NEAR EAST UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF FOOD HYGIENE AND TECHNOLOGY

INHIBITION OF *L.monocytogenes*, *S.aureus*, *E.coli* BY PROBIOTIC DAIRY KEFIR USING ARTIFICIAL INTELLIGENCE BASED MODEL

PhD THESIS

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June, 2022

Approval

We certify that we have read the thesis submitted by Kefyalew Chirkena Bali titled **"Inhibition of Listeria Monocytogenes, Staphylococcus Aureus and Escherichia Coli By Probiotic Dairy Kefir Using Artificial Intelligence Based Model**" and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Doctor of Food hygiene and technology. Thesis defence was held online. The Jury members declared their acceptance verbally which is recorded.

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Declaration

Hereby, I would like to declare that all information, documents, analysis and results in this thesis work have been collected and presented in accordance with the rules and guidance of Institute of Graduate Studies of Near East University, Nicosia. I also confirm that this thesis is original, and no conflict of interest commit.

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Abstract

Foodborne pathogens are the most food contaminating microorganisms that cause foodborne illnesses which is highly associated with the issue of food safety globally. The present study aimed to inhibit the activity of *Listeria monocytogenes*, *Staphylococcus aureus* and *Escheriachia coli* by probiotic lactic acid bacteria in dairy kefir using Artificial intelligence based models. In Artificial Neural Network, the average inhibition result obtained for *Listeria monocytogenes*, *Staphylococcus aureus* and *Escherichia coli* at training stages was found to be 2.4$\log_{10}$CFU/g, 2.0$\log_{10}$CFU/g and 2.4$\log_{10}$CFU/g counts, while it was 2.33$\log_{10}$CFU/g,2.04$\log_{10}$CFU/g and 2.03$\log_{10}$CFU/g in Adaptive Network-based Fuzzy Inference System respectively. The average result obtained in the case of the tested LAB was 4.9$\log_{10}$CFU/g, 4.8$\log_{10}$CFU/g and 4.9 $\log_{10}$CFU/g counts in Artificial Neural Network respectively; relatively similar result was observed in Adaptive Network-based Fuzzy Inference System. The decrease in the number of pathogens was observable at storage days than during fermentation days of the experimental kefir in all the targeted pathogens. Regression analysis had reflected the inhibition of *Listeria monocytogenes* at training, validation and testing stage with R=0.9783, 0.9991, 0.9815 respectively, and with best validation performance of 0.2298 at epoch 4. *Staphylococcus aureus* and *Escherichia coli* were inhibited by regression analysis at training, validation and testing stage *with R*=0.9842, 0.9905, 0.8873 and R=0.9702, 0.9514, 0.9537, with best validation performance of 0.071812 at epoch 21 and 0.18637 respectively. The activity of the all targeted foodborne pathogens were inhibited by the potential probiotic LAB present in dairy kefir milk. Thus, the result of this study indicates that probiotic bacteria of dairy kefir are promising bio controls that can be used in the food industry and agricultural sectors.

Key words: *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli,* kefir, probiotic, lactic acid bacteria

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List of Abbreviations and acronyms

LAB- Lactic acid bacteria

L- Listeria

S- Staphylococcus

E- Escherichia

E-Enterococcus

B-Bacillus

GIT- Gastrointestinal Tract

GMP- Good Manufacturing Practices

HACCP- Hazard Analysis Critical Control Points

WHO- World Health Organization

CDC -Center for Disease Control and Prevention

FDA – Food and Drug Administration

CFU- Colony Forming Unity

MRSA- Methicillin Resistant Staphylococcus aureus

UHT- Ultra Heat temperature

MRD- Maximum Recovery Diluent

MRS- De Man Rogesa and Sharpe

PALCAM- polymyxin Acriflavine LiCl Ceftazidime Aesculin Mannitol

VRB – Violet Red Bile agar

C^0 – degree Celsius

h- hour

ml- milliliter

FD- fermentation day

SD- Storage day

AI – Artificial Intelligence

ANN- Artificial Neural Network

ANFIS- Adaptive Network-based Fuzzy Inference System

CHAPTER I

1. INTRODUCTION

Foodborne pathogens are known to be the most food contaminating microorganisms that cause foodborne illnesses. The contamination of food by pathogens is mostly associated with inappropriate food handling at production, processing, storage and transportation time. Additionally, poor personal hygiene, cleaning and sanitization in terms of cross-contamination are also substantial factors for microbial food contamination (Martinović, 2016). The risk of foodborne pathogens is more evident in developing countries where infrastructure, sufficient information about food safety and adequate surveillance system are not fully addressed (Paudyal et al., 2017). Surprisingly, in the modern lifestyle of human being, particularly in developed countries where the availability of infrastructure and other facilities are secured and well addressed, pathogens are still found to impose a potential risk of foodborne infections and other health problems to the consumers, especially due to the highly increased interest of consumers for ready- to- eat foods and take away foods (Ndieyira et al., 2017; Lopez-Valladares et al., 2018).

Moreover, international trade fair and diverse food supply also widely enhance the spread of foodborne diseases across national and continental borders, resulting in health and related risk to the consumers, especially people of vulnerable groups including pregnant women, elders and the infants. Thus, the development of foodborne illnesses among the consumers due to food poisoning has been becoming the alarming food safety issue (Choi et al., 2020). The impact of foodborne pathogens are not only of the global health issue, but also causes considerable economic burdens, particularly in developing countries, because of the risk associated with the safety and quality of food products (Keba et al., 2020). However, with this in mind, several experts and food industry practitioners have been developing the ways to manage and monitor the impacts of foodborne pathogens within the food products to ensure food safety.

So far, various approaches have been established to control the activity and spread of foodborne pathogens, of which biological controlling approaches have considered to be the effective ways of combating the potential risk of the pathogens to ensure food safety and quality. The present study exclusively conducted an investigation to inhibit the activity of selected bacterial pathogens, namely L. *monocytogens*, *S. aureus* and coliforms/*E. coli* using one of the biological control, which includes probiotic LAB that naturally exist in fermented dairy products, including kefir, yoghurt and other products. It is obvious that a number of research findings have verified the antagonistic effects of probiotic LAB, isolated from various fermented and other food products, against the growth and multiplication of foodborne pathogens (Jara et al., 2020; Rajabi et al., 2020; Camargo et al., 2018; Niaz et al., 2019; Kaya & Simsek, 2019; Abdelhamid & El-Dougdoug, 2020). However, the present study aimed toward the activity of targeted bacteria by the application of LAB in the fermented dairy products, kefir.

1.1. Purpose of the study

Understanding the overall impact of foodborne pathogens in terms of food safety and hygiene helps the food safety experts and associated disciples to work on the mitigation of the pathogens. Thus, the purpose of this study intended against the growth of *L. monocytogens*, *S. aureus* and *Coliform*, more specifically *E. coli* bacteria by using biological approaches, i.e, probiotic LAB naturally present in the dairy kefir milk. This can be determined by evaluating the dynamic multiplication probiotic LAB during fermentation and post fermentation (storage) periods of kefir.

1.2. Research questions and answers

In this regard, some research questions are raised

- Why *L. monocytogenes*, *S. aureus* and Coliforms/*E.coli* are selected for this investigation?
- Why probiotic LAB in kefir are preferred for the inhibition of the activity of the pathogens?
- Can the growth and multiplication of the targeted foodborne pathogens be inhibited by the probiotic LAB within the selected fermented dairy product called kefir?
- What are the feasible benefits that can be obtained from inhibiting these foodborne pathogens from the food safety perspective?

The selection of the present targeted foodborne pathogens is mainly based on their prevalence in the fermented dairy and other food matrices, and responsibility for high morbidity and fatality rates, particularly the pathogenic *L. monocytogens*. Some recently published data have indicated that *L. monocytogenes* survives in wide range of environmental settings and it is the main issue of food safety and quality in the food industry, as it can form biofilms that tolerate the regular sanitation measures (Ulusoy & Chirkena, 2019; Jara et al., 2020; Stratakosa et al., 2020). The other indication could be the emergence of newly recognized pathogenic *L. monocytogenes* as the most food contaminant and serious concern of food safety in recent years (Fox et al., 2018).

Similarly, *S. aureus* is the most commonly identified foodborne pathogen, causing serious foodborne illnesses over the world. It has been clearly verified by recent research findings that the pathogenic *S. aureus* is considered as the third most economically significant foodborne causing pathogen, commonly in human, producing multiple enterotoxins (proteins) that predominantly cause food poisoning (food intoxication) and subsequently becomes serious concern of public health (Tang et al., 2015; Titouche et al., 2018; Rubab et al., 2018; Angelidis et al., 2020; Zhao et al., 2020; Zhao et al., 2021). Regarding coliforms, particularly *E.coli* bacteria, its biological hazards has been reported in dairy food products, as it is the most contaminant of dairy products, such as raw and processed milk (Mhone et al., 2011), and causes more severe and higher rate of illnesses when compared to illnesses associated with other food (Obaidat and Stringer, 2019).

Probiotic LAB are naturally present in different types of fermented dairy products and other food matrices, providing health benefits for the consumers and microbial balance in the gastrointestinal tract (Colombo *et al*., 2018; Kefyalew et al., 2021). Because of the presence of different metabolites such as organic acids, Carbon dioxide, hydrogen peroxide and bacteriocin (nisin), these beneficial microorganisms can able to inhibit the activity of foodborne pathogens; as a result most agricultural sectors and food processing plants use them as alternative bio control agents, incorporate into food products as additives, to mitigate the potential risk of foodborne pathogenic bacteria including their biofilms (Hossain et al., 2017; Camargo et al., 2018; Niaz et al., 2019; Kaya & Simsek, 2019; Abdelhamid & El-Dougdoug, 2020).

Additionally, regarding the inhibition of the targeted foodborne pathogens using probiotic LAB within the fermented dairy products, evidence clearly indicates that LAB possess antagonizing effect against the activity and growth of foodborne pathogens. For instance, the inhibitory activity

of certain species of LAB such as *Pediococcus pentosaceus, Lactobacillus fermentum, Lactobacillus salivarius, Enterococcus faecalis*, *Enterococcus hirae* and *Lactobacillus planturum* against different types of foodborne pathogens was recently reported (Mahdhi et al., 2017; Jara et al., 2020; Tatsaporn & Kornkanok, 2020). In this regard, some experimental data show that the activity of various foodborne pathogens such as *L. monocytogenes* were found to be inhibited by applying potential probiotic LAB isolated from dairy products (Hossain *et al*,. 2020; Sonbol et al., 2020).

Furthermore, inhibiting the effect of the indicator pathogens, *L. monocytogenes, S. aureus and Coliforms/ E.coli*, is for several aspects of which health and economic aspects are the determinant areas of concern. Regarding health aspects, these pathogens have been recognized and well reported as the most serious cause of foodborne diseases over the world due to their presence or their toxins in the food matrices including dairy products, thereby resulting in public health and related problems as a result of consuming contaminated foods (Fox et al., 2018; Keba et al., 2020). Thus, inhibiting the activity of such pathogens and their toxins in the food products using biological approaches, particularly probiotic LAB, helps to protect microbial contamination of food. This approach may be implemented at any level of production processes in the food industry and other agricultural sectors.

In addition, the existence of foodborne pathogens in food items can result in substantial economic losses and costs for agricultural sectors and food processing industries, including farmers, processors, and retailers. Expenses to deal with procedures and regulations, efforts to tracing back the contamination, recalls of food products, closing of processing plants, liability toward the products, and extended effect on the market environment are among the challenges facing the agro-food processing industry (Focker & van der Fels-Klerx, 2020). Several foodborne bacteria including *Listeria monocytogenes*, *Salmonella, Staphylococcus aureus* and others are commonly associated with massive recall of products as a result of their contamination of food products in the agro-food industrial settings (Dey et al., 2013; Herod et al., 2019). This situation generally affects the global economy and international and domestic trade fair.

Yeast are also among the probiotic microbes that naturally present in different fermented food matrices, like dairy milk and meat, and also added into fermented foods for the development of flavor; as a result, more attention is currently given to the microorganisms from the scientists and

industry (Rima et al., 2012). Moreover, it has been suggested that there are global trends regarding consumption of food without preservatives due to the usage of yeasts as a natural alternate to substitute the preservatives to control the growth and multiplication of undesirable microorganisms. This shows that various species of yeasts (e.g. *Saccharomyces cerevisiae var. boulardii*) possess antimicrobial property that can hinder the action of several pathogenic and food spoilage bacteria such as *L. monocytogenes*, *S. typhimurium, S. aureus* and *E.coli* (Rima et al., 2012; Acuña-Fontecilla et al., 2017; Younis et al., 2017).

Hypothesis: The probiotic lactic acid bacteria present in kefir will be effective inhibitory agents against the foodborne pathogenic *L. monocytogenes*, *S. aureus* and *Coliform/ E.coli* bacteria.

1.3. Significance of the study

Various foodborne pathogenic bacteria have been the reason for the food safety and quality issue along the food production chain, including processing, storage and transportation. Inhibition activity toward the growth of these pathogens has been applied by using different approaches for ensuring production of safe and wholesome foods to keep the health of the consumers as general. The use of probiotic bacteria has been approved as an alternative strategy to inhibit the growth of foodborne pathogens. In this study, the inhibition of the growth of foodborne *L. monocytogenes*, *S. aureus* and *Coliform*/ *E.coli* bacteria in dairy kefir milk will be investigated by using the probiotic lactic acid bacteria derived from the kefir grains. There are very limited findings regarding the inhibition these pathogens by using probiotic LAB within the kefir milk. This is to demonstrate the effectiveness of the probiotic lactic acid bacteria against the growth of foodborne bacteria, and in connection with this, the study will indicate that the incorporation of kefir as food additives in food products may provide considerable preservation of food and inhibition of the foodborne bacterial pathogens in food products. Therefore, the objective of the present study was to constrain the activity and growth of foodborne pathogenic *L. monocytogenes, S. aureus and E. coli* using the probiotic LAB within the dairy kefir.

CHAPTER II

2. GENERAL INFORMATION

2.1. General understanding on pathogenic foodborne infections in food safety perspectives

Naturally, foodborne diseases are infectious or toxic that is caused by pathogenic bacteria and other microorganisms entering the body through ingestion of contaminated food. Over the years, there have been remarkable shifts in the key bacterial pathogens which are of course the most food safety concern. The issue of food safety is the usual concern of general public health, which is associated with the risk of food contamination due to the occurrence of pathogens or their toxins in the food products and the production environment (Table 1), the agro-industrial sectors in particular Mota, et al., (2021). Most foods that are sourced from animals, including dairy and their products, meat, poultry and other products are the most perishable products and are commonly involved in most outbreaks of foodborne infections (Garedew et al., 2012; Fox, et al., 2018; Guldimann & Johler, 2018). As a result, several foodborne pathogens are found to be a potential risk in the food safety management system in all food producing sectors.

From food safety perspectives point of view, safe food may include food that is prepared, handled and stored in the way that consumers are not under the risk of adverse effects up on consumption. Contrarily, unsafe food mean that when food is contaminated with any physical, microbiological or chemical hazard and causes negative effects to animals and human health (Focker & van der Fels-Klerx, 2020). In any hazard, when the consumers get the same illness from consuming the same contaminated products, the situation can be known as foodborne illness outbreaks (FDA, 2021). In such condition, several pathogens including *Listeria monocytogens*, *Staphylococcus aureus, Salmonella, Campylobacter jejuni* and Coliform bacteria are responsible and most likely known to cause the outbreaks by contaminating varieties of food products (Martinović et al., 2016). The agro-industrial sectors have frequently faced challenges associated with such pathogens in the areas of food production environment, which can be happened through their persistence on food contact surfaces and food processing environments, as shown in (Table 1). Their persistence, even after the application of regular cleaning procedures, is most likely due to the resistance against antimicrobial agents and disinfectants that have been a major challenge to researchers and experts who deal with food safety and related disciplines (Fleming & Rumbaugh, 2018; Li et al., 2020).

The persistence of foodborne bacteria in the food processing plants, including primary and secondary food processing, is the major cause for the cross-contamination of food, which in turn causes human illnesses (Larsen et al., 2014), and global public health treat in general (Abdelhamid & El-Dougdoug, 2020). In this regard, WHO suggested in its study that about 1 in 10 people gets illness and 420,000 are died by foodborne infections worldwide (Guldimann & Johler, 2018). Additionally, the organization also released that about more than 600 million people are sick by foodborne diseases every year, and children under age of 5 years, exceeding 120,000 in number, die from consuming unsafe food (WHO, 2021). In the United States only, the report released by CDC and FDA shows that foodborne infections are the major risk to public health, in which about 48 million people get sick, 128,000 hospitalized and 3000 die each year (CDC, 2018; FDA, 2020). The other impact of foodborne pathogens is associated with economic burden that mainly face the food industry due to the recall of the products as a result of microbial contamination (Hoffmann et al., 2015).

However, in food industry, understanding the environmental sources of foodborne pathogens and their behavior throughout the food chain and their impact in disease causing potential is essential to leading hygienic best practice aimed at preventing their entry into food production environments and eventually food products (Greppi & Rantsiou, 2016). The implementation of food safety management systems in the agro-industrial sectors is fundamental and best practices that ensure the production environments and food products are safe. Several food safety management systems including HACCP, GMP and traceability are implemented in the agro-industrial sectors to control the hazardous conditions of the pathogens that could emerge at any stage production (Allata et al., 2017; Dzwolak, 2019; Liu, et al., 2021). A number of countries, particularly developed world, have set their own requirements as a prerequisite to mandate foods that entering their country must meet minimum food safety standards, including GMP, HACCP, traceability and other requirements (Allata et al., 2017; Dzwolak, 2019).

Table 1. Some of pathogenic bacteria involved in the contamination of food products

Isolated bacteria	Serotypes or strains	Sources/origin of the agents	Contact surfaces	References
Listeria monocytogenes	1/2a, 1/2b, 1/2c, 3c, 4b	Raw milk and meat, cooked meat, deli meat, milk products, meat products, quick-frozen food, vegetables, aquatic products, bean products, ready-to-eat, cheese	Stainless steel, ceramic tiles, polyethylene, & polyvinyl chloride pipes	Doijad et al., 2015; Wang et al., 2017; Braga et al., 2017
Salmonella	S. Enteritidis, S. enterica serotype Typhimurium, S.Newport, S,Paratyphi B, S.Poona, S.Derby, S. Infantis, S. Virchow, S.Agona	Poultry sources and other cultures collection	Stainless steel, copper, brass, tinned copper, polystyrene	Pontin et al., 2020; Díez-García et al., 2012
	S. Typhimurium DT104	Human	Stainless steel, high density polyethylene, polyvinyl chloride, polycarbonate coupons	Zhu et al., 2014
Escherichia coli	O157:H7, O26: H11, O103: H2 and O103: H25	Ground beef, Ovine feces	stainless steel, polystyrene, glass (the formation of pellicle at air-liquid interfaces)	Wang et al., 2012; Nesse et al., 2014

Staphylococcus aureus and *S. epidemidis*	------	Mastitis milk, cheese before and after packing, raw milk, milk tank, food handler	Stainless steel & polypropylene, dish towels, workers' hands, cutting boards, tables, slicers	Sospedra et al., 2012; Cruzado-Bravo et al., 2019
Campylobacter jejuni	---	Poultry meat	packaging table, dressing table, floor source and washing table	Balogu et al., 2014
	C. jejuni ssp. Jejuni 1, C. jejuni ssp. jejuni 2, and C. jejuni ssp. Doylei	Plant and animal raw materials, finished products, swabs from equipment of the food industry	Glass plates, slides,coverslips, polymeric microtubes and petri dishes, and polystyrene plates	Efimochkina et al., 2017
Pseudomonas species	*P. aeroginosa* ERC-1	Industrial water system	Stainless steel, high density polyethylene, polyvinyl chloride, polycarbonate coupons	Zhu et al., 2014
	P. fluorescens PSD4	Raw milk, milk separator, skim tank, cream tank, homogenizer, pasteurization vat, milk storage vats, cheese vat, cheese ripening room and packaging area	Floor, drains and valves of different milk processing equipment	Aswathanarayan & Vittal, 2014

2.2. Probiotics and their significance associated with foodborne pathogens

Probiotics are live microorganisms exist in naturally fermented dairy and other food products, and also incorporated to other products as enhancements to keep microbial balance in the GIT and hosts' health. Among fermented food products, dairy products are well known in containing probiotic microorganisms, mostly LAB which are considered as desirable and beneficial microorganisms worldwide (Klimko et al., 2020). The benefits of these microorganisms may include potential use as feed additives, food and feed fermentation and as starter cultures (Azat et al., 2016; Edalati et al., 2018; Kim et al., 2019). Probiotic fermented dairy foods are an essential part of human diet because of their nutritional contents Tamang *et al*., (2020), and a predominant source of probiotic bacteria that persist in adequate numbers in the products to protect their physical and genetic constancy during their packaging and storage. One of the characteristic properties of probiotics is their capability to withstand the adverse conditions in the in the GIT, mainly acidic conditions and bile salt secretions. Furthermore, they significantly enhance the health of GIT and other tissues by developing their adherence and colonization potential (Rezac et al., 2018; Kim et al., 2019).

Some literatures express the terminology of fermented foods as “foods or beverages produced through controlled microbial growth and the conversion of food components through enzymatic action” (Dimidi et al., 2019). This expression is similar to the description given by the International Scientific Association for Probiotics and Prebiotics (ISAPP) which is “foods made through desired microbial growth and enzymatic conversions of food components” (Marco et al., 2019). On the other hand, in association with their health benefits apart from the formerly identified terminology, they are categorized in to three naming; explicitly “true probiotics” which indicates the probiotics are viable and active, “pseudo-probiotic” refers to viable and inactive cell and “ghost probiotic” meaning the probiotics are non-viable/dead cells (Zendeboodi et al., 2020).

The other characteristic of probiotic bacteria is associated with their industrial properties where the researchers and experts from different discipline such as medicine, pharmacological, and industry have given attention (Sharifi et al., 2017). The industrial sectors take the primary and prominent role in the production of various probiotic containing foods, which is might be due to well awareness of the consumers regarding their capability to maintain health benefits. In the

process of food fermentation, probiotics such as *Lactobacillus acidophilus* can be incorporated to food items for the characteristic organoleptic of foods as well as their shelf life extension. Moreover, the inhibitory activity of the probiotic foods towards foodborne and spoilage bacteria enhances their usage in the bio-controls and as bio preservatives (Anjum et al., 2014; Abbasiliasi et al., 2017). Regarding technological requirements, the probiotics are mostly considered as appropriate products for addition into food products because they can keep their viability and efficacy in the food products. Hence, they also able to endure industrial applications and can survive sufficiently in the products during their shelf life.

Therefore, probiotic LAB naturally constitute essential compounds that contribute for the functionality of food products, mostly processed in food industry, as they are used as alternative bio-control agents, sometimes known as natural antimicrobials, against potential pathogens (Hossain et al., 2017; Ağagündüz et al., 2020). Thus, the antimicrobial role of the probiotic LAB against various foodborne and food spoilage bacteria is determined by metabolites present in probiotic strains that can actively inhibit the growth of pathogenic foodborne bacteria (Khaneghah et al., 2020). Therefore, for the sake of having safe and fresh-like foods, a combination of two or more natural antimicrobials or with other stressors is currently used worldwide as food preservatives (Abdelhamid et al., 2020). As a result of this, various probiotics can play pragmatic role in large-scale agro- industrial sectors without losing of their viability and functionality. Thus, they are taken as the principal input for the food industry in order to ensuring food safety and in turn the health of the consumers.

Based on the investigations conducted on various probiotic strains isolated from different types of dairy food products, researchers have realized the antimicrobial activity of these strains against various foodborne products. In light of this, the investigation of Kamal et al., (2018) who tested the inhibitory activity of *Lactobacillus rhamnosus* on different foodborne pathogens including *Escherichia coli O157:H7*, *Staphylococcus aureus*, *Yersinia enterocolitica* and Salmonella enterica serovar Typhimurium indicated that probiotics significantly inhibit the growth of the pathogens. In similar situation, Rajabi et al., (2020) reported the antimicrobial effectiveness of spore forming *Bacillus laterosporus* and *Bacillus megaterium,* whereas *Lactobacillus fermentum* MP26 and *Lactobacillus salivarius* MP14 were found to be effective probiotic agents against the growth of *Listeria monocytogenes* (Jara et al., 2020). Probiotic potential of commercial dairy-associated protective cultures and LAB with probiotic potential

becomes highly preferred due to their benefits to food safety in addition to inhibiting the growth and survival of pathogens in foods (Aljasir & D'Amico, 2021; Toushik et al., 2021).

2.3. The pathogenic *Listeria monocytogenes*

2.3.1. Overview on the pathogenic *Listeria monocytogenes*

Listeria monocytogenes is a deadly foodborne pathogen causing serious foodborne infections (WHO, 2003). It is a facultative intracellular gram-positive bacterium, which is ubiquitous in nature, living in the wilderness, farm environment, food production environment, food products, food contact surfaces and other utensils (Fagerlund et al., 2020; Kallipolitis et al., 2020; Kannan et al., 2020). The pathogen is a causative agent of human listeriosis following consumption of either contaminated or undercooked foods (Ranjbar and Halaji, 2018; Cufaoglu et al., 2021), and particularly it causes infections to venerable groups including pregnant women, elders, or people with debilitated immune system or immune-compromised people (Ramaswamy et al., 2007). Furthermore, the pathogen is also responsible for other complications including encephalitis, meningitis, stillbirth and central nervous system infection in newborn and immune-compromised groups (Zhou & Jiao, 2004).

Studies revealed that the case of listeriosis due to *L. monocytogenes* is more prevalent in pregnant women, representing almost 60% of infection in population younger than 40 years (Hunjak et al., 2019). Similarly, a study conducted at Tigray region, Northern part of Ethiopia, regarding pregnant women categorized by socio-demographic appearances showed that *L. monocytogenes* was prevalent in those women of age 20-24 years (18%), residents of rural area (10%), those attending secondary school (9.6%) and home wife (11.4%) (Welekidan et al., 2019). According to the report released by World Health Organization, pregnant women are more likely to get the infection 20 times than that healthy adults as the disease can consequently result in miscarriage or stillbirth, and the report further indicated that individuals with HIV are at least 300 times more likely to contract the disease than that of healthy ones or people with normal immunity (WHO, 2018b).

Among the characteristics of the pathogenic L. *monocytogenes,* the ability to adhere itself to living or non-living surfaces including food contact surfaces to survive in the food production environment or food products is the determinant one that enables the pathogen to withstand the adverse conditions (Jamal et al., 2018). Additionally, the pathogen has also enhanced regulatory

mechanisms, may be genetic determinants that strongly influence its potential to subsist in such environments (Banerji et al., 2022). In this aspect, various food products including pasteurized milk, soft cheese and semi-soft cheese, cooked meat products (Iannetti et al., 2016; Chen et al., 2020; Olaimat et al., 2021) and other foods with low moisture content and food ingredients are mostly involved for the survival of *L. monocytogenes* to cause the outbreak of listriosis (Taylor & Zhu, 2021). Additionally, the pathogen has the capability of growing in temperatures, including cooling temperature; thus, foods that are kept in the refrigerator for more than the recommended time can enhance the chance of survival for the pathogen in the products (Hoffmann et al., 2015).

L. monocytogenes can potentially contaminate food products at any production and processing stages. Thus, after ingestion of the contaminated food, the pathogen can pass the intestinal wall and spread to the body fluid systems, including blood and lymph so that it can easily reach the liver and spleen where its multiplication become high to cause disease that infect the unborn baby (Anderson et al., 2015) (figure 1). Of course when the contaminated food is consumed, the fate of *L. monocytogenes* depends on a complex interaction among the composition of the food matrix, host susceptibility and as well as strain phylogeny. However, the effect of *L. monocytogenes* on food products is massive because of its role for the occurrence disease outbreaks globally (Farber et al., 2021; Maćkiw et al., 2021). The main route of transmission of this pathogen is through contamination along the food chain from farm to fork, and thus, it accounts large number of recalls in the food processing industries (Herod et al., 2019; Duze et al., 2021). It has been suggested that foods that can support the growth *L. monocytogenes* contribute to high risk of infections among the general public; moreover, the emergence of low-moisture foods (foods with water activity < 0.85) is found to be a potential source of the pathogen, which lead to a number of recalls of food products (Ly et al., 2019).

Survival of this pathogen in such kin of food for long time have initiated the researchers to examine the phenotypic and genotypic traits of the pathogen associated with its adaptation to different environmental conditions, stability (tolerance) to desiccation, and thermo tolerance in these foods (Varma et al., 2007; Ly et al., 2019; Taylor & Zhu, 2021). Additionally, manufacturers and distributors look into scientific approaches to mitigate the risk of foodborne illness associated with *L. monocytogenes* Taylor et al., (2019), whereas major food trade associations have come together to develop guiding documents on the control of the pathogen in terms of risk management Farber et al., (2021). Meanwhile, many countries over the world have

established microbiological criteria for *L. monocytogenes* of 100cfu/g for low risk foods that do not support the growth of the organism; but on the contrary side, U.S has currently a "zero-tolerance" approach for all ready-to-eat foods; thus all positive test results lead to a recall which causes huge economic crises and possibly impose a potential risk to public health (Farber et al., 2021). On the other hand, Taylor et al., (2019) suggested that even though accidentally no outbreaks were directly attributed to low moisture foods in the U.S., recent cascades of voluntary recalls addressing producer risk related to potential presence and survival of the pathogen in low moisture foods are relatively new and complex.

2.3.2. The prevalence of the pathogenic *L. monocytogenes* in dairy food products

In addition to the other environmental factors for the widespread of *L. monocytogenes*, animals, particularly dairy cattle can also carry the pathogen without getting sick and shed the bacterium in their milk and feces. Therefore, as it has been well documented, the dairy farm environment is a good reservoir for the pathogen which is responsible for a potential contamination of dairy milk, milking equipment and hand swaps (Mansouri-Najand et al., 2015; Tahoun et al., 2017). It has been obviously described that the pathogen is ranked as the third major foodborne causing organism and negatively affect the dairy production in dairy industry because the prevalence of the pathogen in the dairy farm environment greatly contributes for the microbial risk in milk value chain (El Hag et al., 2021).

Some literatures show that inadequate cleaning of dairy udder, milking person (handlers) and sanitation of all dairy units and shed are also responsible for the microbial contamination of dairy products Mary and Shrinithivihahshini, (2017), which makes human exposure to the pathogen very high (Sonnier et al., 2018). The prevalence of *L. monocytogenes* is known to be appeared not only in raw milk and milk products but also can be present in pasteurized milk Mansouri-Najand et al., (2015), which may probably occur due to insufficient temperature and some procedural faults. According to investigations conducted regarding the prevalence of *L. monocytogenes* in various dairy products, some figures show that these food items are responsible for the occurrence of the pathogen. In light of this, Owusu-Kwarteng et al., (2018) reported that *L. monocytogenes* was prevalent (8.8%) in dairy cow milk of collected from the Northern region of Ghana, while no pathogen was noticed in boiled milk. In the contrary, higher

prevalence of *L. monocytogenes* was reported in pasteurized milk than in raw milk (Navratilova et al., 2004 and Sreeja et al., 2016) as indicated in Table 2.

The characteristic behavior of *L. monocytogenes* in dairy food products may vary among its serovars, a study confirmed. For example, an investigation of Possas et al., (2022), who studied on behavior of *L. monocytogenes* in pasteurized soft milk cheese with different salt concentrations and cured raw sheep milk cheese, realized that there was observable variation among the serovars of the pathogen, particularly 1/2c and 4b, in terms of their survival capacity. On the other hand, according to the report of Seyoum et al., (2015), geographical location determines the prevalence of *L. monocytogenes* isolated from different types of dairy milk obtained from central high land of Ethiopia, representing 3.4% in urban and 1.03% in peri-urban areas. In fact, types of dairy food products also pay a decisive role in the prevalence of *L. monocytogenes* differently as it was indicated in the report of Mary and Shrinithivihahshini (2017), i.e. for branded milk, cheese, ice-cream, milk powder, milk sweets, and yoghurt were recorded 65.9%, 62.5%, 49.2%, 26.6%, 20% and 6.6% respectively. That is why the implementation of legislation and policies in countries like USA and Brazil has been used to decrease the risk associated with the pathogenic *L. monocytogenes* and other foodborne pathogens in different types of dairy products (Farber et al., 2021; Oxaran et al., 2017).

Table 2: The prevalence of *L. monocytogenes* in various dairy milk and milk products

Countries	Dairy food products	Prevalence in %	References
Turkey	Raw dairy milk	5	
	Farm cheese	20	Kevenk and Terzi Gulel, 2016
	White cheese	5	
Ethiopia	Raw dairy milk	2.04	Seyoum et al., 2016
	Pasteurized milk products	20	
	Yoghurt	5	
	Cheese	26.7	
Iran	Raw dairy milk	7.8	Akrami-Mohajeri et al., 2018
	Cheese	32.7	Rahimi et al., 2010
Ghana	Raw dairy milk	8.8	Owusu-Kwarteng et al., 2018
Central Iran	Traditional butter	1	Akrami-Mohajeri et al., 2018
Czech republic	Raw dairy milk	2.1	Navratilova et al., 2004
	Pasteurized milk products	5	
India	Raw dairy milk	16.6	Sreeja et al., 2016
	Pasteurized milk products	25	
	Market milk	6.25	

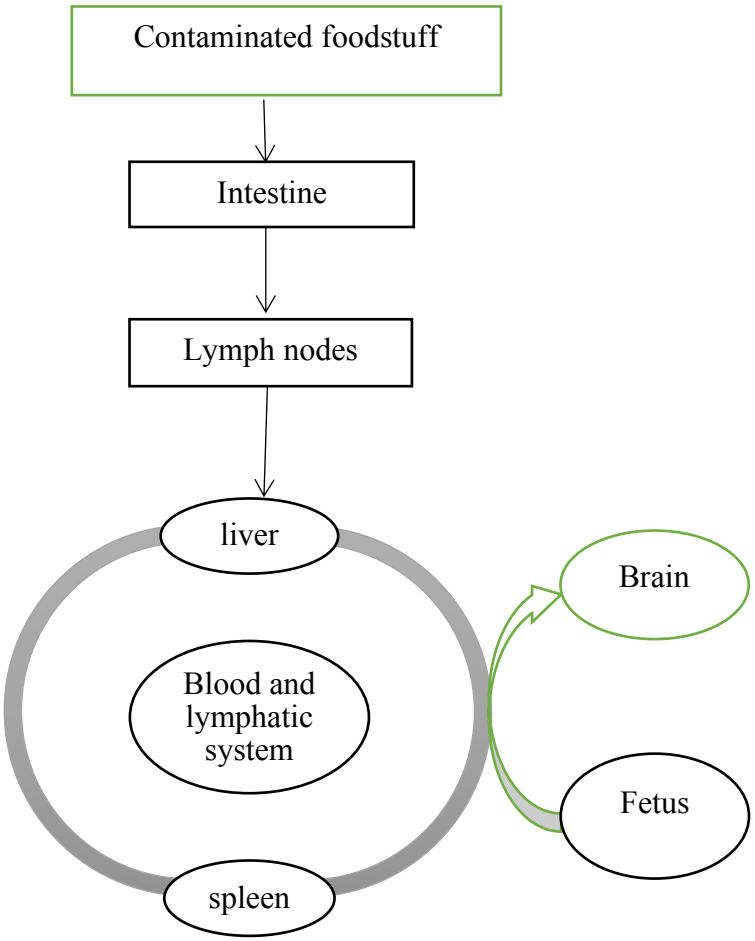


Figure 1: The dissemination of food contaminated *L. monocytogenes* in the body system

2.3.3. The antimicrobial resistance of *Listeria monocytogenes*

Antimicrobials have been used for various purposes both in animals and humans, which include the prevention and control of diseases causing pathogens, production enhancement and growth supplements in animals. However, the misuse or extensive use of antimicrobials in humans and veterinary medicines contributes to the development and spread of antimicrobial resistant foodborne pathogens (Ulusoy & Chirkena, 2019). In fact, the antimicrobial resistance of this pathogen is not intensified only by these factors but also other triggering factors such as microbial biofilm formation on food contact surfaces, food matrix, instrument and other related utensils, which are more responsible to enable the pathogens to resist the activities of antimicrobials Cepas et al., (2019) and any other adverse conditions. In addition to this, different genetic mechanisms have been involved in the development of the antimicrobial resistance of pathogenic *L. monocytogens*, which include self-transferrable plasmids, mobile plasmids, and

conjugative transposons (Kelly et al., 2009; Kohler et al., 2018; Braschi et al., 2018). Among these mechanisms, conjugation is the one that take part in transferring genetic materials between bacterial cells by direct cell to cell communication or by bridging both cells together, one is the donor and the other becomes the recipient (Ulusoy & Chirkena, 2019).

As several study findings suggested the pathogenic *L. monocytogenes* with antimicrobials resistant strains transfer its genetic material to its counterpart with antimicrobial sensitive strains to enable the pathogen tolerate the effect of adverse conditions including the activities of antimicrobials; for example, the streptomycin resistant strain of *L. monocytogenes*, identified as LM35, donates or transfer its genetic materials to streptomycin-sensitive *L. monocytogenes* strains (recipients), identified as LM65 and LM100 (Purwati et al., 2001). Likewise, the strain of this pathogen that harbor tetracycline resistant cell transferred its genes to other listeria strain called *L. ivanovii* through conjugation mechanism (Pourshaban et al., 2002; Jahan & Holley, 2016). Therefore, this mechanism assists the bacterium to persist in any hostile conditions in food products and food processing industries by increasing its antimicrobial resistance, invasion of hosts' cell including intestinal mucosa (Kannan et al., 2020) and enhance overall survival and persistence in the targeted environments.

The other genetic mechanism involved for the adaptability of the pathogenic *L. monocytogenes* toward the effects of antimicrobials and other conditions are the bacterial communication to one another that can be mediated through coordinated communication of bacterial cells among themselves, particularly called Quorum sensing (Frederick et al., 2011; Li & Zhao, 2020; Machado et al., 2020). This system regulates gene expressions and bacterial strong social networks through the production of diffusible signal molecules, auto-inducers (Yang et al., 2018; Brindhadevi et al., 2020). Naturally, the system contains oligopeptides in the cells of gram positive bacteria and N-acyl homoserine lactones in gram-negative bacteria, frequently produced by the bacterium itself largely at the stage of micro-colony formation Zhao et al., (2017), and simply diffuses through the bacterial cell membrane where signal molecules gain high in concentration.

In the bacterial cell- to-cell communication, the process of cell communication is significantly involved not only in modulating the gene expression linked with the production of specific enzymes, virulent factors and metabolites but also in the development of the bacterial community, including detection of the density of other surrounding bacteria (Nadell et al., 2008;

Marić & Vraneš, 2007; Shrout et al., 2011). Additionally, membrane fluidity, modification or change of the target molecules and resistance mediated by Efflux pumps are among the mechanisms by which *L. monocytogenes* enable to tolerate the activity of antibiotic and other adverse conditions (Ebbensgaard et al., 2020; Ndieyira et al., 2017; Kapoor et al., 2017). In general, the antimicrobial resistance of *L. monocytogenes* and other foodborne pathogens becomes the biggest public health challenge of the current time, and the issue of food safety in agro-industrial sectors.

2.3.4. The implication of antimicrobial resistance of *L. monocytogenes* isolated from dairy products

It is obvious that contamination of milk is mostly happened in dairy farm which is associated with product handling, dairy farm management system and personal hygiene, and other related conditions. Thus, it is crucial to evaluate the significance of raw milk and other possible factors, including milking equipment and farm workers, for triggering contamination within the dairy farm, and in turn transmitting the pathogenic *L. monocytogens* to the consumers (Tahoun et al., 2017). In this regard, the challenges associated with the contaminating pathogen are not only restricted to food stuffs and environment but also can antagonize the action of most known antimicrobials used for treatment, including Penicillin, Ampicillin, Tetracycline, and Gentamicin (Yakubu et al., 2012; Olaimat et al., 2018). Additionally, the resistance of *L. monocytogens* toward different antimicrobials such as, Chloramphenicol, Streptomycin, Penicillin G, Kanamycin, Levofloxacin, Amoxicillin, Rifampicin, and Ciprofloxacin has been reported during investigations associated with antimicrobial resistance (Kevenk &Terzi Gulel, 2016; Şanlıbaba et al., 2018; WHO, 2018a; Girma & Abebe, 2018). This can be occurred following the ingestion of dairy food products containing such resistant bacterial strains.

An evaluation of the antimicrobial resistance of *L. monocytogenes* in various dairy-based food products, such as Baladi cheese, Shankleesh, and Kishk, was carried out on some antibiotics including Oxacillin, Penicillin and Ampicillin with prevalence of 93.33%, 90% and 60% respectively (Harakeh et al., 2009). In fact, this shows occurrence or emergence of multidrug resistant *L. monocytogenes* in dairy food products. This finding was also supported by Bouymajane et al., (2021) who investigated on the antimicrobial susceptibility analysis of *L. monocytogenes* isolates with different genes identified including *actA, hlyA, prfA, plcB, inlA,*

inlC and *inlJ* obtained from different foodstuffs including dairy products, and confirmed the multidrug resistant of *L. monocytogenes* in different types of foodstuffs including dairy products. Therefore, the consumption of dairy food products and other food items prepared in inadequate temperature and lack of proper controlling measures may lead to serious health risk (Akrami-Mohajeri et al., 2018).

2.3.5. Factors triggering the Antimicrobial Resistance of *L. monocytogenes*

The use of antimicrobials both in humans and animals has been extensively implemented for different purposes, including disease control and prevention, and animal production enhancement (Hao et al., 2014). However, misusage or inappropriate use of the antimicrobials is the main drivers for the development and spread of antimicrobial resistant pathogens, which in turn causes the outbreak of disease conditions. In addition to the above factors, World Health Organization (WHO) forwarded some factors that trigger the spread of antimicrobial resistance. These include shortage of clean water, poor sanitation and hygienic conditions for humans and animals, poor disease prevention and controlling strategy in health-care centers, inadequate provision of quality and affordable medicines, vaccines and diagnostic kits and lack of awareness and knowledge, and absence of enforcement of legislation (WHO, 2021).

On the other hand, the capability of the pathogenic *L. monocytogenes* to form biofilms on foods contact surfaces, instruments, utensils and other medical settings, because biofilms are importable microbial networks that colonize both biotic and abiotic surfaces in order to endure antimicrobials providing them safeguarding systems (Hall and Mah, 2017; Cepas et al., 2019; Ghosh et al., 2021). Pathogenic *L.monocytogenes* is among some of foodborne pathogens that naturally resistant to various antimicrobials due to both general physiology and mutation or other types of genetic alteration (Olaimat et al., 2018). Mobile genetic elements (plasmids and transposons) are also among the mechanisms that contribute to the increasing antimicrobial resistance of *L. monocytogenes* (Matereke and Okoh, 2020). As result of such mechanisms the pathogen tends to adapt to environmental stress Stratakos et al., (2020) and able to enhance its antimicrobial resistance; but there may be a chance to be exposed to adverse environmental conditions during food production and processing, some of which include physical stressors (heat or hot temperature, high pressure and irradiation), chemical stressors (acids, salts, and oxidants) and biological stressors (microbial agents). Contrary, evidence shows that the persistence of the pathogen in the environment can be assisted by its efficacy to withstand the external stresses

(Matereke & Okoh, 2020). Furthermore, different external and internal factors triggering the antimicrobial resistance of pathogenic bacteria, including *L. monocytogenes* are summarized as follows in Figure 2.

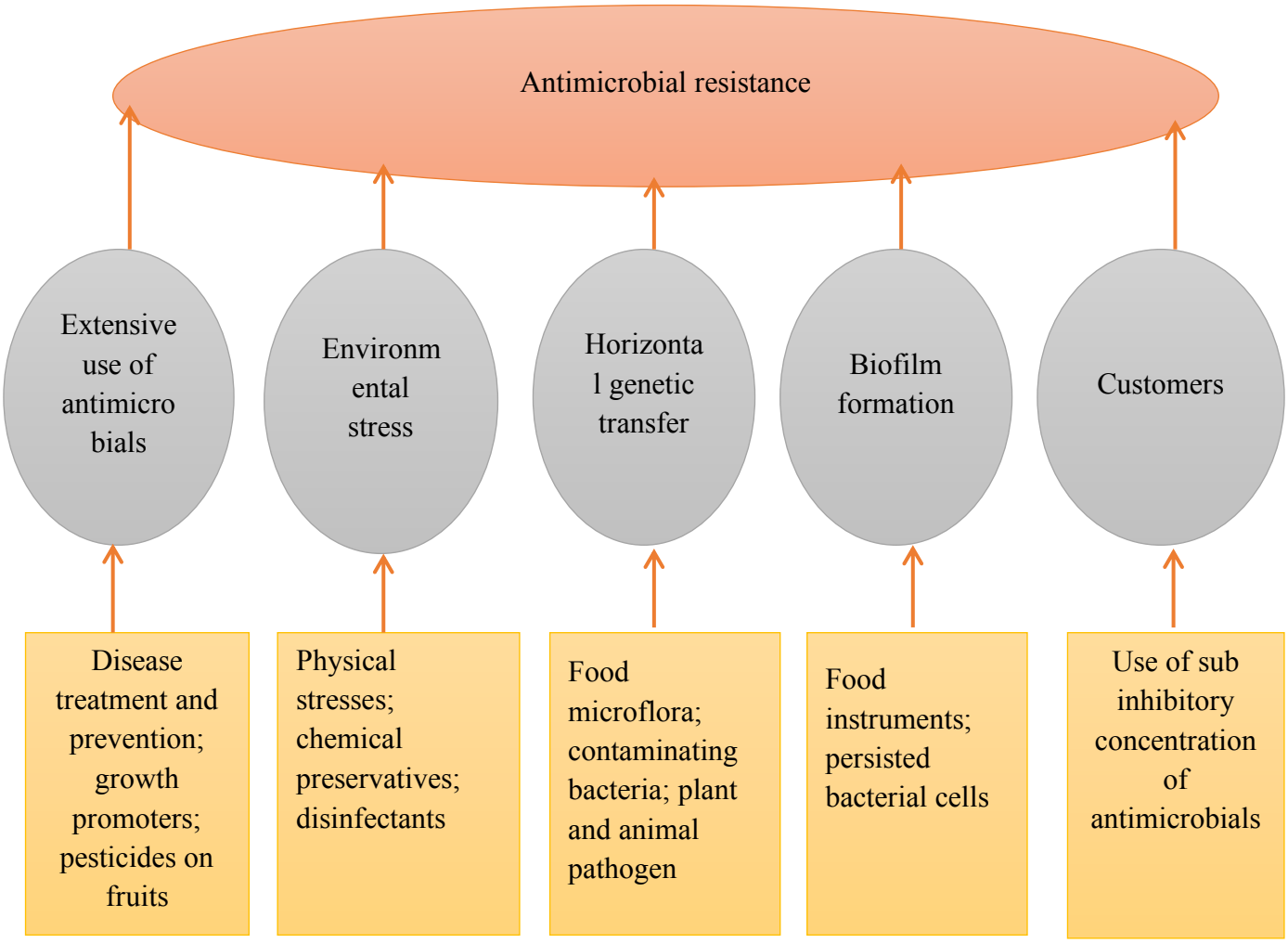


Figure 2. Summary of different factors triggering antimicrobial resistance of *L. monocytogenes*

2.3.6. Significance of *L. monocytogenes* in biofilm formation

In living microbes, biofilms refer to any syntrophic group of microorganisms in which cells adhere to each other and to living or non-living surfaces, including food contact surfaces. The adherence of the cells takes place and implanted within an extracellular matrix which comprises extracellular polymeric substances (EPSs) that contribute for microbial biofilm formation. Indeed, biofilm-forming microorganisms are broad, containing several numbers of known pathogenic fungi and bacteria. The formation of bacterial biofilms can be everywhere in

the environments, such as natural environments, food processing environments, human teeth or/and dental restorative materials Engel et al., (2020), and medical devices (Khatoon et al., 2018). In food processing plants, food contact surfaces and equipment are frequently colonized by biofilms and can serve as a source of cross-contamination, enhancing the reduction of effective food processing strategies and contribute to poor food quality and safety (Brooks & Flint, 2008; Ripolles-Avila et al., 2018; Alvarez-Ordóñez et al., 2019).

Recently published research findings indicate that various foodborne bacteria including *Listeria monocytogens*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella* species, *Escherichia coli* O157: H7 and *Campylobacter jejuni* are all biofilm-forming and persistently cause contamination and spoilage of food products so that it becomes important concern for the food industry (Choi et al., 2013; Choi et al., 2015; Liu et al., 2016; Kocot & Olszewska, 2017; García-Sánchez et al., 2019; Fagerlund et al., 2020; Zou & Liu, 2020; Keba et al., 2020). Therefore, the pathogenic *L. monocytogenes* is recognized as one of the biofilm forming bacteria that enhances its survival and persistence in food processing environments by forming biofilms on food plant surfaces and subsequently contaminates food products (Jiang et al., 2021). Thus, the production of biofilms makes the elimination of the pathogen difficult under regular disinfection and sanitation procedures, leading to high risk of food contamination at any stage of production, and becomes a significant food safety concern Gu T et al., (2021); Rodriguez et al., (2021) and causes huge economic losses (Srey et al., 2013; Gavrilova et al., 2019; Iñiguez-Moreno et al., 2019). Moreover, biofilms are highly involved in the spread of the pathogen and its persistence in the food industries. Therefore, there are various factors associated with the formation of biofilms for the bacterial persistence on food contact surfaces and to stand with the adverse effect of the environments,

2.3.6.1. The production of extracellular polymeric substances (EPSs)

Bacterial biofilms are naturally the aggregated cells to form a colony for metabolic cooperative and subsequently develop into mature biofilms coated in a self-produced matrix of EPSs. EPSs are highly networked and hydrated substances, consisting of polysaccharides, proteins, lipids, extracellular DNA (eDNA) and other metabolites (Das et al., 2013; Costa et al., 2018; Chirkena et al., 2019; Brindhadevi et al., 2020). Among these metabolites, eDNA provides vital part in promoting bacterial to surfaces adhesion and securing the structural stability and

integrity of EPSs which enhance the antimicrobial resistance of bacterial biofilms Okshevsky & Meyer, (2015), and furthermore, both interact to enhance the co-aggregation of bacteria from different environments and their attachment to surfaces. As a result of this, the presence of EPSs throughout the dynamic process of biofilm formation further secures the life of the pathogen by enabling nutrient entrapment, provision of ideal environments for chemical reactions, protection against adverse conditions and reduction of dehydration possibility by retaining moisture (Costa et al., 2018). The contribution of lipids and proteins in the production of EPSs, and thus for biofilm formation also provides a significant means of co aggregation especially when they are exposed to unexpected salinity change and stressed environments (Hede & Khandeparker, 2020).

The overall production of EPSs contributes to the survival and virulence of the pathogen against antimicrobial activities and persistence in industrial settings because of the matrix act as protecting layers against the diffusion of antimicrobials and their accessibility to bacterial cells embedded in the matrix (Seviour et al., 2019). The report of Wang et al., (2018), who investigated the transport of three antimicrobials, namely Sulfamethizole, Tetracycline and Norfloxacin, associated with the response of biofilms showed that there was the vulnerability of biofilms to antimicrobials in the absence of EPSs. This indicates that the presence of EPSs within the biofilms ensures resistance to antimicrobials and tolerance to disinfectants, and thus responsible for the spread of the pathogen. Generally, the complex mechanism behind the formation of biofilms that involved in the persistence of the pathogen in different adverse environments and resistance to various antimicrobials and other chemicals is the existence of polymeric matrix (Shi & Zhu, 2009). As a result, the pathogen gain the ability to sustain in the food processing plants to be responsible for the contamination of food products

2.3.6.2. Bacterial slow growth and stress response

These factors also influence the bacterial biofilm formation based on the availability of nutrients. As the pathogen gets starved for a particular nutrient, it become dormant or undergoes slow growth. During this period, the pathogen becomes persistent and tolerant to antimicrobials, contributing to the difficulty in treating some bacterial infections (Pontes & Groisman, 2019; Gray et al., 2019). The transition from exponential growth to slow or no growth, due to nutrient limitation, is generally accompanied by an increase in antimicrobial resistance of the bacterial biofilms (Mah & O'toole, 2001). During slow growth, bacteria develop the tendency to escape

from the activity of antimicrobial agents because of their effectiveness on actively growing bacteria; thus, lag phase/slow growth offers bacteria survival advantages and promotes regrowth upon the removal of antimicrobials (Li et al., 2016). The stress response, on the other hand, enables the bacteria to withstand the adverse and fluctuating conditions in its immediate surroundings because its survival depends on the ability to sense and respond to changes in the environments with suitable modifications in gene expression and protein activity (Boor, 2006).

2.3.7. Significance of *L. monocytogenes* toward inhibitory effect of probiotic dairy products

It has been well explained that the pathogenic *L. monocytogenes* is the most difficult foodborne bacteria to eradicate due to its resistance properties to extremely adverse conditions (Zadeh et al., 2022) and its biofilm forming ability (Dygico et al., 2020; Mendez et al., 2020; Hossain et al., 2021; Duze et al., 2021). These conditions promote the persistence of the pathogen on surfaces linked with food contact to come up with the contamination of food products. As a result of this, agricultural sectors and the food industry are economically at the risk due to the recall of food products. According to the investigation carried out in thirteen selected food processing manufactories in West Pomeranian region of Poland, which are well known in producing ready-to-eat foods, the prevalence of *L. monocytogenes* in those foods was found to be significant with high level of contamination (Szymczaka et al., 2020). Unlike to this, Oxaran et al., (2017) reported low rate of incidence of *L. monocytogenes* in five dairies and retail products in the Southeast and Midwest regions of Brazil; however, the authors indicated that the pathogen still impose possible health hazard. These all show that the presence of *L. monocytogenes* in food products is mainly associated with huge economic lose due to recall of food products, and a potential risk to public health.

Therefore, to impede the effect of the pathogen, several alternative means of controlling approaches have been found in the side of researches and food industry practitioners. In light of this, some evidence show that various probiotic products have been used as alternative controlling means to overcome the risk associated with effect of the pathogen with in the foods, recently published research works (Muñoz et al., 2019; Hossain et al., 2020; Abdelhamid & El-Dougdoug, 2020; Kouhi et al., 2022; Martín et al., 2022). More specifically, the report of Prezzi et al., (2020) who evaluated the effect of *Lactobacillus rhamnosus* GG on the growth of two foodborne pathogens (*S. aureus* and *L. monocytogens*) by inoculating on the surface of Minas Frescal cheeses indicated that the addition of *Lactobacillus rhamnosus* in the cheese was found to

be responsible for the inhibition of *L. monocytogens*. Similarly, the LAB produced bacteriocin metabolite can also be considered as natural antimicrobial against the activity of *L. monocytogenes* to ensure food safety and quality (Zadeh et al., 2022). Thus, the incorporation of probiotic strains into food products including dairy products can weaken *L. monocytogenes* infection by discouraging its intestinal inoculation and virulence properties (Deng et al., 2020), and in these food products the viability of the probiotics during production and storage can give a significant key quality features (Anihouvi et al., 2022).

In general, probiotic LAB in various dairy products, such as fermented milk, cheese,yoghurt and other products are functioned as starter culture alone or in combination with other traditional starters (Gao et al., 2021). Moreover, probiotics have a great promising environmental friendly and used as alternative biological agents to mitigate the negative effects the pathogen in the food products (Hossain et al., 2020). The reason behind for the effectiveness of probiotic bacteria against the pathogen is associated with the production of metabolites including bacteriocins and organic acids that could be considered as a natural tool in the development of new strategies to prevent or control the risk of *L. monocytogenes* in the food products. In this regard, evidence show that the growth of this pathogen could be controlled by addition of competitive LAB strain-producing bacteriocins that its need has significantly increased in the recent years (Zadeh et al., 2022).

2.4. The pathogenic *Staphylococcus aureus*

2.4.1. Over view on the pathogenicity of *Staphylococcus aureus*

Staphylococcus aureus is among the leading foodborne pathogenic bacteria, and produces varieties of heat stable enterotoxins Ahmed et al., (2019), owing powerful toxins and other virulent factors that assist the bacteria to be highly infectious (Rasheed & Hussein, 2021). It is a gram-positive, catalase-positive, non-spore forming and facultative aerobic-anaerobic bacterium, colonizing the skin and the upper respiratory tract of humans (Flora et al., 2019). The pathogen specifically causes staphylococcal food poisoning from the consumption of staphylococcal enterotoxins produced in the food, consequently cause serious health problem to the public, and huge economic losses to the food industries (Farha et al., 2020). The level of poisoning depends on multiple toxic proteins that can be secreted, especially when they are produced more than 10^5CFU/g representing pathogenic toxins (Zhao et al., 2020).

The staphylococcal enterotoxins are often reported in the dairy milk products, and are observed as the major cause of infections associated with food poisoning in humans and mastitis cases in animals (Zhao et al., 2021). The pathogenic *S. aureus* is a ubiquitous foodborne pathogen. It is the main source of contamination of dairy products, milking equipment and food contact surfaces (Rosengren et al., 2010; Rubab et al., 2018; Titouche et al., 2019). It is also established in raw retail meat products, including pork, poultry and beef meat (Schoen et al., 2020). According to evidences obtained from different sources, one of the characteristic properties of *Staphylococcus aureus* is its ability to develop multiple antimicrobial resistances as it is emerged in health centers, community and animals, and detected in different food products mainly dairy products (Basanisi et al., 2017; Carfora et al., 2015; Zhao et al., 2021). This, furthermore, increases the challenge of controlling the infections, especially methicillin resistant *S. aureus* (MRSA) strains (Rasheed & Hussein, 2021) which could be of the most emergent zoonotic pathogen with public health and veterinary importance (Algammal *et al*., 2020).

The other characteristic of this pathogen is its biofilm formation that enhances to survive and tolerate the situation in the external environment and the host (Torlak et al., 2017; Maia et al., 2020), and including food contact surfaces (Zhang et al., 2021). Like in the other pathogenic bacteria, the formation of biofilm by *S. aureus* is most frequently associated with production of extracellular polymeric substances as the biofilm is considered as part of the normal life cycle of *S. aureus* in the environment. Some research based evidence indicated that the formation of biofilm on food processing utensils is responsible for the survival and spread of foodborne *S. aureus* pathogen in the food products, causing the recalls of the products in food processing industry (Maia et al., 2020; Farha et al., 2021), and makes the treatment of human diseases complicated due to antimicrobial resistance of the biofilm (Odetokun et al., 2018; Abdeen et al., 2020; Bencardino et al., 2021).

2.4.2. The *Staphylococcus aureus* bacterium in the dairy food products

Obviously dairy products are among various food products to be responsible for the transmission of *S. aureus* infection which becomes food safety concern, especially MRSA biofilm isolates, unless serious food safety management systems and strategies are implemented for the prevention of biofilm forming *S. aureus* (Avila-Novoa et al., 2021; Alghizzi & Shami, 2021; Ahmed et al., 2019). That is why the existence of pathogenic *S. aureus* in dairy industry and other agricultural sectors become a potential threat for the community and experts working in

the dairy farm and industry. The investigation conducted associated with the prevalence of *S. aureus* along the production chain of dairy products in north-western Greece show that there could be the involvement of this pathogen in the contamination of dairy products (Papadopoulos et al., 2018). Meanwhile, Shahid et al., (2021) suggested that this pathogen can also be isolated from serving utensils in food processing environments because of its persistence on food contact surfaces due to biofilm forming ability.

Regarding the prevalence of *S. aureus*, some research findings verify that the pathogen can be prevalent in dairy products. Accordingly, Ahmed et al., (2019) reported a prevalence of 26.67% in milk and Egyptian artisanal dairy products, while Zhao et al., (2021) addressed 28.9% in bulk tank milk in Shandong dairy farms. According to the investigation of Alghizzi & Shami, (2021) conducted in Riyadh, Saudi Arabia, it was confirmed that both *S. aureus* and MRSA were found to be prevalent in raw milk, raw Goat milk, raw Horse milk, raw Camel milk, raw Cow milk and Cheese. On the other hand, the prevalence of *S. aureus* was also reported in pasteurized milk in China, imposing potential risk of the pathogen to the public (Dai et al., 2019). In livestock sectors, since *S. aureus* is considered as the most mastitis pathogen in dairy cattle, and exhibits zoonotic potential, the consequences could be huge economic crisis in the dairy industry, and as result of this, public health can be a serious issue (Kümmel et al., 2016).

2.4.3. The significance of *S. aureus* associated with antimicrobial susceptibility

The antimicrobial susceptibility or resistance of *S. aureus* to commonly used antimicrobial agents is associated with its ability to obtain and spread antimicrobial resistant/ susceptibility factors in nature (Akanbi et al., 2017). In dairy farms, milk and milk products are considered as main sources of the pathogenic *S. aureus* bacterium Dai et al., (2019), and thus the use of antimicrobial agents for control of this bacterium, especially in mastitis, is found to be an important alternative approach. However, this pathogen often shows resistance against multiple classes of antimicrobials, which tightens the treatment options for the health professionals (Zhao et al., 2021). The characteristic of the pathogenic *S. aureus* associated with antimicrobial profile involves both resistance and susceptibility to commonly used antimicrobials with considerable variation of resistance in drugs including Penicillin (74.4%), Erthromycin (58.7%) Zhao et al., (2021), Tetracyclin (56.1%), Oxacillin (16.2%), Clindamycin (11.3%), Streptomycin (5.8%), Chloramphenicol (3.7%), whereas susceptibility of the pathogen has been seen in some antimicrobials (Akanbi et al., 2017). Therefore, the spread of the antimicrobial resistant *S.*

aureus, mostly methicillin-resistant one, has been correlated with its potential to establishing new reservoirs, whatever less attention was given to the role of the environment (Ramos et al., 2022).

Of course, the spread of the resistant pathogen is worldwide, and cause serious challenge to the treatment of hospital-acquired infections, through invading community settings and infect the people without predisposing risk factors (Al-Zoubi et al., 2015; Aires-de-Sousa, 2017; Lakhundi & Zhang, 2018). The presence of enterotoxigenic producing *S. aureus* strain, mainly MRSA, in raw dairy milk, unpacked cheese and other products creates serious public health problems, as the products serve as potential vehicle for multidrug resistant MRSA transmission (Titouche et al., 2018; Alghizzi & Shami, 2021). MRSA is one of the utmost significant antibiotic-resistant strains of *S. aureus*, encoding penicillin-binding protein, mediating resistance to the methicillin group and all other β-lactam antibiotics. It is primarily appeared as a major cause of hospital-associated infections, and new incidents in different settings, specifically in the human inhabitants and also in livestock industry (Zhao et al., 2021). In general, MRSA is considered as major problem creating strain to the general public after taking foods containing the pathogen and its toxins. The seriousness of this pathogen is at community level, more specifically hospitalized people, and in animal husbandry (Tsai et al., 2020; Crago et al., 2012).

2.4.4. The significance of *S. aureus* towards inhibitory effects of probiotic dairy products

In dairy industry, the pathogenic *S. aureus* has got attention as contagious causal bacterium of bovine mastitis that can cause huge economic losses to dairy farmers, and it is further responsible to a variety of human diseases including foodborne infections and principal nosocomial in the world, particularly associated with MRSA (Sikorska & Smoragiewicz, 2013). For the sake of ensuring food safety management in food processing industries, it is a mandatory to curb the effects of this pathogen and other foodborne pathogens using various alternatives, of which probiotic LAB is considered the best. Probiotic LAB is responsible for promoting the development of immune system of the host to enhance the protective capacity of the host against foodborne pathogenic *S.aureus* bacterium and other microorganisms. These probiotic bacteria further produce different metabolites hinder the growth of undesirable microorganisms in food and pharmaceutical industries (Nataraj et al., 2021).

The inhibitory effect of probiotic *Enterococcus mundtii* H81, isolated from milk of healthy dairy cows, against S. *aureus*-induced mastitis in mice is an indication of the probiotic LAB as microbiological agents involving in antagonizing the activity of pathogens (Qiu et al., 2022). Similarly, antimicrobial potential of probiotic bacteria isolated from fermented dairy products with optimized level of prebiotic ingredients, including fructooligosaccharide and isomaltooligosaccharide, was also tested to have ant diabetic potential in diabetic rabbits (Shafi et al., 2019). Many strains of probiotic LAB isolated from various food sources, particularly dairy foods, are designated as inhibitory agents against the growth of *S. aureus* and clinical isolates of MRSA. The most active and effective strains include *Lactobacillus reuteri*, *Lactobacillus rhamnosus* GG, *Lactobacillus paracasei*, *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus plantarum*, *Lactobacillus bulgaricus*, *Lactobacillus fermentum* and *Lactococcus lactis* which their effects are mediated both by direct cell competitive exclusion as well as production of bacteriocin inhibitor (metabolite). Moreover, Sikorska & Smoragiewicz, (2013) suggested the potential inhibitory effect of *Lactobacillus acidophilus* against the biofilm formation of the pathogenic *S.aureus*. In general, some identified clinical cases and research articles have confirmed that the interaction between probiotics and MRSA pointed to the viability of elimination or reduction of MRSA colonization with probiotic use.

2.5. The coliform bacteria, *Escherichia coli*

2.5.1. Over view on the Coliform bacteria, the *Escherichia coli*

Coliform bacteria are organisms that abundantly present in the environment and feces of humans and all warm-blooded animals. They are rod shaped gram-negative, non-spore forming and motile or non-motile bacteria which can ferment lactose with the production of acid and gas when incubated at 35-37^{o}c. The presence of total coliforms in food of animal origin indicates environmental sources of contamination since the micro-organisms are plentiful in the environments and various food products (Mhone et al., 2011). Among coliforms, *E. coli* is the most common food contaminant, including raw and processed milk. It is a commensal micro-organism of animals and humans intestines. However, it can be pathological when it is in food and become public health concern due to the possible presence of enteropathogenic and/or toxigenic strains in the food stuffs. Enteropathogenic *E. coli* strains can cause severe diarrhea and vomiting in infants and young children, whereas toxigenic strains like *E. coli* O: 157:H7 cause haemolytic and uremic syndrome (Mhone et al., 2011).

Most strains of *E. coli* like diarrheagenic *E. coli* are common pathogens that transmitted by consumption of contaminated foods, causing acute intestinal diseases in human (Fallah et al., 2021). Food products including dairy products like raw milk are among various food matrices where the pathogens can be detected and contribute the majority of food borne illnesses in human beings (Tian et al., 2022). Evidence show that the involvement of the pathogenic shiga-toxin producing *E coli*, also called verocytotoxic producing *E. coli* in sporadic cases and disease outbreaks is currently increasing due to ingestion of milk and dairy products (Farrokh et al., 2013). Thus, the detection of these harmful bacteria in food products has been the determinant factor for understanding their effects in terms of food safety and public health. Several approachable detection methods, such as biosensors have extended reputations and are considered as an alternative means of detection for pathogens (Rubab et al., 2018), and detection of Coliform bacteria may also be used as a hygienic indicator for dairy products.

2.5.2. Significance of *Escherichia coli* in association with antimicrobial resistance

Antimicrobial resistance is mostly associated with inappropriate use of drugs in the need of treatment for animals and humans. This contributes to the emergence and spread of drug resistance traits among pathogenic and commensal bacteria which eventually become the risk to the general public. For example, the development of antimicrobial resistance in *E. coli* is considered as one of the major public health concern contributing to increased morbidity and fatality rate particularly in countries with low income and economic developed (Najjuka et al., 2016; Sarba et al., 2019). It has been also reported that *E. coli* bacteria that are isolated from different food items have found to develop antimicrobial resistance against various antimicrobials (Godziszewska et al., 2018). In this concern, the antimicrobial resistance of pathogenic *E.coli* isolated from raw milk and Minas Frescal cheeses in Brazil to some antimicrobials including streptomycin could be the indication for the antimicrobial resistance of pathogenic *E.coli* bacteria (Júnior et al., 2019).

Furthermore, the investigation of Yu et al., (2020) on the antimicrobial resistance of *E.coli* strains, isolated from raw milk in the selected four regions of China, against Penicillin (100%), Acetylspiramycin (100%), Lincomycin (98.8%), Oxacillin (98.8%) and Sulphamethoxazole (53%) has also reflected that there is high incidence of the pathogen with a great variation in resistance patterns; this becomes the issue of public and animal health. In addition to antimicrobial resistance, the strain of *E.coli* bacteria isolated from dairy and meat products are

also responsible for biofilm formation, according to the result of research study released from India (Bhardwaj et al., 2021). In general, this bacterial species is characterized by developing a great capacity to accumulate resistance genes, mostly through horizontal gene transfer, and this is why the antimicrobial resistance in *E.coli* has become a restless issue and increasingly observed in human health and veterinary medicine globally.

2.5.3. The significance of *E.coli* towards inhibitory effects of the probiotic dairy products

It is obvious that the effects of various probiotic bacterial strains against the growth and activity of foodborne pathogenic bacteria, most of the time presenting in the food products and the environmental. These microorganisms including *Lactobacillus*, *Bifidobacterium*, *Lactococcus* and *Enterococcus* including metabolic products are responsible for the inhibition of the growth, adhesion activity and even biofilm formation of *E.coli* bacteria (Miyazaki et al., 2010; Darvishi et al., 2021). A study conducted on the antibacterial activity of probiotic bacteria against hemorrhagic *E. coli* O157:H7 obtained that there was considerable antimicrobial effect of the selected probiotic strains on this bacterial strain (Karimi et al., 2018). Likewise, Darvishi et al., (2021) reported the effect of some the strains of *Lactobacillus* such as *L. sakei*, *L. plamtarum*, *L. reuteri*, *L. fermentum*, and *L. casei* with the highest inhibition against the targeted *Escherichia coli* MG1655 when compared to *Bifidobacterium* species, while Wang et al., (2019) demonstrated the stronger probiotic potential of *L. reuteri* inhibiting the growth of enteroinvasive *E.coli* than *L. mucosae*.

Various probiotic LAB that sourced from different food products are found to be responsible for the inhibition of the growth of the pathogenic *E.coli* bacteria in food items. Some evidence show that probiotic LAB, such as *Leuconostoc mesenteroides*, identified as KCTC 13374 and *Lactobacillus plantarum*, identified as KCTC 33133, and isolated from Kimchi, one of the primary source of high sodium content in Korean diet, have exhibited antimicrobial activities against pathogenic *E. coli* (Choi et al., 2021). Similar investigation had conducted on the probiotic potential of LAB isolated from fermented cereal-based foods, and raw goat milk to evaluate their antimicrobial potential, probiotic attributes, technological properties and safety profiles (Xu et al., 2020; Islam et al., 2021). In general, probiotic LAB of various strains are well known as biochemical and safe solution for controlling gastrointestinal pathogens, and create suitable substitutions to antibiotics and chemicals in food technology. Thus, increasing the use of probiotics as a natural and modern agents for prevention of different diseases is recommended.

2.6. The fermented dairy product, kefir

2.6.1. Kefir definition and properties

Kefir is a traditional fermented dairy product, produced by the action of bacteria and yeast that exist in symbiotic association in kefir grains, having a complex probiotic and nutritional composition (Gökmen et al., 2022; Yilmaz et al., 2022). The grains contain casein and other milk solids together with the yeasts that cause the characteristic kefir fermentation, and serve as a starter to induce fermentation in fresh milk. Both kefir and kefir grain have a rich microbiota and their composition is affected by many parameters, which include the origin of kefir, its production method, and kefir grain, i.e. milk ratio, type of milk, fermentation conditions, and equipment used in production and storage conditions (Yilmaz et al., 2022). Naturally, fermented dairy kefir is characterized by well probiotic properties; thus consumer interest in this product has increased due to the accumulating evidence of the effects of kefir microorganisms on the modulation of gut microflora and their antimicrobial activity (González-Orozco et al., 2022).

It is well known that kefir is characterized by possessing an acidic property and antibacterial activity due to the existence of probiotic LAB, e.g, *Lactobacillus lactis* ssp. *lactis*, and yeast in the kefir grains (Yerlikaya, 2019; Wang et al., 2021). These probiotic bacteria produce different metabolites, such as bacteriocins, organic acids and other components that inhibit the growth of pathogenic microorganisms when incorporated into food products. Recent data show that the use of probiotic LAB, isolated from different fermented food products, as the inhibitory or antibacterial agents against various pathogenic bacteria through counteracting their multiplication based on the competitive exclusion principle has been reported (Gómez et al., 2016; Jara et al., 2020; Tatsaporn & Kornkanok, 2020; Rajabi et al., 2020).

2.6.2. Microbial and chemical composition of kefir

The microbiological and chemical composition of kefir and kefir grains indicate that they are very complex probiotic, with lactic acid bacteria and yeasts, generally the predominant microorganisms (Arslan, 2015; Gao & Li, 2016). Kefir is produced when lactic and alcoholic fermentation of milk from kefir grains takes place. Thus, it results in drinking milk with a refreshing flavor, typically acidic, slightly alcoholic and carbonated characteristics (Favilla et al., 2022). Kefir grains have a complex composition of beneficial microbial such as LAB and yeasts. Some LAB include *Lactobacillus paracasei* ssp. *paracasei*, *Lactobacillus acidophilus*,

Lactobacillus delbrueckii ssp. *bulgaricus*, *Lactobacillus plantarum*, and *Lactobacillus kefiranofaciens*, whereas some yeasts include *Saccharomyces cerevisiae*, *Saccharomyces unisporus*, *Candida kefyr*, and *Kluyveromyces marxianus* ssp. *marxianus* are the predominant species in kefir and kefir grains (Prado et al., 2015).

Different metabolites including acetaldehyde, diacetyl, acetone and other compounds are produced from the fermentation of homo-fermentative and hetero-fermentative lactic acid bacteria present in kefir grains. Additionally, in kefir beverages, the formation of ethanol is essentially carried out by conversion of acetaldehyde to ethanol by alcohol dehydrogenase enzyme present in kefir yeasts and lactic acid bacteria (Magalhães-Guedes et al., 2016). So far, kefir grains have been applied for milk fermentation. The fermentation process can change the distribution of several essential elements in kefir milk products. However, the nutritional composition of kefir is variable, and it depends on the source of fat content of milk, the microbial composition and the technological process of kefir production. According to some research findings, kefir is well developed with several chemical compositions or nutritional with different concentration, of which proteins, minerals (Calcium, Copper, Iron, Potassium, Magnesium, Sodium, Phosphorus, Sulfur, and Zinc), essential amino acids and vitamins (A, Carotene, B1, B2, B6, B12, C, D, E) are considered as valuable nutritional composition in kefir (Magalhães-Guedes *et al*., 2016; de Oliveira et al., 2019)

2.6.3. Health benefit of kefir

Kefir has been considered as probiotic food and consumed for several years over the world in terms of its health benefits which include antioxidant, anti-inflammatory, anti-hypocholesterolemic, anti-hypertensive, anti-mutagenic, anti-carcinogenic, and neuroprotective properties (Magalhães-Guedes et al., 2016). Additionally, kefir provides health benefits for other health problems such as colorectal cancer, cardiovascular diseases, type 2 diabetes mellitus, obesity and kidney diseases because of the presence of LAB as a significant part of the kefir's microbial composition (Yilmaz et al., 2022). The mechanisms of kefir against bacterial pathogens involve destabilization of bacterial cell membrane, cell lysis, degradation of nucleic acid, and inhibition of protein synthesis of the bacteria, and thus, this leads to better health, and consequently resistance to infection (Gut et al., 2021).

Studies have suggested that the effects of fermented foods on metabolic syndrome are limited; however, regular kefir become the best alternative agent to improve the anthropometrical measurements, glycemic control, lipid profile, blood pressure, and inflammatory status in patients with metabolic syndrome (da Silva Ghizi et al., 2021; Bellikci-Koyu et al., 2022). Thus, regular consumption of dairy foods is recommendable as they provide favorable effects in the management of metabolic syndrome, and probiotic kefir may deserve a special interest among dairy products. Therefore, the general characteristic properties of probiotic stains of LAB in kefir and kefir grain are regulating and modulating the body system of the host, and used considerably as antibacterial agents (Leite et al., 2015).

The most commonly known probiotic bacteria present in kefir are LAB which mostly capable to tolerate the physiological conditions in the gastrointestinal tract of the host, improving the gastrointestinal tract health and health related problems through adherence and colonization. Thus, consumers greatly incorporate kefir-based dairy foods containing probiotics into their diet, because of their nutritional composition and predominant source of LAB (Vella et al., 2014; Tamang et al., 2020). Moreover, probiotic kefir-based dairy products possess bactericidal and bacteriostatic effects on enteric bacterial pathogens; thus, they are taken as antagonizing agents when applied in dairy technology. Additionally, the products are involved in enhancing economic growth because of their use in the production of dairy products, including cheese, butter, cream and fermented milk (Yerlikaya, 2019).

2.6.4. Antimicrobial activity of kefir

Bacteria and yeast isolated from kefir are found to have in-vivo and in-vitro antimicrobial activity against enteropathogenic bacteria and spoilage fungi (González-Orozco et al., 2022). The inhibitory activity of the probiotic microorganisms in kefir against foodborne pathogens and bacterial spoilage could be driven through their adherence to the intestinal epithelium and their immunomodulation properties. Pathogens including *B. cereus*, *S. aureus*, *L. monocytogenes*, *E. faecalis* and *E. coli* are among the foodborne infections that are inhibited by probiotic bacteria containing kefir milk, a study confirmed (Kim et al., 2016). According to this study, the antimicrobial activity of kefir varies based on its type and fermentation time, as time of fermentation is longer the stronger antimicrobial activity is obtained. Moreover, recently published data indicate that various strains of probiotic LAB including *Pediococcus pentosaceus* and *Enterococcus faecium* are responsible to inhibit the activity of the pathogenic *Bacillus cereus*

ATCC 11778, *Escherichia coli* ATCC8739 and *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 13311 (Tatsaporn & Kornkanok, 2020).

The metabolites of LAB such as bacteriocins (nisin) can also be used as alternative biotechnological means to control the impact of various foodborne pathogens in food processing facilities (Camargo et al., 2018; Niaz et al., 2019; Kaya & Simsek, 2019; Abdelhamid & El-Dougdoug, 2020). In this regard, a study identified that bacteriocins derived from dairy products containing *Enterococcus* species identified as *E. faecalis* (OE-7 and OE-12) and *E. hirae* (OE-9) possess an antibacterial potential against multi-drug resistant foodborne and spoilage pathogens (Sonbol et al., 2020), while the derivative of probiotic *Lactobacillus planturum*, the extracellular polysaccharide, have similar activity against biofilm-forming pathogenic bacteria (Mahdhi et al., 2017). Among various biofilm-forming bacteria, *L. monocytogenes* is the one that is inhibited by probiotic activity of bacteria such as the spore-forming *Bacillus laterosporus*, *Bacillus megaterium, Lactobacillus fermentum* MP26 and *Lactobacillus salivary* MP14 (Rajabi et al., 2020; Jara et al., 2020).

2.7. Artificial Intelligence based approaches and evaluation of experimental results by AI

AI is a model proposed to predict the experimental data of different study findings. In this model, ANN is used to analyze and evaluate the interaction between different input and output variables in certain production system. This model works in data processing technology which involves connecting neurons to one another to develop complex nonlinear input-output relations and functions (Tongal and Booij, 2018; Nourani et al., 2021). Other models, such as ANFIS, Multi-layer perception (MLP) and StepWise-Linear Regression (SWLR) are mostly applied to predict the effect of experimental data. ANFIS is taken as a universal, wide ranging and multipurpose model to estimate all kinds of the problem. It is built by two important layers, namely multi-layer and feed forward networks and comprises input-output variables using fuzzy instruction of Takagi-sugeno type. In this system, the fuzzier and defuzzifier are the key parts of the arrangement in the fuzzy data-base system, modeling the relationship between input and outputs (Abba et al., 2020a).

On the other aspect, multilayer perceptron neural network is among the joint networks of ANN that help anyone to run and solve non-linear systems. Several researchers consider this system as universally accepted predictor when compare with the other classes of the model

(Choubin et al., 2016). Furthermore, the MLP neural network is also built up using an input and output layers where the input layer is considered as a hidden system (Abba, Pham, et al., 2020). However, in the present study, AI model was used to predict the inhibitory effect of probiotic bacteria against the targeted foodborne pathogenic *Listeria monocytogenes*, *Staphylococcus aureus* and *Coliform* bacteria during fermentation and post-fermentation periods within the matrix of probiotic dairy kefir.

CHAPTER III

3. MATERIALS AND METHODS

3.1. Activation of pathogenic bacterial suspensions

Pure cultures of *Listeria monocytogenes* (ATCC 19111) and *Staphylococcus aureus* (ATCC 19212) pathogens were obtained from Ankara University, Turkey. These microorganisms, commonly known as foodborne pathogenic bacteria (Nesse et al., 2014; Martinović et al., 2016; Wang et al., 2017; Bravo et al., 2019; Pontin et al., 2020), were used as target pathogens in the present study. The bacteria were kept in a refrigerator at a temperature of 4^{o}c until laboratory analysis was taken place. Activation of the pathogenic bacteria was performed on nutrient agar (NA) media (Sisco Research Laboratories, Navketan Ind., M.C.Rd., Mumbai, India) through streaking technique and alternatively, about 1 ml of bacterial suspension from each pathogen was suspended in 9 ml of MRD (Lab M Limited, Lancashire BL9 7JJ, United Kingdom). The plates containing bacterial inoculum were incubated at 37^{o}c for 24-48 h. A fecal sample was collected for the cultivation of coliform bacteria which was swabbed on VRB agar (Merck kGaA, Darmstadt, Germany) and incubated at 37^{o}c for 24-48 h. The suspension of each pathogen was adjusted to 0.5 MacFarland turbidity standards to approximate the bacterial concentration to 10^{6} CFU/ml as described by (Prezzi et al., 2020).

3.2. Kefir grains and kefir

Kefir grains were donated by the Department of Food Hygiene and Technology of Near East University, Nicosia (Figure 3). The kefir grains were maintained in low fat (1.5%) UHT milk at a temperature of 4^{o}c with a weekly transfer until further experimental analysis was undertaken. The grains were activated in UHT milk at 25^{o}c for an 18h fermentation period, and then were strained through a sterile sieve and washed with sterile normal saline solution to remove the curdle part of the milk (Angelidis et al., 2020), and following the process of straining the kefir grains, fermented kefir milk was obtained.

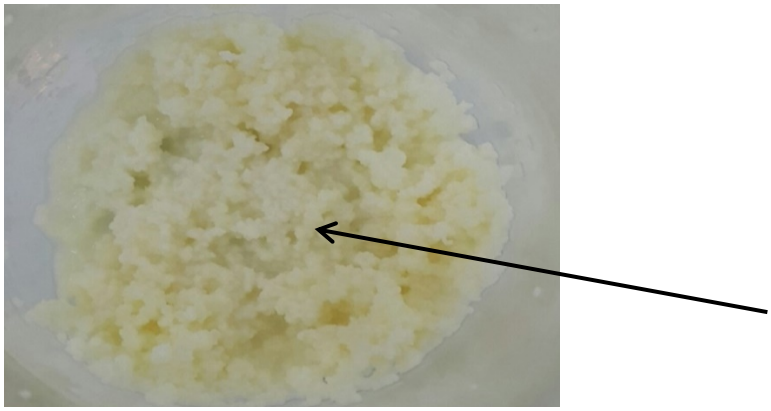
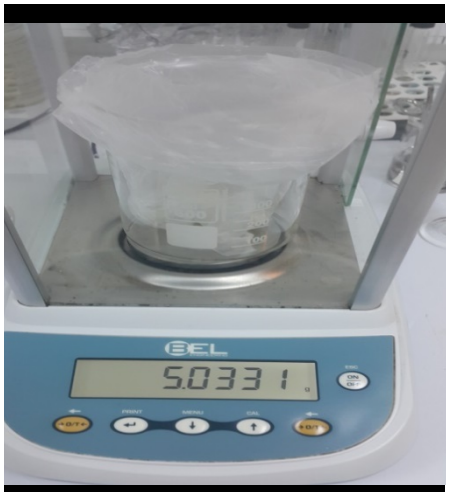


Figure 3. The picture representing kefir grains

3.3. Experimental contamination of kefir

Three glass jars (beakers) were labeled with “L”, “S”, and “C”. The jar labeled with “L” represented for *L. monocytogenes*, “S” for *S. aureus*, “C” for Coliforms, as described in figure 4a. About 5 gram of kefir grains was aseptically weighed on analytical electronic balance (ISO 9001:2015, BEL engineering, Milano, Italy), as shown in figure 4b, and was added to the jar “L”, and then mixed with 50 ml of UHT milk. Similarly, the same amount of kefir grains were added to the glass jar “S” and mixed with the same volume of UHT milk. The same procedure was applied for the glass jar “C”. About 0.1 ml of bacterial suspension of *L. monocytogenes* was added to jar “L” to perform experimental contamination of kefir. Similarly, the same amount of bacterial suspension of *S. aureus* and Coliforms were added to jar “S” and “C” respectively. The same 5 grams of kefir grains were mixed with 50 ml of UHT milk in the other glass jar labeled “D” represent the control group. All the jars containing the homogenized solutions were allowed to stay inside an incubator at 30^0c for two days fermentation process as described by Dimitreli & Antoniou, 2011; Gökmen et al., 2022). Then after, the grains were totally removed, and then the remaining kefir milk in each jar was handled in the refrigerator at 4^0c for the next days of microbiological analysis.

a)



b)

c)

Figure 4. a) Microbial contaminated milk (UHT) with kefir grains, "L" represents *L. monocyotogens*, "C" represents *Coliforms* and "S" represents *S.aureus*

b) Weighting kefir grains on analytical balance

c) Handling kefir grains under aseptic conditions

3.4. Microbiological analysis

3.4.1. LAB

Serial dilutions were prepared in test tubes with MRD (1:9ml) dilution in aseptic conditions for the microbial contaminated solutions. About 0.1 ml of the solutions from each of the test tubes (10^4) representing the targeted microbial was spread on selective agar plate. Specifically, the microbiological analysis of LAB was carried out aseptically by pouring onto the pre-prepared MRS agar media (Merck kGaA, Darmstadt, Germany) from each of the

representative jars under anaerobic conditions, according to the description of Kaban & Kaya, (2008). The agar plates were incubated at 37^{o}c for 48h. After 48h the plates were removed from the incubator for microbial enumeration on the described agar media using colony counting. The enumeration of LAB in each jar was analyzed and compared with the control group.

3.4.2. *L. monocytogenes*

Similar to the procedure done in the case of LAB, the serial dilutions were prepared in the test tubes with MRD for the microbial contaminated solutions presented in the jar "A". About 0.1 ml of the solutions was added onto a selective agar plates, PALCAM agar (Lab M Limited, Lancashire BL9 7JJ, United Kingdom) and spread over the surface of the plates. The spreading of the solution over the agar plates was done by drigalski spatula. The procedure was carried out both during fermentation process and storage time (post fermentation) of the dairy kefir. The plates were collected into an incubator to stay at temperature of 37^{0}c for 24-48h. Following the removal of the plates from the incubator, the enumeration of bacterial colony on the surface of the plates was taken place under colony counting plate.

3.4.3. S. aureus

The homogenized and bacterial contaminated solution in the glass jar "S" was serially diluted (serial dilutions prepared in the test tubes with MRD). About 0.1 ml of the diluted solution was taken by pipette and added onto Baird-Parker agar supplemented with egg yolk tellurite emulsion (20%) (Merck kGaA, Darmstadt, Germany). The solution was then spread over the surface of the plates to evenly distribute the solution for the growth of the colonies of *Staphylococcus aureus*, and then the plates were incubated at 37^{o}c for 24-48h as described by Angelidis et al., (2020) with some modifications. Colony counting on the surface of the agar plates was done for both during fermentation and storage time of kefir milk using the colony counting plate.

3.4.4. Coliformis/ E. coli

The homogenized solution that contaminated with the coliform bacterial suspension was serially diluted in the pre-prepared MRD. Using a 1ml pipette, about 0.1 ml of the solution was taken from the serially diluted solutions and added onto an appropriate selective media, VRB agar (Merck kGaA, 64271 Darmstadt, EMD Millipore Corporation, Germany). After certain

minutes, following the pouring of the solution onto the agar, the plates were allowed to stay in the incubator at 37^{o}c for 24-48h. The enumeration of *Coliforms* was determined by the pour on plate method, cultivating the bacteria onto VRB agar in anaerobic conditions (Kaban & Kaya, 2008). The identification of *Escherichia coli* was determined by incubating the VRB agar plate containing *Coliforms* bacteria, at a temperature of 44^{o}c for 24-48h.

3.5. Data analysis by AI

The experimental analysis of the present study was conducted in triplicate. AI data-based approaches (ANN and ANFIS models) were applied to analyze inhibition of *L. monocytogenes*, *S. aureus* and *E. coli* foodborne pathogenic bacteria by using probiotic lactic acid bacteria present in dairy kefir milk.

CHAPTER IV

4. RESULT AND DISCUSSION

4.1. Result

L. monocytogenes

The ANN model analyzed the inhibition of *L. monocytogenes* by using probiotic dairy kefir, and the inhibition was evaluated at the specified time. The average obtained result in LAB control, tested LAB and *L. monocytogenes* was 5.23, 4.95 and 2.41 $\log_{10}$ CFU/g respectively in training stage; whereas at the testing stage it was produced as 5.80, 5.38 and 2.04 $\log_{10}$ CFU/g respectively. As described in Table 3, the reduction in the number of pathogen was seen at SD1, SD3, SD7 and SD10; whereas the number of the tested LAB was, contrary, increased along with these days respectively.

Additionally, the obtained result in the reduction of the number of *L. monocytogenes* was also supported by the regression analysis at training, validation and testing stage, with R=0.9783, 0.9991, 0.9815 respectively (Fig, 5). The model furthermore revealed that inhibitory activity of the LAB against the pathogen with the best validation performance of 0.2298 at epoch 4 (Fig. 6) and with the error of the model 0.02395 as shown in Fig. 7. Similarly, the ANFIS model simulated with an overall modeling of the inhibition of *L. monocytogenes* using probiotic dairy kefir. In this model, the average number of the bacteria was 5.23, 4.93 and 2.33 $\log_{10}$ CFU/g for LAB control, the tested LAB and *L. monocytogenes* at the training phase, and 5.65, 5.29 and 2.45 $\log_{10}$ CFU/g in the testing stage respectively (Table 4). The number of pathogen in was found to be reduced starting from FD2 to SD10; whereas the number of LAB was increased from FD0 to SD10 unlike that of pathogen

Table 3. ANN model inhibition of *L. monocytogenes* by probiotic dairy kefir in $\log_{10}$ CFU/g

Training stages			
Days	LAB control	Tested LAB	*L. monocytogens*
FD0	3.22	3.1554	2.92988
FD1	4.1	3.81108	3.16429
FD2	4.96	4.70434	3.9069
SD1	5.1	4.95914	2.62325
SD3	5.2	5.03216	2.25576
SD7	5.29	5.04857	2.17457
SD10	5.4	5.0539	2.15349
FD0	4.2	4.09884	3.72184
FD1	5.1	4.95914	2.62325
FD2	5.86	5.25682	2.02536
SD1	5.98	5.41074	1.91552
SD3	6.11	5.52634	1.77089
SD7	6.34	6.01235	1.29927
SD10	6.43	6.25392	1.18377
Average	5.2350	4.9488	2.4106
Testing stages			
FD0	4.18	4.03903	3.6057
FD1	4.97	4.72179	3.81903
FD2	5.78	5.16275	2.08419
SD1	5.88	5.28384	2.00786
SD3	6.1	5.51774	1.78513
SD7	6.23	5.69512	1.53622
SD10	6.41	6.20991	1.20264
Average	5.8081	5.3876	2.0497

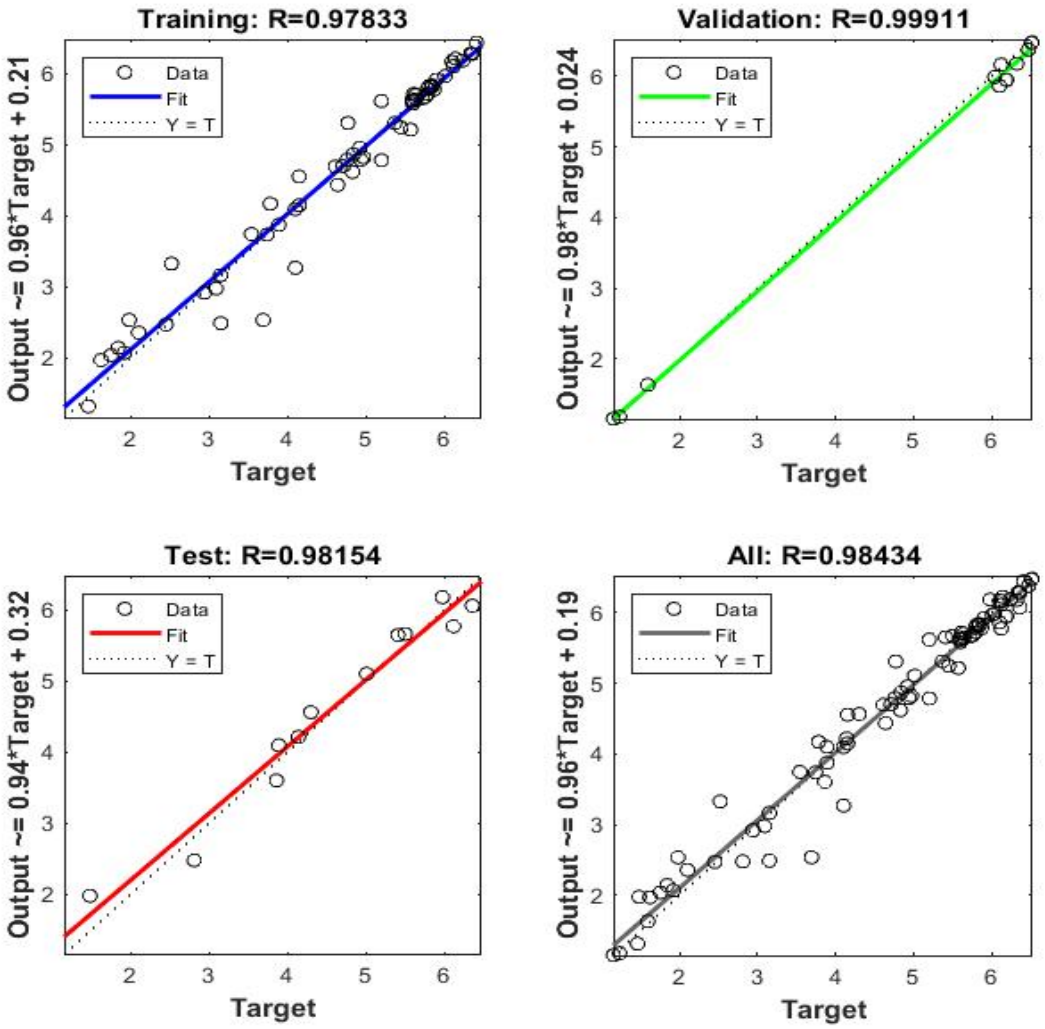


Figure 5. The regression graph of ANN Model prediction of the inhibition of *L. monocytogenes* using probiotic dairy product kefir in $\log_{10}$ CFU/g

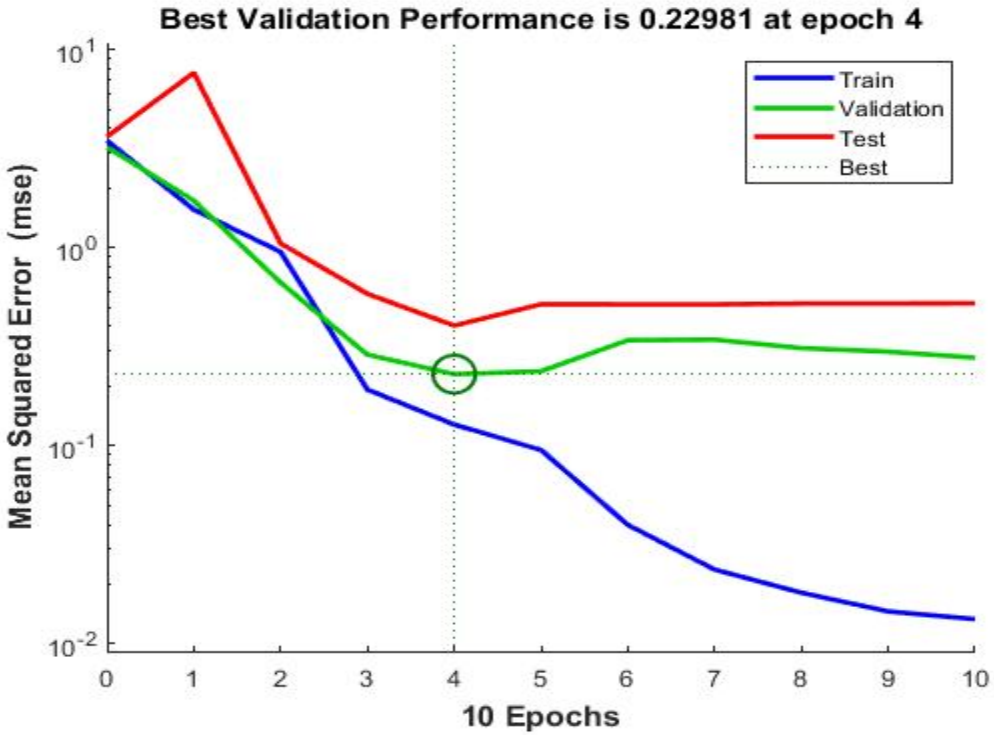


Figure 6. Plot performance graph of ANN Model prediction of the inhibition of *L. monocytogenes* using probiotic dairy product kefir in $\log_{10}$ CFU/g

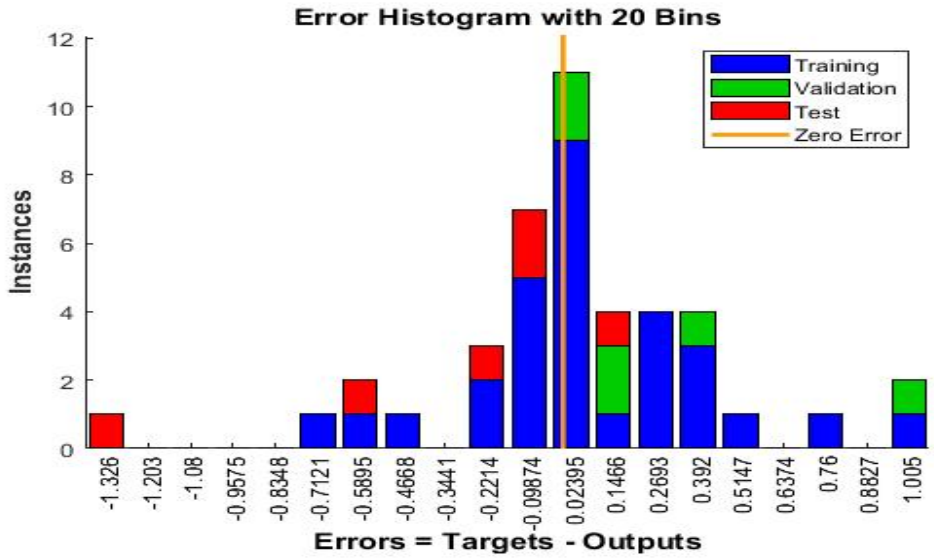


Figure 7. Histogram graph of ANN Model prediction of the inhibition of *L. monocytogenes* using probiotic dairy product kefir in $\log_{10}$ CFU/g

Table 4. ANFIS model inhibition of *L. monocytogenes* by probiotic dairy kefir in $\log_{10}$ CFU/g

Training stages			
Days	LAB control	Tested LAB	*L. monocytogenes*
FD0	3.22	3.15	3.223
FD1	4.1	3.9478	3.2745
FD2	4.96	4.6715	2.7313
SD1	5.1	4.816	2.608
SD3	5.2	4.8571	2.6016
SD7	5.29	4.9144	1.8703
SD10	5.4	5.0067	1.3871
FD0	4.2	4.0791	3.3261
FD1	5.1	4.8143	2.7302
FD2	5.86	5.5105	2.8393
SD1	5.98	5.6223	1.6358
SD3	6.11	5.7867	1.6372
SD7	6.34	5.8936	1.2656
SD10	6.43	5.9725	1.5433
Average	5.2350	4.9316	2.3338
Testing stages			
FD0	4.18	4.1123	3.1567
FD1	4.97	4.611	2.8691
FD2	5.78	5.4321	1.6415
SD1	5.88	5.5083	1.8067
SD3	6.1	5.6885	1.9563
SD7	6.23	5.804	2.7218
SD10	6.41	5.8989	3.0079
Average	5.6500	5.2936	2.4514

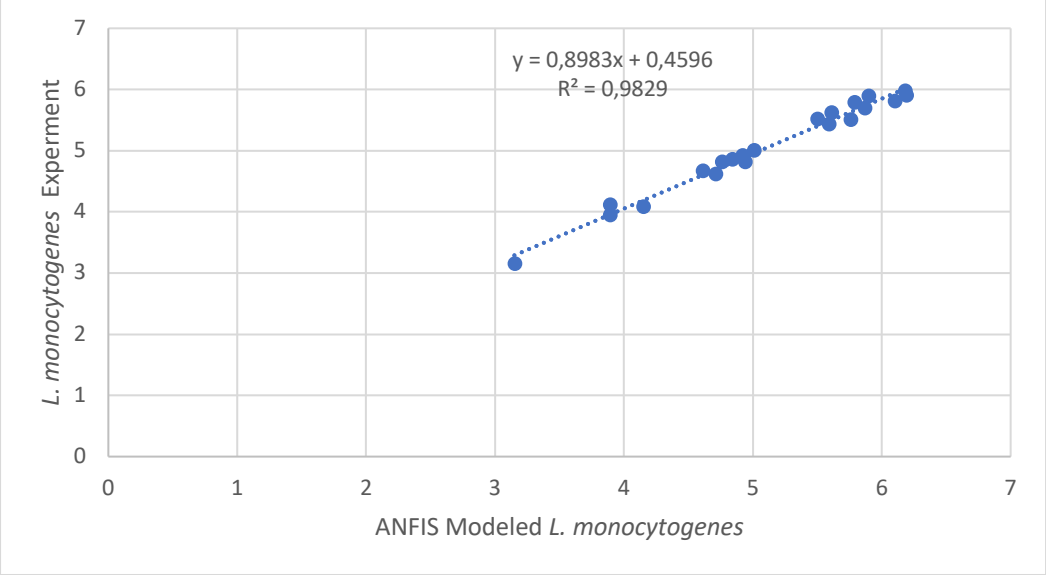


Figure 8. Scatter plots of the inhibition of *L. monocytogenes* using probiotic dairy product kefir using ANFIS model

S. aureus

Like in case of *L. monocytogenes*, the ANN and ANFIS models also analyzed the inhibition of *S. aureus* by using probiotic dairy kefir during fermentation and storage days. The models evaluated at the specified time interval as displayed in Table 5 and 6 respectively. On average *S. aureus* was simulated by ANN model in relation with LAB control ($5.23\log_{10}$CFU/g) and tested LAB ($4.89\log_{10}$CFU/g, getting an average reduction by $2.04\log_{10}$CFU/g. The inhibition of *S. aureus* by probiotic LAB in the dairy kefir was also supported by the regression analysis at training, validation and testing stage, with R=0.9842, 0.9905, 0.8873 respectively as indicated in figure 6, and with the best validation performance of 0.071812 at epoch 21(Figure 7). The inhibition of S. *aureus* pathogenic bacteria by probiotic LAB was also analyzed by ANFIS model, and as it was described in Table 6, the reduction in number of the pathogen was observed from FD2 to SD10 in the training stages; while the number of LAB was found to be increased during the above stated days.

Table 5. ANN Model inhibition of *S. aureus* using probiotic dairy product kefir in log_{10} CFU/g

Training stages			
Days	LAB control	Tested LAB	*S. aureus*
FD0	3.22	3.099997	2.729985
FD1	4.1	3.860021	2.890063
FD2	4.96	4.574421	2.371017
SD1	5.1	4.601372	2.29078
SD3	5.2	4.870955	1.455232
SD7	5.29	5.011625	1.042168
SD10	5.4	5.051125	1.099865
FD0	4.2	4.051899	3.792911
FD1	5.1	4.601372	2.29078
FD2	5.86	5.495737	2.373361
SD1	5.98	5.599042	2.122545
SD3	6.11	5.728212	1.802943
SD7	6.34	5.947762	1.258809
SD10	6.43	6.015005	1.092137
Average	5.2350	4.8935	2.0438
Testing stages			
FD0	4.18	4.010105	3.69915
FD1	4.97	4.574777	2.370089
FD2	5.78	5.437892	2.496509
SD1	5.88	5.511534	2.336036
SD3	6.1	5.717981	1.828292
SD7	6.23	5.84888	1.503898
SD10	6.41	6.001234	1.12627
Average	5.8081	5.4447	2.0041

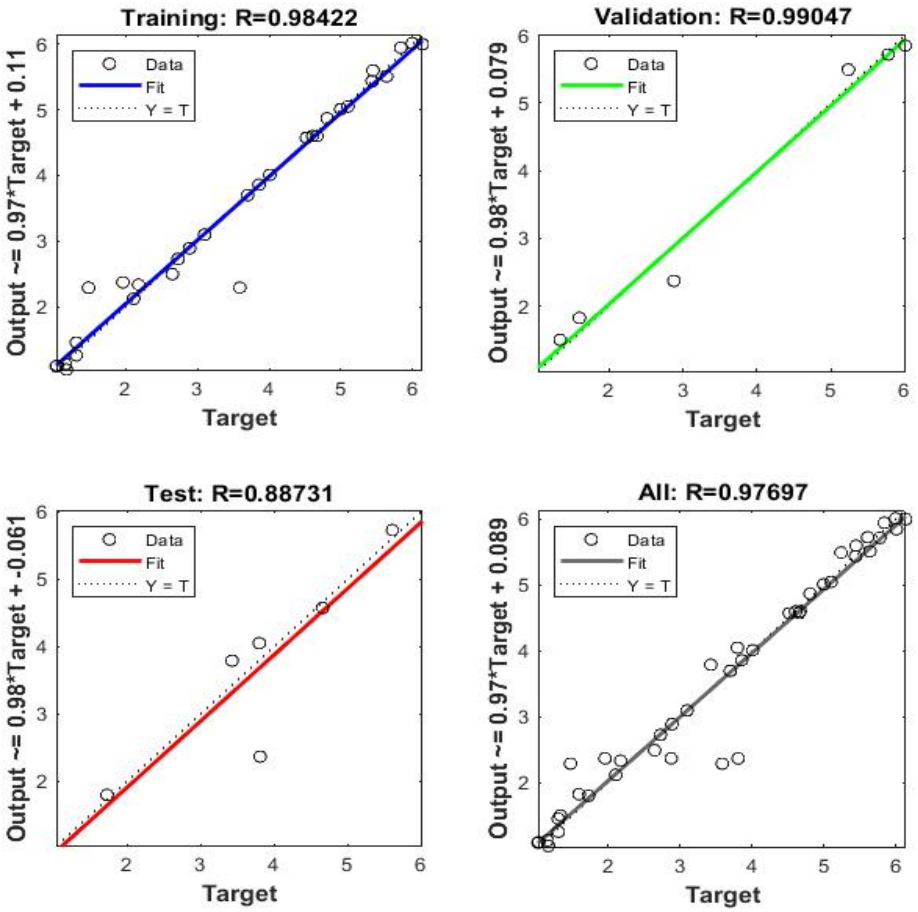


Figure 9. The regression graph of ANN Model prediction of the inhibition of *S. aureus* using probiotic dairy product kefir in $\log_{10}$ CFU/g

Figure 10. Plot performance graph of ANN Model prediction of the inhibition of *S. aureus* using probiotic dairy product kefir in $\log_{10}$ CFU/g

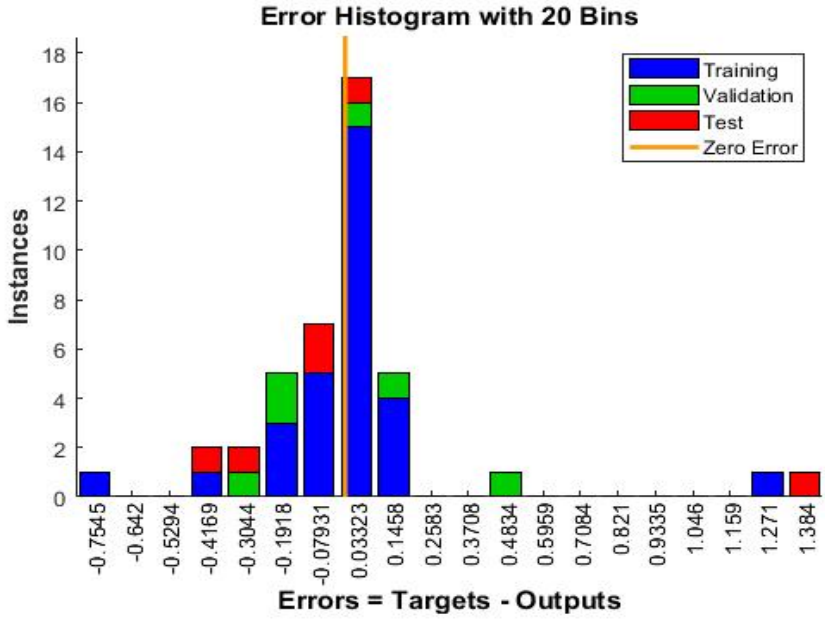


Figure 11. Histogram graph of ANN Model prediction of the inhibition of *S. aureus* using probiotic dairy product kefir in $\log_{10}$ CFU/g

Table 6. ANFIS model inhibition of *S. aureus* by probiotic dairy kefir in $\log_{10}$ CFU/g

Training stages			
Days	LAB control	Test LAB	*S.aureus*
FD0	3.22	3.1	2.73
FD1	4.1	3.7966	3.1944
FD2	4.96	4.5281	2.3997
SD1	5.1	4.6312	2.2107
SD3	5.2	4.8051	1.1803
SD7	5.29	4.951	1.1873
SD10	5.4	5.1009	1.2163
FD0	4.2	3.873	3.081
FD1	5.1	4.6364	2.6065
FD2	5.86	5.2431	2.9766
SD1	5.98	5.5245	1.3546
SD3	6.11	5.6451	1.6865
SD7	6.34	5.854	1.3672
SD10	6.43	5.9209	1.469
Average	5.2350	4.8293	2.0472
Testing stages			
FD0	4.18	3.8731	3.7969
FD1	4.97	4.6098	3.9406
FD2	5.78	5.3633	2.1166
SD1	5.88	5.4137	1.6937
SD3	6.1	5.5426	1.1974
SD7	6.23	5.5781	1.1467
SD10	6.41	5.7215	1.0504
Average	5.6500	5.1574	2.134

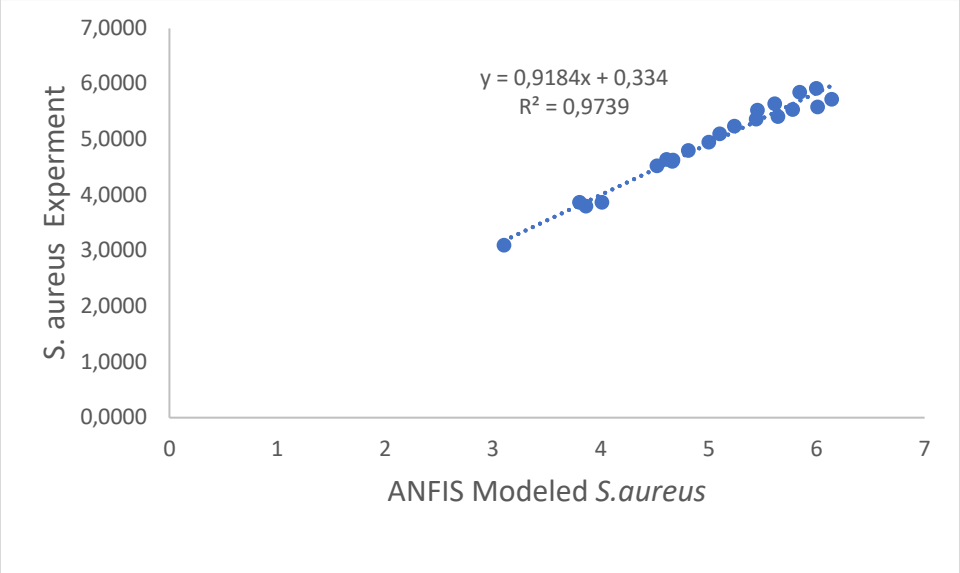


Figure 12. Scatter plots of the inhibition of *S. aureus* using probiotic dairy product kefir using ANFIS model

E. coli

The inhibition of *E.coli* by probiotic LAB present in dairy kefir was analyzed using ANN model, obtaining reduction in the number of the pathogen from FD2 to SD10; whereas an increment in the number of LAB was observed from FD0 to SD10 (Table 7) at training stages. Likewise, the reduction in the number of the pathogen was observed along the days from FD0 to SD10 at testing stages. However, it was revealed that the average result obtained for LAB control, the tested LAB and *E.coli* was 5.23, 4.96 and 2.46$\log_{10}$CFU/g respectively in the training stages; where as in the testing phase it was 5.81, 5.46 and 1.93 $\log_{10}$ CFU/g as displayed in Table 7. Additionally, the inhibition of the targeted pathogen was also braced by the regression analysis with R=0.9702, 0.9514 and 0.9537 at training, validation and testing stages respectively (Fig. 13). The best validation performance for the inhibition of the pathogen was obtained at 0.18637 as shown in Fig. 14.

Similarly, the inhibition of the pathogen by biological means, namely probiotic LAB naturally present in dairy kefir milk and used in the present study, was analyzed using ANFIS model. The model simulated with a complete typical modeling of the inhibition of *E.coli* at training stages with average number of LAB in control (5.23$\log_{10}$CFU/g), the tested LAB (4.93$\log_{10}$CFU/g) and *E.coli* (2.03$\log_{10}$CFU/g) as indicated in Table 8. Likewise, the average value of LAB control, tested LAB and *E.coli* obtained at testing stages were 5.65, 5.25 and 1.34$\log_{10}$CFU/g respectively. In this model, the inhibition of the activity of pathogen was seen along with the fermentation and storage days of kefir milk, which was confirmed by the reduction in the number of the pathogen from FD2 to SD10.

Table 7. ANN Model inhibition of *E.coli* using probiotic dairy product kefir in $\log_{10}$ CFU/g

Training stages			
Days	LAB control	Tested LAB	*E.coli*
FD0	3.22	3.22781	2.61819
FD1	4.1	3.79129	3.17393
FD2	4.96	4.43822	3.23498
SD1	5.1	4.92613	2.95347
SD3	5.2	5.07217	2.88304
SD7	5.29	5.12174	2.85996
SD10	5.4	5.14246	2.84803
FD0	4.2	3.64604	3.42336
FD1	5.1	4.92613	2.95347
FD2	5.86	5.44944	2.14581
SD1	5.98	5.67452	1.62552
SD3	6.11	5.79773	1.36348
SD7	6.34	6.01353	1.25022
SD10	6.43	6.23813	1.20511
Average	5.2350	4.9618	2.4670
Testing stages			
FD0	4.18	3.68151	3.36319
FD1	4.97	4.48445	3.20556
FD2	5.78	5.30451	2.48313
SD1	5.88	5.49074	2.04982
SD3	6.1	5.79159	1.37418
SD7	6.23	5.87139	1.29047
SD10	6.41	6.18071	1.21626
Average	5.8081	5.4569	1.9261

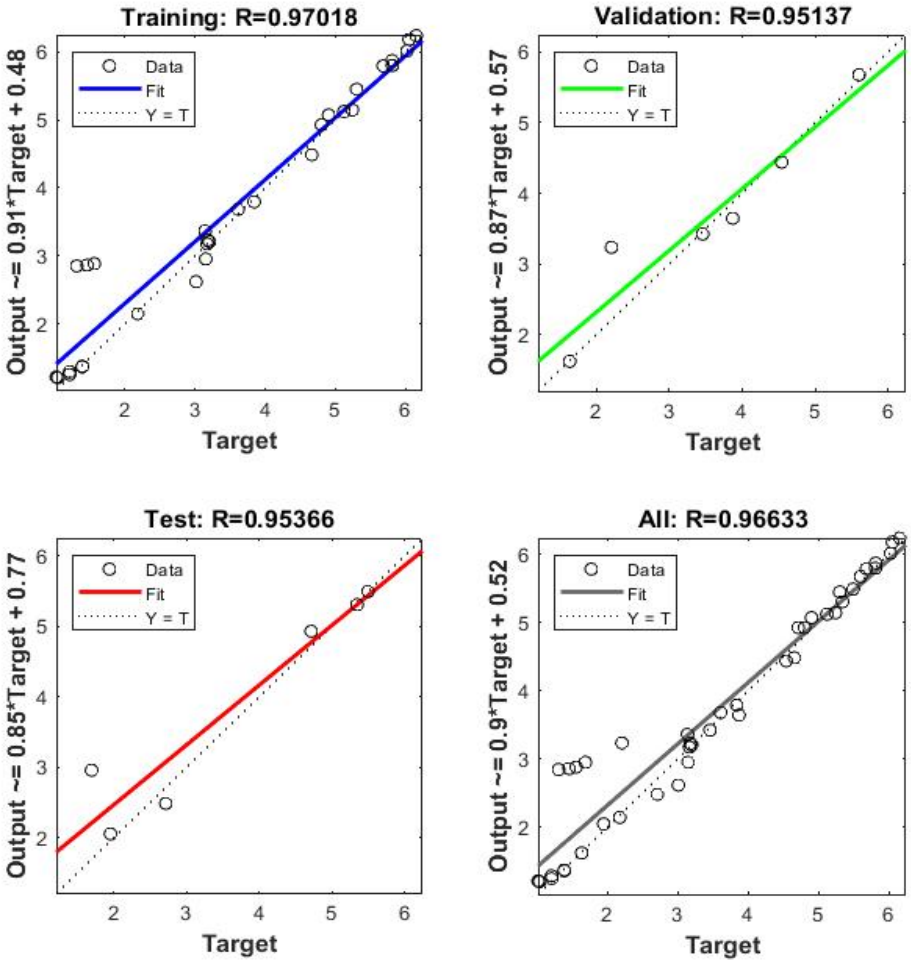


Figure 13. The regression graph of ANN Model prediction of the inhibition of *E.coli* using probiotic dairy product kefir in $\log_{10}$ CFU/g

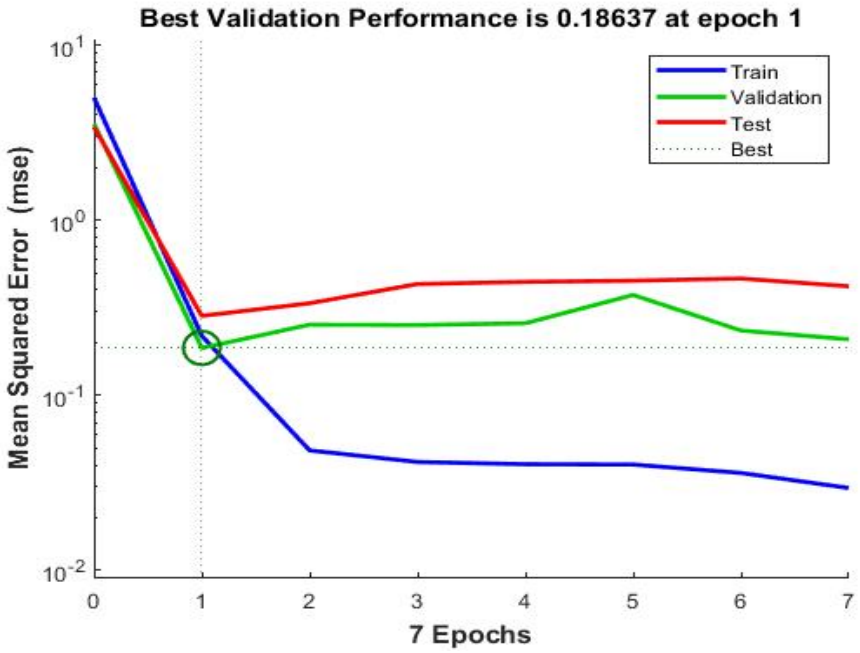


Figure 14. Plot performance graph of ANN Model prediction of the inhibition of *E. coli* using probiotic dairy product kefir in $\log_{10}$ CFU/g

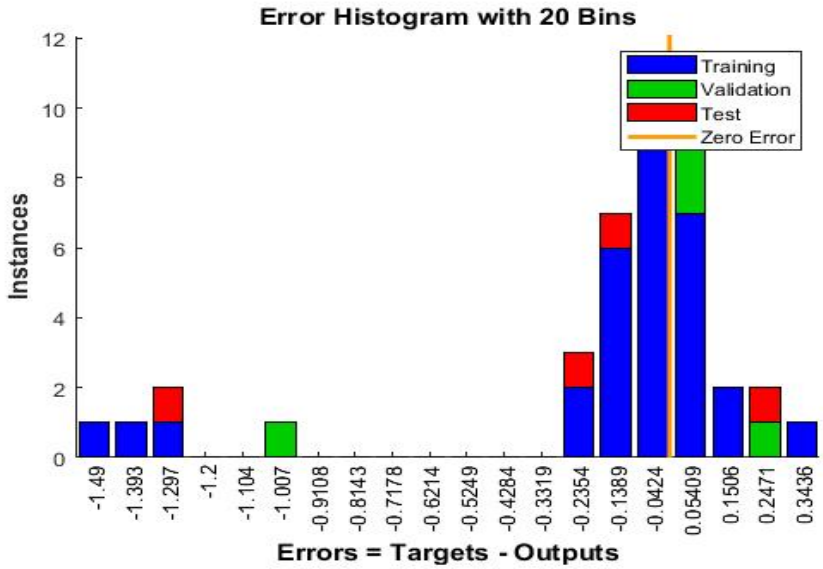


Figure 15. Histogram graph of ANN Model prediction of the inhibition of *E.coli* using probiotic dairy product kefir in $\log_{10}$ CFU/g

Table 8. ANFIS Model inhibition of *E.coli* using probiotic dairy kefir in $\log_{10}$ CFU/g

Training stages			
Days	LAB control	Tested LAB	*E.coli*
FD0	3.22	3.1800	3.0100
FD1	4.1	3.8091	3.3488
FD2	4.96	4.6054	2.5160
SD1	5.1	4.7106	1.9043
SD3	5.2	4.9028	1.4241
SD7	5.29	5.0724	1.4142
SD10	5.4	5.2434	1.4378
FD0	4.2	3.9059	3.2552
FD1	5.1	4.7187	2.7691
FD2	5.86	5.3117	2.2918
SD1	5.98	5.6904	1.2978
SD3	6.11	5.8206	1.3768
SD7	6.34	6.0181	1.2115
SD10	6.43	6.0909	1.2226
Average	5.2350	4.9343	2.0343
Testing stages			
FD0	4.18	3.9910	2.1385
FD1	4.97	4.6777	1.4653
FD2	5.78	5.5236	1.3392
SD1	5.88	5.5680	1.2989
SD3	6.1	5.6617	1.1773
SD7	6.23	5.6334	1.0062
SD10	6.41	5.7466	1.0029
Average	5.6500	5.2574	1.3469

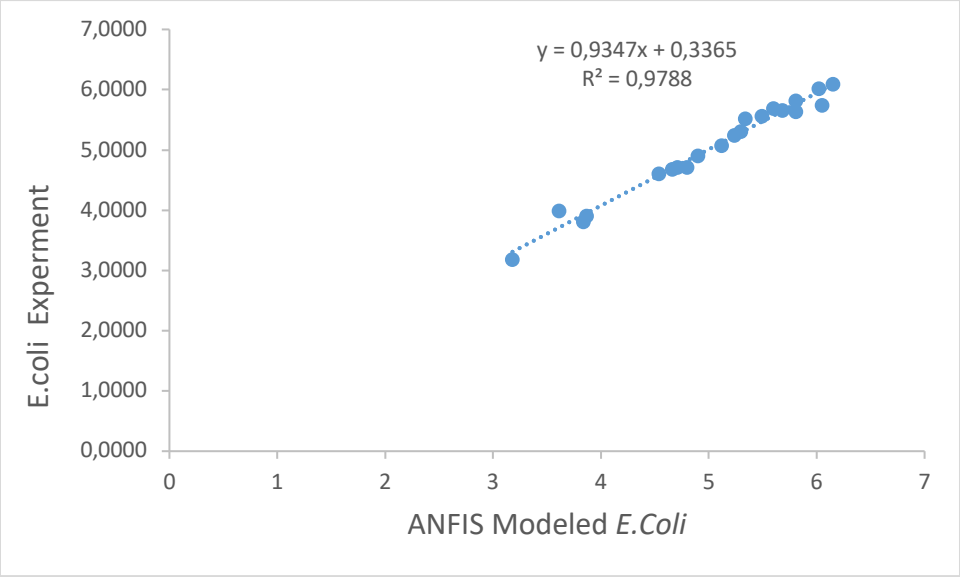


Figure 16. Scatter plots of the inhibition of *E.coli* using probiotic dairy product kefir using ANFIS model

CHAPTER V

5. DISCUSSION

The risk of foodborne pathogens in both health and economical aspects is worldwide, and considered to have negative impact on food safety management systems. Despite, different agricultural and industrial sectors have developed systems, including food safety management systems, which are the fundamental and best practices that ensure the production environments and food products are safe. These systems such as HACCP, GMP and traceability are more implemented in the agro-industrial sectors to control the hazardous conditions of the pathogens that could emerge at any stage production (Allata et al., 2017; Dzwolak, 2019; Liu, et al., 2021). On the other hand, biological means controlling the activity of foodborne pathogens have become attracted the attention of different experts and researchers so far. The present study was conducted to make investigation on the activity of targeted foodborne pathogens, namely *L.monocytogens*, *S. aureus* and *Coliforms/ E.coli* by using biological controlling approach, which include probiotic LAB naturally present in the dairy kefir milk.

According to the present study, the inhibition of these targeted pathogens was evaluated by using ANN and ANFIS models during fermentation and storage days of dairy kefir. The activity of all selected foodborne pathogens were found to be inhibited by probiotic LAB present in dairy kefir particularly during storage time of the kefir milk. Different researches findings have suggested that potential probiotic LAB isolated from various dairy products possess a considerable antagonizing effects against foodborne pathogens including those targeted in the present study. In light of this, the investigation of Kamal et al., (2018) who tested the inhibitory activity of *Lactobacillus rhamnosus* on different foodborne pathogens including *Escherichia coli O157:H7*, *Staphylococcus aureus*, *Yersinia enterocolitica* and Salmonella enterica serovar Typhimurium can be taken as supportive findings with the present study. Furthermore, the antimicrobial activity of probiotic bacterial strains against foodborne pathogenic bacteria may also be a characteristic parameter for probiotics to be included in the composition of probiotic preparations and probiotic foods.

Moreover, in the previous research findings, similar suggestions were forwarded regarding the antagonizing effect of probiotic LAB isolated from different food products. In this regard, Wang et al. (2018) had reported that strains of probiotic LAB, identified as *Lactobacillus*

plantarum PIC33 and *Lactobacillus plantarum* SK5 possess antagonistic effect toward the growth of *S. aureus*, *E. coli* and other foodborne pathogens including *Salmonella enterica*, *Shigella dysenteriae*. Similarly, the demonstration of Mulaw et al., (2019) regarding the inhibitory activity of probiotic strains of LAB against *S. aureus* ATCC 25923, *L. monocytogenes*, and *E. coli* ATCC 25922 confirms that the probiotics are promising agents to possess antibacterial role. Industrially such kinds of probiotic bacteria are more needed in order to incorporate into foods, and are applicable in the development of functional food. Other in vitro investigations have also reported that acid tolerant LAB strains exhibited antagonizing effect on the growth of some foodborne pathogens (Klimko et al., 2020). In addition to their inhibitory activity, probiotics can be supplemented to foods to contribute to their organoleptic characteristics as well as extension of their shelf life during fermentation processes. The probiotics can implement this function by maintaining their viability and efficacy.

It is obvious that various dairy products such as cheese products are involved in the outbreaks of listeriosis due to high consumption and prolonged refrigerated storage. However, according to a study of Lim et al., (2020) two strains probiotic bacteria isolated from Kimchi, namely *Leuconostoc mesenteroides* and *Lactobacillus curvatus* were responsible to antagonize the growth of *L. monocytogenes* in soft cheese. In the present study, the growth and activity of *L. monocytogenes* were tested by LAB present in dairy kefir during fermentation days (FD0-FD2) and storage days (SD1-SD10) by using the analysis of both ANN and ANFIS models. Thus, the count of the pathogen was found to be reduced from SD1 to SD10 at training stage and FD2 to SD10 at testing stage in ANN model; while the number of the same pathogen became reduced along the days FD2 to SD10 at training stage and FD1 to SD10 at testing stage in ANFIS model. Contrary, the number of LAB was increased from day zero of the fermentation to day 10 of storage in the refrigerator in the experimentally contaminated kefir with the pathogen (Table 3 and 4).

The average count obtained in LAB control, tested LAB and *L. monocytogenes* was 5.23, 4.94 and 2.41$\log_{10}$CFU/g at training stage; whereas at the testing stage it was recorded 5.80, 5.38 and 2.04$\log_{10}$CFU/g respectively (Table 3). However, in previous study conducted by Gökmen et al., (2022), the counts of strains of LAB, namely lactobacilli and lactic streptococci, were obtained 9.64–7.91 and 9.64–8.69 log CFU/mL in ranges respectively. This result indicates there is variation in the number of the probiotic LAB when compare to the result obtained in the

present study. The obtained result in the present study shows close agreement with the report of Jara et al., (2020) who identified the potential probiotic *Lactobacillus fermentum* MP26 and *Lactobacillus salivarius* MP14 against the growth of *Listeria monocytogenes.* Additionally, in the investigation of Morandi et al., (2020), the inhibitory activity of LAB against *L. monocytogenes* in Gorgonzola cheese indicated that the counts of the pathogen was found to be < 2.0 $\log_{10}$ CFU/g; and this result is more close to the result obtained during storage days at training and testing stage of the present study.

In previous study, the inhibitory effect of *Lactobacillus rhamnosus* in probiotic Minas Frescal cheeses against *L. monocytogenes* was reported 1.1–1.6 Log CFU/g (Prezzi et al., 2020); however, this result is similar with the result obtained at SD1 (1.6$\log_{10}$CFU/g) and SD3 (1.6$\log_{10}$CFU/g) in ANFIS model, and at SD10 (1.18$\log_{10}$CFU/g) in ANN model in the present study. The inhibitory effect of probiotic LAB was, furthermore, confirmed on biofilm forming pathogenic *L. monocytogenes* present on vegetables and in the food industry without getting risk to consumers (Hossain et al., 2020). Moreover, Martín et al., (2022) suggested that the strain of *Lactiplantibacillus plantarum* B2 alone or combined with the strain *Lactiplantibacillus* spp. B4 are good candidates against *L. monocytogenes* growth in traditional soft cheeses based dairy milk during their storage at refrigeration temperature. The survival of some foodborne pathogens, including both gram positive and gram negative bacteria, in kefir produced by microbial leval and pullulan was determined by the investigation of Gokmen et al., (2022), of which *L. monocytogenes* was found to be the most susceptible bacterium to the metabolites of LAB in kefir during storage, obtaining the highest reduction in the pathogen after 24h fermentation at 30^0c.

The other targeted foodborne pathogen involved in the present study was *S. aureus.* The microbiological profile of the pathogen was evaluated and analyzed by both ANN and ANFIS models at the specified time interval as displayed in Table 5 and 6. The analysis was also carried out for the evaluation of LAB control and the test LAB at both fermentation days and storage days. Based on the analysis done by ANN model, the average number of *S. aureus* was found to be 2.04 $\log_{10}$ CFU/g, while the number of LAB control and the test LAB was 5.23 $\log_{10}$ CFU/g and 4.89 $\log_{10}$ CFU/g at testing stages respectively; whereas in ANFIS model the average number of *S. aureus* was recorded as 2.04$\log_{10}$CFU/g, while the number of LAB control and the test LAB was 5.23 $\log_{10}$CFU/g and 4.82 $\log_{10}$CFU/g.

Recently published articles have indicated that the inhibitory effect of probiotic LAB against the pathogenic *S. aureus* has been confirmed in various food products such as cheese, yoghurt, kefir and milk. In the study of Jiang et al., (2022), it was indicated that strains of LAB isolated from traditional fermented yoghurt was found to have antibacterial and antibiofilm activity against *S. aureus* pathogen, and could have potential for improving safety of dairy products. Thus, the result obtained in this study shows that there is a close agreement in idea with the findings of the present study. On the other hand, another study which was conducted on the efficacy of probiotic LAB, namely *Lactobacillus rhamnosus* isolated from Minas Frescal cheeses, during storage time of 21 days at 7^{o}c, indicated that the probiotic had no inhibitory activity against the pathogenic *S. aureus* (Prezzi et al., 2020), which contradicted with the idea of the present and previous study.

In the present study, the growth and activity of *S. aureus* was evaluated by testing in LAB in dairy kefir during fermentation days (FD0-FD2) and storage days (SD1-SD10) using the analysis of both ANN and ANFIS models. As a result, the count of the pathogen was found to be decreased from FD2 to SD10 at training stage and FD1 to SD10 at testing stage in ANN model (Table 5); while the number of the same pathogen become reduced along the days FD2 to SD10 at both training and testing stages in ANFIS model (Table 6). Contrary, the number of the test LAB and LAB control was increased from FD0 to SD10 during the storage of experimental kefir in the refrigerator. The inhibition of this pathogen may also include the involvement of the different metabolites of LAB, which are the most important compounds that inhibit the growth of undesirable microorganisms, particularly *S. aureus* in food and pharmaceutical industries (Nataraj et al., 2021). Thus, the inhibition of this pathogen enhances food safety and hygiene to ensure the health of the consumers. That is why the present study has given attention to the probiotic LAB strains to curb the effect of those selected pathogens in the dairy kefir products.

Different research studies have revealed that strains of probiotic LAB isolated from dairy food products possess antagonizing activity against the pathogenic *S. aureus*, particularly in food production environments (Jiang et al ., 2021; Nataraj et al., 2021; Folliero et al., 2022; Tarique et al., 2022). For example, the efficacy of probiotic *L. brevis* gp104 which was isolated from Iranian traditional cheese had a promising potential against the growth of *S. aureus* and its potential health benefits for its application as novel bio-therapeutic and bio-preserving agents (Hojjati et al., 2020). Similarly, the antagonizing activity of some probiotic LAB isolated from

traditional high acid and low moisture yogurt-like products, including *Streptococcus thermophilus*, *Lactobacillus delbrueckii*, *Enterococcus faecium*, and *Lacticaseibacillus rhamnosus* was also reported against the pathogenic *S. aureus* in the previous study (Tarique et al., 2022).

Moreover, in the present study the inhibition of *S. auerus* was also confirmed the by the regression analysis at training, validation and testing stage with R=0.9842, 0.9905, 0.8873 respectively as indicated in figure 6. Additionally, the model had reflected the evaluation of the pathogen with the best validation performance of 0.071812 at epoch 21(Figure 7). The evaluation of the inhibitory activity of potential probiotic LAB of dairy kefir against S. *aureus* pathogenic bacteria was also analyzed by ANFIS model as it was described in Table 6. The obtained result showed that the reduction in the count of the targeted pathogen was seen from FD2 to SD10 at both training and testing stages; whereas the number of LAB was found to be increased in these above stated days. In this analysis, the reduction of the count of the pathogen was more observable during storage days than during fermentation days of the experimental kefir. The previous study confirmed that several strains of LAB have exhibited antagonizing potential against foodborne pathogens, among these strains some of which including *Pediococcus acidilactic* and *Lactococcus plantarum* are well described as promising probiotics against *S. aureus* CMCC 26003 (Yan et al., 2019). Thus, the finding of this investigation could have close agreement with the suggestions of the present study.

The inhibition of *E.coli* by probiotic LAB present in dairy kefir was analyzed using ANN model, obtaining the reduction of the number of the pathogen from FD2 to SD10; whereas an increment in the number of LAB was observed from FD0 to SD10 (Table 7) at training stages. Likewise, the decrease in the number of the pathogen was observed along the days from FD0 to SD10 at testing stages. However, it was revealed that the average result obtained for LAB control, tested LAB and *E.coli* was 5.23, 4.96 and 2.46$\log_{10}$CFU/g respectively in the training stages; where as in the testing phase it was found to be 5.80, 5.45 and 1.92$\log_{10}$CFU/g respectively as displayed in Table 7. In this regard, several research findings have been similarly reported in close agreement with the findings of present study regarding the inhibition of *E.coli* in the experimentally contaminated dairy kefir during both fermentation days and storage days. In this perspective, the investigation of de Amorim Trindade et al., (2022) and Darvishi et al., (2021) indicated that different strains of LAB have exhibited probiotic potential against the

growth of *E.coli* and other foodborne pathogens; and thus the findings of these authors have close agreement with the result of the present study.

Additionally, the inhibition of the targeted pathogen was also braced by the regression analysis with R=0.9702, 0.9514 and 0.9537 at training, validation and testing stages respectively (Figure 13). The best validation performance for the inhibition of the pathogen was obtained at 0.18637 as shown in Figure 14. Similarly, the inhibition of the pathogenic *E.coli* by biological means, namely probiotic LAB which are naturally present in dairy kefir milk and used in the present study, was analyzed using ANFIS model. The model simulated with a complete typical modeling of the inhibition of *E.coli* at training stages with average number of LAB in control ($5.23\log_{10}$CFU/g), tested LAB ($4.93\log_{10}$CFU/g) and *E.coli* ($2.03\log_{10}$CFU/g) as indicated in Table 8. Likewise, the average value of LAB control tested LAB and *E.coli* obtained at testing stages were 5.65, 5.25 and $1.34\log_{10}$CFU/g respectively. In this model, the inhibition of the activity of pathogen was seen along with the fermentation and storage days of kefir milk, which was confirmed by the reduction in the number of the pathogen from FD2 to SD10.

The pathogenic *E.coli* is among the well-known and most serious foodborne bacteria, causing severe health problem to the public through adherence to the mucosal membrane of the host's intestine. To combat with the activity of this pathogen, the application of probiotic food products such as dairy products are more reliable as it was demonstrated in the previous studies. As evidence, the strains of some probiotic LAB, reported in the previous studies, including *Lactobacillus sakei, Lactobacillus plamtarum, Lactobacillus reuteri, Lactobacillus fermentum,* and *Lactobacillus casei* have the antagonizing activity toward the pathogenic *E. coli* (Darvishi et al., 2021; Hansen et al., 2021; de Amorim Trindade et al., 2022;). These strains of LAB possess desirable potential for passing through the low pH of stomach and entering intestine to inhibit the adherence activity of infectious *E.coli* (Behbahani et al., 2019). The inhibition of the pathogenic *E. coli* in the present study was more observable during storage days of the dairy kefir stored at 4^0c. This finding is in close agreement with the investigation of Choi et al., (2021) who confirmed the antibacterial influence of probiotic *Leuconostoc mesenteroides* (KCTC 13374) and *Lactobacillus plantarum* (KCTC 33133) isolated from commercially manufactured Kimichi during fermentation at 10^0c and 25^0c.

CHAPTER VI

6. CONCLUSSIONS AND RECOMMENDATIONS

The present study was conducted on the inhibition of pathogenic foodborne pathoens: *L. monocytogenes*, *S. aureus* and *E.coli* by probiotic LAB naturally present in the dairy kefir using artificial intelligence, ANN and ANFIS models. The activity and growth of these foodborne pathogens become repressed by LAB present in dairy kefir. Thus, probiotic dairy kefir products are therefore the biological controlling means and can be applied in the food industry and other agricultural sectors. Based on the present study, the antibacterial activity of probiotic LAB in the kefir was more observable at storage days than during fermentation days. Therefore, based on the conclusions, some points are forwarded as recommendations; firstly, more research work should be emphasized on the investigation of kefir as a potential probiotic antagonizing the activity of the most serious foodborne pathogens. Secondly, Artificial Intelligence based approaches inhibition of these targeted pathogens may be a base line that seek more attention around the research academy

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