Distinguishing the Disinfection Patterns of *Listeria monocytogenes* Contaminated Mozzarella Cheese by Using Gamma Irradiation Treatment

A Dissertation Submitted to East West University in Partial Fulfillment of the Requirements for the Degree of Bachelor of Science in Genetic Engineering and Biotechnology

Submitted By,
Shovan Kundu
ID: 2016-1-77-026

Department of Genetic Engineering and Biotechnology
East West University
Aftabnagar, Dhaka-1212, Bangladesh

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CERTIFICATE

This is to certify that, thesis entitled **"Distinguishing the Disinfection Patterns of *Listeria monocytogenes* Contaminated Mozzarella Cheese by using Gamma Irradiation Treatment"** by **Shovan Kundu,** ID: 2016-1-77-026, to be submitted to the Department of Genetic Engineering and Biotechnology, East West University, Aftabnagar, Dhaka-1212, in partial fulfillment of the requirement for the Degree of Bachelor of Science in Genetic Engineering and Biotechnology. This work was carried out under our supervision and the content of the thesis have been approved and recommended for the award of Bachelor degree.

Suraia Nusrin

Academic Supervisor

Dr. Suraia Nusrin
Assistant professor and Chairperson,
Dept. of Genetic Engineering and Biotechnology,
East West University,
Aftabnagar, Dhaka-1212

Research Supervisor

Dr. Md. Shakhawat Hussain
Principal Scientific Officer,
Food Safety and Quality Analysis Division,
Institute of Food and Radiation Biology (IFRB), Atomic Energy Research Establishment (AERE), Savar, Dhaka

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List of Abbreviations

^{o}C	Degree Celsius
^{60}Co	Cobalt-60
AERE	Atomic Energy Research Establishment
App.	Approximate
Cfu/gm or cfu/g or CFU g^{-1}	Colony forming unit/gram
et al	*et alliori* (and other)
e.g.	exempli gratia and means “for example.”
i.e.	id est and means “in other words.”
g or gm	Gram
IFRB	Institute of Food and Radiation Biology
IAEA	International Atomic Energy Agency
kGy	Killo Gray
OD	Optical density
%	Percentage
pH	Potential of Hydrogen. It refers to the hydrogen ion concentration in a solution. It is the measure of the acidity or alkalinity of a solution

Abstract:

In today's world, food safety is a major concern, as many people throughout the world are becoming infected with foodborne pathogens; such as Listeriosis caused by *Listeria monocytogenes*, which is responsible for death of many children, pregnant women and aged people every year. In this study, the disinfection pattern of *L. monocytogenes* was examined on mozzarella cheese by using gamma irradiation. After bacterial inoculation (10^8 cfu/gm) into sterile (25 kGy) cheese our individual tasted strains as 6, 8, 13, 14, 15 were treated with irradiation doses as 1 kGy, 3 kGy, 5 kGy, 7 kGy, 9 kGy and 10 kGy with a highly pathogenic reference strain *L. monocytogenes* ATCC 7466. The initial bacterial range was 9.5 $\log_{10}$ cfu/gm to 7.9 $\log_{10}$ cfu/gm. Results of irradiation treatments showed that, when the radiation doses were increased as 1 kGy to 10 kGy, the number of colonies were significantly decreased at 3 to 7 logs respectively. But in the middle level, radiation doses such as 5kGy and 7 kGy showed near about similar ranges of deductions for every strain, which were 3.3 $\log_{10}$ cfu/gm to 3.0 $\log_{10}$ cfu/gm. The most effective radiation dose was 10 kGy, where most of the bacteria were killed in all strains. The threshold level of this *L. monocytogenes* is 2 $\log_{10}$ cfu/gm which is much higher than our results. According to "The International Atomic Energy Agency (IAEA)", 10 kGy radiation dose is standard to disinfect foods. This study indicates that, *L. monocytogenes* can grow in prepared dairy foods like cheese, yogurt etc. but this can be potentially disinfected by using gamma irradiation processes for improving food safety of ready-to-eat foods.

CHAPTER 1

1. Introduction:

Food safety is a matter of anxiety these days, as many people throughout the world are infected with foodborne pathogens at a huge rate. These pathogens create serious illness in our body; sometimes even death can be happened.

According to food safety program of World Health Organization (WHO) in Bangladesh, maintenance of food safety is important and it aims to uplift appropriate management framework from food production to consumption for controlling food-borne diseases.

Preventing infection and ensuing the nutritional quality in our healthy diet, food safety is required in the area of handling, storing and preparing foods. Unsafe food and water refer to the contamination of foods or water with hazardous dirt and germs which can cause diseases, like diarrhoea, meningitis, cholera etc. Sometimes, the condition can be severe. Some of these infections also provoke difficulties for our bodies to imbibe the nutrients, what we need to become healthy.

Listeria monocytogenes (L. monocytogenes) is one of the most important food-borne pathogens which causes listeriosis and the main cause of death of many children, Pregnant women, immunocompromised and aged people every year. Mortality rate within this subpopulation is approximately ~20%–40%. The bacterium is able to cross intestinal, placental, and blood-brain barriers within human body and generate severe disease like bacteremia, meningitis, and meningoencephalitis (Drevets and Bronze, 2008).

L. monocytogenes persisting on food-processing surfaces is a gram-positive bacterium under the Phylum Firmicutes. Different dairy products like raw milk, packaged milk, yogurt, cheese, casein, custard, butter is contaminated by this bacterium at a huge rate. The factor which makes the bacterium more perilous is its capability of making biofilms, which give the bacterium some advantages for their survival. In this situation, our study focuses on irradiation treatment to control the contamination of *Listeria monocytogenes* in dairy products like cheese.

1.1: Objectives of the research:

1. Investigation the effect of gamma radiation on *L. monocytogenes* containing mozzarella cheese samples.
2. Identification of the effective doses of gamma irradiation to disinfect *L. monocytogenes* contaminated in mozzarella cheese.
3. Establishment the protocol for complete decontamination of dairy products, like cheese contaminated with *Listeria monocytogenes.*

CHAPTER 2

2. Literature Review:

2.1 Background:

Food contamination connotes the presence of detrimental microorganisms and chemicals in food, which can cause serious illness to the consumers. Food contamination can be divided into three categories. They are: 1) physical, including hair, glass, and insects in foods; 2) chemical, including antibiotics, pesticides, and cleaning agents in foods; and 3) biological; including harmful microbes like bacteria, viruses, fungi, and parasites in foods. There is another type of food contamination named cross contamination; which means transferring of harmful microbes to food, from other foods, cutting boards, utensils, etc., if they are not handled properly. Some of these contaminants are safety concerns, while others are quality concerns (Fsis.usda.gov, 2019).

Bacteria and viruses are the most common cause of food contamination. The symptoms and severity of food poisoning vary, depending on which bacteria or virus has contaminated the food.

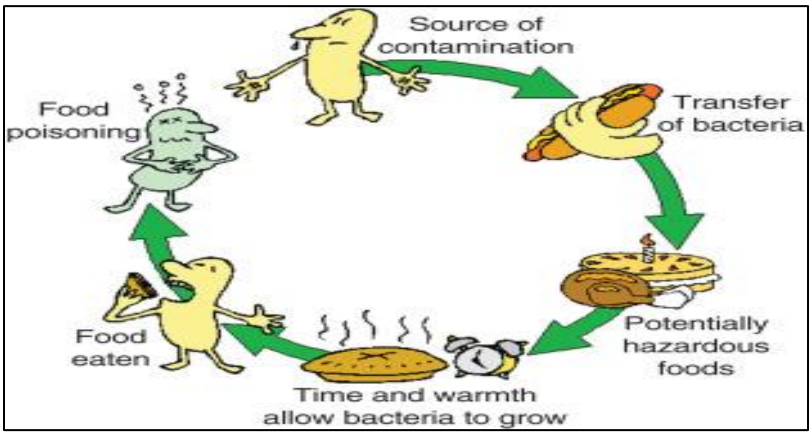

Figure 2.1: Food contamination by harmful bacteria [simplified illustration] (Kamala and Kumar, 2018)

Food contamination in Bangladesh: Among the more common infections that one can get from contaminated foods and drinks are typhoid fever, *Escherichia coli* infections, shigellosis or bacillary dysentery, giardiasis, cryptosporidiosis, other salmonellosis, cholera, rotavirus infections, *Listeria monocytogenes* infection and also a variety of worm infestations are more common (Parveen, Ahmed and Nasreen, 1970) (Ahmed et al., 2010).

Source:

- Unpasteurized (raw) milk and dairy products,
- Soft cheese,
- Raw fruits and vegetables,
- Ready-to-eat meats,
- Refrigerated meat spreads,
- Refrigerated smoked seafood,
- Paper currency notes (Taka),
- Poultry products,
- Water.

(Parveen, Ahmed and Nasreen, 1970) (Ahmed *et al*., 2010)

Among these contaminants, *Listeria monocytogenes* is one of the most hazardous pathogens. The bacterium is becoming more dangerous day by day dur to its biofilm formation and other unique characteristics. *Listeria* can cause fever and diarrhea similar to other food borne germs, but this type of *Listeria* infection is rarely diagnosed.

L. monocytogenes is a Gram-positive bacterium which was first described by E.G.D. Murray in 1924. It was based on six cases of sudden death in young rabbits and he along with his colleagues published a description in 1926 (Murray, Webb and Swann, 1926). Murray referred to the organism as "Bacterium *monocytogenes*" and Harvey Pirie named the genus *"Listeria*" in 1940 (PIRIE, 1940).

In the 1920s, the clinical descriptions of *L. monocytogenes* infection in both animals and humans were first published; but until 1952 in East Germany, it was not recognized as a significant cause of neonatal infection, sepsis, and meningitis (Seeliger, 1952).

Until 1981, *L. monocytogenes* was not identified as a reason of foodborne illness. Then an outbreak of listeriosis was happened in Halifax, Nova Scotia; which involved 41 cases and 18 deaths, mostly in pregnant women and neonates. The incident was epidemiologically linked with the consumption of coleslaw containing cabbage, that had been contaminated with *L. monocytogenes*-contaminated

sheep manure (Schlech et al., 1983). After that, a number of cases of foodborne listeriosis have been reported; and *L. monocytogenes* is now considered as an important hazard in the food industry worldwide (ZHANG, 2007).

Listeria monocytogenes is ubiquitous and an important foodborne pathogen which causes listeriosis. Incidents of food borne listeriosis have been reported involving both immune-compromised and immune-competent persons. Epidemiological evidence shows that dairy products are becoming contaminated by *L. monocytogenes* at a remarkable rate, dairy food processing surfaces are accused as vehicles for some cases of food borne listeriosis. Many outbreaks have involved with pasteurized milk, as well as soft Swiss and Mexican-style cheese (Linnan et al., 1988).

During the last three decades, after the recognition of listeriosis as a foodborne disease, a large number of listeriosis outbreaks and sporadic cases of varying extent have been reported in North America and Europe and diverse food products have been involved in the transmission of the disease. Several outbreaks have been associated with the consumption of dairy products; particularly cheese and ready to- eat foods. In European countries the annual incidence of reported listeriosis cases can vary between 0.3 and 7.5 cases/million inhabitants (Swaminathan and Gerner-Smidt, 2007). Although having a low incidence compared to other foodborne diseases, such as salmonellosis or campylobacteriosis, it is recognized as a major issue to public health authorities due to its high hospitalization rate (94%) and a high case-fatality rate (12.8 to 17% of cases) affecting susceptible groups, such as pregnant women, neonates, children, elderly people and immunocompromised patients (Marder, MPH et al., 2018).

The first articles of *Listeria spp* in Brazil emphasized on food, wastewater, soil and humans. In shrimp, meat and milk products, including raw and pasteurized milk, it was also verified later. Dairy products have been infected with both invasive and non-invasive listeriosis, and serovar 4b is the majority of the isolates of the outbreak. In Europe, dairy products contaminated with *L. monocytogenes* have been implicated in approximately half of the total number of listeriosis outbreaks caused by all types of foods. Lately a number of listeriosis outbreaks associated with cheese prepared from pasteurized milk have been reported. It was found that, in 2% of market cheeses made from raw milk were above the limits specified by the European Commission

(2004/24/EC) and 2.5% of cheeses made with pasteurized milk showed contamination with *Listeria spp* (including *L. monocytogenes*) varying between less than 20 to 20 cfu/gm (Allerberger and Wagner, 2010).

Listeriosis, caused by the bacteria *L. monocytogenes* is a series of diseases; outbreaks of which occur in all countries. There are two main types of listeriosis: a non-invasive form and an invasive form. Noninvasive listeriosis (febrile listerial gastroenteritis) is a mild form of the disease, symptoms include diarrhoea, fever, headache and myalgia (muscle pain). The incubation period is short (a few days). Outbreaks of this disease have generally involved the ingestion of foods containing high doses of *L. monocytogenes*. Invasive listeriosis is a more severe form of the disease and the main cause of death of many children, Pregnant women, immunocompromised and aged people every year (Drevets and Bronze, 2008).

By a previous report, because of consuming *L. monocytogenes* contaminated foods in the United States, approximately 2000 hospitalizations and 500 deaths occur annually (Mead et al., 1999). If this happens in a developed nation, it can be imagined the dangerous effect of this bacterial contamination of a developing nation like us.

2.2 Classification of *Listeria monocytogenes*:

L. monocytogenes is one of the most virulent foodborne pathogens, which can grow and reproduce inside the host cells. It is under the domain of "Bacteria"; "Phylum: Firmicutes"; "Kingdom: Eubacteria"; "Class: Bacilli"; "Order: Bacillales"; "Family: Listeriaceae"; "Genus: Listeria" and "Species: *L. monocytogenes*"

The genus *Listeria* as of 2019 is known to contain 20 species: *L. aquatica, L. booriae, L. cornellensis, L. costaricensis, L. goaensis, L. fleischmannii, L. floridensis, L. grandensis, L. grayi, L. innocua, L. ivanovii, L. marthii, L. monocytogenes, L. newyorkensis, L. riparia, L. rocourtiae, L. seeligeri, L. thailandensis, L. weihenstephanensis*, and *L. welshimeri* (Troxler et al., 2000) (Doijad et al., 2018) (Leclercq et al., 2019).

2.3 Serotypes of *Listeria monocytogenes:*

Based on somatic (O) and flagellar (H) antigens, 13 serotypes were identified in *Listeria monocytogenes* (*L. monocytogenes*) including 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7. By some other research, it is said that, *L. monocytogenes* has 14 serotypes (Borucki and Call, 2003). With the aid of multiplex PCR assay, four major serovars of *L. monocytogenes* strains can be categorized into four distinct serogroups, IIa (serovars 1/2a, 1/2c, 3a, and 3c), IIb (1/2b, 3b, 4b,4d, and 4e), IIc (1/2c and 3c), and IVb (4b, 4d, and 4e) by targeting four marker genes. Food or food production environment is commonly contaminated with serotypes 1/2a, 1/2b, 1/2c, and 4b. By numerous molecular analyses, such as: hybridization patterns of different serotypes by DNA microarray techniques, restriction endonuclease digestion profiles (REDP) by the enzymes AscI and ApaI, comparison in Listeriolysin Gene; *L. monocytogenes* strains haven been classified into two major phylogenetic divisions, with Division I, consisting of serotypes 4b and 1/2b and Division II, consisting of serotypes 1/2a and 1/2c (Borucki et al., 2003) (Brosch et al., 1994) (Borucki et al., 2003). According to a previous paper titled, "Variation in Biofilm Formation among Strains of *Listeria monocytogenes*", the researchers found that, Division I of *L. monocytogenes* (serogroups 1/2b and 4b) produces more biofilms than Division II (serogroups 1/2a and 1/2c); they used the crystal violet microtiter assay for proving their findings (Borucki et al., 2003).

2.4 General description of *Listeria monocytogenes:*

L. monocytogenes is a facultatively anaerobic, rod-shaped bacterium which can grow and reproduce inside the host cells and is one of the most virulent food borne pathogens, with 20 to 30% of foodborne listeriosis infections in high-risk individuals may be fatal. Its ability to grow at low temperatures as 0°C, permits its multiplication at typical refrigeration temperatures, which greatly increases its ability to evade control in human foodstuffs (Linnan et al., 1988).

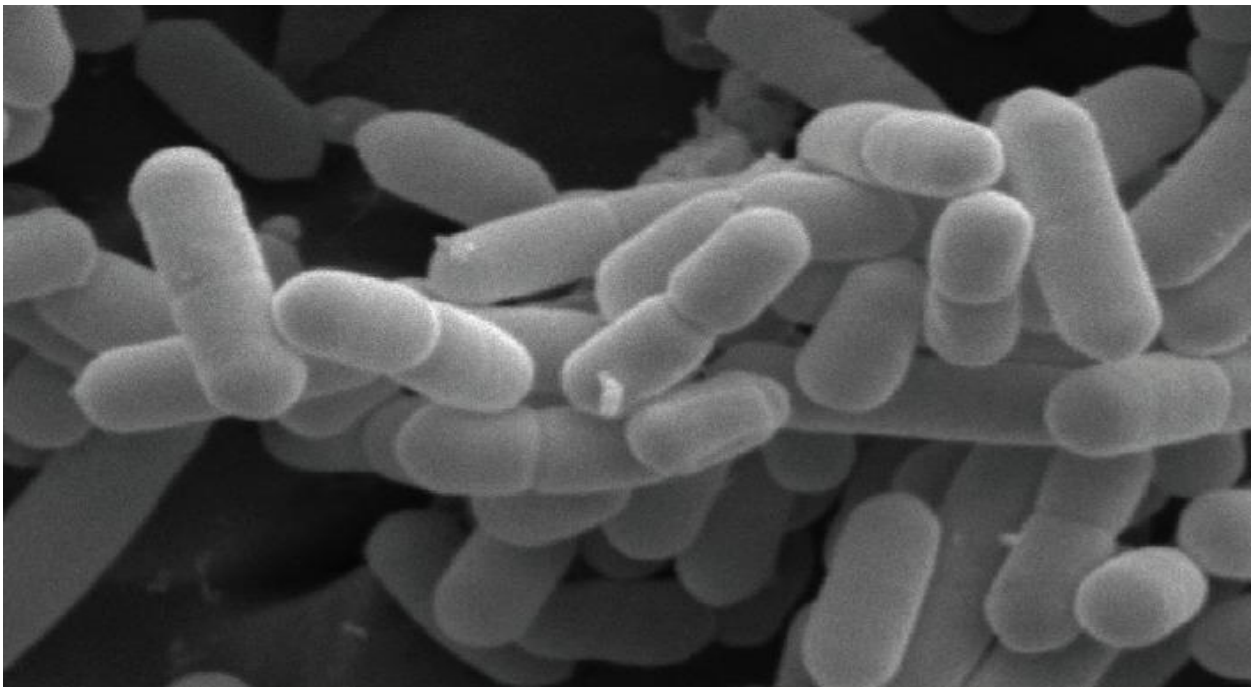

Figure 2.2: Electron micrograph of *Listeria monocytogenes* (Harnessing the Power of Microbes as Therapeutics: Bugs as Drugs, 2015)

Outside host cells, *L. monocytogenes* uses flagellin protein (FlaA) for tumbling, motility to get to the surface, only in a narrow temperature range. Flagellum is also important for initial epithelial cell attachment of the host cells (Gorski, Duhé and Flaherty, 2009) (Lemon, Freitag and Kolter, 2010). When the organism is grown between 20-25°C, flagellin is both produced and assembled at the cell surface, but at 37°C flagellin production is markedly reduced (Linnan et al., 1988).

According to previous studies, 10% of human gastrointestinal tracts may be colonized by *L. monocytogenes.* Because of its recurrent pathogenicity, which causes meningitis in newborns (acquired transvaginal), pregnant mothers are often advised not to eat soft cheeses like Brie, Camembert, feta, and queso Blanco, fresco; which may be infected with *L. Monocytogenes* (Swaminathan and Gerner-Smidt, 2007). It is the third-most common cause of meningitis in newborns. *Listeria monocytogenes* can infect the host's brain, spinal cord and/or bloodstream through the absorption of contaminated food such as unpasteurized dairy or raw foods (Marder, MPH et al., 2018).

The optimum growth temperature of *L. monocytogenes* is 30–37°C; but the bacterium can survive between 0-45°C. Once introduced into the processing plants, it is able to survive and remain for a long period under adverse conditions (Grundling et al., 2004).

2.5 Cell structure of *Listeria monocytogenes:*

The biochemistry of the cell structure of *L. monocytogenes* and other *Listeria spp.* was examined by Fiedler, who proposed a macromolecular demonstrate of the organization of the *Listeria* cell wall (Fiedler, 1988). Electron micrographs of the cell wall appeared it to be that commonplace of gram-positive microbes, i.e., a thick homogeneous structure encompassing the cytoplasmic layer and without the outer membrane characteristic of gram-negative microbes. Confined dry cell walls are composed of around 35% peptidoglycan, comprising of cross-linked meso-diaminopimelic corrosive. The remaining carbohydrate comprises of the cell wall are teichoic acids, which are polymers covalently connected to a particular location on the peptidoglycan. They are ordinarily composed of glycerol or ribitol, impartial sugars, N-acetylamino sugars, and phosphate. Fundamentally, two sorts of cell wall teichoic acids exist among *Listeria* serotypes. Within the to begin with, ribitol buildups are covalently connected by phosphodiester bonds between C-1 and C-5 and are sometimes found with N-acetylglucosamine substituted at C-2; this type is found associated with serotypes (1/2a, b, and c); (3a, b, and c) and 7. In the second, N-acetyl glucosamine is integrated into the chain; this type is found associated with serotypes (4a, b, and d). *Listeria* cell walls also consistently contain lipoteichoic acids, in which a glycolipid moiety, such as a galactosyl-glucosyl-diglyceride, is covalently linked to the terminal phosphomonoester of the teichoic acid. This lipid region anchors the polymer chain to the cytoplasmic membrane. These lipoteichoic acids resemble the lipopolysaccharides of gram-negative bacteria in both structure and function, being the only amphipathic polymers at the cell surface (Paterson, 1940) (Fiedler, 1988).

2.6 Genome of *Listeria monocytogenes:*

The genome of *Listeria monocytogenes* strain EGD-e is fair one of a few stains of the bacterium that have been sequenced. Strain EGC-e is 2,944,528 base pairs long with 2853 open perusing outlines and a GC substance of 39% (NCBI). Encoded proteins uncovered a striking likeness to those of the soil bacterium *Bacillus subtilis*. Qualities were hence classified agreeing to the functional categories characterized for *B. subtilis* (Preprints and Nature, 1997).

Other strains sequenced include *Listeria monocytogenes str.4b F2365*; is a serotype 4b (genomic division II) cheese isolate from the Jalisco cheese outbreak of 1985 in California, which is 2,905,310 base pairs long with a GC content of 38% (NCBI). The genomes of *Listeria monocytogenes str. 1/2a F6854* (genomic division I) was isolated from a sporadic case in 1988 in Oklahoma; and *Listeria monocytogenes str. 4b H7858* was isolated from the multistate outbreak of 1998 to1999 (approximately) in the USA, are being sequenced. *Listeria monocytogenes* has a single circular chromosome. The chromosomes of the serotype 4b strains (F2365 and H7858) lack intact insertion sequence (IS) elements, but contain four copies of transposase ORFA of the IS3 family that are present in homologous locations in both strains. The serotype 1/2a strains (F6854 and EGD-e) contain three copies of the same transposase ORFA in the same location as three of the ORFA insertions in the serotype 4b strains. The additional copy of the transposase ORFA in the serotype 4b strains appears to have resulted from a complete and a partial duplication (along with the associated regions) in strains F2365 and H7858, respectively. In addition, an intact IS element (ISLmo1) is present in the serotype 1/2a strains F6854 (two copies) and EGD-e (three copies), respectively (Mascola et al., 1988) (Marus et al., 2019) (Update: Multistate Outbreak of Listeriosis—United States, 1998-1999, 1999).

The capacity of *Listeria* to possess a wide extend of situations is ascribed to the presence of 331 genes encoding distinctive transport proteins, comprising 11.6% of the entire gene compliment of *L. monocytogenes. Listeria* also has an extensive regulatory range possessing much of the whole genome (Hain et al., 2006).

Listeria monocytogenes is an opportunistic pathogen of people and creatures. *Listeria innocua* is closely related with *L. monocytogenes* but *Listeria innocua* is nonpathogenic. Both of them are habitually separated from cheese, dairy items and other foods.

Table 2.1: Comparisons between two *Listeria* genomes (Buchrieser et al., 2003)

General features	*L. monocytogenes*	*L. innocua*
Size of the chromosome (bp)	2,944,528	3,011,209
G+C content (%)	39	37
G+C content of protein-coding genes (%)	38	38
Total number of protein-coding genes	2853	2973
Average length (codons) of protein-coding genes	306	299
Number of rRNA operons	6	6
Number of tRNA genes	67	66

2.7 Plasmids of *Listeria monocytogenes:*

The presence of plasmid in *Listeria* was to begin with detailed by Pe´rez-D´ıaz (Margolles, 1998). Most plasmids in *Listeria* are cryptic but a few studies have appeared the association of plasmid and transposons in cadmium and anti-microbial resistance in *Listeria*. In spite of a few studies on food and environmental strains there is still exceptionally small data about plasmids in *L. monocytogenes* from nonclinical source such as nourishments. Recently, a few strains of *L. monocytogenes* have been confined from cheese items. Analysis by electrophoresis of *Apa*I and *Sma*I digested chromosomal DNA, it has been defined five clusters in *L. monocytogenes* (m1 to m5). Clusters m1, m2 and m3 of *L. monocytogenes* harbored strains of serogroup1 while clusters m4 and m5 contained strains of serogroup 4 (POYARTSALMERON, 1990) (Margolles, 1998).

According to a study, where they analyzed plasmids isolated from 30 isolates of *Listeria monocytogenes* and 18 isolates of *Listeria innocuao* contaminating short-ripened cheeses. The isolates of *L. monocytogenes* serogroup 1 contained a single plasmid, pLM33 (33.2kbp); whereas the serogroup 4 isolates did not contain any plasmids. One group of *L. innocua* strains contained the plasmid pLI71 (71 kbp) and another one contained two plasmids: pLI59 (59.5 kbp) and pLI56 (56.5 kbp). Plasmids pLM33, pLI71 and pLI59 shared homology regions of at least 20 kbp. Plasmid pLI56 did not encode genes for any known character (such as carbohydrate fermentation; resistance to antibiotics, heavy metals or disinfectants; growth at low pH; NaCl tolerance or thermal inactivation by pasteurization) and displayed different characteristics to the other three plasmids. It was also the only one cured from the parent strain and the sole plasmid not digested by the restriction enzyme PstI. In addition, its lack of homology with pLM33, pLI71 and pLI59 enhanced the possibility of a different origin for plasmid pLI56 (Margolles, 1998).

2.8 Metabolism of *L. monocytogenes:*

The bacterial pathogen, *L. monocytogenes* can grow under aerobic and anaerobic conditions; and is well adjusted to life within the soil and on vegetation as a saprophyte (i.e., extracellular environments), and to life within the cytosol of mammalian cells as a pathogen (i.e., intracellular environment). These situations vary incredibly in their metabolite collection (e.g., carbon, sulfur and nitrogen sources) and abundance (e.g., amino acid accessibility). In this manner, *L. monocytogenes* requires niche-specific adjustments to bolster development, conjointly employments metabolic signals to trigger harmfulness mechanisms, such as master virulence regulator, PrfA (Sauer, Herskovits and O'Riordan, 2019).

According to the studies on carbohydrate fermentations, under anaerobic conditions only hexoses and pentoses supported growth; aerobically, maltose and lactose (but not sucrose) also supported growth of *L. monocytogenes. L. monocytogenes* and *L. innocua* utilize glucose, lactose, and rhamnose under aerobic conditions; *L. grayi* and *L. murrayi* also utilize galactose. *L. ivanovii* and *L. seeligeri* are the only *Listeria* spp. to ferment xylose (Ramaswamy et al., 2007).

2.8.1 Carbon metabolism:

L. monocytogenes can utilize a variety of carbohydrates as carbon sources, but fails to grow with casamino acids. *L. monocytogenes* is able to catabolize glucose via the glycolytic and the pentose phosphate pathways; the genes for the enzymes of the Entner-Doudoroff Pathway (ED Pathway) are absent (Eisenreich et al., 2006).

2.8.2 Nitrogen metabolism:

Glutamine, convertible to glutamate, is the optimal nitrogen source for *L. monocytogenes in vitro and in vivo* nitrogen metabolism of *L. monocytogenes* that is assumed to use ammonium, arginine, and ethanolamine as nitrogen sources during intracellular replication. NrgAB that is activated by TnrA at low concentrations of nitrogen sources is responsible for the uptake of ammonium ions. Gene *nrgAB* is strongly downregulated within murine macrophages, but upregulated in human epithelial cells. These data again suggest a cell-type dependent availability of nitrogen sources and/or cell-type specific metabolic adaptations of *L. monocytogenes*. Ethanolamine lyase EutABC is required for intracellular replication of *L. monocytogenes.* Ethanolamine might be derived from phosphatidylethanolamine by the activity of *Listeria* phospholipases (Buchrieser et al., 2003).

2.9 Pathogenesis of *L. monocytogenes:*

The pathophysiology of *Listeria* disease in people and creatures is still ineffectively caught on. As contaminated food is the major source of disease in both epidemic and sporadic cases, the gastrointestinal tract is thought to be the essential location of section of pathogenic *Listeria* for living into the host (Parveen, 2012). The clinical course of disease as a rule starts almost 20 h after the ingestion of intensely contaminated food in cases of gastroenteritis, whereas the incubation period for the invasive illness is generally much longer, around 20 to 30 days (Dalton et al., 1997) (Linnan et al., 1988). Similar incubation periods have been reported in animals for both gastroenteric and invasive diseases (Wilesmith and Gitter, 1986). The incidence of human listeriosis is very low, normally around 2 to 8 sporadic cases annually per million populations in Europe and the United States (Parveen, 2012).

According to a report, it were 3 of the 12 known serovars of *L. monocytogenes*, 1/2a, 1/2b, and 4b, account for more than 90%of human and animal cases of listeriosis (Schuchat, Swaminathan and Broome, 1991); in spite of the fact that other serovars, such as 1/2c, are frequently found as food contaminants (Rocourt et al., 1992). Among the listeriosis-associated serovars, 4b strains cause over 50% of listeriosis cases around the world, but strains of antigenic bunch 1/2 (1/2a, 1/2b, and 1/2c) prevail in food isolates (Rocourt and Berche, 1987). This suggests that serovar 4b strains are more adapted to mammalian host tissues than strains from serogroup 1/2. Most listeriosis patients have a physiological or pathological defect that affects T-cell-mediated immunity. This justifies the classification of *L. monocytogenes* as an opportunistic pathogen. Listeriosis in nonpregnant grown-ups is related in most cases with at slightest one of the following conditions: malignancies (leukemia, lymphoma, or sarcoma) and antineoplastic chemotherapy, immunosuppressant treatment, persistent liver illness, kidney illness, diabetes, and collagen disease (Rocourt and Brosch 1992). Human immunodeficiency infection (HIV) contamination is additionally a critical hazard factor for listeriosis. AIDS is the fundamental predisposing condition in 5 to 20% of listeriosis cases in nonpregnant grown-ups. It has been assessed that the hazard of contracting listeriosis is 300 to 1,000 times higher for AIDS patients than for the common population (Kales and Holzman, 1990).

2.10 Life cycle of *L. monocytogenes* bacterium inside host cells:

The bacterium maintains two strategies for invasion of host cells. They are:

1. Escaping host immune systems like macrophages, neutrophils.
2. Destruction of the host cells and bacterial proliferation. (Pizarro-Cerdá and Cossart, 2005)

So, the bacterium generates serious illness to our body, which makes it most significant.

Life cycle inside hosts:

Bacterium adhesion to host cell

↓

Phagocytosing by the host cell and phagolysosome formation

↓

Production of listeriolysin O (LLO) toxin by the bacterium

↓

Degrating phagolysosome and free living bacterium starts proliferation

↓

Starting surrounded by actin filaments after actin filaments accumulation

↓

After pushing bacteria forward by the actin filaments, it comes to the terminal of the cell membranes and forms filopod

↓

Attaching another host cells and new cycle begins

(Pizarro-Cerdá and Cossart, 2005)

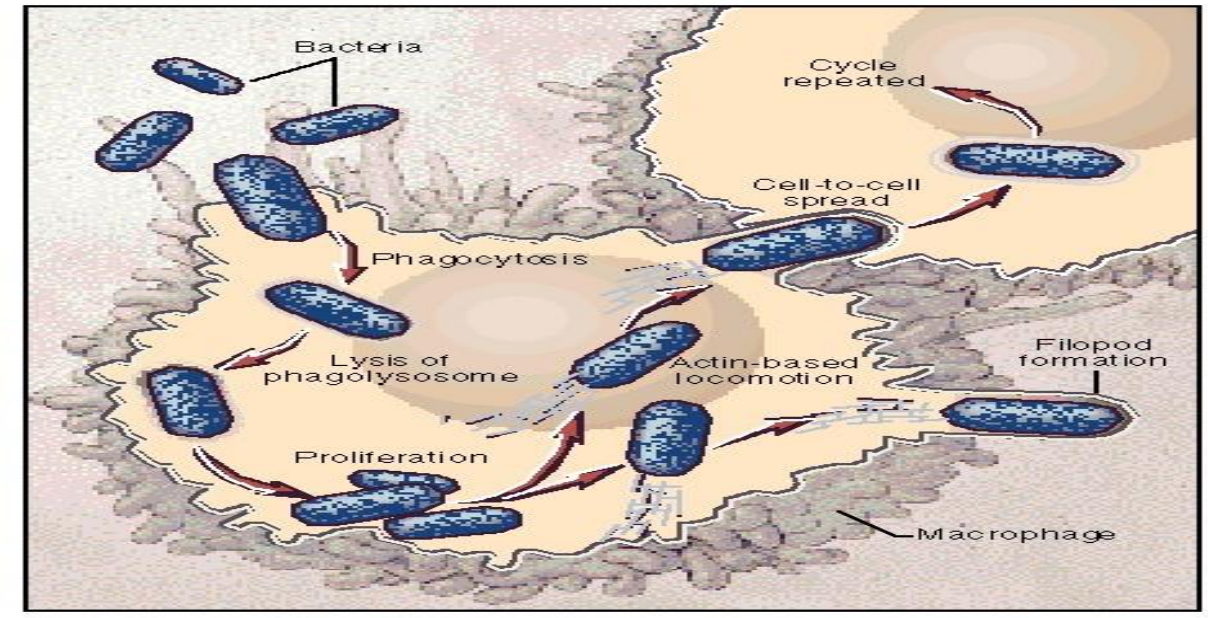

Figure 2.3: Life cycle of *L. monocytogenes* bacterium inside host cells (Southwick and Purich, 1996)

2.11 Biofilm production in food processing surfaces:

The factor which makes the bacterium more hazardous is its capability of making biofilms. The bacterium can survive a long time by this process and can contaminate foods at a huge rate. But biofilm is not formed within host cells, intracellular bacteria are single cells (Pizarro-Cerdá and Cossart, 2005). The biofilm formation gives the bacterium some advantages which makes it more dangerous. It defends the bacteria from different environmental stresses and the bacterium can grow even in refrigeration condition. It becomes hard to remove by cleaning and disinfection than its free-living counterparts. Besides, the biofilms, which are thick and complex becomes more difficult to remove and sometimes are isolated from surfaces even after treatments (Møretrø and Langsrud, 2004)

By biofilm formation, the bacterium gains the ability to persist under UV light and other environmental stresses. Another study showed that, the bacterium is resistant to many chemical disinfectants and sanitizing agents like benzalkonium chloride, sodium hypochlorite, peracetic acid, a mixture of hydrogen peroxide and peracetic acid (Kostaki et al., 2012). Two key enzymes for carbon metabolism (6-phospho-fructokinase and pyruvate dehydrogenase) increased in biofilm cells. (Shi and Zhu, 2009)

The bacterium is becoming antibiotic resistant due to its biofilm formation. It is a matter of concern in the field of controlling *L. monocytogenes* infection. According to a study in Ankara, Turkey, some strains of the bacterium were resistant to ampicillin, nalidixic acid, penicillin G, clindamycin and linezolid. The first multi resistant strain of *L. monocytogenes* (BM4210) was isolated from an 84-year-old patient in 1988 at the Hospital of Dijon. The strain was resistant to erythromycin, chloramphenicol, tetracycline, minocycline and streptomycin (Charpentier et al., 1995).

Process of *L. monocytogenes* biofilm formation in food processing surfaces of dairy foods like cheese:

Depositting organic molecules from diary food products on food processing surfaces.

↓

Attraction of *L. monocytogenes* towards those organic substants of the surface

↓

After disinfection treatment, surviving bacteria becomes stress resistant

↓

Multiplication of persistant bacteria

↓

Expression of cellular genes and quorum sensing

↓

Biofilm formation

(Shi and Zhu, 2009)

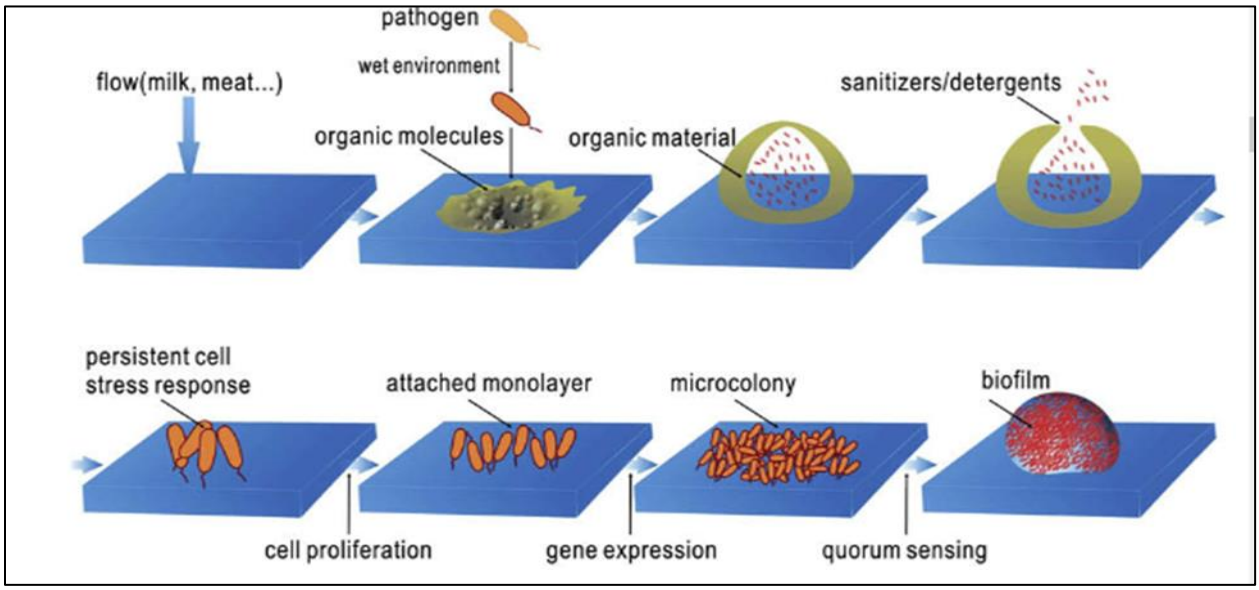

Figure 2.4: Process of *L. monocytogenes* biofilm formation in food processing surfaces of dairy foods (Shi and Zhu, 2009)

2.12 Control of *Listeria monocytogenes* contamination in food:

The control of *L. monocytogenes* contamination in food can be done by three ways. They are:

1) Physical Treatment: Treatment with heat, irradiation, filtration.
2) Chemical Treatment: Treatment with alcohol and other chemical disinfectants.
3) Biological Treatment: Phage and other antimicrobial treatment.

2.13 Food irradiation:

Food irradiation is the process of exposing food and food packaging things to ionizing radiation; such as from gamma rays, x-rays, or electron beams. The radiation can be emitted by a radioactive substance or generated electrically. This treatment is used to improve food safety by extending product shelf-life (preservation), reducing the risk of foodborne illness, delaying or eliminating sprouting or ripening, by sterilization of foods, and as a means of controlling insects and invasive pests. Food irradiation is a technology that improves the safety and extends the shelf life of foods by reducing or eliminating microorganisms and insects.

Irradiation can affect directly, caused by reactive oxygen-centered (OH) radicals originating from the radiolysis of water or indirect on organisms and food products. An indirect effect (the damage to the nucleic acids) occurs when radiation ionizes a neighboring molecule, which in turn reacts with the genetic material. The optimum dose is a balance between that what is needed and that what can be tolerated by the products (Statement summarising the Conclusions and Recommendations from the Opinions on the Safety of Irradiation of Food adopted by the BIOHAZ and CEF Panels, 2011).

2.13.1 Benefits of food irradiation:

- Microorganisms, insect gametes, and plant meristems are anticipated from their propagation, which consequently results in various preservative effects as a function of the absorbed radiation dose (Kilcast, 1989).
- The size of the DNA molecule generally increases with the complexity of an organism; viruses are more radiation resistant than other organisms. The combination of irradiation with heating can be used successfully to inactivation of viruses (Koopmans and Duizer, 2004).
- Insects, mites and other such pests are higher level, multicellular organisms responsible for considerable loss of fresh produce and grains. The best control of insects in agricultural products can be achieved by using fumigants. Radiation has been suggested as an alternative to fumigants.23
- Modification of physical properties by reduces rehydration time of dehydrated vegetables.
- Irradiation extenuates the natural decay of fruits and vegetables, thus extending shelf life.
- Pathogens such as Salmonellae which causes food poisoning can be eliminated from egg, dairy products, poultry and meat (Kilcast, 1989).

Food irradiation is permitted by over 60 countries, with about 500,000 metric tons of food annually processed worldwide. In Austria, Germany, and many other countries of the European Union only dried herbs, spices, and seasonings can be processed with irradiation and only at a specific dose, while in Brazil all foods are allowed at any dose (Deeley et al., 2006).

2.14 Sources of Irradiation:

Irradiation is the process by which an object is exposed to radiation. The exposure can originate from various sources, including natural sources. There are three sources of radiation approved for use on foods.

I. Gamma Irradiation:

Gamma irradiation is produced from the radio-isotopes cobalt-60 and caesium-137, which are derived by neutron bombardment of cobalt-59 and as a nuclear source by-product, respectively. Cobalt-60 is the most common source of gamma rays for food irradiation in commercial scale facilities as it is water insoluble and hence has little risk of environmental contamination by leakage into the water systems. As for transportation of the radiation source, cobalt-60 is transported in special trucks that prevent release of radiation and meet standards mentioned in the Regulations for Safe Transport of Radioactive Materials of the International Atomic Energy Act. Caesium-137 is water soluble and poses a risk of environmental contamination. Insufficient quantities are available for large scale commercial use. An incident where water-soluble caesium-137 leaked into the source storage pool requiring NRC intervention has led to near elimination of this radioisotope. Gamma irradiation is widely used due to its high penetration depth and dose uniformity, allowing for large-scale applications with high through puts. Gamma radiation is used routinely to sterilize medical, dental, and household products and is also used for the radiation treatment of cancer (Fellows, n.d.).

II. Electron beam:

Treatment of electron beams is created as a result of high energy electrons in an accelerator that generates electrons accelerated to 99% the speed of light. This system uses electrical energy and can be powered on and off. The high power correlates with a higher throughput and lower unit cost, but electron beams have low dose uniformity and a penetration depth of centimeters. Therefore, electron beam treatment works for products that have low thickness. Electron beam (or e-beam) is similar to X-rays and is a stream of high-energy electrons propelled from an electron accelerator into food (Fellows, n.d.).

III. X-ray:

x-rays are produced by bombardment of dense target material with high energy accelerated electrons, which gives rise to a continuous energy spectrum. Because of the high atomic numbers

and high melting temperatures, heavy metals, such as tantalum and tungsten are used. Tantalum is usually preferred versus tungsten for industrial, large-area, high-power targets because it is more workable than tungsten and has a higher threshold energy for induced reactions. Like electron beams, x-rays do not require the use of radioactive materials and can be turned off when not in use (Fellows, n.d.).

2.15 Previous studies of treating *L. monocytogenes* contaminated food with radiation:

According to a previous study in India, ready-to-eat sprouts contaminated with *L. monocytogenes* were treated with radiation. Radiation treatment with a 2-kGy dose resulted in complete elimination of 10^3 cfu/gm of *L. Monocytogenes* from all the four varieties of sprouts. No recovery of *L. monocytogenes* was observed in the radiation treated samples stored at normal refrigeration condition up to 12 days (Saroj et al., 2006). By another study, the effective level of irradiation for inactivating *L. monocytogenes* was 16.8 kGy for mozzarella cheese. The research was done for disinfecting different diary diets of immunosuppressed bone marrow transplant patients (HASHISAKA, WEAGANT and DONG, 1989).

CHAPTER 3

3. Methods and Materials:

3.1 *Listeria monocytogenes* isolation from Cheese:

According to the running project of food safety and quality analysis division of Institute of Food and Radiation Biology (IFRB) in Atomic Energy Research Establishment (AERE), Ganakbari, Savar, Dhaka, Bangladesh, cheese samples were collected from different retail shops at Dhaka city, Bangladesh. A total number of 20 cheese samples were collected from those places.

After bacterial culture from these cheese samples and several biochemical tests (carbohydrate test, indole production test etc.), some of the bacterial culture were identified as *L. monocytogenes* and were measured their pathogenicity through biofilm formation capability. The biofilm producing ability refers to the degree of pathogenicity through antibiotic resistance capabilities of the bacterium. Here *L. monocytogenes* strain ATCC 7466 was used as reference strain. The pathogenicity of the identified strains no 6, 8, 13, 14, 15 were compared with this reference strain.

3.2 Bacterial strain collection:

Listeria monocytogenes ATCC 7466 strain, and other isolated strains such as 6, 8, 13, 14, 15 were collected from food safety and quality analysis division, IFRB, AERE. These strains were preserved in nutrients agar slant with glycerol for long time preservation purposes.

3.3 Media preparation:

In this experiment, Nutrient agar (NA) media, nutrient broth media and saline water were used.

3.3.1 Nutrient agar media preparation:

By standard rule, for 1 litter nutrient agar media preparation, desired amounts of nutrient agar power were mixed with remaining amount of distilled water to make 1 litter media. The medium was sterilized by autoclaving at 121°C for 15 min. The medium was dispensed into petri dish and left for cooling to be solidified (Berry, 1984).

3.3.2 Nutrient broth media preparation:

By standard rule, for 1 litter nutrient broth media preparation, desired amounts of nutrient broth powder were mixed with remaining amount of distilled water to make 1 litter media. The medium was sterilized by autoclaving at 121°C for 15 min (Berry, 1984).

3.3.3: Saline water preparation:

By standard rule, for 100 ml saline water preparation, desired amounts of sodium-chloride (NaCl) were mixed with remaining amount of distilled water to make 100 ml saline water. The saline water was sterilized by autoclaving at 121°C for 15 min (Berry, 1984).

3.4 *L. monocytogenes* subculture:

The bacterial strains were freshly sub-cultured in nutrient agar media by using streak plate method. It was needed 24-48 hours incubation at 37°C as it is the optimum temperature for *L. monocytogenes* growth.

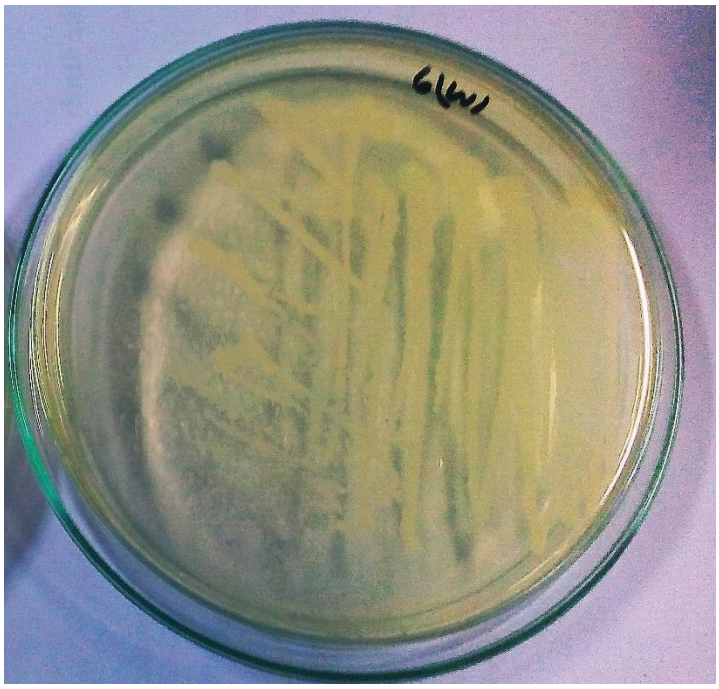

Figure 3.1: Streak plating (subculture) of *L. monocytogenes* strain 6 in nutrient agar media.

3.5. Bacterial culture in nutrient broth media:

For bacterial growth in this experiment, bacterial broth culture was used by using nutrient broth media. Seven test tubes each containing 10 ml of liquid broth media were sterilized by autoclaving at 121^0 C and 15 psi with 15 minutes. After autoclaving process, the sub-cultured *L. monocytogenes* strains were inoculated with the broth medium to make suspension approximate 10^8 cfu/ml and were vortexed properly. The test tubes were capped properly and were stayed it in incubator for growing bacteria into test tubes about 24 hours at 37^0 C.

Figure 3.2: Broth culture of *L. monocytogenes* strains at nutrient broth media

3.6 Cheese Sample Preparation and Bacterial Inoculation:

At first, the cheese samples were sterilized by gamma radiation at 25 kGy. The contamination free samples were measured at 10 grams in a bottle. This pathway was followed for more six bottles. Total seven bottles were used with 90 ml sterilized saline water in each bottle. After that step, the bacterial broth cultures (in nutrient broth) were mixed with the cheese with saline water bottles properly. All these procedures were carried out under laminar airflow cabinet.

Figure 3.3: Bottles containing cheese with saline water contaminated by *L. monocytogenes* inoculates.

3.7 Disinfection process through Irradiation treatment:

This procedure was carried out in sterile condition. The bottles containing cheese with saline water contaminated by *L. monocytogenes* inoculates were kept at incubator for 24 hours incubation at 37°C for bacterial growth. Before radiation treatment, bacterial colonies were counted from each sample by spread plating method.

3.7.1 Irradiation treatment:

Irradiation of the samples were carried out by using Cobalt-60 Panoramic Research Irradiator of Institute of Food and Radiation Biolog10y (IFRB), Atomic Energy Research Establishment (AERE), Bangladesh Atomic Energy Commission, Bangladesh. The irradiation rate was measured

7.89 kGy per hour for doing the radiation treatment. Measurement of dose-rate was performed by Fricke dosimetry system. Fricke dosimeters were prepared following standard protocols; by dissolving 0.392 of ferrous ammonium sulphate (Fe (NH4)2(SO4)2.6H2O) and 0.058 g of sodium chloride (NaCl) in 12.5 mL of 0.4 mol/L sulphuric acid (H2SO4). The volume of the solution was made up to 1 L in a volumetric flask with 0.4 mol/L H2SO4 at 25°C. Therefore, the concentrations of final solution were 0.001 M ferrous ammonium sulphate, 0.001 M sodium chloride and 0.4 mol/L sulphuric acid. Freshly prepared Fricke solution was poured in screw-cap vial and placed in the radiation field with samples for certain amount of time. After completion of irradiation, absorbance of irradiated Fricke solution was measured using spectrophotometer at 303 nm; the unirradiated Fricke solution was used as control.

The equation for the absorbance dose in the Fricke dosimetric solution is

$D = ḍA. NA/(ρḍGεd)$ ……. (1)

Where,

D is the absorbed dose (Gy),

ḍA is the change in absorbance at 303 nm and 25° C (dimensionless),

ḍA = Ai— A0, where Ai and A0 are the absorbance's of the irradiated and non-irradiated solutions respectively,

NA is Avogadro's number (6.02 × 1023 mol–1),

ρ is the density of the dosimetric solution (1.024 × 103 kg/m3),

G is the radiation chemical yield of Fe3+ ions (9.74× 1017 molecules/j) (This G value is valid for electrons or photons in the energy range 0.5–16 MeV at absorbed dose rates of less than 2 × 107 Gy/s), ε is the molar linear absorption coefficient (at 303 nm and 25°C) as measured for the particular spectrophotometer (with a nominal value of 219 m2/mol), and d is the optical path length in quartz cells, usually d = 0.01 m.

For irradiation and absorption measurement temperature of 25°C, with a 1.0 cm path length cuvette, and using the values of e and G given above, equation (1) reduces to

D Fricke (Gy) = 278xḍA. ……. (2)

The Fricke dosimetry system is primarily used in the experiment for dose mapping and determination of dose rate.

After selection of sample position and dose rate by Fricke dosimeters, Amber Perspex dosimeter was used for determination of delivered irradiation dose. The Amber-Perspex dosimeter was prepared by Harwell Laboratory, Oxford shire, United Kingdom. In this experiment, Amber Perspex Dosimeter Type 3042 was used which has a dose detection range between 1 to 30 kGy. Amber 3042 are made from radiation-sensitive poly-methyl methacrylate (PMMA) in the form of optically transparent pieces individually sealed in laminate sachets. They darken when irradiated, and the radiation -induced darkening, accurately measurable by means of a spectrophotometer, is a function of the radiation dose absorbed (Glover et al., 1993). The absorbance (A1) is measured at 603 nm or 651nm. The thickness (T1) of the dosimeter is also measured specific absorbance (A1/T1) is calculated and the determination delivered dose is done by using pre-existing graph /chart produced by Harwell Laboratory (Glover et al., 1993).

Radiation doses (kGy) of 1 kGy, 3 kGy, 5 kGy, 7 kGy, 9 kGy, and 10 kGy were used to observe the disinfection phenomena.

3.8 Bacterial enumeration through Spread plate technique:

After irradiation treatment, the bacterial counts were observed to identify the disinfection process by spread plate method by using nutrient agar media. After spreading the plates were kept at incubator for 24 to 48 hours incubation at 37°C.

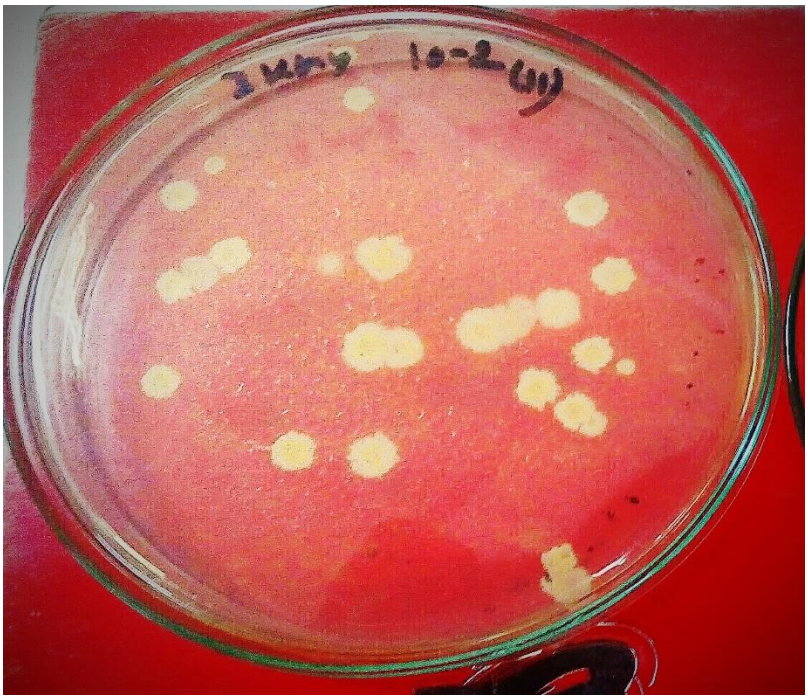

Figure 3.4: Bacterial colonies generating after irradiation treatment

Figure 3.5: Bacterial colonies count (by the unit: cfu/gm)

3.9 Statistical analysis:

The bacterial counts before and after irradiation treatment were converted to $\log_{10}$ cfu/gm unit and Microsoft excel was used to draw the graphs, which represents the statistical comparison of the effectiveness of the irradiation treatment.

CHAPTER 4

4. Results:

Listeria monocytogenes strains (6 strains) were inoculated into cheese with saline water samples and disinfect them by gamma radiation using cobalt-60. Radiation doses as 1 kGy, 3 kGy, 5 kGy, 7 kGy, 9 kGy, and 10 kGy were used to observe the disinfection process. The results are showing statistically by graphs where the vertical lines show the bacterial count in $\log_{10}$ value cfu/gm after radiation doses and horizontal lines shows the radiation doses.

4.1 Disinfection of pathogenic *L. monocytogenes* strain ATCC 7466 in Cheese matrix:

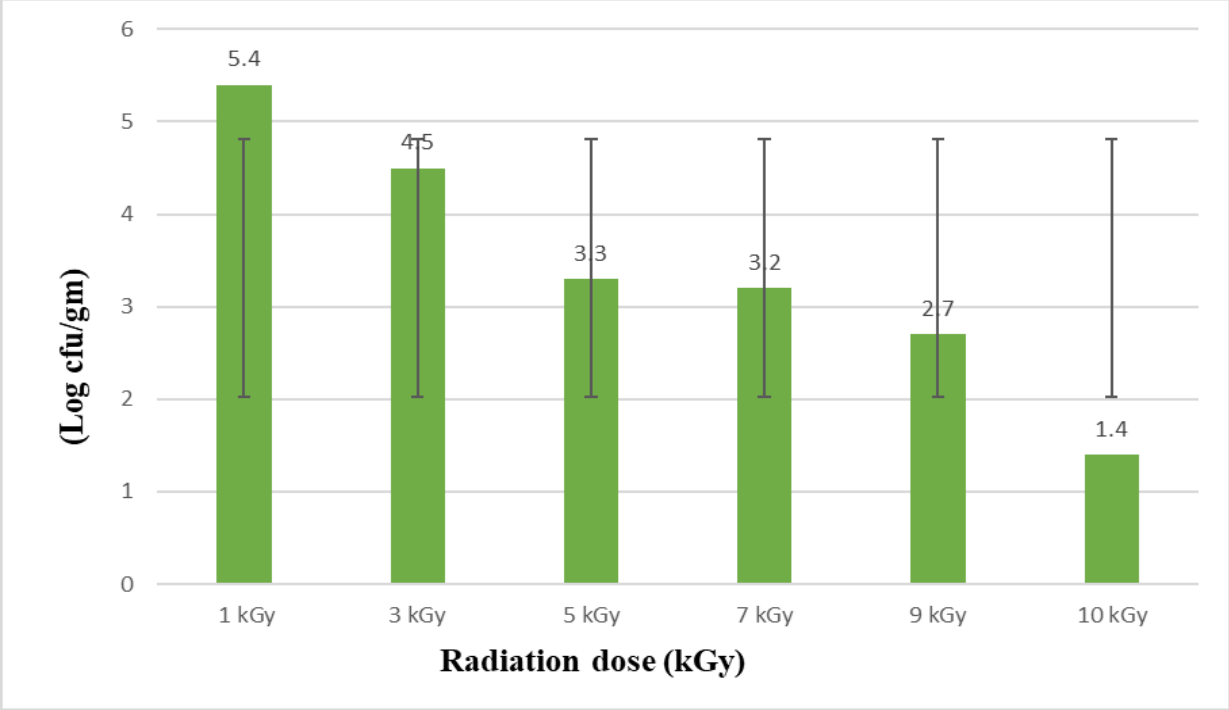

Figure 4.1: Disinfection of pathogenic *L. monocytogenes* strain ATCC 7466 with cheese samples by using gamma radiation

For *L. monocytogenes* strain ATCC 7466, the initial bacterial count was 8.2 $\log_{10}$ cfu/gm without radiation treatment. After applying radiation doses, the bacterial count was 5.4 $\log_{10}$ cfu/gm in 1 kGy, where this range was decreased to 1.4 $\log_{10}$ cfu/gm at radiation dose 10 kGy. In radiation doses 3 kGy and 5 kGy the bacterial counts were gradually decreased near about one log, that were 4.5 $\log_{10}$ cfu/gm, and 3.3 $\log_{10}$ cfu/gm respectively; but in case of radiation dose 7 kGy this count was not much decreased as earlier, which was 3.2 $\log_{10}$ cfu/gm and after 9 kGy radiation dose, this range count also decreased as previous rate, which was 2.7 $\log_{10}$ cfu/gm.

4.2 Disinfection rates of *L. monocytogenes* strain no 6 in cheese matrix:

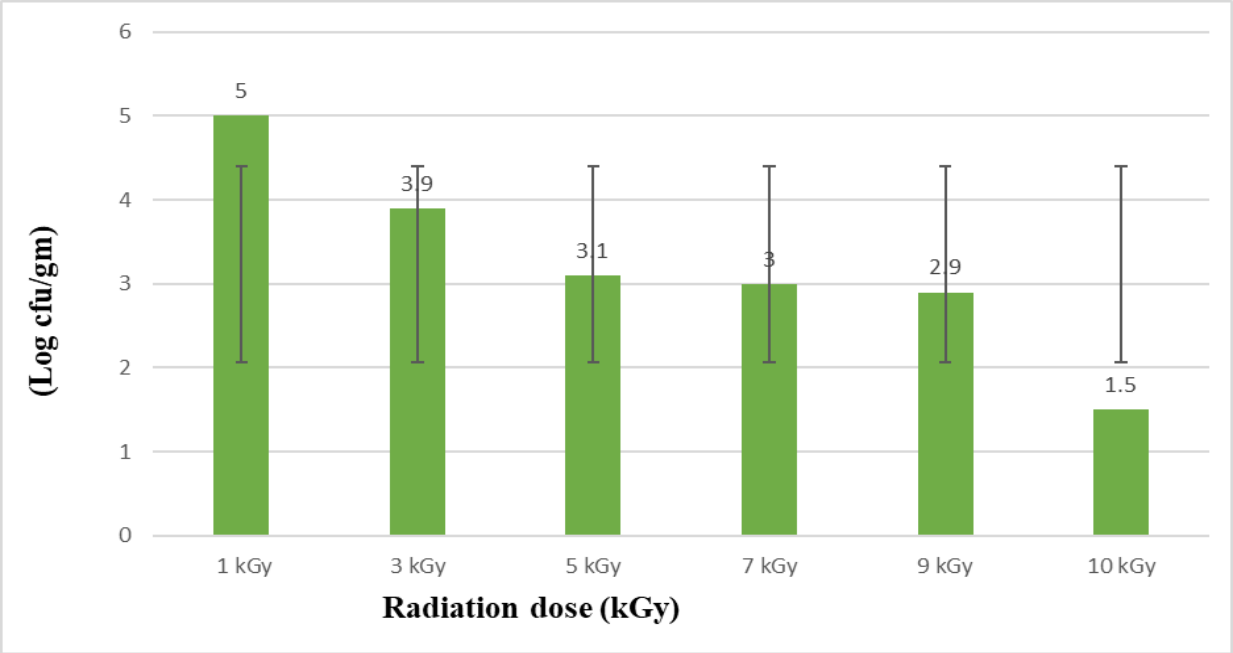

Figure 4.2: Disinfection of *L. monocytogenes* strain no 6 with cheese sample by using gamma radiation

For strain no 6, without radiation treatment the initial bacterial count was 9.5 log_{10} cfu/gm. After applying radiation doses, the bacterial count decreased as 5 log_{10} cfu/gm in 1 kGy, where this range was gradually decreased to 1.5 log_{10} cfu/gm at radiation dose 10 kGy. In radiation doses 3 kGy, and 5 kGy, bacterial counts were observed at 3.9 log_{10} cfu/gm, 3.1 log_{10} cfu/gm respectively, but in dose 7 kGy and 9 kGy, the bacterial counts were 3 log_{10} cfu/gm and 2.9g log_{10} cfu/gm respectively which were not so much effective dose to decrease the bacterial growth rates because both dose rates showed same level of bacterial counts.

4.3 Disinfection rates of *L. monocytogenes* strain no 8 in Cheese matrix:

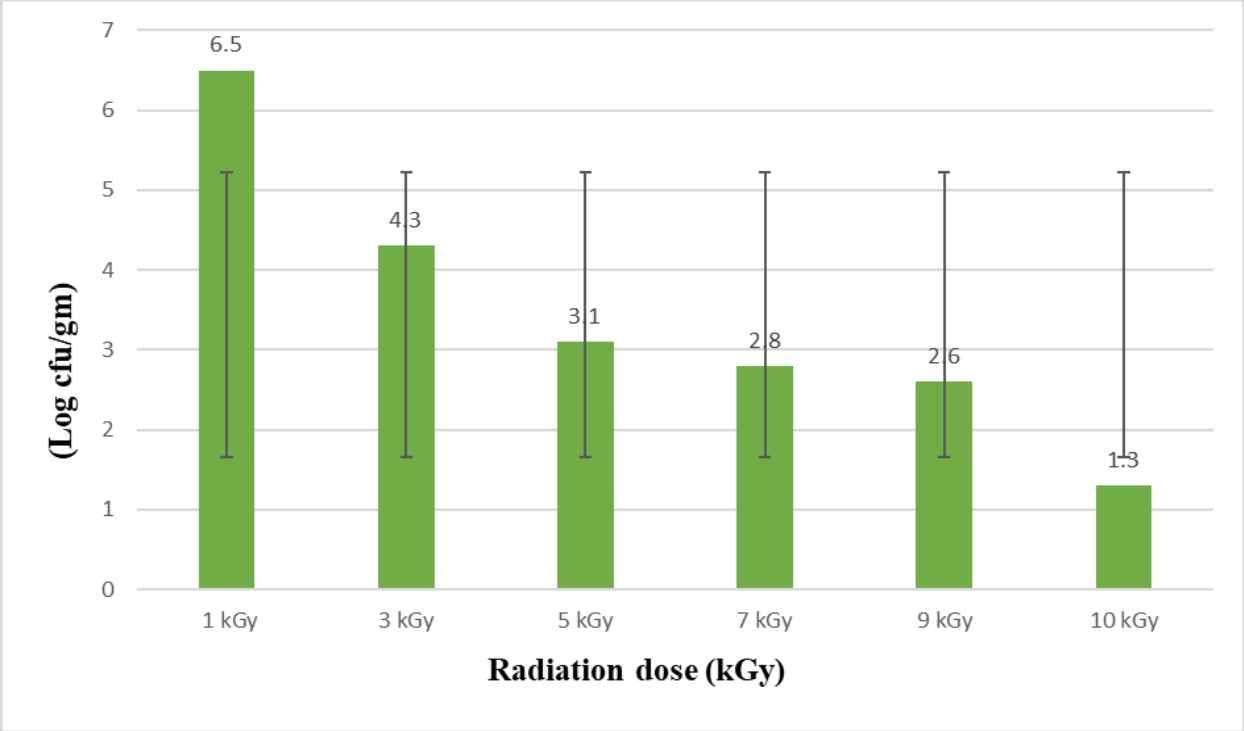

Figure 4.3: Disinfection of *L. monocytogenes* strain no 8 with cheese sample by using gamma radiation

For strain no 8, the initial bacterial count was 9.8 log_{10} cfu/gm in without radiation treatment. After applying radiation doses, the bacterial count was 6.5 log_{10} cfu/gm in 1 kGy, where this range was decreased to 1.3 log_{10} cfu/gm at radiation dose 10 kGy. In radiation doses 3 kGy, 5 kGy, 7 kGy

and 9 kGy, the bacterial counts were observed 4.3 log_{10} cfu/gm, 3.1 log_{10} cfu/gm, 2.8 log_{10} cfu/gm and 2.6 log_{10} cfu/gm respectively. Here, these results indicated that, in 3 kGy bacterial growth rate was decreased one log from 1 kGy; but 5 kGy and 7 kGy dose rates were not much effective on reducing bacterial growths, because they showed near about similar results.

4.4 Disinfection rates of *L. monocytogenes* strain no 13 in Cheese matrix:

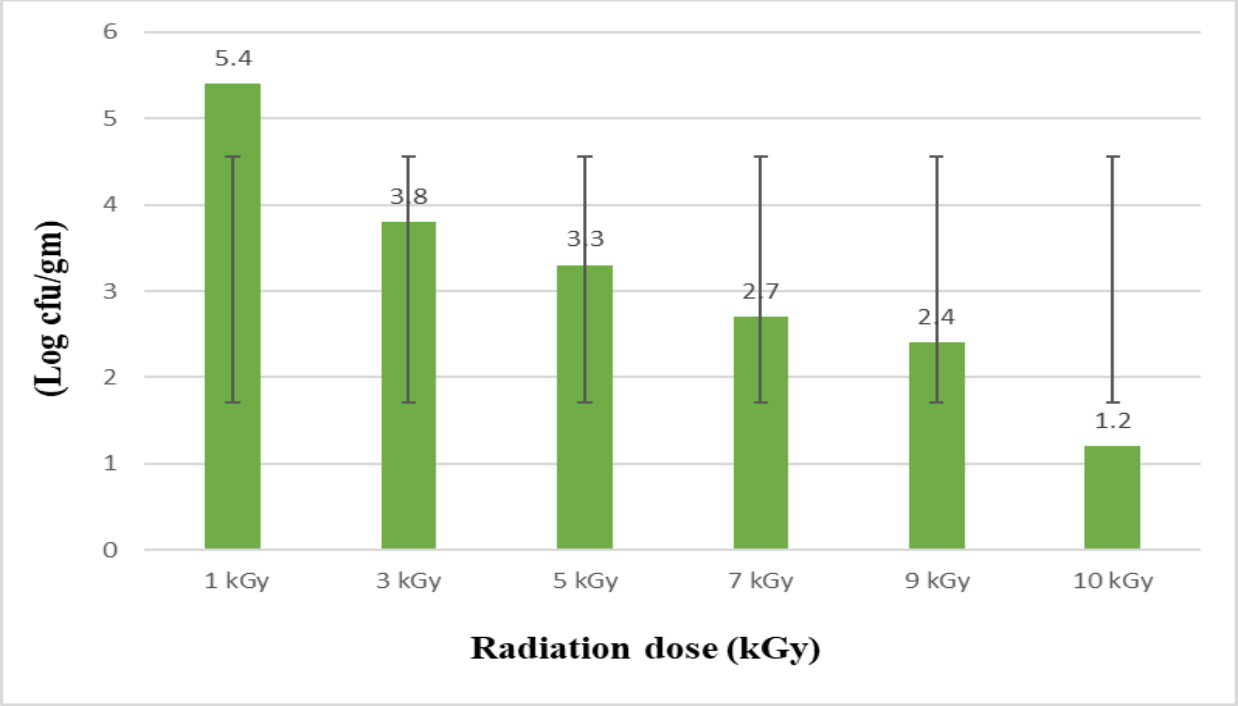

Figure 4.4: Disinfection of *L. monocytogenes* strain no 13 with cheese sample by using gamma radiation

For strain no 13, the initial bacterial count was 7.9 log_{10} cfu/gm without radiation treatment. After applying radiation doses, the bacterial count was 5.4 log_{10} cfu/gm in 1 kGy, where this range was decreased upto 1.2 log_{10} cfu/gm at radiation dose 10 kGy. In radiation doses 3 kGy, 5 kGy, 7 kGy and 9 kGy, the bacterial counts were measured 3.8 log_{10} cfu/gm, 3.3 log_{10} cfu/gm, 2.7 log_{10} cfu/gm and 2.4 log_{10} cfu/gm respectively.

4.5 Disinfection rates of *L. monocytogenes* strain no 14 in Cheese matrix:

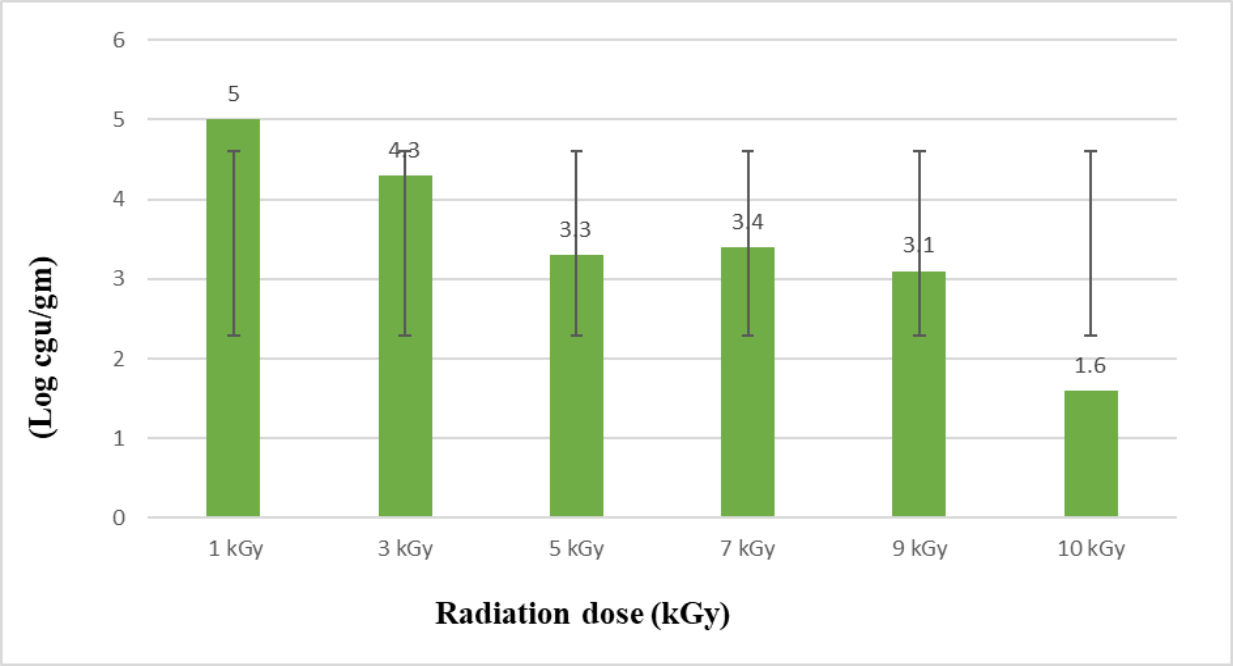

Figure 4.5: Disinfection of *L. monocytogenes* strain no 14 with cheese sample by using gamma radiation

For strain no 14, the initial bacterial count was 8.6 $\log_{10}$ cfu/gm without radiation treatment. After applying radiation doses, the bacterial count was 5 $\log_{10}$ cfu/gm in 1 kGy, where this range was decreased upto 1.6 $\log_{10}$ cfu/gm at radiation dose 10 kGy. In radiation doses 3 kGy, 5 kGy, 7 kGy and 9 kGy, the bacterial counts were measured 4.3 $\log_{10}$ cfu/gm, 3.3 $\log_{10}$ cfu/gm, 3.4 $\log_{10}$ cfu/gm and 3.1 $\log_{10}$ cfu/gm respectively.

4.6 Disinfection rates of *L. monocytogenes* strain no 15 in cheese matrix:

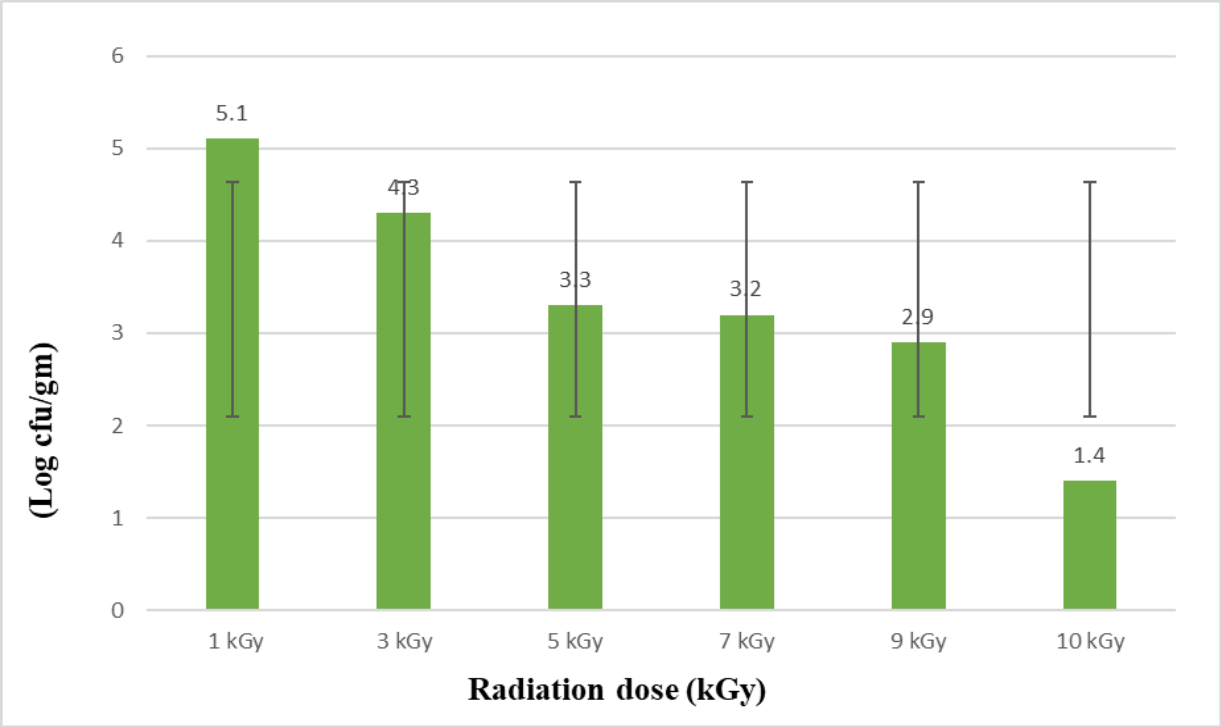

Figure 4.6: Disinfection of *L. monocytogenes* strain no 15 with cheese samples by using gamma radiation

For strain no 15, the initial bacterial count was 8.3 $\log_{10}$ cfu/gm without radiation treatment. After applying radiation doses, the bacterial count was 5.1 $\log_{10}$ cfu/gm in 1 kGy, where this range was decreased to 1.4 $\log_{10}$ cfu/gm at radiation dose 10 kGy. In radiation doses 3 kGy, 5 kGy, 7 kGy and 9 kGy, the bacterial counts were measured 4.3 $\log_{10}$ cfu/gm, 3.3 $\log_{10}$ cfu/gm, 3.2 $\log_{10}$ cfu/gm and 2.9 $\log_{10}$ cfu/gm respectively.

CHAPTER 5

5. Discussion:

The aim of our research was to find out the effectiveness of irradiation treatment for controlling *Listeria monocytogenes* contamination in cheese. *L. monocytogenes* is one of the perilous food-borne pathogens and is becoming more hazardous day by day by antimicrobial resistance, due to its biofilm formation. It causes listeriosis and the main cause of death of many children, Pregnant, immunocompromised and aged people every year; it attacks our immune cells and by host cell destruction, it causes serious illness. For this reason, our study focuses on finding out the killing doses of irradiation treatment to control the dangerous pathogen.

People nowadays are becoming more conscious about the taste of food. They not only take food as the basic need but also for the passion of life. Cheese is one of the most popular food among the people around the world. But by taking contaminated cheese by *L. monocytogenes* as food, people are becoming sick and the smartness of the bacterium creates great hazard for us. So, we choose the contaminated cheese to study the competency of disinfection phenomena by the irradiation treatment.

The bacterial persistence before irradiation treatment was counted. The bacterial counts of pathogenic strain ATCC 7466 were recorded as 8.2 $\log_{10}$ cfu/gm. Similarly, the bacterial counts of strain 6, 8, 13, 14, 15 were 9.5 $\log_{10}$ cfu/gm, 9.8 $\log_{10}$ cfu/gm, 7.9 $\log_{10}$ cfu/gm, 8.6 $\log_{10}$ cfu/gm, 8.3 $\log_{10}$ cfu/gm respectively before irradiation treatment which indicated that the initial bacterial range was 10^8 cfu/gm.

For all identified *L. monocytogenes* strains, including pathogenic reference strain ATCC 7466, the bacterial counts were reduced to approximately (1.2 to 1.6) $\log_{10}$ cfu/gm at radiation dose 10 kGy; where by a previous research the effective level of irradiation for inactivating *L. monocytogenes* was 16.8 kGy for mozzarella cheese (HASHISAKA, WEAGANT and DONG, 1989).

But according to “The International Atomic Energy Agency (IAEA)”, 10 kGy radiation dose is standard to disinfect foods. Above this 10 kGy radiation dose, foods should not be irradiated, according to food standard rule.

As stated by another study on raw milk camembert cheeses, where these cheeses were inoculated with a pathogenic strain of *L. monocytogenes* (V7 serotype ½ a) and were irradiated with gamma or X-rays irradiation. The initial count of *L. monocytogenes*/gm 10^4 inoculates were totally disinfected after 2 kGy radiation dose; But when this initial count was 10^5 bacteria/gm, this inoculates were still viable after the radiation treatment (BOUGLE and STAHL, 1994). So, we can say that, the radiation dose for disinfection processes, higher dose rate is more effective to eliminate higher number of bacterial loads.

By another report, combination of gamma irradiation and refrigeration condition at 4°C used for disinfection of microbial loads in cheese. In that condition, by irradiation doses 2 kGy and 4 kGy, the microbial loads (*L. monocytogenes*) were reduced by approximately 1–2 log cfu/gm (Tsiotsias et al., 2002).

In our study, the initial inoculated bacterial louds range was 9.5 $\log_{10}$ to 7.9 $\log_{10}$ cfu/gm and after treatment with radiation dose 1 kGy, the bacterial counts were reduced to approximately 6.5 $\log_{10}$ cfu/gm (for strain no 8) to 5 $\log_{10}$ cfu/gm (for strain no 6 and 14); besides the reference strain ATCC 7466, the bacterial count was reduced to 5.4 $\log_{10}$ cfu/gm from initial count of 8.2 $\log_{10}$ cfu/gm. The deduction rate of initially was same. After treatment with 3 kGy radiation dose, the bacterial counts were measured 4.3 $\log_{10}$ cfu/gm (for strain no 8, 14 and 15) to 3.9 $\log_{10}$ cfu/gm (for strain no 6) and for strain ATCC 7466, the bacterial count was reduced to 4.5 $\log_{10}$ cfu/gm. Here this value is near about same to our tested strain value. Besides, by 5 kGy radiation treatment, the bacterial counts were reduced to approximately 3.3 $\log_{10}$ cfu/gm (for strain no 13, 14 and 15) to 3.1 $\log_{10}$ cfu/gm (for strain no 6 and 8); where the strain ATCC 7466, the bacterial count was reduced to 3.3 $\log_{10}$ cfu/gm, which is similar range with our tested strains. But the dose rate of this 3 kGy and 5kGy showed not much effectiveness to reduce bacterial growths, because, the deduction rates were much lower than the initial dose rate. By 7 kGy radiation treatment, the bacterial counts were reduced to approximately 3.2 $\log_{10}$ cfu/gm (for strain no 15) to 2.7 $\log_{10}$ cfu/gm (for strain no 13) from the initial counts; where for ATCC 7466 strain, the bacterial count was reduced to 3.2 $\log_{10}$ cfu/gm, which is near about similar deduction rate of 5kGy. That means this dose rate is also not so much effective to reduce bacterial growth pattern. By 9 kGy radiation treatment, the bacterial counts were reduced to 3.1 $\log_{10}$ cfu/gm (for strain no 14) to 2.4 $\log_{10}$ cfu/gm (for strain no 13); and for pathogenic strain ATCC 74666, 2.7 $\log_{10}$ cfu/gm was reduced

from the initial counts, which is approximately higher than our strains. This indicates that, this dose rate is much effective on eliminating bacterial loads.

By 10 kGy radiation treatment, the bacterial counts were reduced to approximately 1.6 $\log_{10}$ cfu/gm (for strain no 14) to 1.2 $\log_{10}$ cfu/gm (for strain no 13), where 1.4 $\log_{10}$ cfu/gm was for pathogenic strain ATCC 7466.

The total bacterial counts need to be 0 $\log_{10}$ cfu/gm for complete deduction of *L. monocytogenes*. But according to food standard rule by the International Atomic Energy Agency (IAEA), higher radiation dose than 10 kGy is not so favorable to consuming some food products. According to the CDC, irradiation does not change a food's nutritional value. The high-energy ray is absorbed as it passes through the food and gives up its energy. The food is slightly warmed and may taste slightly different after irradiation. But higher dose of radiation can breakdown some nutritional elements of food materials; such as fat, protein especially enzymes (heat destroys enzymes, which could be effects on flavor, texture, odor, etc. of food matrixes). From our overall studies we can say that, 10 kGy radiation dose eliminated *L. monocytogenes* at 1.2.to 1.6 $\log_{10}$ cfu/gm, and the threshold level of consuming this bacterium is 2 $\log_{10}$ cfu/gm (BOUGLE and STAHL, 1994), which is much higher than our tested value. So, the bacterium is reduced at a harmless level for consumption of the food. In our study, Cobalt-60 (^{60}Co) has been used, where its main application is at radiotherapy for cancer treatment, food irradiation and industrial applications. The gamma associated radiation emission kills bacteria and other pathogens, without altering the food product or retaining any radioactivity after treatment.

5.1 Concluding Remarks:

Though irradiated foods, both raw and ready-to-eat foods are acceptable in foreign markets by the consumers; but in Bangladesh people's perception of foods treated with irradiation is more negative than those processed by other means due to their lack of proper knowledge. By the declaration of U.S. Food and Drug Administration (FDA), World Health Organization (WHO), Centers for Disease Control and Prevention (CDC) and U.S. Department of Agriculture (USDA), irradiation treatment is safe in food industry (Seidler, 1982). So, for controlling *Listeria*

monocytogenes contamination in food industry like diary product cheese, irradiation treatment can be an effective way.

According to our research, we can conclude that, by 10 kGy radiation dose, the bacterial counts were reduced at a harmless level, which will not generate serious diseases in our body. Thus, we can limit listeriosis and such kind of food borne perilous diseases in an effective way.

CHAPTER 6

6. References:

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Appendix

1. Nutrient agar media preparation:

Nutrient agar is a general-purpose medium supporting growth of a wide range of non-fastidious organisms.

Formula in g/L:

Substance	Amount
Peptone	5g
Beef extract	3g
NaCl	3g
Agar	15g
Distilled water	1000ml
pH	7 ± 0.2

[Nutrient agar media preparation by standard rules: For 1-liter media, nutrient agar powder should be 23 grams; and rest will be distilled water]

After preparation, nutrient broth media was sterilized at 121º C temperature for 15 minutes. The media was used for subculture by steak plating method and for bacterial counts by spread plating method.

2. Nutrient Broth preparation:

Nutrient Broth is the liquid version of the solid medium. It is a classical meat infusion broth that is useful for the routine laboratory purposes.

Formula in g/L:

Substances	Amount (g/L)
Meat extract	1.00
Yeast extract	2.00
Peptone	5.00
Sodium chloride	5.00

Final pH 7.4 ± 0.2 at 25ºC

[Nutrient broth media preparation by standard rules: For 1-liter media, nutrient broth powder should be 8 grams; and rest will be distilled water]

After preparation, nutrient broth media was sterilized at 121º C temperature for 15 minutes. *L. monocytogenes* were inoculated in cooled broth media and incubated for 24-48 hours.

3. Normal Saline solution preparation:

Normal Saline solution growth media provides isotonic medium for growth and dilution procedures.

Substances	Amount
Sodium chloride	0.9 g (0.9%)
Distilled Water	100 ml

[Normal saline water preparation by standard rules: For 1-liter saline water, Sodium chloride (NaCl) should be 9 grams; and rest will be distilled water]

After preparation, the saline water was sterilized at 121º C temperature for 15 minutes.