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Gut-testis axis in roosters: *Lactiplantibacillus plantarum* supplementation improves reproductive performance

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ABSTRACT

Probiotics are widely used in poultry farming and industry, as they offer numerous health and performance benefits for birds. Probiotic *Lactobacilli* maintain gut microbiota balance, aid nutrient utilization, boost the immune system, increase stress resistance and serve as antibiotic alternatives. However, their impact on male reproductive function is not yet fully understood. This study investigated the effect of a novel probiotic strain, *Lactiplantibacillus plantarum* SNI3 (*Lb*SNI3), on the reproductive performance of roosters.

Twenty adult roosters were used. *Lb*SNI3 was administered orally (dose: 2×10^7 CFU/animal/day) for 7 weeks to half of the animals. Control birds (10) received sterile tap water vehicle. Ejaculate volume, sperm concentration, sperm motility, number of IPVL penetration holes and testosterone plasma concentration have been measured weekly. Testis weight, dimensions and histology have been determined at the end of the experiment. mRNA levels of select genes, involved in spermatogenesis and sperm motility, oxidative and steroid synthesis have been measured in the testis samples by qRT-PCR. Total antioxidant capacity, superoxide dismutase (SOD) enzyme activity and malondialdehyde (MDA) levels were also analyzed.

*Lb*SNI3 administration increased the ejaculate volume, sperm concentration and the number of penetration holes, resulting in a significant improvement in the reproductivity index. In contrast, testosterone levels were not statistically different in control versus *Lb*SNI3-treated groups. At the end of the experiment, testis size, the area, and the lumen of seminiferous tubuli were increased in *Lb*SNI3-treated roosters. The testicular expression of *Gpx1, Sepw1, Dio2, Birc5* and *Rec8* genes was elevated following oral administration of *Lb*SNI3. Total antioxidant activity, SOD activity significantly increased, while MDA concentration decreased, indicating enhanced antioxidant capacity in the testis. LbSNI3 produces a bacterial metabolite, γ-glutamyl-glutamate, which enters the glutathione cycle and strengthens the testicular defense mechanisms against oxidative stress.

In conclusion, oral administration of probiotic *Lb*SNI3 enhances antioxidant defense mechanisms in the testis, leading to increased reproductive index in adult roosters. This effect may be mediated through the gut-testis axis and could be utilized to improve productivity in the livestock industry.

Introduction

Male fertility issues are a growing concern in the livestock industry, particularly in modern poultry breeding, where the emphasis on rapid growth and high meat yield often compromises reproductive traits (Hocking et al., 1989; Ansari, 2024). Environmental factors, such as heat

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stress (Karaca et al., 2002), lack of key nutrients (Salas-Huetos et al., 2019), infections Cox et al. (2002) and ageing also negatively affect sperm production and semen quality. Addressing these challenges is crucial in maintaining reproductive efficiency in breeding males.

Probiotic bacteria are widely used in poultry production (Patterson et al., 2003; Juricova et al., 2022) to enhance gut health, prevent pathogen colonization (Markova et al., 2024; Zhang et al., 2024; Xiao et al., 2024; Yang et al., 2024), modulate immune system locally and systemically Pascual et al. (1999), and improve feed efficiency, growth rate and body mass van der Klein et al. (2024) and used as antibiotic alternatives Yaqoob et al. (2022). Beyond their local effects in the gut, emerging evidence highlights that beneficial bacteria influence multiple organs, including the liver Hamid et al. (2019), lungs Le Bras (2024) and brain Huang et al. (2023);Mindus et al. (2021). Recently, a connection between the gut bacterial community and male reproductive function, termed the gut microbiome-testis axis has been recognized in mammals (Li et al., 2022; Leelani, 2023; Zhang et al., 2022). However, how probiotic bacteria may act through the gut microbiome to influence testicular and male reproductive functions in poultry remains unknown.

Birds share a core gut microbiota with other vertebrates, especially at higher taxonomic units (Shang et al., 2018; Lu et al., 2003; Grond et al., 2018), although the genera and species found in the avian gut clusters apart from mammals and human (Ottinger et al., 2024; Grond et al., 2018). One of the most effective ways to modulate the gut microbiome toward beneficial bacteria is through probiotic supplementation, particularly with Lactobacillus species (Xiao et al., 2024; Yang et al., 2024). These rod-shaped, Gram-positive, non-spore forming, anaerobe/aerotolerant bacteria are prevalent throughout the poultry gut. *Lactobacillus* is the dominant genus in the crop and gizzard, significant amount of lactobacilli are present in the small intestine and cecum, while their contribution to bacterial diversity is declining in the colon Shang et al. (2018). While Lactobacilli do not permanently colonize the intestinal tract and their metabolites Eastwood et al. (2023), cell wall components Poornachandra Rao et al. (2019) or extracellular vesicles Mata Forsberg et al. (2019) (postbiotics) have significant local and remote actions.

Recently we identified a unique Lactobacillus strain, *Lactiplantibacillus plantarum* SNI3 (*Lb*SNI3) Otto et al. (2023) which significantly enhances male reproductive performance in rodents (Juhasz et al., 2024). Oral administration of *Lb*SNI3 for 4 weeks in mice and rats led to increased sperm number, improved sperm quality and elevated testosterone plasma levels, ultimately boosting fertilization rate via production of a small dipeptide metabolite, γ-glutamyl-glutamate (Juhasz et al., 2024).

This study aims to investigate whether the probiotic *Lactiplantibacillus plantarum* SNI3 can enhance reproductive functions in roosters. We also address the role of select testicular genes and antioxidant mechanisms in mediating pro-reproductive effects of *Lb*SNI3.

Materials and methods

Lactiplantibacillus plantarum SNI3 (*Lb*SNI3) was provided by J. Kukolya (Institute of Food Science and Technology, Hungarian University of Agriculture and Life Sciences, Hungary). The strain has been deposited in National Collection of Agricultural and Industrial Microorganisms, (NCAIM (P) B 001482), Food Science and Technology, Hungarian University of Agriculture and Life Sciences, Hungary.

*Lb*SNI3 cultures were grown in sterile MRS broth (VWR Chemicals) anaerobically at 37 °C for 48 h to reach bacterial density of 10^8 CFU/mL.

Ethics statement

All procedures in these experiments were complied with the ARRIVE guidelines and performed in accordance with the guidelines of European Communities Council Directive (86/609 EEC), EU Directive (2010/63/EU) and the Hungarian Act of Animal Care and Experimentation (1998; XXVIII, Sect. 243/1998). The applied methods were approved by the Directorate of Food Safety and Animal Health of the Government Office of Pest Country, Hungary (PEI/001/809-2/2015).

Animals

20 adult Yellow Hungarian roosters (52 weeks old) were used (http://www.geneconservation.hu/content/old-hungarian-farm-animals) in this experiment. Animals were kept in individual deep floor cages (0.8 × 1 m) at the National Centre for Biodiversity and Gene Conservation, Institute for Farm Animal Gene Conservation under 14 h Light:10 h Dark photoperiod, the feed contained 16,4 % crude protein, 3,2 % crude fat, 4,2 % crude fiber, 13,7 % crude ash, 11 MJ/kg ME. Feed and water were available ad libitum.

48 h *Lb*SNI3 cultures (10 mL) were then centrifuged, the pellet was washed 2x in sterile tap water and re-suspended in 5 mL water. 100 µl of this bacterial suspension was given *per os* to roosters (dose: 2×10^7 CFU/animal/day) for 7 weeks. Control animals received the same volume of sterile tap water. The roosters were slaughtered at the end of the 7th week.

Semen collection and analysis

Semen from cockerels was collected by abdominal massage technique according to Burrows and Quinn Burrows W.H. (1935) once a week, for 7 weeks. The semen volume was registered and sperm concentration was measured by spectrophotometer (Accucell, IMV, L'Aigle, France), and sperm motility was analyzed by CASA system (Microptic, SCA®,6.6). The ratio of live, intact and morphologically abnormal spermatozoa were determined using aniline blue-eosin staining (Certistain, 115935 Eosin Y, Merck Ltd., Budapest, Hungary). Slides were evaluated microscopically (Zeiss, Axioscope; Carl Zeiss Microscopy GmbH, Germany) using an oil immersion objective at x1000 magnification. A total of 200 spermatozoa were counted per slide Varadi et al. (2019). In addition, DNA integrity was also assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) according to Santiago-Moreno et al. (2019).

Blood sampling

Blood was withdrawn from the wing vein before and 1, 2, 3 weeks after *Lb*SNI3 administration. The last blood sample collection was performed 4 days following the completion of 7 weeks' *Lb*SNI3 treatment. Blood samples were centrifuged (3500 rpm, 20 min, 5 °C) and the plasma stored at −20 °C until assay. For serum steroid analyses, a testosterone ELISA kit (Cat No: DNOV002, Novatec, Dietzenbach, Germany) was used. The immunoassay was performed according to the manufacturer's instructions, and samples were measured in triplicate. Measurements were obtained and data were analyzed using a microplate reader Thermo MultiskanTM FC (Waltham, MA, USA) equipped with SkanIt RE software (version 6.1.1.7). The absorbance was measured at 450 nm with a reference wavelength of 630 nm.

Sperm penetration assay

For the preparation of perivitelline membrane (PVL), after cracking the egg, the yolk was separated from the white and cleaned the remaining white in a 1 % NaCl solution. 0,5 × 0,5 cm pieces of membrane were cut out on the side opposite the germinal disc and washed 3 times in 1 % NaCl solution. The semen was diluted in ratio 1:3 with PBS solution and incubated in a shaking water bath at 40 °C for 30 min. 1 ml of DMEM solution was measured into glass tubes and spermatozoa at a concentration of $1{,}25 \times 10^7$ were placed and carefully mixed. PVL pieces were placed and incubated in a shaking water bath at 40 °C for 5 min. Then the membrane pieces removed and washed three times in 1 % NaCl solution. PVL membranes were mounted on a slide and the number of

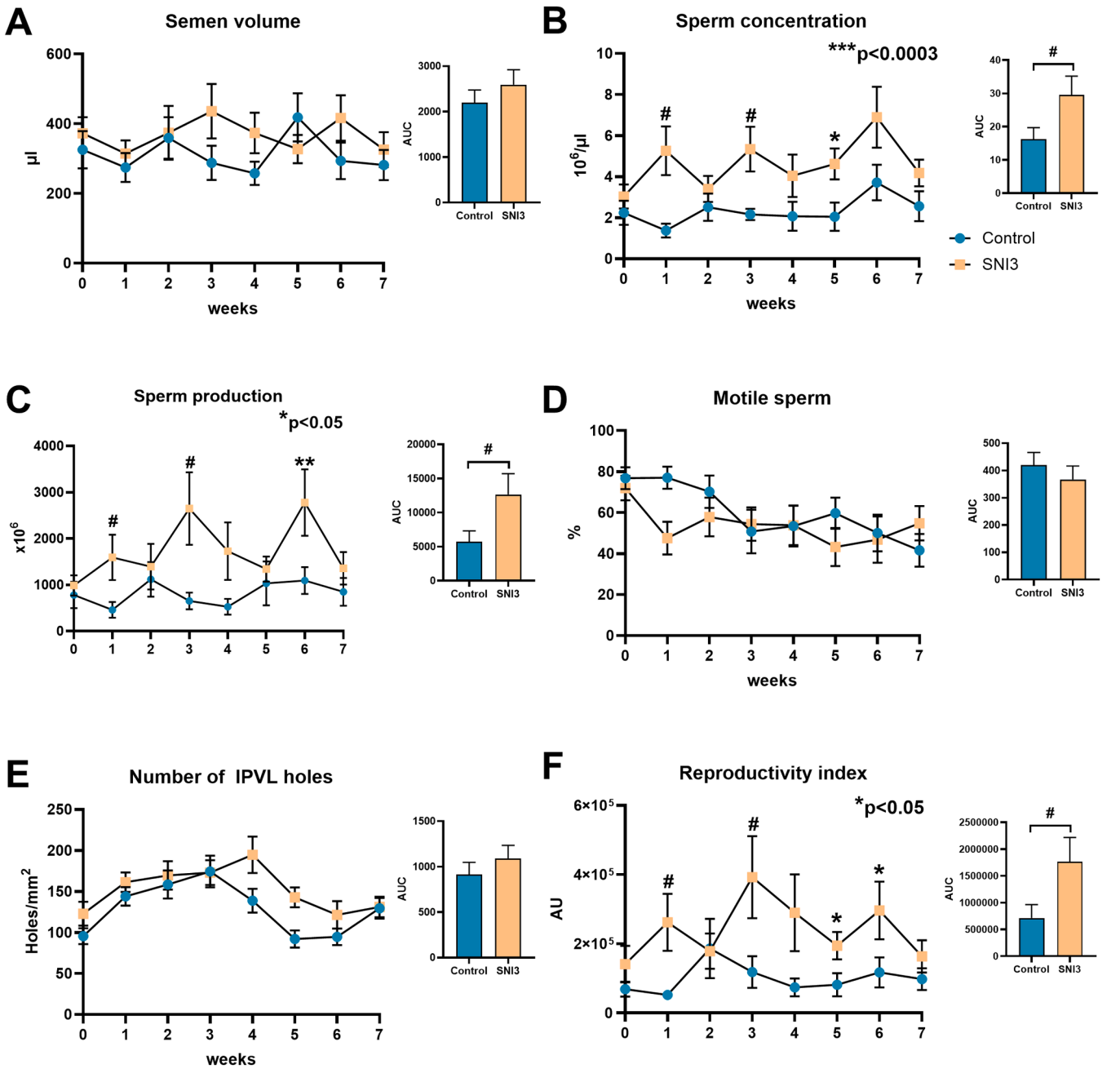

Fig. 1. ***Changes in select semen parameters in*** **Lactiplantibacillus plantarum** ***SNI3 treated roosters.*** *Time course and integrated values of semen volume (A), sperm concentration (B), total sperm number (sperm count x semen volume, C), sperm total motility (D), the number of PVL holes (E) and the reproductivity index (ejaculate volume x sperm number x motile sperm% x penetration holes, F) in control and* Lactiplantibacillus plantarum *SNI3-treated (SNI3) treated animals. All data are expressed as Mean ± SEM values. The area under the curve (AUC) was used to determine the overall effect of L. plantarum SNI3 treatment over the 7 weeks. Repeated measures across time were analyzed using a mixed effects model (REML), where symbols indicate the main effect of treatment: *p < 0.05; ***p < 0.001 vs control. Group comparisons were performed using unpaired, two-tailed t-test (*p < 0.05; **p < 0.01 vs control) and Welch's corrected t-test (# <p0.05 vs control).*

hydrolyzed holes by the spermatozoa were counted in 3 fields of view under a darkfield microscope with a 10 x objective. The membrane test was performed in 3 replicates with each semen sample.

At the 7th week of the experiment, roosters were euthanized and testes were removed and weighed. Right testis was fixed in 10 % buffered formaline, the left testis was frozen in dry ice. Frozen samples were used for either qRT-PCR or measurement of SOD activity and malondialdehyde (MDA) levels.

Histology

Fixed testes were embedded in paraffin, 10 µm sections were cut and stained with hematoxylin-eosin. Digital images were captured at 20x magnification in 3D HISTECH Pannoramic MIDI II. slide scanner. Regions of interest (ROI) were outlined and analyzed with NIS Elements Imaging Software 5.21.01.

qRT PCR and primer sequences

Frozen testis samples (50 mg) were homogenized in TRI Reagent

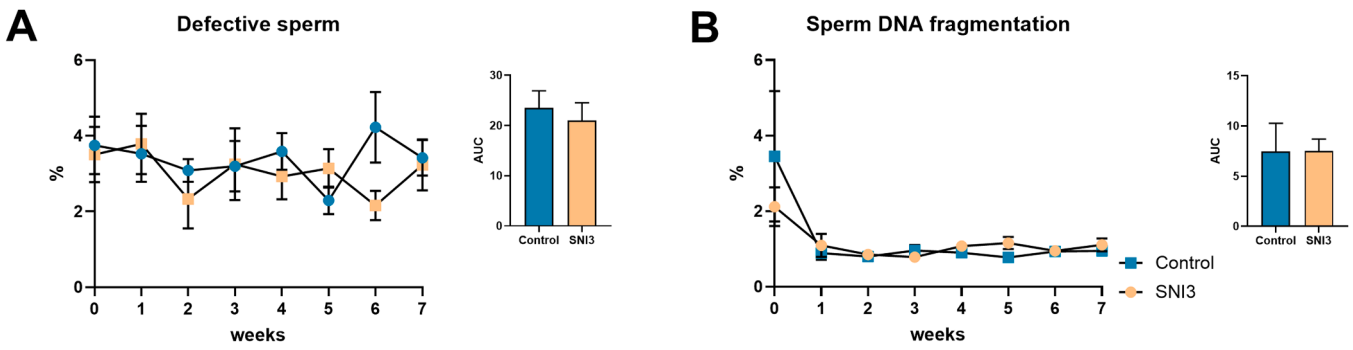

Fig. 2. ***Occurrence of defective sperms and DNA fragmentation.*** *Time course and integrated data on the abundance of morphologically defective sperms (A) and sperm DNA fragmentation. All data are expressed as Mean ± SEM values of control and* Lactiplantibacillus plantarum *SNI3- treated (SNI3) animals. The area under the curve (AUC) was used to determine the overall effect of L. plantarum SNI3 treatment over the 7 weeks. Repeated measures across time were analyzed using a mixed effects model (REML). Group comparisons were performed using unpaired, two-tailed t-test.*

Solution (Ambion, USA) and total RNA was isolated with QIAGEN RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according the manufacturer's instruction. To eliminate genomic DNA contamination DNase I treatment were used and 100 µl RNase-free DNase I (1 unit DNase) (Thermo Scientific, USA) solution was added. Sample quality control and quantitative analysis were performed using a NanoDrop 2000 (Thermo Scientific, USA). Amplification was not detected in the RT-minus controls. cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Primers for the comparative Ct experiments were designed using Primer Express 3.0 Program and Primer Blast software. The primers (Microsynth, Balgach) were used in the Real-Time PCR reaction with Fast EvaGreen® qPCR Master Mix (Biotium, USA) on ABI StepOnePlus (Applied Biosystems, USA) instrument. Primer sequences are listed in (Supplementary Table 1). Gene expression was analyzed using ABI Step One 2.3 software. The amplicons were tested by melt curve analysis using an ABI StepOne Plus instrument. Experiments were normalized to *Gapdh* expressions.

Measurement of total antioxidant capacity in testis samples

Testis samples were homogenized in 60 % ethanol (2–8 °C) at a volume-to-weight ratio of 9:1 (mL:g).The tissue homogenate was centrifuged for 10 min at 10000 g at 4 °C. The supernatant was kept on ice until analysis. The total antioxidant capacity assay was performed in accordance with the manufacturer's instructions with the Total Antioxidant Capacity (T-AOC) Colorimetric Assay Kit (Invitrogen, EEA023). The prepared tissue samples and standards were pipetted into a 96-well plate, and colorimetric measurements were taken using a Thermo MultiskanTM FC (Waltham, MA, USA) equipped with SkanIt RE software (version 6.1.1.7). Absorbance was measured at 414 nm. Data analysis was performed using the SkanIt RE software (version 6.1.1.7).

Measurement of SOD enzyme activity in testis samples

Frozen testis samples (100 mg) were homogenized in 0,5 mL phosphate buffer saline (PBS). Homogenized samples were centrifuged at 1,500 × *g* for 10 min at 4 °C. The collected supernatant was assayed immediately. To measure cytosolic SOD, the supernatants were centrifuged at 10,000 × *g* for 15 min at 4 °C. The supernatants contained cytosolic SOD and the cell pellets contained mitochondrial SOD. The soluble SOD activity assay was performed in accordance with the manufacturer's instructions with the Superoxide Dismutase (SOD) Colorimetric Activity Kit (Invitrogene, EIASODC). Colorimetric measurements were taken using a Thermo MultiskanTM FC (Waltham, MA, USA) equipped with SkanIt RE software (version 6.1.1.7). Absorbance readings were recorded at a wavelength of 450 nm. The absorbance value of the blank (PBS)) was subtracted to account for background interference. Data analysis was performed using the SkanIt RE software (version 6.1.1.7).

MDA assay

The assessment of lipid peroxidation was conducted by measuring malondialdehyde (MDA) concentrations in testis samples using the Thiobarbituric Acid Reactive Substances Assay. The measurements were performed using a Lipid Peroxidation (MDA) Assay Kit (MAK085, Sigma-Aldrich, Merck 2 Merck KGaA, Darmstadt, Germany). Tissue samples (10 mg tissue sample was homogenized on ice in 300 µL of the MDA Lysis Buffer containing 3 µL of butylated hydroxytoluene (BHT). After this procedure, samples were centrifuged at 13,000xg for 10 min to remove insoluble material. The assay was performed in accordance with the manufacturer's instructions. In this process, MDA in the prepared testis tissue sample combines with thiobarbituric acid (TBA) to form an MDA-TBA complex, which is then quantified by colorimetric analysis. For the colorimetric test, a 0.1 mol/L MDA standard was created, and a series of dilutions were prepared to establish a standard curve. The prepared tissue samples and standards were pipetted into a 96-well plate, and colorimetric measurements were taken using a Thermo MultiskanTM FC (Waltham, MA, USA) equipped with SkanIt RE software (version 6.1.1.7). Absorbance readings were recorded at a wavelength of 532 nm. The absorbance value of the blank (molecular biology grade water) was subtracted to account for background interference. Data analysis was performed using the SkanIt RE software (version 6.1.1.7).

Statistical analysis

Data are presented as mean ± standard error of means (SEM). First, F-test was used to test homoscedasticity. In cases, when F-test was not significant (the variances between the two groups are approx. equal), we used two-tailed, unpaired Student's *t*-test (control vs. *L. plantarum* SNI3-treated). When F-test was significant, we performed Welch's corrected t-test. For all analyses GraphPad Prism software (version 9.4.0; San Diego, CA, USA) was used. In all cases, p value$<$0.05 was considered statistically significant.

Results

The animals tolerated the *Lactobacillus* (*Lb*SNI3) treatment, we did not see any sign of gastrointestinal discomfort.

Effect of LbSNI3 on the semen and sperm parameters in roosters

Amount of ejaculate varied between 257 and 418 µl in the control group. In *LbSNI3*-treated roosters the ejaculate volume was higher than

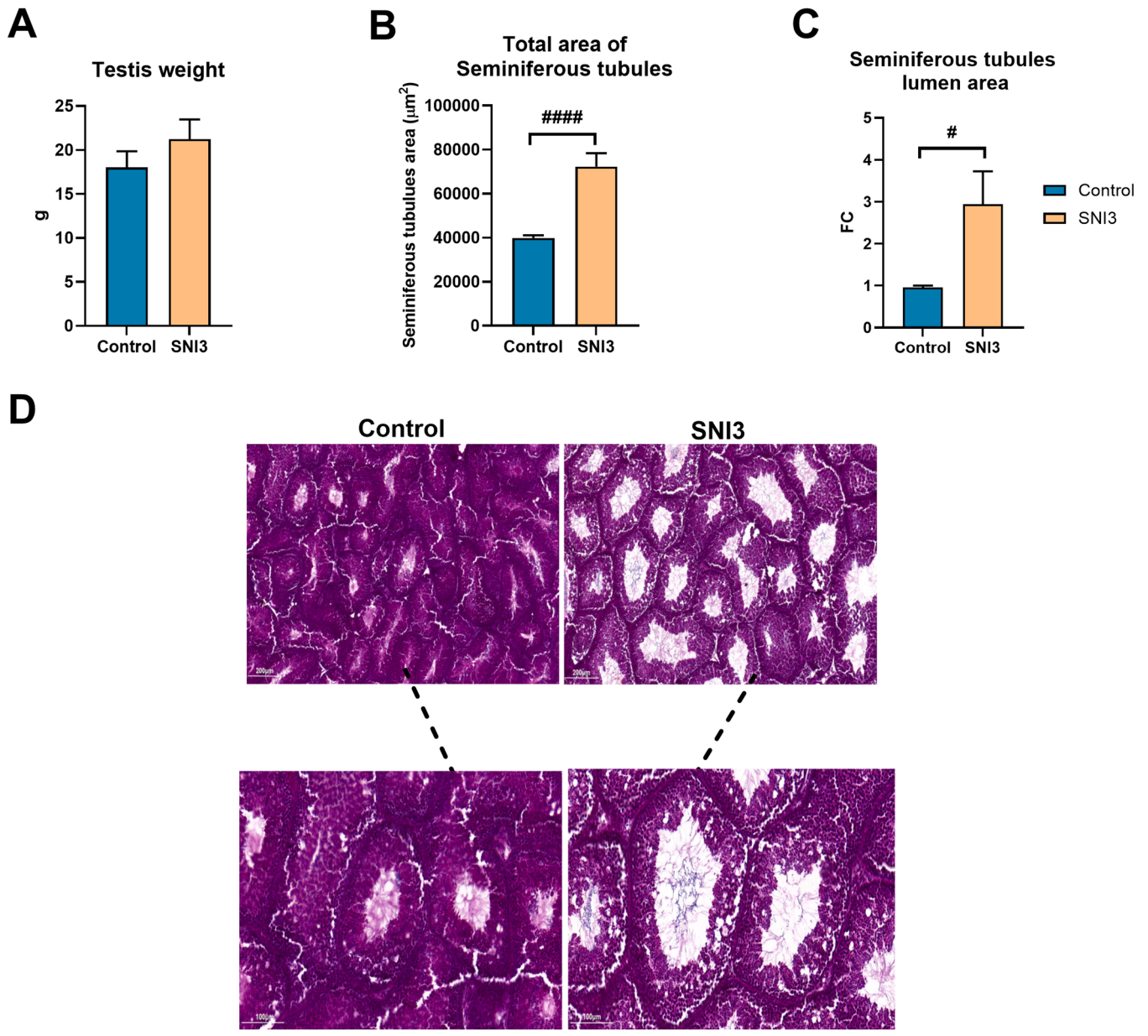

Fig.ure 3. ***Morphological changes in the testis following*** **Lactiplantibacillus plantarum** ***SNI3 treatment.*** *Mean + SEM values of testis weight (A) the area of seminiferous tubules (B) fold change of the lumen area of the seminiferous tubules (C) in control and* Lactiplantibacillus plantarum *SNI3 (SNI3) treated roosters. Data were analyzed by Welch's corrected t-test #p < 0.05; ####p < 0.0001* vs *control. Representative photomicrographs of hematoxylin-eosin stained testis sections from control and* Lactiplantibacillus plantarum *SNI3- (SNI3) treated animals (D).*

controls on the 3rd, 4th and 6th weeks after starting the treatment (Fig. 1A).

The sperm concentration increased in *Lb*SNI3-treated animals throughout the whole experiment, the difference between control and *LbSNI3*-treated groups was statistically significant at 1st, 3rd and 5th weeks of treatment (Fig. 1B). Total sperm number (sperm concentration * semen volume) was significantly higher in *Lb*SNI3-treated group (Fig. 1C).

Sperm total motility was not statistically different between control and *Lb*SNI3-treated groups (Fig. 1D).

There was a tendency for higher number of sperm penetration holes in *Lb*SNI3-treated group compared to controls, the difference was significant at 4th and 5th weeks of treatment (Fig. 1E).

The reproductive index (ejaculate volume * sperm number * motile sperm% * penetration holes) was significantly higher in *Lb*SNI3-treated roosters (Fig. 1F).

Effect of LbSNI3 on the quality of spermatozoa

The percentage of alive, but morphologically abnormal spermatozoa (acrosome-, head-, middle piece- and tail abnormalities) was 10.44 ± 1.19 % in controls and 13.66 ± 0.52 % in *Lb*SNI3-treated animals ($p = 0.11$) (Fig. 2A) Ayeneshet et al. (2024).

DNA fragmentation as measured by TUNEL assay was the highest at the beginning of the experiment in both groups, however was not statistically different throughout the whole treatment period (Fig. 2B).

Effect of LbSNI3 on the testis size and histology

Although the testis weight was higher in *LbSNI3*-treated roosters compared to controls, the unpaired t-test did not reveal significant difference between the groups (Fig. 3A).

Quantitative evaluation of the seminiferous tubules observed on

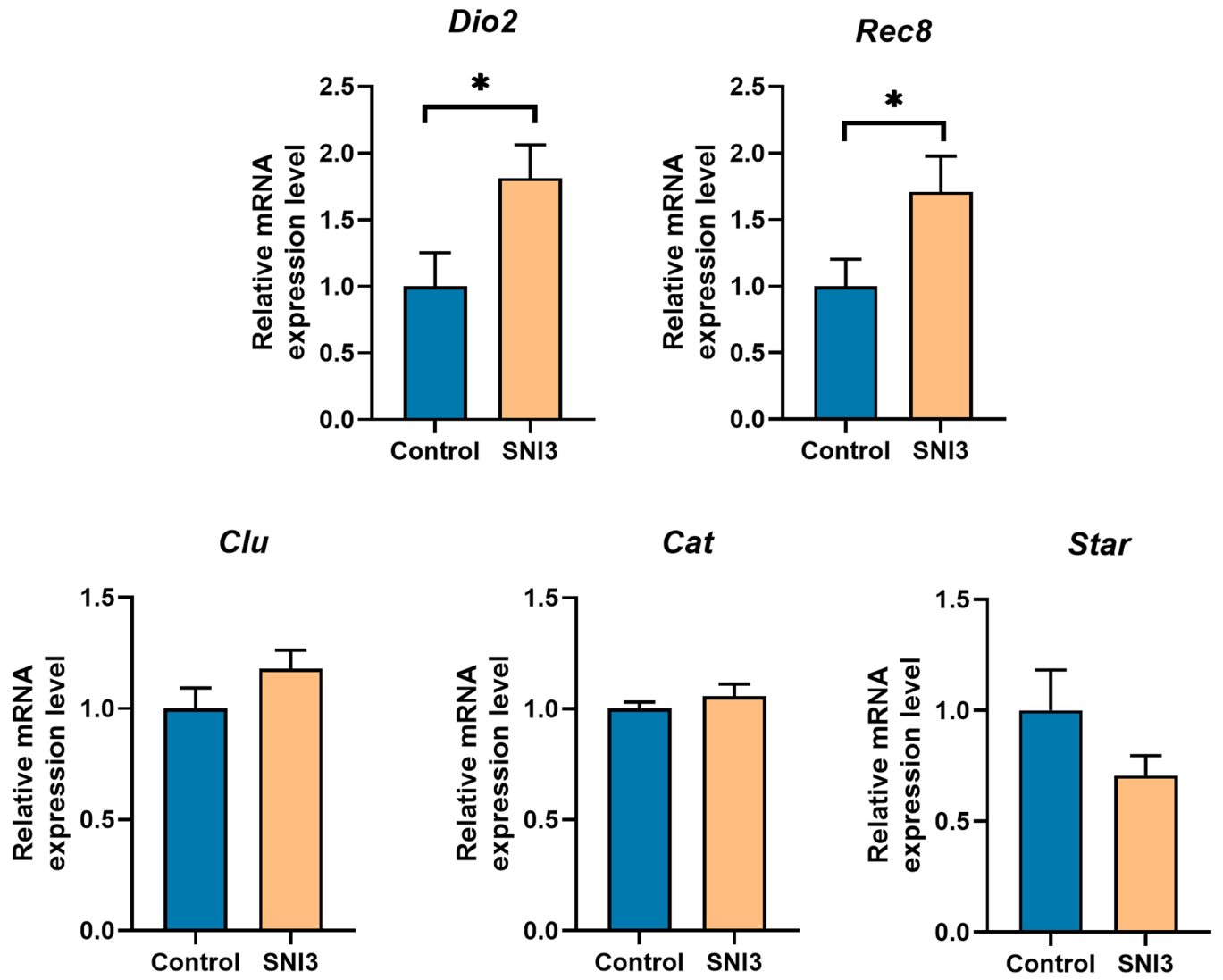

Fig. 4. ***Expression of select genes involved in spermatogenesis and sperm motility in the testis.*** *qRT-PCR analysis of relative mRNA levels in the testis of* Lactiplantibacillus plantarum *SNI3-treated roosters compared to controls. Mean + SEM values. Data were analyzed using an unpaired, two-tailed t-test, * $p < 0.05$. Dio2: Deiodinase, Iodothyronine Type II; 5; Rec8: Meiotic Recombination Protein REC8 Homolog; Clu: Clusterin; Cat: Catalase; StAR: Steroidogenic Acute Regulatory protein.*

hematoxylin-eosine-stained testis sections revealed an increased total area of the tubules and significantly increased lumen area in treated animals, compared to controls (Fig. 3B–D).

Effect of LbSNI3 on spermatogenesis-related genes

Expression of select genes: -Dio2: Deiodinase, Iodothyronine Type II; Birc5: Baculoviral IAP Repeat-Containing 5; Rec8: Meiotic Recombination Protein REC8 Homolog; Clu: Clusterin; Cat: Catalase; StAR: Steroidogenic Acute Regulatory protein- involved in testicular homeostasis, sperm production and oxidative defense are shown on Fig. 4. Relative mRNA levels of Dio2, Birc5 and Rec8 were significantly higher in LbSNI3-treated roosters.

Effect of LbSNI3 on the antioxidant capacity, expression of antioxidant-related markers in the testis

Fig. 5A shows that total antioxidant capacity (T-AOC) of testis tissue samples from LbSNI3-treated roosters was significantly higher than those obtained from control birds. mRNA levels *Gpx1* and *Sepw1* were increased, but that of *Sod1* (cytoplasmic) *Sod2* (mitochondrial) and *Sod3* (extracellular) forms of superoxide dismutase (SOD) do not change upon *Lb*SNI3 treatment (Fig.5B). In spite of unchanged expression of *Sod* mRNAs, SOD enzyme activity (Fig.5C) was significantly higher in the testis of *Lb*SNI3-treated animals. Malondialdehyde (MDA) (Fig. 5D) concentration decreased in the testis of treated roosters.

Effect of LbSNI3 on the serum testosterone level

Large individual fluctuations were seen in weekly sampled serum testosterone levels. Overall, we did not find statistical differences in testosterone concentration between animals receiving *Lb*SNI3 preparation for 7 weeks, compared to controls (Fig 6A, B).

Discussion

In birds, a complete cycle of spermatogenesis takes about 13-14 days (Jones et al., 1993; Lin et al., 1990). The duration of *Lb*SNI3 administration lasted for seven weeks, thus encompassing 3-3.5 spermatogenetic cycles, ensuring the possible effect of the lactobacilli on sperm production to be developed.

Within the testis, the area of seminiferous tubules was increased, indicating increased spermatogenetic activity. In addition, the lumen of the seminiferous tubules was also significantly enlarged. An increase in

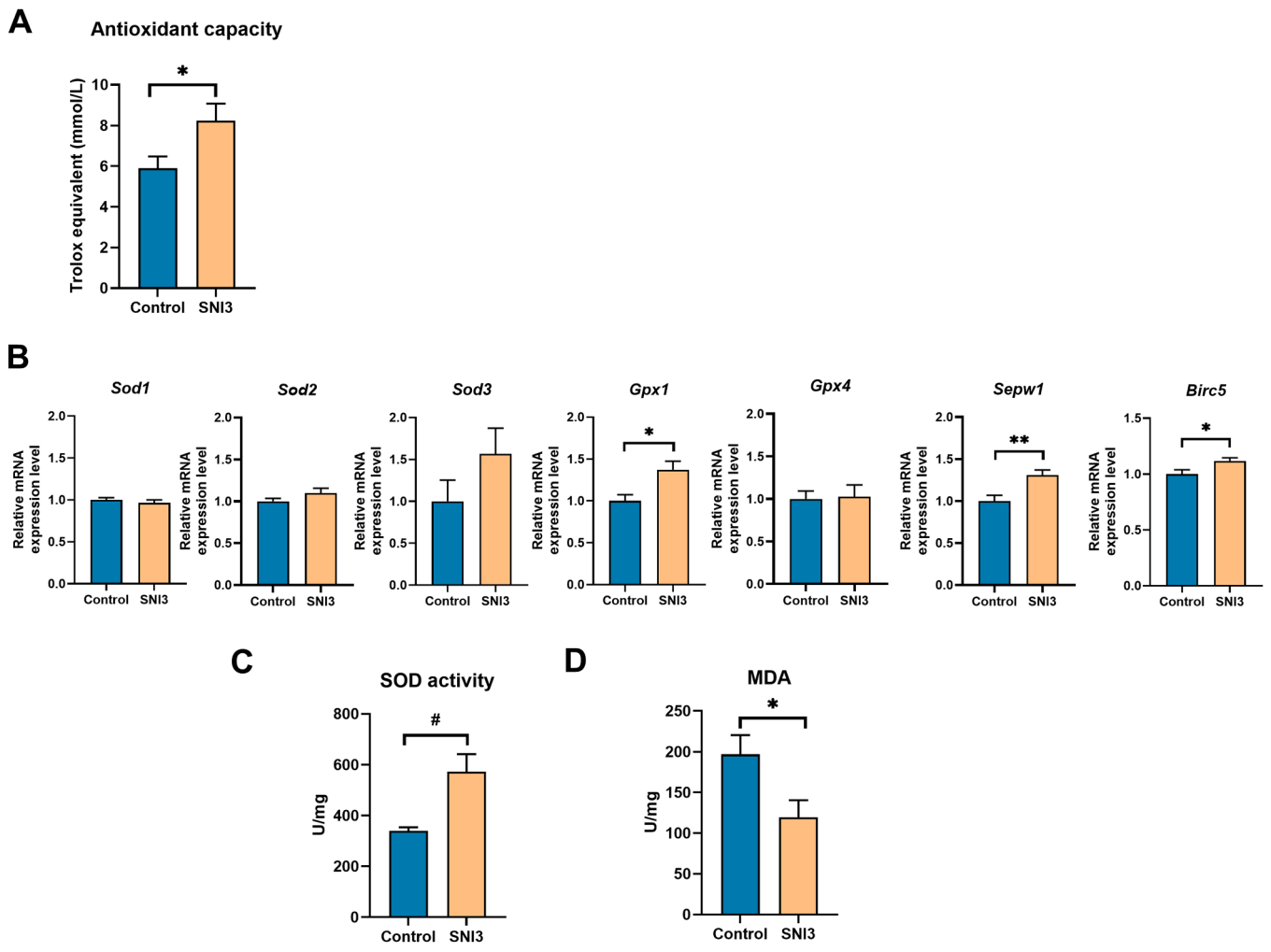

Fig. 5. ***Antioxidant indicators in the testis***. Antioxidant capacity (A) mRNA *levels of antioxidant related genes: Sod1, 2 and 3: Superoxide dismutase −1,2,3; Gpx1: Glutathione Peroxidase 1; Gpx4: Glutathione Peroxidase 4; Sepw1 Selenoprotein W,1; Birc5: Baculoviral IAP Repeat-Containing 5 in the testis of control and* Lactiplantibacillus plantarum *SNI3-treated (SNI3) roosters (B). Superoxide dismutase (SOD) activity (C) and malondialdehyde (MDA) level (D) in the testis of control and* Lb*SNI3 treated roosters. Data are presented as Mean + SEM. Unpaired, two-tailed t-test *p < 0.05; **p < 0.01.*

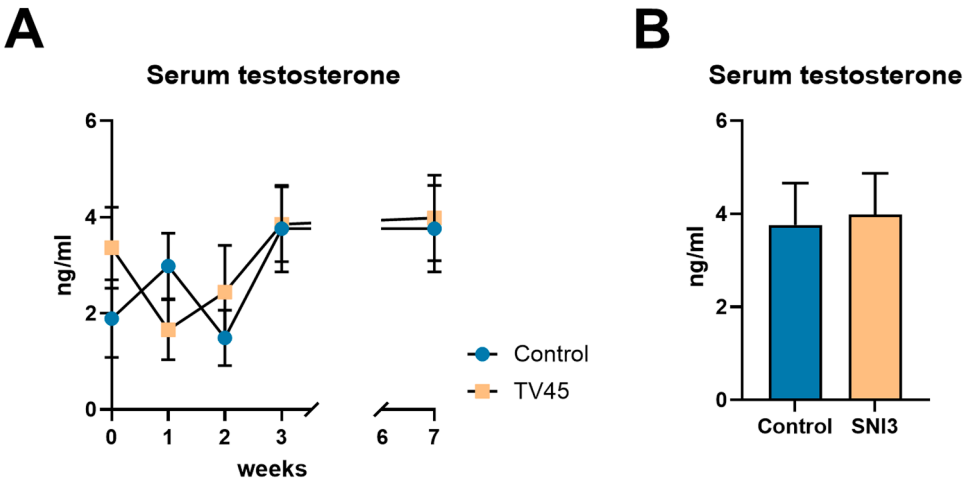

Fig. 6. ***Serum testosterone levels***. *Weekly changes in serum testosterone levels (A) and average values of testosterone production (B) in control and* Lactiplantibacillus plantarum *SNI3-treated roosters. Data are presented as Mean + SEM values. Statistical analysis was performed using an unpaired, two-tailed t-test.*

the lumen size can occur if there is a high sperm output and the sperm accumulates temporarily in the lumen.

In this study, sperm motility values were in the range of the earlier reports on different chicken breeds (Feyisa et al., 2018; Napierkowska et al., 2024; Guo et al., 2023), however, we did not find significant effect of *Lb*SNI3 on sperm motility and quality. By contrast, in male mice, *Lb*SNI3 administration slightly increased sperm motility Juhasz et al. (2024).

Another critical parameter in assessing the quality of sperm is DNA integrity. High levels of DNA fragmentation can negatively affect fertility and embryonic development. In this study, the abundance of TUNEL+ sperms was in the range reported in different breeds of chicken Santiago-Moreno et al. (2019) and more importantly, the number of sperms with DNA fragmentation was low and we did not find differences in sperm DNA fragmentation between control and *Lb*SNI3 treated animals.

The number of IPVL holes is a reliable indirect measure of fertility and breeding performance in birds (Végi et al., 2013; Wishart, 1997). The number of IPVL holes peaked at 56 weeks of life then decreased in both groups, corresponding to the age related decline in fertility. However, in *Lb*SNI3 treated roosters the peak was significantly higher and the decline is slower compared to controls. Overall, the increased sperm production together with the increased IPVL holes emphasize the positive effect of *Lb*SNI3 treatment on the fertility in mature breeders.

Beneficial bacteria, such as lactobacilli are indigenous members of poultry microbiome, have various effects on poultry sperm production through multiple mechanisms. These bacteria improve gut health, increase nutrient absorption, vitamins (vitamin E and B group) and minerals, which support sperm production Fouad et al. (2020). *Lactobacilli* constrain "spermicide" pathogenic bacteria in the lower reproductive tract and semen via lowering the local pH and by producing anti-bacterial compounds Osadchiy et al. (2024).

Due to the unique anatomical arrangement of the cloaca, the lower the genital tract, the higher the possibility of appearance of gut derived bacteria. A unique microbiome has been identified in the seminal fluid of birds and mammals, where lactobacilli dominate and may directly affect semen quality. Exposure of broiler breeders' or turkey semen to various intestinal bacterial strains resulted in decreased sperm motility (Haines et al., 2013; Triplett et al., 2016). Along this line, previous findings from the Kiess' laboratory claimed that orally administered *Lactobacillus acidophilus* resulted in high concentration of lactic acid bacteria in the cloaca of roosters, which may negatively affect sperm quality Kiess et al. (2016). By contrast, a study on local toms by Dim et al. (2020) found significant increase of semen volume, sperm concentration and progressive motility of sperms obtained at the end of a 28 weeks trial. In that study, all three *Lactobacillus* strains (*L. sporogenes, L. delbruckii subspecies bulgaricus* and *L. acidophilus*) were effective in improving sperm quality parameters. A symbiotic, containing *L. rhamnosus, Bifidobacterium longum* and manno-oligosaccharide, through dietary supplementation increased testis weight, testicular cell size and testosterone level in prepubertal quails Aamir Khan et al. (2024). In our study a different *Lactobacillus, Lactiplantibacillus plantarum* SNI3 displayed beneficial effect on sperm production in roosters. The same SNI3 strain also increased spermatogenesis and sperm motility in mice Juhasz et al. (2024).

A recent paper compared the testis transcriptome of roosters with high and low spermatogenesis activity/sperm motility and found set of genes that are differentially expressed Du et al. (2022). Among these genes, we also found *Rec8, Dio2* and *Birc5* genes upregulated in *L. plantarum* SNI3-treated adult roosters. *Rec8* (Meiotic recombination protein REC8 homolog) serves as a marker for meiotic progression in the testis. *Dio2* Iodothyronine deiodinase converts inactive thyroxine (T4) to more active triiodothyronine (T3) in the target tissues and is critical for testicular functions including spermatogenesis (Nittoli et al., 2021; Pan et al., 2022). Central *Dio2* plays a crucial role in mediating the effect of photoperiod on the gonad activity in seasonally breeding birds (and in mammals) Yoshimura (2006). Here, we show that Dio2 expression is upregulated in the testis, supporting local thyroid hormone conversion, which might also be involved in the increased testicular function. *Birc5* (Baculoviral IAP repeate-containing 5) has been implicated in testicular homeostasis and providing protection against oxidative damage.

Excessive oxidative stress has detrimental effects on spermatogenesis and may be a potential cause of decreased fertility Hussain et al. (2023). Lactobacilli or other probiotic strains with antioxidant capacity decrease reactive oxygen species (ROS) and mitigate oxidative stress in sperms R. U. (Khan, 2011; Inatomi et al., 2018). Our present findings that *Lactiplantibacillus plantarum* SNI3 treatment increased total antioxidant capacity in the testis, increased testicular expression of *Gpx1, Spw1, Birc5* and *Dio2* genes, increased SOD activity and decreased MDA levels in the testis, support this mechanism.

Testis is generally considered sterile, therefore gut lactobacilli may act indirectly, through the gut-testis axis Leelani (2023), via production specific bacterial metabolites. For instance, butyrate, an important short chain fatty acid (SCFA), produced by various gut bacteria, including lactobacilli, acts at remote sites, including the testis. Sodium butyrate supplement increases the quality and quantity of breeder rooster's semen Alhaj et al. (2018). Another *Lactobacillus* metabolite, gamma-glutamyl-glutamate (γ-GluGlu) Zhao et al. (2016) has been shown to act in the mammalian testis to boost spermatogenesis Juhasz et al. (2024). Here we show that LbSNI3 produces significant amount of γ-GluGlu to the spent culture medium (Supplementary figure). γ-GluGlu is an intermediate compound in the glutathione cycle Bachhawat et al. (2018) thus supports the protective redox balance resulting improved fertility.

Testosterone, produced by Leydig cells in the testis, support spermatogenesis and play a key role in reproductive physiology. Surprisingly, we did not find significant changes in testosterone levels in *Lb*SNI3 treated roosters. This might be due to a large individual fluctuation, originating from the pulsatile hormone secretion. Alternatively, the age of the roosters might also be a confounding factor.

In conclusion, the novel *Lactiplantibacillus plantarum* strain SNI3 increases sperm production and reproductivity index in adult roosters without significant effect on testosterone plasma concentration. *Lb*SNI3 possesses an antioxidant capacity and promotes antioxidant milieu in the testis to support spermatogenesis. The pro-reproductive effects of this *Lactobacillus* strain can be exploited to increase the fertility of males or prolong the reproductive period in poultry industry using either natural mating or artificial insemination.

Declaration of competing interest

The authors declare no competing financial interests. The *Lactiplantibacillus plantarum* SNI3 and its use on male subjects has been filed for patent application on June 4, 2021 in Hungary. PCT procedure and national phases of PCT are under progress.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.105141.

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