



Enterococcus faecium filtrate suppresses *Salmonella* Heidelberg in broilers

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ABSTRACT: The objective was to investigate the ability of a filtrate produced by the lactic acid bacterium *Enterococcus* sp. to control *Salmonella* Heidelberg *in vivo*. We determined that *Enterococcus* sp. had an antagonistic effect on *Salmonella* Heidelberg, based on *in vitro* testing (spot-on-the-lawn method). For *in vivo* studies, broiler chickens (n = 32), 15 d old, were allocated into 4 groups. Filtrate was given prophylactically once daily orally at 15-19 d of age to 8 birds, followed by an *S. Heidelberg* challenge to 16 birds at 20 d; 5 of 8 and 3 of 8 birds in treated and control groups, respectively (P = 0.32), had < 10 CFU/g of *S. Heidelberg* in their cecum at 21 d. In a therapeutic study with 16 birds challenged with *S. Heidelberg* at 15 d, we gave to 8 birds oral therapeutic filtrate once a day at 16 to 20 d had 5250 cfu/g of the pathogen in the cecum versus 912,950 cfu/g in the control, a 174-fold reduction. We concluded that filtrate has potential as a non-antibiotic approach to control *S. Heidelberg in vivo*.

Key words: avian disease, microbiology, animal production.

O filtrado de *Enterococcus* sp. suprime a *Salmonella* Heidelberg em frangos de corte

RESUMO: O objetivo foi investigar a produção de filtrado pela bactéria de ácido láctico *Enterococcus* sp. e sua capacidade de controlar a *Salmonella* Heidelberg *in vivo*. As bactérias foram submetidas à coloração de Gram e à reação em cadeia da polimerase (PCR) para confirmar a identificação do gênero. O *Enterococcus* sp. teve um efeito antagônico sobre a *Salmonella* Heidelberg com base em testes *in vitro* (método antagonismo de placas). Frangos de corte jovens (n = 32), com 15 dias de idade, foram alocados em quatro grupos para testar os efeitos do filtrado *in vivo*. Quando o filtrado foi administrado profilaticamente uma vez ao dia por via oral aos 15-19 dias de idade a oito aves, seguido por um desafio de *Salmonella* Heidelberg administrado a 16 aves aos 20 dias, cinco de oito e três de oito aves nos grupos tratados e de controle, respectivamente, tinham < 10 UFC/g de *S. Heidelberg* no ceco aos 21 dias de idade. Além disso, em um estudo terapêutico com 16 aves desafiadas com *S. Heidelberg* aos 15 dias, oito aves que receberam terapêuticamente o filtrado uma vez ao dia por via oral dos 16 aos 20 dias de idade apresentaram 5250 ufc/g do patógeno no ceco contra 912.950 ufc/g no controle, uma redução de 174 vezes. Este estudo demonstrou os efeitos protetores do filtrado no controle da *Salmonella* Heidelberg *in vivo*, com potencial como abordagem não antibiótica para o manejo dessa importante doença.

Palavras-chave: doença aviária, microbiologia, produção animal.

Brazil is the world's second largest producer and largest exporter of broiler meat (ABPA, 2022). Poultry are the basis of various products and poultry production and processing provide substantial jobs and revenue to the Brazilian economy, with broiler meat being a key component (VOGADO et al., 2016).

Controlling diseases and infectious agents is an ongoing challenge in poultry production. For example, *Salmonella* Heidelberg is frequently present in digestive and reproductive tracts of broilers, leading to egg contamination (BABU et al., 2016;

SIVARAMALINGAM et al., 2013). Controlling *Salmonella* spp. has become more complicated due to the European Union (EU) prohibiting antibiotic use. Antibiotics were historically used to treat pathogens and increase poultry productivity and this ban has adversely affected poultry health and performance (KURITZA et al., 2014).

Various plant extracts, probiotics, prebiotics, and combinations have been used to control *Salmonella* spp. in poultry. Lactic acid bacteria are common probiotics; they are gram-positive, non-

sporulating, anaerobic cocci that produce lactic acid. These bacteria have been increasingly studied in various contexts, including controlling *Salmonella* spp. in poultry (KURITZA et al., 2014).

Probiotic bacteria can establish bacterial communication, also known as *quorum sensing*, influence bacterial behavior, alter bacterial environments (MORNINGSTAR-SHAW et al., 2016), and produce virulence factors, biofilm, siderophores, and postbiotics (LOURENÇO & BERCHIERI, 2015). Filtrate production by *Enterococcus* has been reported (MORNINGSTAR-SHAW et al., 2016; LOURENÇO & BERCHIERI, 2015). The objective was to investigate the ability of filtrate produced by the lactic acid bacterium *Enterococcus* sp. to control *Salmonella* Heidelberg *in vivo*. We predicted that the filtrate will modulate the bird's microbiota and through bacterial communication, control *Salmonella* Heidelberg.

The *S. Heidelberg* used had resistance to Rif and nalidixic acid (Nal) and was obtained from livers of broiler breeders. The isolate was conditioned in Brain Heart Infusion media supplemented with 30% glycerol and stored at -80 °C. This strain, and the probiotic *Enterococcus* sp., were part of the Avian Pathology Service's Bacteria Collection at Universidade Estadual de São Paulo (UNESP), Botucatu, São Paulo (SP), Brazil.

The sample was collected from cloacal swabs of healthy broiler chickens and the gender was identified prior to being added to the laboratory sample bank. Ten cloacal swabs were collected from each bird and conditioned in Stuart's medium (Merck, Darmstadt, Germany) until sample processing. Swabs were spread on De Man, Rogosa, and Sharpe agar (MRS) (Kasvi, Campinas, Brazil), incubated at 38 °C for 24 h, and single colonies were Gram stained, with genus confirmation using PCR. The DNA was extracted with a commercial kit (QIAamp® DNA Stool, Qiagen, Hilden, Germany) and amplified using the following primers: forward, 5'-AAG CAACGC GAA GAACCTTA- 3' and reverse, 5'-GTC TCG CTA GAG TGC CCA AC- 3', resulting in a 194 bp product (ALLEGRETTI et al., 2014). The final products of the PCR were stained, observed in an ultraviolet light transilluminator, and subjected to 1.4% agarose gel electrophoresis (LGC Biotecnologia, São Paulo, SP, Brazil). The *Enterococcus* species was identified through sequencing as *Enterococcus faecium*.

Enterococcus sp. were subjected to a plate inhibition test, Spot on the Lawn, enabling measurement of inhibition towards *S. Heidelberg*. Inhibition halos formed before and after filtrates were measured and a Wilcoxon test was used to compare medians (1° and 3° quartiles), indicating *Enterococcus* sp. inhibited *S. Heidelberg*.

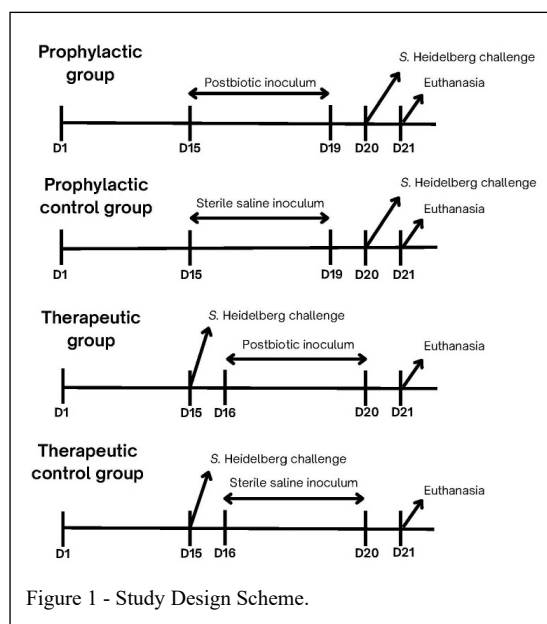
To produce sensitized filtrate, 5 colony-forming units (CFU) of *Enterococcus* sp. and 5 CFU of *Salmonella* Heidelberg were inoculated in 10 mL of sterile media with ½ De Man, Rogosa, and Sharpe (MRS; Acumedia, Neogen, Lansing, MI) broth and ½ brain heart infusion (BHI; Acumedia, Neogen, Lansing, MI), followed by incubation in a Shaker incubator (SP-LABOR 222®, Sao Paulo, SP, Brazil) for 24 h at 38 °C.

A second inoculum was seeded with 1 mL of this mixture in 5 mL of MRS to eliminate *S. Heidelberg*. Flasks were incubated under the same conditions to eliminate *Salmonella* sp. metabolites and isolate lactic acid bacteria sensitized to pathogenic bacteria. Lastly, sensitized *Enterococcus* sp. was centrifuged (10,000 x g, 10 min 4 °C) and the supernatant passed through a 0.22 µm nitrocellulose membrane filter to produce sterile bacteria-free filtrate. Bacteria were quantified using a McFarland nephelometric scale (RIBEIRO et al., 2023), using serial counting plates up to a 10⁻⁸ dilution for all inocula prior to use in dishes containing nutrient agar, to verify no contaminants.

To determine the ability of the filtrate to inhibit *S. Heidelberg* growth *in vivo*, 15 day-old chickens were randomly allocated into 4 groups of 8 birds in wire cages. They were housed in climate-controlled rooms with *ad libitum* access to water and feed (Figure 1).

The prophylactic group received 1 mL of bacteria-free filtrate treatment intraesophageal once daily at 15 to 19 d of age. At 20 d of age, these birds were challenged with resistant *S. Heidelberg*, namely 2 CFU seeded in BHI media with nalidixic acid and rifampicin (Nal/RIF) incubated at 38 °C for 24 h, with 1 mL (6.0 X 10⁹ CFU/mL) given intraesophageally. For the therapeutic group, the same challenge was done at 15 d of age in 8 birds, followed by 1 mL of filtrate daily from 16 to 20 d of age. Prophylactic and therapeutic controls received no treatment and were challenged with resistant *S. Heidelberg* at 20 and 15 d of age, respectively. The *S. Heidelberg* inoculum used in the challenge was quantified using the McFarland nephelometric scale. Confirmation was made through serial decimal counting plates up to a 10⁻⁸ dilution for all inoculums, and they were administered intraesophageally to birds in a single, 1 mL dose (6.0 X 10⁹ CFU/mL).

At 21 d of age, all birds were euthanized by cervical dislocation. Cecae were harvested aseptically and stored at 4 °C pending processing. Each cecum was weighed, macerated and diluted (1:10) with sterile PBS to obtain the first dilution (10⁻¹). Thereafter, 7



serial decimal dilutions were performed (up to 10^{-8}). All dilutions were seeded on duplicate Petri dishes with brilliant green agar containing Nal/RIF and incubated at 38 °C for 24 h, colonies counted, and cfu/g of cecum was calculated. Proportions were compared by Chi square. Furthermore, descriptive analysis was used to make informal comparisons between groups.

The prophylactic group had a higher ($P = 0.32$) percentage (62.5%) of negative (< 10 CFU/g in the cecum), birds than the controls (37.5%) and these 2 groups had ~ 633 and 1040 cfu/g, respectively of the pathogen in the cecum. In the therapeutic study, treated and control birds had 5250 versus 912,950 cfu/g of the pathogen in the cecum, a 174-fold reduction. This decrease was considered relevant, as it reduces the potential for carcass contamination in broiler meat intended for human consumption.

In a previous study, *Lactobacillus helveticus* ATCC 15009-derived postbiotic was just as effective as probiotic administration in reducing *Salmonella* Gallinarum in the cecum and liver of chicks (RIBEIRO et al., 2023). Additionally, *Lactiplantibacillus plantarum* postbiotics inhibited *Salmonella* infection in mice via bacterial pathogenicity modulation, autophagy, and inflammation (HU et al., 2023). The use of a variety of products to reduce *Salmonella* contamination is increasing with the growth of the poultry industry, plus a desire to identify alternatives to antimicrobials. The filtrate studied has potential as an innovative biotechnology for reducing *Salmonella* in avian products, as it is a natural product that leaves

no residue and does not induce bacterial resistance, as is common with antibiotics. It is noteworthy that the filtrate does not act on a single bacterium, but rather on the bird's entire microbiota, modulating it against pathogens (HU et al., 2023).

This was apparently the first trial to use filtrate in broilers. Prophylactic treatment with filtrate had some preventive action against *S. Heidelberg*, profoundly reducing the presence of this pathogen in the cecum and decreasing the proportion of positive birds. The current study provided preliminary evidence for filtrate as a non-antibiotic approach to control *S. Heidelberg in vivo*. This has potential as a highly innovative biotechnology, but substantial further studies are necessary to standardize filtrate extraction, production, conservation, and dose for use in avian species in a variety of production systems and conditions.

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DECLARATION OF CONFLICT OF INTEREST

Authors declare they have no conflicts of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the conception and writing of the manuscript. All authors critically reviewed the manuscript and approved the final version.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

This study was approved by the Ethics Committee on Using Animals in Research of Universidade Estadual de São Paulo (#0032/2021).

REFERENCES

- ASSOCIAÇÃO BRASILEIRA DE PROTEÍNA ANIMAL (ABPA). **Relatório Anual**, 2022. Available from: <<https://abpa-br.org/mercados/>>. Accessed: Jun. 06 2024.
- BABU, U. S. et al. Differential antibacterial response of chicken granulosa cells to invasion by *Salmonella* serovars. **Poultry Science**, v.95, p.1370-1379, 2016. Available from: <<https://doi.org/10.1038/s41575-020-0344-2>>. Accessed: Jun, 06 2024.
- HU, A. et al. Lactiplantibacillus plantarum Postbiotics Suppress *Salmonella* Infection via Modulating Bacterial Pathogenicity, Autophagy and Inflammasome in Mice. **Animals**, v.13, p.3215, 2023. Available from: <<https://www.mdpi.com/2076-2615/13/20/3215>>. Accessed: Jun, 06 2024. doi: 10.3390/ani13203215.
- LOURENÇO, P.; BERCHIERI, Â. **Salmonella Gallinarum As pesquisas, achados e caminhos apontados por grupo de pesquisadores**, 2015. Available from: <<https://www.avisite.com.br/EncarteEspecialSG/>>. Accessed: Jun, 06 2024.
- MORNINGSTAR-SHAW, B. R. et al. **Salmonella Serotypes Isolated from Animals and Related Sources**. 2016. Available from: <<https://www.cdc.gov/national-surveillance/pdfs/salmonella-serotypes-isolated-animals-and-related-sources-508.pdf>>. Accessed: Jun, 06 2024.
- KURITZA, L. N. et al. Probióticos na avicultura. **Ciência Rural**, v.44, n.8, p.1457-1465, 2014. Available from: <<https://www.scielo.br/j/cr/a/MJGzcpy6rWDHk5bywQ88JNJ/?lang=pt>>. Accessed: Jun, 06 2024. doi: 10.1590/0103-8478cr20120220
- RIBEIRO, G. C. et al. Research Note: The effects of a *Lactobacillus helveticus* ATCC 15009-derived postbiotic mitigating *Salmonella* Gallinarum colonization in commercial layer chicks. **Poultry Science**, v.102, n.12, p.103095, 2023. Available from: <<https://www.sciencedirect.com/science/article/pii/S0032579123006144?via%3Dihub>>. Accessed: Jun, 06 2024. doi: 10.1016/j.psj.2023.103095.
- SIVARAMALINGAM, T. et al. A temporal study of *Salmonella* serovars from environmental samples from poultry breeder flocks in Ontario between 1998 and 2008. **Canadian Journal of Veterinary Research**, v.77, n.1, p.1-11, 2013. Available from: <<https://pubmed.ncbi.nlm.nih.gov/23814351/>>. Accessed: Jun, 06 2024.
- VOGADO, G. M. S. et al. Evolução da Avicultura Brasileira. **Nucleus Animalium**, v.8, n.1, p.3, 2016.