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Heat Stress and Cultivable Intestinal Bacteria of Lehmann Brown Hens

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ABSTRACT

The use of genetic methods seems to have briefly pushed back the use of culture methods. Currently, a new technique developed based on the latter – culturomics, enables the discovery and study of expanded bacterial diversity using a large number of culture conditions and media. This study aims to investigate the influence of heat shock on the qualitative and quantitative content of cultivable gut bacteria of Lehmann Brown chickens from the “Arax” chicken factory. The chickens (5 months old, weighing 1.4-1.7 kg) of the “Arax” poultry factory in Armenia were transferred to a sterilized test room with the necessary conditions. After four days of maintenance in a new environment (temperature 25 °C), fecal samples were taken from the hens. After that, the hens were kept at 35 °C for a day, followed by another sampling conducted a day later. The current investigations have emphasized that the bacterial number and diversity of hen gut microbiota undergo changes under the influence of heat stress.

Introduction

In the 19th century, the discovery of culture media stimulated the cultivation of microbiology (Wainwright and Lederberg, 1992). Particularly, this method was the first one to allow the assessment of microbiota structures of humans and animals (Lagier, et al. 2015, 2018). However, the utilization of genetic methods afterward inhibited the use of cultural media for a while (Gramberg, et al., 2023). Nevertheless, the development

of culturomics as a new technique allows to rediscover and investigate the expanding diversity of bacteria mainly due to the method of cultural media, (Abou Chacra, et al., 2024; Wan, 2023; Phelps, et al., 2023; Bonnet, et al., 2019; Lagier, et al., 2018) which is also widely implementing the capacities of microscopy (Ahmad, et al., 2023; Lim, et al., 2019; Kim, et al., 2023; Mulaw, et al., 2019; Hovnanyan, et al., 2015; Ghosh, et al., 2023). This technique, indeed, is competent

with metagenomics (Lian, et al., 2023; Huang, et al., 2023). Illnesses (Pepoyan, et al., 2015a, b) and stressful conditions (Ma, et al., 2023; Tan, et al., 2023) can influence the structure of human and animal (Shevchenko, et al., 2023; Mirzabekyan, et al., 2023; Tanelian, et al., 2023) gut microbiota. It was also illustrated that, for example, there exists a correlation between the qualities of *Escherichia* spp of physiologically robust and ailing individuals' gut microbiota (Shahinyan, et al., 2003; Stepanyan, et al., 2007). In parallel with this investigation, for animals, a correlation was indicated between blood protein and *E. coli* of their gut microbiota (Pepoyan, et al., 2020).

The poultry industry has improved genetics, nutrition, and management practices, resulting in fast-growing chickens; which, however, may have a stressful outcome for the birds. (Kpodo, et al., 2023; Campos, et al., 2023; Stefanetti, et al., 2023). On the other hand, high environmental temperatures are also able to alter poultry health and performance by causing heat stress. Heat stress elicits physiological, behavioral, and production changes in poultry (Wasti, et al., 2020; Garriga, 2006). Reduction in feed intake in heat-stress animals is an adaptive mechanism to minimize metabolic heat production. A significant decrease in feed intake, body weight gain, and feed efficiency has been reported in many studies conducted on birds and other animals (Nawaz, 2021). The birds' gut microbiota is changing, too (Brugaletta, et al., 2023). Considering the latter, the investigation of cultivated bacteria extracted from the gut microbiota of hens exposed to heat stress has served as the aim of the current study.

Materials and methods

The chickens (30 Lehmann Brown hens, 5 months old, weighing 1.4-1.7 kg) of the "Arax" poultry factory of Armenia were transferred to a sterilized test room with the necessary conditions, where they were numbered. Chickens were fed with feed layer TOTAL POL 21 (Arndane Ltd, Armenia) made with a Dutch technique-Nuscience (<https://www.nuscience.eu>).

After 4 days of adaptation to the new conditions, fecal samples were taken of hens held at a temperature of 22-25 °C. Afterward, the birds were exposed to 35-38 °C heat stress. Then, these fecal samples were collected in respective test tubes and were immediately transferred to a microbiological laboratory for further analysis.

To investigate the effect of heat stress on the qualitative content of gut bacteria, the dilution of the fecal material has been organized according to Pepoyan and co-

authors (Pepoyan, et al., 2023; Balayan, et al., 2019). For examining the bacterial contents ENDO (Thermo Scientific Oxoid, USA) (24 hours (h); 37 °C), De Man, Rogosa and Sharpe (MRS) (48 h; 37 °C) and Egg yolk salt (EYS) (prepared on the base of Nutrient agar Himedia (HiMedia Laboratories Pvt. Ltd, India) (48 h; 37 °C) agars were used, and the dominant cultivable isolates (isolates from the most diluted samples) were investigated.

The isolates grown on agars were identified and counted according to Pepoyan and co-authors (Pepoyan, et al., 2023) for *Escherichia coli*. During the preliminary bacterial identification by morphology B-150 optical microscope (B-150 OPTIKA, OPTIKA S.r.l., Italy) has been used, too. The statistical analysis (t-test) was systemized through Excel 2016 and were confirmed by the Mann-Whitney test. The value $p < 0.05$ was judged as significant.

Results and discussions

Bacterial growth on ENDO, MRS, and EYS agars

The studies emphasized that exposure to heat shock does not result in a quantitative disparity in the contents of cultivatable commensal *E. coli* and lactobacilli. In comparison, qualitative and quantitative differences were observed in the bacterial populations growing on EYS agar. Given the high concentration of sodium chloride (approximately 7.5 %) in EYS agar, it facilitates the growth of cocci and bacilli tolerant to sodium chloride in this medium (Ayaz, et al., 2022). On the other hand, the content of egg yolk (lecithin) in the EYS medium indicates the possible differentiation of bacteria with lecithinase activity within that medium (<https://pr.vwr.com>). Lecithinase is a phospholipase which affects lecithin. The presence of lecithinase in bacteria is important due to this ferment's potential role in the:

- pathogenicity of bacteria (Ghannoum, et al., 2000)
- identification of *Clostridium perfringens*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* or *Listeria monocytogenes*
- detection of coagulase-positive *S. aureus*

It can also be used for differentiating some varieties of *Bacillus*.

According to the results, in control samples of EYS agar, bacterial colonies were noticed with up to 10⁻⁶ – 10⁻⁷ dilutions (Photo 1). They were primarily cocci, particularly ovoid diplococci (Figure 1A). Bacilli with different morphologies were present (Figure 2), with some of them also having lecithinase activity.

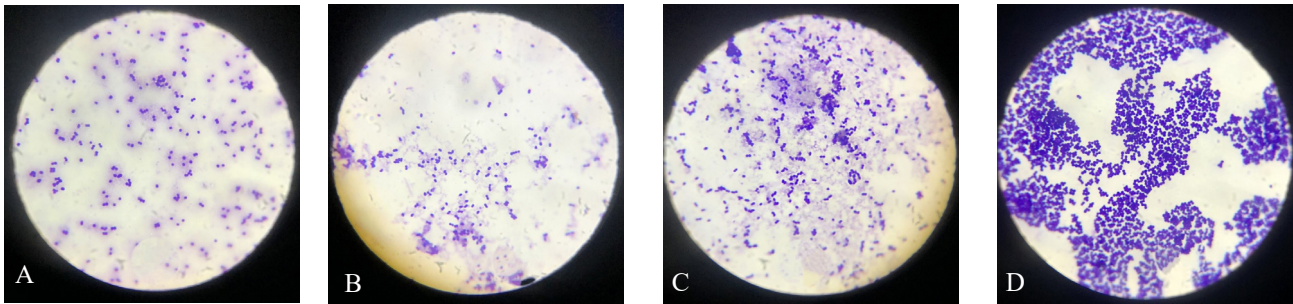


Photo 1. 1000x magnification of bacteria extracted from hens' microbiota (grown on EYS agar). After heat stress, *Staphylococcus spp.* was detected in one of the chickens (not *Staphylococcus aureus*, there is no lecithinase activity (D)).

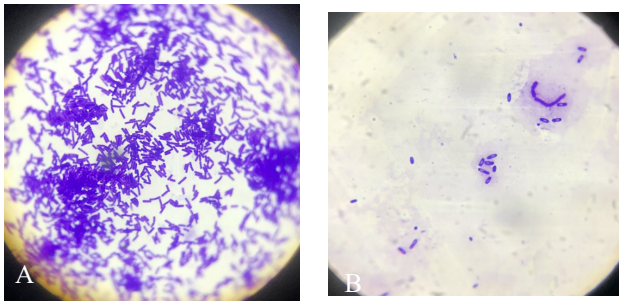


Photo 2. A-*Bacillus subtilis*., magnification 1000x; B-*Bacillus cereus*, magnification 2000x.

In only 1-2 control samples obtained from hens, sporulating bacilli with lecithinase activity were identified. Lecithinase activity is a sign of pathogenicity and is relevant to *Bacillus cereus* and to *Bacillus anthracis*. It is supposed that *Bacillus cereus* was indicated in chickens (Photo 2 B). Scummed, yellowish colonies with mucoid texture that lack lecithinase activity are supposedly other sporulating bacilli, for instance, grass bacillus - *Bacillus subtilis* (Photo 2 A).

Bacterial growth on ENDO, MRS, and EYS agars after heat stress.

The number of bacteria growing on EYS agar decreased until 10^{-3} - 10^{-4} , and in certain instances, the bacterial count from specific hens reached as low as 10^{-1} . According to the literature, in chickens, streptococci and enterobacteria are the first ones to colonize gut microflora within the first few hours of their lives, then completely spread there during 24 hours. On the third day, lactobacilli, streptococci, enterococci, and coliforms are produced in different parts of the gastrointestinal tract (Coloe, et al., 1984).

The studies demonstrated that the decrease in the number of bacteria in birds exposed to heat stress is linked with

a predominance of enterococci, which is similar to the results in the investigated literature (Giraffa, 2022). Among enterococci, *Enterococcus faecalis* showcases quantitative dominance (30-45 %).

Enterococci are gram-positive, non-sporulative, catalase-negative (some strains demonstrate pseudo catalase activity), oxidase-negative, and lactic acid bacteria. Nearly all representatives of enterococci are non-motile and facultative anaerobes. They hydrolyze esculin and L-pyroglutamic acid b-naphthylamide, as well as are able to grow in a medium containing 4 % bile acid, the utilization of which serves as a means of differentiation. When observed under the microscope, enterococci appear as single cells or form pairs or chains. Their metabolism is fermentative, and the primary outcome of glycolysis is the production of L (+) lactic acid. Nowadays, out of 50 varieties identified, the most "popular" in dairy products are *Enterococcus faecalis* and *Enterococcus faecium*. These two strains are quite widespread and are typically resistant to unfavorable external conditions. They grow within a temperature range of 10-45 °C, in the presence of 6.5% NaCl, and at a pH level of 9.6. They are even able to survive at 60 °C after being exposed to heat for 30 minutes, categorizing them as non-sporulating, heat-resistant bacteria. The strains are usually detected in raw or heat-processed food, for instance, in raw or pasteurized milk, cheese, and other dairy products.

Stress (not only limited to heat) brings up various metabolic changes in the organism and affects the composition of gut microbiota. Typically, enterococci exhibit the highest titers, attributed to their exceptional adaptability (Mónica García-Solache, 2019, Oejo et al., 2024; Repoila et al., 2022; Xu et al., 2024). Sporulative bacilli are also able to grow in the medium of EYS agar with a high concentration of sodium chloride. Colonies and bacilli with different morphologies have been discovered. Considering

the widespread utilization of probiotics in combating pathogens across humans (Pepoyan, et al., 2020, 2021; Sajankila et al., 2023; Beghetti et al., 2021; Bodke, et al., 2022; Balayan, et al., 2015; Latif, et al., 2023), animals (Merenstein, et al., 2023; Saha, et al., 2023; Marouf, et al., 2021; Mirzaei, et al., 2022; Pepoyan, et al., 2019), and even plants (Pepoyan, et al., 2020; Du et al., 2021; Khushboo, et al., 2023; de L Agüero et al., 2020; Harutyunyan, et al., 2022; Du, et al., 2021; Yun, et al., 2009), it is prudent to study the influence of various probiotics on the qualitative and quantitative composition of stress-resistant bacteria to avoid potential infections of organisms exposed to stress. Therefore, in hens' gut microbiota, quantitative and qualitative changes occur in response to heat stress, with a particular emphasis on changes related to enterococci.

Conclusion

Current investigations have illustrated that, under heat stress, the bacterial variety and number of gut microbiota of hens undergo changes. Specifically, a decrease in the number of enterococci has been observed under heat stress conditions. Nevertheless, it is vital to acknowledge that these investigations are preliminary and require further genetic confirmation.

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