UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

Colegio de Posgrados

CONTROL OF *Salmonella enterica* serovar Infantis COLONIZATION IN POULTRY USING BACTERIOPHAGES

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Trabajo de titulación de posgrado presentado como requisito
para la obtención del título de Master en Microbiología

Quito, 13 de marzo de 2020

UNIVERSIDAD SAN FRANCISCO DE QUITO USFQ

COLEGIO DE POSGRADOS

HOJA DE APROBACIÓN DE TRABAJO DE TITULACIÓN

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Dedicatoria

A mis padres Carlota y Marcelo, a mi esposo Juan Pablo y a mis hijos Eva Monserrat y Juan Emilio.

Agradecimientos

Sinceros agradecimientos al Instituto de Microbiología de la USFQ, a la USFQ y a PRONACA; al Dr. Alejandro Torres y al Dr. Gabriel Trueba por su acompañamiento y dirección durante el trabajo de tesis, a la Dra. Sonia Zapata por su asesoramiento y al Dr. Patricio Rojas por su acompañamiento en el proceso de titulación, a las excelentes amigas que me dejó el programa MSc. Fernanda Loayza y MSc. Lorena Mejía.

Resumen

En Ecuador, la industria avícola es el principal proveedor de proteína animal, aproximadamente 0.5 toneladas se consumen cada año y la industria crece 3%. Así como en otros países, *Salmonella* es un problema de salud pública permanente asociado con esta industria. Nosotros describimos una estrategia simple para controlar la colonización de *Salmonella enterica* serovar Infantis en pollos de engorde utilizando bacteriófagos nativos administrados en el agua de bebida. Los bacteriófagos fueron aislados desde efluentes, lavado de plumas de aves y las camas en las instalaciones de producción avícola de una granja ecuatoriana. Los fagos nativos aislados fueron evaluados cualitativamente en cuanto a su capacidad de lisis específica de cepas de *Salmonella enterica* serovar Infantis y amplificados en un coctel. La estabilidad del coctel fue demostrada en soluciones de cloro en concentraciones de 0 a 4 ppm y en soluciones suplementadas con un inhibidor de halógenos así como también con un protector viral. Se evaluó la eficiencia del coctel suplementando el agua de bebida de un platel de producción de pollos de engorde con 1000 aves y se comparó la frecuencia de aislamiento de *Salmonella* Infantis en el lote tratado con un lote de igual tamaño en el cual no se administró el coctel de fagos. No se detectó *Salmonella enterica* serovar Infantis en los tamizajes de rutina de la producción de pollos de engorde donde los bacteriófagos fueron aplicados. Por otro lado, en el grupo control, *Salmonella enterica* serovar Infantis fue detectado con una frecuencia del 20% en los ciegos de los pollos, 10% de las muestras de lavado de plumas, 33% de agua de escaldadora y 20% de las muestras de agua del lavado final. En conclusión, el aislamiento, amplificación y aplicación de cocteles de bacteriofagos nativos es una herramienta útil en el biocontrol de la colonización de *Salmonella enterica* serovar Infantis en la industria avícola.

Palabras clave: Salmonella enterica serovar Infantis, granjas avícolas, bacteriófagos nativos, biocontrol

Abstract

In Ecuador, the poultry industry is the main supplier of animal protein, approximately 0.5 tons are consumed each year and the industry grows 3%. As in other countries, *Salmonella* is a permanent public health problem associated with this industry. We describe a simple strategy to control colonization of *Salmonella enterica* serovar Infantis in broilers using native bacteriophages administered in drinking water. The bacteriophages were modified from the effluents, the washing of chicken feathers and the beds in the poultry production facilities of an Ecuadorian farm. Specific native phages were qualitatively evaluated for their specific lysis capacity of *Salmonella enterica* serovar Infantis strains and amplified in a cocktail. The stability of the cocktail was demonstrated in chlorine solutions in dimensions from 0 to 4 ppm and in solutions supplemented with a halogen inhibitor as well as a viral protector. The efficiency of the cocktail was evaluated by supplementing the drinking water from a broiler production plate with 1,000 birds and the frequency of isolation of *Salmonella* Infantis was compared in the treated batch with a batch of the same size in which the phage cocktail. *Salmonella enterica* serovar Infantis was not detected in routine screenings of broiler production where bacteriophages were applied. On the other hand, in the control group, *Salmonella enterica* serovar Infantis was detected with a frequency of 20% in caecas, 10% of the feathers washing samples, 33% of scalded water and 20% of the samples of water from the final wash. In conclusion, the isolation, amplification and application of native bacteriophage cocktails is a useful tool in the biocontrol of colonization of *Salmonella enterica* serovar Infantis in the poultry industry.

***Key* Words/Index Terms: *Salmonella enterica* serovar Infantis, poultry farms, bacteriophages, biocontrol**

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INTRODUCTION

Food borne diseases are an important concern in public health (Havelaar *et al*., 2015; Torgerson *et al*., 2015). Death and diseases caused by contaminated food are a constant threat worldwide (Torgerson *et al*., 2015). The WHO estimated that the most frequent causes for food transmitted diseases are diarrheal disease agents, especially norovirus, *Campylobacter* spp. and non-typhoid *Salmonella enterica*. Other death causes are related to *Salmonella* Typhi, *Taenia solium* and Hepatitis A virus (Torgerson *et al*., 2015). *Salmonella* is a common zoonotic and food born pathogen and the third cause of human death associated to diarrheal diseases worldwide (Ferrari, Rosario, Cunha-neto, Mano, & Figueiredo, 2019; Lei *et al*., 2020).

Salmonella from commensal to intestinal pathogen

Salmonella is a cosmopolitan bacterium genus belonged to *Enterobacteracea* family with high genetic diversity. *Salmonella* are non-spore forming Gram negative bacilli with aerobic metabolism, H_2S production and most of them have peritrichous flagella. *Salmonella* comprises to species *S. bongori* and *S enterica*, according White-Kauffmann scheme based on the surface antigens expressed on lipopolysaccharide (LPS), flagella and capsular polysaccharide, there are about 2,659 serovars of *S. enterica* (Ferrari *et al*., 2019). The most important pathogens are classified among 1,547 serovars of *Salmonella enterica* subsp. *enterica*, but less than 100 serovars are associated with human infections (Centers for Disease Control and Prevention, 2020).

S. enterica colonizes the intestinal tract of almost every animal species wild, domestic (pets) and farm ones. Also, *Salmonella* can survive under non favorable environmental conditions, including desiccation and starvation (Raspoet, 2014) and can cause contamination on poultry, swine and calve derived meat, which can occur in any country, any place and any time (Ferrari *et al*., 2019).

Salmonellosis range from self-limiting gastroenteritis to severe bacteremia and typhoid fever (S. E. Park, 2019; Tegegne, 2019). It depends on *Salmonella* serovar and host interaction (Table 1.) (Lamas *et al*., 2018). There are adapted serovars of *Salmonella* known as specialist because colonize and infect only a

narrow range of host (Kingsley & Ba, 2000). This specialist includes those that cause typhoid fever in humans (Table1). Moreover, there are specialist serovars for animals too, such as *Salmonella enterica* subsp. *enterica* serovar Choleraesuis in pigs, Gallinarum or Pollorum in poultry (Ferrari *et al*., 2019; Tanner & Kingsley, 2018). On the other hand, the generalist serovars are able to infect humans or animals without restrictions, the most important examples are Enteriridis and Thyphimurim serovars, which cause less severe symptoms that could be self-limited with diarrhea and the main symptom (Ferrari *et al*., 2019).

Epidemiology and pathogenesis of salmonellosis

The infection dynamics of *Salmonella* depends on oral-fecal route and its ability of colonize and infect their host. Around 52% of salmonellosis are non-typhoidal and 37% are typhoidal *Salmonella* cases, which represents the 9% of global diarrheal illnesses. However, 41% of all deaths associated with diarrheal diseases are caused by *Salmonella* (Besser, 2018). It is also result of human activities in farm animal industry, and food conservation process (Carrasco, Morales, & García, 2012). Salmonellosis has a special attention for its social and economic impact in productive schemes (Mouttotou, Ahmad, Kamran, & Koutoulis, 2017; Sukumaran, Nannapaneni, Kiess, & Sharma, 2015). Fever, abdominal pain and diarrhea are the most frequent symptoms of *Salmonella* sp. infection. It is an emergent zoonosis with an annual worldwide incidence of 93.8 million people and with a cost that raised to 1000 USD for each case (Evangelopoulou, Kritas, Christodoulopoulos, & Burriel, 2015).

The most common manifestation of *Salmonella* infections is gastroenteritis, with a prevalence of 93.8 million cases worldwide each year (Vinueza, Cevallos, Ron, Bertrand, & De Zutter, 2016). In European Union in 2008 there were 131,468 confirmed cases, representing the second cause of zoonotic diseases in humans (Carrasco *et al*., 2012); in 2015 the United States 94,4625 salmonellosis cases were reported resulting in 26 deaths (Lamas *et al*., 2018). In 2014, in Ecuador 3,373 cases were reported (Vinueza *et al*., 2016).

Virulence and host adaptation of *Salmonella* is due to virulence plasmids (pSLT) and *Salmonella* pathogenicity islands (SPIs) (Fig. 3), which are evolutive acquisitions(Lamas *et al*., 2018). There are five main SPIs (1–5). Proteins with

invasive functions to epithelial cells are codified in SPI-1 and SPI-2 codifies determinants for survival and replication inside host cells in *Salmonella enterica* (Lamas *et al*., 2018; Tanner & Kingsley, 2018). A more efficient host colonization by *Salmonella enterica* subsp. *enterica* is done by the posterior acquisition of SPI-6, 2-aminoethylphosphonate metabolism, Island STM3779-STM3785, Island STM4065-STM4080 and quorum sensing mechanism based on Autoinducer 2 (AI-2) transport and processing that could be involved in communication with gut microbiota, as virulence regulator and genetic exchange facilitator (Gast & Porter, 2020; Lamas *et al*., 2018; Tanner & Kingsley, 2018).

Pathogenesis model of *Salmonella* in humans began with the resistance of *Salmonella* strains to stomach pH. Then *Salmonella* traverses the intestinal mucus layer and adhere to intestinal epithelium by adhesins (codified in SP-3 and SP4). Once attached, *Salmonella* express SPI-1 genes for the multi- protein complex T3SS to be engulfed into the epithelial cell (Lamas *et al*., 2018). The cecal mucose convert The H2S produced by the microbiota is used by cecal mucose cells to produce thiosulphate as a protective response (S. E. Park, 2019). Neutrophiles use thiosulphate and convert it in tetrathionate in the intestinal lumen. *Salmonella* uses tetrathionate as respiratory electron acceptor and grows more than the fermenting commensal bacteria. On the other hand, engulfed *Salmonella* located in *Salmonella* Containing Vacuoles (SCV) express a second T3SS that lets bacteria survive and replicate inside host cells (epithelial cells and macrophages) (Lamas *et al*., 2018). Mature SCV migrate to the Golgi apparatus while *Salmonella* increase their number by replication. Phagocytes and macrophages are also used for replication inside SCVs when bacteria cross the epithelium and then phagocytes facilitate bloodstream dissemination in the host (Lamas *et al*., 2018).

Salmonella in poultry industry

Food-animals, including pigs and poultry, could be colonized for different *Salmonella* serovars (Aabo *et al*., 2002; Magwedere, Rauff, De Klerk, Keddy, & Dziva, 2015). *Salmonella* Typhimurium, Enteritidis and Infantis are most frequently associated with health problems in humans farm (Crim *et al*., 2014; Hugas & Beloeil, 2014; Hungaro, Mendonça, Gouvêa, Vanetti, & Pinto, 2013; C. J. Park & Andam, 2020). Colonized farm animals, without any observable clinic

symptom are a big risk factor for food production industry (Fearnley, Raupach, Lagala, & Cameron, 2011; Stevens, Humphrey, & Maskell, 2009).

Aviculture produce the major human´s source of protein from animal origin (Shepon, Eshel, Noor, & Milo, 2016). In Ecuador, 30-32 Kg/year of chicken meat are consumed *per capita* (Gutierrez, 2017). Around 46 million *Gallus gallus* were reared in Ecuadorian Agricultural Production Units during 2012 (Data from Continuous Agricultural Production and Production Survey. Ecuador). For 2017, the annual production volume reached 250 million broilers (Gutierrez, 2017). The Andean region produce the 68% of chicken and 85% are raised in commercial poultry farms (Corporación Financiera Nacional Ecuador, 2016).

Among farm-animals, broilers production is challenged permanently by *Salmonella* serovars colonization within chicken intestines or in their facilities (Table 2). In Germany from 1991 and 1993, the prevalence of *Salmonella* in poultry meat and its by-products the pathogen was present on 18% of the samples (Hartung, 1993), while in New Zealand, found it on 23/137 (17%) of non-frozen poultry and 2/17 (2%) of frozen poultry meat samples, (Rahman & Othman, 2017). But this dynamic is not exclusive of geographic region or type of processing facilities. *Salmonella* is a cosmopolitan bacterium and can cause contamination on poultry, pork and beef, which can occur in any country, any place and any time. For the European Union countries and 3 nonmembers, the general prevalence of *Salmonella* was reported to be 3.37% within farms with rates varying from 0.08% in Norway to 13.84% in Hungary in 2014 (Vinueza, 2017; Vinueza *et al*., 2016).. In Brazil, 32% of carcases from 4 commercial farms were positive for *Salmonella* (Fuzihara, Fernandes, & Franco, 2000) while United Kingdom found 8% positive samples from analyzed carcasses in 2002. At the east of Azerbaijan, Dehnad, 2004, examined 200 pieces of industrial and semi industrial processed chickens and found 31,5% positivity in the samples. Simmons *et al*, 2003, in the United States, demonstrated 33.9% positivity for *Salmonella* on carcasses. Zeiton and Al-Edi, 2004, in Saudi Arabia, analyzed 360 frozen chickens and revealed the presence of *Salmonella* on 20% of the samples. Other examples include Al Abidy, 2005, in Iraq found 9,72% of frozen products positive for the bacteria. O the other hand, in Sweden, where there is a small poultry industry, the prevalence of contaminated poultry meat with *Salmonella* is low below 1%. In general, all this studies indicate

that *Salmonella* contamination prevalence on poultry meat carcasses extended from 0.16% to 49% in the period from 1991 to 2006 (Rahman & Othman, 2017).

In Latin America, some *Salmonella* outbreaks in humans have been linked to contaminated poultry consumption; nevertheless, data about *Salmonella* prevalence in Latin America is scarce. *Salmonella* in poultry meat is associated with fecal contamination from asymptomatic animals (Hugas & Beloeil, 2014). Other sources are equipment in slaughterhouses, floor, or the manipulation of asymptomatic workers. Final product could be contaminated with the pathogen in any processing stage (Rahman & Othman, 2017). Different *Salmonella* serovars have been detected on poultry final products which can cause disease in humans (Antunes, Mourão, Campos, & Peixe, 2016).

In Pichincha, Ecuador, between 2013 and 2014, according to Vinueza, 15% (n=388) of poultry commercial batches are *Salmonella* positive, besides mentions that Venezuela has a similar incidence of 23% (n=332), unlike prevalence in Brazil which is 5% (n=40) and in Colombia 65% (n=315).

***Salmonella enterica* serovar Infantis**

In recent years, it has been noted a change in the prevalence of *Salmonella* serovar Infantis in broilers raising systems as well as in human infection cases. The reservoir of *S*. Infantis are farm animals in special poultry commercial farms (Miller, Prager, Rabsch, Fehlhaber, & Voss, 2010). Its increased prevalence worldwide is due to an evolutionary acquisition of a mega plasmid pESI that confers bacteria antimicrobial and stress resistance, pathogenicity islands and evolutive advantages to be a dominant serovar. Also confers a virulence factors but with less expression level causing a non-severe symptoms when it infects human hosts (Aviv *et al*., 2014).

The horizontally acquired mega plasmid pESI confers a clearly advantage to this bacterium to become the most prevalent serovar isolated in poultry farm screening (Aviv *et al*., 2014). Despite of *S.* Infantis don't cause any disease in poultry, but its presence in farms increases the probability of poultry carcasses contamination and economical losses to the industry. In South Africa between 2013 and 2014, *Salmonella* Infantis was found among the most common 16 serovars related to poultry farming (Magwedere *et al*., 2015). In the same way, countries

like Cambodia, Vietnam and South Korea have *S.* Infantis as one of the predominant serovars (Cui *et al*., 2016). Nowadays, *Salmonella enterica* subsp. *enterica* serovar Infantis is one of the top ten serovars causing human salmonellosis in both Europe and North America (Gymoese *et al*., 2019).In Ecuador, *S*. Infantis is the most common serovar associated to poultry farms with a 83.9% prevalence (Vinueza *et al*., 2016).

Detection of *Salmonella* requires technical expertise in microbiology and an excellent technical performance due to several steps of enrichment and agar culture of samples (Maddox, 2003). This difficulties and the need of rapid responses and standardization drove the industry to develop more sensitive diagnostics (Hendriksen, Wagenaar, Hendriksen, & Carrique-Mas, 2013). Serotyping methods has been used since 1934 for description of endemic serovars associated to animal colonization or animals or human infections. It consists in the use of antisera specific for somatic or flagella antigens for an agglutination reaction (Hendriksen *et al*., 2013). This method is wide used but it is expensive and focused only in most prevalent serovars or those which presence alert the probabilities of human disease associated with farm animals (Centers for Disease Control and Prevention, 2020; Hendriksen *et al*., 2013).

There has been developed culture independent strategies to detect *Salmonella* serovars. Commercial PCR-based methods like BAX®, ELISA- based systems, Bioline Selecta, Bioline Optima and Vidas, or different strategies using a non-commercial PCR system. The sensibility of methods range from 0.67 to 0.99 with VIDAS and ELISA based system with the poorest sensibility (Eriksson & Aspan, 2007). More recently fluorogenic or real-time PCR methods have been developed to generate quick results in *Salmonella* detection from different sources using specific primers, Itsf and Itsr, for the internal transcribed spacer region of the 16S–23S rRNA gene (Cheung & Kam, 2012). Other isothermal amplification methods under commercially protected protocols like 3M and ANRS are frequently used in the industry (Bird *et al*., 2013; Foti *et al*., 2014).

Moreover, a rapid and precise typing system for *Salmonella* serovar has been developed at the genetic level, commercial kits: Salm SeroGen (Salm Sero-Genotyping AS-1 kit), Check&Trace (Check-Points), xMAP (xMAP *Salmonella* serotyping assay), and *Salmonella* geno-serotyping array (SGSA)

(Yoshida *et al*., 2016). All these protocols need *Salmonella* colonies in pure culture isolation. The identification of *Salmonella* serovars were correct in a range of 75% to 100% of the nontyphoidal *Salmonella* samples. There were included serovars Heidelberg, Hadar, Infantis, Kentucky, Montevideo, Newport, and Virchow (Yoshida *et al*., 2016). The molecular mechanism to *S.* Infantis detection consist of targeted somatic and flagellar genes.

Control strategies of *Salmonella* colonization

Prophylactic antimicrobial administration has been used for elimination or reduction of *Salmonella* sp. within normal microbiota of poultry (Evans & Wegener, 2003). Other strategies are vaccines, prebiotics or probiotics under strict quality control of facilities and workers (Antunes *et al*., 2016; Atterbury *et al*., 2007; Chambers & Gong, 2011). However, none of them has achieved the expected efficiency and the disease continues to be emergent (Laurimar Fiorentin, Vieira, & Barioni, 2005). There are some research carried out with viruses that infect bacteria (bacteriophages), as an alternative, which has been promising in the control of colonization, infection and spread of possible bacteria pathogen strains in poultry (Atterbury et al., 2007; Laurimar Fiorentin et al., 2005; Grant, Hashem, & Parveen, 2016; Spricigo, Bardina, Cortés, & Llagostera, 2013; Thung et al., 2017; Yeh et al., 2017; Zinno, Devirgiliis, Ercolini, Ongeng, & Mauriello, 2014).

In the EU and western countries, poultry and derived products are the main source of food infections caused by *Salmonella*, and from all the range of derived products the main risk factors are uncooked eggs and poultry meat (Yeh *et al*., 2017). To control of *Salmonella* in animals and animal products, several alternatives have been proposed, (Spricigo *et al*., 2013). In the last decade, treating human and animal diseases with antibiotics have become more difficult due to the growing problem of bacterial resistance to antimicrobials (Busani *et al*., 2004), and the risk to human health. The concern about antibiotic resistance has pushed the market to seek alternative treatments. Among these intervention practices to reduce or prevent the spreading of pathogens are the use of different animal genetic lines that control the immune response, chemical treatments to avoid the vertical transmission of *Salmonella* by immersion in oxygen peroxide and phenol of the recent harvested eggs reducing its bacterial load (Doyle & Erickson, 2004), general guides of sanitization by using chemicals, temperatures, pressure for a correct

cleaning and disinfection of all areas. Another alternative practice of raising poultry selecting different bed materials, treatment with different chemicals or composting processes, water sanitization, food supplements with prebiotics, application of probiotics for bacterial competitive exclusion, additives to improve immune responses, vaccines and bacteriophages (Doyle & Erickson, 2004).

Bacteriophages.

Bacteriophages are viruses widely spread in nature, are ubiquitous entities and can be found in sea, soil, deep sea vents, and gastrointestinal tract of humans and animals (Belay, Sisay, & Wolde, 2018). Phage life cycle is strictly associated with the bacterial cell, they have been denominated as molecular parasites because they lack of cell structures and enzymatic systems necessary for food absorption, protein synthesis or new particles construction, and as incomplete organisms they can replicate only inside a living cell (Wernicki, Nowaczek, & Urban-chmiel, 2017).

Bacteriophages were discovered by Twort (1915) as “unidentified molecules which inhibit bacterial growth”, but in 1917 D’Herelle was the first one to isolate and characterize phages (Brown, Lengeling, & Wang, 2017; Kutter *et al.*, 2010). The International Committee on Taxonomy of Viruses, EC 48, Budapest, Hungary, August 2016 (ICTV) imposed some criterion of taxonomy based on genome type and virion morphology (Wernicki *et al.*, 2017). Moreover, the use of proteomics helps to classify viruses in 873 species, 204 genera and 14 subfamilies (Adriaenssens & Brister, 2017). Other factors like host preference, auxiliary structures such as tails or envelopes are considered (Orlova, 2012). Based on nucleic acids, phages can be divided in three groups, double helix DNA, single chain DNA and RNA; most phages described have double helix DNA genome. Other important feature is capsid symmetry, differentiating two groups isometric (polyhedral) and helicoidal (spiral) (Wernicki *et al.*, 2017). It has been proposed that phages are the most abundant form of life in the planet, by 2017 more than 25,000 sequences of nucleotides have been saved on databases, and this abundance of phages in nature is what it makes so great when investigated as they are easily found (Haq, Chaudhry, Akhtar, Andleeb, & Qadri, 2012)

According to the type of infection, phages can be divided in two groups: hose that cause a lytic infection and the other that cause lysogenic, or temperate,

type of infection (Orlova, 2012). The replication of phages is like viruses that infect eukaryotic cells: starts with adsorption, penetration, nucleic acids replication, phage formation and its release from de host cell (Kutter *et al*., 2010; Nelson, 2004; Wernicki *et al*., 2017). During a bacterium infection by lytic phages, DNA is released and induces switching of the protein machinery of the host bacterium. Whit this change, 50-200 of new viral particles could be produced causing the death of host bacterium (Orlova, 2012). On the other hand, lysogenic infection is characterized by integration of the phage DNA into the host cell genome, so that it could be replicated and vertically transmitted to new bacteria cells as a prophage (Orlova, 2012; Wernicki *et al*., 2017) This phase could be reverted under stressful conditions surrounding bacteria population and a lysogenic phage could be a lytic one (Wernicki *et al*., 2017).

Analysis of phages with lysogenic or lytic mode of infection has shown that there is a tremendous variety of bacteriophages. Some phages show specific affinity with unique types of bacteria, while others show affinity for a wider group, the specifics of this affinity is determined by the presence of receptors in the surface of bacterial cells, as LPS fragments, fimbria and other surface proteins. Under lytic phages activity the adhesion process to the bacterial cell consist on the union between phages protein and the cell receptors like teichoic acid and lipoteichoic for Gram positive or LPS for Gram-negatives (Wernicki *et al*., 2017). Then it has a phase of penetration into the genetic material and it reaches the eclipse, where the replication of nucleic acid and the proteins part of the capsid structure occurs, while genetic material replication of the bacteria is on hold, then the phage is formed and mature followed by the release of the newly formed phages producing bacterial lysis. Phages known for lytic activity are T1 and T4. On the other hand, under lysogenic cycle, after forming a prophage, the phage cycle is blocked and enters a latency period which can be interrupted by external factors as sun light, UV radiation or antibiotics. Examples from phages with lysogenic activity include λ, Mυ, MM1 and ϕ11 phages (Kutter *et al*., 2010; Nelson, 2004; Wernicki *et al*., 2017).

Salmonella is an *Enterobacteriacea* that could be easily affected by specific phages. *S.* Thiphymurium for example is the target bacteria for P22 bacteriophage, which belongs to *Podoviridae* family. P22 uses a non-contractile tail to adsorb to

Salmonella surface. Phages L, MG178 y MG40 have *Salmonella* Thiphymurium as target (Labrie, Samson, & Moineau, 2010). Other *Salmonella* serovars could be host for Epsilon 15 bacteriophage (Orlova, 2012). In nature, phages and bacteria are in continuous cycles of co-evolution (Chaturongakul & Ounjai, 2014). Thus, bacteria resistance to phage infection can occur and are studied under controlled laboratory conditions, with a unique bacterial host-phage model. However, at environmental conditions a conjunction of phage resistance mechanism could be working at the same time (Labrie *et al*., 2010). Mechanisms used by bacteria for phage resistance are listed from an interesting review done by (Kurtböke, 2012):

- Phage adsorption inhibition
  - Blocking phage receptors: Changes on three-dimensional conformation of surface structures or their adaptation
  - Extracellular matrix production confers protection by a physical barrier, phage cannot interact with surface molecules
  - Competitive inhibitor production, that mean environmental molecules that are in the bacteria niche and can interact with phages receptors
- Blocking phage DNA entry
  - Proteins from superinfection exclusion system consist of membrane anchored or associated with membrane components causing an inhibition of DNA injection into cells, the transfer of viral DNA into bacterial cytoplasm or by inhibition of phage lysozyme.
- Cutting phage nucleic acids
  - Restriction – modification system classified from type I – type IV groups. This system consists of bacterial enzymes that recognize and degrade non-methylated DNA by restriction enzymes and depend on the restriction methylase enzymes ratio and the number of restriction sites in virion genome.
  - CRISPR-Cas system is acronym of clustered regularly interspaced short palindromic repeats ant its association with *cas* proteins. The CRISPR loci are composed of 21–48 bp direct repeats interspaced by non-repetitive spacers (26–72 bp) and

flanked by *cas* genes. When a virus infects a bacterium, one new repeat-spacer unit at the 5′ end of the repeat-spacer region of a CRISPR locus is acquired. This proto spacer sequence is identical to that found in the viral genome and it is used to identify and degrade incoming viral or plamid DNA.

- Abortive infection systems (Abi)
  - RexA-RexB system: Blocks the replication, transcription or translation of phage through a two-component system (RexA-RexB), that is activated by the viral DNA-protein complex. RexA is an intracellular sensor that forms and homoduplex and activates RexB wich is membrane anchored ion channel. The activation of RexB drives a drop in the cellular ATP level by reduction on membrane potential. In consequence there are decrease in macromolecules synthesis and stops phage infection (Labrie *et al*., 2010)
  - Lit-PrrC system that inhibits the phage translation and probably activates IC R–M system. Activated PrrC cleaves tRNA block phage infection in consequence.
  - Others that involve resistance-induced physiological changes that could destroy bacteria cell too.

The ubiquity of bacteriophages in any environment facilitates their isolation and description of their suitability to be use against bacteria pathogens in human or animal infections or as colonizers (Brown *et al*., 2017; Chaturongakul & Ounjai, 2014). The specificity of isolated phage limits its effectiveness when it is compared with antimicrobial administration (Brown *et al*., 2017), but due to the raising antimicrobial resistance phenotype of interest bacteria phage therapies could be an extraordinary tool in eliminating bacterial infections (Belay *et al*., 2018). In this context, poultry industry has been used bacteriophages to treat animals' diseases (Belay *et al*., 2018; Wernicki *et al*., 2017) whcih improves animal productivity and health. *Salmonella* Infantis in poultry industry is a selected target because its high prevalence (Miller *et al*., 2010). The emergence of new dominant *S.* Infants linage with mega plasmid, potential spread to humans and the fast antimicrobial resistance determinants acquisition is a high relevant challenge. Many publications have

showed the biological control of resistant bacteria with bacteriophage cocktail applications (Belay *et al*., 2018; Borie *et al*., 2008; Rahaman *et al*., 2014; Zhang *et al*., 2010). However, commercial products containing phages could be less effective along time vs. native phages cocktails periodically isolated. The aim of this study was to isolate native bacteriophages from a poultry farm samples including sewage, effluents, bedding, and feathers and evaluate their specific activity against *Salmonella enterica* serovar Infantis as a potential biocontrol tool its colonization in poultry. The bacteriophages have had a special interest and their main applications are: alternative to antibiotics against bacterial pathogens including food pathogens (phage therapy); screening tools based on phage-display; or genetic tools for pathogenic bacteria detection (phage-typing) (Belay et al., 2018). As biologic control agent they had great success rates due to its capacity to infect a wide range of bacterial species, a serotype or a strain. Increasing bacterial resistance to antibiotics and antibiotic use restrictions, create the need of new alternatives such as the isolation of native bacteriophages (Wernicki, Nowaczek, & Urban-chmiel, 2017).

For bacteriophage isolation, the host strains must be those serovars with major prevalence in animal farms to control their own colonization and reduce their prevalence withing the broiler complex., therefore we used *S.* Infantis for phage enrichment culture due to the high relevance of this serovar as the main concern in Ecuadorian poultry industry (Vinueza et al., 2016) as well as other authors used different *Salmonella* serovar including those with some association with human disease like *Salmonella* Enteritidis, *Salmonella* Infantis, *Salmonella* Heidelberg, and *Salmonella* Typhimurium (Rivera et al., 2018). Other authors reported the use of different strains of *Salmonella* as hosts for bacteriophage isolation without restriction of wide range of lytic activity of bacteriophages but focused in isolation from bacteria of public health interest (Petsong, Benjakul, Chaturongakul, Switt, & Vongkamjan, 2019; Phothaworn et al., 2019). Multi-strain *Salmonella* enrichment worked in the bacteriophage isolation as was shown by (Petsong et al., 2019), therefore, it could be an important consideration for future perspectives focus on wide range host bacteriophage isolation. In this study we tested the application of native bacteriophages (using *S.* Infantis host) in drinking water to reduce or eliminate *S.* Infantis in chicken carcasses.

METHODS AND STUDY DESING

This study was approved by the animal health committee and respect the technical and ethical working conditions of animal welfare in the industry, according with universal rules for animal use in experimental proposes. The farm included for this study belonged to an integrated broilers batch followed for the entire production and processing cycle (One day age broiler reception, feed and growing, slaughtering, carcasses processing and marketing) at poultry farms and slaughter facilities in Santo Domingo de los Tsachilas - Ecuador. Three phases were performed during the study: 1) Phage isolation from environmental and chicken samples; 2) phage sensibility and specificity and 3) intervention with a viral cocktail. All broilers used had the same age and were housed on the same day.

1) Phage isolation from environmental and chicken samples

Bacteria strains.

For bacteriophage isolation, we used two strains of *S.* Infantis (identified as U and P strains) were used to select bacteriophages. The bacterial species were confirmed by serologic test with a ready to use rabbit antiserum (SSI Diagnostica A/S, Denmark) and molecular techniques (Kim *et al*., 2006). Additionally, The Microbiology Institute of San Francisco University donated strains of *Salmonella enterica* serovars Typhimurium, *S*. Enteriditis and *Eschericia coli.* For sensitivity test we used 44 *S.* Infantis strains from the bacteria collection kept in the diagnostics laboratory of the industry. We focused on *S.* Infantis*,* for bacteriophage isolation under company demands for the high prevalence of this strain in Ecuadorian poultry farms (Vinueza *et al*., 2016).

Phage isolation.

We used tryptic soy broth, tryptic soy with 0,7% of agar and tryptic soy agar (1.5% of agar) for enrichment and isolation with doble layer method as an economical and technically efficient protocol for phage isolation (Rivera et al., 2018). Previous nonpublished data from de industry let us know that the most efficient place to isolate phages are broiler houses and slaughtering facilities. With completely random sampling design, we selected 5 samples from scalding water from chickens, 5 samples from chicken feathers water, 5 samples from water used

to flush chicken carcasses and 10 samples from chicken bed, moreover 5 samples of turkey carcasses flush water and 5 samples from scalding water from turkeys were collected. Samples were transported in ice to the laboratory facilities withing 2 hour after collection. Twenty-five milliliters of each liquid sample or 25 g of chicken bed were placed in a flask with 225mL of Buffered Peptone Water (BPW). For native bacteriophages isolation, a target bacteria *S.* Infantis culture was prepared 18h before the assay. Briefly, 0.2mL of *S.* Infantis culture and 2mL of each liquid sample prepared with BPW were added to 5mL of Tryptic Soy Broth. These tubes were incubated at 37°C by 24h. Then, each sample was centrifuged at 15,652 g for 10 minutes. One milliliter of each supernatant was dispensed in a 1,5mL sterile tube and 0.2mL of chloroform was added. These bacteriophage cocktail samples (BCS) were stored at -20°C for further analysis. Presence of *S.* Infantis enriched bacteriophages were evaluated as following: tubes with molten semisolid (0.7% agar) tryptic soy culture media at 45 °C were mixed with 0.3mL of 18 hours culture of *S.* Infantis and 0.2mL of the BCS. After mixing, the semisolid medium was dispensed in petri dish containing a solidified layer of tryptic soy agar. Petri dishes were incubated at 37°C for 18 hours. Clear zones (plaques) in the bacterial lawn indicated the presence of lytic phages (Kropinski, Mazzocco, Waddell, Lingohr, & Johnson, 2009). Plaques were collected using a cut sterile plastic pipette tip and suspended in 1mL of salt magnesium (SM) buffer and stored at -20°C (Clokie & Kropinski, 2009). These phage lysates (PL) were used in further assays to test the host specificity and to amplify a phage cocktail. The strong lysis ability was the characteristic that we use to select phages in our cocktail (Petsong et al., 2019). These features were valued in front to the halo diameter, de abundance al transparency of the plaques (Rivera et al., 2018).

2) Isolated phage sensibility and specificity testing

Bacterium host specificity of native phage lysates.

Phage lysates were tested against *S.* Infantis (P and U), *Escherichia coli, S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis and 44 *S.* Infantis strains from a collection kept in diagnostic laboratory of the industry. One strain was isolated from chickens' bed, 10 strains were isolated from chicken meat and the other were isolated from environmental samples. A 24h- bacterial culture was

adjusted to 0.5 McFarland scale using sterile TSB. Then, 100µL of this bacteria dilution were inoculated in 5mL of molten TSA (0.7% agar) and overlaid onto a cell of tri Petri dish. Inoculated culture media plates could solidify for 15 min and were incubated at 37°C for 18h. Each native PL obtained was spotted onto lawns of a host bacterium strain culture using a sterile inoculating loops with 2mm of diameter. Plates were incubated at 37°C for 18h and the appearance of clear zones of lysis were describes as positive bacteriolytic activity (Gencay & Brondsted, 2019). Lytic capability was tested with double layer agar plate using *S.* Infantis isolate as target (Kropinski *et al.*, 2009). Four phage lysates with the best lytic activity (higher clear zone diameter) and greater spectrum against *S.* Infantis were selected to be used in a cocktail.

3) Selection of phage cocktail amplification and administration in water source.

Cocktail preparation in base of selected phage lysates amplification.

To scale up the four selected phage lysates, each one was amplified as follow: 1mL of PL was inoculated in a fresh *S.* Infantis culture, in a final volume of 250mL of TSB. After overnight incubation at 37°C, the culture was filtered with a vacuum system using filter cops (FILTERFLOCKEN MAN 201) which trapped biomass and then 0.2 µm diameter pores filters were used to guaranty any bacteria contamination in the final filtrate solution. Control of non-bacteria contamination were done in nutrient culture media and XLT4 media as selective one. Filtrates were stored under freezing conditions at -20 ° C.

Phages cocktail stability *in vitro* test on water system.

In broilers house, drinking water was treated with chloride solutions to avoid probable pathogens present in water. Then, a commercial halogen neutralizer BALMAR® or a viral protector PROVIR ® was added to water. We tested the lytic ability of phage cocktail in water supplemented with chloride solution at 0, 1 2 3 and 4 ppm as final concentration, then commercial halogen neutralizer, were added following manufacture instructions with a final concentration of 1Kg in 100L of water. A similar batch of chloride water was used to be supplemented with the viral protector under the same conditions and following the manufacture recommendations. An experimental unit was defined as a flask with 500mL of

chloride water (0, 1 2 3 and 4 ppm) treated with the halogen neutralizer or the viral protector and with an inoculum of prepared phage cocktail in a ratio of 1:100. Three repetition of each experimental unit were performed. Chlorinated water at the same concentrations was used as control. The commercial solution used was a factor in this experimental design, the other factor was the time of phage cocktail exposure at 0, 1, 2, 3, and 4 hours.

To evaluate the stability of phage lytic activity, a doble layer assay were performed for each experimental unit. Briefly, 0.2mL of 18h *S.* Infantis culture and 0.1mL of each liquid sample were added to 5mL of molted Tryptic Soy Broth with 0,7% of agar. These mixes were poured on previously prepared tryptic soy agar plates. After 24h incubation at 37°C, the plaques were analyzed.

Bacteriophage cocktail application in drinking water system.

Under farm conditions, drinking water is treated with 1ppm of chloride solution. Previous nonpublished data were used to calculate the water requirement in each growing phase of broilers described in the table 3. Additionally, the continuous surveillance program for *Salmonella* detection in farms environment and the whole poultry productive chain let us know the prevalence of *Salmonella* in different stages. Based in those nonpublished data, we selected two *Salmonella* Infantis positive broiler sheds with 1,000 animals each and we follow them during their whole life until their slaughter process under the strictly protocols of animal welfare and slaughter in chicken production industry.

The experimental design consisted of 1,000 of chickens selected as control group and 1,000 chickens used as treatment group in a different barn. Both were separately managed with the same light and feed programs, with a density of 10 chickens/m2 area. Bacteriophage cocktail suspension was applied 1:100 in the treated water after a 2-hour water starvation for chicken. The viral protector was colored in blue to confirm all chickens take water and the phage cocktail doses included in. Phage cocktail suspension was applied every 5 days for 4 times increasing the total water volume administered according to the chicken age. No screening or control samples were taken either chickens nor barn environment during chickens' life to avoid stressors or non-controlled factors that could affect the chicken's health.

***Salmonella* Infantis screening in slaughtering facility.**

The experimental group (treatment A) was slaughtered at the first turn in the morning and the control group (treatment B) was slaughtered the next day to avoid any cross contamination. Liver, caeca, feathers, scalding water, carcass rinse water and finished product samples. Samples were pre-enriched in BPW during 18 hours in 37°C. The screening for *Salmonella* sp. was done following ANSR (Apracom S.A.) protocols (Foti *et al*., 2014). Positive samples were analyzed to confirm the detection and identification of *Salmonella* Infantis following standard microbiology procedures with XLT4 y BPLS culture media and serology test

RESULTS AND DISCUSSION

Our results indicated that one application of bacteriophage cocktail was effective in the reduction of *Salmonella* in the digestive tract of poultry which is in agreement with previous reports (L Fiorentin, Vieira, Barioni Júnior, & Barros, 2004). The results showed a complete absence of *S*. Infantis in all samples taken from chickens in the treatment group. Meanwhile, in the control group 20% of caeca, 10% of feathers, 33% of scalding water and 20% of carcass rinse water were positive for *S*. Infantis detection. In other studies, periodic application of a combination between a phage cocktails and probiotics mixture, applied orally on poultry showed 10 times less presence of studied *Salmonella* serovars in the liver, spleen, caeca's and ileum than untreated poultry (Laurimar Fiorentin et al., 2005). Also, Filho et al. showed that the oral administration of a phage cocktail prevented *S.* Enteriditis colonization for short period of time (48 hours). In our study, the presence of *S*. Infantis were evaluated after 42 days of production period, taking samples from liver, caeca, feathers, scalding water, carcass rinse water and finished product.

To isolate bacteriophages, we used samples from scalding water (n = 5), chicken feather water (n = 5), flush chicken carcasses water (n = 5) and chicken bed (n = 10). We found that 88% of the samples yielded bacteriophages (31 of 34) that was lower than the reported for Chilean broiler farms in which 97% of processed farm samples were positive for bacteriophages (Rivera *et al*., 2018).

To choose bacteriophage candidates for biocontrol applications, the transparency of litic halos has been taken as qualitative variable (Rivera, et al, 2018). In this study all bacteriophage isolates showed clear plaques with the same or similar transparency. Those mean that all bacteriophage isolates had the same potential to be part of the final control by their lytic activity against *Salmonella* Infantis. To evaluate the sensibility of isolated bacteriophage activity, we used 44 *Salmonella* Infantis strains from our own collection. We did this assay because we wanted to evaluate if the permanent changes on adsorption molecules in bacteria or the development of phage resistance mechanisms could affect the affinity of the native bacteriophages against *Salmonella* Infantis strains (n=44) isolated from different point times in the industry (Wernicki *et al*, 2017, Kurtböke *et al*, 2012). In our

study, 100% of isolated phages (n=30) showed clear plaques on the primary cultures (Figure 1). The plate lysates were evaluated against different strains of *S.* Infantis, 27 out of 44 strains were sensitive to all phages; 6 were sensitive to a cocktail containing a combination of 2 to 29 different phages and 11 strains were resistant to all phages and probably it explains that 25% of *Salmonella* Infantis strain collection were resistant to all phages lysates obtained, highlighting that *Salmonella* strains and phage lysate were isolated in different settings within the industry and the difference among used *Salmonella* strains is only the time of isolation. In Rivera *et al* study, they reported a narrow range of lytic activity of isolated bacteriophage in 9 phage isolates, but they worked with three *Salmonella* serovar strains as target bacteria. We obtained 27 phages with narrow host specificity affecting only *S.* Infantis. The presence of *S.* Infantis lytic phages in high frequency suggest that the environmental presence of this strain along the chicken production chain, since the raising house to slaughtering, but also means that the more dangerous non typhoid pathogen strains like *S*. Thiphymurium and *S.* Enteritidis are not present in the same prevalence (Higgins et al., 2008).

CONCLUSIONS

We demonstrated that the use of native bacteriophages is an efficacious procedure to reduce *S.* Infantis in samples from broilers or environment associated with chicken industry. Successful control of *Salmonella* serovars and other pathogens associated with food animal farms have been shown previously. It is necessary to conduct additional studies to determine the cocktail efficacy overtime. It is known that bacteria acquire immunity to phages, and we assessed that the best and cheaper strategy against this co-evolution is the periodic isolation of new phage collection.

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Table 1. Human and animal diseases associated with host adapted or host generalized serovars of *Salmonella enterica* subsp. *enterica*

Serovars	Host range classification	Natural hosts	Disease	Symptoms or sign(s)	Rare hosts
S. Typhi	Host restricted	Humans	Typhoid fever	Septicemia, fever	-
S. Paratyphi A	Host restricted	Humans	Paratyphoid fever	Septicemia, fever	-
S. Paratyphi B	Host restricted	Humans	Paratyphoid fever		-
S. Paratyphi C	Host restricted	Humans	Paratyphoid fever		-
S. Typhimurium	Host generalised	Humans	Gastroenteritidis	Diarrhea, dysentery, fever	None
		Bovines	Salmonellosis	Diarrhea, dysentery, septicemia, fever	
		Swine	Salmonellosis	Diarrhea	
		Sheep	Salmonellosis	Diarrhea, dysentery, septicemia	
		Horses	Salmonellosis	Septicemia, Diarrhea	
		Rodents	Murine typhoid	Septicemia, fever	
		Poultry			
S. Enteritidis	Host generalised	Humans	Gastroenteritidis	Diarrhea, dysentery, fever	Swine and bovines
		Rodents	Murine typhoid	Septicemia, fever	
		Poultry			
S. Infantis	Host generalised	Human	Gastroenteritidis	Diarrhea, dysentery, fever	
		Poultry			
S. Dublin	Host adapted	Bovines	Salmonellosis	Diarrhea, dysentery, septicemia, fever abortion	Humans
S. Choleraesuis	Host adapted	Swine	Pig paratyphoid	Skin discoloration, septicemia, fever	Humans
S. Gallinarum	Host restricted	Poultry	Fowl typhoid	Diarrhea, comb discoloration, septicemia	None
S. Pullorum	Host restricted	Poultry	Pullorum disease	Diarrhea, septicemia	
S. Typhisuis	Host restricted	Swine	Chronic paratyphoid	Intermittent diarrhea	
S. Abortusovis	Host restricted	Sheep	Salmonellosis	Septicemia, abortion, vaginal discharge Diarrhea, dysentery	
S, Abortusequi	Host restricted	Horses	Salmonellosis	Septicemia, abortion, Diarrhea	

Table 2. Prevalence of *Salmonella* serovars associated to broiler production in different countries

Country/Region	Serovar	Year	%	Source
European Union	*S.* Infantis	2011-2013	26,5%	(Atunnes, 2015) (Vinueza-Burgos et al., 2016)
	S. Infantis	2014	43,4%	(Vinueza-Burgos et al., 2016)
	S. Mbandaka		13,5%	
	S. Livingstone		7,3%	
	S. Enteriditis		7,3%	
Japan	*S.* Infantis	2004-2005		(Assai, 2006)
United States	*S.* Enteritidis	2016	16,8%	(CDC Report, 2016)
	S. Newport		10,1%	
	S. Typhimurium		9,8%	
	S. Javiana		5,8%	
	S. I4(5),12:i:-		4,7%	
	S. Infantis		2,7%	
Venezuela	*S.* Parathyphi B			(Vinueza-Burgos et al., 2016)
	S. Heidelberg			
Colombia	*S.* Paratyphi *B Dt +*			(Vinueza-Burgos et al., 2016)
	S. Heidelberg			
	S. Enteriditis			
	S. Typhimurium			
Peru	*S.* Infantis		84,0%	(Vinueza-Burgos et al., 2016)
	S. Enteritidis		5,0%	
	S. Senftenberg		6,0%	
	S. Debry		1,7%	
	S. Kentucky		1,7%	
Brasil	*S.* Enteritidis			(Vinueza-Burgos et al., 2016)
	S. Infantis			
	S. Typhimurium			
	S. Heidelberg (37)			
Pichincha - Ecuador	*S.* Infantis	2013-2014	83,9%	(Vinueza-Burgos et al., 2016)
	S. Enteriditi*s*		14,5%	
	S. Corvallis		1,6%	

Table 3. Water requirement according to broiler growing phases. Viral protector and phage cocktail supplementation in each phase

Application	Broiler age (Days)	Broiler (n)	Water volume (L)	Phage cocktail volumen (L)	Coloured solution of viral protector (L)
1	20	1,000	60	0.600	0.600
2	25	1,000	60	0.600	0.600
3	30	1,000	80	0.800	0.800
4	35	1,000	100	1,000	1,000

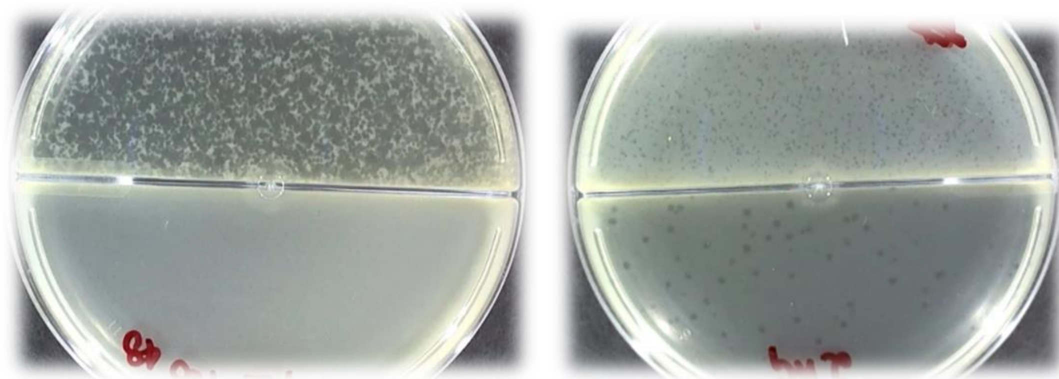

Figure 1. Doble layer analysis from diffent samples within chicken production chain.