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Differentiated *in vitro* Lysozyme Activity in Aves and Mammalia in Response to Seabuckthorn (*Hippophae rhamnoides*) Stimulation

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Abstract

Lysozyme, an intrinsic component of the immune system, is a naturally occurring enzyme with antimicrobial activity (Khorshidian et al., 2022), by hydrolyzing the muramyl dipeptide in the bacteria cell wall. Considered to be an endogenous antibiotic, it differs by species (Ferraboschi et al., 2021). Some of the medicinal plants were cited to inhibit the anti-lysozyme activity and biofilm formation by bacteria (Bukharin et al., 2003). The aim of this research was to evaluate the differences in the in vitro activity of lysozyme between the classes Aves and Mammalia when treated with a protein-carotenoid extract of *Hypopphae rhamnoides* compared to well-known immune modulating preparations (selenium or selenium and copper compounds). The investigations were carried out on serum samples from: a) commercial broiler chickens aged 34 days (n=19) and b) 5-month-old Supercunirom breed male rabbits (n = 19). The agar gel radial immune diffusion method and the Micrococcus lysodeicticus test strain were used to define the in vitro lysozyme activity. The sera were mixed with serial dilutions (1:2, 1:4, etc.) of the tested compounds. The groups were compared by Student's t test for statistical significance of the results. The increase in activity (%) versus control were calculated. Sea buckthorn extract significantly ($t= 7.22$, $p < 0.001$) decreased the in vitro activity of lysozyme at both dilutions used (1:2, 1:4). The concentration of serum lysozyme was higher in rabbits than in chickens and its lytic activity was enhanced by selenium and copper combinations in chickens (183.69 ± 37.91%) and less in rabbits (128.45 ± 84.10%) in a dose dependent manner. At lower dilutions (3:4), the lysozyme activity remained below that of the control treated with saline. The protein-carotenoid extract of sea buckthorn acted inhibiting on lysozyme activity, proving the need for tailored extraction and treatment protocols depending on the bacteria and host species.

Key Words: Aves, Mammalia, sea-buckthorn, lysozyme, innate immune response

1. Introduction

Lysozyme (N-acetyl-muramyl-hydrolase) is a basic mucopeptide of low molecular weight, which exhibits enzymatic activity directed against the structural peptidoglycan of the wall of Gram-positive bacteria, causing cell lysis. The amino acid sequence distinguishes the types of lysozyme, forming structures with different antigenicity. Lysozyme, an intrinsic component of the innate immune system, is a naturally occurring enzyme with

antimicrobial activity. This is expressed by hydrolyzing the glucosidic linkages in the muramyl-dipeptide of the bacteria cell wall, especially in Gram-positive but also, partially, in Gram-negative agents (Khorshidian et al., 2022, Morrison, 2021). Lysozyme was considered an endogenous antibiotic, its type and characteristics differing by species (Callewaert and Michiels, 2010, Ferraboschi et al., 2021).

Lysozyme is widely distributed, being present in high concentrations in hens' egg white, secretions and tissues (tears, saliva, blood serum, nasal mucosa, liver, spleen), in the primary and secondary granulations of the human neutrophils, monocytes, macrophages and some epithelial cells (Bîlbîie and Pozsgi,1987; Potapova et al., 1988; Rainer, 1984; Spinu and Degen, 1999). It intervenes in the oxygen independent phagocytosis, by "digesting" rather than by killing bacteria (Bîlbîie and Pozsgi, 1987; Rainer, 1984; Roitt, 1991; Spinu and Degen, 1999, Baron et al., 2016). The serum level of this mediator with an important role in non-specific immunity was correlated with the functioning state of the granulocytic system (Lollike et al., 1995). Thus, the evaluation of functional levels of the innate immune effectors could represent an objective expression of the reactivity against infectious diseases.

Lysozyme has numerous uses as a dietary supplement, potentially supporting changes in food protein functionalities (Li et al., 2023). As food safety is a subject of broad concern lately, the demand for more natural antimicrobials and less chemical preservatives to extend shelf life of food and beverages is continuously increasing (Juneja et al., 2012; Nawaz et al., 2022). Research was carried out to find such compounds in microorganisms, plants, and even animals. Lysozyme antibacterial properties supported the interest in its use as a preservative in food industry (Mani Lopez et al., 2016; Wu et al., 2019). It was envisaged as a useful agent in packaging (Syngai and Ahmed, 2019) and as a biofilm inhibitor in the food products (Gutiérrez, 2019). The effects of immune modulators, that are either stimulate or inhibit the immune response, are well known. They could be synthetic (sodium selenite, barium selenite) or natural compounds (various proteins, vegetal extracts) of different molecular weights. In vitro experiments facilitate the identification of their action sites and suggest optimal schedules of therapy.

Lysozyme has proven an anti-inflammatory activity in mice by suppressing the LPS-induced responses (Tagashira et al., 2018). Therapeutic enhancement of lysozyme activity in hosts subjected to nutritional, technological, parasitic or infectious stress and therefore immune deficient, could partly restore the "first line of defense", contributing to overcoming the disease (Ragland and Criss, 2017). Such an example is being represented by improved fish health subsequent to dietary control of lysozyme activity and further enhanced immune response (Carbone and Faggio, 2016).

Some of the medicinal plants were cited to inhibit the anti-lysozyme activity of bacteria and biofilm appearance (Bukharin et al., 2003). Polyphenols present in most medicinal and aromatic plants are well-known for their antimicrobial activity (Daglia, 2012; Cushnie and Lamb, 2005). Synergistic effects were noticed between lysozyme and rosmarinic acid most abundant in Lamiaceae (Azhar et al., 2023) and also gentisic acid (Abedi et al., 2020), as lysozyme–phenolics conjugates with antioxidant and antibacterial effect, by destruction of the bacterial cells walls (Li et al., 2023). Research indicated that co-encapsulation of lysozyme and different plant extracts increased the efficacy of the preparations against bacteria (Matouskova et al.,2016).

Hippophae rhamnoides L. (sea-buckthorn) is a deciduous shrubs belonging to order

Rosales, Family: Elaeagnaceae and Genus: *Hippophae* L. It is widespread, being present mainly in the cold to temperate regions of Europe and Asia and has 190 bio-active components, among which sugars, sugar alcohols, fatty acids, vitamins (C, E, and K), phenolic compounds, carotenoids, fiber, amino acids and minerals could be mentioned (Sharma and Kalkal, 2018). The berry extracts are known for their high flavonoid and polyphenol content therefore for their strong antioxidant and antibacterial (anti - S. aureus, B. cereus and P. aeruginosa) activity (Criste et al., 2020). The *in vivo* administration of the plant as feed additive increased the level of immune activity, including that of lysozyme in tilapia fish (Oreochromis niloticus)(Mogodan et al., 2020) and in chickens (Stef et al., 2009). To our knowledge, no other researches investigated the changes in the in vitro lysozyme activity when combined with a protein-carotenoid extract of *Hippophae rhamnoides* berries.

This work intended to monitor the in vitro effects of a protein-carotenoid extract of sea-buckthorn (*Hippophae rhamnoides*) on the in vitro serum lysozyme activity in Aves and Mammalia, in comparison with different microelements' (copper and/or selenium) preparations, which served as controls for their immune stimulating effects.

2. Material and Methods

2.1. Material

2.1.1. Biological material: The investigations were carried out on serum samples from: a) Rock x Cornish commercial crossbreed broiler chickens (n = 19, aged 34 days) and b) 5-month-old Supercunirom breed male rabbits (n = 19). Blood was sampled from the wing vein from the chickens and from the jugular vein in rabbits. The samples were allowed to clot at 37°C, then the sera were separated by centrifugation at 2000rpm/min (Hettich, EBA 200S, Germany) and stored at -20°C till testing.

2.2. Method

The lysozyme contained in biological samples (blood serum or lactose, saliva, milk, urine, conjunctival secretion, etc.) causes cell lysis of the test bacteria Micrococcus lysodeicticus included in the diffusion agar, with the clarification of the reaction medium around the sample well. The diameter of the lysis area is proportional to the concentration of lysozyme contained in the sample.

The radial diffusion technique was performed using Micrococcus lysodeicticus. For that, Micrococcus lysodeicticus was cultivated on Mueller Hinton agar for 24h at 37°C. Using a phosphate buffer (PBS) solution (pH=7.2), the culture was washed away from the culture plates, then centrifuged at 2500 rpm for 10 min. The obtained deposit was diluted with PBS and the washing procedure was repeated 2-3 times.

The bacterial cell pellet was then thoroughly mixed and the suspension density was standardized against a 0.300 optical density control at 1.8 (λ= 535 nm, d=0.5 cm, SUMAL PE, Karl Zeiss, Jena). Six milliliters of this suspension were mixed with the same amount of 2% diffusion agar (agar Noble, phosphate buffer pH 6.2) which was melted and then cooled to 56°C, just before mixing. The mixture was immediately poured in Petri dishes (12 cm diameter) and left to solidify on a perfectly horizontal surface.

Using a 3.5 mm metal puncher, 12 wells were perforated in each Petri dish, where the biological samples were distributed (approximately 37 μl/well). A standard lysozyme powder (Sigma Aldrich, USA) was diluted to 100μg/ml and served as a control, placed in one of the wells of each plate. Serum samples were applied in the wells of the agar plates as whole or diluted: 1:2, 1:4 or 3:4 with selenium and/or copper commercial preparations Cuprosel (selenium and copper mix), Selesol (sodium selenite), Seleretard (barium selenite)(INMV Pasteur, Bucharest) or a protein-carotenoid sea buckthorn

extract. Percentage increase/decrease in activity of each experimental variant was calculated compared to control, represented by experimental sera diluted with saline.

Meanwhile, using the same diffusion method, a standard curve was built by using different concentrations of the standard lysozyme powder (1.25; 2.5; 5; 10; 20; 25 ... 250 μg/ml).

Subsequently, the plates were incubated for 18-20 hours at 37°C. At the end of the incubation period, by using a caliper, the diameters of the clear areas around the wells were measured and lysozyme concentrations (μg/ml) were evaluated by interpolating those diameters on the standard curve.

2.3. Statistical Analyses

Mean values, standard deviation and the statistical significance of the differences (t-Student test) between the values obtained for the two species and for different dilution within the same species were calculated by use of Microsoft Excel program.

3. Results and Discussion

The understanding and interpretation of the involvement of fundamental immunological defense mechanisms in infectious processes allow the selection of the most appropriate techniques of prevention, diagnosis, treatment and eradication of such diseases (Roitt, 1991). The background in controlling infectious diseases is relying on the immune system, through the activity of both innate and adaptive effectors and mechanisms. The immune system has evolved on the phylogenetic scale, reaching its highest competencies in mammals. Nevertheless, the innate immune effectors were present from very first forms of multicellular organisms, i.e., the i-type lysozyme being already encountered in invertebrates (Callewaert and Michiels, 2010)

The antibacterial activity of various multipart structures such as plant extracts, honey or others, rely on the involvement of some enzymes or enzymatic complexes in direct destruction of various bacteria cell structures (mainly the membrane but also polysaccharides or proteins of the cell wall, DNA, autocrine molecules involved in quorum-sensing molecules). These processes jeopardize the bacteria morphology and functionality, thus the entire cell's life (Rutherford and Bassler, 2012, Stefanetti et al., 2024).

The proteolytic and oxidative activities of such enzymes drew the attention of researcher for their potential use not only in therapy but also in food industry, as antimicrobial agents to fight bacterial biofilm forming properties and prolong shelf life of food products (Li et al., 2023, Thallinger *et al.,* 2013). Lysozyme seems to be a "champion" of antibacterial activity keeping i.e., the egg sterile (Baron et al., 2016). International organizations such as FAO and WHO, involved in keeping mankind safe and healthy by providing secure food, defined the lysozyme "as a polypeptide obtained from hen's egg whites consisting of 129 amino acids, having a molar mass of about 14 000 g mol− 1 and an isoelectric point of 10.7" (https://apps.who.int/food-additives-contaminants-jecfa-database/Home/Chemical/3398; https://openknowledge.fao.org /server/api/core/bitstreams/0e4fcdcd-0979-447c-a88f-f357365490a1/content). Similarly, the enzyme was described as being able to hydrolyze the β (1–4) bonds between N-acetylmuramic acid and N-acetylglucosamine, located in the membranes of bacteria, but being mainly active against Gram-positive organisms. The hydrochloride form, as mentioned by FAO/WHO was mainly used for food industry. Clostridium tyrobutyricum, known to be causing the late blowing of cheese is successfully inhibited by lysozyme used as a preservative. The assay used to prove the inhibitory activity of lysozyme against this Gram-positive rod relays an

assay including as reaction support a suspension of Micrococcus luteus ATCC 4698, which changes its turbidity in contact with the enzyme. Lysozyme use in food industry is not only permitted for cheeses but also for various meat products, seafood, vegetables and wine. Nevertheless, no officially approved methodology is available to quantify the presence of lysozyme in food (Mani Lopez, 2016). The *in vitro* activity of the lysozyme (Table 1) was significantly lower ($p<0.05$) in chickens than in rabbits under the same dilution conditions (Serum + Saline 1:2).

Table 1. Lysozyme concentrations for chicken and rabbit sera: experimental variants (µg/ml) (x ± s)

Species	Serum + Saline 1:2	Serum+Cuprosel 1:2	Serum + Selesol 1:2	Serum + Seleretard 1:2
Chickens n = 19	27.28 ± 31.34	31.33 ± 34.35	21.46 ± 24.62	25.46 ± 33.54
Rabbits n = 19	75.55 ± 41.71	91.33 ± 37.03	98.33 ± 37.51	88.44 ± 39.73

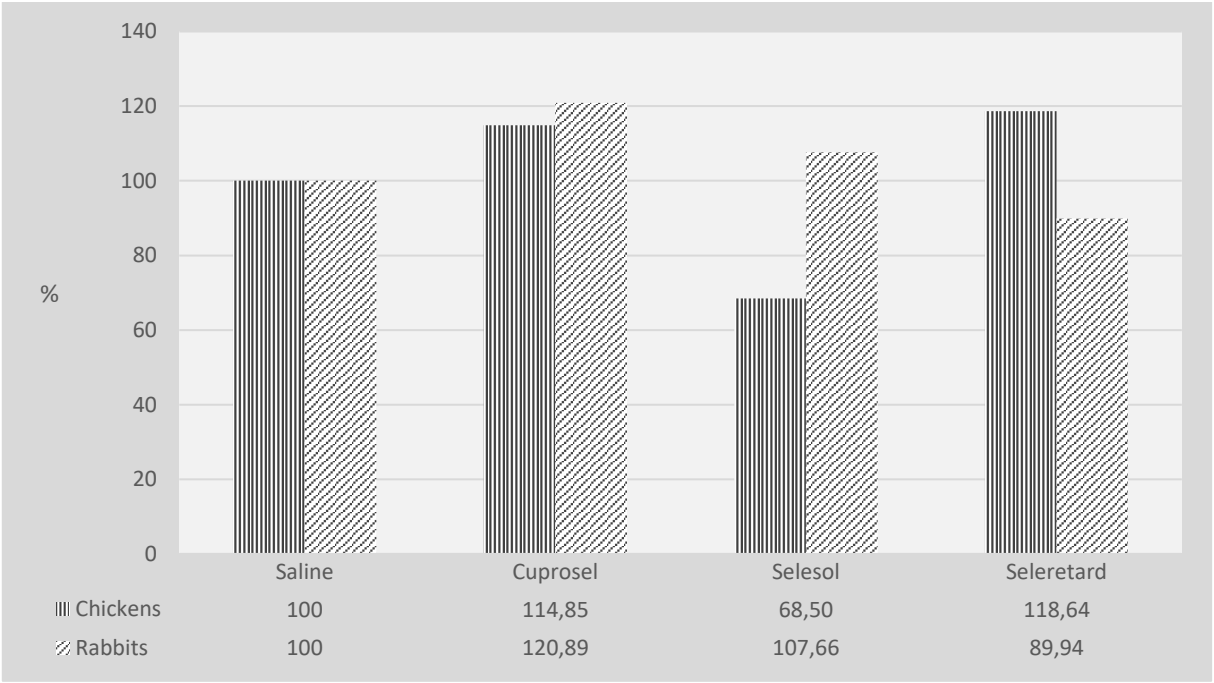

Fig. 1. Percentage of increase in activity of the treated sera when compared to controls in chickens and rabbits

Nevertheless, the distribution of the values was very broad indicated by the high value of the standard deviation (31.34). In rabbits, the lysozyme activity of the non-treated serum control was significantly higher than in chickens (+176.94%, p=0.00012). The values encountered in chickens were somewhat higher than indicated in the literature (24.7 µg/mL in 42 days old chickens, Lebedev et al., 2024). This is paradoxical, while egg white lysozyme is considered to be at the highest level in hen eggs (100 µg/mL, FAO). Similarly, the values recorded for rabbits exceeded those mentioned by other authors (Hrynkiewicz et al., 2020). In chickens (Fig. 1), the Cuprosel increased the activity of the lysozyme with 14.85% (p=NS) and a similar result was observed for the Seleretard (barium selenite) (18.64%, p=NS), while the sodium selenite decreased the lysozyme activity (-31.50%, $p<0.025$). No statistically significant differences were recorded

between the selenium and copper compound in vitro treated serum samples in chickens. Although the lysozyme levels were much higher in rabbits, in non-treated controls but also the immune stimulating compound treated samples, the activity of the later was changed when compared to that noticed in chickens. Thus, although the differences were non-significant between the variants, in rabbits the barium selenite was the one to decrease the lysozyme activity (-10.06%). These results provide information on how different sodium/copper salts have different activities in Aves and Mammalia.

Unexpectedly, the sea buckthorn extract had an inhibiting effect when compared to control at the same dilution (Table 2) that decreased with the further dilution (1: 4 -1.6 ±0.74 µg/ml). The recorded values proved to be very low, standing for a strong inhibiting effect of the protein carotenoid fruit extract on the in vitro lysozyme activity in chickens (-91.31% - II, -99.86% - III). In rabbits, a similar behavior of the lysozyme efficacy was observed, with negative values ranging from 91.60% to 94.28. Interestingly, in both species, the higher dilutions of the sea buckthorn extract induced stronger inhibiting effect, but lesser in rabbits than in chickens (p=NS). These results might be induced by the change in pH of the diffusion medium due to the high concentration of the acid extract. Nevertheless, there is a contradiction between this hypothesis and the fact that in higher dilutions the inhibiting effect was stronger.

Table 2. Lysozyme concentrations in whole and seabuckthorn extract treated serum samples (µg/ml)

Species	Serum + Saline 1:2 (I)	Serum + H. rhamnoides Extract 1:2 (II)	Serum + H. rhamnoides Extract 1:4 (III)
Chickens	27.28 ± 31.34	2.37 ± 8.6	1.6 ± 0.74
Rabbits	75.55 ± 41.71	6.34 ± 9.12	4.32±2.32

Statistical significance of differences between the experimental variants
$t_{I\text{-}II} = 2.64$ $p < 0.005$; $t_{I\text{-}III} = 2.63$ $p < 0.005$; $t_{II\text{-}III} = 7.22$ $p < 0.001$

Further, the acetone-petrol ether- methanol solvent used for extraction could cause these discrepancies between concentrations but also species. Lysozyme activity, an indicator for the responsiveness of innate immune system, is subject to alterations due to either intrinsic, organism dependent or extrinsic, environmental factors (Roitt, 1991). Thus, increased lysozyme levels have diagnostic and/or prognostic value in mucosal infections: digestive, respiratory or urinary infections as well as in infectious diseases or neoplasms (Potapova et al., 1988). The *in vivo* administration of different immune stimulating or immune modulating compounds leads to an increased lysozyme synthesis due to the stimulation of secretory cells, indicating possibilities for an enhanced antimicrobial response (Criste et al., 2020).
In vitro testing of lysozyme activity in treated serum samples is a method easy to perform that can offer valuable indications on stimulating/modulating activity of different compounds.

Conclusions

The experiment proved that representatives of different species react differently to both immune stimulating compounds and plant extracts in terms of in vitro lysozyme activity. The data obtained also allowed a comparison of chicken and rabbit lysozyme activity, proving that chicken lysozyme was a worse in vitro responder to stimulation than rabbit lysozyme. This data underlines the importance of further studies, which could better correlate the chemical composition of the *Hippophae rhamnoides* extract and its biological activity, while tailored experimental variants could support more encouraging results.

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Author Contribution

All authors declare equal contribution to the study design and experimental work, interpretation of the results and editing the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest during the accomplishment of this research. None of the authors has any financial and/or personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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