* and *Streptococcus thermophillus* were used.

3.2.2 Preparation of stock culture. The stock cultures were maintained in glycerol. Glycerol solution (approximately 0.5-0.6 ml) was transferred into 1 ml cryogenic vials using 1 ml serological pipettes. Cryogenic vials were kept on Eppendorf rack and covered with a sheet of aluminum foil to prevent vials popping up during sterilization procedure. The cryogenic vials containing glycerol were autoclaved at 121°C for 15 minutes.

The glycerol vials were kept at room temperature for 1-2 hours after sterilization. After the vials were cooled (approximately room temperature or 37 °F), 0.4 ml of activated culture was transferred into vials. A para film was placed over the rim of vial caps before storage. The vials were stored at -80 °C.

3.2.3 Preparation of media. MRS broth was prepared by diluting 55g of Lactobacilli MRS broth (Difco), 0.05g of Manganese sulphate and 0.5g L-Cysteine (Sigma) in 1 L of distilled water. It was stir boiled at 80 °C until dissolved, and dispensed in to tubes (5ml/tube). Tubes containing MRS broth were sterilized at 121°C for 15 minutes, and they were stored at 4°C until used.

3.2.4 Preparation of yogurt culture. The commercial yogurt culture was sub cultured in skimmed milk. Two loops of each yogurt culture was added separately to tubes containing 5ml of sterilized skim milk (12% W/V). They were incubated at 42 °C for 4 hours and refrigerated one week until used.

3.2.5 Inoculum Preparation. The yogurt culture in skim milk (500 µl) was added to a tube containing 5ml of fresh MRS broth and they were incubated at 42 °C for 18 hours to obtain a sufficient growth of bacteria. The tubes were centrifuged at 10000g for 10 minutes, and the supernatant was removed. The supernatant was removed, and 10 ml of peptone water was added. Finally the prepared inoculum was stored at 4 °C until used.

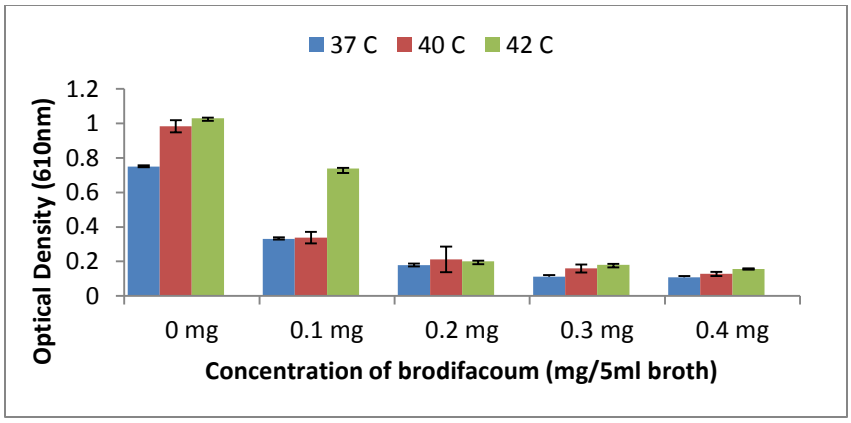
3.2.6 Preparation of Rodenticides. Three selected commercially available rodenticides were used, to test the sensitivity of the yogurt culture to them. They are brodifacoum (Sigma), bromadiolone (Sigma), and diphacinone (Sigma). The stock solutions of rodenticides were prepared by adding 10 mg of rodenticide to 1ml of acetone.

3.2.7 Assay procedure. Tubes containing 5ml of fresh MRS broth were inoculated with activated yogurt culture (300µl/tube). Serially diluted toxins were added (0.025, 0.05, 0.1, 0.2, 0.3, 0.4mg/tube) separately to these test tubes. The tubes containing MRS broth, yogurt culture, and rodenticides were incubated at 37°C, 40°C, and 42 °C for 6 hours. The optical density (OD) of the broth was recorded at 6 hours of incubation.

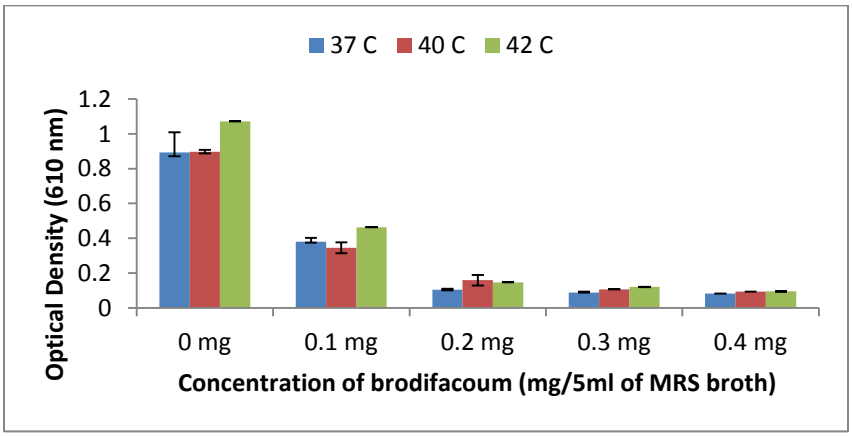
3.3 Results and Discussion

Bioassay techniques are widely used qualitative techniques to screen contaminants in food. They use bacteria as a biomarker. These techniques are based on the principal of growth inhibition of bacteria in response to chemicals. The bioassay techniques are easy to perform and detect a wide range of chemicals with satisfactory sensitivity (Popelka et al., 2003; Rault et al., 2004). A simple on-farm technique has been developed by Yamani et.al, using yogurt culture to detect antibiotic residues in milk. The yogurt culture they used contains *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub-sp. *bulguricus* (Mohammed I. Yamani et al., 1999). It is important to determine the optimum incubation temperature that gives a satisfactory growth of lactic acid bacteria inorder to check it's suitability as a biomarker. This study was focused on selecting the best incubation temperature for the growth of lactic acid bacteria in MRS broth in the presence of rodenticides.

Figures 3-1(a), (b), and (c) show the growth of yogurt culture (a) YF001 (b) YG011 and (c) YI 885 in the presence of brodifacoum in MRS broth at different temperatures. In the control samples as observed by the Optical Density (OD) of the medium, there was a rapid growth of YF001 throughout the incubation period (Figure 3.1(a)). The highest OD was observed at the 42°C indicating the best growth of yogurt culture. Similar results were observed in the growth of YG011 and YI 885 cultures at the incubation temperature of 42 °C (Figure 3-1(b), (c)).



(a)



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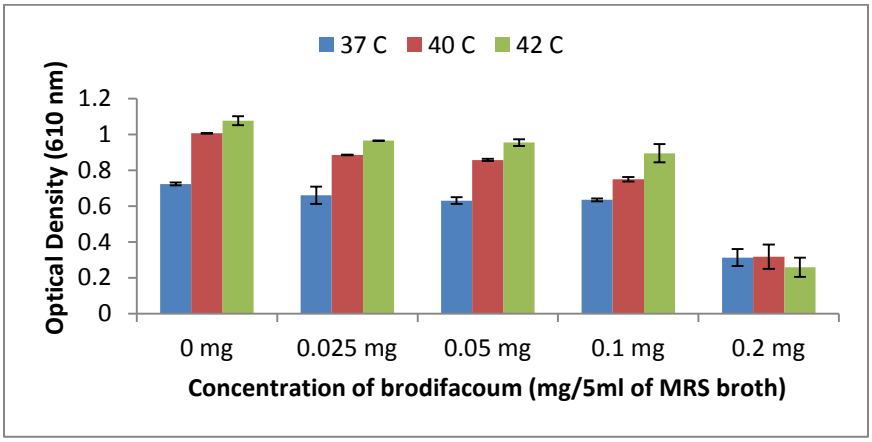


(c)

Figure 3-1. Growth of yogurt culture (a) YF001 (b) YG011 and (c) YI 885 in the presence of brodifacoum, in MRS broth at different temperatures.

The growth of YF001, YG011 and YI 885 was delayed with the increasing concentrations of brodifacoum in the medium indicating the inhibition of bacterial growth in the presence of rodenticides. The highest growth of yogurt culture was observed at 42 °C.

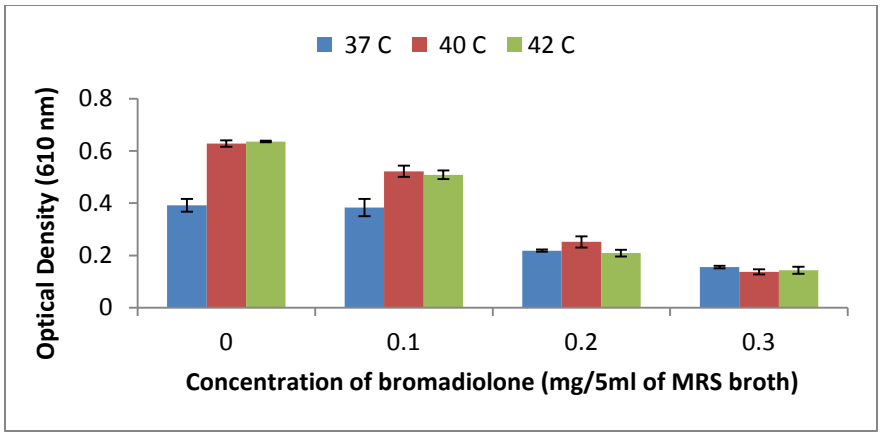
Figure 3-1(a) shows the growth of yogurt culture YF001 in the presence of brodifacoum in MRS broth at different temperatures. In the control sample as observed by the OD of the medium, there was a rapid growth of YF001 throughout the incubation period. The highest growth of YF001was observed at 42 °C (see Figure 3-2).

Figure 3-2. Growth of yogurt culture YF001 in the presence of diphacinone in MRS broth at 42° C.

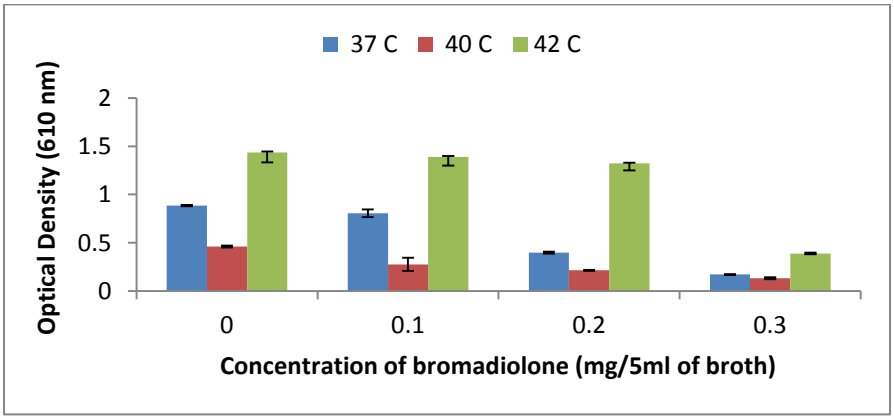
The growth of the YF001 was delayed with the increasing concentrations of rodenticides in the medium indicating the inhibition of bacterial growth in the presence of rodenticides. When brodifacoum was added in the level of 0.1mg/5ml of MRS broth, there was a significant inhibition ($p < 0.05$) of YF001 at 6 hours of incubation compared to the control sample. The best growth of

YF001 was observed at 42° C. The lowest growth of YF001 as observed by OD was observed at 37° C. When brodifacoum was added in 0.2mg, 0.3mg, and 0.4mg/5ml of MRS broth, there was a significant inhibition ($p < 0.05$) of the growth of YF001 at 6 hours of incubation period (Figure 3-1 (a)). However when brodifacoum was added in 0.2mg, 0.3mg, and 0.4mg/5ml of MRS broth, there were no significant differences in the growth of YF001 in different incubation temperatures. This could be due to the higher sensitivity of yogurt culture to brodifacoum. Similar results were observed in the growth of YG011 in the presence of brodifacoum (Figure 3-1 (b)). Among the three yogurt cultures, the best sensitivity for brodifacoum was observed in YI 885 (Figure 3-1 (c)). The minimum inhibitory concentration of brodifacoum was at the level of 0.025mg/5ml (0.005 mg/ml; $p < 0.05$) of MRS broth. The best growth of YI 885 was observed at 42 °C. The lowest growth of YI 885 was observed at 37 °C. The minimum growth of YI 885 (the lowest OD) was observed at the highest concentration of brodifacoum (0.2mg/5ml) in MRS broth. At the highest level of brodifacoum (0.2mg/5ml) in the medium, at 42° C the growth of YI 885 was lower than that at 37 °C and 40 °C. This could be due to the higher sensitivity of YI 885 to brodifacoum at 42 °C.

Figures 3-3 (a), (b), and (c) show the growth of yogurt culture (a) YF001, (b) YG011, and (c) YI 885 in the presence of bromadiolone in MRS broth at different temperatures. There was a rapid growth of all three yogurt cultures in the control samples throughout the incubation period. As observed by the Optical Density (OD) of the medium, the highest OD was observed at the 42 °C indicating the best growth of yogurt culture. The growth of YF001, YG011, and YI 885 was delayed with the increasing concentrations of bromadiolone in the medium indicating the inhibition of bacterial growth in the presence of rodenticides. The highest growth of yogurt culture was observed at 42 °C.



(a)



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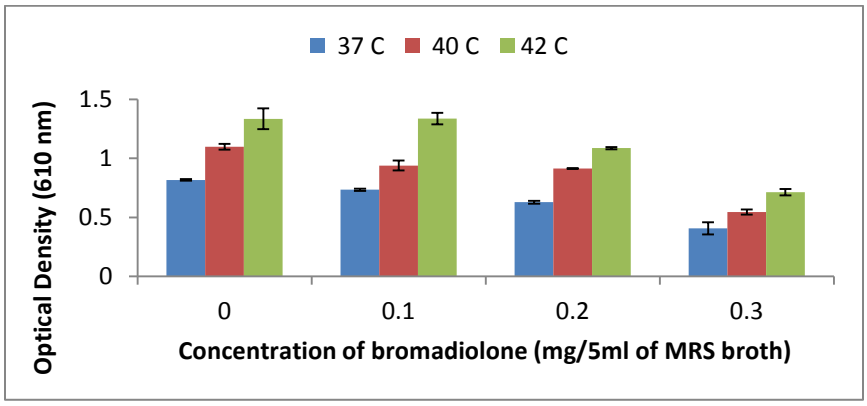
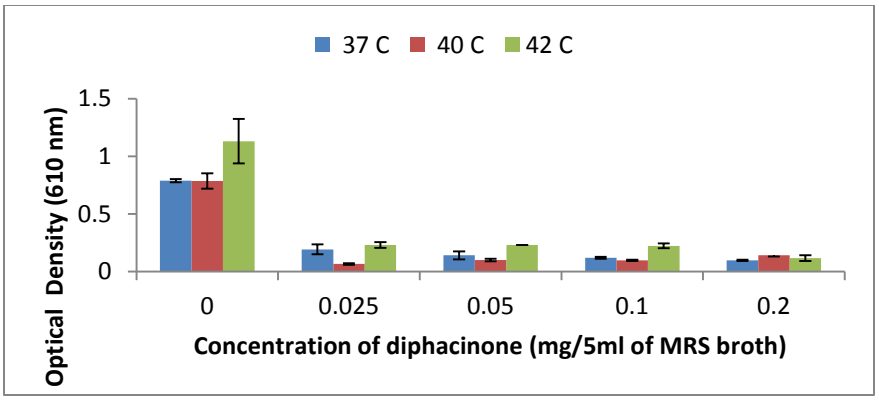


(c)

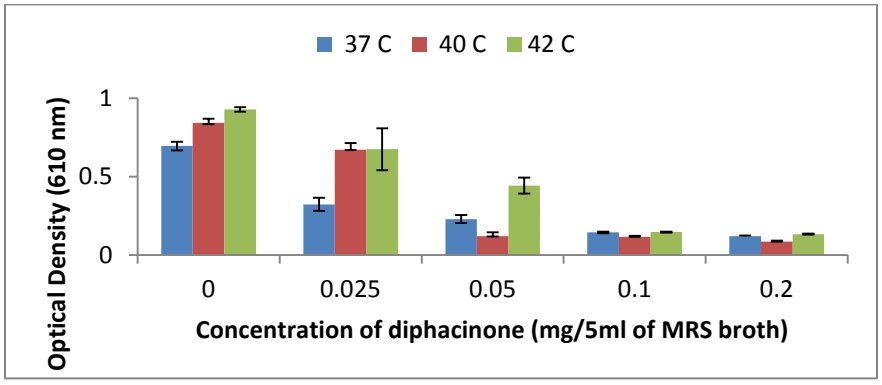
Figure 3-3. Growth of yogurt culture (a) YF001, (b) YG011, and (c) YI 885 in the presence of bromadiolone, in MRS broth at different temperatures.

Figures 3-4(a), (b) and (c) show the growth of yogurt culture (a) YF001 (b) YG011 and (c) YI 885 in the presence of diphacinone in MRS broth at different temperatures. In the control samples as observed by the Optical Density (OD) of the medium, there was a rapid growth of YF001, YG011 and YI 885 throughout the incubation period. The best growth of yogurt culture (highest OD) was observed at 42 °C. The lowest growth of yogurt culture in the control sample was observed at 37 °C. The growth of YF001, YG011, and YI 885 was delayed with the increasing concentrations of brodifacoum in the medium indicating the inhibition of bacterial growth in the presence of rodenticides (Figure 3-2). The highest growth of yogurt culture was observed at 42 °C.

Figures 3-3 (a), (b), and (c) illustrate the growth of yogurt culture (a) YF001, (b) YG011, and (c) YI 885, respectively, in the presence of bromadiolone in MRS broth at different temperatures. The growth of the yogurt cultures YF001, YG011, and YI 885 at 42 °C, in the control samples, as observed by the turbidity (OD at 610 nm) increased constantly (Figure 3-3 (a)) and reached the maximum (OD-0.637, 1.438, and 1.336) at 6 hours of incubation. The best growth was observed at 42 °C. When bromadiolone was added at the levels of 0.1 and 0.2mg/5ml of broth, in all the incubation temperatures, the growth of the yogurt culture was significantly reduced ($p < 0.05$) after 6 hours of incubation. However the highest growth was observed at 40° C. At the level of 0.3mg/5ml of broth, the growth of the yogurt culture was significantly lower than that of the control sample, which indicates the inhibition of yogurt culture in the presence of bromadiolone. The best growth (OD- 0.144) of YF001 at the bromadiolone level of 0.3mg/5ml of broth was observed at 42° C. The same pattern of growth in the yogurt cultures YG011 and YI 885 was observed in the presence of bromadiolone in the medium.



(a)



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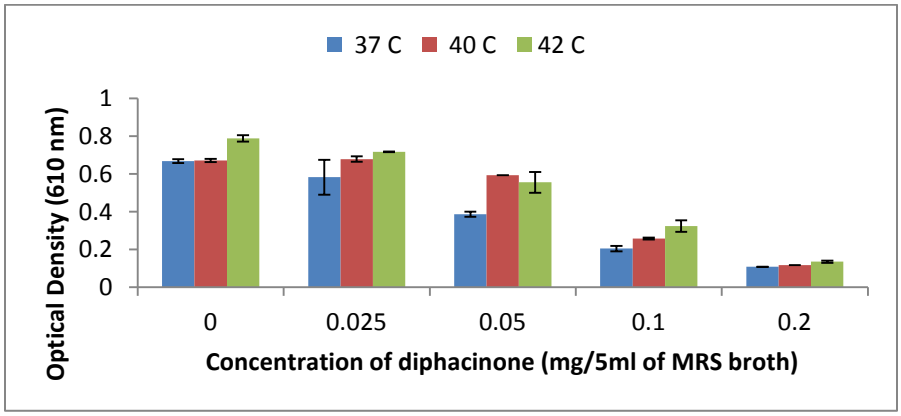


(c)

Figure 3-4. Growth of yogurt culture (a) YF001 (b) YG011 and (c) YI 885 in the presence of diphacinone in MRS broth at different temperatures.

The growth inhibition of YG011 at 42 °C was significant ($p < 0.05$) at 6 hours of incubation (Figure 3-2 (b)). In the control samples, the minimum growth (lowest OD value) of the yogurt culture YG011 (Figure 3-2 (b)) was observed at 40 °C. The highest OD (1.438) was observed at the incubation temperature 42 °C indicating the best growth of YG011. When bromadiolone was added at the levels of 0.1, 0.2, and 0.3mg/5ml of broth, after 6 hours of incubation, the growth of the yogurt culture YG011 was significantly higher ($p < 0.05$) at 42 °C than that of 37° C and 40 °C. Similar pattern of growth in the yogurt culture YI 885 was observed in the presence of bromadiolone (Figure 3-2 (c)) indicating the best growth (highest OD) at 42 °C incubation temperature.

Figures 3-4 (a), (b), and (c) illustrate the growth of yogurt culture (a) YF001 (b) YG011 and (c) YI 885 in the presence of diphacinone in MRS broth at different temperatures.
The growth of the all three yogurt cultures, in the control samples (as measured by OD) rapidly increased with time (at incubation temperatures 37 °C, 40 °C and 42 °C) showing higher OD values at 6 hours of incubation. The best growth of yogurt cultures was observed at 42 °C, YF001, YG011 and YI 885 cultures obtaining OD of 1.132, 0.929, and 0.789, respectively. The growth of yogurt cultures was significantly lowered ($p < 0.05$) at 37 °C, 40 °C, and 42 °C with the increasing concentration of diphacinone in the medium at 6 hours of incubation period. But the OD of the medium at 42 °C was significantly ($p < 0.05$) higher than that of 37 °C and 40 °C, indicating the best growth at 42 °C. Yamani et al. (1999) used yogurt culture containing *Streptococcus thermophilus* and *Lactobacillus delbrueckii* sub-sp. *bulguricus* to detect antibiotic residues in milk. They have also obtained best growth of yogurt culture incubating 2.5 hours at 42 °C (Mohammed I. Yamani et al., 1999).

3.4 Conclusions

All the three yogurt cultures showed significantly ($p < 0.05$) higher growth (as observed by the OD) at 42 °C incubation temperature with few exceptions. The lowest growth of yogurt culture was observed at 37 °C except in YG011 in the presence of bromadiolone. YF001, YG011 and YI 885, showed highest sensitivity to diphacinone at the level of 0.025 mg/5ml of broth (0.005mg/ml). YF001 showed detectable sensitivity to brodifacoum, bromadiolone and diphacinone at the levels of 0.02mg/ml, 0.02mg/ml, and 0.006mg/ml, respectively. Minimum inhibitory concentration of brodifacoum, bromadiolone and diphacinone for YG011 was observed at the levels of 0.02mg/ml, 0.02mg/ml, and 0.006mg/ml respectively. Our results show that the growth of all three yogurt cultures was inhibited in the presence of rodenticides. The best incubation temperature for the yogurt cultures in the presence of rodenticides was 42 °C. Yogurt culture could be used as a biomarker for the early detection of the presence of rodenticides in milk after evaluation of acidic and enzymatic coagulation properties at different concentrations of rodenticides.

CHAPTER 4

Effect of Selected Rodenticides on the Growth and Activity of Different Strains of Lactic Acid Bacteria in MRS Broth

4.1 Introduction

Milk is a widely consumed food, and represents a target that could have major impact on our personal and economic health. The milk can be contaminated unintentionally or intentionally with harmful chemicals at the time of production (M. I. Yamani, L. Al-Kurdi, M. Haddadin, & R. Robinson, 1999). Dairy farms are considered an exceptionally porous industry from a security stand point. The poor level of bio security in majority of the dairy farms today guarantees uninterrupted access to uninvited people. By any standard, food safety and quality inspections represents a monumental task, but the protection of the nation's food supply is vital given the repercussions that intentional contamination of milk could produce in terms of human illnesses and deaths. The contaminated milk also interfere with the starter cultures used in milk processing, especially in yogurt and cheese industry which leads to massive economic losses.

Contamination can be limited if we develop our capacity to use modern detection methods to identify harmful agents and apply rapid methods to determine which stage of processing the contamination occurred because there are multiple vulnerability points in production and processing of milk which a contaminant could be introduced.

Rodenticides are widely used pesticides in the farms and household to control rodents. Superwarfarins are the most commonly used rodenticides in USA. They are long acting, fat soluble, anticoagulants 100 times more potent than warfarin (M. Hadler & A. Buckle, 1992; Park & Leck, 1982). Since the development of these anticoagulant rodenticides many accidental and intentional cases of exposures to humans and animals have been reported. Most of the human

cases of rodenticide exposure have unknown etiology (A. Nelson et al., 2006; J. Spahr et al., 2007) Most of the cases are not documented due to the lack of readily available assays for these compounds, even from the manufacturers (Watts et al., 1990).

Rodenticides are very similar in powder form in regards observational physical characteristics. Due to their odorless and tasteless nature, they must be considered as chemicals of choice for intentional adulteration. Since super warfarin is popular and readily available in the stores there is a possibility of intentional and unintentional contamination of milk by them during production in the farm. Due to multifunctional production process involving converting milk to cheese, there is also a great potential for intentional contamination in any processing stage. It is estimated that toxins concentrations are 3-4 folds higher in cheese curd than in milk. If such products reached consumers, it could create a public health crisis. Therefore, it is important to understand the interaction between chemically adulterating agents and milk proteins during cheese processing to help identify intentional contamination of milk that would likely lead to better isolation of the contaminating substance. This can be critical in reducing time lost to processing an unusable food product as well as reducing the need for unnecessary destruction of usable milk and milk products. Results obtained from this research would be important for interventions and countermeasures in preventing final retail distribution and consumer consumption of intentionally contaminated milk.

During food manufacturing the detection for the presence of toxins in food requires the use of third party laboratory testing which usually take days for final results. Because raw milk represents the most vulnerable step in manufacturing cheese, determination that the milk used in the production of cheese is toxin free is very important.

We have preliminary data from our lab that demonstrates that the lactic acid bacteria (LAB), commonly used in the dairy industry can serve as a simple and rapid tool to identify adulterated milk (Hathurusinghe, Ibrahim, Gyawali, & Tajkarimi, 2011). The principle logic behind this is that LAB are capable of rapid production of lactic acid. A slowed reaction in lactic acid production can be used as a triggered response to changes in the milk content due to the presence of foreign substances. The same logic can be applied to cheese during the rennet or curd production stage of cheese manufacture. The formation of cheese milk is a step by step process which includes three enzymatic coagulation stages. Temperature conditions, pH, and Ca^{2+} must be monitored after rennet has been added to cheese milk. Decreases in pH levels result in increased enzyme activity. This is primarily due to neutralization of charge repulsion between casein micelles. Nájera, De Renobales, and Barron (2003), who did multi factorial study on the factors involved in coagulation time, found that pH showed the greatest effect on rennet coagulation time and temperature was the major influence on gel firmness.

LAB are rod shaped bacilli or cocci that gives numerous beneficial effects to humans and animals. They are involved in fermentation of milk as a functional starter culture contributing to the milk processing industry (Leroy & De Vuyst, 2004). They produce lactic acid as a result of fermentation of carbohydrate in the medium. The faster growth and non-pathogenic properties of LAB increase its suitability to use as a biomarker for early detection of toxins in milk.
The sensitivity of LAB to different chemicals can vary due to several reasons. Previous studies have proved that the survival of different strains of LAB varies with the pH of the medium (Conway, Gorbach, & Goldin, 1987). Some LAB strains are more resistant to the harsh conditions and they can survive in highly acidic medium (Shah & Jelen, 1990). LAB produce bacteriocins that have antibacterial effects against pathogenic bacteria. The survival of them

depends on the ability of different strains of bacteria to produce bacteriocins (Soomro, Masud, & Anwaar, 2002). The genetic variations of LAB strains also contribute to their ability to survive in different environments (Konings et al., 1997). The effect of toxins on the growth and activity of LAB and on the formation of rennet induced milk gels in cheese milk has not been investigated. Our hypothesis is that chemical toxins will slow rennet-induced milk gel formation through enzyme inhibition, chelation of Ca^{2+} and pH increases or shifts in isoelectric points. In a cheese factory an opportune time and place for a terrorist to inflict the most harm to the overall cheese production batch would be during the addition of starter culture and rennet enzymes which is fed to a cheese vat during the standardization process. The sensitivity of different LAB to toxins also has not been investigated, but they may vary with their ability to survive with the concentration and the nature of the toxin. Therefore, it is important to detect the sensitivity of different strains of LAB towards toxins to identify the most sensitive strain. The objective of this study is to determine the effect of selected rodenticides on the growth and activity of different strains of lactic acid bacteria in MRS broth.

4.2 Materials and Methods

4.2.1 Bacterial strains and culture conditions. Three different commercial yogurt cultures (YF001, YG011, and YI 885) consisting of *Lactobacillus bulgaricus* and *Streptococcus thermophillus* were used. The yogurt cultures were obtained from the culture collection of the Food Microbiology and Biotechnology Laboratory at North Carolina A&T State University. The commercial yogurt culture was sub cultured by adding two loops of each yogurt culture to tubes containing 5 ml of sterilized skim milk (12% W/V). This culture was then incubated at 42 °C for 5 hours and kept refrigerated until used. The yogurt culture in skim milk was activated prior to the test using MRS broth (Difco Laboratories, Becton Dickinson, Sparks, MD). The MRS broth

which was prepared by adding 55 g MRS to 1L of deionized water, with 0.5 g cysteine, and then autoclaved at 121 °C for 15 minutes. To activate the yogurt culture, 500 µl of culture from the skim milk was added to a tube containing 5 ml of fresh MRS broth (Difco Laboratories, Becton Dickinson, Sparks, MD) and incubated at 42 °C for 18 hours. Then the tube was centrifuged at 8000g for 10 minutes, and the supernatant was removed. The pellet was resuspended in 10 ml of peptone water. This activated inoculum was used for the assay.

4.2.2 Preparation of rodenticides. Three selected commercially available rodenticides were used, to test the sensitivity of the yogurt culture to them. They are brodifacoum (Sigma), bromadiolone (Sigma), and diphacinone (Sigma). The stock solutions of rodenticides were prepared by adding 10 mg of rodenticide to 1ml of acetone.

4.2.3 Assay procedure. Tubes containing 5ml of fresh MRS broth were inoculated with activated yogurt culture (approximately 3.0 log CFU/ml). Serially diluted rodenticides were added (0.025, 0.05, 0.1, 0.2, 0.3, 0.4mg/tube) separately to these test tubes. The tubes containing MRS broth, yogurt culture, and rodenticides were incubated at 42 °C for 6 hours. The optical density (OD) and the pH of the broth were recorded (using pH meter Model 410A, Orion, Boston, MA) at 0, 2, 4 and 6 hour intervals. The bacterial colonies were counted using the standard enumeration method to determine the viable bacterial population at the end of incubation.

4.2.4 Bacterial growth. The bacterial growth was monitored by measuring OD at two-hour time intervals (0, 2, 4, 6 hours). The OD was measured using spectronic 21 Milton Roy spectrophotometer (Model Genesys 10 Vis, Thermospectronic, Rochester, NY, USA) at the wavelength of 610 nm.

4.2.5 Bacterial enumeration. The bacterial colonies were counted using the standard enumeration method to determine the viable bacterial population at the end of incubation. After incubation the bacteria were serially diluted by adding 1 ml of the broth to 9 ml of 1% peptone water and plated (100 µl) on triplicate plates of MRS agar (Difco Laboratories, Becton Dickinson, Sparks, MD). The MRS agar was prepared by adding 55 g MRS to 1L of deionized water, with 0.5 g cysteine and 14 g agar and then autoclaved at 121 °C for 15 minutes. The bacterial colonies were counted to determine the bacterial population after incubating the plates at 37 °C for 24 hours.

4.2.6 Determination of α and β galactosidase activity. Alpha and beta-galactosidase activity was determined at the end of the incubation. LAB in yogurt culture were harvested after 6 hours of incubation according to the method described in (Alazzeh et al., 2009) with slight modifications. The samples were centrifuged 8000g at 4 ˚C for 10 minutes, discarding the supernatant and washing the precipitated strains twice with phosphate buffer (pH 6.8). Then the bacteria cells were resuspended in 1 ml of phosphate buffer in ependorf tubes containing 0.1 mm glass beads(BioSpec Products Inc., Bartlesville, OK, USA) . The samples were treated with a bead beater (MiniBead Beater-8, BioSpec products Inc., Bartlesville, OK, USA) for 3 minutes. The samples were rested for 15 s in an ice bath after every minute. Samples were then centrifuged at 12000g for 12 minutes and supernatant was stored at -80 ˚C until used as the enzyme source.

4.2.7 α-galactosidase assay. α-galactosidase activity was assayed according to the method described in Food Chemicals Codex (Codex, 2003) by adding 1 ml of p-nitrophenyl-α-D- galactopyranoside substrate and 0.5 ml of sample. The samples were transferred immediately into a 37 °C water bath for 15 minutes. A blank sample was prepared by adding 0.5 ml of

distilled water, 1 ml of substrate. The reactions were stopped at 15 minutes by adding 2.5 ml of Borax buffer. The light absorbance was measured at 405 nm by spectrophotometer (Model Genesys 10 Vis, Thermospectronic, Rochester, NY, USA). One galactosidase activity unit (GalU) is defined as the quantity of the enzyme that will liberate p-nitrophenol for alpha at the rate of 1 mol/min under the conditions of the assay.

Units of α-gal were calculated using the following calculation.

$$\boldsymbol{GalU/g} = \frac{[(\boldsymbol{AS} - \boldsymbol{AB})\ \boldsymbol{X}\ \boldsymbol{F}]}{(\boldsymbol{\varepsilon}\ \boldsymbol{X}\ \boldsymbol{T}\ \boldsymbol{X}\ \boldsymbol{M})}$$

Where

AS is the sample absorbance;

AB is the blank absorbance;

F is the appropriate dilution factor;

T is the reaction time, in minutes;

M is the weight, in grams, of the sample

Epsilon is a factor calculated for the p-nitrophenol (proportional to the millimolar extinction coefficient for p-nitrophenol) standards using the following equation:

Epsilon= AN/C,

in which

AN is the absorbance of the p-nitrophenol standards at 405 nm

C is the concentration, in millimoles per milliliter, of p-nitrophenol.

4.2.8 β-galactosidase assay. β-galactosidase activity was determined according to the method described by Nagy, Kiss, Szentirmai, and Biró (2001) by adding 0.5 ml of sample and 0.5 ml of 15 mM 0-nitrophenyl-β-D-galactopyranoside in 0.03 M sodium phosphate buffer (pH

6.8). The tubes were kept in a shaking water bath at 37 °C for 10 minutes and the reactions were stopped by adding 2 ml of 0.1 M sodium carbonate. The optical density was measured at 420 nm with the spectrophotometer (Model Genesys 10 Vis, Thermospectronic, Rochester, NY, USA). The unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1µmol of *o*-nitrophenyl per minute under assay condition. One galactosidase activity unit (GalU) is defined as the quantity of the enzyme that will liberate 0-nitrophenol for beta at the rate of 1 mol/min under the conditions of the assay.

Units of β-gal were calculated using the following calculation.

$$\boldsymbol{GalU/g} = \frac{[(\boldsymbol{AS} - \boldsymbol{AB})\ \boldsymbol{X}\ \boldsymbol{F}]}{(\boldsymbol{\varepsilon}\ \boldsymbol{X}\ \boldsymbol{T}\ \boldsymbol{X}\ \boldsymbol{M})}$$

Where

AS is the sample absorbance;

AB is the blank absorbance;

F is the appropriate dilution factor;

T is the reaction time, in minutes;

M is the weight, in grams, of the sample

Epsilon is a factor calculated for the 0-nitrophenol (proportional to the millimolar extinction coefficient for 0-nitrophenol) standards using the following equation:

Epsilon= AN/C,

in which

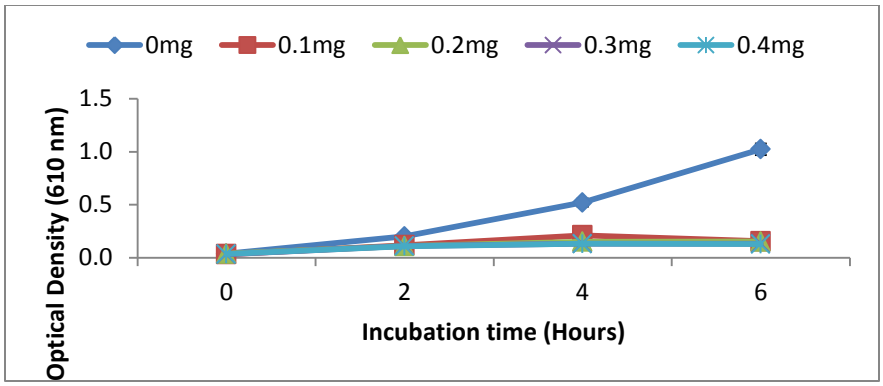
AN is the absorbance of the o-nitrophenol standards at 420 nm

C is the concentration, in millimoles per milliliter, of 0-nitrophenol.

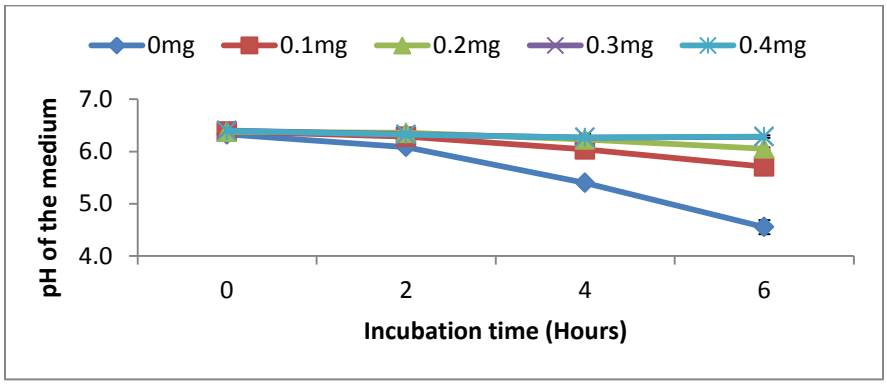
4.2.9 Statistical Analysis. Data analyses were focused on determining if the addition of different concentrations of rodenticides resulted in significantly lower growth of yogurt culture over that of the control group. Statistical analysis of data was performed using the SAS General Linear Model (GLM) procedure of SAS software (SAS Institute Inc., Cary, NC). Duncan's multiple range test was used to find significant differences ($p < 0.05$) between treatments at a particular time. For each growth condition, the test was repeated four times.

4.3 Results and Discussion

Figures 4-1 (a) and (b) show the growth of yogurt culture YF001 in MRS broth in the presence of brodifacoum. In the control sample as observed by the turbidity (OD at 610 nm), there was a rapid growth of the YF001 throughout the incubation period (Figure 4-1(a)). The growth of the yogurt culture was delayed with the increasing concentration of brodifacoum in the medium indicating the inhibition of bacterial growth in the presence of brodifacoum. When bromadiolone was added in 0.1, 0.2, 0.3 and 0.4mg/5ml of broth, the initial growth at 2 hours, as observed by the OD, was low. It continued to grow slowly with the time, but there was a significant difference ($p < 0.05$) of the growth of yogurt culture at 4 hours and 6 hours of incubation period in the presence and absence of brodifacoum. The results indicated that the yogurt culture was highly inhibited after 6 hours of incubation at the rodenticide level of 0.4mg/5ml of MRS broth. The same pattern of growth in the yogurt culture was observed in the samples when the pH values were measured (Figure 4-1 (b)). The lowest growth of the yogurt culture (lowest OD value (Figure 4-1(a)) and the highest pH (Figure 4-1(b)) were observed at the highest concentration of brodifacoum in the medium. The minimum inhibitory concentration was observed at the level of 0.1 mg/5ml (0.02 mg/ml; $p < 0.05$) of broth.



(a)

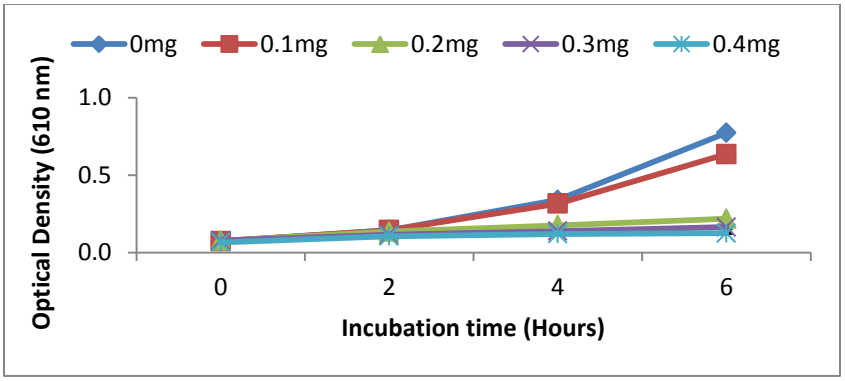


(b)

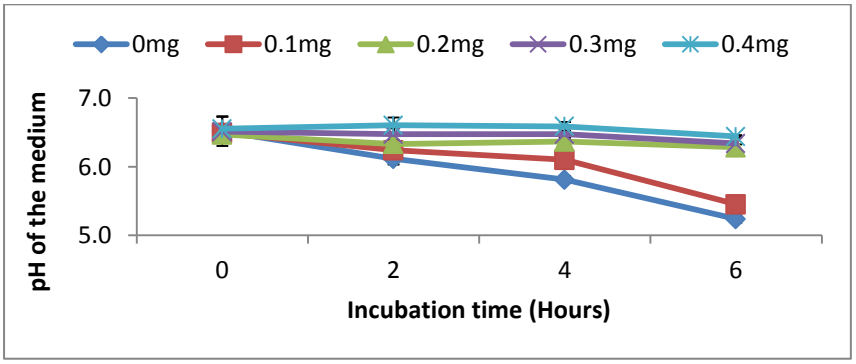
Figure 4-1. Survival and growth of YF001 in MRS broth in the presence of brodifacoum.

Figures 4-2 (a) and (b) illustrate the sensitivity of yogurt culture YF001 in MRS broth in the presence of bromadiolone. The growth of the yogurt culture in the control sample, as observed by the turbidity (OD at 610 nm) increased constantly (Figure 4-2 (a)) and reached the maximum (OD-0.774) at 6 hours of incubation. When the bromadiolone was added at the level of 0.1mg/5ml of broth, there was no significant difference of the growth of the yogurt culture compared to the control sample. However, when bromadiolone was added at the levels of 0.2, 0.3, and 0.4mg /5ml of broth, the growth of the yogurt culture was significantly reduced ($p <$ 0.05) after 6 hours of incubation (OD-0.219, 0.165, and 0.126 at the levels of 0.2, 0.3, and 0.4mg/5ml of broth, respectively). This indicates the inhibition of bacterial growth in the presence

of bromadiolone. The lowest growth of YF001 (highest OD) was observed at the rodenticide level of 0.4mg/5ml of MRS broth. Similar pattern of growth in the yogurt culture was observed in the samples when the pH values were measured (Figure 4-2 (b)). The pH value of the control sample decreased rapidly (pH 5.24) during the 6 hour incubation period. This pH drop indicates the growth and lactic acid production by the yogurt culture. In the presence of bromadiolone in the medium, the growth inhibition of the yogurt culture was significant ($p < 0.05$) at 6 hours of incubation. The minimum growth of the yogurt culture (lowest OD value (Figure 4-2 (a)) and the highest pH (Figure 4-2 (b)) was observed at the highest concentration of bromadiolone (0.4mg/5ml) in the medium. The minimum inhibitory concentration was observed at the level of 0.1mg/5ml (0.02mg/ml) of broth at 6 hours of incubation.



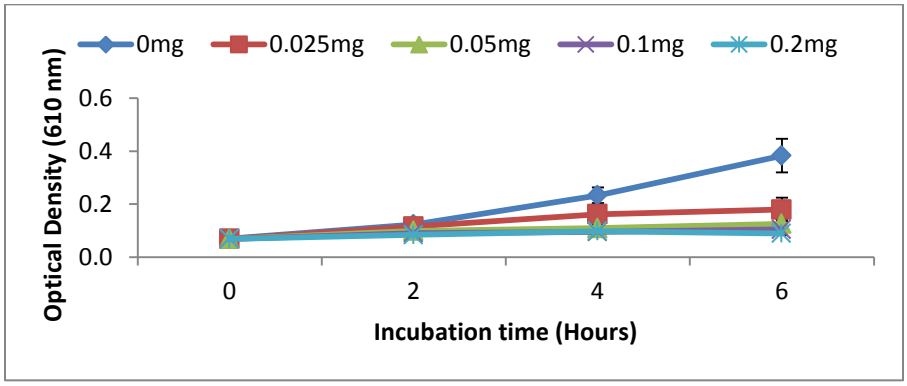
(a)



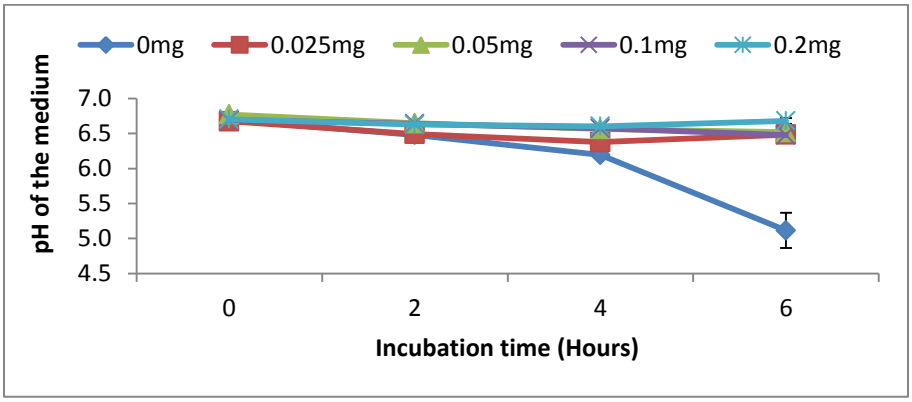
(b)

Figure 4-2. Survival and growth of YF001 in MRS broth in the presence of bromadiolone.

Figures 4-3 (a) and (b) indicate the growth of yogurt culture YF001 (OD and pH values) observed in the presence of diphacinone. The growth of the yogurt culture, in the control sample (as measured by turbidity) rapidly increased with time (Figure 4-3 (a)) showing the maximum OD value of 0.383 at 6 hours of incubation (Figure 4-3(a)). When diphacinone was added to the medium at 0.025mg /5ml of broth, the growth of the yogurt culture at 2 hours of incubation was the same as that of control sample. But the growth of yogurt culture was significantly lowered ($p < 0.05$) with the increasing concentration of diphacinone in the medium at 4 hours and 6 hours of incubation period. This indicates the inhibition of the growth of yogurt culture in the presence of diphacinone.



(a)



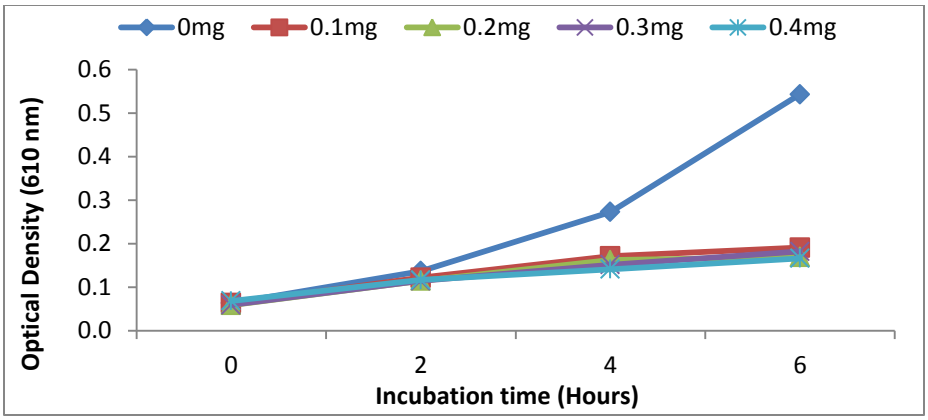
(b)

Figure 4-3. Survival and growth of YF001 in MRS broth in the presence of diphacinone.

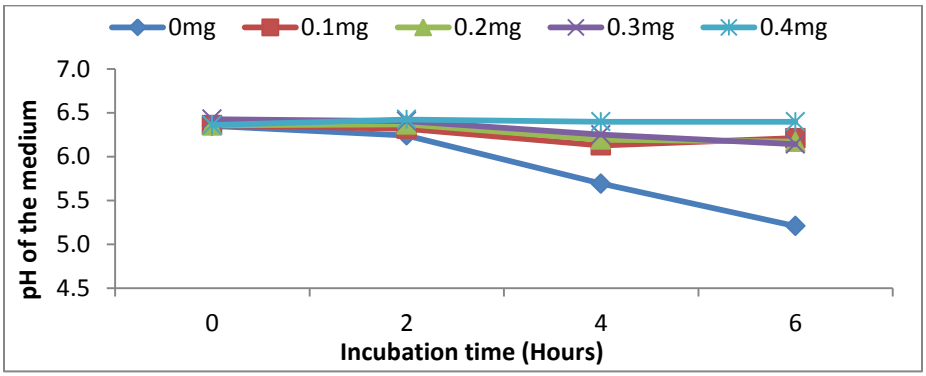
The results indicated that the yogurt culture was highly inhibited after 6 hours of incubation at the rodenticide level of 0.2mg/5ml of MRS broth. The growth of the yogurt culture, when measured by pH of the medium, indicated the same pattern (Figure 4-3 (b)), showing the slower reduction of pH in the presence of rodenticides. The minimum inhibitory concentration for diphacinone was observed at the level of 0.025mg/5ml (0.005mg/ml) of broth. The highest sensitivity of yogurt culture was observed at the diphacinone level of 0.2 mg/5ml of MRS broth.

Figures 4-4 (a) and (b) show the growth of yogurt culture YG011 in MRS broth in the presence of brodifacoum. There was a rapid growth of the YF011 throughout the incubation period in the control sample and the highest turbidity, (OD at 610 nm), was observed (0.543) at 6 hours of incubation period (Figure 4-4 (a)). The minimum inhibitory concentration was observed at the brodifacoum level of 0.1mg/5ml of broth. The lowest growth of YG011 (highest OD) was observed at the rodenticide level of 0.4mg/5ml of MRS broth. Similar pattern of growth in the YG011 was observed in the samples when the pH values were measured (Figure 4-4 (b)).

Figures 4-5 (a) and (b) show the growth of yogurt culture YG011 in MRS broth in the presence of bromadiolone. In the control sample, the growth of the YG011 was low up to 4 hours of incubation period. A rapid growth of YG011 was observed in the control sample after 4 hours of incubation reaching the highest turbidity, (OD at 610 nm) at the end of the 6 hours of incubation period (Figure 4-5(a)). When bromadiolone was added in the concentrations of 0.2, 0.4, 0.6 and 0.8mg/5ml of broth the growth of the yogurt culture was delayed indicating the inhibition of bacterial growth in the presence of bromadiolone. The lowest growth of YG011 (highest OD) was observed at the rodenticide level of 0.8mg/5ml of MRS broth. Similar pattern of growth in the yogurt culture was observed in the samples when the pH values were measured (Figure 4-.5 (b)).



(a)

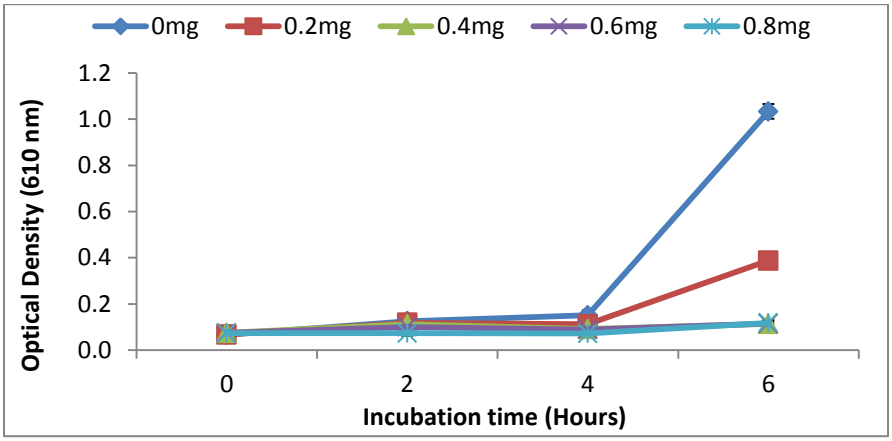


(b)

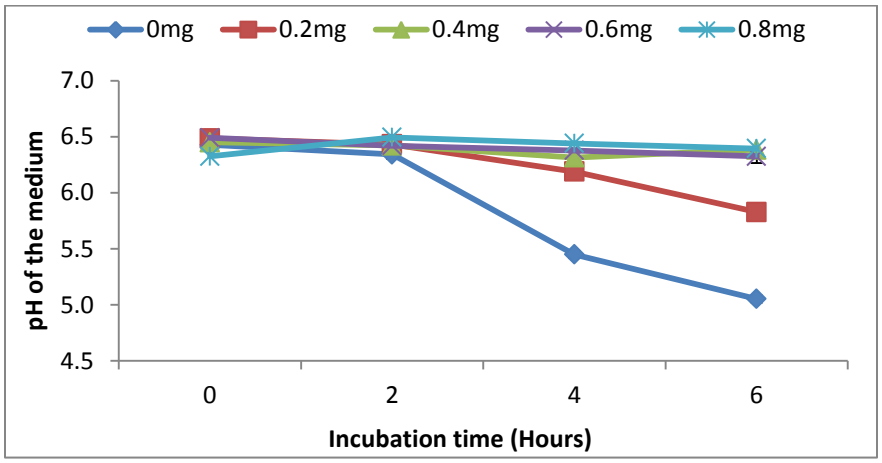
Figure 4-4. Survival and growth of YG011 in MRS broth in the presence of brodifacoum.

Figures 4-6 (a) and (b) show the growth of yogurt culture YG011 in MRS broth in the presence of diphacinone. In the control sample as observed by the turbidity (OD at 610 nm), the growth of the YG011 was slower during the first 2 hours of incubation period. There was a rapid growth of YG011 after 2 hours and reached the maximum (OD-1.071) at 6 hours of incubation (Figure 4-6(a)). The growth of the yogurt culture was significantly delayed with the increasing concentration of diphacinone in the medium indicating the inhibition of bacterial growth in the presence of diphacinone. The results indicated that the yogurt culture was highly inhibited after 6 hours of incubation at the rodenticide level of 0.4mg/5ml of MRS broth. The same pattern of

growth in the yogurt culture was observed in the samples when the pH values were measured (Figure 4-6 (b)).



(a)

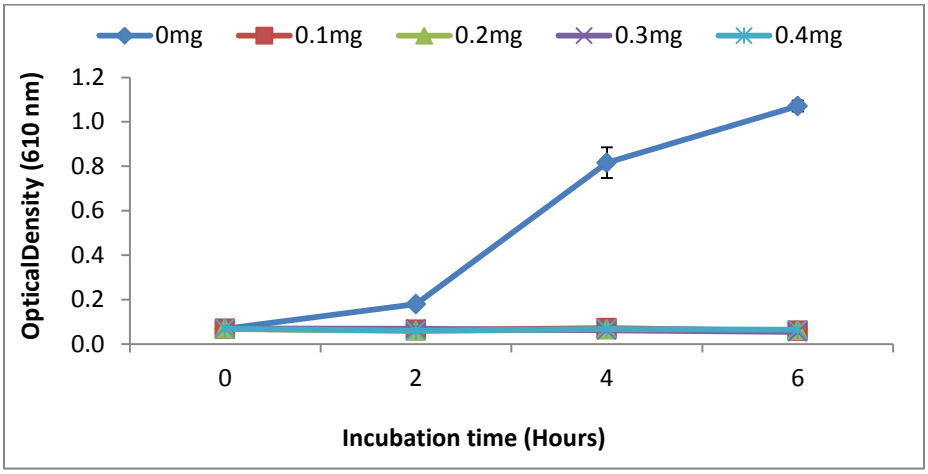


(b)

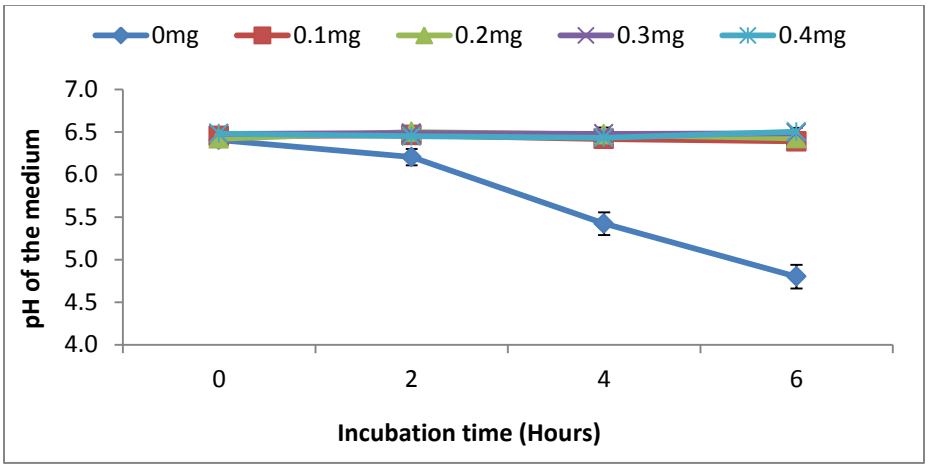
Figure 4-5. Survival and growth of YG011 in MRS broth in the presence of bromadiolone.

Figures 4-7 (a) and (b) show the growth of yogurt culture YI 885 in MRS broth in the presence of brodifacoum. There was a rapid growth of the YI 885 throughout the incubation period in the control samples and the highest turbidity, (OD at 610 nm), was observed at 6 hours of incubation period (Figure 4-7(a)). The growth of YI 885 was inhibited with increasing

concentrations of brodifacoum. The minimum inhibitory concentration of brodifacoum was observed at the level of 0.05mg /5ml of broth. Similar patterns of growth in the YI 885 was observed in the samples when the pH values were measured (Figure 4-7 (b)). When brodifacoum was added there was no difference in the growth of YI 885 (as observed by pH) at 2 hours of incubation (Figure 4-7 (b)) compare to the control sample. However the growth was significantly reduced at 4 hours and 6 hours of incubation.

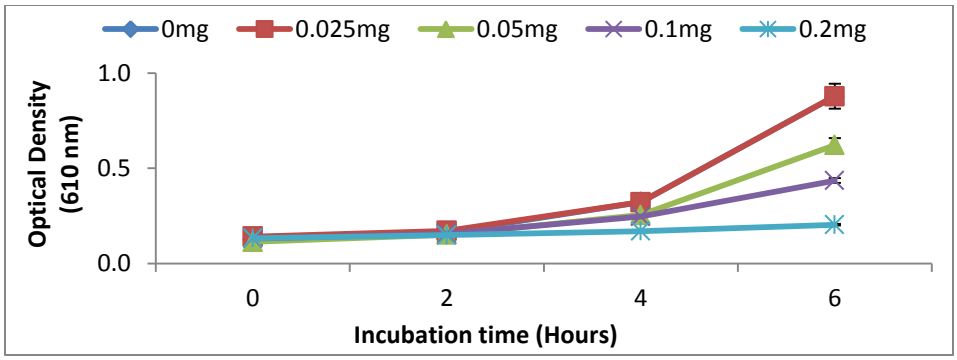


(a)

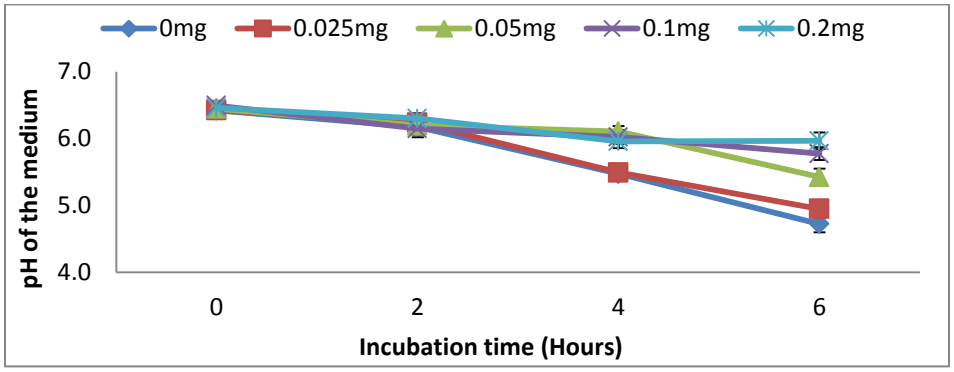


(b)

Figure 4-6. Survival and growth of YG011 in MRS broth in the presence of diphacinone.



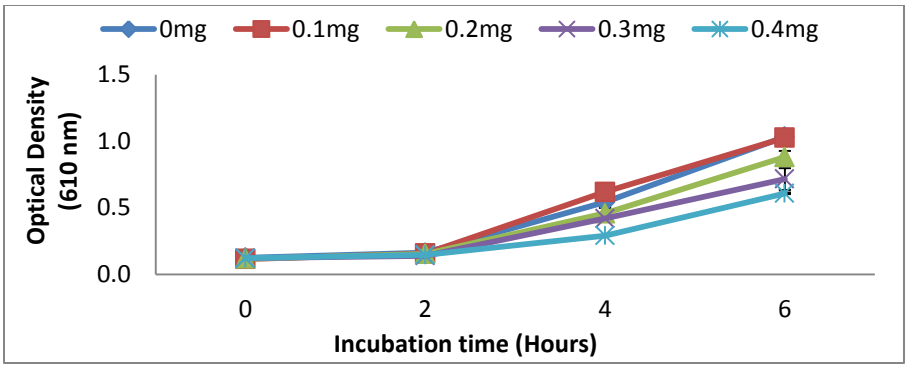
(a)



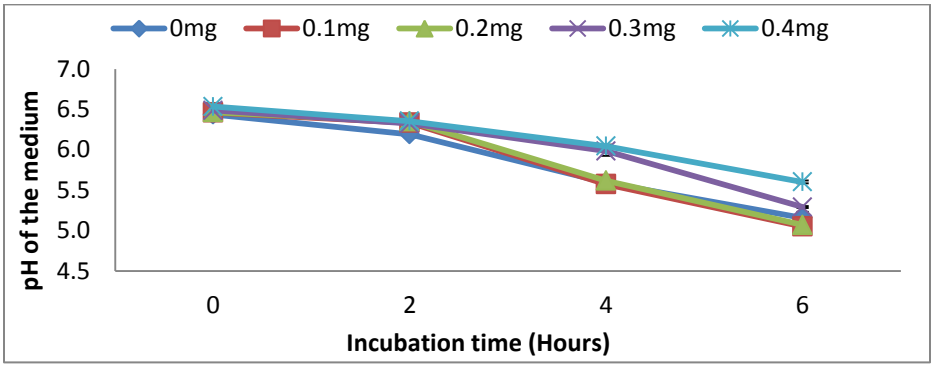
(b)

Figure 4-7. Survival and growth of YI885 in MRS broth in the presence of brodifacoum.

Figures 4-8 (a) and (b) show the growth of yogurt culture YI 885 in MRS broth in the presence of bromadiolone. In the control sample, the growth of the YI 885 was low up to 2 hours of incubation period. A rapid growth of YI 885 was observed in the control sample after 2 hours of incubation reaching the highest OD (1.034) at the end of the 6 hours of incubation period (Figure 4-8(a)). When bromadiolone was added in the concentrations of 0.2, 0.3, and 0.4mg/5ml of broth the growth of the yogurt culture was delayed indicating the inhibition of bacterial growth in the presence of bromadiolone. Similar pattern of growth in the yogurt culture was observed in the samples when the pH values were measured (Figure 4-8 (b)). At the highest concentration of bromadiolone (0.4mg/5ml) in milk, the pH drop was from 6.36 to 5.6 which indicate the growth inhibition.



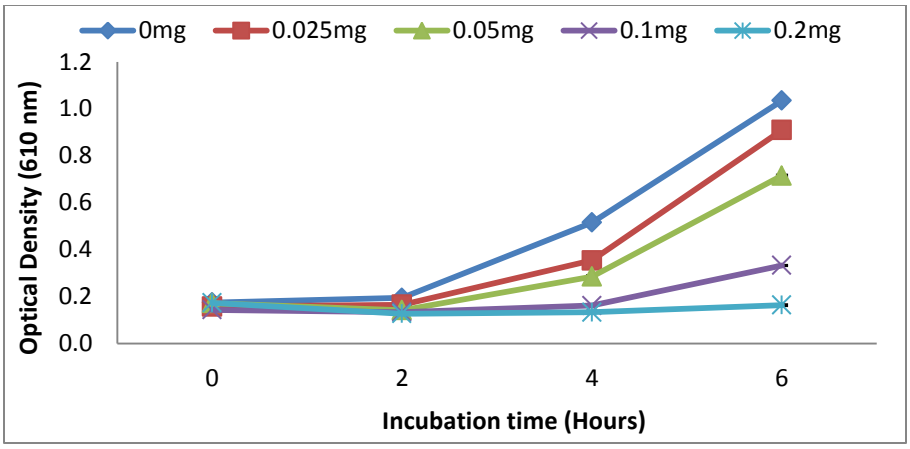
(a)



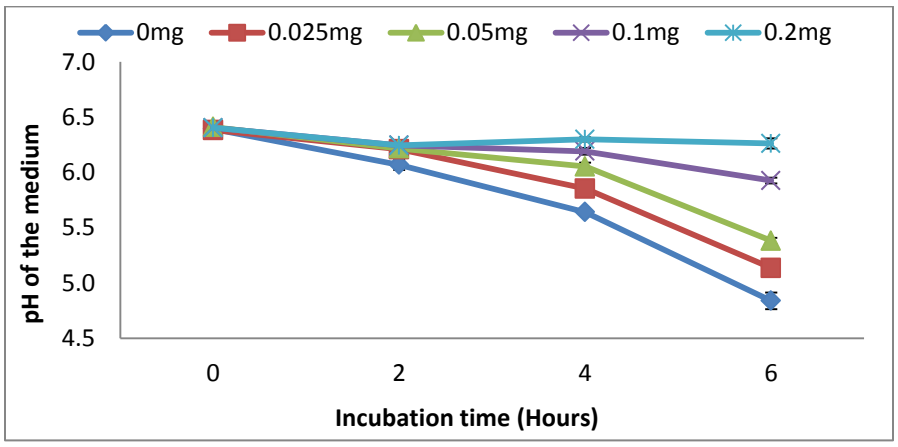
(b)

Figure 4-8. Survival and growth of YI885 in MRS broth in the presence of bromadiolone.

Figures 4-9 (a) and (b) show the growth of yogurt culture YI 885 in MRS broth in the presence of diphacinone. In the control sample as observed by the turbidity (OD at 610 nm), there was a rapid growth of the YI 885 throughout the incubation period (Figure 4-9(a)). The growth of the yogurt culture was delayed with the increasing concentration of diphacinone in the medium indicating the inhibition of bacterial growth in the presence of diphacinone. The results indicated that the yogurt culture was highly inhibited after 6 hours of incubation at the rodenticide level of 0.2mg/5ml of MRS broth. The same pattern of growth in the yogurt culture was observed in the samples when the pH values were measured (Figure 4-9 (b)).



(a)



(b)

Figure 4-9. Survival and growth of YI885 in MRS broth in the presence of diphacinone.

Table 4-1 shows the final population size of yogurt culture in MRS broth in the presence of rodenticides after 6 hours of incubation. In yogurt culture YF001, the bacterial population of control sample showed a population at 6.43 log CFU/ml after 6 hrs of incubation. When brodifacoum was added at the level of 0.1 mg/5ml of broth, there was no significant change of the bacterial population after the incubation when compared to the control sample (see Table 4-1).

Table 4-1

Final Population Size (log CFU/ml) of Yogurt Culture in the Presence of Rodenticides

Rodenticide Concentration (mg/5ml of broth)	YF001			YG011			YI885		
	BR*	BM*	DP*	BR*	BM*	DP*	BR*	BM*	DP*
0	6.43±0.06	8.38±0.01	7.09±0.03	8.4±0.01	8.39±0.01	8.23±0.08	8.94±0.03	9.43±0.08	9.47±0.09
0.025			6.12±0.04				8.91±0.08		8.86±0.02
0.05			5.12±0.00				8.95±0.03		7.81±0.03
0.1	6.14±0.06	7.12±0.01	3.00±0.04	7.11±0.03		4.91±0.03	7.88±0.02	8.53±0.03	7.61±0.01
0.2	5.89±0.11	6.73±0.01	2.06±0.02	6.89±0.25	7.16±0.01	4.83±0.01	6.99±0.01	7.87±0.03	7.52±0.03
0.3	4.87±0.6	6.61±0.02		6.45±0.13		4.73±0.03		7.52±0.02	
0.4	4.64±0.18	6.12±0.02		6.15±0.38	6.80±0.03	4.57±0.08		7.32±0.06	
0.6					6.57±0.05				
0.8					6.16±0.08				

*BR = Brodifacoum, BM = Bromadiolone, DP = Diphacinone.

When brodifacoum was added in the levels of 0.2mg, 0.3mg and 0.4mg/5ml of broth, the bacterial population decreased rapidly during 6 hour incubation period. A significant ($p < 0.05$) growth inhibition (as measured by log CFU/ml) of the yogurt culture YF001 was observed at 6 hours of incubation in the presence of brodifacoum at the level of 0.2, 0.3 and 0.4mg/5ml of broth. At the highest concentration of brodifacoum (0.4mg/5ml) in broth, the bacterial population dropped to 4.64 log CFU/ml (Table 4-1) which indicate the growth inhibition of the YF001. Similar pattern of growth inhibition was observed in YF001 in the presence of bromadiolone and diphacinone. YF001 showed highest sensitivity to diphacinone. At the diphacinone level of 0.2 mg/5ml of broth, the bacterial population dropped to 2.06 log CFU/ml at the end of 6 hours of incubation period.

Same pattern of growth inhibition was observed in yogurt cultures YG011 and YI 885. The population of YG011 and YI 885 was significantly lowered ($p < 0.05$) at 6 hours of incubation period with the increasing concentration of rodenticides in the broth (Table 4-1). This indicates the inhibition of the growth of yogurt culture in the presence of rodenticides in the MRS broth. The minimum growth of YG011 and YI 885 was observed at the highest brodifacoum level of 0.4mg and 0.2mg /5ml of broth, respectively. The minimum inhibitory concentration (as measured by the bacterial population (log CFU/ml) of brodifacoum, bromadiolone and diphacinone for YG011 was at the levels of 0.1, 0.2, and 0.1mg/5ml of broth, respectively.

Tables 4-2 and 4-.3 show the enzyme activity of yogurt culture in the presence of rodenticides. There were statistically significant differences ($p < 0.05$) in α and β-galactosidase activity in the presence of brodifacoum, bromadiolone and diphacinone in MRS broth.

Table 4-2

Alpha Galactosidase Activity (GalU/g) of Yogurt Culture in the Presence of Rodenticides

Rodenticide Concentration (mg/5ml of broth)	YF001			YG011			YI885		
	BR*	BM*	DP*	BR	BM	DP	BR	BM	DP
0	0.12±0.00	0.13±0.01	0.13±0.01	0.14±0.02	0.13±0.01	0.15±0.03	0.11±0.00	0.13±0.03	0.12±0.01
0.025	0.06±0.00		0.06±0.00	0.06±0.00		0.08±0.00	0.09±0.01		0.06±0.00
0.05	0.03±0.00		0.03±0.00	0.03±0.00		0.03±0.01	0.05±0.02		0.03±0.00
0.1	0.01±0.00	0.09±0.00	0.02±0.00	0.02±0.00	0.09±0.01	0.02±0.00	0.04±0.01	0.09±0.00	0.02±0.00
0.2	0.01±0.00	0.05±0.00	0.01±0.00	0.01±0.00	0.05±0.01	0.01±0.00	0.03±0.00	0.05±0.00	0.01±0.00
0.3		0.03±0.00			0.04±0.00			0.047±0.00	
0.4		0.03±0.00			0.03±0.01			0.03±0.00	

*BR = Brodifacoum, BM = Bromadiolone, DP = Diphacinone.

Table 4-3

Beta Galactosidase Activity (GalU/g) of Yogurt Culture in the Presence of Rodenticides

Rodenticide Concentration (mg/5ml of broth	YF001			YG011			YI885		
	BR*	BM*	DP*	BR	BM	DP	BR	BM	DP
0	2.61±0.04	2.49±0.09	2.7±0.09	2.65±0.05	2.65±0.12	2.82±0.07	2.67±0.05	2.36±0.3	2.65±0.09
0.025	2.05±0.2		2.07±0.26	2.14±0.27		2.07±0.13	2.57±0.07		2.07±0.3
0.05	1.71±0.06		1.87±0.35	1.75±0.07		1.94±0.25	2.12±0.03		1.95±0.7
0.1	1.3±0.09	1.87±0.12	1.32±0.23	1.38±0.17	1.89±0.16	1.4±0.22	1.2±0.06	1.87±0.08	1.19±0.02
0.2	0.07±0.00	1.67±0.16	0.10±0.02	0.08±0.01	1.65±0.13	0.12±0.01	0.91±0.01	1.81±0.04	0.07±0.00
0.3		1.58±0.08			1.61±0.11			1.67±0.06	
0.4		1.34±0.21			1.40±0.05			1.36±0.09	

*BR = Brodifacoum, BM = Bromadiolone, DP = Diphacinone.

In the control group α-galactosidase activity was significantly enhanced compared to the α-galactosidase activity in the presence of rodenticides in MRS broth (Table 4.2). The α-galactosidase production was significantly reduced ($p < 0.05$) in YF001 with the addition of brodifacoum to the growth medium compared to the control group (0.01 vs 0.12 GalU/g, respectively). Similarly the α-galactosidase activity was significantly reduced ($p < 0.05$) in YF001 in the presence of bromadiolone and diphacinone in the medium. An enhanced β-galactosidase activity was observed in YG011 and YI 885 in the absence of rodenticides in MRS broth indicating constant growth of the yogurt cultures (Table 4-3). At the brodifacoum level of 0.2mg/5ml of broth, a significant ($p < 0.05$) drop in β-galactosidase production was observed in YG011 and YI 885 (from 0.14 and 0.13 to 0.01 and 0.03 GalU/g, respectively) after 6 hours of incubation indicating the growth inhibition. Similar results were observed in the presence of bromadiolone and diphacinone in the medium. There were no other studies detecting the enzyme activity of lactic acid bacteria in the presence of rodenticides. Several researches (Han, Lim, & Lee, 2007; Ibrahim et al., 2010) have investigated enzyme activity of different strains of lactic acid bacteria in the presence of metal ions, which indicated higher enzyme activity in the presence of metal ions in the medium. The lower enzyme activity of yogurt culture in our study could be due to two reasons. First the yogurt culture may not perform optimally in MRS broth. We used MRS broth because initially it was necessary to detect the sensitivity of yogurt culture to the rodenticides in a basic media. It is expected to have an enhanced enzyme activity of yogurt culture in the milk. The other reason for lower enzyme production is the shorter incubation period of 6 hours. The higher enzyme activity was observed in other studies (Han et al., 2007) at the incubation period of 18 hours.

4.4 Conclusions

The yogurt cultures YF001 and Y1 885 showed highest sensitivity to diphacinone at the level of 0.005 mg/ml. YF001 showed detectable sensitivity to brodifacoum and bromadiolone at the level of 0.02mg/ml. YG011 showed detectable sensitivity to brodifacoum, bromadiolone and diphacinone at the levels of 0.02, 0.04, and 0.02mg/ml, respectively. The growth of YI 885 was inhibited by brodfacoum and bromadiolone at the levels of 0.01 and 0.04mg/ml, respectively. YI 885 was the highest sensitive yogurt culture for all the three rodenticides. Our results show that yogurt culture could be used as a biomarker for the early detection of the presence of rodenticides in milk. The growth of the yogurt culture needs to be studied in the milk in the presence of rodenticides before selecting the most sensitive yogurt culture. This process could be modified to produce a fast, highly sensitive, environmentally safe, and accurate test kit which could be used as a universal marker for early detection of terrorist attacks on food supplies.

CHAPTER 5

Effect of Selected Rodenticides on the Growth and Activity of Different Strains of Lactic Acid Bacteria in Milk

5.1 Introduction

Rodenticides are an important tool for public health pest control, including controlling domestic mice and rats, but the mal practices in marketing and use have been associated with accidental exposures to thousands of children each year (Chua & Friedenberg, 1998; Watt et al., 2005). These products also pose significant risks to non-target wildlife, including both birds and mammals (Fauconnet et al., 1997; Spurr et al., 2005).

Superwarfarins are the most commonly used rodenticides in the United States. They were first introduced in the 1970s after the development of resistant rats to the traditional warfarin rodenticide (M. R. Hadler & A. P. Buckle, 1992). Over 95% of all rodenticides used in United States consist of superwarfarin. Brodifacoum is the commonest rodenticide among them (Watson et al., 2005).

The rodenticides are available to purchase over the counter and they are commonly used to control rodents in agricultural farms and household (J. Spahr et al., 2007). This could lead to unintentional contamination of milk and other food commodities by rodenticides. Their long acting, fat soluble nature (J. Spahr et al., 2007) could make them remain longer periods in food producing animals and their products. The tasteless and odorless nature of rodenticides at their lethal concentrations makes impossible to detect them physically when contaminates food. This is one of the major reasons for the extensive incidences of intoxication in humans and animals (Eason et al., 2001; J. Spahr et al., 2007; Wu et al., 2009). Since the rodenticides are designed to kill mammals, their toxicity is very similar to the target rodents and to the humans.

Numerous studies have been carried out to detect chemicals in milk; most of them are to detect antibiotic and pesticide residues. The methods used for detection of antibiotic residues consist of both qualitative and quantitative techniques. Among the quantitative methods, immunoassays, High Performance Liquid Chromatographic methods, and Gas Chromatography methods are common (Ferguson et al., 2005; Knecht et al., 2004; Zacco et al., 2007). Chromatographic methods are widely used for detection and confirmation of pesticide residues in milk (Bennett et al., 1997; Pagliuca et al., 2005; Schenck & Wagner, 1995). There are only a very few researches have been done to detect rodenticide residues in animal tissues and milk (Huckle, Warburton, et al., 1989; Hunter, 1985). A reversed phase HPLC has developed in 1997 by Fauconnet et al. (1997) to detect anticoagulant rodenticides in animal liver. The existing procedures to detect chemicals and toxins in milk need extensive sample preparation, and they are time consuming. Further, these sophisticated techniques need specific laboratory equipment and trained personnel. Since the milk is distributed among the consumers soon after it is produced, there is a need to screen toxins using a simple on-farm technique, which gives results within few hours, to prevent contaminated milk leaving the farm.

Bioassay techniques, which use bacteria as a biomarker, are widely used qualitative techniques to screen contaminants in food. These techniques are based on the principal of growth inhibition of bacteria in response to chemicals. The bioassay techniques are robust and easy to perform. They detect a wide range of chemicals with satisfactory sensitivity (Popelka et al., 2003; Rault et al., 2004).

Since super warfarin is popular and readily available in the stores there is a possibility of intentional and unintentional contamination of milk by them during production in the farm. If such products reached consumers, it could create a public health crisis. Therefore, it is important

to understand the interaction between chemically adulterating agents and milk proteins during cheese processing to help identify intentional contamination of milk that would likely lead to better isolation of the contaminating substance. This can be critical in reducing time lost to processing an unusable food product as well as reducing the need for unnecessary destruction of usable milk and milk products. Results obtained from this research would be important for interventions and countermeasures in preventing final retail distribution and consumer consumption of intentionally contaminated milk.

During food manufacturing the detection for the presence of toxins in food requires the use of third party laboratory testing which usually take days for final results. Because raw milk represents the most vulnerable step in manufacturing cheese, determination that the milk used in the production of cheese is toxin free is very important.

We have preliminary data from our lab that demonstrates that the lactic acid bacteria (LAB), commonly used in the dairy industry can serve as a simple and rapid tool to identify adulterated milk (Hathurusinghe et al., 2011). The principle logic behind this is that LAB are capable of rapid production of lactic acid. A slowed reaction in lactic acid production can be used as a triggered response to changes in the milk content due to the presence of foreign substances.

The sensitivity of different LAB to toxins also has not been investigated, but they may vary with their ability to survive with the concentration and the nature of the toxin. Therefore, it is important to detect the sensitivity of different strains of LAB towards toxins to identify more sensitive strains. The objective of this study was to determine the effect of selected rodenticides on the growth and activity of different strains of lactic acid bacteria in milk.

5.2 Materials and Methods

5.2.1 Bacterial strains and culture conditions. Three different commercial yogurt cultures (YF001, YG011, and YI 885) consisting of *Lactobacillus bulgaricus* and *Streptococcus thermophillus* were used. The yogurt culture was obtained from the culture collection of the Food Microbiology and Biotechnology Laboratory at North Carolina A & T State University. The commercial yogurt culture was sub cultured by adding two loops of yogurt culture to tubes containing 5 ml of sterilized skim milk (12% W/V). This culture was then incubated at 42 °C for 5 hours and kept refrigerated until used. The yogurt culture in skim milk was activated prior to the test. To activate the yogurt culture, 500 µl of culture from the skim milk was added to a tube containing 5 ml of fresh MRS broth (Difco Laboratories, Becton Dickinson, Sparks, MD), and incubated at 42 °C for 18 hours. Then the tube was centrifuged at 8000g for 10 minutes, and the supernatant was removed. The pellet was resuspended in 10 ml of peptone water. This activated inoculum was used for the assay.

5.2.2 Preparation of rodenticides. Three selected commercially available rodenticides were used, to test the sensitivity of the yogurt culture to them. They are brodifacoum (Sigma) bromadiolone (Sigma) and diphacinone (Sigma). The stock solutions of rodenticides were prepared by adding 10 mg of rodenticide to 1ml of acetone.

5.2.3 Assay procedure. Tubes containing 5ml of pasteurized whole milk were inoculated with activated yogurt culture (approximately 3.0 log CFU/ml). Serially diluted rodenticides were added (0.2, 0.4, 0.6, 0.8, 1.0, mg/tube) separately to these test tubes. The tubes containing milk, yogurt culture, and rodenticides were incubated at 42 °C for 6 hours. The pH of the milk was recorded at 0, 2, 4 and 6 hour intervals. The bacterial colonies were counted using the standard enumeration method to determine the viable bacterial population at the end of

incubation. The lactic acid production of the medium was also determined at the end of the 6 hour incubation period.

5.2.4 Bacterial enumeration. The bacterial colonies were counted using the standard enumeration method to determine the viable bacterial population at the end of incubation. After 6 hours of incubation the bacteria were serially diluted by adding 1 ml of the incubated milk sample to 9 ml of 1% peptone water and was plated (100 µl) on duplicate plates of MRS agar (Difco Laboratories, Becton Dickinson, Sparks, MD). The MRS agar was prepared by adding 55 g MRS to 1L of deionized water, with 0.5 g cysteine and 14 g agar and then autoclaved at 121 °C for 15 minutes. The bacterial colonies were counted to determine the bacterial population after incubating the plates at 37 °C for 24 hours.

5.2.5 Determination of pH. Samples were withdrawn at 2, 4, and 6 hour to measure pH using the pH meter (Model 410A, Orion, Boston, MA). The pH meter was calibrated using pH standard buffers 4.0 and 7.0. The electrode was rinsed with distilled water between different samples.

5.2.6 Determination of lactic acid production. The amount of lactic acid production at the end of the incubation was measured using titration. The 5 ml of milk containing bacteria and rodenticides was added to a flask after 6 hours of incubation. The solution was double diluted using 5 ml of deionized water. It was titrated with 0.1N NaOH until the pH of the medium reaches 8.2. The volume of NaOH used was recorded. The amount of lactic acid production was calculated using the following formula.

Volume of NaOH used x 0.009 = % of lactic acid

5.2.7 Determination of α and β-galactosidase activity. Alpha and beta-galactosidase activity was determined at the end of the incubation. LAB in yogurt culture were harvested after

6 hours of incubation according to the method described in (Alazzeh et al., 2009) with slight modifications. The samples were centrifuged 8000g at 4 ˚C for 10 minutes, discarding the supernatant and washing the precipitated strains twice with phosphate buffer (pH 6.8). Then the bacteria cells were resuspended in 1 ml of phosphate buffer in ependorf tubes containing 0.1 mm glass beads (BioSpec Products Inc., Bartlesville, OK, USA) . The samples were treated with a bead beater (MiniBead Beater-8, BioSpec products Inc., Bartlesville, OK, USA) for 3 minutes. The samples were rested for 15 s in an ice bath after every minute. Samples were then centrifuged at 12000g for 12 minutes and supernatant was stored at -80 ˚C until used as the enzyme source.

5.2.7.1α-galactosidase assay. α-galactosidase activity was assayed according to the method described in Food Chemicals Codex (2003) (Codex, 2003) by adding 1 ml of p-nitrophenyl-α-D- galactopyranoside substrate and 0.5 ml of sample. The samples were transferred immediately into a 37 ˚C water bath for 15 minutes. A blank sample was prepared by adding 0.5 ml of distilled water, 1 ml of substrate. The reactions were stopped at 15 minutes by adding 2.5 ml of Borax buffer. The light absorbance was measured at 405 nm by spectrophotometer (Model Genesys 10 Vis, Thermospectronic, Rochester, NY, USA). One galactosidase activity unit (GalU) is defined as the quantity of the enzyme that will liberate p-nitrophenol for alpha at the rate of 1 mol/min under the conditions of the assay. Units of α-gal were calculated using the following calculation.

$$\boldsymbol{GalU/g} = \frac{[(\boldsymbol{AS} - \boldsymbol{AB})\ \boldsymbol{X}\ \boldsymbol{F}]}{(\boldsymbol{\varepsilon}\ \boldsymbol{X}\ \boldsymbol{T}\ \boldsymbol{X}\ \boldsymbol{M})}$$

Where

AS is the sample absorbance;

AB is the blank absorbance;

F is the appropriate dilution factor;

T is the reaction time, in minutes;

M is the weight, in grams, of the sample

Epsilon is a factor calculated for the p-nitrophenol (proportional to the millimolar extinction coefficient for p-nitrophenol) standards using the following equation:

Epsilon= AN/C,

in which

AN is the absorbance of the p-nitrophenol standards at 405 nm

C is the concentration, in millimoles per milliliter, of p-nitrophenol.

5.2.7.2 β-galactosidase assay. β-galactosidase activity was determined according to the method described by Nagy et al. (2001) by adding 0.5 ml of sample and 0.5 ml of 15 mM 0-nitrophenyl-β-D-galactopyranoside in 0.03 M sodium phosphate buffer (pH 6.8). The tubes were kept in a shaking water bath at 37 °C for 10 minutes and the reactions were stopped by adding 2 ml of 0.1 M sodium carbonate. The optical density was measured at 420 nm with the spectrophotometer (Model Genesys 10 Vis, Thermospectronic, Rochester, NY, USA). The unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1µmol of *o*-nitrophenyl per minute under assay condition. One galactosidase activity unit (GalU) is defined as the quantity of the enzyme that will liberate 0-nitrophenol for beta at the rate of 1 mol/min under the conditions of the assay.

Units of β-gal were calculated using the following calculation.

$$\boldsymbol{GalU/g} = \frac{[(\boldsymbol{AS} - \boldsymbol{AB})\ \boldsymbol{X}\ \boldsymbol{F}]}{(\boldsymbol{\varepsilon}\ \boldsymbol{X}\ \boldsymbol{T}\ \boldsymbol{X}\ \boldsymbol{M})}$$

Where

AS is the sample absorbance;

AB is the blank absorbance;

F is the appropriate dilution factor;

T is the reaction time, in minutes;

M is the weight, in grams, of the sample

Epsilon is a factor calculated for the 0-nitrophenol (proportional to the millimolar extinction coefficient for 0-nitrophenol) standards using the following equation:

Epsilon= AN/C,

in which

AN is the absorbance of the 0-nitrophenol standards at 420 nm

C is the concentration, in millimoles per milliliter, of 0-nitrophenol.

5.2.8 Effect of rodenticides on enzymatic coagulation properties of milk. Changes in viscosity in the presence of toxins during milk coagulation was measured using rotational viscometer (HAAKE Viscotester 7 plus, Thermo Scientific, Waltham MA). Serially diluted toxins were added to (0, 2, 3 and 4 mg) 500 ml of the 3.25% fat milk. The solutions were allowed to thermally equilibrate at 31°C (±1°C) for approximately 35 minutes in a water bath. Undiluted rennet was added at 0.08 µl/ ml of milk and a timer started. The L1 spindle of the viscometer was lowered into the center of the sample and began rotating at 10 rpm. The viscosity was measured every 30 seconds from the addition of rennet until the viscosity reaches

its maximum value. This procedure was performed in triplicate for the control samples and for the contaminated samples.

5.2.9 Statistical Analysis. Data analyses were focused on determining if the addition of different concentrations of rodenticides resulted in significantly lower growth of yogurt culture over that of the control group. Statistical analysis of data was performed using the SAS General Linear Model (GLM) procedure of SAS software (SAS Institute Inc., Cary, NC). Duncan's multiple range test was used to find significant differences ($p < 0.05$) between treatments at a particular time. For each growth condition, the test was repeated four times.

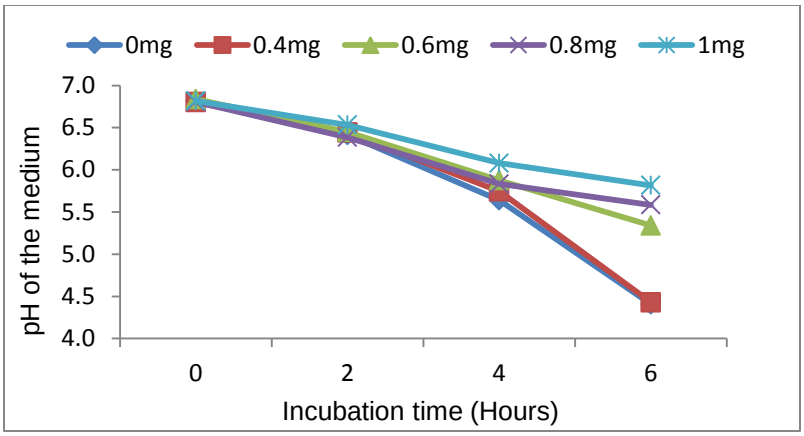
5.3 Results and Discussion

Figures 5-1(a), (b), and (c) show the growth of yogurt culture (YF001) in milk samples in the presence of different concentrations of brodifacoum, bromadiolone and diphacinone. In the control samples as observed by the pH of the medium, there was a rapid growth of YF001 throughout the incubation period. The growth of the YF001 was delayed with the increasing concentrations of rodenticides in the medium indicating the inhibition of bacterial growth in the presence of rodenticides. When brodifacoum was added in the level of 0.4mg/5ml of milk, there was no difference in the growth of YF001 (Figure 5-1 (a)) compared to the control sample. When brodifacoum was added in 0.6mg, 0.8mg, and 1.0mg/5ml of milk, the initial growth at 2 hours, as observed by the pH, was low. It continued to grow slowly with the time, but there was a significant inhibition ($p < 0.05$) of the growth of YF001 at 6 hours of incubation period in the presence of brodifacoum (Figure 5-1 (a)).

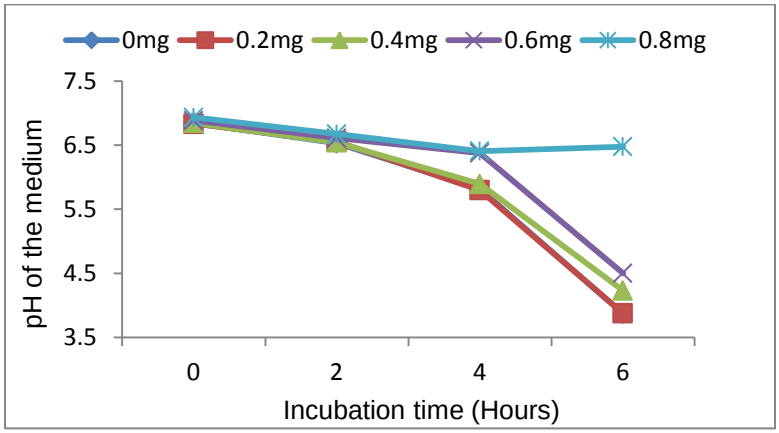
The results indicated that YF001 was highly inhibited after 6 hours of incubation at the rodenticide level of 1.0mg/5ml of milk. The lowest growth of YF001 (highest pH) was observed at the highest concentration of brodifacoum (1.0mg/5ml) in milk. The minimum inhibitory

concentration was observed at the level of 0.6 mg/5ml (0.12 mg/ml; $p < 0.05$) of milk. When bromadiolone was added at the level of 0.6mg/5ml of milk, the growth of YF001 was significantly reduced ($p < 0.05$) after 4 hours and 6 hours of incubation (Figure 5-1 (b)).

At the level of 0.8mg/5ml of milk, the growth of YF001 was significantly lower than that of the control sample, which indicates the inhibition of YF001 in the presence of bromadiolone (Figure 5-1 (b)). The minimum growth of YF001 (the highest pH) was observed at the highest concentration of bromadiolone (0.8mg/5ml) in milk. The minimum inhibitory concentration was observed at the level of 0.4mg/5ml (0.08mg/ml) of milk at 6 hours of incubation (Figure 5-1 (b)). Figure 5-1 (c) shows the growth of YF001 in the presence of diphacinone. When diphacinone was added in the levels of 0.4, 0.6, 0.8 and 1mg/5ml of milk, there was no difference in the growth of YF001 at 2 hours of incubation (Figure 5-1 (c)) compared to the control sample.



(a)



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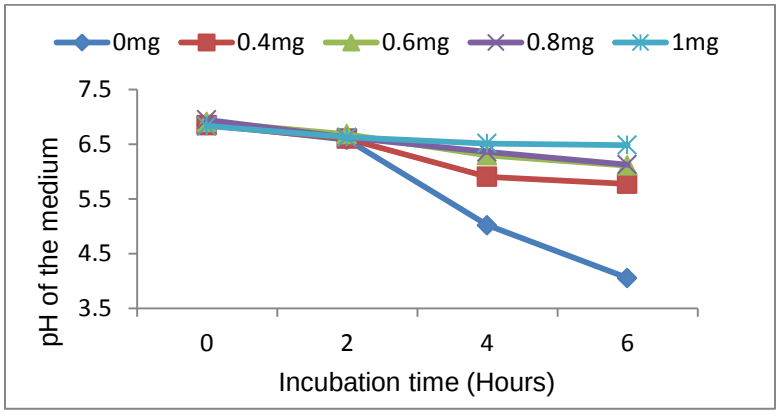


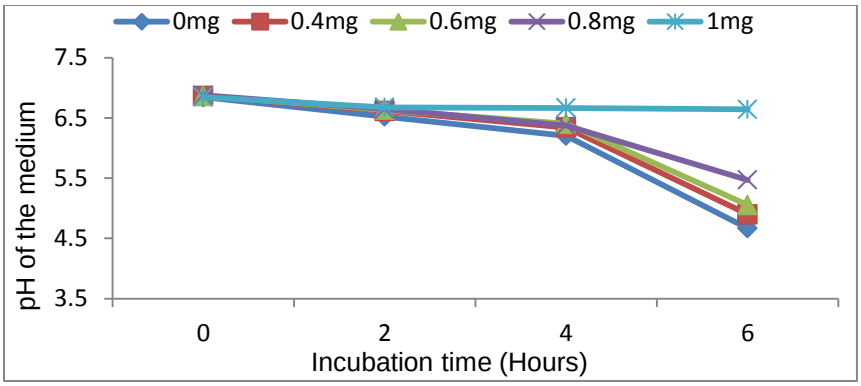
(c)

Figure 5-1. Growth of yogurt culture (YF001) in the presence of (a) brodifacoum, (b) bromadiolone and (c) diphacinone in milk.

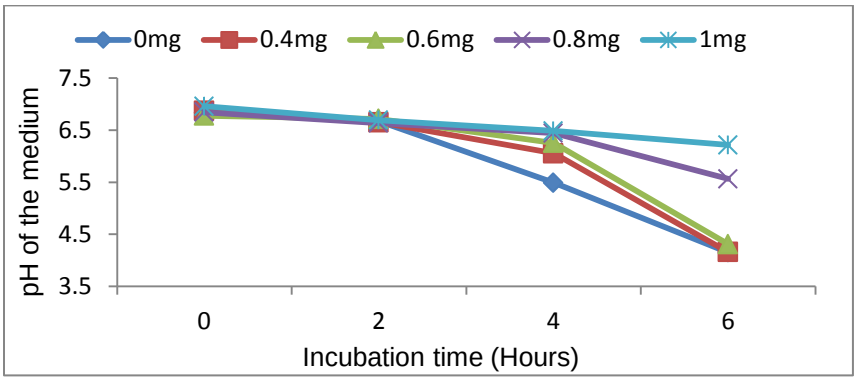
However, the growth of YF001 was significantly reduced ($p < 0.05$) after 4 hours and 6 hours of incubation. The minimum inhibitory concentration of diphacinone was at the level of 0.4 mg/5ml (0.08 mg/ml; $p < 0.05$) of milk. The minimum growth of YF001 (the highest pH) was observed at the highest concentration of diphacinone (1mg/5ml) in milk.

Figures 5-2(a), (b), and (c) show the growth of yogurt culture (YG011) in milk samples in the presence of different concentrations of brodifacoum, bromadiolone and diphacinone. As observed by the pH of the medium, there was a rapid growth of YG011 In the control samples throughout the incubation period. The growth of the YG011 was delayed with the increasing concentrations of rodenticides in the medium indicating the inhibition of bacterial growth in the presence of rodenticides. When brodifacoum was added in the levels of 0.4, and 0.6mg/5ml of milk, there was no difference in the growth of YG011 (Figure 5-2 (a)) compared to the control sample. When brodifacoum was added in 0.8, and 1.0mg/5ml of milk, the initial growth at 2 hours, as observed by the pH, was low. It continued to grow slowly with the time, but there was a significant inhibition ($p < 0.05$) of the growth of YG011 at 6 hours of incubation period in the presence of brodifacoum (Figure 5-2 (a)).

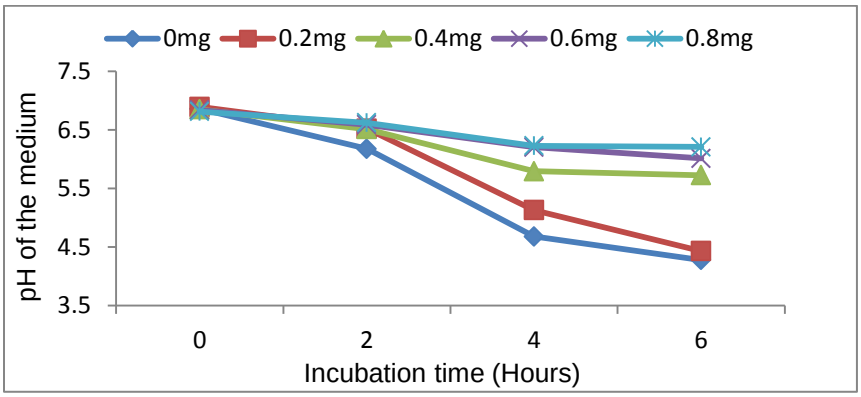
According to the results YG011 was highly inhibited after 6 hours of incubation at the rodenticide level of 1.0mg/5ml of milk. The minimum inhibitory concentration was observed at the level of 0.8 mg/5ml (0.16 mg/ml; $p < 0.05$) of milk. When bromadiolone was added at the level of 0.8mg/5ml of milk, the growth of YG011 was significantly reduced ($p < 0.05$) after 6 hours of incubation (Figure 5-2 (b)) which indicates the inhibition of YG011 in the presence of bromadiolone. The minimum growth of YG011 (the highest pH) was observed at the highest concentration of bromadiolone (1mg/5ml) in milk. The minimum inhibitory concentration was observed at the level of 0.8mg/5ml (0.08mg/ml) of milk at 6 hours of incubation (Figure 5-2 (b)).



(a)



(b)



(c)

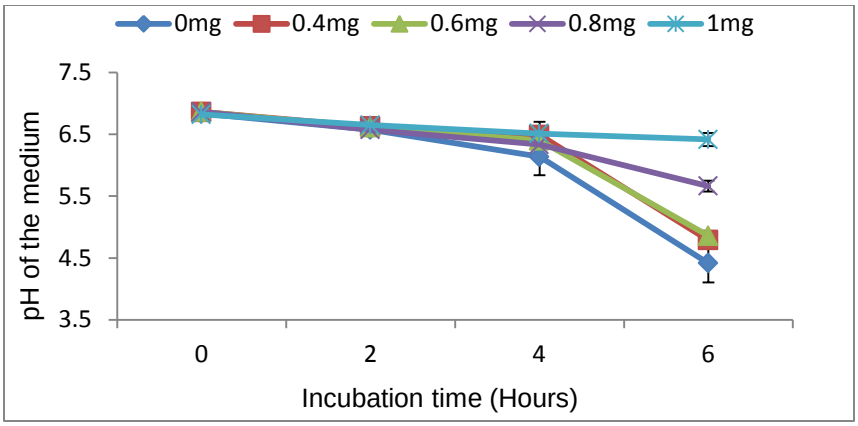
Figure 5-2. Growth of yogurt culture (YG011) in the presence of (a) brodifacoum, (b) bromadiolone, and (c) diphacinone in milk.

When diphacinone was added in the level of 0.2mg/5ml of milk, there was no difference in the growth of YG011 at 6 hours of incubation (Figure 5-2 (c)) compared to the control sample.

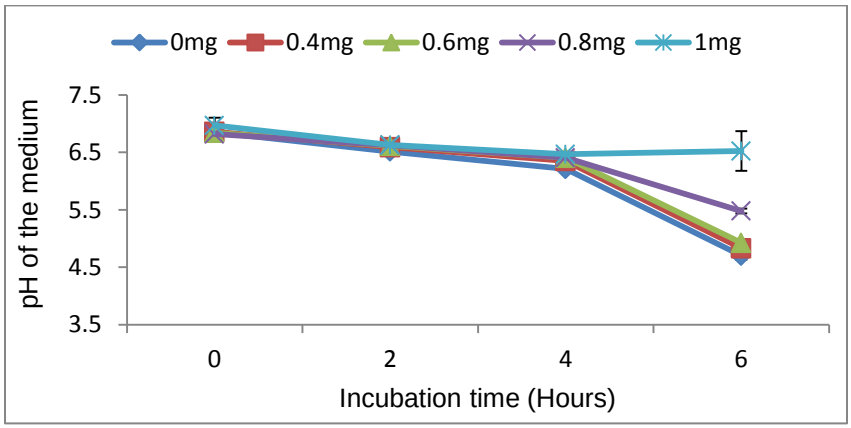
However, the growth of YG011 was significantly reduced ($p < 0.05$) at the diphacinone levels of 0.4, 0.6 and 0.8mg/5ml of milk. The minimum inhibitory concentration of diphacinone was at the level of 0.4 mg/5ml (0.08 mg/ml; $p < 0.05$) of milk. The minimum growth of YG011 (the highest pH) was observed at the highest concentration of diphacinone (0.8mg/5ml) in milk.

Figures 5-3(a), (b) and (c) show the growth of yogurt culture (YI 885) in milk samples in the presence of different concentrations of brodifacoum, bromadiolone and diphacinone. There was a rapid growth of YI 885 in the control samples throughout the incubation period. The growth of the YI 885 was delayed with the increasing concentrations of rodenticides in the medium indicating the inhibition of bacterial growth in the presence of rodenticides. When brodifacoum was added in the levels of 0.4, 0.6, 0.8 and 1mg/5ml of milk, there was no difference in the growth of YI 885at 4 and 6 hours of incubation (Figure 5-3 (a)) compared to the control sample. It continued to grow slowly with the time, but there was a significant inhibition ($p < 0.05$) of the growth of YI 885 at 6 hours of incubation period in the presence of brodifacoum (Figure 5-3 (a)).

The lowest growth of YI 885 (highest pH) was observed at the highest concentration of brodifacoum (1.0mg/5ml) in milk. The minimum inhibitory concentration was observed at the level of 0.4 mg/5ml (0.08 mg/ml; $p < 0.05$) of milk. When bromadiolone was added at the levels of 0.8 and 1mg/5ml of milk, the growth of YI 885 was significantly reduced ($p < 0.05$) after 6 hours of incubation (Figure 5-3 (b)). At the level of 1mg/5ml of milk, the growth of YI 885 was significantly lower than that of the control sample, which indicates the inhibition of YI 885 in the presence of bromadiolone (Figure 5-3 (b)).



(a)



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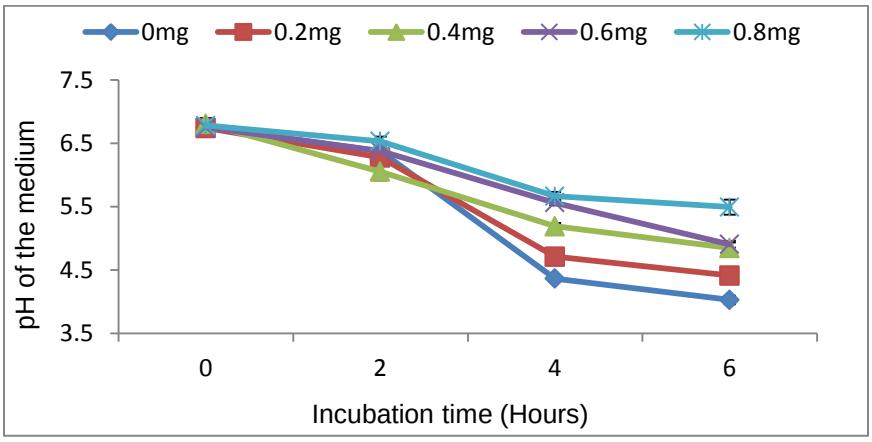


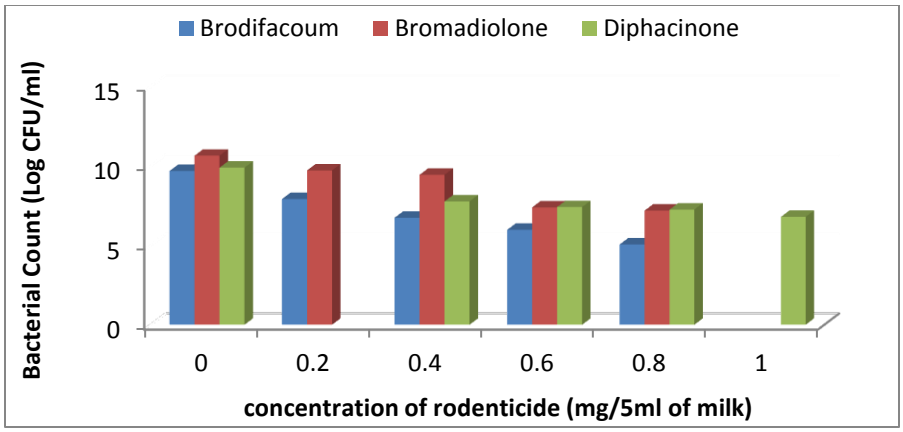
(c)

Figure 5-3. Growth of yogurt culture (YI 885) in the presence of (a) brodifacoum, (b) bromadiolone, and (c) diphacinone in milk.

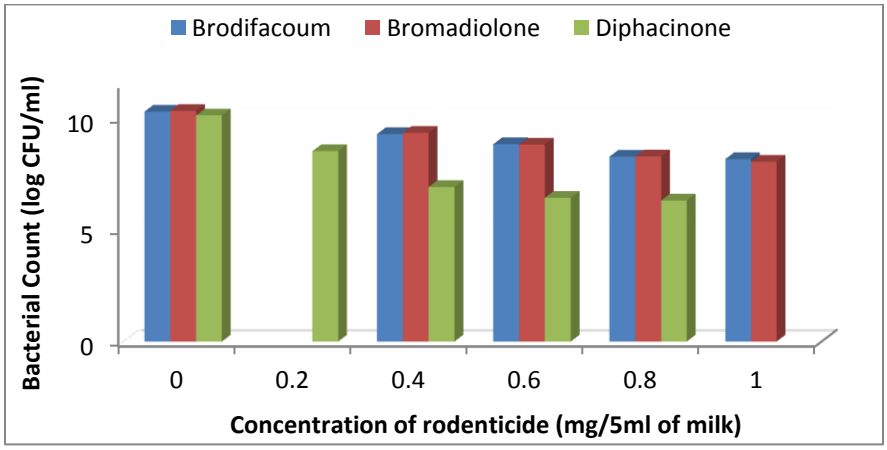
The minimum growth of YI 885 (the highest pH) was observed at the highest concentration of bromadiolone (1mg/5ml) in milk. The minimum inhibitory concentration was observed at the level of 0.8mg/5ml (0.16mg/ml) of milk at 6 hours of incubation (Figure 5-3 (b)). Figure 5-3 (c) shows the growth of YI 885 in the presence of diphacinone. When diphacinone was added in the levels of 0.2, 0.4, 0.6, and 0.8mg/5ml of milk, there was no difference in the growth of YI 885 at 2 hours of incubation (Figure 5-3 (c)) compared to the control sample. However, the growth of YI 885 was significantly reduced ($p < 0.05$) after 4 hours and 6 hours of incubation. The minimum inhibitory concentration of diphacinone was at the level of 0.2 mg/5ml (0.04 mg/ml; $p < 0.05$) of milk. The minimum growth of YI 885 (the highest pH) was observed at the highest concentration of diphacinone (0.8mg/5ml) in milk.

Figures 5-4(a), (b), and (c) show the population of yogurt cultures YF001, YG011 and YI 885 in milk in the presence of brodifacoum, bromadiolone and diphacinone after 6 hours of incubation. When brodifacoum was added in the levels of 0.2, 0.4, 0.6, and 0.8mg /5ml in milk, the bacterial population of YF001 decreased rapidly during 6 hour incubation period (Figure 5-4 (a)). A significant ($p < 0.05$) growth inhibition (as measured by log CFU/ml) of the yogurt culture YF001 was observed at 6 hours of incubation in the presence of brodifacoum at the level of 0.2mg/5ml (0.04mg/ml) of milk. At the highest concentration of brodifacoum (0.8mg/5ml) in milk, the bacterial population dropped to 5.05 log CFU/ml (Figure 5.4 (a)) which indicate the growth inhibition of the YF001 (see Figure 5-5).

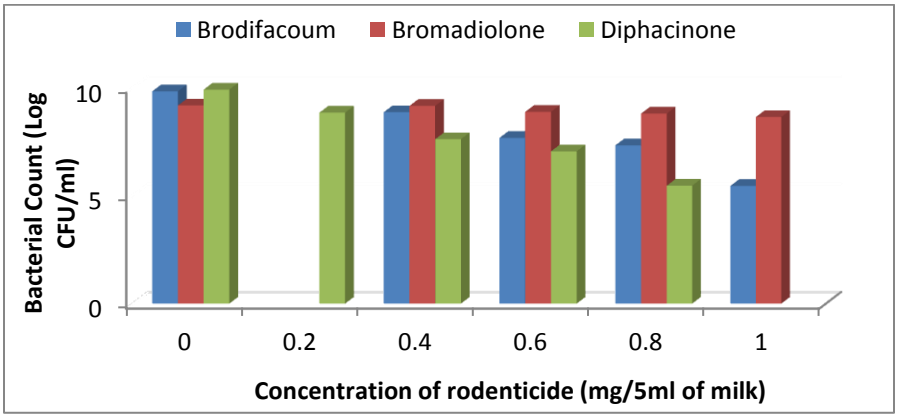
The population of YF001 was significantly lowered ($p < 0.05$) at 6 hours of incubation period with the increasing concentration of bromadiolone and diphacinone in the milk (Figure 5-4 (a)).



(a)



(b)



(c)

Figure 5-4. Population of yogurt culture (a) YF001, (b) YG011, and (c) YI 885 in the presence of rodenticides in milk.

This indicates the inhibition of the growth of YF001 in the presence of bromadiolone and diphacinone in milk. The minimum inhibitory concentration (as measured by the bacterial population (log CFU/ml) of bromadiolone and diphacinone was at the level of 0.2mg/5ml (0.04mg/ml) and 0.4mg/5ml (0.08mg/ml), respectively.

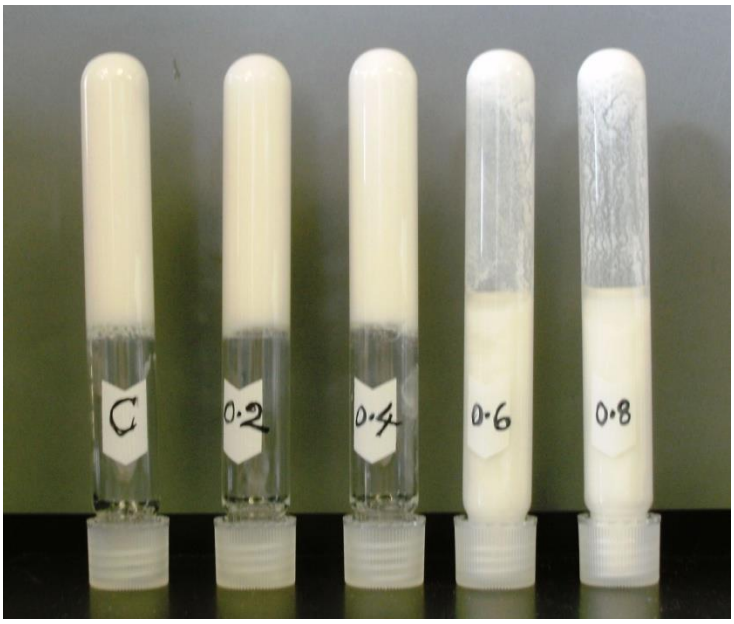


Figure 5-5. Growth inhibition of yogurt culture YF001 in the presence of rodenticides in milk.

Figure 5-4 (b) shows the population of yogurt culture YG011 in the presence of rodenticides. The population of YG011 was significantly lowered ($p < 0.05$) at 6 hours of incubation period with the increasing concentrations of rodenticides in the milk (Figure 5-4 (b)) indicating inhibition of the growth of YG011 in the presence of brodifacoum, bromadiolone and diphacinone in milk. The minimum inhibitory concentration (as measured by the bacterial population (log CFU/ml)) of brodifacoum, bromadiolone, and diphacinone was at the level of 0.4mg/5ml (0.12mg/ml), 0.4mg/5ml (0.12mg/ml), and 0.2mg/5ml (0.12mg/ml), respectively (Figure 5-4 (b)).

Figure 5-4 (c) shows the population of yogurt culture YI 885 in milk in the presence of brodifacoum, bromadiolone and diphacinone after 6 hours of incubation. When brodifacoum was

added in the levels of 0.4, 0.6, 0.8 and 1mg/5ml in milk, the bacterial population decreased rapidly during 6 hour incubation period. A significant ($p < 0.05$) growth inhibition (as measured by log CFU/ml) of YI 885 was observed at 6 hours of incubation in the presence of brodifacoum at the level of 0.4mg/5ml (0.08mg/ml) of milk. When bromadiolone was added at the levels of 0.4, 0.6, 0.8 mg/5ml of milk, there was no significant change of the bacterial population after the incubation when compared to the control sample (Figure 5-4(c)). However at the highest concentration of bromadiolone (1mg/5ml) in milk, the bacterial population dropped to 8.63 log CFU/ml (Figure 5-4(c)) which indicate the growth inhibition of the YI 885. The minimum inhibitory concentration (as measured by the bacterial population (log CFU/ml)) of brodifacoum, bromadiolone and diphacinone was at the level of 1mg/5ml (0.2mg/ml), 0.4mg/5ml (0.12mg/ml), and 0.2mg/5ml (0.12mg/ml), respectively (Figure 5-4 (c)). Overall YI 885 had a higher sensitivity towards diphacinone.

Table 5-1 shows the α-galactosidase activity of yogurt cultures in the presence of rodenticides. There were statistically significant differences ($p < 0.05$) in α-galactosidase activity in the presence of brodifacoum, bromadiolone, and diphacinone in milk. In the control group α-galactosidase activity was significantly enhanced compared to the α-galactosidase activity in the presence of rodenticides in milk. The α-galactosidase activity in YF001 in the absence of brodifacoum, bromadiolone and diphacinone in milk was 0.15, 0.1 and 0.14 GalU/g, respectively. When brodifacoum, bromadiolone and diphacinone was added at the level of 0.4mg/5 ml of milk, α-galactosidase activity in milk was 0.03, 0.01 and 0.03 GalU/g, respectively (Table 5-1).

Table 5-1

Alpha Galactosidase Activity (GalU/g) of Yogurt Culture in the Presence of Rodenticides

Rodenticide Concentration (mg/5ml of broth)	YF001			YG011			YI885		
	BR*	BM*	DP*	BR	BM	DP	BR	BM	DP
0	0.15±0.04	0.1±0.03	0.14±0.02	0.58±0.24	0.49±0.08	0.47±0.03	0.52±0.07	0.47±0.03	0.47±0.04
0.2		0.02±0.00				0.38±0.06			0.42±0.04
0.4	0.03±0.02	0.01±0.00	0.03±0.00	0.39±0.03	0.37±0.06	0.03±0.00	0.44±0.03	0.40±0.03	0.29±0.04
0.6	0.02±0.00	0.01±0.00	0.02±0.01	0.29±0.02	0.2±0.03	0.03±0.01	0.37±0.02	0.36±0.04	0.11±0.03
0.8	0.02±0.00	0.01±0.00	0.03±0.01	0.08±0.01	0.06±0.02	0.01±0.00	0.15±0.05	0.31±0.02	0.03±0.01
1.0	0.01±0.00		0.02±0.01	0.03±0.02	0.02±0.01		0.02±0.02	0.04±0.02	

*BR = Brodifacoum, BM = Bromadiolone, DP = Diphacinone.

This indicates the inhibition of bacterial growth in milk. Similar results were observed in the α-galactosidase activity in YG011 and YI 885. Table 5.2 shows the β-galactosidase activity of yogurt culture in the presence of rodenticides. There was a significant drop ($p < 0.05$) in the β-galactosidase activity of YF001, YG011, and YI 885 cultures in the presence of rodenticides compared to the control sample.

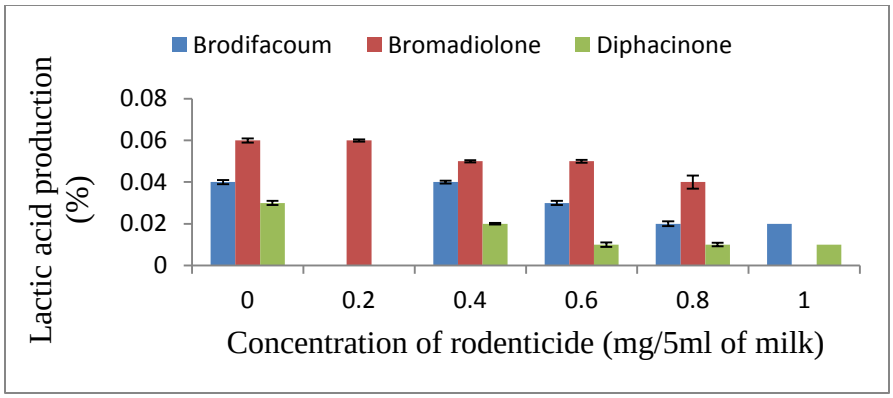
Figures 5-6 (a), (b), and (c) show the lactic acid production of yogurt cultures YF001, YG011 and YI 885 in milk in the presence of brodifacoum, bromadiolone and diphacinone after 6 hours of incubation. When brodifacoum was added in the level of 0.4mg/5ml in milk, there was no significant difference in the lactic acid production compared to that of control sample. When brodifacoum was added in the levels of 0.6, 0.8 and 1mg/5ml in milk, the lactic acid production of YF001 decreased rapidly at the end of 6 hour incubation period (Figure 5-6 (a)). A significant ($p < 0.05$) growth inhibition (as measured by lactic acid production) of the yogurt culture YF001 was observed at 6 hours of incubation in the presence of brodifacoum at the level of 0.2mg/5ml (0.04mg/ml) of milk. At the brodifacoum levels of 0.8, 1mg/5ml of in milk, the lactic acid production dropped to 0.02% (Figure 5-6 (a)) which indicate the growth inhibition of the YF001.

The lactic acid production of YF001 was significantly lowered ($p < 0.05$) at 6 hours of incubation period with the increasing concentrations of bromadiolone and brodifacoum in the milk (Figure 5-6 (a)). This indicates the inhibition of the growth of YF001 in the presence of bromadiolone and diphacinone in milk. The minimum inhibitory concentration (as measured by the lactic acid production) of bromadiolone and diphacinone was at the level of 0.4mg/5ml (0.08mg/ml).

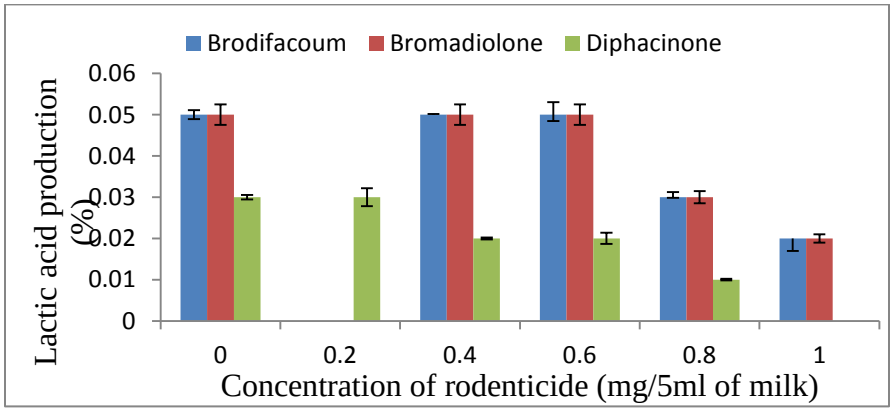
Figure 5-6 (b) shows the lactic acid production of yogurt culture YG011 in the presence of rodenticides. The lactic acid production of YG011 was significantly lowered ($p < 0.05$) at 6

hours of incubation period with the increasing concentrations of rodenticides in the milk (Figure 5-6 (b)) indicating inhibition of the growth of YG011 in the presence of brodifacoum, bromadiolone and diphacinone in milk. The minimum inhibitory concentration (as measured by the lactic acid production) of both brodifacoum and bromadiolone was at the level of 0.8mg/5ml (0.16mg/ml) whereas the minimum inhibitory concentration of diphacinone was at 0.4mg/5ml (0.08mg/ml), respectively and (Figure 5-6 (b)).

Figure 5-6 (c) shows the lactic acid production of yogurt culture YI 885 in milk in the presence of brodifacoum, bromadiolone and diphacinone after 6 hours of incubation. When brodifacoum was added in the levels of 0.4, 0.6, 0.8 and 1mg/5ml in milk, the lactic acid production decreased rapidly during 6 hour incubation period. A significant ($p < 0.05$) growth inhibition (as measured by lactic acid production) of YI 885 was observed at 6 hours of incubation in the presence of brodifacoum at the level of 0.4mg/5ml (0.08mg/ml) of milk. When bromadiolone was added at the levels of 0.4, 0.6mg/5ml of milk, there was no significant change of the lactic acid production after the incubation when compared to the control sample (Figure 5-6(c)). However at the bromadiolone concentration of 0.8mg/5ml in milk, the lactic acid production dropped to 0.03% (Figure 5-6(c)) which indicate the growth inhibition of the YI 885. The minimum inhibitory concentration (as measured by the lactic acid production) of brodifacoum, bromadiolone and diphacinone was at the level of 0.4mg/5ml (0.08mg/ml), 0.8mg/5ml (0.16mg/ml), and 0.6mg/5ml (0.12mg/ml), respectively (Figure 5-6 (c)). Overall YI 885 had a higher sensitivity towards diphacinone. The lactic acid production by the yogurt culture in milk at 6 hours of incubation period was generally low. This could be due to the shorter incubation period.



(a)



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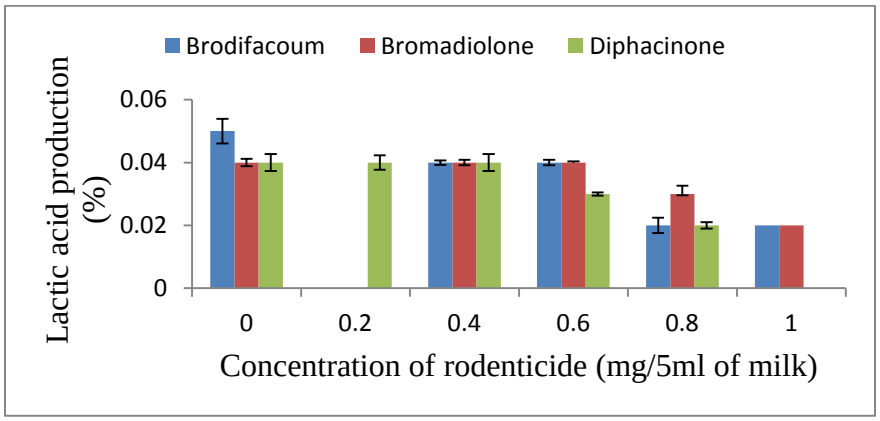

(c)

Figure 5-6 The lactic acid production of yogurt culture (a) YF001, (b) YG011 and (c) YI885 in the presence of rodenticides in milk

Figure 5-7 shows the rennet coagulation in the presence of rodenticides in milk. As seen in Figure 5-7, the control samples coagulated at approximately 30.9 minutes after rennet addition. When brodifacoum was added at 0.002/ml the coagulation of milk delayed to 35.2 minutes. The coagulation was delayed to 45 and 56 minutes at the brodifacoum level of 0.003 and 0.004mg/ml respectively. Similarly, the coagulation of milk delayed in the presence of bromadiolone and diphacinone. One limitation of our work arises because the calculations presented in this study assume homogenous distribution of the contaminants throughout the dairy product as it separates from liquid milk to curd and whey. Since each of the rodenticides is hydrophobic, they may preferentially localize among the hydrophobic casein micelles of the curd compared to the aqueous whey. Therefore, the cheese could have a higher weight ratio of contaminant to dairy than the source milk. Further investigation is required to determine the exact relative concentrations of toxins.

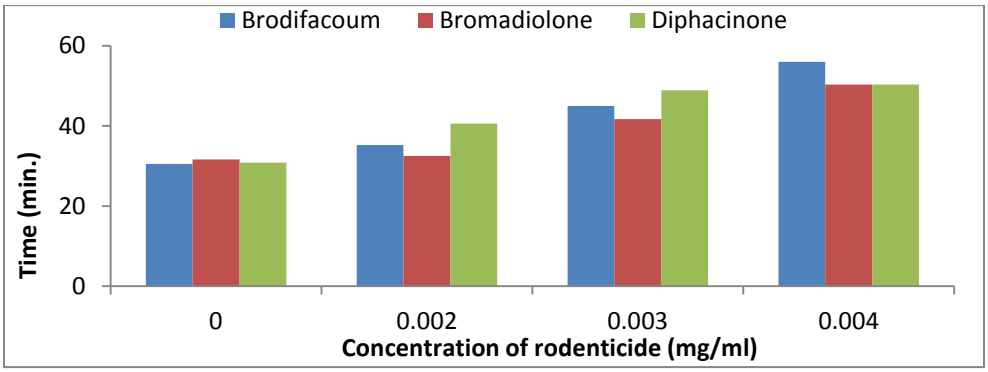

Figure 5-7. Rennet coagulation of milk in the presence of rodenticides.

Table 5-2

Beta Galactosidase Activity (GalU/g) of Yogurt Culture in the Presence of Rodenticides

Rodenticide Concentration (mg/5ml of broth)	YF001			YG011			YI885		
	BR*	BM*	DP*	BR	BM	DP	BR	BM	DP
0	3.76±0.16	3.85±0.03	3.76±0.16	3.98±0.32	4.01±0.18	3.89±0.35	3.99±0.38	0.451±0.04	3.89±0.38
0.2		3.48±0.21				3.44±0.04			3.45±0.22
0.4	3.17±0.11	2.52±0.09	3.16±0.06	2.80±0.11	3.14±0.08	2.89±0.13	3.18±0.07	0.397±0.03	2.46±0.07
0.6	1.23±0.24	2.04±0.14	1.28±0.1	2.10±0.13	2.55±0.16	0.76±0.08	3.03±0.15	0.356±0.04	0.71±0.04
0.8	0.92±0.02	0.10±0.02	0.44±0.05	1.19±0.07	0.83±0.13	0.46±0.10	2.41±0.04	0.313±0.02	0.24±0.06
1.0	0.35±0.07		0.21±0.01	0.47±0.16	0.33±0.03		0.36±0.06	0.041±0.02	

*BR = Brodifacoum, BM = Bromadiolone, DP = Diphacinone.

5.4 Conclusions

The yogurt cultures YG011 and Y1 885 showed highest sensitivity to diphacinone at the level of 0.04 mg/ml. YF001 showed detectable sensitivity to brodifacoum, bromadiolone and diphacinone at the levels of 0.12, 0.08, and 0.08mg/ml, respectively. The growth of YG 011 was inhibited by brodfacoum and bromadiolone at the levels of 0.08 and 0.16mg/ml, respectively. YI 885 showed detectable sensitivity to brodifacoum and bromadiolone at the levels of 0.08 and 0.16mg/ml respectively. A significant growth inhibition of all the yogurt cultures was observed in the presence of all the three rodenticides. Our results show that yogurt culture could be used as a biomarker for the early detection of the presence of rodenticides in milk. This process could be modified to produce a fast, highly sensitive, environmentally safe, and accurate test kit which could be used as a universal marker for early detection of terrorist attacks on food supplies.

CHAPTER 6

Conclusions and Future Directions

All the three yogurt cultures showed significantly ($p < 0.05$) higher growth (as observed by the OD) at 42 °C incubation temperature with few exceptions. The lowest growth of yogurt culture was observed at 37 °C except in YG011 in the presence of bromadiolone. Our results show that the growth of all three yogurt cultures was inhibited in the presence of rodenticides during the 6 hour incubation period. The shortest incubation period that gives satisfactory growth of the yogurt culture was 6 hours. The best incubation temperature for the yogurt cultures in the presence of rodenticides was 42 °C.

The sensitivity of yogurt culture in the presence of rodenticides was detected in MRS broth. The yogurt cultures YF001 and Y1 885 showed highest sensitivity to diphacinone at the level of 0.005 mg/ml. YF001 showed detectable sensitivity to brodifacoum and bromadiolone at the level of 0.02mg/ml. YG011 showed detectable sensitivity to brodifacoum, bromadiolone and diphacinone at the levels of 0.02, 0.04, and 0.02mg/ml, respectively. The growth of YI 885 was inhibited by brodfacoum and bromadiolone at the levels of 0.01 and 0.04mg/ml, respectively. YI 885 was the highest sensitive yogurt culture for all the three rodenticides in the MRS broth. The final population of the bacteria (at the end of 6 hour incubation) reduced with the increasing concentrations of rodenticides. Although α and β-galactosidase activity was low, there were statistically significant differences ($p < 0.05$) in the α and β-alactosidase activity in the presence of rodenticides. The lower enzyme activity could be due to the shorter incubation period of 6 hours.

In milk, the yogurt cultures YG011 and Y1885 showed highest sensitivity to diphacinone at the level of 0.04 mg/ml. YF001 showed detectable sensitivity to brodifacoum,

bromadiolone and diphacinone at the levels of 0.12, 0.08, and 0.08mg/ml, respectively. The growth of YG011 was inhibited by brodfacoum and bromadiolone at the levels of 0.08 and 0.16mg/ml, respectively. YI 885 showed detectable sensitivity to brodifacoum and bromadiolone at the levels of 0.08 and 0.16mg/ml, respectively. A significant growth inhibition ($p < 0.05$) of all the yogurt cultures was observed in the presence of all the three rodenticides. As shown in the results the sensitivity of yogurt culture to the rodenticides in milk was slightly low compared to that in MRS broth. That could be due to the lower availability of rodenticide in the medium due to matrix effect. It could also be due to the higher resistance of yogurt culture in the milk compared to MRS broth. Our results show that yogurt culture could be used as a biomarker for the early detection of the presence of rodenticides in milk. Further studies need to be done using other rodenticides in order to establish the sensitivity and specificity of the test. This process could be modified to develop a fast, highly sensitive, environmentally safe, and accurate on-farm screening test kit which could be used as a universal marker for early detection of terrorist attacks on food supplies.

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