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Abstract

Foodborne *Salmonella* continues to be a major threat for public health, especially from poultry origin. In recent years, an increasing trend of antimicrobial resistance (AMR) in *Salmonella* sp. was noticed due to the misuse of antimicrobials. To find alternatives to this emerging problem, probiotics, particularly lactobacilli, has been proposed. Since data on *Salmonella* in the Lebanese poultry industry is scarce, this study was conducted to determine the prevalence of *Salmonella* at different stages of the broiler production chain and layer flocks in addition to their antibiotic resistance profile and molecular patterns. In addition, the probiotic activity of native poultry-derived *Lactobacillus* strains was tested against the most relevant and drug resistant *Salmonella* sp. Screening of *Lactobacillus* strains for anti-*Salmonella* activity, safety such as antibio-resistance and surface probiotic properties was also done.

Over a period of 3 years, feces samples were collected by a sock method from local Lebanese farms (n=237), while poultry meat was collected from slaughterhouses (n=134) and retail (n=1907). In parallel, ceca (n=115) and neck skins (n=115) were collected from two major slaughter plants. The results highlighted a high prevalence of *Salmonella* in poultry. Considering all samples together, a large diversity of serotypes was identified with predominance among *Salmonella* Infantis (32.9%), *Salmonella* Enteritidis (28.4%) and *Salmonella* Kentucky (21.4%) with high AMR and multi-drug resistance (MDR) in all *Salmonella* isolates. The most prominent resistance was found in nine strains of *S.* Kentucky CIP^R resistant to Extended Spectrum Cephalosporin (ESCs). These strains were genetically characterized by Whole genome sequencing (WGS). The results showed, for the first time in Lebanon, a case of detection and dissemination of the emerging highly drug resistant *S.* Kentucky ST198. Comparing *S.* Enteritidis strains from poultry and humans using PFGE, the results indicated that one persistent clone of *S.* Enteritidis (80% of the strains) is common between poultry and humans in Lebanon. Similar genomic profiles and antimicrobial resistance phenotypes were detected between farms, slaughterhouses and retail suggesting the circulation and transmission of identical clones throughout the food chain and layer flocks.

Results of screening for potential probiotics, four *Lactobacillus* species have been identified as: *L. reuteri* (n= 22, 44 %), *L. salivarius* (n=20, 40 %), *L. fermentum* (n= 2, 4 %) and *L. crispatus* (n=1, 2 %) and two *Enterococcus fecalis* (n=2, 4 %). Eight *Lactobacillus* were chosen depending on their cell surface hydrophobicity capacity and auto/co-aggregation ability for further adhesion assay using Caco-2 cells line. Attachment of the *Lactobacillus* strains varied from 0.53 to 10.78 %. *L. salivarius* A30/i26 and 16/c6 and *L. reuteri* 1/c24 showing the highest adhesion capacity were assessed for their ability to compete and exclude the pathogen for the adhesion site on the caco-2 cell line. *L. salivarius* 16/c6 highly excludes the three *Salmonella* serotypes from adhesion at significant levels.

Résumé

Les salmonelles d'origine alimentaire continuent de représenter une menace majeure pour la santé publique, en particulier celles d'origine avicole. Ces dernières années, une tendance à la hausse de la résistance aux antimicrobiens (AMR) chez les salmonelles a été remarqué en raison de la mauvaise utilisation des antibiotiques. Pour trouver des alternatives à ce problème émergent, des probiotiques, en particulier les lactobacilles ont été proposés. Les données sur les salmonelles dans l'industrie avicole libanaise étant rares, cette étude a été menée pour déterminer la prévalence des salmonelles à différents stades de la chaîne de production des poulets de chair et de poules pondeuses, l'antibiorésistance et leurs profils moléculaires. En outre, l'activité probiotique de souches aviaires de *Lactobacillus* indigènes a été testée contre les salmonelles. Le criblage de l'activité anti-salmonelle, de l'innocuité notamment de l'antibiorésistance, et des propriétés probiotiques de surface des souches de lactobacilles ont également été effectué.

Sur une période de 3 ans, les échantillons de matières fécales ont été collectés par la méthode de la pédichiffonnette dans des fermes libanaises locales (n = 237), tandis que la viande de volaille a été collectée dans des abattoirs (n = 134) et sur le marché (n = 1907). En parallèle, des échantillons de caeca (n = 115) et de peaux de cou (n = 115) ont été collectés dans deux grands abattoirs. Les résultats ont mis en évidence une forte prévalence de *Salmonella* chez les volailles. En tenant compte de tous les échantillons, une grande diversité de sérotypes a été identifiée, avec une prédominance de *Salmonella* Infantis (32,9 %), *Salmonella* Enteritidis (28,4 %) et *Salmonella* Kentucky (21,4 %) avec une antibiorésistance élevée dans tous les isolats de *Salmonella*. La résistance la plus importante a été observée chez neuf souches de *S.* Kentucky résistantes à la ciprofloxacine (CIP^R) et à la céphalosporine à spectre étendu (ESC). Ces souches ont été génétiquement caractérisées par séquençage du génome entier (WGS). Les résultats ont montré, pour la première fois au Liban, un cas de détection et de dissémination du *S.* Kentucky ST198 hautement résistant. La méthode PFGE a montré la présence d'un clone persistant de *S.* Enteritidis (80% des souches) commun entre les souches aviaires et humaines. Des profils génomiques ainsi que des phénotypes de résistance aux antimicrobiens similaires ont été détectés entre les fermes, les abattoirs et le marché, suggérant la circulation et la transmission de clones identiques tout au long de la chaîne alimentaire et des poules pondeuses.

Les résultats du criblage des probiotiques potentiels montrent que quatre espèces de *Lactobacillus* ont été identifiées : *L. reuteri* (n = 22, 44%), *L. salivarius* (n = 20, 40 %), *L. fermentum* (n = 2, 4 %) et *L. crispatus* (n = 1, 2%) et deux *Enterococcus fecalis* (n=2, 4 %). Huit lactobacilles ont été choisis en fonction de leur capacité d'hydrophobicité et d'auto/co-agrégation, pour un test ultérieur d'adhérence sur la lignée cellulaire caco-2. L'attachement des souches de lactobacilles variait de 0,53 à 10,78%. *L. salivarius* A30 / i26 et 16 / c6 et *L. reuteri* 1 / c24 présentant la capacité d'adhérence la plus élevée ont été évalués pour leur aptitude à rivaliser et à exclure l'agent pathogène du site d'adhésion sur la lignée cellulaire caco-2. Il a été démontré que *L. salivarius* 16 / c6 excluait fortement l'adhésion des trois sérotypes de *Salmonella* à des niveaux significatifs.

DEDICATION

To my soul mate and life companion, my dearest husband Elie who endured all the hard and the good times with me.

To the joy of my life, my children, Emmanuelle, Anna-Maria and Georges.

From all of my heart, to my dearest parents, sisters, Joud, Ramia, and Jeanne, and my brother Youssef.

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Table of Contents

List of Figures i

List of Tables iii

List of Abbreviations iv

Introduction 1

Chapter I Bibliographical review 7

1. *Salmonella* 8

1.1. Taxonomy and nomenclature 8

1.2. Adaptation capacity 9

1.3. Pathogenesis and virulence 9

1.3.1. Local inflammatory response 11

1.4. Non-Typhoidal *Salmonella,* a public health concern 12

1.5. *Salmonella* and poultry 14

1.5.1. Poultry production 14

1.5.2. *Salmonella* mode of transmission and pathogenesis 14

1.5.3. *Salmonella* serotypes in poultry 16

1.5.3.1. Serotypes shift 16

1.5.3.2. Factors affecting the dissemination and persistence of specific serotypes 18

1.6. Molecular genotyping 19

1.6.1. Pulse Field Gel Electrophoresis (PFGE) 19

1.6.2. Whole genome sequencing (WGS) 20

1.7. *Salmonella* control at farm level 20

1.7.1. Serotype-specific control programs 21

1.7.2. Vaccination program 22

1.7.3. Antibiotic usage in farms 22

1.8. Antibiotic Resistance, the biggest global threat 24

1.8.1. Antibiotic-resistance Mechanisms 25

1.8.2. Key antibiotic classes, resistance mechanisms with related genes 26

1.8.2.1. β-lactams 27

1.8.2.2. Aminoglycosides: 27

1.8.2.3. Quinolones and fluoroquinolones: 28
1.8.3. Global strategies against AMR 31
2. Promising natural alternative: probiotics 32
2.1. Origin 32
2.2. Types of probiotics 32
2.3. Lactic acid bacteria (LAB) as probiotic: focus on *Lactobacillus* 33
2.3.1. Classification of LAB 33
2.4. *Lactobacillus* classification 35
2.4.1. *Lactobacillus* Niche-Specific Adaptation: The Intestinal Environment 35
2.5. Gut microbiota, probiotics of poultry origin 36
2.6. Anti- *Salmonella* activities 37
2.6.1. Adherence 39
2.6.2. Competition use of nutrients 40
2.6.3. Secretion of active metabolites against *Salmonella* 40
2.6.4. Maintenance of Epithelial Barrier Function. 41
2.6.5. Immunomodulation. 41
2.7. Screening of potential probiotics 42
2.7.1. Safety criteria 43
2.7.1.1. Probiotic identification 43
2.7.1.2. Antimicrobial resistance 43
2.7.2. *In vitro* assays 44
2.7.3. *In vivo* experiments 45
References 46
Chapter II Prevalence, antibiotic resistance and molecular characterization of *Salmonella* serotypes in the Lebanese poultry production 67
Abstract 69
1. Introduction 70
2. Materials and Methods 71
2.1. Sample collection 71
2.1.1. Farm sample collection 71
2.1.2. Processing plant sample collection 71

2.1.3. Poultry meat sample collection 72
2.1.4. Avian and Human Salmonella isolates collection 72
2.2. *Salmonella* isolation and identification 73
2.3. Antimicrobial susceptibility testing 73
2.4. Pulse Field Gel Electrophoresis- PFGE 74
2.5. Statistical Analysis 74
3. Results 74
3.1. Prevalence of *Salmonella* throughout the broiler food chain and laying hen flocks..... 74
3.2. Distribution of *Salmonella* serotypes 76
3.3. Antimicrobial resistance phenotypes 78
3.4. Pulse-Field- Gel Electrophorese (PFGE) 86
4. Discussion..... 90
5. Conclusion 94
References 95
Chapter III_Genomic characterization of Extended-Spectrum β Lactamases (ESBLs) and cephamycinase-producing *Salmonella* Kentucky ST198 in Lebanese broiler production 102
1. Introduction 104
2. Materials and methods 106
2.1. Collection of *Salmonella* Kentucky strains 106
2.2. Antimicrobial sensitivity test 106
2.3. Genome analyses 107
2.4. Phylogenomics 108
3. Results 108
3.1. Multi-Locus Sequence typing (MLST) and detection of plasmids and replicon type (pMLST)..... 108
3.2. Phenotypic and Genotypic antimicrobial resistance and presence of SGI1-K..... 110
3.3. Detection of Insertion Sequence IS*ECP1* 112
3.4. *Salmonella* Pathogenicity Islands and Virulence genes analysis 112
3.5. Phylogenetic Single Nucleotide Polymorphism (SNP) analysis 115
4. Discussion 116

5. Conclusion 119

6. Supplementary Data 120

References 120

Chapter IV Detection of native potential probiotics *Lactobacillus* sp. against *Salmonella* Enteritidis, *Salmonella* Infantis and *Salmonella* Kentucky ST198 of Lebanese chicken origin 128

Abstract 130

1. Introduction 131

2. Materials and methods 133

2.1. Isolation and phenotypic characterization of *Lactobacillus* sp. 133

2.2. *Salmonella* isolates 133

2.3. Assessment of *Lactobacillus* antagonism 134

2.4. Selection of strains depending on their phenotypic aggregation 135

2.5. Species Identification and phylogenetic relations 135

2.6. Antibiotic susceptibility testing 136

2.7. Cell surface properties 136

2.7.1. Auto-aggregation and co-aggregation Assay 136

2.7.2. Hydrophobicity assay 137

2.8. Tolerance to simulated gastrointestinal conditions 138

2.9. Cell Culture 138

2.9.1. Preparation of cell culture 138

2.9.2. Adhesion to Caco-2 cells 139

2.9.3. Inhibition of *Salmonella* adhesion to Caco-2 cell 139

2.10. Co-culture Kinetic study 140

2.11. Statistical Analysis 141

3. Results 141

3.1. Screening of *Lactobacillus* sp. from poultry origin and anti-*Salmonella* activity 141

3.2. Visual aggregation screening 141

3.3. Phenotypic and genotypic identification of *Lactobacillus* isolates with Phylogenetic relatedness. 142

3.4. Antimicrobial resistance 144

3.5. Surface properties assays 145
3.6. Hydrophobicity and auto/co-aggregation correlation 147
3.7. Gastrointestinal tolerance assay 150
3.8. Adhesion Assay 150
3.9. Competition/ Exclusion Assay 152
3.10. Co-culture kinetics 153
4. Discussion 156
5. Conclusion 161
References 162
Conclusion and Perspectives 171
ANNEXES 174

List of Figures

Figure 1: *Salmonella*, the host and its microbiota (Thiennimitr et al., 2012). 12
Figure 2: MoPH PulseNet report, 2015 (unpublished data) 13
Figure 3: Overview of carbohydrate fermentation lactic acid bacteria (Gänzle, 2015). 34
Figure 4: Different mode of action of probiotics against *Salmonella* infection in poultry (Sherman et al., 2009) 39
Figure 5: Strategy for selection of potential probiotics to control *Salmonella* in poultry 42
Figure 6: Percentage of antimicrobial resistance of S. Enteritidis (A), S. Infantis (B) and S. Kentucky (C) from farms, slaughterhouses and retail. The code of antibiotics are: ampicillin (Amp), amoxicillin-clavulanic acid (Amc), piperacillin-tazobactam (Tzp), cefalothin (Kf), cefuroxime (Cxm), cefoxitin (Fox), cefotaxime (Ctx), ceftriaxone (Cro), ceftazidime (Caz), ceftiofur (Eft), cefepime (Fep), imipenem (Imp), gentamycin (Cn), tobramycin (Tob), streptomycin (S), amikacin(Ak), netilmycin (Net), nalidixic acid (Na), ciprofloxacin (Cip), norfloxacin (Nor), trimethoprim (W), trimethoprim-sulfamethoxazole (Sxt), aztreonam (Atm), tetracycline (Te), chloramphenicol (C), enrofloxacin (Enr). 85
Figure 7: Macrorestriction patterns of *S.* Kentucky using the Dice coefficient, and the dendrograms were generated graphically by using unweighted pair group method with arithmetic mean (UPGMA). The codes A, B and K designate the *Salmonella i*solates from slaughterhouse A, slaughterhouse B and retail respectively. The letters C or Q are related to caeca or neck skin respectively. 87
Figure 8: Macrorestriction patterns of *S.* Infantis using the Dice coefficient, and the dendrograms were generated graphically by using unweighted pair group method with arithmetic mean (UPGMA). The code A, B and I designate the *Salmonella* isolates from slaughterhouse A, slaughterhouse B and retail. The letters C or Q are related to caeca or neck skin respectively. .. 88
Figure 9: Macrorestriction patterns of *S.* Enteritidis using the Dice coefficient, and the dendrograms were generated graphically by using unweighted pair group method with arithmetic mean (UPGMA). The code P designate the *Salmonella* isolates. 89
Figure 10: Virulence determinants of the eight Lebanese S. Kentucky isolates, based on the protein sequences of Salmonella sp. database 114
Figure 11: SNP-based Phylogenetic tree of the eight Lebanese Cip ^{R} *S.* Kentucky isolates with *S.* Kentucky CVM29188 as reference genome 115
Figure 12: Evolutionary relationships Tree of *Lactobacillus* sp by the Neighbor-Joining method. The percentage of replicate trees in which the associated species clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). *L.reuteri* KX688655.1, *L. salivarius* MG737855.1, *L. fermentum* KC113207.1, *L. cripatus* MH392998.1, and *Enterococcus fecalis* MK584170.1 were selected as reference sequences. 143
Figure 13: Antimicrobial resistance of the indigenous *Lactobacillus* sp isolated from antibiotic-free (Black columns) and antibiotic-treated broilers (Grey columns). White columns correspond to the total percentage of resistance. Ampicillin (Amp), chloramphenicol (C), erythromycin (Ery), kanamycin (K), gentamycin (Cn) and streptomycin (S). 144

Figure 14: Isolates distribution in defined ranges of percentage of hydrophobicity, auto-aggregation and co-aggregation with the three *Salmonella* sp (*S.* Enteritidis (S.E.), *S.* Kentucky ST198 (S.K.) and *S.* Infantis (S.I.))..... 146
Figure 15: Principal Component Analysis (PCA) of surface proprieties as hydrophobicity and auto/co-aggregation) for the 50 *Lactobacillus* isolates. Isolates underscored were the selected strains 148
Figure 16: Effect of the simile-gastrointestinal conditions on *Lactobacillus* viability. Black and grey columns correspond to lactobacilli subjected to 0.15 % or 0.3 % bile salts respectively... 150
Figure 17: Adhesion of the eight native poultry-derived *Lactobacillus* strains and the three *Salmonella* strains (*S*. Kentucky ST 198 (*S*.K.), *S*. Infantis (*S*.I.) and *S*. Enteritidis (*S*.E.)) to caco-2 cells line. The means and standard deviations of two independent experiments are shown, each with three replicates. The differences between strains adhesion were evaluated separately for *Lactobacillus* strains and *Salmonella* serotypes. *L. salivarius* 16/c6, 16/i4 and A30/i26, and *L. reuteri* 1/c24 revealed no significant differences (*) in their adhesion capacity which is dissimilar from the four remaining tested strains (**). The differences in the adhesion of *S*. Enteritidis, *S*. Infantis and *S*. Kentucky ST198 were also not significant among the three serotypes (†)..... 151
Figure 18: Inhibition of S. Kentucky ST 198 (S.K.), S. Infantis (S.I.) and S. Enteritidis (S.E.) adherence to Caco-2 cells by L. salivarius 16/c6 and A30/i26 and L. reuteri 1/c24 in competition and exclusion assays. The means and standard deviations of three independent experiments are shown, each with three replicates. (*) Lactobacillus strains were fixed and the differences of inhibition were calculated between the three serotypes in the same assay; (*) $p > 0.05$, (**) $p \leq 0.05$. (†) Salmonella serotypes were fixed, and the differences of inhibition were calculated between the three Lactobacillus strains in the same assay. (†) $p > 0.05$, (††) $p \leq 0.05$..... 153
Figure 19: Liquid co-culture assay without agitation: Kinetic growth of pure-cultures and co-cultures of *L. salivarius* 16/c6 and *S*. Enteritidis, *S.* Infantis and *S.* Kentucky ST198 155
Figure 20: Liquid co-culture assay with agitation: Kinetic growth of pure-cultures and co-cultures of *L. salivarius* 16/c6 and *S*. Enteritidis, *S.* Infantis and *S.* Kentucky ST198..... 156

List of Tables

Table 1: List of some antibiotics for therapeutic used in Lebanese poultry production (MoA, unpublished data) 23
Table 2: Drug-resistant *Salmonella* enterica subsp enterica strains isolated from poultry; antibiotic resistance phenotypic pattern and their respective resistance genes 29
Table 3: Sample type and prevalence of Salmonella sp at different points of poultry production chain 74
Table 4: Occurrence of *Salmonella* sp in the 2 slaughter plants A and B at different seasons 75
Table 5: Salmonella serotypes diversity isolated along the chicken production chain 77
Table 6: Antimicrobial resistance patterns of S. Enteritidis, S. Infantis and S. Kentucky isolates 80
Table 7: Antimicrobial resistance, MDR and ESC occurrence of the main serotypes isolated in this study 82
Table 8: Results of Genomic Assembly, SeqSero, MLST, PlasmidFinder and pMLST and Accession Number of the eight Lebanese S. Kentucky isolates 109
Table 9: Phenotypic and Genotypic antimicrobial resistance results of the eight Lebanese CipR *S.* Kentucky isolates using ResFinder 2.1, ResFinder 3.0 and MyDbFinder. 111
Table 10: Results related to the presence/absence of ISEcp1 in the genomes of the Lebanese CipR *S.* Kentucky strains and the co-localized antimicrobial resistance genes in the same contig. 113
Table 11: Type of age, breed, and diet of the broilers and hens deprived of antibiotics and additives coded from 1to16 and antibiotic- treated commercial broilers coded as A. 134
Table 12: Identity, surface properties and antimicrobial resistance pattern of the eight selected *Lactobacillus* sp 149
Table 13: Correlation of Pearson coefficients between hydrophobicity, auto-aggregation, and co-aggregation of the 50 Lactobacillus isolates. The Principal Component Analysis (PCA) was done using. The index of Pearson was used to evaluate the correlation between the six assays, hydrophobicity, auto-aggregation and co-aggregation between the Lactobacillus strains and *S.* Enteritidis, *S.* Infantis and *S.* Kentucky. 149

List of Abbreviations

ACSSUT: ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline

AGPs: Antibiotic Growth Promoters

AMR: Antimicrobial resistance

AP-1: activator protein

AUB: American University of Beirut

Aw: water activity

BRICS: Brazil, Russia, India, China, South Africa

CDC: Centers of Disease and Control and Prevention

Cip: Ciprofloxacin

CSP: cold shock proteins

CU: chaperone-usher

DC: dendritic cells

EC: European Commission

EFSA: European Food Safety and Authority

EPS: Exopolysaccharide

ESBL: Extended- spectrum β-lactamase

ESC: Extended-spectrum cephalosporin

Esumoh: Epidemiological Surveillance Program

EU, European Union

FAO: Food and Agriculture Organization

FDA: Food and Drug Administration

G: Goblet cells

GIT: Gastro-intestinal tract

GRAS: Generally recognized as Safe

IMP: imipenemase

IS: Insertion sequences

KPC: *K. pneumonia* carbapenemase

LAB: Lactic acid bacteria

LARI: Lebanese Agricultural Research Institute

LPS: lipopolysaccharide

MAMPs: Microbe-associated molecular patterns

MAPK: mitogen activated protein kinase

MDR: Multi-drug resistance

MGEs: Mobile genetic elements

MLS: Macrolide-lincosamide-streptomycin

MLST: Multi-locus sequence typing

MoA: Ministry of Agriculture

MoPH: Ministry of public health

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NPIP: National Poultry Improvement Plan

OIE: Organization Internationale des Epizooties

OXA: oxacillinase

P: pathogen

pAmpC: plasmidic AmpC- β-lactamase

PAMPs: pathogen-associated molecular patterns

PB: probiotic

PC: Paneth cell

PCR: Polymerase chain reaction

pESI: plasmid-emerging *S*. Infantis

PFGE: Pulse field Gel electrophoresis

PMQR: plasmid-mediated quinolone resistance

ProP: Proline permease

PRR: pathogen recognition receptors

QRDRs: quinolone resistance determining regions

ROS: Reactive Oxygen Species

Salmonella (S.): *Salmonella* enterica subspecies enterica

SCV: *Salmonella*-containing vacuole

SGI1: *Salmonella* Genomic Island

Slp: surface layer protein

SPI4: *Salmonella* pathogenicity island 4

SPIs: *Salmonella* pathogenicity islands

ST: sequence type

T1SS: Type I secretion systems

T3SS: Type III secretion systems

T6SS: Type VI secretion systems

TC: T lymphocyte

US: United State

VIM: Verona integrin encoded metallo β-lactamase

WGS: Whole Genome Sequencing

WHO: World Health Organization

Introduction

Food safety is a major problem worldwide in both developed and developing countries. The World Health Organization (WHO) estimates that 550 million people fall ill yearly and 230 000 died from diarrheal diseases caused mainly by ingestion of contaminated food or water (WHO, Food safety, 2017). In Lebanon, a total of 294 sporadic food poisoning cases and 109 outbreaks, affecting 765 persons were reported by the Ministry of Public health (MoPH) between 2014 and 2015 (unpublished data, MoPH, PulseNet report, 2015).

Salmonella genus is an important public health concern due to its widespread. This zoonotic foodborne bacterium is one of the leading causes of acute diarrhea in Europe (EFSA/ ECDC, 2017). It is estimated that Non-Typhoidal *Salmonella* causes 93.8 million cases of gastroenteritis and 155,000 annual deaths worldwide (Majowicz et al., 2010). Furthermore, this pathogen is an economic and social burden, resulting in high medical costs and a decrease in productivity. Consequent economic losses due to Non-Typhoidal *Salmonella* have been estimated to exceed 14 billion dollars/ year in the United States (US) alone (Cosby et al., 2015). In Europe, European Food Safety Authority (EFSA) has recently estimated that the overall cost of all salmonellosis is EUR 3 billion per year (EFSA BIOHAZ Panel , 2019). In Lebanon, the heavy contamination and outbreaks caused by *Salmonella* were relayed by the media to the general public and were not without consequences.

The ubiquitous *Salmonella enterica* subsp *enterica*, colonizing indifferently animal and humans intestines, are widely spread in different animal reservoirs (pigs, cattle, and poultry) and foods (Lamas et al., 2018). Poultry meat and eggs remain the major sources of human salmonellosis (Foley et al., 2011). These subspecies contain more than 2500 different serotypes; however, few are responsible for most infections such as *S*. Enteritidis and *S*. Typhimurium (MoPH, PulseNet report, 2015) (including the monophasic variant of Typhimurium 1.4, [5], 12, i :-) (EFSA/ECDC, 2017).

Salmonella can contaminate the poultry products at any stage of the production chain from the primary level to the final stage of retail and handling. Therefore, monitoring, surveillance and prevention programs should be in every step through infection control measures at farm level (biosecurity and vaccination), proper sanitary conditions at slaughterhouse (Good Manufacturing Practices) and appropriate manipulation (Good Hygiene Practices) at retail. In Lebanon, even though that this reservoir is largely contaminated, an effective *Salmonella* surveillance is currently

non-existent, and little is known about its epidemiology in poultry farms, slaughterhouses, and retail stores.

Facing these conditions, the use of antimicrobials in animal production which is introduced as therapeutic, Growth Promoters (AGPs) and disease prevention, have improved animal health and led to higher yields (Pan and Yu, 2014). However, their excessive and indiscriminate practices have contributed to the development and emergence of antibiotic resistant (AMR) or multi-drug resistant (MDR) strains that can reach humans through the food chain (Ferri et al., 2017). Over the past decade, the emergence of MDR *S*. Typhimurium phage DT104, resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSSuT-resistant type) with decreased susceptibility to ciprofloxacin has been linked to the licensing of enrofloxacin (fluoroquinolone) in poultry industry (Threlfall, 2000). In *Salmonella*, the emergence of beta-lactam resistance that was attributed to the expression of a wide variety of Extended-spectrum β-lactamase (ESBL) and AmpC-type β-Lactamases as well as fluoroquinolone resistance was of great concern (Folster et al., 2016, Saliu et al., 2017). These two classes are categorized as critically important antibiotics to treat invasive salmonellosis in elderly and immune-depressed patients and infants' respectively (Medalla et al., 2017). To combat this significant problem, policies and strategies were set at national, regional and international level with either gradual withdraw of several AGPs as in the USA (Patel et al., 2018) or strictly taking it off in the poultry industry as in European Union (EU) (Regulation (EC) No 1831/2003, 2003). As a result, several alternative prophylactic measures to antibiotics have been introduced such as probiotics.

Although the concept of probiotics is not new, their use in animal farming and poultry industry have recently been growing. Research for the development of new products with high probiotic or even antimicrobial potency continues to receive considerable interest. FAO & WHO (2002) defines probiotics as "live microorganisms (bacteria or yeasts) that, when administered in adequate amounts, have beneficial effects on their host". Indeed, multiple beneficial effects such as the balance and the proper functioning of the intestinal flora, reinforcing the intestinal barrier, modulation of the immune system are claimed (Alagawany et al., 2018). Two fundamental mechanisms of inhibition of pathogenic organisms were detected either by direct cell competitive exclusion or by the production of inhibitory compounds, namely lactic and acetic acid, hydrogen peroxide, bacteriocin or bacteriocin-like inhibitors, fat and amino acid metabolites (Ayeni et al.,

2018). Most of the strains used are of enteric origin isolated from the gastrointestinal tract of human and especially poultry. Lactic acid bacteria are considered to be the probiotics of choice both for their great capacity for survival and adhesion in the intestinal environment and their role in the restoration of the gut microbiota (Wang and Gu, 2010).

The aim of this thesis is to identify and characterize circulating *Salmonella* sp. in the Lebanese poultry production and layer hen farms, within a farm to fork approach. The second main objective of our study is discovering possible live lactic acid bacteria (LAB) probiotic to be applied as prophylactic administration to control *Salmonella* dissemination.

To achieve our goals, the work plan proposed in this thesis is divided into two parts:

- ***The first part*** aims to determine *Salmonella* prevalence in Lebanon starting from broiler breeder farms to slaughterhouses and the retail (supermarkets and restaurants) and layer flocks. Serotypes circulation, antibiotic resistance and their genotypic relatedness were also studied. At the end, this work will serve as a database for a national strategy, surveillance programs and intervention measures, set by local authorities (Ministry of Agriculture (MoA)) and regional risk analysis Initiative ("Arab Food Safety Initiative For Trade Facilitation- SAFE) for prevention and control of salmonellosis in human and *Salmonella* dissemination in the poultry industry.

- ***The second part*** targets to isolate and identify native poultry-derived *Lactobacillus* strains and to characterize their probiotic ability against the most relevant and drug-resistant *Salmonella* sp in Lebanese poultry farms.

The manuscript is divided into four main chapters:

Chapter 1 consists in a bibliographical review describing *Salmonella* sp; serotypes, pathogenicity, antimicrobial resistance and the main control strategies applied worldwide at farm level. A detailed view on the latest approach of the use of probiotic in poultry farming focusing on *Lactobacillus* sp. The methods used for screening and evaluating the potential probiotic are also well described.

The results of this scientific research are presented in the form of three chapters where two of them have been submitted:

- Chapter 2: The prevalence, antibiotic resistance and molecular characterization of *Salmonella* serotypes in the Lebanese poultry production have been assessed in this part of the study.
- Chapter 3: This article focuses on genetically characterization of eight strains of *S.* Kentucky resistant to ciprofloxacin (Cip) and ESCs by Whole Genome Sequencing (WGS).
- Chapter 4: It describes the isolation and in-vitro screening of native *Lactobacillus* sp. isolated from the ileum and cecum of broilers and layers. Their anti-*Salmonella* activities are also defined in co-culture as well as by competitive exclusion to caco-2 cell lines.

At the end, a general conclusion and the most relevant perspectives are presented.

Chapter I
Bibliographical review

1. *Salmonella*

1.1. **Taxonomy and nomenclature**

The genus *Salmonella* is a rod, Gram-negative bacterium that belongs to the family of Enterobacteriaceae. It is subdivided taxonomically into two species: *Salmonella bongori* and *Salmonella enterica.* Within *S. enterica*, six subspecies were individualized: *enterica (I), Salamae (II), arizonae (IIIa), diarizonae (IIIb), houtenae (IV) and indica (VI)* (Grimont and Weill, 2007). The majority of strains (99%) isolated in humans and warm-blooded animals belong to *Salmonella enterica subspecies enterica (I)*, abbreviated *Salmonella*.

Currently, 2610 serotypes have been identified, 1547 belonged to *Salmonella enterica subspecies enterica (I)* (Achtman et al., 2012). They were characterized by their cell-surface antigens within lipopolysaccharide (O antigen coded by *rfb* genes) and their flagellar antigens (flagellar 1 and 2 of H antigen coded by *fliC* and *fljB* genes) according to the classification developed by White in 1926 and then by Kauffman 1972 and completed by Minor in 1978. *Salmonella* serotypes of clinical importance are noted in the White-Kauffman-Le Minor scheme (Grimont and Weill, 2007). At this time, names attributed to serotypes are written in roman letters with capital letters and not in italic. Nowadays, the whole genome sequencing (WGS) with multi-locus sequence typing (MLST) approach has been adopted by some Public Health laboratories to replace the traditional serotyping (Ashton et al., 2016). MLST is built on the basis of sequences of several house-keeping genes and isolates with matching alleles for seven gene fragments studied are given a common sequence type (ST). This method affords advance understanding on the real evolutionary relatedness between isolates. Another novel *in-silico* web-based tool is serotyping by SeqSero, to determine *Salmonella* serotypes from *rfb* gene cluster, *fliC* and *fljB* alleles, responsible for *Salmonella* antigenic structure using both raw sequences and assembled data generated from the WGS. It has been successfully introduced confirming the *in vitro* serotyping (Zhang et al., 2015).

1.2. **Adaptation capacity**

Salmonella is a mesophilic bacterium with an optimal growth temperature between 35°C and 37°C, with a pH between 6.5 and 7.5 but can tolerate a higher range from 4.5 to 9 and necessitates a water activity (aw) > 0.93 for growth (Andino and Hanning, 2015). The process of adaptation to new inconvenient environment involves the mechanism of different sigma factors. These alternate factors are structural proteins of prokaryotic RNA polymerase which can increase gene transcription appropriated to the environmental conditions.

When the bacteria is exposed to extreme heat stress, RpoH (heat shock sigma factor) mechanism is triggered (Andino and Hanning, 2015). In *S*. Enteritidis, gene transcription was the highest level when cultured at 42° C. In response to quick adaptation to temperature downshifts, Andino and Hanning (2015) reported that *Salmonella* could well survive due to the expression of cold shock proteins (CSP). As a result, the survival rate of *S*. Enteritidis increases in chicken parts at 2°C, and shell eggs at 4° C.

Salmonella are acid- tolerant due to express acid shock proteins (RpoS -factor, PhoPQ, and Fur) enabling their survival at a low gastrointestinal pH (Foley et al., 2013). Cheng et al.(2014) demonstrated that *S.* Kentucky expresses a high level of *rpoS* (starvation/stationary phase sigma factor) - regulated genes, a potential factor responsible for its new wave of dissemination in poultry.

In dehydrated products, they can survive for a long time, which is related to their ability to survive in outdoor environments such as broiler farms, dry litter, and environmental dust. This protective mechanism against dryness is due to the expression of proP (Proline permease II)(Finn et al., 2013) and *rpoS*- regulated genes (Andino and Hanning, 2015). Maserati et al, (2017) also demonstrated that the virulence factors sopD and sseD are implicated in *Salmonella's* survival during desiccation.

1.3. **Pathogenesis and virulence**

The natural reservoir of *Salmonella* is vast. This bacterium is intestinal parasite, a well-known pathogen associated with both animals and humans. Each *Salmonella* serotype has its characteristic pathogenicity that manifests the variation of the virulence factors among these

different serotypes. Few are strictly host-specific such as *S*. Typhi and *S*. Paratyphi A, B and C in humans, *S*. Gallinarum- Pullorum in poultry, *S*. Cholerasuis in pigs, *S*. Dublin in cattle, and *S*. Abortusequi in horses and *S*. Abortusovis in sheep, while the majority are zoonotic such as *S*. Typhimurium, *S.* Enteritidis.... agents of non-typhoidal salmonellosis (Arya et al., 2017).

The Non-typhoidal *Salmonella* are usually self-limiting foodborne gastroenteritis, but illness becomes complicated and life-threatening for the elderly, infants, and immunosuppressed, and necessitates antimicrobial treatment. Therefore, the host-bacterium status reflects the result of a *Salmonella* infection. While age, genetic and environmental factors determine the host status, the *Salmonella* status is shaped by the virulence factors including the toxins, virulence plasmids, fimbriae and flagella, clusters of virulence genes and type III secretion systems (T3SSs, injectisome-mediated delivery of "effector" proteins from bacteria to host cells) encoded by the horizontally acquired *Salmonella* pathogenicity islands (SPIs) (Foley et al., 2013; Jennings, 2017). There are six secretion systems categorized from type I (T1SS) to type VI (T6SS) in addition to the CU (chaperone-usher) system (Ramos-Morales, 2012). These effector proteins play important role in pathogenicity, biofilm formation, modulation of the eukaryote host, and nutrient acquisition.

After oral infection, *Salmonella* adhered to the intestinal cell surface through fimbriae and other adherence- associated non-fimbrial proteins with SPI4-encoded T1SS and the non-fimbrial giant adhesin SiiE as *Salmonella* contact initiator with host cells (Peters et al., 2017). Within less than 24h, *Salmonella* colonizes the intestinal epithelial cells, triggering gastroenteritis symptoms (Thiennimitr et al., 2012). Motility and two T3SSs are considered as the main *Salmonella* virulence factors necessary for intestinal inflammation. *Salmonella* encodes two virulence-associated T3SSs, namely T3SS-1 and T3SS-2 which are located on two SPI1 and SPI2, respectively. The SPI-1 protein effectors, SipA, SopD, SopB, SopA, SopE2 and SptP mediate the invasion and colonization of epithelial cells causing localized inflammation (Jennings et al., 2017). These effectors remodel the actin cytoskeleton of the host cell inducing the pathogen engulfment by phagocytes in a modified phagolysosome, *Salmonella*-containing vacuole (SCV). *Salmonella* replication and dissemination inside the SCV is assured by approximately thirty T3SS-2 protein effectors encoded by SPI-2 (Figueira and Holden, 2012). The functionality of T3SS-2 helps to distinguish virulent from non-virulent *Salmonella* strains. The reduced virulence

of *S*. Kentucky was therefore partially attributed to the absence of *sopD2, pipB2, sspH2* and *sseI gene*(Cheng et al., 2014).

1.3.1. Local inflammatory response

S. Typhimurium is considered as the most studied pathogen in humans. In this case, its presence in the host is detected through two recognition patterns which are a part of the *Salmonella* structure; pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides, curli and flagella and patterns of pathogenesis via the translocation of the effectors T3SS-1 into the cytosol. These patterns are recognized by pathogen recognition receptors (PRR) of the host expressed by the cytosol (NOD1, NOD2, NLRC4, and NLRP3), the cell membrane (TLR1/TLR2, TLR4, and TLR5) or the humoral compartment (complement). It causes the stimulation of mitogen activated protein kinase (MAPK) transduction pathways that activate the transcription factors, activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) leading to a pro-inflammatory expression (innate immune response). As a result, a cocktail of cyto- and chemokines are produced targeting the pathogen by three major responses: macrophage stimulation (via IFN-$_{\gamma}$), neutrophil recruitment (via the chemokine CXC) and the epithelial release of antimicrobials (via Il-22 cytokine which stimulates the release of the antimicrobial Lipocalin-2). The production of specific antibodies by the adaptive immune response further boosts phagocyte-killing mechanisms (Thiennimitr et al., 2012).

The immune system is activated against *Salmonella* pathogen in its three locations; intracellular (in SVC), extracellular (in epithelial tissue) and luminal gut (Figure 1). The inflammatory response is very effective against the first two. However, it enhanced its growth in the intestinal lumen; the antimicrobial (lipocalin-2) secreted by the epithelial cells sequesters the iron chelator (enterobactin) produced by the microflora, but not the iron chelator (salmochelin) produced by *Salmonella*. In addition, during neutrophils migration, in an attempt to neutralize the pathogen, Reactive Oxygen Species (ROS) are produced and oxidize an endogenous sulfur compound (thiosulfate), that generate a respiratory electron acceptor (tetrathionate) enabling *Salmonella* to growth anaerobically. Bacterial growth in intestine promotes *Salmonella* transmission by the fecal-oral route.

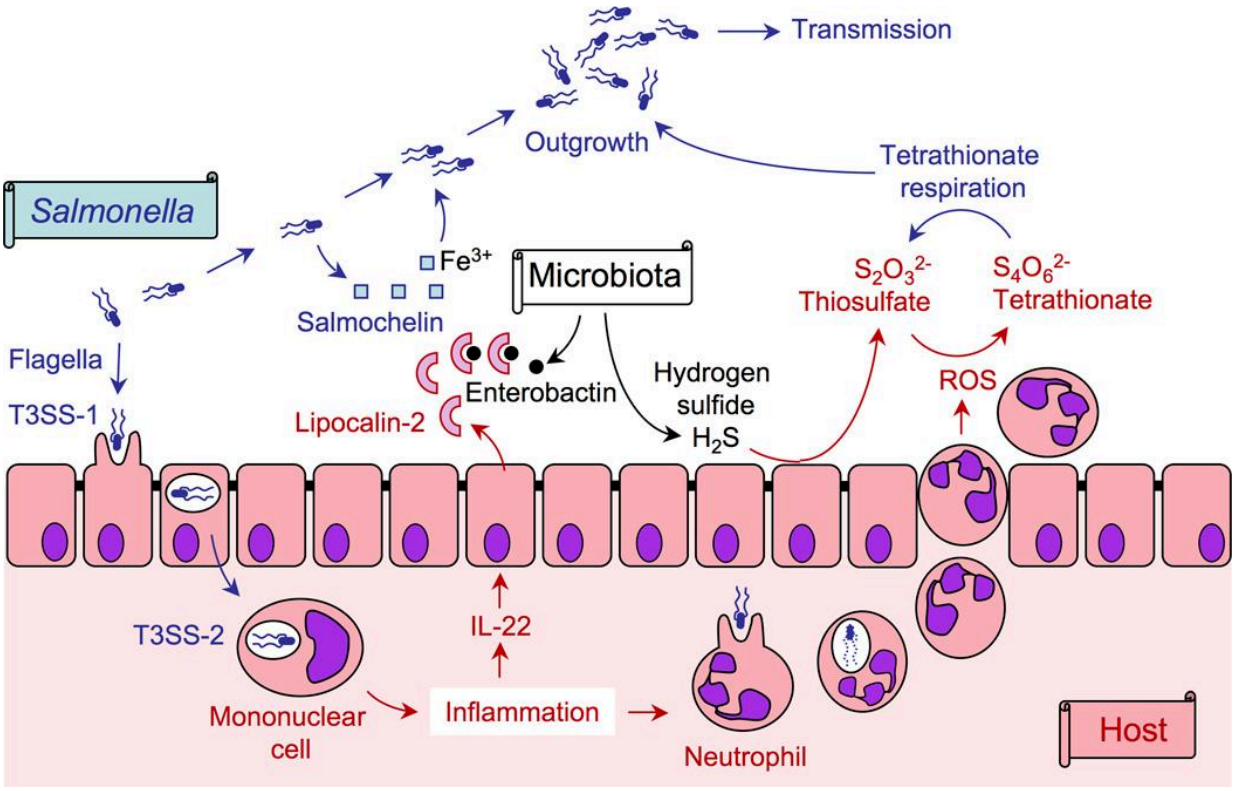

Figure 1: *Salmonella*, the host and its microbiota (Thiennimitr et al., 2012).

1.4. **Non-Typhoidal *Salmonella,* a public health concern**

Non-Typhoidal *Salmonella* is one of the leading pathogens causing foodborne illness with 94,530 confirmed cases reported in European countries in 2016 (EFSA/ECDC, 2017). In USA, Salmonellosis account for approximately 1.2 million cases, 23.000 hospitalizations and a mortality rate of 450 people yearly (CDC, 2019). Few serotypes are responsible for human infections; *S*. Enteritidis and *S.* Typhimurium being the most prevalent and commonly reported worldwide including Lebanon (MoPH, PulseNet report, unpublished data; Fadlallah et al., 2017) (Figure 2). *S*. Enteritidis is frequently associated with eggs and poultry products, whereas *S*. Typhimurium infection is attributed to a broader species range, such as pigs, cattle and poultry. Therefore, foods of animal origin, in particular, contaminated poultry products (eggs and poultry meat) have been considered the primary vehicles of *Salmonella* infection (Antunes et al., 2016). Parallel evolutions of the serotypes in poultry and humans argue in favor of the reality of this concept. The new pandemic *S*. Enteritidis in the 1980s was in line with its high occurrence in

poultry (Rodrigue et al., 1990). In EU, similar evolution was noticed during 2012–2016 between the proportion of foodborne *S.* Enteritidis and their prevalence in laying hens that significantly increased during 2015 and 2016 (EFSA/ECDC, 2017). In Australia, *S.* Typhimurium is a leading cause of foodborne outbreaks linked to the heavily contaminated egg and egg- related products (Pande et al., 2016).

Other poultry-originating emerging serotypes and clones are also reported causing human illness such as *S.* Infantis (EFSA/ECDC, 2015; Nógrády et al., 2007), Cip^{R} *S.* Kentucky ST 198 (Le Hello et al., 2011; 2013), and *S.* Heidelberg (Shah et al., 2017), frequently resistant to antibiotics.

The economic impact of salmonellosis has been repeatedly emphasized. The estimated cost for Non-typhoidal *Salmonella* is more than 14 billion dollars/ year in the US alone (Cosby et al., 2015). In European countries, it has been estimated that the salmonellosis cost is about EUR 3 billion a year (EFSA BIOHAZ Panel, 2019).

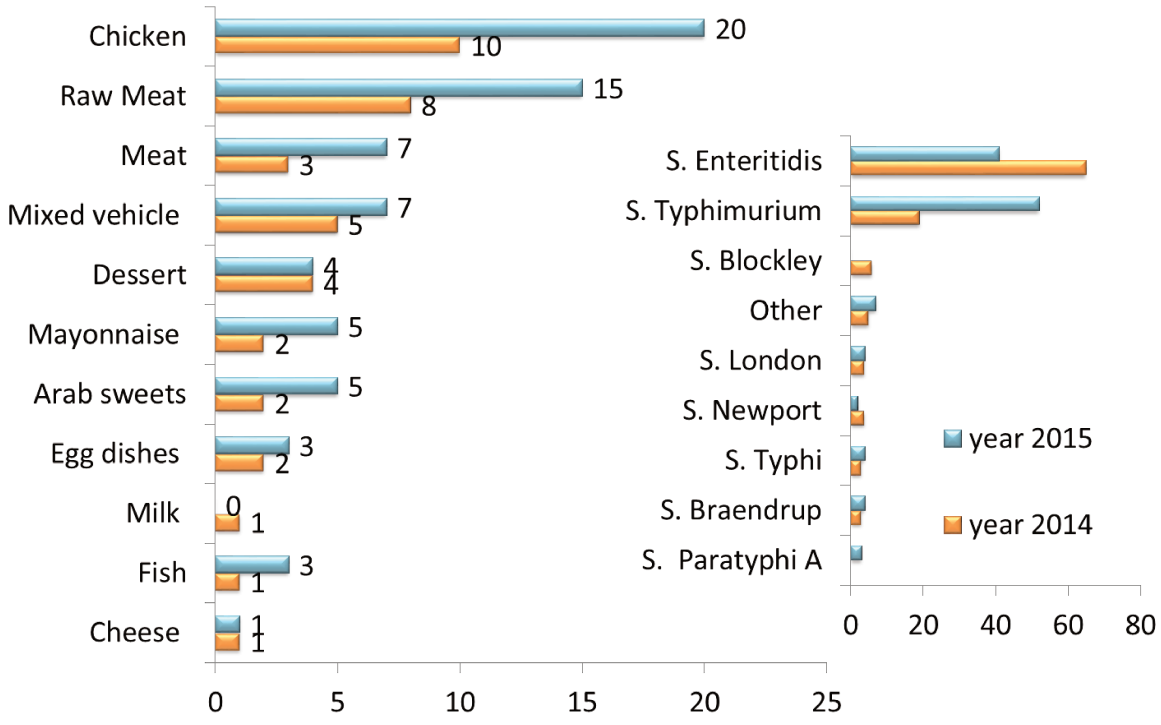

Figure 2: MoPH, PulseNet report, 2015 (unpublished data)

Chicken is the primary vehicle of salmonellosis with 20 and 10 outbreaks have been linked to the consumption of poultry products in 2015 and 2014 respectively. *S.* Enteritidis and *S.* Typhimurium were the most frequent serotypes in these two years.

1.5. *Salmonella* and poultry

The group of *Salmonella* serotypes is considerably large, till present, 2610 serotypes have been identified. However, few are circulating in the food chain causing main outbreaks.

1.5.1. Poultry production

Poultry is one of the most advanced and fasted food industry worldwide. To supply the increasing market demand, more than 90 billion tons of chicken meat are produced yearly where chickens are the most commonly farmed species (FAO, 2018). In the local Lebanese market, broiler production is estimated at 150 million kilos/ year. In the United States, more than 9 billion broilers are processed each year and 77 billion table eggs (Foley et al., 2011). Country members of BRICS (Brazil, Russia, India, China, and South Africa) turned toward a highly cost-effective and vertically integrated intensive livestock production systems (Van Boeckel et al., 2015). This industry is also concentrated in Lebanon where four leading large producers share with more than 50 % of the local poultry market. Concerning egg production, this field comprises two big traders with one sold its output while the other collects the eggs from the medium and small producers.

These new husbandry practices (increased stocking density, larger farms, and bird stress) largely contribute to the risk of *Salmonella* dissemination either vertically or horizontally (Bailey, 1988).

1.5.2. *Salmonella* mode of transmission and pathogenesis

Poultry are commonly known to be *Salmonella* reservoirs, mainly harboring this pathogen in the gastrointestinal tracts. Incidence of *Salmonella* in poultry flocks varied considerably within countries. The EU summary report 2016 concluded that 3.7 % of commercial broiler flocks were positive for *Salmonella*, with values ranging from 0 % to 16.2 % of flocks within individual countries. Similarly, the same study indicated that 2.8 % of European laying flocks were positive for *Salmonella* with values ranging from 0% to 87.5 % of flocks in individual countries (EFSA/ECDC, 2016). In developing countries, higher prevalences were recorded; in Algeria and Constantine, broiler farms were contaminated at 34.4% and 36.6 % respectively (Djeffal et al., 2017; Elgroud et al., 2009). Whereas in Bangladesh, results showed a level of 18 % *Salmonella* contamination at layer farms (Barua et al., 2012). In poultry products, the prevalences were also high ranging from 13 % to 39 % in South America, 35 % to 50 % in Asia and 35 % in Africa

(Antunes et al., 2016). *Salmonella* prevalence on broiler carcasses collected from Lebanese slaughterhouses was about 41.6 % (El Hage, 2013 unpublished data).

At farm level, the route of horizontal transmission occurs via fecal-oral pathway (Foley et al., 2013). Infected animals shed pathogens in the feces which, in turn, contaminate the environment and cause new infections or reinfection. The source of farm infection could be cross-contaminated by feed, humans, domestic, wild animal, insects, contaminated equipment or water (Chousalkar et al., 2018).

In broiler, *Salmonella* can within a few hours colonize and invade the ceca, reaching other internal organs like the liver and spleen (Muyyarikkandy and Amalaradjou, 2017). Poultry carcasses and poultry products can, therefore, be contaminated at the slaughterhouse (Shah et al., 2012). Several production processes could infect the carcasses with *Salmonella* mainly defeathering, evisceration, and chilling operations. Consequently, bacteria can thus survive during all these stages and human consumption and causing subsequent illness.

In laying hens, vertical transmission of *Salmonella* caused by some serotypes such as *S*. Enteritidis , *S*. Typhimurium, and *S*. Heidelberg led to systemic dissemination, colonization, and invasion of the reproductive system and therefore internally contaminated eggs (Ricke et al., 2018; Kaldhone et al., 2017; Chousalkar et al., 2018). Moreover, external eggshell could be contaminated while the egg is laid due to the joint opening of the intestinal, urinary, and reproductive tracts. *S*. Enteritidis is well known for its capacity to survive in the hostile microbicidal properties of egg albumen by producing a capsular-like lipopolysaccharide (LPS). As a result, *S*. Enteritidis is primarily responsible for egg-borne *Salmonella* outbreaks throughout the world (Shah et al., 2012). Eggs could also be contaminated by horizontal route via fecal trans-shell penetration (Pande et al., 2016). Some *Salmonella* serotypes such as *S*. Typhimurium, *S*. Agona and *S*. Infantis might form a biofilm on the eggshell surface (Chousalkar and Gole, 2016).

Colonization mechanisms are so complex that they are variable between hosts, serotypes, and within the serotype (Foley et al., 2011). Except for host-specific *S*. Pullorum and *S*. Gallinarum, which cause Pullorosis and fowl typhoid respectively (Andino and Hanning, 2015) leading to severe flock illnesses and high mortality, other *Salmonella* serotypes establish non- clinical signs of variable duration, which is a potential threat of zoonosis. Such animals (healthy carriers of

Salmonella) could either spread infection between flocks or cause foodborne disease when contaminated poultry products such as meat and eggs, enter the food chain.

1.5.3. *Salmonella* serotypes in poultry

1.5.3.1. ***Serotypes shift***

Population dynamics of *Salmonella* serotypes have been noticed over time, and have been affected by different control programs and strategies (Foley et al., 2011), livestock trade and travel (Barbour et al., 2015).

Until the 1960s, *S*. Gallinarum and *S*. Pullorum were among the most severe diseases in poultry worldwide (Foley et al., 2011). Despite their eradication in most countries, these host-specific biovars still a big challenge in developing countries such as India (Barbour et al., 2015). The sudden rise of ubiquitous *Salmonella* outbreaks recalled that *S*. Gallinarum and *S*. Pullorum were not the only entero-invasive serotype in poultry although not pathogenic to humans. During the period 1950s-1970s, *S*. Typhimurium was well recorded in the most frequently isolated serotypes from poultry origin in many countries including USA (Bullis, 1977) and England (Sojka and Wray, 1975). Similar results were obtained locally as mentioned by Nabbut and Jamal, (1970); *S*. Typhimurium (35.5%) and *S*. Bareilly (25.2%) were the most isolated serotypes from 214 examined chickens. And at lower but significant rate *S*. Pullorum was also isolated (5.1 %).

In the 1980s, began a new wave of serotypes and clones of public health concern. A phenomenon called "pandemic" appeared; the vertical transmission via egg of *S*. Enteritidis. This strain was particularly pronounced in the industrialized countries when lysovar 4 appeared, which was invasive in layer hens and broilers (Rabsch et al., 2000). In Lebanon, 112 proliferating strains of *S*. Enteritidis have been reported in 11 broiler farms (Barbour et al., 1998). At the same period, LARI Microbiological department isolated *S*. Blockley, *S*. Typhimurium, and *S*. Enteritidis from liver of diseased chicken. The work of El Hage et al., (2003), revealed only two serotypes *S*. Enteritidis and *S*. Blockley at a contamination rate of 64.7% and 35.5% respectively. Analyzed samples were broiler ceca collected from Lebanese slaughterhouses (LARI, internal report, 2004).

Due to the outbreaks of *S*. Enteritidis in humans, this serotype has been set as new target in developed countries, namely in USA and EU in the 1989 and 2007 respectively. Whereas *S*. Typhimurium was also added to the list of targeted strains in EU. In the USA, the decline of *S*. Enteritidis prevalence in eggs and poultry meat since the mid-1990s favored the emergence of new serotypes as *S*. Heidelberg from 1997 to 2006 and in 2007 *S*. Kentucky was the most common serotype with contamination of 50 % in retail poultry carcasses (Foley et al., 2011). This latter serotype has highly disseminated worldwide in boilers and layers, in developed (Antunes et al., 2016) and developing countries (Barua et al., 2012). A new clone Cip^R *S*. Kentucky ST198, linked to travel to Africa and the Middle East, has emerged and rapidly disseminated worldwide both in humans and animals, especially in broilers (Le Hello et al., 2013; Ramadan et al., 2018). In EU, the prevalence of *S*. Enteritidis declined significantly reaching 1.0 % and 0.9 % in 2013 and 2014 respectively but still ranked second isolated serotype in broilers. Simultaneously an increase of *S*. Infantis was observed in diverse European countries reaching 38.3% in broiler farms (EFSA/ECDC, 2015). Besides, a Hungarian clone has also been reported worldwide (Nógrády et al., 2007; Hindermann et al., 2017; Tate et al., 2017; Aviv et al., 2014; Franco et al., 2015) possessing a unique megaplasmid (pESI) (plasmid emerging *S*. Infantis).

S. Enteritidis and *S*. Typhimurium remain the most circulating serotypes in both broilers and layers. In Australian layer farms, S. Typhimurium is prevailing (Chousalkar et al., 2018), whereas it is dominant in Chinese broiler industries (Li et al., 2017). In Egypt, high prevalence of *S*. Enteritidis (37.25 %) and *S*. Typhimurium (29.41 %) was recovered from broiler flocks *S*. Infantis (19.6 %), *S*. Kentucky (7.84 %) were also isolated. Another study showed a 15 % contamination of chicken samples was due to *S*. Heidelberg (Barbour et al., 2015)..

Variation of serotypes have been observed between countries; In China, several authors frequently reported *S*. Indiana as the most common serotype in chicken carcasses (Bai et al., 2015). In Australia, Pande et al. (2016) reported that *S*. Mbandaka (54.4 %) was the most frequently recovered serotype along with *S*. Typhimurium (11.5 %) in layer farms. Furthermore, *S*. Heidelberg is mainly isolated from layer and broiler farms from Canada and USA (Edirmanasinghe et al., 2017; Shah et al., 2017).

*1.5.3.2. **Factors affecting the dissemination and persistence of specific serotypes***

Many hypotheses have been raised trying to identify the factors that contributed to the colonization and spread of a particular serotype or new clone in poultry.

The first hypothesis was established by Rabsch et al. (2000) supposing that competitive exclusion plays an essential role in such phenomenon. The authors concluded that *S.* Gallinarum might competitively exclude *S*. Enteritidis in poultry and the eradication of the first one facilitates the dissemination of second. One serotype could yield a cross-immunity against a second one if both organisms share the same immunodominant O-antigen on their cell surface. Indeed, the two serotypes have the same O9 lipopolysaccharide antigen. The presence of *S*. Gallinarum at the beginning of the 20th century may have generated adaptive flock immunity, thereby excluding *S*. Enteritidis strains from circulation in poultry flock. The same concept of competitive exclusion has been thought to be the cause of dissemination of *S*. Heidelberg since it shares same surface antigens with *S*. Enteritidis.

The second hypothesis concerning the persistence of *S.* Enteritidis in the poultry population is thought to be due to its rodent reservoir. Unlike the avian-adapted *S*. Gallinarum, this serotype could be reintroduced into flocks via horizontal contamination by rodents and therefore more challenging to eliminate (Andino and Hanning, 2015). Moreover, the changes in poultry production practices such as higher densities and increased vertical integration may have facilitated *S.* Enteritidis dissemination.

Another hypothesis assumed to be the acquisition of new genetic elements enrolled in the virulence or adaptation in specific clones; in the case of Hungarian *S*. Infantis, megaplasmid (pESI) has been acquired, contributing in significant increase in tolerance to stress factors (e.g. mercury and oxidative stress) and virulence (e.g. biofilm formation, adhesion and invasion into host cell)(Aviv et al., 2014). Others such as *S*. Kentucky through the acquisition of an *E.coli* CoLIV plasmid that encodes for colicins, iron-scavenging genes and the HlyF hemolysin (Johnson et al., 2010). Some mechanisms involve the differential regulation of core *Salmonella* genes via the stationary-phase sigma factor RpoS, to the metabolic adaptation of *S*. Kentucky in the chicken caecum(Cheng et al., 2014).

Human illness and economic consequences of *Salmonella* contamination are not to be neglected; this justifies putting in place methods of fine characterization and means of effective prevention against this pathogen.

1.6. **Molecular genotyping**

Several methods have been used for pathogen identification and characterization including *Salmonella*. While phenotypic characterization such as serotyping and antimicrobial susceptibility testing was still in use, more sensitive genomic methods have been introduced, and there are many.

1.6.1. Pulse Field Gel Electrophoresis (PFGE)

In their early use, through molecular typing, epidemiological surveys use genetic fingerprints for traceability studies to better identify sources of contamination and subsequently the production sectors most implicated in the risks to humans. PFGE technique permits to differentiate bacterial isolates at the strain level. So, it allows, on the one hand, to determine the existing relatedness between the strains during an epidemic and, on the other hand, to identify possible clonal lines. The principle of this technique consists of separating large DNA fragments (between 50 and 1000 Kpb), obtained by the use of enzymes with rare cleavage sites in the genome and known for its high discriminatory power. PFGE was for an extended period the gold reference method used worldwide in epidemiological investigations (Arya et al., 2017). In EU, it has been applied in food poisoning investigation to identify the source of infection due to *S*. Enteritidis (Laconcha et al., 2000) *S*. Typhimurium (Murphy et al., 2008), *S*. Agona (Rabsch et al., 2005). Similarly, the genetic variability of *S*. Typhimurium LT2 from archival cultures dating from 1940 was studied (Edwards et al., 2001). Extensive clonal relatedness studies within multitude serotypes have been carried out, such as MDR *S*. Infantis (Hindermann et al., 2017), Cip^R *S*. Kentucky ST 198 (Le Hello et al., 2013), MDR *S*. California and *S*. Indiana (Wang et al., 2017), and *S*. Heidelberg and *S*. Minnesota (Campos et al., 2018).

PFGE networks such as PulseNET, organized by the US Centers for Disease Control and Prevention (CDC), have been established worldwide with successful standardized methods (Gieraltowski et al., 2016). At national level, this disease tracking network, was formed by the epidemiological Surveillance Program (Esumoh) at the MoPH, LARI and the American

University of Beirut (AUB). This joint effort is responsible for surveillance of foodborne diseases by strain identification, genotyping and establishing the relatedness between clinical cases and their food sources during outbreaks. Fadlallah et al., (2017) demonstrated the clonal relatedness between clinical and food origin *Salmonella* and showed the link of two *Salmonella* outbreaks with their suspected food sources.

1.6.2. Whole genome sequencing (WGS)

Since the first whole bacterial genome sequence in 1995, sequencing technologies have rapidly developed. WGS either by sequencing the chromosome or mobile genetic elements, provided the ultimate discriminatory power (Phillips et al., 2016). It delivers information on pathogen; identification, epidemiological typing, and drug susceptibility. The work of Wang et al., (2017) characterize the sequence of *S*. Indiana at the whole-genome level and verify the transferability of the mobilized colistin resistance gene mcr-1. This technique was also use by Edirmanasinghe et al. (2017) to characterize *S*. Heidelberg isolated from different sources (human, from human, abattoir poultry, and retail poultry). It also allows linking outbreak isolates to attribute sources. In Europe, multi-country outbreaks due to the consumption of eggs contaminated by *S*. Enteritidis have been linked to a persistent contamination of laying hen farms in Poland (EFSA BIOHAZ Panel., 2019). WGS is being adopted in PulseNet surveillance plans, due to its high rate of accuracy and robustness to low-quality assemblies. Making it possible to associate individual isolates with specific geographic locations, allowing for more rapid public health interventions (Arya et al., 2017). The use of Genome sequences has designed a robust framework for large-scale phylogenomic and comparative genomic analyses that can elucidate the bacterial evolution.

1.7. *Salmonella* control at farm level

The eradication of ubiquitous *Salmonella* is almost illusory, given the large number of serotypes to be considered and their ubiquity.

Salmonella can contaminate the food at any stage of the production chain from the primary level to the final stage of retail and handling. Therefore, *Salmonella* surveillance and prevention should be in every step through infection control measures at farm level, proper sanitary conditions at the slaughterhouse (Good Manufacturing Practices) and appropriate manipulation (Good Hygiene Practices) at retail. By reducing the cecal *Salmonella* carriage in poultry during primary

production, fecal shedding will decrease the contamination levels of the carcasses after processing, and eggs, thereby reduce human infection (Muyyarikkandy and Amalaradjou, 2017).

Internationally, at farm level, different strategies were adopted to control *Salmonella* dissemination either by general approaches such as biosecurity enforcement or by targeted policies such as vaccination and *Salmonella* reduction programs. In Lebanon, all farms strengthen their biosecurity measures and vaccination programs abiding the MoA recommendations and related regulations. Despite these efforts, levels of poultry-related *Salmonella* infection remain significantly high.

1.7.1. Serotype-specific control programs

Many control programs have been adopted to target specific *Salmonella* serotypes that are associated with poultry and/ or human salmonellosis; showing increased virulence such as invasiveness or antibiotic-resistance.

In the USA, National Poultry Improvement Plan (NPIP) established in 1935, eradicated *S*. Gallinarum, *S*. Pullorum from commercial flocks, whereas *S*. Enteritidis was targeted in egg - type breeders since 1989 and in broiler meats since 1994 (Foley et al., 2011).

In EU, Commission Regulation (EC) No 2160/2003 (Anonymous, 2003) was implemented in 2007 to reduce the prevalence of the top 5 serotypes (*S*. Enteritidis, *S*. Typhimurium, *S*. Hadar, *S*. Virchow, *S*. Infantis) in breeding hens and the most common serotypes causing human illness in broiler and egg layers (*S*. Enteritidis and *S*. Typhimurium). These strategies were significantly successful in decreasing this prevalence. However, other serotypes emerged, and now it is reconsidered to replace some targeted serotypes (*S*. Hadar, *S*. Virchow) by new ones such as *S*. Kentucky and *S*. Heidelberg or to include all serotypes as a target (EFSA BIOHAZ Panel , 2019).

Indeed, the potential disadvantages in developing a control strategy against one specific serotype are always at the expense of developing another food-poisoning one that may contaminate the flocks. Another issue is that the most relevant serotypes vary between countries and over time (Mead et al., 2010). As mentioned by Foley et al. (2011) successful control of one serotype may raise a concern as to what will fill the potential niche left after the elimination of targeted one from commercial poultry and egg production and potentially cause diseases in humans.

1.7.2. Vaccination program

Due to public health concerns associated mainly to *S*. Enteritidis (serogroup D) and *S*. Typhimurium (serogroup B), commercial vaccines are increasing since, with successful results. For both of them, inactivated (killed) and attenuated (live) vaccines are available, and their particular role is also underlined by the regulation (EC) No 1177/2006 (Methner, 2018) However, vaccination increase selection pressure for other emerging serotypes, including serogroup C such as *S*. Kentucky and *S*. Infantis (Fuche et al., 2016). One of the main drawbacks of vaccines is their reduced/ or absence efficacy against antigenically different serotypes. This enhances new problems caused by emerging serotypes by providing a vacant niche for other serotypes to proliferate (Eeckhaut et al., 2018).

1.7.3. Antibiotic usage in farms

The use of antibiotics was a turning point in the animal industry especially poultry. The antimicrobial practices in Veterinary Medicine took place in the 1940s when *Streptomyces aureofaciens* introduced as animal fed improved performances and accidently it was discovered that it produced chlortetracycline (Alagawany et al., 2018). It comprised disease prevention and treatment as well as AGPs and performance enhancers for livestock. These substances, added at sub-therapeutic doses in poultry feeds, increase productivity and prevent infectious diseases. The use of AGPs, with no need for a veterinary prescription, was approved by the US Food and Drug Administration (FDA) in 1951 (Gouvêa et al., 2015) followed by European approval, in the 1970s (Sanders and Moulin, 2017).

In Lebanon, all imported antibiotics are well controlled. However, surveillance and monitoring procedures which are set, are not implemented. Besides, there are no defined regulations regarding the importation and usage of AGPs (MoA, unpublished data).

Many antimicrobial classes, essential in human treatment, were used in animal husbandry (agyar, 2019). AGPs and prophylactics used in poultry were: tetracyclines (chlortetracycline), β-lactams (penicillin), macrolide (Tylosin, tilmicosin, erythromycin), lincosamide (lincomycin), streptogramins (virginiamycin), glycolipids (bambermycin), polypeptides (bacitracin), ionophores (salinomycin), aminocyclitols (apramycin), amphenicol (florfenicol), chloramphenicol, fluoroquinolone (enrofloxacin, sarafloxacin) and cephalosporin (ceftiofur)

((Angelakis, 2017; Patel et al., 2018). Table 1 shows the list of antibiotics used in Lebanese poultry production.

Table 1: List of some antibiotics for therapeutic used in Lebanese poultry production (MoA, unpublished data)

Antibiotic	Active substances	Target species	Diseases
Spectovet	Lincomycin, Spectinomycin	Sheep and goats, chicken and turkeys, dogs and cats	Pneumo enteritis f non-ruminating calves, respiratory infection, fowl cholera
Precex	Ceftiofur	Bovines, porcines, equines, chicks	Respiratory disease, pneumonia, early chicken mortality
Enrotryl 10%	Enrofloxacin	Cattle, sheep and goats, dogs and cats, poultry	Mastitis, pneumonia, respiratory infections, mycoplasmosis, arthritis
Sulfadoxine and trimethoprim injectin	Sulfadoxin eand trimethoprim	All animals	Pneumonia, bronchitis, diarrhea, colibacilloses, urogenital tract infection

However, Overusing and misusing of such agents have been noticed. More than half of the globally produced antibiotics are used in livestock production with chicken production showing the highest level (Van Boeckel et al., 2015). In 2010, the most five countries with largest global antimicrobial consumption in food animal production were China (23%), the United States (13%), Brazil (9%), India (3%), and Germany (3%). In China, nearly half of the 210, 000 tons of antibiotics produced, were used in livestock as therapeutic drugs and feed additive (Zhu et al., 2017). It has been estimated that by 2030, a total of 105,596 tons of antimicrobials will be consumed in food animal production globally (Suresh et al., 2017).

As a result, concerns on the emergence of AMR and MDR strains started to be voiced, at the end of the 1990s, from different parts of the world, and the use of AGPs became a public health concern (Suresh et al., 2017). The administration of low but in repeated doses of antimicrobial agents (the process in which growth –promoting and prophylactic are used) was the ideal condition to promote the emergence and dissemination of AMR in animals (You and Silbergeld, 2014). Evident links showed the involvement of poultry production as AMR *Salmonella* reservoir and its impacts on public health.

Licensing of fluoroquinolones, enrofloxacin and sarafloxacin for animal use, especially in poultry, in the 1990s headed to increased rates of decreased susceptibility to ciprofloxacin in *S*. Typhimurium DT104 recovered from animal/food (particularly poultry) and humans (Threlfall, 2000). The prophylactic use of fluoroquinolones in African and Asian poultry flocks was thought to be the main causative of the rapid spread of the Cip^R Kentucky ST198 strain (Le Hello et al., 2011).

The use of Extended-spectrum cephalosporin ESC in broilers has also contributed to the spread of ESBL and AmpC-producing *Salmonella* in the poultry sector. Voluntary withdrawal of ceftiofur in Canada and Japanese poultry producers was correlated with a decreasing occurrence of ceftiofur-resistant *S*. Heidelberg and *Salmonella* sp respectively (Shigemura et al., 2018; Dutil et al., 2010).

Another public health issue is the detection of antibiotic residues in poultry products and the emerging environmental pollution by resistant bacteria, antibiotic resistance genes, and antibiotics dissemination. These latter components are shed unmetabolized by poultry at a high rate (75-90 %) in the ecosystem. The spread of AMR from "Farm to fork" via water, manure, food was well reviewed by (Suresh et al., 2017).

1.8. **Antibiotic Resistance, the biggest global threat**

The world is on the edge of a post-antibiotic era where MDR bacteria are a superbug due to an antibiotic apocalypse, and dark ages where people will die from a scratch injury (Bettiol and Harbarth, 2015; Fukuda, 2015). Invasive *Salmonella* infections frequently occur in children, the elderly, and immunocompromised persons who need treatment with either ESC or ciprofloxacin (Diarra and Malouin, 2014). The emerging global antimicrobial resistance (AMR) threat caused 25,000 annual deaths in Europe, 100,000 in the USA and 80,000 in China (Ferri et al., 2017). In the USA, drug-resistant *Salmonella* triggered 100,000 illness cases with high resistance to clinically-relevant antibiotics such as ceftriaxone (36,000 illnesses/year) and ciprofloxacin (33,000 illnesses/year). MDR *Salmonella* (resistance to ≥5 antibiotics) caused 66,000 illnesses (CDC, 2013). Besides, AMR led to an increasing health-care estimated to €1.5 billion and $55 billion yearly in Europe and USA respectively (Ferri et al., 2017). The void of discovering new

antimicrobial agents worsen the situation (Bettiol and Harbarth, 2015). Bacterial resistance is inevitable, warned Fleming when he discovered penicillin.

1.8.1. Antibiotic-resistance Mechanisms

Antibiotics target the lysis of either the bacterial cell wall or membrane or hamper the essential processes related to metabolism (protein synthesis) and replication (nucleic acid synthesis). Consequently, antibiotics must reach the bacterium cytoplasm, without being destroyed or modified, fix on a target and disrupt the bacterial physiology.

The AMR phenomenon, due to selective pressure halt these antibiotic functions. It could be either intrinsic (innate trait) or acquired (Agyare et al., 2019) which is mediated by two fundamental mechanisms, biochemical and genetic.

Biochemical mechanisms include:

1) Decreasing the intracellular antibiotic concentrations either by membrane permeability changes (macrolides or β-lactams antibiotics) or active efflux pump that remove antibiotic from the bacterial cell cytoplasm ((Fluoro) quinolones antibiotics). Some efflux pumps have narrow substrate specificity (for example, the Tet pumps), but many transport a wide range of structurally dissimilar antibiotic substrates and are known as MDR efflux pumps (Coussens et al., 2018).

2) Enzymatic inactivation either by destruction (β-lactamases) or modification of the antibiotic preventing its binding to the target site (aminoglycosides and chloramphenicol).

3) Alteration of the antibiotic target site (e.g., Penicillin Binding protein) so that it does not bind to the bacterial cell. A wide range of antibiotics has been involved in such mechanism including beta-lactams, macrolides, tetracyclines, fluoroquinolones, aminoglycosides, sulfonamide, and vancomycin (Zeng and Lin, 2013).

This acquired biochemical resistance is mediated through genetic mechanisms such as mutation and horizontal transfer. In response to antibiotic selective pressure, this latter can spread the drug resistance between and within species through mobile genetic elements (MGEs) such as plasmids; transposons; or integrons. (Nair et al., 2018).

Plasmids are extrachromosomal self-replicating DNA fragments easily transmitted from one bacterium to another. Defined by their incompatibility (Inc) types (Rozwandowicz et al., 2018), they are the principal source of dissemination of drug resistance genes (Kaldhone et al., 2017). The ability of IncI1 plasmid to carry and spread ESC resistance genes offers a potential explanation for the plasmids' prevalence among MDR *Salmonella* (Folster et al., 2016).

Transposons are known as "jumping genes." It's as small MGEs usually flanked by repeats or insertion sequences that could self-excise and transpose any resistance genes they carry. Insertion sequences (IS) are among the simplest transposons, that don't carry genes other than those required for transposition inactivation affecting virulence, resistance, and metabolism. (Vandecraen et al., 2017). More than 4500 IS belonging to 29 families have been identified to date.

Integrons are a DNA fragment that carries one gene or gene cassettes and may be integrated by site-specific recombination into chromosomal or plasmid DNA of the organism. Class I integrons are the common type recognized among the MDR *Salmonella* which often contain gene cassettes (Gharieb et al., 2015). The *Salmonella* Genomic Island, SGI1 antibiotic resistance gene cluster, which is a complex class 1 integron (*In104*), confers the typical MDR phenotype of epidemic *S*. Typhimurium DT104 (ACSSUT). SGI1 has been described in *S*. Typhimurium DT120 and other Serotypes (*S*. Emek, *S*. Infantis, *S*. Kentucky, *S*. Kiambu, *S*. Kingston, *S*. Meleagridis, *S*. Newport, and *S*. Paratyphi B, *S*. Agona, and *S*. Albany) (Doublet et al., 2008; Beutlich et al., 2011).

In addition, antibiotic resistance increased *Salmonella* virulence and fitness due to the co-localization in the MGEs of the same genomic islands of virulence and antibiotic resistance genes (Qiao et al., 2018).

1.8.2. Key antibiotic classes, resistance mechanisms with related genes

There are three main classes of antibiotics namely β-lactams, aminoglycosides, fluoroquinolones which are regularly used to treat salmonellosis in both human and veterinary medicine (Wang, 2017; Doi et al., 2017).

1.8.2.1. ***β-lactams:***

It contains different subclasses; penicillins, cephalosporins, carbapenems and monobactams with the most essential being β-lactam-β-lactamase inhibitor combinations, third and fourth generation cephalosporin, and carbapenems.

The common mechanism of resistance is the secretion of β –lactamases hydrolyzing the antibiotic. A various range of β-lactamases (active against first-generation β-lactams) was followed by ESBL-producing *Salmonella* mainly from poultry origin (Saliu et al., 2017). This latter enzyme provides high resistance against ESCs (cefotaxime, ceftriaxone, ceftazidime, or cefepime) and monobactams (aztreonam). Among the ESBLs genes, *bla* $_{TEM}$, *bla* $_{SHV}$, and particularly *bla* $_{CTX-M}$ located on IncI1 and IncFIB plasmids were the most frequently observed in poultry and poultry products (Saliu et al., 2017). The explosive dissemination of CTX-Ms worldwide has been stated as the "CTX-M pandemic" (Canton, 2012).

In parallel, plasmidic AmpC β -lactamases, conferring resistance to penicillins, third-generation cephalosporins, cephamycins, and monobactams have also emerged worldwide. These enzymes are encoded by *bla*$_{CMY}$ genes and *bla*$_{DHA}$ genes and frequently carried on IncA/C and IncI1 plasmids. The blaCMY-IncI1 plasmids were common among poultry-derived *Salmonella* serotypes (Folster et al., 2016).

Different MGEs were involved in the mobilization and acquisition of *bla*$_{CTX-M}$ genes, including insertion sequences ISEcp1 and ISCR1 controlling *bla* high -level expression (Ma et al., 2018). The ISEcp1 is also responsible for the spread of *bla*$_{CMY-2}$ by mobilizing the adjacent resistance genes originated from the *Citrobacter freundii* chromosome (Gharout-Sait et al., 2015).

Carbapenemase-producing *Salmonella* closely followed these high resistances. Enzymes responsible for resistance include IMP (imipenemase), VIM (Verona integron encoded Metallo β-lactamase), *K. pneumoniae* carbapenemase (KPC), OXA (oxacillinase) including OXA-48-like enzymes. The carbapenemase gene showed to be located on plasmids or transposons, thereby enabling their dissemination in the ecosystem (Mairi et al., 2018).

1.8.2.2. ***Aminoglycosides:***

Aminoglycosides bind to the 16S rRNA within the 30S ribosomal subunit, and therefore inhibit bacterial protein synthesis. This class includes gentamicin, streptomycin, kanamycin,

tobramycin, amikacin, spectinomycin, and apramycin. Resistance may occur by a multitude of mechanisms. The first mode follows antibiotic modification due to aminoglycoside-modifying enzymes such as aminoglycoside acetyltransferases (encoded by *aacC* and *aacA* genes conferring resistance to gentamycin), adenyltransferases (encoded by *aadA1, aadA2, aadA5, aadA6, aadA7, aadA12, aadA21, aadA22, aadA23, aadA24, aadA26, and aadA27*, resistance to streptomycin and spectinomycin) and phosphotransferases (*strA* and *strB* genes streptomycin) (Michael and Schwarz, 2016); Secondly by increasing efflux; another way of resistance is by decreasing permeability. Modification of the 30S ribosomal subunit could also contribute to resistance preventing aminoglycosides binding (Cameron et al., 2018), and finally by posttranscriptional modification of the 16S rRNA encoded by plasmid-mediated 16S rRNA methylase genes (rmt genes) (Doi et al., 2016).

1.8.2.3. ***Quinolones and fluoroquinolones:***

This class includes nalidixic acid, ciprofloxacin, norfloxacin, enrofloxacin, and sarafloxacin. The resistance is mainly caused by mutations in the quinolone targets, quinolone resistance determining regions (QRDRs), *gyrA*, *gyrB*, *parC*, and *parE* genes, which encoded DNA gyrase and topoisomerase IV. Plasmid resistance, plasmid-mediated quinolone resistance (PMQR) also occurs mainly in the Mediterranean countries (Yanat et al., 2017), but at less extent, including qnr genes, enzymatic inactivation by a variant of an aminoglycoside acetyltransferase gene aac (6')-IB-cr (cr: ciprofloxacin resistant phenotype) (Yanat et al., 2017), and efflux pump encoded by qepA, oqxAB genes (Wang et al., 2017).

It is well known that AMR is a great challenge; however, MDR shows a more severe danger where treatment options became harshly limited and are life-threatening. The resistant strains to third-generation cephalosporins and quinolones are of particular concern since they are considered first choice treatment of salmonellosis. Poultry seems to be a significant vehicle of MDR *Salmonella* (Andino and Hanning, 2015). Distinct MDR patterns have been identified (Table 2). pAmpC resistant gene has been strongly associated with quinolones, and other lactamase genes plasmids are usually found to co- carries ESBLs, aminoglycoside and/or quinolone resistant genes (Wang et al., 2017) and may also transport heavy metals resistance genes (Saliu et al., 2017).

Table 2: Drug-resistant *Salmonella enterica* subsp *enterica* strains isolated from poultry; antibiotic resistance phenotypic pattern and their respective resistance genes

Serotypes	Antibiotic resistance phenotypic pattern	Resistance genes	QRDR point mutation		Country	Reference
			parC	GyrA		
S. Enteritidis	ACSSUT profile+ Caz-Ctx-Ofx-Na	Qnrb		Ser83Y	China	Ma et al., 2018
S. California	Ctx-Ak-Cip	blaCTX-M-90, rmtC, qepA, oqxAB, aac(6')-Ib-cr			China	Wang et al, 2017
S. Indiana		armA, aadA5, aac(6′)-Ib-cr, aac(3)-IVa, aph(4)-Ia, arr-3, blaTEM-1B, blaOXA-1, blaCTX-M-65, catB3, dfrA17, fosA, floR, strB, strA, sul1, sul2, sul2, tet(A), oqxA, oqxB, mcr-1, aph(3′)-IIa, mph(A)			China	Wang et al, 2017
S. Indiana	Ctx-Ak-Cip	blaCTX-M-65, armA, qnrB, qepA, oqxAB, aac(6')-Ib-cr			China	Wang et al., 2017
S. Heidelberg	Amp-Cro	bla CTX-M-2, blaTEM			Brazil	Fitch et al., 2015
S. Minnesota	Amp- Cro	blaCTX-M-14, blaSHV				
S. Heidelberg	Amp-Amc-Caz- Ctx- Fox-Cip-Pef-Na-Smx-Te	blaCMY-2,sul2, tet(A)			EU/ imported chicken meat (gizzards) from Brazil	Campos et al, 2018
S. Minnesota	Amp-Amc-Caz-Ctx-Fox-Kan- Cip- Pef-Na- Smx-Te	blaCMY-2, aphA1, qnrB5, sul2, tet(A)				
S. Mbandaka	Amp- cip	qnrS1/S3			Poland	Hoszowski et al., 2016
S. Mbandaka	Amp-CTx-Caz	blaCMY-2			Poland	
S. Manhattan	Cpdx-Ctx-Caz-Fep	blaCTX-M-15& blaTEM-1			Japan	Noda et al, 2015
S. Infantis	Cpdx-Ctx-Caz-Cfx	blaCMY-2			Japan	
S. Infantis	Cpdx-Ctx	blaTEM52			Japan	
S. Infantis	Amp-C-Te-Cro-Caz-Na-Atm-Ctx	aph(4)-Ia, aph(3')-Ic, aac(3)-IVa, blaCTX-M-65, floR, sul1, tetA, dfrA14		D87Y	USA	Tate et al., 2017
S. Infantis	Ctx-Te- Smx-Tmp- cip	pESI-like megaplasmid carried the ESBL gene blaCTX-M-1, tet(A),			Italy	Franco et al., 2015

		sul1, dfrA1 and dfrA14				
S. Kentucky	Sul-Na-Te-Amp- S-Cn- Cip	sul1, blaTEM-1			Egypt	Abdel-Maksoud et al., 2015
S. Kentucky	Sul-Na-Te – Amp- S,-Ctx-Atm	sul1, sul2, blaTEM-1, blaSHV			Egypt	
S. Kentucky ST198	Cip- Amp-C-Lvx-Na-Sox-Te-S	blaTEM-57, aadA1, aadA2, cmlA1, sul3, tetA/	Thr57Ser, Ser80Ile	Ser83Phe, Asp87Gly	Egypt	Ramadan et al., 2018
S. Kentucky ST198	Cip- Amp-C-Lvx-Na-Sox-Te-S-Sxt	blaTEM-57, aadA1, aadA2, cmlA1, sul3, tetA, dfrA, sul2, floR, aph(30)-Ia	Thr57Ser, Ser80Ile	Ser83Phe, Asp87Gly	Egypt	
S. Kentucky ST198	Amp-Amc-C-Te-Sxt-S-Ka-Na-Cip	blaTEM1-B, cmlA1, tet(A), sul1, sul3, dfrA12, aadA1, aadA2, aph(3=)-la, and mph(A)	Ser80Ile, Thr57Ser,Thr255Ser)	(Ser83Phe, Asp87Gly)	USA	Shah et al., 2018
S. Kentucky	Te-Na-Sxt-S	aadA2, tet (G), sul1, blaCARB-2, floR, dfrA14, erm (42), aph (3′)-Ia			Iraq/ frozen chicken imported from Iran	Harb et al, 2018
S. Typhimurium	Te-Na-Sxt-Cip-Ath-Amc-Amp	aadA7, tet (A),strA, strB, aac(3)-Id,sul1, blaTEM-1B			Iraq/ frozen chicken imported from Iran	
S. Typhimurium	Te-Na-Cn-S-Sxt	aadA2, tet (G), sul1, blaCARB-2, floR			Iraq/ frozen chicken imported from India	
S. Typhimurium	C -Sxt-Te-Ery-Cip-As-Na-Cn-Amc	aac (3)-Id, aadA7			Egypt	Gharieb et al., 2015

ACSSUT: Ampicillin-Chloramphenicol-Streptomycin-Sulfamide-Tetracycline, Amp: Ampicillin, Amc: amoxicillin-clavulanic acid, As: Ampicillin-sublactam, Cfx: cefoxitin, Cro: ceftriaxone, Caz: ceftazidime, Ctx:cefotaxime, Fep: cefepim, Cpdx: cefpodoxime , Eft: ceftiofur, Atm: aztreonam, Cn: Gentamicin, S: streptomycin, Ak: amikacin, Erythromycin, Na: nalidixic acid, Cip: ciprofloxacin, Lvx: levofloxacin, Pef: pefloxacin, Ofx: ofloxacin, Te: tetracycline, Sul: sulfonamide, Smx: sulfamethoxazole, Sox: sulfisoxazole, Tmp: trimethoprim, Sxt: trimethoprim/sulfamethoxazole , C:chloramphenicol, Ath: Azitromycin

1.8.3. Global strategies against AMR

Policies and strategies were set at a national, regional and international level to tackle AMR problem.

One solution was the gradual withdraw of several antibiotics as AGPs and prophylactics. In the USA, these two latter are still allowed where some antibiotic classes were completely banned such as aminocyclitols (apramycin, spectinomycin), amphenicols (florfenicol), and chloramphenicol. In 2005, the fluoroquinolones, enrofloxacin, and sarafloxacin were proscribed, followed, in 2017 by the extra-label usage of medically important antibiotics such as ceftiofur (Patel et al., 2018). Same in Brazil, many classes were phased out. Chloramphenicol and nitrofurans; amphenicols, tetracyclines, beta-lactams (penicillins and cephalosporins), quinolones, and systemic sulfonamides; spiramycin and erythromycin were banned in 2003, 2009 and 2012 respectively (Gouvêa et al., 2015).

The EU took drastic measures by a complete ban of AGPs in 2006. Recommendations are followed in 2011 and 2012, to limit the use, in the veterinary field, of critical third- and fourth-generation antibiotics intended for human therapy and to reduce antimicrobials veterinary drugs usage to 50% by 2018 respectively (Ferri et al., 2017).

In addition, a general awareness campaign against the antimicrobial misuse and AMR has been launched by international organizations, WHO, FAO and Organisation Internationale des epizooties (OIE) (Ferri et al., 2017); in these efforts, Lebanon is an active member in all these joint Committees represented by MoA, MoPH, and LARI.

A back draw of these strategies was a significant increase in the production cost and morbidity rate. As a consequence, a high number of veterinary therapeutic prescriptions was well observed (Suresh et al., 2017) forcing livestock producers to find alternatives such as organic acids with antimicrobial activities; herbs; bacteriophages: spices and other plant extracts; immune-stimulation through cationic peptides and cytokines; prebiotics; fermented feed (Ranjitkar et al., 2016) and probiotics (direct- fed microbial) (Diarra and Malouin, 2014).

2. Promising natural alternative: probiotics

Due to their beneficial characteristics and natural composition, probiotics may be a great alternative to antibiotics in animals including poultry.

2.1. **Origin**

The etymology ‘‘probiotics’’ derived from two Greek words pro and biotos meaning ‘‘for life’’(Ozen and Dinleyici, 2015).

FAO & WHO, (2002) defined “probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host.” In animal use, The US National Food Ingredient Association well-defined probiotics (direct-fed microbial) as “a source of live naturally occurring microorganisms and this includes bacteria, fungi, and yeast.” Others gave a new definition for probiotics “as live microbial feed additives which beneficially affect the host animal via enhancing the balance in the gut and consequently improving feed efficiency, nutrient absorption, growth rate, and economic aspects of poultry”(Abd El-Hack et al., 2017).

Throughout history, probiotic foods have been consumed long before the discovery of microbes, either as natural components of food or as fermented foods. The first discovery in this field was when Metchnikoff (Nobel Prize laureate), in 1905, found that pure cultures of *Lactobacillus bulgaricus* are responsible for milk fermentation and able to eliminate pathogenic toxin-producing bacteria from the colon. Another success story was in 1906 when Henry Tissier isolated *Bifidobacterium* from a human child and could displace harmful microflora in the gut. The first use of probiotics in animals was recorded in the 1940s when the use of *Streptomyces aureofaciens* probiotics in feed resulted in significant weight gain (Angelakis, 2017).

2.2. **Types of probiotics**

There are many sources of probiotics such as bacteria (*Bacillus cereus*, LAB such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*), yeast (*Saccharomyces cerevisiae*, *Saccharomyces boulardii*, and *Candida*), and fungi (*Aspergillus*) (Alagawany et al., 2018). These can be isolated from humans (e.g., gut and breast milk) and animals (e.g., gut) as well as fermented products but the majority is of intestinal origin. Other non-conventional sources of probiotics are used, such as *L. plantarum* and *Leuconostoc mesenteroides* which can be isolated from fruits and vegetables. In

chickens, yeasts (*Saccharomyces boulardii*), and bacteria (*Lactobacillus* sp., *Enterococcus* sp., *Pediococcus* sp., *Bacillus* sp.) are frequently used (Angelakis, 2017). Recently, the focus has been on using lactic acid bacteria as the probiotic of choice because of their natural adaptability to the intestinal environment (Wang and Gu, 2010).

2.3. Lactic acid bacteria (LAB) as probiotic: focus on *Lactobacillus*

At the end of the last century, the term "lactic acid bacteria" gradually emerged (Kandler, 1983). Members of this group are Gram-positive bacteria, non-motile, anaerobic or facultative aerobic cocci or rods, having non-sporulating character. They can ferment carbohydrates (glucose, fructose, sucrose, and lactose) generating lactic acid as one of the primary fermentation products; hence their acid tolerance (Quinto et al., 2014). Their cultivation requires environments rich in sugars, amino acids, fatty acids, salts, and vitamins and low oxygen. Their growth temperature is very variable (20ºC-45ºC) given their ubiquity; mesophilic lactic bacteria have an optimum temperature of growth between 20ºC and 30ºC and thermophilic have an optimum temperature between 30ºC and 45ºC.

2.3.1. Classification of LAB

LAB group belongs to the phylum Firmicutes, class Bacilli, and order Lactobacillale (Quinto et al., 2014). Six families were described; Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae, Leuconostocaceae, and Streptococcaceae and include more than 20 genera.

Various classification schemes have been recognized. The first classification of Orla-Jensen (1919) was based on the following criteria: cellular morphology, growth temperature and mode of glucose fermentation (Heineman, 2010). Kandler (1983) classified the LAB as obligate homofermentative, facultative heterofermentative, and obligate heterofermentative (Figure 3).

Obligate homofermentative metabolism of hexoses via the Emden–Meyerhoff pathway; this group uses the classical pathway of glycolysis to convert one molecule of glucose into two lactate, under optimal growth conditions. The genera belonging to this group are *Streptococcus*, *Lactococcus*, *Pediococcus*, and the majority species of *Lactobacillus* such as *L. salivarius*, *L. bulgaricus, L. casei, L. lactis, L. acidophilus.*

Obligate heterofermentative metabolism of hexoses and pentoses via the phosphoketolase pathway. Lactic acid and ethanol or acetate are the end products, respectively. These are in particular *Leuconostoc*, *Weissella*, *Oenococcus* and some species of the genus *Lactobacillus* such as *L. fermentum* and *L. brevis*.

Facultative heterofermentative metabolism the capacity to adopt one of the two ways according to the environmental conditions. *L. plantarum* is part of it.

The current adopted phenotype-based nomenclature does not notice the pathway for pentose conversion to lactate as the sole end product (Gänzle, 2015). On the other hand, molecular tools like 16S rRNA genes sequences and core genome phylogeny showed that this classification does not reveal the metabolic features of lactobacilli and is inconsistent with the phylogenetic structure of the genus (Zheng et al., 2015).

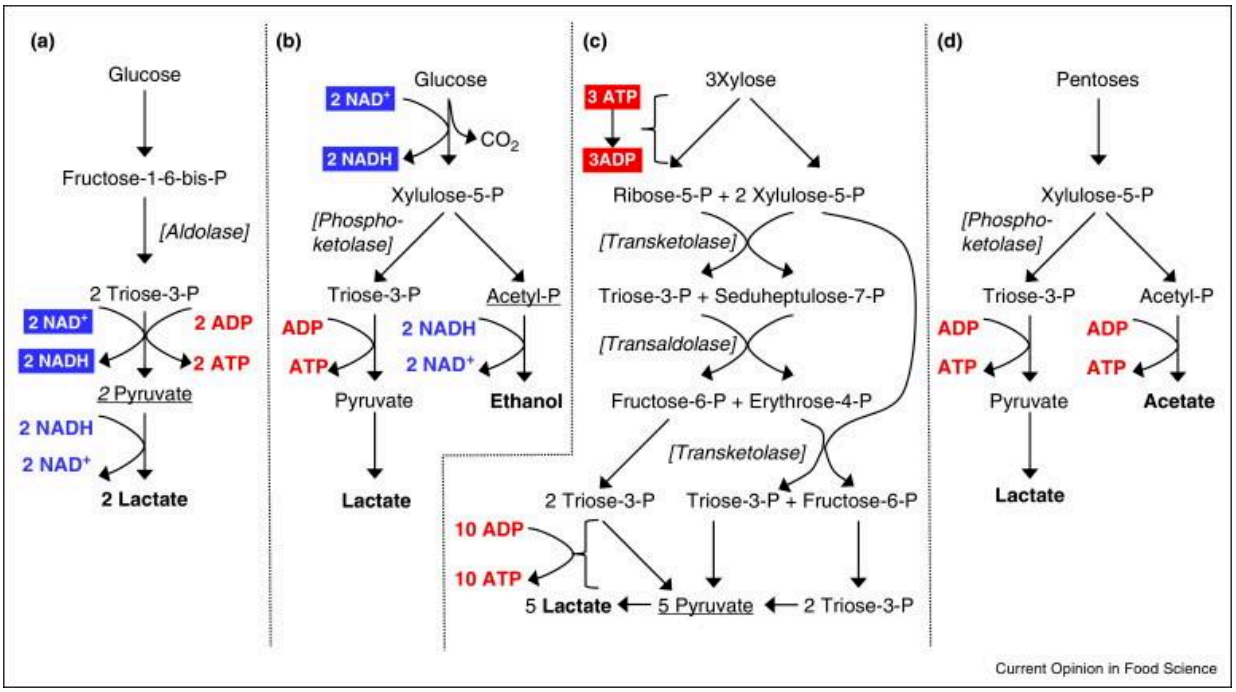

Figure 3: Overview of carbohydrate fermentation lactic acid bacteria (Gänzle, 2015).

(a) Homofermentative metabolism of hexoses via the Emden–Meyerhoff pathway.

(b) Heterofermentative metabolism of hexoses via the phosphoketolase pathway.

(c) Homofermentative metabolism of pentoses via the pentose phosphate pathway.

(d) Heterofermentative metabolism of pentoses via the phosphoketolase pathway

2.4. *Lactobacillus* classification

The large size and high diversity of this genus, close to 200 species, are one of the reasons for its uncertain taxonomy. *Lactobacillus* is an exception among lactic acid bacteria, as it comprises species that employ homolactic metabolism as well as heterolactic metabolism.

Recently, advanced molecular analysis, based on 16S rRNA and robust core genome phylogeny, permit to classify this genus into two major metabolic groups; homofermentative and heterofermentative lactobacilli, which are divided into 24 separate phylogenetic clusters (Zheng et al., 2015). The ecological fitness of heterofermentative lactobacilli is governed by the favored utilization of disaccharides, the capacity use of pentoses and hexoses, and preferential utilization of fructose, phenolic acids, and aldehydes as electron acceptors.

The known term *Lactobacillus* sensu lato includes now pediococci as an integral part of the homofermentative lactobacilli (Zheng et al., 2015) whereas Lactobacillus Genus Complex consists of the heterofermentative lactobacilli and the related genera *Weissella*, *Leuconostoc*, *Oenococcus* and *Fructobacillus* covering *Lactobacillaceae* and *Leuconostococcaceae* (Duar et al., 2017).

2.4.1. *Lactobacillus* Niche-Specific Adaptation: The Intestinal Environment

Lactobacillus species are isolated from nutrient-rich habitats related to food, feed, plants, animals and humans. The first niche of the *Lactobacillus* genus is strongly suggested to be a free-living ancestor (e.g., *L. buchneri* found in Grass/silage) in soil and plants and, subsequently, host adapted to vertebrate (e.g., *L. salivarius* and *L. reuteri*) and insect (e.g., *L. apis* and *L. kunkeei* found in Bees and Flowers, grapes, bees). Some species are defined as “nomadic” (e.g., *L. plantarum*, *L. casei*, *L. paracasei*, and *L. rhamnosus*), and that could be found in different habitats; meat, fish, vegetables and raw or fermented dairy products as well as gut ecosystems (Duar et al., 2017). According to the authors, the preferable habitats of the vertebrate host- adapted *L. salivarius* are a human oral cavity, digestive tract, breast milk and vagina as well as feces of pigs, raccoons, chickens, and hamsters. Other species such *L. reuteri* prefer proximal digestive tract of human and animals (Duar et al., 2017).

In response to the evolution process and niche adaptation, the genome size of lactobacilli was reduced which match with the need to nutrient-rich environments. The shift from soil and plants to the animal gut has three ranges of genomic adaptation; resistance to host barriers such as tolerance to acid and bile acids; adhesion to intestinal cells; and fermentation of some substrates in the gut (Quinto et al., 2014). Additionally, these species could grow at an optimum temperature of 37◦C and higher, body temperatures of most mammals and birds (Duar et al., 2017).

This host adaptation is considered to be symbiotic, and lactobacilli and host are reciprocally affected. It seems that fitness level is completely associated and relevant for the development of probiotics aimed to outcompete pathogens. It is related to higher metabolic activity in the host niche, which could lead to increased production of metabolic compounds that define probiotic activity (Duar et al., 2017).

2.5. **Gut microbiota, probiotics of poultry origin**

At birth the digestive tract of poultry is sterile, but after 6 to 12 hours the cecum will be quickly colonized by the environmental microflora such as Enterobacteriaceae, *Enterococcus* and *Lactobacillus* (Albazaz and Byukunal Bal, 2014). The poultry microbiota, very similar to that of mammals, contains a very diverse microbial population (Oakley et al., 2014) with a significant proportion not cultivable; 52 microbial phyla have been recognized, described as "uncultivated majority"(Shang et al., 2018). In chicken, 29 cultivable genera were identified, each genus is represented by 3 to 4 species, and each species by 3 to 4 different metabolic types, which would make more than 200 different types (Gabriel et al., 2005).

Bacterial communities change drastically between the different anatomical segments of the digestive tract mainly represented by Firmicutes, especially Lactobacillaceae (*Lactobacillus*) at all ages and in all sections of the gut except the cecum where a count decrease in the adult broiler. From the crop to the ileum, the microbial flora consists mainly of facultative anaerobic gram-positive bacteria and at the level of the caeca predominate strict anaerobes. In the crop, mostly lactobacilli are attached to the epithelium, forming almost a thin layer, as well as streptococci, coliforms, and yeasts (Gabriel et al., 2005). *Lactobacillus* is also predominant in the proventriculus and gizzard where the microbial density is relatively low (10^8 / g) due to low pH. The small intestine contains a large number of bacterial species (10^8-10^9 / g), mainly *Lactobacillus*,

Enterococcus, and *Clostridium*. In the duodenum, the presence of many enzymes, the high oxygen pressure, and the presence of bile salts cause the bacterial population to fall (Oakley et al., 2014). Finally, the ceca, the terminal part of the gastrointestinal tract offer a nutrient-rich habitat for the millions of microflora (10^{11} CFU/g). It contains the most diverse microbiota of the gastrointestinal system with the dominant Firmicutes, Bacteroidetes, and Proteobacteria. The development of bacteria is favored by the low frequency of renewal. It is the leading fermentation site (Oakley et al., 2014; Yeoman et al., 2012).

The age also has a significant influence on the diversity of the microflora. The gastro-intestinal tract (GIT) of chicken at three days of age contained mainly *L. delbrueckii*, from 7 to 21 days of age, *L. acidophilus*, and at 28 until 49 days of age, the GIT includes *L. crispatus* (Shang et al., 2018). However, a population of *Lactobacillus* is present in birds of two days of age, and it remains without drastic changes until market age. The main species include *L. acidophilus*, *L. salivarius*, and *L. fermentum*. Ranjitkar et al. (2016) found that a "mature" microbiota occurred from days 15 to 22 where *L. salivarius* (17 to 36 %) and clostridia (11 to 18 %) are the most predominant. Shang et al. (2018) proposed *L. reuteri*, *L. acidophilus*, *L. crispatus*, and *L. salivarius* the four dominant *Lactobacillus* species present throughout the chicken digestive tract.

The composition of the feed influences also the microbiota. Mash feed decreases the number of *Enterococcus* and coliforms but rises *Lactobacillus* and *C. perfringens* in the broiler ileum. When broilers are fed with corn, low percent of clostridia, enterococci, and lactobacilli have been observed, whereas with wheat higher percentage of bifidobacteria were obtained. The addition of antibiotics to feed such as salinomycin inhibited *L. salivarius* in the ileum of two-week-old chickens (Albazaz and Byukunal Bal, 2014).

2.6. **Anti- *Salmonella* activities**

In large-scale rearing facilities, chicks are highly susceptible to *Salmonella* infection, even at low exposure doses, due to the stress and their gradual acquisition of a complete intestinal microflora from their environment. In poultry farming, defined bacterial species (one or mixture of two or more species) or mixed non-defined cultures were used to reduce gastrointestinal colonization by pathogens such as *Salmonella* (Alagawany et al., 2018).

The first use of non-defined strains of probiotics was carried out by Rantala and Nurmi, (1973). In their experiments, the authors observed that day-old chicks administered orally with the intestinal contents of adult birds might have gained a protective effect against *S*. Infantis infection. The defined products as *L. salivarius* CTC2197 appear to prevent *S*. Enteritidis colonization in chickens (Pascual et al., 1999). This phenomenon described as "colonization resistance" or "competitive exclusion" (Yadav et al., 2017) with the highly effective measure to protect newly hatched chicks (Kabir, 2009). It is a strain-dependent trait, mainly includes (Pan and Yu, 2014)(Figure 4):

Direct inhibition of *Salmonella* by:

1. Competition to adhesion to the intestinal binding site
2. Competition of use of nutrients in the gastrointestinal tract
3. Secretion of inhibitory substances against *Salmonella*; bacteriocin/ bacteriocin-like; hydrogen peroxide; and organic acids.

Indirect inhibition by:

4. Strengthening the function of the intestinal barrier
5. Modulating the immune response

Other potential probiotic benefits reside in enhancing growth and productive performance, eggs quality, digestion, and absorption of nutrients (Alagawany et al., 2018).

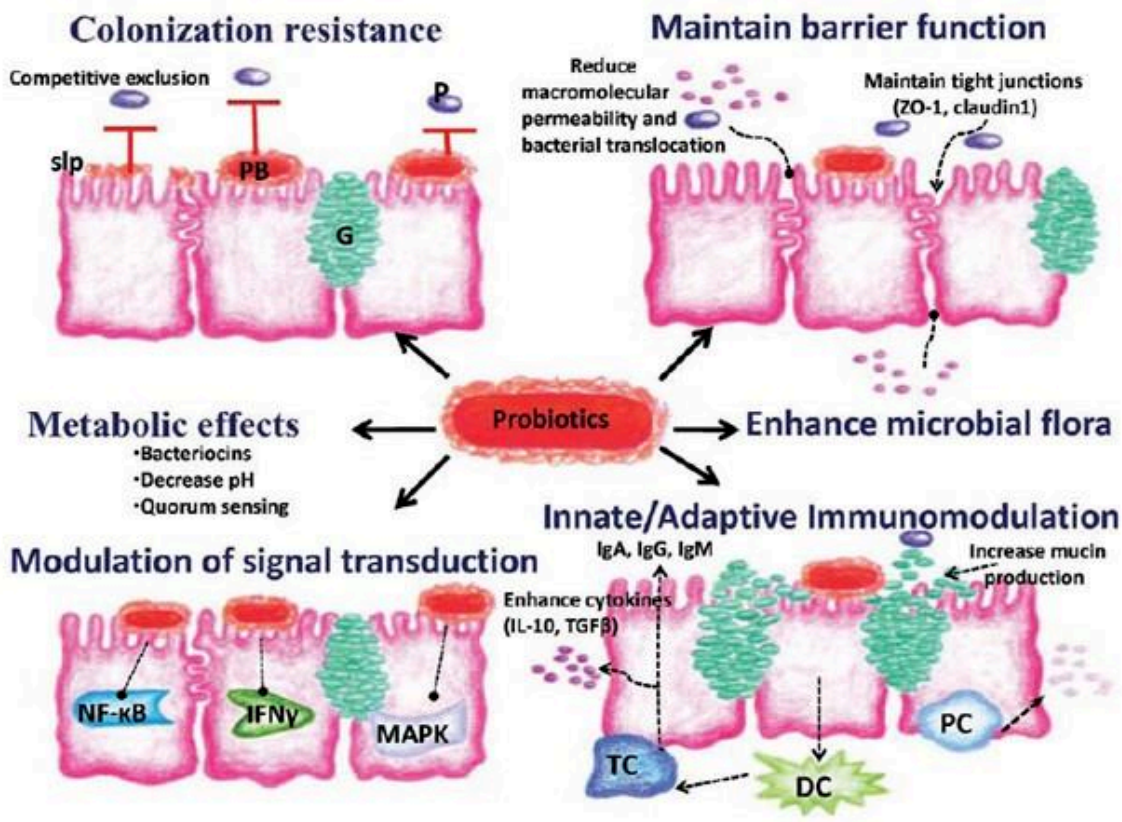

Figure 4: Different mode of action of probiotics against *Salmonella* infection in poultry (Sherman et al., 2009). P: pathogen, PB: probiotic, Slp: surface layer protein, G: Goblet cells, NF-κB: nuclear factor kappa, IFN_{γ}: interferon $_{\gamma}$, MAPK: mitogen-activated protein kinases, TC: T lymphocyte, DC: dendritic cells, PC: Paneth cell

2.6.1. Adherence

Probiotics, by a phenomenon called "barrier effect," embedded in the GIT to form a dense and complex microbial layer, and effectively blocks the attachment and subsequent colonization of *Salmonella* (Gabriel, 2005). Thus, adherence of probiotics to the intestinal epithelial cells is a crucial factor for colonization that can result in competitive exclusion of pathogens and the modulation of host response (Sengupta et al., 2013).

Cell adhesion is a complex process done either specifically via adhesins or nonspecifically controlled by physicochemical reactions of the cell wall including electrostatic and Van der Waals interactions as well as hydrophobic one (García-Cayuela et al., 2014). Surface proteins and (lipo) teichoic acids that cover the peptidoglycan, charge negatively the bacterial surface in

physiological conditions and therefore confer high hydrophobicity character (Babot et al., 2014). This feature is thought to play an essential role in firm adherence to epithelial cells (Mohanty et al., 2019). Several groups of lactobacilli adhesins have been identified; mucus-binding proteins; sortase-dependent proteins; S-layer proteins; non-protein adhesins ((Lipo) teichoic acid and Exopolysaccharide (EPS)). S-layer proteins form the outermost interacting surface in lactobacilli. Chen et al. (2007) confirmed the role of S-layer proteins in adhesion of *L. crispatus* ZJ001 to HeLa cells and their removal reduced auto-aggregation and adhesion. By auto/ and co-aggregation, probiotics could adhere to epithelial cells and form a barrier respectively and therefore inhibit the foodborne pathogens colonization (Kos et al., 2003). The authors demonstrated the role of S-Layer in auto-aggregation and adhesion of *L. acidophilus* M92. A multitude of interrelated surface factors (Fatty acids, surface proteins, LPS, EPS) may have effects on adherence, co-aggregation, and cell to cell interactions (Campana et al., 2017).

The competition for adhesion to epithelial cells has been often demonstrated. Singh et al.(2017) showed the capacity of *L. reuteri* strains to adhere to Caco-2 cells, inhibit and displace the adhesion of *Escherichia coli* ATCC25922, *S*. Typhi NCDC113, *Listeria monocytogenes* ATCC53135, and *Enterococcus faecalis* NCDC115. Mohanty et al. (2019) demonstrated a significant reduction in the adherence of *Salmonella* to the HCT-116 cells when incubated with the *L. plantarum* DM 69 strain.

2.6.2. Competition use of nutrients

Similarly, probiotics intervene through the competitive use of nutrients (Pan and Yu, 2013). This capacity is a non-negligible factor that determines the composition of the microbiota. Thus, an increase in the number of lactobacilli would reduce the substrates available for pathogenic microorganisms leading to these latter inhibition. Abhisingha et al. (2018) suggested that the inhibition of *S*. Enteritidis after 10h of co-culture with *L. johnsonii* was due to competition for limiting nutrients.

2.6.3. Secretion of active metabolites against *Salmonella*

To gain a competitive advantage, lactobacilli modify their environment by producing antimicrobials to make it less suitable for their competitor. These inhibitory compounds are diverse and include organic acids (e.g., lactic acid and acetic acid), oxygen catabolites (e.g., hydrogen

peroxide), and proteinaceous compounds (e.g., bacteriocins) (Ayeni et al., 2018) . Among these activities, the production of organic acids mainly lactic acid acting by its chemical structure and by decreasing the pH, is the main inhibitor metabolite of LAB. The high antibacterial activity of *L. salivarius* C86 and *L. amylovorus* C94 against *Salmonella* in a study done by Adetoye et al. (2018) is related to the high production of lactic acid. *L. fermentum* CS12-1 accumulated hydrogen peroxide in culture broth that inhibits the growth of enterotoxigenic *Escherichia coli* (Kang et al., 2005). Kizerwetter-Świda and Binek, (2016) showed that all poultry- derived *Lactobacillus* were able to produce hydrogen peroxide leading to *S*. Enteritidis inhibition.

Bacteriocins are ribosomally synthesized antimicrobial peptides, produced by Gram-positive and Gram-negative bacteria. They are active mainly against related species and Gram-positive bacteria (*Listeria monocytogenes*), but few studies showed their effectiveness against Gram- negative bacteria such as *Salmonella*. Plantaricin LD1, a bacteriocin produced by *L. plantarum* LD1, inhibit the growth of *E.coli* and *S*. Typhi (Gupta and Tiwari, 2014).

2.6.4. Maintenance of Epithelial Barrier Function.

The gut barrier includes the mucus layers, epithelium, and sub-epithelial immune tissues. The essential function of the epithelium is nutrient absorption while providing a physical barrier to the passage of pro-inflammatory molecules, such as pathogens. This selective permeability could be achieved by the transcellular pathway via specific transporters or channels and by paracellular pathway via intercellular spaces between the adjacent epithelial cells. To create a continuous barrier and regulate paracellular permeability, these spaces are wrapped by Tight Junction complexes (Chelakkot et al., 2018). Lactobacilli might maintain the epithelial barrier function by increasing mucus production, modulation of cytoskeletal and tight junction protein phosphorylation enhancing tight junction function (Sengupta et al., 2013). Yeng et al, (2018) demonstrated that *Lactobacillus* attenuated the barrier disruption of intestinal epithelial cells caused by *Salmonella* LPS. Thus, *Lactobacillus* could maintain the tight junction integrity and appearance.

2.6.5. Immunomodulation.

Lactobacilli can display immunomodulatory responses of the host by interaction with the GIT mucosa. The bacterial surface contains conserved structures known as microbe-associated

molecular patterns (MAMPs) which are lipopolysaccharides, peptidoglycan, lipoteichoic acid, and wall teichoic acids. These MAMPs are recognized by pattern recognition receptors (PRR) leading to a pro-inflammatory expression (innate immune response) such as the production of cytokines (Sengupta et al., 2013). This feature can be significantly different depending on both species and strain. Previous studies have shown the capacity of *L. plantarum*, *L. salivarius* and *Pediococcus acidilactici* to stimulate the innate immunity by producing TNF-α and therefore conferring protection against *Salmonella* infection in Broiler Chicks (Feng et al., 2016). *L. curvatus* DN317 of chicken ceca origin induces an immunomodulatory activity against *Campylobacter* by increasing IL-8 and β-defensin 2 secretion (Zommiti et al., 2017).

2.7. **Screening of potential probiotics**

The general strategy for the selection of probiotic strains requires a set of experiments to identify the most promising candidates (WHO/ FAO, 2002) (Figure 5).

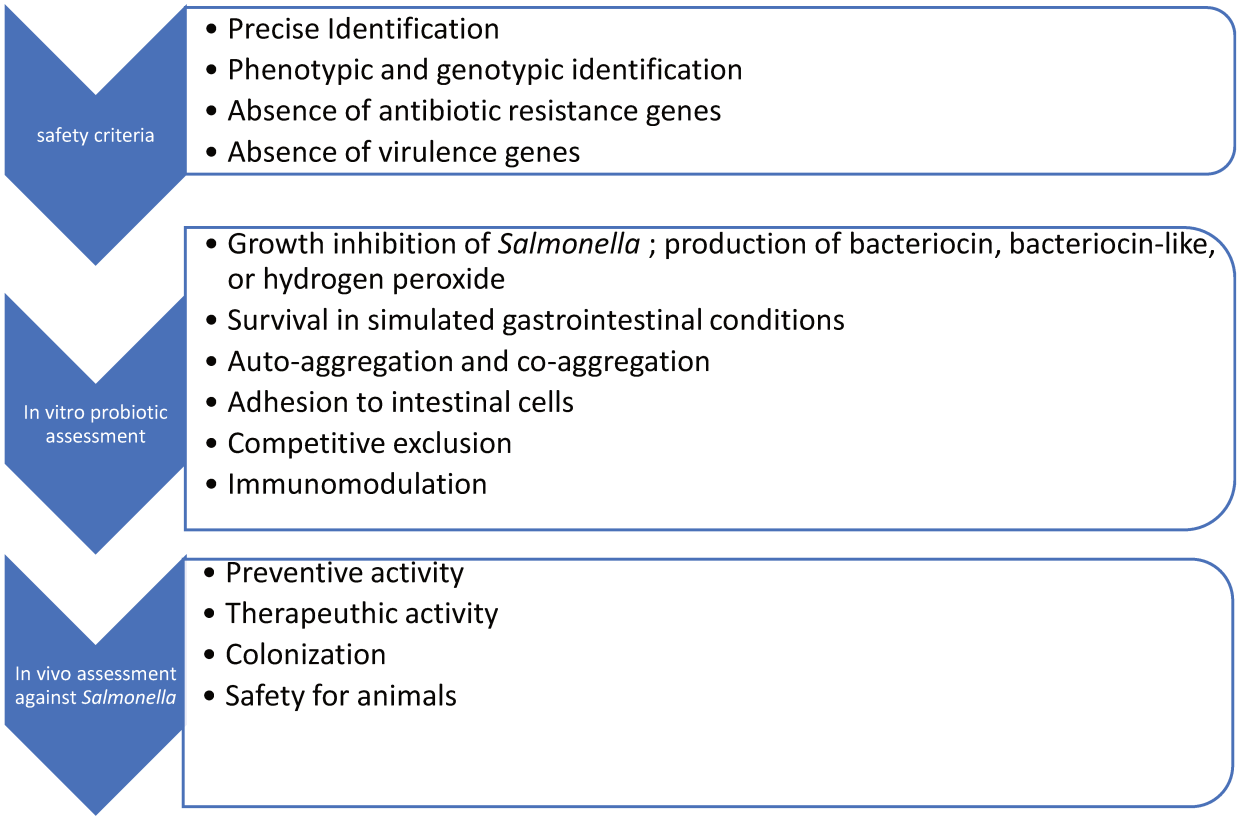

Figure 5: Strategy for selection of potential probiotics to control *Salmonella* in poultry

2.7.1. Safety criteria

To be Generally Recognized as Safe (GRAS), safety evaluations should be performed on a strain-by-strain basis (Saint-Cyr et al., 2016).

2.7.1.1. ***Probiotic identification***

The first step of safety assessment includes proper identification of the strain (Gueimonde et al., 2013). Phenotypical characterization by API system is frequently used. However, this conventional method is not reliable especially for lactobacilli population due to its significant and similar biochemical identifiers. The PCR methodology (mostly on 16S ribosomal RNA) followed by sequencing is frequently adopted for efficient identification (Saint-Cyr et al., 2016).

2.7.1.2. ***Antimicrobial resistance***

As defined by the European Food Safety Authority, requirements for safety assessment of probiotics, such organism shall not possess acquired resistance determinants to antibiotics of medical importance (EfSA, 2012). LAB has three types of resistance; intrinsic (innate), mutational and acquired. This latter, acquired by horizontal gene transfer is of a significant safety concern as antibiotic-resistance could be exchanged between commensal flora of GIT and pathogenic bacteria (Sharma et al., 2014). Whereas, the transfer risk is minimal for intrinsic, or acquired resistance by chromosomal mutation. Lactobacilli are known to have an inherent resistance to aminoglycosides, sulfonamides, and vancomycin as well as to bacitracin, cefoxitin, ciprofloxacin, fusidic acid. Chromosomal mutations have also been gained in lactobacilli. A single mutation in the 23S rRNA gene has been described conferring macrolide resistance in a strain of *L. rhamnosus* (Gueimonde et al., 2013).

This type of resistance is beneficial when need to restore the gut microbiota after antibiotic treatment. Moreover, knowledge of the antibiotic resistance phenotypes is of great importance, and intrinsic resistance might be relevant for the treatment of *Lactobacillus*-related bacteremia (Gueimonde et al., 2013). Both phenotypic and genotypic characterization should be carried out as phenotypically resistant strain could be genotypically "susceptible," and susceptible phenotype could also transport silent genes (Sharma et al., 2014).

Lactobacillus sp. are usually susceptible to antibiotics that inhibit protein synthesis, such as chloramphenicol and erythromycin and to an antimicrobial that inhibit cell wall synthesis such as penicillin and ampicillin (Dec et al., 2017). Due to the use of Macrolide–lincosamide–streptogramin (MLS) antibiotics (tylosin, tilmicosin, lincomycin, and virginiamycin) as growth promoters and/or as prophylactic agents in poultry rearing, gene transfer under antibiotic selective pressure facilitates the spread of MLS resistance in commensal bacteria (Gueimonde et al., 2013).

2.7.2. *In vitro* assays

The ability to survive and adhere to the intestinal cells are the most critical factors that contribute to the survival of probiotic bacteria and thus help them to induce positive health effects on their host. For this reason, adhesive properties have been proposed by many authors as one of the criteria for the selection of new strains for probiotic use (Yadav et al., 2017). In addition, cell envelope is the first target of physicochemical and environmental stress. Lactobacilli encounter several environmental stress factors during their transit through the GIT including low pH, bile salts. By mimicking the GIT conditions, the resistance of the probiotic to pH acidic and bile salts are evaluated (Babot et al., 2014).

Several *in vitro* conventional selection parameters were used to evaluate the surface probiotic properties such as cell surface hydrophobicity, auto-aggregation, co-aggregation, as well as adhesion capacity to epithelial cells. All these features are strain- specific trait; therefore different results have been obtained (Ramos et al., 2013).

Further *in vitro* characterizations are done to evaluate anti-*Salmonella* activities by co-culture assay. This method could be done by agar diffusion (Schillinger and Lucke, 1989) or by liquid co-culture. The probiotic culture supernatant is also assessed for potential bacteriocin secretion. *L. amylovorus* C94 and *L. salivarius* C86 exhibit anti-*Salmonella* activities with total inhibition after 18 hours of co-incubation in liquid medium (Adetoye et al., 2018). Szala et al. (2012) observed complete inactivation of *S.* Heidelberg by *L. plantarum* and *L. brevis* after 48 h of co-culture.

Another screening test is to assess the ability of the probiotic strain to compete or exclude *Salmonella* from the adhesion to intestinal epithelial cells. Several human cell lines have been used to evaluate the potential *Lactobacillus* probiotic for poultry with the human colorectal adenocarcinoma Caco-2 cell line the most common. It has been used to identify the essential genes

in cellular *S*. Enteritidis invasion (Shah, 2012). The chicken LMH, a primary hepatocellular carcinoma cell line is also widely use. By using these cell lines, epithelial cell adhesion was assessed for *L. crispatus* TDCC 75, *L. crispatus* TDCC 76, and *L. gallinarum* TDCC 77 (Spivey et al., 2014). These cell lines are also used to evaluate the immunomodulation activity of probiotic strains, by assaying cytokine production.

2.7.3. *In vivo* experiments

The potential probiotic strains selected in vitro assays were further evaluated in vivo experiments on chickens for highlighting their persistence ability in GIT, their impact on foodborne pathogen colonization and/or their beneficial effects on growth performances in the host. The *in vivo* combined administration of *L. salivarius* 59 and *Enterococcus faecium* PXN33 caused reduction in the colonization of *S.* Enteritidis S1400 in poultry (Carter et al., 2017). Oral administration of *Lactobacillus*-based probiotic culture significantly reduced *S*. Enteritidis recovered from cecal tonsil of neonatal chick (Higgins et al., 2008). A single dose of *L. salivarius* allowed the prevention of *S*. Enteritidis infection in young broilers (Waewdee et al., 2012).

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Chapter II

Prevalence, antibiotic resistance and molecular characterization of *Salmonella* serotypes in the Lebanese poultry production

Prevalence, antibiotic resistance and molecular characterization of *Salmonella* serotypes in the Lebanese poultry production

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Abstract

Since data on *Salmonella* in the Lebanese poultry industry is scarce, this study was conducted to determine the prevalence of *Salmonella* at different stages of the broiler production chain and layer flocks in addition to their antibiotic resistance profile and molecular patterns. Over a period of 3 years, feces samples were collected by a sock method from local Lebanese farms (broiler breeder farms (n= 29), broiler farms (n= 159) and laying hen farms (n= 49)), while poultry meat was collected from slaughterhouses (n=134) and retail (n=1907). In parallel, ceca (n=115) and neck skins (n=115) were collected from two major slaughter plants. Six hundred and seventy-two isolated *Salmonella* strains were serotyped; from which 514 were analyzed for antimicrobial resistance via standard disk diffusion and broth microdilution Method. Pulsed-field gel electrophoresis (PFGE) was used to define the molecular patterns of the main serotypes. The results highlighted a high prevalence of *Salmonella* in poultry. Considering all samples together, a large diversity of serotypes was identified with predominance among *Salmonella* Infantis (32.9%), *Salmonella* Enteritidis (28.4%) and *Salmonella* Kentucky (21.4%). High resistance to nalidixic acid was revealed in all *Salmonella* isolates. The most prominent resistance and multi-resistance was exhibited in *S*. Kentucky and *S*. Infantis. This latter was resistant to both streptomycin and tetracycline at a rate of 88.2% and 99%, respectively. Furthermore, 89.7 % of the strains were multi-drug resistant. All *S*. Kentucky strains were resistant to ciprofloxacin and 62.4% of the strains were multidrug resistant Nine strains of *S*. Kentucky CIP^R were also resistant to Extended Spectrum Cephalosporin (ESCs). Comparing *S*. Enteritidis strains from poultry and humans using PFGE, the results indicated that one persistent clone of *S*. Enteritidis (80% of the strains) is common between poultry and humans in Lebanon. Similar genomic profiles and antimicrobial resistance phenotypes were detected between farms, slaughterhouses and retail suggesting the circulation and transmission of identical clones throughout the food chain and layer flocks. For the first time, this study demonstrates the high prevalence of *Salmonella* in the Lebanese poultry chain, the emergence of new serotypes and the absence of potential barriers preventing such transmission. To control this public health risk, it is of utmost importance to review the current national food safety strategy and to implement effective measures aiming to reduce the prevalence throughout the chain and the transmission of this pathogen to humans.

Keywords: *Salmonella sp.*, prevalence, serotypes, antimicrobial resistance, Pulse Field Gel Electrophoresis.

1. Introduction

Foodborne *Salmonella* continues to be a major threat for public health (EFSA/ ECDC, 2017). It is estimated that non-typhoidal *Salmonella* causes 93.8 million cases of gastroenteritis and 155,000 annual deaths worldwide (Majowicz et al., 2010). Poultry are the primary source of human infection triggered by the consumption of contaminated poultry products, such as meats and eggs (CDC, 2015; Foley et al., 2011). Although *Salmonella* enterica subspecies enterica cover more than 2,500 serotypes, only few are isolated from poultry with *S.* Enteritidis and *S.* Typhimurium being the predominant contaminant implicated in human gastroenteritis (EFSA/ ECDC, 2017; Ricke et al., 2018). Other poultry-associated serotypes have emerged, including *S.* infantis, *S.* Kentucky and *S.* Heidelberg, with this emergence particularly tormenting since these serotypes are frequently resistant to antibiotics (Gieraltowski et al., 2016; Le Hello et al., 2011; Nógrády et al., 2007).

In fact, in recent years, an increasing trend of antimicrobial resistance (AMR) was noticed, causing 25,000 annual deaths in Europe, 100,000 in USA and 80,000 in China (Ferri et al., 2017). Multidrug-resistant (MDR) *Salmonella* strains and extended-spectrum-β-lactamase (ESBL)-producing serotypes are also increasing and constitute an emerging public health concern (Franco et al., 2015; Wasyl & Hoszowski, 2012). Despite being a self-limited infection, the elderly, infants, and immunosuppressed might need antimicrobial therapy to treat salmonellosis. Some of these drugs such as fluoroquinolones and extended spectrum cephalosporin are critically important for human medicine (Medalla et al., 2017), but their effectiveness is questionable and worrisome. This serious public health risk is mainly attributed to the inappropriate use (therapeutic, preventive and growth promoter) of antimicrobials in the animal sector (Ferri et al., 2017).

In Lebanon, chicken is present in every kitchen with a consumption of 30 Kg/person/year. The poultry sector has experienced rigorous growth and is dominated by 10 large-scale slaughterhouses and poultry farms; four of them control more than half of the Lebanese market. Despite this overgrowth of the poultry industry and the risk that *Salmonella* of poultry origin cause on human

health, little or no information is available in Lebanon about this pathogen and its dissemination along the chain.

The objective of this study was to determine *Salmonella* prevalence within a farm to fork approach, starting from broiler breeder farms to slaughterhouses and the retail (supermarkets and restaurants) and layer flocks. Serotypes circulation, antibiotic resistance and their genotypic relatedness were studied. Moreover, this work will serve as a database for a national strategy, surveillance programs and intervention measures, set by local authorities (Ministry of Agriculture) for prevention and control of salmonellosis in human and *Salmonella* dissemination in the poultry industry.

2. Materials and Methods

2.1. Sample collection

Broiler breeder farms, commercial broiler farms, layer farms, slaughterhouses, and retail chicken meats (supermarkets and restaurants) were investigated in this study.

2.1.1. Farm sample collection

For one year (October 2014 / October 2015), a cross sectional study was performed in 29 broiler breeder farms, 159 broiler production farms and 49 egg laying hen farms. In total, 237 farms randomly chosen from all Lebanese districts were enrolled, with only one flock studied at each farm during the rearing period. Fecal samples were collected using boot swabs within the poultry house. To perform sampling, pair of sterile elastic cotton socks were worn over the boots and fecal samples were collected by walking through the entire poultry house. Embedded feces on the cotton socks were put aseptically into sterilized containers and transported within 2 hours to the Lebanese Agricultural Research Institute (LARI) in an ice cooler container for *Salmonella* detection analysis.

2.1.2. Processing plant sample collection

Over one-year period (June 2015/June 2016), two major poultry processing plants (Slaughter plant A and Slaughter plant B), listed among the top four broiler production plants, covering more than the half of the Lebanese chicken production, were included in this study. Both slaughterhouses were fully automated applying Good Hygiene Practices (GHP) and Good Manufacturing Practices (GMP) systems. Both companies are either integrated or contracting with rearing private farms providing them with one- day- age chicks ("Ross 308" and "Hubbard classic" species for

processing plant A and B, respectively) and a diet formula. All breeder birds were vaccinated against *S.* Typhimurium and *S.* Enteritidis. The Slaughter plant A is considered the largest slaughterhouse at a national level with a capacity of almost 22,500,000 broilers slaughtered per year. The evisceration was carried out automatically, Peracetic Acid (PAA) was used as antimicrobial in all the processing steps and chilling was achieved by dry air. On the other hand, slaughterhouse B is considered a small-scale poultry processing plant with a capacity of 3,750,000 broilers slaughtered per year. Contrary to slaughterhouse A, the first step of chilling in slaughterhouse B was performed by immersion system with the addition of chlorine (0.3ppm for 20 min) followed by air chilling for 30 minutes.

Thirty-eight and six farms were randomly chosen from Slaughter plant A and B, respectively. The number of the farms was representatively taken according to the size of the enterprise. At least one sample was taken from each farm during sampling period (autumn, winter and spring seasons).

During processing, one sample of neck skin of post-chilled carcass and 5 to 10 caeca samples during evisceration (pooled in one sample) were taken randomly from each slaughtered flock. In total, 230 samples were collected, with 202 (101 neck skin and 101 caeca) and 28 (14 neck skin and 14 caeca) from slaughter plant A and B, respectively. Samples were coded A or B with the number of sampling from slaughter plants A and B, respectively. A farm was considered to be *Salmonella*-positive if at least one sample was positive whether in caeca or in neck skins.

2.1.3. Poultry meat sample collection

For 3 years, from November 2014 until November 2016, 128 samples of whole chicken carcasses and cuts, and 6 samples of liver were chosen randomly from different slaughterhouses covering all Lebanese regions. In parallel, 1907 samples were collected from Lebanese retail shops (supermarkets and restaurants) including 1156 samples of raw chicken parts (133 liver and 1023 whole chicken carcasses and cuts) and 751 samples of marinated chicken meat.

2.1.4. Avian and Human Salmonella isolates collection

Avian *Salmonella* strains from previous outbreaks in Lebanon and imported raw cuts (16 and 30 isolates, respectively) were included in this study.

For comparison purposes, five strains of clinical *Salmonella* Enteritidis were picked out from the most predominant pulsotype JEGX01.0001 (Fadlallah et al., 2017) collected from a large

repository of *Salmonella* strains in the PulseNet laboratory at the American University of Beirut (AUB).

2.2. *Salmonella* isolation and identification

Salmonella spp. was isolated and identified according to the ISO method NF EN ISO 6579 (2002). Briefly, 25g of sample was homogenized in 225 ml of Buffered Peptone Water (BPW) (Scharlau, Spain). After incubation for 18 h at 37ºC, 1 ml and 0.1 ml of the pre-enrichment suspension were added to 10 ml of Mueller Kauffman Tetrationate broth (Scharlau, Spain) and 10 mL of Rappaport Vassiliadis Soy broth (Scharlau, Spain), and incubated at 37ºC and 41.5ºC, respectively. After 24 h of incubation, 10 µl of each broth was streaked onto Xylose Lysine Desoxycholate (XLD) agar and Salmonella-Shigella agar (SS) plates (Scharlau, Spain) and incubated at 37ºC for 24 h to 48 h. Typical colonies were further confirmed by API® 20E (Biomerieux, France). Confirmed strains were further serotyped by slide agglutination using commercial O and H antisera (Remel, England) in accordance with the Kauffman and White le Minor scheme (2007).

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST) was carried out on the three predominant *Salmonella* serotypes in accordance with the Clinical and Laboratory Standards Institute (CLSI, 2008; 2017). The Kirby-Bauer disc diffusion method was firstly performed, for a panel of 26 antimicrobials (Oxoid, Basingstoke, England) of veterinary and human health importance. The tested antibiotics were: ampicillin (Amp-10 µg), amoxicillin-clavulanic acid (Amc-30 µg), piperacillin-tazobactam (Tzp-110 µg), cephalothin (Kf-30 µg), cefuroxime (Cxm-30 µg), cefoxitin (Fox-30 µg), cefotaxime (Ctx-30 µg), ceftriaxone (Cro-30 µg), ceftazidime (Caz-30 µg), ceftiofur (Eft-30 µg), cefepime(Fep-30 µg), imipenem (Ipm-10 µg), aztreonam (Atm-30 µg), gentamycin (Cn-10 µg), tobramycin (Tob-10 µg), streptomycin (S-10 µg), amikacin(Ak-30 µg), netilmicin (Net-30 µg), nalidixic acid (Na-30 µg), ciprofloxacin (Cip-5 µg), norfloxacin (Nor-10 µg), enrofloxacin (Enr-5 µg), trimethoprim (W-5 µg), trimethoprim-sulfamethoxazole (Sxt-1.25/23.75 µg), tetracycline (Te-30 µg), chloramphenicol (C-30 µg). Antimicrobial MICs for resistant strains were determined using broth microdilution for the following antimicrobials and breakpoint values: Kf (≥32 µg/ml), Cxm (≥32 µg/ml), Fox(≥32 µg/ml), Ctx (≥4 µg/ml), Cro (≥4 µg/ml), Caz (≥16 µg/ml), Eft (≥8 µg/ml), Cn (≥16 µg/ml), Na (≥32 µg/ml), Cip (≥1 µg/ml), Nor (≥16 µg/ml), Enr (≥2 µg/ml).

Escherichia coli ATCC® 25922™ was used as a quality control strain. Antimicrobial resistance to ≥3 classes was considered multi-drug resistance (MDR).

2.4. Pulse Field Gel Electrophoresis- PFGE

A pulsed-field gel electrophoresis (PFGE) analysis of isolates of *S*. Kentucky (n=97), *S*. Infantis (n=64), and *S*. Enteritidis (n=53) was performed using an XbaI restriction enzyme according to the protocol described by Kérouanton et al (2007).

Similarities of PFGE profiles were determined with Bionumerics 7.6 software (Applied Maths, Kortrijk, Belgium) using the Dice Coefficient and dendrograms were generated graphically by using unweighted pair group method with arithmetic mean (UPGMA).

2.5. Statistical Analysis

The differences of *Salmonella spp.* prevalence between the slaughterhouses A and B, and among samples were evaluated by the Chi-Square test using the software R (R x 64 version 3.4.3 (2017-11-30). p-values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Prevalence of *Salmonella* throughout the broiler food chain and laying hen flocks

A total of 237 farms including 29 broiler breeders, 159 broiler farms and 49 laying hens were analyzed for *Salmonella* prevalence. 71 feces samples were identified as positive among the 237 tested farms (30%). Prevalence rates for *Salmonella* were 31 %, 31.4 % and 24.5 % in the breeder farms, broiler farms and laying hen farms, respectively (Table 3).

Forty eight out of 134 raw chicken parts collected from different slaughterhouses were positive for *Salmonella spp.* leading to a prevalence of 35.8 %. In addition, 427 samples out of 1907 retail chicken samples were confirmed positive yielding a *Salmonella* rate of contamination of 22.4 % (Table 3).

Table 3: Sample type and prevalence of *Salmonella* sp at different points of poultry production chain

The global prevalence of *Salmonella spp.* estimated from caeca sampling in the two slaughterhouses was 81.8 % (36/44) indicating the incidence at farm level (*Salmonella spp.* was isolated at least once during seasonal sampling) and 34.8 % at sample level (80/230) having 47.8 % of positive caeca (55/115) and 21.7 % of positive neck skin (25/115) (Table 2). The

Source type	Sample type	Number of samples	Number of contaminated samples/ (%)
Egg laying hens farm	Feces	49	12 (24.5 %)
Broiler breeders farm	Feces	29	9 (31 %)
Broiler farm	Feces	159	50 (31.4 %)
Total farms		237	71 (30 %)
Slaughter house	Raw chicken parts	134	48 (35.8 %)
Retail (restaurant and supermarket)	Raw chicken parts	1907	427 (22.4 %)

prevalence at farm level was very high with 81.6 % (31/38) and 83.3 % (5/6) estimated from caeca samples from slaughterhouses A and B, respectively, and no significant difference ($p>0.05$) was observed between them. Among the 202 samples collected from slaughterhouse A, 65 were positive for *Salmonella* (32.2 %) with 47.5 % caeca contamination (48/101) and 16.8 % neck skin contamination (17/101). At slaughterhouse B, 15 samples among 28 were *Salmonella*- positive (53.6 %), 7 of 14 caeca (50 %) were contaminated comparing to 8 of 14 (57.1 %) neck skin samples. Caeca prevalence was quite similar in both Slaughterhouses A and B ($p>0.05$) on the contrary to neck skin where contamination in slaughterhouse B ($p<0.05$) was higher than slaughterhouse A. Moreover, the seasonal effect was not significant ($p>0.05$) for both slaughterhouses, the prevalence in winter was 41.7 %, in autumn 35.7 % and in summer 28.4 % (Table 4).

Table 4: Occurrence of *Salmonella* sp in the 2 slaughter plants A and B at different seasons

[*] indicates a significant difference of *Salmonella* prevalence in neck skin between slaughterhouses A and B.
[**] indicates no significant differences of *Salmonella* prevalence between the two slaughterhouses at farm level

	Samples type	Summer	Autumn	Winter	Total samples	Total farms
Slaughter plant A	**Caeca**	13/38(34.2%)	16/30 (53.3%)	19/33 (57.6%)	48/101 (47.5%) [***]	31/38 (81.6%) [**]
	Neck skin	6/38 (15.8%)	5/30 (16.7%)	6/33(18.2%)	17/101 (16.8%) [*]	
	Total samples	19/76 (25%)	21/60 (35%)	25/66 (37.9%)	65/202 (32.2%)	
Slaughter plant B	**Caeca**	3/6(50%)	1/5 (20%)	3/3 (100%)	7/14 (50%) [***]	5/6 (83.3%) [**]
	Neck skin	3/6(50%)	3/5 (60%)	2/3 (66.7%)	8/14 (57.1%) [*]	
	Total samples	6/12 (50%)	4/10 (40%)	5/6 (83.3%)	15/28 (53.6%)	
Total Slaughter Plants	**Caeca**	16/44 (36.4%)	17/35 (48.6%)	22/36 (61.1%)	55/115 (47.8%)	36/44 (81.8%)
	Neck skin	9/44 (20.5%)	8/35 (22.9%)	8/36 (22.2%)	25/115 (21.7%)	
	Total samples	25/88 (28.4%)[****]	25/70 (35.7%) [****]	30/72 (41.7%) [****]	80/230 (34.8%)	

[***] indicates no significant differences of *Salmonella* prevalence in caeca between the two slaughterhouses
[****] indicates no significant differences of *Salmonella* prevalence between the seasons in the two slaughterhouses

3.2. Distribution of *Salmonella* serotypes

A total of 672 confirmed *Salmonella* isolates were serotyped. Twenty-three different serotypes were obtained with *S.* Infantis (32.9 %), *S*. Enteritidis (28.4 %) and *S.* Kentucky (21.4 %) being the most predominant ones (Table 5). *S*. Kentucky was found in the broiler production chain from breeders to production farms going through the slaughterhouses until the retail and in laying hen farms. *S*. Enteritidis was isolated from all stages of the poultry production chain except from the 2 investigated slaughterhouses A and B. Furthermore, *S.* Infantis was found from the broiler production farms until retail but not in the broiler breeders and laying hen farms. *S*. Typhimurium was recovered only at retail level with a low prevalence of 2 %.

At the farm level, *S.* Enteritidis (66.7 %, 44.4 %, and 38 %), *S*. Kentucky (16.7 %, 33.3 %, and 22 %) and *S*. Blockely (16.7 %, 22.2 %, and 6 %) were found in the laying hens farms, in the breeder farms and in the broiler farms, respectively. In addition to these three serotypes, *S*. Infantis (22 %), *S.* Emek (8 %) and *S.* Seftenberg (4 %) were isolated from the broiler farms.

Table 5: *Salmonella* serotypes diversity isolated along the chicken production chain

Serotype	Total number	Egg hens Layer farms	Broiler breeder farms	Broiler farms	Slaughter plant A	Slaughter plant B	Slaughterhouse (meat)	Retail	Suspected food (Intoxication)	Imported
S. Infantis	221 (32.9%)			11 (22%)	19 (29.2%)	4 (26.7%)	9 (18.8%)	176 (41.2%)	1 (6.3%)	1 (3.3%)
S. Enteritidis	191 (28.4%)	8 (66.7%)	4 (44.4%)	19 (38%)			32 (66.7%)	112 (26.2%)	15 (93.8%)	1 (3.3%)
S. kentucky	144 (21.4%)	2 (16.7%)	3 (33.3%)	11 (22%)	34 (52.3%)		5 (10.4%)	89 (20.8%)		
S. Heidelberg	31 (4.6%)							6 (1.4%)		25 (83.3%)
S. Newport	15 (2.2%)				2 (3.1%)		1 (2.1%)	12 (2.8%)		
S.Blockley	11 (1.6%)	2 (16.7%)	2 (22.2%)	3 (6%)	1 (1.5%)			3 (0.7%)		
S.Typhimurium	9 (1.3%)							9 (2.1%)		
S. Hadar	9 (1.3%)					7 (46.7%)	1 (2.1%)	1 (0.2%)		
S.Emek	7 (1%)			4 (8%)				3 (0.7%)		
S St paul	4 (0.6%)							2 (0.5%)		2 (6.7%)
S. Munster	3 (0.4%)							3 (0.7%)		
S.Aarhus	3 (0.4%)					2 (13.3%)		1 (0.2%)		
S. Branderup	2 (0.3%)							2 (0.5%)		
S.Virginia	2 (0.3%)							1 (0.2%)		1 (3.3%)
S. Istanboul	2 (0.3%)					2 (13.3%)				
S. Senftenberg	2 (0.3%)			2 (4%)						
S. Glostrup	1 (0.1%)							1 (0.2%)		
S. Mbandaka	1 (0.1%)							1 (0.2%)		
S. Anatum	1 (0.1%)							1 (0.2%)		
S. Montevideo	1 (0.1%)							1 (0.2%)		
S.Agona	1 (0.1%)							1 (0.2%)		
S.Rissen	1 (0.1%)							1 (0.2%)		
S. Lomita	1 (0.1%)							1 (0.2%)		
NT	9 (1.3%)				9 (13.8%)					
Total	672	12	9	50	65	15	48	427	16	30

The *Salmonella* serotypes differed between the two slaughter plants A and B surveyed. At slaughterhouse A, from the 65 *Salmonella* isolates (48 from caeca and 17 from neck skin), four serotypes were identified and 9 *Salmonella* strains untypable. *S.* Kentucky (52.3 %) and *S*. Infantis (29.2 %) were predominant and repeatedly isolated from caeca and neck skin through the seasons sampling followed by *S.* Newport (3 %) and the only *S*. Blockely (1.5 %) which was recovered from a caeca sample. Within slaughterhouse B, four serotypes were identified from the 15 *Salmonella* isolates (8 from neck skin and 7 from caeca). *S.* Hadar (46.7 %) was the predominant one, followed by *S*.Infantis (26.7 %), *S.* Istanbul (13.3 %) and *S.* Aarhus (13.3 %), with the latter was only isolated from neck skin.

Five *Salmonella* serotypes were identified from the 48 *Salmonella* isolates from the chicken meat at the slaughterhouse level. The most frequently isolated was *S*. Enteritidis (66.7 %), *S*. Infantis (18.8 %), and *S*. Kentucky (10.4 %) followed by *S*. Hadar (2.1 %) and *S*. Newport (2.1 %). A very high diversity of serotypes was observed at retail level where 21 *Salmonella* serotypes were recovered. *S*. Infantis (41.2 %), *S*. Enteritidis (26.2 %) and *S*. Kentucky (28.8 %) were the most predominant ones. Rarely isolated *Salmonella* were *S*. Newport (2.8 %), *S*. Typhimurium (2.1 %), *S.* Heidelberg (1.4 %), *S*. Blockley (0.7 %), *S.* Emek (0.7 %), *S*. Munster (0.7 %) and others (3 %).

From the 16 avian *Salmonella* strains isolated from previous outbreaks, 15 were *S*. Enteritidis (93.8 %) and one *S*. Infantis (6.3 %). Five *Salmonella* serotypes were identified from the 30 *Salmonella* strains of imported chicken cuts with *S*. Heidelberg (83.3 %) the most predominant (Table 5).

3.3. Antimicrobial resistance phenotypes

Five hundred and fourteen *Salmonella* strains belonging to the 3 most predominant serotypes throughout the whole broiler food chain and laying hen flocks, *S*. Infantis (n= 204), *S*. Enteritidis (n= 177) and *S.* Kentucky (n=133) were subjected to antimicrobial susceptibility testing. Twenty-six antimicrobials were tested. Great differences in resistance were observed within these serotypes and a multitude of antimicrobial resistance patterns were detected where 1 up to 6 antimicrobial classes were involved. High resistance against nalidixic acid was commonly observed: *S.* Enteritidis (98.9 %), *S*. Intantis (99.5 %) and *S*. Kentucky (100 %). With the exception of this latter antibiotic and nine isolates found to be multi-drug resistant detected only at the retail level, *S*. Enteritidis showed the lowest level of antimicrobial resistance and almost all the other strains were

susceptible to the majority of antimicrobials tested along the broiler food chain and layer flocks (Table 4 and 5). Only two were pan susceptible and few showed resistance to ampicillin (7.9 %), amoxicillin-clavulanic acid (1 %), streptomycin (5.6 %), trimethoprim (3.9 %), trimethoprim-sulfamethoxazole (2.3 %), tetracycline and chloramphenicol (1.7 % each), ciprofloxacin, norfloxacin and enrofloxacin (1 % each) (Figure 6). On the other hand, *S*. Infantis isolates were characterized by their high resistance rates to tetracycline (99 %) and streptomycin (88.2 %) followed by chloramphenicol (9.3 %), ampicillin (9.3 %), trimethoprim (2.4 %) and trimethoprim-sulfamethoxazole (1 %) (Figure 6). The majority of *S.* Infantis (89.7 %) were multi-drug resistant with 14 different antimicrobial profiles having the "S-Na-Te" pattern (71.6 %) as the most predominant one circulating in all over the broiler food chain (Table 6 and 7).

In parallel, all the *S.* Kentucky isolates were resistant to quinolones and fluoroquinolones with very high ciprofloxacin MIC levels observed (6.25->32 µg/ml). Very high resistance was found to ampicillin (71.4 %), amoxicillin–clavulanic acid (56.4 %), tetracycline (58.7 %), streptomycin (54.9 %) and gentamycin (53.4 %). Low resistance was observed against chloramphenicol (7.5 %), cefalothin (7.5 %), cefuroxime (6.8 %), cefoxitin (6.8 %) and trimethoprim (0.7 %). These resistances were shown to persist in all stages of the broiler production chain and layer flocks. Resistance against third generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime and ceftiofur) was observed in 9 strains representing 6.8 % of the total *S.* Kentucky isolates and were isolated from slaughterhouse A (n=2) and retail (n=7). A very large diversity of antimicrobial resistance profile (n=36) was observed among *S.* Kentucky with 27 (62.4 % of the strains) considered as a multi-drug resistant patterns disseminated along the food chain. The most prevalent were "Amp-Amc-Cn-S-Na-Cip-Nor-Te-Enr" (14.3 %), "S-Na-Cip-Nor-Enr" (9.8 %), "Na-Cip-Nor-Enr (9 %), "Amp-Amc-Na-Cip-Nor-Enr" (8.3 %) and "Amp-Amc-Cn- Na-Cip-Nor-Te-Enr (8.3 %) (Figure 6, Table 6 and Table 7).

Table 6: Antimicrobial resistance patterns of *S.* Enteritidis, *S.* Infantis and *S*. Kentucky isolates

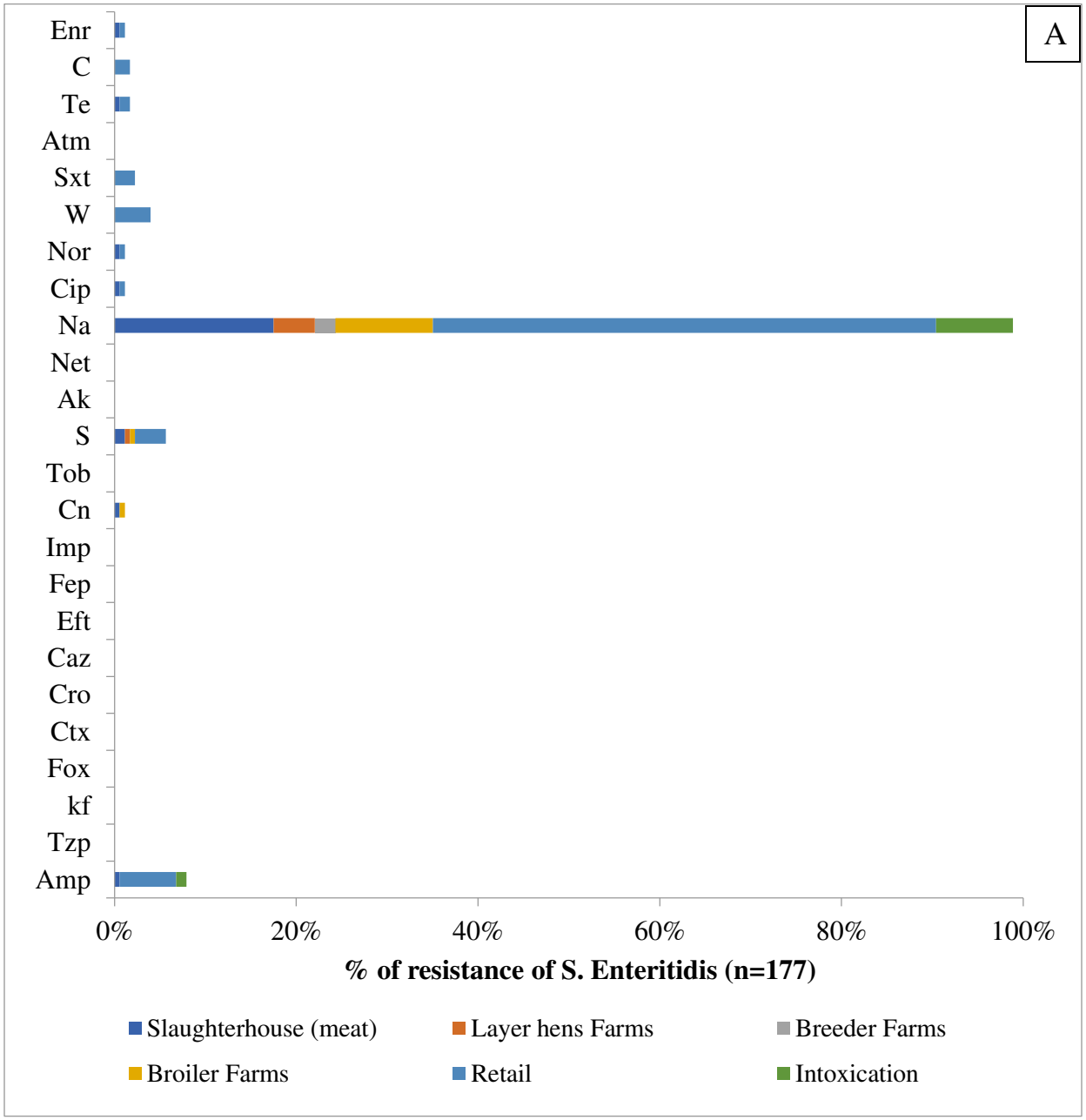
Serotypes	Antimicrobial resistance patterns	Laying hen farms	Breeder farms	Broiler farms	Slaughterhouse A and B	Slaughterhouse (meat)	Retail	Total	%
***Salmonella* Enteritidis**	Pan susceptible					1	1	2	1.1%
	Na	8	4	19		28	99	156	88.1%
	Na-W						1	1	0.6%
	S-Na	1		1				2	1.1%
	Amp-Na					1	4	5	2.8%
	S-Na-Te					1		1	0.6%
	Amp-Amc-Na						1	1	0.6%
	Amp-S-Na-C						1	1	0.6%
	Amp-S-Na-W						2	2	1.1%
	Amp-Amc-S-Na-W						1	1	0.6%
	Amp-S-Na-W-Sxt						1	1	0.6%
	Cn-S-Na-Cip-Nor-Enr					1		1	0.6%
	Amp-S-Na-W-Sxt-C						1	1	0.6%
	Amp-Na-W-Sxt-Te-C						1	1	0.6%
	Amp-Na-Cip-Nor-Te-Enr						1	1	0.6%
***Salmonella* Infantis**	Na						2	2	1.0%
	Te						1	1	0.5%
	Na-Te				1	1	16	18	8.8%
	S-Na-C				1			1	0.5%
	Na-Te-C						2	2	1.0%
	S-Na-Te			9	16	6	115	146	71.6%
	S-Na-Te-C						10	10	4.9%
	S-Na-W-Te				2			2	1.0%
	Amp-Na-Te-C						1	1	0.5%
	Amp-S-Na-Te			1	2	2	9	14	6.9%
	Na-Nor-W-Te				1			1	0.5%
	Amp-S-Na-Te-C						4	4	2.0%
	S-Na-W-Sxt-Te						1	1	0.5%
	Amp-S-Na-W-Sxt-Te-C						1	1	0.5%
***Salmonella* Kentucky**	Na-Cip-Nor-Enr			2	2	2	6	12	9.0%
	S-Na-Cip-Nor-Enr				1	1	11	13	9.8%
	Cn-Na-Cip-Nor-Enr			1	2		1	4	3.0%
	Na-Cip-Nor-Te-Enr				1		1	2	1.5%
	Amp-Na-Cip-Nor-Enr						2	2	1.5%
	S-Na-Cip-Nor-C-Enr						1	1	0.8%

	Cn-S-Na-Cip-Nor-Enr					1	2	3	2.3%
	Amp-Amc-S-Na-Cip-Nor-Enr						1	1	0.8%
	Cn-Na-Cip-Nor-Te-Enr						1	1	0.8%
	Cn-S-Na-Cip-Nor-Te-Enr				1			1	0.8%
	Amp-S-Na-Cip-Nor-Enr						1	1	0.8%
	Amp-Na-Cip-Nor-Te-Enr						1	1	0.8%
	Amp-Amc-Na-Cip-Nor-Enr			2	3		6	11	8.3%
	Amp-S-Na-Cip-Nor-Te-Enr						7	7	5.3%
	Amp-Cn-Na-Cip-Nor-Te-Enr		1				2	3	2.3%
	Amp-Amc-Cn-Na-Cip-Nor-Enr						1	1	0.8%
	Amp-Amc-Kf-Na-Cip-Nor-Enr			1				1	0.8%
	Amp-Amc-Na-Cip-Nor-Te-Enr						3	3	2.3%
	Amp-Cn-S-Na-Cip-Nor-Te-Enr				1		6	7	5.3%
	Amp-Amc-Cn-S-Na-Cip-Nor-Enr				1		1	2	1.5%
	Amp-Amc-S-Na-Cip-Nor-Te-Enr						3	3	2.3%
	Amp-Amc-Cn-Na-Cip-Nor-Te-Enr	1		1	3		6	11	8.3%
	Amp-Amc-Kf-Na-Cip-Nor-Te-Enr				1			1	0.8%
	Amp-Amc-Cn-Na-Cip-Nor-Te-C-Enr						1	1	0.8%
	Amp-Amc-Cn-S-Na-Cip-Nor-Te-Enr				11	1	7	19	14.3%
	Amp-Amc-Cn-Na-Cip-Nor-Atm-Te-Enr				1			1	0.8%
	Amp-Amc-Cn-S-Na-Cip-Nor-Te-C-Enr				1		6	7	5.3%
	Amp-Amc-Cn-S-Na-Cip-Nor-W-Te-Enr				1			1	0.8%
	Amp-Amc-Tzp-Cn-Na-Cip-Nor-Te-Enr				1			1	0.8%
	Amp-Amc-Cn-S-Na-Cip-Nor-Te-Enr						2	2	1.5%
	Amp-Amc-Kf-Cxm-Fox-Ctx-Cro-Caz-Eft-Na-Cip-Nor-Enr						2	2	1.5%
	Amp-Amc-Kf-Cxm-Fox-Ctx-Cro-Caz-Eft-Na-Cip-Nor-C-Enr						1	1	0.8%
	Amp-Amc-Cxm-Fox-Cro-Caz-Eft-Cn-S-Na-Cip-Nor-Atm-Te-Enr						1	1	0.8%
	Amp-Amc-Kf-Cxm-Fox-Ctx-Cro-Caz-Eft-Cn-S-Na-Cip-Nor-Te-Enr						1	1	0.8%
	Amp-Amc-Kf-Cxm-Fox-Ctx-Cro-Caz-Eft-Cn-Na-Cip-Nor-Atm-Te-Enr				1			1	0.8%
	Amp-Amc-Kf-Cxm-Fox-Ctx-Cro-Caz-Eft-Cn-S-Na-Cip-Nor-Atm-Te-Enr				1		2	3	2.3%

The code of antibiotics are: ampicillin (Amp), amoxicillin-clavulanic acid (Amc), piperacillin-tazobactam (Tzp), cefalothin (Kf), cefuroxime (Cxm), cefoxitin (Fox), cefotaxime (Ctx), ceftriaxone (Cro), ceftazidime (Caz), ceftiofur (Eft), gentamycin (Cn), streptomycin (S), nalidixic acid (Na), ciprofloxacin (Cip), norfloxacin (Nor), trimethoprim (W), aztreonam (Atm), tetracycline (Te), chloramphenicol (C), enrofloxacin (Enr).

Table 7: Antimicrobial resistance, MDR and ESC occurrence of the main serotypes isolated in this study

	Number of antimicrobial classes/ (%)								
				Multi drug Resistance					
Serovars	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**Total MDR**	**ESCs**
***Salmonella* Enteritidis(n= 177)**	2 (1.1%)	156 (88.1%)	10 (5.6%)	2 (1.1%)	5 (2.8%)	2 (1.1%)	0 (0%)	9 (5.1%)	0 (0%)
***Salmonella* Infantis (n=204)**	0 (0%)	3 (1.5%)	18 (8.8%)	150 (73.5%)	28 (13.7%)	4 (2%)	1 (0.5%)	183 (89.7%)	0 (0%)
***Salmonella* Kentucky (n=133)**	0 (0%)	12 (9%)	38 (28.6%)	14 (10.5%)	60 (45.1%)	9 (6.8%)	0 (0%)	87 (62.4%)	9 (6.8%)
Total (n= 514)	2 (0.4%)	171 (33.3%)	66 (12.8%)	166 (32.3%)	93 (18.1%)	15 (2.9%)	1 (0.2%)	279 (53.5%)	9 (1.8%)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40%
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% of resistance of S. Enteritidis (n=177)
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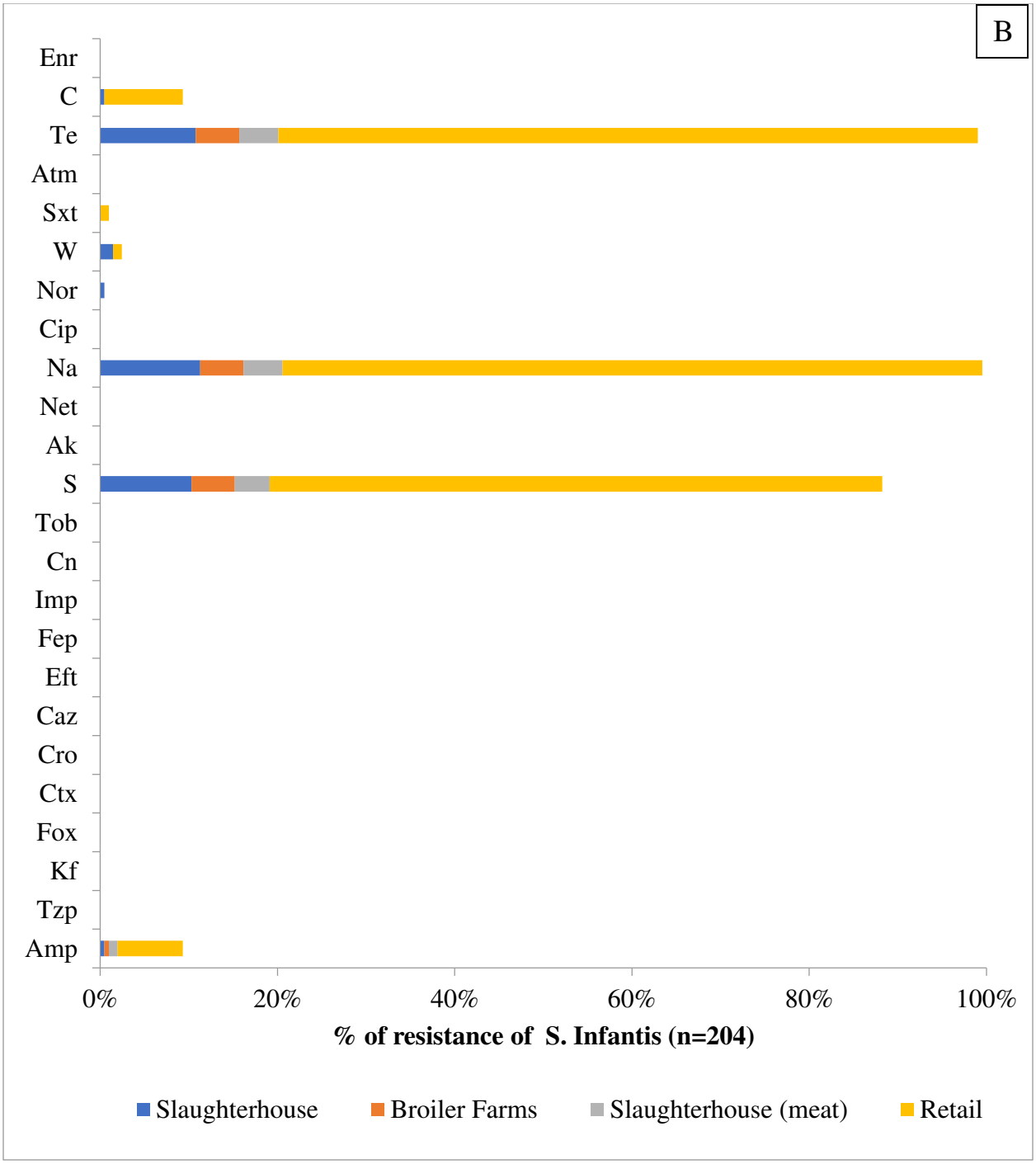
Layer hens Farms
Breeder Farms
Broiler Farms
Retail
Intoxication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% of resistance of S. Infantis (n=204)
Slaughterhouse
Broiler Farms
Slaughterhouse (meat)
Retail

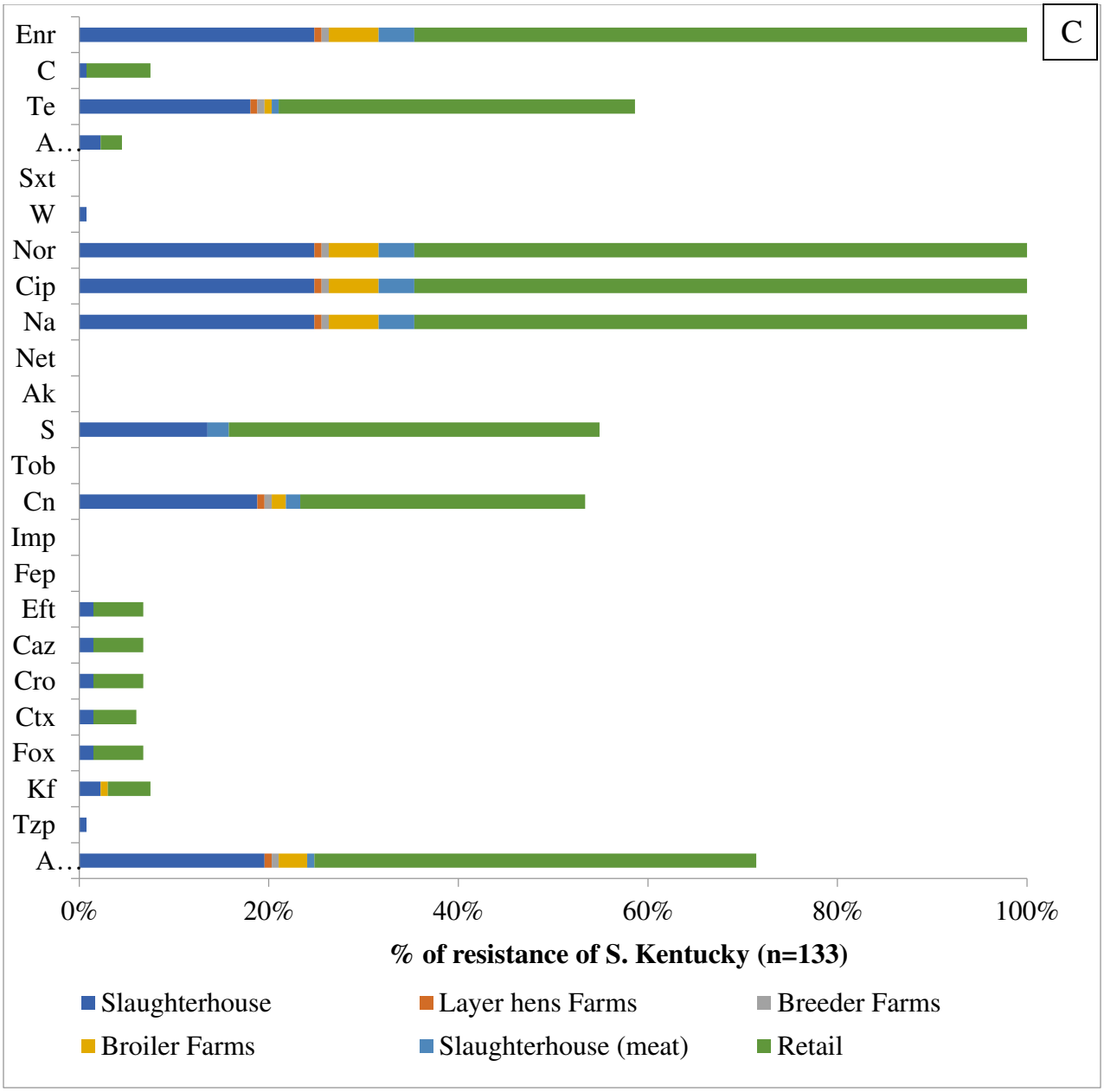

Figure 6: Percentage of antimicrobial resistance of S. Enteritidis (A), S. Infantis (B) and S. Kentucky (C) from farms, slaughterhouses and retail. The code of antibiotics are: ampicillin (Amp), amoxicillin-clavulanic acid (Amc), piperacillin-tazobactam (Tzp), cefalothin (Kf), cefuroxime (Cxm), cefoxitin (Fox), cefotaxime (Ctx), ceftriaxone (Cro), ceftazidime (Caz), ceftiofur (Eft), cefepime (Fep), imipenem (Imp), gentamycin (Cn), tobramycin (Tob), streptomycin (S), amikacin(Ak), netilmycin (Net), nalidixic acid (Na), ciprofloxacin (Cip), norfloxacin (Nor), trimethoprim (W), trimethoprim-sulfamethoxazole (Sxt), aztreonam (Atm), tetracycline (Te), chloramphenicol (C), enrofloxacin (Enr).

3.4. Pulse-Field- Gel Electrophorese (PFGE)

Among the 97 isolates of *S.* Kentucky, 10 different pulsotypes stand out, with a diversity index of 0.767. Genotyping with one restriction enzyme showed 5 different clusters with a degree of similarities ≥ 95.7 % between all *S.* Kentucky isolates. The main cluster includes 35 *S.* Kentucky isolates and covered the broiler food chain, from broiler breeder farm (n=1), broiler farm (n=1), slaughterhouse A (skin neck=3, caeca= 13) to retail (n=17) (Figure 7).

With a diversity index of 0.966, *S.* Infantis showed a great diversity among the isolates and established a contamination at all steps of the broiler production and not only at the slaughterhouse. Among the 64 isolates, 36 pulsotypes were distinguished. Interestingly, associate isolates with 100% of similarity originated from farm, slaughterhouse, supermarket and restaurant (Figure 8).

Seven pulsotypes were demonstrated for *S.* Enteritidis with a 0.369 diversity index. A relationship between several isolates from farm to fork is also present for this serotype. One dominant profile (grouping 80 % of the strains) contains sporadic human and poultry isolates from different sources: broiler breeder farm, layer farm, broiler farm, slaughterhouses, retail, and food suspected of intoxication (Figure 9).

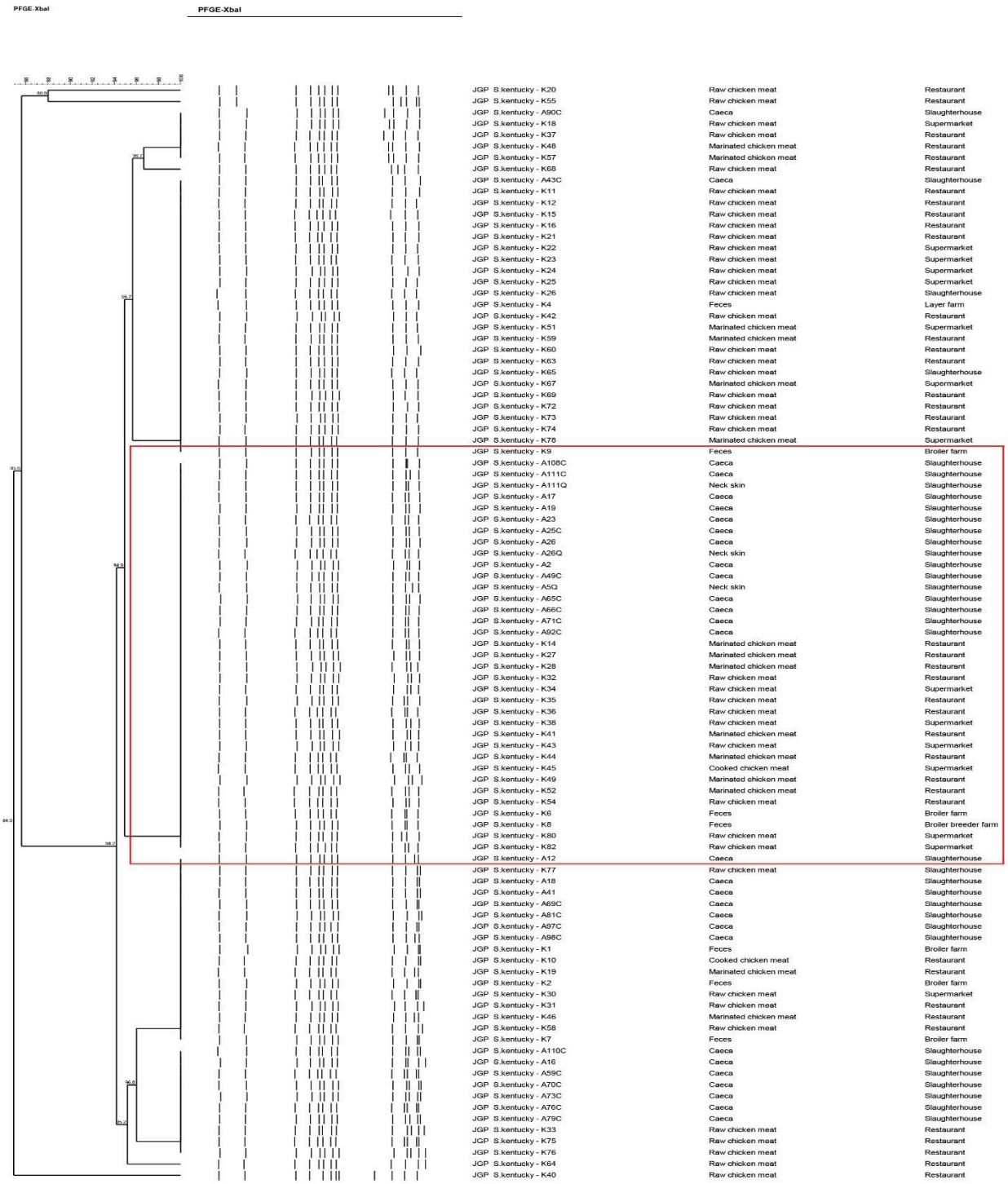

Figure 7: Macrorestriction patterns of *S.* Kentucky using the Dice coefficient, and the dendrograms were generated graphically by using unweighted pair group method with arithmetic mean (UPGMA). The codes A, B and K designate the *Salmonella i*solates from slaughterhouse A, slaughterhouse B and retail respectively. The letters C or Q are related to caeca or neck skin respectively.

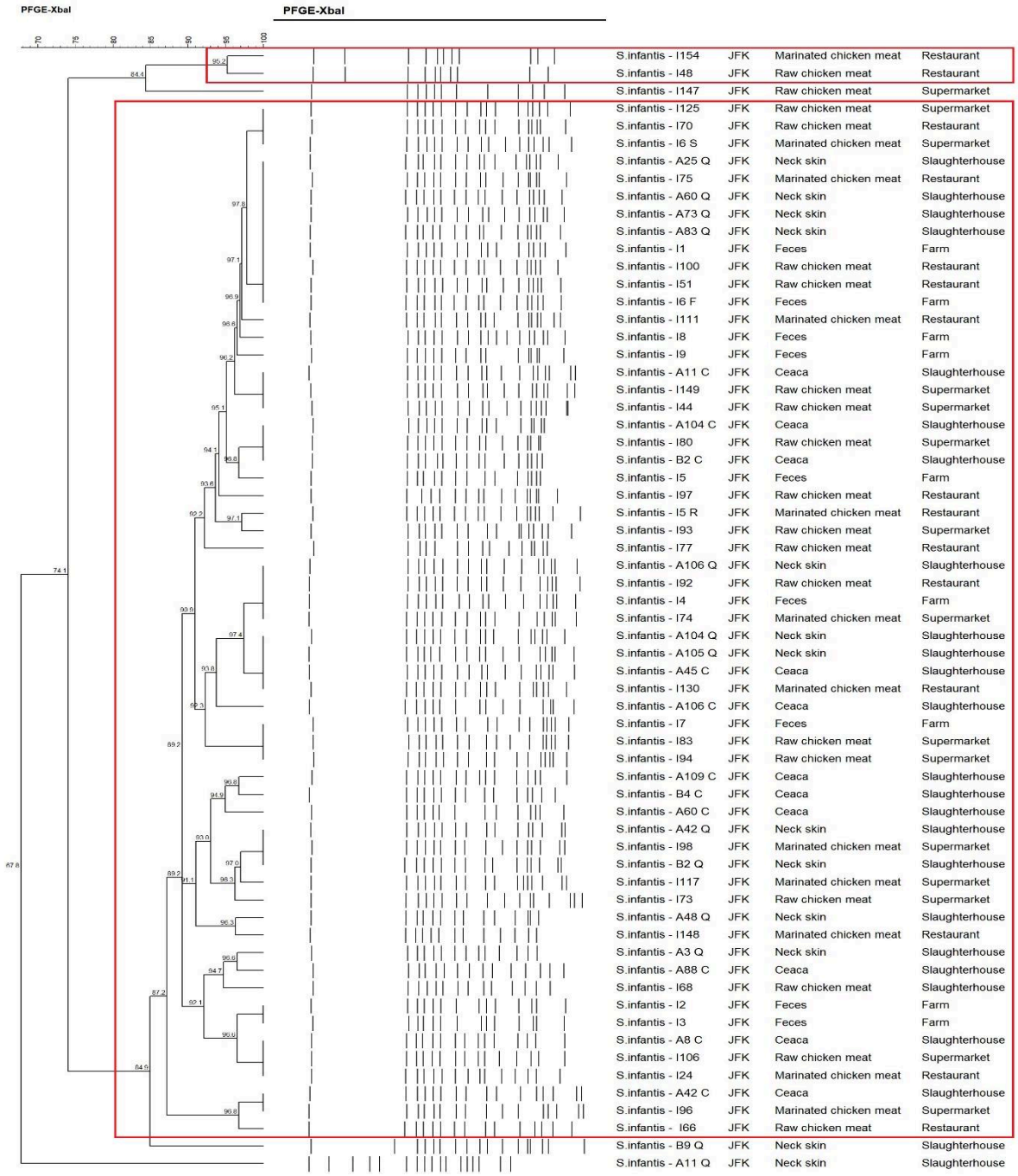

Figure 8: Macrorestriction patterns of *S.* Infantis using the Dice coefficient, and the dendrograms were generated graphically by using unweighted pair group method with arithmetic mean (UPGMA). The code A, B and I designate the *Salmonella* isolates from slaughterhouse A, slaughterhouse B and retail. The letters C or Q are related to caeca or neck skin respectively.

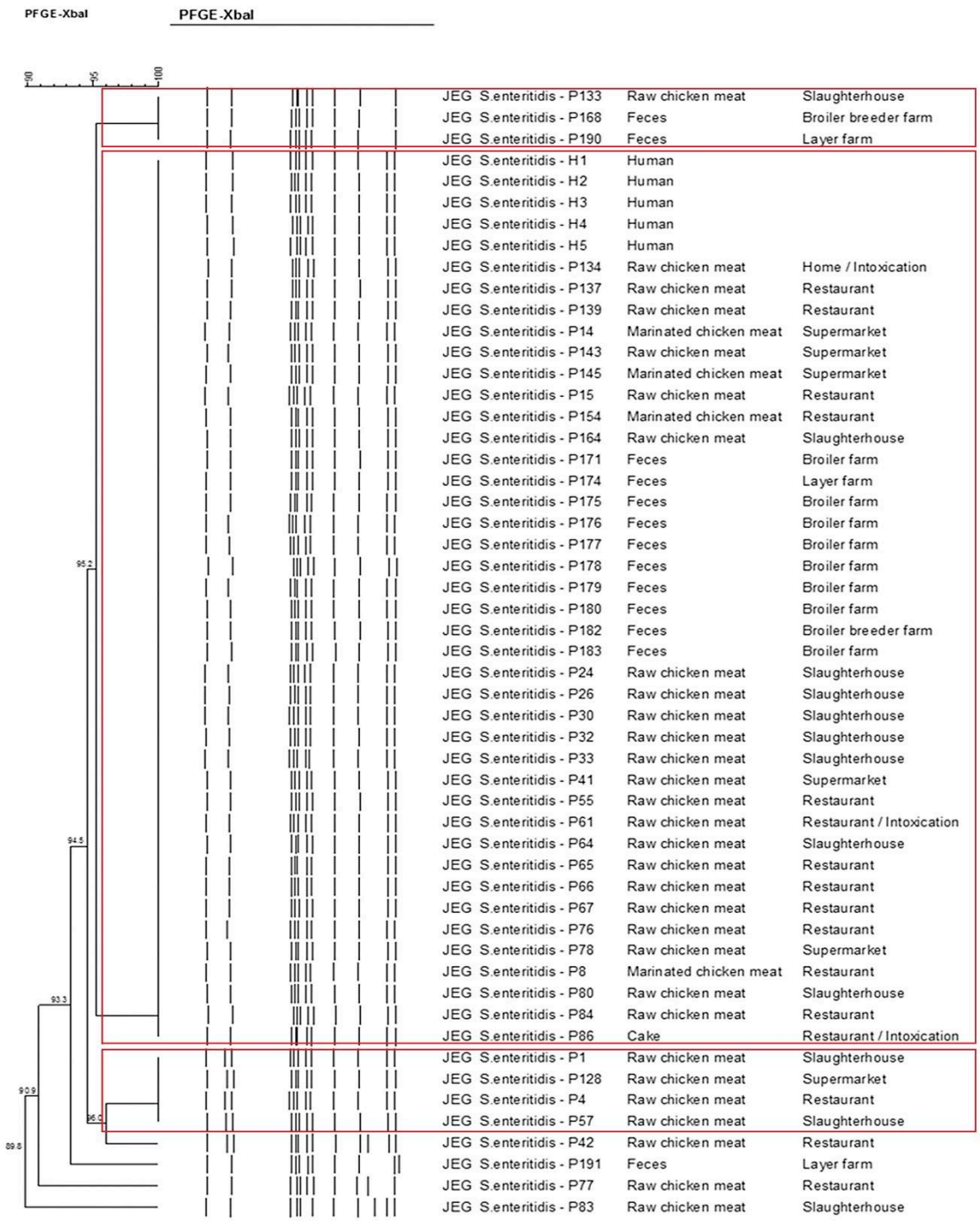

Figure 9: Macrorestriction patterns of *S.* Enteritidis using the Dice coefficient, and the dendrograms were generated graphically by using unweighted pair group method with arithmetic mean (UPGMA). The code P designate the *Salmonella* isolates.

4. Discussion

The global prevalence of *Salmonella* in the farms is 30 %. Infected breeder farms (31 %) and layers (24.5 %) are of great concern since contamination can be disseminated to commercial broiler flocks and eggs, respectively, via vertical or horizontal transmission. This result as well as the prevalence in broilers (31.4%) is similar to previous studies done in developing countries such as Bangladesh, Algeria and Constantine (Barua et al., 2012; Djeffal et al., 2017; Elgroud et al., 2009). However, it is exceedingly higher than reports from the EU where, in the context of mandatory National Control Programs, the reported flock prevalences were of 1.47 %, 2.6 % and 3.71 % in breeding flocks, broilers and laying hens, respectively (EFSA/ ECDC, 2017). This high occurrence could be attributed to the absence of *Salmonella* reduction plan at the farm level in Lebanon.

The high global prevalence of 47.8 % of *Salmonella* isolated from caeca suggesting that the major source of contamination is mainly at the farm level (fully integrated) rather than at slaughterhouse is in agreement with other studies (Zhu et al., 2017). Most of the farms did not implement good farming practices since 81.3 % of the farms are *Salmonella* positive. The lower prevalence of *Salmonella* observed on neck skins from Slaughterhouse A (16.83 %) comparing to slaughterhouse B (57 %) highlighted the differences in their practices: indeed, the first one, in line with the high number of slaughtered poultry, performed air chilling with Peracetic Acid (PAA) (Slaughterhouse A) while the second performed chilling by immersion with chlorine (0.3ppm) (Slaughterhouse B). In fact, carcass chilling is considered a critical step to avoid the cross-contamination of pathogens. Commercial immersion chilling is widely used in the United States but has been often criticized because of the potential cross-contamination risk (Sukted et al., 2017). Furthermore, and despite that chlorine is widely used in the USA as an effective antimicrobial, the presence of high organic materials during the processing often reduces its antimicrobial efficacy, while organic concentration above 5 % can cause complete inactivation (Paul et al., 2017). Another antimicrobials such as PAA, used in Slaughterhouse A, were often chosen and have proved to be more effective in chillers (Blevins et al., 2017).

Salmonella prevalence in poultry meat in slaughterhouses and retail was 35.8 % and 22.4 %, respectively. The retail contamination is lower than other regions in Asia as reported in Japan (54.1 %) (Shigemura et al., 2018) and in Korea (42 %) (CHOI et al., 2015). This rate is quite steady in

Lebanon when compared to a previous study carried out by our institute with 41.6 % of contamination in chicken carcass in slaughterhouses (EL Hage, 2013 unpublished results).

Twenty-three *Salmonella* serotypes were identified along the chain. The main diversity, higher at the retail level, 21 serotypes compared to only three at the farm level, indicated a higher risk of cross-contamination and other contaminating sources that should be investigated further. The three main serotypes were *S*. Infantis (32.9%), *S.* Enteritidis (28.4%), and *S*. Kentucky (21.4%) which is in accordance with the poultry-associated *Salmonella* serotypes distribution worldwide. *S*. Enteritidis and *S*. Infantis were still among the most prevalent serotypes in laying hens and broilers and broiler meat, respectively, in Europe (EFSA/ECDC, 2016). *S.* Kentucky ranks among the 12 most prevalent poultry-associated *Salmonella* serotypes worldwide (Shah et al., 2017). In fact, many studies mentioned a shift in *Salmonella* serotypes in poultry production, which is related to the control measures against specific serotypes (Antunes et al., 2016; Rabsch et al., 2000).

The type of broiler species, the management system adopted, rearing at farm level and its geographical location might explain the obviously different *Salmonella* serotypes diversity in the two slaughterhouses, where only *S.* Infantis is detected as a common serotype. It seems that horizontal contamination of this serotype has occurred from the broiler farm level until retail level. Although being classified as the fourth serotype causing human salmonellosis in the EU and predominating in local poultry production, it could not be associated to foodborne outbreak in Lebanon (*MOPH/ Pulse Net report,* 2015).

The high prevalence of *S*. Enteritidis, especially at breeders and layers farms is very concerning. This serotype, leading cause of human salmonellosis in Lebanon (*MOPH/ Pulse Net report,* 2015) and in the world (EFSA/ECDC, 2016; Foley et al., 2011), is known to be vertically transmitted and therefore leads to the contamination of broiler flocks and eggs (Cox et al., 2000). In the EU, outbreaks due to *S.* Enteritidis in eggs have caused the highest number of outbreak cases in 2016. Moreover, when performing PFGE analysis on *S*. Enteritidis isolates, in addition to farms, retail, and human isolates shared the same pulsotype at a rate of 80 %. This same pulsotype has been circulating in Lebanon since 2010 and was linked to three outbreaks (Saleh et al., 2011) and isolated in clinical and food samples in 2017 (Fadlallah et al., 2017). Previous studies have demonstrated that this serotype is highly clonal (Campioni et al., 2012; Fardsanei et al., 2017). The presence of *S*. Enteritidis was confirmed along the broiler production chain and layer flocks;

however, it was not detected in both slaughterhouses A and B. This might be due to the vaccination programs against *S*. Enteritidis and *S*. Typhimurium at the broiler breeder level, which is known to be effective in controlling *Salmonella*.

One of our major findings is the high prevalence of *S*. Kentucky circulating all over the poultry production chain, and it has been the most predominant in the biggest slaughterhouse in the country (52.3%). In fact, this serotype is highly present in poultry worldwide (Shah et al., 2017) but not commonly associated with human illness in Lebanon (*MOPH/ Pulse Net report,* 2015) and in USA (CDC, 2017) and only 1% of human salmonellosis in EU (EFSA/ECDC, 2016). Its capacity to grow in moderate acidic environment provides it an advantage over other serotypes to proliferate in chicken caecum (Foley et al., 2013). The route to broiler flocks contamination remains unclear. Mostly horizontally transmitted, Papadopoulou et al. (2009) indicated that protein concentrates in animal feed are source of contamination by *S.* Kentucky.

Most isolates from imported chicken were *S*. Heidelberg, which has also been detected in retail at 1% suggesting that the source of contamination of this serotype was derived from Brazilian chicken meat products. This was the case in the EU where contaminated chicken cuts, notified by the Rapid Alert System for Food and Feed were imported from Brazil (*RASFF,* 2017).

The improper use, overuse or misuse of antimicrobials in agriculture have contributed to the dissemination of drug- resistant non-typhoidal *Salmonella* that may be transmitted to humans via the food chain (Aarestrup, 2015). This *Salmonella* resistance represents a serious global threat to public health (CDC, 2013). In Lebanon, fluoroquinolones (enrofloxacin), third-generation cephalosporins (ceftiofur) and trimethoprim are widely used in the therapy of poultry production. In this investigation, an extremely high rate of nalidixic acid resistance and highly worrisome MDR among the isolates were obtained. *S*. Enteritidis showed the lowest resistance, which is in accordance with international findings (Michael and Schwarz, 2016). Since the introduction of the (fluoro)quinolone class in poultry production, nalidixic acid resistance of *Salmonella* has been often reported worldwide (Fei et al., 2017; Gouvêa et al., 2015). This is of great concern since this resistance may be an indicator of reduced susceptibility to other quinolones of clinically great importance such as ciprofloxacin (Choi et al., 2005). Our results showed that antimicrobial resistance has increased in all *Salmonella* serotypes tested at the end of the production chain, especially at retail level, suggesting that these serotypes gained resistance not only from bad

practices at the farm, but also from resistance genes in the environment (Ferri et al., 2017) and therefore the dissemination of more AMR to consumers.

Despite the recent emergence of antimicrobial resistance among *S.* Infantis in Europe (EFSA/ECDC, 2015), it is still reported as pan-susceptible in USA poultry (Shah et al., 2017). Our results showed a remarkable total resistance to NA (99.5 %), tetracycline (99 %) and streptomycin (88.2 %), and to a lesser extent, trimethoprim (2.4 %) and trimethoprim-sulfamethoxazole (1 %). High incidence of MDR *S.* Infantis (89.7 %) having the "S-Na-Te" pattern (71.6 %) was the most predominant one circulating throughout the broiler food chain. Two of 14 antimicrobial profiles displayed a pattern "S-Na-W-Te" and "S-Na-W-Sxt-Te." Our results might be related to similar clonal spread of *S.* Infantis in broiler and humans detected in Hungary (Nógrády et al., 2007). The typically emerging pattern "Na-S-Sul-Te" became widely disseminated in European countries (Nógrády, 2012). Aviv et al. (2014) declared a unique megaplasmid (pESI) (plasmid emerging *S.* Infantis) conferring high antimicrobial resistance, virulence and stress tolerance. Later on, Extended Spectrum Cephalosporin (ESC) Resistance has been announced in Italy (Franco et al., 2015), in Switzerland (Hindermann et al., 2017) and USA (Tate et al., 2017). None of our isolates were ESBL producers. High genetic relatedness (87.8 %) has been found among these isolates in accordance to other studies (Franco et al., 2015, Vinueza-Burgos et al., 2016) suggesting that this strain is clonal. PFGE analysis with high rate of individual subtypes, especially at the retail level, suggests frequent possibilities of cross-contamination. In addition, similar PFGE patterns have been detected across isolates from different sources where slaughterhouse A was mainly implicated, suggesting that this slaughterhouse may be the source of *Salmonella* Infantis contamination.

All *S.* Kentucky strains were highly ciprofloxacin-resistant (MIC level: 6.25->32µg/ml) and showed large, diverse antimicrobial resistance profiles. Our *S.* Kentucky strains in both MDR, mainly "Amp-Amc-Cn-S-Na-Cip-Nor-Te-Enr" (14.3 %), and AMR (Amp, Amc, Te, S, Cn) are very similar to the emerging ST198-X1 strain and coherent with the rapid and extensive global epidemic Ciprofloxacin resistant ability of this subtype described by Le Hello et al. (2011). The source of this contamination is very variable, Le Hello et al. (2011) strengthened on poultry as the main niche in Africa (Ethiopia, Nigeria Morocco,) and Europe (Poland, Germany and France) other on reptiles (Zajac et al., 2013) and the environment (Le Hello et al., 2013). This multiple

class resistance might be attributed to the acquisition of an integrative mobilizable element "*Salmonella* genomic island 1" (SGI-1) that confer resistance to different classes of antimicrobials, amoxicillin, gentamycin and sulfonamide (Doublet et al., 2008) followed by cumulative mutations in the *gyrA* and *parC* genes leading to resistance to nalidixic acid and then to ciprofloxacin in 2002. It is also shown that nine different strains of *S.* Kentucky are ESCs comparable to those detected in the Mediterranean basin (Le Hello et al., 2013). Two of them (A 66C-B108C) were isolated from slaughterhouse A and seven (K12, K24, K31, K32, K38, K43, K48) from retail, indicating that this slaughterhouse is the causative dissemination of ESCs *S.* Kentucky. In addition, according to PFGE analysis, these strains are highly related, proposing that this strain is also clonal. Strains from farms (layers or /and broiler breeders or/ and broilers), slaughterhouse A and retail were grouped within one pulsotype suggesting that S. Kentucky is circulating throughout the broiler food chain and layer flocks. It should be noted that slaughterhouse A has its farms, and therefore its role in the dissemination of *S.* Kentucky is important.

5. Conclusion

This work highlighted *Salmonella* prevalence in the Lebanese poultry production and the relatedness between different stages of the food chain, through a "Farm to fork" approach. The great incidence of *Salmonella* compared to developed countries is very alarming in the Lebanese poultry industry, urging the establishment of an effective prevention and control program along the food chain.

S. Enteritidis is highly predominant with human illnesses attributed to only one poultry-associated clone that has been persistent since 2010 in Lebanon. Moreover, this is the first time that *S*. Kentucky and *S.* Infantis are reported to be spread in Lebanon. These two strains are exceedingly Multi-Drug resistant to the key antibiotic classes circulating all over the Lebanese poultry chain and therefore could be a potential threat to consumers. The miss and / over use of uncontrolled drugs in Lebanese animal production is the leading origin of emergent MDR bacteria, but also the circulation of resistant strains from other countries via human travels and good trades. AMR is a global public health problem that requires national, regional and international sustainable solutions. To develop countermeasures that will have lasting effects, new ideas complementary to traditional approaches are needed. Thus, the discovery of new antimicrobial agents from natural origins, along with their biocontrol, present alternative approaches.

6. ACKNOWLEDGMENTS

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Chapter III

Genomic characterization of Extended-Spectrum β Lactamases (ESBLs) and cephamycinase-producing *Salmonella* Kentucky ST198 in Lebanese broiler production

Genomic characterization of Extended-Spectrum β Lactamases (ESBLs) and cephamycinase-producing *Salmonella* Kentucky ST198 in Lebanese broiler production

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Abstract

Despite the low incidence of salmonellosis due to *Salmonella enterica*, subsp. *enterica* serovar Kentucky in humans, this serovar has been associated with a worldwide spread of a particular epidemic clone, Ciprofloxacin-resistant (Cip^R) *S*. Kentucky ST198, being mostly recovered from poultry farms and products. Here we report, for the first time in Lebanon, a case of detection and dissemination of the emerging highly drug-resistant *S.* Kentucky ST198. A number of eight strains of *S.* Kentucky isolated from broilers were genetically characterized by whole genome sequencing (WGS). Phylogenetic analysis revealed a close relatedness between the isolates. They all harbored mutations in chromosomal-quinolone resistance genes *gyrA* and *parC* with double substitutions in GyrA (S83F and D87N) and a single substitution in ParC (S80I). Resistance genes against third generation β-lactams, bla_{TEM-1B} and plasmid-encoded cephamycinase bla_{CMY-2} were common, six out of eight isolates were shown to carry both of them. Aminoglycosides (aadA7 and aac (3)-Id), tetracyclines (tet (A)) and sulfonamides (sul1) resistance genes were detected in five strains among which four were positive for the presence of Salmonella Genomic Island 1 variant SGI1–K. The insertion sequence ISEcp1 was detected in six strains downstream of the bla_{CMY-2} gene. All studied isolates harbored a variety of Salmonella Pathogenicity Islands (SPIs) as well as regulatory and virulence genes. In this study, all evidence points to one or several factors implicated in the multidrug resistance (MDR) and virulence of *S.* Kentucky ST198 in Lebanon. These findings are alarming and shed new light on *S.* Kentucky ST198 as a potential public health threat that should be an integral part of surveillance programs in the chain of Lebanese poultry production.

Keywords: Whole Genome Sequencing, Cip^R *S.* Kentucky ST198, ESBLs, cephamycinase, virulence genes, poultry

1. Introduction

Uncommon in human salmonellosis, *Salmonella enterica* subsp. *enterica* serovar Kentucky is however widespread in poultry meat (CDC, 2017), (Shah et al., 2017). An emerging highly Cip^R *S.* Kentucky ST198 subtype was well described by (Le Hello et al., 2011), causing human infections linked to travelers returning from Middle-East, Southeast Asia or Africa (Le Hello et al., 2013b; Mulvey et al., 2013).

Since the 1990s, Cip^R *S.* Kentucky ST198 has accumulated numerous chromosomal resistance determinants with the integration of the mobilizable "*Salmonella* genomic island 1" (SGI1). This latter was described in the MDR *S.* Typhimurium DT 104 (Boyd et al., 2001), as responsible for the global spread of MDR *Salmonella*, mainly to amoxicillin, gentamicin, and sulfonamides (Doublet et al., 2008). Single mutation of topoisomerase-encoding *gyrA* chromosomal gene, followed by double mutations in *gyrA* and *parC* genes, have led to high-level resistance to nalidixic acid and later generations of fluoroquinolones, such as ciprofloxacin.

Firstly recorded in Egypt from 2002 to 2005, Cip^R *S.* Kentucky has promptly spread throughout Africa, the Middle East, Europe, and North America causing a global establishment of a challenging bacterial clone (Haley et al., 2016; Le Hello et al., 2013a; Ramadan et al., 2018). Le Hello et *al.* (Le Hello et al., 2013a) identified this strain from different sources (environment, humans, animals, and food) and different locations, particularly from several new countries in the Indian sub-continent and Southeast Asia.

Mediterranean isolates belonging to this emerging serovar, have become producers of various carbapenemases (*bla*$_{VIM-2}$; *bla*$_{OXA-48}$), cephamycinase (*bla*$_{CMY-2}$), Extended Spectrum β-Lactamases (ESBL) (*bla*$_{CTX-M-1}$; *bla*$_{CTX-M-15}$; *bla*$_{CTX-M-25}$) and a mix of carbapenemases and ESBL (*bla*$_{OXA-48}$ and *bla*$_{VEB-8}$) which pose an imminent threat to public health (Le Hello et al., 2013b; Seiffert et al., 2014). Cephamycinase CMY-2 is the most prevalent pAmpC β-lactamase distributed among IncI1 and IncA/C plasmids (Fricke et al., 2009). The IS*Ecp1* insertion sequence also plays an important role in the spread of ESBL and *bla*$_{CMY-2}$ by mobilizing the adjacent resistance genes originated from the *Citrobacter freundii* chromosome by ISEcp1-mediated transposition (Verdet et al., 2009).

Some studies have shown that MDR *Salmonella* and ESBLs-producing isolates became more pathogenic by co-carrying several virulence genes (Khoo et al., 2015; Li et al., 2017). These genes are located on plasmids, prophages, SPIs, and fimbrial clusters (Li et al., 2017) Some virulence genes were identified to confer pathogenicity more than others. Yang et al. (2015) reported that *sodC1*, which encodes a periplasmic Cu-Zn superoxide dismutase for the survival of *Salmonella* in the macrophage, was detected only in highly pathogenic strains. *S.* Kentucky is thought to be unharmful to humans due to the lack of virulence genes such as *grvA, sseI, sopE, and sodC1* (Cheng et al., 2014) or *sopD2, pipB2, sspH2,* and *srfH* (Dhanani et al., 2015).

The relevant concern to *S*. Kentucky is its accelerated dissemination in chicken, referred to a better acid response than other serotypes (Joerger et al., 2009). Others attributed the differential regulation of core *Salmonella* genes via the stationary-phase sigma factor RpoS, to the metabolic adaptation in the chicken caecum (Cheng et al., 2014).

In Lebanon, *S.* Kentucky was among the most predominant serovars in the broiler production chain (Broiler breeder farms, broiler farms, slaughterhouses, and retail) and layer flocks. The global prevalence of this serovar was 21.4% among the total identified ones (unpublished results), although it was not related to human intoxication (*MOPH/ Pulse Net report/ Study case report in Lebanon*, 2015). It has been shown that all isolated strains were ciprofloxacin resistant, 65.4 % were multidrug resistant, and 6.8 % were also Extended Spectrum Cephalosporin (ESCs) resistant. The aim of this study was, therefore, to determine whether these Cip^R and ESC resistant *S.* Kentucky strains belong to the expanding ST198-SGI1. In line with this, deep genomic characterization of these isolates was performed.

2. Materials and methods

2.1. Collection of *Salmonella* Kentucky strains

Eight ciprofloxacin and ESCs resistant *S.* Kentucky strains were previously recovered as follows: seven strains from retail chicken cuts (17-70328 (K12), 17-70460 (K24), 17-70462 (K31), 17-70464 (K32), 17-70468(K38), 17-70469 (K43) and 17-70472 (K48)) and one strain from commercial slaughterhouse caeca broiler (17-70474 (A66C)) (unpublished results).

2.2. Antimicrobial sensitivity test

Antimicrobial susceptibility testing (AST) was carried out referring to the Clinical and Laboratory Standards Institute (CLSI, 2008; CLSI, 2017). The Kirby-Bauer disc diffusion method was firstly performed, for a panel of 26 antimicrobials (Oxoid, Basingstoke, England) of veterinary and human health importance. The tested antibiotics were: ampicillin (Amp-10 µg), amoxicillin-clavulanic acid (Amc-30 µg), piperacillin-tazobactam (Tzp-110 µg), cephalothin (Kf-30 µg), cefuroxime (Cxm-30 µg), cefoxitin (Fox-30 µg), cefotaxime (Ctx-30 µg), ceftriaxone (Cro-30 µg), ceftazidime (Caz-30 µg), ceftiofur (Eft-30 µg), cefepime (Fep-30 µg), imipenem (Ipm-10 µg), aztreonam (Atm-30 µg), gentamycin (Cn-10 µg), tobramycin (Tob-10 µg), streptomycin (S-10 µg), amikacin(Ak-30 µg), netilmicin (Net-30 µg), nalidixic acid (Na-30 µg), ciprofloxacin (Cip-5

µg), norfloxacin (Nor-10 µg), enrofloxacin (Enr-5 µg), trimethoprim (W-5 µg), trimethoprim-sulfamethoxazole (Sxt-1.25/23.75 µg), tetracycline (Te-30 µg), chloramphenicol (C-30 µg). Antimicrobial Minimum Inhibitory Concentrations (MICs) for resistant strains were determined using broth microdilution for the following antimicrobials and breakpoint values: Kf (≥32 µg/ml), Cxm (≥32 µg/ml), Fox(≥32 µg/ml), Ctx (≥4 µg/ml), Cro (≥4 µg/ml), Caz (≥16 µg/ml), Eft (≥8 µg/ml), Cn (≥16 µg/ml), Na (≥32 µg/ml), Cip (≥1 µg/ml), Nor (≥16 µg/ml), Enr (≥2 µg/ml). *Escherichia coli* ATCC® 25922™ was used as a quality control strain. Antimicrobial resistance to ≥3 classes was considered MDR.

2.3. Genome analyses

Isolates were characterized by Whole Genome Sequencing. Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Valencia, CA) and quantified with a Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA). Libraries for sequencing were prepared using Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA). High-throughput sequencing was performed on Illumina MiSeq with 2 × 250 paired-end reads. Raw sequence data were submitted to the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under accession number PRJEB27597. Raw reads were assembled in contigs using Assembler 1.2 (https://cge.cbs.dtu.dk/services/Assembler/) (Larsen et al., 2012) or SPAdes 3.9 (https://cge.cbs.dtu.dk/services/SPAdes/) (Nurk et al., 2013). All samples were then subjected to *in-silico* serotyping using SeqSero 1.2 (https://cge.cbs.dtu.dk/services/SeqSero/all.php) (Zhang et al., 2015) starting from assembled data to confirm *in-vitro* serotyping. When concordance was not verified, analysis was repeated starting from raw reads. To verify the presence of acquired antimicrobial resistance genes, assembled genomes were analyzed using ResFinder2.1 (https://cge.cbs.dtu.dk/services/ResFinder/, Selected threshold for %ID = 90%; Selected minimum length = 60 %), while ResFinder3.0 (https://cge.cbs.dtu.dk/services/ResFinder-3.0/) (Zankari et al., 2012) was used to detect known chromosomal point mutations that can confer antimicrobial resistances. MLST (Multi-Locus Sequence Type), plasmids and plasmid typing were performed using MLST 1.8 (https://cge.cbs.dtu.dk/services/MLST/) (Larsen et al., 2012), Plasmid Finder 1.3 (https://cge.cbs.dtu.dk/services/PlasmidFinder/, Selected threshold for %ID = 85%), and pMLST 1.4 (https://cge.cbs.dtu.dk/services/pMLST/) (Carattoli et al., 2014), respectively. MyDbFinder (https://cge.cbs.dtu.dk/services/MyDbFinder/) was used to investigate the presence of SGI1-K

(*Salmonella* genomic island 1 variant K, GenBank accession number: AY463797.8) (Levings et al., 2005), which is frequently integrated into *S*. Kentucky genome. The reference used to find IS*Ecp1* was the deposited sequence of *S*. Typhimurium strain 110516 [KX377449.1:780-1276].

2.4. Phylogenomics

Assembled genomes and a reference genome (*S*. Kentucky CVM29188, (Fricke et al., 2009)) were used to build a SNP-based phylogenetic tree using CSIPhylogeny 1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny/) (Kaas et al., 2014) with default parameters for SNP filtering and SNP pruning.

3. Results

3.1. Multi-Locus Sequence typing (MLST) and detection of plasmids and replicon type (pMLST)

All isolates submitted to WGS belonged to the Sequence Type (ST) ST198, except one isolate 17-70472 (K48) for which it was not possible to assign a MLST sequence-type. This is most likely due to a bad assembly comparing to other samples since the sample 17-70472 (K48) showed a high number of contigs (3495 contigs) and a bad N50 value (Table 8).

All plasmids recovered from all isolates belonged to replicon type IncI1 and ColRNAI. Using pMLST based on WGS data, two IncI1 type plasmid were ST12, and two other plasmids were identified as belonging to ST2 and ST65. Four others were non-typable, but two of them closely match ST12 and ST23 (Table 8).

Table 8: Results of Genomic Assembly, SeqSero, MLST, PlasmidFinder and pMLST and Accession Number of the eight Lebanese *S.* Kentucky isolates

ID IZSVe (Ref. Lebanon)	Source	Genome size (bp)	Number of contigs	N50 [a]	Serotype	MLST	Plasmids	pMLST [a]	accession Number
17-70328 (K12)	chicken cuts/retail	4922807	93	534536	*S.* Kentucky	ST198	IncI1, ColRNAI	IncI1[ST65]	ERR2681948
17-70460 (K24)	chicken cuts/retail	5002563	251	238030	*S.* Kentucky	ST198	IncI1, ColRNAI	IncI1[ST12]	ERR2681949
17-70462 (K31)	chicken cuts/retail	4967065	105	534536	*S.* Kentucky	ST198	IncI1, ColRNAI	IncI1[ST12]	ERR2681950
17-70464 (K32)	chicken cuts/retail	4916713	80	450673	*S.* Kentucky	ST198	IncI1, ColRNAI	IncI1[unknown, closest match ST23]	ERR2681951
17-70468 (K38)	chicken cuts/retail	4980697	458	26113	*S.* Kentucky	ST198	IncI1, ColRNAI	IncI1[unknown, closest match ST12]	ERR2681952
17-70469 (K43)	chicken cuts/retail	4896047	93	293715	*S.* Kentucky	ST198	IncI1, ColRNAI	IncI1[unknown]	ERR2681953
17-70472 (K48)	chicken cuts/retail	4417617	3495	1670	*S.* Kentucky	unknown	IncI1	IncI1[unknown]	ERR2681954
17-70474 (A66C)	chicken caeca/ slaughterhouse	4942747	149	120678	*S.* Kentucky	ST198	IncI1, ColRNAI	IncI1[ST2]	ERR2681955

[a] = N50 statistic defines assembly quality. Given a set of contigs ordered from the shortest to the longer, N50 is defined as the shortest sequence length among contigs which cover at least half of genome size

[a] = plasmid sequence type (pMLST) is defined only for schemed plasmids (i.e., IncI1): plasmid replicon and identified alleles in square brackets are given

3.2. Phenotypic and Genotypic antimicrobial resistance and presence of SGI1-K

Antimicrobial sensitivity testing showed high ciprofloxacin MIC levels (12.5->32 µg/ml). Six out of eight strains were multidrug resistant. Phenotypic antimicrobial resistance patterns are reported in Table 9.

WGS analysis confirmed the presence of antimicrobial resistance genes that conferred the phenotypic resistance. Indeed, there was a correlation between the resistance phenotype and the corresponding gene encoding it. Quinolone resistance-determining regions (QRDRs) of the target genes *gyrA, gyrB, parC*, and *parE* showed that all strains harbored double amino acid substitutions in GyrA, serine to phenylalanine at codon 83 (S83F) and aspartic acid to asparagine at codon 87 (D87N), and single substitution in ParC, serine to isoleucine at codon 80 (S80I). These mutations are responsible for high resistance levels to ciprofloxacin (Table 9). All isolates displaying ESCs resistant phenotype were found to carry resistance genes to third generation β-lactam, $bla_{\text{TEM-1B}}$ (Class A) and plasmid-encoded cephamycinase $bla_{\text{CMY-2}}$ (Class C) with 6 out of 8 isolates carrying both of them. Resistance genes to aminoglycosides (*aadA7* and *aac (3)-Id*), tetracyclines (*tet (A)*) and sulfonamides (*sul1*) were detected in 5 out of 8 strains. Except for sample 17-70460, isolates harboring resistance genes *aadA7* and *aac(3)-Id*, *tet(A)* and *sul1* are also positive for the presence of SGI1-K variant. Only one sample, 17-70462, also presents *floR* gene which confers cross-resistance to chloramphenicol and florfenicol (Table 9).

Table 9: Phenotypic and Genotypic antimicrobial resistance results of the eight Lebanese CipR *S.* Kentucky isolates using ResFinder 2.1, ResFinder 3.0 and MyDbFinder.

ID IZSVe (Ref. Lebanon)	**AMR Pattern**	**AMR genotype**	**QRDR point mutations**		**SGI1-K**
			gyrA	*parC*	
17-70328 (K12)	Amp-Amc-Kf-Cxm-Fox-Ctx-Cro-Caz-Eft-Na-Cip-Nor-Enr	*blaCMY-2, blaTEM1B*	S83F, D87N	S80I	absence
17-70460 (K24)	Amp-Amc-Kf-Cxm-Fox-Ctx-Cro-Caz-Eft-Cn-S-Na-Cip-Nor-Atm-te-Enr	*aadA7,aac(3)-Id,blaCMY-2, blaTEM1B,sul1, tet(A)*	S83F, D87N	S80I	absence
17-70462 (K31)	Amp-Amc-Kf-Cxm-Fox-Ctx-Cro-Caz-Eft-Na-Cip-Nor-C-Enr	*blaCMY-2, blaTEM1B, floR*	S83F, D87N	S80I	absence
17-70464 (K32)	Amp-Amc-Cxm-Fox-Cro-Caz-Eft-Cn-S-Na-cip-Nor-Atm-Te-Enr	*aadA7,aac(3)-Id,blaCMY-2, blaTEM1B,sul1, tet(A)*	S83F, D87N	S80I	presence
17-70468 (K38)	Amp-Amc-Kf-Cxm-Fox-Ctx-Cro-Caz-Eft-Cn-S-Na-Cip-Nor-te-Enr	*aadA7,aac(3)-Id, blaTEM1B,sul1, tet(A)*	S83F, D87N	S80I	presence
17-70469 (K43)	Amp-Amc-Kf-Cxm-Fox-Ctx-Cro-Caz-Eft-Cn-S-Na-Cip-Nor-Atm-te-Enr	*aadA7,aac(3)-Id,blaCMY-2, blaTEM1B,sul1, tet(A)*	S83F, D87N	S80I	presence
17-70472 (K48)	Amp-Amc-Kf-Cxm-Fox-Ctx-Cro-Caz-Eft-Na-Cip-Nor-Enr	*blaCMY-2*	S83F, D87N	S80I	absence
17-70474 (A66C)	Amp-Amc-Tzp-Kf-Cxm-Fox-Ctx- Cro-Caz-Eft-Cn-S-Na-cip-Nor-Atm-Te-Enr	*aadA7,aac(3)-Id,blaCMY-2, blaTEM1B,sul1, tet(A)*	S83F, D87N	S80I	presence

3.3. Detection of Insertion Sequence IS*ECP1*

Nucleotide sequence analysis of the eight *S*. Kentucky strains revealed the presence of IS*Ecp1* in six strains, at approximately 600 bp downstream of the bla_{CMY-2} gene, excepting strains (17-70468(K38) and 17-70472 (K48)) (Table 10).

3.4. *Salmonella* Pathogenicity Islands and Virulence genes analysis

A screening of SPIs, virulence genes, *rpoS*-regulated core genes, as well as other genes related to pathogenicity and survival in these *S.* Kentucky strains was performed (Figure 10).

As presented in Figure 10, SPI-5, SPI-13, SPI-14 were not detected in all studied strains. C63PI was identified in all strains except in 17-70472(K48). SPI-1 is absent from four *S.* Kentucky strains (17-70328 (K12), 17-70468 (K38), 17-70472 (K48), 17-70474(A66C)). SPI-2 is only present in 3 strains (17-70328 (K12, 17-70462 (K31), 17-70469 (K43)). SPI-3 is present in 6 strains and is absent from both 17-70468 (K38) and 17-70472 (K48). SPI-4 was detected in S. Kentucky strains 17-70328 (K12), 17-70462 (K31), 17-70464 (K32), and in 17-70474 (A66C).

The fimbrial genes *fimA* (type 1 fimbriae), *lpfD* (long polar fimbriae), *csgAB* (thin aggregative fimbriae), *steB*, *tcfA,* and *stjB* as well as the non fimbrial gene *SiiE* were identified in all sequenced *S.* Kentucky strains, except for strain 17-70472 (K48) in which *lpfD*, *stjB,* and *siiE* are absent. Moreover, all isolates carry *sitC,* and six strains harbored *iroN*. *sipA* and *avrA* are present in all genomes, and *sopE2* is absent from 17-70468 (K38) and 17-70472 (K48) strains. All isolates of serovar Kentucky lacked SPI-2 associated gene *sspH2* while *ssek2* was identified in 6 isolates but was missing in the all *sopE2*-lacking strains. Pathogenicity island two effector *sseC* was detected in all strains. The *pipA* gene was identified in all isolates except in 17-70472 (K48) that lacks the *pipD*. This latter is also absent from the 17 -70468 (K38) strain. Putative transcriptional regulator MarT-encoding gene was detected in all strains.

Concerning the *rpoS* regulated genes, all but 17-70472 (K48) strain, contain the *narZYV* operon that lacks the *nazW* gene. However, this latter is present alone in the strain 17-70472(K48). The *prpBCDE* operon implicated in propionate catabolism was identified in all strains except in the 17-70472(K48) strain where *prpD* was missing. The galactose transporter operon *mglABC* was

Table 10: Results related to the presence/absence of ISEcp1 in the genomes of the Lebanese CipR *S.* Kentucky strains and the co-localized antimicrobial resistance genes in the same contig.

ID_IZSVe (Ref Lebanon)	insertion sequence IS*Ecp1*	Resistance genes in the same contig
17-70328 (K12)	NODE_1; Position: 771647..772143	**blaCMY-2**: NODE_1; Position: 772260..773405
17-70460 (K24)		None
17-70462 (K31)	NODE_14; Position: 71183..71679	**blaCMY-2**: NODE_14; Position: 71796..72941
17-70464 (K32)	NODE_74; Position: 20105..20476	**blaCMY-2**: NODE_74; Position: 20593..21738
17-70468(K38)	NODE_315; Position: 579..1075	None
17-70469 (K43)	NODE_70; Position: 4743..5118	**blaCMY-2**: NODE_70; Position: 5235..6380
17-70472 (K48)	NODE_1431; Position: 400..895	None
17-70474 (A66C)	NODE_54; Position: 9143..9518	**blaCMY-2**: NODE_54; Position: 9635..10780

Legend:

presence (100% Identity; 497/497 Query/HSP length)
presence (100% Identity; 376/497 Query/HSP length)
presence (100% Identity; 372/497 Query/HSP length)
presence (98,79% Identity; 497/497 Query/HSP length)
absence

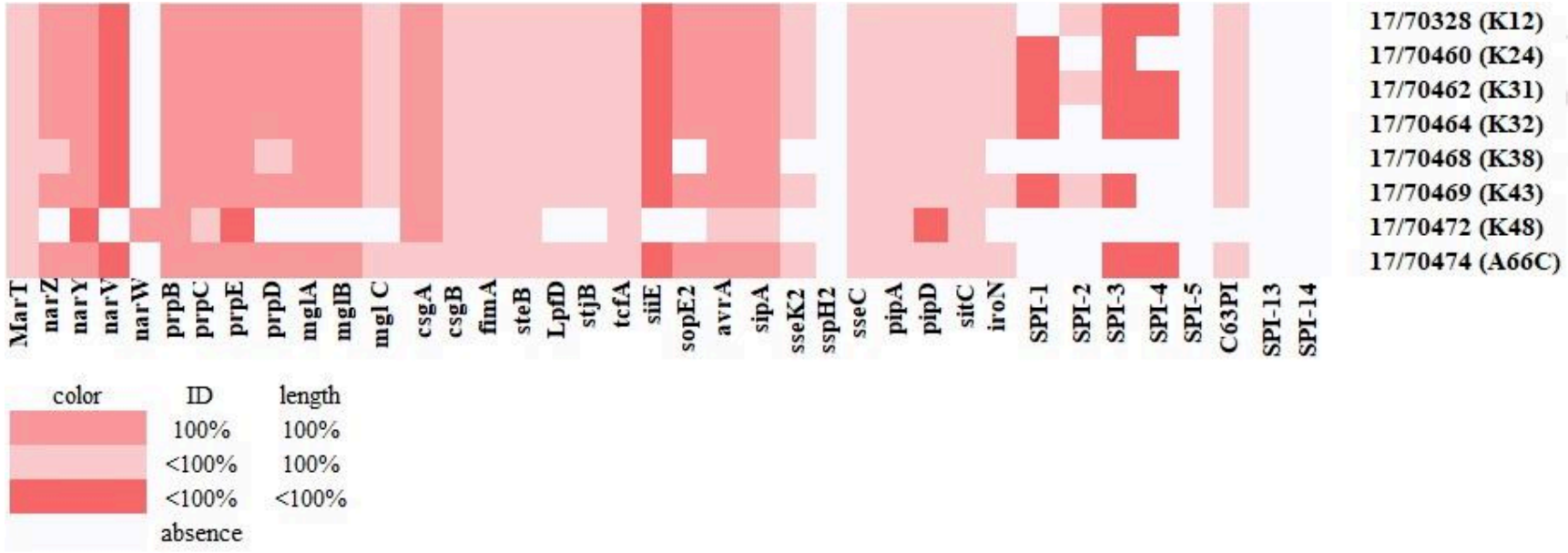

Figure 10: Virulence determinants of the eight Lebanese *S.* Kentucky isolates, based on the protein sequences of *Salmonella* sp. database

detected in all strains except in 17-70472 (K48). All sequenced isolates harbored the curli-encoding genes *csgA* and *csgB,* involved in the attachment of *Salmonella* to the mucosa.

3.5. Phylogenetic Single Nucleotide Polymorphism (SNP) analysis

Assembled genomes were used to build a phylogenetic tree with *S.* Kentucky CVM29188 selected as reference genome. As shown in Figure 11, where only shared SNP in the genomes – a close phylogenetic relatedness between all strains was observed with the only exception of sample 17-70472 (K48). Once again, this is most probably due to the bad assembly achieved for this particular sample. SNP difference among isolates varies between 12 and 7491 nucleotides.

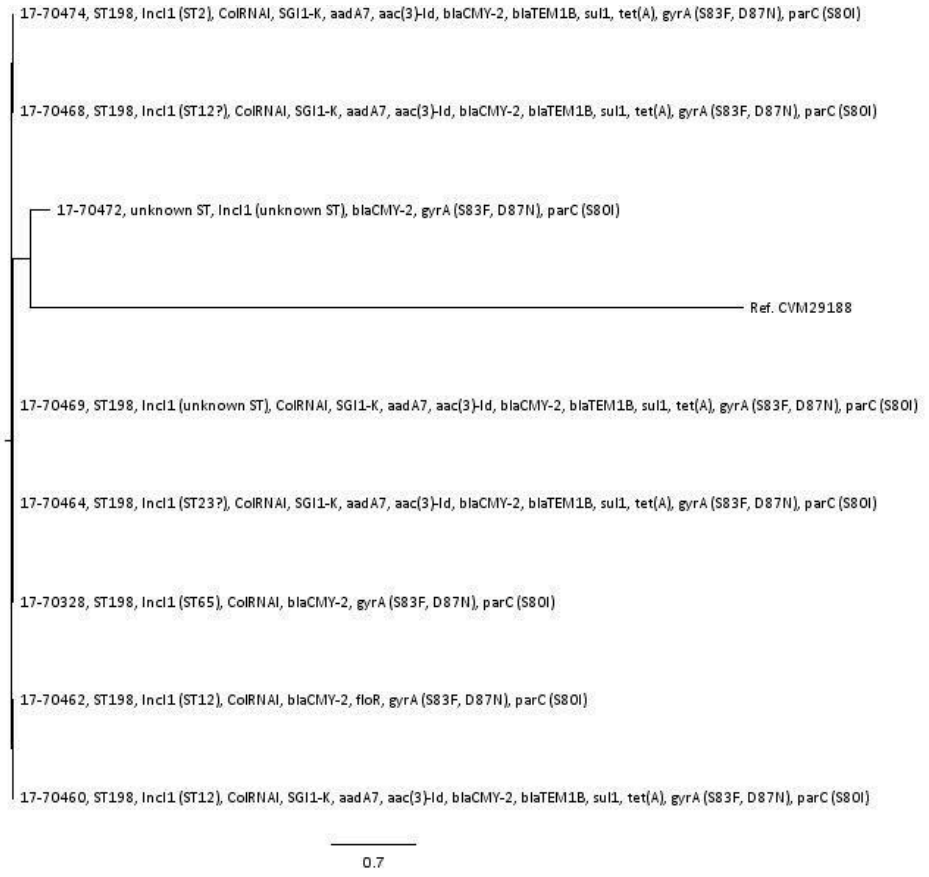

Figure 11: SNP-based Phylogenetic tree of the eight Lebanese Cip ^{R} *S.* Kentucky isolates with *S.* Kentucky CVM29188 as reference genome

4. Discussion

In this study, for the first time in Lebanon, *MLST* analysis performed on eight *S.* Kentucky *isolates from poultry* showed that seven isolates were belonged to the international emerging ST198-Cip^R Kentucky clone, out of them six were identified as MDR. All isolates harbored the already described mutations in *gyrA* and *parC* genes, linked to high-level fluoroquinolone resistance (Le Hello et al., 2013b). According to the authors, double substitutions in GyrA (Ser83 and Asp87) and a single ParC substitution (Ser80) are frequently identified in ciprofloxacin-resistant isolates. Different mutations in codon Asp87 are possible depending on the geographical origin of isolates, among them the Asp87Arg (D87N) that is commonly found in strains collected from South-East Asia, North Africa and the Middle East. Interestingly, two mutations in *gyrA* (Ser83Phe and Asp87Gly) and three mutations in *parC* (Ser80Ile, Thr57Ser, and Thr255Ser) were recently described in a Cip^R *S.* Kentucky ST198 isolated from a human patient in Washington state (Shah, DH., Paul, NC., Guard, 2018).

The *bla*$_{\text{TEM-1B}}$ (conferring resistance to third generation β-lactams) or *bla*$_{\text{CMY-2}}$ (encoding a cephamycinase) genes were detected in all isolates, among them six were interestingly shown to carry both. The Cip^R *S.* Kentucky ST198 isolates from the Mediterranean area have acquired β-lactamases (CTX-M, CMY-2, VIM-2, OXA- 48 and OXA- 204) encoding-genes conferring resistance to ESCs and carbapenems (Collard et al., 2007; Ktari et al., 2015). ESBLs and cephalosporinases are repeatedly encoded by 90-200 kb plasmids from the IncI1, IncL/M or IncA/C incompatibility groups (Le Hello et al., 2013b). Liakopolous et al.(2016) showed that the emergence of ESC resistant *Salmonella* in the Netherlands was due to the presence of *bla*$_{\text{CMY}}$ gene on IncI1 plasmids. Similarly, the IncI1 plasmid replicon was found in all isolates investigated. Moreover, plasmid sequences were diverse within these isolates; among them, two were identified as the IncI1/ ST12 plasmid. This latter has been disseminated worldwide, being related to the spread of *bla*$_{\text{CMY}}$–type pAmpC genes among *Enterobacteriaceae* (Hansen et al., 2016).

The MICs of cefotaxime, ceftiofur, and ceftazidime are known to be low in the natural habitats of bacteria. IS*Ecp1* are often integrated by transposition at the 5' ends of β-lactamase genes and may provide both 35 and 10 promoter sequences, located within the IS proximal to its right IR (IRR), for expression (Vandecraen et al., 2017). This event significantly enhances the *bla*$_{\text{CMY-2}}$ gene expression, thereby enabling an increase in these MICs of 2 to 8-fold (Fang et al., 2018). In our

study, all detected IS*Ecp1* insertions were located upstream the bla_{CMY-2} gene except for 17-70468 (K38) and 17-70472 (K48) strains, where the resistance gene was most probably not situated in the same contig. Moreover, most of the samples (5 out of 8) carried resistance genes to aminoglycosides (*aadA7* and *aac(3)-Id*), tetracyclines (*tet(A)*), and sulfonamides (*sul1*). *S.* Kentucky isolates commonly harbored an SGI, i.e., the SGI1-K, initially detected in *S.* Kentucky strains isolated in Australia (Levings et al., 2005)·(Levings et al., 2007). It comprises an MDR region including *aac(3)-Id*, *aadA7*, *tet(A)*, and *sul1* resistance genes as well as a mercury resistance module and other antimicrobial resistance genes. All but 17-70460 (K24) strains, containing resistance genes *aadA7* and *aac(3)-Id*, *tet(A)* and *sul1* were also positive for the presence of SGI1-K.

Gene transfer under antibiotic selective pressure facilitates the *spread* of drug *resistance (Ferri et al., 2017).* This could explain the dissemination of the highly MDR ST198-Cip^R Kentucky clone following the excessive therapeutic use of fluoroquinolones (enrofloxacin), third-generation cephalosporins (ceftiofur), and trimethoprim in the Lebanese poultry industry. These findings are in accordance with other reports in Africa and some parts in Asia (Ktari et al., 2015). In agreement with all these, it is noteworthy to mention that *S*. Kentucky is well known for its genomic plasticity leading to genetic rearrangements by horizontal acquisition of plasmids or genomic islands which account for antibiotic resistance pattern diversity (Wasyl et al., 2015). Indeed, 36 different resistance profiles were detected among 133 Cip^R *S*. Kentucky isolated along the Lebanese broiler production chain and layer flocks (unpublished data). Moreover, other *non-negligible* contributors could trigger this MDR such as the free trade and travel as well as the usage of contaminated feeds of aquaculture origin in poultry farms (Le Hello et al., 2011).

The virulotyping results revealed a little gene variability among seven *S*. Kentucky strains. The number of SPIs varied from one to five islands per isolate with C63P1 being the most predominant. The 17-70462 strain harbored the five SPIs detected. This SPIs variability among strains was also reported by (Roer L, Hendriksen RS, Leekitcharoenphon P, Lukjancenko O, Kaas RS, Hasman H, 2016).

Adherence to the cell surface is one of the first route to infection through fimbriae and other adherence- associated non-fimbrial proteins. Fimbria also plays an important role in biofilm formation, adhesion, and colonization (Lasaro et al., 2009). The SPI4-encoded T1SS and the non-

fimbrial giant adhesin SiiE were shown to initiate contact of *Salmonella* with host cells (Peters et al., 2017). Indeed, deletion of *siiE* gene resulted in a substantial decrease of the adhesion ability of *Salmonella* (Gerlach et al., 2007). All strains investigated in this study harbored the common *Salmonella* adhesion genes.

Salmonella encodes two virulence-associated T3SSs, namely T3SS-1 and T3SS-2 which are located on two *Salmonella* pathogenicity islands SPI1 and SPI2, respectively. The SPI-1 protein effectors SipA, SopD, SopB, SopA, and SopE2 mediate *Salmonella* invasion and colonization of epithelial cells (Raffatellu et al., 2005), while SPI-2 is implicated in *Salmonella* intracellular replication and dissemination (Figueira and Holden, 2012). Many T3SS-translocated effectors are encoded by genes located outside these pathogenicity islands. The *sipA* and *sopE2* genes located on SPI-1 and outside of it, respectively, play a significant role in *Salmonella* invasion of epithelial cells (Zhang et al., 2018). Here, these genes were detected in all strains. T3SS-1 *avrA* gene involved in apoptosis suppression of infected macrophages according to Lamas et al. (2018) was identified in all strains, thereby confirming the findings of Tasmin et al., (2017). The functionality of T3SS-2 helps to distinguish virulent from non-virulent *Salmonella* strains. The absence of the *sspH2* gene from *S*. Kentucky, encoding the SPI2-restricted translocated protein, was systematically reported in many studies (Dhanani et al., 2015; Tasmin et al., 2017). However, this gene was shown to be highly conserved among *Salmonella* serovars (Ramos-Morales, 2012). The reduced virulence of *S.* Kentucky was therefore partially attributed to the absence of *sspH2* gene as described by (Dhanani et al., 2015).

SPI-5 encodes at least *five genes*, *pipD, sigD/sopB, sigE/pipC, pipB, and pipA*, all of which contribute to enteropathogenesis of *Salmonella*. The deletion of *pipA, pipB,* and *pipD* genes resulted in a reduction of inflammatory responses and fluid secretion rate in infected hosts (Wood et al., 1998). Both *pipA* and *pipD* genes were identified in all of the eight studied strains, thereby corroborating results of Beutlich et al. (2011).

All studied isolates carried the putative iron transport gene *sitC,* but six of them harbored the salmochelin associated protein-encoding *iroN* gene. These findings are in accordance with other results carried out by (Dhanani et al., 2015). The authors found out that among different serotypes of *Salmonella* studied, only *S*. Kentucky harbored both the *iroN* and the *sitABCD* genes. The putative iron transport system SitABCD and IroN were considered to be essential factors in the

virulence mechanisms of *Salmonella* and their presence in *S.* Kentucky isolates deserves attention. They linked this finding to the emerging pathogenic *S.* Kentucky isolates associated with human infection worldwide. The work of Borges et al. (2017) showed that the virulent *S.* Enteritidis ST4 strain, which is associated with salmonellosis outbreaks, harbors the *IroN* gene.

Another explanation to *S*. Kentucky's emergence, as a predominant colonizer of the chicken caecum, might be the high expression levels of *RpoS*-regulated genes when compared to *S*. Typhimurium (Cheng et al., 2014). Indeed, this study highlighted the role of genes involved in galactose catabolism and curli production in colonization of *S*. Kentucky in the caeca. These *RpoS*-regulated genes have been detected in all studied strains.

5. Conclusion

This report addresses the first complete approach done in Lebanon that confirms the emergence of the highly drug-resistant, Cip^R *Salmonella enterica* serovar Kentucky ST198. Although infrequently associated with illness in human, *S.* Kentucky remains the most common non-clinical, non-human serovar reported worldwide.

The present study showed a co-possessed multidrug resistance and some virulence determinants that could be involved in the pathogenicity of *S*. Kentucky, which are likely to cause foodborne outbreaks and an imminent threat to public health. These ESBLs and cephamycinase-producing strains are the first evidence in Lebanon, thereby highlighting their high dissemination in the Mediterranean basin.

Our findings that *S*. Kentucky ST198 isolates harbor an *arsenal of virulence factors* suggest that these could be deployed to promote host-cell infection. In this regard, further functional and transcriptional studies should be carried out to elucidate the contribution of these virulence genes to the pathogenicity of S. Kentucky isolates and/or to predict the extension of their virulence potential.

Further efforts are needed from health, food, and agricultural authorities to control the emergence of this epidemic ST198-Cip^R Kentucky clone. Thus, its inclusion as a target strain in any national reduction plan of *Salmonella* in poultry is worth fully to be implemented.

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8. Transparency declarations

The data gathered for this article are assembled as part of R.E.H.'s Ph.D. thesis at INP of Toulouse, France. All other authors: none to declare.

9. Author contributions

All authors contributed to the project conception and interpretation of data. R.E.H. and Z.A.K. led on manuscript construction and writing, while Y.E.R. and F. M. contributed to critical revision. All data were generated and analyzed by R.E.H., C.L., A.L., S.P., and A.R.

10. Supplementary Data

S1 File. Reference SPIs sequences and matching results of the eight Lebanese *Salmonella* Kentucky isolates

S2 File. Virulence genes and matching results of the eight Lebanese *Salmonella* Kentucky isolates.

The S1 and S2 files are attached at the end of the manuscript

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Chapter IV

Detection of native potential probiotics *Lactobacillus* sp. against *Salmonella* Enteritidis, *Salmonella* Infantis and *Salmonella* Kentucky ST198 of Lebanese chicken origin

Detection of native potential probiotics *Lactobacillus* sp. against *Salmonella* Enteritidis, *Salmonella* Infantis and *Salmonella* Kentucky ST198 of Lebanese chicken origin

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Abstract

Salmonella continues to be a major threat for public health, especially from poultry origin. In recent years, an increasing trend of antimicrobial resistance (AMR) in *Salmonella* sp. was noticed due to the misuse of antibiotics. To overcome this emerging problem, probiotics, particularly within the genus *Lactobacillus*, could be proposed. Due to the benefits of the indigenous microbiota, *Lactobacillus* from poultry origin were isolated from hens and broilers ileum and cecum, and their probiotic potential was further studied. Four *Lactobacillus* species have been identified as: *L. reuteri* (n= 22, 44 %), *L. salivarius* (n=20, 40 %), *L. fermentum* (n= 2, 4 %) and *L. crispatus* (n=1, 2 %) and two *Enterococcus fecalis* (n=2, 4 %). Eight *Lactobacillus*; *L. salivarius* (n=4), *L.reuteri* (n=2), *L.crispatus* (n=1) and *Lactobacillus* sp.(n=1) isolates were chosen on the basis of their cell surface hydrophobicity and auto/co-aggregation ability for further adhesion assays using Caco-2 cells line. Their attachment varied from 0.53 to 10.78 %. *L. salivarius* A30/i26 and 16/c6 and *L.reuteri* 1/c24 showing the highest adhesion capacity were assessed for their ability to compete and exclude *Salmonella* adhesion to the caco-2 cells line. *L. salivarius* 16/c6 exclude greatly the three *Salmonella* serotypes (*S*. Enteritidis, *S*. Infantis and *S*. Kentucky ST 198) from adhesion and that at significant levels. Results of the liquid co-culture assays showed a complete *Salmonella* growth inhibition after 24h. As a result, *L. salivarius* 16 / c6, an indigenous strain isolated from poultry, could constitute a preventive probiotic added directly to the diet as an antimicrobial agent against *Salmonella* sp.

Keywords: *Salmonella* sp., poultry, probiotic, *Lactobacillus salivarius*, inhibition, adhesion

1. Introduction

Non- typhoidal *Salmonella* is the leading cause of foodborne gastroenteritis (EFSA/ ECDC 2017). Poultry products are primarily consumed worldwide and are commonly known to be reservoirs for a variety of microorganisms. *Salmonella* is the most encountered pathogen in poultry products and the most prominent in harboring avian gastrointestinal tracts (GIT) (Tan *et al.,* 2014). In developing countries, high prevalences were recorded, ranging from 13% to 39% in South America, 35% in Africa and 35% to 50% in Asia (Antunes et al., 2016). In Lebanon, according to our recent study, the percentage of contamination of poultry meat at the retail level (supermarket and restaurant) was 22% (unpublished data).

Several control strategies have been adopted to reduce or eliminate this pathogen at the farm level. It is known that the use of Antibiotic Growth Promoters (AGPs) and other prophylactic treatments improve the animal health and productivity rate in livestock farming (Pan and Yu, 2014). However, the massive use of antibiotics as feed additive have led to the emergence and spread of antimicrobial resistant (AMR) pathogens and epidemic multi-drug resistant (MDR) clones or genes in poultry reservoirs (Ferri et al., 2017). Recently, resistance to critical antibiotics, namely fluoroquinolones and Extended-Spectrum B-Lactamases (ESBLs) have spread worldwide and reach humans through the food chain (Franco et al., 2015). As a result and since 2006, AGPs in animal industry have been completely banned in EU (Regulation (EC) No 1831/2003, 2003) and reduced in many countries, including the United States.

Another plan was the implementation of *Salmonella* control programs in poultry farms in many countries, including USA (National Poultry Improvement Plan (NPIP) eradication in eggs (1989) and meats (1994)) and EU (Commission Regulation (EC) No 2160/2003). Targeted *Salmonella* sp. have been successfully reduced, but unfortunately, it cleared the way to more resistant less common serotypes and new clones as *S*. Heidelberg and *S*. Kentucky (Foley et al., 2011).

A promising alternative strategy against pathogens is the use of lactic acid bacteria (LAB) as probiotics. Probiotics are “non-pathogenic live microorganisms when ingested in adequate quantity exert health benefit on their host” (FAO & WHO, 2002). The use of probiotics as broiler growth promoters, direct-fed microbial (Spivey et *al.*, 2014, Saint-Cyr *et al.*, 2017) improve the animal health and might reduce the emergence of AMR (Ouwehand *et al.*, 2016). Lactobacilli

and Bifidobacteria were the most studied probiotic strains against gastrointestinal microbial pathogens (Muñoz-Quezada et al., 2013), especially against *Salmonella* infection in broiler tract (Feng *et al.*, 2016; Rantala and Nurmi, 1973). Two fundamental mechanisms of inhibition of pathogenic organisms were detected either by direct cell competitive exclusion or by production of inhibitory compounds, namely lactic and acetic acid, hydrogen peroxide, bacteriocin or bacteriocin-like inhibitors, fat and amino acid metabolites (Ayeni et *al.,* 2018).

Intestinal adhesion and colonization are the first steps in *Salmonella* infection in poultry. As a consequence, the adhesion property is an essential prerequisite as well as one of the main criteria for selecting potential probiotic strains (FAO & WHO, 2002). The probiotic ability prevents the selected strains from direct elimination by peristalsis and inhibits the colonization of enteric pathogens in chicken by competitive exclusion (Yadav et al., 2017). Means to evaluate adherence capacity of LAB to poultry epithelia may include *in vitro* analysis such as cell aggregation, cell wall hydrophobicity, adhesion to human colorectal adenocarcinoma cells line (Caco-2) and chicken hepatocellular carcinoma cells line (LMH) (Spivey et *al.,* 2014). Since bacterial populations of GIT are particular for different animals, therefore poultry-specific probiotics could be more effective than non-specific microbial agents (Vineetha et al., 2016).

This study aims to develop an effective probiotic derived from broilers and hens' gastrointestinal tract (GIT). In this regard, *in vitro* experiments were achieved to reveal the probiotic activity of native poultry-derived *Lactobacillus* strains against the most relevant and drug-resistant *Salmonella* sp. (*S*. Enteritidis, *S*. Infantis and *S*. Kentucky ST198) in Lebanese poultry farms. Screening of *Lactobacillus* strains for anti-*Salmonella* activity, safety and surface probiotic properties will also be assessed. Finally, potential *Lactobacillus* probiotics will be selected for further *in vitro* characterization such as adhesion and co-culture kinetics. Their adhesion and abilities to exclude, and compete with *Salmonella* serotypes for epithelial tissue using Caco-2 cells line as an experimental model were evaluated as well as their capacity to inhibit the pathogen growth in a co-culture broth.

2. Materials and methods

2.1. Isolation and phenotypic characterization of *Lactobacillus* sp.

Lactobacillus sp. were isolated from the digestive tract (ileum and cecum) of 16 antibiotic-free healthy broilers chosen according to the type of age (Four levels), breed (Four species) and diet (four levels) (Table 11) and ten antibiotic-treated commercial broilers. Samples were coded from **1 to 16 (antibiotic-free broiler) and A (antibiotic-treated commercial broilers) with the origin of** sampling as "i" (ileum) and "c" (cecum).10 g of ileum and cecum content of each broiler were homogenized in 90 ml of Buffered peptone water. The homogenate was diluted to 10^{-7} fold and 0.1 ml were plated onto de Man, Rogosa and Sharpe (MRS agar) (Sigma). Plates were incubated anaerobically for 3 to 4 days at 37°C. In total, 212 strains randomly selected, were first characterized by Gram staining, motility and the detection of catalase activity. Gram-positive, negative catalase bacilli were presumptively considered as *Lactobacillus* for further identification. Isolates were preserved in MRS broth with 20% glycerol at −70°C until use. Later, strains were sub-cultured at least two times before the assays.

2.2. *Salmonella* isolates

Antagonistic activity and co-aggregation ability of *Lactobacillus* strains were tested on three native avian *Salmonella*, isolated from our previous study. *S*. Enteritidis was the most predominant avian pulsotype causing human illness, whereas and in addition to their high prevalences in Lebanese poultry production, *S*. Kentucky ST198 and *S*. infantis were chosen for their multidrug-resistance pattern. Strains were inoculated into 15 ml Tryptic Soy Broth (TSB) (Sigma) and incubated at 37°C for 18 h for further studies.

Table 11: Type of age, breed, and diet of the broilers and hens deprived of antibiotics and additives coded from 1to16 and antibiotic- treated commercial broilers coded as A.

Experiment number	Age	Breed	Diet
1	Broiler, 35 days	Cobb	High starch diet: Corn 60%, Soya 20%, wheat 20%
2	Broiler, 35 days	Cobb	High protein diet: Soya 40 %, corn 40 %, wheat 20%
3	Broiler, 35 days	Cobb	High gluten diet: Wheat 60%, soya 20%, corn 20%
4	Broiler, 35 days	Ross	High starch diet: Corn 60%, Soya 20%, wheat 20%
5	Broiler, 35 days	Ross	High protein diet: Soya 40 %, corn 40 %, wheat 20%
6	Broiler, 35 days	Ross	High gluten diet: Wheat 60%, soya 20%, corn 20%
7	Broiler, 1 day old	Cobb	High starch diet: Corn 60%, Soya 20%, wheat 20%
8	Broiler, 1 day old	Cobb	High protein diet: Soya 40 %, corn 40 %, wheat 20%
9	Broiler, one day old	Cobb	High gluten diet: Wheat 60%, soya 20%, corn 20%
10	Broiler, one day old	Ross	High starch diet: Corn 60%, Soya 20%, wheat 20%
11	Broiler, one day old	Ross	High protein diet: Soya 40 %, corn 40 %, wheat 20%
12	Broiler, one day old	Ross	High gluten diet: Wheat 60%, soya 20%, corn 20%
13	Layer,69 weeks old	Isa Brown	Normal feed: Corn 40%, soya 32%, wheat 20%
14	Layer, 69 weeks old	Isa White	Normal feed: Corn 40%, soya 32%, wheat 20%
15	Layer, 27 weeks old	Isa Brown	Normal feed: Corn 40%, soya 32%, wheat 20%
16	Layer, 27 weeks old	Isa White	Normal feed: Corn 40%, soya 32%, wheat 20%
A	Broiler, 35 weeks old	Ross	Normal feed:Corn 40%, soya 32%, wheat 20%

2.3. **Assessment of *Lactobacillus* antagonism**

The anti-*Salmonella* activity of 212 presumptive *Lactobacillus* was preliminarily screened using the Spot on the lawn and agar well diffusion methods (Schillinger and Lucke, 1989) with minor modifications. Briefly, 10µL of the overnight *Lactobacillus* cultures were spotted onto the surface of MRS agar plates and incubated anaerobically for 18 h at 37˚C. In parallel, an overnight

culture of each chosen *Salmonella* isolates was inoculated at 10^5 CFU/ml in 7ml of TSB soft agar (0.7% agar) and then poured onto previously cultured plates with *Lactobacillus.* After solidification, the plates were additionally incubated for 18h at 37˚C under anaerobic conditions. The inhibition zone around the *Lactobacillus* spot was recorded.

To identify the inhibitory substances secreted in the cell-free culture supernatants, agar well diffusion assay was used. *Lactobacillus* isolates presenting antagonism were grown overnight at 37 °C in 15 ml MRS broth. The cell-free supernatant (CFS) was obtained by centrifugation (4000 ×g, 20 min, 4 °C), filtered with 0.22 µm-pore-size Hi-MED syringe filters and then adjusted to pH 6.5 by 1 N NaOH. *Salmonella* isolates were added at 10^6 CFU/ml to 20 ml TSB supplemented with 0.75% agar-agar (semi-solid) and then poured onto an empty Petri-dish. After complete solidification, 6 mm wells were punched, and 50 µL of the CFS were added to each well. The plates were left to settle at 8˚C for 24 hours to allow the diffusion of the secreted antimicrobial substances, then incubated at 37˚C for 24h. The absence or presence of any inhibitory zone was recorded after 24 h of incubation at 37 °C. The two assays were done in triplicate.

2.4. **Selection of strains depending on their phenotypic aggregation**

Lactobacilli strains (n=50) were chosen according to their high anti-*Salmonella* activity in spot-on- the- lawn test. A preliminary visual aggregation screening was done according to Del Re *et al.* (2000) with minor modifications. Briefly, all lactobacilli were grown in MRS broth at 37 °C under anaerobic conditions for 18 hours. Three categories were identified: 1) Strains with aggregation phenotype (Agg+) with visible aggregates even after vigorous vortex, 2) Strains with constant turbidity without precipitate (Agg-) and 3) Strains with mixed phenotype forming a precipitate and a clear or little turbid supernatant (Agg+/Agg-)

2.5. **Species Identification and phylogenetic relations**

The 50 selected isolates were identified by API50CHL (Biomérieux) and 16S rRNA gene sequence analysis. DNA extraction was achieved with a Qiamp DNA mini Kit. Amplification of 16S rRNA gene was performed in Veriti device (Applied Biosystem, USA) and included: denaturation at 95 °C for 15 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 2 min followed by another extension at 72 °C for 7 min. Reaction mixtures (50 µl) were prepared as follows: reaction buffer 10x (5 µl), 10mM dNTPs

mix (1 µ1), 0.5 mM of primer (27F (5′-GTGCTGCAGAGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CACGGATCCTACGGGTACCTTGTTACGACTT-3′), bacterial DNA (5 µl), and 2.5 U of HotStarTaq DNA polymerase (Qiagen, Germany). Amplicons separation was completed by electrophoresis at 100 V on 1% (w/v) agarose, stained with Ethidium Bromide in 1 × TBE buffer and purified by using GenElute ^{TM} PCR Clean-Up Kit (Sigma-Aldrich) according to the manufacturer's instructions. DNA sequencing was carried out on SeqStudio Genetic Analyzer (Applied Biosystem, USA). Editing was performed with Bioedit (version 7.2.5, 2013) and 16S rDNA sequences were compared with other sequences using NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree was assembled by using the neighboring methods (Saitou and Nei, 1987) with the tree builder function of MEGA7 (Kumar et al., 2016). *L.reuteri* KX688655.1, *L. salivarius* MG737855.1, *L. fermentum* KC113207.1, *L. cripatus* MH392998.1, and *Enterococcus fecalis* MK584170.1 were selected as reference sequences.

2.6. **Antibiotic susceptibility testing**

Antibiotic resistance of the different *Lactobacillus* isolates was assessed by broth microdilution procedure following the Clinical and Laboratory Standards Institute (CLSI, 2012) in Mueller Hinton broth supplemented with 10% MRS broth. The following antimicrobials (Oxoid, England) were used: ampicillin (Amp), gentamicin (Cn), kanamycin (K), streptomycin (S), erythromycin (Ery) and chloramphenicol (C). The cut-off values for the assessment of Lactobacillus sp. as feed additives were determined according to the European Food Safety Authority guidance (EfSA, 2012)

2.7. **Cell surface properties**

2.7.1. Auto-aggregation and co-aggregation Assay

Auto-aggregation and co-aggregation capacities of the selected lactobacilli strains, chosen according to their auto-aggregation visual features, were further assessed (Collado et al., 2007) with minor modifications. Overnight *Lactobacillus* culture (10^8 CFU/ml) was centrifuged (4000 ×g, 20 min, 4 °C). The pellet was washed with phosphate-buffered saline (PBS) pH 7.1 and re-suspended in the same buffer. Then, the cell culture (4 ml) was placed in glass bijoux bottles and

incubated at room temperature for 24 h. The absorbance values (OD600) were measured at different times (t_0, t_4, and t_{24}).

The auto- aggregation percentage was calculated using the formula: $1 - (A_t/A_0) \times 100$ where A_t represents the absorbance at different times (4 and 24 h) and A_0 absorbance at time =0 (t_0). The aggregation ability was classified (Del Re et al., 2000) with minor modification: Isolates with aggregation values ≥ 65 % were classified as highly auto-aggregative, and ≤10 % were classified as non-auto- aggregative.

For the co-aggregation assay, mixed cultures of equal volumes (2ml) of each *Lactobacillus* and each of the three *Salmonella* strains, as well as monocultures (4ml), were prepared and incubated at room temperature without agitation. Absorbance values (OD600) were measured at 24h.

The percentage of co-aggregation was calculated as follow (Handley et al., 1987): $(1- A_{mix}/ (A_{Sal}+ A_{Lac})/2) \times 100$, where A_{Sal} and A_{Lac} represent the absorbance of monocultures, *Salmonella* and *Lactobacillus* respectively, and A_{mix} represents the absorbance of the mixed culture at 24h. Values below 20% are indicative of weak co-aggregation capability (Solieri et al., 2014)

2.7.2. **Hydrophobicity assay**

The microbial adhesion to hydrocarbons (MATH) test was evaluated as defined by Rosenberg et *al.*, (1980) with slight changes. *Lactobacillus* cultures were centrifuged, the pellet was washed with PBS buffer pH= 7.1 and re-suspended in the same buffer to adjust the concentration at10^8 CFU/ml. An equal volume of 2 ml of cell culture and xylene (apolar solvent) were mixed and vigorously vortexed for 5 min before measuring the absorbance at 600 nm (A_0). After incubation at Room Temperature for 1 h, the aqueous phase was cautiously removed, and its absorbance at 600nm (A_1) was measured.

The cell surface hydrophobicity (H) was calculated as follows: $H\ \% = (1- A_1/A_0) \times 100$. Isolates with (H) values greater than 70% were classified as highly hydrophobic, between 50–70% were classified as moderate, and Hydrophobicity lower than 50% were classified as low hydrophobicity (Buahom *et al.,* 2018).

2.8. **Tolerance to simulated gastrointestinal conditions**

The gastrointestinal tolerance of the eight *Lactobacillus* strains, chosen according to their hydrophobicity and auto/ co-aggregation capacity, was assessed (Babot et al., 2014) with minor modifications. Overnight *Lactobacillus* cultures in MRS broth were centrifuged (4000 x g, 4ºC, 20 min) and adjusted to approximately 10^8 CFU/ml in PBS buffer. A volume of 1.75 ml was inoculated in 2.25 ml of a simulated gastric juice (125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$, 3 g/l pepsine pH 2.0); After incubation at 41.5º C (poultry corporal temperature) for 1h (mean retention time in proventriculus and gizzard), the suspension was centrifuged and washed twice with PBS buffer. The pellet was then re-suspended in 3 ml of simulated intestinal juice (NaCl 22 mM, KCl 3.2 mM, $NaHCO_3$ 7.6 mM, pancreatin 0.1% w/v, bile salts 0.15% or 0.3% w/v , pH = 8.00) and incubated at 41.5 °C during 2 h (mean retention time in the small intestine). The concentrations of bile salts were selected to simulate 0.1 to 1% bile concentration range of the poultry gastrointestinal tract (GIT), with approximately 0.25% in the ileum and 0.1 % in the cecum (Spivey et al., 2014). After serial dilutions, 0.1 ml of the suspensions were plated onto MRS agar and incubated anaerobically for three days at 37ºC.

The ability of isolates to tolerate the GIT conditions was as follows: % survival = ($\log_{10} N_1/\log_{10} N_0$) × 100 Where $\log_{10} N_0$ is the number of bacterial cells in PBS, and $\log_{10} N_1$ is viable cells after the simile-gastrointestinal assay.

2.9. **Cell Culture**

2.9.1. Preparation of cell culture

The human colorectal adenocarcinoma Caco-2 cell line was used to perform adhesion assays. Cells were grown in a 75 cm^2 flask containing Dulbecco's Modified Eagle's Medium (DMEM) (1x DMEM, 1M-1Glutamax, Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Eurobio), 1x Non Essential Amino Acids (NEAA), 100 U/ml penicillin and 10 mg/ml streptomycin (Sigma-Aldrich). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 until 80% confluence. Prior to the adhesion assay, 5×10^4 cells were seeded in 24-well tissue culture plates and incubated at the same conditions as above for 16 days (fully

differentiation). At the end of the incubation time, cell lines monolayers were washed twice with Dubelcco's PBS to remove antibiotics before adding bacterial suspension.

2.9.2. Adhesion to Caco-2 cells

Overnight cultures of the *Lactobacillus* strains (16/c6, 16/c2, 16/i10, 16/c4, 14/i8, 12/c8, 1/c24, A30/i26) and *Salmonella* serotypes were centrifuged, washed twice in Dubelcco's PBS (Eurobio) and re-suspended in an antibiotic-free DMEM medium at a concentration of 10^8 CFU/ml. Then, 1 ml of bacterial culture was added to each cell well and incubated during 1 h at 37 °C in a humidified atmosphere containing 5 % CO_2. After incubation period, supernatant were removed and cell well were gently washed three times with Dulbecco's PBS buffer to eliminate non-adherent bacteria. Finally, Caco-2 cell line monolayers were trypsinized with 0.25 % trypsin-EDTA solution (Eurobio) and adherent bacteria were enumerated by plating serial dilutions onto MRS agar medium for *Lactobacillus* and TSA agar medium for *Salmonella.*

Adhesion ability was calculated as (N_1/N_0) x 100 where N_1 and N_0 represent the total bacteria adhered (CFU) and total bacteria added (CFU) respectively. Two independent experiments were conducted with triplicate for each condition.

2.9.3. Inhibition of *Salmonella* adhesion to Caco-2 cell

Two different protocols were followed to evaluate the ability of the selected *Lactobacilli* strains to inhibit *Salmonella* adhesion to Caco-2 cells. *L. salivarius* (16/c6 and A30/i26) and *L. reuteri* (1/c24) strains were chosen according to their adhesion properties.

The competition adhesion assay was performed by seeding Caco-2 cells monolayers with a mix culture of each of the selected *Lactobacillus* (10^8 CFU/ml) with each of *Salmonella* strain (10^7 CFU/ml) in complete DMEM. *Salmonella* monocultures were used as controls. After an incubation period of 2 h at 37°C in a humidified atmosphere containing 5% CO_2, supernatant with the non-adherent bacteria were removed, and then Caco-2 cells were trypsinized. The adherent bacterial cells were serially diluted and plated on TSA agar medium and MRS agar medium to enumerate *Salmonella* and *Lactobacillus* respectively.

The ability of *Salmonella* strains to adhere to Caco-2 cells in the absence (N_{Sal}) and the presence (N_{Mix}) of the *Lactobacillus* was calculated as follows:

Anti-adhesion ability % = 1− (N_{Mix}/N_{Sal}) % (Son et al., 2017)

For exclusion assays, Caco-2 cells monolayers were pre-exposed to *Lactobacillus* strain (10^8 CFU/ml) for 1 h (Singh et al., 2017). Then, Caco-2 cells monolayers were gently washed three times with Dulbecco's PBS and *Salmonella* strains (10^7 CFU/ml) were added and incubated for two hours. At the end of incubation time, supernatant with the non-adherent bacteria were removed, and then Caco-2 cells were trypsinized. The adherent bacterial cells were serially diluted and plated on TSA agar medium and MRS agar medium to enumerate *Salmonella* and *Lactobacillus* respectively. Two independent experiments for each strain were conducted with triplicate for each condition.

2.10. **Co-culture Kinetic study**

Two series of experiments were carried out to evaluate the effect of *L. salivarius* 16 / c6 on the growth of *Salmonella* strains under co-culture conditions.

In the first co-culture experiment, the 18 h old *Lactobacillus* strain (10^7 CFU/ml) and each culture of the three strains of *Salmonella* (approximately 10^5 CFU/ml) were co-inoculated into 100ml Laptg medium (Peptone 15g/L-tryptone 10g/L- yeast extract 10g/L-glucose 10g/L- tween 80 0.1%) (All media were purchased from Sigma-Aldrich) at pH 6.9 and incubated in a shaker – incubator at 100rpm, at 37°C for 24 h. Pure cultures of each of the strains serve as controls. Before enumeration, the culture was left for 10 min without shaking to evaluate the auto / co-aggregation capacity of *L. salivarius*. Then 0.1ml were taken from the supernatant and plated out at different time (0h, 4h, 8t h, and 24 h) on selective media ((XLD agar for *Salmonella* and MRS agar for *Lactobacillus*) for counting. The pH of the culture medium was also measured. Three independent replicates were performed for each assay.

In the second experiment, the bacterial cultures were prepared as described above. Before enumeration, the culture was vigorously vortexed; then 0.1ml were taken and plated out at different time (0h, 4h, 8t h, and 24 h) on selective media ((XLD agar for *Salmonella* and MRS agar for *Lactobacillus*) for counting .

2.11. **Statistical Analysis**

The results for hydrophobicity, auto-aggregation and *Salmonella* inhibition by competition/ exclusion as well as liquid co-culture assay are given as the mean ± Standard Deviation (SD) of three independent experiments. The results for adhesion are expressed as the mean ± SD of two experiments each done in triplicate. Statistical analysis were performed using XLSTAT 2014 software. Lactobacillus surface properties (n=3) for fifty strains were assessed by the Principal Component Analysis (PCA). The index of Pearson was used to evaluate the correlation between the six assays, hydrophobicity, auto-aggregation and co-aggregation between the *Lactobacillus* strains and *S*. Enteritidis, *S*. Infantis and *S*. Kentucky. Differences among the results of adhesion and inhibition by competitive/ exclusion was performed by one-way ANOVA. P-values ≤ 0.05 were considered statistically significant.

3. Results

3.1. **Screening of *Lactobacillus* sp. from poultry origin and anti-*Salmonella* activity**

A total of 212 isolates which showed to be bacillus, gram-positive with no catalase activity were collected from broiler ceca and ileum samples. A number of 157 *Lactobacillus* sp. were isolated and identified from chickens that were subjected to 16 different trails where the chickens weren't treated with antibiotics. In addition, 55 *Lactobacillus* isolates were selected from chickens that were previously treated with antibiotics. The strains were preliminarily tested for inhibitory activity against *S.* Enteritidis, *S.* Kentucky ST198 and *S.* Infantis by agar spot test and well diffusion. All *Lactobacillus* isolates were found to produce inhibition zones against the three strains of *Salmonella* based on the agar spot test (spot-on-the-lawn). The radii of their inhibition zones ranged from 1.2 to 4.4 cm (data not shown). However, the CFSs of all *Lactobacillus* isolates, neutralized to pH 6.8 did not display any antimicrobial effect against *Salmonella* strains.

3.2. **Visual aggregation screening**

Fifty *Lactobacillus* isolates were chosen for visual screening. Three auto-aggregation phenotypes were well-defined as follows (Annex I): Category 1- (Agg+) strains (n=7, 14 %) aggregated rapidly displaying a clear supernatant and visible aggregates even after vigorous vortex. Group 2- Non-auto-aggregating (Agg-) strains (n=7, 14 %) showed a turbid supernatant. Group 3-

Mixed (Agg+/Agg-) strains (n= 36, 72 %) revealed both a precipitate and turbidity/or clear supernatant.

3.3. **Phenotypic and genotypic identification of *Lactobacillus* isolates with Phylogenetic relatedness.**

The biochemical results of the fifty *Lactobacillus* strains by API 50CHL are shown in Annex I. Eight *Lactobacillus* species were identified as follow: *L. fermentum* (n = 22, 44 %), *L. salivarius* (n = 13, 26 %), *Leuconostoc lactis* (n = 9, 18 %), *L. brevis* (n = 2, 4 %), *L. acidophilus* (n = 1, 2 %), *Lactococcus raffinolactis ou L. crispatus* (n = 1, 2 %), *L. plantarum* (n = 1, 2 %), and *L. delbrueckii* sp *delbrueckii* ((n = 1, 2 %). According to the Api 50CHL, *L. fermentum* and *L. salivarius* were the most common species among the isolates. The 16S rRNA gene sequence results showed four *Lactobacillus* species, *L. reuteri* (n= 22, 44 %), *L. salivarius* (n=20, 40 %), *L. fermentum* (n= 2, 4 %) and *L. crispatus* (n=1, 2 %) and two *Enterococcus faecalis* (n=2, 4%) (Figure 12). The three remaining *Lactobacillus* isolates (16/i10, 14/i15, A30/c2) were non-typable. The most common species were *L.reuteri* and *L.salivarius*. The phylogenetic tree demonstrated a close relatedness among the same species.

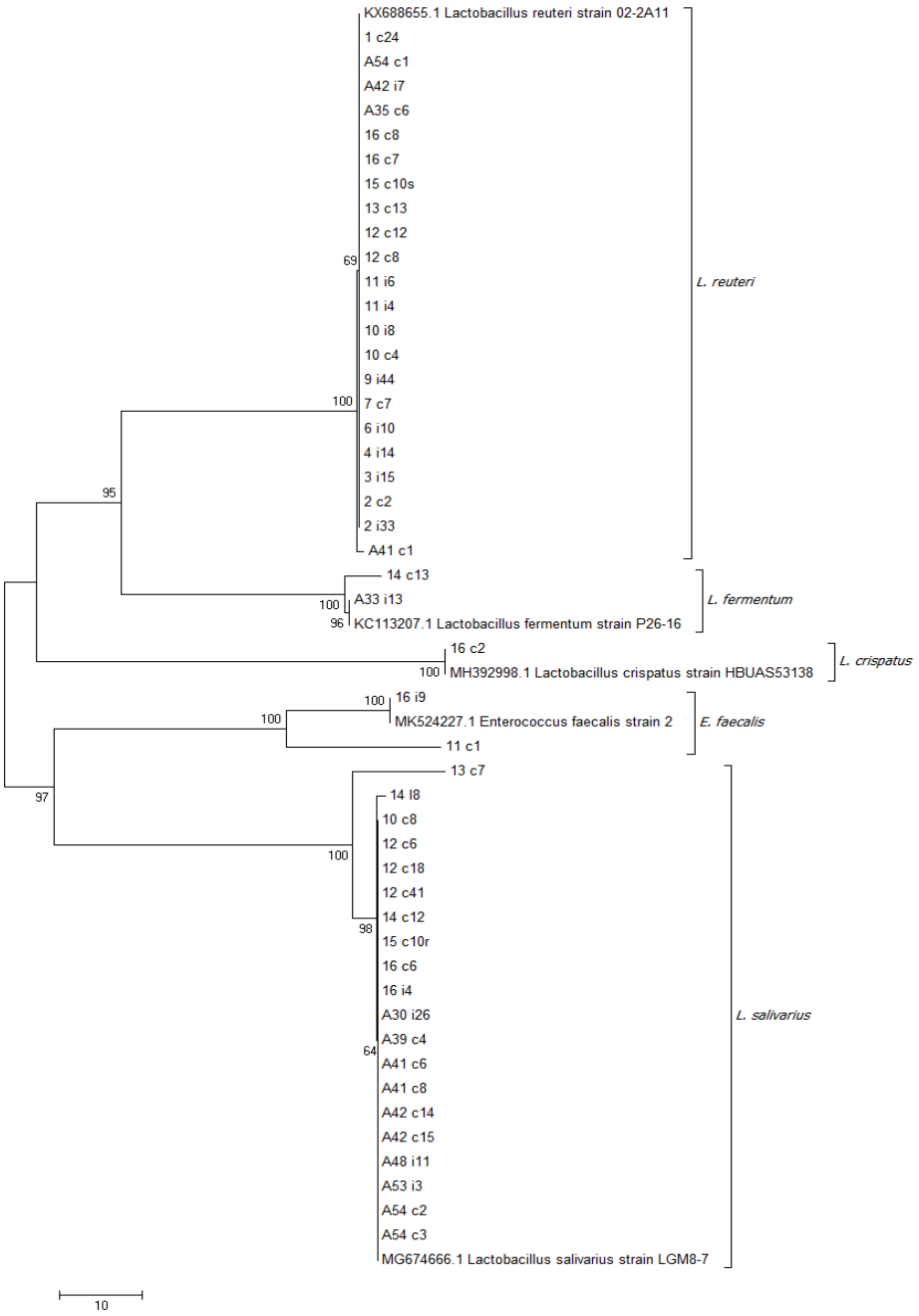

Figure 12: Evolutionary relationships Tree of *Lactobacillus* sp by the Neighbor-Joining method. The percentage of replicate trees in which the associated species clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). *L.reuteri* KX688655.1, *L. salivarius* MG737855.1, *L. fermentum* KC113207.1, *L. cripatus* MH392998.1, and *Enterococcus fecalis* MK584170.1 were selected as reference sequences.

3.4. **Antimicrobial resistance**

The resistance of lactobacilli isolates to the six antibiotics tested by the micro-dilution procedure for the determination of MIC was determined (Figure 13). A very high AMR was observed among the isolates independently of the farms (antibiotics free or with antibiotic) with a total resistance against ampicillin (100 %), high resistance to chloramphenicol (96 %), kanamycin (88 %), streptomycin (76 %) and gentamicin (64 %). Resistance to erythromycin was shown to be higher in antibiotic-treated farms than in antibiotic-free farms with a percentage of resistance of 75% and 20% respectively. None of the strains was pan-susceptible.

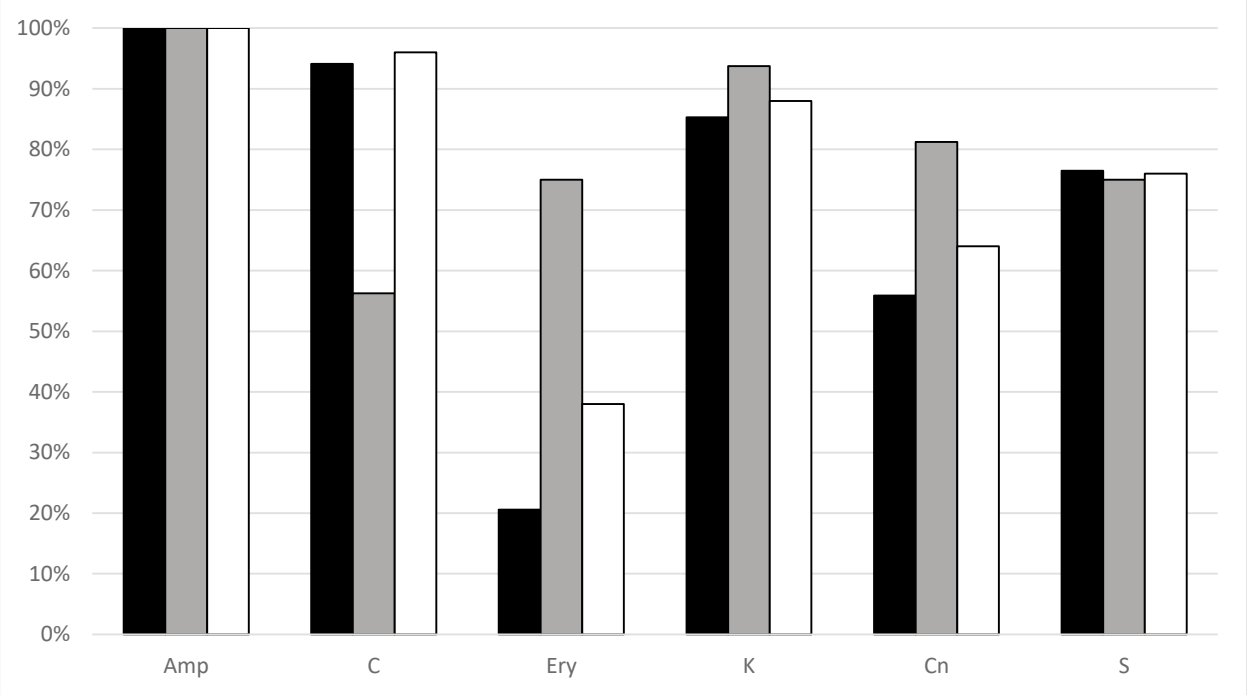

Figure 13: Antimicrobial resistance of the indigenous *Lactobacillus* sp isolated from antibiotic-free (Black columns) and antibiotic-treated broilers (Grey columns). White columns correspond to the total percentage of resistance. Ampicillin (Amp), chloramphenicol (C), erythromycin (Ery), kanamycin (K), gentamycin (Cn) and streptomycin (S).

3.5. **Surface properties assays**

The most fifty anti-*Salmonella* strains (according to spot-on-the-lawn test) were selected and tested for their surface properties. A visual screening primarily evaluated the auto-aggregation ability of fifty *Lactobacillus* strains after 18 h of incubation, followed by spectrophotometric analysis at 4h and 24h (Figure 14). The visual screening was confirmed by auto-aggregation assay at 4h. Category 1 (Agg+) demonstrated high auto-aggregation percentage (≥ 65 %) whereas the category 2 (Agg-) showed a deficient percentage (≤10 %). All *Lactobacillus* belonging to category 3 (Agg+/Agg-) exhibited a range between 10 and 65 % of auto-aggregation except three strains. One of them revealed high auto-aggregation ability (>65 %) and two others were non-aggregative (≤10 %). Almost all *Lactobacillus* (n= 45, 90 %) possessed this feature at 24h.

The co-aggregation properties of *Lactobacillus* strains with the three *Salmonella* serotypes tested differed considerably among the strains ranging from 0 % to 94.6 % (Annex: I). A percentage of 54 %, 60 % and 64 % of *Lactobacillus* strains co-aggregated (percentage of co-aggregation >20 %) with *S*. Kentucky ST198, *S.* Enteritidis and *S*. Infantis respectively.

The hydrophobic property of the *Lactobacillus* strains was assessed by xylene extraction (Annex: I, Figure 14). The results revealed that 62% of the isolates showed high affinity for xylene (H>70 %) and 34 % were non-hydrophobic (H< 50 %).

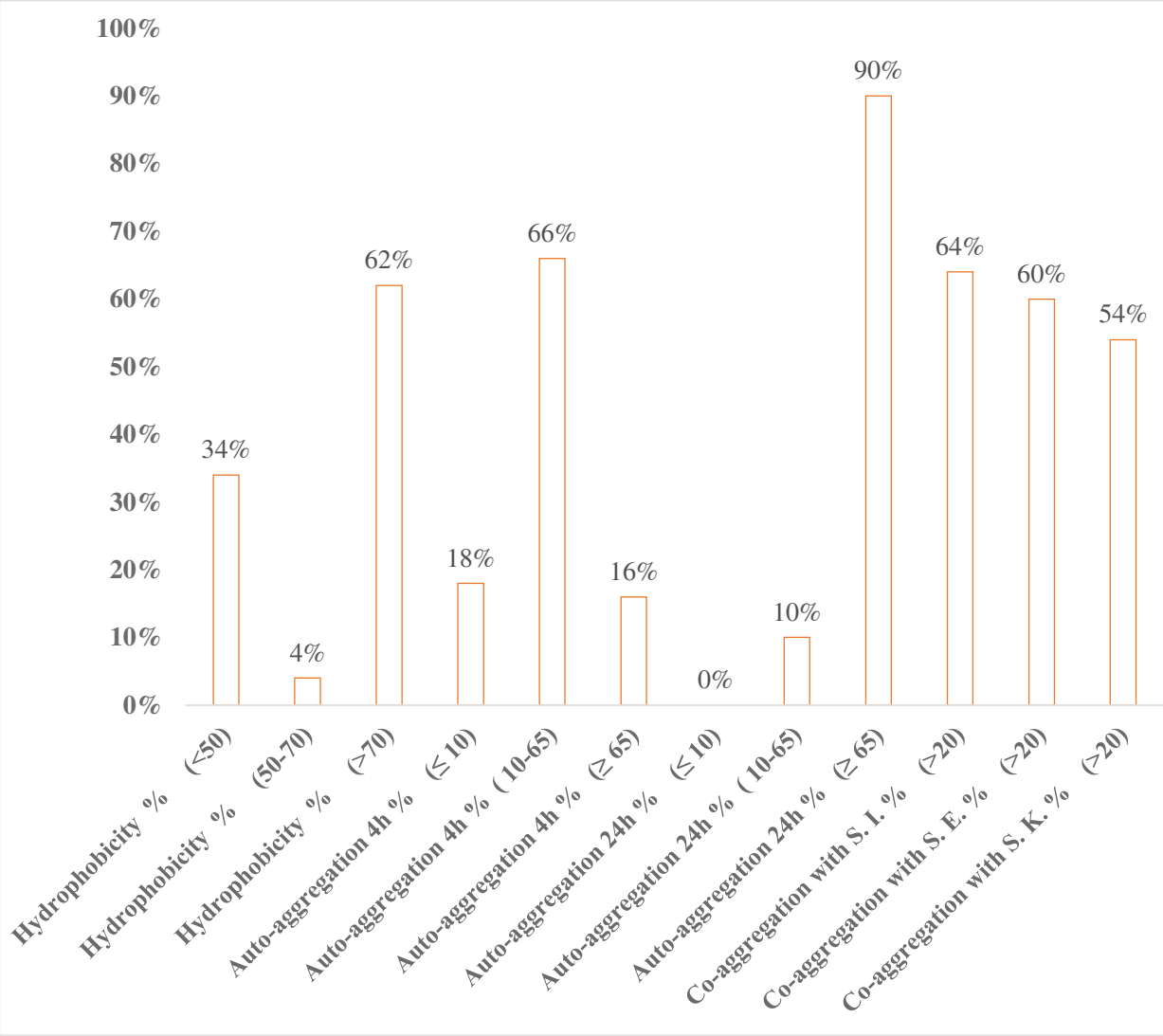

Figure 14: Isolates distribution in defined ranges of percentage of hydrophobicity, auto-aggregation and co-aggregation with the three *Salmonella* sp (*S.* Enteritidis (S.E.), *S.* Kentucky ST198 (S.K.) and *S.* Infantis (S.I.))

3.6. **Hydrophobicity and auto/co-aggregation correlation**

The results obtained from the surface *Lactobacillus* assays were subjected to Principal Component Analysis (PCA) (Figure 15). The first PC1 and the second PC2 principal components explain 48 % and 27.5 % of the total variance respectively. *L. salivarius* A30/i26 showed to be highly hydrophobic (98.84 % ± 1.34), possess an aggregation phenotype (Agg+) and ability to aggregate rapidly at 4h (76.15 % ± 3.93). According to the PCA analysis, the most co-aggregative strains were *L. crispatus* 16/c2, *L. salivarius*16/c4, 16/c6 and 14/i8, and *L. reuteri* 12/c8. In addition to these properties, *L. salivarius* 16/c6 showed to be non- auto-aggregative at 4h but revealed this feature at 24 h (9.89 % ± 3.63 and 95.91 % ± 2.58 respectively). *L. salivarius* 16/c4 possess an aggregation phenotype (Agg^{+}) and rapidly auto-aggregate at 4h (76.23 % ± 3.38). *Lactobacillus sp.*16/i10 and *L. reuteri* 1/c24 were highly hydrophobic (98.36 % ± 3.63 and 91.81 % ± 7.78 respectively) but showed no and moderate auto-aggregation capacity respectively at 4h of assay (6.16 % ± 5.53 and 13.76 % ± 1.87 respectively) (Table 12).

No significant correlation between hydrophobicity, auto-aggregation, and co-aggregation has been detected among the fifty tested strains (Table 13). On the contrary, the co-aggregation results between the three *Salmonella* serotypes and *Lactobacillus* isolates were significantly correlated, since the correlation coefficient value could reach 0.890.

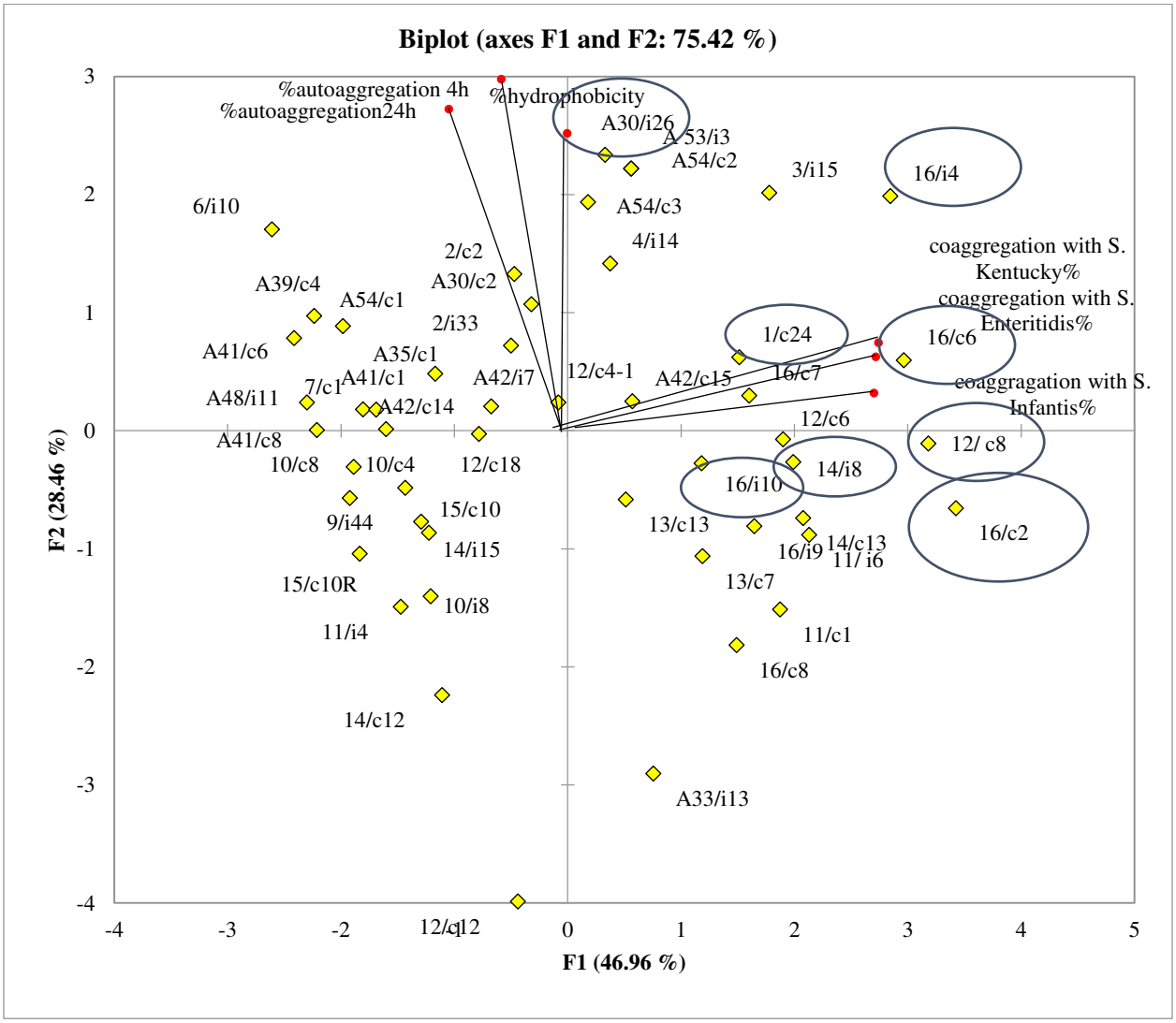

Figure 15: Principal Component Analysis (PCA) of surface proprieties as hydrophobicity and auto/co-aggregation) for the 50 *Lactobacillus* isolates. Isolates underscored were the selected strains

Table 12: Identity, surface properties and antimicrobial resistance pattern of the eight selected *Lactobacillus* sp

Isolates	Visual aggregation	Auto-aggregation 4h (%)	Auto-aggregation 24h (%)	% Co-aggregation with			Hydrophobicity (%)	Antimicrobial resitance pattern
				S. Enteritidis	*S.* Infantis	*S.* Kentucky		
L.crispatus 16/c2	Agg+/Agg-	14.46 ± 2.78	58.67 ± 7.62	89.36	75.06	69.66	84.58 ± 1.92	Amp
L. salivarius 16/c6	Agg-	9.89 ± 3.63	95.91 ± 2.58	71.07	69.55	94.55	90.26 ± 3.91	Amp-C-K-S
L. salivarius 16/i4	Agg+	76.23 ± 3.38	92.95 ± 10.5	82.49	80.45	79.94	82.25 ± 5.84	Amp-C-K-Cn-S
Lactobacillus sp.16/i10	Agg+/Agg-	6.16 ± 5.53	79.46 ± 1.18	45.60	34.32	63.51	98.36 ± 0.75	Amp
L. salivarius 14/i8	Agg+/Agg-	23.14 ± 5.29	73.47 ± 3.67	62.30	70.35	47.54	81.63 ± 1.2	Amp
L.reuteri 12/c8	Agg+/Agg-	33.93 ± 6.44	71.86 ± 1.89	83.47	73.87	80.00	52.66 ± 2.98	Amp-C-Ery-K-Cn-S
L.reuteri 1/c24	Agg+/Agg-	13.76 ± 1.87	91.81 ± 7.78	50.43	62.47	58.93	97.53 ± 0.96	Amp-C-K-S
L.salivarius A30/i26	Agg+	76.15 ± 3.93	99.63 ± 0.26	49.54	25.71	60.00	98.84 ± 1.34	Amp-K-Cn-S

Values of auto-aggregation and hydrophobicity are means of triplicate assays with their standard deviations

Table 13: Correlation of Pearson coefficients between hydrophobicity, auto-aggregation, and co-aggregation of the 50 Lactobacillus isolates. The Principal Component Analysis (PCA) was done using. The index of Pearson was used to evaluate the correlation between the six assays, hydrophobicity, auto-aggregation and co-aggregation between the Lactobacillus strains and *S.* Enteritidis, *S.* Infantis and *S.* Kentucky.

Variables	Hydrophobicity (%)	Auto-aggregation 4h (%)	Auto-aggregation 24h (%)	Co-aggregation with S. Infantis (%)	Co-aggregation with S. Enteritidis (%)	Co-aggregation with S. Kentucky (%)
Hydrophobicity (%)	1					
Autoaggregation 4h (%)	0.302	1				
Autoaggregation24h (%)	0.277	0.525	1			
Co-aggregation with S. Infantis (%)	-0.033	-0.125	-0.180	1		
Co-aggregation with S. Enteritidis(%)	0.098	-0.015	-0.187	0.873	1	
Co-aggregation with S. Kentucky (%)	0.104	-0.051	-0.219	0.831	0.890	1

3.7. **Gastrointestinal tolerance assay**

The eight chosen *Lactobacillus* sp. were further evaluated for their capacity to survive in the simulated GIT of chicken (Figure 16). All strains were able to tolerate the acidity and 0.1 % (w/v) bile salts. However, at 0.3 % bile salts, two strains of *L. salivarius* 16/i4 and A33/i26 count declined considerably from 8 Log10 CFU /mL in control to 0 and 3 Log10 CFU /mL respectively leading to a low percentage of survival of 0 % and 37 % respectively.

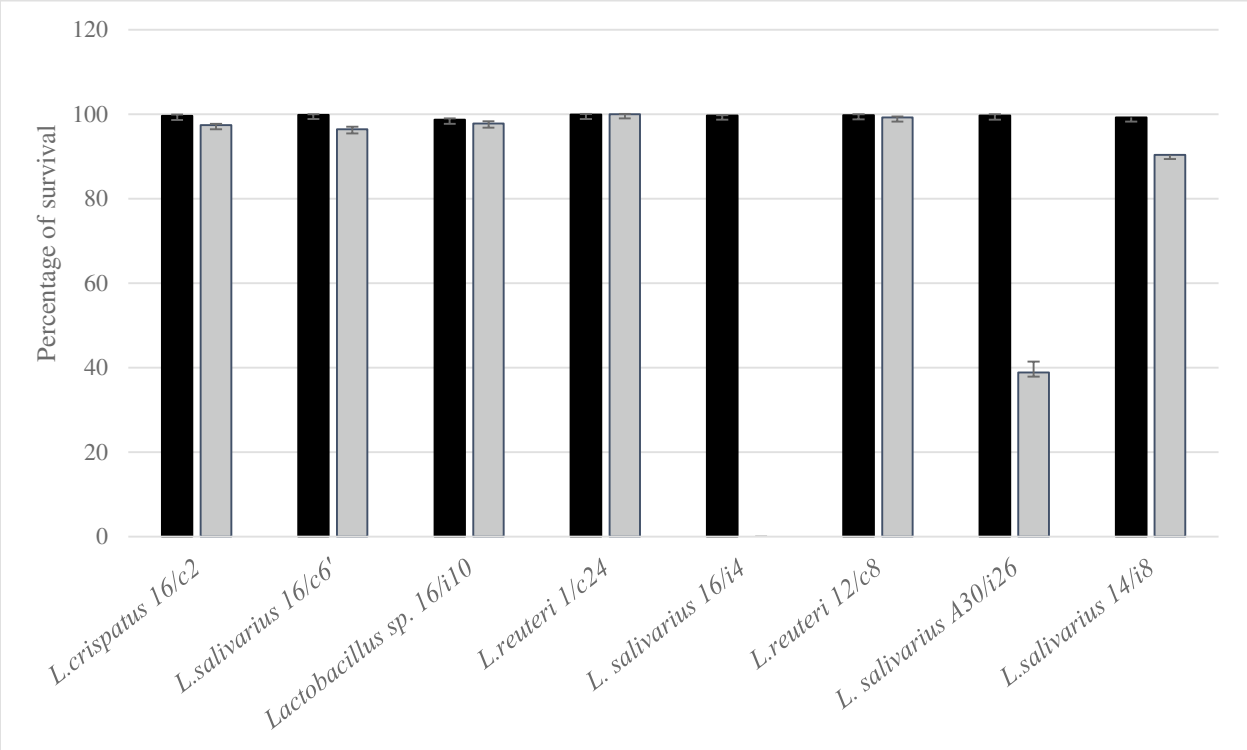

Figure 16: Effect of the simile-gastrointestinal conditions on *Lactobacillus* viability. Black and grey columns correspond to lactobacilli subjected to 0.15 % or 0.3 % bile salts respectively.

3.8. **Adhesion Assay**

The ability of the selected *Lactobacillus* and the three *Salmonella* strains to adhere to Caco-2 cell line was also studied (Figure17). Attachment of *Lactobacillus* isolates varied from 0.53 to 10.78 %. *L. salivarius* A30/i26, 16/c6 and 16/i4, *L. reuteri* 1/c24 were the highest adhesive strains with an adhesion ability of 10.78 % ± 4.2, 6.5 % ± 1.82, 5 % ± 0.99 and 6.43 % ± 2.26 respectively with no significant differences. The remaining strains *Lactobacillus* sp 16/i10, *L. salivarius* 14/i8,

L. reuteri 12/c8 and *L. crispatus* 16/c2 showed no significant differences with a low adhesion capacity of 3.61 % ± 1.14, 2.35 % ± 0.86, 1.99 % ± 0.66 and 0.53 % ± 0.21 respectively.

S. Infantis, *S*. Enteritidis and *S.* Kentucky ST198 attached to Caco-2 cells at a percentage of 8.81 % ± 0.87, 7.81 % ± 1.41 and 6.77 % ± 0.89 respectively. No significant difference was found between serotypes (Figure 17).

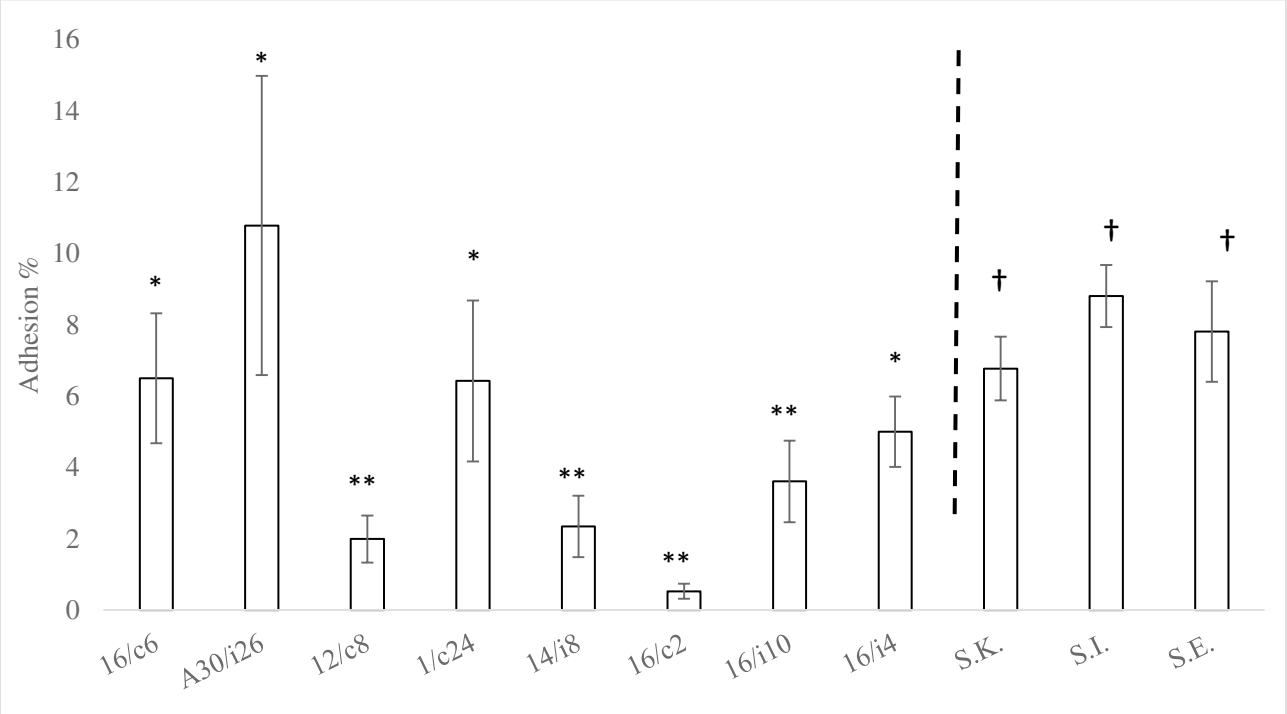

Figure 17: Adhesion of the eight native poultry-derived *Lactobacillus* strains and the three *Salmonella* strains (*S*. Kentucky ST 198 (*S*.K.), *S*. Infantis (*S*.I.) and *S*. Enteritidis (*S*.E.)) to caco-2 cells line. The means and standard deviations of two independent experiments are shown, each with three replicates. The differences between strains adhesion were evaluated separately for *Lactobacillus* strains and *Salmonella* serotypes. *L. salivarius* 16/c6, 16/i4 and A30/i26, and *L. reuteri* 1/c24 revealed no significant differences (*) in their adhesion capacity which is dissimilar from the four remaining tested strains (**). The differences in the adhesion of *S*. Enteritidis, *S*. Infantis and *S*. Kentucky ST198 were also not significant among the three serotypes (†).

3.9. **Competition/ Exclusion Assay**

Three *Lactobacillus* strains showing the most adhesion capacity, *L. salivarius* A30/i26 and 16/c6 and *L.reuteri* 1/c24 were assessed for their ability to compete with the pathogen for the adhesion site on the Caco-2 cell line (Figure 18). The three *Lactobacillus* isolates displayed no significant effect on the adhesion of the pathogens to Caco-2 cells.

In exclusion assay, the adhesion site occupied by the probiotic bacteria becomes inaccessible to the pathogen. *L. salivarius* 16/c6 can highly exclude the pathogens than *L.salivarius* A30/i26 and *L. reuteri* 1/c24. The anti-adhesion percentages of *S*. Enteritidis, *S*. Infantis and *S*. Kentucky ST198 to Caco-2 cells were 70.30 % ± 6.22, 86.57 % ± 9.22, and 79.54 % ± 9.26 respectively with no significant difference between them.

L.salivarius A30/i26 and *L. reuteri* 1/c24 indicated low exclusion of the three serotypes from adhesion to caco-2 cells with *S*. Kentucky being significantly the least inhibited.

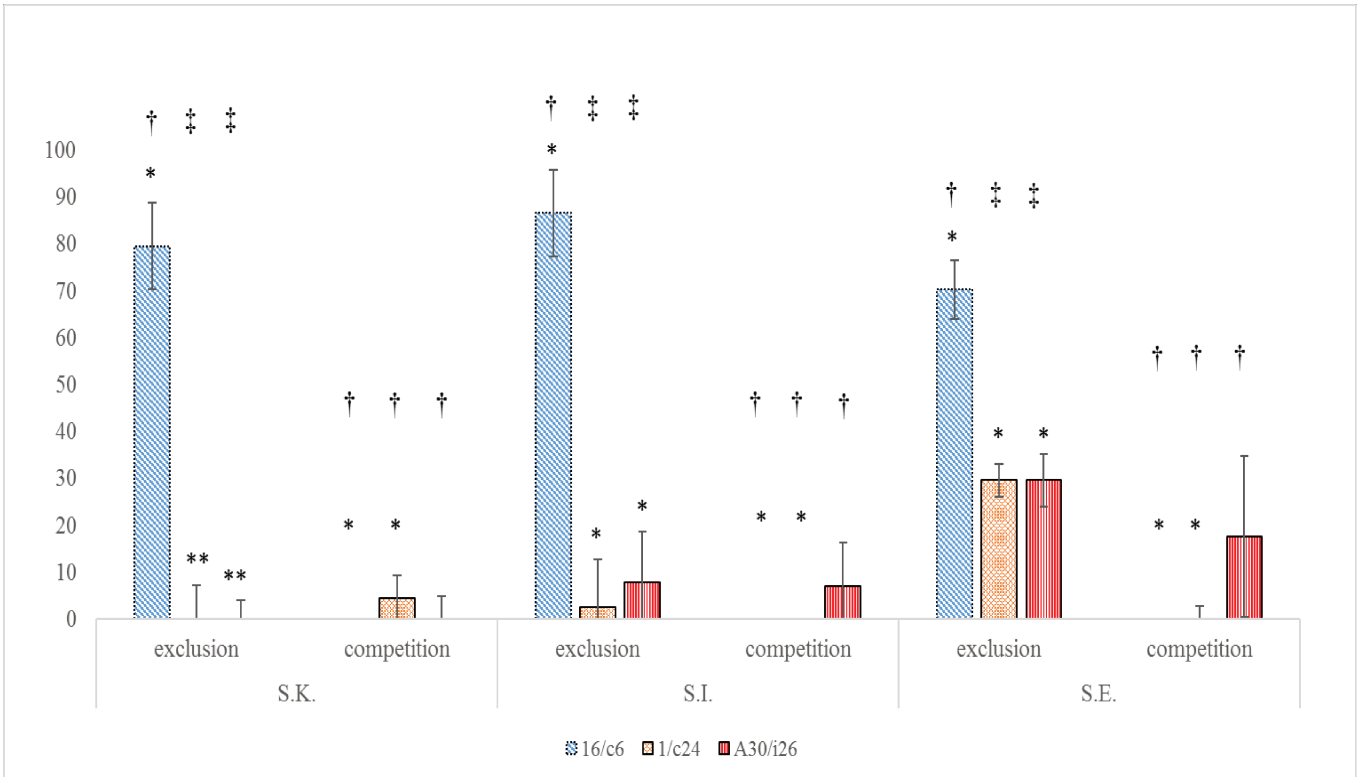

Figure 18: Inhibition of *S.* Kentucky ST 198 (S.K.), *S.* Infantis (S.I.) and *S.* Enteritidis (S.E.) adherence to Caco-2 cells by *L.* salivarius 16/c6 and A30/i26 and *L.* reuteri 1/c24 in competition and exclusion assays. The means and standard deviations of three independent experiments are shown, each with three replicates. (*) *Lactobacillus* strains were fixed and the differences of inhibition were calculated between the three serotypes in the same assay; (*) $p > 0.05$, (**) $p \leq 0.05$. (†) *Salmonella* serotypes were fixed, and the differences of inhibition were calculated between the three *Lactobacillus* strains in the same assay. (†) $p > 0.05$, (††) $p \leq 0.05$

3.10. **Co-culture kinetics**

L. salivarius 16/c6, which was able to inhibit *Salmonella* adhesion to Caco-2 cell line by exclusion assay, was further studied to evaluate its inhibition capacity of *Salmonella* serotypes by liquid co-culture assay (Figure 19, 20). The pure cultures of the *Lactobacillus* (16/c6) and the three *Salmonella* serotypes (*S*.E., *S*.K., and *S*.I.) showed that both strains grew very well in Laptg medium.

In the two experiments, with and without agitation, differences in CFU between the control cultures of *Salmonella* (*S*.E., *S*.K., and *S*.I.) and co-cultures (*S*.E./LAB, *S*.K./LAB, and *S*.I./LAB)

were observed from the initial first hours. However, CFUs from the co-cultures without agitation became significantly lower than those from co-cultures with agitation and from the control cultures at 8h. Indeed, the *Salmonella* count of co-cultures increased from 5 log10 to 6 log10 CFU /ml in the first 4h then the number of *Salmonella* sharply decreased to 2 log 10 and 1log 10 CFU/ml in the co-cultures of *S*. Infantis, *S*. Enteritidis, and *S*. Kentucky respectively (Figure 19). There was a drastic reduction in value to no viable *Salmonella* cell count between 8 h and 24 h of analysis. At the end of the experiments, undetectable level (<10 CFU/ml) was obtained. In the line, *L. salivarius* count decrease from 7 log10 to 6 log 10 at 8h then reduced to almost 4 log10 at 24h in monoculture (16/c6) and co-cultures (LAB/*S*.E., LAB/*S*.K, and LAB/*S*.I.).

In the second experiments, as shown in Figure 20, the *Salmonella* counts of co-cultures (*S*.E./LAB, *S*.K./LAB, and *S*.I./LAB) slightly increased from 5 log10 to 6 log10 CFU/ml in 4 h and remained constant until 8 h then decreased to an undetectable level (<10 CFU/ml) at 24h. However, the *Salmonella* number in the pure culture (*S*.E., *S*.K., and *S*.I.) increased from approximately 5 log10 to 8 log10 CFU/ml at 8h and remain constant at the end of the experiments. The *Lactobacillus* count was not affected by the pathogens. There was no difference in the *Lactobacillus* count in the LAB-*Salmonella* mix (LAB/*S*.E., LAB/*S*.K, and LAB/*S*.I.)as compared with the *Lactobacillus* monoculture controls (16/c6).

Monitoring the pH of the mono- and co-cultures revealed that the pH gradually decreased from approximately 6.97 to approximately 3.9 at 24h.

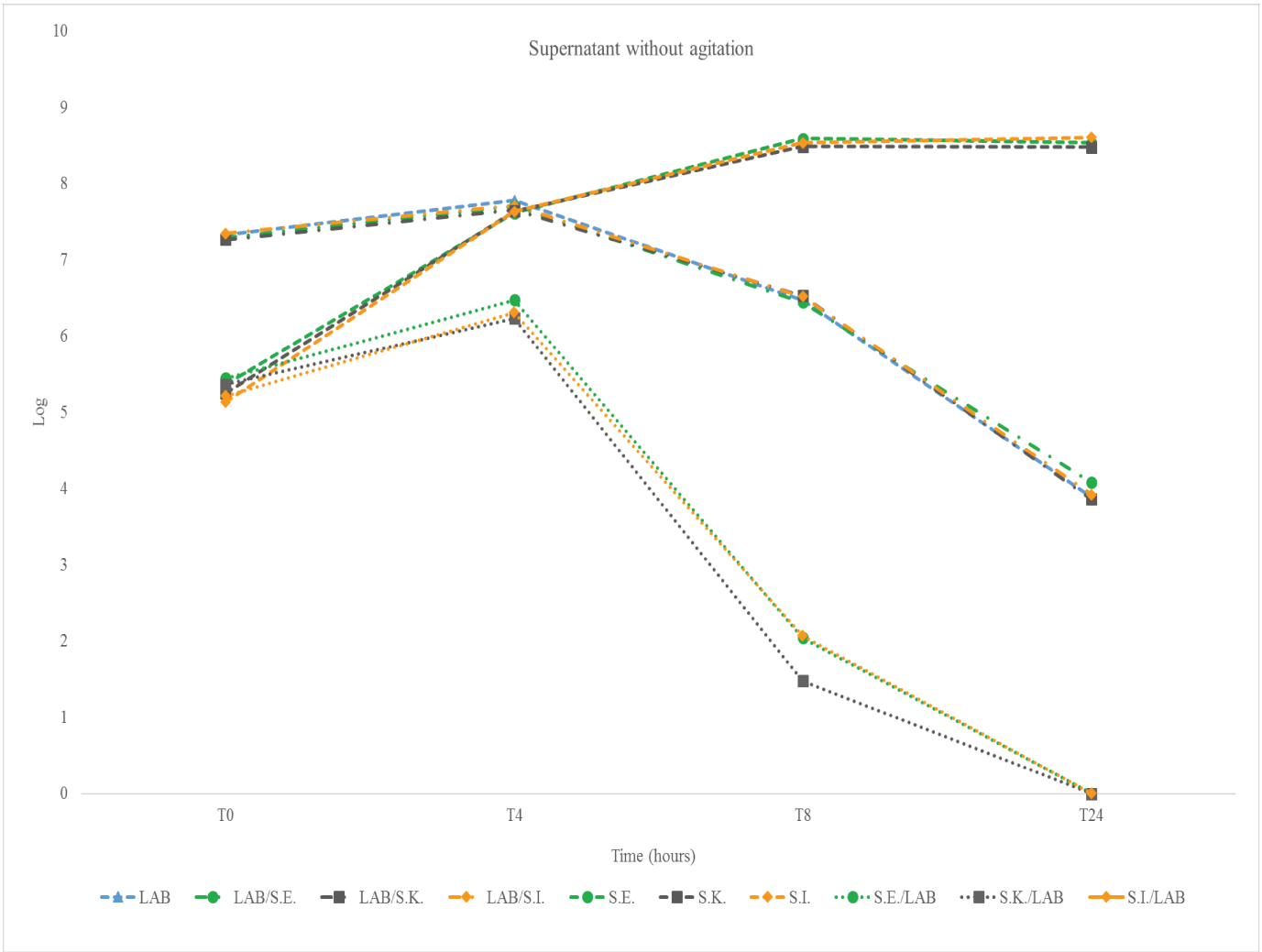

Figure 19: Liquid co-culture assay without agitation: Kinetic growth of pure-cultures and co-cultures of *L. salivarius* 16/c6 and *S.* Enteritidis, *S.* Infantis and *S.* Kentucky ST198

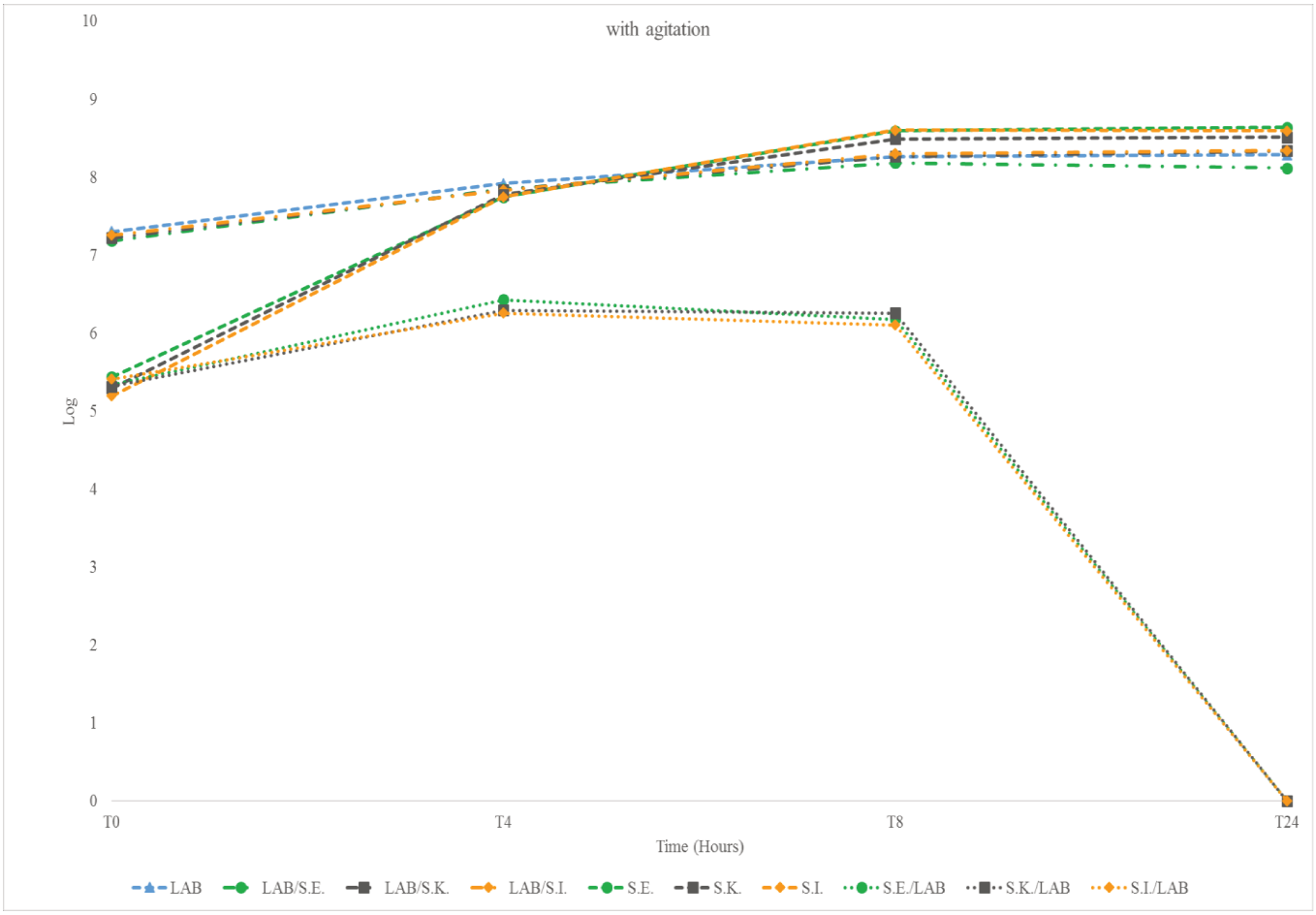

Figure 20: Liquid co-culture assay with agitation: Kinetic growth of pure-cultures and co-cultures of *L. salivarius* 16/c6 and *S.* Enteritidis, *S.* Infantis and *S.* Kentucky ST198

4. Discussion

LAB constitute principal resident of the GIT that provide host protection against enteric pathogens colonization, and resistance, that includes the competition for nutrients and the secretion of inhibitory substances. Recently, the interest in using probiotics, particularly *Lactobacillus* sp., has been reassured by current proposals to find alternatives to using antibiotics and reducing their sub-therapeutic doses in animal feeds to improve the growth and health status of the livestock (Chen et al., 2007). Numerous factors affect the microbial biodiversity of poultry GIT such as breed, diet, and age of the chicken and the section of the GIT (ileum or caeca). This microbiota changes significantly in the first 2-3 weeks until is stabilize at 5-6 weeks of age. It is found that in broilers

fed with corn-soy diet without antibiotics and additives, 70% of the ileum population belong to *Lactobacillus* sp. The use of antibiotics in broilers induced changes in the composition of the intestinal bacterial community, namely *L. salivarius* (Albazaz and Buyukunal Bal, 2016). In this regard, different experiments were conducted to cover microbiota variability in *Lactobacillus* strains.

In most cases, phenotypic strain identification by API 50 CHL was found inconsistent with the 16S rRNA sequencing method in accordance with other studies (Kao, Liu, and Shyu, 2007; Sakaridis *et al.*, 2014). Lactobacilli population is large and have similar biochemical identifiers which caused a lack in distinguishing them by API 50 CHL test; yet, isolates that were identified as *L. salivarius* were also found to belong to the same species by sequencing. On the contrary, all *Leuconostoc lactis* were identified *as L. salivarius* by sequencing. Moreover, *L. fermentum* revealed to be *L. reuteri* by sequencing except two strains A33/i13 and 14/c13 that matching results were observed in the two methods in accordance with Sakaridis et al., (2014). Most of the intestinal strains recognized as *L. fermentum* are now classified as *L. reuteri* (Yadav et al., 2017), which is regarded as the most prevalent *Lactobacillus* species in the intestinal tract in poultry (Wang et al., 2014). Even though the studied *Lactobacillus* isolates were obtained from different experiments, low diversity was observed among species. Permanent strains (*L. acidophilus*, *L. salivarius*, and *L. fermentum*) were found in all birds of two days until the market age. The study of Babot *et al.* (2014) showed that the most common *Lactobacillus* species were *L. crispatus*, *L. reuteri* and, *L. salivarius* per our findings.

As defined by the European Food Safety Authority, requirements for safety assessment of probiotics, such organism shall not possess acquired resistance determinants to antibiotics of medical importance (EFSA, 2012). Antibiotic-resistant is acquired by horizontal gene transfer between commensal flora of gastrointestinal and antibiotic-resistant pathogenic bacteria. In the present study, the fifty Lactobacilli strains tested for antibiotic susceptibility showed to be resistant to at least one antibiotic. High AMR was detected against all used antibiotics (ampicillin, chloramphenicol, kanamycin, streptomycin, and gentamicin) with less extend to erythromycin which was shown to be higher in farms treated with antibiotics. Lactobacilli have intrinsic resistance to aminoglycosides due to the absence of cytochrome-mediated electron transport, which mediates drug uptake (Charteris et al., 2016). In this line, aminoglycosides resistance strains

do not represent a significant safety concern, considering that intrinsic resistance presents a minimal potential for horizontal transfer (Fraqueza, 2015).

On the contrary, these strains are usually susceptible to antibiotics that inhibit protein synthesis, such as chloramphenicol and erythromycin and to an antimicrobial that inhibit cell wall synthesis such as penicillin and β-lactamase inhibitors. The high incidence rate of ampicillin resistance (100%) recorded in this study is higher than that observed by other researchers (Dec et al., 2017). Due to the use of Macrolide–lincosamide– streptogramin (MLS) antibiotics (tylosin, tilmicosin, lincomycin, and virginiamycin) as growth promoters and/or as prophylactic agents in poultry rearing, gene transfer under antibiotic selective pressure facilitates the spread of MLS resistance in commensal bacteria. This various antibio-resistances have been observed from different sources (Sharma et al., 2014).

In vitro tests have been used to assess the probiotic potential of lactobacilli. The production of hydrogen peroxide, organic acids by decreasing the pH and bacteriocin are a useful mode of action of *Lactobacillus* to inhibit *Salmonella* growth. However, in the present study, hydrogen peroxide production was unlikely to be the cause of the *Salmonella* inhibition in the agar diffusion test because all lactobacilli were grown under anaerobic conditions (Schillinger and Lucke, 1989). Furthermore, the well-diffusion antagonism method did not show any inhibition excluding the possibility of bacteriocins or bacteriocin-like being the reason for the *Salmonella* inhibition. The production of organic acids by decreasing the pH was likely being the cause of such effect (Adetoye et al., 2018). Although the bacteriocin or bacteriocin-like activity produced by LAB is commonly more effective against Gram-positive bacteria such as *Listeria monocytogenes* (Ramos et al., 2013), however, the inhibition of *Salmonella* (Gram-negative) has also been reported (Gupta and Tiwari, 2014).

The adhesion behavior of bacteria is a complex multistep process; it includes non-specific and specific ligand-receptor mechanisms (García-Cayuela et al., 2014). The non- specific adhesion is controlled by physicochemical reactions of the cell wall including electrostatic and Van der Waals interactions as well as hydrophobic properties. These latter are the most reliable long-range non-covalent interactions (Lewis acid-base) due to the surface proteins and (lipo) teichoic acids that cover the peptidoglycan and that by conferring a net negative bacterial surface charge in physiological conditions (Babot *et al.*, 2014). According to the authors, this feature is strain-

specific and vary depending on the medium, age and surface structures of bacteria. Indeed, considerable variability of hydrophobicity capacity has been observed with 62 % of the isolates showing high hydrophobicity (70%).

Auto-aggregation and co-aggregation of a probiotic strain are necessary for adhering to the intestinal tract and inhibiting the foodborne pathogens colonization by forming a defensive barrier (Kos et al., 2003). Moreover, the LAB co-aggregating ability might regulate pathogens microenvironment and stimulate the excretion of antimicrobial substances (Potočnjak et al., 2017). *Lactobacillus* sp. also favors many aggregation- promoting factors (APFs) involved in auto-aggregation and/or adhesion in a strain-specific manner (Nishiyama et al., 2016). Furthermore, Exopolysaccharides (EPS) are believed to play an essential role in cell aggregation, biofilm formation and adhesion. Polak-Berecka *et al.*, (2014) concluded that *L. rhamnosus* adherence/ or co-aggregation ability was strongly related to specific interactions based on surface proteins and specific fatty acids, whereas polysaccharides (hydrophilic nature) hinder adhesion and aggregation by masking protein receptors.

Aggregation values increased over time typically at 20h of incubation in a strain-dependent way (Collado et al., 2007). Indeed, all our strains possessed this feature at 24h of the auto-aggregation assay. All isolates with (Agg+) phenotype were identified as *L.salivarius* in agreement with Ait Seddik *et al.* (2017) who demonstrated the highly auto-aggregation ability of this strain. According to Solieri *et al.* (2014), co-aggregation values below 20% are indicative of weak co-aggregation capability. Our isolates differed in the co-aggregation ability (0 to 94.6 %) with indicating once again the strain-specific characteristics.

Another probiotic protective mechanism involves competition for adhesion sites (Singh et al., 2017). *L. salivarius* (16/c6, 16/i4, 14/i8, A30/i26), *L. reuteri* (1/c24), *L. crispatus* (16/c2), *L. fermentum* (12/c8) and 16/i10 were chosen according to their cell hydrophobicity and auto/co-aggregation abilities. The adherence capacity differed significantly between the *Lactobacillus* strains which is consistent with other studies showing that this ability was species and strain-dependent (Campana, Van Hemert and Baffone, 2017). The highest adhesion was shown by four strains of *Lactobacillus; L. salivarius* A30/i26 and 16/i4 being highly auto- aggregative and hydrophobic and *L salivarius* 16/c6 and *L. reuteri* 1/c24 showing a great co-aggregation and hydrophobicity abilities. *L.crispatus* 16/c2, *L. reuteri* 12/c8, *L. salivarius* 14/i8 revealed the lowest

adhesion percentage despite their high co-aggregation capacity. Interestingly, *Lactobacillus* sp.16/i10, a high hydrophobic strain, exhibited also a low adhesion percentage.

The studied parameters (hydrophobicity, aggregation and co-aggregation, adhesion) illustrated no interrelation. However, some mentioned that hydrophobic nature is related to the attachment to the epithelial cells (Handley et al., 1987, Salotti de Souza, 2018), but denied by others (Ramos et al., 2013). García-Cayuela *et al.*, (2014) revealed a correlation between auto-aggregation and co-aggregation in contradiction to our results. Del Re *et al.* (2000) proposed that auto-aggregation and hydrophobicity are independent characters, but both of them are necessary for adhesion. Multitude interrelated surface factors (Fatty acids, surface proteins, LPS, EPS) may have unpredictable effects on adherence, co-aggregation, and cell to cell interactions (Campana et al., 2017).

Survival in the GIT is a critical probiotic property. Bile tolerance is strain specific related to the hydrolase activity (Zommiti et al., 2017). By mimicking the GIT conditions, all the eight *Lactobacillus* strains were capable of growing at 0.1 % (w/v) of bile salt, but two *L. salivarius* A30/i26 and 16/i4 were affected by 0.3%. This concentration is considered as critical for resistant probiotic screening (Ramos et al., 2013). The bile salt hydrolyzes genes, *bsh-1* and *bsh-2*, were found to be responsible for acid and bile tolerance in *L. salivarius* UCC118 (Adetoye et al., 2018). In favor of our findings, significant decreasing cell count in most of *L. salivarius* isolates has been observed when incubated with a high concentration of bile salts (0.5%) whereas most of *L. reuteri* isolates showed high tolerance (Abhisingha et al., 2018).

L. salivarius A30/i26 and 16/c6 and *L. reuteri* 1/c24 have been chosen for their high adhesion and were further evaluated for their abilities to compete/exclude the three *Salmonella* serotypes from epithelial adhesion using Caco2 as an experimental model. The inhibition of the pathogen adhesion by the three probiotic strains indicated a high variability in a strain-dependent property. *L. salivarius* 16/c6 significantly inhibited the adhesion of the three *Salmonella* serotypes to Caco-2 cell monolayers exclusively by exclusion assay in accordance with the study done by Campana, Van Hemert and Baffone, (2017). The authors suggested that *L. salivarius* W24 could prevalently inhibit the adhesion of pathogens to caco-2 cells exclusively by exclusion. Jankowska et al., (2008) showed that *L. paracasei* reduces *Salmonella* adhesion to caco-2 cells by 4 and 7-fold in competition and exclusion experiments respectively. However, the inhibition of the attachment of

Salmonella to caco-2 cells by exclusion as well as by competition was frequently reported (Jessie Lau and Chye, 2018; Singh et al., 2017)

The inhibition of the three *Salmonella* serotypes by *L. salivarius* 16/c6 was similarly demonstrated by liquid co-culture assay and that by two different significant ways. When the co-cultures were tested without agitation, the kinetic growth results of *Lactobacillus* and the pathogens confirmed what has been previously distinguished by auto-aggregation and co-aggregation assay and showed the ability of these features over time. Indeed, the co-cultures and *L. salivarius* monoculture revealed a clear supernatant after 8h of incubation. Efficient aggregation and proper settling of flocs are essential in the management of effluent in the activated sludge process (Malik et al., 2003). In this regard, this feature in our strain might be promising in the purification and decontamination of wastewater of the slaughterhouse mainly polluted by pathogens and organic materials.

When *L. salivarius* 16/c6 and the three *Salmonella* serotypes were subjected to the same co-culture assay but with agitation, the reduction of *Salmonella* counts in mix cultures co-occurred with the decrease in pH in accordance with other studies (Abhisingha et al., 2018) until complete growth inhibition of the three *Salmonella* serotypes after 24 hours of co-incubation. Szala, Paluszak and Motyl, (2012) observed complete inactivation of *Salmonella* Heidelberg by *L. plantarum* and *L. brevis* after 48 h of co-culture whereas other study showed that *L. plantarum* was not active in co-culture with *E.coli* (Ayeni et al., 2018). *Salmonella* could adapt to extreme acidic environments (pH= 3); some strains have acid-adaptation systems that enable them to survive at pH < 2 (Tan et al., 2014). Other non-negligible antimicrobial factors are involved in *Salmonella* inhibition, like competition for nutrients (Abhisingha et al., 2018) and the contact-dependent inhibition (CDI) mechanism (Bian et al., 2016). This latter, where contact cell to cell is needed could be explained by the exchange of information between bacteria such as conjugation, secretion systems, contact-dependent inhibition, allolysis, and nanotubes. In fact, in our study, the low count has been observed at 4 h between *Salmonella* monocultures and mixed cultures.

5. Conclusion

The native poultry-derived *L. salivarius*16/c6 is a candidate to be a potent probiotic. Its use in dietary supplement reinforces the intestinal microbiota of newly hatched chicken due to its

viability, persistence in poultry intestinal tract and ability to block the adhesion sites against *Salmonella* sp.

Adhesion of *Lactobacillus* strains to epithelial cells should also be investigated using the chicken LMH cell line to evaluate its probiotic potential in poultry.

The study of these parameters is a preliminary tentative to discover native probiotic strains; however further in vivo experiments are necessary to confirm our hypothesis.

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Conclusion and Perspectives

The issue of salmonellosis is a major problem worldwide including Lebanon. According to MoPH, *Salmonella* is considered the main contributor in sporadic food poisoning and outbreaks related to chicken. Strategies and plans against *Salmonella* infection are hampered due to the lack of sufficient data on *Salmonella* prevalence, circulation serotypes and their antibiotic resistance patterns from farm to fork chain. These information are the basis for any local normative decree (Lebanese standards and MoA directions) or common regional within the Arab League "Arab Food Safety Initiative For Trade Facilitation" (SAFE)) for the prevention and control of salmonellosis in human and *Salmonella* dissemination in the poultry industry. This is the first integrated approach in Lebanon trying to answer decision makers concerns on *Salmonella* control. This project discovers the possibility of the use of native *Lactobacillus* as probiotic against this pathogen.

Not only a high rate of *Salmonella* incidence was detected, but also a multitude of MDR strains and clones against critical antibiotics were observed along the food chain. *S*. Enteritidis is highly predominant with human illnesses attributed to only one poultry-associated clone that has been persistent since 2010 in Lebanon. Moreover, this is the first time that AMR and MDR *S*. Kentucky and *S.* Infantis are reported. This study confirms the spread of the notorious highly drug-resistant, Cip^R *S.* Kentucky ST198 in both a major slaughterhouse and retail market. These strains were ESBLs and cephamycinase-producers emphasizing their high spreading in the Mediterranean basin. A native poultry-derived *Lactobacillus* with a high potential probiotic characteristic against *Salmonella* was isolated, identified and characterized.

These data are sufficient enough to establish a *Salmonella* risk assessment and hence eight years control strategies and plans. The collection of these *Salmonella* strains in this study will enrich our established *Salmonella* repository since the sixties of the last century. These conserved strains constitute a national reference on *Salmonella* basis for further analysis like complete genotyping. More studies may include but not limited to strain serotypes, subtypes, virulence and antimicrobial resistance profile and evolution trend. A WGS of *S*. Infantis will show a clear vision of relatedness of this strain to the Hungarian clone B circulating in Europe. Comparative work with other national Gene Bank might be also valuable globally.

This valued information might serve international strategies carried out by international organizations such as WHO, FAO and OIE.

This work opens the way for a global approach for combating *Salmonella* dissemination. A 'One Health' approach might include integrated surveillance (collaboration between human health, food safety and animal health) and containment plans (farms, retail and consumers) to reduce or minimize *Salmonella* transmission. Continuous surveillance and monitoring to detect the emergence of any serotype or new clone resistant *Salmonella* along the poultry food chain is critical to establish an effective control campaign on national, regional and global level especially in the era of world Trade organization legislations.

Knowledge of the diversity of circulating strains and their resistance patterns can guide the development of poultry stakeholder's awareness programs on how to prevent this pathogen.

It is very crucial to adopt Lebanese legislative decrees on antibiotics use and handling in poultry to reduce and minimize the selection of resistant *Salmonella* (from the top of the poultry production pyramid and within flocks). Especially those critically important for human treatment like fluoroquinolone and ESC. On the other hand, alternative solution in *Salmonella* control should be more evaluated like the use of live probiotic in poultry feeds.

In fact, this study is a preliminary tentative to discover native probiotic strains; however further experiments are necessary to confirm our hypothesis like:

- Adhesion of *Lactobacillus* strains to epithelial cells using the chicken LMH cell line to evaluate its probiotic potential in poultry.
- *In vivo* experimentation on broiler and layer hens fed on the native proposed probiotic matrix and compared to control group.

ANNEXES

ANNEXE 1

16S rRNA gene sequencing	APi 50 cHL identification	Visuel aggregation	Auto-aggregation 4h %	Auto-aggregation 24h %	Hydro-phobicity %	co-aggregation with S. infantis%	co-aggregation with S. Enteritidis%	co-aggregation with S. Kentucky%
L. crispatus 16/c2	L. acidophilus	Agg+/Agg-	16.06 ± 0.18	54.79 ± 5.05	85.42 ± 1.78	75.06	89.36	69.66
L. salivarius 16/c6	L. salivarius	Agg-	8.47 ± 3.76	94.42 ± 0.13	91.91 ± 3.78	69.55	71.07	94.55
L. reuteri 16/c7	L. fermentum1	Agg+/Agg-	16.34 ± 2.64	92.12 ± 4.01	90.49 ± 0.47	45.84	60.16	65.23
L. reuteri 16/c8	L. fermentum1	Agg-	7.77 ± 0.76	67.98 ± 3.74	15.52 ± 2.25	47.93	51.03	51.97
L. salivarius 16/i4	Leuconostoc lactis	Agg+	76.93 ± 4.47	89.81 ± 12.72	79.13 ± 3.19	80.45	82.49	79.94
Enterococcus faecalis16/i9	L. plantarum2	Agg-	7.38 ± 3.24	89.22 ± 1.5	24.62 ± 0.98	75.43	53.11	42.56
16/i10 (Not -typed)	Lactococcus raffinolactis ou L. crispatus	Agg+/Agg-	9.24 ± 2.09	80.01 ± 1	97.99 ± 0.57	34.32	45.60	63.51
L. Salivarius 15/c10R	L. salivarius	Agg+/Agg-	18.8 ± 3.27	0.0	96.58 ± 4.27	0.00	0.00	0.00

L. reuteri 15/c10	L. brevis2	Agg+/Agg-	25.35 ± 2.53	84.95 ± 2.51	44.63 ± 4.26	18.11	9.40	11.18
L. salivarius 14/c12	Leuconostoc lactis	Agg+/Agg-	-3.64 ± 6.41	52.37 ± 6.35	72.71 ± 0.48	0.00	21.06	0.00
L. fermentum 14/c13	L. fermentum1	Agg-	24.26 ± 3.27	61.93 ± 0.17	82.57 ± 2.21	59.15	52.64	63.27
L. salivarius 14/i8	L. salivarius	Agg+/Agg-	20.59 ± 4.15	75.4 ± 2.14	81.3 ± 1.49	70.35	62.30	47.54
14/i15 (Not-typed)	L. salivarius	Agg+/Agg-	40.64 ± 6.1	96.75 ± 0.08	0 ± 0	26.50	18.26	2.04
L. salivarius 13/c7	L. salivarius	Agg+/Agg-	18.64 ± 7.78	88.29 ± 4.98	0 ± 0	55.61	55.73	41.42
L. salivarius 13/c13	L. fermentum1	Agg+/Agg-	26.72 ± 4.84	95.28 ± 4.22	18.63 ± 3.81	40.36	39.95	44.14
L. salivarius 12/c4-1	Leuconostoc lactis	Agg+/Agg-	24.92 ± 1.96	94.97 ± 2.74	82.87 ± 0.73	49.58	30.50	17.32
L. salivarius 12/c6	Leuconostoc lactis	Agg+/Agg-	27.92 ± 2.91	68.65 ± 2.3	96.75 ± 1.23	47.52	62.42	63.29
L. reuteri 12/ c8	L fermentum1	Agg+/Agg-	36.77 ± 5.86	72.43 ± 2.28	51.01 ± 1.22	73.87	83.47	80.00
L. reuteri 12/c12	L. fermentum1	Agg+/Agg-	9.03 ± 0.35	38.93 ± 4.19	1.8 ± 2.55	13.07	6.02	19.13

L. salivarius 12/c18	L. salivarius	Agg+/Agg-	24.19 ± 1.92	91.21 ± 0.47	82.49 ± 3.18	23.63	23.03	17.04
L. reuteri 11/i4	L. fermentum1	Agg+/Agg-	17.35 ± 4.76	86.64 ± 2.07	21.31 ± 2.99	2.55	7.53	16.24
L. reuteri 11/ i6	L. fermentum1	Agg+/Agg-	26.73 ± 2.44	73.79 ± 0.87	24.5 ± 3.67	63.37	60.31	63.22
Enterococcus faecalis 11/c1	L. delbrueckii ssp delbrueckii	Agg-	2.97 ± 1.83	84.62 ± 1.36	0 ± 0	66.71	57.06	53.26
L. reuteri 10/c4	L. fermentum2	Agg-	7.17 ± 6.64	82.25 ± 14.37	78.42 ± 2.28	9.35	14.94	8.21
L. salivarius 10/c8	L. salivarius	Agg+/Agg-	53.47 ± 1.72	96.12 ± 1.22	2.02 ± 2.86	8.37	0.00	15.75
L. reuteri 10/i8	L. fermentum1	Agg+/Agg-	21.23 ± 6.66	82.85 ± 5.87	0 ± 0	16.10	11.60	14.25
L. reuteri 9/i44	L. fermentum1	Agg+/Agg-	47.48 ± 2.15	95.23 ± 3.9	0.03 ± 0.04	12.99	1.64	6.35
L. reuteri 7/c7	L. fermentum1	Agg+/Agg-	21.06 ± 2.97	95.56 ± 1.69	96.66 ± 1.38	5.62	5.76	9.44
L. reuteri 6/i10	L. fermentum1	Agg+/Agg-	83.61 ± 3.24	98.96 ± 0.45	90.9 ± 0	0.00	0.00	0.00

L. reuteri 4/i14	L. brevis	Agg+/Agg-	64.22 ± 12.07	98.92 ± 0.86	50.19 ± 0.03	40.78	49.50	44.47
L. reuteri 3/i15	L. fermentum1	Agg+/Agg-	65.49 ± 4.59	95.59 ± 2.97	76.72 ± 9.77	53.38	67.75	76.55
L. reuteri 2/i33	L. fermentum1	Agg+/Agg-	32.95 ± 4.17	99.12 ± 0.46	79.54 ± 5.41	28.16	28.17	29.46
L. reuteri 2/c2	L. fermentum1	Agg+/Agg-	49.28 ± 2.9	99.03 ± 0.37	97.71 ± 1.11	33.50	25.46	31.22
L. reuteri 1/c24	L. fermentum1	Agg+/Agg-	13.74 ± 2.65	98.02 ± 0.6	89.57 ± 9.52	62.47	50.43	58.93
A30/c2	Leuconostoc lactis	Agg+/Agg-	41.89 ± 0	99.07 ± 0.27	94.75 ± 0	25.26	32.91	37.23
L. salivarius A30/i26	Leuconostoc lactis	Agg+	76.07 ± 5.56	99.48 ± 0.08	98.42 ± 1.6	25.71	49.54	60.00
L. fermentum A33/i13	L. fermentum1	Agg-	2.33 ± 3.3	61.38 ± 9.77	0 ± 0	46.47	24.77	38.37
L. reuteri A35/c6	L. fermentum1	Agg+/Agg-	32.25 ± 5.22	98.75 ± 0.25	79.85 ± 2.51	20.00	19.10	15.38
L. salivarius A39/c4	L. salivarius	Agg+	70.13 ± 1.52	98.04 ± 0.34	70.86 ± 5.13	12.17	0.00	0.00
L. salivarius A48/i11	L. salivarius	Agg+/Agg-	21.88 ± 2.95	99.24 ± 0.16	96.8 ± 1.55	0.00	0.00	0.00

L. reuteri A41/c1	L. fermentum1	Agg+/Agg-	18.86 ± 1.53	99.01 ± 0.84	90.52 ± 4.35	0.00	13.01	14.52
L. salivarius A41/c6	L. salivarius	Agg+	66.4 ± 15.24	99.2 ± 0.31	73.37 ± 8.5	1.98	0.00	0.00
L. salivarius A41/c8	L. salivarius	Agg+/Agg-	38.7 ± 2.95	98.93 ± 0.46	46.85 ± 13.54	0.00	3.25	3.76
L. salivarius A42/c14	Leuconostoc lactis	Agg+/Agg-	24.02 ± 2.18	99.36 ± 0.34	76.17 ± 2.38	29.64	1.08	0.00
L. salivarius A42/c15	Leuconostoc lactis	Agg+/Agg-	23.13 ± 4.09	99.21 ± 0.07	49.32 ± 1.44	34.92	74.58	24.28
L. reuteri A42/i7	L. fermentum 1	Agg+/Agg-	21.5 ± 4.07	97.43 ± 0.52	90.24 ± 0.69	42.47	10.65	18.16
L. salivarius A 53/i3	L. salivarius	Agg+	66.4 ± 13.07	99.26 ± 0.13	99.7 ± 0.1	47.15	54.43	42.20
L. reuteri A54/c1	L. fermentum 1	Agg+/Agg-	47.89 ± 1.99	99.22 ± 0.15	86.9 ± 6.62	2.33	0.00	18.12

Supplementary Data: S1

	SPI-2 S. Typhimurium LT2	SPI-3 S. Choleraesuis SC-B67	C63PI S. Typhimurium SL1344	SPI-1 S. Choleraesuis SC-B67	SPI-14 S. Gallinarum SGC-8	SPI-14 S. Gallinarum SGA-8
17-70328 (K12)						
17-70460 (K24)						
17-70462 (K31)						
17-70464 (K32)						
17-70468 (K38)						
17-70469 (K43)						
17-70472 (K48)						
17-70474 (A66C)						

	SPI-5 S. Typhimurium LT2	SPI-13 S. Gallinarum SGD-3	SPI-13 S. Gallinarum SGG-1	SPI-13 S. Gallinarum SGA-10	SPI-4 S. Thypi CT18
17-70328 (K12)					
17-70460 (K24)					
17-70462 (K31)					
17-70464 (K32)					
17-70468 (K38)					
17-70469 (K43)					
17-70472 (K48)					
17-70474 (A66C)					

	ID	length
	100%	100%
	<100%	100%
	<100%	<100%

Supplementary Data: S2

			Sample							
			17/7032 8 (K12)	17/7046 0 (K24)	17/7046 2 (K31)	17/7046 4 (K32)	17/7046 8 (K38)	17/7046 9 (43)	17/7047 2 (K48)	17/7047 4 (A66C)
DNA translation number	Gene name	rev/ unrev								
gar73237	Respiratory nitrate reductase 2 alpha chain narZ NGUA18_01113	unrev	vs	vs	vs	vs	vc	vs		vs
gar73236	Respiratory nitrate reductase 2 beta chain narY NGUA18_01112	unrev	vs	vs	vs	vs	vs	vs	gri	vs
aax65484	Nitrate reductase 2, gamma subunit narV SCH_1578	unrev	gr	gr	gr	gr	gri	gri		gri
kto49248	Nitrate reductase subunit alpha narZ A7S24_23185 A7S72_00240 CBI64_02880 IN36_11310 IN69_03795 IN77_19665 IN95_16340	unrev	vc	vc	vc	vc	vc	vc		vc
pap00907	Propionate--CoA ligase	unrev	vs	vs	vs	vs	vs	vs		vs
gar74426	2-methylcitrate dehydratase	unrev	vs	vs	vs	vs	vc	vs		vs
gar74425	Citrate synthase	unrev	vs	vs	vs	vs	vs	vs	vc	vs
gar74424	2-methylisocitrate lyase (2-MIC) (MICL) (EC 4.1.3.30) ((2R,3S)-2-methylisocitrate lyase)	unrev	vs	vs	vs	vs	vs	vs	vs	vs
esg61438	Beta-methylgalactoside transporter inner membrane component	unrev	vc	vc	vc	vc	vc	vc		vc

gar72600	Galactose/methyl galactoside import ATP-binding protein MglA (EC 3.6.3.17)	unrev	vs	vs	vs	vs	vs	vs		vs
gar72599	D-galactose-binding periplasmic protein	unrev	vs	vs	vs	vs	vs	vs		vs
aal09832	Serine/threonine-protein phosphatase 2 (EC 3.1.3.16)	rev	vc	vc	vc	vc	vc	vc	gri	vc
cfw71692	Nucleation component of curlin monomers	unrev	vc	vc	vc	vc	vc	vc	vc	vc
gar73668	Curlin (Major curlin subunit CsgA)	unrev	vs	vs	vs	vs	vs	vs	vs	vc
gar73235	Putative nitrate reductase molybdenum cofactor assembly chaperone NarW	unrev							vs	
cnt79762	Propionate--CoA ligase (EC 6.2.1.1) (EC 6.2.1.17)	unrev							gri	
phi63044	Propionate--CoA ligase	unrev								
phi63043	2-methylcitrate dehydratase	unrev								
aav78238	Citrate synthase	unrev								
phi63041	2-methylisocitrate lyase (2-MIC) (MICL) (EC 4.1.3.30) ((2R,3S)-2-methylisocitrate lyase)	unrev								
aal21094	D-galactose-binding periplasmic protein (GBP) (D-galactose/ D-glucose-binding protein) (GGBP)	rev								
phi63439	Galactose/methyl galactoside import ATP-binding protein MglA (EC 3.6.3.17)	unrev								
phi63438	Beta-methylgalactoside transporter permease	unrev								
aav77238	Respiratory nitrate reductase 2 beta chain	unrev								

phi60655	Nitrate reductase	unrev								
phi56380	Nitrate reductase	unrev								
aal20074	Major curlin subunit (Fimbrin SEF17)	rev								
acn46709	Nucleation component of curlin monomers	unrev								
pap02408	Effector protein YopJ	unrev	vs	vs	vs	vs	vs	vs	vc	vs
gar75962	Cell invasion protein SipA	unrev	vs	vs	vs	vs	vs	vs		vs
akg95516	Invasion A (Fragment) sipA	unrev	vs	vs	vs	vs	vs	vs		vs
gar72945	Guanine nucleotide exchange factor sopE2	unrev	vs	vs	vs	vs		vs		vs
aal23387	Putative fimbrial usher protein stjB STM4572	unrev	vc	vc	vc	vc	vc	vc		vc
aad41067	Membrane protein (Permease) (SitC)	unrev	vc	vc	vc	vc	vc	vc	vc	vc
agq74824	Type-1 fimbrial protein subunit A fimA CFSAN002050_25170	unrev	vc	vc	vc	vc	vc	vc	vc	vc
aih08902	LpfD (Fragment)	unrev	vc	vc	vc	vc	vc	vc		vc
ajq18275	Pathogenicity island 2 effector protein SseC (Translocation machinery component)	unrev	vc	vc	vc	vc	vc	vc	vc	vc
aav78334	Putative fimbrial protein tcfA SPA2455	unrev	vc	vc	vc	vc	vc	vc	vc	vc
avd49329	Type III secretion system effector SteB	unrev	vc	vc	vc	vc	vc	vc	vc	vc
ege33635	Dipeptidase (EC 3.4.-.-) pipD SG9_1005	unrev	vc	vc	vc	vc	vc	vc		vc
avd46272	Type III secretion system effector protease PipA	unrev	vc	vc	vc	vc	vc	vc	vc	vc
adx19548	Putative transcriptional regulator MarT	unrev	vc	vc	vc	vc	vc	vc	vc	vc

aty38589	iroN	unrev	vc	vc	vc	vc		vc		vc
cbw18209	Type III secretion system effector protein sseK2 SL1344_2113	unrev	vc	vc	vc	vc		vc		vc
abl63534	FimA (Fragment)	unrev	vc	vc	vc	vc	vc	vc		vc
aax66796	Outer membrane usher protein steB SCH_2890	unrev	vc	vc	vc	vc	vc	vc		vc
ajq16843	Giant non-fimbrial adhesion protein (Ig-like domain repeat protein) siiE CD793_02290	unrev	gri	gri	gri	gri	gri	gri		gri
aal20547	Secreted effector protein SteB (Salmonella translocated effector B)	rev								
ege36237	Protein lpfD	unrev								
aao70134	Putative fimbrial protein (TsaA) tsaA tcfA t2550	unrev								
acy87732	Dipeptidase (EC 3.4.-.-) pipD STM14_1240	unrev								
aav77681	Uncharacterized protein pipA SPA1763	unrev								
ajq16893	Giant non-fimbrial adhesion protein siiE	unrev								
aty38582	iroN	unrev								
aax65758	Guanine nucleotide exchange factor sopE2 SCH_1852	unrev								
aaf00615	E3 ubiquitin-protein ligase SspH2 (EC 2.3.2.27) (RING-type E3 ubiquitin transferase SspH2) (Salmonella secreted protein H2) (Secreted effector protein sspH2)	rev								
aax66702	Iron transporter: fur regulated sitC SCH_2796	unrev								

asz36104	Effector protein YopJ avrA CK947_05725	unrev								
ajq18182	Translocation machinery component sseC	unrev								
aal58882	Cell invasion protein SipA (Effector protein SipA)	rev								
caa57991	Cell invasion protein SipA (Effector protein SipA)	rev							vc	
cnt80311	Outer membrane usher protein steB ERS008207_01012	unrev							vc	
cnu41015	Dipeptidase (EC 3.4.-.-) pipD ERS008198_02707	unrev							gri	

	ID	length	
	100%	100%	vs= dark green
	<100%	100%	vc = light green
	<100%	<100%	gri= grey

empty boxes mean no gene found