Assessment of Potential Probiotic LAB strains to control non-typhoidal *Salmonella enterica* from poultry

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A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science in

Industrial Biotechnology

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2019.

IN THE NAME OF ALLAH, THE BENEFICENT

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DEDICATION

All My Effort is dedicated to the

"MY BELOVED FAMILY"

Especially my Ammi, Abbu

And Phuphoo

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All praises be to ALLAH Almighty the most Merciful, the most Gracious and the all-knowing. Due to His mercy and love, all worthy and great things are possible. Peace and blessings of Allah be upon the last and final Messenger **Muhammad (S.A.W)** who has always been the source of guidance and knowledge to the humanity forever. And blessed me the courage to get higher education and to complete this manuscript.

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SARA AZIM

CONTENTS

Chapter 1 1
INTRODUCTION 2
Chapter 2 5
LITERATURE REVIEW 5
2.1 NON TYPHOIDAL *Salmonella enterica* 6
2.1.1 *Salmonella enterica* as pathogen 7
2.2 Anatomy and Physiology of Poultry Gut 8
2.3 Recent Treatment of Non Typhoidal *S. enterica* 10
2.3.1 Antibiotics 10
2.3.2 Phage Therapy 12
2.4 Alteration of Gut Microbiota 13
2.4.1 Probiotics 13
2.4.1.1 Definition of probiotics 13
2.4.2 Lactic Acid Bacteria 14
2.5 CHARACTERISTICS OF PROBIOTIC BACTERIA 15
2.5.1 Resistance to Gastrointestinal Stress 15
2.5.2 *In vitro* safety of LAB strains 16
2.6 Possible Mechanisms of Lactic Acid Bacteria to Control Non Typhoidal *Salmonella enterica* 16
2.6.1 Competitive Exclusion of Pathogenic Microorganism 16
2.6.2 Antagonistic Effect on Pathogenic Microorganism 17
2.6.3 Non Immunological Effect 17
2.6.4 Stimulation of Immune Response 17
2.7 Utilization of Lactic Acid Bacteria in Poultry Industry as Probiotics 18
2.7.1 Effect on Growth Stimulation, Feed Consumption and Metabolism 19
2.7.2 Effect of probiotics on intestinal morphology 20
2.7.3 Effect of probiotics on meat quality 20
Chapter 3 21
MATERIAL AND METHODS 21
3.1 SAMPLE COLLECTION 21
3.2 ISOLATION OF BACTERIA 21
3.3 SELECTION OF *LACTOBACILLUS* AND *ENTEROCOCCUS* SPECIES 21
3.4 PHENOTYPIC IDENTIFICATION 22

3.4.1 Colony Morphology 22
3.4.2 Gram Staining 22
3.4.3 Catalase Test 22
3.5 GENOTYPIC IDENTIFICATION 23
3.5.1 Isolation of Genomic DNA 23
3.5.2 Universal primers 23
3.5.3 Quantification of DNA 24
3.5.4 Amplification of 16S rDNA Using PCR 24
3.5.4.1 PCR Conditions 24
3.5.5Confirmation of DNA Extraction 25
3.5.6 PCR Product Purification 25
3.6 ASSESSMENT OF PROBIOTIC POTENTIAL 26
3.6.1 Acid Tolerance 26
3.6.2 Bile Tolerance 26
3.6.3 Lysozyme tolerance 27
3.6.4 Phenol tolerance 27
3.7 SAFETY ASSESSMENT OF LAB STRAINS 27
3.7.1 Antibiotic Resistance Profiling 27
3.7.2 Hemolytic Test 28
3.7.3 DNASE Test 28
3.8 *IN VITRO* ASSESSMENT OF ANTI- *SALMONELLA* ACTIVITY 29
3.8.1 Co- Aggregation Assay 29
3.8.2 Mucin Adhesion Assay 29
3.8.3 Time Kill Assay 30
3.8.4 Cell Surface Hydrophobicity Assay 31
CHAPTER 4 32
RESULTS 32
4.1 Sample collection and Isolation of *Lactobacillus* 32
4.1.2 Selection of *Lactobacillus* 34
4.2 Phenotypic Identification 34
4.2.1 Colony Morphology 34
4.2.2 Gram Staining 34
4.2.3 Catalase test 35
4.3 Assessment of Probiotic Potential 36
4.3.1 GIT related stress tolerance 36

4.3.1.1 Acid tolerance 36
4.3.1.2 Bile tolerance 38
4.3.1.3 Phenol Tolerance 38
4.3.1.4 Lysozyme tolerance 39
4.3 Safety Assessment of Selected Strains 40
4.3.1 Antibiotics Susceptibility assay 40
4.3.2 DNase activity 41
4.3.3 Hemolytic activity 42
4.4 *In vitro* assessment of potential LAB strains for non typhoidal *Salmonella enterica* control 42
4.4.1 Co-aggregation Assay 42
4.4.1.1 Co-aggregation assay of LAB strains with *S. typhimurium* 42
4.4.1.2 Co-aggregation assay of LAB strains with *S. enteritidis* 44
4.4.2 Cell Surface Hydrophobicity Assay 47
4.4.3 Time Kill Assay 48
4.4.4 Mucin adhesion Assay 50
4.5 GENOTYPIC IDENTIFICATION OF LAB STRAINS 52
4.5.1 Amplification of 16S rDNA through PCR 52
Chapter 5 54
DISCUSSION 54
CHAPTER 6 59
CONCLUSION AND FUTURE PROSPECTS 59

LIST OF FIGURES

Figure 1: Route of Salmonella enterica in poultry _____ 7

Figure 2: Anatomy and physiology of poultry gut _____ 8

Figure 3: Possible Proposed Mechanism of LAB Strains_____ 18

Figure 4: Modes of action and beneficial activities of probiotics in poultry _____ 19

Figure 5: PCR Cycles for 16S rDNA amplification _____ 25

Figure 6: Colony morphology of LAB strains _____ 32

Figure 7: Image showing the halo zone formation around bacterial colonies on MRS agar plates _____ 34

Figure 8: Different gram positive, rod and coccus shape morphologies of isolates reveled observed under compound microscope at 100X resolution. _____ 35

Figure 9: Catalase activity of LAB isolates and positive control _____ 35

Figure 10: Comparative analysis of acid tolerance of LAB strains at 0hr to 4hr _____ 37

Figure 11: Comparative analysis of bile tolerance of isolates time interval 0h to 4h_____ 38

Figure 12: Comparative analysis of phenol tolerance of LAB strains at 0hr to 24hr _____ 39

Figure 13: Comparative analysis of lysozyme tolerance of LAB strains at 0min to 90 min ___ 40

Figure 14: a) and b) probiotic culture shows no DNAse activity whereas c) *Salmonella enterica* shows zone around the colony on DNAse agar _____ 42

Figure 15: a) and b) probiotic culture on blood agar shows no formation of hemolysis zone c) Staphylococcus aureus forms a zone of hemolysis on blood agar _____ 42

Figure 16: Co-aggregation Analysis of 17 LAB isolates with *S. typhimurium* PA17 _____ 43

Figure 17: Co-aggregation Analysis of 17 LAB isolates with *S. typhimurium* PA40 _____ 44

Figure 18: Co-aggregation Analysis of 17 LAB isolates with *S. typhimurium* PA48 _____ 44

Figure 19: Co-aggregation Analysis of 17 LAB isolates against *S. enteritidis* PA51 _____ 45

Figure 20: Co-aggregation Analysis of 17 LAB isolates against *S. enteritidis* PA71 _____ 46

Figure 21: Co-aggregation Analysis of 17 LAB isolates against *S. enteritidis* PA76 _____ 46

Figure 22: Comparative Co-aggregation of Selected LAB isolates with selected *S. enterica* strains _____ 47

Figure 23: Cell surface hydrophobicity assay of selected LAB strains using three organic solvents _____ 48

Figure 24: Comparative time kill assay analysis of selected LAB strains against *S. enteritidis* PF76 _____ 49

Figure 25: Comparative time kill assay analysis of LAB strains against *S. typhimurium* PF40 50

Figure 26: Competitive inhibition analysis of selected LAB strains against selected *S. enterica* strains on porcine mucin Ⅲ _____ 51

Figure 27: a) positive control S. enteritidis PA76 b) competitive adhesion of combined LAB strains treated with *S. enteritidis* PA76 c) competitive adhesion of PFS 13 against *S. enteritidis* PA76 d) competitive adhesion of PFS 9 against *S. enteritidis* PA76 _____ 51

Figure 28: The confirmation of 16S rDNA amplification as reveled by the presence of 1200bp fragment on 1% agarose gel._____ 52

LIST OF TABLES

Table 1: Traditional pathogens versus emerging pathogens in poultry meats 10

Table 2: Major Alternatives to Antibiotics and Their Advantages and Disadvantages 12

Table 3: Bacteriophages That Are Approved for Clinical Used Against Salmonellosis 13

Table 4: Universal primers used to amplify the DNA 24

Table 5: Isolation of LAB strains from poultry feces and stock preparation 33

Table 6: The phenotypic characteristics of isolated bacterial strains from poultry 36

Table 7: Antibiotic susceptibility profile of isolated LAB strains from poultry 41

Table 8: The results of nucleotide similarity for 16S rDNA sequences for specie level identification 53

LIST OF ABBREVIATIONS

M	Molar
N	Normality
µL	Microliter
mL	Millilitre
µg	Microgram
bp	base pair
H_2O_2	Hydrogen peroxide
TAE	Tris-acetate EDTA
HCl	hydrochloric acid
WHO	World Health Organization

ABSTRACT

Globally multidrug resistant *Salmonella enterica* is alarming for public health and is major economic concern for animal farming. It is mainly associated with gastroenteritis infection both in humans and animals specially poultry. The irrational use of an antibiotics as a growth promotor in a poultry feed develops potential antimicrobial resistance in non-typhoidal *Salmonella enterica.* This demands an efficient alternative strategy among which probiotics has been explored as safe substitute. The aim of the study was to isolate potential probiotic LAB strains from poultry gut. Total 17 strains were isolated, all strains were tested for their survival rate including pH 2, 0.3% bile, 10 µg/mL lysozyme and 0.4% phenol concentration that are associated with GIT. All isolates were found resistant to gastric stress conditions. Safety assessment of LAB strains was confirmed by absence of DNase, non- hemolytic assay and antibiotic susceptibility assay. Four selected LAB strains showed remarkable inhibition of non typhoidal *Salmonella enterica* including *S. enteritidis* and *S. typhimurium* after 18hours of co-incubation. *L. reuteri* PFS4, *L. reuteri* PFS9, *Enterococcus faecium* PFS 13 and *Enterococcus faecium* PFS14 exhibited high co-aggregation, time kill assay, mucin competitive adhesion assay and cell surface hydrophobicity individually as well as in combination. In our study selected indigenous strains shows effective results against *S. enteritidis* and *S. typhimurium* isolated from poultry sources. These four strains can be further used as feed additives for poultry to control non typhoidal *Salmonella enterica* and confirm by *in vivo* poultry trial.

Chapter 1

INTRODUCTION

The most dynamic and vibrant section of livestock sector of Pakistan is poultry. Around the globe non typhoidal *Salmonellae are* major public health concerns. Avian Salmonellosis has been identified as the substantial and most significant disease in the poultry especially broiler bird (Hussain *et al.,* 2015). In Pakistan, second largest industry is poultry industry during 2017-2018 survey, poultry approximately contributes 1.4% in the GDP, 7.5% in agriculture whereas 12.7% in livestock of Pakistan (Ministry of finance, 2018). Poultry considered as one of the major risk factor of non typhoidal *Salmonella* as it easily resides in the intestinal tract and excreted out through feces and contaminate the environment (Antunes *et al.,* 2016). Prevalence of avian Salmonellosis ranges from 16.10 % to 53.25% which causes heavy economy loss to poultry world by increasing mortality rate and dropping poultry production rate. *Salmonella enteritidis* and *Salmonella typhimurium* are one of the most common *Salmonella* infections and due to their zoonotic potential they are capable to easily communicate and transmit the infection to humans. Mismanagement and mishandling of poultry products at the farm level transmit the infection at fork level as commercial meat product is the one of the finest and more consumable product Mortality rate in young birds is most often higher and restricted to the first few weeks of age, whereas mortality rate is quite low in adult birds. Spreading of *Salmonella* infection generally happens horizontally from infected birds, contaminated environments or infected rodents (Cohen *et al.,* 2019). Primary *S. enterica serotype enteritidis* can infect the interior of the egg through trans-ovarial or trans-shell transmission. Hallmarks of the infection in young birds are depression, low feed consumption rate, increased drowsiness, white diarrhea and retard growth. Moreover lesions occurrence in adult birds are infected organs i.e. infected liver or intestine,

ruffled feathers, pale shrunken combs and low production rate of eggs. In severe cases, death may occur at 7-10 days after infection (Murakami *et al.,* 2017).

Salmonella is the major contributor towards illness to farm animals as antibiotic resistance mainly due to intrinsic characteristics, excessive use of antimicrobial, irrational usage of antibiotics, mutation or due to the presence of transferable genes are significant and vital factors responsible for occurrence and spreading of pathogen which ultimately leads to development of multi drug resistance in the strains (Shakoor *et al.,* 2019). Antimicrobial resistance is major concern for humans and food producing animals, as they are continuously spreading throughout the countries including Pakistan due to the over dosage of antibiotics, misuse of antimicrobials. These all are contributing in proliferating the infection rate and dwindling the treatment rate (National AMR Action Plan for Pakistan, 2017). Antibiotics generally administrated to entire flocks of animals in order to treat the disease, prevent the animals from disease or as growth promotors, by enhancing meat production through elevation of feed conversion ratio (Roth *et al.,* 2018). In Pakistan, now a days it is discovered that numerous types of antibiotics are using as growth promoters i.e**.** β-lactam antibiotics (comprising mainly penicillin), lincosamides, and macrolides including erythromycin and tetracycline, oligosaccharide, avilamycin, virginiamycin etc (Rahman and Mohsin, 2019). These antibiotics also modifies the composition of avian microbiome.

Consumer pressure, burden of antibiotics and their harmful effect on avian health, alternative therapies are under consideration to maintain low mortality and morbidity rate, to increase feed, growth efficiency there are certainly a number of non-therapeutics alternatives that can substitute antibiotics use. Among these probiotics are most popular (Diarra and Malouin, 2014). Probiotics are nonpathogenic microorganisms that confers positive effect in animals. Till now, they don't transfer antibiotic resistance genes to another organisms and maintain genetic stability. These

microbes mostly reside in crop, gizzard and ileum region of poultry, having ability to tolerate gastric acid, alkalinity of intestine and lysozyme enzyme. Probiotics have strong adhesion power to confer intestinal wall and face off with gastroenteritis pathogens (Mousav *et al.,* 2018) various probiotics like *Lactobacillus reuteri, Enterococcus lactis* and *Enterococcus faecium* were demonstrated for inhibiting pathogenicity of the pathogens in the poultry. Mechanisms of probiotics executes in the poultry are as follows: competitive adhesion to intestinal site, competition for survival, maintaining gut microflora by competitive exclusion, mucin adhesion and production of metabolites (Yadav *et al.,* 2016). Moreover, the goal behind the usage of probiotics is to compete and inhibit the growth of pathogen i.e. *Salmonella enterica* serotype *Salmonella enteritidis* and *Salmonella typhimurium* and compete with adherent sites present in intestinal mucosa.

The research objectives of the study are as follows:

RESEARCH OBJECTIVES

1) Isolation and identification of potential LAB strains isolated from poultry.
2) *In vitro* assessment of potential LAB strains to control non typhoidal *Salmonella enterica* in poultry.

Chapter 2

LITERATURE REVIEW

Poultry industry is leading industry in Pakistan after textile industry and contributes a major portion i.e. $3.25 dollars in the GDP of Pakistan (GOP, 2015). Some serious and severe diseases affecting the poultry birds that includes, Salmonellosis, Collibacillosis and Newcastle Disease virus causes heavy economic loses in poultry and possesses a serious threat to the public health (Mustafa and Ali, 2005). *Salmonella enterica* is substantial and significant food borne pathogen causing food safety issue for public health worldwide. In poultry industry *Salmonella* is foremost leading foodborne pathogen especially in the poultry products (meat and egg) (Hessel *et al.*, 2019). *Salmonella* can be easily spread from chicken intestinal contents and during slaughtering process. (Rouger *et al*., 2017). Now a day's chicken meat is considered as one of the finest healthy food products, enriched in nitrogenous compound i.e. proteineous element (Bhaisare *et al.*, 2014). Poultry skin and muscles provides remarkable support for bacterial growth therefore helps in the accommodation of *Salmonella* on the meat or in their gastrointestinal tract and excrete the pathogens through feces in the environment, making environment contaminated. Body fats have the tendency to shield *Salmonella* from harsh environment and physiological stresses in poultry meat. So lessening of fat deposition in body organs is essential in order to reduce existence and survival of *Salmonella sp.* (Morita *et al.,* 2006). In animals and food production units *Salmonella* is recognized as endemic (Sanchez *et al.,* 2002). There is strong association between contaminated chicken products and colonization of *Salmonella* in live poultry birds. Therefore, in order to diminish or reduce the occurrence or re-occurrence of *Salmonella*, in poultry farm there is a need

to keep an eye on poultry products environment and final products to decrease the *Salmonella* contamination (Sanchez *et al.,* 2002).

Avian and mammalian intestinal tract is the main reservoir of non typhoidal *Salmonella enterica* which ultimately leads to food stuff contamination (Barrow *et al.,* 2012). It is estimated that around 93.8 million illness and 155000 mortality rate came across the world, associated with non typhoidal *Salmonella* (Majowicz *et al.,* 2010).

The zoonotic potential of *Salmonella* causes serious health issues like gastroenteritis known as Salmonellosis that's lasts for 4 to 7 days mainly caused by non-typhoidal *Salmonella* spp. but mortality rate is very rare (Crump *et al.,* 2015).

2.1 NON TYPHOIDAL *Salmonella enterica*

Non typhoidal *Salmonella* frequently adhered and colonized in the poultry gut through horizontal or vertical transmission without having detectable signs and symptoms. At primary production level of the poultry population, it simply integrated and became part of poultry system and transmit the infection through contaminated table eggs or contaminated water into humans and causes gastroenteritis diseases (Barrow *et al.,* 2012).

Route of *Salmonella enterica* in poultry is:

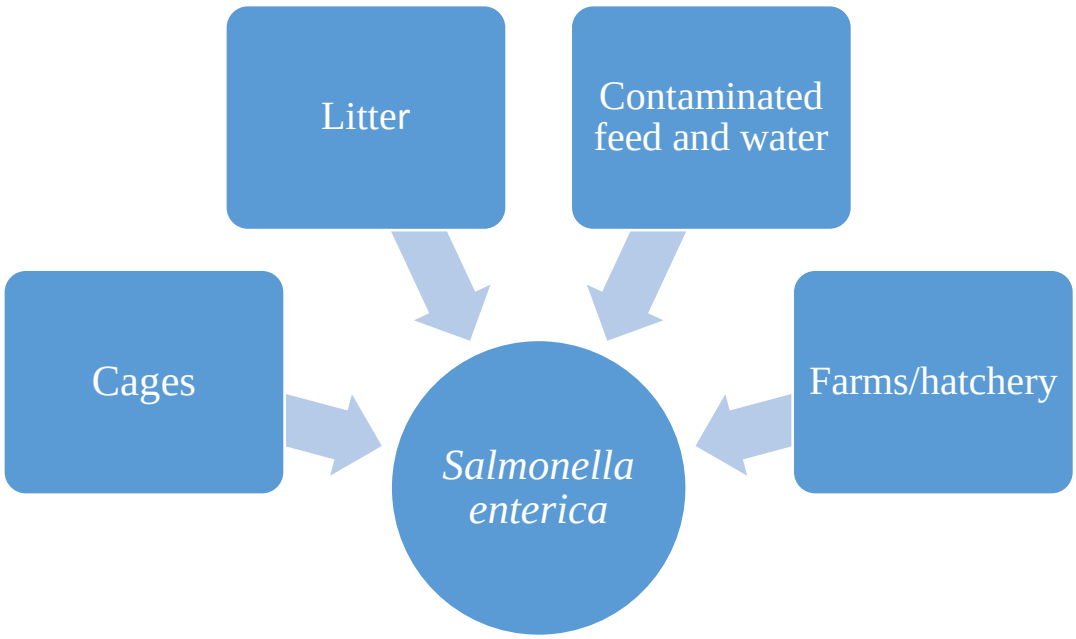

Figure 1: Route of *Salmonella enterica* in poultry

2.1.1 *Salmonella enterica* as pathogen

Salmonella sp. is gram negative, non-spore forming and rod shaped bacteria, belonging to family *Enterobacteriaceace.* They are facultative anaerobes, means they can grow in both aerobic and anaerobic conditions. Optimum temperature and pH for its growth ranges from 35-45℃ and 6.5-7.5 respectively (Jajere, 2019). *Salmonella sp.* are DNase and catalase positive, whereas urease negative. *Salmonella enterica serotype enteritidis* and *S. enterica serotype typhimurium* causes significant financial loss due to high treatment cost and replacement of diseased flocks. It's an alarming and health concern issues in developing countries (Pui *et al.*, 2011). Food borne Salmonellosis majorly outbreaks in human caused by contaminated poultry eggs and meat, usually egg associated Salmonellosis is caused by *Salmonella enterica serotype enteritidis*. *S.enteritidis* can easily imparts in normal appearing eggs, and if these eggs eaten undercooked may cause illness (Harker *et al.*, 2014).

2.2 Anatomy and Physiology of Poultry Gut

The poultry gut is mainly composed of esophagus, crop, proventriculus, gizzards, small intestines includes duodenum, jejunum, and ileum, large intestine comprise of cecum, colon, and cloaca. Poultry GI tract is considerable as shorter GI tract than other mammalian GI tract but have faster digestive system. Though intake of nutrients and feeding can respond and delay the passage rate, the average transit time is not more than 3.5hours; however short retention time helps for the selection of bacteria that can easily adhere to the mucosal layer in small intestine and readily grow there. Moreover, the ceca, have two blind pouches which results in lessened the transit rate of nutrients and feed are ultimate territories for a varied and diverse microbiome that has significant and substantial outcome on host nourishment and healthiness of the animals (Deng Pan and Zhongtang Yu., 2014). Anatomy and physiology of poultry gut is observed in figure 2.

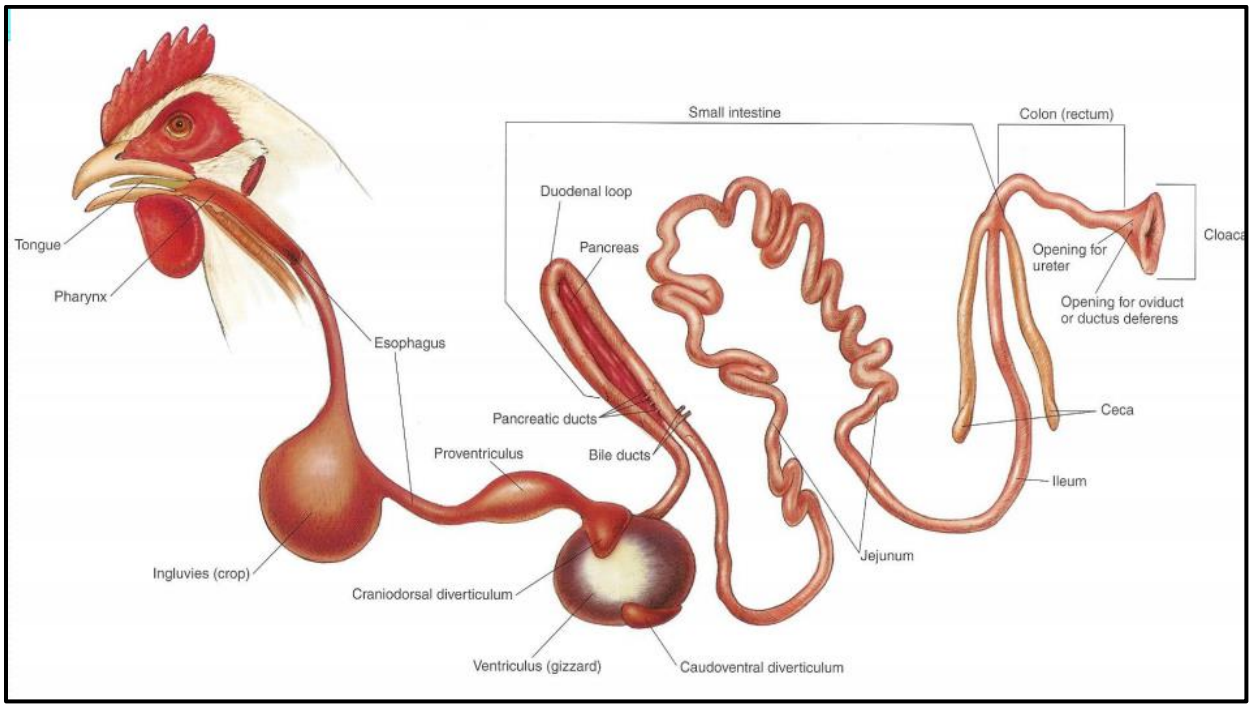

Figure 2: Anatomy and physiology of poultry gut

2.2.1 Interaction of *S. enterica* with Intestine in Poultry

Poultry microbiome is an initiator of infectious illness to humans (Salmonellosis) and also act as a pool of many antibiotics resistance determinants. The pathogenic micro-organisms cause infectious diseases in the intestinal tract by communicating with the host cell triggering many physio pathological mechanisms resulting in the imbalance of intestinal absorption and exertion of water (Rubino, 1989). Microorganism like *Salmonella* and other species have a significant feature i.e. flagella which helps in the colonization and adhesion to the mucosal layer of intestine. The integrity and connection between host and gut microflora is the mucosal layer and cell coat which normally composed of glycol-calix constitutes of glycol-conjugates on the acute surface of the epithelial layer of the host (Holzapfel *et al., 1*998) mainly epithelial glycol-conjugates serves as receptors for the attachment of pathogenic microorganisms. Several studies showed that adhesion mechanism of pathogen is due to flagella and pilus (Simpson *et al., 1992*; Purushothaman *et al.,* 2001). The factor affecting colonization of *Salmonella sp.* in poultry mainly depend upon age, feed intake, survival of *Salmonella sp.* in gastric barrier, use of antimicrobial substance, these all factors affect the invasion and colonization of pathogen to the poultry gut (Dunkley *et al.,* 2018). Traditional pathogens and emerging pathogens in poultry meat (Satin 2002*;* Ellerbroek 2009**)** illustrated in table 1.

Table 1: Traditional pathogens versus emerging pathogens in poultry meats

"Traditional" pathogens	Emerging pathogens
Campylobacter spp.	*Campylobacter jejuni* (O:19, O:4, O:1), *Campylobacter lanienae*
Salmonella spp.	*Salmonella* Typhimurium (DT104, DTU302), *S.* Enteritidis (PT4, PT8, PT13, PT14b)
Escherichia coli	Enterohemorrhagic *Escherichia coli* (EHEC)
Yersinia enterocolitica	*Listeria monocytogenes*
Staphylococcus aureus	*Arcobacter butzleri*
Clostridium perfringens	*Mycobacterium avium* subsp. *paratuberculosis*
Clostridium botulinum	*Aeromonas hydrophila*
Bacillus cereus	*Enterobacter sakazakii*
	Helicobacter pylori, *Helicobacter pullorum*

A significant amount of all emerging infections is associated with animals present in hatchery and product of meat level. The numerous occurrences of infection mainly related with absorption of diseased poultry meats caused by mostly Salmonella *sp., S. aureus*, and rarely by *Bacillus sp*.

2.3 Recent Treatment of Non Typhoidal *S. enterica*

2.3.1 Antibiotics

Globally, drug resistant infections are projected to cause 10 million human deaths at a cost of 100 trillion USD annually by 2050 if current trends continue (Neill, 2014). In poultry, antibiotics are commonly used in poultry feed for the treatment of Salmonellosis. In poultry industry they also used as growth promotors to increase the performance and enhanced feed efficacy. Many

Salmonella sp. shows antibiotics resistance pattern to quinolones, fluoroquinolones and nalidixic acids (Su *et al.,* 2004). In Pakistan no rules and regulation are there for the eradication of *Salmonella* from the poultry industry (Wajid *et al.,* 2019). By the order of Supreme Court of Pakistan, antibiotics should be banned in livestock and poultry industry due to increase resistance pattern of antibiotics. However biosecurity and pyramidal industrial structure is required to eradicate multi-drug resistant pathogen from the poultry (Andres *et al.,* 2014). Bacteriophages, inhibitory bacteria, plant extract, nanoparticles and probiotics are alternatives treatment to control salmonellosis in poultry industries (Ahmed *et al.,* 2016). Major advantages and disadvantages of antibiotics (Ghosh *et al*., 2018) shown in table 2.

Table 2: Major Alternatives to Antibiotics and Their Advantages and Disadvantages

Strategy	Advantages over conventional antibiotics	Possible disadvantages
Phage therapy	• Self-replicating pharmaceuticals • Selective towards specific strains of bacteria • Amenable to genetic engineering	• Immunogenicity • Pharmacokinetics • Release of bacterial endotoxins • Inadequate preparations – failure to remove endotoxins and pyrogenic substances • Resistance development
Lysins	• Amenable to genetic engineering • Selective towards specific strains of bacteria • Not prone to resistance development	• Production • Lack of sufficient knowledge
CRISPR/Cas9	• Can be tuned for a variety of antimicrobial applications • Reversal of antibiotic usage • Specificity towards pathogenic strains	• Expensive large-scale production • Toxicity
Antimicrobial peptides	• Not prone to resistance development • Broad-spectrum activity is an advantage, depending upon application	• Expensive large-scale production • Susceptible to proteolysis • Toxicity
Bacteriocins	• Specificity towards pathogenic strains of bacteria • Resistance to heat and UV	• Expensive large-scale production • Susceptible to proteolysis
SMAMPs[a]	• Ease of synthesis • Not prone to resistance development • Broad-spectrum activity is an advantage, depending upon application	• Toxicity • Route of administration
IDR peptides[b]	• Work by modulating the immune system • No resistance development as no direct antimicrobial activity	• Expensive large-scale production • Susceptible to proteolysis
Probiotics	• Easy availability	• Used mostly for intestinal infections
Antibodies	• Selective towards specific strains of bacteria • Do not damage the microflora	• High cost of production • Poor shelf life

2.3.2 Phage Therapy

Bacteriophages used to reduce the infection in infected live stocks ranging from *Staphylococcus Aureus, Pseudomonas Aeruginosa, Salmonella and Shigella* species by propagation and anchorage onto the microbial cell, helps in the injection of DNA material into the cell cytoplasm, taking over

the host machinery helps in the formation of new phage component. Some bacteriophage approved for the treatment of *Salmonella sp.* are clinically approved displayed as follows: (Ghosh *et al.,* 2018).

Table 3: Bacteriophages That Are Approved for Clinical Used Against Salmonellosis

PRODUCT	**COMPANY**	**CONDITIONS**	**STATUS**
Eco shield	Intralytix	Food industries (*S.enterica*)	Approved
Phagelyph	biochimpharma	Treatment and prophylaxis of enteric fever and Salmonellosis	Approved

2.4 Alteration of Gut Microbiota

2.4.1 Probiotics

2.4.1.1 Definition of probiotics

Definition of probiotics varies time to time with the increasing and development of knowledge about these microorganisms. According to Schrezenmeir and De Vresea: product that having ability to colonized, integrate in host body and employ positive effect on host health_as reported by Sander in 2014.

According to Kerry in 2018 suggested that Microbes named as probiotics that must fulfil these requirements, as reported by Sanders, M.E., 2014.

- Candidate must be microorganisms and at the time of administration must be viable and living.
- They must be feed in a dose which is adequately high and promoting beneficial health to the host.

2.4.2 Lactic Acid Bacteria

Lactic Acid bacteria (LAB) are gram-positive, non-spore forming cocci, coccobacilli or bacilli. All lactic acid bacteria grow anaerobically, yet distinctive to most anaerobes, they enhance inside the sight of oxygen as "aero tolerant anaerobes" (*Floch,* 2011). This bacterial family contains both rods and cocci shape bacteria. Different species of lactic acid bacteria (such as *Streptococcus, Leuconostoc, Pediococcus, Aerococcus, Enterococcus, Vagococcus, Lactobacillus, Carnobacterium*) have modified to grow differently in different environmental conditions (Stanley *et al.,* 2003).

The original statement of the constructive function of some bacteria can be attributed to the revolutionary work of Metchnikoff in the early 1900s, which prompt that these helpful microorganism could be administered with a view to exchanging dangerous microbes with useful ones. The term probiotic, which means for life, was once first coined by Lilly and Stillwell (Senok *et al*., 2005). Many reviews have proven the effectiveness of probiotics to offer a right alternative to the use of antibiotics in the therapy of enteric or gastro enteric illness or to reduce the symptoms of antibiotic associated diarrhea (Millette *et al*., 2008). Viability and survival of probiotic microorganism are predominant traits with the intention to provide health benefits.

The natural habitats of these bacteria are dairy products, mucosal membranes of humans and animals, including birds. Mostly lactic acid bacteria isolated from the GIT (gastrointestinal tract)

of chickens, geese, ducks and pigeons. The most commonly identified species in these birds are *L. salivarius, L. johnsonii, L. crispatus, L. reuteri* and *L. agilis* (Stephensonet *et al.,* 2010) whereas, *E. faecium, E. faecalis, E. hirae, E. durans, E. cecorum* and *E. lactis* are also natural residents of the farm animal's intestinal tract, which helps in rapid performance of broiler chickens (Royan *M,* 2018).

2.5 CHARACTERISTICS OF PROBIOTIC BACTERIA

2.5.1 Resistance to Gastrointestinal Stress

The viability and survival of probiotic microorganism are the essential parameters for offering therapeutic features. A few factors were claimed to affect the viability of probiotic bacteria including low pH and bile salts. The low pH is effective to provide a mighty barrier in opposition to the entry of bacteria into the intestinal tract. The pH of the gizzard and crop commonly ranges from pH 2.5 to pH 3.5 (Holzapfel *et al.,* 2001). Bile secreted within the small intestine reduces the survival of bacteria by means of destroying their cell membranes, the essential components of which can be lipids and fatty acids. Resistance to bile salts is regarded an essential parameter for settling on probiotic strains (Succi *et al.,* 2005). A suitable and effective probiotic should be viable, safe for consumption and tolerant to bile and gastric juices. It should also be able to survive through the gastrointestinal tract and adhere to the epithelial cells of the gut (Yadav & Shukla, 2017). Phenol tolerance is another important selection criterion for the probiotics survival in the gut region, as gut bacteria can de-aminate amino acids leading to the formation of phenolic compound.

2.5.2 *In vitro* safety of LAB strains

Naturally lactic acid bacteria is the part of healthy gut microflora or GIT of the animals especially avian. Now a days this population of microbes acts as an enormous reservoir of antibiotic resistance genes. These microorganisms can trigger the presence and occurrence of antibiotic resistance bacteria in the body of the host. When the bacteria are naturally resistant to an antibiotic and this property is inherent to a particular bacterial species then this type of resistance is regarded as 'intrinsic resistance'. This type of resistance to a particular antibiotic is then present in all of the strains of that species. The intrinsic resistance is assumed to have a very little potential for horizontal spread, furthermore acquired resistance which is mediated by the addition of genes is considered to have a high potential for lateral gene transfer (Devirgiliis e*t al*., 2011). Conversely, acquired resistance occurs when a strain belonging to a class of typically susceptible species to a particular antibiotic is resistant to a given antimicrobial drug. Acquired resistance can be due to the genes that are acquired by the bacteria by gaining exogenous DNA or by the mutation of bacteria's already present genes (Ammor *et al*., 2007)*;* (Van Reenen & Dicks, 2011). Published data of resistance profiles was taken into account by The European Food Safety Authority (EFSA) for the development of the safety scheme for probiotics. This assessment is a prerequisite for all strains that are to be used as feed additives in poultry (Cano Roca, 2014).

2.6 Possible Mechanisms of Lactic Acid Bacteria to Control Non Typhoidal *Salmonella enterica*

2.6.1 Competitive Exclusion of Pathogenic Microorganism

Competitive exclusion plays a vital role in the adhesion, attachment and colonization of microorganisms. The colonization of pathogenic microorganisms can be lessened by probiotics

mainly through the adherence of probiotics to specific receptors present in epithelium of intestine (Lloyd *et al.,* 1999).

2.6.2 Antagonistic Effect on Pathogenic Microorganism

Lactic acid bacteria prevent pathogenic colonization in the gizzard and cecum region by the production of antimicrobial components i.e. organic acids, hydrogen peroxide, low molecular weight components, these components have positive effects against pathogens which are mainly causing illness in avian.

2.6.3 Non Immunological Effect

Probiotics proposed non immunological mechanism in which there is increased in gut defense barrier due to which intestinal absorptivity and ecology of gut microbiota stabilized (Isolauri *et al.,* 2001).

2.6.4 Stimulation of Immune Response

Immunity is stimulated by probiotics either through migration or multiplication of gut probiotics. Some lactic acid bacteria are proficient in the production of cytokines which helps in (Havenaar and Spanhaak, 1994). Possible proposed mechanism of LAB strains is demonstrated in figure 3 (Carmo *et al.,* 2018).

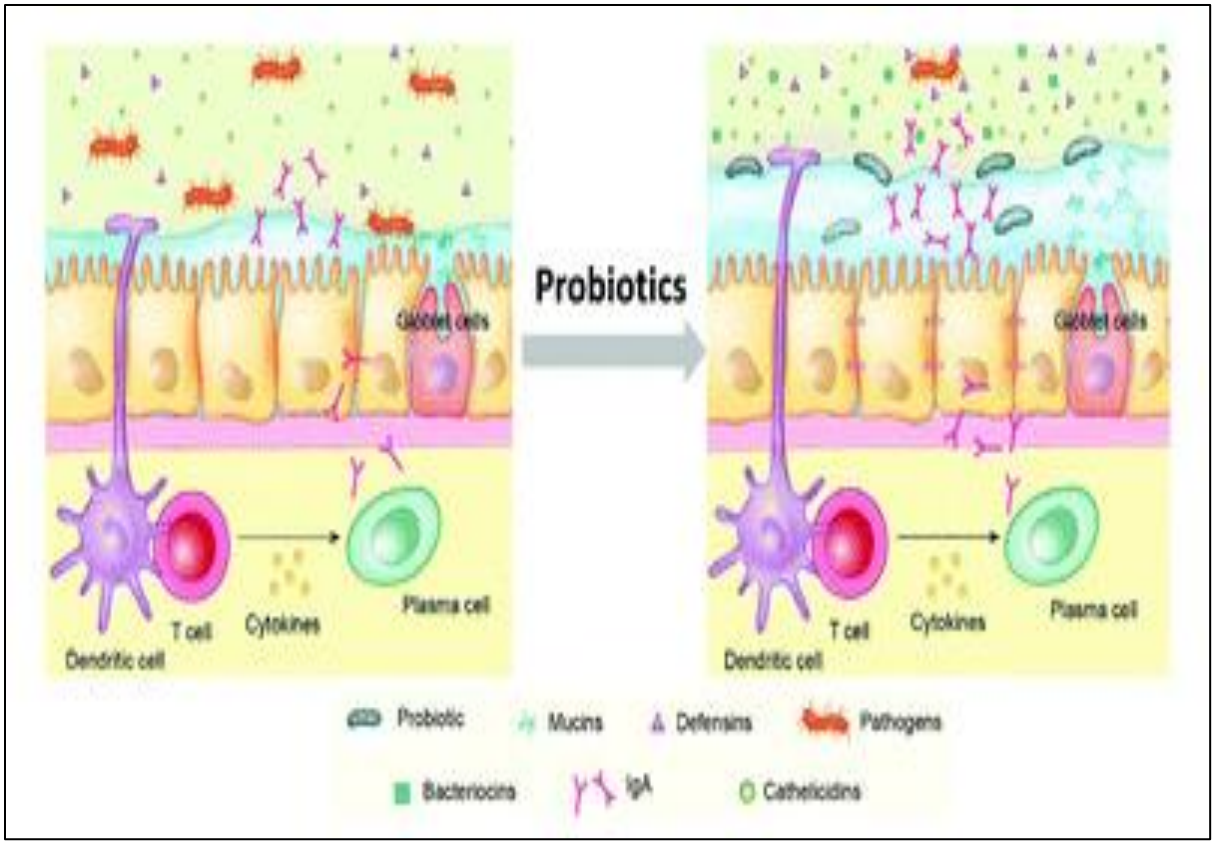

Figure 3: Possible Proposed Mechanism of LAB Strains

2.7 Utilization of Lactic Acid Bacteria in Poultry Industry as Probiotics

Probiotics known as growth and health promoters due to their biochemical, physiological and immunological influential effect on the host and also capable of resisting the harmful disease they are found in various kind of food, pharmaceutical product, sea foods and in poultry also. Modes of action and beneficial activities of probiotics in poultry is illustrated in figure 4.

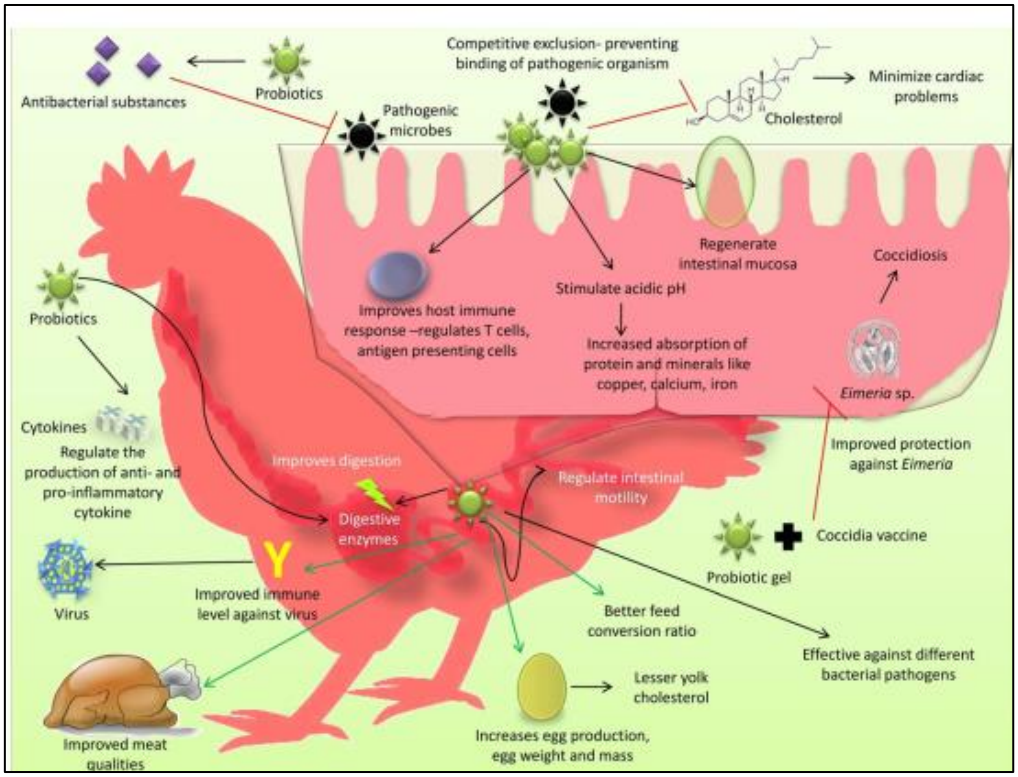

Figure 4: Modes of action and beneficial activities of probiotics in poultry

2.7.1 Effect on Growth Stimulation, Feed Consumption and Metabolism

A diversity of microbial species like *Bacillus, Lactobacillus, Bifidobacterium Enterococcus,* and *Saccharomyces* frequently used in livestock and poultry (Simon *et al.*, 2001). However, there has been a growing trend on feeding *Lactobacillus* to poultry (Tellez *et al.*, 2001; Kawakami *et al.*, 2011). A cocktail of selected bacteria of *Lactobacilli, Enterococcus, Streptococci* and *Bacillus* are known to be beneficial probiotics to animals especially for livestock. Probiotics, as a feed additive have been acclaimed to be a nontoxic, harmless and growth enhancers in animals (O'Dea *et al.* 2006; Sabatkova *et al.*, 2008; Bansal *et al.*, 2011). It is reported that *Lactobacillus* claimed to increase body weights and feed to gain ratio when analyzed with control broilers (Jin *et al*., 2008).

2.7.2 Effect of probiotics on intestinal morphology

Studies have been carried out to explore the effects of probiotic intake on the histology and morphology of the intestine. It is reported that probiotic *Lactobacillus sp*. influence the villi height and crypt depth in the small intestine of broilers (Bai *et al*., 2013). The consumption of *Lactobacillus sakei* Probio-65 helps in the increase villi height and crypt depth in jejunum of broilers as compared to chickens fed with antibiotic, therefore probiotics are suggested to increase the length of villi by triggering cell mitosis and induce gut epithelial-cell propagation (Samanya and Yamauchi, 2002). Enhancement of villi with the help of probiotics is beneficial to the broilers as the increased and enlarged surface area of the villi improved the nutrition absorption (Caspary, 2002).

2.7.3 Effect of probiotics on meat quality

According to widespread and extensive knowledge probiotics supplements could enhanced meat quality. Characteristics of meat quality that includes odor, taste, flavor, tenderness, nutrition determined by intramuscular lipid content of the chicks. In 2009, according to Endo and Nakano in pectoral and thigh meat there is much conversion of unsaturated fatty acids to saturated fatty acids meat of broilers nourished with supplemented diet comprising of *Bacillus, Lactobacillus, Streptococcus, Clostridium, Saccharomyces* and *Candida*. The outcomes proposed that due to the presence of probiotic strains meat fat is converted into favorable fat.

Chapter 3

MATERIAL AND METHODS

3.1 SAMPLE COLLECTION

10 poultry fecal samples were collected from different poultry farms, kept in sterile falcon tubes and stored at 4℃ before use.

3.2 ISOLATION OF BACTERIA

1 mL of the fecal sample was diluted with 9 mL of sterile PBS up to 10^{-7} dilutions. 100 µL were spread on MRS agar and M17 agar incubated at 37℃ and 30℃ for 48 hours in anaerobic condition using anaerobic jars, paraffin and candle. Strains were purified and grown in MRS broth and M17 broth with pH 6.5 and then with 40% glycerol, stocks were prepared and those stocks were stored at -20°C and -80°C in box number 175.

3.3 SELECTION OF *LACTOBACILLUS* AND *ENTEROCOCCUS* SPECIES

Selection of lactic acid bacteria was confirmed when 70 µL of inoculum grown in MRS broth was spread on MRS agar plates supplemented with 1% calcium carbonate and 0.05% of L-cysteine. Plates were incubated at 37°C for 48hrs. Clear zone was observed around bacteria which confirmed their production of lactic acid.

3.4 PHENOTYPIC IDENTIFICATION

3.4.1 Colony Morphology

Pure cultures were platted on MRS and M17 agar and incubated at 37°C and 30 ℃ for 48hrs in anaerobic environment. Color and shape of the colonies were observed under microscope.

3.4.2 Gram Staining

Standard protocol was used to stain the isolated bacteria. Single colony from MRS agar and M17 agar plates were selected. Droplet of distilled water was dispensed and sterile loop was used to transfer bacterial colony on the slide to prepare smear. The smear was then heat fixed and treated with crystal violet for 1min. After washing with distilled water, iodine solution was added for 40 sec and then again washed with distilled water. Ethanol was added as decolorizing agent for 5 sec. Again washed with distilled water and safranin was added for 40 sec and washed with distilled water. After air drying, slides were observed under microscope at 100X objective with immersion oil. Purple stained bacteria were gram positive and pink stained bacteria were gram negative. After staining of bacterial isolates, cells were observed to study their structural morphology.

3.4.3 Catalase Test

Standard protocol was used for catalase test. Single colony from MRS and M17 agar was picked and transfer to a dry clean slide by sterile loop. 3% H2O2 was added on the colony and mix well. *Salmonella enterica* was used as positive control. No bubble production was catalase negative and bubble production indicates the catalase positive results (Somashekaraiah *et al*., 2019).

Strains were purified and grown in MRS broth and M17 broth having pH 6.5, incubated at 37℃ and 30℃for 24 hours. Then with 40% glycerol, stocks were prepared and stored at -20°C and -80°C isolates were placed in box number 175.

3.5 GENOTYPIC IDENTIFICATION

3.5.1 Isolation of Genomic DNA

The protocol for DNA extraction of Gram positive bacteria was adapted from (Lindback and Granum, 2006). At 37°C bacterial cultures were grown overnight. The cultures were centrifuged at 10000 rpm for 2 minutes in order to get pellets of the cells. The cells were re-suspended in 495 µL SET Buffer (25 mM EDTA, 20 mM Tris-Hcl, 75 mM NaCl). 50 µL Lysozyme (10 mg/mL) was added and incubated for 1 hour at 37°C. This step was followed by the addition of 10% SDS and 5 µL proteinase K, incubated for 2 hours at 55°C. After incubation, 200 µL 5M NaCl and 700 µL Chloroform: iso-amyl alcohol (24:1) were added and incubated at room temperature with frequent inversions for 30 minutes. The Eppendorf tube was centrifuged for 30 minutes at 4500g at 4°C. The aqueous phase was transferred to a fresh tube. DNA was precipitated by adding equal volume of isopropanol. In a table top centrifuge, the tubes were centrifuged at maximum speed for 10 mins. The precipitated DNA was washed with 70% ethanol, let the pelleted DNA air dried. The DNA was re-suspended in 30 µL nucleus free water and immediately stored at -20°C.

3.5.2 Universal primers

Universal Primers 9F and 1510R used for the PCR optimization, sequences of the universal primers showed as follows:

Table 4: Universal primers used to amplify the DNA

PRIMERS	SEQUENCE5'to 3'	REFERENCE
9F	GAGTTTGATCCTGGCTCAG	(Takahashi *et al.*, 2014)
1510R	GGCTACCTTGTTACGA	(Takahashi *et al.*, 2014)

3.5.3 Quantification of DNA

The isolated DNA was quantified using nano drop- spectrophotometer (Thermo Scientific) as per user manual.

3.5.4 Amplification of 16S rDNA Using PCR

3.5.4.1 PCR Conditions

For amplification of DNA PCR protocol was optimized, 25 µL was the total volume prepared for each reaction, containing 2.5 µL PCR buffer, 0.75 µL MgCl2, 1 µL dnTPs, 1 µL Taq polymerase, 1 µL each primer, 14.75 µL PCR water and 3 µL of DNA template. PCR cycles and conditions for 16S rDNA is shown in figure 5

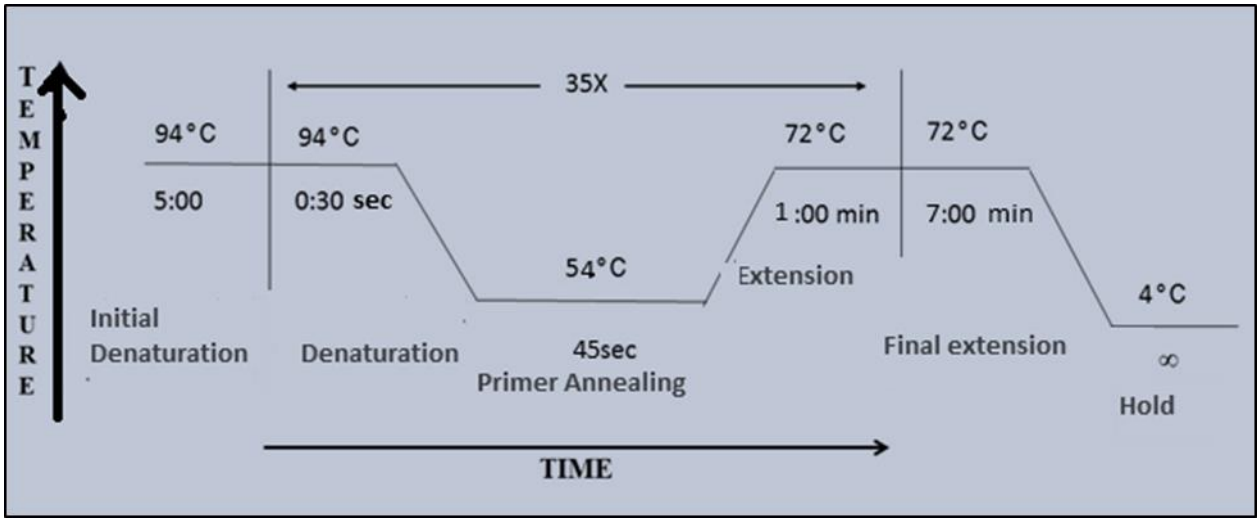

Figure 5: PCR Cycles for 16S rDNA amplification

3.5.5Confirmation of DNA Extraction

Amplified PCR products were separated on 1% agarose gel in 1X TAE buffer. 0.50g of agarose gel (Vivantis) is added in 50 mL of TAE buffer. Heat the mixture until solution became clear. 5 µL of Ethidium bromide was added after dissolving gel in buffer. 100bp of DNA ladder was used as marker. Wells were loaded with loading dye and sample mixture. Voltage was set at 80V, current 220A for 50 minutes. The gel was visualized under UV light using UV Trans illuminator of Labnet.

3.5.6 PCR Product Purification

Thermo scientific Gene JET PCR purification Kit was used for the purification of PCR product. For the purification of PCR product binding buffer was added in 1:1 volume. This solution was then added to the Gene JET purification column and centrifuged for 30-60s. Flow through was discarded. 700 µL of wash buffer was added to the purification column and centrifuged. Residual wash buffer was removed by more centrifugation. 50 µL

of elution buffer was added and stored at -20℃ in micro-centrifuge tube. After purification, DNA was quantified using Nano Drop then the product was sent for sequencing to Eurofins USA.

3.6 ASSESSMENT OF PROBIOTIC POTENTIAL

3.6.1 Acid Tolerance

The tolerance of LAB strains against Acid were tested using sterile flat-bottom 96-well microtiter plates. All the isolates were grown in MRS and M17 broth for 24 hours anaerobically To check pH tolerance, pH of MRS and M17 broth was adjusted to 2 with (1.0) molar of hydrochloric acid. 150 µL of MRS broth was added to each well whereas 30 µL of bacterial cultures with optimized optical density (i.e. OD ~ 0.1) were dispensed in wells of micro-titer plate, whereas broth with 2 pH was used as positive control. Microplates were incubated aerobically at 37^{o}C and 30 ℃ for continuous 4hrs. Optical densities were read at 620 nm using a microplate reader for 4 hours. All experiments were performed in triplicates (Turchi *et al.*, 2013).

3.6.2 Bile Tolerance

Isolated LAB strains were grown in MRS and M17 broth for 24hrs under anaerobic conditions, 0.3% oxbile was added in MRS and M17 broth.150 µL of MRS and M17 broth having 0.3% bile salt was added to each well whereas 30 µL of bacterial cultures with optimized optical density (i.e. OD ~0.1) were dispensed in wells of micro-titer plate. 0.3% oxoid bile broth was used as negative control. Microplates were incubated anaerobically at 37^{o}C and 30 ℃ for continuous 4hours. Samples were analyzed at 620nm using a microplate reader. All experiments were performed in triplicates (Turchi *et al.*, 2013).

3.6.3 Lysozyme tolerance

To assess the survivability of isolated strains in lysozyme, cells were grown in MRS and M17 broth overnight at 37℃ and 30℃, cells were pelleted out by centrifugation (10min, 3500rpm) and re-suspended in sterile PBS solution containing lysozyme (100 mg/L). Samples were incubated at 37℃ and 30℃after 30 and 90 mins, optical density were measured at 620nm. Experiment was performed in triplicates. Survival rate of isolates that maintained at least 80% of their growth rate after 90mins of treatment with lysozyme were considered for further testing (Turchi *et al*., 2013).

3.6.4 Phenol tolerance

To assess the phenol tolerance of the isolated bacteria .Cultures were grown in MRS and M17 broth having pH 6.5 anaerobically for 24hrs. Cultures were further inoculated in MRS and M17 broth supplemented with 0.4% phenol. 150 µL of broth and 50 µL culture inoculated in phenol was added to sterile 96 well microtiter plate, whereas broth with 0.4%phenol was used as negative control. Optical density was measured at 620nm at 0hour interval and after 24 hours to evaluate the phenol tolerant ability of isolates (Turchi *et al*., 2013).

3.7 SAFETY ASSESSMENT OF LAB STRAINS

3.7.1 Antibiotic Resistance Profiling

Antibiotic resistance profiling of all isolates were assessed. Antibiotics were available in disc (oxoid) form. Resistance pattern were checked against few antibiotics which are commonly used in poultry mainly includes were TE-30 (Tetracycline), AMP-10 (Ampicillin), K-30 (Kanamycin), CIP-5 (Ciprofloxacin), C-30 (Chloramphenicol), CN-10 (Gentamicin), AMC-30 (Amoxycillin/ clavulanic acid), VA-30 (Vancomycin), RD (Rifampicin), MEM (Meropenem), NA (Nalidixic acid), ENR (Enrofloxacin), FEP (Cefepime), IPM (Imipenem), SXT

(Sulphamethoxazole), CFM (Cefixime), TEC (Tecoplanin). Cultures were inoculated in MRS and M17 broth for 24 hours under anaerobic conditions. 100 µL Bacterial suspension were swabbed on MRS and M17 agar plates with the help of sterile cotton swab. Antibiotic discs were then placed on the agar plates and incubated at 37°C and 30℃ for 24 hours anaerobically. By measuring the zone of inhibition, and using zone diameter interpretive criteria **(**CLSI guidelines 2018) isolates were deliberated as resistance, intermediate susceptible or sensitive to the antibiotics.

3.7.2 Hemolytic Test

Isolates were scrutinized by streaking the overnight growth on blood agar (oxoid) plates having 7% v/v sheep blood (collected from animal sciences lab, NARC Islamabad.) *Staphylococcus aureus* (National analytical lab, NUST) was used as a positive control. Agar plates were incubated at 37℃ and 30 ℃ for 2–3 days anaerobically and observed the zone of hemolysis around the bacterial colonies (Yadav *et al*., 2016).

3.7.3 DNASE Test

Overnight growth of isolates were streaked on DNase agar (oxoid) plates to check the production of DNase enzyme. Plates were incubated at 37◦C and 30℃ anaerobically for 2-3 days. *Staphylococcus aureus* was used as a positive control. Plates were observed for the zone of DNase activity by spreading 1N HCl throughout the plate. Clear and pinkish zone around colonies were considered as positive for DNase activity (Yadav *et al.*, 2016).

3.8 *IN VITRO* ASSESSMENT OF ANTI- *SALMONELLA* ACTIVITY

3.8.1 Co- Aggregation Assay

Bacterial cells from overnight growth of the LAB strains were harvested and centrifuge in table top centrifugation apparatus (5000g, 20 min, 4℃), remove the supernatant, wash the cells twice with sterile Phosphate-buffered saline (pH7.2), again centrifuge for 10min at 5000xg, re-suspend the cell in sterile phosphate-buffered saline. Optical densities were adjusted 0.25±0.05 in order to sustain the number of bacterial cells (10^7–1 0^8 CFU/mL) in sterile phosphate buffer. For co-aggregation assay ,bacterial suspensions of LAB strains(4 mL) were mixed with equal volume of the overnight growth of pathogenic *Salmonella* strains (4 mL) (previously isolated in food microbiology lab, source of the strains were infected poultry fecal, by Abubakar Siddique in 2018) in sterile falcon tube, mixtures were incubated at 37℃ and 30℃ without vortexing and agitation. After 24hr absorbance (600nm) were measured. The percentage of co-aggregation was calculated as

$$\frac{\textbf{A (Pathogen+ LAB strain)/2 - A (mix)}}{\textbf{A (pathogen+ LAB strain)/2}} \quad \textbf{X 100}$$

Whereas A pathogen and LAB strains are the overnight culture of pathogenic strains and adjust absorbance of LAB strains respectively in the sterile falcon tubes and A (mix) represents the absorbance of mixed LAB strains and pathogenic strains after 24 hours (Gomez *et al.,* 2016).

3.8.2 Mucin Adhesion Assay

LAB strains were evaluated for adhesion to immobilized mucin in sterile flat bottomed 96 well microtiter plate. Microtiter plates were coated with 300 µL porcine mucin type Ⅲ (10 mg/mL,

Sigma-Aldrich) in sterile phosphate buffer solution (PBS), kept overnight in 4℃. Plate were washed thrice with the help of sterile PBS to remove unbound mucin from the wells. The overnight grown inocula were centrifuged in table top centrifugation apparatus (5000xg for 2 min at 4℃). The cells were harvested and washed thrice with the sterile PBS. The absorbance of the cells adjusted to 0.25±0.05 in order to maintain 1 x10^8 CFU /mL in sterile at 600nm.

For competitive mucin adhesion, 100 µL of LAB strains whose absorbance were adjusted to 0.25±0.05 and 100 µL of *Salmonella* species were added to mucin coated well at the same time and co-incubated for 90 min. Adhered bacterial cells, then treated with 300 µL Triton X in sterile phosphate buffered solution. LAB strains and pathogenic strains then enumerated by plating on MRS, M17 and SS agar respectively. Co-culture of pathogenic strains and LAB strains were also performed whereas monoculture of pathogenic and LAB strains alone considered as positive controls (Dhanani and Bagchi, 2014).

3.8.3 Time Kill Assay

Lab strains were cultured in MRS and M17 broth at 37℃ and 30 ℃ for 24 hours. The overnight cultures were then centrifuged in table top centrifugation machine for 30 min at 5000rpm. The supernatant was neutralized at 7 pH with 1M HCl and 1M NaOH. The supernatant then sterilized by filtration through 0.45 µm filter and immediately stored at -20℃

Salmonella enterica and LAB strains were grown in their respective broth for 24 hours at 37℃ and 30℃. 100 µL of pathogenic strains and cell free supernatant of LAB strains were co-culture in 96 well sterile microtiter plate. OD was measured after every 4hour intervals till 24 hours. Monoculture of pathogenic strains and cell free supernatant of LAB strains were taken as positive control. Experiment was performed in triplicates (Prabhurajeshwar and Chandrakanth, 2019).

3.8.4 Cell Surface Hydrophobicity Assay

Log phase of LAB strains were harvested and centrifuged at 8000xg for 10 min. Supernatant discarded and cell pellets were collected, cell pellets were washed thrice with sterile PBS and re-suspend in PBS. Optical densities were maintained 1±0.05 at 600nm. Initially 3 mL of cell suspension was transferred to 15 mL sterile falcon tube by adding 1 mL of hydrocarbons. Three hydrocarbons were tested i.e. toluene (non polar, aromatic hydrocarbon), xylene (non polar, aromatic hydrocarbon) and chloroform (non polar hydrocarbon). Falcon tubes were incubated at 37℃for 10 minutes for temperature establishment, followed by 15sec vortexing, then incubated for 20 minutes at 37℃ for phase separation. Co-culture of LAB strains against pathogens were also performed. Lower aqueous phase were collected in separate glass tube, and OD at 600nm was recorded. Percent hydrophobicity was calculated by the formula as follows (Rokana *et al.,* 2018).

$$\frac{ODi - ODt \times 100}{ODi}$$

Where ODi is initial OD of cell suspension, ODt is OD of aqueous phase recorded at 600nm after 20 minutes.

CHAPTER 4

RESULTS

4.1 Sample collection and Isolation of *Lactobacillus*

10 poultry fecal samples were collected from NARC Islamabad, samples were homogenized and spread on specific media i.e. MRS and M17 agar then isolates were purified in order to obtain single pure colony of *Lactobacillus* and *Enterococcu*s. Colony morphology of LAB strains revealed in figure 6. Sample performa and stock preparation is illustrated in table 5.

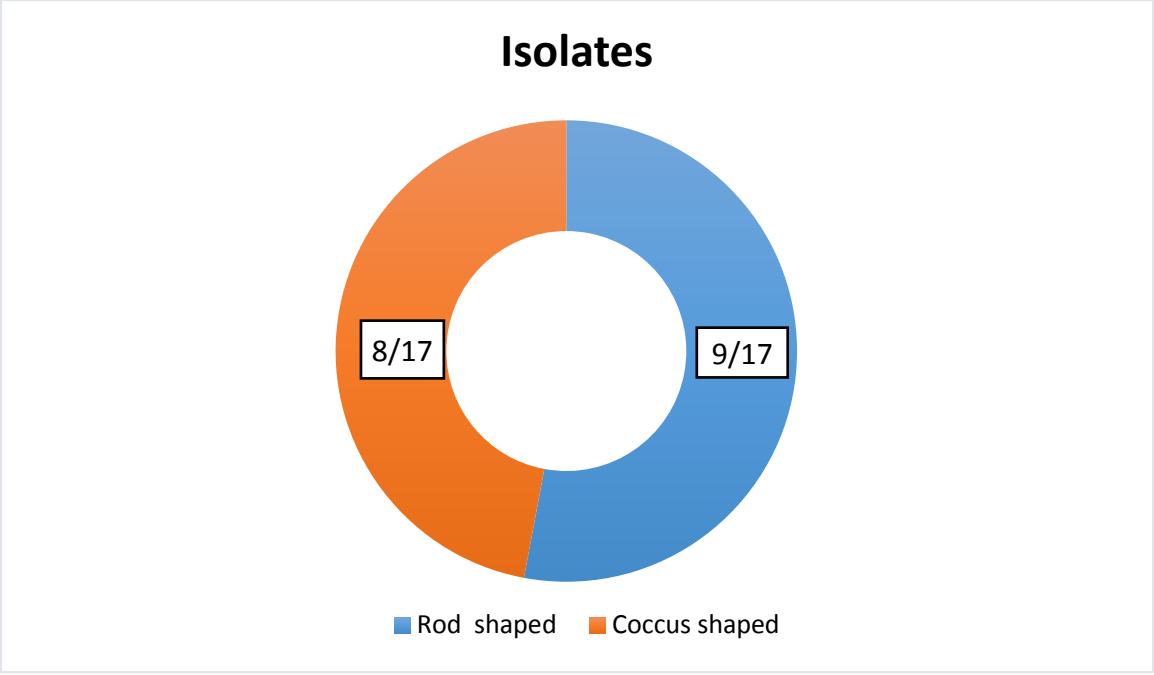

Figure 6: Colony morphology of LAB strains

Table 5: Isolation of LAB strains from poultry feces and stock preparation

SAMPLE CODE	SAMPLE	LOCATION	ISOLATES	STOCK PREPARATION	BOX NO.
PFS1	Poultry feces	NARC	PFS1	17/1/2019	175
PFS2	Poultry feces	NARC	PFS2	17/1/12019	175
PFS3	Poultry feces	NARC	PFS3	17/1/2019	175
PFS4	Poultry feces	NARC	PFS4	17/1/2019	175
PFS5	Poultry feces	NARC	PFS5	20/1/2019	175
PFS6	Poultry feces	NARC	PFS6	20/1/2019	175
PFS7	Poultry feces	NARC	PFS7	20/1/2019	175
PFS8	Poultry feces	NARC	PFS8	20/1/2019	175
PFS9	Poultry feces	NARC	PFS9	20/1/2019	175
PFS10	Poultry feces	NARC	PFS10	22/1/2019	175
PFS11	Poultry feces	NARC	PFS11	22/1/2019	175
PFS12	Poultry feces	NARC	PFS12	22/1/2019	175
PFS13	Poultry feces	NARC	PFS13	22/1/2019	175
PFS14	Poultry feces	NARC	PFS14	24/1/2019	175
PFS15	Poultry feces	NARC	PFS15	24/1/2019	175
PFS16	Poultry feces	NARC	PFS16	24/1/2019	175
PFS17	Poultry feces	NARC	PFS17	24/1/2019	175

4.1.2 Selection of *Lactobacillus*

Clear halo zones (Figure 7) appeared around the colonies of *Lactobacillus* on MRS and M17 agar supplemented with calcium carbonate. Lactic acid produced by the *Lactobacillu*s and *Enterococcus* species. Lactate reacts with calcium and form calcium lactate, so clear zone appeared around *Lactobacillus* colonies.

Figure 7: Image showing the halo zone formation around bacterial colonies on MRS agar plates

4.2 Phenotypic Identification

4.2.1 Colony Morphology

The purified bacterial isolates were culture on MRS and M17 agar plates. Colonies were observed for their appearance, color and shape.

4.2.2 Gram Staining

Gram staining was performed for all the bacterial isolates. All isolates were gram positive bacterium. Shape morphology of the isolates reveled under 100X resolution compound microscope is shown in figure 8.

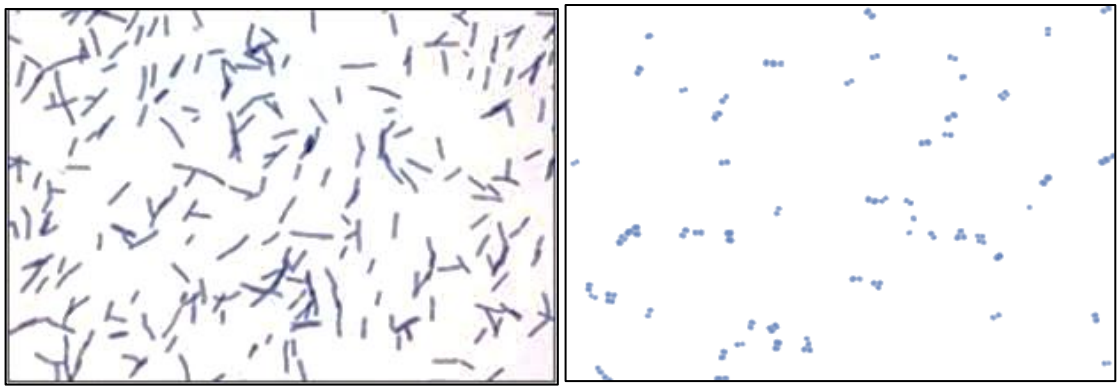

Figure 8: Different gram positive, rod and coccus shape morphologies of isolates reveled observed under compound microscope at 100X resolution.

4.2.3 Catalase test

Figure 9 illustrate that no bubble formation was observed when single bacterial colony was treated with 3% hydrogen per oxide, whereas bubble formation observed in positive control. So, all LAB isolates were catalase negative.

Figure 9: Catalase activity of LAB isolates and positive control

Table 6: The phenotypic characteristics of isolated bacterial strains from poultry

Isolates	Halo zone	Catalase test	Gram Staining	Colony morphology
PFS1	+	-	Short rods	Round, white
PFS2	+	-	Long rods	Round, white
PFS3	+	-	Short rods	Round, white
PFS4	+	-	Long rods	Round, white
PFS5	+	-	Long rods	Round, white
PFS6	+	-	Short rods	Round, white
PFS7	+	-	Long rods	Round, white
PFS8	+	-	Short rods	Round, white
PFS9	+	-	Short rods	Round, white
PFS10	Nil	-	Coccus	Round, white
PFS11	Nil	-	Coccus	Round, white
PFS12	Nil	-	Coccus	Round, white
PFS13	Nil	-	Coccus	Round, white
PFS14	Nil	-	Coccus	Round, white
PFS15	Nil	-	Coccus	Round, white
PFS16	Nil	-	Coccus	Round, white
PFS17	Nil	-	Coccus	Round, white

4.3 Assessment of Probiotic Potential

4.3.1 GIT related stress tolerance

4.3.1.1 Acid tolerance

All the 17 isolates 9 *Lactobacillus* isolates and 8 *Enterococcus* isolates were showing high tolerance for 2 hours and then their growth decreases as their incubation time increases. Most of the strains

showed normal growth pattern that is their growth decreases at the start and then start increasing after some period of time and then start decreasing. But some of the strains showed exceptionally well growth even after 4 hours at pH 2. In comparison of *Lactobacillus* and *Enterococcus, Lactobacillus* PFS1, PFS2, PFS3, PFS4, PFS5, PFS6, PFS7, PFS8 and PFS9 shows good pH tolerance then *Enterococcus* strains as *Lactobacillus* mostly present in gizzard and crop region of poultry whereas *Enterococcus* are inhabitants of cecum region of poultry. OD of isolates were checked at 5 different time interval i.e. 0 minute, 60 minutes, 120 minutes, 180 minutes and 240 minutes in microplate reader (fig. 10). Experiment were performed in triplicates.

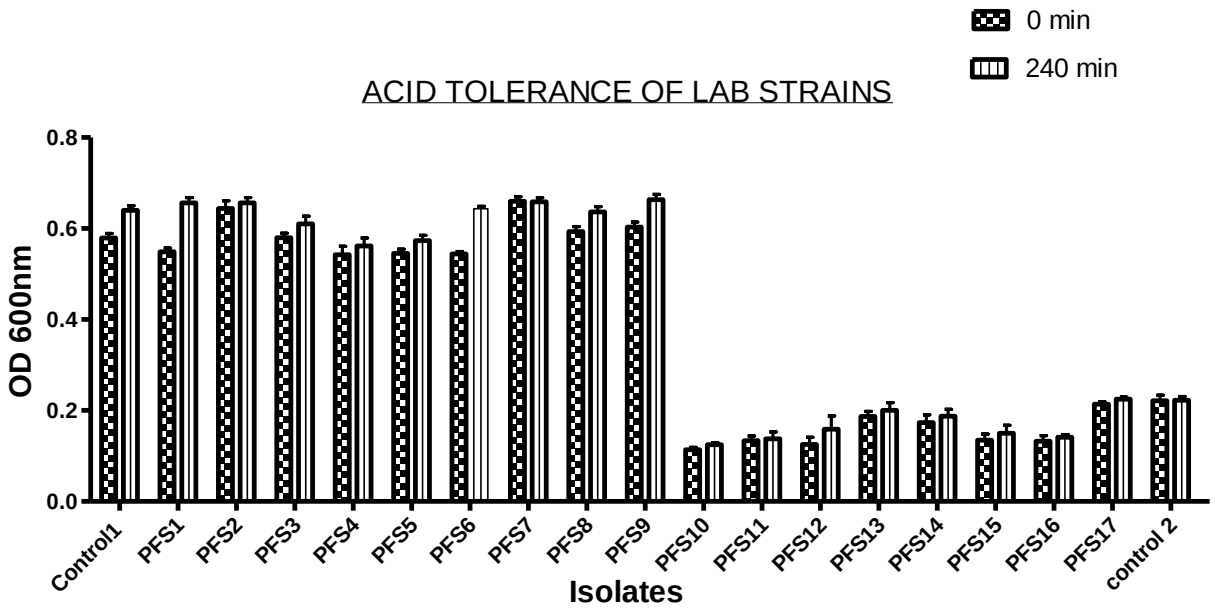

Figure 10: Comparative analysis of acid tolerance of LAB strains at 0hr to 4hr

4.3.1.2 Bile tolerance

All isolated strains were tested against 0.3% bile salt. All strains showed normal pattern of growth, OD was measured at 5 different time intervals i.e. 0 minute, 60 minutes, 120 minutes, 180 minutes and 240 minutes in microplate reader (fig. 11).

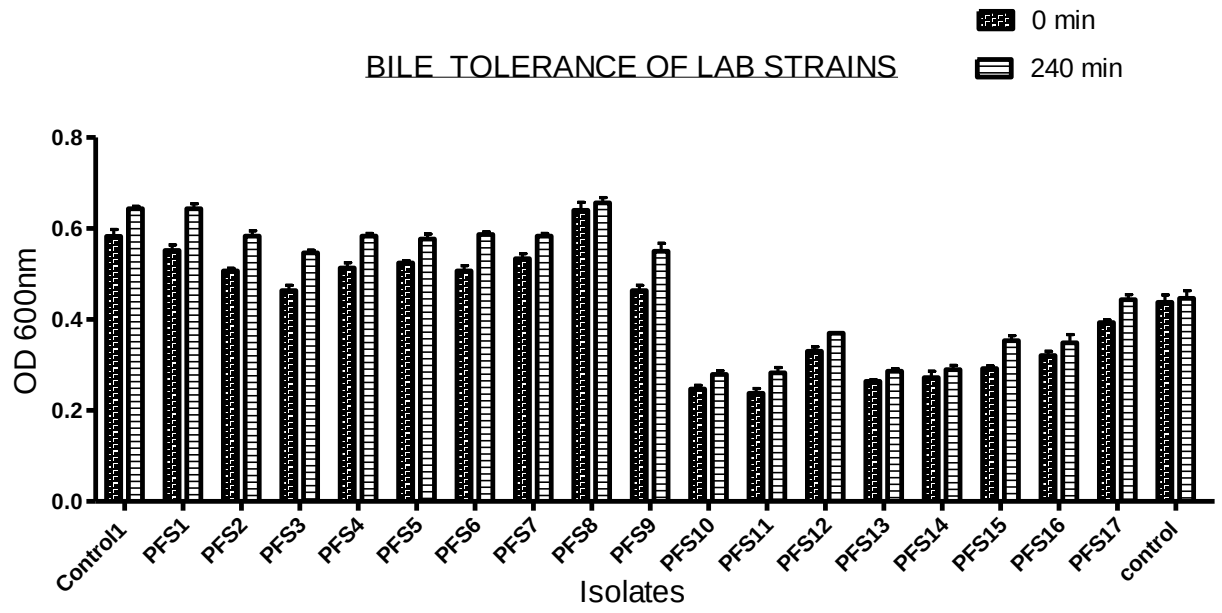

Figure 11: Comparative analysis of bile tolerance of isolates time interval 0h to 4h

4.3.1.3 Phenol Tolerance

All 17 isolates were further evaluated for phenol resistance at 0hr and 24hr (fig. 12). All the strains showed good survival rate towards phenol. Isolates were showing different degree of sensitivity towards phenol. All isolates showed good tolerance toward phenol.

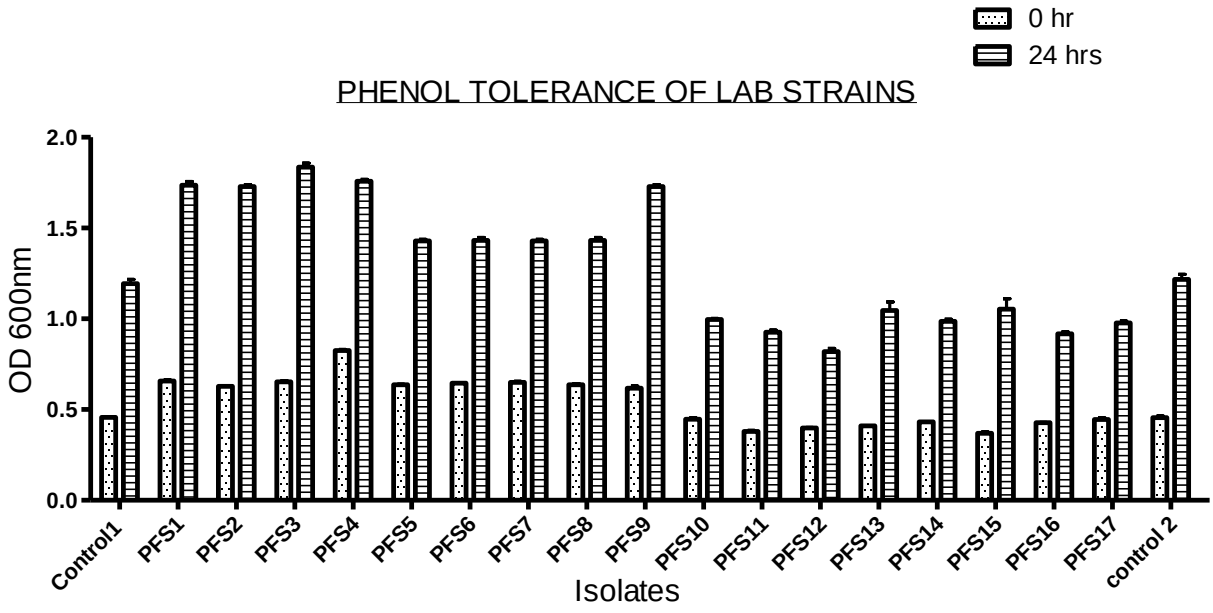

Figure 12: Comparative analysis of phenol tolerance of LAB strains at 0hr to 24hr

4.3.1.4 Lysozyme tolerance

Lysozyme resistance test was performed to analyze the ability of strains to survive into the oral cavity of poultry (fig. 13). Isolates that maintained their survival rate at least 80% of their growth rate after 90mins of treatment with lysozyme were considered for further testing.

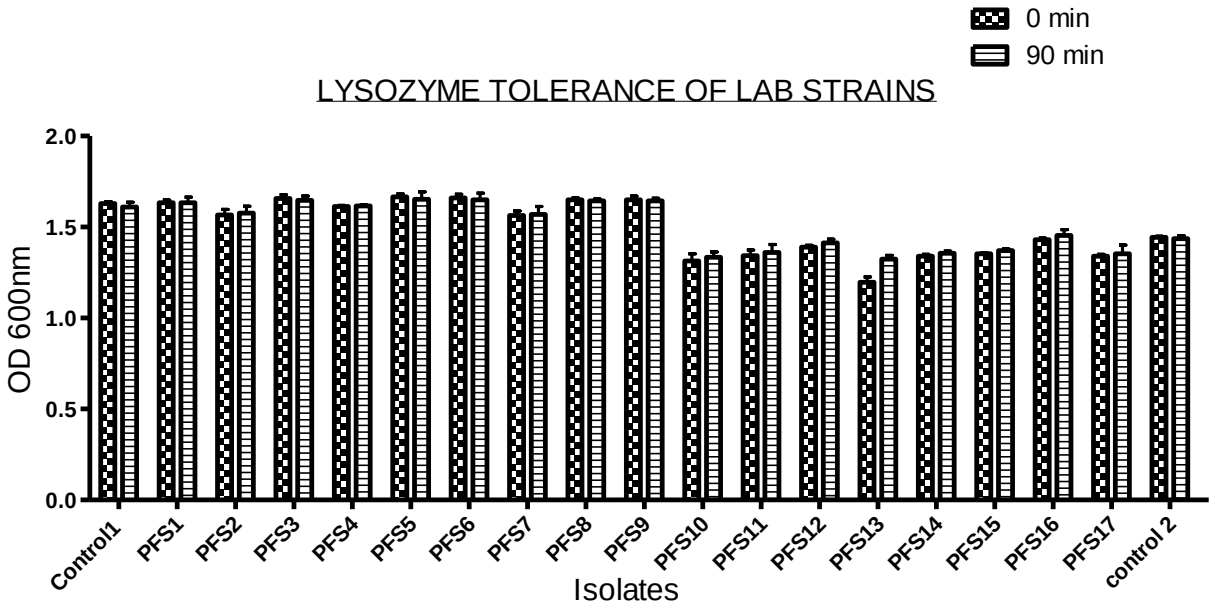

Figure 13: Comparative analysis of lysozyme tolerance of LAB strains at 0min to 90 min

4.3 Safety Assessment of Selected Strains

4.3.1 Antibiotics Susceptibility assay

All isolates were checked for antibiotic susceptibility against 19 antibiotics, which are most commonly used in poultry industry as growth promoters. No zone was observed against Vancomycin, Kanamycin and Streptomycin, whereas all LAB strains except *Lactobacillus reuteri* PFS4 and *Lactobacillus reuteri* PFS9 are resistant to 3rd and 4th generation antibiotics. In table 7, antibiotic susceptibility profiling of LAB strains is illustrated where Black Square represents resistance and White Square represents sensitivity to the antibiotics.

Table 7: Antibiotic susceptibility profile of isolated LAB strains from poultry

ISOLATES	CN	CIP	K	VA	C	S	IPM	MEM	NA	CFM	DA	RD	LZD	SXT	FEP	AK	TE	CT	AMC
PFS101	■	□	■	■	□	■	□	□	□	■	□	□	□	□	■	□	□	■	□
PFS102	■	□	■	■	□	■	□	□	□	■	□	□	□	□	■	□	□	■	□
PFS103	■	■	■	■	■	■	□	□	□	■	□	□	□	□	■	□	□	■	□
PFS104	■	□	■	■	■	■	□	□	□	□	□	□	□	□	□	□	□	□	□
PFS105	■	■	■	■	□	■	□	□	□	■	□	□	□	□	■	□	□	□	□
PFS106	■	■	■	■	□	□	□	□	□	■	□	□	□	□	■	□	■	■	□
PFS107	■	■	■	■	□	■	□	□	□	■	□	□	□	□	■	□	□	□	□
PFS108	■	□	■	■	□	■	□	□	□	■	□	□	□	□	■	□	□	■	□
PFS109	■	□	■	■	□	■	□	□	□	□	□	□	□	□	□	□	□	□	□
PFS110	■	□	■	□	□	■	□	□	□	■	□	□	□	□	□	□	■	■	□
PFS111	□	□	■	□	□	■	□	□	□	□	□	□	□	□	□	□	■	■	■
PFS112	□	□	■	□	□	■	□	□	□	□	□	□	□	□	□	□	■	■	□
PFS113	□	□	■	□	□	■	□	□	□	□	□	□	□	□	□	□	■	□	□
PFS114	□	□	■	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□	□
PFS115	□	□	■	□	□	■	□	□	□	□	□	□	□	□	□	□	□	■	□
PFS116	□	□	■	□	□	■	□	□	□	□	□	□	□	□	□	□	■	■	□
PFS117	□	□	■	□	□	■	□	□	□	□	□	□	□	□	□	□	■	□	□

White square indicates sensitivity and Black Square indicates resistance to antibiotics. Abbreviation of antibiotics are as follows :TE-30 (Tetracycline), AMP-10 (Ampicillin), K-30 (Kanamycin), CIP-5 (Ciprofloxacin), C-30 (Chloramphenicol), CN-10 (Gentamicin), AMC-30 (Amoxycillin/ clavulanic acid), VA-30 (Vancomycin), RD (Rifampicin), MEM (Meropenem), NA (Nalidixic acid), ENR (Enrofloxacin), FEP (Cefepime), IPM (Imipenem), SXT (Sulphamethoxazole), CFM (Cefixime), TEC (Tecoplanin).

4.3.2 DNase activity

All isolates have shown no zone formation on DNase agar plate, whereas *S. enterica* shows zone around the colony (fig. 14).

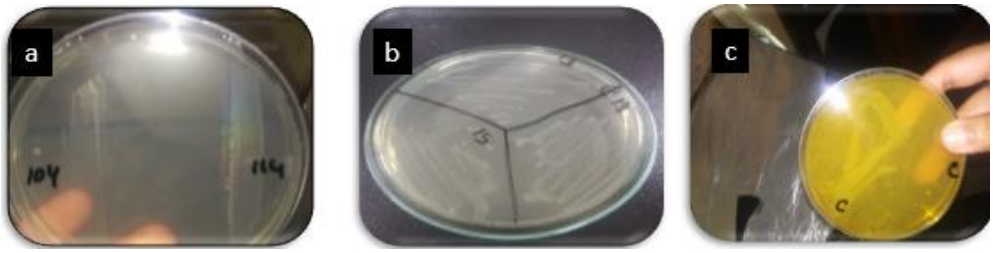

Figure 14: a) and b) probiotic culture shows no DNAse activity whereas c) *Salmonella enterica* shows zone around the colony on DNAse agar

4.3.3 Hemolytic activity

Growth of probiotic isolates on blood agar did not show any zone of hemolysis whereas *Staphylococcus aureus* formed a hemolysis zone on blood agar (fig. 15).

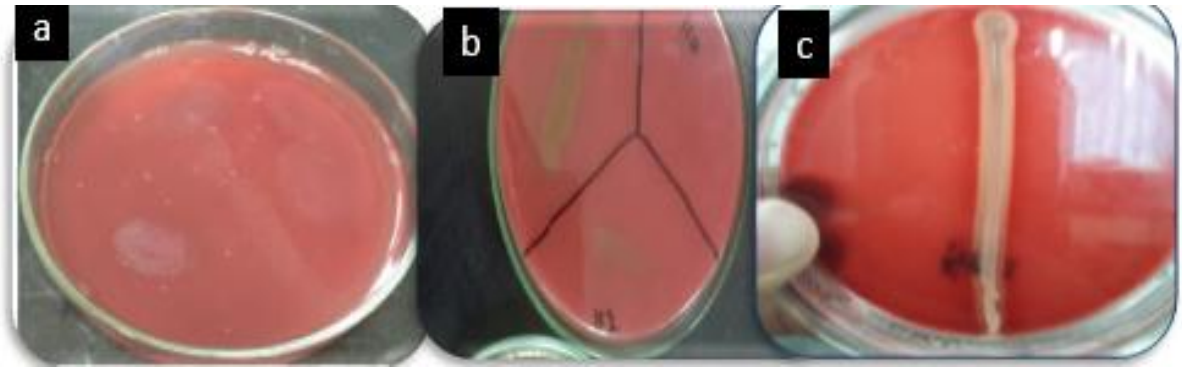

Figure 15: a) and b) probiotic culture on blood agar shows no formation of hemolysis zone c) Staphylococcus aureus forms a zone of hemolysis on blood agar

4.4 *In vitro* assessment of potential LAB strains for non typhoidal *Salmonella enterica* control

4.4.1 Co-aggregation Assay

4.4.1.1 Co-aggregation assay of LAB strains with *S. typhimurium*

All 17 LAB isolates were further evaluated for co-aggregation assay against three *S. typhimurium* strains i.e. *Salmonella typhimurium* PA17, *S. typhimurium* PA40, *S. typhimurium* PA48 illustrated in figure 16, 17 and 18 respectively.

PFS2, PFS11 showed least % co-aggregation and PFS4, PFS9, PFS13 showed higher % co-aggregation of S*almonell*a *typhimurium* PA17 i.e. 33%, 35%, 59% and 58% respectively. PFS8, PFS10, PFS11, PFS12, PFS16 showed least % co-aggregation values whereas PFS4, PFS9, PFS13, PFS14 showed highest % co-aggregation against *S. typhimurium* PA40 i.e. 36%, 43%, 59% and 45% respectively. PFS8, PFS15 showed least % co-aggregation whereas PFS4, PFS9, PFS13, PFS14 showed highest % co-aggregation against *S. typhimurium* PA48 i.e. 37%, 39%, 58%, 57% respectively.

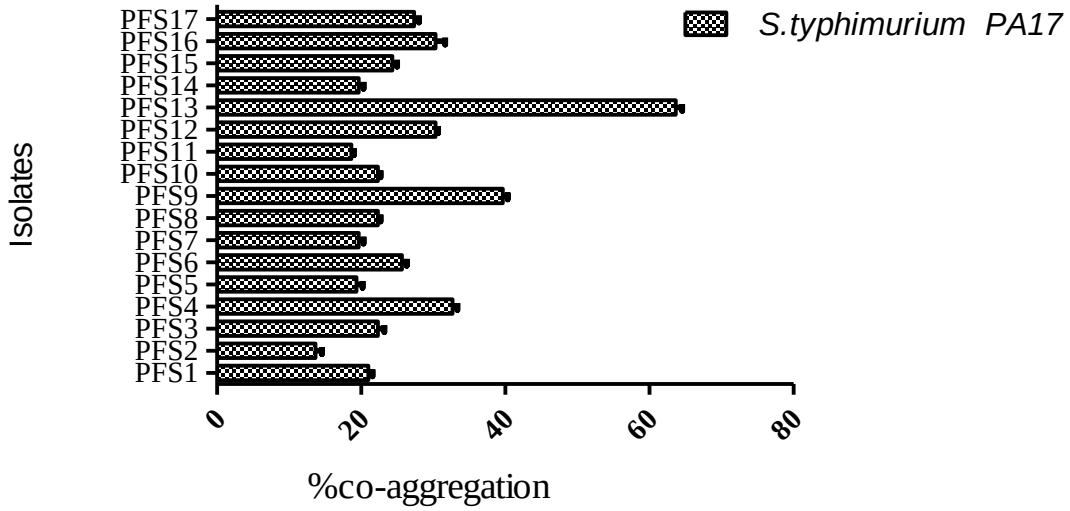

Figure 16: Co-aggregation Analysis of 17 LAB isolates with *S. typhimurium* PA17

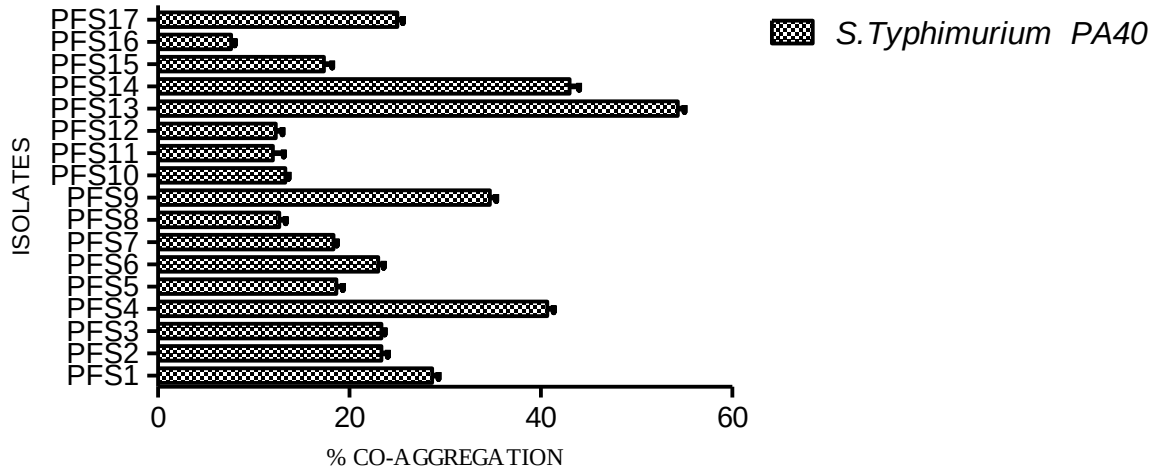

Figure 17: Co-aggregation Analysis of 17 LAB isolates with *S. typhimurium* PA40

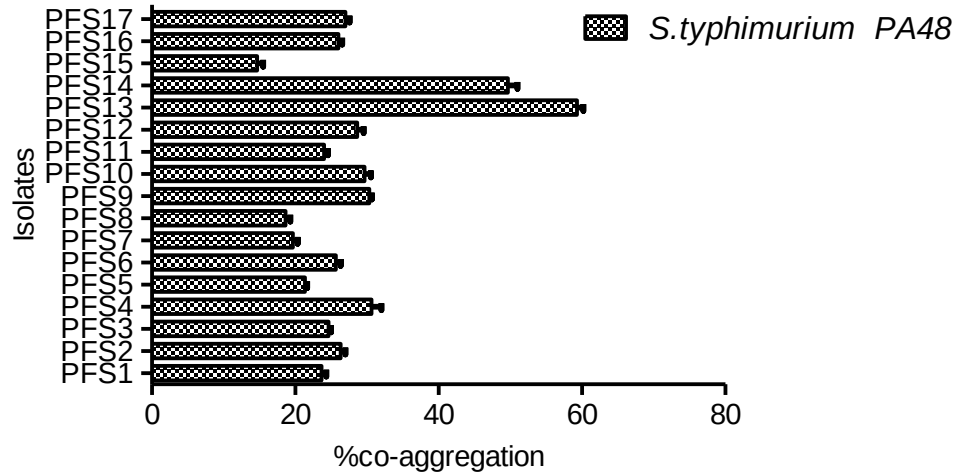

Figure 18: Co-aggregation Analysis of 17 LAB isolates with *S. typhimurium* PA48

4.4.1.2 Co-aggregation assay of LAB strains with *S. enteritidis*

All 17 LAB isolates were further evaluated for co-aggregation assay with three *S. enteritidis* strains i.e. *S. enteritidtis* PA51, *S. enteritidis* PA71, *S. enteritidis* PA76.

LAB isolates PFS2, PFS8, PSF12, and PSF15 showed least % co-aggregation with *S. enteritidis* PA51, whereas PFS13, PFS14 showed remarkable % co-aggregation of *S. enteritidis* PA51 i.e. 57% and 45% respectively (fig.19).

PFS2, PFS7, PFS17 showed least % co-aggregation values with *S. enteritidis* PA71, whereas PFS4, PFS9, PFS 13 recorded high co-aggregation values with *S. enteritidis* PA71 i.e. 38%, 42% and58% respectively (fig. 20).

PFS3, PFS7, PFS10, PFS11, PFS16 showed least % co-aggregation values with *S. enteritidis* PA76 whereas PFS4, PFS9, PFS13 and PFS14 showed highest % co-aggregation with *S. enteritidis* PA76 i.e. 42%, 59%, 60% and 42% (fig.21).

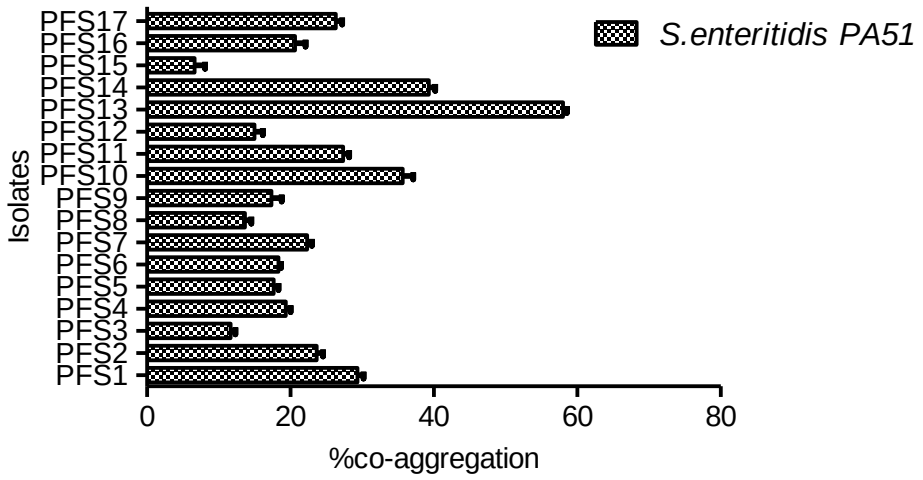

Figure 19: Co-aggregation Analysis of 17 LAB isolates against *S. enteritidis* PA51

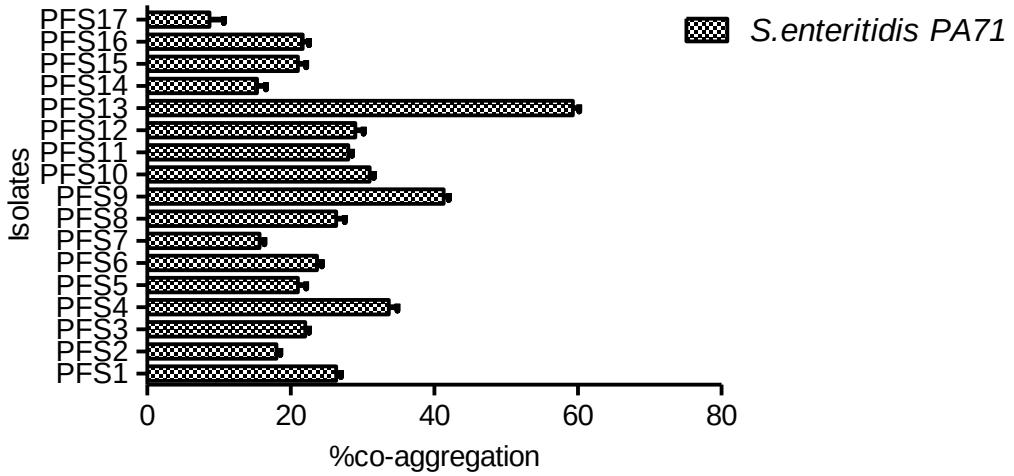

Figure 20: Co-aggregation Analysis of 17 LAB isolates against *S. enteritidis* PA71

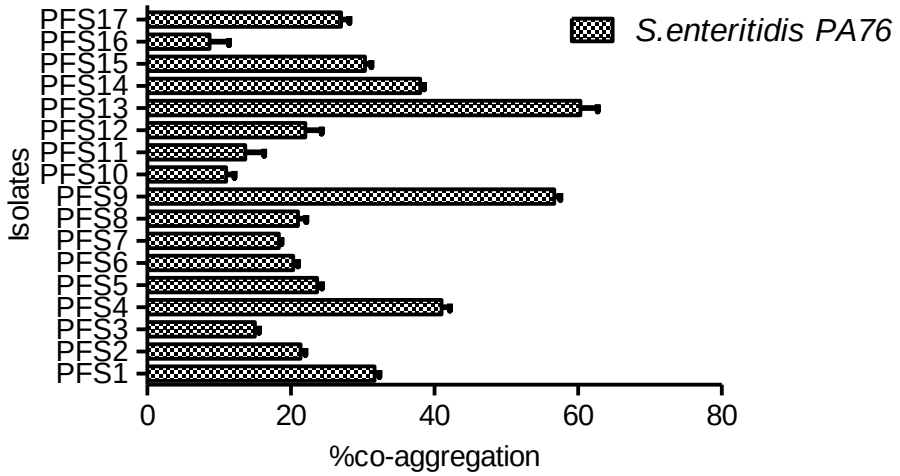

Figure 21: Co-aggregation Analysis of 17 LAB isolates against *S. enteritidis* PA76

On the basis of highest % co-aggregation values of LAB strains with of *S.enterica* strains. *Lactobacillus reuterii* PFS4, *Lactobacillus reuterii* PFS9, *Enterococcus faecium* PFS13 and *Enterococcus faecium* PFS14 were furthur evaluated to control *Salmonella* infection from poultry. Co-aggregation assay of these four selected strains were also performed with *S.typhimurium* PA 40 and *S.enteritidis* PA76 as these pathogens were least resistant to antibiotics as they were

resistance to 3rd generation cephlosporins and these selected strains showed highest % co-aggregation values with these two pathogens as well. Combination of LAB strains significantly show % co-aggregation values with *S. enteritidis* upto 68% whereas 45% co-aggregation recorded with *S. typhimurium* (fig.22). So our strains showed promising results with *S. enterica.*

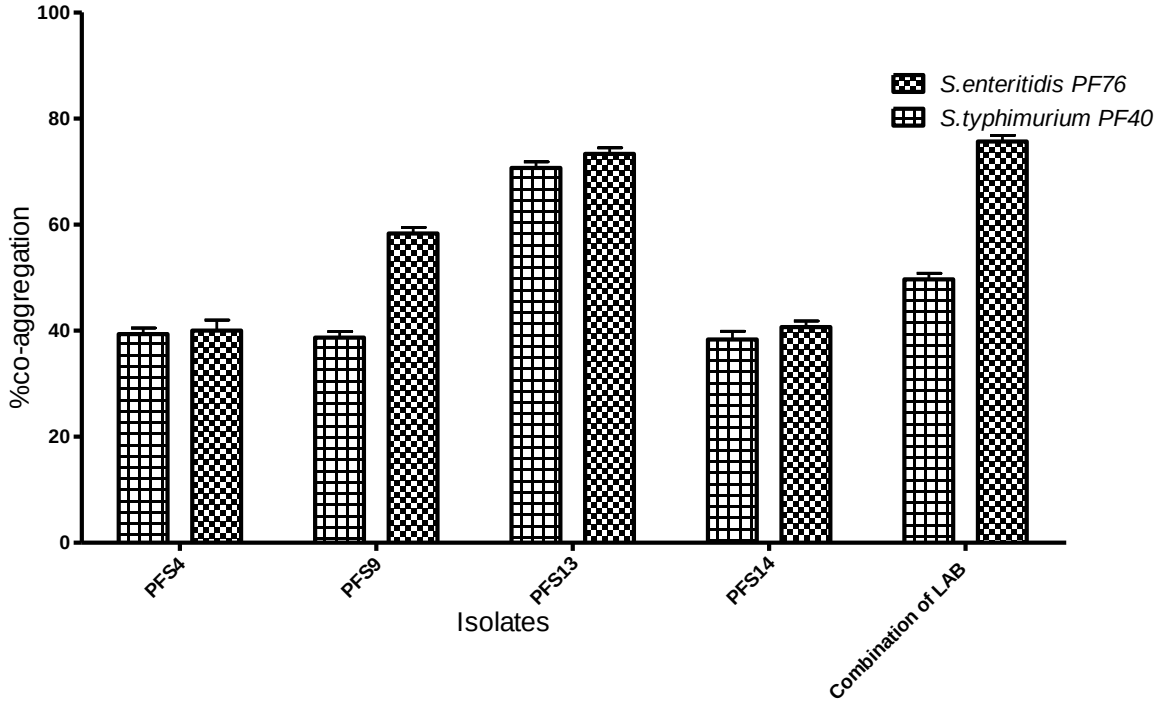

Figure 22: Comparative Co-aggregation of Selected LAB isolates with selected *S. enterica* strains

4.4.2 Cell Surface Hydrophobicity Assay

Four selected LAB strains and combined LAB strains were investigated for cell surface hydrophobicity assay. All selected strains exhibited high hydrophobicity with all the three hydrocarbons i.e. chloroform, xylene and toluene. Notably highest hydrophobicity disposition showed by PFS9 and combined LAB strains among other strains. The percent hydrophobicity values of *Lactobacillus reuteri* PFS4, *Lactobacillus reuteri* PFS9, *Enterococcus faecium* PFS13,

Enterococcus faecium PFS14 with chloroform were 79%, 87%, 74%, 82% respectively indicates that presence of exopolysaccharide around the bacterial membrane for the survival of bacterial strains in GIT tract (fig. 23).

Additionally, hydrophobic interactions of LAB strains with xylene: PFS4 56%, PFS9 87%, PFS13 54%, PFS14 60%, indicates that *Lactobacillus reuteri* PFS4, *Lactobacillus reuteri* PFS9 are hydrophobic in nature whereas, *Enterococcus faecium* PFS14 and *Enterococcus faecium* PFS13 are amphiphilic. Hydrophobic interaction of PFS4, PFS9, PFS13and PFS14 with toluene were 56%, 87%, 84%, 60% and 84% respectively.

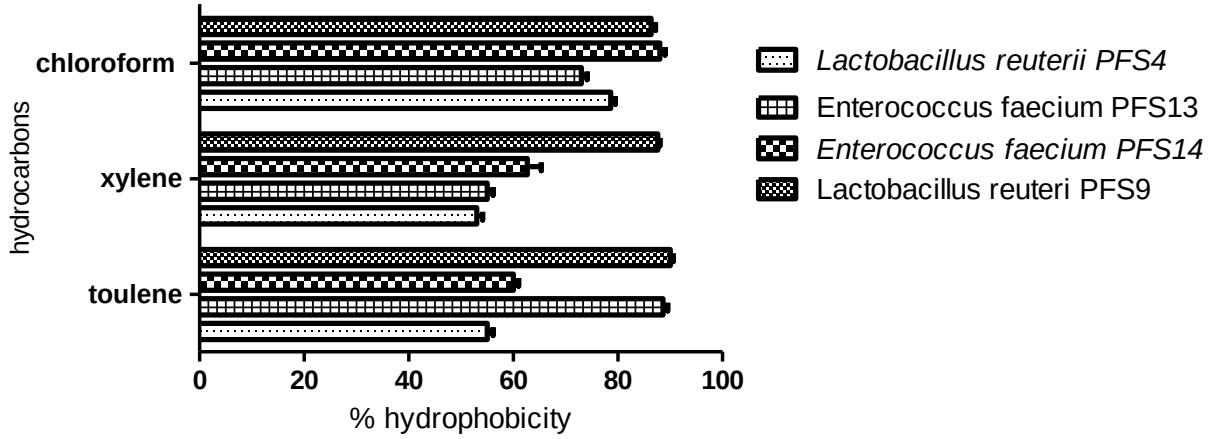

Figure 23: Cell surface hydrophobicity assay of selected LAB strains using three organic solvents

4.4.3 Time Kill Assay

Time kill assay was illustrated the reduction of cell count of *S. enterica* (*S. typhimurium* and *S. enteritidis*) in the presence of cell free supernatants of each LAB strains (PFS4, PFS9, PFS13, PFS14) covering the 2-3 segments of different incubation period (0hr, 4hr, 8hr, 16hr and 24hrs) . The reduction activity was clearly shown in both *S. enteritidis* and *S. typhimurium* by all selected strains especially by the combination of LAB strains (fig.24). At 8 hours more than 50% optical

densities were observed, whereas after 16hrs, the decrease in optical densities indicates the unavailability of nutrients to pathogenic strains and utilization of nutrients by LAB strains in *S. enteritidis* and *S. typhimurium* (fig. 25).

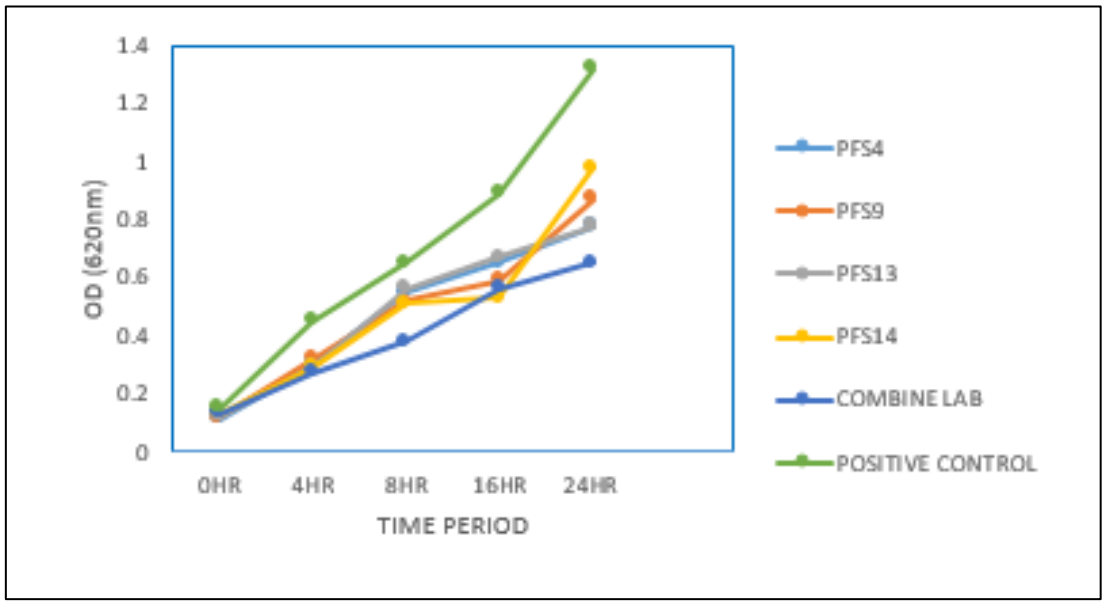

Figure 24: Comparative time kill assay analysis of selected LAB strains against *S. enteritidis PF76*

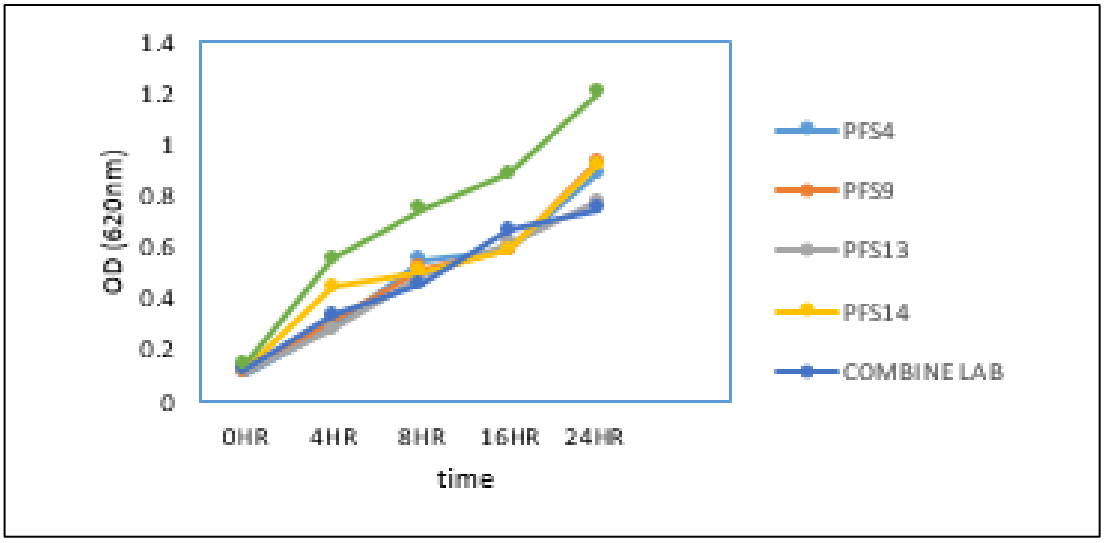

Figure 25: Comparative time kill assay analysis of LAB strains against *S. typhimuriumPF40*

4.4.4 Mucin adhesion Assay

Mucin adhesion assay was performed to check the adhesion and competitive inhibition of *S. enteritidis* and *S. typhimurium* (fig. 26). Adhesion to mucin is essential requirement for the colonization. Four selected strains were tested on porcine mucin to evaluate the % adhesion competitive inhibition. On average all four selected isolates showed remarkable competitive inhibition against *S. enteritidis* as compared to *S. typhimurium* i.e. 60% and 55.7% respectively (fig. 27). Moreover, competitive inhibition of combined LAB strains were less as compared to individual strains indicated that LAB strains not only compete with pathogenic strains but they also compete with other LAB strains for the adhesion to the epithelium receptors.

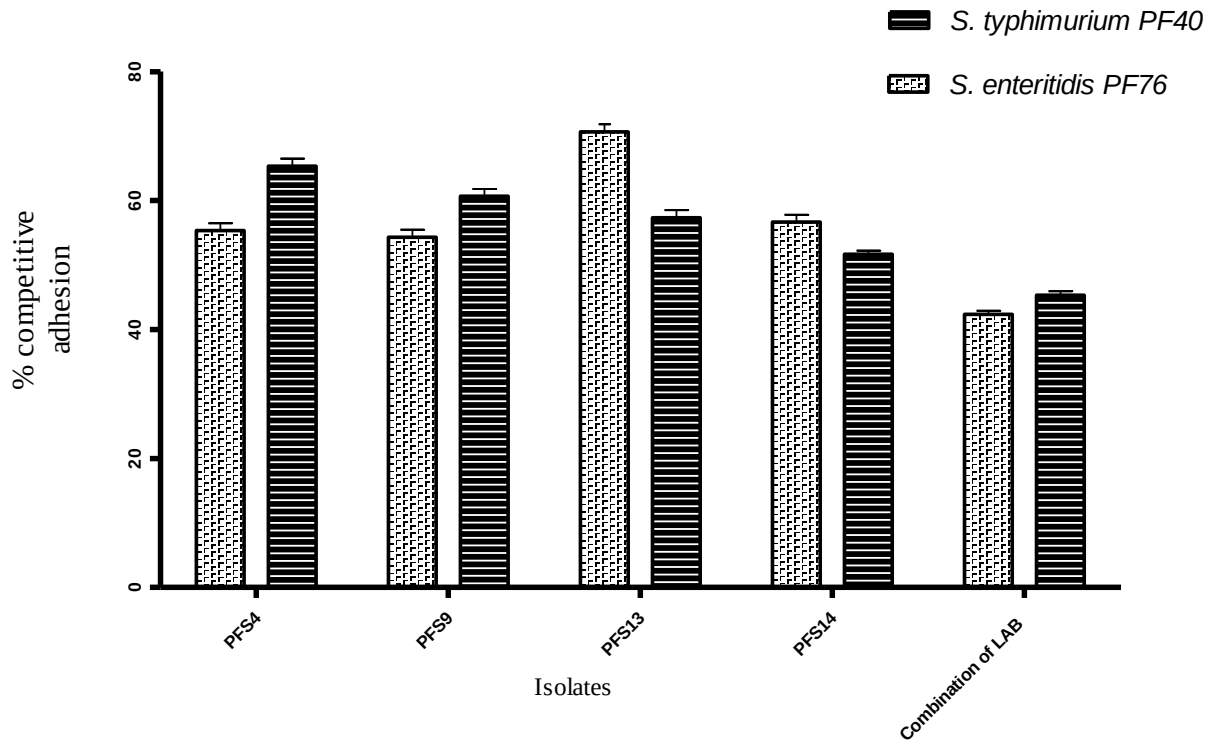

Figure 26: Competitive inhibition analysis of selected LAB strains against selected *S. enterica* strains on porcine mucin Ⅲ

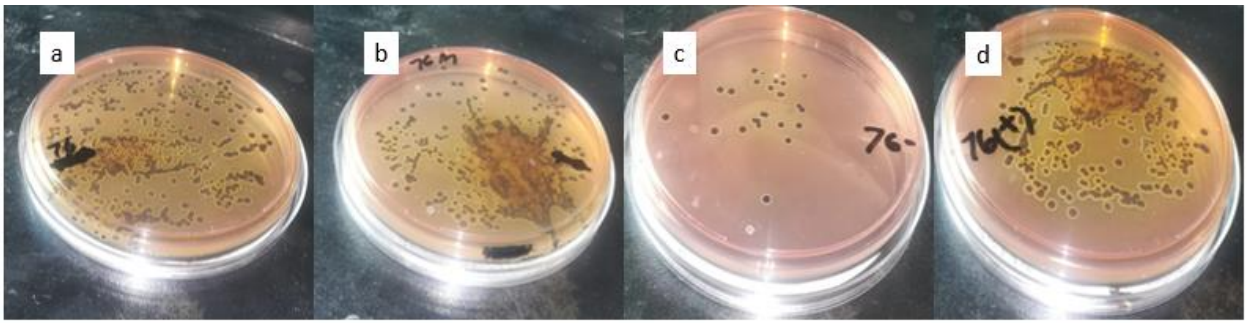

Figure 27: a) positive control *S. enteritidis* PA76 b) competitive adhesion of combined LAB strains treated with *S. enteritidis* PA76 c) competitive adhesion of PFS 13 against *S. enteritidis* PA76 d) competitive adhesion of PFS 9 against *S. enteritidis* PA76

4.5 GENOTYPIC IDENTIFICATION OF LAB STRAINS

4.5.1 Amplification of 16S rDNA through PCR

PCR product of 16S rDNA was 1200 bps approximately. It was amplified by using primers 9F and 1510R. PCR product was observed on agarose gel because of electrophoretic separation. A fragment of 1200 bps was observed for all the samples (fig.23).

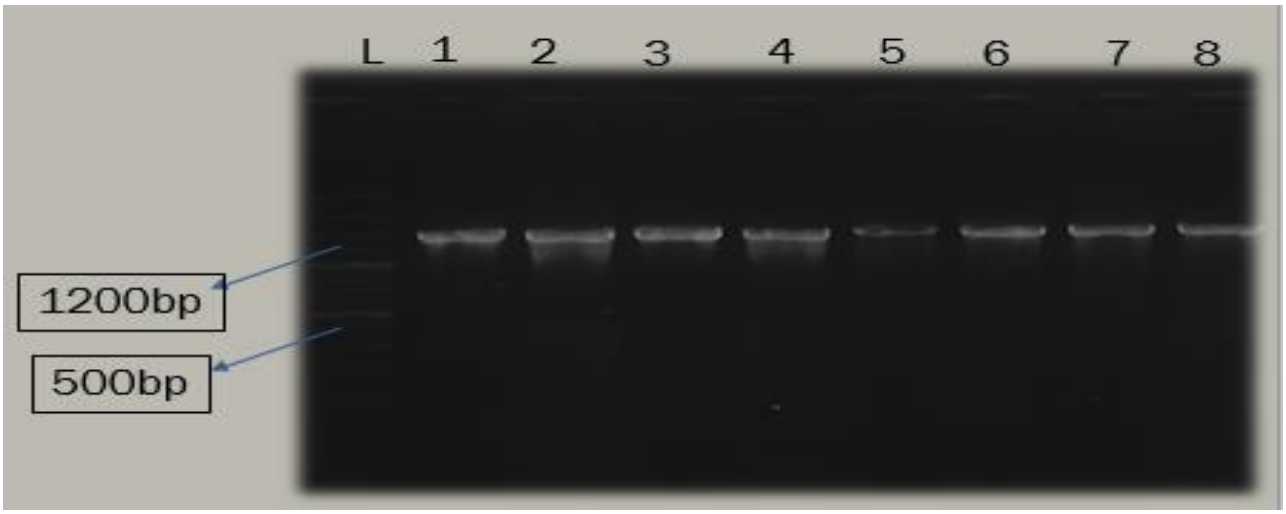

Figure 28: The confirmation of 16S rDNA amplification as reveled by the presence of 1200bp fragment on 1% agarose gel.
L: ladder, 1: PFS4, 2:PFS9, 3: PFS13, 4: PFS14, 5:PFS11, 6: PFS2, 7: PFS 15, 8: PFS8

PCR purified products of LAB isolates were delivered for sequencing to Eurofin USA. All isolates results were retrieved by making consensus sequence. The consensus sequence of all strains is approximately 500 to 1120 bps. All the consensus sequence were subjected to blast for strain identification.

Table 8: The results of nucleotide similarity for 16S rDNA sequences for specie level identification

Isolate.no	Identification after MEGA BLAST	Sequence size	Similarity
PFS4	*L. reuteri*	520	100%
PFS9	*L. reuteri*	597	100%
PFS11	*Enterococcus lactis*	1136	100%
PFS14	*Enterococcus faecium*	635	100%
PFS13	*Enterococcus faecium*	1100	100%

Chapter 5

DISCUSSION

Lactic acid bacteria are usually part of healthy gut flora of animals .The diversity of poultry gut mainly composed of pathogenic microbes as well as beneficial bacterium that's have shielding and protective effect against pathogens. The purpose of the present study was to evaluate and identify the indigenous Lactic *acid bacteria that* has the potential to control non typhoidal *Salmonella enterica* serovars *S. enteritidis* and *S. typhimurium* which was already isolated from disease poultry feces. In the present study, we have isolated, identified and characterized potential *LAB strains* from poultry feces.17 strains were isolated among that 4 best potential isolates were chosen for the further *in vitro* anti-*Salmonella* testing against *Salmonella enterica serovars S. typhimurium* and *S. enteritidis.* Strains selected were *L. reuteri* PFS4, *L. reuteri* PFS9, *Enterococcus faecium* PFS14 and *Enterococcus faecium* PFS13, which correlates with the previous studies that's same species identified but from different sources like raw shrimps i.e. *Lactobacillus reuteri* isolated from cattle feces (Adewale *et al.,* 2018; Duar *et al.,* 2017), *Enterococcus faecium* isolated from poultry samples (Stępien-Pysniak *et al.,* 2016) and *Enterococcus lactis* isolated from raw shrimps (Braiek *et al.*, 2018).

In the first phase of the study, the initial screening of the isolates was done towards lysozyme, acid and bile salts which are the imitators of gastrointestinal track of the poultry. The pH of the gizzard and crop is 2 and the probiotics should be able to survive in a pH as low as 2. To evaluate the GIT related stress tolerance of the LAB strains, acid, bile, phenol and lysozyme tolerance tests

were carried out. Firstly, the survival of the isolates was checked at pH 2. All isolated strains survived in acidic conditions specially *Lactobacillus sp.* as they are resident of gut flora especially gizzard region, so tolerance of *Lactobacilli* was higher as compared to *Enterococcus* species, as E*nterococcus* mostly found in caeca region of the poultry gut. So the different trends of *Lactobacillus* and *Enterococcus sp*. survival in low pH attributed towards the great genetic diversity as a consequence of differentiation at specie level.

In some case the resistance to bile salts by LAB strains are generally intrinsic and also depends on genetic variability (Ruiz *et al.*, 2013). Results showed that acid and bile have separate effects on the growth of bacteria as we know that bile stress takes place after pH stress in the stomach, so a probiotic candidate must be able to tolerate both. The results of bile tolerance of *Enterococcus sp*. was comparable with *Lactobacilli sp*. For phenol tolerance, gut microflora have the potential to de-aminate aromatic amino acids. These aromatic amino acids are acquired from dietary proteins and can result in the formation of phenols. These phenolic compounds can inhibit the growth of the probiotics in the gastrointestinal tract. Therefore, it is important for the probiotics to show resistance to phenol in order to survive in the GIT. The result suggests that all the isolated strains can tolerate the stress to phenols and this phenol resistance of the isolates can also be exploited such that these phenol resistant probiotics can be used in functional foods rich in proteins with aromatic amino acids. To analyze the ability of strains to survive into the oral cavity, Lysozyme resistance test was also evaluated. Isolates that maintained their survival rate at least 80% of their growth rate after 90mins of treatment with lysozyme were considered for further testing (Turchi *et al*., 2013). All 17 strains were considered for assessment after giving good results in GIT related stress tolerance.

In the second phase, safety of LAB strains were evaluated on the basis of DNase, hemolytic and antibiotic susceptibility assay. None of the strains exhibited hemolysis and DNase activity. It means the selected isolates do not exhibit apparent pathogenicity, whereas in accordance to previous studies the probiotics especially *Lactobacilli sp.* showed antibiotic resistance pattern against 3rd but this resistance is not transferable to another organism (Abriouel *et al.,* 2015).

LAB strains were further evaluated anti-*Salmonella* activity to flush out the pathogen from the gut region to reduce the disease burden and helps in the assessment of colonization of probiotics in poultry (Shahbazi *et al.,* 2016). Microbial cell surface adhesion, competitive exclusion, adhesion to mucosal membranes or mucin protein and competition for the nutrients or survival in limiting nutrients are determinant key factors which helps in decreasing the pathogen load in the intestine and crop region (Robyn *et al.,* 2012). The electrostatic and other small forces helps in the strong interaction, attachment and adhesion of cells to their respective receptors. Confined growth of microbes to the epithelium surface mostly determined by strong associations between surface charges and hydrophobicity of cell surface. The presence of (glycol) proteinaceous compound showed higher hydrophobicity to survive in gastro intestinal tract, whereas a hydrophilic surface was linked with hydrophilic environment of the intestinal region. Lipoteichoic, teichoic acids and other outer cell wall elements might have an effect on hydrophobicity as well, but it is unclear. (Abdulla *et al.,* 2014). Bacterial adhesion to hydrocarbons especially xylene reflects the hydrophobicity of the bacterial strains. When % hydrophobicity value is <33%, bacterial strain is hydrophilic,>66% indicates that strains are hydrophobic. So, in present study, affinity of hydrocarbons (chloroform, toluene and xylene) were assessed against selected probiotics strains. The highest affinity was recorded against chloroform and toluene which indicates the presence of proteineous materials on the cell surfaces of the selected strains as compared to xylene. Our results

of *L. reuteri* and *Enterococcus faecium* were in line and comparable with the previous results where strains were *L. fermentum, L. casei* and *L. rhamnosus* against *S. enterica* (*R*okana *et al.,* 2018).

All LAB strains showed deposition in co-aggregation assay with non typhoidal *Salmonell*a, whereas *L. reuteri* PFS4, *L. reuteri* PFS9, *Enterococcus fecium* PFS14 and *Enterococcus faecium* PFS13 exhibited highest % co-aggregation with *S. enteritidis* and *S. typhimurium*. The results were significantly related and associated with previous studies (Gomez *et al.,* 2016).

Mucin adhesion is correlated with co-aggregation and cell surface hydrophobicity. The capacity for adherence to components of the mucosa and to avoid rapid exclusion from a beneficial environment must be a high-priority task for an intestinal organism (Jonsson *et al.,* 2001). Mucin adhesion competition assay of selected strains resulted from 42.5% to 69.9 % against non typhoidal *Salmonella* species, which is significant and comparable with the mucin adhesion of L. *plantarum* against *Salmonella* pathogen (Dhanani *et al.,* 2013*;* Tallon *et al.,* 2007*).* Antimicrobial activity of selected LAB strain was evaluated individually and in combination revealed promising results against *S. enteritidis* and *S. typhimurium.* Interestingly, *L. reuteri* PFS9 and combination of LAB strains in this study were capable to inhibit the growth of both tested non typhoidal *Salmonella* strains completely between 8hrs to 16hrs. Hence this proves the ability of LAB strains to survive in the limited nutrients and to flush out the pathogens efficiently. Several authors also demonstrated the strong inhibition of non typhoidal *Salmonella* by co-culture activities of LAB strains (Lima *et al.,* 2007*;* Nakphaichit *et al.,* 2019). Our results implies with previous results.

Isolated LAB strains have the ability to reduce the non typhoidal *Salmonella enterica in-vitro* testing, therefore these four selected strains *L. reuteri* PFS4, *L. reuteri* PFS9, *Enterococcus*

faecium PFS13 *and Enterococcus faecium* PFS14 will be further evaluated by *in vivo* trials for the better results.

CHAPTER 6

CONCLUSION AND FUTURE PROSPECTS

The current study indicated that all of the indigenous four strains are safe and have the potential probiotic characteristics. Isolation of *Lactobacillus and Enterococcus* strains with significant ability against non typhoidal *Salmonella enterica* serovars *S. enteritidis* and *S. typhimurium* demonstrate poultry dropping as a good reservoir for potential *Lactobacillus and Enterococcus sp.* The strains were capable to colonize in the harsh environment of the GIT i.e. crop and caecum region and did not show any DNase or hemolytic. Further cell surface hydrophobicity, co aggregation assay indicates that selected strains found in different region of poultry gut and in combination of these LAB strains have the ability to adhere, compete and restrict the growth of *Salmonella enterica*. Therefore, these strains can be used as feed additives, growth promotors and alternative to antibiotics commonly used in the poultry industry which can be confirmed by in vivo trials.

Further we can go for whole genome sequencing for their complete characterization. On the basis of whole genome, we can predict their possible mechanism for the reduction of MDR non typhoidal *Salmonella enterica.* Additionally, these strains can be assessed for their potential use in human health. Safety, adhesion, mucus binding and immuno-modulation can be further evaluated on cell lines.

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