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Intergenerational and transgenerational effects of epigenetic factors applied in early developmental stages – insights from in ovo model

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Preface

This thesis presents a novel investigation into the transgenerational effects of bioactive compounds, utilizing a chicken model to explore changes in the transcriptomes of both somatic and germline tissues. To the best of our knowledge, this is the first study to adopt such a model for examining how dietary and environmental factors can influence gene expression across multiple generations.

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Mariam Ibrahim, September 2025

Abstract

Epigenetic modifications, shaped by environmental inputs, regulate gene expression and contribute to phenotypic and clinical variability. Some of these changes are transgenerationally transmitted, though their persistence may differ across tissues. This thesis aimed to investigate how prenatal exposure to potential epigenetic modulators can influence the transcriptome of somatic and germline tissues across successive generations, and to evaluate the stability of primordial germ cells (PGCs) as an experimental model for studying epigenetic transmission by comparing the potential epigenetic effects of different conditions (freezing–thawing and in vitro cultivation) on the expression of germ cell–specific markers. Using an *in ovo* chicken model, we applied a synbiotic (PoultryStar®) alone or with choline to F1 embryos on day 12 of incubation and tracked effects in subsequent generations (F2–F4 generations). Treatment groups included control, synbiotic alone, or synbiotic plus choline, with lineages receiving either a single F1 exposure (to assess inter- and transgenerational effects) or repeated exposures in every generation (to test cumulative effects of repetitive injections). Cecal tonsils, cecal mucosa, and gonads were sampled from adult males (21 weeks old), while embryonic blood was collected at Hamburger–Hamilton (HH) stages 14–16. RNA sequencing was performed on all tissues, and RRBS was applied to gonads. *In ovo* exposure induced both intergenerational (F2) and transgenerational (F3 and F4) transcriptomic changes that were tissue-specific. Cecal tonsils exhibited robust and persistent transgenerational responses in F3, cecal mucosa showed transient intergenerational effects in F2, and embryonic blood displayed moderate effects in F3 that declined in F4. Gonads were particularly sensitive to synbiotic plus choline, demonstrating pronounced transcriptomic and epigenetic alterations in F2 and F3 generations. In general, enriched pathways included metabolism, immune signaling, proteostasis, stress responses, cytoskeletal dynamics, and cell growth and development. These findings highlight dynamic transmission patterns, indicating that epigenetic effects are non-linear. Notably, repeated exposures did not consistently amplify effects across generations. In parallel, we investigated the stability of chicken PGCs under short- and long-term cryopreservation. Cryopreserved PGCs maintained viability, germline competence, and transcriptomic stability, confirming their utility for biobanking and as a model for studying epigenetic transmission. Overall, this thesis demonstrates that prenatal stimulation with bioactive compounds (synbiotic and choline) can

program gene expression across generations, while cryopreserved PGCs provide a robust platform for germline preservation and functional studies. Together, these findings highlight the influence of microbial and nutritional factors on long-term metabolic and immune outcomes and reinforce the central role of germline biology in both experimental and applied contexts of transgenerational epigenetic regulation.

Resumé

Modyfikacje epigenetyczne, kształtowane przez czynniki środowiskowe, regulują ekspresję genów i przyczyniają się do zmienności fenotypowej oraz klinicznej. Niektóre z tych zmian są przekazywane międzypokoleniowo, choć ich trwałość może różnić się w zależności od tkanki. Celem niniejszej pracy było zbadanie, w jaki sposób prenatalna ekspozycja na potencjalne modulatory epigenetyczne może wpływać na transkryptom tkanek somatycznych i zarodkowych w kolejnych pokoleniach, oraz ocena stabilności pierwotnych komórek płciowych (PGC) jako modelu eksperymentalnego do badania przekazywania potencjalnych zmian epigenetycznych poprzez porównanie wpływu różnych warunków (zamrażanie–rozmrażanie i hodowla in vitro) na ekspresję markerów specyficznych dla komórek płciowych. W modelu in ovo zastosowano synbiotyk (PoultryStar®) samodzielnie lub w połączeniu z choliną u zarodków F1 w 12. dniu inkubacji i monitorowano efekty w kolejnych pokoleniach (F2–F4). Grupy eksperymentalne obejmowały kontrolę, synbiotyk podany samodzielnie lub z choliną, przy czym zarodki F1 poddawano pojedynczej ekspozycji na czynniki epigenetyczny (w celu oceny efektów między- i transpokoleniowych) lub powtarzanej ekspozycji w każdym pokoleniu (w celu sprawdzenia kumulacyjnych efektów wielokrotnych wstrzyknięć). Migdałki jelitowe, błona śluzowa jelita grubego oraz gonady pobierano od dorosłych samców (21 tygodni), natomiast krew zarodkową pobierano w stadiach Hamburger–Hamilton 14–16. We wszystkich tkankach wykonano sekwencjonowanie RNA, a w gonadach zastosowano RRBS. Ekspozycja in ovo wywołała zarówno efekty międzypokoleniowe (F2), jak i transpokoleniowe (F3 i F4), które były specyficzne dla tkanki. Migdałki jelitowe wykazały silne i trwałe odpowiedzi transgeneracyjne w F3, błona śluzowa jelita grubego wykazała przejściowe efekty międzygeneracyjne w F2, a krew zarodkowa wykazała umiarkowane efekty w F3, które zmniejszyły się w F4. Gonady były szczególnie wrażliwe na połączenie synbiotyku i choliny, wykazując wyraźne zmiany transkryptomiczne i epigenetyczne w F2 i F3. Ogólnie, wzbogacone szlaki obejmowały metabolizm, sygnalizację immunologiczną, proteostazę, reakcje na stres, dynamikę cytoszkieletu oraz wzrost i rozwój komórek. Wyniki te podkreślają dynamiczne wzorce dziedziczenia, wskazując, że efekty epigenetyczne nie są liniowe. Należy zauważyć, że powtarzane ekspozycje nie powodowały konsekwentnego wzmocnienia efektów w kolejnych pokoleniach. Równolegle badano stabilność PGC kur w warunkach krótkoterminowej i długoterminowej kriokonserwacji. Mrożone-rozmrożone PGC zachowały

żywotność, kompetencje germinalne oraz stabilność transkryptomu, potwierdzając ich przydatność do biobankowania oraz jako model do badania dziedziczenia epigenetycznego. Podsumowując, niniejsza praca wykazała, że prenatalna stymulacja związkami bioaktywnymi (synbiotyk i cholina) może programować ekspresję genów w kolejnych pokoleniach, a PGC poddane kriokonserwacji stanowią solidną strategię do zachowania linii zarodkowych i badań funkcjonalnych. Razem wyniki te podkreślają wpływ czynników mikrobiologicznych i żywieniowych na długoterminowe wyniki metaboliczne i immunologiczne oraz wzmacniają znaczenie biologii komórek zarodkowych w kontekście zarówno eksperymentalnym, jak i praktycznym transmisji zmian epigenetycznych.

List of Publications

This thesis is based on the following papers:

1. **Paper I**

Primordial Germ Cells as a Potential Model for Understanding (Nutri) Epigenetic-Metabolic Interactions: A Mini Review

Mariam Ibrahim, Ewa Grochowska and Katarzyna Stadnicka

Published – Frontiers in Cell and Developmental Biology. 2025, 13, 1576768; http://dx.doi.org/10.3389/fcell.2025.1576768

Impact factor: 4.6, 100 Ministerial Points

2. **Paper II**

The Effect of Short- and Long-Term Cryopreservation on Chicken Primordial Germ Cells

Mariam Ibrahim, Ewa Grochowska, Bence Lázár, Eszter Várkonyi, Marek Bednarczyk, Katarzyna Stadnicka

Published – Genes. 2024, 15(5), 624; https://doi.org/10.3390/genes15050624

Impact factor: 2.8, 100 Ministerial Points

3. **Paper III**

Inter- and Transgenerational Effects of In Ovo Stimulation with Bioactive Compounds on Cecal Tonsils and Cecal Mucosa Transcriptomes in a Chicken Model

Mariam Ibrahim, Marek Bednarczyk, Katarzyna Stadnicka and Ewa Grochowska

Published – International Journal of Molecular Science. 2025, *26*(3), 1174; https://doi.org/10.3390/ijms26031174

Impact factor: 4.9, 140 Ministerial Points

4. **Paper IV**

Transgenerational Effects of In Ovo Stimulation with Synbiotic and Choline on Gonadal Tissue Across Three Generations

Mariam Ibrahim, Ewa Grochowska, Marek Bednarczyk and Katarzyna Stadnicka

Published – Scientific Reports. 2025, 15, 30940; https://doi.org/10.1038/s41598-025-16387-6

Impact factor: 3.9, 140 Ministerial Points

5. **Manuscript V**

Multi-generational transcriptomic changes in embryonic blood following in ovo stimulation with nutriepigenetic factors

Mariam Ibrahim, Katarzyna Stadnicka, Marek Bednarczyk, and Ewa Grochowska

Draft

Abbreviations

ATP	Adenosine triphosphate
BMP4	Bone morphogenetic protein 4
BP	Biological process (Gene Ontology term)
BH	Benjamini-Hochberg correction
C	Control group
CC	Cellular component (Gene Ontology term)
CRE	Cis-regulatory element
CpG	Cytosine-phosphate-Guanine
CpG-ODNs	CpG-Oligodeoxynucleotides
CVH	Chicken vasa homolog
cDNA	Complementary DNA
DAZL	Deleted In Azoospermia Like germ cell marker
DDT	Dichloro-diphenyl-trichloroethane
DHEA	Dehydroepiandrosterone
DEGs	Differentially expressed genes
DHR	Differential histone retention
DMLs	Differentially methylated loci
DMGs	Differentially methylated genes
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
DHA	Docosahexaenoic acid
ED	Embryonic day
EGK X	Eyal-Giladi and Kochav stage X
EPA	Eicosapentaenoic acid
EMA-1	Epithelial membrane antigen-1
EWAS	Epigenome-wide association study
F0	Parental generation
F1	First generation
F2	Second generation
F3	Third generation
FACS	Fluorescence-activated cell sorting
Fto	FTO Alpha-Ketoglutarate Dependent Dioxygenase gene
GOS	Galactooligosaccharide
GO	Gene Ontology
GSEA	Gene set enrichment analysis

Gly	Glycine
HH	Hamburger–Hamilton stage
H3K27me3	Histone H3 lysine 27 trimethylation
H3K4me2	Histone H3 lysine 4 dimethylation
H3K9me3	Histone H3 lysine 9 trimethylation
IOF	In ovo feeding
IGF2R	Insulin-like growth factor 2 receptor
Irx3	Iroquois homeobox 3 gene
Irx5	Iroquois homeobox 5 gene
KEGG	Kyoto Encyclopedia of Genes and Genomes
lncCPSET1	Chicken-PGC-specifically-expressed transcript 1
lncPGCAT-1	Long non-coding RNA PGC transcript-1
lncPGCR	Long non-coding RNA PGC regulator
lncRNA	Long non-coding RNA
LP	Lactiplantibacillus plantarum
miRNA	MicroRNA
NANOG	Nanog Homeobox
NaCl	Physiological saline
ncRNA	Non-coding RNA
OCT4	Octamer-binding transcription factor 4
ORA	Over-representation analysis
PCOS	Polycystic ovary syndrome
PGC	Primordial germ cell
PIWI	PIWI proteins
POUV	Class V POU (POUV) transcription factors
PUFA	Polyunsaturated fatty acids
RNA	Ribonucleic acid
RRBS	Reduced representation bisulfite sequencing
RT-qPCR	Reverse transcription–quantitative polymerase chain reaction
Se	Selenium
SSEA-1	Stage-specific embryonic antigen-1
SSEA-3	Stage-specific embryonic antigen-3
SSEA-4	Stage-specific embryonic antigen-4
SOX2	SRY (sex determining region Y)-box 2
SYN	Group receiving in ovo injection of 2 mg synbiotic/embryo
SYNCH	Group receiving in ovo injection of synbiotic (2 mg) + choline (0.25 mg)/embryo
SYNs	Group receiving single in ovo injection of 2 mg synbiotic/embryo in F1

SYNCHs	Group receiving single in ovo injection of synbiotic (2 mg) + choline (0.25 mg)/embryo in F1
SYNr	Group receiving repeated in ovo injections of 2 mg synbiotic/embryo in F1, F2, F3
SYNCHr	Group receiving repeated in ovo injections of synbiotic (2 mg) + choline (0.25 mg)/embryo in F1, F2, F3
TE	Transposable elements
TET	Ten-eleven translocation enzyme
Zn	Zinc
ZGA	Zygotic genome activation

TABLE OF CONTENTS

1. Introduction

Over recent years, there has been increasing academic interest in how environmental factors, including diet, stress, and chemical exposures, can affect gene expression by inducing changes in epigenetic mechanisms [1]. Unlike genetic variants, epigenetic modifications do not change the underlying DNA sequence but can affect gene expression and phenotype [2]. Epigenetic stimulations such as nutritional inputs during early developmental windows, at which the genome is highly sensitive, can shape long-term physiological traits, influence disease risk, and, in some cases, transmit effects to subsequent generations [3]. Dissecting the causal links between specific dietary components, epigenetic modifications, and phenotypic outcomes is important to research developmental biology, disease prevention, and adaptive responses [1, 2].

The establishment of model systems that enable researchers to accurately trace how environmental factors acting as epigenetic modifiers induce epigenetic changes and alter gene expression is one of the main challenges in epigenetics research. A good model must be capable of capturing both direct responses and the potential for these induced changes to be passed on to future generations.

The chicken embryo provides a valuable model for epigenetic research [4, 5]. It enables researchers to perform controlled timing of environmental exposure to a selected substance and to study its effect in the absence of maternal influences that can confound results [4, 6].

The long-term stability and transmission of induced epigenetic modifications remain controversial. While some research supports the idea that nutritional interventions can have lasting, transgenerational impacts, others remain skeptical, raising concerns about the underlying molecular mechanisms and questioning their relevance.

1.1. Outline

This Ph.D. thesis investigates the intergenerational and transgenerational effects of epigenetic factors applied during early developmental stages, using a chicken *in ovo* stimulation model. The research forms part of the project titled "Research on the intergenerational and multigenerational phenotypic and epigenetic effect of *in ovo* stimulation of chicken embryo", funded by the National Science Centre, Poland (project no. 2020/37/B/NZ9/00497). The study was conducted using Green-legged Partridgelike chickens maintained at a commercial hatchery in Wągrowiec, Poland. The project was initiated at the Bydgoszcz University of Science and Technology (2022–2023) and then continued at the Faculty of Health Sciences, Collegium Medicum, Nicolaus Copernicus University in Bydgoszcz (2023–2025). Bioinformatic analyses were carried out using the computing resources of the Poznań Supercomputing and Networking Center (PSNC, https://pcss.plcloud.pl/). This thesis is based on a cycle of thematically interconnected publications (I-IV) and the manuscript prepared for submission (V), developed throughout the course of the project.

2. Aims and hypothesis

The aim of this thesis is to investigate the potential of prenatal *in ovo* stimulation with synbiotic and choline applied at embryonic day 12 to affect the transcriptome (and methylome) of both the directly exposed (F1 generation) and subsequent generations (F2, F3 and F4).

This study hypothesizes that:

(1) *In ovo* application of epigenetic modifiers induces inter- and transgenerational effects in somatic and germline cells, altering transcriptomic profiles across generations. Early-life epigenetic modifications are expected to trigger heritable gene expression changes via both inter- and transgenerational transmission mechanisms (Paper III, Paper IV, Manuscript V).

(2) The extent and pattern of these changes are anticipated to vary between tissues and generations (Paper III, Paper IV, Manuscript V).

(3) Repeated exposure across generations (context-dependent transmission) is hypothesized to produce more pronounced transcriptional changes than a single ancestral exposure in the F1 generation (germline-dependent transmission) (Paper III, Paper IV, Manuscript V).

(4) Primordial germ cells (PGCs) may provide a stable model system for investigating inter- and transgenerational epigenetic transmission (Paper II).

To address these hypotheses, the study aims to:

(1) Investigate intra-, inter-, and transgenerational effects of early developmental exposure to synbiotic and choline on gene expression (Paper III, Paper IV, Manuscript V) and DNA methylation (Paper IV).

(2) Identify differentially expressed genes (DEGs) and differentially methylated genes (DMGs), gene ontology terms (GOs), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in germline and somatic tissues across generations following *in ovo* treatment (Paper III, Paper IV, Manuscript V).

(3) Analyze tissue-specific dynamics of gene expression changes across generations under single and repeated epigenetic exposure (Paper III, Paper IV, Manuscript V).

(4) Study the stability of PGCs through comparing the effects of different conditions (freezing-thawing and in vitro cultivation) on the expression of germ cell specific markers (Paper II).

3. Thematic coherence of the thesis publications

The five studies form a coherent body of research investigating the impact of early-life nutritional interventions on gene expression and epigenetic programming, particularly in the context of intergenerational and transgenerational transmission.

The first paper (Paper I), "Primordial germ cells as a potential model for understanding (nutri)epigenetic-metabolic interactions", introduces primordial germ cells (PGCs) as a valuable model to study how early-life nutritional cues can influence epigenetic reprogramming and metabolic outcomes. PGCs arise early in embryonic development and are the precursors to gametes (sperm and ova). As the only cells that transmit genetic and epigenetic information to the next generation, they represent a critical window through which environmental factors, such as diet, can shape both immediate and inherited traits. Thus, this paper establishes the theoretical and mechanistic basis for using PGCs to investigate intergenerational (context-dependent: resulting from direct or continuous exposure to an environmental factor within or across generations) and transgenerational (germline-dependent: epigenetic modifications passed through the germline, affecting offspring beyond the directly exposed generation) transmission of traits shaped by diet and bioactive compounds acting as epigenetic factors.

The second study (Paper II), “The effect of short- and long-term cryopreservation on chicken primordial germ cells”, complements the research by addressing the technical feasibility of preserving PGCs for long-term studies. It examines how cryopreservation (which can induce epigenetic effects), affects PGC basic pluripotency and germ cell-specific markers. The findings show that PGCs retain their identity after both short- and long-term freezing, with increased expression of marker genes following long-term cryopreservation. These results support the use of PGCs as a robust model in germline-focused and transgenerational research.

This concept of inter- and transgenerational transmission is supported by three experimental studies using a chicken model, in which *in ovo* stimulation with bioactive compounds was applied (synbiotics alone (as a potential epigenetic factor) or in combination with choline (a known epigenetic factor). The third study (Paper III), "Inter- and transgenerational effects of *in ovo* stimulation with bioactive compounds on cecal tonsils and cecal mucosa transcriptomes in a

chicken model" explores how *in ovo* exposure to synbiotic and choline influences gene expression in immune tissues across generations. This paper provides evidence of transgenerational effects at the transcriptomic level, highlighting the gut-immune interface as a target of nutritional programming.

The fourth study (Paper IV), "Transgenerational effects of *in ovo* stimulation with synbiotic and choline on gonadal tissue across three generations", directly links back to the PGC-based model proposed in the first paper. By examining the gonadal tissues (the origin of germ cells), this study investigates how bioactive compound (synbiotic and choline) exposure affects the methylome and transcriptome of reproductive (germline) tissues over multiple generations.

The fifth study (Manuscript V, draft manuscript to be submitted), "Multi-generational transcriptomic changes in embryonic blood following *in ovo* stimulation with nutriepigenetic factors", contributes a systemic perspective by analyzing transcriptomic changes in embryonic blood, which notably contains circulating PGCs during early development. By examining this tissue, the study may capture both somatic and germline-related transgenerational responses in F3 and F4 embryos to *in ovo* nutritional stimulation applied in F1 embryos.

Together, these studies form a cohesive narrative, advancing our understanding of how nutritional interventions during embryonic development can exert multi-generational effects, with PGCs as a central model for unraveling these complex interactions.

4. Background

4.1. Epigenetics

Epigenetics is a rapidly growing field that investigates the mechanisms by which environmental factors can influence gene expression, turning genes on and off, without altering the underlying DNA sequence to regulate and maintain differentiated cell types [7]. A broad spectrum of environmental exposures, including diet and pollutants, can alter the epigenome, allowing an individual's environment to modulate gene expression and phenotypes, as well as clinical outcomes [7].

One of the most intriguing aspects of epigenetics is its intersection with nutrition, leading to the emerging field of nutriepigenetics. Nutriepigenetics is a growing area of research focused on how certain nutrients can act as epigenetic modulators [8]. Folate and B vitamins (B12, B6, and riboflavin) are essential for one-carbon metabolism, which supplies methyl groups for DNA methylation [9]. These nutrients act as cofactors or substrates for enzymes involved in epigenetic processes. For instance, folate deficiency can disrupt DNA methylation patterns, leading to genomic instability and increased disease risk [9]. Maternal folate status during pregnancy is particularly critical, as it influences fetal brain development and long-term health outcomes [10]. Polyphenols, found in plant-based foods, exert epigenetic effects by modulating DNA methylation and histone modifications [11]. They can inhibit DNA methyltransferases and histone deacetylases, thereby activate silenced genes and suppress oncogenes [11]. For example, resveratrol and curcumin have been shown to induce epigenetic changes that contribute to their anti-inflammatory and anticancer properties [12, 13]. Fatty acids, particularly omega-3 polyunsaturated fatty acids (PUFAs), influence epigenetic mechanisms by altering the availability of substrates for DNA methylation and histone modifications. Omega-3 PUFAs, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have been associated with improved metabolic outcomes by modulating inflammation-related genes [14]. Conversely, excessive intake of omega-6 PUFAs and saturated fatty acids can lead to pro-inflammatory epigenetic changes, contributing to chronic diseases [14].

Over the past few decades, nutriepigenetics has advanced rapidly, thanks in large part to improvements in genomic and epigenomic research tools. The Dutch Famine studies provided

early evidence of the impact of maternal nutrition on the health of offspring, highlighting the role of epigenetic mechanisms in transgenerational health [15, 16]. The development of genome- and epigenome-wide association studies (GWAS and EWAS) has enabled researchers to identify genetic and epigenetic markers associated with disease risk and dietary responses [17]. Current research trends are centered on decoding the molecular mechanisms by which food interacts with our genes, with the aim of leveraging this knowledge to develop personalized approaches to nutrition and health [18].

4.2. Inter- and transgenerational epigenetic effects transmission

Epigenetic modifications caused by gene-environment interactions can be transmitted across generations, raising the possibility that ancestral exposure to environmental factors may be transmitted to progeny, exerting a long-lasting effect [7]. This epigenetic transmission happens when epigenetic changes are present in the germline and persist in germ cells during development until conception [19]. It's critical to distinguish between intergenerational and transgenerational transmission when discussing epigenetic effects (Figure 1).

Intergenerational effects arise when environmental exposures experienced by a parent (F0) directly affect their offspring (F1) and potentially the germ cells that will form the next generation (F2) [20]. Maternal exposure during pregnancy or during formation of eggs in oviparous species as in chicken can simultaneously influence the developing fetus (F1) and its germ cells (future F2). On the paternal side (or unpregnant mother), in both species, environmental exposures prior to conception can directly affect the germ cells (sperm in males, ova in females), which contribute to the F1 generation. These effects are considered intergenerational, as the exposed germ cells are part of the resulting offspring. A true transgenerational effect, where epigenetic changes are inherited without direct exposure, can only be demonstrated if altered phenotypes appear in generations that were not directly exposed. This means observing effects in the F3 generation through the maternal line (because F1 and F2 were directly exposed), or in the F2 generation through the paternal line [20].

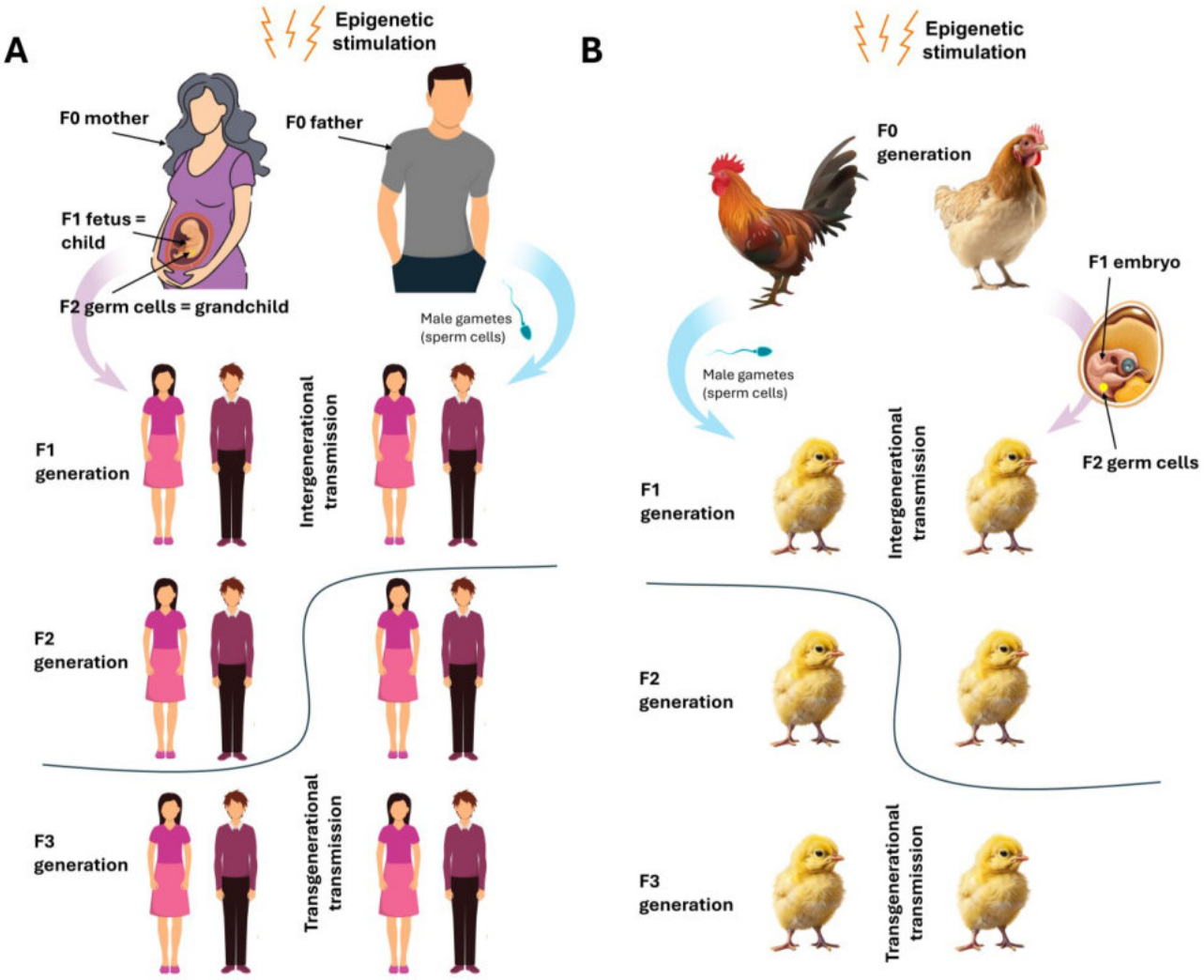


Figure 1. Intergenerational and transgenerational epigenetic transmission in (A) human and in (B) chicken. Transmission to the immediate offspring of an individual in which the change arose by an environmental stimulus is termed intergenerational transmission. In the case of transgenerational transmission, environmental influences are maintained even in the absence of the initial stimulus or epigenetic trigger.

A growing body of evidence supports the idea that the effects of nutriepigenetic factors are passed down across generations. This means that the nutritional condition of parents can affect the health and risk of disease in later generations through epigenetic mechanisms. Evidence for inter- and transgenerational transmission of epigenetic effects primarily stems from animal models. For example, Hardikar *et al.* have used a Wistar rat model subjected to undernutrition over 50 generations and found that the undernourished rats had lower birth weights, greater visceral fat accumulation, and developed insulin resistance, when compared to age- and sex-matched controls [21]. They also showed elevated levels of circulating insulin, homocysteine, endotoxin, and leptin, along with reduced levels of adiponectin, vitamin B12, and folate. Notably,

these rats were eight times more susceptible to Streptozotocin-induced diabetes than controls. These metabolic disruptions persisted even after two subsequent generations were provided with unrestricted access to a standard, nutrient-rich diet. Another research was conducted in which pregnant female mice were exposed to bisphenol A, and it was found that this exposure resulted in obesity in the F2 generation due to increased food intake [22]. This phenotype was observed to be transmissible up to the F6 generation. Chromatin accessibility was analyzed in sperm from generations F1 to F6, and epigenetic changes were identified at two cis-regulatory elements (CREs) of the *Fto* gene, specifically at sites containing CCCTC-binding factor (CTCF) motifs. These CREs were found to exhibit increased interactions with the *Irx3* and *Irx5* genes, which are involved in the differentiation of appetite-regulating neurons. When the CTCF-binding site in *Fto* gene was deleted, normal food intake was maintained and the obesity phenotype was not observed, even after ancestral bisphenol A exposure. Through this study, it was demonstrated that epigenetic modifications of *Fto* gene could reproduce phenotypes typically associated with genetic variants [22]. In a rat model, transgenerational epigenetic transmission was observed following transient exposure of F0 generation females to the environmental toxicants vinclozolin or dichloro-diphenyl-trichloroethan (DDT) [23]. It was demonstrated that epigenetic marks, including differentially methylated regions (DMRs), non-coding RNAs (ncRNAs), and differential histone retention (DHR), were maintained and co-localized across the F1 to F3 generations, indicating that integrated epigenetic mechanisms mediated the transmission of environmentally induced phenotypes. A broader selection of research on inter- and transgenerational epigenetic transmission is presented in Table 1.

Given that germ cells are known to undergo substantial epigenetic reprogramming [24, 25], it is crucial to demonstrate how induced epigenetic modifications that are created in germ cells are able to withstand reprogramming and be passed on to subsequent generations. How environmental stimuli transforms into epigenetic changes is another important question.

Table 1. Summary of some studies on inter- and transgenerational epigenetic effects of environmental and nutritional exposures in different models.

Species	Transmission Type	Exposure Substance	Exposure Stage	Last Assessed Generation	Effect	Main conclusion	Ref.
mice	Trans	Folic acid	F0 mice	F3	Spinal axon regeneration	Transgenerational differentially methylated regions are observed in each consecutive generation	[26]
chicken	Inter	Folic acid	F0 males	F1	Lipid metabolism disorder	Sperm DNA N6-methyladenine and 5-methylcytosine methylation were involved in epigenetic transmission. Paternal dietary excess folic acid leads to hepatic lipid accumulation in offspring.	[27]
Drosophila	Trans	Cadmium	From eggs to adults	F4	Wing development defects	Cadmium exposure caused wing development defects, and this was transmitted to F1-F4 generations.	[28]
mice	Trans	Phthalate	F0 males	F2	Metabolic health	Paternal exposure to endocrine disrupting phthalates can induce intergenerational and transgenerational metabolic disorders in the offspring.	[29]
rats	Trans	Lipopolysaccharides	Prenatal (during gestation)	F3	Hypertension	Lipopolysaccharides exposure can induce the transgenerational transmission of natriuresis dysfunction and hypertension. The underlying mechanism is related to an altered landscape of histone modification and transcriptome expression.	[30]

rats	Trans	Environmental toxicants	Prenatal (during gestation)	F3	Phenotypic abnormalities	Environmental exposures to toxicants can lead to heritable epigenetic modifications, resulting in the transgenerational transmission of disease susceptibility.	[31]
sheep	Trans	Methionine supplement	F0 males	F2	Growth and male fertility phenotypes	Environmentally altered epigenetic marks are transmitted to subsequent generations.	[32]
Drosophila	Trans	Cadmium	Egg stage	F4	Development	The developmental toxicity caused by cadmium could be transmitted to offspring.	[33]
rats	Trans	Dehydroepiandrosterone (DHEA)	F0 females	F2	Polycystic ovary syndrome	Female F1 and F2 offspring with ancestral DHEA exposure exhibited PCOS-like reproductive and metabolic phenotypes. Male offspring with ancestral DHEA exposure exhibited lower quality of sperms.	[34]
rat	Trans	DDT and vinclozolin	Prenatal (during gestation)	F4	Pathological incidence for kidney, obesity and multiple types of diseases	DDT and vinclozolin have the potential to promote the epigenetic transgenerational transmission of disease and sperm epimutations to the outcross F4 generation in a sex specific and exposure specific manner.	[35]
chicken	Inter	Folate supplementation	F0 males	F1	Growth and metabolic traits	Paternal folate could regulate lipid and glucose metabolism in broiler offspring. Epigenetic transmission may involve altered spermatozoal miRNAs and lncRNAs.	[36]

quail	Trans	Genistein	Prenatal (F0 embryos)	F3	Sexual maturity, weight, Behavioral traits	Embryonic environment affects the phenotype of offspring three generations later in quail.	[37]
duck	Trans	Methionine deficiency	F0 females	F2	Growth and lipid metabolism	The mother's diet is able to affect traits linked to growth and to lipid metabolism in the offspring of the sons.	[38]
pigs	Trans	Methylating micronutrients	F0 males	F2	Carcass traits, gene expression (liver, kidney, muscle), DNA methylation	Paternal exposure to a methyl-rich diet can lead to transgenerational effects in F2 pigs via epigenetic modifications.	[39]

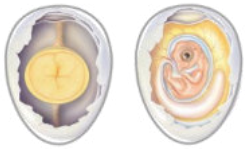
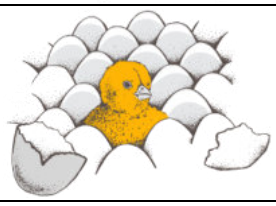
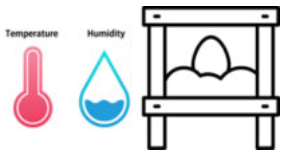
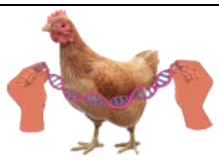
Inter: intergenerational transmission; Trans: transgenerational transmission; DHEA: Dehydroepiandrosterone; DDT: dichloro-diphenyl-trichloroethan; F: filial generation.

4.3. Chicken model

Referring to the International Chicken Genome Sequencing Consortium [40], the chicken genome contains about 1 billion DNA base pairs, making it roughly one-third the size of the human genome, which has around 2.8 billion base pairs. Despite this size difference, chickens have approximately 20,000 to 23,000 genes, comparable to the estimated 20,000 to 25,000 genes found in humans. Interestingly, about 60% of chicken genes have human counterparts or orthologues. These genes are evolutionarily conserved and often carry out similar functions in both species. Moreover, researchers found that chicken and human gene pairs share about 75% sequence identity on average. The extensive blocks of conserved synteny between the chicken and human genomes makes the chicken a valuable model for investigating the regulation of gene expression in vertebrates [41]. Furthermore, key epigenetic mechanisms such as histone post-translational modifications and DNA modifications, are highly conserved between chicken and human cells, supporting its relevance in comparative epigenetic studies [41].

Unlike mammalian models, chickens offer unique advantages for studying the effects of epigenetic factors and their transgenerational transmission (Table 2, Discussed in Paper I). Different studies have highlighted the usages of chicken embryo as a powerful and practical model for studying transgenerational epigenetic transmission due to their biological features and experimental flexibility [5, 42, 43]. The chicken is an especially valuable model for studying how nutrition during early life can shape health across generations [44]. Chickens have proven to be valuable models for studying nutritional rehabilitation, especially through dietary intervention studies in broilers, underscoring their relevance as translational models for human nutrition [45]. They have significantly advanced our understanding of how specific nutrients, such as omega-3 fatty acids, can influence early-life nutritional programming, offering insights that inform strategies to promote human health and development [46]. Additionally, chickens have been recognized as effective models for exploring adipokine-mediated regulation in metabolic and reproductive diseases, with findings showing strong parallels to similar conditions in humans [47]. They also provide a robust system for studying human lipid metabolism disorders, including non-alcoholic fatty liver disease [48].

Table 2. Advantages of transgenerational transmission research in chicken model.

Advantage		Description & Implications
Oviparity (Egg-laying)		Embryonic development occurs outside the mother, allowing precise control over embryonic environment, and minimizing maternal influences. Researchers can investigate nutritional influences without the interference of maternal hormones, leading to a clearer understanding of how diet affects growth and metabolism.
Independent Offspring (Precociality)		Many bird species (e.g. chickens, quail) are self-sufficient at hatching, reducing postnatal confounding factors from parental care.
High Offspring Numbers		Hens can lay up to 300 eggs per year. Scientists can produce enough offspring to thoroughly examine the long-term impacts of nutritional interventions (enhanced statistical power for detecting transgenerational effects).
Short Generation Time		Early sexual maturity. Short interval between generations. Facilitates multi-generational studies within a practical timeframe. Make it easier to track how dietary changes affect future generations.
Reduced Cage Effects		The temperature of incubation and humidity can be easily and strictly controlled to minimize the interindividual environmental variability. Group rearing reduces environmental variability among siblings.
Well-Studied Genome		Detailed knowledge about its genome, allowing for integration of epigenomics and transcriptomics.
Cost effectiveness and less-ethical consideration		Chick embryos can be incubated and manipulated both *in ovo* and *ex ovo* at low cost, with minimal infrastructure, space, or feed requirements. Less ethical consideration in research than mammals Experiments on chick embryos up to day 14 do not fall under the EU Directive.

More recently, chicken primordial germ cells (PGCs) have emerged as a powerful model for investigating the interplay between metabolism and epigenetics, particularly in relation to how

prenatal nutritional and environmental exposures can shape epigenetic transmission associated with metabolic disorders (Reviewed in Paper I [4]).

4.4. Chicken PGCs

Chicken PGCs are first seen at the center of the blastodisc at Eyal-Giladi stage X [49, 50]. Following the development of the primitive streak, these cells translocate anteriorly to the germinal crescent. By Hamburger-Hamilton (HH) stages 10–12, PGCs enter the vascular system and actively migrate through the dorsal mesentery to reach the genital ridges [49, 50]. Chicken PGCs are characterized by several molecular markers that are crucial for their identification and study such as *SSEA-1*, *EMA-1*, *SSEA-4*, and *SSEA-3* [51]. The expression of pluripotency markers such as *POUV*, *SOX2*, and *NANOG*, along with germ cell markers like *DAZL* and *CVH*, further defines the molecular profile of chicken PGCs [52]. These markers are consistently expressed across various conditions, including fresh isolation, cryopreservation, and in vitro culture, indicating the cells' stability and resilience [53-55].

The early developmental migration of avian PGCs through the bloodstream makes them uniquely accessible for collection, an advantage not easily achievable in mammalian models [56]. Chicken PGCs can be isolated from embryos at various stages of development, each offering unique advantages for research and application. The isolation of PGCs from embryonic blood is commonly performed at HH stages 14 to 16, where they are abundant in circulation before migrating to the gonadal regions [57]. Additionally, PGCs can be isolated from the embryonic gonadal regions at later stages, such as HH 26-28, where they have migrated and begun to settle [58]. The choice of stage for isolation depends on the intended application, such as genetic engineering or germplasm preservation, and the specific characteristics of PGCs at each stage. For instance, Gong et al. demonstrated that PGCs isolated at different time points (embryonic day (ED) 3.5, 4.5, and 5.5) showed notable variations in migration and proliferation abilities, with ED 3.5 being optimal for *in vitro* long-term culture cell line establishment due to higher proliferation and migration capabilities [59]. Interestingly, female and male PGCs at ED 5.5 exhibits robust DNA damage repair capability, making them particularly suitable for long-term in vitro cryopreservation [59].

Chicken PGCs represent a unique model for in vitro culture, as chickens are currently the only vertebrate species whose PGCs can be stably maintained in culture over extended periods [60]. This capability has been extensively documented, with numerous studies demonstrating that chicken PGCs retain their germline identity and functionality during long-term culture and even after cryopreservation [53, 61]. As a result, developing optimized culture systems for chicken PGCs has become an active area of research, with comparative studies exploring different media formulations to enhance cell growth and expansion [57].

PGCs in chicken exhibit a unique epigenomic landscape that, while sharing some conserved features with mammalian PGCs, also displays distinct epigenetic signatures shaped by avian-specific evolutionary and developmental processes (Reviewed in [24]). In chickens, PGCs are specified through preformation, relying on maternally inherited determinants, in contrast to the inductive specification mechanism observed in mammals [62]. Unlike mammalian PGCs, which undergo global DNA demethylation, chicken PGCs do not exhibit such extensive demethylation. Instead, they display a reduction in 5-hydroxymethylcytosine levels and chromatin decompaction [62].

The epigenetic regulation of chicken PGCs involves a combination of DNA methylation, histone modifications, and non-coding RNAs, all of which play critical roles in germ cell development and differentiation [24, 63]. Figure 2 summarizes the epigenetic reprogramming events in chicken germ cells, highlighting the main features across different molecular layers (DNA methylation, histone modifications, and non-coding RNAs) [24].

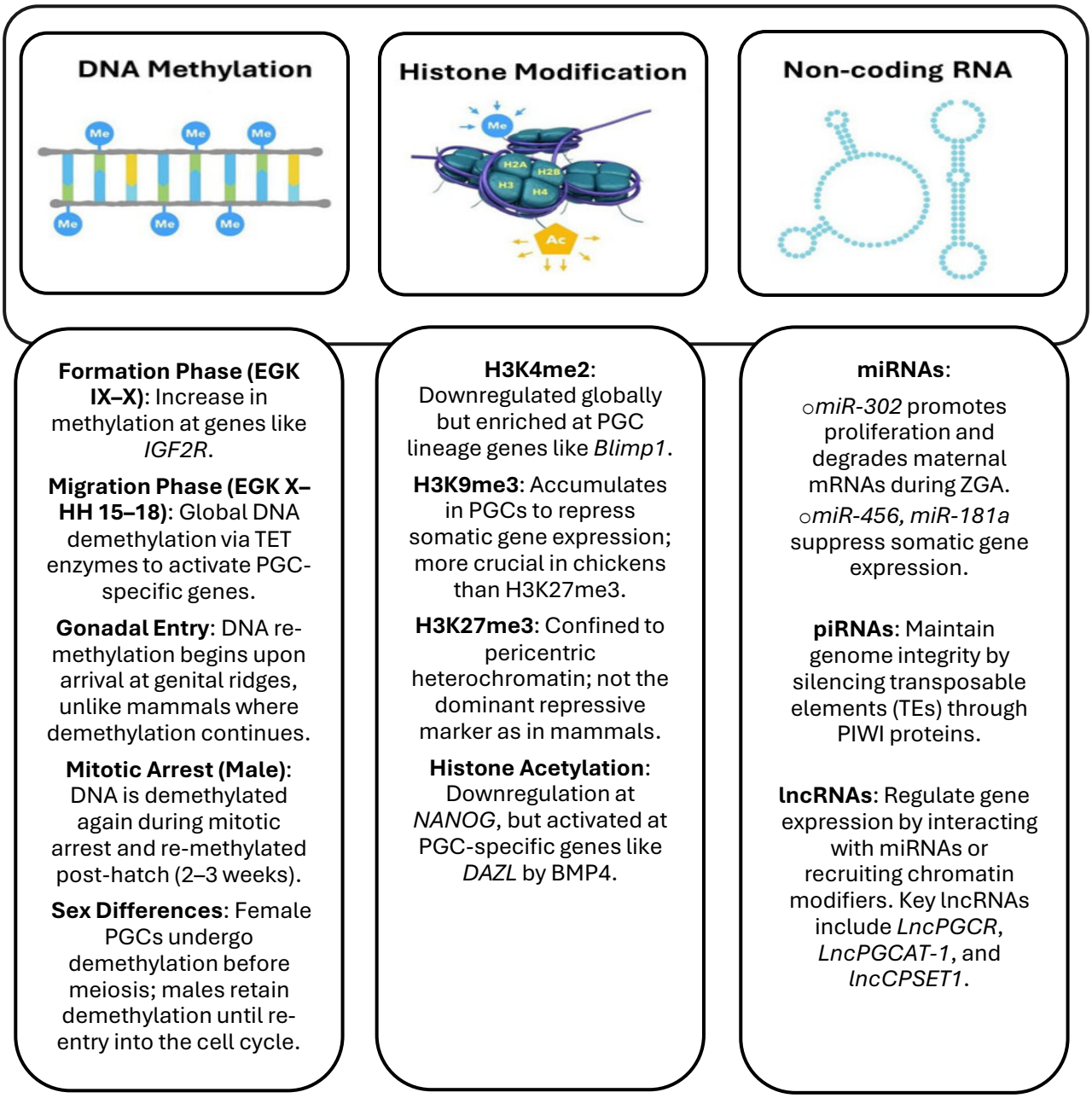


Figure 2. Epigenetic reprogramming in chicken germ cells. H3K27me3: Histone H3 lysine 27 trimethylation; H3K4me2: Histone H3 lysine 4 dimethylation; H3K9me3: Histone H3 lysine 9 trimethylation; EGK: Eyal-Giladi and Kochav stage; IGF2R: Insulin-like growth factor 2 receptor; TET: Ten-eleven translocation enzyme; *NANOG*: Nanog Homeobox gene; *DAZL*: Deleted In Azoospermia Like gene; *BMP4*: Bone morphogenetic protein 4; TEs: transposable elements.

4.5. *In ovo* stimulation

In ovo feeding (IOF) is a cost-effective method for researching early nutrition or epigenetic stimulations in chickens (Discussed in Paper I). It was initially developed for vaccine delivery in broiler hatcheries [64]. Nowadays, this approach includes a wide range of substances, including nutrients such as glucose, amino acids, and vitamins, as well as supplements like probiotics, prebiotics, exogenous enzymes, hormones, vaccines, drugs, and nutraceuticals [64]. *In ovo* injection into the air cell is considered safe and is recommended for delivering various bioactive compounds. The air cell is particularly suitable for administering prebiotics, probiotics, and their combinations (synbiotics) on ED 12 [65]. At this stage, the chorioallantoic membrane is well vascularized, allowing efficient passive transfer of water-soluble prebiotics from the air cell into the embryonic bloodstream [65]. Probiotics, on the other hand, are likely ingested by the embryo during the hatching process. On the other hand, IOF involves injecting nutritional substances into the amniotic fluid between ED 14 and ED 18 [64]. Since the embryo naturally ingests amniotic fluid prior to hatching, IOF enables the direct delivery of essential nutrients at a critical stage, supporting the chick's metabolic demands during the intensive hatching period [64]. ED 14 is preferable for administering carbohydrates, hormones, and similar substances, while ED 17.5 is more suitable for probiotics, CpG-Oligodeoxynucleotides (CpG-ODNs), vitamins, amino acids, and related bioactives [64].

IOF has been extensively utilized to explore how various compounds influence embryonic growth, hatch success, post-hatch performance, and the development of the digestive and immune systems. Since embryonic nutrition plays a pivotal role in shaping the formation and function of organs and tissues, both IOF and *in ovo* injection serve as precise methods for delivering targeted nutritional interventions or other epigenetic factors. These techniques also enhance the utility of the chicken embryo as a model for epigenetic studies, offering researchers a controlled way to examine how specific substances influence gene expression and long-term developmental outcomes (Paper I [4]). Recently, the *in ovo* injection of bioactive compounds is one of the most investigated approaches. Table 3 shows a summary of some *in ovo* applications of bioactive substances in chicken.

Table 3. A summary of some recent *in ovo* application studies of different bioactive substances in chicken.

Injected substance	ED	Main Aim	Main findings	Ref.
Carvacrol	17.5	To improve early immune function in chicken	Enhanced anti-pathogenic and pro-inflammatory responses in the yolk sac via upregulation of antimicrobial peptides, and NOD-like receptor pathways	[66]
Vitamin C	11	To investigate the effects of *in ovo* feeding of Vitamin C on embryonic development, egg hatching time, and chick rectal temperature	Accelerated hatching process and reduced chicks' rectal temperature	[67]
Formula product	18	To determine the effect of *in ovo* feeding of the formula product on hatching parameters, some organ characteristics and ileal histology	Positive effects on hatching parameters, small intestinal development and ileum histology	[68]
Glutamine	18	To evaluate the short- and long-term effects of *in ovo* administration of glutamine on intestinal epithelial development and functions	Increased intestinal villus morphology, epithelial cell proliferation, and differentiation, and altered epithelial cell population toward absorptive cells	[69]
Bacillus subtilis (probiotic), raffinose (prebiotic), and their combinations	12.5	To assess the response of chicks to *in ovo* injection of Bacillus subtilis, raffinose, and their combinations	Positive effects on hatching traits, cecal microbial populations, intestinal histomorphometry, nutrient transport- and intestinal function-related genes, and chick quality of newly hatched chicks	[70]
Oregano essential oil	17.5	To investigate the metabolic impacts of oregano essential oil	Elevated expression of key enzymes and receptors involved in detoxification pathways and lipid metabolism in the jejunum of hatchling chicks	[71]
selenium (Se) and zinc (Zn)	14	To assess the effect of *in ovo* feeding of selenium and zinc on hatchability, production performance, liver, intestinal morphology, antioxidant levels and expression levels of immune-related genes in broiler chickens	Enhanced cellular immunity in the broiler chickens	[72]

Organic selenium	10	To investigate the effects of *in ovo* injection of organic selenium on the hatching traits of broiler chickens and their performance	Positive effects on performance of broiler chickens. No effect on immune response or microbial population	[73]
Grape pomace extract	17.5	To evaluate the impacts of *in ovo* feeding during early embryonic development using grape pomace extract as a natural antioxidant on hatchability, productive performance, immune response, and antioxidant status in broilers	Enhanced growth performance, immune response, and antioxidant status in hatched chicks	[74]
Lactoferrin	15	To investigate the effects of *in ovo* lactoferrin injection on some physiological parameters and immune response of post hatch chicks	Improved hatchability, lipid profile, immune response and antioxidant indices	[75]
Galactooligosaccharide (GOS) or *Lactiplantibacillus plantarum*	12	To investigate the modulatory impacts of *in ovo* delivery of prebiotic and probiotic on oxidative stress, the intestinal transcriptome, and various plasma metabolites in chickens	Enhanced immune system and improved antioxidant status and gut health of chickens with no negative impact on plasma blood metabolite indices	[76]
Betaine	12	To investigate the effects of *in ovo* inoculation of betaine on hatchability, hatching weight, and intestinal development, as well as serum and expression levels of some antioxidants in the post hatched chicks	Positive effects on intestinal morphometry by ameliorating the jejunal villus length, the ratio of villus height to villus width, and absorptive surface area	[77]
Zinc glycine chelate (Zn-Gly) and a multistrain probiotic	17	To determine the effect of *in ovo* administration of zinc glycine chelate (Zn-Gly), and a multistrain probiotic on the hatchability and selected parameters of the cellular and humoral immune response of chickens	Effect on lymphocyte proliferation and stimulation of cellular immune mechanisms in birds	[78]
Lactobacillus Plantarum	14	To explore the effects of broiler embryonic injection of Lactobacillus Plantarum on the growth performance, lipid metabolism of serum and liver, microbial diversity, and short-chain fatty acids of broiler intestines after hatching	Promoted production performance and altered serum metabolism based on modulation of the intestinal microbiota and its metabolites	[79]

Trace elements zinc (Zn) and selenium (Se)	14	To investigate the effect of trace elements Zn and Se supplementation on histomorphology, immunological role, and functional activity of goblet cells of the small intestine	Positive effect on histomorphology and functional activity of goblet cells of the small intestine	[80]
Copper	10	To assess impacts of early *in ovo* injection of copper on histomorphometric parameters of small intestine and growth performance of post-hatched chicks.	Improved growth performance and small intestine histomorphometry parameters	[81]
Manganese	9	To study the effect of *in ovo* manganese injection on the embryonic development, antioxidation, hatchability, and performances of offspring broilers under normal and high temperature	Improved antioxidative ability in the chick embryonic heart, with no effect on other performances of embryos and performances of offspring boilers under different temperatures	[82]
L-Arginine	18	To evaluate the effects of *in ovo* feeding of L-arginine on the hatchability, growth performance, antioxidant capacity, and meat quality of slow-growing chickens	Increased antioxidant capacity of the breast muscle in the starter period	[83]
synbiotic (Galacto-oligosaccharide and *Lactobacillus salivarius*) / synbiotic (RFO (lupin-based oligosaccharides of the raffinose family) and *Lactobacillus plantarum*)	12	To investigate whether injecting synbiotics into the egg air chamber of embryo incubation will affect the processes of angiogenesis, and thus the share of histopathological changes in superficial pectoral muscle	Positive effect on the capillarity of the pectoral muscles of chickens, with a lower share of degenerative changes, such as muscle fiber necrosis or splitting due to better nutrition and oxygenation	[84]
Vitamins (A and D) and probiotic *Lactobacilli*	18	To determine the effects of *in ovo* inoculation of vitamins A and D either alone, or in combination	Developed immune competence	[85]

		with probiotic *lactobacilli* on chicken immune responses		
Betaine	11	To investigate the effect of *in ovo* injection of betaine on adrenal steroidogenesis in chicken fetuses	Promoted adrenal glucocorticoid synthesis in chicken fetuses before hatching, involving alterations in DNA methylation	[86]
Vitamin C	11	To explore effects of *in ovo* feeding of vitamin C on splenic development	Regulated splenic development and maturation by affecting purine nucleotide metabolism pathway and promoting apoptosis	[87]
L-arginine	14	To evaluate the effect of *in ovo* injection of arginine on hatchability, immune system and caecum microflora of broiler chickens	Improved effect on caecal microflora	[88]

4.6. Summary of the background

The study of the literature showed that research on genome-environment interactions is gaining more attention as early life experiences and environmental cues are found to leave lasting marks on phenotype, with potential effect on future generations. Prenatal life is a critical time when organisms are especially sensitive to their surroundings. The chicken model offers unique advantages for studying genome-environment interactions, as it allows precise temporal control over environmental exposures without maternal confounding effects.

5. Methodology

5.1. Ethical consideration

The study received approval from the Local Ethical Committee for Animal Experiments in Bydgoszcz, Poland (Approval No. 15/2022, dated 20.04.2022), and was conducted in compliance with Directive 2010/63/EU and Regulation (EU) 2019/1010. The research is reported in line with the ARRIVE guidelines [89] (https://arriveguidelines.org). Throughout the study, animal welfare was closely monitored. The birds were raised under standard poultry farming conditions, cared for by trained personnel, and regularly checked by a veterinarian.

5.2. Selection and dosage testing of choline and synbiotic

Two separate experiments were carried out to identify the optimal dose and pairing of choline with synbiotic supplement (detailed in Paper III, Figure 3).

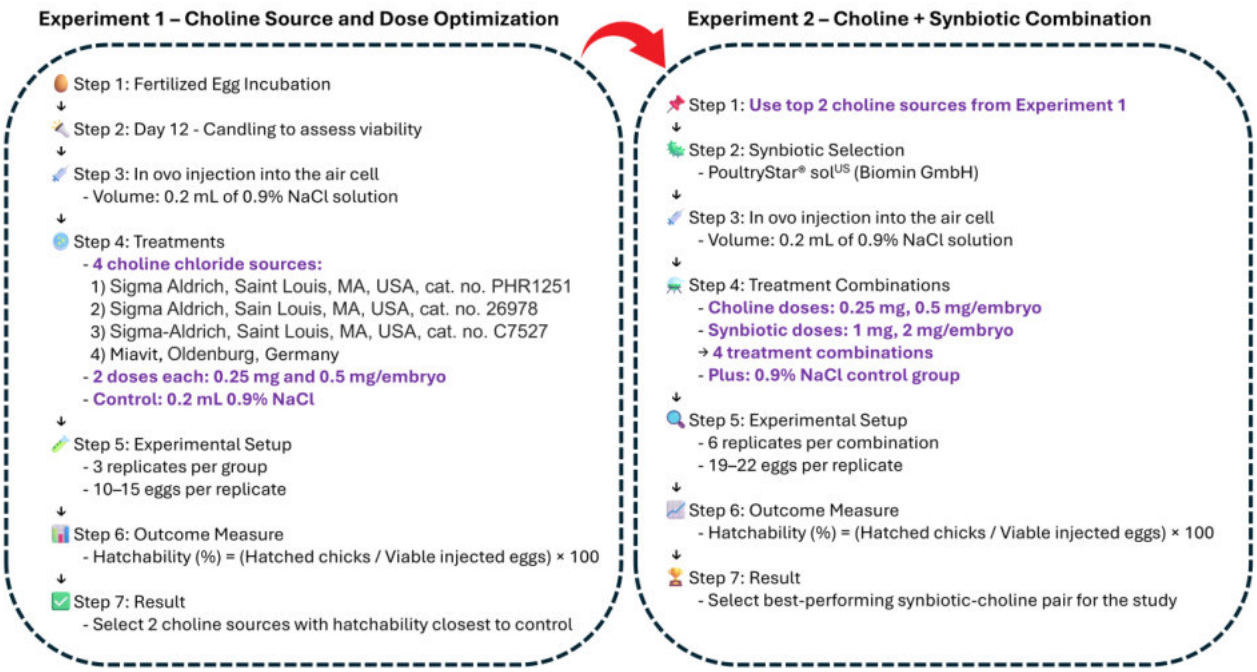


Figure 3. Selection and dosage testing of choline and synbiotic.

The synbiotic used in this study, PoultryStar® sol^{US} (Biomin GmbH, Herzogenburg, Austria), is detailed in Table 4. The objective was to find a combination that would maintain hatchability rates on par with those observed in the untreated control group. The *in ovo* injection method employed 0.2mL of a 0.9% NaCl solution, following a protocol optimized by Bednarczyk et al. to deliver compounds safely during incubation without interfering with embryo development [90, 91].

Table 4. Synbiotic product description (PoultryStar® sol^{US}).

Component	Details
Type	Commercial synbiotic (prebiotic + probiotic) formulation
Prebiotic	Inulin
Probiotic Strains	- *Pediococcus acidilactici* (cecum)
	- *Bifidobacterium animalis* (ileum)
	- *Enterococcus faecium* (jejunum)
	- *Lactobacillus reuteri* (crop)
CFU	5.0×10^9 CFU/g
Solubility	Water-soluble, suitable for *in ovo* delivery

5.3. Birds

The research was conducted using Green-legged Partridgelike chickens (Figure 4), a traditional Polish breed (Zielononóżka kuropatwiana) known for its hardiness and ability to adapt to various environmental conditions. These slow-growing birds are well-suited to different climates thanks to their low dietary needs, strong health, and natural resistance to environmental stressors [92]. They also display strong maternal instincts. Unlike commercial poultry lines, this breed hasn't been heavily subjected to selective breeding, which has helped maintain a broad genetic pool. This genetic diversity makes them especially valuable for studies focused on transgenerational effects. Green-legged Partridgelike chickens are outbred lines. According to Guerrero-Bosagna outbred lines may manifest higher susceptibility to epigenetic modifications when compared to inbred counterparts, rendering them a good model for observing effects across generations [93].

Figure 4. Green-legged Partridgelike chickens post-hatching. A breed-characteristic black striping of the hatchlings is clearly exposed.

5.4. Experimental design

Table 5 shows the details of the *in ovo* based experimental design (detailed in Paper III, paper IV, Manuscript V).

Table 5. Experimental design and conditions summary.

Category	Details
Species & Breed	Green-legged partridgelike chicken
Location	Commercial hatchery, Wągrowiec, Poland
Incubation Conditions	- Initial 18 Days: 37.5 °C, 55% relative humidity, eggs rotated every 2 hours
	- Final 3 Days (Hatcher): 36.9 °C, 65% relative humidity
Injection timepoint	Day 12 of embryonic development
Injection Method	Manual air cell injection using 0.2 mL solution (0.9% NaCl-based), sealed with non-toxic adhesive
Choline Source	Sigma Alrich, Sain Louis, MA, USA, cat. no. C7527
Synbiotic Source	PoultryStar®, sol^{US} (Biomin GmbH, Herzogenburg, Austria)
Embryo Injection (F1 embryos)	Candled and randomly assigned to: 1. SYN (Synbiotic): 2mg PoultryStar® in 0.2mL of 0.9% NaCl 2. SYNCH (Synbiotic + Choline): 2 mg synbiotic + 0.25 mg choline in 0.2mL NaCl 3. Control (C): 0.2mL 0.9% NaCl only

Experimental Groups (F2/F3)	1. SYNs: Descendants of SYN (no further injection) 2. SYNCHs: Descendants of SYNCH (no further injection) 3. SYNr: SYN with repeated injection in F2 and F3 4. SYNCHr: SYNCH with repeated injection in F2 and F3 5. Control (C)
F4 Assessment	F4 embryos (embryonic blood samples)
Birds per Generation	30 birds per group in each generation, 3 groups in F1 and 5 groups (F2 & F3), 2 replicates per group
Housing Conditions	Semi-intensive floor pens with chopped wheat straw and perches
Temperature Regime	Maintained at 16–18 °C during colder months (adjusted according to breed requirements)
Lighting Conditions	Natural daylight supplemented with artificial light - Growth period: 12 h light / 12 h dark - Reproduction period: Gradual increase to 16–17 h light (from 20 to 36 weeks of age)
Diet	Standard commercial feed (Golpasz, De Heus, Golub-Dobrzyń, Poland); free from antibiotics, probiotics, prebiotics
Laying Hen Diet	75% winter wheat + 25% De Heus layer concentrate (Product Code: 1957 - HD660X00S-W00)
Water	Clean drinking water provided *ad libitum*
Body Weight Assessment	At 21 weeks of age: 10 randomly selected adult birds/group 12-hour fasting period prior to measurement

The project was carried out over three generations with additional assessment of F4 embryos. Three experimental groups were established in F1 generation: synbiotic (SYN), synbiotic with choline (SYNCH) and the control group (C, 0.9% physiological saline). The SYN and the SYNCH groups were further split into two groups in later generations, such that two new groups were formed: repeatedly injected synbiotic (SYNr) group and repeatedly injected synbiotic plus choline (SYNCHr) group. Additionally, the original SYN and SYNCH groups continued with the initial single injection established in F1 embryos, referred to as the SYNs and SYNCHs groups, respectively (Paper III, Paper IV, Manuscript V, Figure 5).

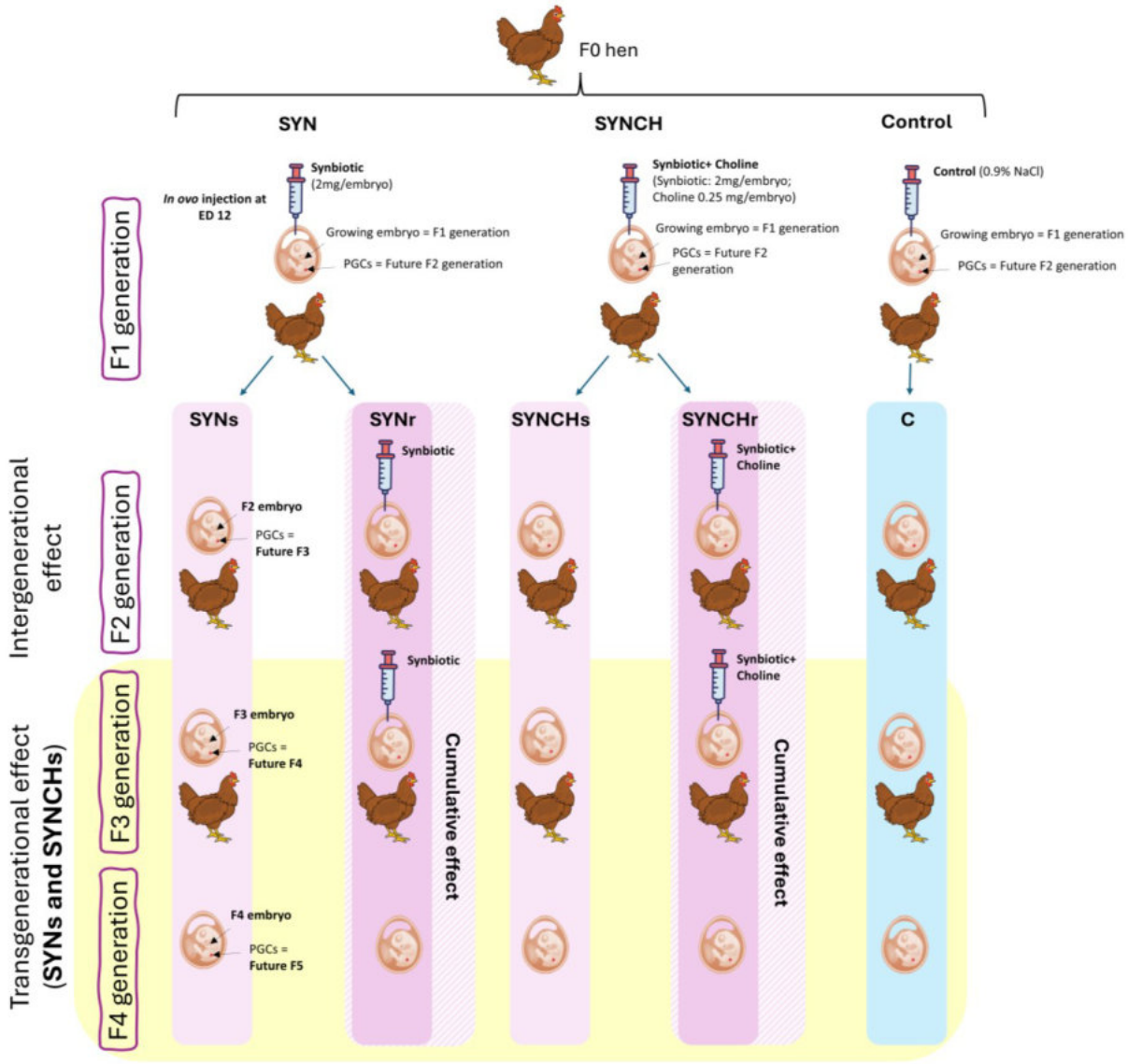


Figure 5. The experimental design of the study (Manuscript V).

5.5. Samples preparation

Samples were obtained from adult male chickens (21 weeks old) and 2.5-day-old embryos (Figure 6A). In adult chickens, the collected tissues included cecal tonsils (Paper III), cecal mucosa (Paper III), and gonads (Paper IV). From the embryos, blood samples containing circulating PGCs were isolated (Manuscript V). The presence of PGCs in the embryonic blood was validated through fluorescence-activated cell sorting (FACS), as illustrated in Figure 6B.

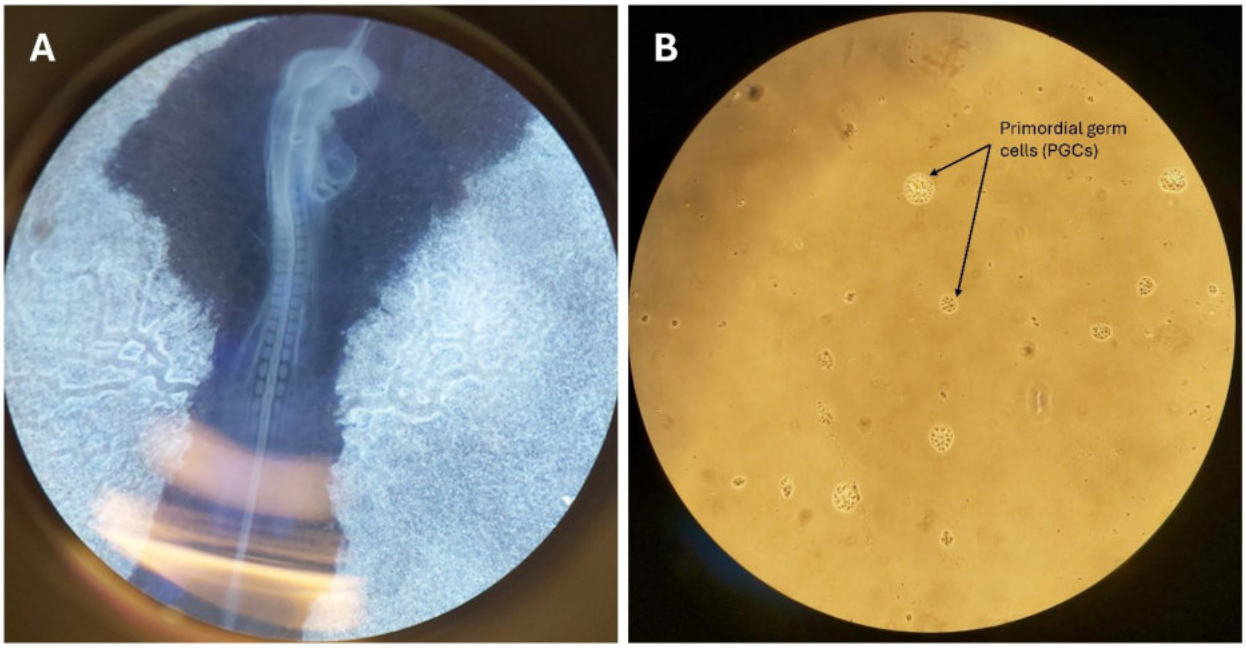


Figure 6. Visualization of a 2.5-day-old chicken embryo and detection of circulating primordial germ cells (PGCs). (A) Microscopic image of a 2.5-day-old chicken embryo at HH stage 14–16 (approximately 22 somites), captured after subgerminal cavity ink injection to enhance anatomical contrast. (B) Microscopic image of PGCs isolated from embryonic blood at the same stage, following fluorescence-activated cell sorting (FACS).

Figures 7 and 8 illustrate the experimental designs employed for multi-generational sample collection in this study. Figure 7 outlines the approach used to collect samples reflecting both intergenerational (from F2 generation) and transgenerational effects (from F3 and F4 generations) of synbiotic and choline injections. In contrast, Figure 8 presents the setup for collecting samples to evaluate the impact of repeated injections of the same treatments across successive generations. Detailed information regarding the samples collected is provided in Table 6.

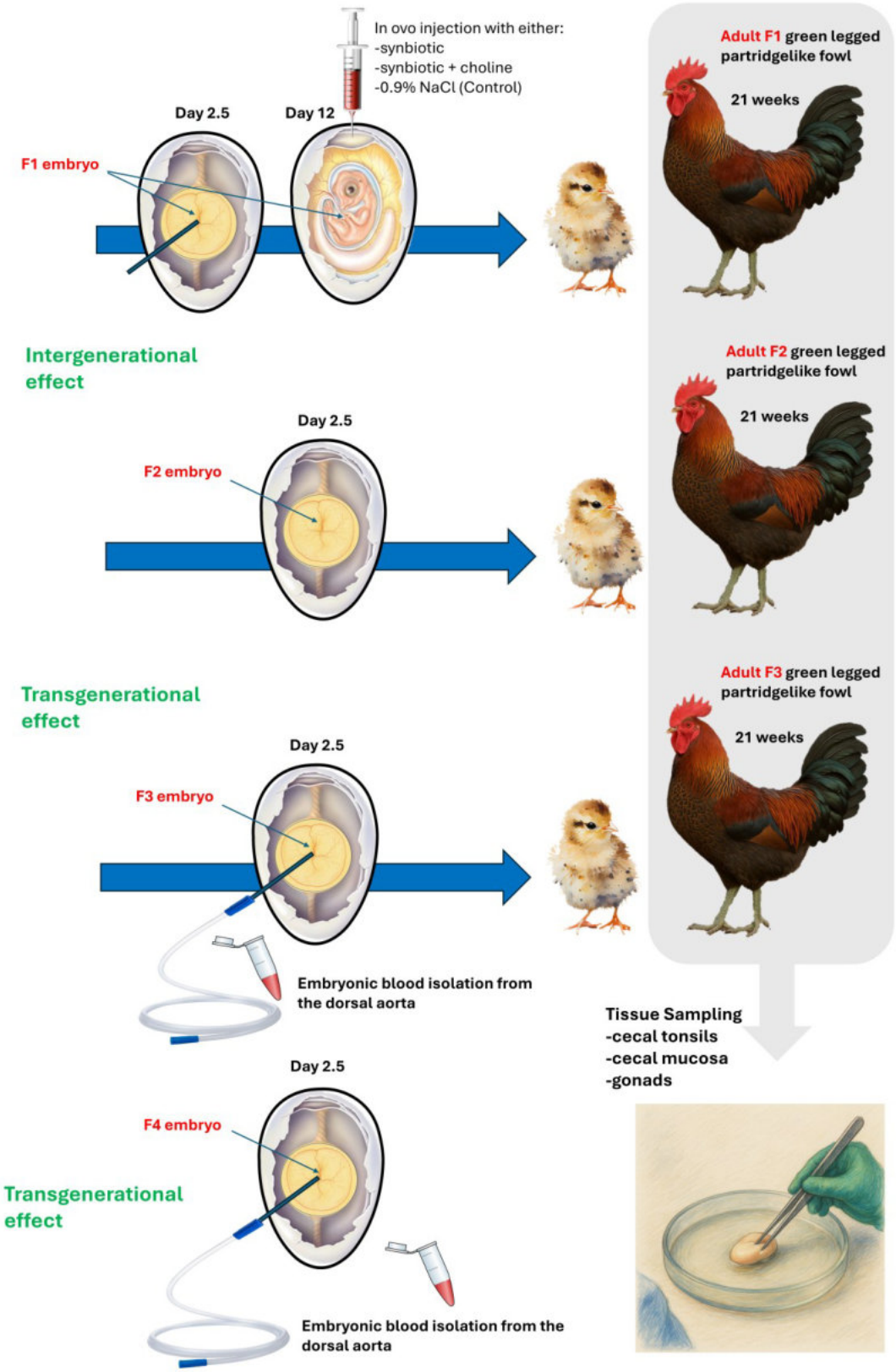


Figure 7. Experimental design for multi-generational sample collection in Green-legged Partridgelike fowls to study the inter- and transgenerational effects of the *in ovo* treatment.

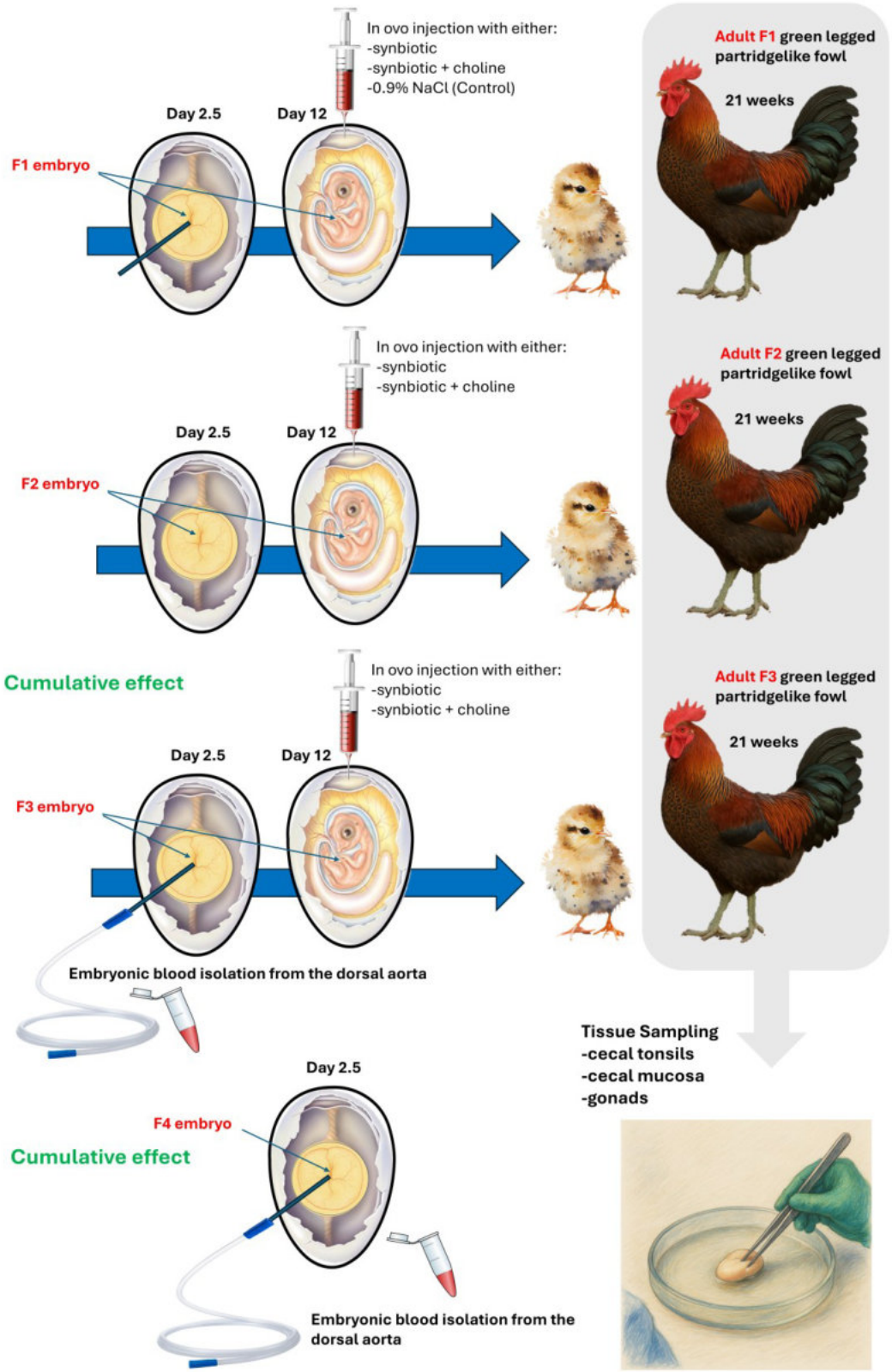


Figure 8. Experimental design for multi-generational sample collection in Green-legged Partridgelike fowls to study the cumulative effects of repeated *in ovo* treatments.

Table 6. Summary of tissue collection, processing, and analysis methods.

Sample Type	Generation/ Source	Collection Method	Extraction Kit / Method	Assessment Method	Purpose
Cecal Tonsils (Paper III)	F1, F2 and F3 21-week-old male chickens	Dissected from male chickens post-mortem. Stored in RNAlater (ThermoFisher Scientific, Waltham, MA, USA) at 4°C (initially), then at –80°C for later RNA isolation or frozen directly at -20°C for DNA isolation	RNA: GeneMATRIX Universal RNA Purification Kit (EURx, Gdańsk, Poland, cat. no. E3598) with RNA Extracol (EURx, Gdańsk, Poland, cat. no. E3700) DNA: Tissue DNA Purification Kit (EURx, cat. no. E3550)	RNA: Agilent Bioanalyzer 2100 with RNA Nano 6000 Assay Kit (Agilent Technologies, Santa Clara, CA, USA); 1% agarose gel DNA: Agilent Bioanalyzer 2100 with DNA 1000 Kit; 1% agarose gel	Transcriptome, RRBS Methylome sequencing
Cecal Mucosa (Paper III)	F1, F2 and F3 21-week-old male chickens				
Gonads (Paper IV)	F2 and F3 21-week-old male chickens				
Embryonic Blood (Manuscript V)	F3 and F4 embryos 2.5 days male embryos (HH stage 14–16)	Blood containing circulating PGCs collected from the dorsal aorta of embryos using a stereomicroscope (Figure 9). A fine glass microcapillary pipette (inner diameter: 30 µm, outer diameter: 40 µm) connected to a mouth pipette was used for precise blood collection. Blood samples from 60 embryos per group were individually transferred into Eppendorf tubes containing RNAlater and stored at 4°C.	RNA: GeneMATRIX Universal RNA Purification Kit (EURx, Gdańsk, Poland, cat. no. E3598)		Transcriptome sequencing

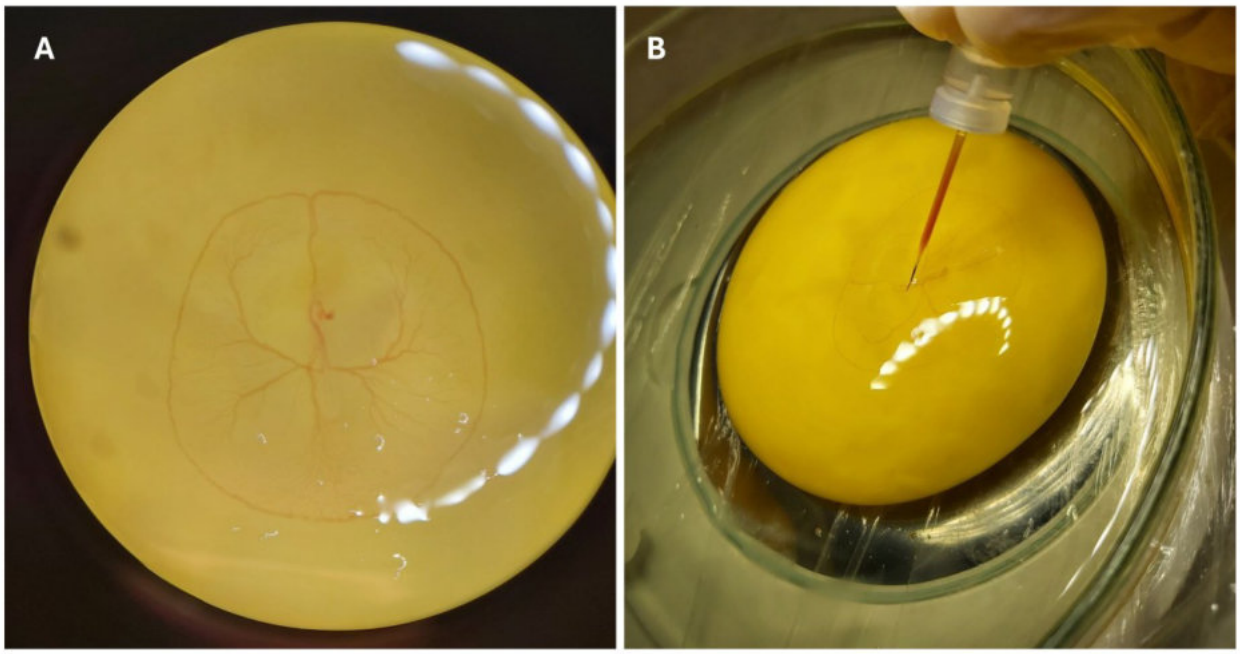


Figure 9. Embryonic blood isolation. (A) HH stage 14–16 embryo under a stereomicroscope; (B) Blood isolation using a fine glass microcapillary pipette connected to a mouth pipette.

5.6. Sex determination

Sex determination was performed to identify the sex of the embryo from which the blood sample was isolated (Detailed in Paper II and Manuscript V). DNA was extracted from each embryo, corresponding to its respective isolated blood sample. Sex determination of the embryos was performed as previously described by Clinton et al [94] and is summarized in Table 7.

Table 7. DNA extraction and embryo sex determination protocol.

Step	Details
DNA Extraction Kit	QIAamp Fast DNA Tissue Kit (Qiagen, Cat. No. 51404)
Sample Homogenization	Vortexed in lysis buffer for 30 sec
Lysis Conditions	Incubated in thermomixer at 56 °C, 1000 rpm for 5 min (TS-100C, Biosan, Riga, Latvia)
Sex Determination Primers	- Female-specific: *XhoI* W-repeat (415 bp) - Internal control: 18S ribosomal gene (256 bp)
Primer Sequences	- *XhoI* W-repeat: F: 5’-CCCAAATATAACACGCTTCACT-3’ R: 5’-GAAATGAATTATTTTCTGGCGAC-3’ - 18S rRNA: F: 5’-AGCTCTTTCTCGATTCCGTG-3’ R: 3’-GGGTAGACACAAGCTGAGCC-3’
PCR Product Analysis	Electrophoresis on 2% agarose gel stained with MIDORI Green Advance (NIPPON Genetics, Düren, Germany, Cat. No. MG04)

Electrophoresis Conditions	110 V for 35 minutes
Visualization System	G:Box Chemi XR5 (SYNGENE, Cambridge, UK)
Sex Identification Criteria	- Female: Two bands (*XhoI* W-repeat and 18S) - Male: Single band (18S only)
Sample Inclusion	Only male samples were included in the study

5.7. RNA-Sequencing and analysis

For every tissue, three RNA-seq libraries per treatment and control group in every studied generation were prepared using the Novogene NGS Stranded RNA Library Prep Set (PT044, Novogene, Cambridge, UK). Sequencing was conducted at a depth of 20M per sample on the Illumina Novaseq6000 platform by Novogene (Cambridge, United Kingdom), using a 150 paired-end sequencing kit for data generation. Table 8 lists all the bioinformatic tools used to identify differentially expressed genes (DEGs) between the treatment and control groups. DEGs were identified using an adjusted p-value ≤ 0.05, with a log2 fold change cutoff of 0 for cecal tonsils and cecal mucosa, and 0.58 for gonads and embryonic blood.

Table 8. Summary of RNA-sequencing workflow and bioinformatics analysis pipeline.

Step	Details	Tool/Platform	Version	Ref.
Quality Control	Quality assessment of raw data	FastQC	v0.12.1	[95]
Read Trimming	Removal of adapters and low-quality sequences	fastp	v0.23.4	[96]
Read Mapping	Reads were mapped to the chicken genome (bGalGal1.mat.broiler GRCg7b or Gallus gallus genome assembly GRCg6a, galGal6)	STAR	v2.7.11b	[97]
Differential Expression Analysis	Normalization and DEG identification (adj. p ≤ 0.05, |log2 fold change|=0 for cecal tonsils and mucosa or 0.58 for gonads and embryonic blood)	DESeq2	v1.42.0	[98]
Statistical Environment	DESeq2 Analysis	RStudio	v2024.09.0+375.pro3 and v2025.5.0.496	

Functional Enrichment	GO and KEGG analysis using ClusterProfiler and SRplot	SRplot/ clusterProfiler	–	[99], [100]
Pathway Visualization	KEGG pathways	Pathview	–	[101], [102]
Venn Diagrams	Overlap of DEGs visualized	jvenn	–	[103]

DEGs: differentially expressed genes; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

5.8. Reduced representation bisulfite sequencing (RRBS) library preparation

RRBS libraries were generated with the Zymo-Seq RRBS Library Kit (Cat. No. D5461, Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions (Paper IV). In total, 18 libraries were prepared from control, SYNCHs, and SYNCHr groups in both the F2 and F3 generations (n = 3 per group). Each library was constructed from 300 ng of genomic DNA with a 2% spike-in of *E. coli* genomic DNA (5 ng/µl). Library concentrations were quantified using a Qubit 4 fluorometer with the Qubit 1X dsDNA HS Assay Kit (Cat. Nos. Q33238 and Q33230, Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA). Quality assessment was performed on an Agilent 2100 Bioanalyzer using the Agilent DNA 1000 Kit (Cat. No. 5067-1504, Agilent Technologies, Santa Clara, CA, USA).

5.9. RRBS-sequencing and bioinformatic analysis

Table 9 shows the summary of RRBS-sequencing workflow and bioinformatic analysis. Details can be found in Paper IV.

Table 9. Summary of RRBS-sequencing workflow and bioinformatics analysis pipeline.

Step	Details	Tool/Platform	Version	Ref.
Sequencing	75-cycle paired-end sequencing	AVITI platform (Element Biosciences, San Diego, CA, USA); Genomed (Warszawa, Poland)	—	—
Quality control	Raw read quality assessment	FastQC	v0.12.1	[95]
Read trimming	Adapter/quality trimming	https://github.com/FelixKrueger/TrimGalore	v0.6.10	—

Alignment & methylation calling	Mapping to chicken genome (GRCg6a, galGal6)	Bismark (Bisulfite Read Mapper and Methylation Caller)	v0.24.2	[104]
Differential methylation analysis	Identification of DMLs and DMRs; coverage ≥10, Δmeth ≥20%, p ≤0.05	DSS package in RStudio	RStudio v2025. 5.0.496	[105]
Annotation of DMRs	Genomic annotation; TSS defined as ±3 kb	ChIPseeker TxDb.Ggallus.UCSC.galGal6.refGene	—	[106]
Functional enrichment analysis	GO and KEGG enrichment of DMGs (p ≤0.05, q ≤0.10, BH correction)	clusterProfiler	—	[100]
Integration with gene expression (RNA-seq)	Matching DMGs with DEGs by ENTREZID identifiers	org.Gg.eg.db annotation package	—	—

Δmeth ≥ 20%: the difference in DNA methylation levels between the treatment group and control; BH: Benjamini-Hochberg correction; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

5.10. Validation of RNA-seq data by quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

To verify the reliability of the RNA sequencing data, five up- and five downregulated DEGs for every tissue involved in different KEGG pathways were selected for RT-qPCR analysis. Six biological replicates were performed for each sample. The cDNA was prepared using the smART First strand cDNA Synthesis kit (Eurx, Gdańsk, Poland, cat.no. E0804). The cDNA was amplified by real time qPCR with primers (listed in Paper III, Paper IV) designed using Primer Blast [107]. Table 10 shows the parameters used for the RT-qPCR reaction. Amplification was performed in CFX Opus 96 real-time PCR system (BIO-RAD, CA, USA). The Pffafl (or standard curve) method was used to analyze the relative expression levels of the studied genes [108]. SRplot was used to visualize the PCR vs RNA-seq expression double Y axis plot.

Table 10. RT-qPCR reaction parameters.

Parameter	Details
Reaction Volume	20 µL
Template Input	50 ng cDNA
Enzyme Additive	0.25 U UNG (uracil-N-glycosylase)
Primers	15 pmol each (forward and reverse)
Master Mix	1× SG qPCR Master Mix (EURx, Gdańsk, Poland, cat. no. E0401)
qPCR Instrument	CFX Opus 96 Real-Time PCR System (BIO-RAD, CA, USA)
Thermal Cycling	- 50 °C for 2 min (UNG pre-treatment) - 95 °C for 10 min (initial denaturation) - 40 cycles of: • 94 °C for 15 s • 60 °C for 30 s • 72 °C for 30 s
Melting Curve	- 95 °C for 5 s - 70 °C for 5 s - Ramp to 95 °C at 0.5 °C/5s

5.11. Isolation, cryopreservation, and molecular characterization of chicken PGCs

Chicken embryonic blood containing circulating PGCs was collected at HH 14–16 from Green-legged Partridgelike embryos, with sex determined and samples pooled into male and female groups. Three experimental conditions were analyzed: freshly isolated blood containing PGCs, PGCs subjected to short-term cryopreservation involving freezing and thawing after two days, and PGCs maintained in culture for three months before undergoing long-term cryopreservation for two years. For cell culture, approximately 1–2µL of blood from individual embryos was cultured *in vitro* using the selective PGC medium developed by McGrew and colleagues [109]. To evaluate the effects of preservation, RNA was extracted from fresh samples using the GeneMATRIX Universal RNA Purification Kit (Eurx, Gdańsk, Poland, cat.no. E3598) and from thawed samples using the GeneElute Single Cell RNA Purification kit (Sigma-Aldrich, St. Louis, MO, USA, cat.no. RNB300). The expression of key germline markers (*SSEA-1*, *CVH*, *DAZL*) and pluripotency markers (*OCT4*, *NANOG*) was assessed by RT-qPCR. Cryopreservation and thawing were performed following established PGC freezing protocols (detailed in Paper II). Briefly, avian KO-DMEM was prepared by mixing DMEM (Thermo Fisher Scientific, Waltham, MA, USA, 21068-028) and sterile water (Thermo Fisher Scientific, Waltham, MA, USA, 15230-089) at a 2:1 ratio, supplemented with sodium pyruvate (Thermo Fisher Scientific, Waltham, MA, USA, 11360-039)

at 4 µL per 1 mL of medium. The resulting solution was divided into two portions; to one portion, dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA, 276855, final concentration 8%), chicken serum (Sigma-Aldrich, St. Louis, MO, USA, C5405, 10%), and $CaCl_2$ (Sigma-Aldrich, St. Louis, MO, USA, C-34006, 0.75%) were added to generate the DMSO-based freezing medium. The cell pellet was resuspended in 250 µL of avian KO-DMEM, after which 250 µL of the DMSO-based freezing medium was added slowly. The final cell suspension was transferred into cryovials and initially stored at −80 °C and subsequently moved to liquid nitrogen for long-term preservation after overnight storage. The detailed protocols for freezing-thawing and culturing can be found in Paper II.

6. Results

6.1. Highlights from Paper III: Effect of treatments on body weight

As part of the phenotypic assessment, we evaluated the long-term effects of *in ovo* bioactive compound administration on adult body weight. No significant differences in body weight were observed between the control and experimental groups across all three generations (F1–F3), regardless of whether the birds received synbiotic alone or synbiotic combined with choline. While a general decline in body weight was noted in the F3 generation compared to F2, likely due to seasonal production effects, this change was independent of the *in ovo* treatment. These results suggest that the administered bioactive compounds did not affect the long-term growth performance of chickens.

6.2. Highlights from Paper III, Paper IV and Manuscript V: Differential expression

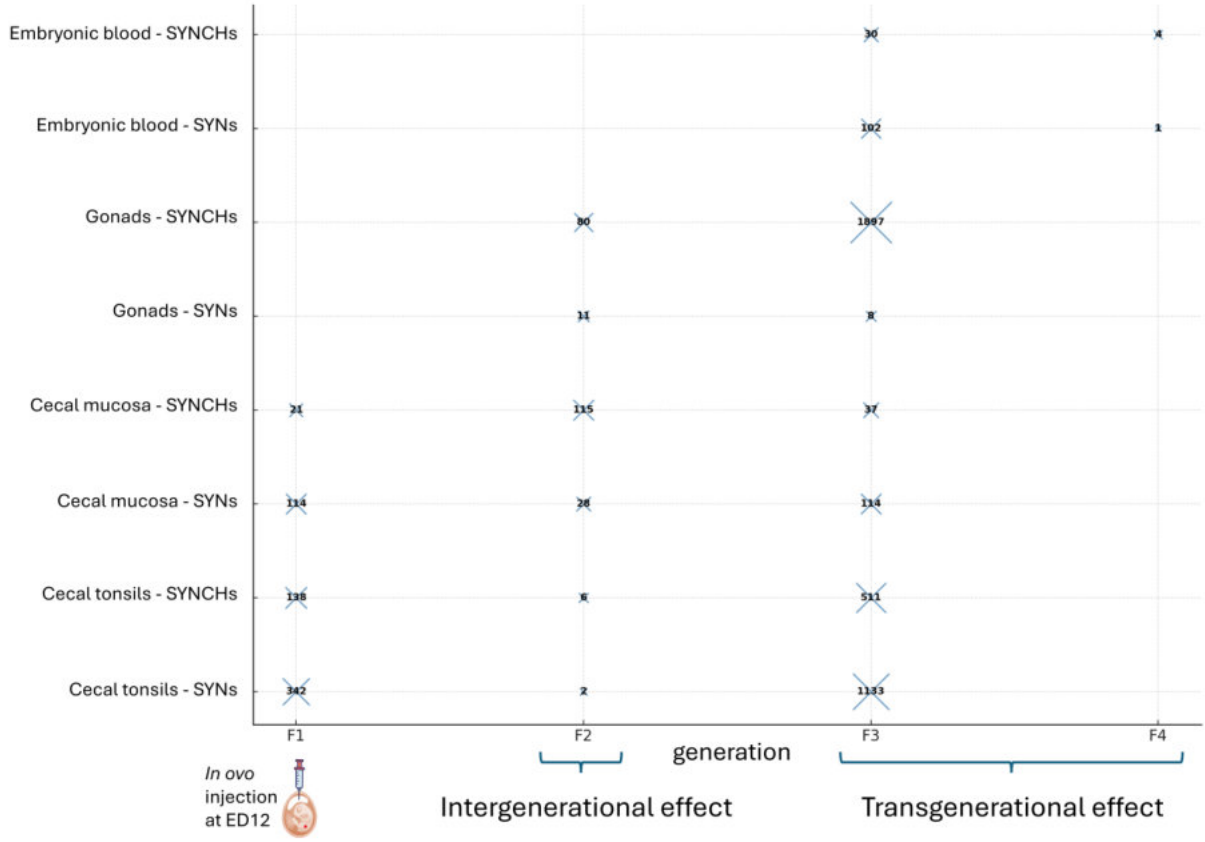


Figure 10. Comparative transcriptomic effects across generations in SYNs vs. SYNCHs (Scaled X-Marker Plot). Scatter plot showing the number of differentially expressed genes (DEGs) across generations (F1–F4) in cecal tonsils, cecal mucosa, gonads and embryonic blood tissues. Marker size is scaled by DEG count, with numeric values shown at each position. The plot was generated

in Python (version 3.10) using the Matplotlib tool (version 3.10.6) and subsequently modified manually to add additional information.

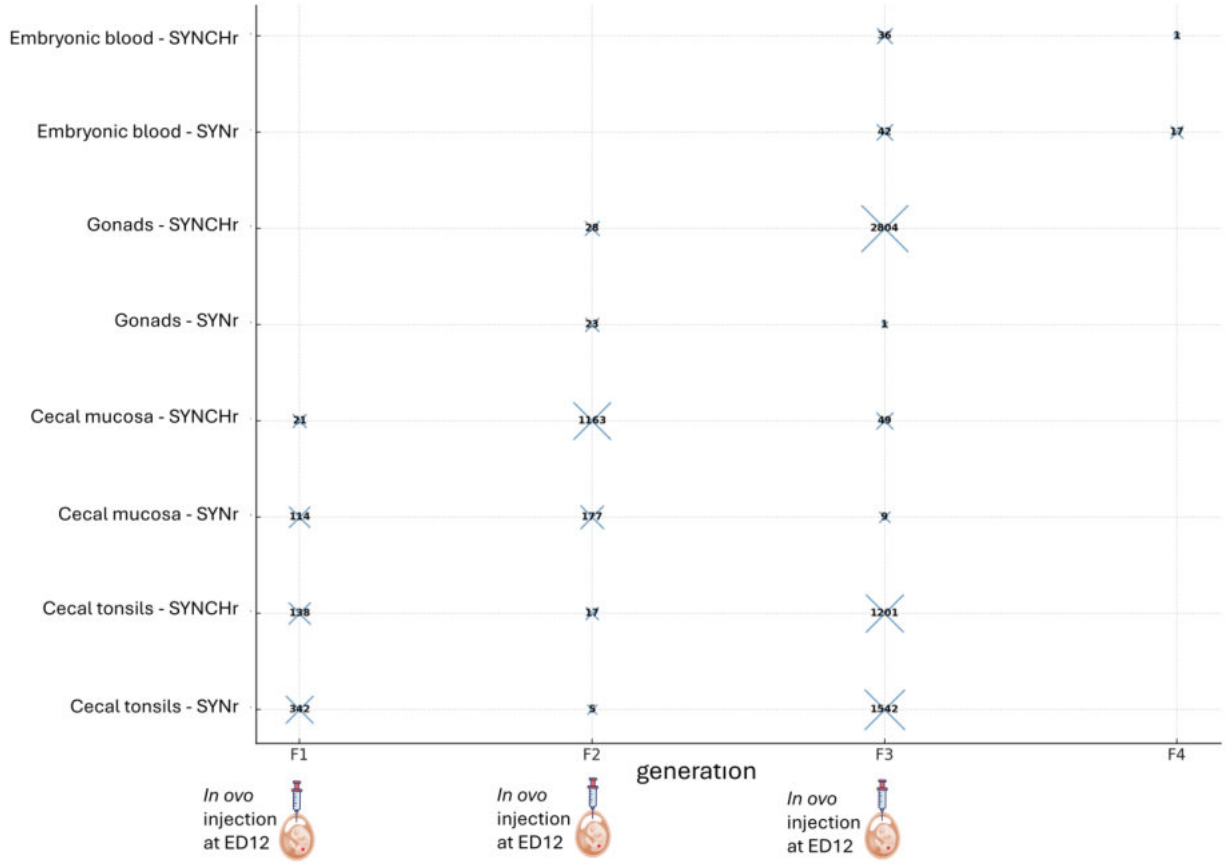


Figure 11. Cumulative transcriptomic effects across generations in SYNr vs. SYNCHr (Scaled X-Marker Plot). Scatter plot illustrating cumulative DEGs across generations (F1–F4) for cecal tonsils, cecal mucosa, gonads and embryonic blood tissues. Marker size is proportional to DEG counts, with values displayed inside the markers. The plot was generated in Python (version 3.10) using the Matplotlib tool (version 3.10.6) and subsequently modified manually to add additional information.

Across generations, both single *in ovo* injections of synbiotic and synbiotic plus choline in F1 embryos induced changes in gene expression compared to control, likely resulting in transgenerational transcriptomic effects in F3, though the magnitude and persistence varied by tissue (Figure 10). In the single-exposure design, synbiotic generally produced stronger effects than synbiotic plus choline except for gonads, where synbiotic plus choline triggered markedly higher numbers of DEGs. Embryonic blood also showed transgenerational responses extending into F4, though at lower magnitude. Repeated injections did not produce a clear or consistent cumulative effect (Figure 11), but the response pattern resembled that observed in the single-injection groups.

6.3. Highlights from Paper III, Paper IV and Manuscript V: GO and KEGG enrichment

Table 11. Summary of over-representation analysis (ORA) results for cecal tonsils, cecal mucosa, and gonads, and gene set enrichment analysis (GSEA) results for embryonic blood (general functional categories in each tissue).

Tissue	Group	F1	F2	F3	F4
Cecal tonsils (Paper III)	SYNs	GO: metabolism, homeostasis, immune signaling KEGG: metabolic pathways	Waning enrichment	GO: metabolism, homeostasis, immune signaling. KEGG: metabolic pathways	
	SYNr			GO: metabolism, homeostasis, immune signaling. KEGG: metabolic pathways, protein synthesis	–
	SYNCHs	GO: homeostasis, immune signaling KEGG: metabolic and immune-related pathways	Waning enrichment	GO: translation, metabolism KEGG: ribosome, cytoskeleton and immune-related pathways	
	SYNCHr			GO: translation, metabolism, enzyme activities. KEGG: metabolic and protein related pathways	–
Cecal mucosa (Paper III)	SYNs	GO: metabolic processes KEGG: metabolic and immune-related pathways	GO: Cell cycle, genomic regulation, metabolism KEGG: metabolism	GO: Immune-related processes	
	SYNr		GO: Cell cycle, genomic regulation, signal transduction KEGG: metabolic pathways, extracellular matrix, cell cycle	Waning enrichment KEGG: metabolism	–

	SYNCHs	GO: metabolism, response to reactive oxygen species, immune signaling KEGG: metabolic pathways, ribosome, phagosome	GO: morphogenesis and growth-related pathways KEGG: metabolism, cytoskeleton	GO: immune signaling and response, signal transduction KEGG: metabolic pathways	
	SYNCHr		GO: cell cycle, signal transduction KEGG: metabolic pathways, DNA replication, cytoskeleton, lysosome	GO: metabolism KEGG: metabolic pathways, extracellular matrix	–
Gonads (Paper IV)	SYNCHs	–	–	GO: Extracellular matrix, collagen binding, chromosomal organization KEGG: Motor proteins, cytoskeleton, Extracellular matrix -receptor interaction	–
	SYNCHr	–	GO: Motility, response to stimuli	GO: Cytoskeleton, Extracellular matrix organization, tissue migration KEGG: Motor proteins, cytoskeleton, extracellular matrix-receptor interaction, biosynthesis of nucleotide sugars.	–
Embryonic blood (Manuscript V)	SYNs	–	–	GO: metabolic processes, detoxification, protein related processes KEGG: metabolic, cytoskeletons and protein-related pathways.	GO: signal transduction. KEGG: ribosome, cell cycle, steroid biosynthesis

SYNr	–	–	GO: Protein synthesis and maturation, metabolic processes. KEGG: ribosome biogenesis, cytoskeleton	GO: structural constituent of ribosome, growth factor activity KEGG: ribosome, immune related pathways, signaling pathways
SYNCHs	–	–	GO: detoxification, protein-related processes, metabolism KEGG: ribosome, oxidative phosphorylation, cytoskeleton and extracellular matrix	GO: morphogenesis, transcription KEGG: ribosome, metabolic pathways, cytoskeleton
SYNCHr	–	–	GO: detoxification, Response to reactive oxygen species, protein-related processes KEGG: metabolic pathways, cytoskeleton, ribosome	GO: translation, detoxification, antioxidant activity. KEGG: ribosome and protein-related pathways, cell cycle, metabolic and immune-related pathways

Table 11 summarizes the top 10 enriched GO and KEGG functional categories identified by ORA and GSEA. Detailed lists of enriched terms and pathways are provided in Paper III (cecal tonsils and mucosa), Paper IV (gonads), and Manuscript V (embryonic blood). Enrichment patterns were tissue- and generation-specific, with metabolic and immune pathways predominating in cecal tissues, and extracellular matrix, cytoskeleton, and protein-related processes highlighted in gonads and embryonic blood.

6.4. Highlights from Paper IV: RRBS results

The RRBS analysis performed for SYNCHs and SYNCHr groups compared to control in gonadal tissue demonstrated a methylation profile broadly consistent with the RNA-seq output, with stronger effects observed in F3 compared to F2. Differentially methylated loci (DMLs) and regions (DMRs) across generations are summarized in Figure 12. In SYNCHs, methylation changes were mainly enriched in the TGF-β pathway, whereas in SYNCHr, changes were more pronounced and enriched in Wnt, focal adhesion, melanogenesis, and adipocytokine signaling pathways in F3. Integration of RRBS and transcriptomic data revealed overlap between methylation and gene expression in F3, involving 37 genes (47 DMRs) in SYNCHs, and 194 genes (306 DMRs) in SYNCHr. The majority of DMRs were intergenic (>70%), though promoter-associated DMRs were proportionally higher in SYNCHr (14.58% in F2 and 12.78% in F3) compared to SYNCHs groups (5.26% in F2 and 10.16% in F3).

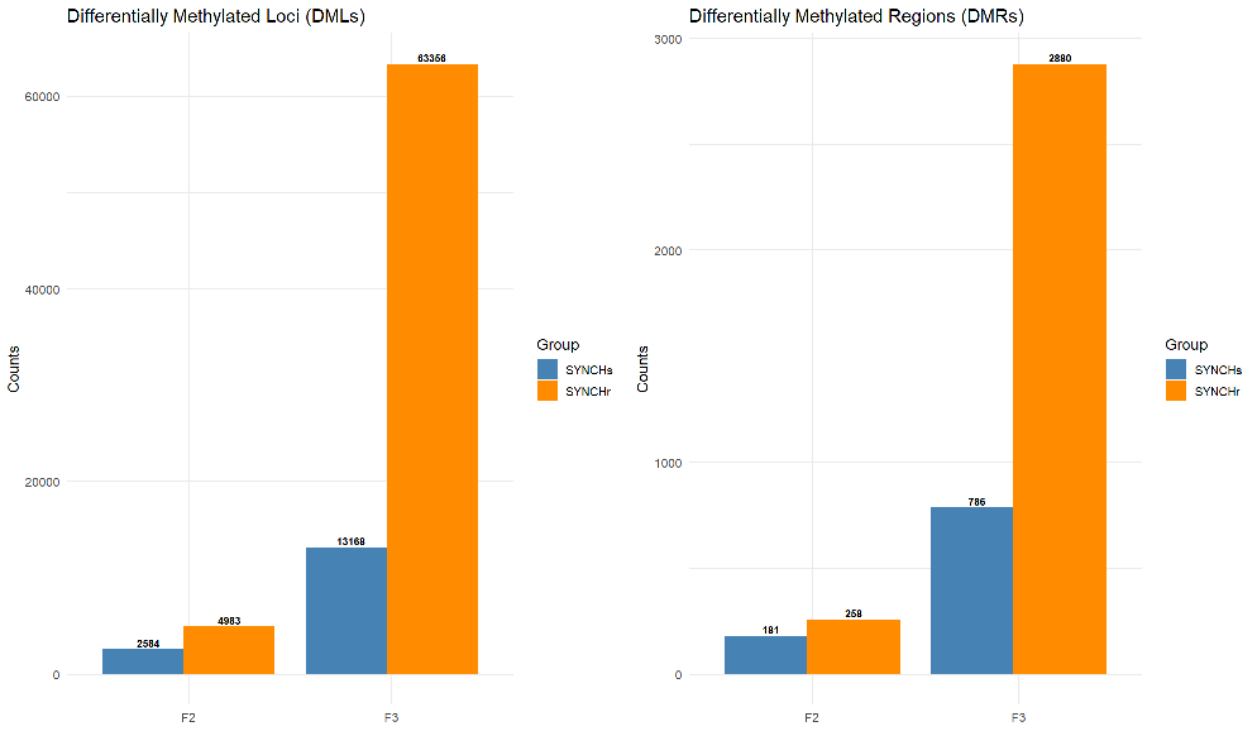


Figure 12. Bar plots showing counts of differentially methylated loci (DMLs, left panel) and differentially methylated regions (DMRs, right panel) in F2 and F3 generations for SYNCHs and SYNCHr groups. Plots were generated in R (ggplot2) based on counts of DMLs and DMRs identified across F2 and F3 generations.

6.5. Highlights from Paper III and Paper IV: Validation of RNA-seq results.

In cecal tonsils, cecal mucosa, and gonads, RT-qPCR validation confirmed the RNA-seq findings. Specifically, the analysis showed upregulation of genes with positive log2 fold changes and downregulation of genes with negative log2 fold changes among the 10 DEGs selected in each tissue (5 upregulated and 5 downregulated).

6.6. Highlights from Paper II: Cryopreservation of primordial germ cells

Chicken PGCs maintained stable expression of both germ cell-specific markers (*SSEA-1*, *CVH*, *DAZL*) and pluripotency markers (*OCT4*, *NANOG*) across various handling conditions: fresh isolation, short-term cryopreservation (2 days), and long-term cryopreservation (2 years after *in vitro* culture). There were no significant differences between male and female samples. Surprisingly, PGCs that had undergone freezing consistently exhibited higher levels of marker gene expression compared to freshly isolated cells. These results underscore the resilience of chicken PGCs to cryopreservation over time. The preservation of lineage-specific and pluripotency traits supports the viability, germline competence, and transcriptomic integrity of cryopreserved PGCs.

7. Discussion

7.1. Evaluation of the hypothesis

The hypotheses of this project were partially confirmed. *In ovo* application of epigenetic modifiers (synbiotic and choline) appears to induce both inter- and transgenerational effects in somatic tissues [cecal tonsils (Paper III), cecal mucosa (Paper III), and embryonic blood (Manuscript V)] and germline tissues [gonads (Paper IV)], altering transcriptomic profiles across generations. Early life epigenetic modifications (prenatal) can trigger transmissible changes in gene expression through intergenerational (F2) and transgenerational (F3 and beyond) transmission mechanisms. The magnitude and pattern of these changes varied depending on the injected substance, tissue, and generation. For example, cecal mucosa exhibited a strong effect in F2 that diminished by F3, whereas cecal tonsils showed a modest effect in F2 but a pronounced effect in F3. Embryonic blood displayed modest changes in F3 that decreased in F4, and gonads were more responsive to combined synbiotic and choline treatment than to synbiotic alone. Contrary to our hypothesis, repeated injections did not produce a consistently cumulative effect across generations; however, the transcriptomic profiles of repeated injection groups largely paralleled those of single injections (Paper III, Paper IV, Manuscript V). The remarkable resilience of chicken PGCs to both short- and long-term cryopreservation, with retention of their germline identity and viability, underscores their utility as a robust model for studying inter- and transgenerational transmission (Paper II).

7.2. Distinct transcriptomic trends in cecal tonsils and cecal mucosa

Our data reveal distinct inter- and transgenerational patterns in cecal tonsils and cecal mucosa following *in ovo* stimulation with synbiotic and synbiotic plus choline (Paper III). Cecal tonsils showed modest transcriptomic changes in F2 but a sharp increase in DEGs in F3, suggesting transgenerational effects. This pattern seems to be consistent with “generational skipping,” where epigenetic modifications are not consistent in every generation, and can be inherited silently and reappear in later generations. Evidence from mouse studies indicates that transgenerational effects may involve “generational skipping”, where phenotypes do not manifest in every generation. For example, Weber-Stadlbauer et al. [110] reported that

behavioral despair appeared in F2 and F3 offspring of immune-challenged ancestors, but not in the direct F1 generation, suggesting that F1 may act as a “silent carrier” of latent traits that re-emerge under specific conditions. Similar patterns have been observed in models of chronic stress [111, 112]. In our study, the modest changes in cecal tonsils in F2 may likewise reflect a silent carrier state. The reduction of DEGs in F2 could also represent a “washout effect” [113], whereas the resurgence in F3 may have been reinforced by environmental factors such as seasonal conditions, which were similar in F1 and F3 but differed in F2, potentially amplifying latent epigenetic signals. Seasonal differences between cohorts (F1 and F3 in autumn–winter vs. F2 in spring–summer) may have acted as environmental triggers, amplifying the re-emergence of effects in F3.

In contrast, cecal mucosa displayed a different trajectory: strong transcriptomic alterations in F2 (possible intergenerational), followed by attenuation in F3. Only in the SYNs group did effects in F3 approach F1 levels, suggesting variability across treatments. This trajectory resembles “washout” dynamics described in other transgenerational models, where epigenetic changes emerge strongly in one generation but fade or partially persist in the next [113].

A research on glyphosate exposure have supported the non-linear nature of transgenerational transmission [114]. Negligible effects were observed in F0 and F1, but a significant impact emerged in F2, with some of these changes persisted into F3, while others diminished or disappeared [114]. Although the pattern differs from ours, these findings support the broader principle that epigenetic effects are dynamic, variable, and may emerge, wane, or reappear across generations [113]. This parallels our observations, where environmental stimulation triggered generation-dependent transcriptomic changes of varying intensity, underscoring the unpredictable and evolving character of transgenerational transmission.

These divergent patterns may reflect the functional specialization of the two tissues. Cecal tonsils, as immune organs with continuous antigen exposure and a role in immune memory [115, 116], may be more prone to stable and re-emerging transgenerational effects. In contrast, cecal mucosa, with its primary functions in absorption and barrier maintenance [117], may show more transient responses. Importantly, such differences highlight the tissue-specific nature of epigenetic regulation, as demonstrated in other animal models where identical exposures produce distinct transcriptional and phenotypic outcomes across tissues. Such tissue-specific responses align with evidence that the same epigenetic stimuli can produce distinct outcomes

depending on the tissue context. For example, developmental exposure to diethylstilbestrol (DES) in mice induced tissue-specific DNA methylation and histone modification patterns in seminal vesicles and uterine tissues, driving divergent gene expression and phenotypes [118]. This underscores the importance of tissue-specific epigenetic regulation [119] in shaping inter- and transgenerational transmission patterns.

Overall, the findings emphasize that transgenerational epigenetic transmission is non-linear and context-dependent, with effects that may skip generations, diminish, or reappear depending on tissue type and environmental factors.

7.3. Distinct gonadal responses to synbiotic and synbiotic plus choline supplementation

The transcriptomic effects on gonadal tissue observed in the SYNCH groups, compared to SYN alone, may be explained by two main factors (Paper IV). First, choline is an essential nutrient involved in numerous physiological and epigenetic processes, including DNA methylation, neurotransmitter synthesis, cell membrane integrity, lipid metabolism, protein homeostasis, and the regulation of inflammation [120, 121]. These mechanisms are likely to exert stronger and more persistent influences on gene expression than the microbiota-mediated effects of synbiotics. While synbiotics can broadly modulate gut microbial composition and activity [122], their direct impact on reproductive tissues such as the gonads may be limited relative to choline. Second, the combination of synbiotics with choline may generate a synergistic effect, whereby choline amplifies the epigenetic influence of synbiotics, leading to the more pronounced and durable changes observed in SYNCH groups. Nutritional interactions of this type have been reported previously; for example, Handy et al. showed that nitrate and resveratrol supplementation each independently improved glucose tolerance and reduced cellular stress in high-fat-fed mice, but when co-supplemented, these effects were attenuated, highlighting the complexity of nutrient interactions [123].

Several studies further support the role of nutriepigenetic factors in shaping male gonadal gene expression and transgenerational transmission. In Atlantic salmon, micronutrient supplementation altered gonadal gene expression, up-regulating cytokine receptor interaction while down-regulating mismatch repair and DNA replication pathways and influenced DNA methylation of genes essential for embryonic and synaptic signaling [124]. Similarly, Chan et al.

demonstrated that lifetime dietary exposure to methyl donor folic acid in male mice induced hypomethylation in neurodevelopment-related genes across F1–F3 germ cells [125]. While differentially methylated cytosines declined in F2 sperm compared to F1, they unexpectedly increased again in F3, and young LINE elements were significantly affected across three generations [125]. These findings underscore that nutritional epigenetic modifications can exert variable yet enduring effects on the male germline, consistent with the transgenerational patterns we observed in SYNCH groups.

7.4. Transgenerational transcriptomic changes in embryonic blood

The detection of DEGs in the SYNs and SYNCHs groups in F3 generation following a single F1 injection indicates that an ancestral exposure can induce gene expression changes that persist across multiple generations, consistent with a transgenerational response (Manuscript V). Similar patterns have been reported across species, where environmental stressors trigger heritable epigenetic modifications. For example, vinclozolin exposure in pregnant F0 rats caused disease transmission in unexposed F3 offspring [126], while in Drosophila, stress-induced heterochromatin disruption was passed through several generations before gradually reverting to baseline [127]. This aligns with our results, a sharp reduction in DEGs was observed in F4 embryos suggesting that the transcriptomic impact of ancestral exposure weakens with generational distance. This attenuation aligns with previous findings showing that while germline epigenetic alterations can be robustly established during fetal development, their functional effects may diminish over time [128].

7.5. Embryonic blood vs. adult tissues

The comparatively weaker transgenerational effects observed in embryonic blood, relative to adult tissues such as the gonads, may reflect the transient and dynamic nature of this compartment during development. At the stage of sampling, chicken embryonic blood contained not only hematopoietic cells but also PGCs migrating to the gonads. This cellular heterogeneity may dilute or mask stable transmission signals. The high turnover and short lifespan of blood cells may dilute early transcriptomic or epigenetic changes, while tissues with long-lived or self-renewing populations can be more likely to preserve such modifications [129]. By contrast, adult tissues represent more differentiated and stable niches that are continuously shaped by

microbial, metabolic, and immune interactions, enabling inherited signals to be reinforced or amplified over time [7]. Together, these factors may suggest that adult tissues may better capture the lasting, tissue-specific consequences of early nutritional and microbial programming.

7.6. Mapping enrichment to synbiotic and choline

The enriched GO terms and KEGG pathways identified in somatic and germline tissues can be linked to the functions of synbiotics (prebiotics and probiotics) and choline based on previously reported literature (Paper III, Paper IV, Manuscript V). For example, synbiotics and microbiota-derived metabolites can influence host physiology through multiple pathways. Microbial fermentation enhances the production of short-chain fatty acids (SCFAs), such as butyrate and propionate, which support epithelial tight junction assembly, cellular homeostasis, and energy metabolism [130]. Probiotic surface molecules, including pili and flagella, interact with host receptors to reinforce barrier integrity and modulate immune and signaling pathways [131]. In addition, microbiota influence protein synthesis, heat shock protein expression, and cellular stress responses, thereby linking gut microbial activity to host proteostasis and immune regulation [132, 133].

Choline exerts direct molecular and epigenetic effects. It is an essential nutrient for phospholipid synthesis, preserving membrane structure, fluidity, and organelle integrity [134, 135]. Choline-derived metabolites, including phosphatidylcholine, betaine, and acetylcholine, regulate diverse cellular functions, from neurotransmission to lipid transport and immune signaling [136]. As a methyl donor, choline contributes to one-carbon metabolism and DNA methylation, influencing transcriptional regulation and epigenetic programming across generations [137]. Choline supplementation was also shown to modulate antioxidant defense systems [138]. Importantly, maternal choline availability can impact fetal vascularization, stem cell proliferation, and brain development, underscoring its pleiotropic roles in both somatic and germline physiology [139].

7.7. Integration of germline preservation with transgenerational epigenetic studies

By demonstrating that chicken PGCs retain their germline identity and viability after both short- and long-term cryopreservation, Paper II [53] may provide a methodological foundation for long-term and multigenerational nutriepigenetic research. The ability to preserve PGCs ensures experimental continuity, reproducibility, and the possibility of accessing specific developmental

stages across extended timelines [55, 140]. If germline cells can be preserved and revived without loss of identity, researchers can standardize experiments across time and generations. Importantly, cryopreservation itself may constitute an environmental stressor capable of influencing epigenetic states, since chemical cryoprotectants like dimethyl sulfoxide (DMSO) can alter DNA methyltransferase activity and potentially reshape epigenetic landscapes [141, 142]. The observed upregulation of pluripotency and germ cell marker genes after long-term storage suggests that PGCs not only withstand cryogenic stress but may actively reprogram in response to it. PGCs are a robust model for investigating inter- and transgenerational transmission [4]. Their resilience under cryopreservation supports the interpretation that nutritional or environmental signals detected in experimental settings may reflect true programming rather than instability of the model system.

8. Limitations

Although our study spanned multiple generations and included both single- and repeated-treatment lineages, we did not directly assess specific epigenetic modifications such as DNA methylation, histone marks, or non-coding RNA expression. Therefore, we cannot definitively attribute the observed transcriptomic differences to epigenetic transmission. We did perform RRBS on gonadal tissue, which confirmed the RNA-seq results. RRBS analyses for other tissues are planned for the future but are not included in this thesis due to the extensive time requirements. Nevertheless, because each generation was compared to its respective control group in every generation, the changes observed in tissues can reasonably be interpreted as responses to the *in ovo* administration of synbiotic alone or in combination with choline. Accordingly, the transcriptomic shifts seen in SYNs and SYNCHs groups may reflect transgenerational effects.

It is important to note that transcriptomic data were not collected from all generations in gonads and embryonic blood, as our primary aim was to evaluate whether the effects of *in ovo* stimulation persist into later generations, and comprehensive multi-generational sampling is cost prohibitive. This limitation restricts our ability to track the temporal progression or persistence of gene expression changes across generations. Without these intermediate datasets, it remains unclear whether the patterns observed in grand-offsprings represent gradual changes, stable transmission, or re-emergence of gene expression alterations.

9. Main conclusions

From this thesis work, we can conclude that:

a. *In ovo* exposure to synbiotic and synbiotic plus choline induces both intergenerational (F2) and transgenerational (F3, F4) effects in somatic (cecal tonsils, cecal mucosa, embryonic blood) and germline (gonads) tissues (Papers III, Paper IV, Manuscript V).
b. These effects are tissue-specific and generation-dependent, with some signals waning in intermediate generations and re-emerging or amplifying in later generations (Paper III).
c. Cecal tonsils, with their immune functions, exhibited robust transgenerational responses, whereas cecal mucosa showed more transient intergenerational changes (Paper III).
d. Embryonic blood demonstrated moderate transgenerational effects in F3 that declined in F4 (Manuscript V).
e. Gonads were particularly sensitive to the combination of synbiotic and choline, showing more pronounced and lasting transcriptomic and putative epigenetic responses compared to synbiotic alone (Paper IV).
f. Enriched pathways included metabolism, immune signaling, proteostasis, stress responses, cytoskeletal dynamics, and cell growth/development, reflecting both choline and synbiotic-mediated effects of the treatments (Papers III, Paper IV, Manuscript V).
g. Observed transcriptomic changes are consistent with generational skipping and washout phenomena, indicating that epigenetic effects can be latent, re-emerge, or attenuate across generations (Paper III, Manuscript V).
h. While direct epigenetic modifications were not comprehensively assessed in all tissues, RRBS in gonads supports RNA-seq findings, suggesting that the observed transcriptomic changes likely reflect epigenetic regulation (Paper IV).
i. Repeated *in ovo* stimulation did not consistently amplify transcriptomic effects across generations (no linear cumulative effect of DEG with repeated *in ovo* injection in every generation). Instead, repeated exposures generated patterns largely similar to those observed after single treatments, suggesting that the magnitude of transgenerational programming may plateau following the initial exposure (Paper III, Paper IV, Manuscript V).

j. The findings underscore the importance of nutritional and microbial factors in shaping long-term metabolic and immune outcomes via inter- and transgenerational mechanisms (Paper III, Paper IV, Manuscript V).
k. Chicken PGCs demonstrate remarkable resilience under both short- and long-term cryopreservation, retaining their germline identity, pluripotency, and viability. This stability underscores their value as a robust experimental model for studying inter- and transgenerational transmission (Paper II).

10. Perspectives

The results of this study suggest that early-life nutritional and microbial interventions can shape gene expression and physiological outcomes across generations. Translating these findings to human health, prenatal and early-life modulation of the microbiome and epigenetic landscape may offer novel strategies to influence metabolic, immune, and developmental trajectories. Using the chicken model provides valuable insights into how diet and metabolic programming affect long-term health and disease risk across generations. Future research should prioritize longitudinal studies to evaluate the sustained impact of parental diet, microbial supplementation, and methyl donor availability on offspring health, with particular attention to epigenetic markers, gene expression, and functional outcomes. Ultimately, uncovering how early environmental exposures regulate gene activity across generations could inform preventive and personalized approaches in human health, supporting strategies to reduce disease susceptibility and optimize developmental potential.

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Primordial germ cells as a potential model for understanding (Nutri) epigenetic - metabolic interactions: a mini review

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Primordial germ cells (PGCs) are the progenitors of gametes (sperm and eggs), making them crucial for understanding germline transmission and epigenetic modifications, which are critical for studying transgenerational effects of nutrition and metabolic diseases. This is particularly relevant given the growing evidence that environmental factors, such as diet, can influence metabolic disease risk across generations through modulating epigenetic mechanisms, as seen in both human and animal studies. The unique biological and experimental attributes make PGCs in the chicken embryo a potential model for exploring the complex interactions between nutrition, epigenetic inheritance, and metabolic diseases, providing insights that are translatable to metabolic health and disease prevention tactics. This brief review emphasizes the potential of chicken PGCs as a model system to investigate the mechanisms underlying transgenerational metabolic programming.

KEYWORDS

nutritional programming, nutriepigenetic, metabolic processes, PGCs, transgenerational effects

1 Introduction

Epigenetic regulation during development plays a crucial role in cell fate determination, lineage specification, and the establishment of cellular identity. Metabolic diseases such as obesity, type 2 diabetes, and non-alcoholic fatty liver disease are affected by epigenetic mechanisms including DNA methylation, histone modification, and non-coding RNA expression (Nicoletti et al., 2024; Rivera-Aguirre et al., 2023; Gómez de Cedrón et al., 2023). Nutritional factors such as vitamin B12, folate, and choline act as methyl donors or coenzymes for one-carbon metabolism, and their dietary intake can modulate the epigenetic patterns, impacting the onset and progression of metabolic diseases (Nicoletti et al., 2024; Rivera-Aguirre et al., 2023). Endocrine disruptors like phthalates, bisphenol A, pesticides, polychlorinated biphenyls, and dioxins, as well as nutritional imbalances, can induce epigenetic changes in primordial germ cells (PGCs), potentially resulting in altered phenotypes in later generations (Rizzo et al., 2023;

Brehm and Flaws, 2019; Brieño-Enríquez et al., 2016). Studies have shown that exposure to metabolic disruptors during prenatal or early life stages can cause metabolic diseases in future generations, underlining the need to understand the epigenetic memory and molecular determinants of these effects (Feroe et al., 2017). A key challenge in the field is identifying model systems that allow researchers to track how specific environmental factors, such as nutrition, trigger epigenetic modifications and subsequent changes in gene expression patterns. These models must enable the study of both immediate effects and potential transmission across generations. Potent models are essential for developing nutritional programming strategies to produce desired traits and implement efficient preventive measures for metabolic diseases. The chicken embryo model offers unique advantages for studying these interactions, as it allows precise temporal control over environmental exposures without maternal confounding effects. However, debate persists regarding the stability and inheritance of environmentally-induced epigenetic changes. While some studies demonstrate transgenerational effects of nutritional interventions (Wu et al., 2019), others question the molecular mechanisms and evolutionary significance of such inheritance (Verdikt and Allard, 2021). This brief review aims to shed light on the potential of chicken PGCs as a model for studying how prenatal nutritional and environmental factors influence epigenetic inheritance in metabolic disorders, and the mechanisms linking environmental signals to specific epigenetic modifications.

2 The chicken model for metabolic processes research

Chickens have been considered a useful model to explore the role of adipokine mediated regulation in metabolic and reproductive diseases, with parallels to metabolic diseases in humans (Mellouk et al., 2018). Key adipokines, including adiponectin, visfatin, and chemerin, demonstrate conserved regulatory functions across both species (Mellouk et al., 2018). Chickens constitutively exhibit hyperglycemia despite having normal levels of hyperactive endogenous insulin, requiring large doses of exogenous insulin to induce hypoglycemia, mirroring the insulin resistance seen in human type 2 diabetes pathology (Mellouk et al., 2018; Haselgrübler et al., 2017). Moreover, chickens have been genetically selected for traits such as fatness, which is associated with phenotypic variations in adiposity and metabolic disorders (Resnyk et al., 2017). Additionally, the metabolic genes in chickens are largely conserved with those in humans, and several quantitative trait loci (QTLs) connected to fatness in chickens include genes that link to human obesity or diabetes susceptibility (Mellouk et al., 2018; Nadaf et al., 2009). The chicken's metabolic system allows for the insights into nutrient metabolism particularly through hepatic lipogenesis and tissue-specific insulin signaling patterns (Mellouk et al., 2018). In both humans and chickens, the liver is the primary site for *de novo* lipogenesis (90%) (Liu et al., 2018). Furthermore, the post-hatch period in chickens is especially useful for studying metabolic programming, as it involves substantial changes in liver metabolism that are comparable to human metabolic processes (Van Every and Schmidt, 2021). Besides, chickens offer a well-established model for researching human lipid metabolism disorders, including non-alcoholic fatty liver disease (Ayala et al., 2009). The robustness of chicken metabolic pathways is demonstrated by the genome-scale metabolic model iES1300, which demonstrates substantial homology with human carbohydrate metabolism networks (Salehabadi et al., 2022).

3 Current limitations in understanding metabolic-epigenetic interactions

Current limitations in metabolic-epigenetic research center on three key challenges. The incomplete knowledge about how specific metabolites induce epigenetic changes, like histone acetylation and methylation, and how these changes in turn control metabolic pathways is one of the main limitations. This bidirectional interaction is key in a variety of biological contexts, encompassing embryonic development, cancer, and chronic diseases, however, it is difficult to characterize due to the complexity of these processes and their heterogeneity between cell types and conditions (Milazzotto et al., 2023; Ge et al., 2022; Gómez de Cedrón et al., 2023). Furthermore, the field is impeded by the limited understanding of how epigenetic changes caused by metabolic alterations can be passed down between generations, as seen in studies of paternal transgenerational inheritance of metabolic diseases (Pepin et al., 2022). The potential for targeted nutritional and lifestyle interventions to modulate epigenetic marks and maintain metabolic homeostasis is promising, yet the precise mechanisms and long-term effects of such interventions have yet to be fully understood (Gómez de Cedrón et al., 2023).

4 Nutritional programming in chicken model

Growth and development-related metabolic pathways can be optimized through prenatal dietary stimulation. Maternal nutrition, for example, β-carotene supplementation, can influence embryonic development through the growth hormone-insulin-like growth factor axis, promoting liver development and affecting metabolism-related gene expression (Wang et al., 2024). Contrariwise, prenatal protein undernutrition, induced by albumen removal, has been shown to cause long-term alterations in body weight, reproductive performance, and hepatic metabolism, underscoring the vital role that proper prenatal nutrition plays in metabolic programming (Willems et al., 2015).

Understanding the epigenetic changes driven by nutrients is necessary to gain deeper insight into diet-gene interactions. Nutriepigenetics provides insights into improving poultry health and performance by modulating genes associated with immunity, metabolism, and growth (Hassan et al., 2022). The in ovo feeding (IOF) technique, originally designed for vaccine delivery in broiler hatcheries, has evolved into a cost-effective approach for studying early nutrition in chickens (Das et al., 2021). This method now incorporates a variety of substances, including nutrients such as glucose, amino acids, and vitamins, as well as supplements like probiotics, prebiotics, exogenous enzymes, hormones, vaccines, drugs, and nutraceuticals (Das et al., 2021). Given the critical role of embryonic nutrition in regulating tissue and organ development in

later stages, in ovo injections and IOF are recognized as powerful tools for implementing targeted nutritional strategies at early developmental stages, and to investigate the effects of injected chemicals and the epigenetic changes they cause. For instance, the administration of L-leucine in ovo has been found to stimulate lipid metabolism and enhance thermotolerance in male chicks under heat stress, indicating a sex-dependent metabolic response (Han et al., 2018).

Dietary methyl donors such as folate, choline, and B vitamins are crucial for DNA methylation, influencing gene expression and disease risk (Anderson et al., 2012). *In ovo* folic acid supplementation has been reported to improve immune function and growth in broilers by modifying histone methylation in immune gene promoters (Li et al., 2016). Furthermore, feeding-based dietary betaine supplementation has been shown to modulate DNA methylation in response to corticosterone-induced hepatic cholesterol accumulation. Key cholesterol gene expression (*HMGCR*, *CYP7A1*) was normalized by reversing corticosterone-induced methylation changes, highlighting the epigenetic influence of diet (Wu et al., 2024). Paternal folate supplementation in chickens has been shown to affect the growth and metabolic profiles of offspring, with changes in lipid and glucose metabolism linked to alterations in spermatozoal and hepatic miRNAs and lncRNAs (Wu et al., 2019). Guo et al. (2024) found that excessive folic acid intake in male chickens can alter sperm DNA methylation (6 mA and 5 mC), increasing hepatic lipogenesis and lipid accumulation while reducing lipolysis in both roosters and their offspring. This study highlights environment-sensitive regions in the sperm epigenome that respond to dietary factors and transmit an epigenomic map, potentially shaping metabolic health in offspring.

Despite the advantages of embryonic manipulations in avian species, there have been relatively few studies on PGCs concerning the transgenerational inheritance effects of epigenetic stimuli.

5 Main metabolic-epigenetic crosstalk in chicken germ cells

PGCs in chicken possess unique epigenomic landscape, which, despite sharing some conserved features with mammals, exhibit distinct epigenetic signatures that reflect their evolutionary and developmental pathways, reviewed in (Woo and Han, 2024). In chickens, PGCs are specified by preformation and are influenced by maternally inherited factors, contrasting with the inductive specification seen in mammals (Kress et al., 2024). Unlike mammalian PGCs, chicken PGCs do not experience genome-wide DNA demethylation or a decrease in histone H3K9me2, which are typical features of extensive epigenetic programming in mammals (Kress et al., 2024). Instead, chicken PGCs maintain high levels of 5mC and exhibit a unique epigenetic signature characterized by high global levels of H3K9me3, particularly in inactive genome regions. This signature is progressively established during migration and remains stable in the gonads, indicating a divergence from the basal state resetting observed in mammals. The processes in chicken PGCs are more about chromatin reconfiguration rather than *bona fide* programming, as seen in mammals (Kress et al., 2024). Additionally, the transcription factor Zeb1 and histone methylation regulate *BMP4* expression, highlighting the interplay between genetic and epigenetic factors in PGC development (Zhou et al., 2021). LncRNAs also contribute significantly to chicken PGC development (Jiang et al., 2021). Furthermore, during mitotic arrest, chicken prospermatogonia undergo unique epigenetic programming, characterized by gradual DNA demethylation and histone acetylation, which differs from the mammalian pattern (Choi et al., 2022). These findings underscore the distinct epigenetic landscape of chicken PGCs, which involves a combination of DNA methylation, histone modifications, and non-coding RNAs, all contributing to the regulation of germ cell development and differentiation (Woo and Han, 2024; Rengaraj et al., 2022).

Metabolic regulation in chicken PGCs involves a complex interplay of pathways and factors that ensure proper development and function. Glycolysis is a critical metabolic pathway, with glucose phosphate isomerase (GPI) being essential for maintaining glycolysis and energy supply in chicken PGCs. Knockdown of GPI significantly reduces the expression of glycolysis-related genes and endogenous glucose levels, underscoring its role in PGC proliferation (Rengaraj et al., 2012). Additionally, the transition from glycolysis to oxidative phosphorylation is a key event in PGC formation, indicating a shift in energy metabolism as these cells develop (Zuo et al., 2023). The *C1EIP* gene, regulated by STAT3 and histone acetylation, promotes PGC formation by interacting with ENO1 and inhibiting the Notch signaling pathway (Jin et al., 2020). The TGF-β and Wnt signaling pathways are also activated during PGC formation *in vitro* and *in vivo*, further emphasizing the metabolic and signaling intricacies involved in PGC regulation (Ding et al., 2024). Autophagy, as indicated by the increased number of autolysosomes, is another metabolic process that is enhanced in PGCs, especially following BMP4 induction (Ding et al., 2024). The piRNA pathway also plays a protective role in PGCs, with piRNA pathway genes such as *CIWI* and *CILI* being crucial for maintaining genomic integrity and preventing DNA double-strand breaks (Rengaraj et al., 2014). These pathways collectively underscore the complex metabolic network that supports the development and function of chicken PGCs, integrating energy metabolism, signaling, and genomic protection mechanisms.

Metabolic pathways are intricately linked to epigenetic changes, as metabolites can influence epigenetic mechanisms, and conversely, epigenetic modifications can regulate metabolic processes (Verdikt and Allard, 2021). This metabolic-epigenetic interplay is crucial during early germ cell development, affecting cell fate determination and potentially playing a role in transgenerational epigenetic inheritance (Verdikt and Allard, 2021).

6 Chicken PGCs: a tool for transgenerational studies

Chicken PGCs may offer a window into the epigenetic mechanisms that mediate the transgenerational effects of prenatal nutritional interventions. Growing evidence suggests that dietary influences can significantly impact epigenetic marks in PGCs, which are crucial for transgenerational inheritance. The application of nutritional programming in chickens, unlike in mammals, allows for the isolation of nutritional effects without hormonal interference, providing a clearer understanding of its impacts on growth and metabolism (Willems et al., 2015). The unique accessibility of avian PGCs during early development, due to their migration via blood circulation, provides an opportunity for their collection, which is not as easily achievable in mammalian models (Nakamura et al., 2013). Chicken

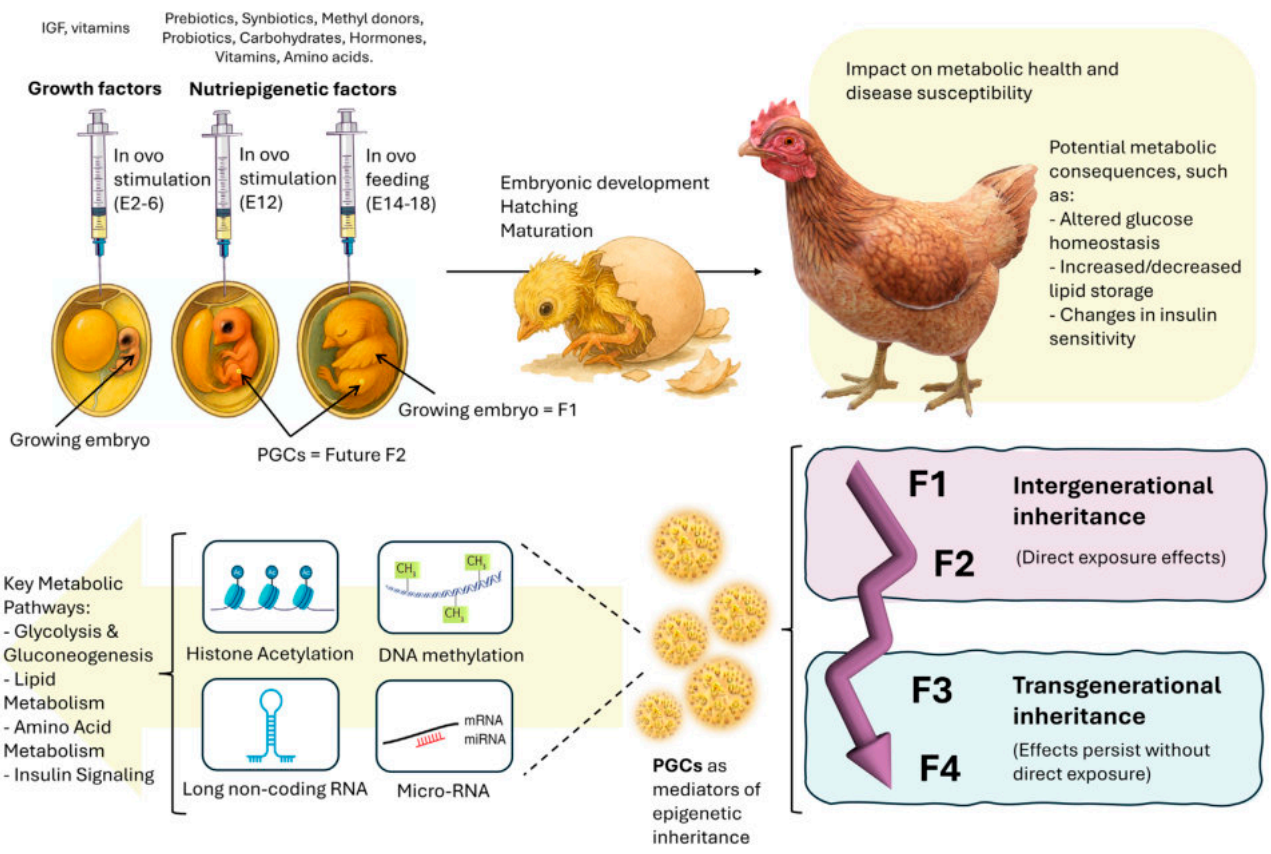

FIGURE 1
The in ovo model for investigating nutrient-induced metabolic programming in chickens through PGCs. In ovo stimulation (E12) and in ovo feeding (E14-18) introduce nutripigenetic factors (e.g., prebiotics, probiotics, methyl donors, carbohydrates, hormones, vitamins, and amino acids) that influence embryonic development (F1). These interventions can induce epigenetic modifications (including histone acetylation, DNA methylation, long non-coding RNA, and miRNA regulation) that affect key metabolic pathways such as glycolysis, gluconeogenesis, lipid metabolism, amino acid metabolism, and insulin signaling. Changes in metabolic regulation may alter glucose homeostasis, lipid storage, and insulin sensitivity, potentially leading to metabolic disorders. Through primordial germ cells (PGCs), these epigenetic and metabolic effects can be inherited across generations, contributing to intergenerational (F1-F2) and transgenerational (F3-F4) inheritance of metabolic traits. This highlights the potential of chicken PGCs as a valuable model for studying the epigenetic basis of nutrition-induced metabolic diseases.

PGCs can be isolated from embryos at various stages of development, each offering unique advantages for research and application. The isolation of PGCs from embryonic blood is commonly performed at HH stages 14–16, where they are abundant in circulation before migrating to the gonadal regions (Dehdilani et al., 2023). Additionally, chicken PGCs can be isolated from the embryonic gonadal regions at later stages, such as HH 26–28, where they have migrated and begun to settle (Zare et al., 2023). Chicken PGCs are characterized by several molecular markers that are crucial for their identification and study such as *SSEA-1*, *EMA-1*, *SSEA-4*, and *SSEA-3* (Mathan et al., 2023). Pluripotency markers such as *POUV*, *SOX2*, and *NANOG*, along with germ cell markers like *DAZL* and *CVH* markers are consistently expressed across various conditions, including fresh isolation, cryopreservation, and *in vitro* culture, indicating the cells' stability and resilience (Ibrahim et al., 2024). Chicken PGCs are a model for *in vitro* culture. The chicken is the only vertebrate whose PGCs can be stably cultured *in vitro* for an extended period of time (Ichikawa and Horiuchi, 2023). The ability to culture chicken PGCs *in vitro* has been well-documented, with various studies highlighting their resilience and the maintenance of their germline characteristics during long-term culture and cryopreservation (Kong et al., 2018; Ibrahim et al., 2024). The development of optimal culture systems for chicken PGCs has been a focus of several studies comparing the efficiency of different media dedicated to cell expansion and differentiation (Dehdilani et al., 2023). One of the most efficient systems is the feeder-free culture method developed by for expanding chicken PGCs, applied in the research over the last decade (Whyte et al., 2015). Despite the advancements, challenges remain in establishing standardized culture conditions. A primary issue is the inconsistency in protocols across different laboratories, leading to variations in success rates for cell growth and maintenance. These discrepancies make it difficult to replicate and reproduce results reliably. The derivation, expansion, and long-term culture of PGCs appear to depend on multiple factors, including the quality of materials, embryos and incubation quality, the breed of chickens from which PGCs are derived, and the specific combination of culture components essential for PGC survival (Dehdilani et al., 2023). Successful cultivation of chicken PGCs requires specific growth factors and supplements to maintain their developmental potency, stemness, survival, and proliferation (Dehdilani et al., 2023). The absence of these essential components can

impair cell growth and viability. Key growth factors include Fibroblast Growth Factor 2 (FGF2), Activin A, BMP4, Insulin-like Growth Factor 1 (IGF-1) and B27 supplement (Miyahara et al., 2016; Whyte et al., 2015; Barkova et al., 2022; Choi et al., 2010).

Additionally, the short-term interval between generations enables tracking the transgenerational effect of studied dietary factors. Artificial insemination technology and the high reproductive capacity of hens, producing up to 300 eggs annually, allow for the generation of enough offspring broilers to study the potential transgenerational impacts of nutritional interventions (Ibrahim et al., 2025). Chickens provide a unique model due to their ability to minimize maternal confounding effects through direct manipulation of egg content, which is not possible in mammalian models (Morisson et al., 2017). This allows for precise control over the nutritional environment during critical developmental periods, facilitating the study of nutritional programming and its transgenerational effects (Morisson et al., 2017). The success of nutritional interventions heavily depends on the selection of suitable delivery techniques and platforms, a condition fulfilled through the application of in ovo injection in chicken embryos. The use of chickens as a model for nutritional rehabilitation, as demonstrated in studies involving dietary interventions in broilers, further underscores their potential as a translational model for human nutritional studies (Baxter et al., 2018). Chickens have been instrumental in advancing knowledge about the role of specific nutrients, such as omega-3 fatty acids, in early life nutritional programming, which can inform strategies to improve human health and productivity (Cherian, 2013).

Recent research by Verdikt et al. has highlighted the interplay between metabolic and epigenetic regulation of PGCs in mammals, particularly in the context of transgenerational epigenetic inheritance (Verdikt and Allard, 2021). Their review suggested that environmental factors may influence epigenetic remodeling in PGCs through metabolic pathways, thereby affecting gene expression. While most studies have focused on mature germ cells, such as sperm and eggs, PGCs remain relatively understudied despite their potential sensitivity to environmental changes. This sensitivity makes PGCs a crucial window for investigating how epigenetic information is transmitted across generations. Another study also hypothesized that the DNA methylome of sperm may show changes in its expression profile in response to high paternal folic acid intake, which has been widely suggested as a methyl donor for the DNA methylation process, and then the altered sperm DNA methylome could transmit certain metabolic and developmental changes from father to offspring (Guo et al., 2024). Although chickens may not serve as an ideal translational model for studying germline programming mechanisms in humans due to species-specific differences, they are highly valuable for investigating multigenerational effects of nutrients, particularly in the context of metabolic processes. The in ovo model allows researchers to explore how nutrients impact epigenetic regulation of metabolic processes, gene expression, and development across generations (Figure 1). This approach provides critical insights into the inheritable effects of key nutrients, which are relevant to human health and the development of other vertebrates.

Overall, the investigation into transgenerational inheritance in chicken PGCs not only enhances our understanding of evolutionary biology and adaptation but also holds potential implications for improving animal breeding and addressing metabolic health issues in broader contexts.

7 Conclusion and perspectives

In agreement with Diniz et al. (2024), further advances are essential for translating findings into applications for developmental disorders and understanding the broader implications of early-life nutrition for long-term health outcomes. Therefore, investigation of nutriepigenetic effects transmission through the chicken PGC model has revealed important insights, while also highlighting critical areas for future research: (1) elucidating the molecular mechanisms underlying nutrient-induced epigenetic modifications in PGCs, (2) understanding how these modifications are maintained and transmitted across generations, and (3) determining the conservation of these mechanisms across species. The chicken PGC model offers unique advantages for addressing these questions, particularly through its experimental accessibility and ability to control environmental exposures precisely. It is important to note that this model system should be applied carefully and serve primarily at the very early stages of preclinical trials, providing an initial overview of basic pathways (particularly metabolic pathways) at a general, conserved annotation level. The simplicity and ethical advantages of the in ovo model make it particularly valuable as a preliminary screening tool prior to more comprehensive studies using established animal preclinical models.

Author contributions

MI: Conceptualization, Data curation, Formal Analysis, Writing – original draft. EG: Conceptualization, Funding acquisition, Supervision, Writing – review and editing, Project administration. KS: Conceptualization, Funding acquisition, Supervision, Writing – review and editing, Formal Analysis, Resources.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Glossary

PGC	Primordial germ cell
QTLs	Quantitative trait loci
IOF	In ovo feeding
HMGCR	3-Hydroxy-3-Methylglutaryl-CoA Reductase
CYP7A1	Cytochrome P450 Family 7 Subfamily A Member 1
miRNAs	MicroRNAs
lncRNAs	Long Non-Coding RNAs
6mA	*N6*-methyladenine
5mC	5-Methylcytosine
H3K9me2	Histone 3 Lysine 9 Dimethylation
H3K9me3	Histone 3 Lysine 9 Trimethylation
Zeb1	Zinc Finger E-Box Binding Homeobox 1
BMP4	Bone Morphogenetic Protein 4
GPI	Glucose Phosphate Isomerase
C1EIP	Chromosome 1 Expression in PGCs
STAT3	Signal Transducer and Activator of Transcription 3
ENO1	Enolase 1
TGF-β	Transforming Growth Factor Beta
piRNA	PIWI-Interacting RNA
CIWI	PIWI-Like Protein 1
CILI	PIWI-Like Protein 2
HH	Hamburger-Hamilton Stages
SSEA-1	Stage-Specific Embryonic Antigen-1
SSEA-4	Stage-Specific Embryonic Antigen-4
SSEA-3	Stage-Specific Embryonic Antigen-3
POUV	POU Class 5 Homeobox 1 (also known as OCT4 in mammals)
SOX2	SRY-Box Transcription Factor 2
NANOG	NANOG Homeobox (Pluripotency-Associated Transcription Factor)
DAZL	Deleted in Azoospermia-Like
CVH	Chicken Vasa Homolog
FGF2	Fibroblast Growth Factor 2
IGF-1	Insulin-like Growth Factor 1
E	Embryonic day
F1	First Filial Generation
F2	Second Filial Generation
F3	Third Filial Generation

genes

MDPI

Article

The Effect of Short- and Long-Term Cryopreservation on Chicken Primordial Germ Cells

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Abstract: Primordial germ cells (PGCs) are the precursors of functional gametes and the only cell type capable of transmitting genetic and epigenetic information from generation to generation. These cells offer valuable starting material for cell-based genetic engineering and genetic preservation, as well as epigenetic studies. While chicken PGCs have demonstrated resilience in maintaining their germness characteristics during both culturing and cryopreservation, their handling remains a complex challenge requiring further refinement. Herein, the study aimed to compare the effects of different conditions (freezing-thawing and in vitro cultivation) on the expression of PGC-specific marker genes. Embryonic blood containing circulating PGCs was isolated from purebred Green-legged Partridgelike chicken embryos at 14–16 Hamburger–Hamilton (HH) embryonic development stage. The blood was pooled separately for males and females following sex determination. The conditions applied to the blood containing PGCs were as follows: (1) fresh isolation; (2) cryopreservation for a short term (2 days); and (3) in vitro culture (3 months) with long-term cryopreservation of purified PGCs (~2 years). To characterize PGCs, RNA isolation was carried out, followed by quantitative reverse transcription polymerase chain reaction (RT-qPCR) to assess the expression levels of specific germ cell markers (*SSEA1*, *CVH*, and *DAZL*), as well as pluripotency markers (*OCT4* and *NANOG*). The investigated genes exhibited consistent expression among PGCs maintained under diverse conditions, with no discernible differences observed between males and females. Notably, the analyzed markers demonstrated higher expression levels in PGCs when subjected to freezing than in their freshly isolated counterparts.

Keywords: cell culture; cryopreservation; genes; markers; primordial germ cells

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1. Introduction

The study of avian primordial germ cells (PGCs) dates back to 1870 when they were first described by Waldeyer. Since then, researchers have focused on understanding the origin, migration, differentiation, and molecular markers of PGCs in birds, notably in species like chicken (*Gallus domesticus*) and Japanese quail (*Coturnix japonica*) [1]. PGCs offer a lot of potential as genetic resources for avian research, especially when studying genetically modified animals [2]. PGCs are the earliest group of germ cells to appear during development and are responsible for generating both oocytes and spermatogonia in adult organisms [1]. These cells are capable of transmitting genetic information to the

next generation through gametogenesis [1]. Avian PGCs exhibit distinctive developmental features, such as their unique circulation within the embryonic bloodstream (13 HH–17 HH) before ultimately settling in the genital ridges (28 HH–30 HH) [3,4]. Respectively, during these stages, PGCs can be sourced either from the circulating blood (cPGCs) or from the developing gonads (gPGCs). However, the limited number of PGCs that can be obtained from a single embryo presents a challenge for widespread implementation [4]. Several research endeavors have provided insight into the self-renewal capacity of chicken PGCs, which has resulted in the establishment of protocols for maintaining their growth and proliferation in defined in vitro culture systems for extended periods of time while maintaining their germline characteristics [3]. While the existing protocols for cultivating chicken PGCs can be reproducible, their efficacy differs among breeds, and they are unable to sustain PGCs derived from avian species other than chickens [3,5,6]. A generic protocol remains to be developed for all avian PGCs. Cultivation of PGCs not only makes them readily available in laboratory settings but also allows their use as carriers in transgenic bioreactors and provides a valuable model for studying transgenic chickens [7,8]. Because PGCs allow for the acquisition of the full genetic makeup of the stock, the advent of technologies to manipulate PGCs has provided insights into ex situ conservation [9]. The development of long-term culture systems for chicken PGCs has offered the chance to greatly increase the number of PGCs before cryopreservation and storage for future use [10]. Cryopreservation of PGCs provides support for commercially or industrially important poultry lines or breeds that have undergone extensive selection, serving as a backup in the event of their loss due to pathogen outbreaks, genetic issues, breeding cessation, or natural disasters [9].

The successful development of in vitro cultivation and cryopreservation techniques relies on the acquisition of pluripotency and germline characteristics of PGCs, which in turn are essential for the success of future applications. Various methods of cryopreservation of stem cells across a range of species have been conducted so far (Table 1). PGCs are distinguished by the expression of specific markers that distinctly identify their germ cell lineage apart from somatic cells. Stage-specific embryonic antigen-1 (SSEA-1), a well-established cell surface glycoprotein antigen, serves as a valuable marker for identifying and isolating PGCs within avian embryos [11,12]. This marker is intertwined with the essential roles of PGCs, including cell adhesion, migration, and differentiation [13]. Chicken VASA homologue (CVH) and deleted in azoospermia-like (DAZL), both conserved RNA-binding proteins, exhibit targeted expression exclusively within germ cells throughout germline development [3,14]. Numerous studies have highlighted the pivotal role played by these markers in germline commitment and the intricate process of gametogenesis in invertebrates [3,15,16]. These RNA-binding proteins are essential for sustaining germ cell survival, migration, proliferation, and differentiation [17–20]. Furthermore, PGCs express several pluripotency-related core transcription factors such as nanog homeobox (NANOG), octamer-binding transcription factor 4 (OCT4), and SOX2, whose expression controls transcription of germness-related genes in these cells [21]. These transcription factors exert precise control over the fate of cells by inhibiting differentiation, thus preserving the cells' stem cell properties. PGCs lacking these transcription factors may undergo programmed cell death [22] or exhibit compromised migratory capacity, rendering them unable to successfully establish colonies within the gonadal regions after being reintroduced into the embryo's bloodstream [23]. Studying germ cell-specific genes in depth can reveal their functions in germ cell development and survival, advancing the potential for generating PGC-like cells and in vitro gamete production [24].

Previous studies revealed that PGCs cultured for shorter durations demonstrated better germline competence [6,25]. Hence, cryopreservation of PGCs may also influence their competency, necessitating further analysis of how freezing and thawing cultures may affect PGCs. To our knowledge, the differences in gene expression of germline and pluripotency markers between cryopreserved chicken PGCs and freshly isolated PGCs have not been illustrated. Additionally, no studies have investigated the differences in

the impact of short-term and long-term cryopreservation on chicken PGCs. The current study was conducted on the Green-legged Partridgelike chicken, a native Polish breed that demonstrates remarkable adaptability to adverse environmental conditions and exhibits heightened disease resistance compared to other breeds [26]. We aimed in this study to examine how various conditions, namely in vitro cultivation, freezing-thawing, and length of freezing period, affect the expression of marker genes specific to PGCs in Green-legged Partridgelike chickens.

Table 1. Overview of cell cryopreservation success by species.

Species	Cell Type	Method(s) of Cryopreservation	Main Cryopreservation Success Indicators	Reference
Chicken	Primordial germ cells	Slow freezing	Gonadal colonization and sperm differentiation post-transplantation	[27]
Drosophila	Primordial germ cells	vitrification	Production of donor-derived gametes	[28]
Rats	Spermatogonial stem cells	Slow freezing	Production of all germ cell types after long-term cryopreservation	[29]
Fish	Germline stem cells	slow freezing	Gonadal colonization post-transplantation	[30]
Human	Induced pluripotent stem cells	slow freezing	Retention of pluripotency and differentiation capacity post-cryopreservation	[31]
Chicken	Primordial germ cells	Slow freezing	Successful migration into gonads	[32]
Horse	Spermatogonial stem cells	vitrification/slow-freezing/fast-freezing	Metabolic activity and spermatogonial stem cell's protein expression comparable to fresh cells	[33]
Chicken	Primordial germ cells	stored at −150 °C (vitrification)	Viable gametes and offspring produced post-transplantation	[34]
Bovine	Spermatogonial stem cells	Slow freezing	Colonization and proliferation in recipient testes post-transplantation	[35]
Human	Embryonic stem cells	vitrification/slow-freezing	Maintenance of pluripotency	[36]
Rhesus macaques	Spermatogonial stem cells	slow freezing	Retention of engraftment potential post-cryopreservation	[37]

2. Materials and Methods

2.1. Ethical Considerations

All experimental procedures adhered to the guidelines for the care and use of experimental animals of the University of Science and Technology. The experimental protocols were approved by the Local Ethical Committee for Animal Experiments in Bydgoszcz, Poland (Approval No. 15/2022 from 20.04.2022 r.).

2.2. Fertilized Eggs and Incubation

Fertilized eggs from Green-legged Partridgelike chickens were purchased from Zofia i Gracjan Skórniccy-Hodowla Kur Zielononóżek (Duszniki, Poland). Eggs were incubated at a temperature of 37.8 °C and a relative humidity of 60% for 60 h to obtain cPGCs from embryos at the 14–16 HH stage. The eggs were periodically tilted at a 45° angle every 120 min during the incubation process.

2.3. Derivation of Embryonic Blood Containing cPGCs

Embryonic blood containing cPGCs was isolated from the dorsal aorta of individual embryos under a stereomicroscope using a mouth pipette with fine transfer glass microcapillary of inner diameter 30 µm and outer diameter 40 µm. The isolated blood underwent three different processes (Figure 1): (1) fresh isolation; (2) cryopreservation for a short term (2 days); and (3) in vitro culture (3 months) with long-term cryopreservation of cultured

PGCs (2 years). Following isolation, embryos were collected for sex determination and stored at −20 °C until further use.

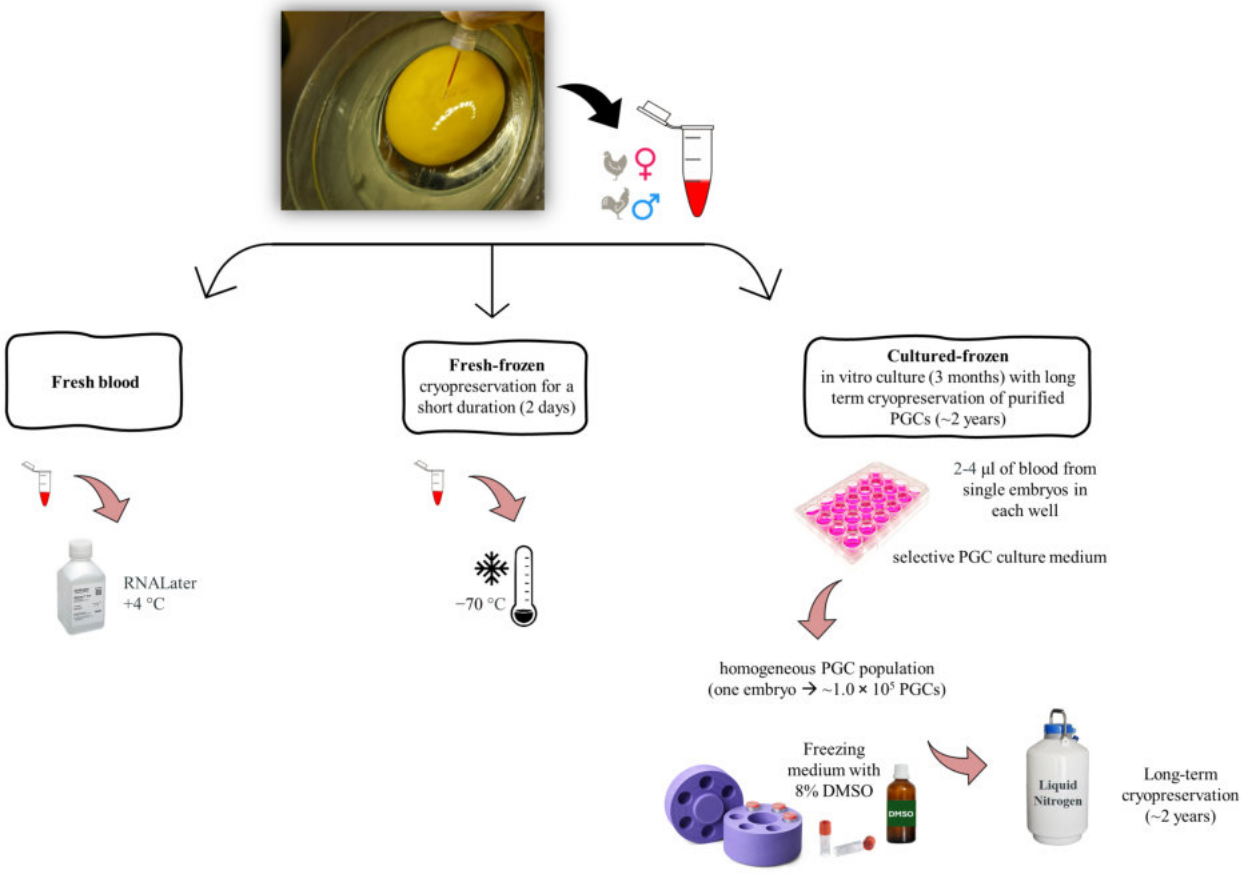

Figure 1. Preparation of samples under three different conditions. PGCs: Primordial germ cells; DMSO: Dimethyl sulfoxide.

For freshly isolated blood samples, blood from 20 embryos was placed individually in tubes with RNALater (ThermoFisher, Waltham, MA, USA) and stored at 4 °C until later usage. Once sex determination was done, the samples were pooled into male and female groups. The cells were separated by centrifugation in RNase-free water at 10,000× *g* for three minutes. Subsequently, RNA isolation was carried out using the GeneMATRIX Universal RNA Purification Kit (Eurx, Gdańsk, Poland, cat.no. E3598) following the instructions provided by the manufacturer. For samples cryopreserved for short term, the blood drawn from single embryos was frozen separately as described below. On the other hand, approximately 1–2 µL of blood from single embryos were cultured in vitro in the selective PGC culture medium developed by McGrew and colleagues [38]. The medium consisted of: Calcium-free DMEM (Gibco, Billings, MT, USA, 21068-028), tissue culture-grade water (Gibco, Billings, MT, USA, A12873-01), Sodium Pyruvate (Gibco, Billings, MT, USA, 11360039), MEM vitamin solution (Gibco, Billings, MT, USA, 11120052), MEM amino acids (Sigma, St. Louis, MO, USA, M5550), B27 supplement (Gibco, Billings, MT, USA, 17504044), Glutamax (Gibco, Billings, MT, USA, 35050038), nonessential amino acids (Gibco, Billings, MT, USA, 11140035), nucleosides (EmbryoMax, Munich, Germany, ES-008-D), β-mercaptoethanol (Gibco, Billings, MT, USA, 31350010), $CaCl_2$ (Sigma, St. Louis, MO, USA, C4901-100G), ovalbumin (Sigma, St. Louis, MO, USA, A5503), Na heparin (Sigma, St. Louis, MO, USA, H3149-25KU), penicillin–streptomycin mixture (Gibco, Billings, MT, USA, 15070-063), chicken serum (Sigma, St. Louis, MO, USA, C5405), human activin (Invitrogen, Waltham, MA, USA, PHC9564), bFGF2 (Gibco, Billings, MT, USA, 13256-029), and ovotransferrin (Sigma, St. Louis, MO, USA, C7786). While in culture, one-third of

the medium was replaced with fresh medium every two days. The cells were cultured for 3 months until a homogeneous PGC population was obtained (Figures S1 and S2). Male and female cell lines were established and then 1.0×10^5 PGCs from each sample were used for long-term cryopreservation. RNA samples were retrieved from resuscitated thawed samples (Figure S3) using the GeneElute Single Cell RNA Purification kit (Sigma-Aldrich, St. Louis, MO, USA, cat.no. RNB300) following the manufacturer's protocol.

2.4. Freezing and Thawing of Cells

Freshly prepared freezing media for PGCs was used for freezing both the established PGC lines and the freshly isolated blood. The cryopreservation steps are outlined in Figure 2. The freezing medium was formulated with a 2:1 ratio of DMEM (Thermo Fisher Scientific, Waltham, MA, USA, 21068-028) and sterile water (Thermo Fisher Scientific, Waltham, MA, USA, 15230-089). Additionally, 4 µL sodium pyruvate (Thermo Fisher Scientific, Waltham, MA, USA, 11360-039) was added per 1 mL of medium. To a part of this avian KnockOut DMEM (KO-DMEM) medium, 8% dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA, 276855), 10% chicken serum (Sigma-Aldrich, St. Louis, MO, USA, C5405), and 0.75% 20 mM $CaCl_2$ (Sigma-Aldrich, St. Louis, MO, USA, C-34006) were added. The freezing process was done as previously described [32]. Briefly, PGCs containing samples were suspended in 250 µL of DMSO free freezing medium, followed by gentle addition of 250 µL of PGCs freezing medium. The cultured PGCs were kept in nitrogen for up to two years. Fresh blood was kept for two days at −70 °C. For the thawing of PGCs, a solid bead bath at 37 °C was used, and then the total content of the tube was pipetted into 2 mL of culturing media for PGCs. After centrifugation (1000× *g*, 3 min) the supernatant was removed.

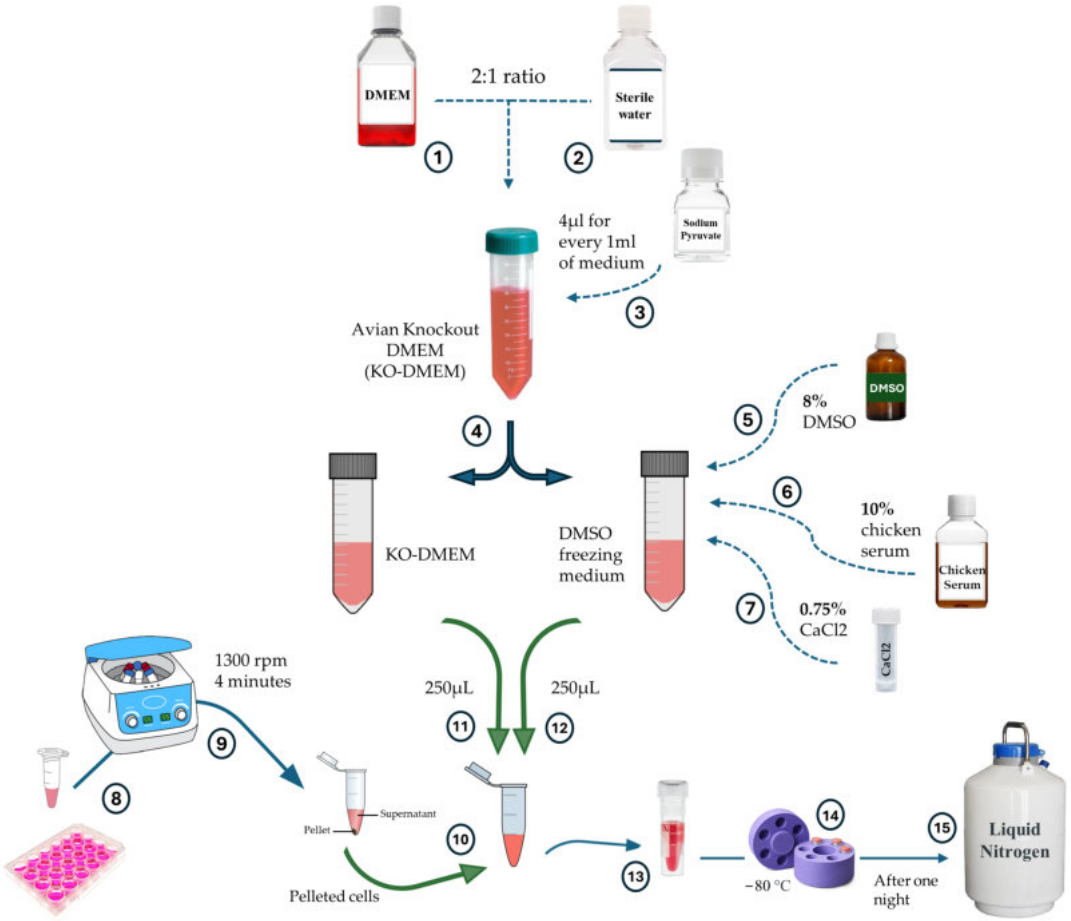

Figure 2. A scheme showing the steps for PGCs cryopreservation. Avian KO-DMEM was prepared by 2:1 ratio of DMEM and sterile water. Then 4 µL of Sodium pyruvate was added to every 1 mL of

the medium. The volume of the prepared medium is then divided into two; to one of these parts, DMSO (final concentration 8%), chicken serum (final concentration 10%) and $CaCl_2$ (final concentration 0.75%) were added to form the DMSO freezing medium. After pelleting the cells to be frozen and removing the supernatant, the cells were resuspended in 250 µL of avian KO-DMEM. Then, 250 µL of DMSO freezing medium were added slowly. The cell suspension was then transferred to a cryovial which was then placed into −80 °C. For long term storage, the cells were moved to liquid nitrogen after one night.

2.5. Sex Determination

The DNA extraction from each embryo was performed using the QIAamp Fast DNA Tissue Kit (Qiagen, Hilden, Germany, Cat. No. 51404), according to the manufacturer's instructions. The embryos were homogenized by vortexing with lysis buffer for 30 s followed by incubation in a thermomixer (TS-100C, Biosan, Riga, Latvia) at 1000 rpm for 5 min at 56 °C. The sex of the donor embryos were determined using two pairs of primers: the female-specific *XhoI* W-repeat sequence primer set (5′primer: 5′CCCAAATATAACACGCTTCACT3′; 3′primer: 5′GAAATGAATTATTTTCTGGCGAC3′) and the 18S ribosomal gene sequence (5′primer: 5′AGCTCTTTCTCGATTCCGTG3′; 3′primer: 3′GGGTAGACACAAGCTGAGCC 3′), as described previously by Clinton et al. [39]. The PCR products were separated by electrophoresis, using 2% agarose gel stained with MIDORI Green Advance (NIPPON Genetics, Düren, Germany, cat.no. MG04), at 110 V for 35 min. The DNA bands were then visualized and photographed under G:Box Chemi XR5 (SYNGENE, Cambridge, UK). In female samples, two bands are observed: one corresponding to the female-specific *XhoI* W-repeat sequence with a product size of 415 base pairs, and the other to the 18S ribosomal gene, which is 256 base pairs in size and serves as internal control of PCR. In contrast, male embryos are expected to show only the 18S ribosomal gene sequence (Figure S4).

2.6. Quantitative Reverse Transcription PCR (RT-qPCR)

The cDNA was prepared using the smART First strand cDNA Synthesis kit (Eurx, Gdańsk, Poland, cat.no. E0804). The cDNA was amplified by real time qPCR with the primers shown in Table 2. Primers for *SSEA-1*, *CVH* and *DAZL* were designed using Primer3 (v.0.4.1) [40]. The reactions were performed in a 20-µL volume containing 10 ng cDNA; 0.25U UNG (uracil-N-glycosylase); and 15 pmol of each forward and reverse amplification primer in 1× SG qPCR master mix (Eurx, Gdańsk, Poland, E0401). Thermocycling conditions for real time qPCR were as follows: 1 cycle for UNG pre-treatment at 50 °C for 2 min, 1 cycle for initial denaturation at 95 °C for 10 min; and 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Melting-curve profiles were analyzed for all amplicons using the following thermal conditions: 95 °C for 5 s, 70 °C for 1 min, and then a gradual temperature increase to 95 °C at a ramp rate of 0.11 °C/s. Amplification was performed in Roche Light Cycler 480 v. II real-time system (Roche, Basel, Switzerland).

Table 2. Information about primers used for RT-qPCR.

Gene Abbreviation	Gene Name		Primer Sequences	Amplicon Size (bp)	Source
OCT4	Octamer-binding transcription factor 4	F	TCAATGAGGCAGAGAACACG	144	[41]
		R	TCACACATTTGCGGAAGAAG		
CVH (DDX4-VASA)	Chicken Vasa homologue (DEAD-Box Helicase 4)	F	AAGAGGAGCAGTTGGAGGTC	210	This study
		R	AGTAATGGTGCTGGAGGGTC		
DAZL	Deleted In Azoospermia Like	F	TTCGTCAACAACCTGCCAAG	144	This study
		R	TTCACCTCCTTCACAGTACCA		
NANOG	Nanog Homeobox	F	CAGCAGACCTCTCCTTGACC	149	[42]
		R	AAAAGTGGGGCGGTGAGATG		
SSEA-1	Stage-specific embryonic antigen-1	F	GCCACCTACCTGAAGTTCCT	104	This study
		R	TGCTCATCCCAGAAAGACGT		
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	F	ACACAGAAGACGGTGGATGG	193	[42]
		R	GGCAGGTCAGGTCAACAACA		

2.7. Statistical Analysis

Each sample was measured in triplicate, and fold change gene expression was determined for male and female PGCs in different conditions relative to male fresh-frozen cells, with the male fresh-frozen samples serving as the control/reference ($2^{-\Delta\Delta Ct}$ method, where control/reference = 1). All data from RT-qPCR analyses were presented as the mean ± standard deviation (SD) from three independent experiments. GraphPad Prism (version 10.0.1) software (GraphPad Software, La Jolla, CA, USA) was employed for data analysis. Significant differences in relative gene expression were assessed using a two-way ANOVA with Tukey's multiple comparison test. A p-value of less than 0.05 was considered statistically significant.

3. Results

To investigate the impact of freezing on the expression of the core pluripotent markers and germ-cell specific markers by PGCs, we present Figure 3, which illustrates the relative fold-change of gene expression in male and female PGC samples maintained in the different studied conditions compared to PGCs in fresh-frozen male samples (control). Remarkably, no significant difference in gene expression was observed between male and female samples in all studied conditions. PGCs in female fresh-frozen samples showed consistent expression pattern across all conditions, with no significant deviation from the reference. When comparing PGCs in fresh blood to those in the referenced fresh-frozen samples, it's observed that PGCs in fresh blood samples generally showed lower expression levels of the studied genes. Cultured-frozen PGCs showed higher expression of the studied genes compared to fresh-frozen cells, but without marked significance, except for the *CVH* gene, which stands out with a significant increase in expression ($p < 0.0001$), particularly in cultured-frozen male PGCs, with a mean equal to 14.5. Overall, fresh-frozen PGCs, frozen for short duration, cultured-frozen PGCs, frozen for long duration, and freshly isolated PGCs showed persistent expression of pluripotency and germline-specific markers. PGCs in fresh blood showed the lowest levels of expression for the studied markers, whereas those cultured-frozen revealed the highest levels of expression.

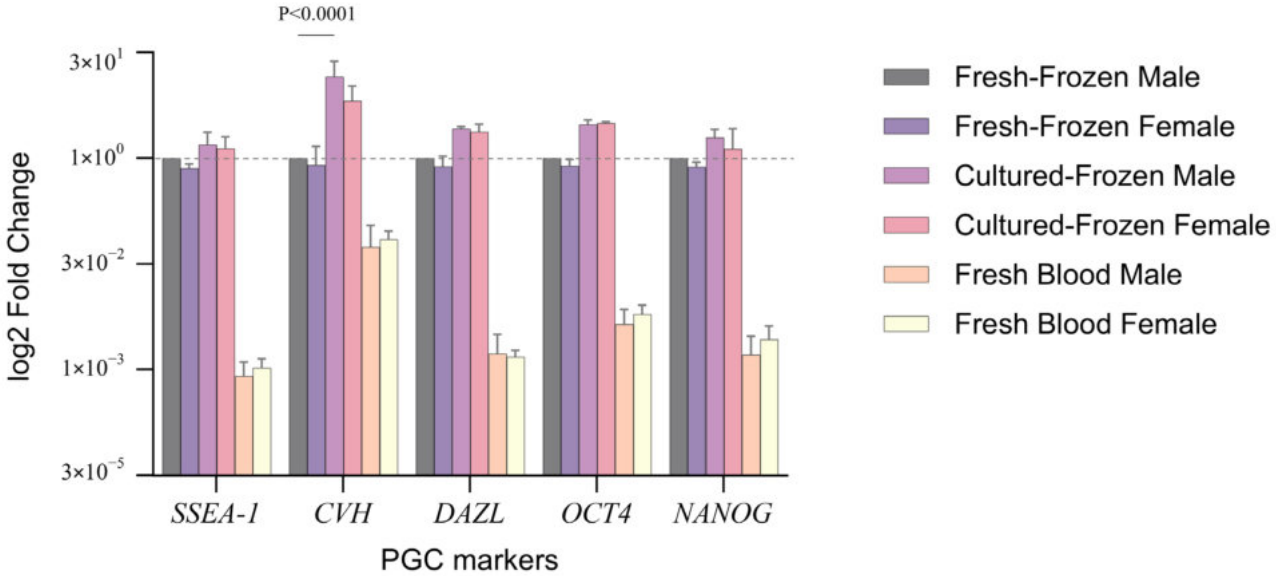

Figure 3. Fold change gene expression was determined for male and female PGC samples in different conditions relative to fresh-frozen male PGCs, with the fresh-frozen male PGC sample serving as the control/reference ($2^{-\Delta\Delta Ct}$ method, where control/reference = 1). All data from RT-qPCR analyses were presented as the mean ± standard deviation (SD) from three independent experiments. A *p*-value of less than 0.05 was considered statistically significant. Plotted data are $\log_2$ transformed.

4. Discussion

In this study, we aimed to investigate the impact of different conditions on the expression of pluripotency and PGC-specific marker genes (*SSEA-1*, *NANOG*, *OCT4*, *DAZL*, and *CVH*) in PGCs subjected to either immediate analysis after isolation, cryopreservation for a short term (2 days), or long-term cryopreservation (2 years) after in vitro culturing. We showed that male and female PGCs retained germ cell identity even under conditions of freezing-thawing and in vitro cultivation. No significant differences were observed between the sexes. Furthermore, PGCs subjected to freezing showed higher levels of expression of the aforementioned marker genes than the freshly isolated PGCs.

Altgilbers et al. have examined the expression of PGC-specific genes, including *OCT4*, *NANOG*, *DAZL*, and *CVH*, in both PGCs and chicken embryo fibroblasts [4]. Their findings demonstrated that the pluripotency markers *OCT4* and *NANOG*, along with the specific PGC stem cell markers *SSEA-1*, *DAZL*, and *CVH*, were exclusively expressed in PGCs [4]. In contrast, no expression of these markers was observed in chicken embryo fibroblasts [4]. These results clearly distinguish the gene expression patterns between PGCs and other somatic cells, highlighting the unique expression profiles characteristic of pluripotency and stemness in PGCs. Based on the information available, the expression of the mentioned genes in this study is specifically associated with PGCs found in embryonic blood obtained from 14–16 HH stage embryos.

In line with our study, Tonus et al. have shown that PGC lines, maintained for an extended period in culture (151–540 days), consistently manifested a high proportion of cells expressing SSEA-1 (90–99%), even after cryopreservation [43]. Noteworthy as well, they have unveiled the persistent expression of vital germline-specific markers—*CVH*, *DAZL*, *OCT4*, *NANOG*, *CXCR4*, and other essential genes crucial for effective gametogenesis—across the prolonged cultivation and cryopreservation stages of various cell lines [43]. This cumulative evidence implies the retention of germline competency, thereby maintaining an in vivo-like phenotype.

The higher expression of PGC markers in frozen samples compared to those in unfrozen samples may be attributed to the onset of epigenetic changes, likely caused by DMSO. The cryopreservation of chicken PGCs has been routinely conducted utilizing

DMSO as a penetrable cryoprotectant, either individually or in combination with serum as a non-penetrable cryoprotectant, through the method of gradual freezing [27]. The standard method for assessing the effectiveness of cryopreservation is to measure the survival rate of cells after thawing [44]. However, an increasing body of evidence suggests that DMSO may result in alterations to the original epigenetic markers of cells [44]. Although epigenetic mechanisms are pivotal in determining cell fate, there is a limited amount of research available on how various cryobiological factors impact these epigenetic processes. It was demonstrated that in vitro DMSO treatment of mouse embryonic stem cells upregulated pluripotency markers' mRNA expression [45]. Cryopreserving zebrafish PGCs using cryoprotectants including DMSO, polyvinylpyrrolidone (PVP), and ethylene glycol have been demonstrated to result in downregulation of *CXCR4*, *OCT4*, *VASA*, and *SOX2* transcripts, along with an increase in the expression of heat shock proteins [46]. Notably high levels of DNA methylation were observed only in the promoters of *VASA* (83.6%) and *CXCR4B* (62.1%) [46]. This suggests that DNA methylation may have played a role in reducing the expression of certain genes, like *VASA* and *CXCR4B*. However, for other transcripts like *OCT4* and *SOX2*, reduced transcript levels were not found to be linked to increased promoter methylation [46]. Similarly, another report suggested that cryopreservation with DMSO can reduce the expression of pluripotency markers such as *OCT4* in human embryonic stem cells [47]. However, such changes were not detected in specific types of stem cells, indicating that certain cell types may be less susceptible to the DMSO effect [48].

Research has indicated that DMSO can induce changes in the DNA methylation profile across the genome, particularly at specific gene loci [49]. It was found to induce alterations in the gene expression of DNA methylation enzymes [50]. Existing literature indicates that DMSO can lead to an elevation in the expression of DNA methyltransferases (DNMTs) [49,50]. Following DMSO treatment of cardiac human microtissues, *DNMT1*, a key factor for maintenance of DNA methylation, and *DNMT3A*, which facilitates both de novo and maintenance of DNA methylation, were found to be upregulated while ten-eleven translocation methylcytosine dioxygenase 1 (*TET1*), which plays a key role in active demethylation, was found to be downregulated [49]. Interestingly, no significant disruption in DNA methylation was observed when analyzing hepatic pathways. Conversely, when mouse embryonic stem cells and embryoid bodies were subjected to DMSO treatment, it was observed that *DNMT1* and *DNMT3B* expression remained unaffected, whereas the expression of *DNMT3A* increased [50]. DMSO can enhance protein levels and catalytic activity through interactions with enzyme substrates, particularly DNA and S-Adenosyl-l-methionine (AdoMet) [51]. Alternatively, DMSO might serve as a methyl donor, potentially inducing hypermethylation [52].

Different results presented by different studies may indicate species-specific and cell-specific effects of DMSO. Hence, investigating the epigenetic consequences of cryopreservation in different models can contribute to a more comprehensive understanding of the cellular mechanisms that can be induced by DMSO upon cryopreservation. To further support the hypothesis that the observed changes in gene expression stem from epigenetic mechanisms, particularly DNA methylation, it is crucial to conduct quantitative analyses of gene expression levels for pivotal enzymes engaged in epigenetic regulation. Additionally, assessing epigenetic markers, with a focus on DNA methylation patterns and histone modifications at pertinent genomic sites, is essential. Furthermore, employing bisulfite sequencing would offer a comprehensive and quantitative evaluation of DNA methylation across gene loci of interest. Alternatively, methylation arrays could provide a feasible method for high-throughput analysis of the methylation status in these crucial regions.

In this study, we have explored the effects of cryopreservation on the gene expression of Green-legged Partridgelike chicken PGCs. The significance of our findings lies in their contribution to avian germplasm conservation. This is particularly relevant for the Green-legged Partridgelike chicken breed, where maintaining genetic diversity is of utmost importance.

To build upon the current study and fully ascertain the utility of PGCs for cryopreservation, we propose several avenues for future research. Firstly, assessing the post-thaw functionality of PGCs will be critical to ensuring they can differentiate into functional gametes. Secondly, long-term viability studies are necessary to monitor the survival and developmental competence of PGCs over extended periods. Thirdly, a comparative analysis of cryoprotectants will help identify the most effective conditions for PGC preservation. Lastly, an examination of the epigenetic impacts of cryopreservation will provide deeper insights into the cellular changes induced by this process.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/genes15050624/s1, Figure S1: Chicken PGCs from a representative culture imaged at (A) seeding day, (B) 20 days of culture, and (C) after 50 days of culturing; Figure S2: A representative image of the PGCs in culture during the purification process; Figure S3: Thawed chicken PGCs after long term cryopreservation; Figure S4: Example of the PCR reactions visualization for sex determination of embryos.

Author Contributions: Conceptualization, M.B. and K.S.; Funding acquisition, M.B.; Investigation, E.G., B.L., E.V. and K.S.; Methodology, M.I. and E.G.; Writing—original draft, M.I.; Writing—review & editing, E.G., B.L., E.V. and K.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Local Ethical Committee for Animal Experiments in Bydgoszcz, Poland (Approval No. 15/2022 from 20.04.2022r).

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this article and the Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest.

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Inter- and Transgenerational Effects of In Ovo Stimulation with Bioactive Compounds on Cecal Tonsils and Cecal Mucosa Transcriptomes in a Chicken Model

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Abstract: Exploring how early-life nutritional interventions may impact future generations, this study examines the inter- and transgenerational effects of in ovo injection of bioactive compounds on gene expression in the cecal tonsils and cecal mucosa using a chicken model. Synbiotic PoultryStar® (Biomin) and choline were injected in ovo on the 12th day of egg incubation. Three experimental groups were established in the generation F1: (1) a control group (C) receiving 0.9% physiological saline (NaCl), (2) a synbiotic group (SYN) receiving 2 mg/embryo, and (3) a combined synbiotic and choline group (SYNCH) receiving 2 mg synbiotic and 0.25 mg choline per embryo. For the generations F2 and F3, the SYN and SYNCH groups were each divided into two subgroups: (A) those injected solely in F1 (SYNs and SYNCHs) and (B) those injected in each generation (SYNr and SYNCHr). At 21 weeks posthatching, cecal tonsil and cecal mucosa samples were collected from F1, F2, and F3 birds for transcriptomic analysis. Gene expression profiling revealed distinct intergenerational and transgenerational patterns in both tissues. In cecal tonsils, a significant transgenerational impact on gene expression was noted in the generation F3, following a drop in F2. In contrast, cecal mucosa showed more gene expression changes in F2, indicating intergenerational effects. While some effects carried into F3, they were less pronounced, except in the SYNs group, which experienced an increase compared to F2. The study highlights that transgenerational effects of epigenetic modifications are dynamic and unpredictable, with effects potentially re-emerging in later generations under certain conditions or fading or intensifying over time. This study provides valuable insights into how epigenetic nutritional stimulation during embryonic development may regulate processes in the cecal tonsils and cecal mucosa across multiple generations. Our findings provide evidence supporting the phenomenon of epigenetic dynamics in a chicken model.

Keywords: choline; cecal tonsils; cecal mucosa; in ovo stimulation; intergenerational effect; epigenetic dynamics; transcriptome; transgenerational effect

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1. Introduction

A bioactive compound is a substance with biological activity that affects a living organism. The effect of these compounds on organisms can be positive or negative depending on the substance, the dose, and its bioavailability [1]. In the concept of nutrigenetics and nutrigenomics, these substances can transfer information from the external environment

and can influence gene expression in the cell, thus modulating metabolic processes and the function of the whole organism [2]. Epigenetic mechanisms can modulate gene expression without altering the underlying DNA sequence. These mechanisms regulate how genes are turned on and off, allowing cells to respond to environmental signals and maintain cell-specific gene expression profiles. Major epigenetic mechanisms include DNA methylation, histone modifications, chromatin remodeling, and non-coding RNAs [3].

Epigenetic inheritance phenomena assume that epigenetic modifications can affect not only the phenotypes of exposed individuals but also their progeny and further subsequent generations through inter- and transgenerational effects occurring either via epigenetic changes during embryonic development or through the inheritance of epigenetic marks from the gametes [4,5]. Epigenetic effects can be classified as inter- or transgenerational. Intergenerational inheritance refers to the transmission of traits or phenotypes between generations that is influenced by environmental factors, often observed in the context of parental experiences affecting offspring [6]. Parental effects are also classified as an example of "context-dependent" epigenetic inheritance [7]. The latter term has a broader meaning. "Context-dependent" epigenetic inheritance is defined as that which results from direct and continuous exposure to an environmental stressor within or across generations [7]. In contrast, transgenerational (so-called "germline-dependent") inheritance involves the passing of epigenetic changes through the germline, allowing these modifications to affect multiple generations beyond the immediate offspring. As such, only the altered phenotypes occurring in the second (in the case of male transmission) or third (in the case of female transmission) generation after a trigger can truly be described as transgenerational effects [6].

Studies on mammalian models have shown that DNA methylation patterns can be transmitted for generations after exposure to an environmental perturbation (such as toxins, deficient dietary supplements, heat stress, oxidative stress, metabolic disorders, and hormonal exposure) by escaping the transgenerational erasure mechanisms [8]. Importantly, the timing of stress impact has been found to play an important role in determining epigenetic outcomes, with changes occurring early in life potentially having a greater impact than those that occur later [9].

Taking this into consideration, bird models have several advantages over mammalian ones when studying inter- and transgenerational epigenetic inheritance [5]. Chickens are characterized by early sexual maturity, a high rate of egg production (300 eggs/year), and shorter intervals between generations, as well as requiring small floor space and less feed. However, one major advantage is that a bird's embryo develops outside of the mother, and the maternal influence is reduced only to the egg composition. Other environmental factors, such as the temperature of incubation and humidity, could be strictly controlled to minimize interindividual environmental variability [5]. Moreover, the in ovo technique makes it possible to impact an embryo by direct injection of the studied substance into an egg. Despite these advantages, the chicken model has not been often utilized in inter- and transgenerational studies; therefore, the knowledge in this field needs further exploration.

Currently, synbiotics are widely used to improve health both in humans and animals [10]. Many years of research, including that conducted by our group, have shown that bioactive substances such as prebiotics, probiotics, and synbiotics, administered in ovo to the embryo on day 12 of incubation, may directly affect exposed individuals in the following terms: composition of the microbiota in chickens [11,12], physiological traits [13–15], immunological traits [16,17], intestinal development [18,19], performance traits [12,20], and immune-related gene expression in chickens [21,22].

It was observed that epigenetic mechanisms such as DNA methylation and histone modification can be influenced by dietary intake of nutrients like choline and other methyl

donors [23]. Prenatal exposure to betaine, a choline metabolite, can modulate hypothalamic cholesterol metabolism in chickens through epigenetic modifications, affecting gene expression and brain function in offspring [24]. Additionally, choline influences the gut microbiome and immune status, promoting beneficial bacteria and improving disease resistance in broiler chickens [25]. Choline supplementation has been shown to alter the gut microbiome composition, increasing the abundance of beneficial bacteria and activating pathways associated with steroid hormone biosynthesis and degradation of environmental pollutants [25].

Taking into consideration the facts mentioned above, for the first time, we stated the hypothesis that a single in ovo injection of bioactive compounds (a synbiotic and its combination with choline) may induce inter- and transgenerational effects on immune-related tissues, altering the transcriptome of both the directly exposed generation and subsequent ones. Therefore, our study aimed to investigate, for the first time, if transcriptome changes that were acquired in one generation, as a result of the prenatal in ovo impact on embryonic and long-term postembryonic development, can be inherited and propagated in the future generations. It should be noted that the novelty of this study is the use of in ovo technology and a chicken model to conduct a three-generational experiment on the effects of bioactive compounds, such as a synbiotic (PoultryStar® solUS, Biomin GmbH, Herzogenburg, Austria) and choline, on immune system tissue transcriptomes, namely cecal tonsils and cecal mucosa. Furthermore, the experimental design was the first of its kind. In parallel, we reproduced birds that received a single in ovo injection in F1 as well as individuals with repeated in ovo injections in each successive generation to investigate both "germline-dependent" and "context-dependent" inheritance.

2. Results

In this study, slow-growing local Green-legged partridgelike chickens were used to study inter- and transgenerational effects of bioactive compounds, choline and synbiotic, administered in ovo. Two groups, SYNs and SYNCHs, were designed to investigate the transgenerational impact of the single in ovo synbiotic as well as synbiotic + choline stimulation applied to the eggs laid by F0 hens. In contrast, the SYNr and SYNCHr groups, where chickens received repeated in ovo stimulation in every generation, aimed to explore the cumulative effects of repeated stimulation across generations. We examined the resulting changes in gene expression patterns within immune system tissues, i.e., the cecal mucosa and cecal tonsils, in the generations F1, F2, and F3, following in ovo stimulation at embryonic day 12 with bioactive compounds.

2.1. Dose Selection of Synbiotic and Choline

The results of Experiment 1, focused on selecting the choline source and dosage, are presented in Supplementary File S2, Table S1. The highest hatchability rates were observed with choline (Sigma Aldrich, Sain Louis, MA, USA, cat. no. C7527) at both dosages, 0.25 mg and 0.5 mg, achieving 93.3% and 100% hatchability, respectively. A two-way ANOVA was performed to examine the effects of choline source, dose, and their interaction on hatchability (Supplementary File S3, Table S1). None of the factors—choline source, dose, or their interaction—significantly influenced hatchability. Although choline source accounted for 13.8% of the variance in hatchability ($\eta^2p = 0.138$), this effect was not significant. Similarly, dose accounted for only 1.1% of the variance ($\eta^2p = 0.011$), and the interaction term explained 2.2% ($\eta^2p = 0.022$), both of which were also non-significant. For further evaluation, we selected choline (Sigma Aldrich, Sain Louis, MA, USA, cat. no. C7527) and choline (Miavit, Oldenburg, Germany) at both dosages because we observed the highest hatchability for these two products (Supplementary File S2, Table S2).

In Experiment 2, the combination of choline (Sigma Aldrich, Sain Louis, MA, USA, cat. no. C7527) at a dosage of 0.25 mg/embryo and PS synbiotic at a dosage of 2 mg/embryo achieved a 96% hatchability rate across six trials, consistently performing well. Choline (Miavit, Oldenburg, Germany) produced similar results, 96% hatchability, with 0.5 mg choline and 1 mg/embryo synbiotic. A three-way ANOVA was performed to evaluate the effects of choline source, choline dose, synbiotic dose, and their interactions on hatchability (Supplementary File S3, Table S2). None of the main effects (choline source, choline dose, or synbiotic dose) was statistically significant ($p > 0.05$). However, there were significant interactions between choline source and synbiotic dose ($p = 0.008$) and between choline dose and synbiotic dose ($p = 0.010$). Post hoc pairwise comparisons were conducted to investigate the interaction effects of choline source and synbiotic dose, as well as choline dose and synbiotic dose, on hatchability (Supplementary File S3, Tables S3 and S4, respectively). No statistically significant differences were observed between any combinations of choline source and synbiotic dose (Ptukey > 0.05). In contrast, the post hoc analysis for the interaction between choline dose and synbiotic dose revealed a significant difference between 0.25 mg choline with 2 mg synbiotic and 0.5 mg choline with 2 mg synbiotic, with the former showing significantly higher hatchability ($p = 0.033$, mean difference = 7.783%, 95% CI [0.527, 15.040]). Other comparisons within this interaction did not reach statistical significance.

Based on these findings, we selected choline (Sigma Alrich, Sain Louis, MA, USA, cat. no. C7527) at a dosage of 0.25 mg/embryo and PS synbiotic at a dosage of 2 mg/embryo for the three-generational study. While the selected combination of choline and PS synbiotic resulted in the highest hatchability rates, the differences between this combination and others were not statistically significant ($p > 0.05$).

2.2. Effect of the In Ovo Stimulation on Body Weights of Adult Chickens

The average body weights of the chickens in each group of each generation are shown in Figure 1. No significant differences in body weights were observed in the experimental groups compared to controls in F1, F2, and F3 (Figure 1). Across all groups, body weights were consistently lower in the generation F3 compared to F2. Although the natural effect of a production season on chicken body weights was observed, the injected bioactive compounds did not affect the body weights of chickens within the same generation.

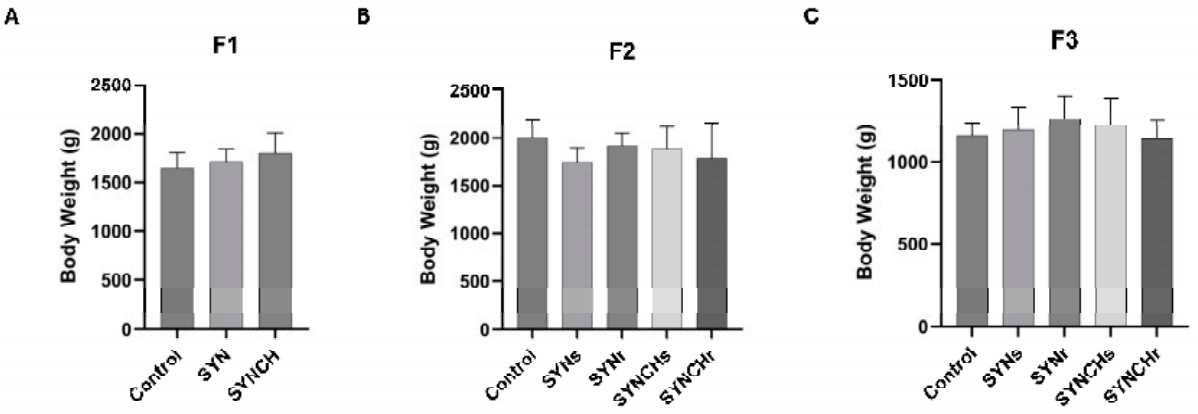

Figure 1. Body weights (in grams) of adult chickens in the different groups of F1 (**A**), F2 (**B**), and F3 (**C**) generations (n = 10 per group in each generation). All data were presented as the mean ± standard deviation (SD). SYN: synbiotic group; SYNCH: synbiotic and choline group; SYNs: single injection (F1) of synbiotic group; SYNr: repeated injections (F1, F2, F3) of synbiotic group; SYNCHs: single injection (F1) of synbiotic + choline group; and SYNCHr: repeated injections (F1, F2, F3) of synbiotic + choline group.

2.3. Gene Expression Changes Induced in Chickens by In Ovo Stimulation with Bioactive Compounds

The input read counts and the uniquely mapped reads to the chicken genome (bGal-Gal1.mat.broiler. GRCg7b) generated from each group in generations F1, F2, and F3 are summarized in Supplementary File S4. Using datasets derived from these uniquely mapped reads, differential expression analysis was performed, identifying genes with statistically significant changes in expression (adjusted p-value of $\leq$0.05). Differential expression gene (DEG) profiles are presented in Supplementary File S5, showcasing volcano plots and heatmaps.

Figure 2 presents the DEG counts across generations F1, F2, and F3 following in ovo synbiotic and synbiotic + choline stimulation for the cecal tonsils (Figure 2A) and the cecal mucosa (Figure 2B). The identified DEGs across all comparisons in both tissues are provided in Supplementary Files S6 and S7 for cecal tonsils and cecal mucosa, respectively. In generation F1, we observed that both synbiotic and synbiotic + choline administration resulted in notable changes in gene expression compared to the control, with the SYNCH group resulting in fewer DEGs than SYN in both tissues. In the cecal tonsil tissue, by generation F2, the number of DEGs drops across all groups, with the SYNs group showing two DEGs and the SYNr group five DEGs. The SYNCH groups maintained 6 DEGs in SYNCHs and 17 DEGs in SYNCHr. In the cecal mucosa in the generation F2, we observed a much larger increase in DEGs, particularly in the SYNr (177 DEGs) and SYNCHr (1163 DEGs) groups. In comparison, the SYNs and SYNCHs groups maintained 28 and 115 DEGs, respectively. In generation F3, we observed a resurgence of DEGs in the cecal tonsils, particularly in the SYNr group with 1542 DEGs and the SYNs group with 1133 DEGs, followed by the SYNCHr group with 1201 DEGs and the SYNCHs group with 511 DEGs. In the cecal mucosa; however, the number of DEGs decreased in F3, except for that of the SYNs group, which increased to 114 DEGs. The SYNr group exhibited 9 DEGs, while the SYNCHs and SYNCHr groups showed 37 and 49 DEGs, respectively. Overall, the data demonstrate that synbiotic and synbiotic + choline treatments have distinct effects on gene expression in both the cecal tonsils and cecal mucosa. The results suggest a strong transgenerational effect in F3 (SYNs and SYNCHs) on gene expression in the case of cecal tonsils despite the decrease in DEGs in F2 which is linked to the intergenerational effect of the stimulation. Repeated in ovo stimulation amplifies these effects, particularly in generation F3. On the other hand, the results observed in the case of cecal mucosa indicate an intergenerational effect in F2 and a potential transgenerational effect on gene expression in F3 (SYNs and SYNCHs). Repeated injections across generations intensify gene expression changes, particularly in F2, but may stabilize by F3.

Figure 3 shows the Venn diagrams illustrating the distribution and the overlapping of DEGs across different comparisons in the three generations for cecal tonsils and cecal mucosa, respectively. The overlapping genes are listed in Supplementary Files S8 and S9 for cecal tonsils and cecal mucosa, respectively.

2.4. Functional Clustering Based on Gene Ontology (GO) and KEGG Pathways

Functional information was extracted from the DEG datasets using Gene Ontology (GO) enrichment analysis. The enriched GO terms were categorized into three groups: biological process (BP), cellular component (CC), and molecular function (MF). The complete lists of significant GO terms across all comparisons for the two tissues—cecal tonsils and cecal mucosa—are provided in Supplementary Files S10 and S11, respectively. Likewise, the lists of significant KEGG pathways across all comparisons for cecal tonsils and cecal mucosa can be found in Supplementary Files S12 and S13, respectively.

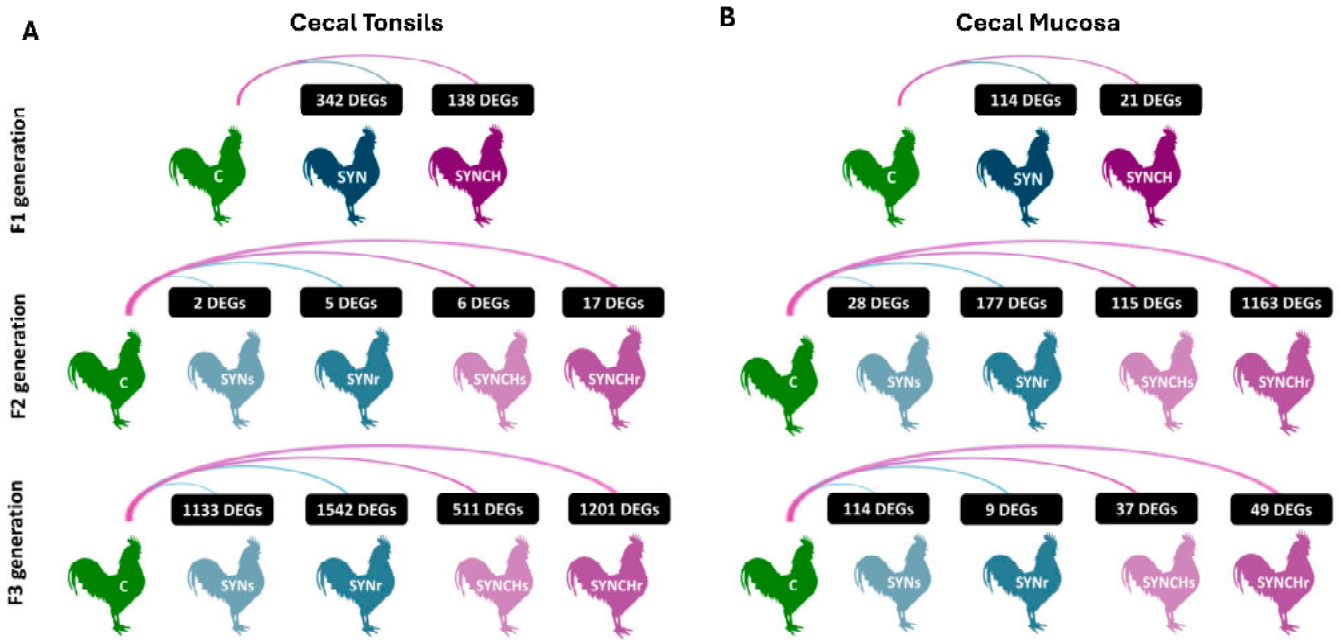

Figure 2. A diagram presenting the number of differentially expressed genes (DEGs) obtained by comparing experimental groups with the control group across three generations: F1, F2, and F3 (n = 3 per group in each generation). The figure is divided into two parts: (**A**) shows the results from the analysis of cecal tonsils, while (**B**) displays the results from the analysis of cecal mucosa. C: control; SYN: synbiotic group; SYNCH: synbiotic and choline group; SYNs: single injection (F1) of synbiotic group; SYNr: repeated injections (F1, F2, F3) of synbiotic group; SYNCHs: single injection (F1) of synbiotic + choline group; and SYNCHr: repeated injections (F1, F2, F3) of synbiotic + choline group.

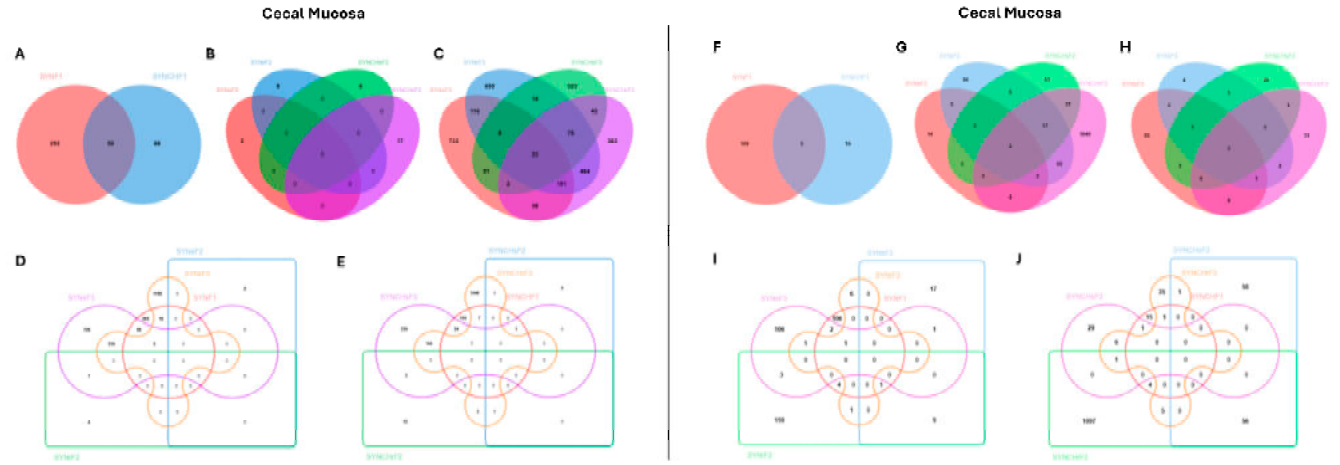

Figure 3. Venn diagrams illustrating the distribution of differentially expressed genes (DEGs) across comparisons in F1 (**A**,**F**), F2 (**B**,**G**), and F3 (**C**,**H**) generations, all synbiotic groups (**D**,**I**), and all synbiotic + choline (**E**,**J**) groups for cecal tonsils and cecal mucosa.

2.4.1. GO Terms and KEGG Pathways Enrichment Related to Cecal Tonsils

Figure 4 shows the top ten GO term enrichment analysis in cecal tonsils across three successive generations, comparing the control and synbiotic-injected groups. In the first generation (F1), biological processes were primarily related to cellular homeostasis. The second generation (F2) showed a reduction in gene expression enrichment. Both single (SYNs) and repeated injection (SYNr) groups exhibited minimal functional enrichment across biological processes, cellular components, and molecular functions. In the third generation (F3), gene expression dramatically increased. Biological processes re-emphasized cellular homeostasis and metabolic activities. Molecular functions expanded to include

transmembrane transporter activity, chemoattractant activity, and chemokine receptor binding. The repeated injection groups (SYNr) demonstrated additional enrichment in specific cellular transition processes and metabolic pathways, particularly in the third generation.

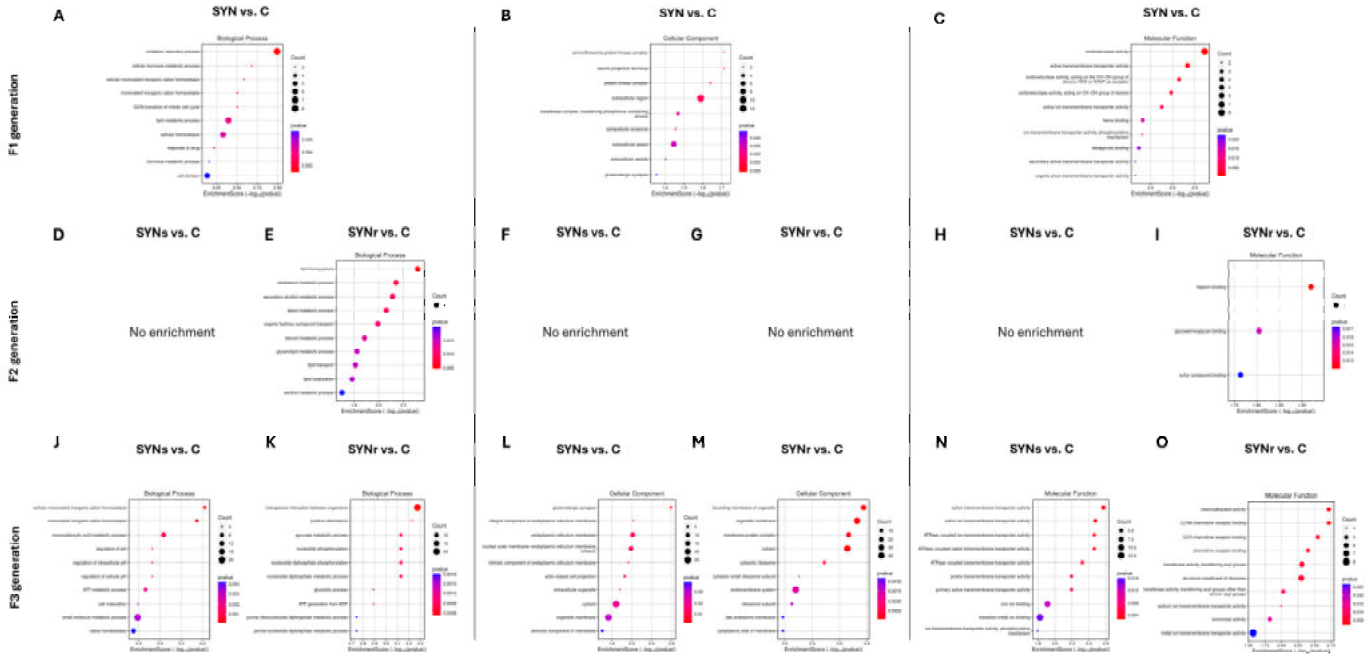

Figure 4. Gene Ontology (GO) enrichment analysis of DEGs in cecal tonsils across F1, F2, and F3 generations. (**A**–**O**) Bubble plots showing top 10 enriched terms for biological processes (**A**,**D**,**E**,**J**,**K**), cellular components (**B**,**F**,**G**,**L**,**M**), and molecular functions (**C**,**H**,**I**,**N**,**O**) in SYN groups. The size of the bubbles represents the number of enriched genes, and the color gradient indicates the enrichment significance. F1 results demonstrate response to direct exposure to synbiotic treatment, F2 shows intergenerational effects, and F3 reveals transgenerational effects.

Figure 5 displays the GO term enrichment analysis in cecal tonsils across three successive generations, comparing the control and synbiotic + choline-injected groups. In F1, the SYNCH group showed enrichment in chemical homeostasis, lipid metabolism, and hormone transport ($p < 0.05$). F2 demonstrated reduced enrichment, with the SYNCHs group showing enrichment in immune system development and the SYNCHr group in cellular transitions ($p < 0.05$). F3 exhibited increased enrichment in both SYNCHs (511 terms) and SYNCHr (1201 terms) groups compared to control, with translation and biosynthetic processes dominating in SYNCHs and pyruvate metabolism and ATP generation prominent in the SYNCHr group. For molecular functions, F1 showed enrichment in hormone and receptor activities, while F3 displayed significant enrichment in ribosomal structure and RNA binding (SYNCHs) and oxidoreductase activity (SYNCHr). Both F3 groups showed enrichment in translation regulation compared to control.

Figure 6 presents the KEGG pathway enrichment analysis in cecal tonsils across the generations F1, F2, and F3. In the F1 SYN group, metabolic pathways including retinol metabolism and steroid hormone biosynthesis showed significant enrichment ($p < 0.05$). The peroxisome proliferator-activated receptor (PPAR) signaling pathway was enriched in both F1 SYN and F3 SYNs groups compared to control. In F2, pathway enrichment was limited, though PPAR signaling persisted in the SYNs group. F3 SYNs and SYNr groups shared a common enrichment profile in oxidative phosphorylation and glycolysis pathways versus control. For SYNCH groups, F1 showed enrichment in oxidative phosphorylation, phagosome, and lysosome pathways. The cytokine–cytokine receptor interaction pathway was enriched in both F1 SYNCH and F3 SYNCHs groups, while the carbon metabolism

pathway appeared in both F2 SYNCHr and F3 SYNCHr groups. In F3, both SYNCHs and SYNCHr groups showed significant enrichment in the ribosome pathway compared to control. Significant KEGG pathways, visualized with Pathview, are shown in Supplementary File S14.

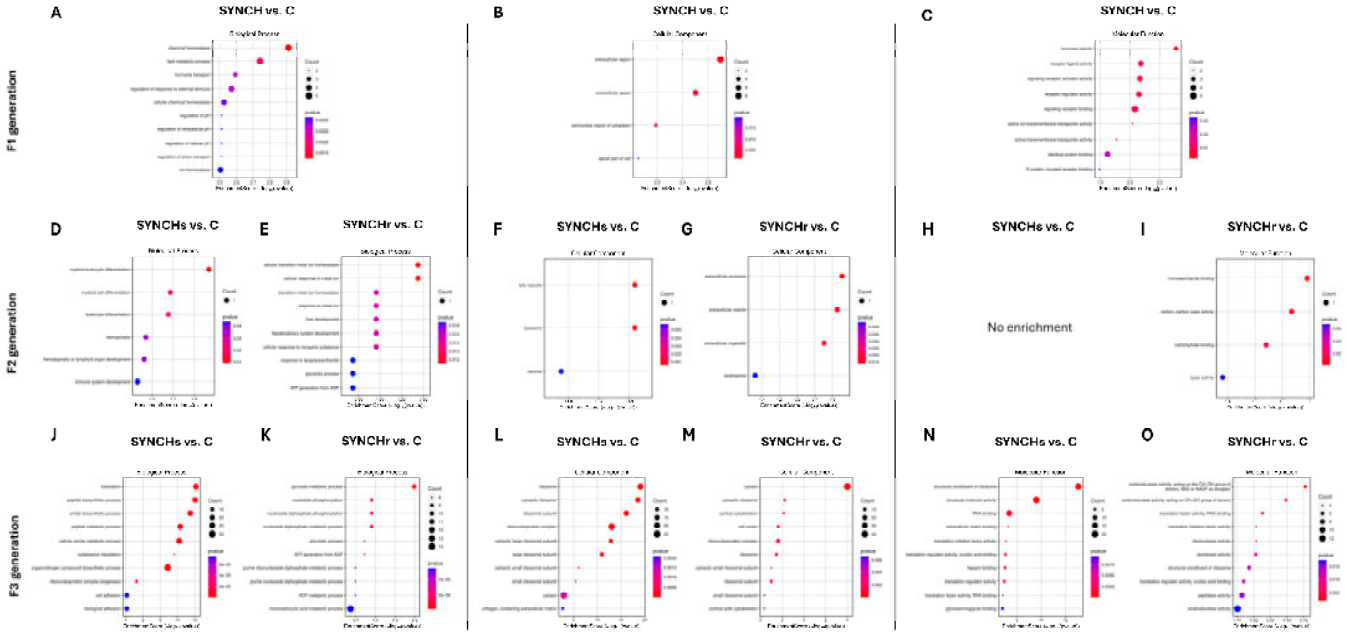

Figure 5. Gene Ontology (GO) enrichment analysis of DEGs in cecal tonsils across F1, F2, and F3 generations. (**A**–**O**) Bubble plots showing top 10 enriched terms for biological processes (**A**,**D**,**E**,**J**,**K**), cellular components (**B**,**F**,**G**,**L**,**M**), and molecular functions (**C**,**H**,**I**,**N**,**O**) in SYNCH groups. The size of the bubbles represents the number of enriched genes, and the color gradient indicates the enrichment significance.

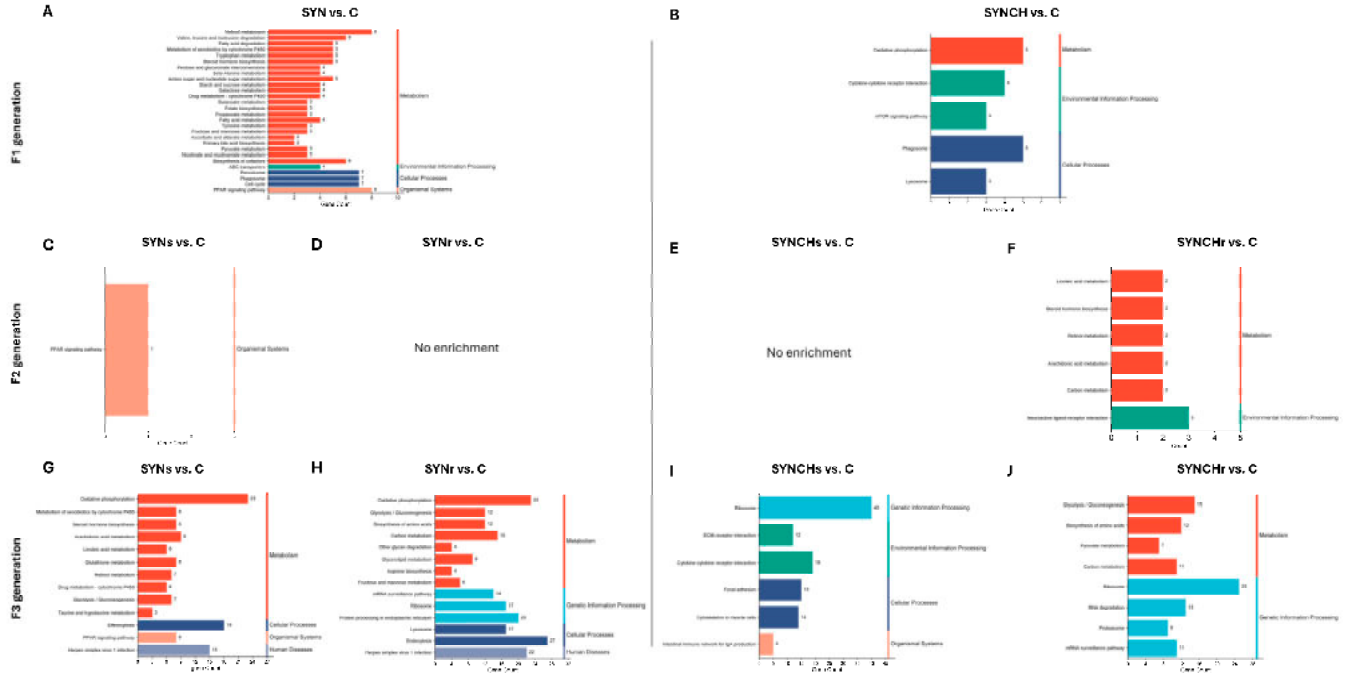

Figure 6. KEGG pathway enrichment analysis of DEGs in cecal tonsils across F1, F2, and F3 generations. (**A**–**J**) Bar plots depict the enriched KEGG pathways in SYN and SYNCH groups. Enrichment is shown for SYN groups in F1 (**A**), F2 (**C**,**D**), and F3 (**G**,**H**) and for SYNCH groups in F1 (**B**), F2 (**E**,**F**), and F3 (**I**,**J**). Each bar represents a pathway, with bar length corresponding to the number of enriched genes.

2.4.2. GO Term and KEGG Pathway Enrichment Related to Cecal Mucosa

Figure 7 shows the top ten GO term enrichment analysis in cecal mucosa across three successive generations, comparing the control and synbiotic-injected groups. The F1 SYN treatment demonstrated significant enrichment ($p < 0.05$) in pathways associated with catabolic processes and metal ion response. F2 generation analysis revealed enrichment in cell cycle and genomic regulation pathways in both SYNs and SYNr groups ($p < 0.05$ vs. control). The F3 SYNs treatment group exhibited significant enrichment in immune system-associated processes. Analysis of cellular components identified cytoskeletal element enrichment in F1 SYN, while F2 SYNs and SYNr groups displayed enrichment in chromosomal components and heterochromatin regions. Molecular function assessment demonstrated significant enrichment in kinase and phosphotransferase activity (F1 SYN) and purine ribonucleoside triphosphate binding (F1 SYN, F2 SYNs). Additionally, DNA-dependent ATPase activity showed consistent enrichment in F2 SYNs and SYNr groups relative to control.

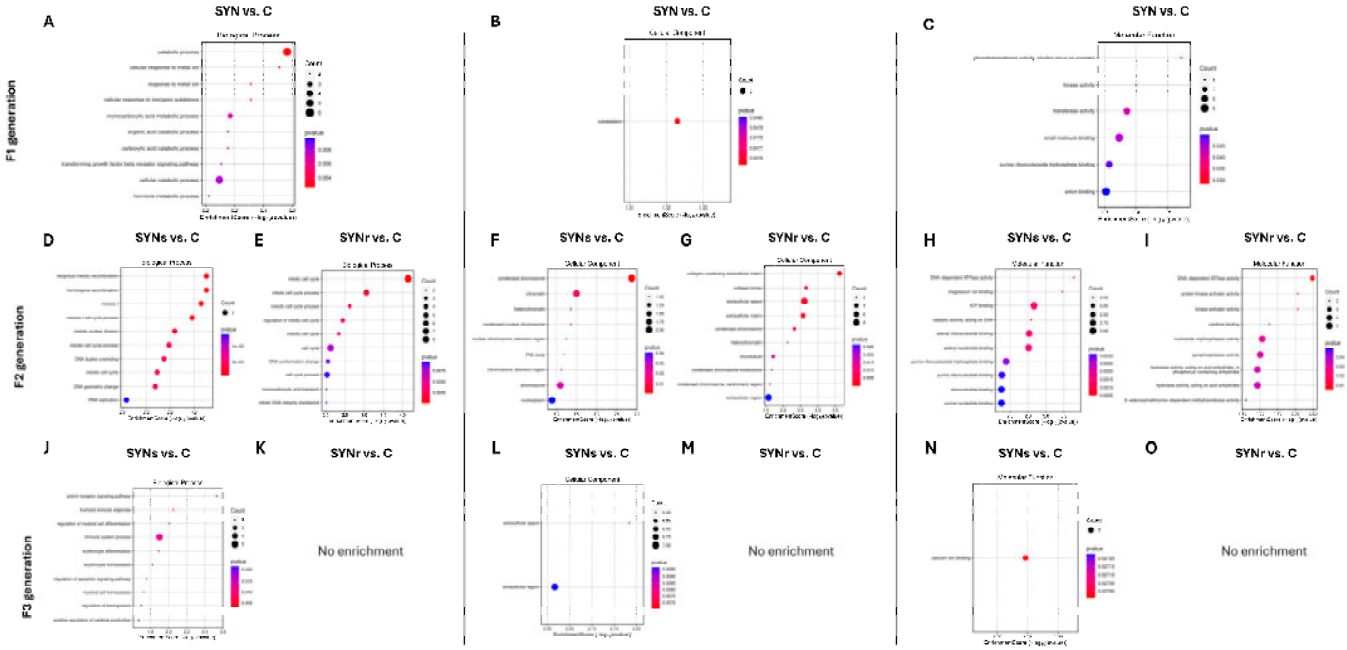

Figure 7. Gene Ontology (GO) enrichment analysis of DEGs in cecal mucosa across F1, F2, and F3 generations. (**A**–**O**) Bubble plots showing top 10 enriched terms for biological processes (**A**,**D**,**E**,**J**,**K**), cellular components (**B**,**F**,**G**,**L**,**M**), and molecular functions (**C**,**H**,**I**,**N**,**O**) in SYN groups. The size of the bubbles represents the number of enriched genes, and the color gradient indicates the enrichment significance.

Figure 8 presents the top ten GO term enrichment analysis of the cecal mucosa across three successive generations, comparing control and synbiotic+choline groups. The F1 SYNCH treatment exhibited significant enrichment ($p < 0.05$) in monocarboxylic acid metabolism and reactive oxygen species response pathways. Analysis of the F2 generation revealed enrichment in cell adhesion processes in both SYNCHs and SYNCHr groups, with additional enrichment in cell cycle and phagocytosis pathways specific to F2 SYNCHr ($p < 0.05$ vs. control). Cellular component assessment identified enrichment in apical cell regions and organelle membrane components in F1 SYNCH, while cytoskeletal components showed significant enrichment in both F2 SYNCHs and SYNCHr groups. Molecular function analysis demonstrated enrichment in oxidoreductase and transmembrane transporter activities in F1 SYNCH, sulfur compound and glycosaminoglycan binding in F2 SYNCHs, and ion binding and hydrolase activity in F2 SYNCHr relative to control ($p < 0.05$).

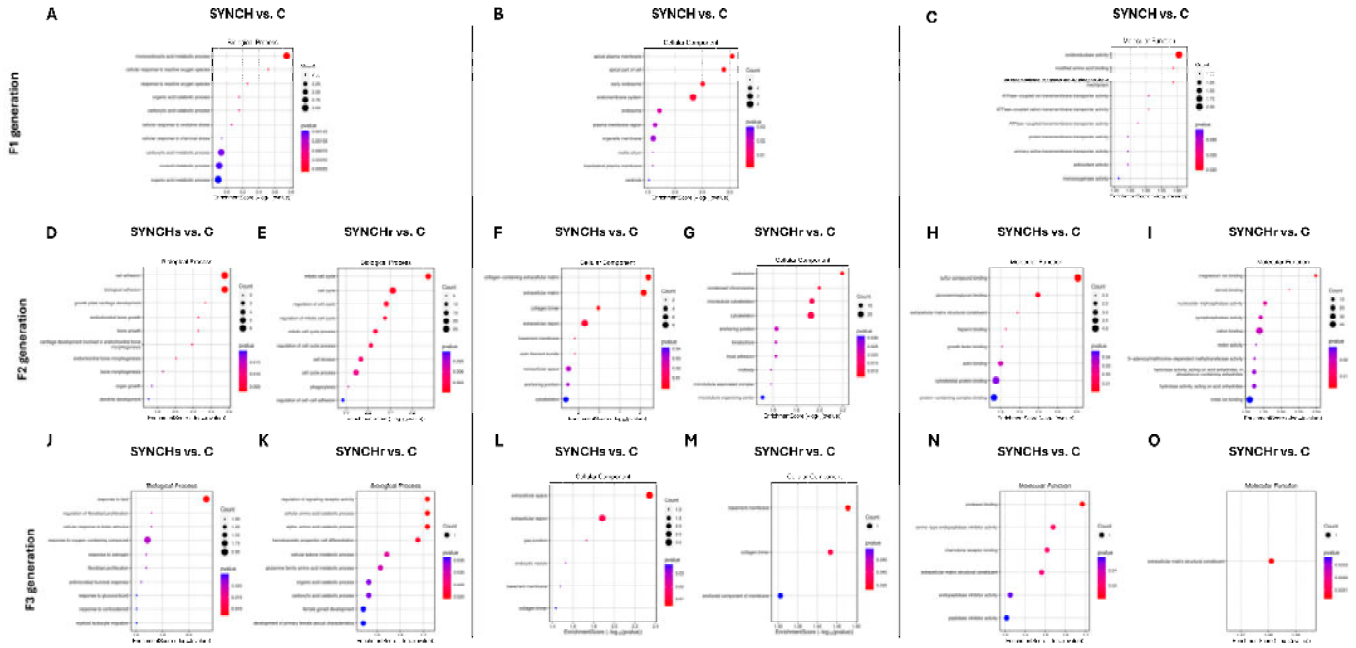

Figure 8. Gene Ontology (GO) enrichment analysis of DEGs in cecal mucosa across F1, F2, and F3 generations. (**A**–**O**) Bubble plots showing top 10 enriched terms for biological processes (**A**,**D**,**E**,**J**,**K**), cellular components (**B**,**F**,**G**,**L**,**M**), and molecular functions (**C**,**H**,**I**,**N**,**O**) in SYNCH groups. The size of the bubbles represents the number of enriched genes, and the color gradient indicates the enrichment significance.

Figure 9 presents the KEGG pathway enrichment analysis in cecal mucosa across the generations F1, F2, and F3. In the synbiotic groups, the KEGG pathway enrichment analysis revealed a strong focus on metabolism across generations. The F1 SYN treatment demonstrated significant enrichment ($p < 0.05$) in nucleotide sugar biosynthesis, amino sugar metabolism, sphingolipid metabolism, and retinol metabolism pathways. Toll-like receptor signaling pathways showed concurrent enrichment. F2 analysis identified enrichment in glutathione metabolism and drug metabolism pathways in both SYNs and SYNr groups, with the PPAR signaling pathway specifically enriched in F2 SYNr and persisting in F3 SYNr ($p < 0.05$ vs. control). The F3 SYNs group exhibited significant enrichment in lipid-associated pathways, notably linoleic acid and arachidonic acid metabolism. In SYNCH groups, F1 treatment showed enrichment in oxidative phosphorylation pathways, while F2 SYNCHs demonstrated enrichment in extracellular matrix (ECM)–receptor interaction and cytoskeletal components. F2 SYNCHr maintained similar pathway enrichment with additional PPAR signaling pathway activation. F3 analysis revealed significant enrichment in ether lipid metabolism and glycosphingolipid biosynthesis pathways in both SYNCHs and SYNCHr groups relative to control ($p < 0.05$).

Significant KEGG pathways were visualized using Pathview, highlighting potentially affected genes (Supplementary File S15).

2.5. Validation of Sequencing Data by RT-qPCR

Figure 10 presents the log2 fold change of the ten selected DEGs in each tissue, analyzed using both RT-qPCR and RNA sequencing. In the cecal tonsils (Figure 10A), RT-qPCR showed upregulation of *SRSF5*, *LAMB2*, *PLA2G10*, *MVB12B*, and *AWAT1*, along with downregulation of *RPS12*, *ADH1C*, *ATP6V0A4*, *ASS1*, and *GSTA4*. These results align with the RNA-sequencing data, demonstrating the reliability of the sequencing approach. Similarly, in the cecal mucosa (Figure 10B), RT-qPCR indicated upregulation of *FN1*, *CCNB3*, *SCD*, *ITGB3*, and *DES* and downregulation of *GSTA4*, *FABP1*, *MCOLN3*, *SLC17A5*, and

FABP2. The strong concordance in gene expression patterns and log2 fold change values between RT-qPCR and RNA sequencing further supports the accuracy and reliability of the RNA-seq data.

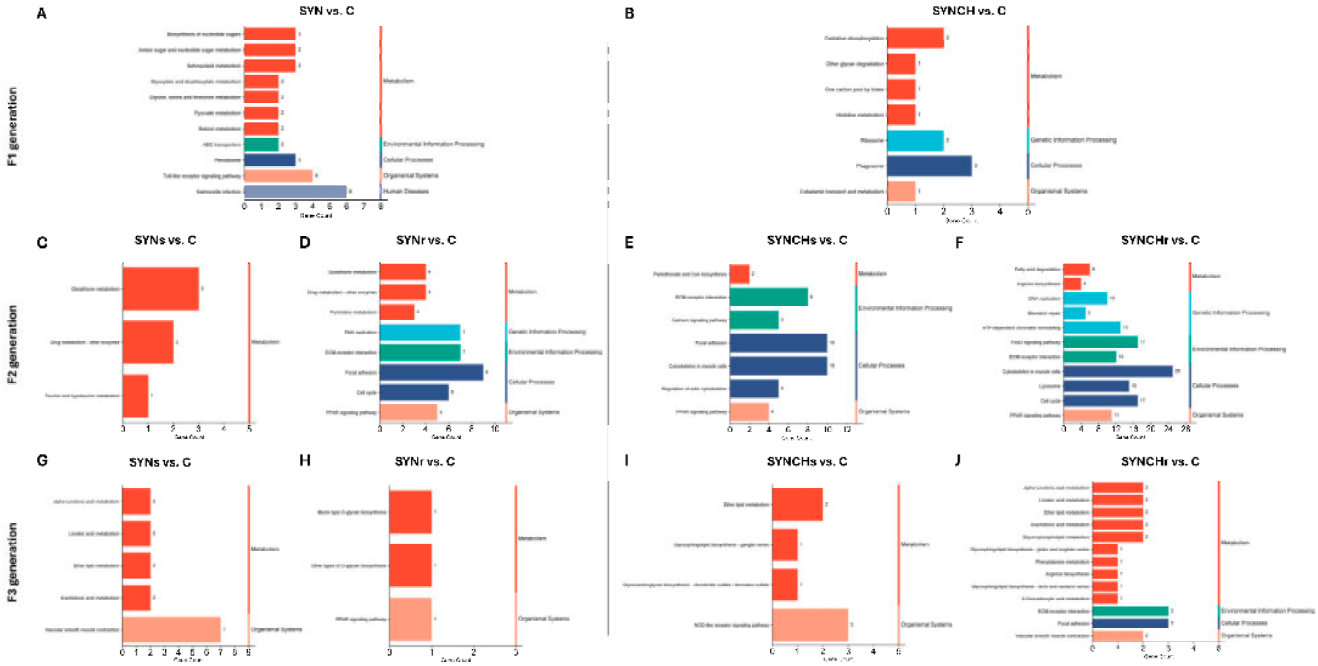

Figure 9. KEGG pathway enrichment analysis of DEGs in cecal mucosa across F1, F2, and F3 generations. (**A**–**J**) Bar plots depict the enriched KEGG pathways in SYN and SYNCH groups. Enrichment is shown for SYN groups in F1 (**A**), F2 (**C**,**D**), and F3 (**G**,**H**) and for SYNCH groups in F1 (**B**), F2 (**E**,**F**), and F3 (**I**,**J**). Each bar represents a pathway, with bar length corresponding to the number of enriched genes.

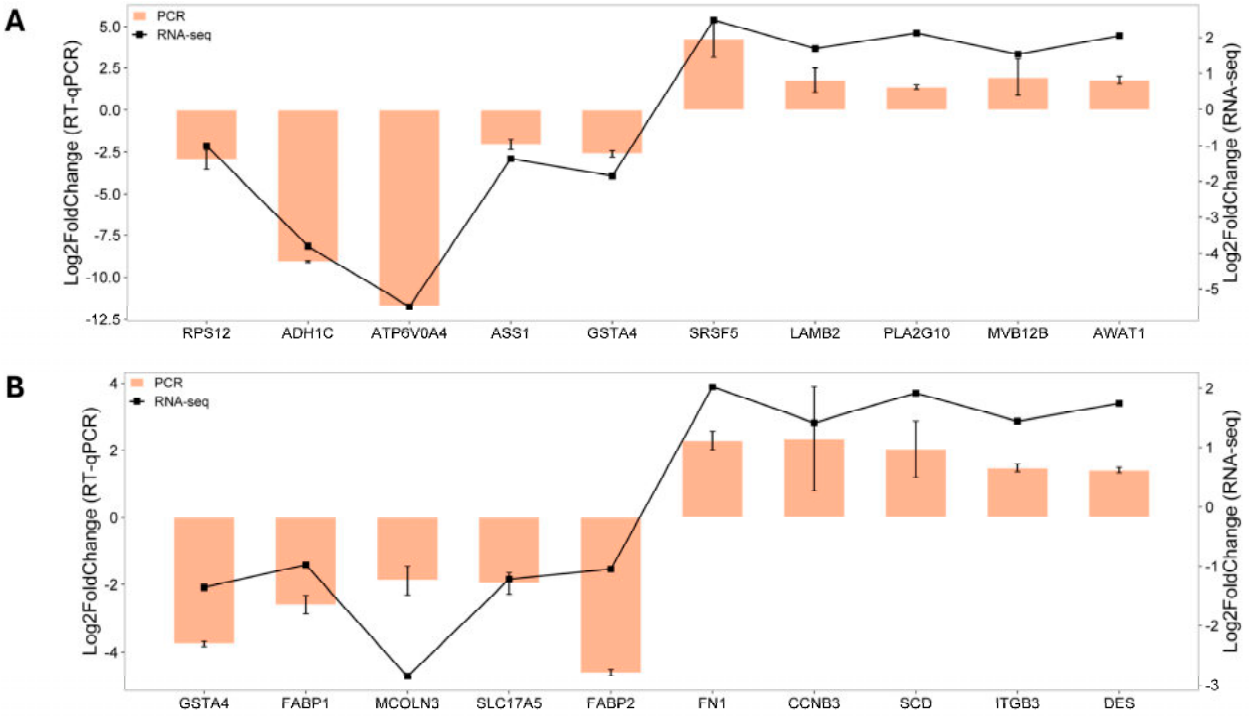

Figure 10. RT-qPCR validation of 10 selected genes for each tissue. PCR vs. RNA-seq dual y-axis plot for the genes differentially expressed in the (**A**) cecal tonsils and (**B**) cecal mucosa. All data from RT-qPCR analyses were presented as the mean ± standard error of the mean (SEM).

3. Materials and Methods

3.1. Ethical Consideration

The animals were handled following the decision of the Local Ethical Committee for Animal Experiments in Bydgoszcz, Poland (Approval No. 15/2022 on 20 April 2022), Directive 2010/63/EU and Regulation (EU) 2019/1010. Welfare monitoring was applied. Birds were kept in standard environmental conditions on a poultry farm. Qualified personnel carried out the rearing of birds. A veterinarian at the facility provided oversight of animal welfare. The study complies with the 3Rs principles and ethical standards. No suitable in vitro alternatives exist for studying transgenerational epigenetic effects in avian models.

3.2. Animals

The study involved Green-legged partridgelike chickens, a local Polish slow-growing breed known for its minimal environmental and nutritional demands, hardiness, resistance to harsh conditions, and well-developed maternal traits [26]. This breed has not undergone extensive selective breeding [26], maintaining a wider range of genetic traits.

3.3. Selection and Dosage Testing of Choline and Synbiotic

Fertilized eggs obtained from the F0 hens were incubated in standard conditions in a commercial hatchery, Wagrowiec, Poland (37.5 °C, 55% relative humidity, turned every 2 h, for 18 days, then in the hatcher for 3 days at 36.9 °C, 65% relative humidity). On the 12th day of embryonic development, after candling, bioactive compounds suspended in 0.2 mL of NaCl were manually injected into the air chamber of 10–15 eggs (Experiment 1) or 19–22 eggs (Experiment 2) with viable embryos per replicate. After injection, the hole was sealed with non-toxic glue to avoid embryo contamination and prevent moisture loss. The eggs were then returned to incubation under the same standard conditions. The in ovo injection protocol, using 0.2 mL of 0.9% NaCl, was adapted from the method optimized by Bednarczyk et al. [11,12] to ensure effective compound delivery without harming embryonic development.

3.3.1. Experiment 1

Experiment 1 aimed to select a proper choline source and dosage. Four different choline sources were tested: (1) choline chloride (Sigma Aldrich, Saint Louis, MA, USA, cat. no. PHR1251); (2) choline chloride (Sigma Aldrich, Sain Louis, MA, USA, cat. no. 26978); (3), choline chloride (Sigma-Aldrich, Saint Louis, MA, USA, cat. no. C7527), and (4) choline chloride (Miavit, Oldenburg, Germany). Two dosages, 0.5 mg/embryo and 0.25 mg/embryo, were evaluated for their effects on the eggs' hatchability. For each group three repetitions were tested separately. A control group received 0.9% NaCl. The results from the three repetitions were summed up. Hatchability was calculated for each group from the following formula: total number of hatched chicks to the number of viable eggs, candled and injected at day 12 of incubation multiplied by 100. Two choline sources from the groups with the highest hatchability were selected for the second experiment.

3.3.2. Experiment 2

The aim of the second experiment was to select the proper combination of choline and synbiotic for further study in the project. Two choline sources that showed the best results in Experiment 1 were combined with the synbiotic (PoultryStar® sol^{US}, Biomin GmbH, Herzogenburg, Austria). The two choline products were administered at dosages of 0.25 mg/embryo and 0.5 mg/embryo, and each dosage was cross-combined with two dosages of the synbiotic, 1 mg/embryo and 2 mg/embryo. Each combination was tested in six repetitions, with 19–22 eggs per repetition. A control group receiving 0.9% NaCl

was also included in this phase. After injection, eggs were further incubated under the standard conditions as described before. The results from the six repetitions were summed up. Hatchability was calculated according to the formula described in Experiment 1. Based on the hatchability of the eggs, the optimal combination of choline and synbiotic doses was selected for further experiments in the project. The combined solution of synbiotic and choline was administered manually into the air chamber of fertilized viable eggs on embryonic day 12. The synbiotic preparation used for in ovo administration, PoultryStar® sol^{US} (PS; Biomin GmbH, Herzogenburg, Austria), consisted of a prebiotic (inulin) and a probiotic mixture of four microbial strains (5.0×10^9 CFU/g): *Pediococcus acidilactici* from the cecum, *Bifidobacterium animalis* from the ileum, *Enterococcus faecium* from the jejunum, and *Lactobacillus reuteri* from the crop. The PS synbiotic is a commercial, well-defined, poultry-specific, multi-species synbiotic product that promotes a beneficial gut microbiota through the combined action of carefully selected probiotic microorganisms and prebiotic fructooligosaccharides [27]. It is also easily soluble in water, so it can be used for in ovo injections.

Statistical analyses were performed using JASP (version 0.19.3, JASP Team (2025), Amsterdam, The Netherlands). Hatchability data were analyzed using both two-way and three-way analyses of variance (ANOVAs) to examine the effects of choline source, choline dose, synbiotic dose, and their interactions on hatchability rates. For the two-way ANOVA, we assessed the effects of choline source and dose on hatchability. The three-way ANOVA included choline source, choline dose, and synbiotic dose as independent factors to evaluate potential interaction effects among these variables. Post hoc pairwise comparisons were conducted using Tukey's HSD test to identify specific differences within significant interactions. Effect sizes were reported as partial eta-squared (η^2p) and confidence intervals for mean differences were adjusted for multiple comparisons. Significance was determined at $p < 0.05$.

3.4. Experimental Design

Fertilized eggs from F0 green-legged partridgelike hens were incubated under standard conditions as described before at a commercial hatchery in Wagrowiec, Poland. On the 12th day of embryonic development, viable embryos identified by candling were randomly assigned to one of three experimental groups: (1) the synbiotic group (SYN), which received an injection of 2 mg PS synbiotic suspended in 0.2 mL NaCl; (2) the synbiotic and choline group (SYNCH), which received an injection of 2 mg PS synbiotic and 0.25 mg choline (Sigma Aldrich, Sain Louis, MA, USA, cat. no. C7527) suspended in 0.2 mL NaCl; and (3) the control group (C), which received an injection of 0.2 mL NaCl (0.9%). This rearing scheme was continued through three generations (F2 and F3). In F2 and F3, treatment groups were split into four subgroups: two groups continued with the single injection (without repeated injection in F2 and F3), one with synbiotic alone (SYNs) and the other with synbiotic and choline (SYNCHs). The other two groups received repeated injections of synbiotic alone (SYNr) and synbiotic with choline (SYNCHr) in F2 and F3.

After hatching, all chickens of each generation were raised in the same local poultry farm under semi-intensive conditions in floor pens with a bedding made of chopped wheat straw, enriched with perches, with 30 birds per experimental and control group (allowing natural behaviors) in two rearing replicates per experimental group and generation. Indoor parameters were maintained according to breed-specific requirements, with ambient temperature stabilized in cold seasons at 16–18 °C. Photoperiod management combined natural light exposure through facility windows with supplementary artificial lighting. During the growth phase, a 12:12 light:dark cycle was implemented. Upon reaching reproductive maturity, the photoperiod was gradually extended to maximally 16–17 h of

light (20–36 weeks of age), initiated at dawn, to optimize egg production for generational progression.

All birds of each generation were fed the same commercial diet free from antibiotics, probiotics, and prebiotics, purchased from a feed company (Golpasz, De Heus, Golub-Dobrzyń, Poland). Laying hens were fed a diet prepared on the farm consisting of 75% winter wheat and 25% concentrate for laying hens from De Heus (manufacturer's code: 1957—HD660X00S-W00). Birds had free access to fresh water. Individual body weights of 10 randomly selected adult chickens (after a fasting period of 12 h) per group were measured in week 21 of life across the five groups in each generation. GraphPad Prism (version 10.0.1) software (GraphPad Software, La Jolla, CA, USA) was employed for data analysis using one-way ANOVA.

3.5. Tissue Collection and RNA Isolation

Samples of cecal tonsils and cecal mucosa were collected from randomly selected 21-week-old chickens (n = 6 per group per generation). Samples were preserved in RNAlater buffer (ThermoFisher, Waltham, MA, USA) and then stored at −80 °C until use. To homogenize the tissue samples, metal beads (2.4 mm, cat. no. 10032-370, OMNI International, Tulsa, OK, USA) were employed. RNA isolation was performed using the GeneMATRIX Universal RNA Purification Kit (EURx, Gdańsk, Poland, cat. no. E3598), following the manufacturer's protocol for animal tissues with RNA Extracol reagent (EURx, Gdańsk, Poland, cat. no. E3700). RNA quantity and purity were assessed on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The integrity of the isolated RNA was assessed using an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) with an RNA Nano 6000 Assay Kit (Agilent Technologies, Santa Clara, CA, USA). Furthermore, RNA degradation and contamination were monitored on 1% agarose gel. All the extracted RNA samples passed the quality control requirements (RNA integrity number (RIN) $\geq$ 7.5) and were processed for downstream applications.

3.6. RNA-Sequencing and Bioinformatic Analysis

In total, 78 RNA-seq libraries (n = 39 per tissue) were prepared using Novogene NGS Stranded RNA Library Prep Set (PT044, Novogene, Cambridge, UK). All cDNA libraries were sequenced using a paired-end strategy with a reading length of 150 bps on an Illumina NovaSeq 6000 sequencing platform (Illumina, San Diego, CA, USA) at a depth of 20 million reads per sample by Novogene (Novogene, Cambridge, UK). FastQC v0.12.1 was used to perform the raw sequencing data's quality control [28]. Next, the raw data were processed using fastp tool v0.23.4 [29] to remove adapter sequences and trim low-quality reads to obtain clean data for downstream analyses. Simultaneously, the Q20, Q30, and GC contents of the clean data were calculated. All the paired-end reads (n = 3 per group and per generation in each tissue) passed the quality control and were mapped to the chicken reference genome (bGalGal1.mat.broiler.GRCg7b) using STAR v.2.7.11b aligner [30]. The DESeq2 v.1.42.0 program in RStudio v.2024.09.0+375.pro3 was used to perform the differential expression analysis [31]. DESeq2 was used to normalize the raw counts. A fold change criterion of less than (for downregulated genes) or greater than 0 (for upregulated genes) and an adjusted *p*-value less than or equal to 0.05 were used to define differentially expressed genes. The Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathway enrichment analysis was carried out with the Scientific and Research plot tool (SRplot, http://www.bioinformatics.com.cn/SRplot, accessed on 7 October 2024) [32], which utilizes clusterProfiler [33]. Significantly enriched KEGG pathways were visualized using Pathview [34]. Jvenn, https://jvenn.toulouse.inrae.fr/app/index.html, accessed on 7 October 2024, was used to construct the Venn diagrams [35].

3.7. Validation of Sequencing Data by Reverse Transcription–Quantitative Polymerase Chain Reaction (RT-qPCR)

Five up- and five downregulated significantly differentially expressed genes involved with different KEGG pathways were chosen for RT-qPCR assessment to validate the RNA sequencing output (Supplementary File S15). The smART First strand cDNA Synthesis kit (Eurx, Gdańsk, Poland, cat. no. E0804) was used to prepare the cDNA. Primers for the selected genes were designed using Primer Blast [36]. Supplementary File S1 shows the list of primers used for the real-time qPCR amplification of the cDNA. Reference genes were selected according to the results of the reference gene stability experiment [37]. First, 50 ng of cDNA, 0.25U of uracil-*N*-glycosylase (UNG), and 15 pmol of each forward and reverse amplification primer were added to a 1 × SG qPCR master mix (Eurx, Gdańsk, Poland, E0401) in a 20 µL volume for each reaction. Thermocycling conditions for RT-qPCR were as follows: 1 cycle for UNG pretreatment at 50 °C for 2 min, 1 cycle for initial denaturation at 95 °C for 10 min, and 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. All amplicons' melting curve profiles were examined under the following thermal conditions: 95 °C for 5 s, 70 °C for 5 s, and then a gradual rise in temperature to 95 °C at a ramp rate of 0.5 °C/5 s. The CFX Opus 96 real-time PCR equipment (BIO-RAD, Hercules, CA, USA) was used for the amplification. The relative expression levels of the studied genes were examined using the Pffafl (or standard curve) approach [38]. The double y-axis plot of PCR expression versus RNA-seq expression was visualized using the SRplot tool [32].

4. Discussion

To the best of our knowledge, we are the first to utilize a chicken model in this study to conduct a comprehensive inter- and transgenerational experiment investigating the effects of bioactive compounds, i.e., PS synbiotic and choline on immune system tissue transcriptomes.

Many studies on pre-, pro-, and synbiotics have focused on their effect on exposed individuals and/or their immediate offspring [39–41]. However, little is known about the effects of pre-, pro-, and synbiotic supplementation on further generations. Taking into account the potential of pre-, pro-, and synbiotics in building the body's immunity, we found it interesting to study if the alterations introduced by a synbiotic as well as a synbiotic combined with choline in the transcriptome of immune system tissues can be observed in further generations, i.e., F2 and F3. The changes in tissue gene expression or transcriptome often act as precursors or direct contributors to phenotypic changes. These alterations in gene expression can arise from a variety of factors, broadly categorized as genetic, epigenetic, and environmental influences [42,43]. Epigenetic factors, in particular, involve modifications that affect gene expression without altering the DNA sequence itself. Such mechanisms include DNA methylation, histone modification, and regulation by non-coding RNAs. These epigenetic changes can influence chromatin structure and gene accessibility, potentially altering gene expression. Importantly, epigenetic modifications are reversible and can be influenced by environmental conditions, lifestyle, and other external factors [42]. Herein, we decided to study immune system transcriptomes as a link between epigenetic alterations and an individual's phenotype due to the fact that it is generally accepted that changes in the epigenetic mechanism can alter phenotypic characteristics [44]. In our study, we established treatment groups which received single injections in eggs laid by generation F0 hens to study the phenomenon of transgenerational (germline-dependent) epigenetic inheritance in successive generations. In parallel, we also reproduced the treatment groups that received repeated injections in each generation to investigate the multigenerational effects of introduced bioactive compounds directly on the exposed generation as well as their cumulative effects in the successive generations.

We observed that the whole-genome gene expression profiles showed distinct intergenerational and transgenerational patterns in cecal tonsils and cecal mucosa stimulated in ovo with a synbiotic and a synbiotic combined with choline. In cecal tonsils, we revealed a very high increase in the DEG number in F3 between treated groups and the control, suggesting a transgenerational effect of synbiotic and choline injection. Interestingly, the effects were less pronounced in the generation F2, showing a sharp reduction in DEGs before the spike in F3. However, in cecal mucosa, the gene expression effects were more prominent in the generation F2, indicating intergenerational effects. In F3, some of these effects carried over, suggesting the potential for transgenerational influence, although the DEGs did not reach the same levels as in F2. An exception was seen in the SYNs group, where the effect increased in F3 compared to F2. Hence, cecal tonsils demonstrated robust transgenerational effects by F3, while cecal mucosa had intergenerational changes in F2 with the potential for continued, though less pronounced, transgenerational effects in F3. This observation can be supported by considering the specialized immune functions and intricate architecture of cecal tonsils, which likely render them more susceptible to transgenerational programming due to their continuous exposure to diverse antigens and their crucial role in shaping the immune system [45,46]. In contrast, the cecal mucosa, primarily involved in nutrient absorption and barrier function [47], may exhibit less pronounced and persistent transgenerational effects. This difference could be attributed to the transient nature of mucosal changes compared to the role of cecal tonsils in establishing long-lasting immune memory. Additionally, the differences in gene expression profiles in both tissues, despite the same epigenetic stimulation, may be due to the tissue-specific nature of epigenetic regulation [48]. For instance, in mice, developmental exposure to diethylstilbestrol (DES) induces distinct, tissue-specific patterns of DNA methylation and histone modifications in seminal vesicles and uterine tissues, driving differential gene expression and resulting in unique phenotypic outcomes [49]. This reflects the crucial role of tissue-specific epigenetic regulation in driving the observed intergenerational and transgenerational gene expression patterns.

Our findings demonstrate that the in ovo stimulation of F1 embryos with bioactive compounds can induce dynamic, non-linear intergenerational and transgenerational shifts in both cecal tonsil and cecal mucosal tissues [4].

4.1. Cecal Tonsils

The results of DEGs and enrichment analysis in the cecal tonsils seem to support our hypothesis that even a single in ovo injection of synbiotic or synbiotic + choline is able to induce potential epigenetic effects on immune-related tissues, which impacts not only the exposed individual's transcriptome but has the potential to modulate gene expression in generation F3. It is well established that embryos containing primordial germ cells (PGCs)—the precursor cells that give rise to the germline cells— are sensitive to external factors, which can introduce epigenetic marks resulting in altered gene expression of selected genes [5].

Interestingly, the whole-genome gene expression did not differ between single-injection groups (SYNs and SYNCHs) and control in F2. However, the effect in F3 was well observed. This interesting observation may be explained by "generational skipping", a phenomenon in which epigenetic modifications regulating gene expression are inherited across generations but may not manifest consistently in each. A study by Weber-Stadlbauer et al. [50] provides evidence for generational skipping in the context of transgenerational inheritance in mice. The research found that increased behavioral despair emerged in the F2 and F3 offspring of immune-challenged ancestors but not in the direct F1 descendants. This suggests that the generation F1 may act as a "silent carrier" of certain traits, which

do not manifest until later generations. This also suggests that certain effects of prenatal immune activation may skip a generation, becoming latent and potentially re-emerging under specific environmental conditions or in later generations. In our study, the generation F2 may similarly act as a "silent carrier". This pattern of inheritance is similar to other studies where a "silent carrier" phenomenon has been observed in response to chronic stress exposure [51,52]. Furthermore, the observed decrease in the number of DEGs from generation F1 to F2 could also be attributed to a "washout" effect [4]. On the other hand, the DEG increase in the generation F3 could be due to additive effects or shifts in environmental conditions (e.g., season) that reintroduce or amplify the initial epigenetic signals. In our study, generations F1 and F3 experienced similar conditions, being reared in autumn–winter season, while the F2 birds were raised during the spring–summer season. We suppose that this shift back to autumn–winter in generation F3 may potentially trigger a resurgence in gene expression effects.

Our hypothesis is further supported by the enrichment of GO terms and KEGG pathways in the cecal tonsils, which correspond to the specific treatments administered in each group. In the synbiotic groups, these enrichments are attributed to the effects of synbiotics alone, while in the synbiotic + choline groups, they reflect the combined influence of synbiotics and choline. While some enriched terms and pathways were consistently affected across generations in each group, other pathways and terms appeared uniquely in specific generations. For instance, within the SYNCH group, in KEGG pathway analysis, the phagosome pathway was enriched only in the F1 generation, while the ribosome pathway was exclusively affected in the F3 generation. In the SYN group, the KEGG pathway of ABC transporters was enriched in F1 but not in subsequent generations. Conversely, in the SYNr group, the KEGG pathway of endocytosis was uniquely enriched in the F3 generation and absent in earlier generations. This observation aligns with findings from other studies. For instance, Beck et al. demonstrated that while certain epigenetic marks, such as differentially methylated regions (DMRs), are transmitted across generations, distinct epimutations were observed in each generation in response to the epigenetic stimulation [53]. In their study, generation F3 exhibited a more integrated and overlapping epigenetic profile compared to the earlier generations. This included a higher overlap of DMRs with differentially hydroxymethylated regions (DHRs) and non-coding RNAs (ncRNA), suggesting a cumulative effect of epigenetic alterations over generations. Their findings indicate that the epigenetic landscape of generation F3 may be more complex and impactful for transgenerational inheritance.

Among the top ten enriched BPs in the synbiotic-injected groups are those related to cation homeostasis, which was seen in F1 SYN and then in F3 SYNs. Indeed, probiotics within synbiotics stabilize intestinal microbiota, which is essential for maintaining cation homeostasis [54]. This stabilization helps reduce toxic metabolites, protect the gut lining, and improve the absorption and regulation of ions like calcium and magnesium [54]. Additionally, synbiotics may influence the host's ionic balance by affecting cation transport and homeostasis mechanisms [55]. We also observed effects of the F3 SYNs group on BPs related to monocarboxylic acid metabolism, ATP metabolism, and small-molecule metabolism. This is probably related to synbiotics' ability to increase the production of short-chain fatty acids like acetate, butyrate, and propionate, which are crucial for energy metabolism and gut health [56]. By modulating the gut microbiota, synbiotics enhance the biosynthesis of small molecules, contributing to better metabolic health and a reduced risk of metabolic disorders [57]. Moreover, in our study in the F3 SYNr group, pathways related to pyruvate metabolism, nucleotide diphosphate metabolism, and purine nucleoside diphosphate metabolism were enriched. Synbiotics have been shown to increase bacterial-derived metabolites, including pyruvate, enhancing metabolic pathways [58]. Synbiotic

modulation of gut microbiota also upregulates key pathways involved in carbohydrate, nucleotide, and amino acid metabolism, essential for growth and immune responses [59]. The enriched CCs in SYN groups included the endoplasmic reticulum membrane, extracellular organ, cytosol, organelle membrane, and extrinsic component of the membrane. Synbiotics modulate the gut microbiome, influencing cellular compartments and improving nutrient absorption and immune responses [60]. They enhance intestinal barrier function by modulating cytoskeletal and tight junctional protein phosphorylation [60]. In our study, the glutamatergic synapse was enriched in both F1 and F3 SYN groups, with synbiotics affecting glutamatergic neurotransmission and potentially influencing mood, behavior, and stress responses [61]. Prebiotics like galacto-oligosaccharides also enhance glutamatergic signaling, with long-term benefits of early-life prebiotic supplementation [62]. In terms of MFs, we observed enrichment of synbiotic-injected groups in transmembrane transporter activity, which is important for nutrient absorption [63]. Moreover, synbiotics can enhance membrane fluidity and transporter function [64]. In the F3 SYNr group, we observed that enriched functions included chemoattractant activity and chemokine receptor binding, influenced by synbiotics modulating gut microbiota and short-chain fatty acid production [56]. Synbiotics may also regulate the CCR6 receptor, important for mucosal immunity, through microbiota modulation [65].

In the F3 SYNCHs group, synbiotics affected gene expression in cecal tonsils, influencing BPs related to protein synthesis, peptide metabolism, and cellular amide processes. Choline, a key component of cell membranes, plays a role in maintaining cellular homeostasis and protein synthesis through methylation processes [66]. Synbiotics also alter gut microbiota composition, improving nutrient absorption, including amino acids, and biosynthetic processes [39]. We observed that, in the F3 SYNCHr group, PS synbiotic influenced cellular metabolism, including pyruvate metabolism, nucleoside phosphorylation, and ATP generation. Choline plays a key role in lipid metabolism, energy balance, and nucleotide metabolism, supporting processes like ATP generation and nucleotide phosphorylation through its involvement in phosphatidylcholine synthesis and as a precursor for S-adenosylmethionine [67]. In both SYNCHs and SYNCHr groups of F3, MFs related to translation regulation and initiation factor activity were observed. Choline is essential for ribosomal integrity, particularly in the intestinal mucosa, and its deficiency impairs ribosomal function [68]. Choline supplementation restores polysome profiles and enhances protein synthesis by supporting ribosomal membrane binding and aggregation [68].

In synbiotic-injected groups, particularly in F1 and F3, we observed significant enrichment in metabolic pathways including retinol and steroid hormone metabolism, drug metabolism, and cytochrome P450 pathways. Synbiotics influence gut microbiota, aiding in the conversion of vitamin A [69] and steroid hormone metabolism [70]. Probiotics have also been shown to alter the expression of cytochrome P450 (CYP) enzymes throughout the gastrointestinal tract [71]. Moreover, our analysis revealed enrichment of the PPAR signaling pathway in F1, F2, and F3 SYNs groups. Indeed, synbiotics were shown to activate the PPAR signaling pathway, reducing neuroinflammation [72]. Fructose and mannose metabolism pathways were enriched in F1 SYN and F3 SYNr, as synbiotics can modulate the host's biochemistry, lipid, carbohydrate, and amino acid metabolism [73,74]. The F3 SYNs and SYNr groups also shared common metabolic pathways such as oxidative phosphorylation and glycolysis/gluconeogenesis. Probiotics have been shown to alter carbon metabolism through phosphorylation and glycolysis [75]. Synbiotics also reduce oxidative stress markers and increase antioxidant levels, enhancing oxidative phosphorylation efficiency by protecting mitochondria from oxidative damage [76].

In the SYNCH groups, we observed enrichment of pathways such as phagosome and lysosome pathways in F1. Lysophosphatidylcholine (LPC), a choline derivative, enhances

phagosome maturation and bactericidal activity, indicating a role for choline metabolites in immune responses [77]. Moreover, in our study, F1 and F3 SYNCHs groups revealed enrichment in KEGG pathways related to cytokine–cytokine receptor interactions. Synbiotics have been shown to reduce inflammatory markers in intestinal models, which could affect cytokine signaling pathways [78]. They may also boost the gut microbiota's ability to process choline, potentially altering inflammatory metabolite production through cytokine modulation [57,79]. Additionally, carbon metabolism pathways were enriched in our study in F2 SYNCHr and F3 SYNCHr groups. Choline plays a role in one-carbon metabolism, serving as a precursor to betaine, which is involved in the methylation of homocysteine to methionine, a key process in one-carbon metabolism [80]. In F3, both SYNCH and SYNCHr groups showed a resurgence of enriched pathways, particularly the ribosome pathway. The gut microbiome impacts protein synthesis, cellular homeostasis, and stress responses [81]. Choline is essential for phospholipid synthesis, which maintains cell membrane integrity and supports ribosome function for efficient protein synthesis [79].

Our findings are based on the whole-genome gene expression. Genome-wide studies have an advantage over single-gene expression because they allow the study of multiple genes and pathways. They are also a good tool in exploratory studies like the one we present in this work. In our study, a genome-wide approach allowed us to observe complex effects of injected substances on the transcriptome of the cecal tonsil tissue in a three-generational context. We found several proofs for the impact of in ovo synbiotic and choline stimulation on the cecal tonsil transcriptome. The effective action of the injected substances was also observed through their influence on the specific GO terms and KEGG pathways, which are related to previously observed biochemical and physiological effects of these substances on the organism. Our results indicate the potential of in ovo synbiotic and choline injections to modulate the transcriptome of adult chicken cecal tonsils, as well as their potential to influence the tissue transcriptome in subsequent generations.

4.2. Cecal Mucosa

In the cecal mucosa, another scenario of transgenerational dynamics was observed, where an initial increase is followed by a "washout" effect [4]. The change becomes more pronounced in F2 but then starts to recede, highlighting the non-linear nature of epigenetic effects across generations [4]. Except for the SYNs group, the number of DEGs decreased in F2 then increased in F3 to the same level as in F1. Research on the transgenerational effect of glyphosate exposure demonstrated negligible impacts on the generations F0 and F1, but a significant effect emerged in the generation F2 [82]. By the generation F3, some of these effects persisted, though with variations; certain effects seen in the generation F2 decreased or no longer appeared in the generation F3, while others continued to manifest [82]. These findings collectively underscore the complexity and non-linear nature of epigenetic inheritance. While the pattern observed in the glyphosate study differs from ours, it supports the overarching idea that transgenerational effects are dynamic and may emerge, diminish, or reappear in subsequent generations. This aligns with our findings, which show that environmental exposures can trigger epigenetic modifications with variable impacts across generations, highlighting their unpredictable and evolving nature.

Similar to cecal tonsils, we observed enriched GO terms and KEGG pathways which were related to the synbiotic and choline. In the F1 SYN group, synbiotics primarily enhance catabolic and metabolic processes. Probiotics break down complex carbohydrates into simpler sugars, which are then fermented into short-chain fatty acids [83,84]. Using specialized transport systems and enzymes, these bacteria metabolize prebiotics, supporting overall gut catabolic activity [84]. In the F2 SYNs and F2 SYNr groups, we observed a notable shift towards cell-cycle-related processes, as synbiotics improve gut barrier function by

decreasing gut permeability and reinforcing intestinal wall integrity [85]. This enhancement reduces the likelihood of pathogen translocation and inflammation, supporting regulated cell proliferation and potentially reducing disease risk [86]. Additionally, we showed that immune-system-related processes were enriched in the F3 SYNs group. Indeed, synbiotics are well known to increase both innate and adaptive immunity by stimulating natural killer cells, macrophages, antibody production, and T-cell responses [87]. The interaction of probiotics with intestinal cells induces cytokine production, helping to balance pro- and anti-inflammatory responses in the gut [87]. Moreover, we found that F2 SYNs and SYNr groups exhibited enrichment in chromosomal components like chromosomes and heterochromatin among other CC terms. This is probably due to synbiotics' ability to support gut health, which may improve chromosomal stability by reducing inflammation and oxidative stress, thus helping to prevent DNA damage [88]. This protective effect suggests synbiotics could play a role in maintaining DNA integrity and managing conditions like colorectal cancer [88]. Regarding MFs in our study, the F1 SYN and F3 SYNr groups showed enriched kinase and phosphotransferase activities. Previously, probiotics have been shown to influence adenosine-monophosphate-activated protein kinase (AMPK) activity [89]. Prebiotics can enhance intestinal barrier integrity through protein kinase C (PKC)-dependent mechanisms [90]. Moreover, phosphotransferase enzyme activity can be affected by substrate availability and specific bacterial strains, both of which synbiotic supplementation can modulate [91]. We also observed that both F1 SYN and F2 SYNs groups show purine-ribonucleoside-triphosphate-binding enrichment, which can be influenced by probiotics' effects on purine metabolism, affecting the availability and binding of purine ribonucleoside triphosphates [92].

In synbiotic + choline groups, we showed a slightly different profile of enriched GO terms in comparison with the SYN group. For instance, the F1 SYNCH group was enriched in BPs related to responses to reactive oxygen species (ROS). This is in line with results of other studies in which synbiotics were found to enhance antioxidant enzyme activity, which helps mitigate oxidative stress [93,94]. We also observed that BPs related to cell adhesion were enriched in both F2 SYNCHs and SYNCHr. Choline phosphate was reported to promote cell adhesion [95], and synbiotics are also known to improve bacterial adhesion to host cells [96]. Moreover, the F1 SYNCH group showed enrichment in CCs related to the apical cell region and organelle membranes. Synbiotics may improve gastrointestinal barrier integrity by influencing tight junctions between epithelial cells, which helps maintain the apical environment and prevent pathogen translocation [83]. In poultry, choline-enriched probiotics have been shown to enhance intestinal histological parameters, such as villus length and crypt depth, indicating better nutrient absorption and gut health [84]. In our study, the F2 SYNCHs group revealed enrichment in MFs such as glycosaminoglycan binding. Choline is essential for lipid metabolism and DNA methylation, influencing cellular interactions with glycosaminoglycans [97]. Additionally, synbiotics were found to influence lipid profiles, which can indirectly affect glycosaminoglycan interactions [98]. We also showed that the F2 SYNCHr group was enriched in ion binding and hydrolase activity functions. Choline transport in the intestine involves a carrier-mediated system that may interact with cation-binding sites [99]. Synbiotics can affect hydrolase activity, such as the bile salt hydrolase activity, which plays a key role in cholesterol metabolism [100].

In our study, KEGG pathway enrichment analysis in synbiotic groups across generations F1, F2, and F3 highlighted a strong focus on metabolism. This result is in agreement with the findings of other authors. For instance, synbiotic interventions have been shown to reverse high-fat-diet-induced changes in microbial populations, enhancing beneficial species while reducing harmful ones, which improves metabolic parameters like reduced body weight gain and glucose and lipid metabolism [101,102]. A study on diet-induced

obese mice has demonstrated that synbiotics can regulate glucose metabolism by modulating the insulin–IGF-1 signaling pathway through the overexpression of glucose transporters GLUT-1 and GLUT-4, which are essential for glucose uptake and metabolism [103]. Additionally, synbiotic supplementation in obese individuals has resulted in significant improvements in obesity-related biomarkers, including reductions in cholesterol and cytokines, highlighting their positive effects on metabolic pathways linked to lipid metabolism [104].

In the synbiotic + choline-injected groups, we observed a more diverse set of enriched pathways across generations. KEGG pathways related to metabolism were enriched in each group and each generation. In addition to the above-described effect of synbiotics on gut tissue metabolism, choline plays a critical role in lipid metabolism, particularly in lipoprotein synthesis and secretion [105]. Choline deficiency impairs intestinal lipid metabolism, leading to reduced plasma triacylglycerol and cholesterol levels and altered intestinal morphology, affecting fat absorption [105]. Besides metabolic pathways, the F2 SYNCHs group showed enrichment in ECM–receptor interaction and muscle cell cytoskeleton, which was also seen in the SYNCHr group. Probiotics can modulate immune responses in cecal tonsils, potentially affecting ECM–receptor interactions through changes in cytokine expression and immune cell activity [106]. The bioavailability of choline and its conversion to trimethylamine-*N*-oxide can influence intestinal health and disease, impacting ECM–receptor interactions via changes in cellular communication and immune responses [79]. Additionally, synbiotics can enhance intestinal villi height and surface area, which could indirectly affect muscle cell cytoskeletons [107]. Synbiotic supplementation also increased tight junction protein expression, such as Claudin-1 and Occludin, critical for the intestinal barrier and cytoskeletal dynamics [108]. In broiler chickens, choline combined with probiotics can improve intestinal histological parameters, potentially enhancing the structural integrity of intestinal and muscle cells in the cecal tonsils [109].

In both tissues, we observed a higher number of implicated genes within the potentially affected GO terms and KEGG pathways in the repeated injection groups. This finding aligns with our expectations, suggesting a cumulative effect of synbiotic injections across successive generations.

While this study involving bioactive compounds' effects on immune tissue transcriptomes was conducted using a chicken model, the findings provide valuable insights into epigenetic mechanisms and their transgenerational effects that are broadly applicable across vertebrate species, including humans. Epigenetic regulatory processes, such as DNA methylation, histone modification, and non-coding RNA activity, are conserved across vertebrates [110]. These mechanisms underpin the ability of environmental factors, including nutrition, to modulate gene expression [111]. The controlled nature of the chicken model allows for precise examination of these processes, offering a foundational understanding that can inform studies in humans [112]. Similar to the in ovo injections used in this study, early-life nutritional interventions in humans—such as maternal dietary supplementation during pregnancy—are known to influence offspring health [42]. For instance, studies have demonstrated how maternal intake of methyl-group donors, including folate and choline, can modulate epigenetic markers associated with immune and metabolic functions [113]. These parallels suggest that the bioactive compounds used in our study could have analogous effects in humans, warranting further investigation. Human studies, such as the Dutch Hunger Winter cohort, have shown that prenatal exposure to environmental factors can result in epigenetic modifications that persist across generations [114]. Our findings align with this phenomenon, demonstrating that nutritional stimulation during embryonic development can lead to both inter- and transgenerational effects on gene expression.

5. Conclusions

To the best of our knowledge, this study is the first to use a chicken model for a transgenerational experiment on the impact of bioactive compounds on immune system tissues transcriptomes. Our findings contribute to the growing body of evidence suggesting that dietary and environmental factors can influence gene expression across multiple generations. We observed that PS synbiotic and choline supplementation affected gene expression in both the cecal tonsils and cecal mucosa, with distinct effects on each tissue. The synbiotic- and synbiotic + choline-injected groups demonstrated transgenerational influences on gene expression, although the patterns varied. In the cecal tonsils, the reappearance of effects in the generation F3, after a skipped effect in F2, highlights the complex interplay between epigenetic mechanisms and environmental factors. This underscores the importance of considering the potential for latent effects to be reactivated under changing conditions. In the cecal mucosa, the results suggest that induced epigenetic modifications can trigger transgenerational effects that are not uniform or predictable, with some impacts emerging or diminishing in subsequent generations. These findings emphasize the need for further research into the complex epigenetic mechanisms through which epigenetic factors influence gene expression across generations.

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Abbreviations

PS	Synbiotic PoultryStar® (Biomin)
NaCl	Physiological saline
F0	Parental generation
F1	First generation
F2	Second generation

F3	Third generation
C	Control group
SYN	Bird group receiving in ovo injection of 2 mg synbiotic/embryo
SYNCH	Bird group receiving in ovo injection of synbiotic (2 mg) combined with choline (0.25 mg) per embryo
SYNs	Bird group receiving a single in ovo injection of 2 mg synbiotic/embryo in F1
SYNCHs	Bird group receiving a single in ovo injection of synbiotic (2 mg) combined with choline (0.25 mg) per embryo in F1
SYNr	Bird group receiving repeated in ovo injections of 2 mg synbiotic/embryo in F1, F3, and F3
SYNCHr	Bird group receiving repeated in ovo injections of synbiotic (2 mg) combined with choline (0.25 mg) per embryo in F1, F2, and F3
CD4+	Cluster of differentiation 4 positive (marker for helper T cells)
CD8+	Cluster of differentiation 8 positive (marker for cytotoxic T cells)
CD20+	Cluster of differentiation 20 positive (marker for B cells)
miRNA	MicroRNA
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
GO	Gene Ontology
BP	Biological process (Gene Ontology term)
CC	Cellular component (Gene Ontology term)
MF	Molecular function (Gene Ontology term)
RT-qPCR	Reverse transcription– quantitative polymerase chain reaction
cDNA	Complementary DNA
DEG	Differentially expressed gene
ATP	Adenosine triphosphate
PPAR	Peroxisome proliferator-activated receptor
ECM	Extracellular matrix
SRSF5	Serine- and arginine-rich splicing factor 5
LAMB2	Laminin subunit beta 2
PLA2G10	Phospholipase A2 group X
MVB12B	Multivesicular body subunit 12B
AWAT1	Acyl-CoA wax alcohol acyltransferase 1
RPS12	Ribosomal protein S12
ADH1C	Alcohol dehydrogenase 1C
ATP6V0A4	ATPase H+ transporting V0 subunit A4
ASS1	Argininosuccinate synthase 1
GSTA4	Glutathione S-transferase A4
FN1	Fibronectin 1
CCNB3	Cyclin B3
SCD	Stearoyl-CoA desaturase
ITGB3	Integrin beta 3
DES	Desmin
FABP1	Fatty-acid-binding protein 1
MCOLN3	Mucolipin TRP cation channel 3
SLC17A5	Solute carrier family 17 member 5
FABP2	Fatty-acid-binding protein 2
5azaC	5-azacytidine
PGC	Primordial germ cell
DMRs	Differentially methylated regions
DHRs	Differentially hydroxymethylated regions
ncRNA	Non-coding RNA

CCR6 receptor	C-C chemokine receptor type 6
CYP enzymes	Cytochrome P450 enzymes
AMPK	Adenosine-monophosphate-activated protein kinase
PKC	Protein kinase C
ROS	Reactive oxygen species
GLUT-1	Glucose transporter type 1
GLUT-4	Glucose transporter type 4

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Transgenerational effects of in ovo stimulation with synbiotic and choline on gonadal tissue across three generations

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Epigenetic mechanisms play a pivotal role in hereditary processes, shaping phenotypic outcomes across generations. This study investigates the transgenerational impacts of in ovo injection of bioactive substances on gene expression and DNA methylation in the male gonads using the Green-legged Partridgelike chickens as a model organism. Synbiotic PoultryStar® (Biomin; PS) and choline were injected in ovo on the 12th day of egg incubation. In the F1 generation, three groups were established: (1) control (C, 0.9% physiological saline); (2) PS synbiotic (SYN, 2 mg/embryo); and (3) PS synbiotic combined with choline (SYNCH, 2 mg/embryo of synbiotic and 0.25 mg/embryo of choline). In subsequent F2 and F3 generations, groups SYN and SYNCH were further divided into two subgroups each: (A) only injected in F1 embryos (SYNs and SYNCHs); and (B) repeatedly injected in every generation (SYNr and SYNCHr). At 21 weeks post-hatching, gonadal tissues were sampled from F2 and F3 male chickens for transcriptomic and reduced representation bisulfite sequencing (RRBS). Synbiotic alone produced minimal and diminishing changes in gene expression across generations. In contrast, the single co-administration of synbiotic and choline in F1 embryos (SYNCHs) led to 1,897 differentially expressed genes (DEGs) and 786 differentially methylated regions (DMRs) in F3. Repeated administration across generations (SYNCHr) resulted in an even greater number of DEGs (2,804) and DMRs (2,880) in F3, reflecting a cumulative exposure effect. DEGs in SYNCH groups were mainly enriched in pathways related to cytoskeletal organization and extracellular matrix. In SYNCHs, methylation changes were associated with TGF-beta signaling, whereas SYNCHr showed additional enrichment in Wnt signaling, focal adhesion, and adipocytokine signaling pathways. Integrative analysis revealed coordinated changes in gene expression and DNA methylation, particularly in the F3 generation, identifying 37 genes (47 DMRs) in SYNCHs and 194 genes (306 DMRs) in SYNCHr. This study highlights the potential of prenatal epigenetic interventions to induce gene expression and DNA methylation changes across generations in reproductive tissues.

Keywords Choline, DNA methylation, Gene expression, Gonads, in ovo stimulation, Transcriptome, Transgenerational effect

The impact of epigenetic information extends beyond mitotic cell-to-cell inheritance to include meiotic intergenerational and transgenerational inheritance[1]. It is widely recognized that genetic information can undergo epigenetic reprogramming in both the maternal and paternal germlines, potentially leading to inherited phenotypic alterations in offspring[2]. Nutrition plays a significant role as one of the primary external influencers of epigenomes[3]. Nutriepigenetics, the study of how dietary factors influence gene expression through epigenetic mechanisms, unveils a new layer of complexity in understanding the interplay between diet, the gut microbiome, and host health[4]. Interventions with bioactive substances, such as synbiotics and choline supplementation, known for their ability to modulate the gut microbiota, may exert nutriepigenetic effects, ultimately impacting the development and long-term health of the host organism[5,6]. Building on this, early nutritional reprogramming through such nutriepigenetic factors offers a promising avenue for identifying specific epigenetic changes linked to growth and metabolic outcomes[7]. This line of research has been made feasible by advancements in in ovo

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technology, which allows for the precise administration of various substances, including nutrients, hormones, vaccines, prebiotics, probiotics, and synbiotics as early as day 12 of egg incubation[8]. Diverse lines of evidence converge to suggest that epigenetic markers hold the capacity to be transmitted from parents to offspring through gametes[9,10]. In mammals, research has revealed that the dietary habits of progenitors can influence the inherited epigenetic information passed on to the next generation[11]. One of the pioneering studies linking molecular epigenetic alterations to transgenerational disease inheritance in mammals investigated the impact of administering the agricultural fungicide vinclozolin to pregnant rats[12]. Subsequent generations, F1 to F4, exhibited reproductive abnormalities including elevated testicular germ cell apoptosis and reduced sperm motility. These transgenerational phenotypes were found to be associated with alterations in DNA methylation within sperm cells[12]. Leroux et al. were pioneers in demonstrating transgenerational inheritance in birds, showing that the embryonic environment can influence the phenotype of offspring up to three generations later in quail[13]. Sexual maturity of females, adult body weight and behavioral traits were affected by a single injection of genistein at the onset of egg incubation of the first generation[13]. Similarly, in ducks, providing a diet deficient in methionine resulted in grand-offspring with modified weight gain and metabolic parameter alterations[14].

In the present study, we used the Green-legged Partridgelike chicken, which is a slow-growing breed, primarily distinguished by its low environmental and nutritional demands, to investigate the transgenerational impact of bioactive substances on male germline. Unlike commercial poultry lines, this breed has undergone limited selective breeding[15]. As an outbred population, it serves as a valuable model for transgenerational epigenetic studies, as it may be more sensitive to epigenetic modifications compared to inbred strains[16]. The knowledge about the transgenerational impact of nutriepigenetic factors in the male gonads remains limited despite their importance in inheritance. While both male and female gonads serve as primary reproductive organs for gamete production, the yolk from the female gonad potentially contains additional factors contributing to intergenerational and transgenerational inheritance compared to the male gonad[17]. Consequently, the male gonad presents an ideal target for studying the direct effects of epigenetic stimulation on transgenerational inheritance. Observing changes in gene expression patterns within gonads is particularly intriguing since these organs are known to exhibit lower metabolic activities compared to active organs, e.g., the liver[17].

Although extensive research in mammalian models has documented evidence of germline inheritance of epigenetic markers in response to nutritional stimuli[18]our understanding of the intergenerational and transgenerational mechanisms underlying prenatally induced epigenetic stimulations and their impacts in chickens, particularly within less-explored tissues such as the gonads, remains incomplete. The present study aims to investigate the transgenerational effects of in ovo injection of bioactive substances (synbiotic and choline) on gene expression and DNA methylation within the male gonads of Green-legged Partridgelike chickens.

Materials and methods

Ethical consideration

All experiments were performed in accordance with relevant guidelines and regulations. Approval for the experimental protocols was granted by the Local Ethical Committee for Animal Experiments in Bydgoszcz, Poland, under Approval No. 15/2022 on 20.04.2022, Directive 2010/63/EU and Regulation (EU) 2019/1010. The study is reported in accordance with ARRIVE guidelines[19] (https://arriveguidelines.org).

Birds and experimental design

The experiment was conducted across three successive generations of Green-legged Partridgelike chickens, with 300 eggs allocated per generation across treatment and control groups. Fertilized eggs obtained from F0 hens were incubated in standard conditions in a commercial hatchery, Wagrowiec Poland (37.5 °C, 55% relative humidity, turned every two hours, for 18 days, then in the hatcher for 3 days at 36.9 °C, 65% relative humidity). The selection of choline source and dosage, along with the synbiotic dosage, was based on hatchability results reported in our previous manuscript[20].

The experimental design is illustrated in Fig. 1 and described in detail in our previous studies[20,21]. In brief, on the twelfth day of embryonic development, after candling, eggs with viable F1 embryos were randomly divided into the following three experimental groups: (1) synbiotic group (SYN) injected with a single dose of synbiotic (PoultryStar® sol^{US}, Biomin GmbH, Herzogenburg, Austria; further referred to as PS); 2 mg/embryo suspended in 0.2 mL of physiological saline (NaCl)); (2) synbiotic and choline group (SYNCH) injected with a single dose of the PS synbiotic (2 mg/embryo) and choline (0,25 mg/embryo, Sigma-Aldrich, Saint Louis, MA, USA, cat. no. C7527) suspended in 0.2 mL of NaCl; (3) control group (C) injected with 0.2 mL of NaCl (0.9%). The rearing scheme was continued till F3 generation. In F2 and F3, the treatment groups were split into four, such that two groups were continuously bred without receiving any further injections in F2 and F3. These groups were designated as SYNs (received a single dose of synbiotic in F1 embryos), and as SYNCHs (received a single dose of synbiotic combined with choline in F1 embryos). For the other two groups, the injection of synbiotic and synbiotic with choline was repeated in every generation. These groups were referred to as SYNr and SYNCHr, respectively. The PS synbiotic preparation administered in ovo included a prebiotic (inulin) and a probiotic mixture of 4 microbial strains (5.0×10^9 CFU/g) selected from 4 different sections of the poultry gastrointestinal tract: *Pediococcus acidilactici* isolated from the cecum, *Bifidobacterium animalis* from the ileum, *Enterococcus faecium* from the jejunum and *Lactobacillus reuteri* from the crop. Chickens were reared under semi-intensive rearing conditions in floor pens, $n = 150$ birds divided into five experimental groups (30 birds/group), in two rearing replicates per group per generation. Birds were kept in pens, with a bedding made of chopped wheat straw, enriched with perches. The reared birds were fed a commercial diet free from antibiotics, probiotics and prebiotics, purchased from a feed company (Golpasz, De Heus, Golub-Dobrzyń, Poland) and had a free access to fresh water. The laying hens received feed prepared directly on the farm, based on 75% winter wheat and 25% concentrate for laying hens from De Heus Polska (Manufacturer's code: 1957 - HD660 × 00 S-W00).

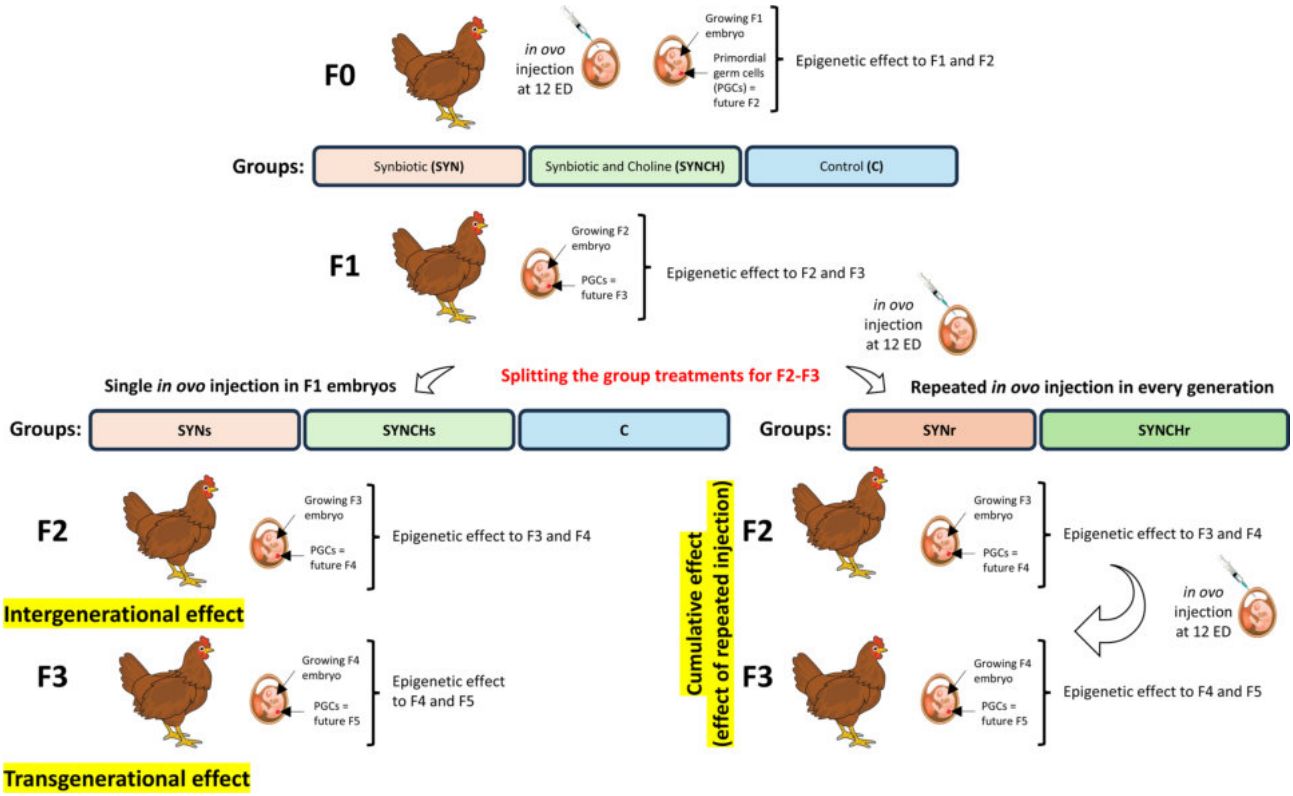

Fig. 1. The experimental design of the study. Three experimental groups were established at F1: synbiotic (SYN), synbiotic with choline (SYNCH) and the control group (C, 0.9% physiological saline). The SYN and the SYNCH groups were further split into two groups in F2 and F3, such that two new groups were formed: repeatedly injected synbiotic (SYNr) group and repeatedly injected synbiotic with choline (SYNCHr) group. Additionally, the original SYN and SYNCH groups continued with the only single-injection established in F1, referred to as the SYNs and SYNCHs groups, respectively. SYNs and SYNCHs groups are designed to study the transgenerational effects in F3 chickens. SYNr and SYNCHr groups are designed to study the cumulative effects due to repeated stimulation.

Sample preparation

Testicular samples were collected from 21-week-old male chickens ($n = 6$). For RNA isolation, samples were fixed in RNAlater buffer (ThermoFisher, Waltham, MA, USA, cat. no. AM7021). These samples were then stored at − 80 °C until later usage. For DNA isolation, separate portions of the same tissue samples were immediately placed on dry ice and then stored at − 20 °C. Metal beads (2.4 mm, cat.no 10032-370, OMNI International USA) were used to homogenize the tissues. RNA was isolated using the GeneMATRIX Universal RNA Purification Kit (EURx, Gdańsk, Poland, cat.no. E3598) following the instructions provided by the manufacturer for animal tissues with the use of RNA Extracol reagent (EURx, Gdańsk, Poland, cat. no. E3700). RNA integrity was evaluated on an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) using an RNA Nano 6000 Assay Kit RNA (Agilent Technologies, Santa Clara, CA, USA). In parallel, DNA was isolated using the Tissue DNA Purification Kit (EURx, Gdańsk, Poland, cat. no. E3550), following the manufacturer's protocol. DNA concentration was measured using a Qubit 4 Fluorometer (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, U.S., Cat. No. Q33238) with the Qubit dsDNA BR Assay Kit (ThermoFisher Scientific, Waltham, MA, USA, Cat. No. Q32850). DNA integrity was evaluated by agarose gel electrophoresis.

RNA-sequencing and analysis

A total of 30 RNA-seq libraries ($n = 15$ per generation (F2 and F3), $n = 3$ per group) were prepared using the Novogene NGS Stranded RNA Library Prep Set (PT044, Novogene, Cambridge, UK). Sequencing was conducted at a depth of 20 M per sample on the Illumina Novaseq6000 platform by Novogene (Cambridge, United Kingdom), using a 150 paired-end sequencing kit for data generation. Quality control assessment of the raw sequencing data was performed using FastQC v0.12.1 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/)[22]. Reads underwent a trimming process using the fastp tool v1.0.1[23]. The Q20, Q30, and GC contents of the clean data were analyzed. Following data preprocessing, the paired-end reads were mapped to the chicken genome (Gallus gallus genome assembly GRCg6a (galGal6), Genome Reference Consortium [GCA_000002315.5 GCF_000002315.6]) using STAR 2.7.11b software[24]. Differential expression analysis was conducted using DESeq2 (version 1.48.1)[25] on RStudio (2025.5.0.496)[26]. The raw counts were normalized using the DESeq2 package. Defined differentially expressed genes (DEGs) were called using adjusted p-value less

than or equal to 0.05 and a log2 fold change cutoff of less than or greater than 0.58. Upregulated genes were determined by a log2 fold change greater than 0.58 and an adjusted p-value of ≤ 0.05, while downregulated genes were identified by a log2 fold change less than 0.58 and an adjusted p-value of ≤ 0.05. Enrichment analysis for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG, developed by Kanehisa Laboratories)[27–29] pathways was performed using clusterProfiler[30]. Multiple testing correction was applied using the Benjamini-Hochberg (BH) method (pAdjustMethod = "BH"). Significance thresholds were set at a p-value ≤ 0.05 and a false discovery rate (q-value) ≤ 0.10. Only terms and pathways with at least three implicated DEGs were considered.

Validation of sequencing data by quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

To verify the reliability of the RNA sequencing data, five up- and five downregulated DEGs in SYNCH groups in F3 generation were selected for RT-qPCR analysis. Six biological replicates were performed for each sample. The cDNA was prepared using the smART First strand cDNA Synthesis kit (Eurx, Gdańsk, Poland, cat.no. E0804). The cDNA was amplified by real time qPCR with the primers listed in Table 1. Primers were designed using Primer Blast[31]. The reactions were performed in a 20 µL volume containing 50 ng cDNA; 0.25U UNG (uracil-N-glycosylase); and 15 pmol of each forward and reverse amplification primer in 1 × SG qPCR master mix (Eurx, Gdańsk, Poland, E0401). Thermocycling conditions for real time qPCR were as follows: 1 cycle for UNG pre-treatment at 50 °C for 2 min, 1 cycle for initial denaturation at 95 °C for 10 min; and 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. Melting-curve profiles were analyzed for all amplicons using the following thermal conditions: 95 °C for 5 s, 70 °C for 5 s, and then a gradual temperature increase to 95 °C at a ramp rate of 0.5 °C/5s. Amplification was performed in CFX Opus 96 real-time PCR system (BIO-RAD, CA, USA). The Pffafl (or standard curve) method was used to analyze the relative expression levels of the studied genes[32]. SRplot was used to visualize the PCR vs. RNA-seq expression double Y axis plot[33]. Pearson's correlation analysis was performed to evaluate the linear association between log2 fold changes obtained from RNA-seq and qPCR experiments. The correlation coefficient (r), corresponding p-value, and 95% confidence intervals were computed using RStudio (2025.5.0.496)[26]. Statistical significance was assessed at a threshold of $p \leq 0.05$.

Reduced representation bisulfite sequencing (RRBS) library preparation

RRBS libraries were prepared using the Zymo-Seq RRBS Library Kit (Irvine, California, U.S., cat. no. D5461) following the manufacturer's protocol. A total of 18 libraries were prepared from the control, SYNCHs and SYNCHr groups in F2 and F3 generations ($n = 3$ per group). A total of 300 ng of genomic DNA was utilized for library preparation with 2% spike-in using *E.coli* genomic DNA (5ng/µl). The concentrations of the prepared libraries were assessed using Qubit 4 fluorometer (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, U.S., Cat.

	Gene symbol	Gene name	Direction	Primer sequence	Reference
Upregulated genes	SOSTDC1	Sclerostin domain containing 1	Forward	CATTCACTTCTACGGCTTACTCC	This study
			Reverse	CAACTTGAACGCGATTGTTACGG	
	RGS2	Regulator of G-protein signaling 2	Forward	ACCACACCTACTTCAGACCTTC	This study
			Reverse	GTTCTCTTCGCAGAACTCAGAC	
	ELOVL3	ELOVL fatty acid elongase 3	Forward	AAGTCCTGGAACTGGGTGATAC	This study
			Reverse	CACCAGACAACATCTCCTTGTAG	
	STAR	Steroidogenic acute regulatory protein	Forward	AGGAGAAGCCCTTCAGCGAGA	This study
			Reverse	CACTTTGTCTCCGTTGTCCGCC	
	IL21R	Interleukin 21 receptor	Forward	ACATGCAGTGTCTGCGGTCC	This study
			Reverse	GGTTCTGACTGGATGTCCTTGCC	
Downregulated genes	CKMT2	Creatine kinase, mitochondrial 2	Forward	GGTCGATCAGAGGTGGAACT	This study
			Reverse	CAAACTGTGGCAATGGTGGT	
	C1orf158	Chromosome 1 open reading frame 158	Forward	CGAGGAGCCGACATTTGGTA	This study
			Reverse	TAGTTGGTGGGTGGTTCCAG	
	NME4	NME/NM23 nucleoside diphosphate kinase 4	Forward	ATGCACGTCAGCAGGAACG	This study
			Reverse	TCCCTTTGGAACCAGAAGCC	
	SPERT	Spermatid associated	Forward	CAACTCCCACAAGCAGTCCTAATC	This study
			Reverse	GCTTTGTACACCGTGTGCTCTG	
	NEUROD1	Neuronal differentiation 1	Forward	GCTGAGAACGGAGGCGCT	This study
			Reverse	GTCCTCCTCCTTCTTGTCGG	
Reference genes	PPIA	Peptidylprolyl isomerase A	Forward	CCAACCCCGTCGTGTTCTTC	[34]
			Reverse	GTTATGGGCACCTTGTCAGCG	
	ACTB	Actin beta	Forward	TGAACCCCAAAGCCAACAGAG	[35]
			Reverse	TCACCAGAGTCCATCACAATACCA	

Table 1. Primers for RT-qPCR.

No. Q33238) with Qubit 1X dsDNA HS Assay Kit (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA; Cat. No. Q33230). Libraries were validated using the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) with the Agilent DNA 1000 Kit (Agilent Technologies, Santa Clara, CA, USA, cat. no. 5067 – 1504).

RRBS-sequencing and bioinformatic analysis

Sequencing was performed on the AVITI platform (Element Biosciences, San Diego, CA, USA) using a 75-cycle paired-end sequencing kit by Genomed (Warszawa, Poland). FastQC v0.12.1 was used to assess the raw sequencing data's quality control[22]. Reads were trimmed with Trim Galore (https://github.com/FelixKrueger/TrimGalore). Bismark (Bisulfite Read Mapper and Methylation Caller software, version v0.24.2, https://www.bioinformatics.babraham.ac.uk/projects/bismark/) was used to map the reads to the chicken genome (GRCg6a (galGal6))[36]. Differential methylation analysis was performed using the DSS package[37] in RStudio (version 2025.5.0.496)[26]. A minimum coverage threshold of 10 reads per CpG site was applied. CpG methylation levels were compared between control and treatment groups using DMLtest() with smoothing enabled to improve robustness. Differentially methylated loci (DMLs) and differentially methylated regions (DMRs) were identified with a minimum methylation difference of 20% and p-value threshold of 0.05. DMRs were annotated using the ChIPseeker package[38]employing the TxDb.Ggallus.UCSC.galGal6.refGene transcript annotation database. The transcription start site (TSS) region was defined as ± 3 kb. Genes associated with DMRs (differentially methylated genes, DMGs) were subjected to GO and KEGG[27–29] enrichment analysis using the clusterProfiler package[30] with parameters set to pvalueCutoff = 0.05, qvalueCutoff = 0.10, pAdjustMethod = "BH". To investigate the relationship between DNA methylation and gene expression, DEGs from RNA-seq analysis were matched with genes associated with DMRs based on shared gene identifiers (ENTREZID). Overlap was determined by comparing DMR-annotated gene IDs with DEGs mapped to ENTREZID using the org.Gg.eg.db annotation package.

Results

In this study, slow-growing Green-legged Partridgelike chickens were used as a model organism to study the effects of single and repeated in ovo stimulation with potential dietary epigenetic modulators on gonadal tissues across three generations. Groups SYNs and SYNCHs were designed to investigate the transgenerational impact (in F3 generation) of synbiotic alone and with choline following a single in ovo stimulation in F1 embryos. Furthermore, groups SYNr and SYNCHr, underwent in ovo stimulation in every generation, aimed to explore the cumulative effects of repeated stimulation. We presented the resulting changes in gene expression and DNA methylation patterns in the male gonads of F2 and F3 following in ovo stimulation with bioactive compounds acting as potential epigenetic modulators.

Summary of RNA-seq data

A summary of RNA-seq data quality is provided in Supplementary File S1 (Table S1). The number of raw reads per sample ranged from 19,690,535 to 58,384,722 across all F2 and F3 group samples. After trimming low-quality reads and adapters, the number of clean reads ranged from 19,418,168 to 57,573,407 per sample. Across all samples, over 97% of bases had a quality score of Q20, and over 92% had a quality score of Q30, indicating high sequencing accuracy. GC content varied between 48% and 51%. Clean reads were mapped to the chicken reference genome assembly galGal6 (GRCg6a), and the mapping summary is provided in Supplementary File S1 (Table S1).

Gene expression changes associated with nutriepigenetic factor supplementation

Using datasets derived from uniquely mapped reads, totaling 25,466 identified genes, differential expression analysis was performed, identifying genes with statistically significant changes in expression (|log2 fold change| threshold = 0.58, adjusted p-value of ≤ 0.05).

Figure 2 shows the discrepancy in the number of DEGs when comparing treatment groups with the control in both F2 and F3 generations. The identified DEGs in the gonads across all comparisons are provided in Supplementary file S2. Groups treated with PS synbiotic alone, SYNs and SYNr, showed higher numbers of DEGs in F2 than in F3. There were 11 DEGs for SYNs and 23 DEGs for SYNr in F2 generation, whereas this count was reduced to 8 and 1 DEG for SYNs and SYNr in F3 generation, respectively. Conversely, in the PS synbiotic plus choline-treated groups, we observed an inverse trend. In SYNCHs and SYNCHr in F2, we identified 80 and 28 DEGs, respectively, which markedly escalated to 1,897 and 2,804 DEGs for SYNCHs and SYNCHr in F3, respectively. Notably, administering synbiotic and choline together resulted in a greater number of affected genes compared to PS synbiotic supplementation alone. The results obtained provide compelling evidence that synbiotic supplementation alone, whether administered as a single injection in F1 (SYNs) or repeatedly in F1, F2 and F3 (SYNr), exhibited minimal effect, which even diminished by the F3 generation. However, when synbiotic supplementation was combined with choline, the impact became more pronounced.

In F2, the SYNCHs group shared only 3 overlapping DEGs with SYNCHr, while the SYNs and SYNr shared just one DEG. No common DEGs were found between SYNs and SYNr in F3. In contrast, SYNCHs and SYNCHr in F3 showed substantial overlap, sharing 1,339 DEGs. Across generations (homologous groups in F2 and F3), one common gene was found in SYNs, none in SYNr, 14 in SYNCHs, and 6 in SYNCHr. Overlapping DEG lists are provided in Supplementary File S3.

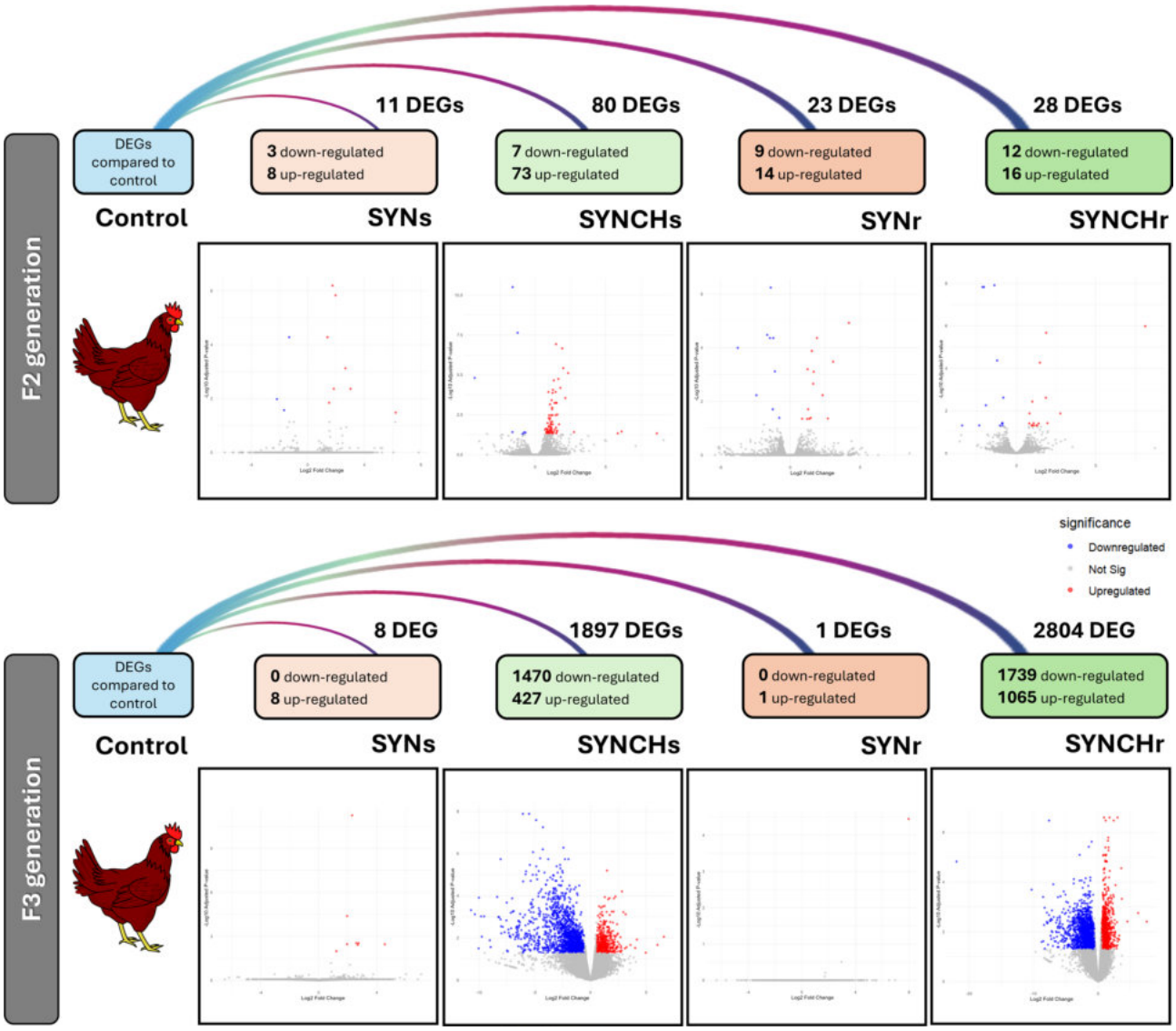

Fig. 2. Differentially expressed genes (DEGs) identified in F2 and F3 generations. The diagram summarizes the number of DEGs detected in each experimental group compared to the control. Volcano plots illustrate the expression profiles, where red dots indicate upregulated genes, blue dots indicate downregulated genes, and grey dots represent non-significant genes. DEGs: differentially expressed genes; C: control; SYNs: group of single injection of synbiotic in F1; SYNr: group of repeated injection of synbiotic in F1-F3; SYNCHs: group of single injection of synbiotic with choline in F1; SYNCHr: group of repeated injection of synbiotic with choline in F1-F3.

Functional clustering by gene ontology (GO)

Functional information was extracted from the DEG datasets using gene ontology (GO) enrichment analysis. The enriched GO terms were categorized into three groups: biological process (BP), cellular component (CC), and molecular function (MF). The lists of all significant GO terms across all comparisons are provided in Supplementary file S4. Significant enrichment in SYN groups in F2 and F3 was not possible due to the low number of DEGs in these groups. No significant enrichment was observed in the SYNCHs group in the F2 generation, whereas the SYNCHr group showed enriched biological processes primarily related to cellular motility and the regulation of responses to external stimuli. In F3, SYNCHs group resulted in 10 significantly enriched CCs, primarily associated with extracellular matrix structure and chromosomal organization (Fig. 3A-B). Only one MF term, collagen binding, was significantly enriched in the SYNCHs group (Fig. 3C-D). On the other hand, the SYNCHr group showed significant enrichment of BP terms related to cytoskeletal organization, extracellular matrix organization, and tissue migration (Fig. 4A–B). Enriched CC terms in this group were associated with supramolecular structures, including the collagen-containing extracellular matrix, cytoskeletal fibers, and actin filament bundles (Fig. 4C–D). The enriched MF terms included actin binding and cytoskeletal protein binding (Fig. 4E–F).

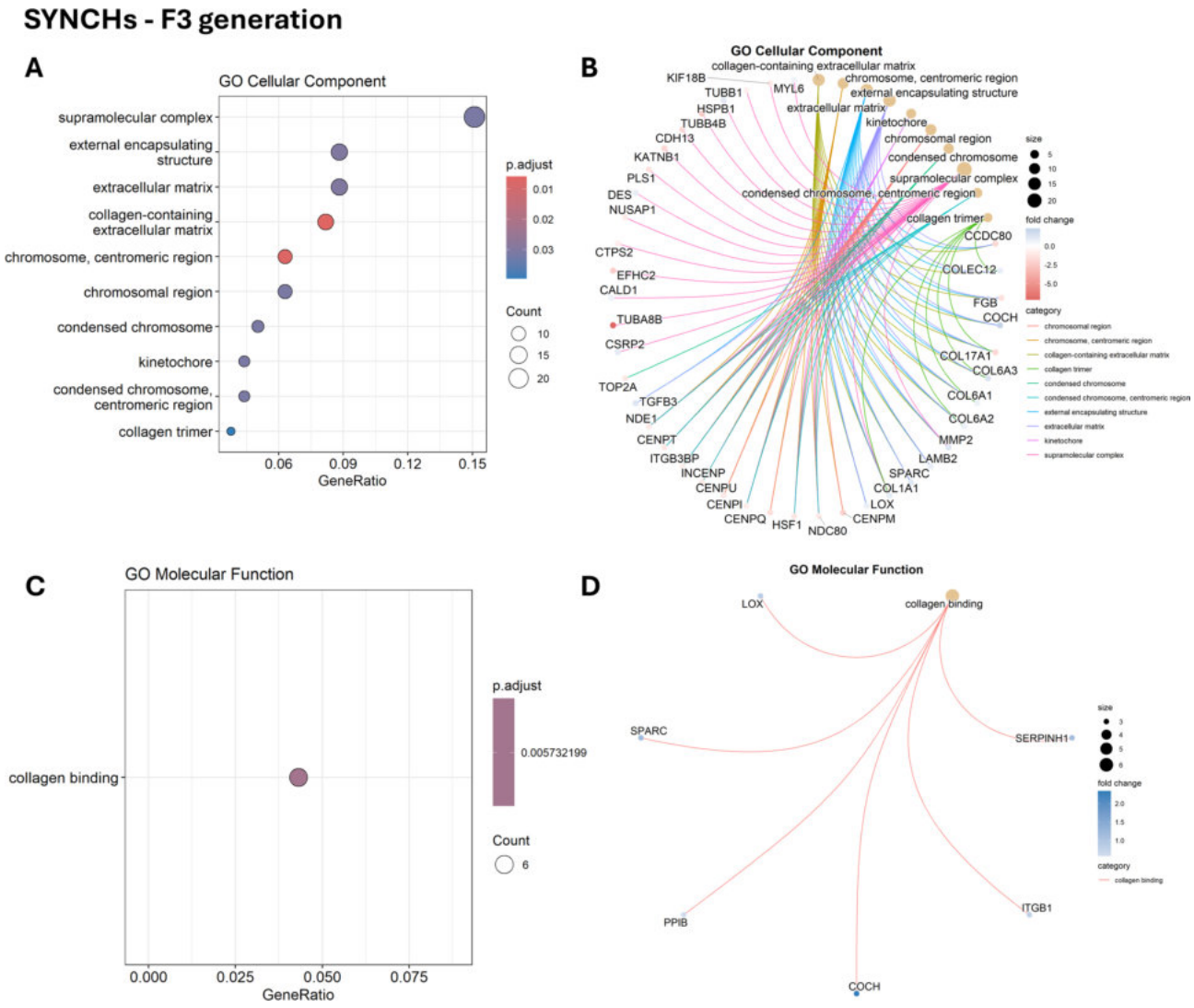

Fig. 3. Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in SYNCHs compared to control in F3 generation. (**A**, **C**) Dot plots showing the top 10 enriched GO terms (cellular components and molecular functions, respectively). (**B** and **D**) Cnet plots showing the relationship between the DEGs and GO terms for the enriched cellular components and molecular functions, respectively.

KEGG enrichment analysis

KEGG pathway enrichment was observed exclusively in the SYNCHs and SYNCHr groups in F3 generation (Fig. 5). Both groups shared enrichment in motor proteins, cytoskeleton in muscle cells, and ECM–receptor interaction pathways. Additionally, the SYNCHr group showed further enrichment in focal adhesion, regulation of actin cytoskeleton, and biosynthesis of nucleotide sugars.

Validation of sequencing data by RT-qPCR

Figure 6 presents the log2 fold change of the ten selected DEGs in the gonadal tissue, analyzed using RT-qPCR and RNA sequencing. RT-qPCR showed the upregulation of *SOSTDC1*, *RGS2*, *ELOVL3*, *STAR* and *IL21R* and the downregulation of *CKMT2*, *C1orf158*, *NME4*, *SPERT* and *NEUROD1* which is consistent with the RNA-sequencing results. Pearson's correlation test showed a strong, statistically significant positive correlation between RNA-seq and qPCR log2 fold changes ($r = 0.96$, $n = 10$, $p < 0.001$), indicating a high degree of agreement between the two methods. The consistency of the log2 fold change changes (qRT-PCR) and log2 fold changes (RNA-seq) further confirmed the reliability of the RNA-seq data.

RRBS-based analysis of DNA methylation in synbiotic + choline groups

Since RNA-seq analysis revealed a stronger effect on gene expression following the co-administration of synbiotic and choline compared to synbiotic alone, we performed differential DNA methylation analysis using RRBS data for the SYNCHs and SYNCHr groups compared to their respective controls in the F2 and F3 generations, to

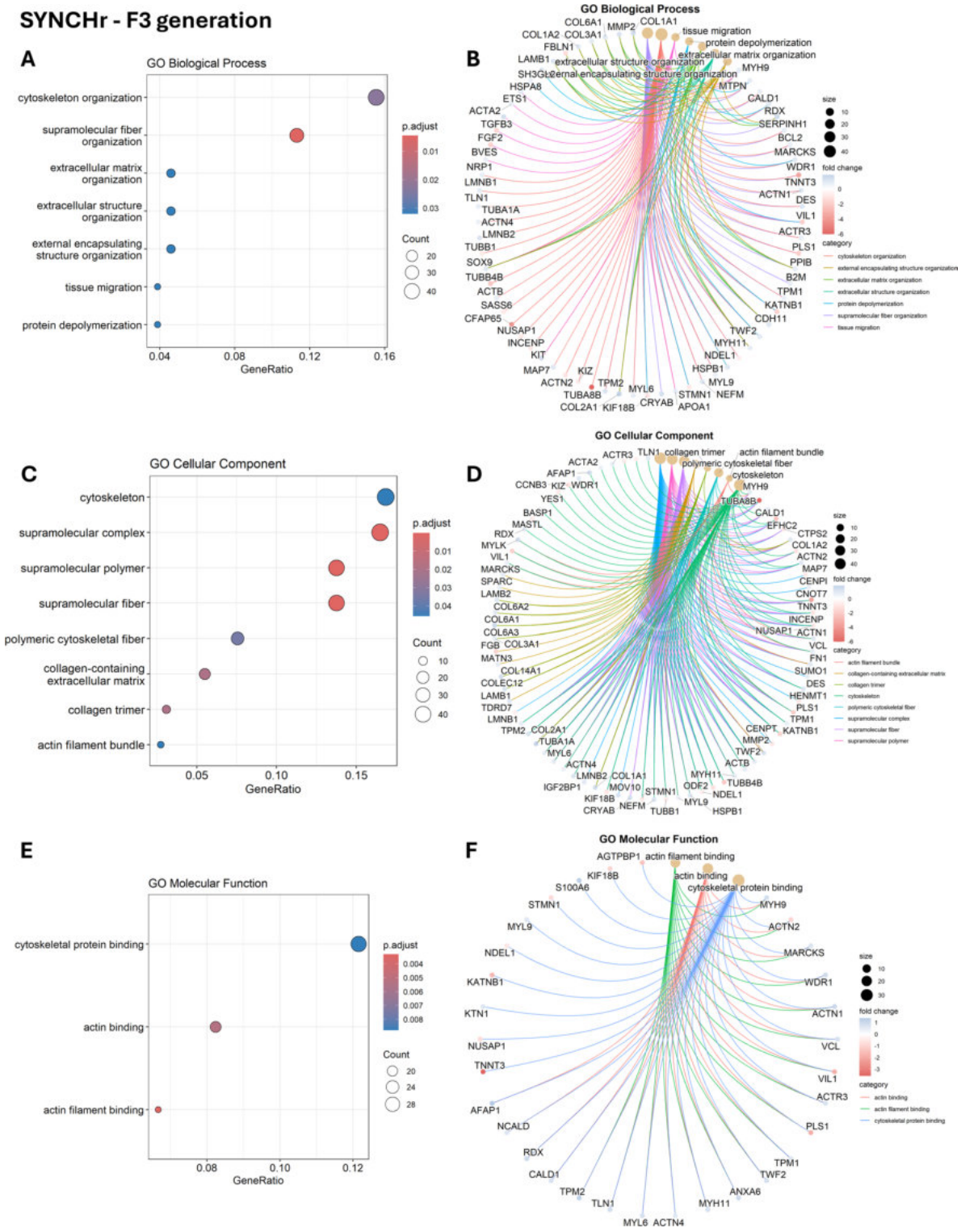

Fig. 4. Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) in SYNCHr compared to control in F3 generation. (**A**, **C**, **E**): dot plots showing the top 10 enriched biological processes, cellular components and molecular functions, respectively. (**B**, **D**, **F**) Cnet plots showing the relationship between the DEGs and GO terms for biological processes, cellular components and molecular functions, respectively.

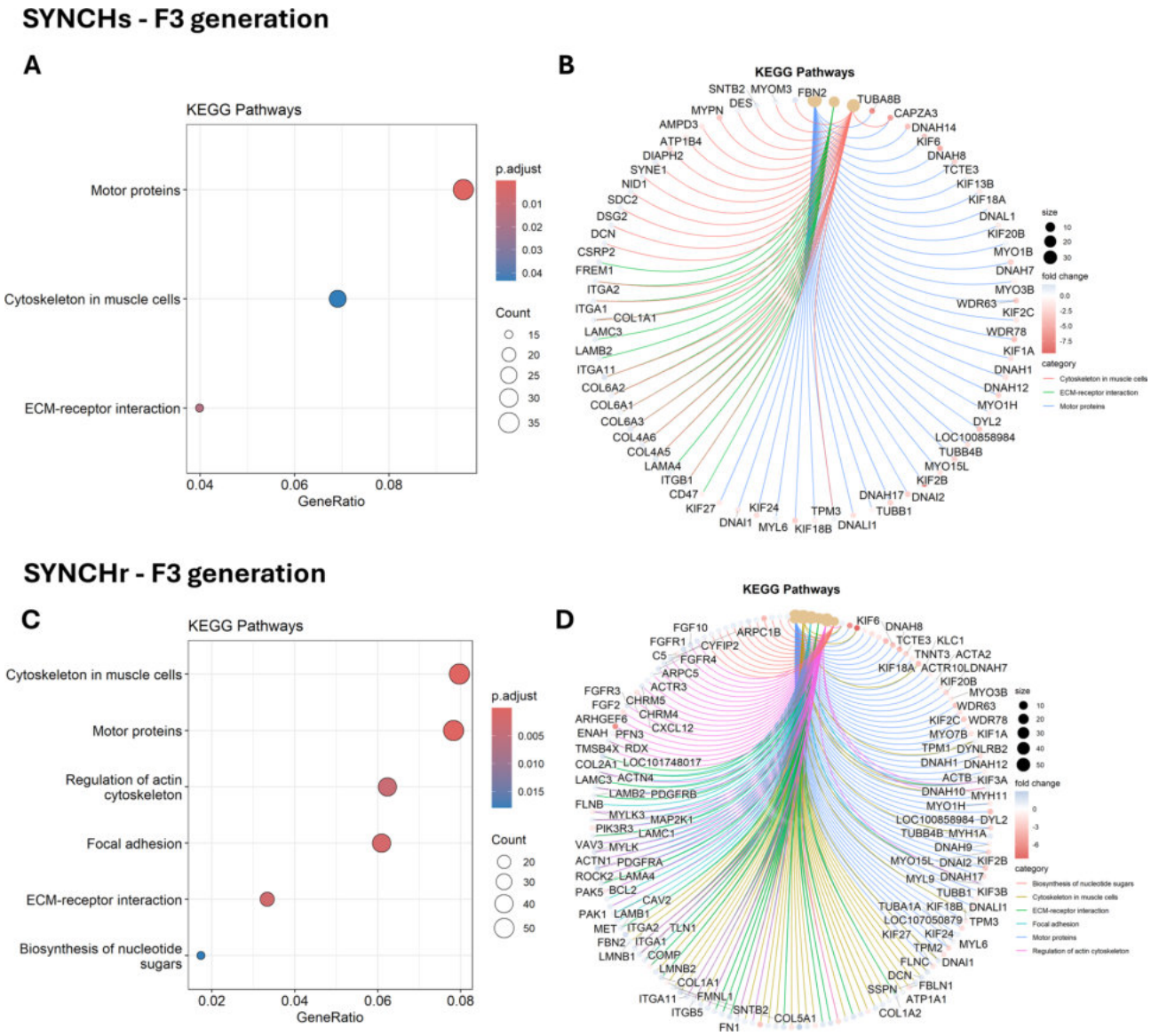

Fig. 5. KEGG pathways analysis in F3 generation. (**A**, **C**): significant KEGG pathways in SYNCHs and SYNCHr groups in F3, respectively. (**B**, **D**): Cnet plots showing the relationships between the DEGs and the KEGG pathways in SYNCHs and SYNCHr groups, respectively. Pathway data sourced from the KEGG PATHWAY database – © Kanehisa Laboratories. Used with permission[27–29].

assess the effects of in ovo treatment on gonadal methylation profiles. A total of 18 RRBS libraries from male gonads were analyzed, with uniquely mapped reads ranging from about 39.5–66.8% (Supplementary File S5).

Differentially methylated loci (DMLs) were identified based on a false discovery rate (FDR) ≤ 0.05 and a methylation difference ≥ 20% (Fig. 7). In the SYNCHs group, 2,584 DMLs were detected in F2, increasing to 13,168 in F3. In the SYNCHr group, 4,983 DMLs were identified in F2, which increased substantially to 63,356 DMLs in F3. These results reflect a pattern similar to the RNA-seq profiles. In the SYNCHs group, 181 differentially methylated regions (DMRs) were detected in F2, of which 171 were annotated to 157 differentially methylated genes (DMGs). By F3, the number of DMRs increased more than fourfold to 786, with 768 annotated, mapping to 629 DMGs. In the SYNCHr group, 258 DMRs were identified in F2, with 247 annotated to 222 DMGs, whereas in F3 this number increased to 2,880 DMRs, of which 2,824 were annotated, corresponding to 1,606 DMGs. In all groups, the majority of DMRs (> 70%) were located in distal intergenic regions (Supplementary file S6). Promoter-associated DMRs (≤ 3 kb from TSS) comprised a higher proportion in SYNCHr groups (14.58% in F2 and 12.78% in F3) compared to SYNCHs groups (5.26% in F2 and 10.16% in F3).

Genes associated with DMRs in the SYNCHs group were enriched in the Salmonella infection KEGG pathway in F2, and the TGF-beta signaling pathway in F3 (Fig. 8). In the SYNCHr group, no pathway enrichment was observed in F2, while in F3, enriched pathways included Wnt signaling, focal adhesion, melanogenesis, and the adipocytokine signaling pathway (Fig. 8). Supplementary file S7 shows the detailed list of enriched pathways with implicated differential methylated genes. No significant enrichment was seen for GO terms in all groups.

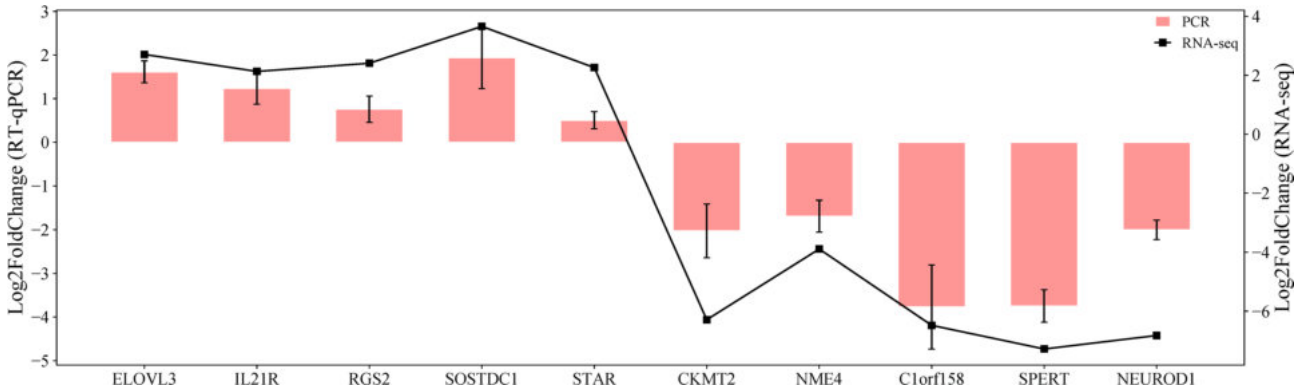

Fig. 6. RT-qPCR validation of 10 selected genes. PCR vs. RNA-seq dual y-axis plot for the genes differentially expressed in the SYNCH groups in F3. All data from RT-qPCR analyses were presented as the mean ± standard error of the mean (SEM).

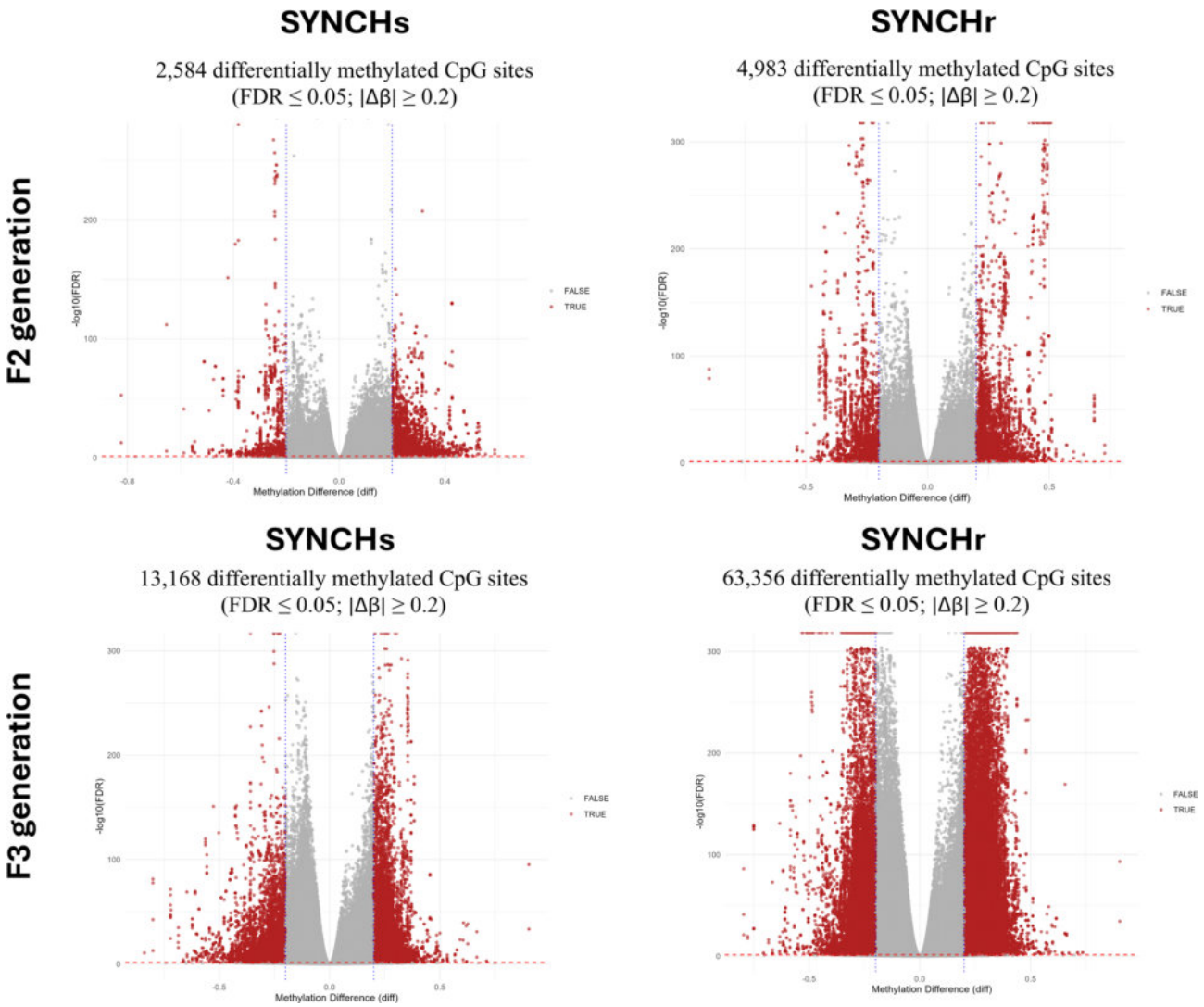

Fig. 7. Volcano plots showing differentially methylated CpG loci (DMLs) in SYNCH groups compared to control in F2 and F3 generations (FDR ≤ 0.05, |Δβ| ≥ 0.2). |Δβ| ≥ 0.2: absolute methylation difference of at least 20%.

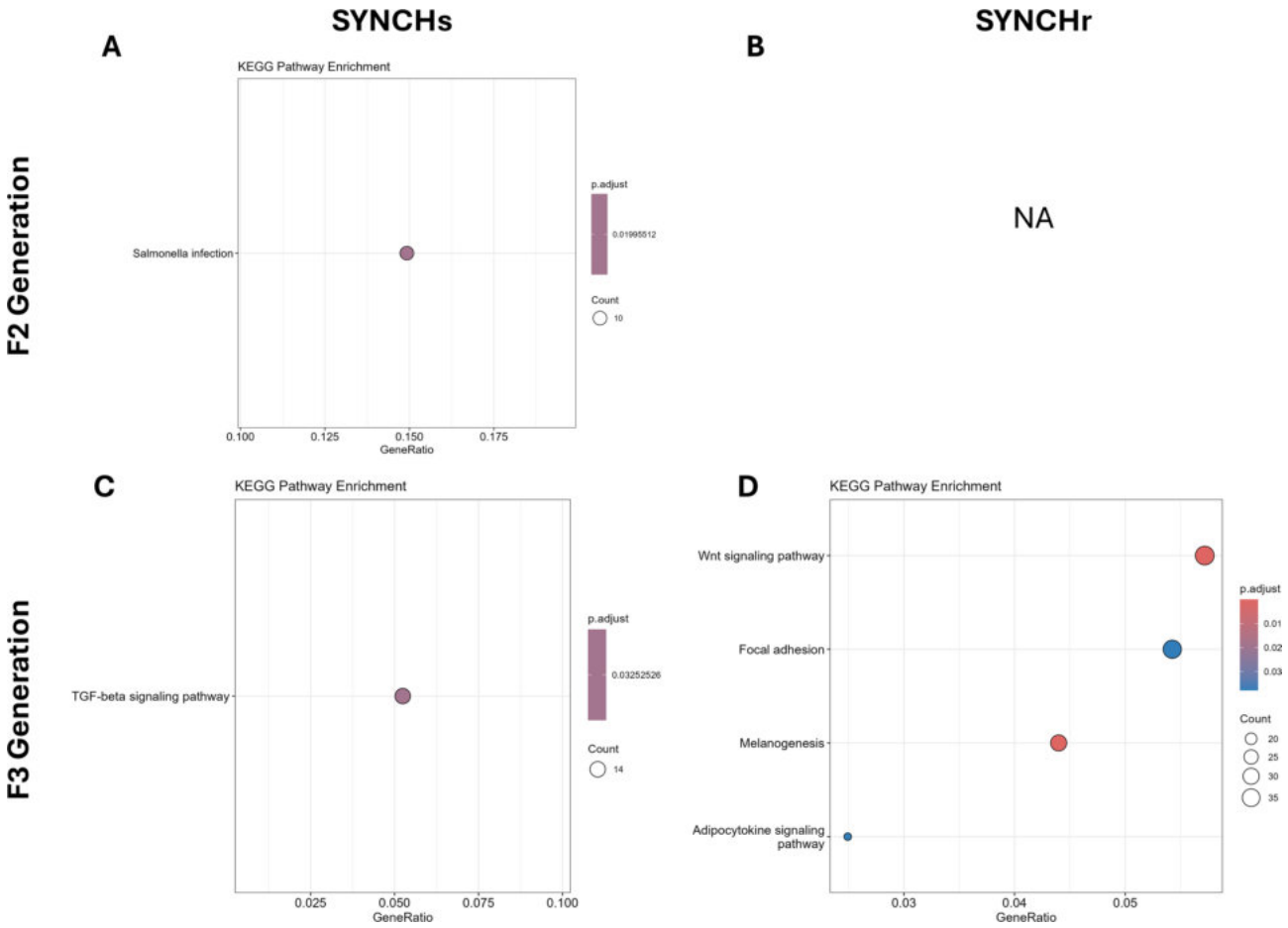

Fig. 8. Significantly enriched KEGG pathways associated with differentially methylated genes in the SYNCH groups of F2 and F3 generations. Pathways were identified using an enrichment analysis with a p-value cutoff of 0.05, q-value cutoff of 0.10, and Benjamini-Hochberg (BH) adjustment for multiple testing. Only pathways containing at least three genes were retained. NA: not available (no enrichment). Pathway data sourced from the KEGG PATHWAY database – © Kanehisa Laboratories. Used with permission[27–29].

Integrative analysis of methylation and transcriptomic data identified concordant methylation-expression changes in 1 gene (1 DMR) in SYNCHs-F2, 37 genes (47 DMRs) in SYNCHs-F3, and 194 genes (306 DMRs) in SYNCHr-F3, while no such overlap was detected in SYNCHr-F2 (Fig. 9). Supplementary File S8 summarizes the overlap between DMGs and DEGs and examines the relationship between the direction of methylation and corresponding gene expression changes in SYNCHs-F2 (Table S1), SYNCHs-F3 (Table S2), SYNCHr-F3 (Table S3). Overall, the integrative results revealed a mixed pattern of methylation–expression associations. In SYNCHs-F2, the only gene with concordant changes was hypermethylated in a distal intergenic region and exhibited upregulated expression. In the SYNCHs group in F3, the majority of DMRs ($n = 25$) showed an inverse correlation with gene expression (e.g., hypermethylation with downregulation or hypomethylation with upregulation), whereas 22 DMRs exhibited concordant changes. In contrast, the SYNCHr group in F3 showed a higher number of DMRs with concordant changes ($n = 190$), while 116 DMRs followed the canonical inverse relationship.

Discussion

Our analysis of gene expression profiles in male gonads following in ovo stimulation revealed distinct effects, with clear differences between the PS synbiotic alone and PS synbiotic combined with choline. When administered alone to F1 embryos, the synbiotic treatment in the SYNs group induced a modest effect in gene expression in F2 male gonads, which largely diminished by F3 generation, suggesting a weak and fading transgenerational effect. Contrary to our hypothesis, repeated administration of PS synbiotic did not enhance the transcriptional response in F3. However, the single co-administration of PS synbiotic with choline to eggs containing F1 embryos led to a strong effect on gene expression in F3 male gonads. Due to the fact that in each generation all groups were compared to the control, we deduce that the observed effect in generation F3 (in SYNCHs group) can be a response to co-administration of PS synbiotic with choline to eggs containing F1 embryos and therefore can be regarded as a transgenerational effect. Notable, repeated administration of both PS synbiotic with

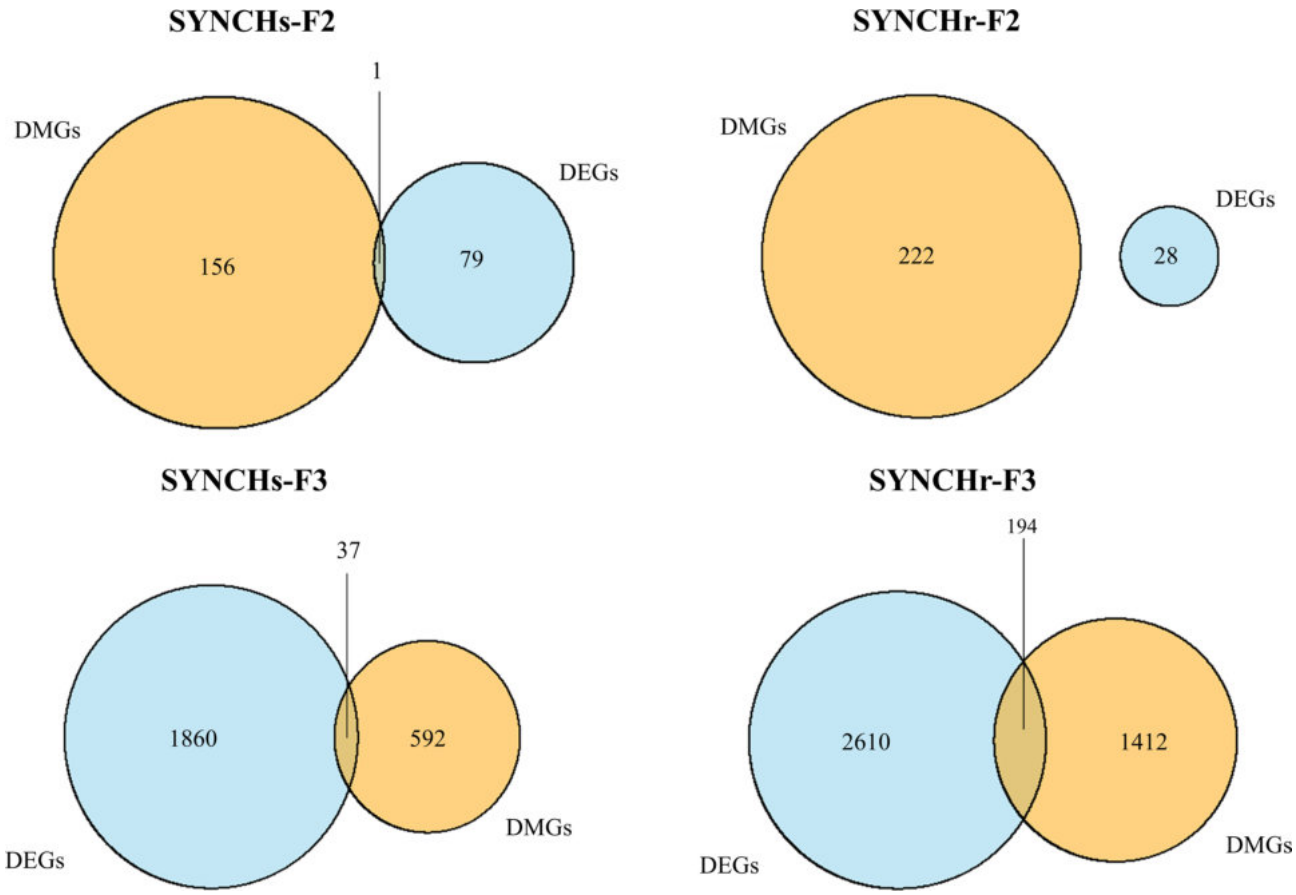

Fig. 9. Overlap between differentially methylated genes (DMGs) and differentially expressed genes (DEGs). SYNCHs: group of single injection of synbiotic with choline in F1; SYNCHr: group of repeated injection of synbiotic with choline in F1-F3; F2: second generation; F3: third generation.

choline supported our hypothesis of a cumulative effect, showing a pronounced transcriptional response in F3 generation, though the effect in F2 remained moderate. To further investigate the effects of synbiotic combined with choline, DNA methylation profiling revealed a pattern consistent with gene expression results, specifically, a substantial increase in the number of DMLs from F2 to F3. Integrative analysis identified a subset of genes in the SYNCHs and SYNCHr F3 groups showing concurrent changes in both methylation and expression, suggesting potential epigenetic regulation in response to the in ovo intervention. Taking this into consideration, it can be deduced that the effects observed in the SYNCHs group of the F3 generation likely represent a transgenerational response initiated by the single in ovo injection of synbiotic and choline in F1 embryos.

The sustained effects seen in the SYNCH groups, in contrast to the SYN groups, may be attributed to different explanations: (1) Choline is an essential nutrient that plays crucial roles in various physiological and epigenetic processes, including DNA methylation, neurotransmitter synthesis, cell membrane integrity, muscle fat metabolism, muscle proteins homeostasis, and the modulation of inflammation and autophagy[39,40]. These mechanisms might lead to more pronounced and lasting effects on gene expression compared to the mechanisms associated with synbiotics. While the components of synbiotics may have wider-reaching effects on the gut microbiota[41]they might not have a potent impact on the gene expression in reproductive tissues such as the gonads to the same degree as choline. (2) The interaction between synbiotics and choline could lead to a synergistic effect, where choline enhances the epigenetic impact of synbiotics, resulting in a more pronounced transgenerational effect as seen in the SYNCHs group. Different types of supplementations can indeed have varying effects, even on the same tissue. For instance, a study by Handy et al. investigated the effects of independent and combined supplementation with nitrate and resveratrol on metabolic adaptations in high-fat-fed male mice[42]. Their main findings highlighted that both supplementations independently improve glucose tolerance and reduce markers of cellular stress. However, when nitrate and resveratrol were co-supplemented, the improvement in glucose tolerance was attenuated.

Several research studies have illustrated the impact of nutriepigenetic substances on male gonadal gene expression and DNA methylation and their transgenerational effects through the male germline. Saito et al. have explored the impact of micronutrient supplementation on gene expression and DNA methylation profiles in the male gonads of Atlantic salmon[17]. Notably, the supplementation influenced the expression of genes associated with three biological pathways in gonads: up-regulation of cytokine receptor interaction and down-regulation of mismatch repair and DNA replication[17]. In terms of DNA methylation, micronutrient supplementation affected

the methylation status of genes linked to critical pathways for embryonic development, including cell signaling and synaptic signaling. Chan et al. showed that lifetime exposure of male mice to methyl donor folic acid diets resulted in changes in the DNA methylation, primarily exhibiting hypomethylation, affecting genes involved in neurodevelopmental pathways across F1, F2, and F3 male germ cells[43]. The number of differentially methylated cytosines decreased in F2 sperm compared to F1 but unexpectedly increased in F3 sperms. Although there was no significant retention of inter- and trans-generational inheritance of differentially methylated cytosines, young long interspersed nuclear elements (LINEs) were notably impacted up to the third generation[43].

In our study, a potential transgenerational effect was observed in the F3 generation following a single injection of PS synbiotic with choline into F1 embryos (SYNCHs group). We found 14 DEGs common between SYNCHs group in F2 and F3 generation. Among these, *PTCHD3* (patched-domain containing 3) is a male germ cell–specific gene, expressed in the midpiece of sperm in mouse, rat, and human, and has been proposed to function as a receptor for Hedgehog (Hh) signaling to regulate sperm development and/or function[44]. However, functional tests revealed that it is not critical for spermatogenesis or fertility in mice[45]. Another shared DEG, *LOC107050879* (also known as kinesin heavy chain *KIF5A*), encodes a motor neuron protein involved in the intracellular transport of organelles, proteins, and RNA, and is predominantly expressed in neurons[46]. However, analysis of mouse testis shows that *KIF5A* is expressed in somatic cells of the testis[47]. *SCARA5* (Scavenger receptor class A member 5) is implicated in iron homeostasis[48]. SCARA5 is expressed in embryonic male gonadal somatic cells, where it mediates ferritin-based iron uptake essential for activating the *Sry* (Sex-determining Region Y) gene[49]. Maternal iron deficiency disrupts this pathway, leading to impaired iron-dependent epigenetic regulation, which in turn causes male-to-female sex reversal by inhibiting Sertoli cell differentiation and proper testis development in mouse embryos[49]. This highlights the essential role of *SCARA5*-mediated iron acquisition in the epigenetic control mechanisms governing male gonadal differentiation[49]. *MIR22* is a microRNA known for its cytoprotective effects, including anti-oxidative, anti-inflammatory, and anti-apoptotic functions[50]. During fetal testicular development in sheep, *MIR22* is upregulated, suggesting a potential role in male gonadal differentiation[51]. This is supported by predictions that *MIR22* represses estrogen signaling pathways, which are commonly associated with ovarian development[51]. In situ hybridization studies have localized *MIR22* expression specifically to Sertoli cells within fetal testicular cords[51]. *CXorf65*, a poorly characterized open reading frame located on the X chromosome (Gene ID: 101748108; *CXorf65* homolog), is highly expressed in the testis of both mice and humans[52]. Its mouse ortholog, *Gm614*, has been shown through knockout studies to impair sperm binding and fertilization, underscoring its functional importance in male fertility[52]. Additionally, *ANKRD60* (Ankyrin repeat domain 60) is classified as reproductive tract-specific in humans and mice, suggesting a potential role in reproduction[53].

The enrichment was mainly seen in F3 generation for SYNCH groups. The only molecular function affected in the SYNCHs group in the F3 generation is collagen binding, which may be attributed to the effect of synbiotic. Probiotic and synbiotic treatments can accelerate extracellular matrix (ECM) remodeling by stimulating fibroblast activity and enhancing collagen deposition, thereby promoting faster tissue repair and re-epithelialization[54]. Consistent with these findings, CCs associated with collagen and ECM structure were also enriched in the SYNCHs group.

The SYNCHr group of F3 showed enrichment in GO terms mainly related to cytoskeletal and ECM organization. Choline lipids, particularly phosphatidylcholine species with saturated fatty acids, contribute to ECM organization by enhancing membrane rigidity, supporting focal adhesion formation, and facilitating stable cell-ECM interactions[55]. Additionally, probiotic treatments have been shown to support ECM remodeling by stimulating fibroblast activity and increasing collagen deposition[54].

Both the SYNCHs and SYNCHr groups showed enrichment in similar KEGG pathways, primarily involving motor proteins, cytoskeletal components in muscle cells, and ECM-receptor interactions. Notably, the SYNCHr group also exhibited additional enrichment in pathways related to focal adhesion and nucleotide sugar biosynthesis. The known involvement of choline in membrane biosynthesis, cell adhesion, and one-carbon metabolism lends biological support to the KEGG pathway enrichments observed[56].

On the other hand, in the SYNCHs group in the F2 generation, DMGs were enriched in the KEGG pathway associated with Salmonella infection, which is indicative of immune system-related processes. Appropriately selected probiotics and prebiotics can exert potent immunomodulatory effects[57]. Additionally, cholinergic signaling can contribute to immune regulation and maintenance of homeostasis[58]. In the F3 generation, DMGs in the SYNCHs group were enriched in the TGF-β signaling pathway. Choline has been shown to induce an anti-fibrotic effect both in vivo and in vitro by regulating the TGF-β1/Smad2/3 and p38MAPK pathways[59]. KEGG enrichment in the SYNCHr group highlighted pathways including Wnt signaling, focal adhesion, melanogenesis, and adipocytokine signaling. Acetylcholine receptors are known to regulate immune-related genes, including those involved in Wnt-mediated host immune response, thereby highlighting a gut-brain-microbial axis driven by cholinergic signaling and Wnt pathway activation[60]. Additionally, dietary choline has been demonstrated to reduce body fat mass gain, prevent adipocyte hypertrophy, and attenuate adipose tissue inflammation, processes regulated by adipocytokines[61]. Acetylcholine and acetylcholinesterase inhibitors, both linked to choline metabolism, have been shown to inhibit light-induced melanogenesis in vitro in melanocytes and ex vivo in mouse skin[62].

The limitation of this study is the incomplete annotation of the chicken genome, which impacts both gene mapping and downstream analyses[63]. During gene ID conversion (e.g., from gene symbols to ENTREZIDs) for pathway enrichment, a substantial number of genes could not be mapped due to missing or inconsistent entries in public databases. This limitation may have affected the integrative analysis by reducing the apparent overlap between DMR-associated genes and DEGs. Inconsistent gene identifiers likely led to the omission of valid gene matches, thereby underrepresenting potential epigenetic regulation.

Conclusion

To the best of our knowledge, this study is the first to employ a chicken model to explore the transgenerational effects of in ovo administration of bioactive compounds on reproductive tissues. While transgenerational inheritance remains a debated topic, our findings show that a single exposure of F1 embryos to synbiotic combined with choline (SYNCHs) can induce changes in gene expression and DNA methylation detectable in the F3 generation, supporting the potential occurrence of transgenerational effects of these combined substances. Nonetheless, further additional research, both in vivo and in silico, is required to enhance the identification of intergenerational and transgenerational epigenetic marks responsive to nutritional signals.

Data availability

Sequence data that support the findings of this study have been deposited in the National Center for Biotechnology Information (NCBI) under the primary accession code: PRJNA1142492 for RNA-seq data (http://www.ncbi.nlm.nih.gov/bioproject/1142492) and PRJNA1303698 for RRBS data (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1303698).

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Author contributions

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Multi-generational transcriptomic changes in embryonic blood following in ovo stimulation with nutriepigenetic factors

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Abstract

Background and aim: Epigenetic modifications regulate gene expression and are influenced by environmental factors, shaping phenotypic and clinical outcomes. These changes can persist across generations, though their stability can vary by tissue. This study aims to observe the effects of prenatal stimulation with potential epigenetic factors in F1 embryos on the transcriptome of embryonic blood across generations. Since primordial germ cells (PGCs) circulate in embryonic blood before settling in the gonads, this tissue is likely to represent both somatic and germline lineages.

Method: We established an *in ovo* model over three generations of Green-legged Partridgelike chickens with an additional assessment of F4 embryos. Synbiotic PoultryStar® (PS) and choline were injected *in ovo* on the 12th day of egg incubation. F1 embryos were divided into control (0.9% NaCl), synbiotic (SYN, 2 mg PS), and synbiotic plus choline (SYNCH, 2 mg PS + 0.25 mg choline). In F2 and F3, SYN and SYNCH were split into two subgroups each: A) injected only once in F1 embryos (SYNs and SYNCHs); and B) repeatedly injected in every successive generation (SYNr and SYNCHr). Fertilized eggs from all groups laid by F2 and F3 hens were incubated until HH stages 14–16, at which point embryonic blood was collected from the dorsal aorta of embryos. Embryos were sexed using PCR-based sex determination, and blood samples were pooled by sex. RNA was isolated from male samples for RNA sequencing.

Results and conclusion:

Administration of synbiotic and synbiotic plus choline induced transcriptomic changes in F3 embryonic blood. A single ancestral in ovo exposure triggered detectable transcriptomic changes in F3, which largely diminished in F4, suggesting effects attenuate with generational distance. Repeated injections in SYNr and SYNCHr groups did not produce cumulative effects. Gene set enrichment analysis indicated that the most affected functional categories involved metabolism, detoxification, cytoskeletal organization, and protein regulation. These findings highlight that targeted prenatal interventions can induce multigenerational transcriptomic modifications, though their persistence may be limited and context-dependent.

Introduction

Epigenetic mechanisms, including DNA methylation, histone modification, and regulation by non-coding RNAs, are crucial in linking the environment to gene expression [1]. These mechanisms are highly sensitive to environmental inputs, especially during critical developmental windows such as prenatal and early postnatal periods [2].

In some cases, the consequences of epigenetic modulation can be transferred to the germline and passed on beyond the directly exposed individual, influencing the phenotype of subsequent generations [3, 4]. However, such transgenerational effects remain difficult to conclusively demonstrate, particularly in vertebrates, where inherited molecular changes can be difficult to distinguish from direct environmental influences [4]. Epigenetic transmissions are dynamic, involving non-linear, time-dependent changes that challenge a simplistic on–off model, with effects that can gradually "wash in" or "wash out" across generations in response to environmental stressors [5]. Understanding these dynamics requires sensitive, quantitative methods and a focus on how epigenetic patterns evolve over time [5].

Avian models such as the chicken provide unique advantages for studying inter- and transgenerational effects of epigenetic factors without the confounding effects of in utero influences, due to their external embryonic development and accessibility of egg content for manipulation [6, 7]. Of particular interest is the chicken embryonic blood, which in early stages contains both somatic blood cells and circulating primordial germ cells (PGCs) before they migrate to the gonads [8]. As such, embryonic blood offers a valuable snapshot of systemic gene regulation that may include components relevant to both somatic and germline lineages. The epigenome of chicken erythrocytes responds to both internal factors, such as metabolism, and external influences like the environment, affecting chromatin structure and gene expression [9]. With a compact genome that retains a similar gene order to humans, chicken red blood cells serve as an effective model for exploring how environmental conditions shape the epigenome and for drawing parallels to human health [9].

Despite increasing interest, few studies have explored transcriptomic changes in transgenerational transmission in birds, and even fewer have examined gene expression in

embryonic blood at later generations such as F3 and F4. The objective of this study was to examine changes in gene expression patterns in the embryonic blood of F3 and F4 chicken generations following ancestral in ovo exposure to potential epigenetic modulators, namely, a synbiotic (PoultryStar®, PS) and choline.

2. Materials and Methods

2.1. Ethical Consideration

The study adhered to the ethical standards outlined in Directive 2010/63/EU and Regulation (EU) 2019/1010. The Local Ethical Committee for Animal Experiments in Bydgoszcz, Poland, approved the experimental protocols under Approval No. 15/2022, issued on April 20, 2022, in accordance with these documents. This research has been reported following the ARRIVE guidelines [10] (https://arriveguidelines.org). Throughout the study, animal welfare was monitored by trained staff and a veterinarian. The birds were raised under standard environmental conditions in a poultry farm.

2.2. Birds and Experimental Design

This study was conducted using Green-legged Partridgelike chickens, a native Polish slow-growing breed known for its adaptability and resilience. These birds are well-suited to various environmental conditions due to their minimal nutritional needs, strong immunity, and natural resistance to harsh climates [11, 12]. Additionally, they exhibit strong maternal traits. Unlike commercial poultry breeds, this breed has undergone minimal selective breeding, preserving greater genetic diversity, making it a valuable model for transgenerational research [11]. Green-legged Partridgelike chickens are outbred lines. According to Guerrero-Bosagna et al. outbred lines may manifest higher susceptibility to epigenetic modifications when compared to inbred counterparts, rendering them a good model for observing effects across generations [13].

Figure 1 presents the study design, which was detailed in our previous papers [14, 15]. Briefly, the experiment spanned three generations (F1–F3), starting from fertilized eggs (F1 embryos) of Green-legged Partridgelike hens from the F0 generation. In this study, F4 embryos were also included. On embryonic day 12, eggs with viable F1 embryos were injected manually into the air

cell with one of the following treatments (3 groups): (1) Synbiotic group (SYN) – received an injection of 2 mg/embryo of synbiotic PoultryStar® sol^{US} (Biomin GmbH, Herzogenburg, Austria; further referred to as PS) suspended in 0.2 mL of 0.9% NaCl; (2) Synbiotic and choline group (SYNCH) – received 2 mg/embryo of synbiotic PS combined with 0.25 mg/embryo of choline (Sigma Alrich, Sain Louis, MA, USA, cat. no. C7527), suspended in 0.2 mL of 0.9% NaCl; (3) Control group (C) – received an injection of 0.2 mL of 0.9% NaCl. From the F2 generation onward, the treatment groups were divided into four subgroups: (1) SYNs – single synbiotic PS injection applied only in F1 embryos; (2) SYNCHs – single synbiotic PS + choline injection applied only in F1 embryos; (3) SYNr – repeated synbiotic PS injections in F2 and F3 generations; (4) SYNCHr– repeated synbiotic + choline injections in F2 and F3 generations. In addition to a control group (0.9% NaCl). The selection of the choline source, its dosage, and the combined synbiotic and choline dosages was guided by findings from two experiments described in our previous study [14]. The in ovo injection protocol was based on the optimized method of Bednarczyk et al. [16, 17]. Housing and feeding protocols for chickens were described previously [14, 15].

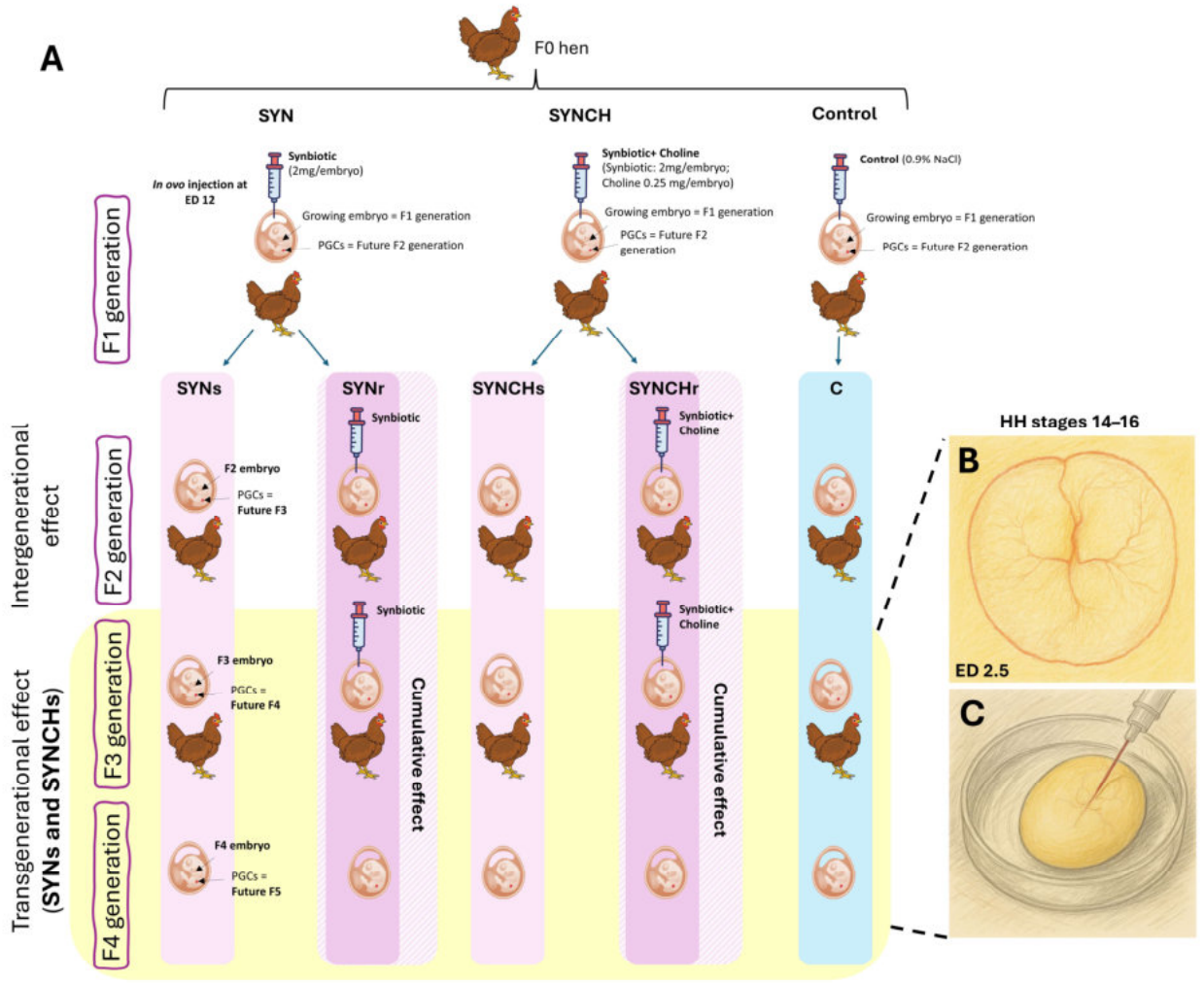

Figure 1. Experimental design of the study. (A) The study was conducted over three generations in addition to F4 embryos. F1 embryos were injected at embryonic day (ED) 12 with either a synbiotic, a combination of synbiotic and choline, or 0.9% physiological saline (NaCl). In subsequent generations, fertilized eggs from each treatment group were divided into two subgroups: one that continued without further injections (SYNs and SYNCHs), and another that received repeated injections at ED12 in every generation (SYNr and SYNCHr). (B) Fertilized eggs from the F2 and F3 generations were incubated for 2.5 days, reaching Hamburger-Hamilton (HH) stages 14–16. (C) Embryonic blood was collected from the dorsal aorta using a fine glass microcapillary pipette.

2.3. Embryonic blood isolation

A total of 100 eggs (n=20 eggs per group) were incubated under standard conditions (37.5 °C, 55% relative humidity, turned every 2 hours) for 2.5 days, until the embryos reached HH-stage

14-16. Embryonic blood containing cPGCs was extracted from the dorsal aorta of individual F3 and F4 embryos under a stereomicroscope. A fine glass microcapillary pipette (inner diameter: 30 µm, outer diameter: 40 µm) connected to a mouth pipette was used for collection (Sigma Alrich, Saint Louis, MA, USA, cat. no. A5177).

Blood samples from every embryo were individually transferred into Eppendorf tubes containing RNALater (ThermoFisher, Waltham, MA, USA, cat. no. AM7021) and stored at 4°C until later usage. After determining the sex of the embryos (methodology is described in the next subchapter), samples were pooled into male and female groups, with only male samples being used in this study (n=6/group). The collected blood was separated through centrifugation in RNase-free water at 10,000× g for three minutes. RNA extraction was then performed using the GeneMATRIX Universal RNA Purification Kit (Eurx, Gdańsk, Poland, cat. no. E3598) following the manufacturer's protocol. Three replicates were obtained for each group in F3 and F4 generations.

RNA integrity was assessed using an Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) with an RNA Nano 6000 Assay Kit. Additionally, RNA quality was checked by electrophoresis on a 1% agarose gel. All extracted RNA samples met the required quality standards, achieving an RNA integrity number (RIN) of 9.0 or higher, confirming their suitability for downstream applications.

2.4. Sex determination

DNA was extracted from each embryo, corresponding to its respective isolated blood sample, using the QIAamp Fast DNA Tissue Kit (Qiagen, Hilden, Germany, Cat. No. 51404), following the manufacturer’s instructions. Embryo samples were homogenized by vortexing in lysis buffer for 30 seconds, followed by incubation in a thermomixer (TS-100C, Biosan, Riga, Latvia) at 56°C with a shaking speed of 1000 rpm for 5 minutes.

Sex determination of the embryos was performed using two pairs of primers: one specific to the female *XhoI* W-repeat sequence (5’-*CCCAAATATAACACGCTTCACT*-3’ and 5’-*GAAATGAATTATTTTCTGGCGAC*-3’), and another targeting the 18S ribosomal gene (5’-*AGCTCTTTCTCGATTCCGTG*-3’ and 3’-*GGGTAGACACAAGCTGAGCC*-3’), as previously described by Clinton et al [18].

The PCR-amplified products were separated using electrophoresis on a 2% agarose gel stained with MIDORI Green Advance (NIPPON Genetics, Düren, Germany, cat. no. MG04). The gel was run at 110 V for 35 minutes, and DNA bands were visualized and photographed using the G:Box Chemi XR5 imaging system (SYNGENE, Cambridge, UK). In female samples, two distinct bands are observed: one corresponding to the female-specific *XhoI* W-repeat sequence (415 base pairs) and the other to the 18S ribosomal gene (256 base pairs), which serves as an internal PCR control. In contrast, male embryos exhibit only the 18S ribosomal gene band. Only male samples were included in this study.

2.5. RNA-Sequencing and Analysis

A total of 30 RNA-seq libraries were generated (15 libraries per generation (F3 and F4), with 3 libraries per treatment and control group) using the Novogene NGS Stranded RNA Library Prep Set (PT044, Novogene, Cambridge, UK). Sequencing was performed on the Illumina NovaSeq 6000 platform (Novogene, Cambridge, UK) at a depth of 20 million forward and 20 million reverse reads per sample, utilizing a 150 paired-end sequencing kit. Quality control of the raw sequencing data was assessed using FastQC v0.12.1 [19]. Adapter sequences and low-quality reads were removed using fastp v0.23.4 to obtain high-quality clean data for further analysis [20]. The Q20, Q30, and GC content of the processed reads were then evaluated. Following preprocessing, paired-end reads were aligned to the chicken genome (bGalGal1.mat.broiler.GRCg7b) using STAR 2.7.11b [21]. Differential gene expression analysis was performed using DESeq2 v1.42.0 [22] in RStudio (2025.5.0.49) [23]. Raw counts were normalized within the DESeq2 package, and differentially expressed genes (DEGs) were identified based on an adjusted p-value (≤ 0.05) and log2 fold change threshold of 0.585. Over-representation analysis (ORA) for Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was performed using clusterProfiler [24]. Multiple testing correction was applied using the Benjamini-Hochberg (BH) method (pAdjustMethod = "BH"). Significance thresholds were set at a p-value ≤ 0.05 and a false discovery rate (q-value) ≤ 0.10. Only terms and pathways with at least three implicated DEGs were considered. Gene set enrichment analysis (GSEA) for KEGG pathways and GO terms were also performed [25] using RStudio (2025.5.0.496) [23]. Enrichment was assessed with a p-value cutoff of 0.05, and multiple testing correction was applied using the BH method. KEGG pathways and

GO terms were considered significant if the adjusted p-value was below 0.05. Additionally, only gene sets with an absolute normalized enrichment score |NES| ≥ 1.5 and at least three core enrichment genes were retained for downstream analysis and visualization. The function and expression information of the common genes were retrieved from UniProt (https://www.uniprot.org/uniprotkb) [26], NCBI (https://www.ncbi.nlm.nih.gov/gene/) and Bgee (Gene Expression Evolution Database, https://bgee.org/) [27].

3. Results

3.1. RNA Sequencing and Alignment Summary Statistics

A total of 30 embryonic blood samples were sequenced using the Illumina NovaSeq 6000 sequencing platform. After removing adapter sequences and low-quality reads, the average number of clean reads obtained in the experimental groups ranged from 16,846,058 to 30,562,766 in F3, and from 17,557,189 to 34,060,498 in F4. Across all samples, the quality metrics were high, with 99.99% of bases achieving Q20 and at least 97.9% reaching Q30. The GC content ranged from 48% to 54%. Additionally, 84% to 89.85% of clean reads uniquely mapped to the chicken reference genome (bGalGal1.mat.broiler.GRCg7b; NCBI RefSeq assembly: GCF_016699485.2, annotation release 106).

3.2. Identification of differentially expressed genes (DEGs)

Following mapping and quality control, a total of 25,470 expressed genes were retained for differential expression analysis. Figure 2 presents the number of differentially expressed genes (DEGs, adjusted p-value ≤ 0.05; absolute log2 fold change (|log2FC|) cutoff of 0.585) identified in comparisons between the experimental groups and the control group in F3 and F4 embryos.

In the F3 generation, the SYNs group exhibited the highest number of DEGs (n = 102), while the SYNCHs group showed 30 DEGs. Groups subjected to repeated stimulation showed 42 DEGs in SYNr and 36 in SYNCHr. In the F4 generation, the number of DEGs declined markedly in all groups. Only one DEG was identified in the SYNs group, while the SYNCHs group had four. The SYNr and SYNCHr groups showed 17 and one DEG, respectively.

When comparing gene expression between F3 treatment groups—one receiving a single F1 injection versus another receiving injections across multiple successive generations (F1-F3), we found eleven shared genes between SYNs and SYNr (*HIST1H4D*, *LOC124417784*, *DSE*, *CYP3A4*, *COX14*, *LOC112531967*, *LOC112532140*, *J6367_mgt12, SERF2*, *PHKA1* and *MITD1*), and five shared between SYNCHs and SYNCHr (*LOC124417784*, *TMEM151B*, *BMP5*, *CYP3A4* and *SPECC1*). No overlapping DEGs were detected across the experimental groups in F4 embryos. Table 1 summarizes the putative functions of the common DEGs identified. Cross-generational comparisons of corresponding groups in subsequent generations revealed one common gene shared between SYNs group in F3 and SYNs group in F4 (*HBBA*), and no overlapped DEGs between SYNCHs in F3 and SYNCHs in F4. The SYNr group showed one overlapping gene between F3 and F4 (*BG8*), whereas no shared DEGs were found in the SYNCHr group across generations. Interestingly, the expression of the aforementioned genes (*HBBA and BG8)* was downregulated in F3 embryos while upregulated in F4 embryos. Table 2 outlines the potential functions of the two shared DEGs observed across comparable groups in the F3 and F4 generations.

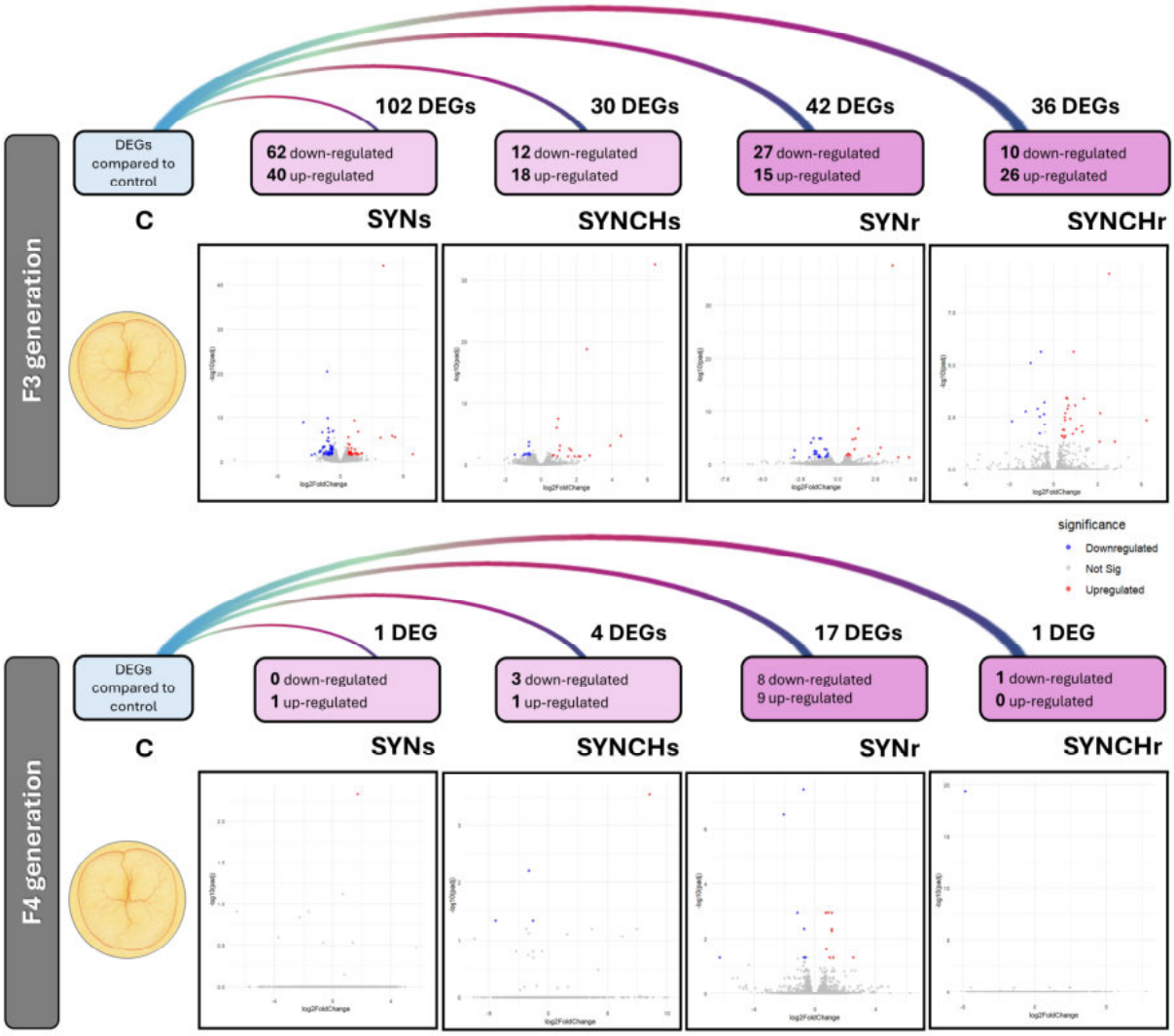

Figure 2. Diagram showing the number of differentially expressed genes (DEGs) identified by comparing the experimental groups with the control group in F3 and F4 generations (n = 3 samples per group per generation). C: control group; SYNs: group that received a single injection of synbiotic PS in F1 embryos; SYNr: group that received repeated synbiotic PS injections (F1-F3); SYNCHs: group that received a single injection of synbiotic PS and choline in F1 embryos; and SYNCHr: group that received repeated injections of synbiotic PS and choline (F1-F3).

224 *Table 1. Shared differentially expressed genes between SYNs and SYNr groups, and between SYNCHs and SYNCHr groups in F3.*

comparison	Gene ID	Description	Expression	Function[a]
SYNs-SYNr	HIST1H4D	Histone Cluster 1 H4 Family Member D	↑	Core component of nucleosome; plays a central role in transcription regulation, DNA repair, DNA replication, and chromosomal stability.
	LOC124417784	Uncharacterized LOC124417784	↑	Function not characterized.
	DSE	Dermatan Sulfate Epimerase	↑	Converts D-glucuronic acid to L-iduronic acid residues; important in dermatan sulfate biosynthesis.
	CYP3A4	Cytochrome P450 Family 3 Subfamily A Member 4	↑	Involved in metabolism of sterols, steroid hormones, retinoids, fatty acids, and xenobiotics.
	COX14	Cytochrome C Oxidase Assembly Factor COX14	↓	Regulates cytochrome c oxidase assembly; essential for mitochondrial function.
	LOC112531967	Uncharacterized LOC112531967	↓	Potentially associated with viral processes.
	LOC112532140	Uncharacterized LOC112532140	↓	Potentially involved in immune responses to viral infections.
	J6367_mgt12	Uncharacterized Gene J6367_mgt12	↓	Related to tRNA-Ser.
	SERF2	Small EDRK-rich factor 2	↓	Positive regulator of amyloid protein aggregation and proteotoxicity.
	PHKA1	Phosphorylase b kinase regulatory subunit alpha 1	↑	Phosphorylase b kinase catalyzes the phosphorylation of serine in certain substrates. The alpha chain may bind calmodulin.
	MITD1	Microtubule-Interacting and Trafficking Domain Protein 1	↓	Required for efficient abscission at the end of cytokinesis, together with components of the ESCRT-III complex.
SYNCHs-SYNCHr	LOC124417784	Uncharacterized LOC124417784	↑	Function not characterized.
	TMEM151B	Transmembrane Protein 151B	↑	Encodes a protein with two transmembrane domains; function not well-defined.
	BMP5	Bone Morphogenetic Protein 5	↑	Growth factor from the TGF-beta superfamily; involved in cartilage and bone formation, and neurogenesis.
	CYP3A4	Cytochrome P450 Family 3 Subfamily A Member 4	↑	Involved in metabolism of sterols, steroid hormones, retinoids, fatty acids, and xenobiotics.
	SPECC1	Sperm Antigen with Calponin Homology and Coiled-Coil Domains 1	↑	Involved in cytokinesis and spindle organization; may play a role in actin cytoskeleton organization and microtubule stabilization.

[a] Retrieved from the UniProt (https://www.uniprot.org/uniprotkb/). Uncharacterized genes were searched on NCBI (https://www.ncbi.nlm.nih.gov/gene/). SYNs: group that received a single injection of synbiotic PS in F1 embryos; SYNr: group that received repeated synbiotic PS injections (F1-F3); SYNCHs: group that received a single injection of synbiotic PS and choline in F1 embryos; and SYNCHr: group that received repeated injections of synbiotic PS and choline (F1-F3).

Table 2. Common differentially expressed genes between analogous SYNs and SYNr Groups in F3 and F4.

Comparison	Gene ID	Description	Expression	Function[a]
SYNsF3-SYNsF4	HBBA	Hemoglobin Beta, Subunit A	↓ in F3 ↑ in F4	Involved in oxygen transport from the lung to the various peripheral tissues
SYNrF3-SYNrF4	BG8	BG Gene 8 (Major Histocompatibility Complex Class IV)	↓ in F3 ↑ in F4	Involved in the immune system, particularly in antigen presentation and immune response modulation

[a] Retrieved from the UniProt (https://www.uniprot.org/uniprotkb/). SYNs: group that received a single injection of synbiotic PS in F1 embryos; SYNr: group that received repeated synbiotic PS injections (F1-F3).

Functional clustering of DEGs

Gene Ontology (GO) enrichment analysis was performed on DEGs from the embryonic blood of F3 and F4 embryos to investigate the functional significance of transcriptional changes. Enriched GO terms were classified into three categories: biological process (BP), cellular component (CC), and molecular function (MF). Over-representation analysis (ORA) using the KEGG and GO databases revealed no significant enrichment in F3. In F4 embryos, the limited number of identified DEGs was insufficient to support robust functional annotation.

We further performed gene set enrichment analysis (GSEA) for GO terms and KEGG pathways to investigate the systemic regulation of the entire gene sets in embryonic blood across F3 and F4 generations (Figures 3 and 4). In F3 embryos, SYNs and SYNr are predicted to activate protein-related and metabolic processes. By F4, SYNs showed activation of MFs linked to enzyme, protein, and DNA/nucleic-acid binding, whereas SYNr activated structural molecule activity and growth

factor activity. For the SYNCH groups in F3, SYNCHs activated BPs related to translation and metabolic processes, and both SYNCHs and SYNCHr showed suppression of detoxification and response to toxic substances; at the MF level they shared decreased structural molecule, ribosomal, actin and antioxidant activities with increased ATP-dependent chaperone and heat-shock protein binding. In F4, SYNCHs showed suppressed morphogenesis and DNA-binding transcription factor activity, while SYNCHr showed activation of detoxification/response to toxic substances and translation-related functions.

Table 3. The number of enriched KEGG pathways and GO terms by ORA and GSEA in chicken embryonic blood in F3 and F4 generations.

generation	comparison	GSEA							
		KEGG		GO: BP		GO: CC		GO: MF	
		Up	Down	Up	Down	Up	Down	Up	Down
F3	SYNs vs. C	15	5	12	7	0	12	12	33
	SYNr vs. C	1	1	15	5	4	4	3	1
	SYNCHs vs. C	1	4	8	3	2	9	4	5
	SYNCHr vs. C	1	2	5	37	0	7	5	23
F4	SYNs vs. C	5	2	0	0	6	0	5	2
	SYNr vs. C	7	17	0	0	0	5	0	3
	SYNCHs vs. C	1	7	0	79	0	7	0	17
	SYNCHr vs. C	24	1	59	6	31	0	30	1

C: control group; SYNs: group that received a single injection of synbiotic in F1 embryos; SYNr: group that received repeated synbiotic injections (F1-F3); SYNCHs: group that received a single injection of synbiotic and choline in F1 embryos; and SYNCHr: group that received repeated injections of synbiotic and choline (F1-F3). ORA: over-representation analysis; GSEA: gene set enrichment analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology; BP: biological process; CC: cellular component; MF: molecular function.

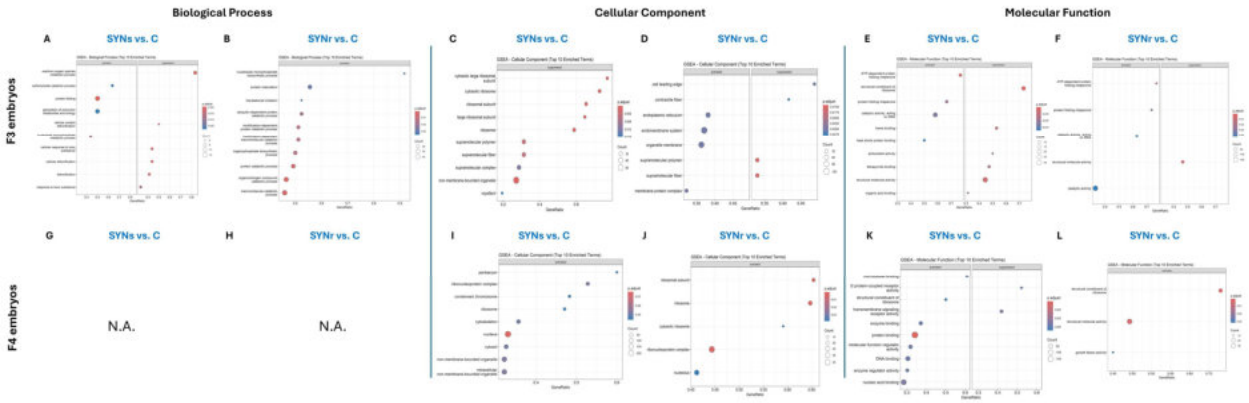

Figure 3. Enriched GO terms in single and repeatedly synbiotic injected groups by GSEA in chicken embryonic blood in F3 and F4 generation. C: control group; SYNs: group that received a single injection of synbiotic PS in F1 embryos; SYNr: group that received repeated synbiotic PS injections (F1-F3); GSEA: gene set enrichment analysis.

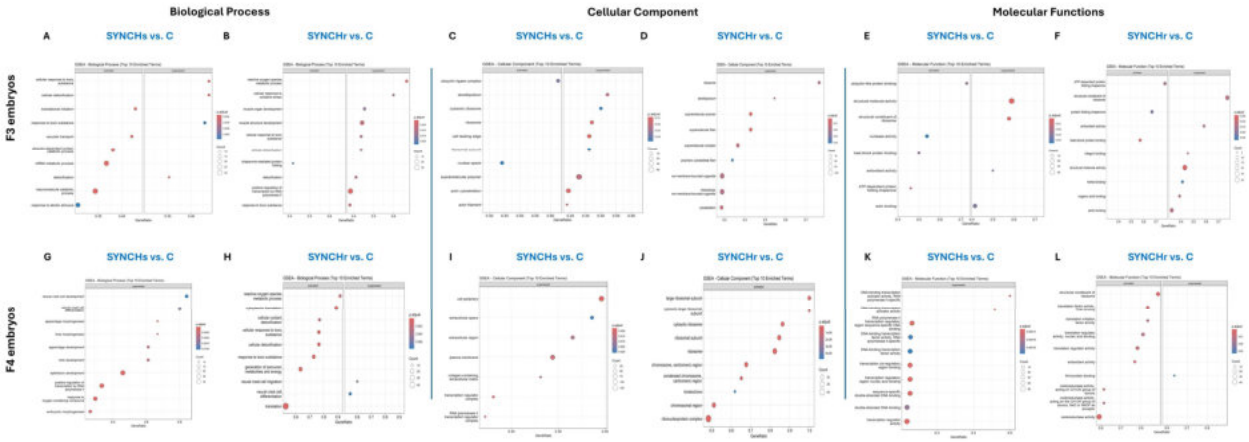

Figure 4. Enriched GO terms in single and repeatedly synbiotic + choline injected groups by GSEA in chicken embryonic blood in F3 and F4 generation. C: control group; SYNCHs: group that received a single injection of synbiotic PS and choline in F1 embryos; SYNCHr: group that received repeated injections of synbiotic PS and choline (F1-F3). GSEA: gene set enrichment analysis.

Figure 5 shows the significant KEGG pathways identified by GSEA, providing insights into the transcriptional shifts induced by each treatment. In F3 embryos, SYNs showed significant positive enrichment in metabolic pathways, while SYNr exhibited strong positive enrichment for ribosomal biogenesis. Among the enriched pathways in SYNCH groups in F3, SYNCHs was negatively enriched for oxidative phosphorylation, whereas SYNCHr showed positive enrichment in pyrimidine metabolism. Cytoskeleton-related pathways were consistently negatively regulated across all treatment groups in F3 embryos. In F4 embryos, GSEA revealed sustained positive enrichment of ribosome-related pathways in all treatment groups, with more pronounced enrichment observed in SYNr and SYNCHr groups.

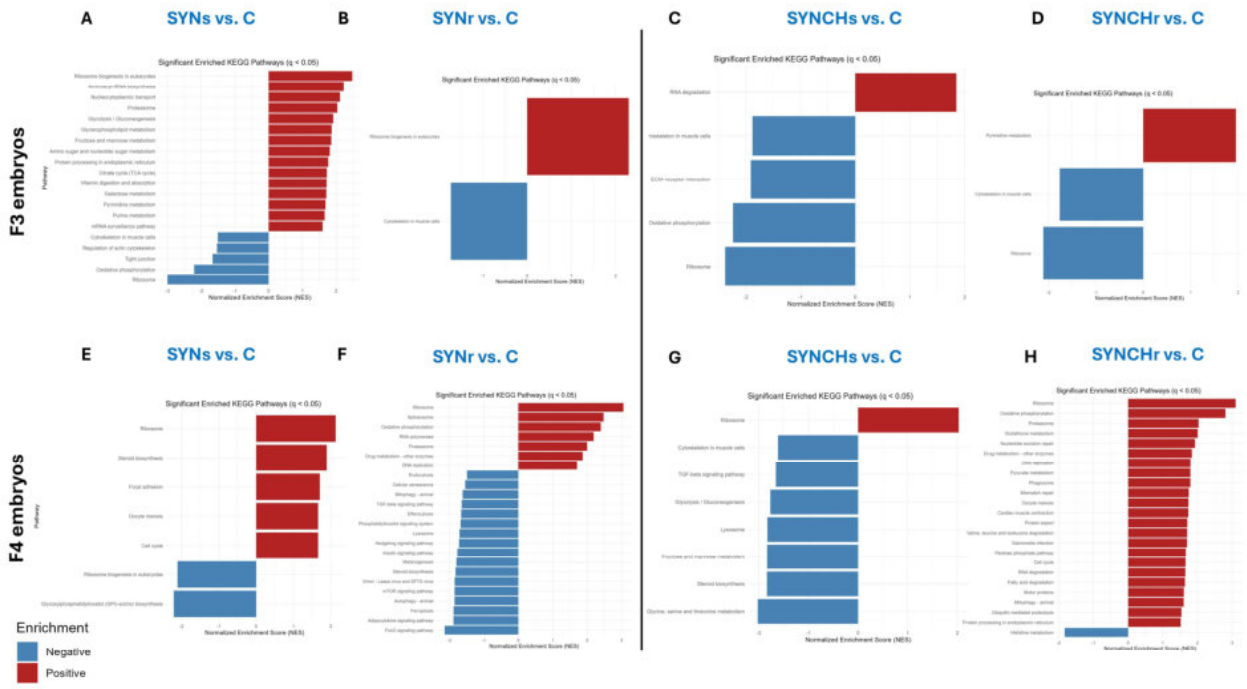

Figure 5. Enriched KEGG pathways by GSEA. (A–H) Bar plots showing the enriched KEGG pathways in SYN and SYNCH groups. Enrichment is shown for SYN groups in F3 embyos (A, B) and F4 embryos (E, F) and for SYNCH groups in F3 embryos (C, D) and F4 embryos (G, H). Each bar represents a pathway, with bar length corresponding to the number of enriched genes. C: control group; SYNs: group that received a single injection of synbiotic in F1 embryos; SYNr: group that received repeated synbiotic injections (F1-F3); SYNCHs: group that received a single injection of synbiotic and choline in F1 embryos; and SYNCHr: group that received repeated injections of synbiotic and choline (F1-F3). KEGG: Kyoto Encyclopedia of Genes and Genomes; GSEA: gene set enrichment analysis

4. Discussion

In this study, slow-growing Green-legged Partridgelike chicken embryos were used to investigate the effects of bioactive compounds (choline and synbiotic PS) administered in ovo (either as a single injection in F1 embryos or in every generation) on the embryonic blood transcriptome of F3 and F4.

The presence of DEGs in the SYNs and SYNCHs groups in F3 following a single F1 injection suggests that a single ancestral exposure can trigger significant changes in gene expression two generations later. This may reflect a transgenerational response. Consistent with our results, studies across a range of species have increasingly demonstrated that environmental factors can induce epigenetic modifications that are transmitted across generations. For example, exposure

of gestating F0 generation rats to vinclozolin led to disease transmission to the unexposed F3 generation [28]. In Drosophila, stress-induced heterochromatic disruption has been shown to be transmitted to multiple subsequent generations, though it gradually reverted to the normal state [29]. In line with these observations, the striking decline in the number of DEGs observed in the SYNs and SYNCHs F4 embryos in our study further supports the notion that the transcriptomic impact of ancestral exposure diminishes with increasing generational distance from the initial F1 exposure. As previously suggested by Iqbal et al., even when robust epigenetic alterations arise in germ cells during fetal development and are transmitted to the next generation, their functional consequences are likely to attenuate over time [30].

Contrary to what we hypothesized, repeated injections in the SYNr and SYNCHr groups did not result in a cumulative effect. The presence of eleven shared DEGs between SYNs and SYNr and five between SYNCHs and SYNCHr in the F3 generation suggests that certain gene networks respond consistently to synbiotic or choline-based stimulation, regardless of exposure frequency. For example, among the shared genes, *CYP3A4* is upregulated in all the groups of F3 embryos compared to control. This gene is involved in xenobiotic metabolism and has been linked to environmental and dietary exposures, and its persistent regulation across treatments indicates a potential core metabolic response [31]. *HIST1H4D*, a histone gene, and *COX14*, involved in mitochondrial function, suggest modulation of chromatin dynamics and cellular energy metabolism, key processes often targeted by epigenetic regulation [32]. Among the shared DEGs between SYNCHs and SYNCHr, *BMP5* stands out for its established role in developmental pathways, including skeletal and neural development, and is important in regulating embryogenesis, skeletal development, and the maintenance of adult-tissue homeostasis [33, 34].

Interestingly, *HBBA*, a gene involved in hemoglobin synthesis [9], and *BG8*, a hematopoietic BG gene associated with immune regulation [35]**,** were the only DEGs shared between F3 and F4 in SYNs and SYNr, respectively. Notably, both exhibited reversed expression patterns: they were downregulated in F3 but upregulated in F4. This bidirectional regulation may reflect compensatory mechanisms or homeostatic feedback in later generations, as the host attempts to restore baseline gene expression.

Our GSEA suggested that prenatal synbiotic alone or in combination with choline modulated distinct processes and function categories. Among these, key metabolic processes and protein-related processes, including synthesis, folding, maturation, and chaperone activity, were commonly enriched in the embryonic blood of F3 and F4 embryos. Such changes map with the reported effects of synbiotics and choline in literature. Choline is an essential nutrient involved in biosynthesis of phospholipids, neurotransmitters, and one-carbon metabolism, with a critical step being its import into mitochondria [36]. It participates in multiple biosynthetic pathways, especially phospholipid metabolism critical for membrane structure and function [37]. Choline acts as an important methyl donor, a precursor for membrane formation, and is necessary for acetylcholine biosynthesis [38]. In a study with mice, a methionine and choline deficient diet led to a hypermetabolic state, weight loss, and improved insulin sensitivity and glucose tolerance, indicating choline's influence on energy metabolism [39]. Synbiotic supplementation modulates functional metabolic pathways in the intestinal microbiota, impacting host metabolism [40]. The gut microbiota significantly influences intestinal lipid and lipoprotein metabolism, affecting systemic metabolic health [41]. Microbial metabolites such as short-chain fatty acids (SCFAs) play key roles in regulating energy intake, energy harvesting, glucose and lipid metabolism, adipogenesis, immune responses, and the pathophysiology of obesity and related metabolic disorders [41]. Both animal and human studies support a strong relationship between gut microbiota composition, SCFA production, and the development or prevention of metabolic disorders [41]. The gut microbiome influences protein synthesis, cellular homeostasis, and stress response pathways [42]. Synbiotics regulate heat shock proteins via gut microbiota interactions, enhancing mucosal immunity and stress resilience [43]. Choline is essential for phospholipid synthesis, which preserves cell membrane integrity and supports ribosomal function necessary for efficient protein synthesis [44].

5. Limitations

Although our experimental design followed multiple generations and included both single- and repeated-treatment lineages, we did not directly assess epigenetic modifications such as DNA methylation, histone marks, or non-coding RNA expression. Therefore, we cannot conclusively attribute observed transcriptomic differences to stable epigenetic transmission mechanisms.

However, due to the fact that in each generation all groups were compared to the control, we can deduce that the observed effect in embryonic blood can be a response to the administration of PS alone or with choline to eggs containing F1 embryos. Therefore, the changes seen in SYNs and SYNCHs groups can be considered transgenerational effect. Additionally, although the experimental model spans multiple generations, transcriptomic data were not collected from F1 and F2 embryos. This limits our ability to track the temporal progression or persistence of gene expression changes across generations. Without these intermediate datasets, it is difficult to determine whether observed patterns in F3 and F4 represent gradual changes, stable transmission, or re-emergence of gene expression shifts.

6. Conclusion

This study revealed distinct transcriptomic profiles in two successive generations following in ovo administration of the PS synbiotic, either alone or combined with choline. Transcriptomic changes in embryonic blood were particularly pronounced in the F3 generation of Green-legged Partridgelike chickens following a single injection in F1 embryos, suggesting potential transgenerational effects of the intervention in SYNs and SYNCHs groups. The reduced transcriptomic alterations observed in the subsequent F4 generation may indicate that the intervention's impact is strongest in earlier generations and gradually diminishes over time.

7. Author Contributions

M.I.: Writing—original draft, Data curation, Investigation, Methodology, Formal analysis, Visualization, Writing—review and editing. E.G.: Data curation, Methodology, Formal analysis, Conceptualization, Supervision, Writing—review and editing, Project administration, Resources. M.B.: Conceptualization, Funding acquisition. K.S.: Methodology, Conceptualization, Supervision, Writing—review and editing.

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9. Data Availability Statement

All data supporting the findings of this study are included in the main text and Supplementary Files, which will accompany the published version (Not included in this draft). The raw sequencing data, in FASTQ format, have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA). Accession numbers will be provided prior to submission.

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11. Conflicts of Interest

The authors declare no conflicts of interest.

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