Novel bacterial species from the chicken gastrointestinal tract and their functional diversity

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Novel bacterial species from the chicken gastrointestinal tract and their functional diversity

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List of abbreviations

AAI: Average Amino acid Identity

AMR: Antimicrobial Resistance

ANI: Average Nucleotide Identity

ARDRA: Amplified Ribosomal DNA Restriction Analysis

ATP: Adenosine Triphosphate

BA: Biogenic Amines

BCE: Before the Common (or current) Era

CAZymes: Carbohydrate Active Enzymes

CD cells: Cluster of Differentiation, system used to differentiate cell surface molecules

DAPI: 4′,6-Diamidine-2′-phenylindole dihydrochloride

DNA: Deoxyribonucleic Acid

EIEC: Enteroinvasive *Escherichia coli*

FTU: Phytase Units

GALT: Gut-Associated Lymphoid Tissue

GIT: Gastrointestinal Tract

GPRs: G protein-coupled receptors

HDP: Host Defence Peptides

HEPA: High Efficiency Particle Arresting

IEC: Intestinal Epithelium

Ig: Immunoglobulin

JAM: Junctional Adhesion Molecule

mRNA: messenger-RNA

NADH: Nicotinamide Adenine Dinucleotide

PAMP: Pathogen-Associated Molecular Patterns

PPAR: Peroxisome Proliferator Activated Receptor

RNA: Ribonucleic Acid

SCFA: Short-Chain Fatty Acids

Serovar: Serological Variants

TJ: Tight Junction

TLR: Toll-Like Receptors

Tregs: T regulatory cells

TSHR: Thyroid Stimulating Hormone Receptor

ZO: Zonula Occludens, a protein that occurs exclusively on the cytoplasmic side of the tight junctions

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1. Introduction

Domesticated chicken *Gallus gallus domesticus* [1] is the most numerous bird species in the world with a population of 23.7 billion, followed by ducks with approximately 1.1 billion and turkeys and geese with 0.3 billion each [2]. Poultry production provides one of the best sources of quality protein and plays a crucial role in sustaining livelihoods in developing countries [3]. Demand of egg increased from 61.7 million tonnes in 2008 to 76.7 million tonnes in 2018 with the Netherlands, China and Mexico leading consumption per capita (19.8 to 22.2kg in 2020) [2]. Corresponding annual projections for poultry meat were 2.9 % on underdeveloped countries and 1.6 % for developed countries, respectively [4]. The main reasons of such increased demand are its low cost, high nutritional value, low-carbon impact, and lack of religious taboos upon consumption [3, 5]. The amount of quality digestible protein that chicken provides is up to 60% and 87% for chicken eggs. It contains lutein and important amino acids for human nutrition such as lysine, threonine, methionine, and cysteine that cereals or grains lack. Consequently, chicken flesh is regarded as a healthy meat with high nutritional value [6].

Human intensive selection breeding has shaped a highly productive animal in terms of growth rate and efficient feed conversion ratios (feed conversion into muscle) with easy handling requirements compared to bigger animals, making it an affordable source of protein in comparison to other livestock [7]. The breeding selection methods for the two main growth traits, meat production, and reproductive traits (egg production) combined in the same bird have been extremely successful. However, modern chicken genomes present adaptive mutations to the domestic lifestyle among them, overlapped genes associated with growth, appetite, and metabolic regulation [8]. For instance, a missense mutation at the locus for thyroid stimulating hormone receptor (TSHR) in one allele, which regulates hormone secretion and photoperiod control of reproduction in vertebrates, might be explained by an absence of the strict seasonal reproduction compared to the natural behaviour [8]. These genomic outputs enrich the specie´s functional knowledge and provide a successful model of domestication. Beyond nutrition, chickens have served as experimental model organism for research in genetics, embryology, oncology, virology, immunology and microbiology [9, 10].

Archaeological studies suggest that chicken domestication started to occur in multiple independent events ca 8000 to 5400 years ago from Junglefowl species in southeast Asia. Bones of domesticated fowls are dated from the Indus valley as early as 2500–2100 BCE [11]. The spread of this species started with the establishment of trade routes to Africa, Europe and America later in 1500 [12]. Nowadays, the chicken farming industry is widespread around the globe and has enabled the rapid rise of broiler production up to 65.8 billion of slaughtered individuals per year [2]. Such unprecedented event of domestication and phenotypic changes, together with the enormous population size, the global distribution and the future sedimentary record of such consumption, highlights chicken industry as an important event of the current “Anthropocene” epoch (yet unofficial geological time) [11].

In the industry, the success of chicken production is a consequence of a coordinated chain-integrated system that connects farms with slaughterhouses, packing areas and markets. All steps keep strict hygienic practices including disinfection, constant litter changes and separation of the facilities from outside contact maintaining biosecurity protocols [13, 14]. Moreover, fertilised eggs are transported to hatcheries and maintained under climate-controlled housing separated from the parents [15, 16]. This axenic maintenance and isolation from early contact with adults alter the process of microbial colonisation [17] and impacts the physiology and nutrition of the animal, increasing the susceptibility to digestive impaired functionality [18]. The banning on use of antibiotics in animal feed of 2006 by the European Regulation 1831/2003/EC [19] has rephrase chicken breeding strategies to understand how microbiome structure and immunity play a role in its health [20].

1.1 Gastrointestinal microbiome of chicken

The anatomy of gastrointestinal system in birds, considering its body size, is rather small and shorter compared to mammals. This conformation means a shorter retention of food on the gastrointestinal tract (GIT), yet its function does not decrease digestion efficiency [21]. This functional compensation may be partially explained by the fact that the chicken GIT hosts a complex microbial community that improve the diet profits to the bird benefit [22]. In this sense, the structure and functionality of the intestinal microbiome is crucial for the health of poultry.

It is known that the process of acquisition and maturation of the gut microbiome throughout its lifecycle its crucial and has a strong influence on the development of enteric epithelium and the regulation of physiological functions that maintain intestinal homeostasis (i.e., immunity, nutrient digestion, intestinal barrier integrity). Consequently, these functions optimise resources and energy to the host [23]. Gut microbiome also plays an important role assimilating nutrients from food, producing cofactors, vitamins, essential amino acids, and avoiding harmful pathogens establishment (see Section 1.2.1).

The taxonomic and functional diversity explorations in complex microbial communities utilise two successful methodologies that have produced accurate descriptions of the community structure and its ecology [24, 25]. Culture-independent studies, that base the information acquisition on direct metagenomic sequencing of DNA extracted directly from the samples, combined with bioinformatic and statistical analysis based on databases, references, and community profiling [22, 26, 27]. Culture-dependent procedures target the isolation of microorganisms in pure cultures followed by whole genome sequencing and phylogenomic analysis [28]. The correct interpretation of metagenome datasets and functional analyses guidance requires experimental validation and reference genome sequences obtained from cultivated bacteria [24, 25, 27]. In this sense, both efforts feed references to genes and genome catalogues and improve the description of microbiome functions by broadening the panorama of the biological components (bacterial, archaeal, fungal and bacteriophage), its dynamics and estimation of their ecological function.

Studies on the taxonomic composition of poultry digestive tract agree on the marked differences within the bacterial colonization throughout the different GIT sections. Both culture-dependent and independent approaches reported the presence of microorganisms belonging to seven different phyla: *Bacillota*, *Bacteroidota*, *Pseudomonadota*, *Actinomycetota*, *Verrucomicrobiota*, *Synergistota* and *Fusobacteriota* [29-34]. The following table (Table 1) lists the current reports of phyla and genera of bacteria detected inside the gut of chickens.

The chicken GIT tract starts with the beak, mouth and oesophagus where some salivary glands secret amylases, the main purpose of these organs is to swallow and move food towards the crop [42]. Crop contains longitudinal folds that distend to store food and gathers the conditions for hydrolysis of sugars and lactate fermentation to take place [51]. In this section, a clear dominance of *Lactobacillaceae* members has been reported together with *Enterobacteriaceae, Streptococcaceae* and *Bifidobacteria* in lower amount [35]. These groups are thought to adhere the crop epithelium, hydrolase starch and ferment sugars to lactate. This organ represents a biocontrol checkpoint, putting pressure on selecting metabolically fitted bacteria that can survive the fermentation conditions and be afterwards seeded to the next intestinal sections [39]. Crop gets first colonised after hatch [52] and the bacterial profile will be influenced by the diet and litter. Yet, the physiology of crop and the competence for adhesion sites will shape the colonisers profile inhibiting saprophytes and pathogens [53]. Former anti-bacterial agents such as penicillin and monensin, would eliminate *Lactobacillaceae* population increasing the number of *Enterobacteriaceae* [54].

The GIT continues with the stomachs where food is grinded. It consists of two chambers termed proventriculus, or glandular stomach which secrets mucus and hydrochloric acid; and the gizzard or mechanical stomach which consists of two pairs of opposing muscles composed by circular muscle that grind food and secrete mucus to protect from the strong acidic conditions [42]. Being so close to the crop, gizzard is dominated by the same bacterial genera but due to the presence of gastric juices and pepsin the population rate might reduce one to two orders [42]. The mean retention time is estimated to vary between half an hour and an hour and the pH ranges from 2.5 to 5 depending on the diet content, pellet size and age [55-57].

Table 1. Physicochemical conditions of different portions of the chicken gastro-intestinal tract and spatial distribution of most abundant bacteria (phylum and genus).

	Digestion stage (enzymatic activity)	pH	Substrates	Oxygen conditions	Microorganisms reported		Colonization rate	References
					Phyla	**Genus**		
Crop	Amylases	5–6.5	starch, sucrose, maltose, SCFA, lipids	Anaerobic Microaerophilic Aerobic	*Bacillota* *Actinobacteriota*	*Clostridium, Enterococcus, Eubacterium, Lactobacillus, Pseudomonas, Staphylococcus, Streptococcus, Sarcina Bifidobacterium, Micrococcus*	10^8-10^9	[35-39]
					Pseudomonadota	*Aeromonas, Enterobacter, Klebsiella, Pseudomonas, Escherichia/Shigella*		
Gizzard	Mechanical and chemical breakage by muscular contractions, hydrochloric acid and pepsin	3 - 4	peptones carbohydrates, lipids	Anaerobic Microaerophilic	*Bacillota*	*Clostridium, Enterococcus, Lactobacillus, Ligilactobacillus (L. aviarius, L. salivarius)*	10^5 -10^7	[35, 36, 40, 41]
					Actinobacteriota *Pseudomonadota*	*Bifidobacterium* *Escherichia/Shigella*		
Duodenum	Hydrolysis and absorption. Biliary salts, trypsin, chymotrypsin, amylase, carboxypeptidases, sucrase, isomaltase, Peptidases and phosphatases High osmotic pressure (Na Mg)	6-7	glucose, monosaccharides, disaccharides, peptones, amino acids, organic acids, SCFA, vitamins	Anaerobic	*Bacillota*	*Lactobacillus, Ligilactobacillus, Enterococcus Lactococcus, Streptococcus*	10^8-10^9	[29, 32, 36, 41, 42]
					Pseudomonadota	*Escherichia/Shigella, Pseudomonas Burkholderia Ralstonia*		
					Bacteroidota	*Bacteroides, Alistipes*		
					Actinobacteriota	*Corynebacterium*		
Jejunum **Ileum**		6-7	glucose, monosaccharides, disaccharides, peptones, amino acids, organic acids, vitamins	Anaerobic	*Bacillota*	*Ligilactobacillus*, Peptostreptococcus, Streptococcus, Turicibacter, Veillonella*	10^8-10^9	[29, 31, 36, 37, 41-43]
					Pseudomonadota *Campylobacteriota*	*Alcaligenes, Escherichia/Shigella, Hafnia, Ochrobactrum* *Campylobacter*		
					Bacteroidota *Fusobacteriota*	*Bacteroides, Flavobacterium,* *Fusobacterium*		

Caecum	Absorption of water, carbohydrates and amino acids	6	glucose, monosaccharides, amino acids, uric acid, propionate, butyrate, acetate, vitamins	Anaerobic	*Bacillota*	*Anaeromassilibacillus*, Anaerotignum*, Anaerotruncus, Bacillus, Butyricicoccus*, Clostridium*, Drancourtella*, Enterococcus*, Erysipelothrix, Eubacterium*, Faecalibacterium*, Faecalicatena*, Flavonifractor*, Gallibacter*, Gemmiger*, Lactobacillus*, Ligilactobacillus*, Limosilactobacillus*, Megamonas*, Monoglobus*, Paenibacillus*, Phascolarctobacterium, Pseudoflavonifractor*, Ructibacterium*, Ruminococcus, Sellimonas*, Staphylococcus, Streptococcus, Veillonella*, Weisella*	10^8-10^{11}	[26, 28, 29, 31, 32, 37, 44-48]
					Bacteroidota	*Alistipes*, Butyricimonas, Bacteroides*, Barnesiella*, Flavobacterium*, Gallalistipes*, Mediterranea*, Odoribacter*, Phocaeicola**		
					Fusobacteriota	*Fusobacterium*		
					Pseudomonadota Desulfobacterota	*Bilophila*, Ochrobactrum, Alcaligenes, Escherichia* (E. fergusoni*), Desulfovibrio*, Biophila*		
					Verrucomicrobiota Synergistes Mycoplasmatota	*Akkermansia *, Cloacibacillus *, Spiroplasma sp.*		
					Actinobacteriota	*Bifidobacterium*, Collinsella *, Enorma*, Olsenella *, Rubneribacter*,*		
Cloaca Faeces	Water reabsorption, digestive waste and reproductive purposes	6-7	glucose, carboxylic acids, ammonia, uric acid	Anaerobic Microaerophilic Aerobic	*Bacillota* *Actinobacteriota*	*Clostridium*, Enterococcus*, Eubacterium*, Faecalibacterium*, Lactobacillus*, Ligilactobacillus*, Limosilactobacillus* Bifidobacterium*, Olsenella**	10^9 -10^{10}	[27, 31, 32, 42, 49, 50]
					Bacteroidota	*Alistipes*		
					Pseudomonadota	*Escherichia*, Pseudomonas, Salmonella**		

*Culture-based report

The small intestine (duodenum, jejunum, and ileum) is a large constant-diameter tube, whose walls consist of villi that decrease length towards the distal regions (1.5 mm in the duodenum to 0.4–0.6 mm in the ileum). In this section, many enzymes are secreted and much of the digestion and nutrient absorption takes place [42]. Transition time of digesta lasts approximately 2.5 hours [58]. The group that dominates colonisation of small intestine belong to the phyla *Bacillota:* that include *Lactobacillus*, *Ligilactobacillus Enterococcus*, *Lactococcus* and *Streptococcus;* additionally some other minor abundant groups including *Turicibacter, Clostridium* and *Peptostreptococcus*; from *Bacteroidota*: *Bacteroides*, *Flavobacterium* and *Alistipes* and from Pseudomonadota*: Escherichia*, *Shigella*, *Burkholderia*, *Pseudomonas, Hafnia, Ochrobactrum and Alcaligenes* [29, 31, 36, 37, 42]*.* Towards the distal sections, members of the *Campylobacteriota* phylum, *Campylobacter* and *Helicobacter* have been detected in ileum [22]. However, its presence has been debated to be an indicator of compromised immunity.

At the moment, studies of 16S rRNA gene microbiota analysis, report a clear dominance of *Lactobacillaceae* that seems to be a fit group that adapts to the acidic-fermentative and dynamic conditions of the upper parts [29, 35-37, 42]. The importance of the *Lactobacillaceae* group started to gain attention due to its metabolic plasticity, ecological diversity and novel taxonomic descriptions in the recent years; leading to a taxonomic reorganisation in 2020 that clarified the overly heterogeneous genus *Lactobacillus* sp., resulting in 25 novel genera re-assignations that included the existing *Lactobacillus, Paralactobacillus,* and the *novel genera: Holzapfelia, Amylolactobacillus, Bombilactobacillus, Companilactobacillus, Lapidilactobacillus, Agrilactobacillus, Schleiferilactobacillus, Loigolactobacilus, Lacticaseibacillus, Latilactobacillus, Dellaglioa, Liquorilactobacillus, Ligilactobacillus, Lactiplantibacillus, Furfurilactobacillus, Paucilactobacillus, Limosilactobacillus, Fructilactobacillus, Acetilactobacillus, Apilactobacillus, Levilactobacillus, Secundilactobacillus and Lentilactobacillus* [59]*.* This re-classification has brought more clarity on the study of metabolic and functional adaptations that are now easily explained by a genetic and taxonomic relatedness. This encourages a re-evaluation of former conclusions about the population structure and ecology of a given community and prevents a sub-estimation of the functional properties between members of the group.

The caeca comprise two blind-ended pouches that receive digesta from ileum. The retention time of digesta at this point lasts around (12 to 20 h) and is the site of greatest water absorption [60]. Fermentation of carbohydrates and breakdown of nutrients that escape total enzymatic hydrolysis are completed by bacteria, which might be the reason why these sections gather the most densely populated microbial community with great taxonomic diversity providing the last recovery of nutrients [22].

The majority of chicken microbial studies focus primarily on caecal communities; hence, the highest diversity of microorganisms has been described in this region. Caecal microorganisms ferment complex polysaccharides and produce SCFAs (acetate, butyrate, lactate, and propionate) that can be absorbed by the bird and used as an energy source [61]. Caecal microbiome in the first few weeks of life is predominantly colonised by members of *Bacillota*,

mostly of the order *Clostridiales* [47, 62]. Bacteria from the chicken caeca have been successfully cultured and sequenced, including 133 gut anaerobe strains representing a wide range of metabolic potentials and ecological niches [28]. However, the entire functional diversity of the chicken caecal microbiome is not yet totally described. An extended list of microorganisms detected up to date in this region and along the GIT of chicken is detailed in Table 1 and Figure 1 [63].

At the final portion of the GIT, cloaca and excreta, dominate *Lactobacillus, Limosilactobacillus, Ligilactobacillus, Clostridium, Faecalibacterium, Ruminococcus, Enterococcus* (phylum *Bacillota*)*, Alistipes* (phylum *Bacteroidota*)*, Escherichia, Pseudomonas* and *Salmonella* (phylum *Pseudomonadota*) [32, 49, 50]. The different abundance and taxonomic fluctuation of this community is a collection of planktonic bacteria that colonise the upper regions, therefore, a temporal reflection of the diversity of GIT bacteria in that moment [27].

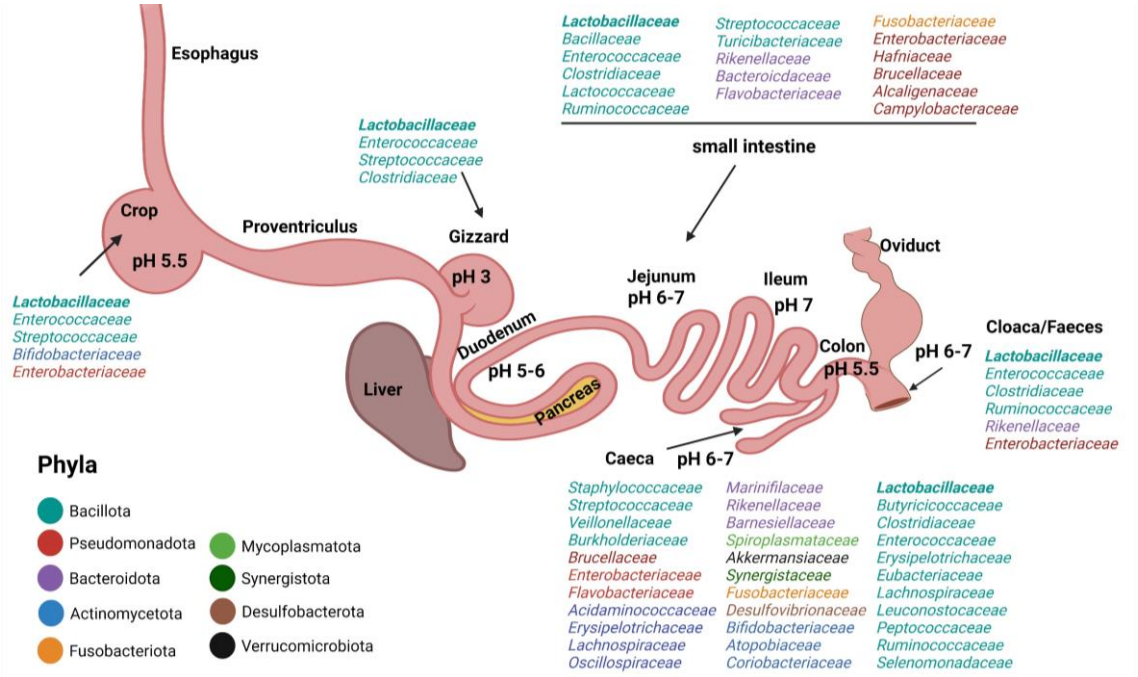

Figure 1. Structure of the bacterial community throughout the gastrointestinal tract of chicken. Modified from Bindari and Gerber (2022) and Maki (2019).

1.2 Function of the microbiome

The presence of microorganisms in the gastrointestinal tract improves the host's nutrition by facilitating nutrients availability [54, 65]. Their presence trains the chickens' adaptive immune response by interacting with the intestinal epithelium, maintains homeostasis against colonization of pathogens [20, 66], and stimulates the absorptive capacity of the epithelia by producing cofactors, antimicrobial compounds, amino acids, SCFA, and producing mucosa layers in the *lamina propria* [67-69].

Knowledge on these functions have driven performance improvements on the use of probiotics and growth promoting prebiotics [70, 71]. However, since some species differ in their growth requirements and substrate preferences deeper functional studies are needed to understand how the chemical composition, physicochemical factors and structure of the digesta determine the species distribution of the microbial community in the gastrointestinal tract [72].

1.2.1 Nutrition

Microorganisms in the gastrointestinal tract represent an added value to the metabolic potential. All digestive processes are strongly linked to gut microbiome and influenced by its composition and diversity. They obtain most of their carbon and energy sources from dietary compounds that are either resistant to the host's enzymatic activity or slowly absorbed by the host [72].

Most of intestinal bacteria are capable to hydrolyse polysaccharides, oligosaccharides, and disaccharides into primary sugars and ferment these sugars into lactate, acetate, propionate and butyrate [73, 74]. These SCFAs are absorbed across the mucosa through the epithelium by passive diffusion and once they reach circulation, have an effect on the upper gut musculature, blood flow regulation and growth stimulation and proliferation of enterocytes among others [75, 76]. Their presence in the digestive lumen lowers pH and improves mineral absorption [54] and might positively influence the maintenance of bone health according to recent studies [77-79]. An improvement of the mineral absorption help to relieve common non-infectious leg diseases in broilers (external rotation of the tibia and femur or chondromalacia) caused by nutritional imbalances of calcium, phosphorus, vitamin D, and poor feeding management [80].

Most of these sugar fermentation takes place at caeca [26, 61, 81] where molecular hydrogen produced during the fermentation process is removed by hydrogen-consuming microorganisms. Although the presence of these microorganisms is not directly providing nutrients to the host or other bacteria, the oxidation of NADH is not possible at high H_2 pressure, therefore, the presence of hydrogen consuming microorganisms is essential to the continuous formation of SCFA [82]. This might explain the increase on diversity and abundance of bacteria with different ecological niches in this GIT section.

The enormous potential of the chicken microbiome to hydrolyse structural polysaccharides is reflected in the highly diverse number of carbohydrate active enzymes (CAZymes) detected so far encoded in genomes recovered from GIT bacteria [22].

Intestinal bacteria also contribute to the nitrogen metabolism. Dietary protein is hydrolysed by the host's peptidases and assimilated as amino acids and small peptides by the host and its microbiota. All *Lactobacillaceae* are auxotroph to amino acids and need an access of external amino acids, therefore they are thought to compete with the host for amino acids capture [83]. Colonisers from caeca and cloaca are capable to produce ammonium from the metabolism of urea, which is used by the host and other bacteria for the synthesis of amino acids and vitamins [84]. Additionally, low concentration of amino acids in regions like caeca, favours bacteria that are

not dependent on available amino acids (like all *Enterobacteriaceae*) and reduce the proportion of amino acids dependants (*Lactobacillaceae*) [83].

The metabolism of amino acids via decarboxylation, produce a large range of compounds known as biogenic amines [85]. Biogenic amines (BAs) are active molecules that poses a wide array of biological functions including gene regulation, stress resistance, cell to cell communication, cell proliferation and differentiation and cellular signalling on both eukaryotic and prokaryotic cells [86]. However, many of these benefits depend on the concentration. For instance, putrescine and spermine (product of the ornithine decarboxylation) have shown positive effect on growth of chickens at low non-toxic concentrations [87] but an excess absorbed in circulation is undesirable since BAs are food quality and freshness indicators in raw meat, and such metabolites are related to putrefaction [85].

A special case of attention are the aromatic amino acids that produce toxic end-products during decarboxylation. Indoles like 3-methyl-indole (skatole), p-cresol and phenol uncouple the proton gradient of biological lipid membranes inhibiting ATP production by oxidative phosphorylation [88]. On the other hand, an excess of amino acids available in the intestine also induces deamination reactions. Released ammonia serves as a source of nitrogen to many bacteria. However, higher concentrations of ammonia, have been described to have adverse effects on epithelial cells, including altering epithelial morphology and DNA synthesis [83, 89].

1.2.2 Sites of microbiome-host interaction: epithelia maturation and immune-modulation

Along the GIT tract, microorganisms establish and interact with the architecture of the gut barrier which form one of the four interconnected barriers that collectively separate and protect the lumen intestine from the body: the bacterial barrier, the mucosa layer, the epithelial mechanical barrier, and the immunological barrier. The first layer, already discussed in the previous sections, refers to the “microbial barrier”, microorganisms attached to the intestinal surface that provide nutrients and compete with pathogens for nutrients and attachment sites [90]. The next layer is a chemical barrier called intestinal mucosa, it represents a line of defence of the immune response and prevents commensal and pathogenic bacteria from trespassing the intestinal barrier [91]. The mucosa consists of mucin, a mucus layer made of glycoproteins secreted by calceiform epithelial cells called goblet cells [91] it protects epithelia against invasion of pathogens and contributes to lubricate and prevent intestinal cells from dehydration. Mucin also represents a source of carbon, nitrogen, and energy for commensal bacteria and gives an advantage against bacteria that cannot adhere to the mucosal surface [72] so its presence is associated with good intestinal health [74].

Bacterial SCFAs production regulates the expression of mucin in goblet cells by affecting the histone acetylation at the promoter region of *muc*2 [92], which improves the chemical barrier of the mucus layer that covers the intestinal epithelia. [90]. SCFAs also induce the gene expression of endogenous host defence peptides (HDPs), a group of peptides that are able to disrupt the membrane structure, electron transport or proton gradient of a broad range of microorganisms

including bacteria, fungi and enveloped viruses [93]. HDPs also bind and neutralize bacterial endotoxins and promote wound healing by activating immune cells [94]. Studies of expression of HDPs genes in chicken demonstrated that acetate, propionate and butyrate enhanced HDP expression and reduced bacterial colonisation [95]. Some of these peptides are result of a basal expression, while others are induced by bacteria [90]. Glycans of the mucosa layer bind luminal antigens and imprint dendritic cells located in deeper levels of the barrier, making the mucus a selective barrier able to tolerate continual exposure to commensal bacteria and food antigens without triggering an inappropriate inflammatory immune response to gut antigens [96].

Enterocytes at intestinal epithelia form a mechanical barrier of tight connections between cells, creating a monolayer that control the transit of different materials including ions, solutes, and water as well as bacteria, antigens, and xenobiotics [97]. This cellular monolayer of epithelial cells, stem cells and goblet cells are connected by tight junctions of transmembrane and peripheral proteins called tight junction [98] proteins, that regulate the permeability of the intestinal barrier [99]. The presence of SCFA such as propionate, tributyrin and butyrate promotes the production of ZO-1, claudin-1, claudin-4, junctional adhesion molecule-3 (JAM-3) and occluding proteins involved in gut barrier function [90]. Although the mechanism is yet not clear, it is suggested to be downregulated by intracellular mRNA signals [100]. In some cases, the epithelial damage to the intestinal barrier function have been observed to be reduced and reversed. The cellular disruption and disorganization of the actin cytoskeleton caused by *Escherichia coli* (EIEC) could be reversed by incubating the epithelial cells with *L. plantarum*, which increased the density of actin filaments and tight junction proteins [98, 99]. In broilers, the butyrate-producing bacteria *Clostridium butyricum* was observed to improve the intestinal mechanical barrier by upregulating genes for tight junction proteins claudins 2, 15, 19, and 23, improving consequently the broilers growth performance [101]. Some biphenyl and flavonoid compounds, such as thymol and carvacrol found in some essential oils, have been proved to up-regulate the mRNA expression of occludin, ZO-1, and claudin-1 in the small intestine of broiler chickens, strengthening the intestinal barrier junctions and improving intestinal integrity [102, 103]. All the former interactions stimulate the mechanical barrier of cells and train their immune and chemical response.

Finally, the immunologic barrier of gut consists of a layer of immune cells T cells, B cells, macrophages and dendritic cells, located at the basolateral site of the intestinal epithelium (IECs) that collectively protect against pathogen invasion and maintain gut health [97]. The immunological system of chickens as many other animals includes both the innate and the adaptive immunity, innate immune response represents the first line of protection and provides the recognition of pathogen-associated molecular patterns (PAMPs) retained by Toll-like receptors (TLRs), which trigger the activation and proliferation of cytokines and innate immune cells: macrophages, epithelial cells, dendrocytes, natural killers, and heterophils. The adaptive immune response is, on the other hand, specific to the antigen and stimulates lymphocytes B cells (immunoglobulins production) and T cells (T-cell receptors) [104]. In both cases, the importance of a healthy balanced microbiome lies on three main strengths: the competitive exclusion for epithelial receptors, the proper training and signal promotion of the

immunomodulatory response, and the production of antimicrobial compounds after sensing the invasion.

Microbiome plays an important role modulating the activation of both responses [105]. Immediately after hatch, the first colonisation and changes in the microbiome, influence the maturation of the gut-associated lymphoid tissue (GALT), found underdeveloped in germ-free animals [106]. These lymphoid aggregates receive antigens derived from the intestines and influence the development of B cells. The maturation of CD4+ and CD8+ cells is therefore acquiring a better activated phenotype compared to those of germ-free animals [91, 105]. It has been reported that by binding SCFAs to receptors, such as Toll-like receptors (TLR) and G protein-coupled receptors (GPRs) the differentiation of T cells into T regulatory cells (Tregs) is promoted [107]. In chickens exposed to maternal faeces after hatching, increased levels of IgA and IgY and an increased gut bacterial diversity were observed when compared with control hygienic conditions [63]. However, these mechanisms are not yet completely determined [91, 108]. In germfree chickens it was demonstrated that microbiota has a dramatic effect on the repertoire of intestinal T cells and their expression of cytokines [108, 109]. Similarly, various studies have shown that chickens treated with probiotics produce a greater number of antibodies in response to a given antigen [91].

1.2.3 Pathogens control

The role of the microbiome in maintaining gut health is key and relies on the modulation and maturation of defence responses: T and B cells maturation, cytokines expression, production of antimicrobial peptides, SCFA supply that regulates the epithelia maturation and mediate immune response, and the competitive restriction to the establishment of pathogens [107]. The avian industry has faced several challenges since the ban of antibiotic use that go from investment loses after negative performance of the animals, costs to improve animal welfare, re-emergence of enteric diseases and zoonotic risk increases [110, 111].

Among common bacterial taxa that are detected abundant during events of dysbiosis are *Campylobacter* (*Campylobacter jejuni* and *Campylobacter coli*), *Salmonella enterica, Escherichia coli*, *Proteus* sp. and *Clostridium perfringens* [108, 112]. Some of these species are found in high concentrations in the intestinal microbiota (10^7 UFC/g) therefore, they are generally accepted to be opportunistic species for poultry [113]. However, some authors consider taxa of low abundance and transitional distribution such as *Salmonella enterica* and *Clostridium perfringens* to be active pathogens that cause disease in chickens, and whose events of infection depend on the age, immune status of the host, and type of serovar [114, 115].

Enterobacteriaceae dysbiosis expansion is well documented to be inhibited by the intervention of microbial signalling of the intracellular butyrate sensor peroxisome proliferator–activated receptor γ (PPAR-γ), that limits the bioavailability of oxygen (electron acceptor) in the lumen, supporting ß-oxidation metabolism on epithelial cells [116]. The surveillance of these infections owes the human interest to avoid foodborne illness and zoonotic diseases.

Other pathogens of veterinary interest are coccidiosis and necrotic enteritis, two diseases that affect the growth and feed conversion. Both innate and adaptive immunity respond against avian coccidiosis, coccidiosis is caused by any of the seven species of *Eimeria*, a genus of apicomplexan *Chromista* parasites that complete its lifecycle in the intestine of many animals including chicken and whose infection symptoms are related to high mortality, bloody diarrhoea, and weight loss reduced feed efficiency and poor weight gain [117, 118]. Intestinal colonisers compete with *Eimeria* sporozoites (motile phase) for adhesion at the epithelial receptors preventing enterocytes invasion [119]. Other actions to reduce parasite colonisation include co-aggregation to pathogens and expression of compounds with antimicrobial effect like SCFAs, hydrogen peroxide and cytokines [117].

A recent strategy used to modulate the microbiome include the use of bacteriophages, intracellular viral particles that invade and, in some cases, lyse the target cell. The advantage on the use of bacteriophages as a modulatory therapy is the specificity of invasion to a determined group of bacteria, the lack of secondary effects to its use and their self-replicate nature [120]. The poultry industry has a special interest on controlling dysbiosis and foodborne pathogens, therefore some studies that target the control of *Salmonella* spp., *Campylobacter* spp., *Escherichia* spp. and *Clostridium* spp. have started to test phage therapies with promising results that are still testing effective ways of application and dose [121, 122]

1.2.4 Factors that modulate the microbiome of chicken

Many physicochemical factors shape the community establishment such as the changes on pH, low concentrations of oxygen, osmotic pressure, temperature, and even hostile biochemical conditions such as lytic enzymatic activities, cofactors, metallophores competition and oxidant conditions [123]. Their abundance will depend on the anatomical region, bird age, diet, geography, metabolism, immunity, and breeding conditions among others [22, 23, 26, 35, 37, 124-126].

There is a significant successional increase in the taxonomic composition diversity as the chicken ages [50, 127]. Bacteria from the genera *Escherichia*, *Clostridium* and *Enterococcus* have been detected at high abundance at one-day old chicken faecal samples, however after 34 days of growth, this dominance decreased together with an abundance increase of *Lactobacillaceae* family members [126]. Weekly sampling analysis showed a diversity increase of caeca colonisers, a decrease of diversity on faeces and an establishment of bacterial diversity at duodenum through time. The dominance of *Lactobacillaceae* seems to be stablished after the first week of life and restricted to the upper sections, while caeca gain taxa diversity of *Lachnospiraceae*, *Ruminococcaceae*, *Oscillospiraceae* among others [128]. In laying hens, four stages of caeca microbial development have been proposed after observing 16S gene sequences shifts. Immediately after hatching, the caecum is colonised by members of the *Enterobacteriaceae* family. However, one week after, *Lachnospiraceae*, *Ruminococcaceae* and members of *Bifidobacteriaceae* dominate caeca. This profile is maintained until the 4th week, which is the usual slaughter time point for broilers. The third stage goes from the 6th week until the 6th month and is

characterised by a dominance of *Rikenellaceae*, *Porphyromonadaceae* and *Bacteroidaceae* (*Bacteroidota*); *Ruminococcaceae*, *Lachnospiraceae*, *Faecalibacterium*, (*Bacillota*). Besides the aforementioned taxa, *Desulfovibrio, Succinivibrio* and representatives of *Pseudomonadota*, appear in hens older than seven months at the 4th stage [62]. Age plays a strong role on defining the pattern of colonization and might be due to the maturation level of the immune response and epithelia. However, among individual birds, especially at an early age, colonisation varies greatly and depends on the levels of maternal IgY carried in the egg and sources of microorganisms at an early exposition [22, 43, 129].

The nutrient content of diets also modulates the establishment of microorganisms, among them, the pellet size, the fibre content, the quantity of water-soluble polysaccharides and the sources of fat, starch and proteins [130]. Additionally, several supplements have been used in an attempt to shape the GIT microbiome and to support bird's performance using prebiotics, probiotics (see Section 1.2.1.1 Nutrition), enzymes like phytases and mineral supplements such as phosphorus and calcium. For phytase and phosphate supplementation tests, low dietary phosphorus diets have been observed to decrease microbial diversity on six-week-old chicken and it is suggested that supplementation with phytase to high-phosphorus content diets may have adverse effects on the gut microbiota (dysbiosis) [131]. Moreover, a decrease in the SCFA production at phosphorus deficiency conditions was detected, potentially caused by a decrease in the hydrolytic activity [131]. In the other hand, supplementation with phytase and $CaCO_3$ + formate at high concentration (1500 FTU/kg and 8.2 g/kg dry matter), increase the abundance of *Lactobacillus johnsonii* in crop and *Limosilactobacillus reuteri* and *L. johnsonii* in ileum, two heterofermentative strains that might be profiting the availability of phosphate to produce SCFA, while at low concentration of $CaCO_3$ *Streptococcus alactolyticus* was highly abundant [132].

Very few experiments have addressed the question of how housing and environmental conditions influence the structure of intestinal microbiome. The maintenance of similar conditions along pens in the industry might take for granted this variable and if considered, it is analysed throughout the view of animal welfare and hygiene [133]. Besides the impact of nutritional intervention on the intestinal microbiome, a study evaluated three different levels of biosecurity at the housing conditions: [134] a standard feed facility, (2) a facility with floor pens for small-scale experiments previously cleaned and disinfected with vaporized hydrogen peroxide, and (3) negative pressure HEPA filtered isolators previously disinfected with vaporized hydrogen peroxide [23]. Changes on the caecal microbial composition and functionality were significantly influenced by the housing conditions rather than with the diet intervention. Housing conditions with the lowest biosecurity level presented the highest phylogenetic diversity. Pen contact effects were also detected for birds raised in the same isolator, for instance relative higher abundance of *Blautia* sp. were detected in one of the pens under the highest biosecurity level housing [23]. Studies on the influence of the first contact environment during the microbiota acquisition demonstrated that eggshell plays an important role in the development of the chicken intestinal microbiome, especially in the jejunum and ileum during establishment and succession [33]. Based on this, the

housing conditions regarding hygiene and hatching first contact have to be considered during the interpretation of intestinal microbiota shifts in poultry.

A study on the microbial colonisation differences regarding gender, found a positive correlation between the abundance of *Bacteroides*, *Lactobacillus* and *Megamonas* in the caeca of male chickens and the metabolism of glycan and lipids. In female chickens, however, *Ruminococcaceae* and *Enterococcaceae* were positively correlated to lipid metabolism. Moreover, gene expression levels of genes associated to glycan and lipid metabolism were different between male and females [135].

1.3 Cultivation of microorganisms from the GIT of chicken: The importance of culture collections

There has been a global effort to preserve genetic material and germplasm of some organisms such as important crops, animal resources, human genetic material, and microorganism. The target of such collections is to broaden and improve the study conservation and sustainable management of these resources in biotechnological applications, research developments and future demands of the growing world [136]. Microorganisms are involved in an endless number of pathways and reservoirs recycling nutrients and stablishing mutualistic relationships within all the known environments where they play a niche-role [137].

Gut microbiota of animals has been an important topic for researchers interested on the improvement of animal growth and production. The study of gut microorganisms follows some highlights through time and has been strongly influenced by technological developments. In the early years, microbiology focused its research on the study and cultivation of pathogens. The study of intestinal bacteria in the 1960s and 1970s boosted the utilization of anaerobic cultivation techniques on both human and animal hosts [138]. Along with the studies on gut microbiota, researchers started to interest on probiotic bacteria and the elucidation of their effect on gut´s health for both humans and domestic animals [139]. Following the novel developments on molecular biology, several approaches adapted to the study of microorganisms by handling DNA, RNA, and proteins first from isolated microorganisms (PCR amplification, library sequences, microarrays, SDS-page and Western blot analysis among others) but recently from metagenomic material coming from any different environment. These strategies detect microorganisms missed by cultivation, through massive molecular sequence methods and have the advantage to become cheaper and faster [140]. After a decade of high throughput sequencing studies, understanding of gut microbial function has improved by allowing the integration of data from the entire ecosystem and revealing ecological niches that cannot be described by the study of single organisms. Nonetheless, this information is mainly descriptive and in most cases the interpretation remains speculative and untested [141]. Elucidation of the amount of data obtained by massive sequencing demands very robust and vast genomic databases to precise taxonomic classification, improve functional assignment and test structural hypothesis. Additionally, the

amplicons number depend on the abundance of certain population, being restricted to the highest and most abundant. Culturomics has emerged as a new approach to the study of complex microbial ecosystems providing cultured strains for a better extensive characterization and downstream studies [142, 143].

In 2014, the Hungate1000 project started to collect information from culture-dependent and culture-independent rumen studies in order to compile and catalogue rumen microbial genome sequences from every former work and homogenize the deep average state of the rumen microbiota information including: a core bacterial species that includes novel taxa, species remaining uncultured, and several cultured rumen bacteria without a reference genome sequence in the databases [144]. Further livestock collections include pig (*Sus scrofa*) and chicken (*Gallus gallus*) initiatives from the RTWH Aachen (Prof. Thomas Clavel) and the Leibniz-Institut DSMZ-German Collection of Microorganisms and Cell Cultures that have helped to describe chicken and pig microbiome building a repository of cultivable bacterial strains isolated from the intestine of chicken (chiBAC) and pigs (piBAC) [63, 145]. Furthermore, a collection of 133 different strains of bacteria from the caeca of chicken was published, describing encoded genes that may interact with the host such as collagenase, hyaluronidase, and sulfatases among others [28]. Material provided by these efforts aims to sustain future research on microbial-host interactions, facilitate targeted colonization and improve molecular studies. However, big-picture projects that include the upper regions dominated by different bacterial taxa are still missing specially for species under high demand of consumption (chicken, pigs, or fish/seafood).

Previous cultivation reports have focus on the use of few media (one or two) to isolate microorganisms throughout the whole tract which may have narrowed the isolation diversity description. In chickens, mostly caeca and faeces have been used as an isolation source to stablish culture collections, neglecting the diversity in the upper part of the GIT. The importance of each GIT section and the physicochemical conditions of crop that serves as a checkpoint that puts pressure on selecting the entrance of microorganisms, highlights the importance of including the upper regions into the study and cultivation of bacteria that colonise these regions. A broader organ-specific culture media formulation that mimics the physicochemical conditions of the different GIT sections (tract portion) that satisfies the nutritional requirements of the bacteria and improve the isolates diversity is proposed on the Table 2.

Table 2. Nutritional conditions found within different parts of the digestive tract of chicken and the proposed media formulation.

GIT tract	Crop	Gizzard	Jejunum Ileum	Cecum	Cloaca
Source of C	starch, sucrose, glucose	starch, sucrose, maltose	glucose (high concentration), maltose, fructose, organic acids, CO_2	glucose (low concentration), organic acids, starch, cellulose	organic acids, amino acids,
Source of N	peptones	peptones	amino acids, ammonia, nitrates	uric acid, ammonia, nitrates	uric acid, ammonia
Source of P	phytate, nucleic acids, phospholipid starch phosphate	phytate, nucleic acids, phospholipid starch phosphate	pyrophosphate, ion phosphates, nucleic acids,	pyrophosphates, ion phosphates	pyrophosphates, ion phosphates
Cofactor	vitamins, ions	vitamins, ions	histidine, haematin, vitamins, ions	histidine, haematin, vitamins, ions	ions, bilirubin
pH	5.5	3-4	6-7	6	6-7
Culture condition	Changes on redox conditions	acidic and proteolytic conditions, anaerobic conditions	High osmotic pressure, anaerobic conditions, biliary salts	sudden alkalization by uric acid entrance, high osmotic pressure, anaerobic conditions	Changes on redox conditions (oxygen pressures changes)

1.4 Objectives and hypothesis

The digestive system of chicken presents different physicochemical conditions along the GIT, shaping an individual microbial profile along sections with different metabolic capacities and divergence on the adaptations to the environment. The exponential interest of the scientific community and the industry to understand how gut microbiome contributes to an optimal development of chickens, demands the inclusion of information of the entire digestive system sections including microbial genes, genomes, metagenomes, proteins, metabolites, and viral particles. In this sense, the objective of this work was to build a collection of culturable bacteria isolated from the upper sections of the digestive system of chickens: crop, jejunum, and ileum, which broadens the description of taxa, genes, and genomes, improving the repository of chicken microorganisms. Being an unexplored environment, a second and third objective consisted of a functional description of the microbial collection that increase the gene catalogue and repertoire of carbohydrate-active enzymes (CAZymes) peptidases and interactive molecules, as well as to contribute with the taxonomical description of potentially novel species undetected by cultivation efforts of chicken microbiome in the past.

The best way to observe environmental adaptations is through the study of complete genomes from organisms that inhabit different GIT sections. In this sense, the establishment of a culture collection from crop and small intestine of chicken will represent the first bacterial repository obtained from these regions. The functional genomic analysis of the bacterial isolates would provide information about their enzymatic potential in the environment, host-interactive adaptability, and the metabolic value that these bacteria represent within the host. In parallel, the accessory annotation might provide detailed information about the environmental dynamics in each region representing a biotechnological potential tool for studying chicken gut function through its microbial diversity.

2. Novel taxonomic and functional bacterial diversity of the upper digestive tract of chicken

Abstract

The digestive system of chicken presents different physicochemical conditions along the gastrointestinal tract (GIT), shaping an individual microbial profile along sections with different metabolic capacities and divergence on the adaptations to the environment. Efforts to obtain cultivable bacteria originating from the upper region of chicken GIT enrich the reference genome database and provide information about the site- specific adaptations of bacteria colonizing such GIT sections allowing to understand the metabolic profile and adaptive strategies to the environment. However, the lack of sufficient reference genomes limits the interpretation of sequencing data and restrain the study of complex functions. In this study, 43 strains obtained from crop, jejunum and ileum of chicken were isolated, characterised and genome analysed to observe their metabolic profiles, adaptive strategies and to serve as future references. Eight isolates represent new species that colonise the upper gut intestinal tract and present consistent adaptations that enable us to predict their ecological role, expanding our knowledge on the adaptative functions. Strains of *Limosilactobacillus* were found to be more abundant in the crop, while *Ligilactobacillus* dominated the ileal digesta. Isolates from crop encode a high number of glycosidases specialised in complex polysaccharides compared to strains isolated from jejunum and ileum. While isolates from jejunum and ileum encode a higher number of genes that interact with the host such as collagenases and hyaluronidases, indicating preferential persistence and adaptations along the GIT. These results represent the first repository of bacteria obtained from the crop and small intestine of chicken using culturomics, improving the potential handling of chicken microbiome with biotechnological applications.

Keywords: chicken, crop, ileum, jejunum, Lactobacillaceae, microbiome, novel taxa

2.1 Introduction

Poultry production provides one of the best sources of quality protein and plays a crucial role in sustaining livelihoods in developing countries. The availability of organic carbon along the intestine, and the relatively short transition time of digesta (around 8–12 h) [37, 124] favours the colonisation of heterotrophic fermentative microorganisms with a total or partial sensitivity to oxygen. In general, a healthy chicken microbiome profile will be dominated by the phyla *Bacillota*, *Bacteroidota*, *Actinomycetota*, and *Pseudomonadota*, and its abundance will depend on the anatomical region, bird age, diet, geography and breeding conditions among others [35].

Molecular-based studies have improved our understanding of the diversity, composition, and gene content of the gut microbiome in chickens. The recent description of Metagenome Assembled Genomes (MAGs) representing more than 1000 novel species from public chicken gut microbiome samples, and caeca samples of two commercial bird genotypes highlights how yet unexplored is the microbial diversity of chicken [26, 146]. Although most of these studies have a high-throughput sequencing approach and good reproducibility, the lack of sufficient reference genomes and genes limits the interpretation of sequencing data and restrains deeper analysis of detailed functions and gene catalogues construction. Studies comparing bacteria along the different anatomical sections enhance how the anatomy of the gastrointestinal tract (GIT) and its physiology influence bacterial colonisation [39, 126, 147].

The importance of handling colonizing species from crop, jejunum and ileum represents an advantage to observe the dynamics of nutrient digestibility, pathogen exclusion or diet additives interaction within microbiome of the upper digestive tract sections [39]. The capacity of crop to store food under constant provision, allows microorganisms to initiate digestion throughout fermentation and consequently the absorption of lactic acid and butanoic acid via organic acid diffusion by the host [67]. Furthermore, crop can play a role in chicken's health by improving nutrient digestibility and regulating the entrance of pathogenic organisms to the digestive system [39]. The importance of the small intestine lay on the high enzymatic activity enhanced by the pancreatic discharge at the anterior jejunum. In this GIT section, the density of the microbiota increases towards the ileum and enzymes of bacterial origin such as trehalase and lactase have been detected [42]. Moreover, bacterial amino acid absorption at ileum prevents the formation of consequent toxic end-products such as putrescine, cadaverine and other cytotoxic biogenic amines that could spoil meat quality [87]. Despite the anatomical differences between digestive regions, the role of crop and small intestine's microbiome within the whole digestive process have not yet been given the same relevance and most studies of the chicken microbiome have been performed using samples from the lower digestive regions, caeca and faeces [27, 28, 48, 63].

Functional analyses have described the chicken microbiome to be capable of fermenting carbohydrates and complex polysaccharides present in the host diet, producing short-chain fatty acids, organic acids, vitamins and antimicrobial compounds (e.g., bacteriocins) [45, 148]. This knowledge has improved culturomic studies, enhancing the growing conditions after observing the ecological role of groups of microorganisms within the environment. In caeca, functional

studies of cultivated bacteria have described the ecology and niche occupation redundancy of strains of *Ruminococcaceae*, *Erysipelotrichaceae* or *Enterobacteriaceae*, that converge on the production of butyrate through different metabolic pathways of fermentation (acetyl-CoA, butyrate kinase or lysine fermentation) [28].

The added value of cultivating microorganisms is the further information that provides the functional and accessory genes annotation. The codification and expression of host-guest interactive molecules, accessory mechanisms, carbohydrate transport systems, unique genes, singleton genes, or defence systems such as antiviral or antimicrobial compounds, helps to indirectly observe the organisms' response to the ecosystem dynamics. Bacteria cultivated from caeca (members of *Bacteroidota* and *Bacillota)* was found to encode genes for the adaptation of the host-life style such as hyaluronidase, heparinase, chondroitinase and mucin-desulfating sulfatase [28, 48]. The presence and expression of these molecules can indicate the level of adaptability and specialisation within the environment.

Despite the anatomical differences among the digestive regions, and the key role of crop and small intestine in the digestive process, most of the aforementioned studies have been done with samples from the lower digestive regions, caeca and faeces. Therefore, studying cultivable bacteria originating from the upper digestive regions will enrich the reference genome database and provide information about the site-specific adaptations of strains colonising such GIT sections. In this study, we successfully cultivated and characterised 43 bacterial strains obtained from the upper digestive tract of chickens. The screening, annotation and taxonomic analysis of strains originating from different regions allowed us to understand the metabolic profile and adaptive strategies of the strains. Among the collection, eight strains represent new species and one of each represents a novel genus, expanding our knowledge of the specialized function of the chicken gut microbiome.

2.2 Methods

2.2.1 Animal sampling

Animals were maintained and fed ad libitum with a commercial corn-based diet and housed at the Lindenhof experimental station of the University of Hohenheim. Ten 47–67-week-old Lohmann Brown classic laying hens and six 21-day-old Ross 308 broilers were euthanized by hypoxia induction with CO_2 and immediately decapitated. The digestive tract was clamp-closed before being dissected and placed into a reductive solution of 0.5 % cysteine for transportation.

2.2.2 Ethical approval

The use of animals in this study was reviewed and approved by the Regierungspräsidium Tübingen, Germany (approval number HOH50/17 TE).

2.2.3 Culture media

All culture media used in this work were prepared following Tanaka, Kawasaki & Kamagata recommendations [149-151] . Phosphate, protein, carbohydrate and agar solutions were prepared and autoclaved separately to avoid the formation of reactive oxygen species and ketosamines during sterilisation [152]. Afterwards, solutions were equally mixed before solidification. Media with high sugar content such as de Man–Rogosa–Sharpe (MRS) medium and poultry-feed agar (PFA) were sterilized under different conditions than the rest (110 °C for 30 min) to avoid sugar oxidation. Protein and peptone solutions were prepared using peptones from soybean plants. These measures were taken to improve the isolation of a greater diversity of organisms than when using traditional plate-dependent cultivation.

Table 3. List of media used for the isolation of bacteria in the digestive tract of poultry and its developed formula.

	TSA	PFA	GMM	MRS	PSM
Media	Tryptic soy agar	Poultry feed agar 2%	Gut Microbiota Medium	Man Rogosa Sharpe medium	Postgate Standard Medium
Source of C	Dextrose 0.2% Starch 0.5%	Heteropolysaccharides, homopolysaccharides	Dextrose 0.04%, Cellobiose 0.1%, Maltose 0.1%, Fructose 0.1%, Acetic acid 0.17%, Propionic acid 0.2%, Butyric acid 0.2%	Dextrose 5%	Na-DL-lactate, Ascorbic acid
Source of N	Amino acids from casein and soybean	Proteins present in soy plants	Amino acids from Tryptone and meat extract	Amino acids from soy peptones, yeast extract, (NH4)3 citrate	NH_4Cl
Source of P	phosphate compounds from Yeast extract	phosphate compounds soy plants	K_2HPO_4	K_2HPO_4	K_2HPO_4
Cofactor	Yeast extract	Mineral traces	histidine, hematin, vitamin mix, mineral mix, menadione	Hemin, menadione, yeast extract	Yeast extract
Inhibitors	none	none	Tween 80	Tween 80	C source restriction
Reducing agents	Dithiothreitol	L-Cysteine-HCl x H_2O Na_2S	L-cysteine	L-Cysteine-HCl x H_2O	Na-thioglycolate
Target group	*Bacillota Pseudomonadota Verrucomicrobiota*	*Bacteroidota Bacillota Fusobacteriota Pseudomonadota Verrucomicrobiota*	*Bacteroidota Bacillota Actinomycetota*	*Bacillota Actinomycetota*	Organotrophic microorganisms: *Desulfobacterota*

- Nonselective media. Contain complex nutrients such as peptones, polysaccharides, and fatty acid chains
- Nonselective media enriched by soy plant-based chicken feed. Contain complex nutrients, polysaccharides, and proteins.
- Formulated media selective for digestive colonizers. Contain fewer complex nutrients and cofactors which are disposable during the digestion
- Selective media for gram-positive lactic-acid bacteria that can resist the effect of surfactant compounds.
- Selective media for organotrophs sulphate-reducing bacteria that oxidize organic compounds, organic acids, and alcohols.

2.2.4 Bacterial isolation

Samples were taken to an anaerobic station (Don Whitley Scientific) that contained a mixture of 80 % N_2 (quality level 5.0), 15 % CO_2 (quality level 3.0) and 5 % H_2 (quality level 5.0), where

digesta content was extracted from the digestive tract and 10-fold serially diluted with a sterile physiological solution (0.85 % NaCl). Isolates were obtained from direct cultivation of the samples and enrichment cultures by plating 0.1 ml dilutions of 10^{-4} to 10^{-7} into TSA, GMM, MRS, PSM and PFA (2 %) (Table 4).

Dilution plates were monitored every 24 h and new colonies were collected into time batches at 48, 96, 144, 216 and 360 h. In parallel, enrichment cultures were obtained by inoculating 1 g sample into each culture media and plating 0.1 ml diluted enrichment broth on solid agar at the same time batches and dilutions used during the direct isolation. All isolates were serially cultured into the correspondent media plates to obtain axenic cultures. Plates were incubated at 39 °C under anaerobic conditions.

Table 4. Isolation strategy for each sample.

Organ	Isolation media*	pH	Dilution rate	Incubation time	Incubation temperature
Crop	TSA, PFA, GMM	6	10^{-8}, 10^{-9}, 10^{-10}	48, 96, 144, 216 and 360 h	39°C
	MRS, PSM		10^{-6}, 10^{-7}		
Jejunum	TSA, PFA, GMM	7	10^{-8}, 10^{-9}, 10^{-10}		39°C
	MRS		10^{-7}, 10^{-8}		
Ileum	TSA, PFA, GMM		10^{-8}, 10^{-9}, 10^{-10}		39°C
	MRS, PSM		10^{-7}, 10^{-8}		

Plates were monitored until growth appearance. Colonies having different macroscopic and microscopic morphologies were isolated into new plates according to their previous growth media. After the isolation Gram stain was performed to assess their, microscopic aggrupation. These tests were helpful after activation from storage at -80° C to confirm the viability and identity of the microorganism.

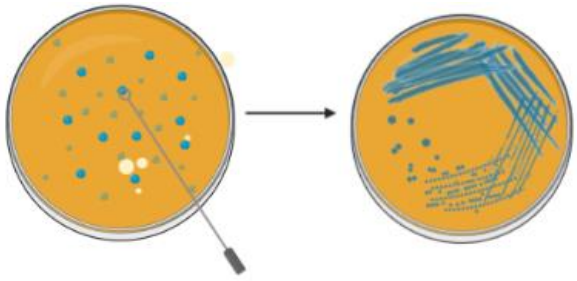

Figure 2. Example of the obtaining of a pure culture of bacteria.

2.2.5 Isolates storage

To maintain the genetic stability of the isolates, a maximum of two subcultures were allowed for each isolate. Isolates were stored in the same media (liquid) where they were cultivated. Cells were suspended within the liquid media, adjusted to an optical density of 0.8 OD at 600nm and distributed into the cryovials. An equal volume of adjusted media and glycerol solution (50%) was mixed to a final concentration of 25% of glycerol. Both glycerol and media were pre-reduced with

N_2 before the mixture. All cryovials were be secured and stored at -80°C. Once frozen, exposure to oxygen did to have much of an effect on the bacteria as long the vials remained frozen.

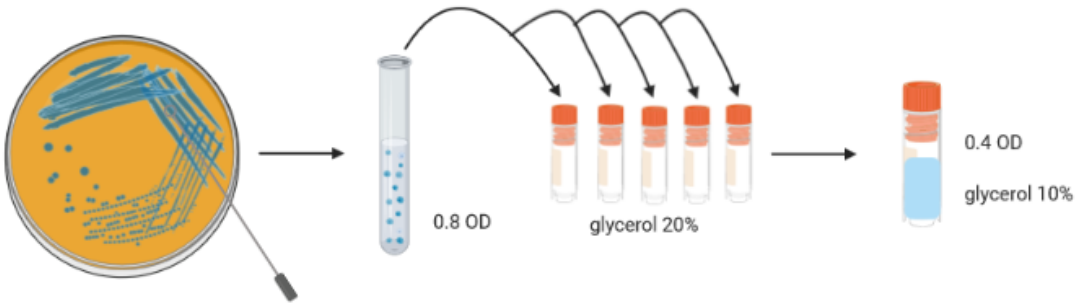

Figure 3. Procedure for the conservation of isolated bacteria.

2.2.6 DNA extraction

DNA extraction was performed on fresh bacterial cultures, between 48 h and 96 h of incubation, following an enzymatic lysis protocol [153]. In brief, cells were grown to a density of 1.0 (A600nm) and washed twice with phosphate buffered saline (PBS). The cell pellet was suspended on 0.2 ml of PBS buffer and incubated at 37°C with 1 U of lysozyme and 1 U of recombinant mutanolysin (A&A Biotechnology Lot.130721) for 30 minutes, followed by an incubation of 30 min at 56°C with 5 µL of proteinase K. A unit of RNAse A was added to each tube and incubated at room temperature for 10 minutes. Both enzymes were part of the MagAttract HMW DNA extraction Kit (Qiagen). The tubes were centrifuged at 21168 x g for one minute and the supernatant was recovered and purified using magnetic beads according to the MagAttract HMW DNA Kit manufacturer's instructions. Quantification was performed with a fluorometric method using Qubit dsDNA HS assay (Thermo Scientific, Waltham, MA, USA) in a Qubit 4 Fluorometer (Thermo Fisher Scientific, USA). DNA samples were stored at −20°C.

2.2.7 Strain identification by 16S rRNA gene sequencing

Isolates (n=689) were screened using ARDRA-fingerprinting, comparing restriction patterns of the gene 16S rRNA digested with the enzyme MspI (NEB Inc., Lot. R0106). Different digestion profiles (n=160) were selected and identified by amplifying and sequencing the 16S rRNA gene using the primers 27F and 1492R [154]. Amplicons were compared using BLAST tool [155] and aligned with the closely related species at the non-redundant GenBank 16S ribosomal RNA database from the National Center for Biotechnology Information (NCBI). Phylogenetic trees were generated based on maximum-likelihood using FastTree and iTOL [156, 157]. Once identified, isolates with an identity percentage lower than 99.7 % with type strains on the database were selected for whole genome sequencing. The complete list of the 160 isolates identified by 16S sanger sequencing is available at the supplementary material in Table 9.

2.2.8 Genome sequencing and processing

Among the collection, 43 genomes were sequenced using a 150-bp paired-end run from Illumina NovaSeq 6000 and PacBio Sequel II platforms (Pacific Biosciences) using P6 chemistry. Reads

from Illumina were quality-controlled, decontaminated, and merged using BBtools (version 37.62) [158]. Assembly was performed using SPAdes (version 3.15.0) [159] under a high-coverage isolate tag for trimmed reads. Long reads from PacBio were assembled using Trycycler (version 0.5.3) [160] utilizing post-filtered reads from PacBio, and short-read corrected Illumina reads. All sequencing services were carried out by Novogene Company Ltd. (Cambridge, UK). The final genome assemblies were submitted to the ENA database under the BioProject number PRJEB56193. Individual genome accession numbers are given in the supplementary section in Table 10.

2.2.9 Genome annotation

Annotation and gene prediction was done with Prokka (v1.14.5) [161]. Further metabolic pathway analyses, motif validation, and participation in individual biogeochemical transformations annotations were mapped with METABOLIC (METabolic And BiogeOchemistry anaLyses In miCrobes) (v4.0) [162]. Both annotation strategies were used to predict the main metabolic pathways in the collection.

Accessory features were also annotated in the genomes. Secondary metabolite biosynthetic gene clusters were detected by mining the genomes against antiSMASH 6.0. database [163]. Antiviral systems presence was identified utilising Prokaryotic Antiviral Defence LOCator [164] [164] using HMM-based homologue searches and gene presence/absence/synteny criteria. Prophage inclusions within the genomes were detected with the PHASTER search tool [165]. Only complete phage clusters, including genes related to capsid, tail proteins, proteases, and genetic material were considered complete prophage insertions.

2.2.10 Taxonomic analysis

Proteomes were used to phylogenetically place the genomes based on 400 marker genes with PhyloPhlAn (v3.0.2) [166] and visualised in iTOL (v6.5.8) [157]. Genomes were taxonomically classified with GTDB- Tk (version 2.1.0) [29] and GTDB (R214 [167]. Isolates that were not assigned to a reliable taxonomic identity were further analysed using genomic comparison of core genes using the concatenated- gene- alignment fasta tool from Anvi'o version 7.1 (Eren, 2021 #213). Phylogenetic trees were inferred by maximum- likelihood using FastTree [156] and visualized in iTOL [157]. Identity parameters for taxonomic delineation, namely digital DNA–DNA hybridization (dDDH), average amino acid identity (AAI) and average nucleotide identity (ANI), were calculated using the Genome- to- Genome Distance Calculator from the DSMZ [168], EzAAI (version 1.2.1) [169] and FastANI (version 1.33; https://github.com/ParBLiSS/FastANI). Sequences for the type strains of the genera *Limosilactobacillus*, *Ligilactobacillus* and *Clostridium*, as well as the family *Oscillospiraceae*, were collected using the NCBI genome browser (www.ncbi.nlm.nih.gov/datasets/genome/) considering genus type strains for each taxonomic group according to the List of Prokaryotic Names with Standing in Nomenclature (LPSN). Trees were visualized and annotated in iTOL (version 6.5.8) [33].

2.2.11 Large-scale genome analyses

To evaluate the relative abundance and occurrence of the isolates within the host, the assemblies were mapped against 106 chicken metagenome samples from the project PRJEB60928, obtained from crop, ileum and caeca using CoverM (v0.6.1) (https://github.com/wwood/CoverM). The list of samples is available at Table 11. The obtained relative abundance per region was then used to estimate the prevalence of each strain at the three regions through all the samples.

2.2.10 Metabolic characterisation

Cellular fatty acids and biochemical profile of novel taxa strains were carried out by DSMZ services, Leibniz-Institut DSMZ–Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; Braunschweig, Germany. In total, 300 mg of frozen wet-weight biomass was used for the Microbial Identification System (MIDI Inc., version 6.1). The composition of cellular fatty acids was identified by comparison with the TSBA40 naming table.

The MicroPlate phenotypic profile Biolog GEN III was used to determine 71 different carbon source utilisation assays and 23 chemical sensitivity assays. In brief, the isolate was grown on an agar medium and then suspended in a special "gelling" inoculating fluid (IF) at the recommended cell density. The cell suspension was inoculated with 100 µL per well into the GEN III MicroPlate incubated to allow the phenotypic fingerprint to form. Increased respiration reduces the tetrazolium redox dye, giving a purple colour. Negative wells and the negative control (a well with no carbon source) remain colourless. After incubation, the phenotypic fingerprint of purple wells is compared to BIOLOG's species library to assign an identity.

2.2.11 Data availability

All sequences generated in the present study are available via ENA database under the project number PRJEB56193 and the individual accession numbers provided in Supplementary Data, table 10. The codes and data for genome and metagenome analyses are publicly available at https://github.com/Bibi888/chicken_gut.

2.3 Results

In this work, a total of 689 isolates from crop, ileum and jejunum of broilers and laying hens were obtained in pure culture, 131 isolates originated from broilers and 558 isolates from laying hens. All isolates were screened with ARDRA-fingerprinting, resulting in 160 isolates selected for identification with 16S rRNA Sanger sequencing. Once identified, the selection of strains for further analysis (genome sequencing) was based on the exclusion of risk pathogens and the inclusion of prevalent strains with the same identity but obtained from different digestive sections. Finally, the genome of 43 different strains, including eight novel species and one novel genus, was sequenced, and functionally annotated. The collection's diversity and source of isolation are detailed in figure 4 and table 9 (Supplementary data 8.1).

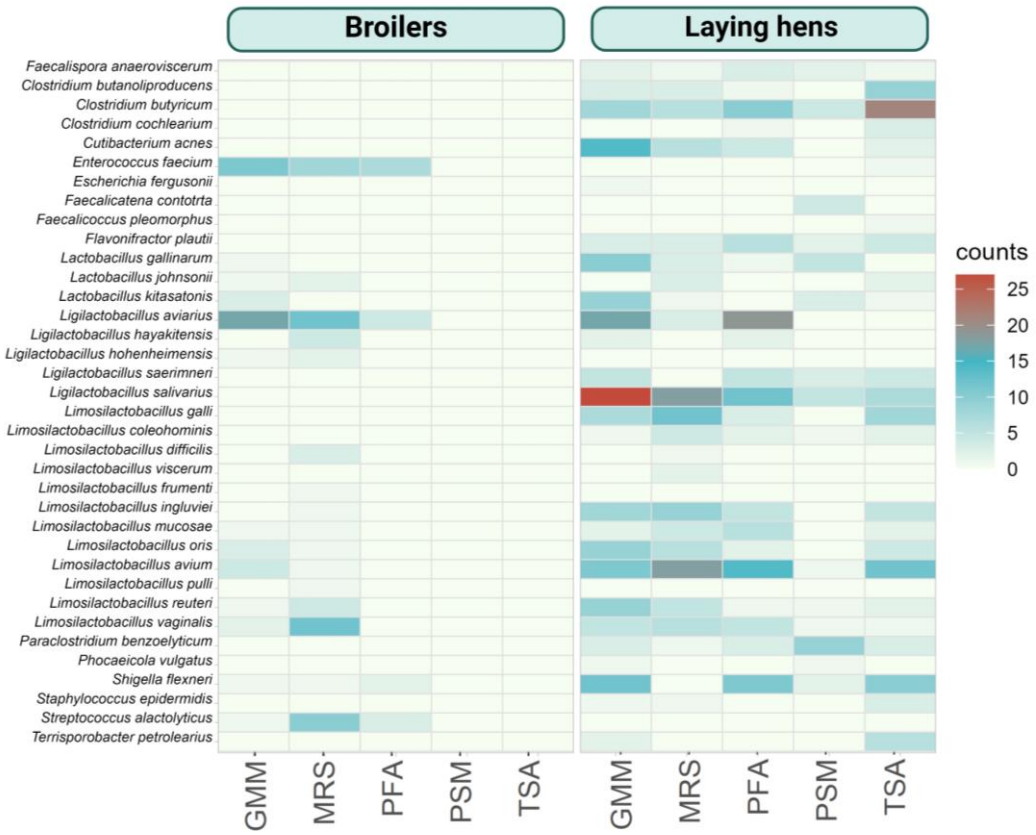

Figure 4. Heat map of the number of bacteria isolated from crop, jejunum and ileum obtained in five different culture media: gut microbiota medium (GMM), Man Rogosa Sharpe (MRS), Poultry Food Agar (PFA), Postgate Medium (PSM) and Tryptic Soy Agar (TSA).

2.3.1 The upper digestive tract isolates collection

The cultured collection encompasses 11 strains obtained from crop, classified within the families *Clostridiaceae, Lactobacillaceae, Propionibacteriaceae* and *Streptococcaceae*. Fourteen strains from jejunum belonging to the families *Lactobacillaceae* and *Enterococcaceae*, and 18 strains from ileum, including the families *Acutalibacteraceae*, *Clostridiaceae*, *Enterococcaceae*, *Lactobacillaceae* and *Peptostreptococcaceae*. Their phylogenetic relation is shown in figure 5 depicting the generated phylogenomic tree based on the proteome of 400 gene markers. More than half of the strains within the collection (n = 33) were detected at a prevalence of 50% in at least one anatomical region of the 106 metagenome sequences obtained from crop, ileum, and caeca of chicken, indicating their presence in dominant communities within the chicken gut microbiome with variable relative abundance, depicted in the outer stripe. In general, all strains belonging to the genus *Limosilactobacillus* were highly prevalent across the intestine (>60%) and more abundant in crop (>1%) than in ileum and caeca, while for *Ligilactobacillus*, strains were highly prevalent only in ileum and caeca and more abundant in ileum (>1%) than in crop, indicating an anatomical section preference (Inner coloured stripe). However, an exception was observed for *Lactobacillus gallinarum*, which was highly prevalent and abundant (>95% and 7.0%, respectively) in all sections.

Within the collection, the species of *Lactobacillus gallinarum* and *Limosilactobacillus reuteri* were recovered from the three different sections. Both showed a higher abundance in the crop and ileum (>5.0 and 0.2 respectively), than in caeca (>0.3 and 0.02) and were present in all metagenomic datasets (100% prevalence) along the intestine. In addition to the abundant species, *Streptococcus alactolyticus* isolated from jejunum was highly abundant in crop (7.5 %) and highly prevalent (>75%) in crop and ileum, suggesting being a dominant member of the upper regions.

Minor abundant species *Enterococcus faecium* and *Paraclostridium bifermentans* (< 0.02 %) were mapped exclusively at ileum with a prevalence of 4%. The presence of these species is particularly low throughout the intestine. Contrastingly to the expected abundance of the strains at the upper regions, the species of *Flavonifractor plauti*, originally isolated from ileum was found to be abundant with 0.28 % and highly prevalent at caeca (100%) compared to crop (10%) and ileum (4%). Such differences indicate a persistent species along the intestine with a higher abundance at the lower digestive parts. Finally, all *Clostridium* sp. and *Cutibacterium* acnes species were not detected in the metagenomic dataset.

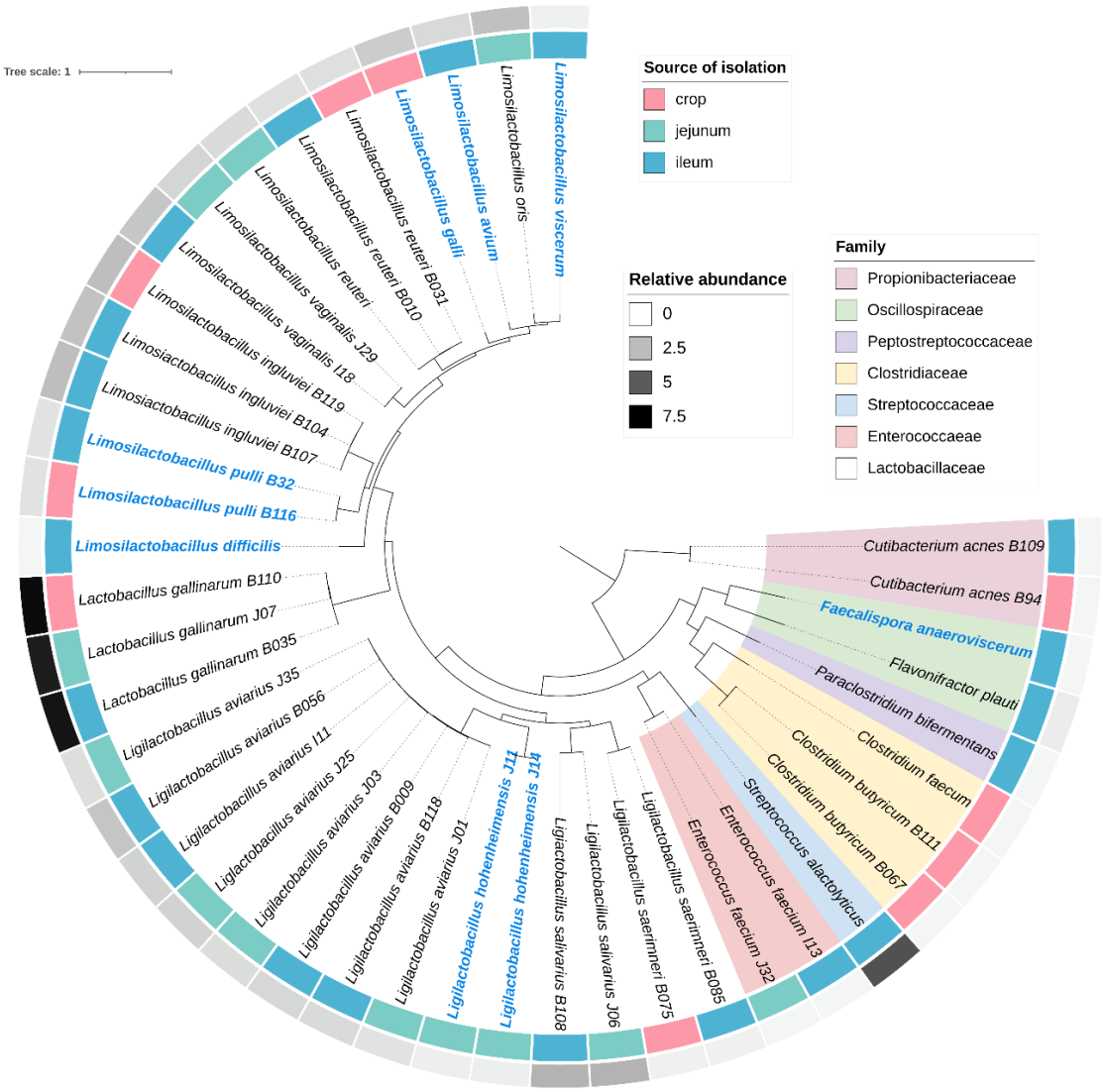

Figure 5. Phylogenetic tree of strains isolated from the upper digestive system of chicken based on 400 amino-acid bacterial marker sequences using PhyloPhlAn. The tree was visualized using iTol and rooted to the middle point. Novel taxa are highlighted in blue. The inner stripe depicts the source of isolation. The outside grey gradient stripe indicates the mean relative abundance of each species along gut-derived chicken DNA samples from the same chicken from where strains were obtained.

2.3.2 Novel taxa within the collection

The cultivation effort provided eight novel species according to the 16S rRNA gene sequence analysis and genome-based taxonomic assignment. Among them, two species identified as *Limosilactobacillus galli* sp. nov. and *Clostridium butanoliproducens* sp. nov. were obtained from crop samples; one species characterized as *Ligilactobacillus hohenheimensis* sp. nov. was obtained from jejunum; four species of *Limosilactobacillus avium* sp. nov., *Limosilactobacillus viscerum* sp. nov., *Limosilactobacillus difficilis* sp. nov. and *Faecalispora anaeroviscerum* gen. nov. sp. nov., obtained from ileum, represent three novel species and one novel genus,

respectively. Finally, two isolates obtained from samples of crop and ileum were identified and named as *Limosilactobacillus pulli* sp. nov. All species belong to the families *Clostridiaceae*, *Lactobacillaceae* and *Oscillospiraceae.* These species are shown in blue letters in figure 5 and listed in table 5. Most of the novel taxa descriptions are based on species isolated at least twice, indicating the reproducibility of the isolation method. However, further reclassifications may occur in the future with the addition of new isolates.

All novel species of *Lactobacillaceae* were detected on at least one of the digestive sections of crop, ileum or caeca, except for the novel species of *L. difficilis* that was not detected in any sample (0% of abundance). *L. pulli* isolated from crop and ileum of laying hens, had a prevalence of more than 60% and a relative abundance of more than 0.5% at crop ileum and caeca, suggesting the detection of a new key members of the chicken microbiome (Figure 6). As well as for *L. avium*, *L. viscerum* and *Limosilactobacillus galli,* that were prevalent with more than 50% and abundant at ~0.05% at crop and ileum (not at caeca). The novel species of *L. hohenheimensis* was more abundant (0.1%) and prevalent (>75%) at ileum than at crop or caecum, similar to its closest related species *Ligilactobacillus aviarius.*

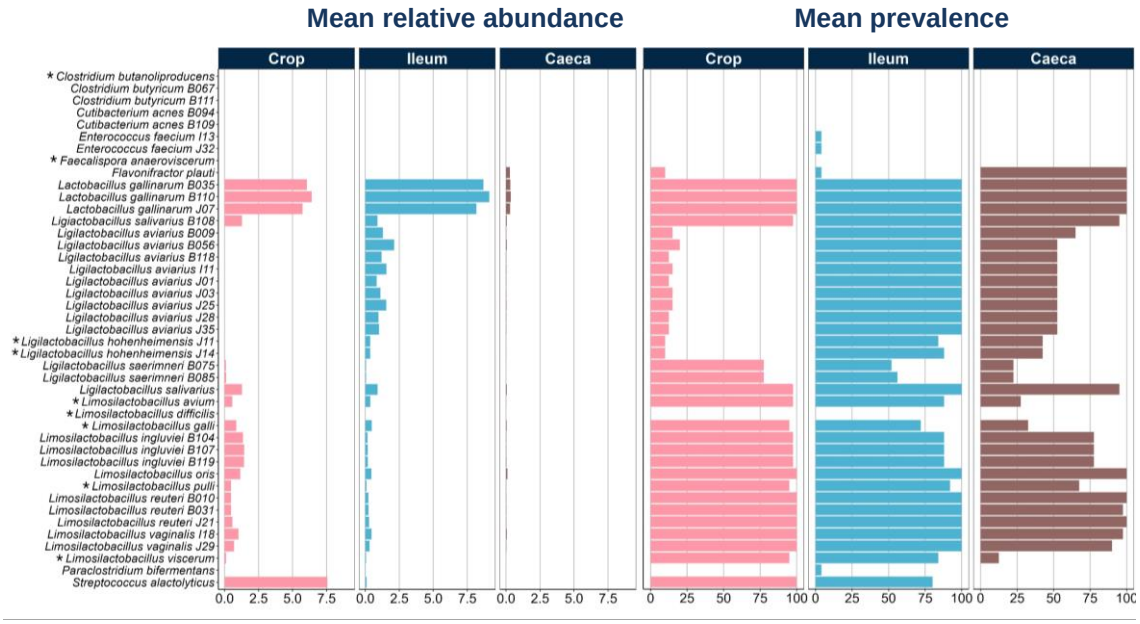

Figure 6. Mean relative abundance and prevalence of bacterial strains from the collection along 106 metagenome datasets from chicken. The two panels depict the mean relative abundance and prevalence, expressed as the percentage of samples where the strain was detected at a given section. Each column represents one section: crop, ileum, and caeca. Strains of the same species are included to highlight the differences on presence and relative abundance among them.

2.3.3 Phylogenetic analysis of 16S rRNA gene and genomic sequences

The analysis of 16S rRNA genes revealed a similarity of below 99.7%, considered as a threshold for species delineation, for all the isolates when compared to closely related type strains Whole genome phylogenetic analysis was performed using a concatenated sequence of core genes and considering the pairwise indexes ANI (>95–96%), AAI (>96%) and cAAI (>97%) at intra-species

level, and AAI (>65%), cAAI (>65%) and a G+C content difference of above 1% at intra-genus level. Annotation of trees reconstructed with 16S rRNA gene and genomic sequences depicts the species cluster in the first stripe (Figure 7 to 9). The genomes of *C. butanoliproducens* (Figure 7), *Limosilactobacillus galli* (Figure 8) and *Ligilactobacillus hohenheimensis* (Figure 9) share a species cluster with accession numbers available in the databases [27].

The taxonomic assignment and pairwise index calculation analysis of strain CIES53^T identified as *Faecalispora anaeroviscerum* sp. nov., revealed that the related species '*Clostridium merdae*', '*Clostridium minihomine*', *Clostridium sporosphaeroides* and *Clostridium jeddahense*, assigned to the genus *Clostridium*, family Clostridiaceae [170], belong to the family *Oscillospiraceae* and share a common ancestor. These species are related to the type species *Caproicibacter fermentans* EA1^T, *Caproiciproducens galactitolivorans* BS-1^T, *Neglectibacter timonensis* SN17^T, *Clostridium sporosphaeroides* ATCC 25781^T and *Clostridium jeddahense* JCD^T. The last two were previously classified as *Clostridium* but suggested to be reclassified according to [171], naming them *Faecalispora sporosphaeroides* DSM 1294^T and *Faecalispora jeddahensis* DSM 27834^T. The analysis in figure 10 depicts the phylogenetic and pairwise analysis that supported the description of the novel genus and the further combination of the species to the genus *Faecalispora*.

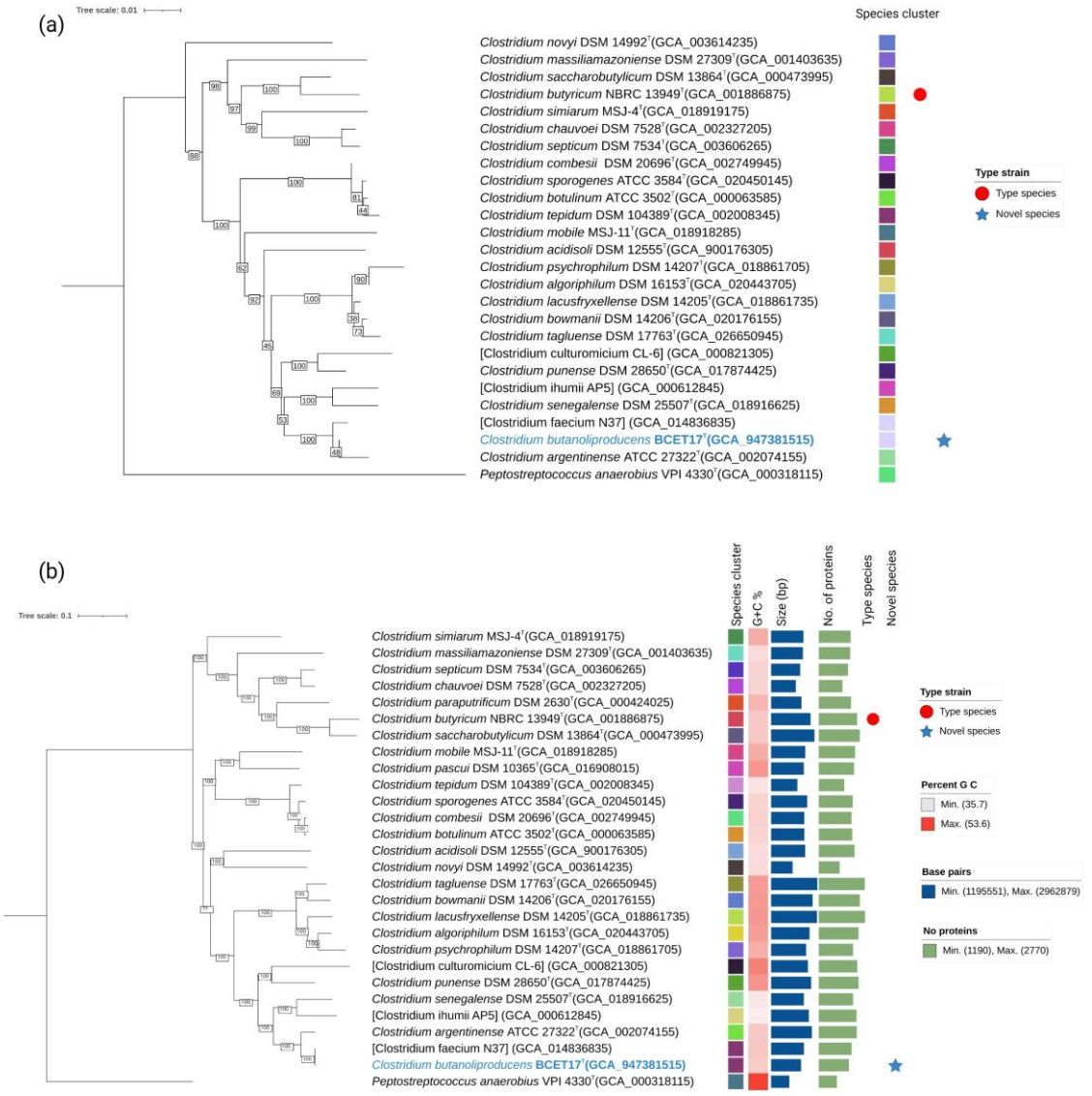

Figure 7. Phylogenetic trees of species from the genus *Clostridium* including the novel species *Clostridium butanoliproducens* and annotation of the related species. Novel species are highlighted in blue. (a) Phylogenetic tree based on the 16S rRNA gene sequences. (b) Phylogenetic tree based on 87 concatenated core genes obtained from each genome. Both trees were inferred by the maximum-likelihood method and visualized with iTol using *Clostridium butyricum* NBRC 13949^T as the genus type species and *Peptostreptococcus anaerobius* VPI 4330^T as an outgroup. Bootstrap values (1000 replicates) are depicted above branches and branch lengths correspond to sequence differences indicated by the scale bar above.

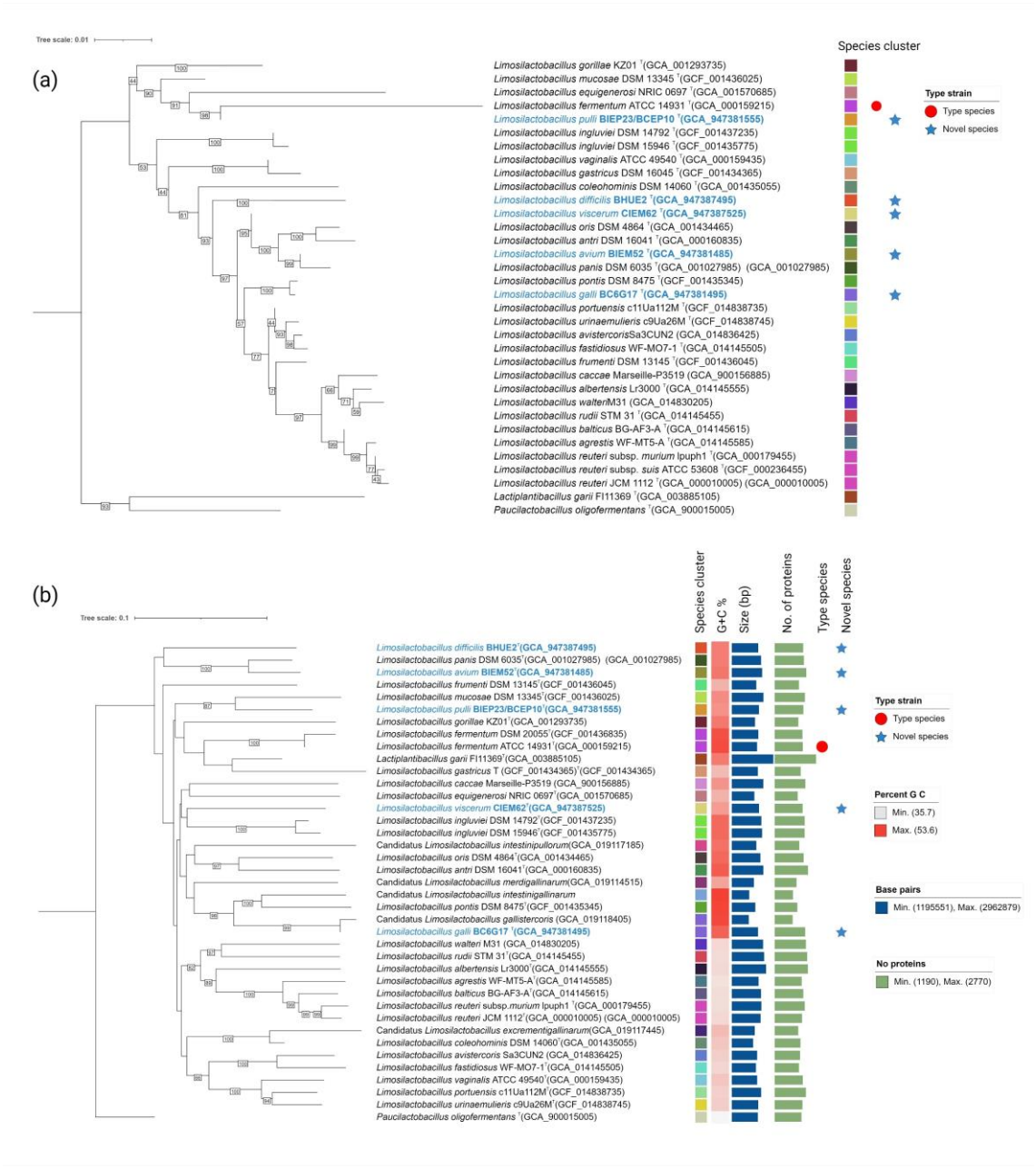

Figure 8. Phylogenetic trees of species from the genus *Limosilactobacillus* and annotation of the related species. Novel species are highlighted in blue. (a) Tree based on the 16S rRNA gene sequences. (b) Tree based on 71 concatenated core genes obtained from each genome. Both trees were inferred by maximum-likelihood method and visualized with iTol using *Limosilactobacillus fermentum* ATCC 14931^T as genus type species and *Paucilactobacillus oligofermentans* AMKR18^T as external group. Bootstrap values (1000 replicates) are depicted above branches, branch lengths correspond to sequence differences indicated by the scale bar above.

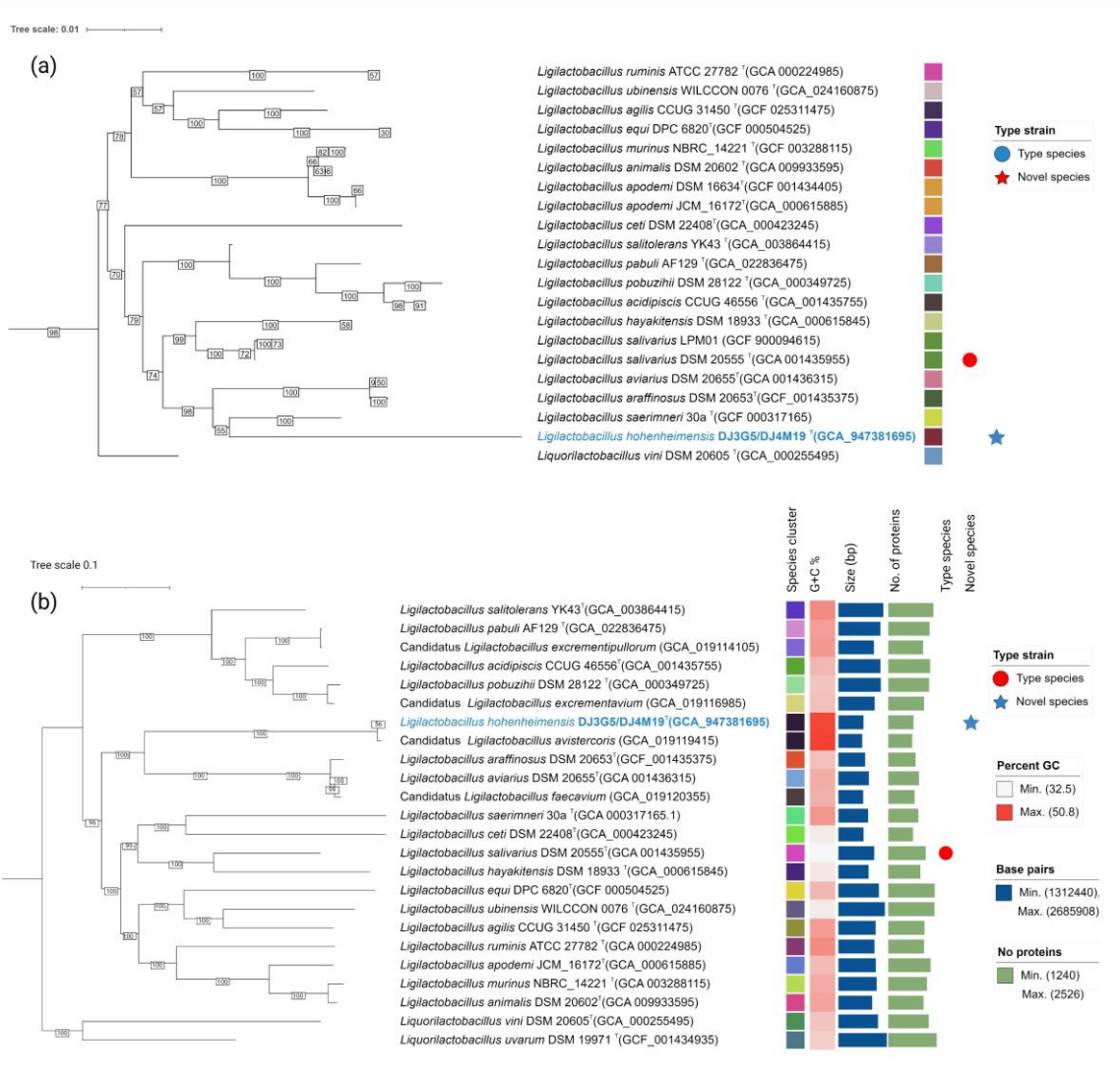

Figure 9. Phylogenetic tree of species from the genus *Ligilactobacillus* including the novel species *Ligilactobacillus hohenheimensis* and annotation of the related species. Novel species is highlighted in blue. (a) Tree based on 16S rRNA gene sequences. (b) Tree based on 91 concatenated core genes obtained from each genome. Both trees were inferred by maximum-likelihood method and visualized with iTol using *Ligilactobacillus salivarius* DSM 20555^T as genus type species and *Liquorilactobacillus vini* DSM 20605^T as external group. Bootstrap values (1000 replicates) are depicted above branches, branch lengths correspond to sequence differences indicated by the scale bar above.

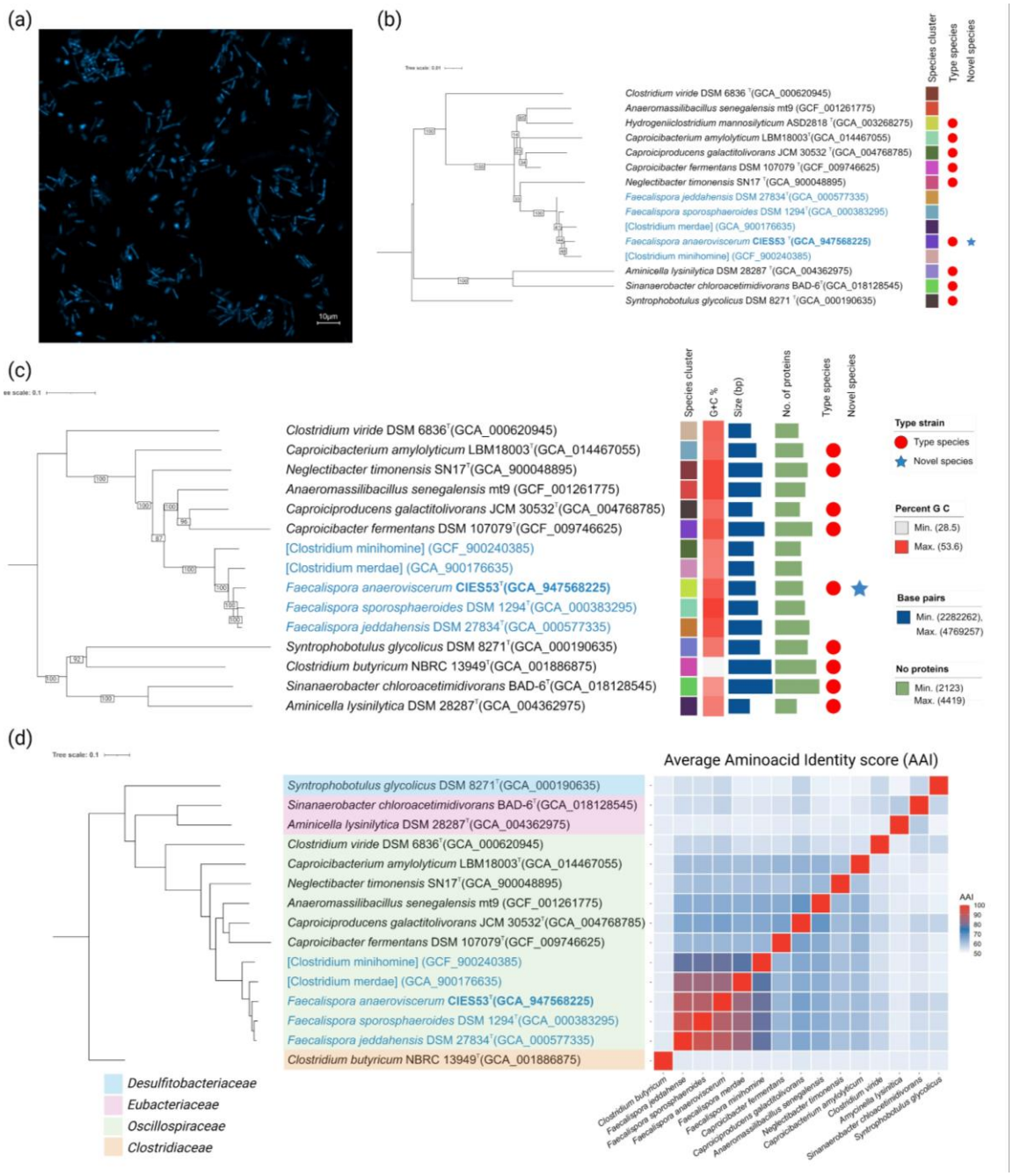

Figure 10. Phylogenetic analysis of species closely related to the novel genus description *Faecalispora* gen. nov. (a) The type species of the genus *F. anaeroviscerum* CIES53^T at 48 h of incubation, stained with DAPI and detected by fluorescent microscopy (Zeiss cLSM900). (b) Tree based on 16S rRNA gene sequences. (c) Tree based on a concatenated sequence of 49 core genes. Both trees were inferred by maximum-likelihood method, annotated according to genome features of each strain, and visualized with iTol. The type species of each genus are marked with a red dot and the clade of *Faecalispora* gen. nov. is marked in blue letters. *Clostridium butyricum* NBRC 13949^T, *Syntrophobotulus glycolicus* DSM 8271^T, *Sinanaerobacter chloroacetimidivorans* BAD-6^T *and Aminicella lysinilytica* DSM 28287^T were used as external groups from the families *Clostridiaceae, Desulfitobacteriaceae* and *Eubacteriaceae* respectively, both figures show monophyly and high similarity among species of *Faecalispora* gen. nov. (d) Correlation matrix of average-amino acid identity (AAI) scores calculated among reference species closely related to *F. anaeroviscerum* CIES53^T. Branch lengths correspond to sequence differences indicated by the scale bar above each tree.

2.3.4 Functional annotation

All strains within the collection, except for *Clostridiaceae* and *Oscillospiraceae* members, can produce lactic acid via lactate dehydrogenase (LDH), the most common fermentative pathway, followed by the acetogenesis pathway, present in all the strains from the collection except for *Ligilactobacillus* species (Figure 11). Across the collection, acetogenesis was detected solely by the acetate kinase, which tells about the community's preference for the reversible nature of the enzyme acetate kinase at the conversion and use of acetate. Additionally, this enzyme that has been reported to stimulate the chemotaxis signal system CheA-CheYBV [172], was detected in species of our collection: *P. bifermentans, F. anaeroviscerum, C. butanoliproducens* and *Clostridium butyricum*.

Further, less common fermentative pathways mapped along the collection were butyrate fermentation, detected in *C. butyricum*, *F. anaeroviscerum*, *C. acnes* and *F. plautii*, followed by succinate and formic acid fermentation, observed only in *C. butyricum* and *C. acnes* (Figure 11). All *Limosilactobacillus* and two strains of *C. butyricum* encode genes to produce ethanol from the fermentation of glucose. Only the novel taxa *C. butanoliproducens* codify genes for butanol production via butanol dehydrogenase during butanoate metabolism.

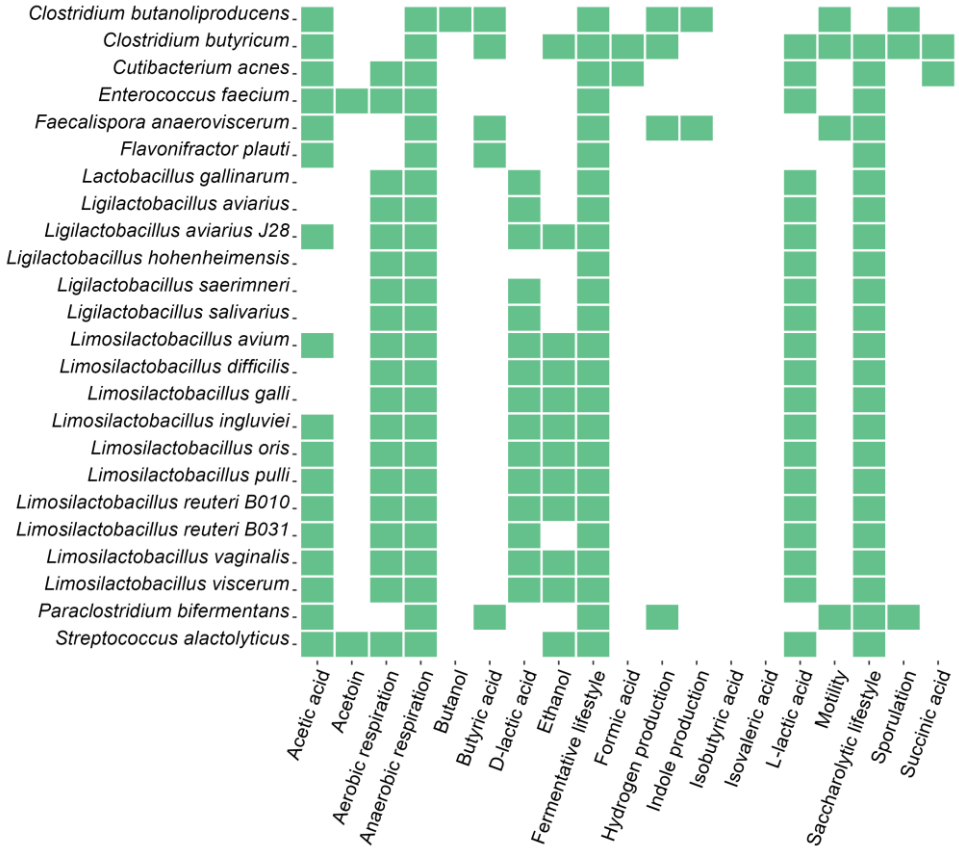

Figure 11. Fermentation pathways mediated by species from the bacterial collection of the upper digestive system of chicken. Presence of fermentation pathways annotated on the genome of each strain. Strains of the same species are included to highlight the differences encoded among them.

The following approach revealed functional differences by the number and type of genes for carbohydrate-active enzymes (CAZyme database) and peptidases (MEROPS database) throughout strains colonising different digestive sections. Despite the diverse origins of the bacterial isolates, the profile of abundant enzymes detected along the three digestive regions was conserved (Figure 12). Punctual differences were highlighted by the presence of taxa in each GIT section.

Among all genes encoding glycosidic enzymes, 51% were detected in all strains and include amylases, glucosidases, muramidases and xylanases. As for peptidase genes, 49% of signal and posttranslational peptidases and amino and carboxypeptidases were shared by all strains from the collection. Abundant genes with the respective predicted enzymatic functions are depicted in Figure 12.

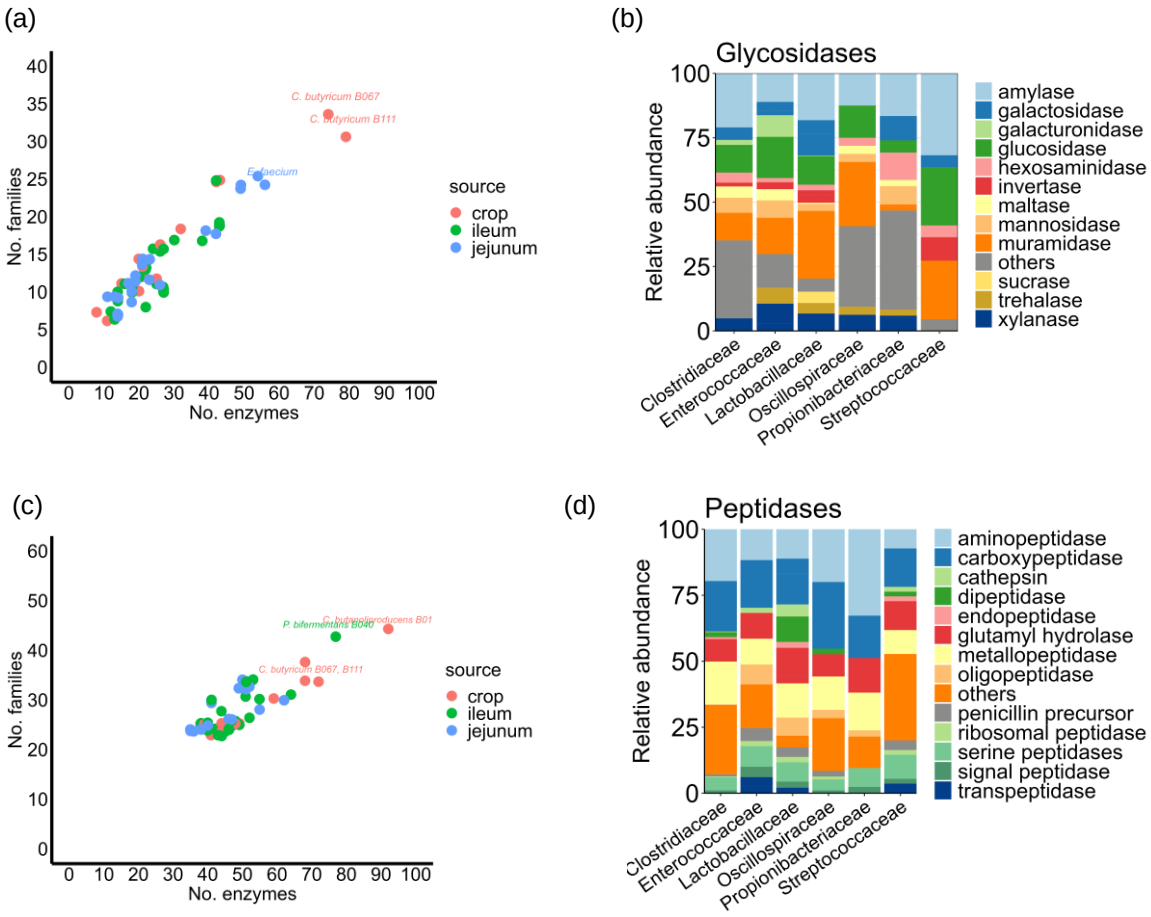

Figure 12. Functional enzymatic annotation across the collection of bacteria from the upper digestive system of chicken. (a and c) Total number of families of glycoside hydrolases and peptidases and (b and d) relative abundance of glycosidase and peptidases encoded by bacteria from the collection at family level.

Commonly shared genes were higher among strains isolated from crop and ileum (22% glycolytic and 27% peptidic) than strains from jejunum and ileum (2% glycolytic and 5% peptidic). These genes included cellobiosidases, fucosidases, galactosidases, glucosaminidases, glycogenases, hyaluronate lyases, mannosidases, peptidoglycan lyases as well as aspartyl proteases,

glutamate synthases, transposases and proteasomes components found in strains from *Lactobacillaceae*, *Clostridiaceae*, *Oscillospiraceae*, *Propionibacteriaceae* and *Streptococcaceae*. Contrastingly, no shared genes for glycolytic nor peptidic enzymes were found between jejunum and crop and only one type of glucuronidase enzyme was found exclusively in jejunum strains. This means that the majority of enzymes encoded by jejunum strains might be present either in crop or ileum.

C. butyricum and *C. butanoliproducens* isolated from crop harbour the highest counts of genes assigned to glycosidases and peptidases (Figure 12A and 12D). Strains recovered from this digestive section encode the highest proportion of section-specific glycosidases (19%) that were not detected on strains from jejunum or ileum. Such enzymes are specialised in the degradation of dextrins, furanosides, glucans, mannans and xyloglucans which underlines the potential of the local microbial community to obtain carbon from complex carbohydrate hydrolysis. Genes encoding peptidases exclusively found at crop isolated strains, were related to sporulation factors from *C. butyricum* and hydrogenase-processing endopeptidases encoded by the novel species of *C. butanoliproducens* and represent 5% of the total of peptidases detected along the collection.

Strains recovered at jejunum belong either to *Enterococcaceae* or to *Lactobacillaceae* family, with *E. faecium* having the highest count and diversity of active enzymes at jejunum compared to *E. faecium* strains recovered from the ileum. Such functional diversity included chitosanase, chitinase, iduronidase, glucuronidase and hexosaminidase enzymes, all devoted to the hydrolysis of complex carbohydrates like chitin, a common polysaccharide from the exoskeleton of insects. At family level, only the group of *Enterococcaceae* presented a higher count of genes for CAZymes at jejunum compared to crop or ileum.

As expected by the anatomy of the GIT section, ileum strains presented less genetic diversity for exclusive glycolytic enzymes than crop (5%) but higher abundance of amylases, glucosidases and muramidases, which indicates the specialisation of these bacteria to the degradation of carbohydrates previously hydrolysed at the upper parts. In this region, 14% of the exclusive peptidases found, included posttranslational peptidases, serine-protease, immune inhibitors, and a protease insulin-like degrading enzymes encoded in the novel species *F. anaeroviscerum*.

2.3.5 Genes encoding host- interactive molecules.

Genes for enzymatic adaptations that allow host-microbe interactions, such as collagenases, pitrilysin and homologues of neprilysin, were detected in strains from *Clostridiaceae*, *Enterococcaceae*, *Lactobacillaceae*, *Oscillospiraceae*, *Propionibacteriaceae* and *Streptococcaceae* (Figure 13). High counts of neprilysin and pitrilysin were found in crop strains of *C. butyricum* and *C. butanoliproducens*, suggesting a higher interaction of these strains to the host endothelins and glucagon/insulin hormones at regions where these strains colonise. Furthermore, collagenase encoding strains were detected in higher numbers in jejunum and ileum, having *E. faecium*, *P. bifermentans* and *S. alactolyticus* the highest counts of collagenase gene clusters detected per genome. Genes encoding aminidases were found mainly as hexosaminidase and sialidases at *C. acnes* and *Lactobacillaceae* strains isolated from all

sections, some other less common aminidases like glucosaminidase and galactosaminidase were exclusively found at crop and ileum encoded by *F. plautii* and *C. acnes*.

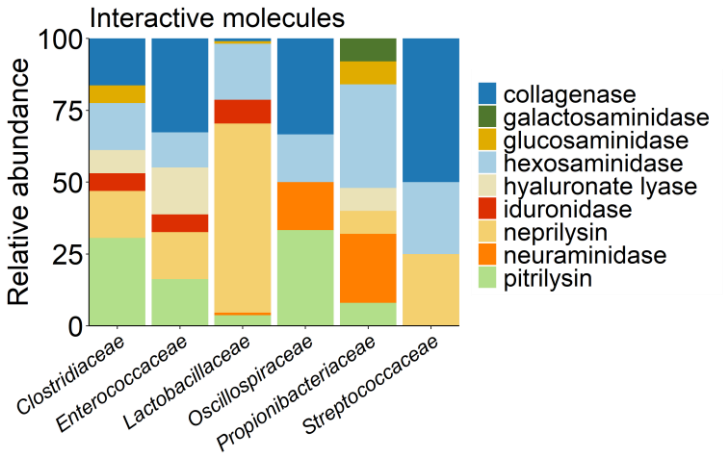

Figure 13. Distribution of genes encoding proteins that mediate interactions with the host across strains from the collection of bacteria from the upper digestive system of chicken grouped at family level.

The presence of hyaluronate lyase clusters along genomes of *C. butyricum*, *E. faecium* and *C. acnes* from the three sections talks about the interaction of these strains to the host digestive extracellular matrix (mucosa layer), and how they are potentially able to hydrolyse hyaluronate chains through a β–hydrolysis process. Such capability is known to be a pathogenic factor for bacterial spreading either by facilitating bacterial invasion breaking the polysaccharide matrix architecture or as an antigenic disguise that prevents the recognition of bacteria by phagocytes. However, such adaptations might represent a possible specialisation to colonise mucosa.

All novel species in this work encoded gene clusters with various phosphorylation activities including protein phosphatases, inorganic phosphatases, nucleotidases, sugar phosphatases and signalling phosphatases involved in chemotaxis response. Some of which might potentially provide a source of available phosphate to the host (Figure 14).

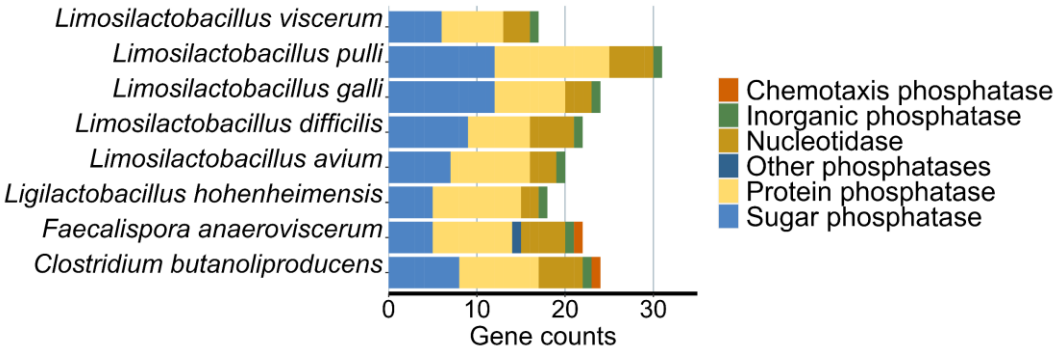

Figure 14. Number of phosphatases group genes present in the novel species of bacteria from the collection of bacteria from the upper digestive system of chicken.

2.3.6 Accessory annotation

Beyond functional genes, bacteria develop additional genome adaptations that reflect the complexity of interactions within members of the community and the environment (host) that assure their individual persistence in the community. The presence and transference of such features and their study, contribute to better understand the bacterial community dynamics, especially in a high transitional environment such as the chicken upper digestive system. In this case, antiviral mechanisms (Figure 15), prophage inclusions (Figure 16), second metabolites defence genes (Figure 17) and antimicrobial resistance were explored using tools for accessory annotation.

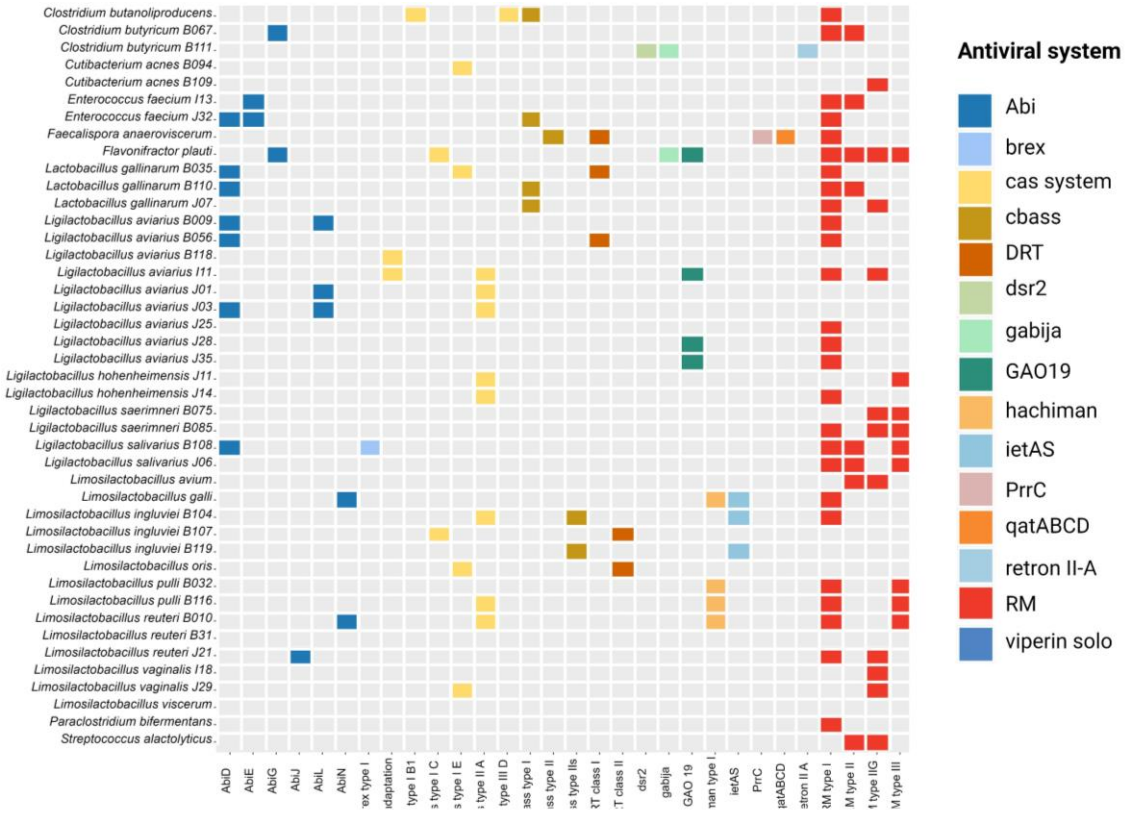

Figure 15. Presence of antiviral proteins and antiviral defence systems detected along the bacterial collection. Strains of the same species are included to highlight the differences encoded among them. Antiviral systems are colour-coded listed in the following order: Abi (Abortive system), brex (Bacteriophage Exclusion), cas system (CRISPR/Cas systems), cbass (Cyclic oligonucleotide-Based Anti-phage Signaling System), DRT (defense-associated reverse transcriptase), dsr2 (defense-associated sirtuin), Gabija (GajA and GajB proteins), GAO19 (enzyme hat mediates antiviral immunity in prokaryotes), Hachiman (genes hamABC), ietAS (satellite antivital protein), PrrC (anticodon nuclease), qatABCD (ATPase system), retron II-A (reverse transcriptase system), RM (Restriction-modification system) , viperin (anti-viral ISGs).

Defence systems to viral infections such as the most common restriction-modification systems were found in strains of *Limosilactobacillus* and *Ligilactobacillus*; whereas the bacterial suicide programming was encoded in all the members of the collection except for all the species of

Clostridium, *C. acnes*, *L. hohenheimensis*, *L. difficilis* and *Limosilactobacillus oris*. Genes for toxin-antitoxin mechanisms (TA) were found in *Enterococcus*, *Lactobacillus* and *Ligilactobacillus*. Strains from ileum presented a higher number of antiviral defence mechanisms compared to their crop or jejunum homologues. Most of these processes involve nonspecific mechanisms that target a broader range of viral invasions by blocking the infection cycle via nucleases, toxins, abortive mechanisms, or the interruption of viral replication/transcription. Contrastingly, few complete prophage insertions were found, in the species *C. butyricum*, *L. aviarius*, *Ligilactobacillus saerimneri*, *L. difficilis*, *Limosilactobacillus reuteri* and *S. alactolyticus,* isolated mainly at ileum and crop.

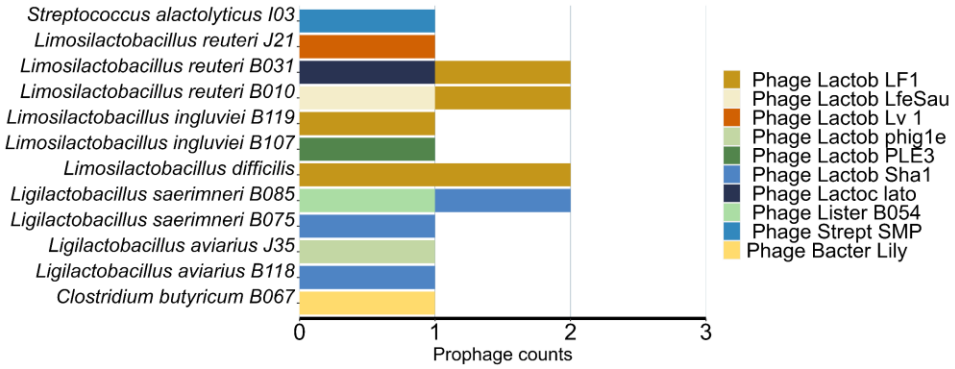

Figure 16. Annotation of prophage insertions detected along the bacterial collection. Strains of the same species are included to highlight the differences encoded among them.

Analyses of genes involved in the potential production of antimicrobials and secondary metabolites revealed one common trait found in all the strains of *Lactobacillaceae*, the type III polyketide synthase (PKSs) gene *mva*S that codifies a catalytic enzyme involved in the biosynthesis of polyketides.

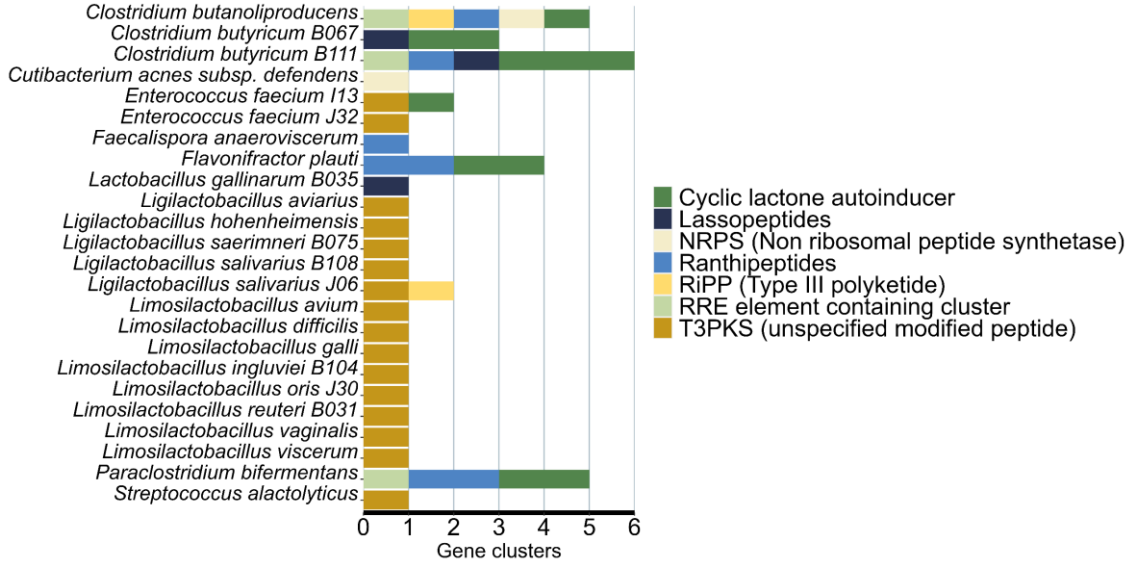

Figure 17. Accessory annotation for antimicrobial peptide precursors detected along the bacterial collection.

This enzyme called hydroxymethylglutaryl-CoA synthase, is involved in the catabolism of fatty acids, and enters the mevalonate pathway producing a precursor on the biosynthesis of isoprenoid compounds with the potential to inhibit pathogenic microorganisms [173, 174]. In addition, a common antimicrobial trait detected in *Clostridium* and *Flavonifractor* strains was the cluster for ranthipeptides related to the synthesis of biologically active molecules with antimicrobial activity [175].

Very few drug resistance mechanisms were found along the collection and most of them included the genes for drug efflux transporters ABC, *Emr*AB, *Mep*A, *Mdl*AB or *Pat*A, and the gyrase-protecting protein that confers resistance against quinolones, present on the species of *Clostridium*, *Limosilactobacillus*, *P. bifermentans* and *S. alactolyticus*. Interestingly, the ABC multidrug efflux pump *Efr*AB was detected in all the strains from the collection, suggesting it to be a useful trait within this environment.

2.3.7 Taxonomic descriptions

The classification of the isolates was done based on genome sequences using the GTDB database (R207_v2) with the tool GTDB-Tk and constructing phylogenomic trees to either confirm or deny the novel delineations. Thresholds of 16S rRNA gene sequence identities were in most cases not considered as indication of a novel species. However, dDDH values <70% and ANI values <95% were considered as thresholds for separated species. Additionally, differences within-species in the G+C content of DNA (>1%) also supported the status of distinct species although not in all cases.

Description of *Limosilactobacillus galli* sp. nov.

Limosilactobacillus galli (gal'li. L. gen. n. *galli*, of a chicken).

Cells are anaerobic facultative, stain positive to Gram stain and have a short rod shape. The genome encodes for aminotransferase class I and II, the Complex V (ATP synthase) and for genes predicting the utilization of lactate, cellulose, and oligosaccharides as carbon sources. Formation of ethanol and lactic acid after fermentation of sugars, and glutaredoxin are predictable. Sugar phosphatases *ybi*V, *ybj*I and *yid*A, dITP/XTP pyrophosphatase, oligoribonuclease and PAP phosphatases, pyrophosphatase *ppa*X, phosphoserine phosphatase, tyrosine phosphatase, phosphoglycolate phosphatase, threonine phosphatase, exopolyphosphatase are encoded. The biochemical test reported that on Biolog agar media with 5% sheep blood incubated at 37°C under microaerophilic conditions, the species assimilated D-maltose, sucrose, D-raffinose, fructose, lactic acid, serine, alpha keto-glutaric acid, acetic acid, butyric acid, sodium butyrate and acetoacetic acid, and tolerate minocycline, tetrazolium violet, tetrazolium blue potassium tellurite, Tween 40, 8% of NaCl and sodium bromate. The predominant cellular fatty acids were $C_{16:0}$, $C_{18:0}$ and $C_{19:0}$. The type strain BC6G17^T (DSM 113833 ^{T} and LMG 32623^T) was isolated in Gut Microbiota Medium from crop of Brown Lohmann laying hens. Genome sequence and 16S rRNA gene are available at the NCBI database under the accessions GCA_947381495 and OM760982 respectively.

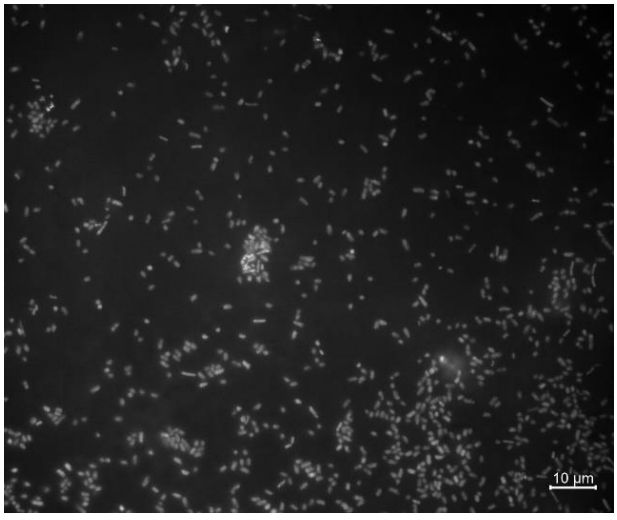

Figure 18. *Limosilactobacillus galli* stained with DAPI and detected by fluorescent microscopy (Zeiss cLSM900).

Description of *Limosilactobacillus avium* sp. nov.

Limosilactobacillus avium: (a' vi. um. L. gen. pl. n. avium, of birds).

Cells are anaerobic facultative, Gram-stain-positive and have a short rod shape. The genome encodes flavin prenyltransferase, aminotransferase class I and II, complex V (ATP synthase) and subunit I of cytochrome ubiquinol oxidase. Utilization of lactate, cellulose, rhamnosides and xylosides from hemicellulose and oligosaccharides as carbon substrates and formation of ethanol, acetate and lactic acid after fermentation of sugars is encoded. Genes for sugar phosphatases ybiV, ybjI and yidA, dITP/ XTP pyrophosphatase, oligoribonuclease and PAP phosphatases, pyrophosphatase PpaX, phosphoserine phosphatase, tyrosine phosphatase, phosphoglycolate phosphatase, threonine phosphatase, and exopolyphosphatase are identified. On Biolog agar media with 5 % sheep blood incubated at 37 °C under microaerophilic conditions, the species assimilates trehalose, gentiobiose, glucose, raffinose, fructose, lactic acid, serine, sorbitol, glucoronamide, α-keto-glutaric acid, α-keto-butyric acid, acetic acid, butyric acid, propionic acid, formic acid, acetoacetic acid, sodium butyrate and sodium lactate, and tolerates rifamycin, minocycline, potassium tellurite, guanidine HCl, vancomycin, tetrazolium, nalidixic acid, 8 % NaCl and sodium bromate. The predominant cellular fatty acids are C16: 0 and summed feature 7 ($C_{19:0}$ cyclo ω10c/19 ω6 and $C_{19:1}$ ω6c). The type strain, BIEM52T (=DSM 113849T=LMG 32671T), was isolated on MRS medium from ileum of Brown Lohmann laying hens. The genome and 16S rRNA gene sequences are available from the NCBI database under the accessions GCA_947381485 and OM760983, respectively.

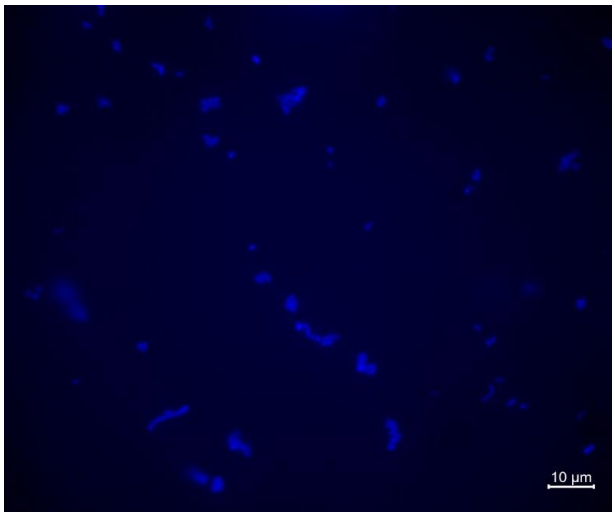

Figure 19. *Limosilactobacillus avium* stained with DAPI and detected by fluorescent microscopy (Zeiss cLSM900).

Description of *Limosilactobacillus pulli* sp. nov.

Limosilactobacillus pulli (pul'li. L. gen. n. pulli, of a chicken).

Cells are anaerobic facultative, Gram-stain-positive and have a small rod shape. The genome encodes for phosphoserine and the aromatic aminotransferases for histidinol, aspartate and tyrosine aromatic aminotransferase, aminotransferase class I and II, complex V (ATP synthase), and subunit I of cytochrome ubiquinol oxidase. Utilization of lactate, cellulose and oligosaccharides through β-glucosidases and β-galactosidases as possible carbon substrates and formation of ethanol acetate and lactic acid after fermentation of sugars is encoded. Genes encoding acylphosphatases, alkaline phosphatase, sugar phosphatases YbiV, YbjI and YidA, dITP/XTP pyrophosphatase, oligoribonucleases, pyrophosphatase PpaX, phosphoserine phosphatase, tyrosine phosphatase, phosphoglycolate phosphatase, threonine phosphatase and exopolyphosphatase are identified. On Biolog agar media with 5 % sheep blood incubated at 37 °C for 48 h under anaerobic conditions, the species assimilates dextrin, d-glucose, d-fructose, d-galactose, d-glucuronic acid, maltose, turanose, fucose, gentiobiose, glucuronamide, inosine, pectin, sucrose, stachyose, sodium lactate, sodium butyrate, d-serine and l-arginine, and tolerates fusidic acid, lincomycin, minocycline, nalidixic acid, potassium tellurite, rifamycin, tetrazolium, troleandomycin, Tween 40, vancomycin and 1–4 % NaCl. The predominant cellular fatty acids are $C_{16:0}$, $C_{18:0}$ and summed feature 7 ($C_{18:1}$ ω7c and $C_{18:1}$ ω6c).

The type strain, BIEP23/BCEP10T (=DSM 115077T=LMG 32877T), was isolated in PFA medium from the ileum of Brown Lohmann laying hens. Genome and 16S rRNA gene sequences are available from the NCBI database under the accessions GCA_947381555 and OQ831033, respectively.

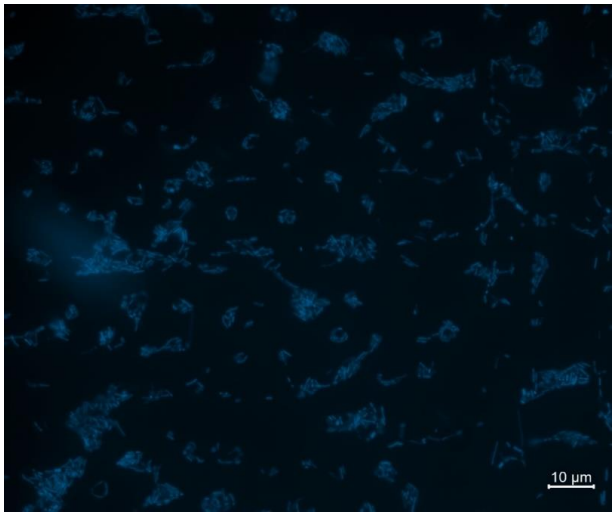

Figure 20. *Limosilactobacillus pulli* stained with DAPI and detected by fluorescent microscopy (Zeiss cLSM900).

Description of *Limosilactobacillus viscerum* sp. nov.

Limosilactobacillus viscerum (vis' ce. rum. L. gen. pl. n. *viscerum*, from the internal organs).

Cells are anaerobic facultative, Gram-stain-positive and have a short rod shape. The genome encodes for aminotransferase class I and II, and the ATPase proton pump type F (complex V). Utilization of lactate, cellulose and xylose from hemicellulose as possible carbon substrates and formation of formate, acetate, ethanol and lactic acid from the fermentation of sugars is encoded. Genes for exopolyphosphatases, nucleosidases, tyrosine phosphatases, uracil phosphatase, threonine and serine phosphatase, sugar phosphatases YbiV and YbjI, dITP/XTP pyrophosphatase, and acylphosphatase were identified. On Biolog agar media with 5 % sheep blood incubated at 37 °C under microaerophilic conditions, the species assimilates maltose, raffinose, lactose, d-glucose, d-mannitol, d-serine, α-keto-glutaric acid, acetoacetic acid and sodium butyrate. Tolerates minocycline, tetrazolium violet, tetrazolium blue, Tween 40, potassium tellurite and sodium bromate. The predominant cellular fatty acids are $C_{16:0}$, $C_{18:0}$ and cyclopropane acid $C_{19:0}$ ω8c.

The type strain, CIEM62T (=DSM 113835T=LMG 32625T), was isolated in MRS medium from the ileum of Brown Lohmann laying hens. Genome and 16S rRNA gene sequences are available from the NCBI database under the accessions GCA_947387525 and OM760985, respectively.

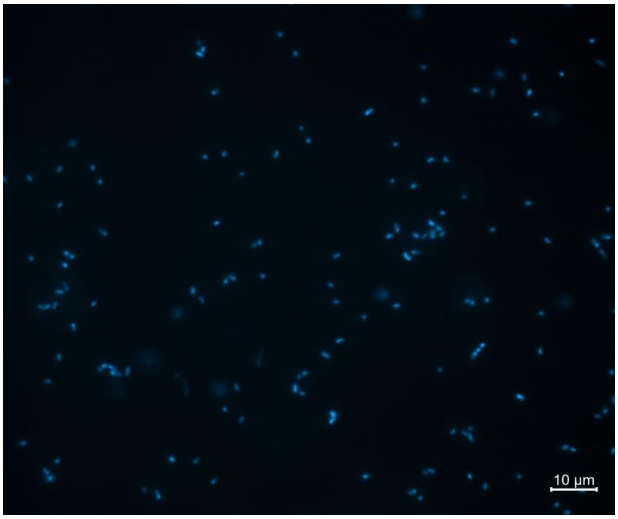

Figure 21. *Limosilactobacillus viscerum* stained with DAPI and detected by fluorescent microscopy (Zeiss cLSM900).

Description of *Limosilactobacillus difficilis* sp. nov.

Limosilactobacillus difficilis (dif. fi' ci. lis. L. masc. adj. *difficilis*, difficult).

Cells are anaerobic facultative, Gram-stain- positive and have a short rod shape. The genome encodes for phosphoserine aminotransferase, aminotransferase class I and II, branched-chain amino acid aminotransferase, histidinol aminotransferase, the ATPase proton pump type F (complex V), subunit I of cytochrome ubiquinol oxidase, and chlorite dismutase. Utilizes lactate, cellulose, rhamnose and xylose from hemicellulose debranching and oligosaccharides as

possible carbon substrates and forms acetate, ethanol and lactic acid from the fermentation of sugars. Genes encoding tyrosine phosphatases, sugar phosphatases YidA, exopolyphosphatases, uracil phosphatase, tyrosine-phosphatase, dITP/XTP pyrophosphatase, histidinol phosphatase, phosphoglycolate phosphatase, acylphosphatase, threonine and serine phosphatase, pyrophosphatase PpaX, and nucleosidase are identified. On Biolog agar media with 5 % sheep blood incubated at 37 °C for 18 h under microaerophilic conditions this species can assimilate cellobiose, dextrin, d-fructose, d-fucose, d-galactose, d-galacturonic acid, d-glucose, d-glucuronic acid, lactose, maltose, d-mannose, melibiose, raffinose, S-salicin, d-serine, d-sorbitol, trehalose, turanose, glucuronamide, inosine, l-fucose, l-galactonic acid lactone, l-rhamnose, N-acetyl neuraminic acid, N-acetyl- d- galactosamine, N-acetyl- d- glucosamine, N-acetyl-d-mannosamine, pectin, stachyose, sucrose, acetic acid, acetoacetic acid, hydroxy-butyric acid, keto-butyric acid, keto-glutaric acid, methyl-d- glucoside, formic acid, propionic acid, sodium butyrate and sodium lactate. Tolerates aztreonam, fusidic acid, gentiobiose, guanidine HCl, lincomycin, lithium chloride, minocycline, nalidixic acid, niaproof 4, potassium tellurite, rifamycin, tetrazolium, troleandomycin, Tween 40 and vancomycin. The predominant cellular fatty acids are $C_{16:0}$, cyclopropane acid $C_{19:0}$ ω8c and summed feature 8 ($c_{18:1}$ ω7c).

The type strain, BHUE2T (=DSM 114195T=LMG 32875T), was isolated in MRS medium from the ileum of broilers Ross 308. Genome and 16S rRNA gene sequences are available from the NCBI database under the accessions GCA_947387495 and OQ832131, respectively.

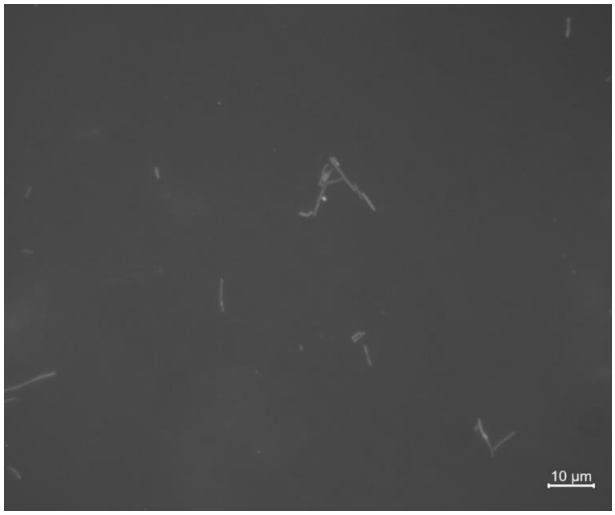

Figure 22. *Limosilactobacillus difficilis* stained with DAPI and detected by fluorescent microscopy (Zeiss cLSM900).

Description of *Ligilactobacillus hohenheimensis* sp. nov.

Ligilactobacillus hohenheimensis (ho. hen. heim. en'sis. N.L. masc. adj. *hohenheimensis*, pertaining to Hohenheim, Germany; the place of isolation). Cells are anaerobic facultative, Gram-stain- positive and have a short rod shape. The genome encodes β-glucoronidases, β-galactosidases and hexosaminidases, and the ATPase proton pump type F (Complex V).

Utilization of lactate and cellulose, and formation of acetate, ethanol and l-lactic acid from the fermentation of sugars is predicted by the genes. Genes encoding acylphosphatase, nucleosidase, dITP/XTP pyrophosphatase, threonine and serine phosphatase, exopolyphosphatases, pyrophosphatase ppaX, tyrosine phosphatases, tyrosine-phosphatase, and phosphoglycolate phosphatase are identified. On Biolog agar media with 5 % sheep blood incubated at 37 °C for 48 h under anaerobic conditions, the species assimilates maltose, d-serine, d-fructose-6-PO4, l-histidine, glucuronamide, sodium lactate, sodium butyrate, quinic and mucic acid. Tolerates 8 % NaCl, minocycline, guanidine HCl, lithium chloride, potassium tellurite, Tween 40, tetrazolium violet and tetrazolium blue. The predominant cellular fatty acids are $C_{18:0}$ and $C_{16:0}$.

The type strain, DJ3G5/DJ4M19T (=DSM 113870T=LMG 32876T), was isolated in MRS medium from the jejunum of broilers Ross 308. Genome and 16S rRNA gene sequences are available from the NCBI database under the accessions GCA_947381805 and OM760988, respectively.

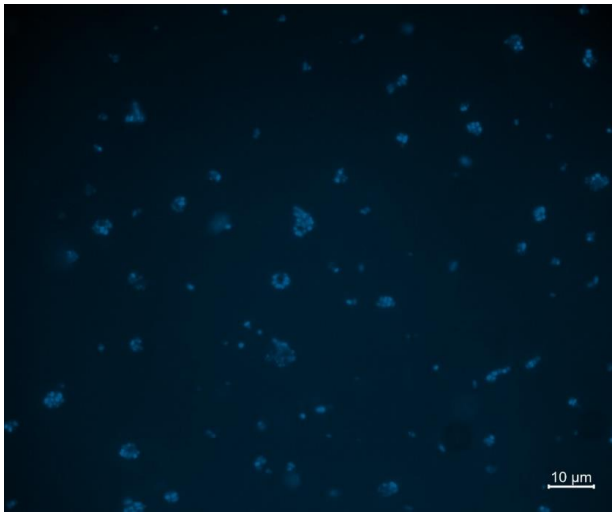

Figure 23. *Ligilactobacillus hohenheimensis* stained with DAPI and detected by fluorescent microscopy (Zeiss cLSM900).

Description of *Clostridium butanoliproducens* sp. nov.

Clostridium butanoliproducens (bu.ta.no.li.pro.du'cens. L. neut. n. *butanol*, butanol; L. pres. part. *producens*, producing; N.L. part. adj. *butanoliproducens*, butanol producing).

Cells are strictly anaerobic, stain positive to Gram stain and have a rod shape with endospore production. The genome encodes branched-chain amino acid aminotransferases, aspartate and tyrosine aminotransferases, acyl-CoA dehydrogenase, formate C-acetyltransferase and the Wood Ljungdahl pathway. It presents gene clusters for nitrogen fixation (*nif*DK), sulphur oxidation (*dsr*AB), sulphite reduction (*asr*ABC), a cytoplasmic Fe-Fe hydrogenase and the ATPase proton pump type V and F (Complex V). Degrade chitin and produce ethanol butanol and acetate. Encode nucleoside triphosphatases, phosphoglycolate phosphatase, dTTP/UTP pyrophosphatases, phosphosulfolactate phosphatase, arginine-phosphatases, histidinol

phosphatases, CheY-P phosphatase (chemotaxis), sugar phosphatases *ycd*XY and *yid*A, dITP/XTP pyrophosphatase, and pyrophosphatases *ppa*X. The biochemical profile test reported that on Biolog agar media with 5% sheep blood incubated at 37°C for 47.25 h under anaerobic conditions, the species assimilated dextrin, D-galacturonic acid, D-glucose, D-glucuronic acid, D-fructose, fucose, glucuronamide, L-galactonic acid lactone, L-rhamnose, pectin, acetoacetic acid, aminobutyric acid, hydroxy-butyric acid, keto-butyric acid, keto-glutaric acid, p-hydroxy-phenylacetic acid, sodium lactate, sodium butyrate, butyric acid and D-serine. It tolerates aztreonam, fusidic acid, gentiobiose, guanidine HCl, lincomycin, lithium chloride, minocycline, nalidixic acid, niaproof 4, potassium tellurite, rifamycin, tetrazolium, troleandomycin, Tween 40, vancomycin and NaCl at 1, 4 and 8% concentration. The predominant cellular fatty acids are $C_{16:0}$ and the summed feature 4 ($_{17:1}$ iso I/anteiso B).

The type strain BCET17^T (DSM 115076^T and LMG 32878^T) was isolated in Tryptic Soy-bean Agar medium from the crop of Brown Lohmann laying hens. Genome sequence and 16S rRNA gene are available at the NCBI database under the accessions GCA_947381515 and OQ831034 respectively.

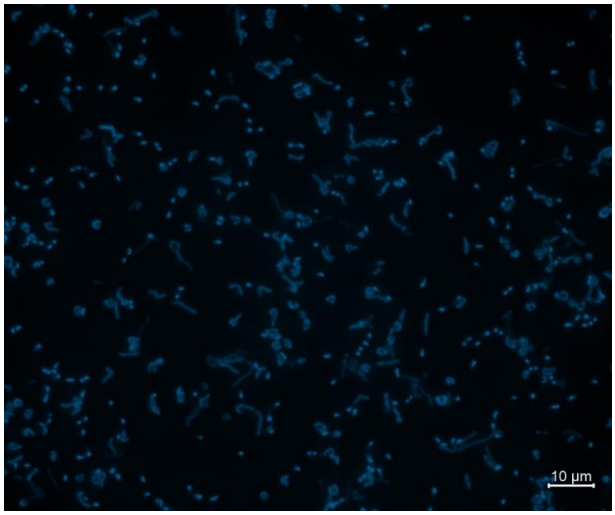

Figure 24. *Clostridium butanoliproducens* stained with DAPI and detected by fluorescent microscopy (Zeiss cLSM900).

Description of *Faecalispora* gen. nov.

Faecalispora (Fae.ca.li.spo'ra. N.L. masc./fem. adj. *faecalis*, pertaining to faeces; Gr. fem. n. *spora*, a seed, in biology, a spore; N.L. fem. n. Faecalispora, a spore-forming organism from faeces).

A bacterial genus identified by genomic analysis of the species *F. anaeroviscerum*, assigned to the family *Oscillospiraceae*. The species *Clostridium jeddahense* and *Clostridium sporosphaeroides*, formerly assigned to *Clostridium* [34, 36], are closely related species that cluster together as members of the novel genus *Faecalispora* and share a common ancestor according to the phylogenetic analysis (Figure 10).

Description of *Faecalispora anaeroviscerum* sp. nov.

Faecalispora anaeroviscerum (an. a. e. ro. vis' ce. rum. Gr. pref. *an*, not; Gr. masc. n. *aer*, air; L. neut. pl. n. *viscera*, internal organs of the body; N.L. gen. pl. n. *anaeroviscerum*, anaerobic from the internal organs).

Cells are strictly anaerobic, Gram-stain- positive, rod-shaped and do not produce endospores. The genome encodes for aminotransferase class I and II, phosphoserine aminotransferase, ornithine aminotransferase branched-chain amino acid aminotransferase, aspartate, and tyrosine aromatic aminotransferase, histidinol aminotransferase, and acyl-CoA dehydrogenase. Presents gene clusters for sulphur oxidation (dsrAB), sulphite reduction (asrABC), a cytoplasmic Fe-Fe hydrogenase, and the ATPase proton pump type V and F (complex V). Degrades cellulose and chitin and utilizes ethanol, butanol, and lactate as carbon sources. Transforms pyruvate to formate. Genes for histidinol phosphatases, dITP/XTP pyrophosphatase, cheY-P phosphatase (chemotaxis), Undecaprenyl-diphosphatase, pyrophosphatases ppaX, phosphoglycolate phosphatase, tyrosine-phosphatase, phosphoserine phosphatase, arginine phosphatases, phosphoglycolate phosphatase and nucleoside phosphatases are encoded. On Biolog agar media with 5 % sheep blood incubated at 37 °C for 22 h under anaerobic conditions, the species assimilates maltose, cellobiose, gentiobiose, turanose, raffinose, melibiose, d-glucose, d-mannose, d-fructose, d-galactose, 3-methyl glucose, d-fucose, l-fucose, l-rhamnose, sodium lactate, d-serine, d-glucose-6-PO_4, d-fructose- 6- PO_4, l-histidine, l-serine, pectin, d-galacturonic acid, l-galactonic acid lactone, d-glucuronic acid, glucuronamide, mucic acid, amino-butyric acid, β-hydroxy-d, l-butyric acid, α-keto-butyric acid, acetoacetic acid, propionic acid, acetic acid, formic acid and sodium butyrate. Tolerates troleandomycin, rifamycin, minocycline, lincomycin, guanidine HCl, vancomycin, tetrazolium violet, tetrazolium blue, p-hydroxy-phenylacetic acid, methyl pyruvate, nalidixic acid, lithium chloride, potassium tellurite, aztreonam and sodium bromate. The predominant cellular fatty acids are $C_{14:0}$, $C_{16:0}$ N OH and summed feature 4 ($C_{17:1}$ iso I/anteiso B).

The type strain, CIES53T (=DSM 113860T=LMG 32675T), was isolated in Postgate standard medium from the ileum of Brown Lohmann laying hens. Genome and 16S rRNA gene sequences are available from the NCBI database under the accessions GCA_947568225 and OM760984, respectively.

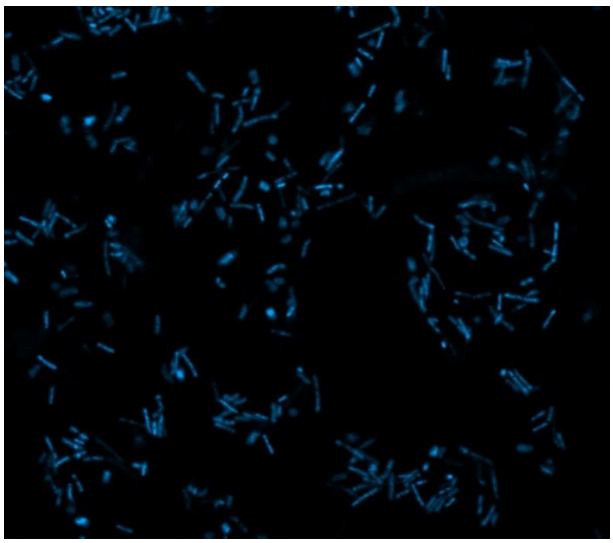

Figure 25. *Faecalispora anaeroviscerum* stained with DAPI and detected by fluorescent microscopy (Zeiss cLSM900).

2.3.8 Strains deposit

Novel species cultures were deposited to the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) and to the Belgian Coordinated Collection of Microorganisms (BCCM) and are available to public access under the numbers provided at the table 5. Biological material was shared in agreement to the Nagoya protocol published in February 2018.

Table 5. Novel bacterial species isolated from crop and small intestine of chicken.

Novel taxonomy assignation	Genome size	GC %	ANI %	Gut region	DSMZ accession number	BCCM accession number	16S accession number
Limosilactobacillus galli	1861581	50.43	83.25	crop	DSM 113833	LMG32623	OM760982
Limosilactobacillus avium	2158112	48.49	92.86	ileum	DSM 113849	LMG 32671	OM760983
Limosilactobacillus pulli	1924125	45.87	86.82	ileum	DSM 115077	LMG 32877	OQ831033
Clostridium butanoliproducens	3507657	28.34	87.39	crop	DSM 115076	LMG 32878	OQ831034
Faecalispora anaeroviscerum	2921858	50.36	85.44	ileum	DSM 113860	LMG 32675	OM760984
Limosilactobacillus viscerum	1993658	44.9	77.84	ileum	DSM 113835	LMG32625	OM760986
Ligilactobacillus hohenheimensis	1381484	50.47	69.45	jejunum	DSM 113870	LMG32876	OM760988
Limosilactobacillus difficilis	1998683	47.38	69.75	ileum	DSM 114195	LMG 32875	OQ832131

2.4 Discussion

The anatomical and physiological differences along the gut intestinal tract (GIT) of chickens have biased the study of gut microbiomes as independent environments with poor-traceable approaches. The upper digestive parts play a key role in the efficient utilisation of nutrients. It also represents the first defence line against pathogenic organisms that enter the digestive system with both the adaptive-innate immune functions and the presence of *Lactobacillaceae* capable of antagonising these organisms [39]. Yet, cultivation of bacteria from the chicken digestive system has focused on caeca and faeces samples and the search for pathogenic strains, leaving the upper regions less explored. Although cultivation methods carry a selection bias to microorganisms that adapt better to the culture conditions, and their prevalence and abundance do not always reflect the ecology and dynamics of the community, efforts in cultivating chicken bacteria help to broaden the pool of chicken gut microbiomes and manipulate strains formerly represented only by metagenomic sequencing. In this work we succeeded in isolating two *Candidate* novel species described only as metagenomes [27].

Former studies have described *Bacillota* as a major representative of dominant groups at the crop, gizzard and small intestine [35, 36, 38, 49]. The initial screening of our isolates with ARDRA revealed that 80% belonged to *Bacillota* and, among them, 20% to the *Lactobacillaceae* family. This information, together with the knowledge provided by the strain genome sequences, improves the study of community dynamics through the eyes of ecology, providing an extent of the niche role within the community even though all representatives are not yet cultured.

2.4.1 The ecology of the collection along the digestive tract

The collection is composed of heterotrophic fermentative and anaerobic bacteria that obtain energy and carbon from the oxidation of carbohydrates (from glucose, pentose, maltose, cellobiose and cellulose), aliphatic amino acids (glutamine, lysine, arginine, serine) and organic acids oxidation (acetate, lactate, butyrate, and long chain fatty acids). Niches might differ along the intestine depending on the availability and complexity of nutrients at the digestion stage. Still, the primary energy and carbon fluxes lead to the formation of lactate, acetate, butyrate, succinate, ethanol, and butanol. The prevalence and relative abundance of our isolates in the hosts metagenomes, highlighted a successful isolation strategy of abundant strains within the environment. In this sense, the functional description of such genomes and their accessory genes potentially reflect their ecological role along the digestive tract.

In crop, the high substrate availability comes along with the high number of genes encoding enzymes specialised in the degradation of complex carbohydrates (dextrins, furanosids, glucans, mannans and xyloglucans) found in genomes isolated from that section. Although caecum is considered a region with complex carbohydrate fermentation [28], polysaccharide hydrolysis might be taking place in significant amounts already in the upper parts. However, due to the low absorption capacity of crop [42], substrate availability might be profited either at lower digestive regions or consumed by other commensal microorganisms. The colonisation of fermentative microorganisms such as *Lactobacillus, Ligilactobacillus* and *Limosilactobacillus* might supply

fermentation products and simpler carbohydrates to the digesta content, and the detection of these microorganisms at ileum and caeca assessed by metagenome mapping, confirms the role of crop on providing metabolically active bacteria to the lower GIT sections.

The dominant groups in crop were *Clostridiaceae*, *Streptococcaceae* and *Lactobacillaceae*, specifically *Lactobacillus* and *Limosilactobacillus* genera, which dominated above *Ligilactobacillus*. Strains from these groups encoded a high number of glycolytic enzymes and antiviral mechanisms, most of which had a broad protection mechanism less specific to the target. Such metabolically active potential with less specific antiviral system profile suggests a highly transitional section, with frequent environmental changes and high niche occupation dynamics. In contrast, strains isolated from crop from the Family *Lactobacillaceae*, *Clostridiaceae* and *Propionibacteriaceae*, presented the highest diversity of genes codifying antimicrobial compounds, which expression might prevent the further access of pathogenic organisms to the digestive system.

Two novel taxa of *Limosilactobacillus* were cultivated for the first time. *L. galli* isolated from the crop, and *L. pulli* obtained from crop and ileum. Both species seem to have a colonisation preference to crop but are present along the intestine in different proportions. They both presented a high count of genes encoding sugar and protein phosphatases including phosphoserine phosphatases, tyrosine-phosphatases and exopolyphosphatase, a linear polymer of residues of orthophosphate involved in energy storage, that might compete with the host for phosphate scavenging but can also be a colonisation advantage involved in bacterial motility or biofilm formation [176].

The third new species description of *C. butanoliproducens* was isolated from crop at relatively high redundancy. Interestingly, this species was not found in any of the metagenomes, which can either be explained by a presence of such organisms in a dormant state (spore), a low abundance of the species not covered by the metagenome sequencing, or a cultivation selectivity of the isolation strategy from which these strains were obtained. *S. alactolyticus,* isolated initially from jejunum, depicted very high abundance at the crop, suggesting to be a dominant member of the upper regions. Although not detected in this work, *S. alactolyticus* has also been isolated from the caecum and evaluated as a potential probiotic for chicken [177].

In jejunum, the diversity of strains isolated had the least representatives regarding taxonomic diversity therefore, metabolic functionality observations were minor than at crop or ileum. Compared to the enzymatic adaptability, the amount and diversity of genes assigned to interactive molecules with the host and the accessory adaptations encoded by strains isolated in this region are the lowest of the three GIT sections. They encoded the lowest number of glycosidase families and did not possess any exclusive enzyme, except for an α-glucoronidase detected in isolates of *E. faecium* from jejunum, but not present at the homologues of ileum. This enzyme would hydrolase glucuronosyl bonds in the main chain of "hardwood" xylans which might be an adaptation of *Enterococcus* strains in this section. This species was redundantly isolated in higher

amounts from jejunum and ileum of broilers which suggests a high colonisation rate in this specific region.

All isolates presented a cluster involved in the secondary bile acid metabolism; an important adaptation of this region due to the high concentration of bile secretions that enter the anterior jejunum. Previous descriptions consider *E. faecium* a safe probiotic with strong intestinal adhesion and colonisation ability that can inhibit harmful microorganisms in the intestine [178]. An adhesive related protein (collagen adhesin) was found in all strains of *E. faecium*, facilitating bacterial adhesion to tissue structures with the corresponding ligand. It may also explain why *E. faecium* was not detected when mapped at other regions like crop or caeca, making it a region-specific coloniser.

Studies have shown that *E. faecium* is a preventive treatment against *Salmonella* Typhimurium infection to broilers that reduces the pathological changes in the liver and intestine and the levels of inflammatory factors such as IL-1β, TNF-α, and IL-8. Additionally, when given as an additive to chicken feed, growth performance and absorption of nutrients were significantly improved [179]. Such evidence of host and strain specificity has thriven *E. faecium,* a functional probiotic for chicken approved by the EU since 2011 [180].

An important contribution of this work is the cultivation of *L. hohenheimensis*, a novel species of *Ligilactobacillus* with a very reduced genome (1.3 mbp) compared to the rest of the members of the genus that were isolated with low redundancy from the jejunum of broilers (3 isolates). The success of its isolation may be a consequence of the enrichment of the samples that preceded isolation. It had the highest abundance and prevalence at ileum, although it was also mapped at crop and caeca with lower prevalence. Functionally has a very narrow potential of partially degrading cellulose, lactose, lactate and a complete absence of amino acid utilisation genes. Such functional carbon utilisation reduction suggests an adaptation to a commensal member of the community that depends on the nutrient supplies from the host or the environment.

The GIT section with the highest cultivation diversity and metabolic functionality in this work was ileum. Strains at this section presented the highest diversity of genes encoding peptidases and host-interactive molecules, antiviral mechanisms, and prophage inclusions. The dominant group was the *Lactobacillaceae* family, specifically *Lactobacillus* and *Ligilactobacillus* genera, which were recovered in very high redundancy from both cultivation strategies (direct and enrichment), indicating anatomical dominance and colonisation preference. The highest number of novel descriptions were found in this section including *F. anaeroviscerum*, not mapped at any metagenome dataset but isolated eight times under different isolation strategies; *L. avium, L. difficilis, L. viscerum,* and *L. pulli*, isolated with a redundancy of two to four times and detected at an abundance of ~0.1%, a low presence compared to *Ligilactobacillus* strains. The increase of glucose absorption in the small intestine might influence substrate intake diversification to other sources of carbon such as peptides and fatty acids where these strains might find an advantage [181].

The highest number of antiviral mechanisms was found in strains of this region compared to the homologue isolates of jejunum or crop. For instance, *L. gallinarum* isolated from ileum possess the CRISPR CAS system type I-E and the abortive system AdiDL while the homologue strains of crop and jejunum presented only the cbass nuclease mechanism, indicating different adaptations to the community dynamics and higher interaction with viruses at this region. Regarding defence mechanisms against other bacteria, the type III polyketide synthase (PKSs) enzyme, detected on all the *Lactobacillaceae* family members of the collection, was related to the enzyme hydroxymethylglutaryl-CoA synthase in the synthesis of isoprenoids. However, its presence has been also associated to the lactic fermentation in fermented vegetables [182] and it is involved in the regeneration of NAD+ during lactic acid fermentation [183]. The presence of genes related to pyruvate metabolism and lactate fermentation on the collection's genomes, suggests that hydroxymethylglutaryl-CoA synthase might interfere in both pathways, reducing NADH during lactic acid fermentation and catalysing the formation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) at the mevalonate pathway during the integration of secondary metabolites [174].

Genomes of all collection members included the drug resistance efflux transporter EfrAB, which provides resistance to structurally unrelated antimicrobial agents such as quinolones, tetracyclines or anthracyclines [184]. Although antibiotic resistance tests were performed only on the novel species descriptions, the presence of such a spread resistance mechanism with a broad spectrum suggests a valuable tool within the environment against different antimicrobial molecules. However, further gene expression tests must be performed to recognise this mechanism's activity.

Finally, the presence of different groups of phosphatases on all the novel species descriptions provides insights about the potential contributions of these strains to the supply of available phosphate from insoluble sources such as proteins, nucleotides, inulin, phytates and other organic sources of unavailable phosphate present in the plant-based diet given to chickens, that is not accessible to its metabolism [37, 38].

2.5 Conclusion

This culture collection represent the first deep isolation approach of bacteria from the chicken's upper digestive system. It presents a wide enzymatic and host-interactive capacity that hold a metabolic value to the host. The redundancy of isolation from more than one source demonstrated that, although a significant portion of the genomic content is conserved, isolates hold different genomic features (e.g. carbohydrate usage, antiviral defence systems, prophage occurrence) to prosper in different environmental niches. Beyond the differences in diversity and colonisation rate through the upper digestive tract, the easiest way to observe environmental adaptations is through the comparison of similar organisms that inhabit different GIT sections, such as *L. gallinarum* or *E. faecium*, that contributed to understand the adaptive response of such strains along the different regions of the digestive system. The newly obtained isolates are present along the intestine, and they are consistently found within the upper GIT sections (crop to ileum). The novel species of *Limosilactobacillus and Ligilactobacillus* represent key members of the chicken microbiome of crop or ileum and its isolation success lies in the exploration of these sections. These results describe the unexplored taxonomic and functional diversity of bacteria inhabiting crop, jejunum and ileum, improving the potential handling of chicken microbiome with biotechnological applications. Collectively, these findings emphasize the value of combining cultivation with metagenomics to study microbial diversity.

3. Host specific adaptations of *Ligilactobacillus aviarius* to poultry

Abstract

The genus *Ligilactobacillus* encompasses species adapted to vertebrate hosts and fermented food. Their genomes encode adaptations to the host lifestyle. Reports of gut microbiota from chicken and turkey gastrointestinal tract have shown a high persistence of *Ligilactobacillus aviarius* along the digestive system compared to other species found in the same host. However, its adaptations to poultry as a host has not yet been described. In this work, the pan-genome of *Ligilactobacillus aviarius* was explored to describe the functional adaptability to the gastrointestinal environment. The core genome is composed of 1179 gene clusters that are present at least in one copy that codifies to structural, ribosomal and biogenesis proteins. The rest of the identified regions were classified into three different functional clusters of orthologous groups (clusters) that codify carbohydrate metabolism, envelope biogenesis, viral defence mechanisms, and mobilome inclusions. The pan-genome of *Ligilactobacillus aviarius* is a closed pan-genome, frequently found in poultry and highly prevalent across chicken faecal samples. The genome *of L. aviarius* codifies different clusters of glycoside hydrolases and glycosyltransferases that mediate interactions with the host cells. Accessory features, such antiviral mechanisms and prophage inclusions, variate among strains from different GIT sections. This information provides hints about the interaction of this species with viral particles and other bacterial species. This work highlights functional adaptability traits present in *L. aviarius* that make it a dominant key member of the poultry gut microbiota and enlightens the convergent ecological relation of this species to the poultry gut environment.

Keywords: *Ligilactobacillus aviarius,* pan-genome, poultry

3.1 Introduction

There has been a growing interest on studying host specificity of the gut microbiome to understand to which extent factors such as diet, environment, host phylogeny and its evolutionary story impact the composition of the gut microbiome [185]. Ecology and evolution play a long-term role shaping microbial dynamics and this idea has supported the emergence of "phylosymbiotic studies" defined as the observance of patterns in microbial communities that summarize and reflects the phylogeny of their host linking the microbiome to the host´s evolutionary story [186]. In some mammals, the study of phylosymbiosis has related microbial dispersal to their phylogeny [187, 188]. However, for non-mammalian vertebrate taxa, including birds and amphibians, studies still discuss the presence of phylosymbiosis [189-191].

Chicken gastrointestinal colonisation has been described as a stochastic process driven by the contact with environmental microorganisms present in food and water [20]. It is highly diverse and changes along the gastrointestinal tract (GIT) [26, 35]. Yet, previous studies typically convey on a constant presence of *Enterobacteriaceae* and *Lactobacillaceae*, suggesting that some microorganisms are more persistent to the gastrointestinal environment and are adapted to the physicochemical changes along poultry GIT environment, than others. Studies using 16S rRNA amplicon sequencing and metagenome-assembled genomes showed the dominance of *Ligilactobacillus aviarius, Lactobacillus crispatus* and *Ligilactobacillus salivarius* as host-associated commensal lactic acid bacteria in chickens representing 18% of the faecal clone sequences and present in over half of the chicken samples [35, 192].

The genus *Ligilactobacillus* (formerly referred to as *Lactobacillus salivarius* group) encompasses 16 motile, homofermentative rod-shaped species with a G+C content of around 32.5 to 43.3 %. Most *Ligilactobacillus* species are adapted to vertebrate hosts and have been obtained from mammals, reptiles, and amphibians' digestive samples [59]. Some other species of *Ligilactobacillus* are also present in fermented foods, silage, and soil [193]. Several strains of *Ligilactobacillus* express urease gene clusters dependent on low pH to mediate the resistance to gastric acids and buffer the environmental changes [194]. *L. aviarius* has been reported to encode an alpha-glucanotransferase that converts amylose starch into isomalto-/malto-polysaccharides (IMMP) [195, 196]. These highly branched α-glucans linked through α1-4 and α1-6 bonds are resistant to digestion by the host's α-amylase and therefore, represent a supply of soluble dietary fibre [196]. Such digestibility resistance improves IMMP distribution to the large intestine and serves as a prebiotic source for the bacterial community to ferment and produce short-chain fatty acids (SCFAs) [71]. Leading to the assumption that the production of IMMP from an active *L. aviarius* colonisation in the small intestine of birds, improves digestion and represents a potential supply of feed for fermentative bacteria in the lower part of the GIT. Experiments of probiotics co-feeding mixtures of *L. aviarius* with *L. salivarius* and *Ligilactobacillus agilis* reported an increase of Lactobacilli, a decrease of *Escherichia coli* on the small intestine lumen of laying hens and an increase on the egg weight and laying performance, favoured by the increase of mucosal absorption and cytokine expression [128].

The frequent reports of *L. aviarius* to poultry highlights its persistence as a well-adapted coloniser of the avian GIT, and some studies agree on its presence as an indigenous *Ligilactobacillus* species along with *L. agilis* and *L. salivarius* [128, 197, 198]. However, the identification approach must be taken cautiously due to the intra-species similarity and recent taxonomic re-assignations [59]. Additionally, many works focus on detecting and identifying poultry-associated species, but they lack a functional description [199]. Therefore, this work aims to describe the encoded functional adaptability of *L. aviarius* and the features that might make it host-specific adapted to the poultry gut environment. This is the first comparative genomic analysis for this species, which describes the genomic diversity and functional adaptability of *Ligilactobacillus aviarius,* not explored previously.

3.2 Methods

3.2.1 Phylogenomic analysis of the genus *Ligilactobacillus*

Reference genomes of the genus *Ligilactobacillus* (Table 6) were collected from public databases and previous studies. Briefly, 17 reference genomes were downloaded from the National Center for Biotechnology Information (NCBI) database (genomes obtained in August 2022). The genome of *Ligilactobacillus hohenheimensis* DSM 113870 was used from our collection [200]. All reference genomes were phylogenetically placed based on 487 universal markers using Phylophlan 3.0 [166] and annotated using iTOL (v6.5.8) [157]. *Liquorilactobacillus vini* [59, 201] was used as an external group. A codon usage analysis was run in ATGme (http://atgme.org/) to detect the presence of less common codons.

Table 6. Reference genomes of species from the genus *Ligilactobacillus*.

Genome	Accession number	Source of isolation	Reference
Ligilactobacillus hohenheimensis	GCA_947381805	jejunum of broilers	This work
Ligilactobacillus aviarius	GCA_001436315	faeces of chicken	[59, 202]
Ligilactobacillus araffinosus	GCA_001435375	duodenum of chicken	[59, 202]
Ligilactobacillus salitolerans	GCA_003864415	Spent mushroom substrates	[59, 193]
Ligilactobacillus pobuzihii	GCA_000349725	fermented cummingcordia	[59, 203]
Ligilactobacillus acidipiscis	GCA_002117995	fermented fish	[59, 204]
Ligilactobacillus saerimneri	GCA_000423265	pig faeces	[59, 205]
Ligilactobacillus ceti	GCA_001438785	lungs of a beaked whale	[59, 206]
Ligilactobacillus salivarius	GCA_002736025	human saliva	[59, 207]
Ligilactobacillus hayakitensis	GCA_001434455	faeces of thoroughbred (horse)	[59, 208]
Ligilactobacillus equi	GCA_001435735	faeces of horses	[59, 209]
Ligilactobacillus agilis	GCA_001436215	municipal sewage	[59, 210]
Ligilactobacillus ruminis	GCA_900113455	bovine rumen	[59, 211]
Ligilactobacillus apodemi	GCA_001434405	faeces, wild Japanese wood mouse	[59, 212]

Ligilactobacillus murinus	GCA_001436015	intestine of rat	[59, 213]
Ligilactobacillus animalis	GCA_000183825	dental plaque of baboon	[59, 214]
Ligilactobacillus pabuli	GCA_022836475	silage	[215]
Liquorilactobacillus vini	GCA_001435395	grape must	[59, 201]

*Species reported in chicken intestine

3.2.2 Prevalence and abundance of poultry related *Ligilactobacillus* in chickens

To evaluate the prevalence and abundance of species of *Ligilactobacillus* related to poultry, 603 DNA sequences obtained from poultry samples were downloaded from the SRArun selector browser of the NCBI database. Samples of crop where obtained from the project PRJEB60928, (n= 48) obtained by our research group (internal communication, not yet public), samples from duodenum, jejunum, ileum, caeca and colorectum where obtained from the project PRJNA417359 (crop=38, duodenum=99, jejunum=98, ileum=97, caeca=99 and colorectum=98) [216]. Finally, samples from faeces were obtained from the project PRJEB22062 “Gut microbiomes from 359 European pig and poultry herds” (n=159) [217-219]. Details of each sample are listed at the Supplementary material file S1. The collected raw reads were mapped against the genomes of *L. aviarius* DSM 20655 (GCA001436315), *L. aviarius* J01 (GCA947381835), *L. hohenheimensis* J14 DSM 113870 (GCA947381805) and *L. araffinosus* DSM 20653 (GCA001435375) using CoverM (v0.6.1) (https://github.com/wwood/CoverM).

3.2.3 Genomes collection and selection

Genome assembled sequences (n=21) of *L. aviarius* available at NCBI database as of July 2022 were collected from the projects PRJEB56193, PRJNA222257, PRJNA316009 and PRJNA377666 [200, 220, 221]. Metagenome-Assembled Genomes (MAGs) with more than 90% completeness available at the Integrated Chicken Reference Genomes and Gene Catalogue (ICRGGC, https://nmdc.cn/icrggc/) (n = 12) were collected [192]. All genomes were taxonomically analysed, screened and selected according to the index of completeness (>90%) and no contamination using Anvi’o v7.1 [222], and a pairwise genome comparison to discard clonal genomes (dereplication cluster >99% ANI) using dRep 3.2.2 [223] retaining 26 genomes.

3.2.4 Pan-genome analyses

The pan-genome of *L. aviarius* was calculated in Anvi’o v7.1 [222] using the set of 26 genomes (Table 7). Genomes were pre-treated to discard contigs shorter than 2.5 kb. Gene prediction was made with HMMS included in the workflow of Anvi’o. The resulting pan-genome output was visualised through the interactive interface of Anvi’o. The core genome calculation was performed considering the presence of 90% of single-copy genes and an index of geometric homogeneity of 0.95, obtaining 1179 genes. The predicted annotation of ORFs of each genome was used to identify the number of total genes, core genes, and new genes present in the further pan-genome analysis and the resulting pan-genome output files were visualised using RStudio v1.1.463. Clusters were assigned following the microbial pan genomics workflow of Anvi’o (https://merenlab.org/2016/11/08/pangenomics-v2/) that considers Euclidean distance of orthologous genes using linkage Ward method, defined by the gene tree in the centre of the pan-

genome. The core genes shared among the 26 strains were concatenated and aligned using the workflow for homogeneity indices in pan-genomes of Anvi'o. The tree was visualised with iTOL [157].

Table 7. Metadata of genomes on *L. aviarius* used for the pan-genome analysis.

Genome name	Digestive part	Source	BioProject	Genome type (Mb)	Genome size
B009	Ileum	*Gallus gallus domesticus*	PRJEB56193	genome	1633176
B056	Ileum	*Gallus gallus domesticus*	PRJEB56193	genome	1587309
B118	Ileum	*Gallus gallus domesticus*	PRJEB56193	genome	1547249
I11	Ileum	*Gallus gallus domesticus*	PRJEB56193	genome	1676028
J01	jejunum	*Gallus gallus domesticus*	PRJEB56193	genome	1600094
J03	jejunum	*Gallus gallus domesticus*	PRJEB56193	genome	1615470
J25	jejunum	*Gallus gallus domesticus*	PRJEB56193	genome	1602748
J28	jejunum	*Gallus gallus domesticus*	PRJEB56193	genome	1550506
J35	jejunum	*Gallus gallus domesticus*	PRJEB56193	genome	1574759
GCA_001436315	faeces	*Gallus gallus domesticus*	PRJNA222257	genome	1543068
GCA_016902635	cecum	*Gallus gallus domesticus*	PRJNA377666	genome	1517028
GCA_001652025	Ileum	*Meleagris gallopavo*	PRJNA316009	genome	1547880
GCA_001652055	Ileum	*Meleagris gallopavo*	PRJNA316009	genome	1490430
GCA_001654615	Ileum	*Meleagris gallopavo*	PRJNA316009	genome	1602376
MAGs_co_10232	faeces	*Gallus gallus domesticus*	PRJEB22062	metagenome	1419430
MAGs_co_10238	faeces	*Gallus gallus domesticus*	PRJEB22062	metagenome	1465364
MAGs_co_10952	faeces	*Gallus gallus domesticus*	PRJEB22062	metagenome	1334687
MAGs_co_2168	faeces	*Gallus gallus domesticus*	PRJEB22062	metagenome	1503872
MAGs_co_2235	faeces	*Gallus gallus domesticus*	PRJEB22062	metagenome	1413800
MAGs_co_5238	cecum	*Gallus gallus domesticus*	PRJEB22062	metagenome	1616609
MAGs_co_5566	faeces	*Gallus gallus domesticus*	PRJEB22062	metagenome	1493014
MAGs_co_6121	Ileum	*Gallus gallus domesticus*	PRJEB22062	metagenome	1313075
MAGs_co_7371	jejunum	*Gallus gallus domesticus*	PRJEB22062	metagenome	1521009
MAGs_co_8896	cecum	*Gallus gallus domesticus*	PRJEB22062	metagenome	1472325
MAGs_co_9092	faeces	*Gallus gallus domesticus*	PRJEB22062	metagenome	1467841
MAGs_co_9747	faeces	*Gallus gallus domesticus*	PRJEB22062	metagenome	1503870

3.2.5 Phenotype prediction and functional analysis

The annotation file of strains of *Ligilactobacillus*' species isolated from small intestine of chicken (PRJEB56193): *L. aviarius* J01 (GCA947381835), *L. hohenheimensis* J14 (GCA947381805), *L. saerimneri* (GCA947381505) and *L. salivarius* (GCA947381595) were compared to observe absence and presence of metabolic pathways encoded within the genomes to assess genome reduction within species related to chicken.

Functional analysis and gene absence/presence of the 26 genomes *L. aviarius* were performed using gene prediction in Anvi'o, to identify the protein domains present in each genome. Differences in presence and absence along the genome were subdivided into four regions

(clusters) according to the protein domains present in that region. The identified regions were classified into different functional clusters of orthologous groups (COGs), and the differences among each strain were selected and highlighted in a heatmap.

Different functional features of the interaction of *L. aviarius* with the host were investigated using DRAM annotation (Distilled and Refined Annotation of Metabolism) [224]. Briefly, DRAM annotates contigs using UniRef90 [225], PFAM [226], dbCAN [227], RefSeq viral [228], VOGDB (https://vogdb.org/), and the MEROPS peptidase database [229] and curates these annotations into useful functional categories. The results were hand curated to show the positive presence of metabolic pathways and subunits completion.

3.2.6 Prophage identification

Prediction of prophage genes and regions insertions in the 26 genomes of *L. aviarius* was performed using PHASTER [165] (www.phaster.ca). The prophage regions were compared against a bacterial and phage/prophage database available within PHASTER by October 2022. The insertion was considered complete when the region contained known phage sequences and when more than >90% of the proteins in the detected regions were associated with known phage sequences. Proteins present within the operon insertion and its distribution were directly exported from PHASTER's website.

3.2.7 Antiviral defence systems detection

Antiviral systems presence was identified utilising Prokaryotic Antiviral Defence LOCator (PADLOC) [230] using HMM-based homologue searches and gene presence/absence/synteny criteria. CRISPR–Cas systems were detected using CRISPR-Cas++ 1.1.2 using the genome assemblies as input [231]. The occurrence of defence systems in strains from different origins was compared.

3.3 Results and Discussion

3.3.1 Phylogenomic analysis of *Ligilactobacillus* strains related to poultry

The genus *Ligilactobacillus* encompasses homofermentative organisms frequently associated with the gut environment of different hosts or fermented substrates such as silage, food, or soil. The size of genomes used for the phylogenomic analysis (reference genomes) ranges between 1.4 and 2.3 Mb with a G+C content of 32.5 to 43.3%. The species of *L. aviarius, L. araffinosus* and *L. hohenheimensis* (Figure 26) have been reported on poultry and show a smaller genome size (1.46 Mb) than the average of the genus (1.87 Mb).

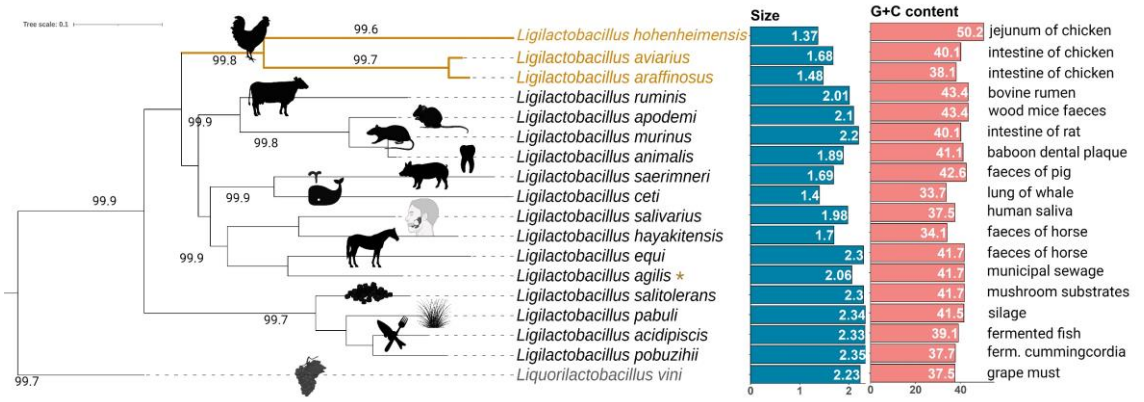

Figure 26. Phylogeny of the core genome sequences of taxa belonging to the genus *Ligilactobacillus* (database available genomes). Reconstruction of a maximum-likelihood tree based on 497 single-copy core protein sequences concatenated. The tree is rooted on *Liquorilactobacillus vini* DSM 20605. Bootstrap percentages (1000 replicates) are shown at the branch points. Bar depicts 0.1 substitutions per nucleotide position.

Such reduction might indicate a reductive adaptation to the gut environment of poultry (genome reduction) [232]. Other species found in poultry like *L. saerimneri*= 1,525,592 bp (GCA944326145) [233] (assembly not considered in the phylogenomic analysis) have a relatively similar genome size, while some other species such as *L. agilis* = 2,168,186 bp (GCA025311455) (PRJNA880302-no publication related*), L. salivarius* = 1,807,598 bp (GCA002159345) [221] and *L. animalis* = 1,940,664 bp (GCA001705475) (PRJNA337943 no publication related), also reported in poultry do not share such size. *L. aviarius* and *L. hohenheimensis* lack a complete operon of repair system genes and peptides transport (Figure 27) compared to other Ligilactobacilli, which might partly explain the differences in genome size. The loss of repair DNA genes might be explained by the Proteomic Constraint theory, that propose that in order to minimize mutations (errors), organisms reduce its proteome size leading to a reduction of selection pressure [234]. Among the group of poultry-related species, *L. hohenheimensis* has a relatively high content of G+C (50.1%) compared to the rest of *Ligilactobacillus* species, with an average of 40%. The particular traits of this species contradict the general understanding that larger bacterial genomes tend to have higher G+C contents [235]. Other factors should be considered to explain the higher G+C content, such as the organism's normal optimal temperature range, a restriction of the genetic code (where encoding certain amino acids requires at least some usage of A/T or G/C), or the presence of rare codons in genes. Although some codons resulted less frequent such as CGG instead of AGA (15.6‰) or CGU (13.2‰), or the stop codon UAG instead of UAA (2.3‰), the presence of rare codons must be taken with care since the codon usage table used to compare was based on the codon usage of *L. salivarius* available at the databases, a relatively distant member of the genus *Ligilactobacillus.* Moreover, the genomic GC content of *L. hohenheimensis* (50%) is less common within the bimodal GC content distribution of bacteria, with peak values either below 45% or above 60%, and 10% higher than the average GC content observed in *Bacillota* [236]. Although all species share the same ancestor

and similar fermentation pathways, the clade of *L. salitolerans, L. acidipiscis* and *L. pobuzihii* show a bigger genome size (~2.3 Mb) and have been reported in associations with fermented food and mushroom substrates, which is a different lifestyle from the host associated environment.

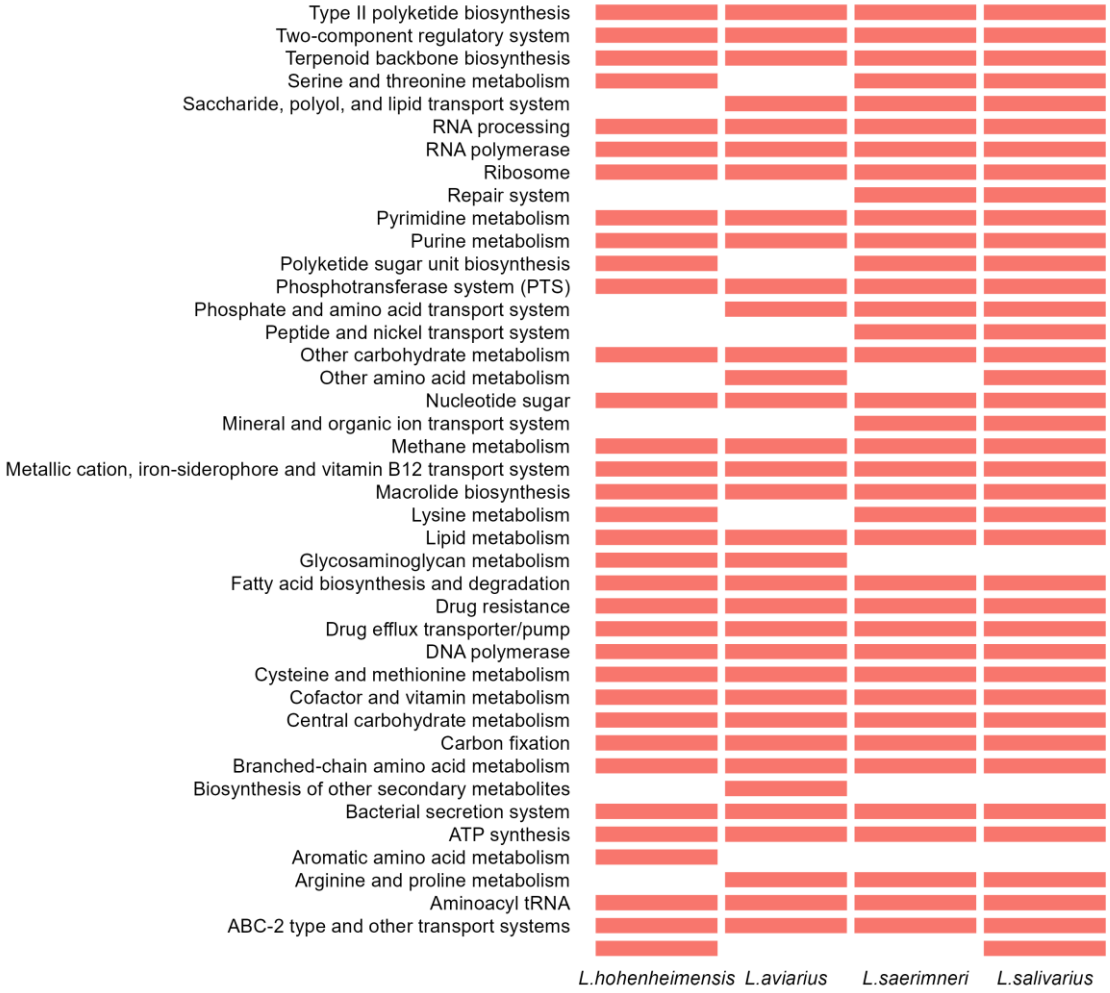

Figure 27. Comparison of presence-absence of main metabolic pathways in four species of *Ligilactobacillus* found within the intestinal environment of chicken: *L. aviarius, L. hohenheimensis, L. salivarius* and *L. saerimneri.*

3.3.2 Presence of *Ligilactobacillus* related species on poultry

To observe the prevalence and abundance of *L. aviarius, L. araffinosus,* and *L. hohenheimensis* as relevant community members in the digestive tract of poultry, its presence was estimated using a large-scale comparison of the genomes against samples of chicken digesta available at public databases. Both strains of *L. aviarius* (GCA947381835 and GCA001436315) that were detected abundant above 0.1%, depicted a prevalence of at least 80% on duodenum, jejunum, ileum, and faecal samples. Its abundance ranged between 0.5 to 40% (Figure 28). In both cases, our isolate of *L. aviarius* J01 and the reference genome DSM 20655, maintained a prevalence between 20 and 60% in crop, colorectum and caeca, where its abundance reduces, suggesting to be a persistent member of the intestinal community with a dominance in small intestine. The presence

of *L. araffinosus* follows the same pattern as *L. aviarius* along the chicken intestine. However, its presence in crop and caeca depicts lower percentage of prevalence and abundance than the rest of the strains considered in the analysis. This recently separated species from *L. aviarius* [59] appears to have a bigger sensitivity to the environmental conditions of crop and caeca, dropping its abundance to 0.1%, which highlights its individual environmental adaptations from those of *L. aviarius*. The lowest prevalence and abundance were detected for *L. hohenheimensis* DSM 113870, which presented the highest prevalence at small intestine (50%) and the lowest (10%) in crop and caeca, above *L. araffinosus*. The high occurrence of *L. aviarius* in poultry samples (high abundance and dominance along the GIT) and the availability of genomic/metagenomic assemblies in the databases further supported the functional analyses to understand the adaptability of this species to the chicken's gut environment.

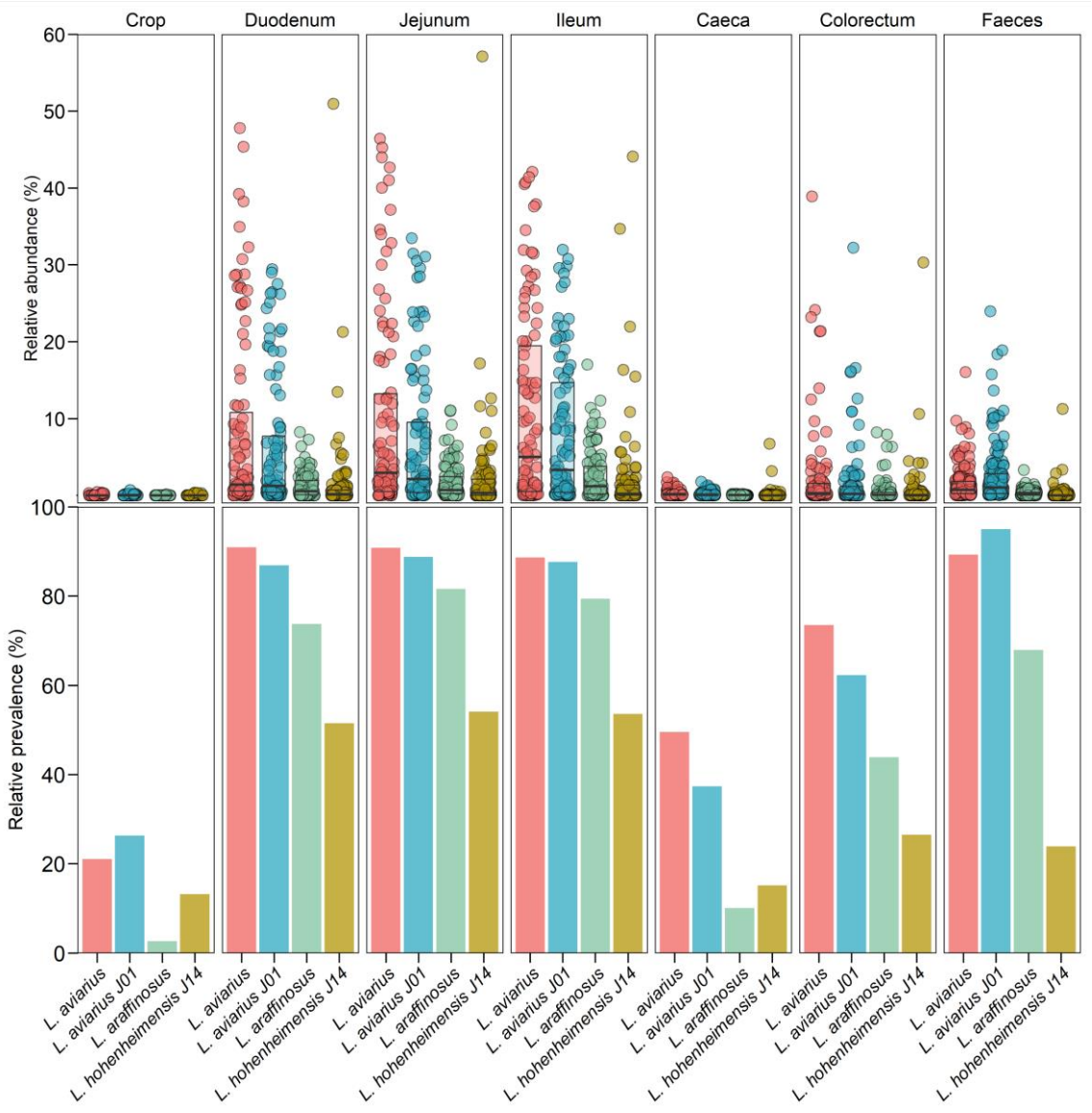

Figure 28. Relative abundance and prevalence of species of *Ligilactobacillus* related to poultry across chicken faecal samples obtained from public databases. Dots represent the relative

abundance of each sample (n=159). Bars depict the mean prevalence of each species across all faecal samples.

3.3.3 Pan-genome determination of *Ligilactobacillus aviarius*

The pan-genome of 26 dereplicated genomes of *L. aviarius* was determined, including 17 publicly available genomes from the NCBI (July 2022) and nine newly sequenced genomes from our bacterial collection (PRJEB56193). From this dataset, three genomes correspond to isolates from the ileum of turkeys, and the others originate from the different intestinal sections of the chicken: six from the jejunum, five from the ileum, three from caeca, and nine isolated from faeces (Table 7). The average genome size was 1.52 Mb with a standard deviation of ± 0.88 considering the size of metagenomic assemblies (MAGs) and of ± 0.48 considering only isolated genomic assemblies. Such difference was considered in the pan-genome analysis lowering the core gene threshold and predicting genes in metagenome mode (included in Anvi'o´s protocol) to attenuate the loss of precision in the analysis [237].

The number of genes detected in each genome varied by 5.36% (1456 ± 78.18) (mean ± SD), with no correlation observed between the number of genes and the number of contigs nor the length of the contigs, showing that the quality of the genome did not influence the genome annotation and the pan-genome analysis. The pan-genome of *L. aviarius* cannot be considered closed since the number of pan-genome genes represented against the number of genomes does not reach the plateau. In this analysis, the addition of the last two genomes decreased the number of total genes and reduced the standard deviation (Figure 29A). Despite the number of new genes did not increase, and the number of core genes was close to reach a constant with the addition of genomes (Figure 29 B, C), a future addition of novel genomes will be necessary to reach a possible plateau and enrich the pan-genome analysis.

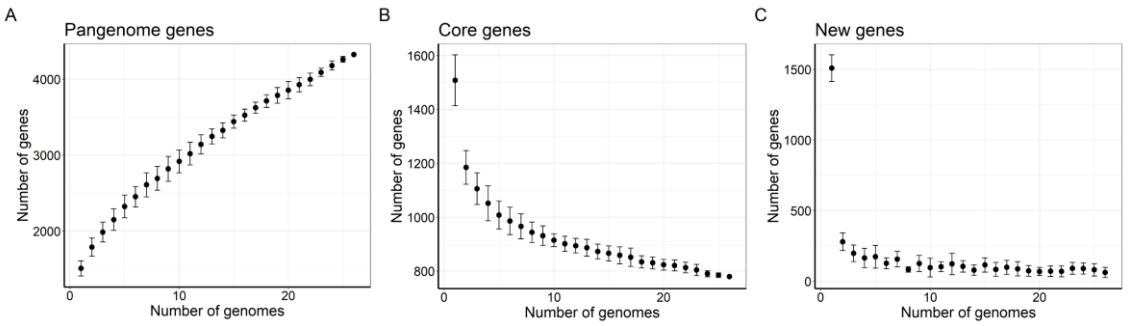

Figure 29. Number of core genes and pan-genome of 26 genomes *L. aviarius*. (A) The number of genes of the pan-genome increases and flattens as a function of the number of genomes included in the analysis while (B) the core genome and (C) number of new genes decreases. Bars depict standard deviation.

The core genome threshold in this analysis was lowered to a presence of 90% of single copy genes shared throughout the dataset. 90% of the genes resulted in 1,179 genes (cluster named core90) present at least in one copy in 23 out of 26 of the genomes (Figure 30). The analysis of

strict core genes shared among 100% of the genomes reduced the number to 857 core genes (cluster named core). The functional annotation displayed that most core genes are related to basic biological functions such as translation, ribosomal structure, replication, cell wall/envelope biosynthesis, carbohydrate transport, and metabolism, as expected. The rest of the identified regions were classified into three different functional clusters of orthologous groups (COGS) that varies according to the type of genome assembly and source of isolation. The first cluster detected (cluster 1, 58 genes) included genes encoding carbohydrate metabolism and transporters. Carbohydrate metabolism was represented by genes for glycoside hydrolases and sugar transporters of arabinose, lactose, melibiose, maltose, sucrose, and starch, together with some glycosyltransferases that are predicted to mediate interactions with the host cell mucosa layer (glucans, dextrins, galactans) and digestive cell receptors. In addition, genes assigned to peptidases were detected in core and cluster 1, which might serve as possible indicators for host-specific bacterial interactions. Further points are discussed in the paragraph 3.5.

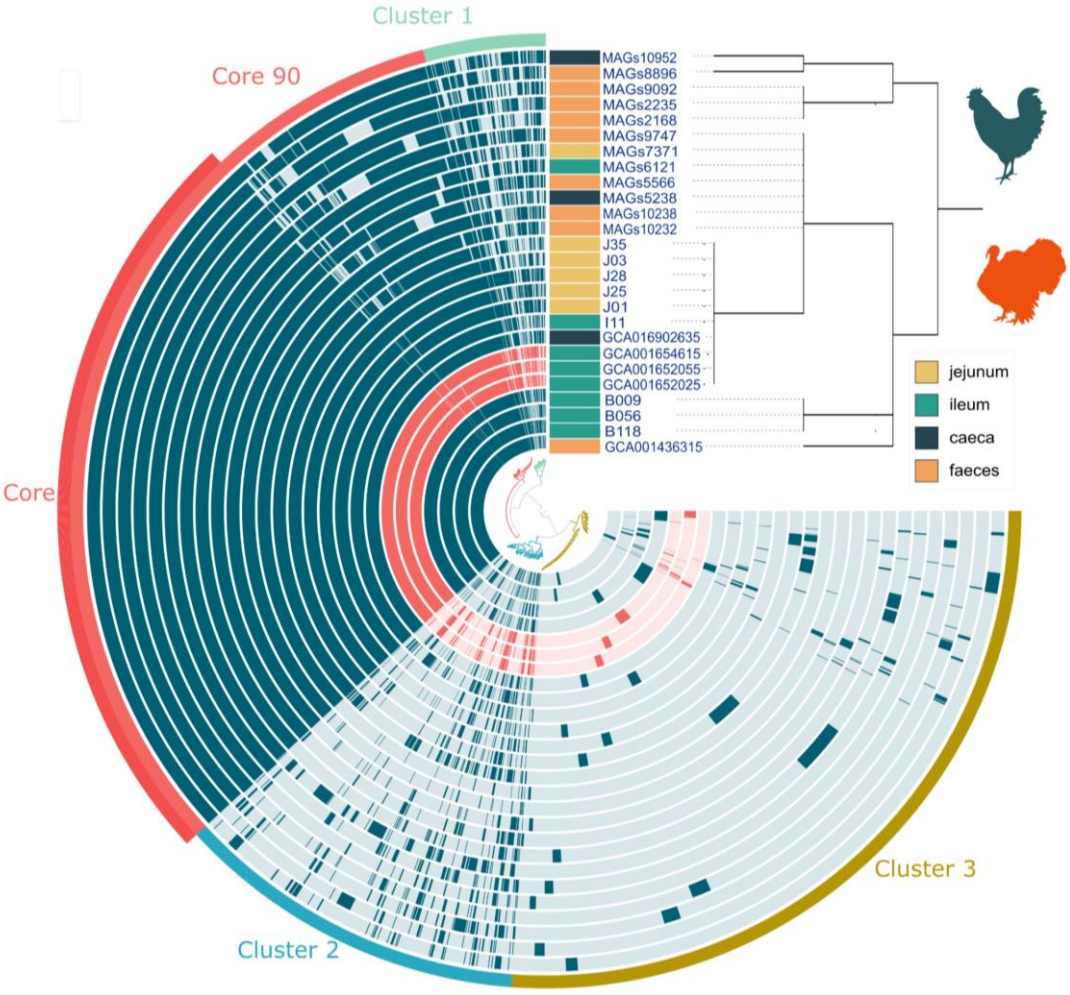

Figure 30. Pan-genome analysis of *L. aviarius*. Comparative genomic analysis of the 26 non-clonal strains of *L. aviarius* available in the databases. The inner layers represent individual genomes obtained from chicken (blue) or turkey (orange), arranged according to their phylogenetic relation. Genomes are depicted, organised by clusters of orthologous genes where each colour shadow indicates a cluster: Core genes in orange, Cluster 1 genes in green, Cluster

2 genes in blue, and Cluster 3 genes in yellow. An absence of colour depicts an absence of genes. The origin of each genome (either jejunum, ileum, caeca or faeces) is displayed next to the genome label.

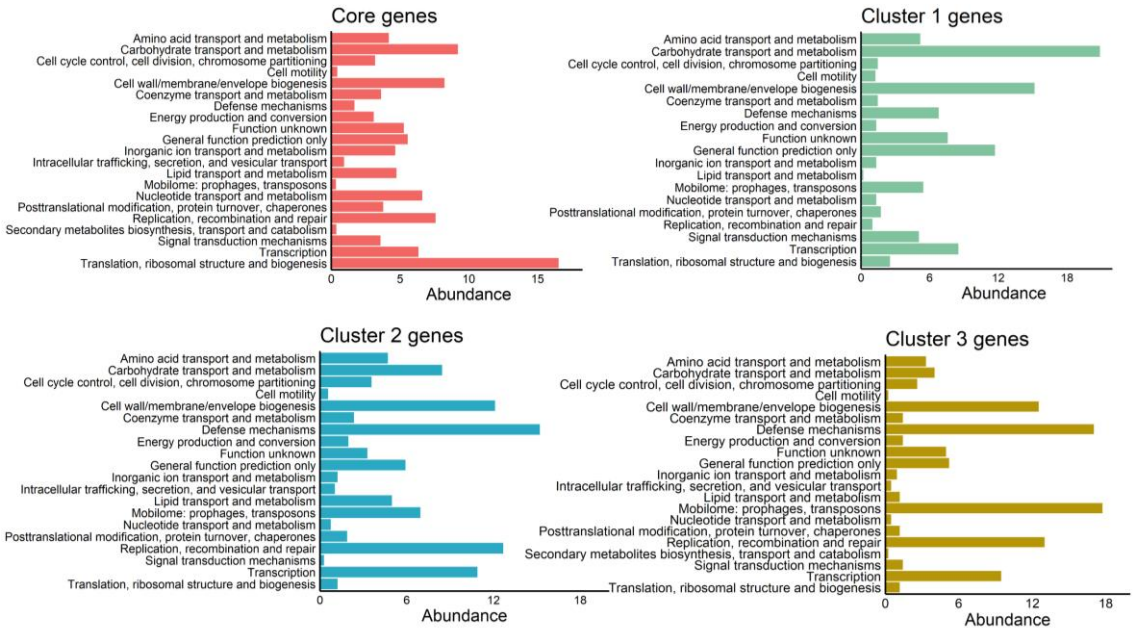

Figure 31. Relative abundance of genes within different functional categories found on each pan-genome cluster. The corresponding number of genes to each functional category is: 1179 in core genes, 150 genes in cluster 1, 140 genes in cluster 2 and 80 genes in cluster 3 shared among the 26 genomes of *L. aviarius*.

The distribution of genes assigned to carbohydrate metabolism and transport showed that these genes are constitutive and present in all genomes (core). However, some genes codifying for cellulose and maltodextrin utilization and transportation were exclusively found in cluster 1, representing an individual adaptation. Within these two clusters (core and cluster 1), some peptidase genes such as insulin protease (ptrA2/M16B), bleomycin hydrolase (C01B) and endothelin protease (M13), related to the maturation and degradation of peptide hormones were detected. The presence of these genes might indicate a host-lifestyle adaptation of *L. aviarius* whose peptidase activity might crosstalk the signalisation of the host. However, no evidence has been demonstrated in chicken. Cluster 2 encompass 41 genes encoding viral defence and replication mechanisms such as the abortive infection strategy (Abi), the Cispr/Cas type IIA system, the defence reverse transcriptase (DRTs) mechanism and the restriction-modification (RM) systems that recognize and cleaves foreign DNA which will be discussed in detail at the accessory features section. This information hints at the species' constant interaction with phage and foreign DNA. Finally, cluster 3 comprehended 16 genes encoding mainly mobilome inclusions (prophages/transposons), viral defence mechanisms, and cell wall envelope biogenesis/transcriptional genes. In general, the repertoire of genes included in all clusters might illustrate the importance of such housekeeping genes to persist in the chicken gut environment.

3.3.4 Metabolism of *Ligilactobacillus aviarius*

The species of *L. aviarius* is a heterotrophic bacterium able to obtain energy and carbon by oxidating glucose and arabinose (pentose) via the Embden-Meyerhof glycolysis and the pentose phosphate cycle. ATP is hydrolysed to generate ion gradients across the membranes by the F-type ATPase unit (Figure 32) that couples with H^+ transport across a membrane to obtain energy.

Figure 32. FoF1-type ATP synthase genes cluster present on the strain B118 of *L.aviarius* isolated from ileum of chicken.

Although relevant genes were only partially found, Entner-Doudoroff pathway (glucose-6-phosphate to pyruvate) and citrate cycle might be also important to oxidise carbon and reduce cofactors. Across all genomes, whole clusters of arabinose and chitin metabolism were detected codified, while genes for carbohydrate active enzymes (CAZymes) for cellulose and maltodextrin utilisation (*bcs*Z and *yvd*J) in cluster 1, and mannose, galactose, and xylose utilisation (nucleotide sugar metabolism) in cluster 2 were individually found in some genomes. Finally, only three genomes of strains isolated from ileum and one of caeca presented carbohydrate active enzyme genes for the hydrolysis of the plant polysaccharides galactan and mannan. The presence of genes related to carbohydrate transport and metabolism, exclusively found in some genomes (genes clustered in cluster 1) might be an indication of an adaptive nutrient utilisation strategy of *L. aviarius* within the intestine environment. Their potential expression would provide the strain a metabolic advantage on the use of these sugars at a specific GIT section. However, a clear separation regarding carbohydrate utilisation among strains originated from different GIT sections was not observed (Figure 33 and 34).

Genes for acetate production including *pta* and *ack*A (Figure34) were also detected, these genes convert acetyl-CoA into acetate forming ATP and present a reversible reaction that depends on the environmental conditions. In nutrient-rich conditions, acetyl-CoA is converted to acetate, while under starving conditions acetyl-CoA is generated allowing the cell to dump the excess of acetylation potential in exchange for ATP formation [238]. These genes were constitutively detected in all genomes. Both, lactate and acetate production can contribute to the production of butyrate and propionate when coupled with butyrate cycle via a NAD-independent d-lactate dehydrogenase (d-iLDH) also present within all genomes of *L. aviarius* (*ldh*) [239].

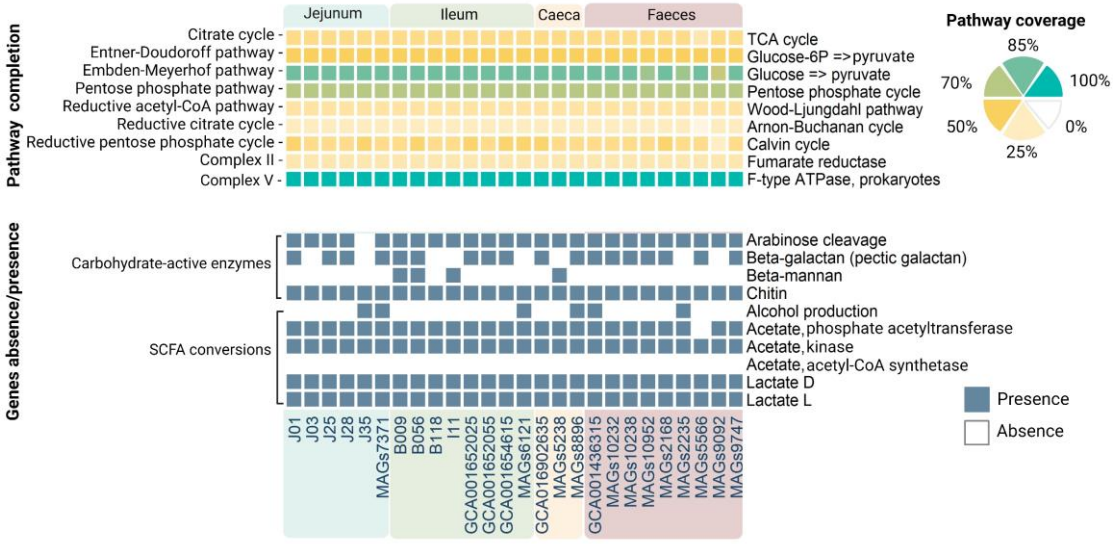

Figure 33. Metabolic pathways detected along the chicken GIT and across the pan-genome input genomes of L.aviarius. The figure is separated in two parts to highlight pathway completion (superior heatmap) and genes presence/absence (inferior heatmap). Tiles are coloured by pathway coverage according to the scale in the superior right corner. Presence/absence in the second heatmap represent the presence of genes necessary to express a particular process. An absence of colour indicates absence genes.

A cysteine biosynthesis gene was present in all genomes, meaning that the species is auxotroph to the rest of amino acids. A dihydrolipoamide dehydrogenase gene to hydrolyse leucine, lysin and methionine was constitutive in all genomes as well as many catalytic peptidases including aspartic peptidases (A08, A24A), cysteine peptidases (C01, C10, C26, C40, C56, C59, C60 C69 and C82), metallo-peptidases (M01, M03, M13, M16, M20, M24, M29, M38, M41, M48, M50, M60, M78, M79), serine peptidases (S01, S08, S09, S11, S12, S14, S15, S16, S24, S26, S33, S54) and threonine peptidase (T01B). The presence of the majority of these genes within the core cluster reflects the essential adaptations that *L. aviarius* gathers to efficiently colonise and adapt the chicken intestinal environment abundant on organic matter. The additional genes found individually represent a signature element of this strain that might help during niche occupation.

3.3.5 Functional adaptations of *Ligilactobacillus aviarius* to the host

Intestinal bacteria have been described to hold some adaptations that help them to survive in the intestine, such as transforming primary bile salts into secondary bile salts, degrading urea as a survival mechanism in acidic conditions, or producing large protein domains involved in extracellular matrix binding [240]. The pan-genome of *Ligilactobacillus aviarius* did not encode a urease cluster, and no biliary salt dihydroxylation gene was detected. Its strategy to survive the acidic conditions of the GIT lays on an adaptive presence of an FoF1-type ATP synthase (*atp* ABCDEFGH) (Figure 32 and 33) present in the core cluster, generating a constant gradient between extracellular and cytoplasmic pH due to protons exclusion. These adaptations have been described on the homofermentative species of *Lacticaseibacillus rhamnosus*, a well-known

human probiotic [241]. Across the pan-genome, different genes related to surface structures that interact with the host epithelia were detected, such as the pilus assembly protein *pul*E and the ATPase *pil*B protein domains that provide motility to the cell and might mediate interaction and adherence to the chicken gut epithelia [242]. Glycosyltransferases genes related to EPS (*gal*M) and glucan binding domain (yg repeat) (Figure 34) were detected in all genomic assembled genomes and partially in the metagenomic assemblies, meaning that completeness has to be taken into account in downstream analysis [237]. Expression of genes of mucin hydrolase (peptidase M60) that targets complex host glycoproteins, such as mucins [243], or peptidase M24 related to collagen recycling, might play a role in the host cross-talk interaction between bacteria and the glycolipids of the host cell surface.

Some of the key genes detected along the genomes that might mediate the interaction of *L. aviarius* were detected at the core cluster and cluster 1 and encode peptidases that can interact with host molecules such as endothelins (neprilysin/neutral endopeptidases, *pep*O), insulin (insulinase, *ptr*A2), collagen (Xaa-Pro aminopeptidase), mucin (enhancing-like peptidase), anti-inflammatory glycopeptides (bleomycin hydrolases), and peptide hormones maturation (prolyl oligopeptidase) (Figure 35), enzymes described to be involved in the maturation and degradation of peptide hormones and neuropeptides such as alpha-melanocyte-stimulating hormone, luteinizing hormone-releasing hormone (LH-RH) or insulin [244, 245]. Although the sole presence of those genes does not ensure an interaction, a possible expression might be related to a host lifestyle adaptation where the formed molecules, if absorbed, can interact with the host signalling. Being present along all the genomes, these interactions would affect the host's biological response and might be the key to the successful colonisation of *L. aviarius* across the intestine of poultry.

The potential to hydrolyse and assimilate carbohydrates with different chemical natures suggests a dynamic and planktonic lifestyle even in distal regions where absorption and competence would limit carbon intake. Additionally, the dominance and presence across the gut epithelium require fast multiplication rates that seem to be supported by the number of genes involved in replication, envelope biogenesis, and transcription, which represent 30% of total genes.

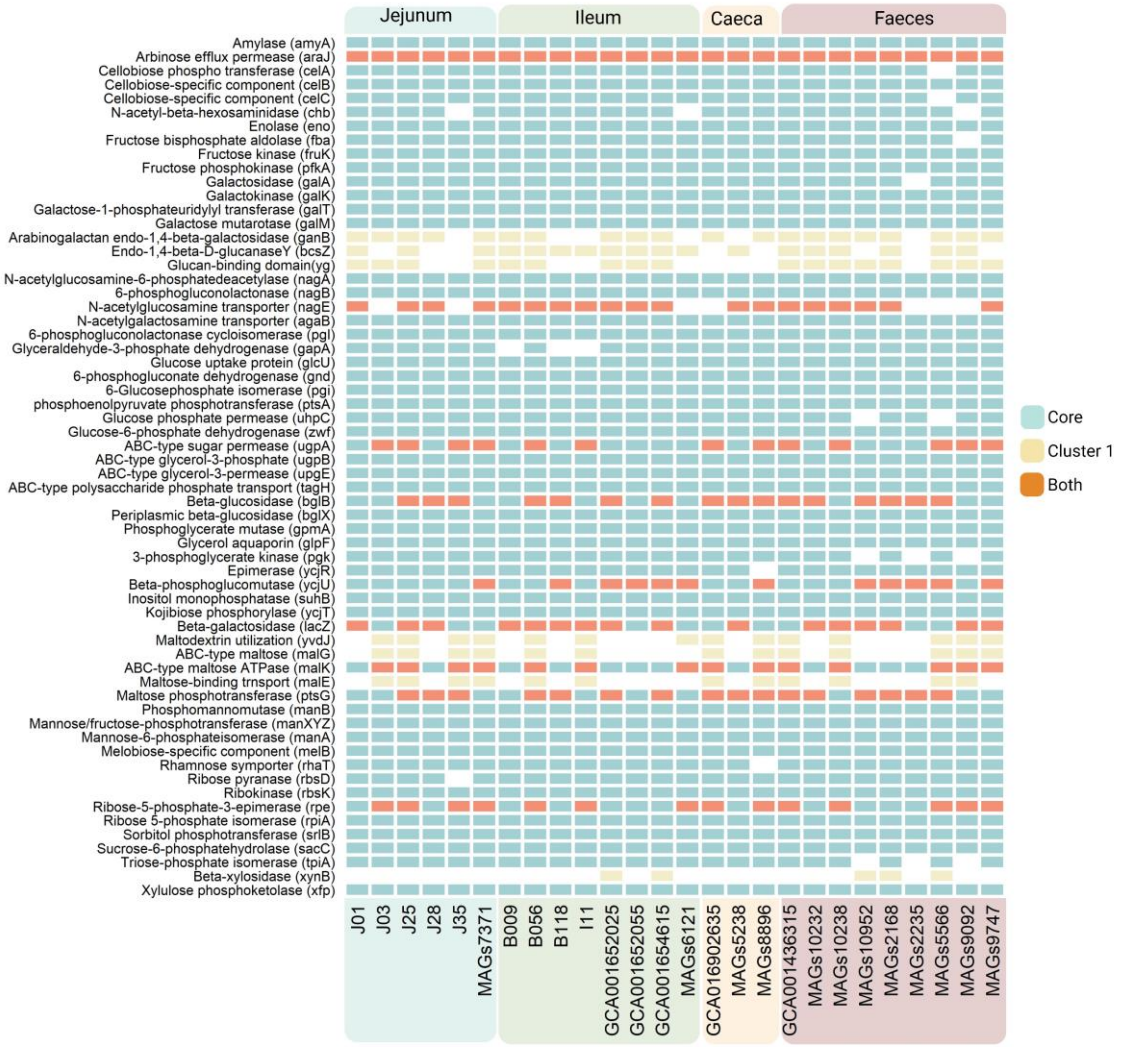

Figure 34. Distribution of carbohydrate metabolism and transport genes codified at the core cluster, cluster 1 or both across all input genomes of *L. aviarius* originating from four different origins along the chicken GIT.

Studies on the pan-genome of *L. salivarius* have shown two ways of adaptation within the intestinal environment, either by gaining adhesion abilities or by developing efficient utilization of nutrients [246]. In the case of *L. aviarius*, the homogeneous presence of genes for carbon utilisation, enzymes that potentially interact with the host, and its persistence along the whole GIT indicate that despite its narrow genome size, the gene repertoire of *L. aviarius* provides the necessary tools for efficiently colonise and adapt to the chicken intestinal environment. However, studies of gene expression and interaction are still needed.

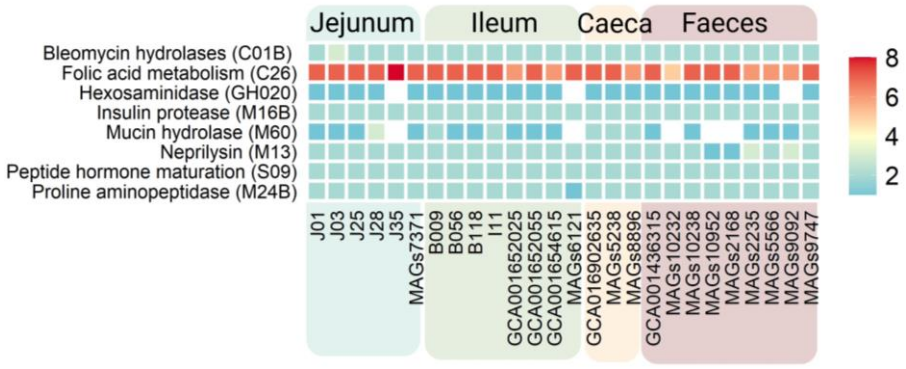

Figure 35. Distribution of peptidases with a potential to interact with the host structures and signalisation across all input genomes of *L. aviarius* originating from four different origins along the chicken GIT. The scale represents the number of gene-copies detected in each genome.

3.3.6 Accessory features in the genome of *Ligilactobacillus aviarius*

The detected accessory genes differed from genome to genome and were mainly found in clusters 2 and 3 of orthologous genes. Among these inclusions, several genes encoded antiviral defence, such as the abortive system (Abi) that prevents phage maturation by autolyzing the infected cell. In this case, the genes for *abi*D and *abi*L were more abundant among most of the genomes. Both proteins provide resistance by avoiding the phage's replication cycle completion and interfering with the phage endonuclease [247]. Additional to a suicidal strategy, two enzymatic mechanisms that involve the detection and modification of foreign DNA were detected: the restriction-modification system (RM) that signals endonucleases to degrade double-strand DNA, and the defence reverse transcriptase system (DRTs) that signal retro transcriptase enzymes that suppress the expression of phage genes. Some of these mechanisms have a non-specific activity against specific phages and might be active against a broad range of foreign DNA at an early stage of the infection after the intrusion of foreign DNA into the cell, before transcription [248].

Genes for the adaptive immune system CRISPR CAS type II and the ATPases/protease were detected among all strains while antiviral system GAO 19 was found codified only at strains obtained from jejunum and ileum. In this case, the defence locus herA-SIR2 of the protease-helicase system GAO 19 [248], might play a protective role on strains that colonise jejunum and ileum (Figure 36). In general, the diversity of antiviral systems detected in this single species present across the GIT, emphasizes the adaptation plasticity of *L. aviarius* and the importance of counting on multiple defence mechanisms to succeed within this environment.

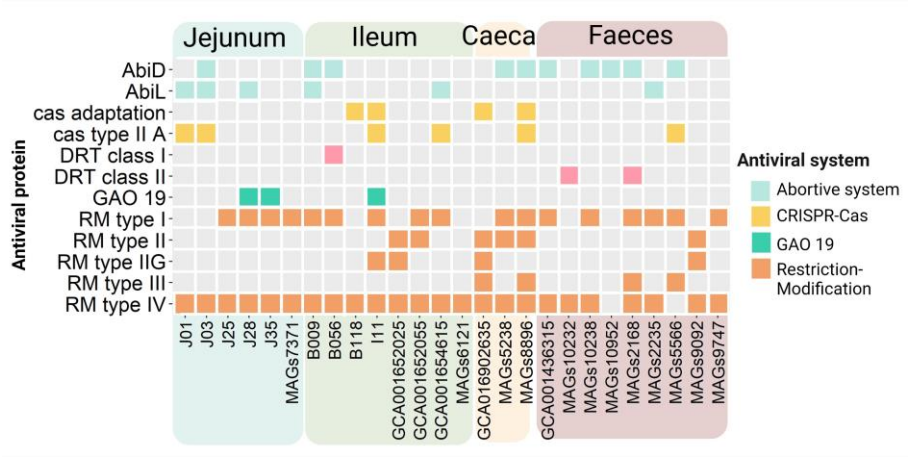

Figure 36. Distribution of antiviral defence mechanisms observed across genomes and metagenomes obtained from different chicken GIT sections.

Finally, four genomes were detected to carry complete prophage inclusions (tail, head, capsid, protease, portal, and terminase). All the detected inclusions are detailed in table 8. Despite the taxonomic distance, these prophages have been found in genomes of *Ligilactobacillus equi* [131], a member of the genus *Ligilactobacillus* that colonises the intestine of horses. Previous studies have shown that the number of prophages (phages in a lysogenic state) might be larger in a healthy gut, which allows for inferring the physiological state of the host by observing the composition and number of prophages [249]. Moreover, studies on humans have reported that people with similar diets converge to a similar viral community [250]. In this sense, the presence of similar prophages in different species of the same genus might result from the availability of similar nutrients and an adaptation of the genus *Ligilactobacillus* to the intestinal environment. Such inclusions play a key role in the exchange of genetic material and serve as a repository of mobile elements to maintain balance in the GIT.

Table 8. List of complete prophage insertions found in genomes of *L.aviarius*.

Phage name	Type of genome	Host	No. of genes	GC %	Length
PHAGE_Lactob_Sha1	Genomic from ileum	chicken	19	40.54	16.4
PHAGE_Lactob_Lj928	Genomic from faeces	chicken	49	40.00	31.6
PHAGE_Lactob_LfeSau	Genomic from faeces	chicken	47	39.51	29.8
PHAGE_Bacill_vB_BtS	Genomic from ileum	turkey	19	40.05	16.7
PHAGE_Lactob_phig1e	Genomic from jejunum	chicken	62	40.10	43.7

3.4 Conclusion

This work highlights functional adaptability traits in *L. aviarius* making it a persistent key member of the poultry gut microbiota. The metabolic potential predicted on *L. aviarius* genomes reflect a constitutive utilization of glucose, arabinose, chitin, acetate, and lactate, with the potential to hydrolyse additional plant polysaccharides and nucleotide sugars. The description of some genome signature elements of *L. aviarius* can improve the optimization of diets for the host, fermentation conditions, implementation of health biomarkers or enhance the production of specific metabolites, especially for a persistent specie along the digestive tract.

The comparison of genomes and identification of orthologous genes of commensal organisms such as *L. aviarius*, improves search and description of genes and pathways at unexplored microbial communities with similar environmental conditions. This approach facilitates the discovery of novel enzymes, biosynthetic gene clusters, or metabolic capabilities. Additionally, these genes can be used as markers to infer the relatedness of species and their evolutionary divergence, especially to understand dynamics of colonisation at a highly domesticated animal like chicken. Finally, a deep characterisation of proteins that interact with the host such as peptidases that potentially interact with hormone peptides can be utilized in various biotechnological applications, including bioregulation of communities or heterologous expression in other species of interest. In addition, the study and characterization of accessory genes provide information on the environmental dynamics and evidence the adaptation plasticity of *L. aviarius* to the GIT of poultry. Such characterization and functional descriptions broaden the understanding of the close host-interactions and provide information for further biotechnological applications.

4. General discussion

Deep knowledge about the chicken microbiome composition and robust references are based on colonisers on the distal regions, despite the main entrance of gut colonisers is through the proximal sections [42]. Most studies focus on the exploration of distal sections, even at an early stage of life when colonisation starts from proximal to distal sections. Up to date, most cultivation efforts sample caeca, colon and faeces [27, 28, 63] and a couple of scarce isolates of *L. aviarius* from ileal samples are available [63, 202]. Breeding conditions and farming maintenance, as well as the end of the use of antibiotics as growth promoters in poultry production, make chicken an exceptional case of study to understand how microbiome stablishes and contributes to maintain the host homeostasis. It has been stated that the diversity of microbial colonisers at the distal sections is metabolically and taxonomically more diverse. However, explorations on the microbial community at the first stages of digestion, are as important as the finals. The observance of their capacity to adapt to environments with faster transition time, drastic physicochemical changes, and their capacity to modify the epithelia architecture and obtain nutrients under such conditions, contributes to complete the picture of the complex system's ecology and interactions to the hosts not yet completely described.

The establishment of a collection of cultured bacteria from the first portion of the GIT of chicken: crop, jejunum, and ileum, improved the gene and genome catalogue of microorganisms related to the chicken gut environment and enriched the reference database to support further analysis and taxonomic assignations from the not-yet well explored upper GIT sections. To our knowledge, this collection represents the first repository of bacteria associated to crop and small intestine of chicken and provides the first description of eight novel species of the genus, *Faecalispora*, *Clostridium*, *Ligilactobacillus* and *Limosilactobacillus* not yet identified and handled in culture.

4.1 The small letters in culture media preparation

Being an unexplored environment, the physicochemical conditions of crop, jejunum and ileum, as well as the availability of carbon, nitrogen and cofactors in each section were taken into consideration at formulating the culture media. Former works make use of a relatively narrow selection of media that typically includes Wilkins-Chalgren anaerobe agar (WCHA), brain heart infusion (BHI), Gifu anaerobic medium (GAM), de Man Rogosa Sharpe (MRS) or the gut microbiota medium (GMM); all of them supplemented with compounds found at the intestinal environment such as biliary salts, rumen fluid or mucins [28, 63]. However, compared to bacterial culture collections of pigs, humans, or mice intestine [143, 145, 251] the selection of media reported on chickens is rather narrow and possibly underestimated even at the sections of high interest such as faeces and caeca. Therefore, the selection of media used in this work, contemplated five different media (Figure 37) that would satisfy the metabolic demands of microorganisms previously detected by 16S rRNA gene sequencing in other works at this GIT sections.

Far from a precise selection on the number of culture media, its formulation must contemplate an accurate interaction of the ingredients upon preparation. In this work, the preparation of each media considered the recommendations made by the group of Yoichi Kamagata [149, 151, 252] that included the sterilisation of phosphates and agar in a separated container to avoid the formation of H_2O_2 that cannot be eliminated by catalase negative bacteria, narrowing the spectrum of isolates. Moreover, carbohydrates were also autoclaved separated from peptides to avoid the formation of ketosamines, furfurans, melanoidins, Stecker aldehydes or other Maillard reaction products (MRP) during sterilisation [253]. MRP impact the availability of essential amino acids, certain vitamins, some nutritionally important metals such as copper, zinc or iron, and proteins whose hydrolytic access is reduced after the e-amino residue is modified by a carbonyl compound [254]. During the experiment design and selection of culture media it is important to consider that every formulation carry a broad spectrum of effects depending on the chemical nature, its individual reactivity, transformation recalcitrance and biological availability, especially when using pre-formulated lyophilised media. Improvements in the field of culturomics using artificial intelligence and sensor detectors [255] still relate the bacterial proliferation on the use of culture media, therefore, its optimal preparation is key on the positive development of bacteria and defines the profile of microorganisms that might prosper with ease.

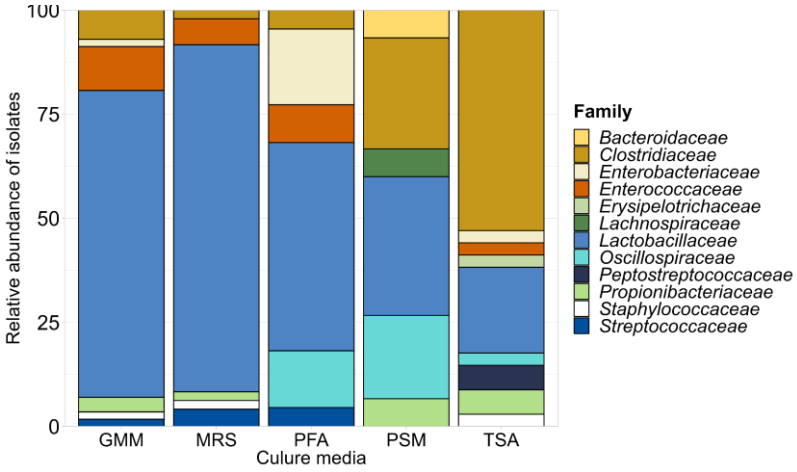

Figure 37. Relative abundance of bacteria isolated from crop, jejunum and ileum obtained in five different culture media: gut microbiota medium (GMM), de Man Rogosa Sharpe (MRS), Poultry Food Agar (PFA), Postgate Medium (PM) and Tryptic Soy Agar (TSA).

All media within the selection supported the growth of at least six different taxonomic families being *Lactobacillaceae* and *Clostridiaceae* two of the most abundant. The proportion of carbon and nitrogen sources variated throughout media in order to stimulate the development of different groups. Higher concentrations of glucose, fructose, and maltose in MRS, as well as starch and cellobiose in GMM and enrichment with a mixed vitamin solution, favoured the isolation of *Lactobacillus*, *Limosilactobacillus*, *Ligilactobacillus*, *Enterococcus* and *Streptococcus*. The media PFA formulated using the chicken's granulated feed supported the growth of *Escherichia* and *Shigella* at the highest proportion and allowed the development of species of *Flavonifractor*

(*Oscillospiraceae*), although the exact nutrient formulation is not known, the feed is based in an equally proportioned soy-corn meal that contains ~250g/kg of crude protein. The complex nature of nutrients within this media may have selectively influence the development of prototrophic bacteria able to synthesise their own amino acids and cofactors such as *F. plautii* and *E.coli/S.flexnerii* whose genome codifies for amino acid biosynthesis including cysteine, histidine, isoleucine, leucine, lysine, ornithine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine, as well as the cofactors proto and siroheme, cobalamin, pyridoxal, riboflavin and tetrahydrofolate.

Postgate Medium (PM) included exclusively lactate as a source of carbon and was selected to broaden the spectrum of isolation to chemoautotrophic bacteria that produce ATP from the oxidation of inorganic compounds like hydrogen sulphide (H_2S), elemental sulphur, ferrous iron (Fe^{2+}) and ammonia (NH_3), hydrogen (H_2) or methane (CH_4). This media was enriched with sodium thioglycolate to help maintaining thiol groups in a reduced state. The presence of chemoautotrophic microorganisms within the chicken gut environment, as in many other ecosystems deprived from sunlight, complete the cycle of the energy flux and contribute to the synthesis of organic compounds used by the heterotrophic community and the host. Although the constant feed intake provides organic compounds for the community to obtain energy and carbon, and the group of chemoautotrophs is a minority, their presence might represent a metabolic sink that prevents the accumulation of inorganic compounds. Within the 15 isolates obtained in this media, incomplete operons related to nitrogen fixation and carbon fixation were encoded within the genomes of *Clostridiaceae* and *Oscillospiraceae*. Although not conclusive of an actual expression nor functionality, chemoautotrophy and chemoheterotrophy in a single organism are not mutually exclusive and represent an advantage of energy obtainment [256]. Organisms whose growth was favoured exclusively in this media encompass *Faecalicatena contorta* and *Phocaeicola vulgatus* from the families *Bacteroidaceae* and *Lachnospiraceae*.

The use of Tryptic Soy Agar (TSA) supported the largest group of isolates from *Clostridiaceae* and was the only media where isolates from *Terrisporobacter petrolearius* grew (*Peptostreptococcaceae*). Although the media formulation included glucose as source of carbon from carbohydrates, the main source of carbon included peptones from the soy extract that might have favoured the development of proteolytic organisms that can hydrolyse proteins as substrates for growth under anaerobic conditions. Despite the harm of *Clostridium perfringens* in chicken's health, other species of *Clostridium* such as *C. butyricum*, can act as a probiotic that increases interleukin IL-17A gene expression and reduces Claudin-1 gene triggered by *C. perfringens* and prevent an infection [257]. In addition, all *Clostridiaceae* isolates in this work encode for acetate as a major end-product of metabolism and additionally are able to use sulphite as an alternative electron acceptor.

To our knowledge, very few authors justify the selection of culture media nor discuss its direct influence on their results. Moreover, a strong influence of the cultivation strategy of clinical microbiology remains at the formulation of media used to isolate commensal microorganisms,

that in fact are more susceptible to the use of antibiotics or selective compounds such as dyes or surfactants, and present a narrowed adaptation capacity to develop on different environments compared to pathogens [258]. In the other hand, some conclusions obtained from human and other animal gut studies have stated that: either a pre-incubation in blood culture, the addition of rumen fluid, or the addition of sheep blood have increased the isolation of novel species in human stool samples [46, 259]. In chicken, whose diet has been lately based exclusively on plants, the use of enriched nutritive agars with major components of meat extract and casein peptones should rather be formulated with plant based components that mimic the environment and consider the auxotrophy to some amino acids and cofactors [260].

4.2 The watershed of the taxonomic reorganisation among *Lactobacillaceae*

The taxonomic reorganisation of the genus *Lactobacillus* into 25 genera stated a new way of observing the microbial composition and its ecology [59]. The polyphasic approach on which is based, allow to separate organisms that occupy similar niches with different metabolic capacities reflected in their abilities of homofermentative or heterofermentative products, improving the analysis of environments where these species dominate such as fermented substrates, cavities and gut environments [59]. In chicken, the former reports of the dominance of *Lactobacillus* sp. on the proximal GIT sections did not provide detailed information about fermentation pathways, secondary metabolites, ecological roles or how its complex nutritional requirements are satisfied.

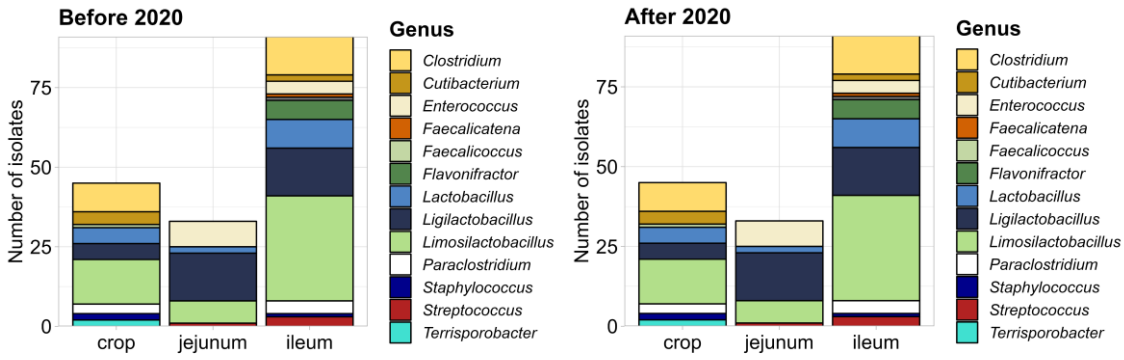

Figure 38. Number of bacterial isolates from the phylum *Bacillota* obtained in this work annotated under the taxonomic classification before and after the reorganisation of the genus *Lactobacillus* sp.

Three genera of *Lactobacillaceae: Lactobacillus*, *Ligilactobacillus* and *Limosilactobacillus* are of importance within the ecological analysis of chicken gut microbiome and resolve the former generalisation of multiple niche occupation by the emended genus *Lactobacillus.* In this case, the high abundance of *Limosilactobacillus* and *Lactobacillus* in crop, showed a clear advantage given at the environment for the colonisation of bacteria with a wide spectrum of carbohydrate utilisation [59]. Both *Limosilactobacillus* and *Lactobacillus* colonise in higher abundance this environment

rich in fermentable substrates. In contrast, homofermentative bacteria *Ligilactobacillus,* with a moderate less-wide range of carbohydrate fermentation, were found more abundant at jejunum and ileum samples, where substrates might have been previously hydrolysed by the host or other commensals. These results highlight the importance of the taxonomic re-assignations seen through the eyes of ecology, bringing more clarity on the different niche occupation of the now-separated species grouped within different genera, as well as a detailed approach of its adaptations to the environment.

The advantage of cultivation in this work provided robust references that support the taxonomic delineations of *Limosilactobacillus* and *Ligilactobacillus*. Moreover, the addition of the species *L. hohenheimensis* to the genus, opens the possibility to alleviate the heterogeneity of *Ligilactobacillus* which gathers a cluster of poultry related bacteria that differs from the rest of the genus grouping criteria (AAI, cAAI). However, more isolates are needed to support a further reclassification.

4.3 The importance of the upper colonisers and the key role of ecology

In chicken GIT, the first sections (crop, proventriculus, gizzard) are susceptible to be colonised by a wide variety of bacteria due to the availability of substrates at a high concentration, oxygen concentration and the proximity to the feed entrance. However, these environments maintain extreme conditions such as low pH, low redox potential, high osmotic pressure, and antibacterial secretions from the host, that shape the profile of colonisers to the most fit and adaptable microbes [240, 261, 262]. Bacteria from the collection at these regions, encode an ATPase proton exclusion pump to resist acid fluctuations, that despite to be a high energetic investment, provides the advantage to be present during the hosts' digestion and obtain first hand components including amino acids, minerals, fibre, carbohydrates, and starch. The decline of the redox potential influences the energy metabolism strategy to a "more affordable" fermentation of organic substrates or inorganic oxidation, to obtain energy and carbon under anaerobic conditions [83]. Bacteria that successfully colonise at this early point of digestion, form a variety of SCFAs that indirectly help to regulate the colonisation of saprophytic and pathogenic microorganisms by reducing the pH. There has been reported an indirect correlation between *Enterobacteriaceae* and the SCFA concentrations, proving that an increase of SCFAs significantly reduces the population of potential enteropathogens [263].

The capacity to ferment carbohydrates, resist low pH, osmotic pressure and produce SCFAs, encoded at genomes from the isolates of *Clostridiaceae*, *Lactobacillaceae*, *Enterococcaeae* and *Streptococcaceae* obtained from crop in this work, gives an advantage over other bacteria with a structural sensitivity to low pH and high osmotic pressure. In addition, the retention time of digesta in crop, influences and regulates the inoculum of microorganisms that will continue the colonisation in distal regions [51]. Approaches using 16S rRNA gene sequencing have reported a similar microbial population profile between crop and gizzard dominated by *Lactobacillaceae* and *Clostridiaceae* [81, 264], meaning that microorganisms that survive crop conditions are potentially able to resist those from proventriculus and gizzard. In this sense, determining the

microbial composition and its metabolic potential at these sections is crucial to understand the role of the proximal digestive sections selecting and seedling fit microorganisms.

Conditions in small intestine sections continue having high osmotic pressure, pH variations and increased enzymatic activities due to enhanced secretions of host-related substances. The prevailed dominance of Gram positive bacteria in this region reported by some studies [43, 126, 265, 266] was also confirmed by the isolation rate of *Lactobacillaceae* and *Clostridiaceae* species that remain colonising jejunum and ileum despite the increased competence for nutrients with the host. However, from ileum, a broader taxonomic and metabolic diversity was recovered represented by species from the families *Bacteroidaceae, Oscillospiraceae, Peptostreptococcaceae* and *Propionibacteriaceae.* The strict anaerobic conditions and the less acidic and variable conditions at this section provide opportunities to protein fermenters, butyrate and propionate producers as well as to H_2 and CH_4 oxidisers among other niches, the potential of these microorganisms to synthesise their own cofactors does not compete with *Lactobacillaceae* and *Clostridiaceae* for amino acids, enriching the diversity of metabolic products [267, 268].

Former explorations of the microbiota in proximal regions (crop to ileum) have reported a dominance of *Lactobacillus* sp [126, 264, 269, 270]. However, few have been ecologically re-interpreted after the taxonomic re-assignation of the genus in 2020, to bring more clarity to the former idea of the ecology and functions of the community. The presence of heterofermentative bacteria represents a broader supply of SCFAs and might indirectly indicate that the environment provides a wider range of carbohydrates and cofactors available for fermentation at crop and gizzard. By contrast, homofermentative organisms (that produce mainly lactate), whose metabolic plasticity and nutrient demands are less variable, find an advantage at environments with less availability of resources, such as duodenum and ileum, obtaining energy using a more efficient strategy and even competing with the host. This can be observed at the genome size difference and glycosidase genes present on *Limosilactobacillus* and *Lactobacillus*, larger than in *Ligilactobacillus* genomes, as well as from the large-scale genomes search along crop, small intestine, caecal and faecal samples. The advantage of sequencing, assembling, and annotation of genomes from pure microbial cultures is to provide better quality data compared to environmental sequencing or metagenome analyses. The information obtained from these genomes is therefore a reflection of the adaptations each microorganism develops within the environment and data generated can substantially improve gene database of chicken microbiome. The identified microbial functions encoded within the strain collection may have many effects within the chicken intestinal tract including:

4.2.1 Functions encoded within the collection: Nutrient availability supply

The metabolic potential of carbohydrate catabolism observed through carbohydrate active enzymes present in the isolates represents a key process that triggers downstream mechanisms within the same bacterial community, the adjacent microbial communities, the epithelia, the host's digestion and immune responses. In general, carbohydrate's metabolisms encoded by isolates

from these sections, was found more extended and specialised, indicating to have a more important role than the metabolism of proteins. Little information is available on the ratio of metabolism of carbohydrates and proteins of bacteria in chickens. Yet, metabolism of carbohydrates at distal sections is more desirable since it does not lead to putrefactive reactions that carry negative health effects on the host [83] such as biogenic amines, ammonia or volatile fatty acids (See section 1.2.1.1). Genomes of proximal colonisers from the collection, encoded glycosylases (hydrolases and transferases) that transform carbohydrates and represent a source of soluble fibre. The synthesis of malto, xylo, galacto among other oligosaccharides by upper colonisers resist the host's digestion and reach the lower sections caeca and colon, favouring its metabolism over proteins [196, 271-275]. In pig and human intestinal systems, the presence of fermentable carbohydrates in the intestinal digesta has been shown to "suppress" protein fermentation due to a preference of bacteria to ferment carbohydrates from which more energy is obtained [276, 277]. In the other hand, enzymes that facilitate protein digestion in the upper intestine deplete proteins and prevent them to reach further sections, in this sense, the metabolic activity of bacteria at crop and small intestine are crucial at providing substrates to the microbial community in caeca.

The carbohydrate fermentation potential encoded indicates a highly adaptive mechanism to the digestive environment. SCFA produced after bacterial fermentation serve as energy source for enterocytes and stimulate the immune layer development [90]. Butyrate, acetate, and lactate detected as main fermentation products within the strain collection have been shown to stimulate the generation of mucosa layer, epithelia maturation, regulatory T cells production, cytokines expression (see section 1.2.1.2) and even shape the environmental profile due to its antimicrobial properties [90, 278], highlighting its value as a prebiotic, hypothesis already tested for humans at *in vitro* tests and mice [274, 279].

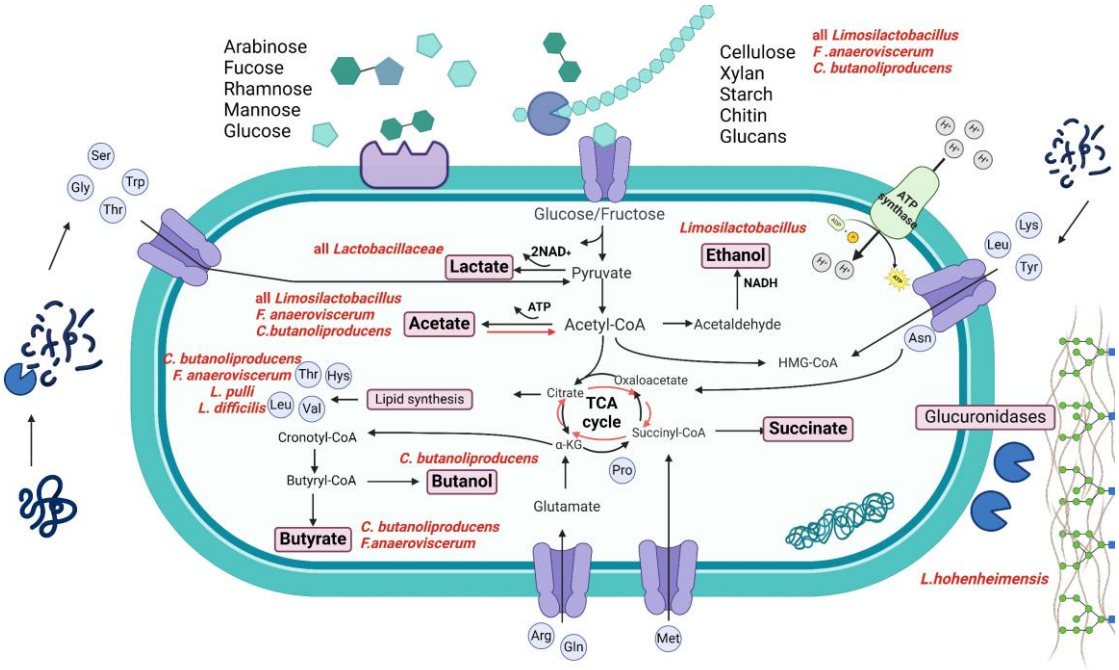

Figure 39. Outline of the main fermentation pathways encoded among bacteria from the collection.

Peptidases in the other hand would provide amino acids to the host and the bacterial community, the latest having an essential demand to some of them for its development (*Lactobacillaceae* and *Propionibacteriaceae*). The added value of bacteria during the digestion of proteins might lay on the late processing of amino acids such as tryptophan that can be transformed into intermediates including indole and serotonin [47]. These molecules communicate with the adaptive and innate immune cells via the aryl hydrocarbon receptor [280] producing interleukin IL-22 which among other effects strengthens the epithelial barrier, induce the production and secretion of antimicrobial peptides and stimulates the proliferation of intestinal goblet cells and mucins (MUC-2) [47, 98, 99].

Beyond all the further benefits from the catabolism of carbohydrates and proteins, the collection also encoded for carbohydrate active enzymes that build long chained branched glycosides and glycans through carbohydrate anabolism. These oligosaccharides are important for adhesion of bacteria to the gut epithelial cells, represent fermentable carbohydrates to feed other microbial groups located in distal regions and are an indirect nutrient supply of soluble fibre for the host's digestion [195].

4.2.2 Functions encoded within the collection: Molecules of interaction with the host

Unlike SCFA or IMMPs which are a result of the primary metabolism of bacteria, some enzymes encoded within the collection have to potential to interact directly with the host's epithelial architecture and hormone signalisation. Molecules such as hyaluronate lyase and collagenase interfere with the structural proteins on the epithelial surface and modify it. In the other hand, peptidases with the potential to interact with peptide hormones and peptide signals from the host

were widely distributed in all the strains particularly in *Lactobacillaceae*. The peptidase PepO, a neutral endopeptidase homologue of the eukaryotic membrane-bound protease “neprilysin” can catalyse the conversion of big endothelin to endothelin, signalling the host via the activation of such signals. Endogenous neprilysin (NEP) in humans play a key role in regulating the levels and activity of a variety of peptide hormones and neuropeptides, including natriuretic peptides, bradykinin, substance P, and amyloid-beta peptide [281]. However, research on the role of interaction of bacterial peptidases with peptide hormones in chickens is limited. At *in vitro* observations, the endopeptidase PepO of *Streptococcus pyogenes* is able to degrade an active form of peptide signalisation pheromone, inhibiting quorum-sensing activation under low metal conditions [282]. Whether bacterial endopeptidases interact with host peptides is not clear due to its location and lack of evidence of such peptidases reaching bloodstream. However, studies on molecular dynamic of NEPs from the bacterial source *Streptococcus suis* GZ1 docked with Aβ peptide accumulated in Alzheimer's disease have confirmed a positive stable interaction at the active site of bacterial NEP residue Glu-538 with the region His-13 of the Aβ peptide [283], providing information about the interaction spectrum of these molecules.

To this extent, future differences observed on taxonomic groups between studies, should also be interpreted from an ecological point of view where one taxon can represent the ecological equivalent of other in its absence, being able to cover the niche and keeping the environment's resilience and the host’s physiology unaltered.

4.3 The novel species within the collection

Microbial explorations on novel environments often include the description of not yet characterised microorganisms. The isolation of bacteria from samples of crop, jejunum and ileum succeeded on the first cultivation and description of *L. galli* and *L. hohenheimensis,* two bacterial species formerly detected by metagenome analysis of faeces [27], and six novel species not reported before, either due to the bias of sampling distal GIT sections and their decrease of abundance at lower sections, or in the case of *Clostridium* and low abundant bacteria, due to their resistance to be detected by sequencing methods.

The colonisation specificity of many of the species from the collection remains in the upper sections. The large-scale search analysis of the collection’s genomes in samples of crop, ileum and caeca of the same birds and the redundancy of its isolation, confirmed a preference to either crop or jejunum and ileum.

The novel species of *Limosilactobacillus* presented a low redundancy of isolation, but its abundance was observed higher in crop, especially for *L. avium* and *L. pulli*. However, the strain *of L. difficilis*, from which only two isolates were obtained, reported no detection at any GIT section, remaining so far undetected at the tested samples together with *F. anaeroviscerum* and *C. butanoliproducens*. Although future explorations of poultry samples might detect the presence of these strains, its isolation frequency was of at least two clones, which supports the hypothesis

that these specie's dormant phase cannot be easily detected by direct sequencing. Nonetheless, these isolation reports from ileum account for their condition as members of the small intestine community and highlight their preferential colonisation to the upper sections. The handling of these microorganisms enriches the diversity knowledge of the community and represents a potential source of beneficial microorganisms to modulate the homeostasis of chicken intestine.

4.4 *Ligilactobacillus aviarius*, an indigenous member of the chicken microbiome

When building up the collection, the species L. aviarius stood out as a redundant isolate from the small intestine due to its persistence: From this specie, the biggest number of isolates and strains was obtained (72 isolates, 9 different strains) coming either from jejunum or ileum. Due to its occurrence in this work and its presence in all former poultry studies, *L. aviarius* can be considered an indigenous specie of the avian intestine occurring naturally inhabiting the intestine of chicken [35, 196, 197, 202], turkey [284, 285], gull [286] and recently in takahē an endemic bird from New Zealand [287]. Unlike other host related lactic acid bacteria, *L. aviarius*, seems to have a preference to colonise the avian intestine, being so far not yet related to other hosts-group like *L. salivarius* to humans [128, 288, 289], *L. agilis* to sewage, mice and pig [210, 290] or *Lactobacillus kitasatonis* to pigs and humans [265, 291, 292]. Moreover, genomes and metagenomes availability deposited in reference collections report jejunum, ileum, caeca and faeces as the main source of isolation [27, 63, 146] representing a well-adapted coloniser to avian intestine, being poultry so far, the most common environment of occurrence. The isolation of nine strains of *L. aviarius* from jejunum and ileum of chicken, enrich our knowledge about the persistence and specific adaptations of *L. aviarius* to the whole intestine of chicken.

The pan-genome construction of *L. aviarius* [293] contemplated the inclusion of all non-clonal genomes available in the databases up to July 2022. Despite the low availability of genomes of *L. aviarius*, each GIT section, from jejunum to faeces, was represented by at least three genomes. However, the phylogenomic analysis did not show any GIT section-derived clustering, meaning that despite the strain was isolated or assembled from a given section, its distribution is not restricted to a region and presented only minor specific adaptations to the GIT sections. These characteristics can be also observed at the functional annotation, where genomes from all sections presented similar gene clusters codifying to carbohydrate transport, synthesis of oligosaccharides or envelop biogenesis that unlike other species recovered in the collection, have a high prevalence along the GIT with higher abundance at ileum (section 3.3.4).

Besides the dominance and adaptability within the chicken intestine, the ecological role of *L. aviarius* within the environment is to metabolise carbohydrates and supply oligosaccharides to the lower sections. Its advantage over other ileum colonisers stands out by its capacity to interact with the host encoding key molecules of communication with the intestinal epithelia (endopeptidases insulinases, collagenases and anti-inflammatory glycopeptides) that ease its

establishment and adaptability. Moreover, the amount of genes devoted to envelop biogenesis suggest a resilient adaptation to constant changes in the environment, and the codification of folic acid antagonist molecules, a competitive trait against other colonisers to deplete their sources of folates, its DNA synthesis and replication [294].

4.5 The chicken hologenome

The microbial component of every multicellular organism has evolved with the host as a result of their coexistence, to a point of mutual dependence that favours the optimal development of all the components. The concept of holobiont, introduced by Lynn Margulis in 1991 [295], encompass a functional unit of related elements that persist within the same space. The term describes the extension of metabolic networks that a single organism would be unable to cover, offering a variety of tools to persist over time and surpass natural selection. The total codification of genomes involved in these symbiotic relations was introduced in 2008 by Ilana Zilber-Rosenberg and Eugene Rosenberg [296] under the concept of hologenome, to describe the sum of host genome and associated microbial genomes.

In the case of chicken, the nutritional diversity of the holobiont (chicken + its microbiome) expand the ability of utilising feed components unavailable to the digestive system of the host, thus improving the overall energy capture from the diet [297]. This concept highlights the important role of microbes during the adaptation process of artificial selection [296]. In fact, the close relation of chicken to its microbiome seems to be a synergistic result shaped during domestication. Among the most successful traits of the “chicken holobiont” are: the energy efficient trait of fast weight gaining related to a high production of SCFA by microbial colonisers that increases the energy availability to the host, promoting weight gaining [297, 298]; the improvement of epithelia development [299] and the immune response stimulation and signalisation [63, 104]. The absence of some metabolites in germ-free animals, such SCFAs, and quorum-sensing molecules [300] confirms the closed dependence of chicken to its microbiome to maintain homeostasis.

Since carbohydrate hydrolysis seems to be an important added value that the microbiome offers to the host, observations on the amount and types of CAZymes dedicated to the hydrolysis, biosynthesis, and modification of complex carbohydrates have been annotated within the chicken gut microbiome of caeca and faeces. The CAZyme repertoire encoded by our collection, broadened the hydrolytic capabilities known for “the chicken holobiont” (Figure 40) expanding the digestive potential of approximately 57 enzymatic families encoded exclusively at the genome of *Gallus gallus*. This enzymatic potential is however partially described, hence deeper metagenomic and culturomic explorations will help to identify new pathways within the chicken hologenome.

These interactions are demonstrated to be important not only at a “local- metabolic” level, but to impact social behaviour [301]. Behavioural studies in chicken have related feather pecking (FP), a negative behaviour in chickens that reduces animal welfare, to the microbial composition by

observing FP behaviour of microbial transplanted chicken from high (HFP) and low feather pecking (LFP) during the first two post-hatch weeks [302]. Observing that behavioural responses of HFP birds seemed to be influenced by receiving microbiomes from HFP adults, compared to LFP microbiome or the control treatment. However, the results were not sufficient to alter conduct responses after the treatment. Although behavioural responses in birds were partially demonstrated, the physiological pathway remains unclear, and more research need to be done addressing impact of gut microbial composition in complex social animals such as chicken.

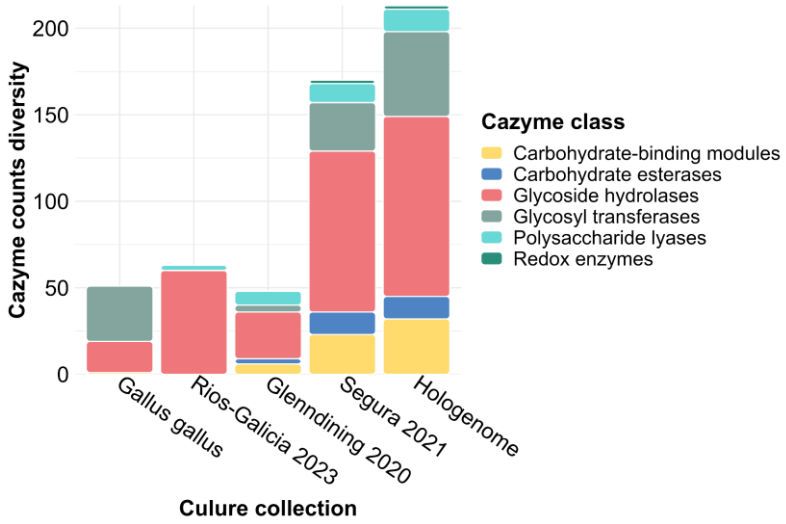

Figure 40. Culture collection's diversity of CAZymes encoded by members of the chicken hologenome along. The genome of *Gallus gallus* (CAZyme database), the bacterial collection from crop and small intestine [200], a caecal metagenomic assembly approach [26], an ileal, caecal and colon metagenomic study [303] and the hypothetical hologenome constructed by collecting all non-redundant CAZymes detected encoded by the former studies plus the endogenous CAZymes of *Gallus gallus*, depicting the carbohydrate metabolic potential that the hologenome of chicken might gather.

5. Summary

The modern morphotype of chicken used in the poultry industry nowadays is characterized by an efficient energy transformation ratio that makes it one of the most convenient protein sources for human consumption. The aseptic breeding conditions in farming and the limited parental contact with offspring, make of chicken an exceptional case of study to understand how microbiome stablishes and contributes to maintain the host's homeostasis. It has been stated that the process of acquisition and maturation of the gut microbiome throughout its lifecycle its crucial and plays an important role assimilating nutrients from food, producing cofactors, vitamins, and essential amino acids, but also influences the regulation of physiological functions such as immune response, pathogen establishment and intestinal barrier integrity. In this sense, research must be directed to maintain the gastrointestinal tract (GIT) to a state of symbiotic equilibrium between the microbiome and the host. Along the GIT, the availability of substrates decreases and the accumulation of complex nutrients resistant to digestion is favoured towards the distal sections. In contrast, the taxonomic and metabolic diversity of caeca and faeces is bigger compared to those at crop and small intestine. Despite the transition time of digesta plays a crucial role on the distribution of the microbial population, the genomic analysis of bacterial colonisers in distal sections has shown a specialisation of some members to degrade complex nutrients in the diet. Highlighting the importance to understand the influence of the microbial population colonising the proximal sections and the host's digestion, during nutrients transformation.

Considering the improvements in the use of culturomics to approach microbial ecology diversity, and the amount of useful information encoded at genomes of cultured bacteria from caeca and faeces of chicken, a collection of microbial colonisers from the proximal digestive sections: crop and small intestine was established. The generated information broadened the description of taxa, genes and genomes, improving the repository of chicken's microbiome. As main results, the functional description of the collection contributed to enrich the gene catalogue and repertoire of carbohydrate-active enzymes, peptidases, interactive molecules, and accessory adaptations, increasing the metabolic potential assumed for chicken as an holobiont. Additionally, one species from *Clostridium,* the genus *Faecalispora*, one species from *Ligilactobacillus*, and five species from *Limosilactobacillus* from the family *Lactobacillaceae* were described and handled in pure culture for the first time, expanding the diversity of taxonomic descriptions formerly undetected by sequencing approaches. The advantage of counting with DNA sequences of the samples of crop, ileum and caeca from the same chickens where the collection was obtained from, provides a panoramic picture of the colonisation preference, prevalence and abundance of each strain within the environment. Moreover, the calculated relative abundance was reflected at the isolation redundancy. Finally, the different genomic adaptations found along the collection are reflected in their advantages to dominate at a given section, as well as in their colonisation plasticity.

Within the collection, the isolation redundancy and relative abundance of *L. aviarius* along all GIT sections, highlighted its persistence and particular adaptability as a dominant key member of the poultry gut microbiome. Compared to other colonisers, its small genome size and functional

adaptations, suggest a close ecological relation to the poultry gut environment. The codification and characterization of accessory genes provide information of its dynamics with other members of the community and evidence its adaptation plasticity, broadening our knowledge about the close host-microbe interactions. All these efforts target to improve our general understanding of the microbiome structure and dynamics within the host and provides robust references to the GIT database. In addition, cultured microorganisms represent a biotechnological tool that might increase the production efficiency improving chicken's health and prioritising animal welfare.

6. Zusammenfassung

Der moderne Morphotyp des Huhns, der heutzutage in der Geflügelindustrie verwendet wird, ist durch ein effizientes Energieumwandlungsverhältnis zu einer der besten Proteinquellen für den menschlichen Verzehr geworden. Die aseptischen Zuchtbedingungen in der Landwirtschaft und der fehlende Kontakt der Eltern mit dem Nachwuchs machen das Huhn zu einem außergewöhnlichen Studienobjekt, um zu verstehen, wie sich das Mikrobiom aufbaut und zur Aufrechterhaltung der Homöostase des Wirts beiträgt. Es wurde festgestellt, dass der Prozess des Erwerbs und der Reifung des Darmmikrobioms während des gesamten Lebenszyklus von entscheidender Bedeutung ist und eine wichtige Rolle bei der Assimilation von Nährstoffen aus der Nahrung, der Produktion von Cofaktoren, Vitaminen und essenziellen Aminosäuren spielt, aber auch die Regulierung physiologischer Funktionen wie die Immunantwort, die Ansiedlung von Krankheitserregern und die Integrität der Darmbarriere beeinflusst. In diesem Sinne muss die Forschung darauf ausgerichtet sein, den Darmtrakt in einem Zustand des symbiotischen Gleichgewichts zwischen Mikrobiom und Wirt zu erhalten. Entlang des GIT nimmt die Verfügbarkeit von Substraten ab, und die Anhäufung von komplexen, verdauungsresistenten Nährstoffen wird in den distalen Abschnitten begünstigt. Im Gegensatz dazu ist die taxonomische und metabolische Vielfalt in Zäkum und Fäkalien größer als in Kropf und Dünndarm. Obwohl die Übergangszeit im Verdauungstrakt eine entscheidende Rolle für die Verteilung der mikrobiellen Population spielt, hat die genomische Analyse der bakteriellen Besiedler in den distalen Abschnitten eine Spezialisierung einiger Mitglieder auf den Abbau komplexer Nährstoffe gezeigt, die nicht in der Nahrung enthalten sind. Dies unterstreicht, wie wichtig es ist, den Einfluss der mikrobiellen Population, die die proximalen Abschnitte besiedelt, und die Verdauung des Wirts während der Nährstoffumwandlung zu verstehen.

In Anbetracht der Verbesserungen bei der Nutzung der ‚culturomics' zur Erforschung der mikrobiellen Ökologie und der Menge nützlicher Informationen, die in den Genomen kultivierter Bakterien aus Zäkum und Kot von Hühnern kodiert sind, wurde eine Sammlung mikrobieller Besiedler aus den proximalen Verdauungsabschnitten - Kropf und Dünndarm - erstellt. Die gewonnenen Informationen erweiterten die Beschreibung von Taxa, Genen und Genomen und verbesserten das die Zugänglichkeit von Bakterien aus Hühnern. Als wichtigste Ergebnis trug die funktionelle Beschreibung der Sammlung dazu bei, den Genkatalog und das Repertoire an kohlenhydrataktiven Enzymen (CAZyme), Peptidasen, interaktiven Molekülen und akzessorischen Anpassungen zu bereichern, wodurch das metabolische Potenzial des Huhns als Holobiont vergrößert wurde. Darüber hinaus wurden ein Art der Gattung *Clostridium*, der Gattung *Faecalispora*, eine Art von *Ligilactobacillus* und fünf Arten von *Limosilactobacillus* aus der Familie der *Lactobacillaceae* erstmals in Reinkultur beschrieben und bearbeitet, wodurch die Vielfalt der taxonomischen Beschreibungen erweitert wurde, die zuvor durch Sequenzierungsansätze unentdeckt blieb. Der Vorteil der Zählung mit DNA-Sequenzen der Proben von Kropf, Ileum und Zäkum von denselben Hühnern, von denen die Sammlung stammt, bietet einen großen Einblick in die Kolonisierungspräferenz, Prävalenz und Abundanz jedes Stammes in der Umgebung.

Darüber hinaus spiegelt sich die berechnete relative Häufigkeit in der Isolationsredundanz wider. Schließlich stellen sich die verschiedenen genomischen Anpassungen, die in der Sammlung gefunden wurden, in ihren Vorteilen, in einem bestimmten Abschnitt zu dominieren, sowie in ihrer Kolonisierungsplastizität dar.

Innerhalb der Sammlung unterstrichen die Isolationsredundanz und die relative Häufigkeit von *L. aviarius* in allen GIT-Abschnitten seine Persistenz und besondere Anpassungsfähigkeit als dominantes Schlüsselmitglied des Geflügeldarm-Mikrobioms. Im Vergleich zu anderen Kolonisatoren deuten die geringe Genomgröße und die funktionellen Anpassungen auf eine enge ökologische Beziehung zur Darmumgebung von Geflügel hin. Die Kodierung und Charakterisierung akzessorischer Gene gibt Aufschluss über ihre Dynamik mit anderen Mitgliedern der Gemeinschaft und belegt ihre Anpassungsplastizität, wodurch unser Wissen über die engen Wirt-Mikroben-Interaktionen erweitert wird. All diese Bemühungen zielen darauf ab, unser allgemeines Verständnis der Mikrobiomstruktur und -dynamik innerhalb des Wirts zu verbessern, und liefern robuste Referenzen für die GIT-Datenbank. Darüber hinaus stellen kultivierte Mikroorganismen ein biotechnologisches Instrument dar, das die Produktionseffizienz steigern, die Gesundheit der Hühner verbessern und dem Tierschutz Vorrang einräumen könnte.

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8. Supplementary material

8.1 Supplementary data

Table 9. Database of the160 isolates obtained from the cultivation of bacteria from crop, jejunum and ileum of chicken, identified by 16S sanger sequencing.

	Internal sequenc	*Specie*	ID%	iginal strain designati	Risk Grp.	hit with 16S rRNA	Gut region	Isolation media	Oxygen resistance	solation time	ate of isolatio	lew species
1	B001	*Lactobacillus johnsonii*	96.14	BC7T2	1	ATCC 33200	crop	TSA	facultative	48h	Jan-20	no
2	B002	*Limosilactobacillus pontis*	99.85	BC6G17	1	LMG 14187	crop	GMM	facultative	48h	Jan-20	yes
3	B003	*Limosilactobacillus alvi*	99.58	BC6M1	1	R54	crop	MRS	facultative	48h	Jan-20	no
4	B004	*Ligilactobacillus salivarius*	97.64	BCEP5	1	JCM 14209	crop	PFA	facultative	48h	Jan-20	no
5	B005	*Lactobacillus kitasatonis*	100	BI6G6	1	JCM 1039	ileum	GMM	facultative	96h	Apr-20	no
6	B006	*Limosilactobacillus panis*	99.24	BCEM4	1	DSM 6035	crop	MRS	facultative	48h	Jan-20	no
7	B007	*Ligilactobacillus aviarius*	99.78 .	BCEP25	1	DSM 20655	crop	PFA	anaerobic	72h	Jan-20	no
8	B008	*Clostridium cochlearium*	99.85	BCET54	1	NCTC 13027(T)	crop	TSA	anaerobic	96h	Jan-20	no
9	B009	*Ligilactobacillus saerimneri*	99.79	BIEG38	1	DSM 16049	ileum	GMM	facultative	48h	Apr-20	no
10	B010	*Limosilactobacillus reuteri*	99.43	BIEM5	1	JCM 1112	ileum	MRS	facultative	48h	Apr-20	no
11	B011	*Limosilactobacillus ingluviei*	99.93	BI5M2	1	DSM 15946	ileum	MRS	facultative	48h	Apr-20	no
12	B012	*Limosilactobacillus mucosae*	99.50	BIEG8	1	S32	ileum	GMM	facultative	48h	Apr-20	no
13	B013	*Lactobacillus kitasatonis*	99.86	BCEG54	1	JCM 1039	crop	GMM	anaerobic	48h	Jan-20	no
14	B014	*Ligilactobacillus aviarius*	99.86	BIEP40	1	DSM 20655	ileum	PFA	anaerobic	2w	Apr-20	no
15	B015	*Limosilactobacillus panis*	99.93	BIEM52	1	DSM 6035	ileum	MRS	facultative	1w	Apr-20	yes
16	B016	*Terrisporobacter petrolearius*	96.74	BCET23	1	LAM0A37	crop	TSA	anaerobic	1w	Jan-20	no
17	B017	*Ligilactobacillus saerimneri*	99.86	BIEP27	1	DSM 16049	ileum	PFA	facultative	72h	Apr-20	no
18	B018	*Clostridium butyricum*	100	BIES2	1	CP013239	ileum	PSM	anaerobic	48h	Apr-20	no
19	B019	*Enterococcus faecium*	98.04	BIET4	1	LMG 11423	ileum	TSA	facultative	48h	Apr-20	no
20	B020	*Faecalicoccus pleomorphus*	99.24	BCET49	1	DSM 20574	crop	TSA	anaerobic	1w	Jan-20	no
21	B021	*Paraclostridium benzoelyticum*	99.85	BIES1	1	JC272	ileum	PSM	anaerobic	4w	Apr-20	no
22	B022	*Terrisporobacter petrolearius*	99.93	BCET37	1	LAM0A37	crop	TSA	anaerobic	120h	Jan-20	no
23	B023	*Ligilactobacillus saerimneri*	99.86	BCEP57	1	DSM 16049	crop	PFA	facultative	48h	Jan-20	no
24	B024	*Ligilactobacillus aviarius*	99.86	BCEG14	1	DSM 20655	crop	GMM	anaerobic	48h	Jan-20	no
25	B025	*Clostridium butyricum*	99.35	BIEG40	1	DSM 10702	ileum	GMM	anaerobic	72h	Apr-20	no
26	B026	*Limosilactobacillus alvi*	97.63	BIEM28	1	R54	ileum	MRS	facultative	120h	Apr-20	no
27	B028	*Limosilactobacillus panis*	99.93	BIEM40	1	DSM 6035	ileum	MRS	facultative	48h	Apr-20	no
28	B029	*Limosilactobacillus oris*	99.65	BCEG74	1	DSM 4864	crop	GMM	facultative	144h	Jan-20	no
29	B030	*Limosilactobacillus vaginalis*	99.51	BIES8	1	AF243177	ileum	PSM	facultative	48h	Apr-20	no
30	B31	*Limosilactobacillus reuteri*	99.65	BC6G16	1	JCM 1112	crop	GMM	facultative	72h	Jan-20	no
31	B32	*Limosilactobacillus mucosae*	99.50	BIEP23	1	S32	ileum	PFA	facultative	1w	Apr-20	no
32	B33	*Limosilactobacillus mucosae*	99.37	BC7P1	1	S33	crop	PFA	facultative	72h	Jan-20	no
33	B34	*Lactobacillus kitasatonis*	99.86	BCES7	1	JCM 1039	crop	PSM	facultative	120h	Jan-20	no
34	B35	*Lactobacillus gallinarum*	99.79	BIEG45	1	JCM 2011	ileum	GMM	facultative	120h	Apr-20	no
35	B36	*Staphylococcus epidermidis*	100%	BI5M5	1	NCTC 11047	ileum	MRS	facultative	48h	Apr-20	no
36	B37	*Lactobacillus gallinarum*	99.79	BCEG40	1	JCM 2011	crop	GMM	facultative	48h	Jan-20	no
37	B38	*Cutibacterium acnes subsp. acnes*	99.92	BCEM41	1	DSM 1897	crop	MRS	facultative	48h	Jan-20	no
38	B39	*Cutibacterium acnes subsp. acnes*	99.78	BCET35	1	DSM 1897	crop	TSA	facultative	48h	Jan-20	no
39	B40	*Paraclostridium benzoelyticum*	99.23	BIET41	1	JC272	ileum	TSA	anaerobic	72h	Apr-20	no
40	B41	*Clostridium butyricum*	99.76	BCES2	1	DSM 10702	crop	PSM	anaerobic	1w	Jan-20	no
41	B42	*Clostridium butyricum*	99.77	BIET49	1	DSM 10702	ileum	TSA	anaerobic	120h	Apr-20	no
42	B43	*Clostridium butyricum*	99.1	BCET25	1	DSM 10702	crop	TSA	anaerobic	72h	Jan-20	no
43	B44	*Staphylococcus epidermidis*	99.72	BCEG32	1	NCTC 11047	crop	GMM	anaerobic	48h	Jan-20	no
44	B45	*Limosilactobacillus panis*	99.37	BIEG51	1	DSM 6035	ileum	GMM	facultative	48h	Apr-20	no
45	B47	*Clostridium argentinense*	99.05	BCET15	1	ATCC 27322	crop	TSA	anaerobic	48h	Jan-20	no
46	B48	*Clostridium argentinense*	98.69	BIET14	1	ATCC 27322	ileum	TSA	anaerobic	48h	Apr-20	no
47	B49	*Limosilactobacillus reuteri*	99.58	BI6G18	1	JCM 1112	ileum	GMM	facultative	48h	Apr-20	no
48	B50	*Clostridium argentinense*	99.01	BCET16	1	ATCC 27322	crop	TSA	anaerobic	48h	Jan-20	no
49	B52	*Limosilactobacillus oris*	99.63	BIEG13	1	DSM 4864	ileum	GMM	facultative	48h	Apr-20	no
50	B53	*Ligilactobacillus aviarius*	99.7	BIES11	1	DSM 20655	ileum	PSM	facultative	72h	Apr-20	no
51	B54	*Limosilactobacillus vaginalis*	99.72	BCEG35	1	ATCC 49540	crop	GMM	facultative	72h	Jan-20	no
52	B55	*Staphylococcus epidermidis*	99.44	BCET29	1	NCTC 11047	crop	TSA	anaerobic	2w	Jan-20	no
53	B56	*Ligilactobacillus aviarius*	99.91	BIES18	1	DSM 20655	ileum	PSM	facultative	1w	Apr-20	no
54	B57	*Cutibacterium acnes subsp. acnes*	99.93	BIET26	1	DSM 1897	ileum	TSA	facultative	72h	Apr-20	no
55	B58	*Flavonifractor plauti*	99.54	CIET9	1	ATCC 29863	ileum	TSA	anaerobic	72h	Apr-20	no
56	B59	*Flavonifractor plauti*	99.64	CIES36	1	ATCC 29863	ileum	PSM	anaerobic	1w	Apr-20	no
57	B61	*Clostridium butyricum*	99.3	BCET31	1	DSM 10702	crop	TSA	anaerobic	72h	Jan-20	no
58	B62	*Faecalicatena contorta*	94.22/ 99.54	CIES54	1		ileum	PSM	facultative	1w	Apr-20	no
59	B63	*Clostridium argentinense*	98.77	BCET17	1	ATCC 27322	crop	TSA	anaerobic	1w	Jan-20	no
60	B64	*Clostridium argentinense*	98.91	BIET16	1	ATCC 27323	ileum	TSA	anaerobic	96h	Apr-20	no
61	B65	*Paraclostridium benzoelyticum*	99.86	BCET4	1	JC272	crop	TSA	anaerobic	48h	Jan-20	no
62	B66	*Paraclostridium benzoelyticum*	99.71	BCET6	1	JC272	crop	TSA	anaerobic	72h	Jan-20	no
63	B67	*Clostridium butyricum*	98.92	BCET32	1	DSM 10702	crop	TSA	anaerobic	1w	Jan-20	no
64	B68	*Clostridium argentinense*	99.04	BIET15	1	ATCC 27322	ileum	TSA	anaerobic	72h	Apr-20	no
65	B71	*Clostridium butyricum*	98.50	BIEG32	1	DSM 10702	ileum	GMM	anaerobic	96h	Apr-20	no
66	B73	*Paraclostridium benzoelyticum*	99.92	BCES22	1	JC272	crop	PSM	anaerobic	1w	Jan-20	no
67	B74	*Limosilactobacillus coleohominis 99.06%*	99	BIEM44	1	DSM 14060	ileum	MRS	facultative	6w	Apr-20	no
68	B75	*Ligilactobacillus saerimneri*	99.74	BCEP31	1	DSM 16049	crop	PFA	facultative	96h	Jan-20	no
69	B76	*Cutibacterium acnes subsp. acnes*	99.82	BIEG54	1	DSM 1897	ileum	GMM	facultative	120h	Apr-20	no
70	B77	*Paraclostridium benzoelyticum*	99.93	BIEG64	1	JC272	ileum	GMM	anaerobic	48h	Apr-20	no
71	B78	*Paraclostridium benzoelyticum*	99.78	BIEG78	1	JC273	ileum	GMM	anaerobic	48h	Apr-20	no
72	B79	*Clostridium butyricum*	98.85	BIET1	1	DSM 10702	ileum	TSA	anaerobic	96h	Apr-20	no
73	B80	*Shigella flexneri*	99.92	CIET29	2	X96963	ileum			48h	Apr-21	no
74	B81	*Clostridium butyricum*	98.10	BIET25	1	DSM 10703	ileum	TSA	anaerobic	1w	Apr-20	no
75	B82	*Clostridium butyricum*	99.2	BIET27	1	DSM 10702	ileum	TSA	anaerobic	2w	Apr-20	no
76	B83	*Phocaeicola vulgatus*	99.47	CIES55	1	ATCC 8482	ileum	PSM	anaerobic	72h	Apr-20	no
77	B84	*Clostridium butyricum*	98.68	BIET47	1	DSM 10702	ileum	TSA	anaerobic	96h	Apr-20	no
78	B85	*Ligilactobacillus saerimneri*	99.71	CIET55	1	DSM 16049	ileum	TSA	facultative	48h	Apr-20	no
79	B86	*Shigella flexneri*	99.92	CIEP53	2	X96963	ileum	PFA	facultative	1-2 days	Apr-20	no
80	B87	*Escherichia coli*	100	CIEG61	2	ATCC 11775	ileum	GMM	facultative	1-2 days	Apr-20	no
81	B88	*Ligilactobacillus saerimneri*	99.56	CIEG64	1	DSM 16049	ileum	GMM	facultative	72h	Apr-20	no
82	B89	*Limosilactobacillus panis*	99.56	BCEM1	1	DSM 6035	ileum	MRS	facultative	1w	Apr-20	no
83	B90	*Clostridium butyricum*	99.03	BCEP62	1	DSM 10702	ileum	PFA	anaerobic	72h	Apr-20	no
84	B91	*Shigella flexneri*	99.90	CIEP55	2	ATCC 29903	ileum	PFA	facultative	1-2 days	Apr-20	no
85	B92	*Flavonifractor plautii*	99.89	CIES11	1	ATCC 29863	ileum	PSM	anaerobic	48h	Apr-20	no
86	B93	*Flavonifractor plautii*	99.39	CIEP6	1	ATCC 29863	ileum	PFA	anaerobic	48h	Apr-20	no
87	B94	*Cutibacterium acnes subsp. defendens*	99.87	BCEG64	1	AB573714	crop	GMM	facultative	72h	Jan-20	no
88	B95	*Clostridium jeddahense*	99.68	CIES53	1	JCD	ileum	PSM	anaerobic	1w	Apr-20	yes
89	B96	*Flavonifractor plautii*	99.79	CIEP52	1	JH417629	ileum	PFA	anaerobic	72h	Apr-20	no
90	B97	*Flavonifractor plautii*	99.73	CIEP51	1	ATCC 29863	ileum	PFA	anaerobic	48h	Apr-20	no
91	B98	*Limosilactobacillus panis*	99.84	CIEM62	1	DSM 6035	ileum	MRS	anaerobic	48h	Apr-20	yes
92	B99	*Limosilactobacillus coleohominis*	99.06	CIEM16	1	DSM 14060	ileum	MRS	facultative	4w	Apr-20	no

93	B101	*Limosilactobacillus reuteri*	99.64	BCET61	1	JCM 1112	crop	TSA	facultative	8w	Jan-20	no
94	B102	*Ligilactobacillus aviarius*	98.62	**BI6G3**	1	*DSM 20655*	ileum	GMM	facultative	16w	Apr-20	no
95	B104	*Limosilactobacillus ingluviei*	99,81	BI6G16	1	DSM 15946	ileum	MRS	facultative	4w	Apr-20	no
96	B105	*Limosilactobacilus mucosae*	99,24	BCEG9	1	DSM 6035	crop	MRS	facultative	11w	Jan-20	no
97	B106	*Limosilactobacillus mucosae*	99,06	BCEG12	1	S32	crop	PSM	anaerobic	2w	Jan-20	no
98	B107	*Limosilactobacillus ingluviei*	99,24	CIEP8	1	DSM 15946	ileum	MRS	facultative	12w	Apr-20	no
99	B108	*Ligilactobacillus salivarius*	99,71	BI5M10	1	BCRC 14759	ileum	TSA	facultative	4w	Apr-20	no
100	B109	*Cutibacterium acnes*	99,92	BCET8	1	DSM 1897	crop	PSM	facultative	1w	Jan-20	no
101	B110	*Lactobacillus gallinarum*	99,9	BC6G7	1	JCM 2011	crop	GMM	anaerobic	5w	Jan-20	no
102	B111	*Clostridium butyricum*	98,9	BCET24	1	DSM 10702	crop	MRS	facultative	8w	Jan-20	no
103	B112	*Limosilactobacillus mucosae*	**99.11**	BCET9	1	S32	crop	TSA	facultative	2w	Jan-20	no
104	B113	*Ligilactobacillus aviarius*	99,86	BIEG36	1	DSM 20655	ileum	GMM	anaerobic	6w	Apr-20	no
105	B114	*Limosilactobacillus panis*	99,42	CIEG42	1	DSM 6035	ileum	GMM	anaerobic	72h	Apr-20	yes
106	B115	*Limosilactobacillus vaginalis*	99,61	BC7G23	1	ATCC 49540	crop	GMM	anaerobic	7w	Jan-20	no
107	B116	*Limosilactobacillus mucosae*	100	BCEP10	1	S32	crop	PFA	anaerobic	7w	Jan-20	no
108	B117	*Limosilactobacillus mucosae*	99,56	CIET56	1	S32	ileum	TSA	anaerobic	8w	Apr-20	no
109	B118	*Ligilactobacillus aviarius*	99,72	BI5M8	1	DSM 20655	ileum	MRS	anaerobic	9w	Apr-20	no
110	B119	*Limosilactobacillus ingluviei*	99,93	BCET48	1	DSM 15946	crop	TSA	anaerobic	3w	Jan-20	no
111	J1	*Ligilactobacillus aviarius*	99,9	DJEG3	1	DSM 20655	jejunum	GMM	facultative	1w	Jul-21	no
112	J2	*Lactobacillus johnsonii*	99,82	DJ4G12	1	ATCC 33200	jejunum	GMM	anaerobic	96h	Jul-21	no
113	J3	*Ligilactobacillus aviarius*	99,64	DJ4G10	1	DSM 20655	jejunum	GMM	anaerobic	72h	Jul-21	no
114	J4	*Enterococcus durans*	99,47	DJ5G6	1	NBRC 100479	jejunum	GMM	facultative	48h	Jul-21	no
115	J5	*Limosilactobacillus vaginalis*	99,0	DJEM6	1	ATCC 49540	jejunum	MRS	anaerobic	1w	Jul-21	no
116	J6	*Ligilactobacillus salivarius*	99,37	DJ3M5	1	BCRC 14759	jejunum	MRS	anaerobic	48h	Jul-21	no
117	J7	*Lactobacillus gallinarum*	99,39	DJ3M1	1	JCM 1039	jejunum	PFA	facultative	48h	Jul-21	no
118	J8	*Limosilactobacillus vaginalis*	99,5	DJ4M16	1	ATCC 49540	jejunum	MRS	anaerobic	72h	Jul-21	no
119	J9	*Shigella flexneri*	99,38	DJ2P2	2	ATCC 29903	jejunum	PFA	facultative	48h	Jul-21	no
120	J10	*Enterococcus faecium*	99,47	DJ3M3	1	ATCC 9790	jejunum	GMM	facultative	48h	Jul-21	no
121	J11	*Ligilactobacillus sp.*	93,36	DJ3G5	1	DSM 19972	jejunum	GMM	anaerobic	2w	Jul-21	yes
122	J12	*Enterococcus faecium*	99,83	DJEG2	1	ATCC 9790	jejunum	GMM	facultative	1w	Jul-21	no
123	J13	*Ligilactobacillus aviarius*	99,43	DJ2G1	1	DSM 20655	jejunum	GMM	facultative	48h	Jul-21	no
124	J14	*Ligilactobacillus sp.*	93,68	DJ4M19	1	DSM 20655	jejunum	MRS	facultative	2w	Jul-21	yes
125	J15	*Ligilactobacillus aviarius*	99,91	DJ4G15	1	DSM 20655	jejunum	GMM	facultative	96h	Jul-21	no
126	J16	*Enterococcus faecium*	99,08	DJ4G16	1	ATCC 9790	jejunum	GMM	facultative	96h	Jul-21	no
127	J17	*Ligilactobacillus aviarius*	99,91	DJ4G11	1	DSM 20655	jejunum	MRS	facultative	1w	Jul-21	no
128	J18	*Ligilactobacillus aviarius*	99,83	DJEM4	1	DSM 20655	jejunum	MRS	facultative		Jul-21	no
129	J19	*Ligilactobacillus aviarius*	99,82	DJ3G4	1	DSM 20655	jejunum	GMM	facultative		Jul-21	no
130	J20	*Shigella flexnerii*	99,38	DJ2P3	2	ATCC 29903	jejunum	PFA	facultative	3w	Jul-21	no
131	J21	*Limosilactobacillus reuteri*	94,1	DJ3M12	1	DSM 19972	jejunum	MRS	facultative	1w	Jul-21	yes
132	J22	*Ligilactobacillus aviarius*	93.09	DJ3M10	1	DSM 20655	jejunum	MRS	facultative	1w	Jul-21	yes
133	J23	*Enterococcus faecium*	99,25	DJ3M13	1	ATCC 9790	jejunum	MRS	facultative	1w	Jul-21	no
134	J24	*Enterococcus faecium*	98.01	DJEP1	1	JCM 2011	jejunum	PFA	facultative	72h	Jul-21	no
135	J25	*Ligilactobacillus aviarius*	99,55	DJ4G7	1	DSM 20655	jejunum	MRS	facultative	2w	Jul-21	no
136	J28	*Ligilactobacillus aviarius*	99,73	DJ3M7	1	S32	jejunum	MRS	facultative	1w	Jul-21	no
137	J29	*Limosilactobacillus vaginalis*	99,0	DJEM6	1	ATCC 49540	jejunum	MRS	facultative	1w	Jul-21	no
138	J30	*Limosilactobacillus oris*	99,64	DJ3M4	1	DSM 4864	jejunum	MRS	facultative	48h	Jul-21	no
139	J31	*Limosiactobacillus vaginalis*	99,8	DJ4M14	1	DSM 4864	jejunum	MRS	facultative	2w	Jul-21	no
140	J32	*Enterococcus faecium*	98,02	DJEG5	1	LMG 11423	jejunum	MRS	facultative	2w	Jul-21	no
141	J33	*Enterococcus faecium*	99,82	DJEM3	1	NBRC 100479	jejunum	MRS	facultative	2w	Jul-21	no
142	J34	*Ligilactobacillus aviarius*	99,78	DJ3M9	1	DSM 20655	jejunum	MRS	facultative	2w	Jul-21	no
143	J35	*Ligilactobacillus aviarius*	99,55	DJ4G7	1	DSM 20655	jejunum	MRS	facultative	2w	Jul-21	no
144	J36	*Ligilactobacillus aviarius*	99,72	DJEG8	1	DSM 20655	jejunum	GMM	facultative	1w	Jul-21	no
145	J37	*Ligilactobacillus aviarius*	99,71	DJEG7	1	DSM 20655	jejunum	GMM	facultative		Jul-21	no
146	J39	*Limosilactobacillus vaginalis*	99,61	DJEG9	1	c11Ua_112_M	jejunum	GMM	facultative	3w	Jul-21	no
147	I1	*Enterococcus faecium*	100	DI4M4	1	ATCC 33200	ileum	MRS	facultative	96h	Jul-21	no
148	I2	*Ligilactobacillus aviarius*	99,82	DI5G1	1	DSM 20655	ileum	GMM	facultative	48h	Jul-21	no
149	I3	*Streptococcus alactolyticus*	99.75	DI5M12	1	ATCC 43077	ileum	MRS	facultative	48h	Jul-21	no
150	I4	*Enterococcus faecium*	99.79	DI5M7	1	ATCC 43077	ileum	MRS	facultative	48h	Jul-21	no
151	I5	*Limosiactobacillus mucosae*	99,51	DI5G2	1	S32	ileum	GMM	facultative	48h	Jul-21	no
152	I6	*Enterococcus hirae*	99,24	DI5G3	1	ATCC 9790	ileum	GMM	facultative	48h	Jul-21	no
153	I7	*Ligilactobacillus aviarius*	99,1	DI4M6	1	DSM 20655	ileum	MRS	facultative	48h	Jul-21	no
154	I9	*Lactobacillus gallinarum*	99,4	DI5M16	1	JCM 2011	ileum	MRS	facultative	1w	Jul-21	no
155	I10	*Ligilactobacillus aviarius*	99,25	DI5M19	1	DSM 20655	ileum	MRS	facultative	2w	Jul-21	no
156	I11	*Ligilactobacillus aviarius*	99,59	DI3P1	1	AP3	ileum	PFA	facultative	48h	Jul-21	no
157	I12	*Enterococcus durans*	99,39	DI5M20	1	NBRC 100479	ileum	MRS	facultative	2w	Jul-21	no
158	I13	*Enterococcus faecium*	99,41	DI3P4	1	JCM 2011	ileum	PFA	facultative	48h	Jul-21	no
159	I14	*Streptococcus alactolyticus*	100	DI4G10	1	ATCC 43077	ileum	GMM	facultative	96h	Jul-21	no
160	I15	*Limosilactobacillus reuteri subsp. kinnaridis*	100	DI4G11	1	AP3	ileum	GMM	facultative	96h	Jul-21	no
161	I16	*Enterococcus faecium*	99,28	DIEG2	1	NBRC 100479	ileum	GMM	facultative	72h	Jul-21	no
162	I17	*Lactobacillus kitasatonis*	99.73	DIEG5	1	JCM 1039	ileum	GMM	facultative	2w	Jul-21	no
163	I18	*Limosilactobacillus vaginalis*	99,98	DIEG6	1	ATCC 49540	ileum	GMM	facultative	2w	Jul-21	no
164	I19	*Lactobacillus kitasatonis*	99,43	DIEG8	1	JCM 1039	ileum	GMM	facultative	2w	Jul-21	no
165	I20	*Limosilactobacillus reuteri subsp. kinnaridis*	100	DIEM6	1	AP3	ileum	MRS	facultative	1w	Jul-21	no
166	I21	*Limosilactobacillus sp*	96,75	DIEM7	1	Marseille-P3825	ileum	MRS	facultative	1w	Jul-21	yes
167	I22	*Limosilactobacillus frumenti*	99,75	DIEM8	1	DSM 13145	ileum	MRS	facultative	1w	Jul-21	no
168	I23	*Ligilactobacillus aviarius*	99,64	DI5G16	1	DSM 20655	ileum	GMM	facultative	2w	Jul-21	no
169	I24	*Limosilactobacillus panis*	99,24	DIEG4	1	DSM 6035	ileum	GMM	facultative	72h	Jul-21	no
170	I25	*Limosilactobacillus vaginalis*	99.5	DIEM4	1	ATCC 49540	ileum	MRS	facultative	72h	Jul-21	no
171	I26	*Limosilactobacillus sp*	94,73	DIEM9	1	Marseille-P3825	ileum	MRS	facultative	2w	Dec-21	yes
172	I27	*Limosilactobacillus sp*	97,19	DIEM12	1	DSM 16041	ileum	MRS	facultative	3w	Dec-21	yes
173	I29	*Limosilactobacillus panis*	99,44	DIEG9	1	DSM 6035	ileum	GMM	facultative	2w	Dec-21	no
174	I30	*Lactobacillus gallinarum*	98,68	DIEG10	1	JCM 2011	ileum	GMM	facultative	3w	Dec-21	no
175	I31	*Limosilactobacillus panis*	98,24	DIEG11	1	DSM 6035	ileum	GMM	facultative	3w	Dec-21	no
176	E2	*Limosilactobacillus sp*	95,24	E2	1	DSM 20655	ileum	MRS	facultative	18w	Dec-21	yes

Table 10. Database of the 43 bacterial culture-collection genomes obtained from crop, jejunum and ileum of chicken.

	Code	Isolate	Tax	ID%	Phylum	Family	Source	Media	enome stat	Time	ANI%	Genome Tax	w type str	eplication s	Accession no.	Biosample
1	B002	BC6G17	Limosilactobacillus gallus	99.85	Firmicutes	tobacillac	crop	GMM	done	48h	N/A	*osilactobacillus ga*	yes	17_0	ERS14260881	SAMEA112150981
2	B009	BIEG38	Ligilactobacillus aviarius	99.79	Firmicutes	tobacillac	ileum	GMM	done	48h	96,13	*ilactobcaillus aviar*	no	19_8	ERS14260882	SAMEA112150982
3	B010	BIEM5	Limosilactobacillus reuter	99.43	Firmicutes	tobacillac	ileum	MRS	done	48h	97,06	*osilactobacillus reu*	no	13_3	ERS14260883	SAMEA112150983
4	B015	BIEM52	Limosilactobacillus aviun	99.93	Firmicutes	tobacillac	ileum	MRS	done	1w	92.88	*osilactobacillus av*	yes	15_0	ERS14260884	SAMEA112150984
5	B031	BC6G16	Limosilactobacillus reuter	99.65	Firmicutes	tobacillac	crop	GMM	done	72h	97,36	*osilactobacillus reu*	no	13_1	ERS14260885	SAMEA112150985
6	B032	BIEP23	Limosilactobacillus pullum	99.50	Firmicutes	tobacillac	ileum	PFA	done	1w	97.56	*osilactobacillus pul*	yes	11_1	ERS14260886	SAMEA112150986
7	B035	BIEG45	Lactobacillus gallinarum	99.79	Firmicutes	tobacillac	ileum	GMM	done	120h	98.17	*ctobacillus gallinar*	no	20_1	ERS14260887	SAMEA112150987
8	B040	BIET41	aclostridium benzoelytic	99.23	Firmicutes	treptococc	ileum	TSA	done	72h	97.11	*lostridium bifermer*	no	5_0	ERS14260888	SAMEA112150988
9	B056	BIES18	igilactobacillus aviariu	99.91	Firmicutes	tobacillac	ileum	PFA	done	72h	96:05:00	*ilactobcaillus aviar*	no	19_4	ERS14260889	SAMEA112150989
10	B058	CIET9	Flavonifractor plauti	99.54	Firmicutes	illospirace	ileum	TSA	done	72h	98.47	*Flavonifractor plauti*	no	6_0	ERS14260890	SAMEA112150990
11	B063	BCET17	ostridium butanolproduce	98.77	Firmicutes	ostridiacea	crop	TSA	done	1w	99.65	*idium butanolprodu*	yes	21_1	ERS14260891	SAMEA112150991
12	B067	BCET32	Clostridium butyricum	98.92	Firmicutes	ostridiacea	crop	TSA	done	1w	97.54	*lostridium butyricu*	no	1_1	ERS14260892	SAMEA112150992
13	B075	BCEP31	igilactobacillus saerimne	99.74	Firmicutes	tobacillac	crop	PFA	done	96h	98,58	*actobacillus saerim*	no	18_1	ERS14260893	SAMEA112150993
14	B085	CIET55	*gilactobacillus saerimne*	99.74	Firmicutes	tobacillac	ileum	TSA	done		98,52	*actobacillus saerim*	no	18_1	ERS14260894	SAMEA112150994
15	B094	BCEG64	terium acnes subsp. def	99.87	Actinobacte	onibacteria	crop	GMM	done	72h	97.08	*Cutibacterium acnes*	no	2_2	ERS14260895	SAMEA112150995
16	B095	CIES53	Clostridium anaerovisceri	99.68	Firmicutes	ostridiacea	ileum	PSM	done	2w	93.91	*ctridium anaerovisc*	yes	7_0	ERS14260896	SAMEA112150996
17	B098	CIEM62	mosilactobacillus digestu	99.84	Firmicutes	tobacillac	ileum	MRS	done	48h	N/A	*silactobacillus dige*	yes	14_1	ERS14260897	SAMEA112150997
18	B104	BI6G16	mosilactobacillus ingluvi	98.33	Firmicutes	tobacillac	ileum	MRS	done	4w	97,51	*silactobacillus ingl*	no	10_1	ERS14260898	SAMEA112150998
19	B107	CIEP8	*mosilactobacillus ingluv*	99,24	Firmicutes	tobacillac	ileum	PFA	done	12w	97,28	*silactobacillus ingl*	no	10_2	ERS14260899	SAMEA112150999
20	B108	BI5M10	Ligilactobacillus salivariu	98.0	Firmicutes	tobacillac	ileum	TSA	done	4w	97,44	lactobacillus saliva	no	22_1	ERS14260900	SAMEA112151000
21	B109	BCET8	Cutibacterium acnes	99,06	Firmicutes	onibacteria	crop	PSM	done	1w	99,51	*Cutibacterium acnes*	no	2_1	ERS14260901	SAMEA112151001
22	B110	BC6G7	Lactobacillus gallinarum	99.35	Firmicutes	tobacillac	crop	GMM	done	5w	98,25	ctobacillus gallinar	no	20_2	ERS14260902	SAMEA112151002
23	B111	BCET24	Clostridium butyricum	98.9	Firmicutes	ostridiacea	crop	MRS	done	8w	97,72	*lostridium butyricu*	no	1_1	ERS14260903	SAMEA112151003
24	B116	BCEP10	mosilactobacillus pullum	98.99	Firmicutes	tobacillac	crop	PFA	done	7w	97,6	osilactobacillus pul	yes	11_1	ERS14260904	SAMEA112151004
25	B118	BI5M8	Ligilactobacillus aviarius	99,72	Firmicutes	tobacillac	ileum	MRS	done	48h	95,88	ilactobacillus aviar	no	19_9	ERS14260905	SAMEA112151005
26	B119	BCET48	*mosilactobacillus ingluvi*	97,54	Firmicutes	tobacillac	crop	TSA	done	3w	97,56	osilactobacillus ingl	no	10_3	ERS14260906	SAMEA112151006
27	E2	E2	limosilactobacillus difficil	94,15	Firmicutes	tobacillac	ileum	E2	done	12w	N/A	osilactobacillus diff	yes	9_1	ERS14260907	SAMEA112151007
28	I03	DI4M4	treptococcus alactolyticu	99.75	Firmicutes	tobacillac	ileum	MRS	done	48h	99,27	otococcus alactoly	no	4_0	ERS14260908	SAMEA112151008
29	I11	DI3P1	*Ligilactobacillus aviarius*	99,59	Firmicutes	tobacillac	ileum	PFA	done	48h	95,72	Ligilactobacillus av	no	19_3	ERS14260909	SAMEA112151009
30	I13	DI3P4	*Enterococcus faecium*	99,41	Firmicutes	tobacillac	ileum	PFA	done	48h	99	nterococcus faeciu	no	3_1	ERS14260910	SAMEA112151010
31	I18	DIEG6	mosilactobacillus vaginal	99,98	Firmicutes	tobacillac	ileum	GMM	done	2w	98,89	silactobacillus vagi	no	12_2	ERS14260911	SAMEA112151011
32	J01	DJEG3	Ligilactobacillus aviarius	99,56	Firmicutes	tobacillac	jejunum	GMM	done	1w	96,15	ilactobacillus aviar	no	19_5	ERS14260912	SAMEA112151012
33	J03	DJ4G10	*Ligilactobacillus aviarius*	99,64	Firmicutes	tobacillac	jejunum	GMM	done	72h	96	ilactobacillus aviar	no	19_7	ERS14260913	SAMEA112151013
34	J06	DJ3M5	Ligilactobacillus salivariu	99.17	Firmicutes	tobacillac	jejunum	MRS	done	48h	97,49	lactobacillus saliva	no	22_2	ERS14260914	SAMEA112151014
35	J07	DJ3M1	Lactobacillus gallinarum	99.24	Firmicutes	tobacillac	jejunum	PFA	done	48h	98,36	ctobacillus gallinar	no	20_3	ERS14260915	SAMEA112151015
36	J11	DJ3G5	lactobacillus hohenheim	93.23	Firmicutes	tobacillac	jejunum	GMM	done	48h	N/A	ctobacillus hohenhe	yes	8_2	ERS14260916	SAMEA112151016
37	J14	DJ4M19	lactobacillus hohenheim	93.06	Firmicutes	tobacillac	jejunum	MRS	done	120h	N/A	ctobacillus hohenhe	yes	8_1	ERS14260917	SAMEA112151017
38	J21	DJ3M12	Limosilactobacillus albus	94,1	Firmicutes	tobacillac	jejunum	MRS	done	1w	97,37	osilactobacillus all	yes	13_2	ERS14260918	SAMEA112151018
39	J25	DJ2P5	*Ligilactobacillus aviarius*	99.34	Firmicutes	tobacillac	jejunum	PFA	done	48h	96,09	ilactobacillus aviar	no	19_6	ERS14260919	SAMEA112151019
40	J28	DJ3M7	*Ligilactobacillus aviarius*	99,73	Firmicutes	tobacillac	jejunum	MRS	done	72h	96,13	ilactobacillus aviar	no	19_2	ERS14260920	SAMEA112151020
41	J29	DJEM5	*mosilactobacillus vaginal*	99	Firmicutes	tobacillac	jejunum	MRS	done	1w	98,78	silactobacillus vagi	no	12_3	ERS14260921	SAMEA112151021
42	J30	DJEM6	Limosilactobacillus oris	98,64	Firmicutes	tobacillac	jejunum	MRS	done	2w	95,83	mosilactobacillus o	no	16_0	ERS14260922	SAMEA112151022
43	J32	DJ4M14	Enterococcus faecium	99,02	Firmicutes	erococcac	jejunum	MRS	done	2w	98,97	nterococcus faeciu	no	3_1	ERS14260923	SAMEA112151023
44	J35	DJ4G7	*Ligilactobacillus aviarius*	99,55	Firmicutes	tobacillac	jejunum	MRS	done	1w	96,25	ilactobacillus aviar	no	19_1	ERS14260924	SAMEA112151024

Table 11: Metadata of chicken samples obtained from the project PRJEB60928.

sample	Section	Week	Strain	Host
LH1_241	Caeca	16	LB	1.03
LH1_242	Caeca	16	LSL	7.36
LH1_243	Caeca	16	LB	1.55
LH1_244	Caeca	16	LSL	3.52
LH1_245	Caeca	16	LSL	0.74
LH1_246	Caeca	16	LB	6.43
LH1_247	Caeca	16	LSL	0.29
LH1_248	Caeca	16	LB	5.28
LH1_249	Caeca	16	LSL	0.51
LH1_250	Caeca	16	LB	11.66
LH1_251	Caeca	16	LB	4.16
LH1_252	Caeca	16	LSL	2.26
LH1_253	Caeca	16	LSL	2.86
LH1_254	Caeca	16	LB	3.37
LH1_255	Caeca	16	LSL	6.96
LH1_256	Caeca	16	LB	4.76
LH1_257	Caeca	16	LB	1.9
LH1_258	Caeca	16	LSL	9.2
LH1_259	Caeca	16	LSL	8.58
LH1_260	Caeca	16	LB	18.04
LH1_343	Ileum	16	LB	76.61
LH1_348	Ileum	16	LB	74.99
LH1_352	Ileum	16	LSL	35.36
LH1_353	Ileum	16	LSL	76.27
LH1_358	Ileum	16	LSL	80.64
LH1_359	Ileum	16	LSL	68.05
LH1_381	Crop	16	LB	1.7
LH1_382	Crop	16	LSL	4.37
LH1_383	Crop	16	LB	1
LH1_384	Crop	16	LSL	7.3
LH1_385	Crop	16	LSL	1.12
LH1_386	Crop	16	LB	1.44
LH1_387	Crop	16	LSL	7.04
LH1_388	Crop	16	LB	3.79
LH1_389	Crop	16	LSL	9.45
LH1_390	Crop	16	LB	2.51
LH1_391	Crop	16	LB	1.87
LH1_392	Crop	16	LSL	0.93
LH1_393	Crop	16	LSL	1.21
LH1_394	Crop	16	LB	2.79
LH1_395	Crop	16	LSL	3.8
LH1_396	Crop	16	LB	1.2
LH1_397	Crop	16	LB	3.63
LH1_398	Crop	16	LSL	1.06
LH1_399	Crop	16	LSL	0.7
LH1_400	Crop	16	LB	2.11
LH1_441	Caeca	24	LB	1.24
LH1_442	Caeca	24	LSL	11.76
LH1_443	Caeca	24	LB	1.86
LH1_444	Caeca	24	LSL	9.07
LH1_445	Caeca	24	LB	1.41
LH1_446	Caeca	24	LSL	23.83

sample	Section	Week	Strain	Host
LH1_447	Caeca	24	LB	11.32
LH1_448	Caeca	24	LSL	6.52
LH1_449	Caeca	24	LSL	3.41
LH1_450	Caeca	24	LB	8.77
LH1_451	Caeca	24	LB	0.19
LH1_452	Caeca	24	LSL	55.11
LH1_453	Caeca	24	LB	4.46
LH1_454	Caeca	24	LSL	53.8
LH1_455	Caeca	24	LSL	21.08
LH1_456	Caeca	24	LB	1.29
LH1_457	Caeca	24	LB	26.97
LH1_458	Caeca	24	LSL	29.44
LH1_459	Caeca	24	LSL	23.34
LH1_460	Caeca	24	LB	2.12
LH1_542	Ileum	24	LSL	71.89
LH1_543	Ileum	24	LB	75.62
LH1_544	Ileum	24	LSL	48.57
LH1_545	Ileum	24	LB	66.05
LH1_546	Ileum	24	LSL	68.31
LH1_547	Ileum	24	LB	80.11
LH1_548	Ileum	24	LSL	14.68
LH1_549	Ileum	24	LSL	42.96
LH1_550	Ileum	24	LB	36.97
LH1_551	Ileum	24	LB	64.22
LH1_552	Ileum	24	LSL	32
LH1_553	Ileum	24	LB	56.44
LH1_554	Ileum	24	LSL	67.64
LH1_555	Ileum	24	LSL	29.81
LH1_556	Ileum	24	LB	66.49
LH1_557	Ileum	24	LB	45.64
LH1_558	Ileum	24	LSL	5.1
LH1_559	Ileum	24	LSL	42.28
LH1_560	Ileum	24	LB	43.28
LH1_581	Crop	24	LB	14.76
LH1_582	Crop	24	LSL	0.21
LH1_583	Crop	24	LB	3.28
LH1_584	Crop	24	LSL	0.32
LH1_585	Crop	24	LB	0.64
LH1_586	Crop	24	LSL	1.49
LH1_587	Crop	24	LB	2.2
LH1_588	Crop	24	LSL	0.49
LH1_589	Crop	24	LSL	0.79
LH1_590	Crop	24	LB	2.69
LH1_591	Crop	24	LB	5.27
LH1_592	Crop	24	LSL	9.81
LH1_593	Crop	24	LB	2.63
LH1_594	Crop	24	LSL	0.64
LH1_595	Crop	24	LSL	0.7
LH1_596	Crop	24	LB	2.89
LH1_597	Crop	24	LB	0.7
LH1_598	Crop	24	LSL	0.82
LH1_599	Crop	24	LSL	8.07
LH1_600	Crop	24	LB	0.46

8.2 Supplementary figures

Figure S1. Phylogenetic tree of species from the family *Oscillospiraceae* that include the novel genus description *Faecalispora* gen. nov. Species of the novel genus *Faecalispora* gen. nov. are highlighted in blue letters. The tree is based on 49 core genes concatenated obtained from each genome and using members of the families *Clostridiaceae* (orange), *Desulfitobacteriaceae* (blue) and *Eubacteriaceae* (pink) as external groups. Bootstrap values (1000 replicates) are depicted above branches, branch lengths correspond to sequence differences indicated by the scale bar above.

8.3 Culture media

Gut microbiota medium (GMM)

Solution A	250 ml
Solution B	250 ml
Solution C	480 ml
Solution D	20.00 ml

Solution A

- Meat Extract	5 g
-Soy Peptone	2 g
-Yeast Extract	1 g
- Distilled water	250 ml

Solution B

- D-glucose	0.4 g
- Cellobiose	1 g
- Maltose	1 g
- Fructose	1 g
- Acetic acid (30 mM)	1.7 ml
- Isovaleric acid (1 mM)	0.1 ml
- Propionic acid (8 mM)	2 ml
- Butyric acid (4 mM)	2 ml
- KH_2PO4	1.36 g
- $MgSO_4$-$7H_20$	0.002 g
- $NaHCO_3$	0.4 g
- NaCl	0.08 g
- $CaCl_2$	0.08 g
- $FeSO_4$	0.4 mg
- Tween 80 (25%)	2 ml
- Sodium resazurin (0.1% w/v)	0.50 ml
- Distilled water	250 ml

Solution C

- Agar	15g
- Distilled water	480 ml

Solution D*

- Vitamin Mix 1%	10 ml
- Trace Mineral Mix 1%	10 ml
- L-cysteine	0.5 g
- Hemin solution	10 ml
- Vitamin K1 solution	1 ml

*Solution sensitive to heat, do not autoclave.

Prepare each solution in a separated bottle. Dissolve the ingredients in the indicated volume of distilled water. Sterilize by autoclaving at 15 lbs pressure at 110°C for 30 minutes except for the solution D that is sterilised with a cellulose filter of 0.2 nm. Cool down and mix the solutions at around 50°C before adding the cysteine, the vitamin mix and the traces solution. Mix gently by

sparging filtered 100% N_2 gas using a hydrophobic filter and pour into sterile plates under anaerobic conditions.

Man Rogosa Sharpe Medium (MRS)

Solution A	250 ml
Solution B	250 ml
Solution C	490 ml
Solution D	10.00 ml

Solution A

- Casein peptone, tryptic digest 10.00 g
- Meat extract 10.00 g
- Yeast extract 5.00 g
- Distilled water 250 ml

Solution B

- Glucose 20.00 g
- Tween 80 1.00 g
- K_2HPO_4 2.00 g
- Na-acetate 5.00 g
- $(NH_4)^3$ citrate 2.00 g
- $MgSO_4$ x 7 H_2O 0.20 g
- $MnSO_4$ x H_2O 0.05 g
- Sodium resazurin (0.1% w/v) 0.10 ml
- Distilled water 250 ml

Solution C

- Agar 15g
- Distilled water 490 ml

Solution D*

- Vitamin Mix 1% 10 ml

Prepare each solution in a separated bottle. Dissolve the ingredients in the indicated volume of distilled water. Sterilize by autoclaving at 21 lbs pressure at 110°C for 30 minutes. Cool down and mix the solutions at around 50°C with the vitamin mix solution. Mix gently by sparging filtered 100% N_2 gas using a hydrophobic filter and pour into sterile plates under anaerobic conditions.

Postgate Medium (PM)

Solution A	980.00 ml
Solution B	10.00 ml
Solution C	10.00 ml

Solution A

- K_2HPO_4 0.50 g
- NH_4Cl 1.00 g
- Na_2SO_4 1.00 g
- $CaCl_2$ x 2 H_2O 0.10 g
- $MgSO_4$ x 7 H_2O 2.00 g
- Na-DL-lactate 2.00 g

- Yeast extract 1.00 g
- Sodium resazurin (0.1% w/v) 0.10 ml

- Agar 15 g
- Distilled water 980.00 ml

Solution B

- $FeSO_4$ x 7 H_2O 0.50 g
- Distilled water 10.00 ml

Solution C

- Na-thioglycolate 0.10 g
- Ascorbic acid 0.10 g
- Distilled water 10.00 ml

Prepare each solution in a separated bottle. Dissolve the ingredients in the indicated volume of distilled water. Sterilize by autoclaving at 15 lbs pressure at 121°C for 15 minutes. Cool down and mix the solutions at around 50°C. Mix gently by sparging filtered 100% N_2 gas using a hydrophobic filter and pour into sterile plates under anaerobic conditions.

Poultry-Feed Medium 2% (PFA)

Solution A 500 ml
Solution B 500 ml

Solution A

- Poultry feed mixture (PFM) 10g
- Thioglycolate 0.5g
- Sodium resazurin (0.1% w/v) 0.50 ml
- Distilled water 500 ml

Solution B

- Agar 15g
- Distilled water 500 ml

Dissolve PFM in 500 ml of water, boil for 5 minutes and cool to room temperature. Filter the infusion with a cheese cloth to discard big particles. Add thioglycolate and resazurin solution and add water to reach 500 ml. Dissolve agar in distilled water to prepare solution B to sterilise in separated bottles. while Adjust pH and autoclave 30 min at 110 °C. Spare sterile solutions with 100% N_2 using a hydrophobic filter and dispense under anaerobic and sterile conditions plates.

Tryptic Soy Agar (TSA)

Solution A 250 ml
Solution B 250 ml
Solution C 500 ml

Solution A

- Trypticase soy broth 5.5 g
- Resazurin solution (0.1% w/v) 0.5 ml

- Distilled water 250.0 ml

Solution B

- D-Glucose 2.0 g
- Distilled water 250.0 ml

Solution C

- Agar 20.0 g
- Distilled water 500.0 ml

Prepare each solution in a separated bottle. Dissolve the ingredients in the indicated volume of distilled water. Sterilize by autoclaving at 15 lbs pressure at 121°C for 15 minutes. Cool down and mix the solutions at around 50°C. Mix gently by sparging filtered 100% N_2 gas using a hydrophobic filter and pour into sterile plates under anaerobic conditions.

8.4 Solutions

Trace solution

- $CaCl_2$ x 2 H_2O 0.25 g
- $MgSO_4$ x 7 H_2O 0.50 g
- K_2HPO_4 1.00 g
- KH_2PO_4 1.00 g
- $NaHCO_3$ 10.00 g
- NaCl 2.00 g
- Distilled water 1000 ml

Vitamin mix

- Folic acid 2.0 mg
- Thiamine 5.0 mg
- Riboflavin 5.0 mg
- Nicotinic acid 5.0 mg
- Pyridoxine hydrochloride 10.0 mg
- Calcium Pantothenate 5.0 mg
- p-Aminobenzoic acid 5.0 mg
- Biotin 2.0 mg
- Vitamin B12 0.1 mg
- Monopotassium phosphate 900 mg
- Thioctic acid (lipoic acid) 5.0 mg

Hemin solution:
Dissolve 50 mg hemin in 1 ml 1 N NaOH, make up to 100 ml with distilled water. Store refrigerated.

Vitamin K1 solution:
Dissolve 0.1 ml of vitamin K1 in 20 ml 95% ethanol and filter sterilize. Store refrigerated in a brown bottle.

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Thank you God, for guiding my steps.

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PROFILE

I am a motivated and responsible graduate on Microbiology who puts quality on her work and enjoys developing her own ideas and improve both my research and communication skills.

My friendly nature and responsibility sense are traits I bring to my research duty. I have worked within multicultural groups developing adaptive and cooperative skills. I consider myself able to handle research through independent work and self-initiative.

EDUCATION

2019 – 2023 **Doctoral candidate** at the Faculty of Agricultural sciences
University of Hohenheim

2015 - 2017 **Master's in Chemical-Biological Sciences**
Instituto Politécnico Nacional, Mexico City, Mexico

2010 - 2014 **Bacteriologist-Parasitologist Chemist** (Bachelor of Science in Microbiology)
Instituto Politécnico Nacional, Mexico City, Mexico

MAIN SKILLS

- Isolation and culture of microorganisms (yeast and bacteria)
- Storage of microorganisms for short, medium and long term periods.
- Phylogenetic identification of microorganisms
- Genotyping of microbial communities/consortia (RFLP, BOX, DGGE)
- DNA, RNA, plasmid extraction, PCR and related molecular biology techniques
- Genome assembly, annotation and phylogenomic analysis
- Expression and purification of recombinant proteins
- Data analysis: statistics, data visualisation, coding
- Software: R, Python, SQL
- Writing of scientific articles and scientific reports

ACADEMIC/RESEARCH TRAINING

August 2012 Biodegradation tests of cleaning products by microorganisms present in sewage.
Trainee internship

August 2013 Isolation and molecular identification of colonizing bacteria of digestive tract of the beetle *Dendroctonus valens*, involved in the nitrogen cycle (urea degraders and nitrogen fixers).
Trainee internship

September 2015 Characterization of *Pantoea agglomerans* as a plant growth promoting bacteria (PGPR) in maize plants.
Bachelor's degree project

August 2017	Isolation and molecular characterization of plant growth promoting bacteria (PGPR) from the maize environment (phosphate solubilizing bacteria, phytohormone producers and nitrogen fixers). ***Master's degree project***
February 2018	Research technician at the Microorganisms Collection of the National Centre of Genetic Resources (**CM-CNRG**) at Tepatitlán de Morelos, Jalisco, Mexico.
October 2019	Doctorate candidate at the Department of Functional Microbiology of Livestock at the University of Hohenheim, Stuttgart, Germany

RESEARCH PRODUCTS

Guerrero-Chavez, Ana Carolina, et al. "Diesel Impacts on Functional Bacterial Groups and Collembolans During Phytoremediation in a Mesocosm System." Water, Air, & Soil Pollution 231.10 (2020): 489.

Rios-Galicia, Bibiana, et al. "The Mexican giant maize of Jala landrace harbour plant-growth-promoting rhizospheric and endophytic bacteria." 3 Biotech 11.10 (2021): 447.

Sáenz, J. S., Rios-Galicia, B., Rehkugler, B., & Seifert, J. (2023). Dynamic Development of Viral and Bacterial Diversity during Grass Silage Preservation. Viruses, 15(4), 951

ACADEMIC AWARDS

2013	Scholar of the Alfredo Harp Helú foundation for students of excellence.
2017	Offered with an institutional training scholarship for young researchers BEIFI (Institutional scholarship for training researchers).

LANGUAGE PROFICIENCE

- **English** - Advanced level C1. Certified by IELTS on March 2017.
- **French** - Intermediate level B2. Certified by DELF on March 2008.
- German – Intermediate level B1. Certified by UNIcert®-Zertifikat, Universität Hohenheim

ADDITIONAL INFORMATION

First-aid and CPR training

Co-management of an entertainment-science web-page with microbiological content.

Stuttgart, 17.04.2023

Place, Date

Signature

Annex 3

Declaration in lieu of an oath on independent work

according to Sec. 18(3) sentence 5 of the University of Hohenheim's Doctoral Regulations for the Faculties of Agricultural Sciences, Natural Sciences, and Business, Economics and Social Sciences

1. The dissertation submitted on the topic

Novel bacterial species from the chicken gastrointestinal tract and their functional diversity

is work done independently by me.

2. I only used the sources and aids listed and did not make use of any impermissible assistance from third parties. In particular, I marked all content taken word-for-word or paraphrased from other works.

3. I did not use the assistance of a commercial doctoral placement or advising agency.

4. I am aware of the importance of the declaration in lieu of oath and the criminal consequences of false or incomplete declarations in lieu of oath.

I confirm that the declaration above is correct. I declare in lieu of oath that I have declared only the truth to the best of my knowledge and have not omitted anything.

Stuttgart 17.04.2023

Place, Date

Signature

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