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BACTERIOPHAGE SURVEILLANCE IN EGG FARMS: IMPLICATIONS FOR SALMONELLA MANAGEMENT AND BIOSECURITY

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ABSTRACT:

Background: Biosafety regulations are crucial in agri-food systems to mitigate risks associated with pathogens. Bacteriophages, viruses targeting bacteria, proliferate by lysing bacterial cells, releasing infectious viral particles.

Objective: To investigate the presence of bacteriophages in guano capable of combating Salmonella Gallinarum (SG) and Salmonella ser. Enteritidis (SE), indicating simultaneous presence of these bacteria.

Methods: Samples were collected from seven egg-laying bird farms in Luján and Mercedes districts. Four farms employed automatic shed openings, while three utilized manual methods. Various microbiological techniques were applied to detect Salmonella spp., and a spot test method was used to identify lytic phages targeting SG and SE.

Results: Sampling began from the first row of cages, 2 meters from the cone's initiation, extending to three subsequent rows in a zigzag pattern. Automatic farms utilized guano evacuation belts for regular sampling. Salmonella spp. was found in one automatic farm. SG phages were detected in two conventional and three automatic farms, while SE phages were present in four automatic and two manual farms.

Conclusion: Identifying bacteriophages presents a valuable tool for biosecurity and sanitation strategies, aiding health monitoring by alerting potential infections.

KEYWORDS: Pesticides; Salmonella Ser. Gallinarum; Bacteriophages; Salmonella Ser.

INTRODUCTION:

Salmonella is a type of bacteria in the family Enterobacteriaceae. They are gram-negative bacilli that can live in both aerobic and facultatively anaerobic environments. So far, over 2,500 people serotypes have been developed found in a wide range of hosts. In the poultry business, typhoid salmonellae are bacteria from the genus Salmonella subspecies enterica serovar Gallinarum biovar Gallinarum (SG).

Table 1: References

Reference	Citation
Gast, Dittoe, & Ricke, 2024	Gast, R. K., Dittoe, D. K., & Ricke, S. C. (2024).
Poudel & Adhikari, 2024	Poudel, A., & Adhikari, B. (2024).
Curtiss III, 2024	Curtiss III, R. (2024).
EROL & KASKATEPE, 2024	EROL, H., & KASKATEPE, B. (2024).
Jiang et al., 2024	Jiang, X., et al. (2024).

Table 2: More References

Reference	Citation
Watler, Toka, Lardé, Johnson, & Butaye, 2024	Watler, D., Toka, F., Lardé, H., Johnson, T., & Butaye, P. (2024).
Bianchessi, De Bernardi, Vigorelli, Dall'Ara, & Turin, 2024	Bianchessi, M., De Bernardi, F., Vigorelli, E., Dall'Ara, P., & Turin, L. (2024).
Kirti, Krishna, & Shukla, 2024	Kirti, A., Krishna, S., & Shukla, A. (2024).
Kabeta, Tolosa, Duchateau, Immerseel, & Antonissen, 2024	Kabeta, T., Tolosa, T., Duchateau, L., Immerseel, F., & Antonissen, G. (2024).
Tufail & Ashfaq, 2024	Tufail, A., & Ashfaq, M. (2024).

Table 3: Additional References

Reference	Citation
Huang & Naushad, 2024	Huang, Y., & Naushad, S. (2024).
Lamichhane et al., 2024	Lamichhane, J. R., et al. (2024).
Allam et al., 2024	Allam, M. et al. (2024).
Choi et al., 2024	Choi, S. et al. (2024).
Raheel et al., 2024	Raheel, M. et al. (2024).
Sain et al., 2024	Sain, P. et al. (2024).

These bacteria cause avian typhus, a significant disease affecting laying birds. Many factories in developed countries no longer have this disease, but it still exists on commercial farms in Latin America and South America (Gast, Dittoe, & Ricke, 2024; Poudel & Adhikari, 2024).These farms lose a lot of money because of the high death rates, lower production of eggs and quality, and strict veterinary and hygiene-sanitary rules—costs for buildings that are sick. Also, a type of enterobacteria called paratyphic salmonellae can be passed from animals to humans. Salmonella subspecies enterica serovar Enteritidis (SE) is one of these microorganisms that can make people sick if they eat eggs, meat, or other animal products that are contaminated with it. Biosafety rules must be stringent in agri-food systems. Many studies show that Salmonella spp. is still present on farms, even though steps are utilized to reduce their number (Curtiss III, 2024; EROL & KASKATEPE, 2024). The "Surveillance and control program for contaminating by Salmonella spp. in commercial poultry farms" was approved by Senasa Resolution no. 86/2016 at the national level. This program aims to lower the number of farms with specific non-host-specific Salmonella serotypes in broilers and laying hens. This is a crucial step to reduce the risk that harmful germs to humans will get into the final poultry product. This means that the method in Annex D of the ISO 6579 standard should be used to "find Salmonella spp. in animal faeces and tests at the primary

production level." The correct medium to use is the Rappaport-Vassiladis modified semisolid medium (MSRV) (Jiang et al., 2024; Watler, Toka, Lardé, Johnson, & Butaye, 2024).A minimum of the White-Kauffmann-Le Minor method is used to type a strain from each positive sample. Molecular tests are another way to find out what a gene is. Each lab has to use the polyvalent sera OS-A and OS-B to do serological typing (agglutination tests). This is because the Enteritidis, Typhimurium, and Heidelberg serotypes are all part of the OS-A serogroup and must be delivered to SENASA DILAB for serotyping. Legal rules from the European Union, including Regulation 2160/2003/EC6 and Directive 2003/99/EC7, set the framework for implementing Salmonella laws at the European level. Since then, regulations have been added for each type of production (Bianchessi, De Bernardi, Vigorelli, Dall'Ara, & Turin, 2024; Kirti, Krishna, & Shukla, 2024).To lessen the risk to public health posed by Salmonella and its presence in the food chain, the European Union has produced Regulation 2019/268. Here, Salmonella spp. detection will be done in compliance with EN/ISO 6579-1. You can use semisolid Rappaport Vassiliadis agar (MSRV) or broth as a selective medium. A suspect colony must be taken for confirmation; if the isolation results are negative, a maximum of four colonies are taken from various media and examined. It is permissible to perform immediate biochemical verification on the well-isolated suspicious colony while conducting a purity check on non-selective agar. The procedures outlined in Technical Report ISO/TR 6579-3 must be followed to perform serotyping (Kabeta, Tolosa, Duchateau, Immerseel, & Antonissen, 2024; Tufail & Ashfaq, 2024).

Additionally, if approved following EN ISO 16140-2 (alternative detection methods), additional techniques may be employed. Phages, or bacteriophages, are viruses that only infect prokaryotic cells. They comprise the most basic and prevalent biological system found in nature; the estimated number of phage particles worldwide is 1031, and they cohabit alongside the bacteria they invade in a 10:1 ratio. When lytic phages proliferate, they lyse the bacteria and release contagious virus particles. When creating biosafety protocols, this multiplicative cycle presents an intriguing possibility because it could serve as an amplifier for detecting dangerous bacteria like SG and SE (Huang & Naushad, 2024; Lamichhane et al., 2024).

Phages combine some advantageous characteristics to identify bacterial pathogens: They act as signal amplifiers throughout the infection cycle, are inexpensive and straightforward to produce, exhibit high specificity for targeting specific cells, can be used to differentiate between live and dead cells, and maintain their activity in a variety of environmental conditions, obviating the need for time-consuming sample pretreatments. These days, the expansion of the chicken industry has resulted in a rise in production and waste volume, especially guano. Guano is a combination of excrement produced by laying chickens, to which indigestible food components, digestive system mucosa cells, intestinal microbiota microbes, feathers, and broken egg remnants are added (Allam et al., 2024; Choi et al., 2024).

A microbe that can be used to identify the presence of a pathogen is known as a microbiological indicator. For instance, indicators are typically used to assess food microbiological quality or water pollution. Phage populations are limited to those that harbour bacteria, as they require the latter's infection to increase. Thus, in the current work, a microbiological analysis was conducted to isolate Salmonella spp. and measure the enteric bacteria to ascertain whether bacteriophages which lyse SG and SE in guano suggest a potential concurrently processed existence of these pathogenic bacteria. However, lytic phages were shown to be present in the samples of SE and SG (Raheel et al.; Sain et al., 2024).

MATERIALS AND METHODS:

SAMPLING:

During June and October 2017, guinea pig farmers in the Buenos Aires province of Argentina's regions of Luján and Mercedes provided samples of their ganano. Seven farms, three manual and four automatic, were selected among a total of seventeen farms that comprise both districts because of their proximity to the National University of Luján, located in Luján, Buenos Aires, Argentina (Choudhary, Midha, Gulati, & Baranwal, 2024).

Seven samples were gathered, one for each farm, since all farms had fewer than five sheds, as SENASA Resolution 86/2016 required. Sampling started in the first row of cages in manual houses with three rows; this was two meters from the beginning of the cone, four meters from the second, and six meters from the third. It was taken for sample in a zigzag fashion using a sanitized jigsaw. The guano evacuation belts in the automated sheds were turned on, and material samples were collected every ten minutes (Barrow, 2024; Sang, Ren, & Yao, 2024).

BACTERIAL STRAINS:

The SG INTA90 strain, kindly provided by Dr. Horacio Terzolo of the Balcarce Agricultural Experimental Station of the National Institute of Agricultural Technology, INTA-EEA Balcarce, Argentina, and the SE Inc strain, which was isolated from a hatchery and antigenically characterized by the Malbrán Institute, served as the host cells used for phage isolation. To find out the viral preciseness of the isolated phages, strains of SG 88 (isolated coming from a hen sick with typhus and offered by Dr. Ricardo Anselmo of the National University of Luján) were employed (Alves et al., 2024; Kong et al., 2024).

SE 9 along with SE 15 (isolated from the Luján River and contributed by Dr. Ricardo Anselmo of the National University of Luján); SE INTA Nal R 12 (protocol 285/94 receptive to nalidixic acid, offered by Dr. Horacio Terzolo, of INTA-EEA Balcarce, Argentina); SE int (reisolated from the intestinal tract after four different sections in birds); SE high (reisolated from liver after four passages in poultry); Pseudomonas spp 1 (from the General Microbiology subject strain gathering, The department of Basic Sciences, National University of Luján); E. coli (from INTA-EEA Balcarce, Argentina) (da Costa, Carciofi, de Aragão, & Ienczak, 2024; Han et al., 2024).

DETERMINATION OF SALMONELLA SPECIES:

IDENTIFYING SALMONELLA SPECIES:

To identify Salmonella spp., 225 ml of Britania peptone water was mixed with 25 g of guano, and the mixture was incubated for 24 ± 2 hours at 37°C. After that, 0.1 ml of the mixture was added to 10 ml of Britania Rapapport Vassiliadis broth, and the mixture was incubated for 24 ± 1 hour at 42°C. After that, one batch of enrichment broth was separated by depletion using an XLD agar plate (Oxoid). Biochemical assays, including three sugar and iron agar (TSI) (Oxoid), lysine and iron agar (LIA) (Britania), sulfur-indole mobility medium (SIM), Indole (Britania), Methyl red, Voges - Proskauer (Oxoid), and Citrate (Britania) (I.M.Vi.C.), were used to confirm colonies positive for Salmonella. A polyvalent somatic sera test (OS-A and OS-B) was conducted employing these sera for serological type (Araújo et al., 2024; Dunislawska, Pietrzak, Bełdowska, & Siwek, 2024).

ENTEROBACTERIACEAE QUANTIFICATION:

CALCULATING ENTEROBACTERIACEAE QUANTITATION:

Using the most probable number (MPN) technique, the following media were used to determine the levels of total coliforms, faecal coliforms, and E. coli: lauryl sulfate tryptose a broth (Oxoid), lactose-brilliant green-bile broth (Oxoid), methylene blue eosin agar (Britania), and nutrient agar (Britania). The acquired data were presented as NMP/gram (NMP/g). The method's maximal detection limit was more significant than 1,100 NMP/g (Leisner & Larsen, 2024).

LYTIC PHAGE ISOLATION AND DETECTION:

LITHIC PHAGE ISOLATION AND DETECTION:

To isolate phages, 5 g of guano disappeared in 10 ml of nutritional broth (Britania). To isolate SG or SE phages, 500 µl of the host strain 108 colony-producing units/millilitre (CFU/ml) was added and cultured for 24 hours at 37°C. After centrifuging the supernatant for ten minutes at 3,500 rpm, the process was repeated until no longer precipitate was visible. The centrifuge volume was acquired by stirring, and an equivalent amount of chloroform was added. Spot testing was used to confirm the presence of the lytic phages SE and SG. The procedure to identify the presence of bacteriophages by spot test involved mixing 0.5 ml of host bacteria (SE or SG) with 5 ml of 0.8%

(m/v) nutrient agar (Britania) that had been dissolved and tempered at 50 ± 2°C (Chen et al., 2024; Lupia et al., 2024).

The combination was then put into a Petri dish with nutrient agar (Britania). After the phage suspension solidified, a grid was created at the Petri dish's base, and one drop (10 µl) of each phage suspension isolated from each farm was deposited in each square, along with the label indicating the guano sample's origin. After allowing it to dry in a laminar flow, the plates were placed in incubators for twenty-four hours at 37°C. If there was evidence of lysis where a decrease had been planted, it indicated the presence of phages. The bilayer approach was used to select individual plates and purify them three steps out of the diluted faecal samples that tested positive for lytic phages. Different bacterial strains were examined and plated on nutritional agar (Britania) to ascertain the viral specificity of the isolated phages (Sarrami et al., 2023; Thu et al., 2023).

After incubating the plates for 48 hours at 37°C, it was observed if whole or partial lysis occurred. To accomplish this, a nutritional agar plate (Britania) was divided into four sections with two lines drawn on its bottom using a permanent marker. A circle was created in the core of each of the four sectors. A sterile loop was streaked on the bacterial strain in the industry enclosed by the indicated circle. Using a micropipette, one drop (10 µl) of each phage isolate was applied to each sector inside the circle-delimited area. After letting it dry in a laminar flow, the plate was incubated at 37°C for 24 hours, after which a first reading and a second check were taken. After 48 hours, total lysis was considered (Lublin & Farnoushi, 2023; Montoro-Dasi, Lorenzo-Rebenaque, Marco-Fuertes, Vega, & Marin, 2023).

RESULTS:

QUANTIFICATION OF ENTEROBACTERIACEAE AND IDENTIFICATION OF SALMONELLA SPECIES:

One of the automated farms had motile Salmonella found in any of the examined guano specimens. It was concluded by the use of standard biochemical procedures and agglutination tests that the recovered bacteria were Salmonella spp. They were responding to the OS-B serum, ruling out the possibility that they were Enteritidis, Typhimurium, or Heidelberg serotypes. The findings of the seven examined samples showed that more than 1,100 enteric bacteria were present. The results reported for enterobacteria are shown in Table 1 (Additives et al., 2023; Brenner, 2023).

***Table 1**. Quantitation of enteric bacteria (NMP/g) in guano samples from laying hens*

NMP/g			
Farms	**Fecal Coliforms**	**Total Coliforms**	**E.coli**
(A) Manual	210	>1,100	9.1
(B) Manual	>1,100	>1,100	9.1
(C) Automatic	>1,100	>1,100	7.3
(D) Automatic	>1,100	>1,100	44
(E) Manual	28	>1,100	11
(F) Automatic	>1,100	>1,100	210
(G) Automatic	>1,100	>1,100	7.3

ISOLATION AND DETECTION OF LYTIC PHAGES

They were using SG and SE as host cells, which allowed for the isolation of phages in six of the seven investigated farms. Seven lytic phages had been separated in the guano samples from the automatic sheds, a few of which generated full or partial lysis in SE or SG strains in the spot test; four bacteriophages were isolated with SE and three with SG. Two lytic phages were isolated from the hand sheds using SE as the host cell and two from the automatic sheds using SG. This yielded four phages that generated partial lysis of SE or SG in the spot test. In the spot assay, only two phages isolated from two distinct auto breeders and their host cell was SE exhibited total lysis plaques (Pereira et al., 2023).

After being purified, the two bacteriophages that completely lysed the SE strain faced difficulties with Salmonella strains that originated in the environment, in birds, in one of the automated farms

that were sampled, as well as a Pseudomonas and an E. Col strain. Table 2 provides a breakdown of the 48-hour plate readings. Both phages were found to be able to fully lyse the SG as well as SE strains that were isolated from birds while partially separated from the environment, in addition to totally lysing the SE strain that was used to isolate them (Inbaraj, Agrawal, Thomas, Chaudhuri, & Chaturvedi, 2023).

Table 2. *Lytic activity of phages A and B against various bacterial strains.*

Bacterial Strain	**Source and Location Of Isolation**	**Phage A From Automatic Farm F**	**Automatic Farm Phage B G**
SG INTA90[a]	Balcarce, Argentina	+	+
SE 9	Lujan River	-	Q
SE in	Intestine	+	-
Salmonella spp A[b]	Stool	-	-
Pseudomonas spp. 1	Farmland	-	-
SG 88	Stool	+	+
SE Inc[a]	Hatchery	+	+
SE 15	Lujan River	Q	Q
SE high	Liver	+	Q
E. coli INTA	Balcarce, Argentina	-	-
INTA Nal R12	Balcarce, Argentina	-	-

DISCUSSION:

This investigation aimed to ascertain whether the existence of bacteriophages that lyse SG and SE in guano suggests that these pathogenic bacteria may also be present concurrently. Only one of the seven guano samples examined by standard microbiological methods revealed the existence of Salmonella spp. Serology proved that Salmonella reacted to the OS-B serum and was not one of the serotypes Gallinarum or Enteritidis. The inherent resistance which birds possess against enteric pathogens as their immune systems and gut microbiota grow could account for the lower isolation of Enterobacteriaceae (Farhat et al., 2023).

Similarly, a healthy diet combined with thorough cleaning and disinfecting of the surroundings may help lower the chance of bird contamination. Because of the challenges some authors have had identifying Salmonella in guano, environmental sampling is done in addition to animal faeces examination. When the total coliform count using the NMP technique is more significant than 1,100 NMP/g, a wide range of Enterobacteriaceae, not just Salmonella, coexist in the guano samples. For this reason, it could be more practical to use additional, more targeted techniques, like the PCR (polymerase chain reaction) method, for the detection of Salmonella spp. in guano in bird production systems layers (Shaji, Selvaraj, & Shanmugasundaram, 2023).

However, concerning the phages found in chicken guano, it was feasible to identify lytic Salmonella bacteriophages of the Enteritidis and Gallinarum serotypes from six out of the seven farms in which the guano was collected. As a disease that inhabits animals' gastrointestinal systems, Salmonella can be isolated from phages that infect this bacteria by searching the animal's intestines or excrement. The existence of these phages in guano should receive special attention as it may be a sign that their target cells are still present in the surrounding environment. Nevertheless, these six farms tested negative for Salmonella spp. But positive for bacteriophages (Chacón et al., 2023).

Similar findings have been reported by some authors in their research conducted in other environments (pig and cattle farms), emphasizing that, despite the small number of samples collected making generalizations impossible, the existence of Salmonella phages indicates that all of these environments have made contact with Salmonella at a particular time, even though the host cell may currently be negative. We were unable to conduct a statistical analysis due to the small number of guano samples (7 samples overall); therefore, we were unable to conclude that Salmonella spp. are still present in the environment, but they did show that they were present at some point (Asrore et al., 2023).

Because bacteriophages are strict intracellular parasites that require bacterial infection to reproduce, it is essential to remember that both have population dynamics that the predator-prey model characterizes. Bacteria may exhibit various defence mechanisms against the phage, which may lead to new variants that can get past the barriers the bacteria present and optimize their infection cycle. Consequently, if a sample tests negative for host bacteria but positive for phages, this could suggest that phages regulate the amount of Salmonella in the environment (Raut, Maharjan, & Fouladkhah, 2023).

It is well known that Salmonella can last extended periods in the surroundings and can adjust to specific conditions by, for instance, forming biofilms. Given their proximity and shared environment, the bacteriophages that invade the target cell must also be present for the phage to survive. Similarly, as noted before, it is essential to remember that washing and sterilization do not ensure the complete eradication of microorganisms. The presence of bacteriophages that lyse SG and SE in guano might be a test that would supplement the isolation as well as observing of Salmonella in birds and allow for more extraordinary authority strategies and pathogen prevention, which are crucial steps to prevent the risk of contamination of by-products derived from poultry production, even though the study only examined a small number of guano samples (Martins, Contreras, Furian, Borges, & do Nascimento, 2023).

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