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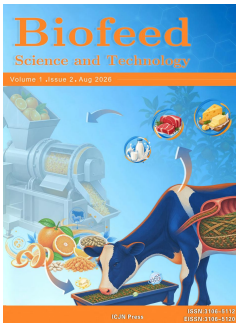

ARTICLE

Effects of Sodium Diformate on Immune Organs, Inflammatory Cytokines, Intestinal Morphology, and Cecal Microbiota of Hy-Line Brown Laying Hens

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Abstract

Background: Organic acidifiers are promising alternatives to in-feed antibiotics, but the effects of sodium diformate (NaDF) on intestinal health and cecal microbiota in laying hens remain unclear. **Objective:** This study aimed to investigate the effects of dietary NaDF supplementation on immune organ indices, pro-inflammatory cytokines, intestinal morphology, and cecal microbiota in Hy-Line Brown laying hens. **Methods:** A total of 720 twenty-one-week-old hens were randomly assigned to 6 groups (6 replicates of 20 hens each). Hens were fed a basal diet supplemented with 0 g/kg, 1 g/kg, 2 g/kg, 3 g/kg, 4 g/kg, or 5 g/kg NaDF for 24 weeks following a 1-week pre-feeding period. **Results:** Dietary NaDF supplementation showed no significant effect on spleen coefficient or the expression of pro-inflammatory cytokines across intestinal segments ($P > 0.05$). Notably, 1 g/kg to 4 g/kg NaDF significantly increased duodenal villus height ($P < 0.05$). The ratio of villus height to crypt depth was not significantly affected, with a linear increasing trend observed ($P < 0.05$), peaking at 4 g/kg. Ileal villus height was significantly increased ($P < 0.05$), with linear and quadratic responses ($P = 0.028$, $P = 0.004$). Cecal microbiota analysis revealed no significant changes in α diversity or microbial composition at the phylum level ($P > 0.05$). However, supplementation with 4 g/kg NaDF significantly reduced the abundance of *Parabacteroides* and *Mordavella massiliensis* ($P < 0.05$), while *Alistipes* and *Prevotella* showed increasing trends ($P = 0.083$, $P = 0.055$). **Conclusion:** Dietary NaDF supplementation improved duodenal and ileal morphology and exerted minor effects on specific bacterial genera, thereby promoting intestinal health in laying hens. However, it did not significantly affect the spleen index or inflammatory cytokine expression. The 4 g/kg level showed the most consistent improvements in intestinal morphology.

Keywords

Sodium diformate; Laying hens; Immune organ; Pro-inflammatory cytokines; Intestinal morphology; Cecal microbiota

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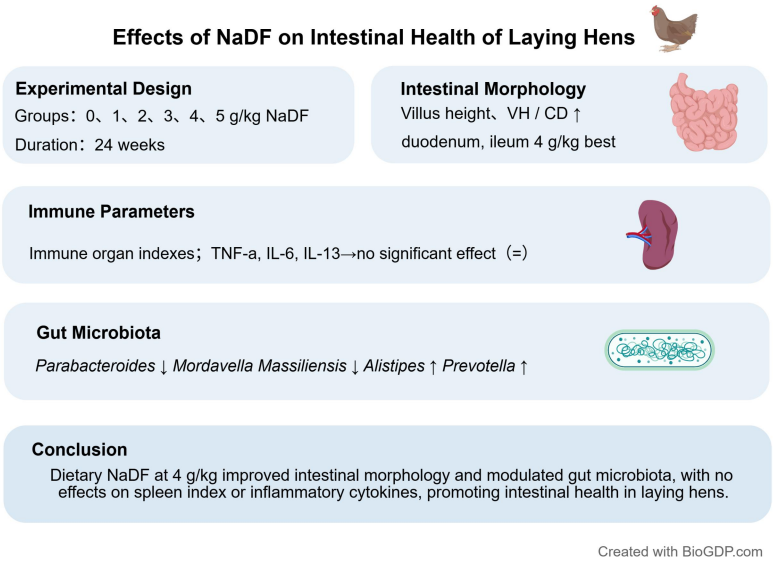
Graphical Abstract



Highlights

- NaDF at 4 g/kg improved duodenal and ileal morphology in laying hens.
- NaDF preserved α-diversity and phylum-level microbial composition.
- NaDF selectively reduced *Parabacteroides* and *Mordavella massiliensis*.
- NaDF increased SCFA-producing genera *Alistipes* and *Prevotella*.

1. Introduction

The rapid intensification and large-scale development of the laying hen industry have led to increasingly prominent issues, including reduced immune function[1], chronic inflammatory responses[2], impaired intestinal barrier function, and gut microbiota dysbiosis[3], all of which directly affect the health and disease resistance of birds, ultimately impacting production efficiency. High-density rearing has been shown to increase corticosterone levels via the hypothalamic-pituitary-adrenal axis, reduce plasma immunoglobulin G concentrations and macrophage phagocytic activity, and suppress immune organ development[4]. Furthermore, intensive production practices render poultry more susceptible to pathogen and toxin infections, with excessive pro-inflammatory cytokine production under immune stress leading to impaired intestinal structure and barrier function, thereby increasing the risk of intestinal inflammation[2]. In addition, oxidative stress compromises the intestinal epithelial barrier and tight junctions, altering intestinal barrier function and consequently affecting nutrient absorption and utilization, ultimately reducing growth performance[5]. Therefore, in the context of green livestock production, there is a critical need to develop safe and sustainable feed additives capable of improving immune function, modulating inflammatory responses, and maintaining intestinal health in laying hens.

Organic acidifiers have garnered considerable attention due to their ability to lower gastrointestinal pH, inhibit pathogenic bacterial proliferation, and regulate intestinal immunity, while offering advantages such as absence of drug resistance and residue concerns[6]. Among organic acids, formic acid exhibits the strongest antimicrobial activity; however, its volatility, irritancy, and corrosiveness limit its direct application in animal feed. Sodium diformate (NaDF), a novel acidifier, not only retains the antimicrobial advantages of formic acid but also overcomes its practical limitations. NaDF possesses sustained-release formic acid properties and has been demonstrated to regulate intestinal immunity, improve mucosal morphology, and modulate gut microbiota composition[7,8].

Recent studies have revealed that NaDF exerts multiple beneficial effects on intestinal health in poultry. Regarding anti-inflammatory effects, dietary supplementation with 1 g/kg to 5 g/kg NaDF significantly reduced jejunal tumor necrosis factor alpha (TNF-α) levels in broilers, indicating its anti-inflammatory potential[7]. In terms of intestinal morphology, NaDF supplementation significantly increased the ratio of villus height to crypt depth in the duodenum, thereby enhancing the intestinal absorptive surface area[8]. Concerning gut microbiota modulation, NaDF linearly increased ileal *Lactobacillus* counts while reducing the populations of *Escherichia coli* and *Salmonella*, and selectively enriched immunostimulatory microbiota, including *Jeotgalicoccus* and *Tetragenococcus* species[7]. Furthermore, Sun *et al*.[8] demonstrated that NaDF improves immune competence by modulating the gut microbiota.

Although NaDF, as a formate-based compound, has shown promising effects on growth performance in broilers[7], systematic studies investigating its effects on immune organ development, inflammatory cytokine expression, intestinal morphology, and cecal microbiota composition in laying hens remain limited. The underlying mechanisms of action in laying hens are still not fully elucidated. Therefore, the present study was conducted to investigate the effects of dietary supplementation with graded levels of NaDF on immune organ indices, intestinal morphology, intestinal inflammatory cytokines, and cecal microbiota in Hy-Line Brown laying hens, aiming to provide a theoretical basis for the application of NaDF in laying hen production.

2. Materials and Methods

2.1 Experimental Design

The experiment on laying hens was approved by the IACUC of Shenyang Agricultural University (SNLL24073103) in accordance with the Chinese National Guidelines for Laboratory Animal Welfare (GB/T 35892-2018). Hens were housed in three-tier stacked cages under a 16 h of light: 8 h of dark cycle with ad libitum access to feed and water. The house temperature was maintained at 24–26 °C. The 3Rs principles were strictly followed to minimize animal pain and distress. A completely randomized single-factor design was employed in this study. A total of 720 twenty-one-week-old healthy Hy-Line Brown laying hens were randomly assigned to 6 dietary treatments, each consisting of 6 replicates with 20 hens per replicate. The pre-experimental period lasted for 1 week, and the experimental period lasted for 24 weeks.

NaDF (purity ≥ 95%) was provided by Jiangsu Zhongdan Chemical Technology Co., Ltd. (Jiangsu, China). Hens in the control group were fed a basal diet without NaDF supplementation, while those in the experimental groups were fed the basal diet supplemented with 1 g/kg, 2 g/kg, 3 g/kg, 4 g/kg, or 5 g/kg NaDF. The dosage levels were selected based on previous studies demonstrating beneficial effects of NaDF supplementation within this range on intestinal health and growth performance in poultry[7]. NaDF was added to the experimental diets by replacing the corresponding proportion of zeolite powder in the basal diet. The sodium chloride content in the experimental diets was adjusted according to the sodium provided by NaDF supplementation. The basal diet was a corn-soybean meal-based diet formulated according to the Chinese Agricultural Industry Standard NY/T 33-2004 (Feeding Standard of Chicken) Available online: https://openstd.samr.gov.cn/. The composition and nutrient levels of the basal diet are presented in **Table 1**.

Table 1. Composition and nutrient levels of the basal diet (air-dry basis)

Items	Content (%)
Ingredients	
Corn	60.35
Vegetable Oil	1
Soybean Meal	25.70
Rice Husk	1.32
NaCl	0.32
$CaHPO_4$	1.02
Limestone Powder	9.19
Methionine	0.11
Choline Chloride	0.10
Premix(1)	0.39
Zeolite Powder	0.50
Total	100
Nutrient Levels	
ME (MJ/kg)(2)	11.08
CP	16.05
Lys	0.77
Met	0.38
SAA	0.66
Thr	0.65
Trp	0.22
Ca	3.80
TP	0.49
AP(2)	0.28

NOTE: (1) The premix provided the following per kg of diets: VA 8000 IU; VD_3 1600 IU; VE 5 IU; Vitamin K_3 0.50 mg; VB_{12} 0.004 mg; Folic acid 0.25 mg; biotin 0.10 mg; Thiamin 0.80 mg; Riboflavin 2.50 mg; Pantothenic acid 2.20 mg; Niacin 20 mg; Choline 500 mg; Pyridoxine 3 mg; Cu (as copper sulfate) 8 mg; Fe (as ferrous sulfate) 60 mg; Mn (as manganese sulfate) 60 mg; Zn (as zinc sulfate) 80 mg; Se (as sodium selenite) 0.30 mg; I (as potassium iodide) 0.35 mg; (2) ME and AP were calculated values, while the other were measured values. The contents of crude protein, amino acids, calcium, and total phosphorus were determined according to the methods specified in GB/T 6432-2018, GB/T 18246-2019, GB/T 6436-2018, and GB/T 6437-2018, respectively. CP: crude protein; Lys: lysine; Met: methionine; SAA: sulfur amino acids; Thr: threonine; Trp: tryptophan; Ca: calcium; TP: total phosphorus; AP: available phosphorus; VA: Vitamin A; VB: Vitamin B; VE: Vitamin E.

2.2 Feeding Management

The present study was conducted at the Experimental Farm of Shenyang Agricultural University. The laying hens were housed in three-tier stepped battery cages. The lighting schedule provided 16 h of light and 8 h of dark throughout the experimental period. All birds had ad libitum access to feed and water. The facility was heated by a water-circulating heating system and ventilated by axial-flow fans. The room temperature was maintained between 24 ℃ and 26 ℃. The feeding management, immunization protocols, and hygiene management of the experimental birds were carried out in accordance with the Hy-Line Brown laying hen management guidelines.

3. Measurement Indices and Methods

3.1 Immune Organ Indices

On the final day of the experimental period, two hens from each replicate were randomly selected and euthanized. The spleens were collected immediately after euthanasia, rinsed with physiological saline, blotted dry with absorbent paper, and weighed. The immune organ index was calculated as the percentage of spleen weight relative to live body weight (%).

3.2 Intestinal Mucosal Histomorphology

On the final day of the experimental period, two hens from each replicate were randomly selected and euthanized. Tissue samples were collected from the duodenum, jejunum, and ileum, and immediately fixed in 4% paraformaldehyde. The fixed intestinal samples underwent trimming, dehydration, embedding, sectioning, staining, and sealing using standard histological procedures. Target areas of the tissues were selected for photomicrography and imaging using CaseViewer image scanning software (CaseViewer 2.2, 3DHISTECH Ltd., Budapest, Hungary). Villus height (VH) and crypt depth (CD) were measured using Image Pro Plus 6.0 image analysis software (Image-Pro Plus 6.0, Media Cybernetics, Inc., Rockville, Maryland, USA), and the ratio of villus height to crypt depth (VH / CD) was subsequently calculated.

3.3 Intestinal Inflammatory Cytokines

On the final day of the experimental period, two hens from each replicate were randomly selected and euthanized. Total RNA was extracted from intestinal tissue samples using the TRIzol method according to the manufacturer's protocol. The RNA concentration and purity were assessed by measuring the OD260/280 ratio using a NanoDrop-2000 spectrophotometer (ThermoFisher Scientific Co., Waltham, MA, USA), and RNA integrity was verified using agarose gel electrophoresis. The extracted RNA was then reverse-transcribed into complementary DNA (cDNA) using a First-Strand cDNA Synthesis Kit (Tiangen Biotech Co., Ltd., Beijing, China) following the manufacturer's recommended protocol. Primers were designed using Primer-Blast (NCBI), and their specificity was verified. All primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The cDNA samples were amplified by real-time quantitative polymerase chain reaction (qPCR) using the SuperReal PreMix (Probe) kit (Tiangen Biotech Co., Ltd., Beijing, China). The qPCR cycling conditions were as follows: 95 ℃ for 5 min, followed by 40 cycles of 95 ℃ for 10 s, 60 ℃ for 30 s, and a final extension step at 72 ℃ for 5 min. The specificity of the qPCR products was evaluated by melting curve analysis. β-Actin was used as the internal reference gene, and the relative expression levels of target genes were calculated using the 2^-ΔΔCt method. All kits were obtained from (Tiangen Biotech Co., Ltd., Beijing, China).

3.4 Cecal Microbiota

On the final day of the experimental period, two hens from each replicate were randomly selected and euthanized. Cecal contents were collected immediately after euthanasia, snap-frozen in liquid nitrogen, and stored for subsequent metagenomic analysis. All experimental procedures were performed according to the standard protocols provided by Illumina, including sample quality assessment, library preparation, library quality control, and sequencing. After confirming the quality of the genomic DNA extracted from the samples, the DNA was fragmented. The fragmented DNA then underwent end repair, A-tailing, adapter ligation, purification and size selection of ligation products, library amplification, and product purification to construct the sequencing library. Following library quality validation, sequencing was performed on an Illumina sequencing platform. The raw reads obtained from sequencing were subjected to quality control and filtered to obtain clean reads for subsequent bioinformatics analysis. The clean reads were assembled, coding genes were predicted, and a non-redundant gene set was constructed. Functional annotation and taxonomic analysis of the non-redundant gene set were performed using universal and specialized databases. The species composition and abundance information of the samples were statistically analyzed. All bioinformatics analyses were conducted using the BMKCloud platform (www.biocloud.net).

3.5 Statistical Analysis

Experimental data were collated and preliminarily calculated using Excel 2019, followed by one-way analysis of variance (ANOVA) using SPSS 26.0 statistical software (SPSS Inc., Chicago, IL, USA). Duncan's multiple range test was used for post-hoc comparisons among treatment groups. The results are presented as means with standard error of the mean (SEM). Statistical significance was declared at $P < 0.05$, and $P < 0.01$ was considered extremely significant.

4. Results and Analysis

4.1 Immune Organ Indices

As presented in **Table 2**, dietary supplementation with different levels of NaDF had no significant effect on the spleen index of laying hens ($P = 0.350$). A linear decreasing trend ($P = 0.048$) and a quadratic change ($P = 0.092$) were also observed. The highest spleen index was observed in the 2 g/kg NaDF supplementation group.

Table 2. Effects of dietary NaDF supplementation on immune organ indexes of laying hens

Items	Dietary NaDF Supplementation Levels (g/kg)						SEM	*P*-Values		
	0	1	2	3	4	5		ANOVA	Linear	Quadratic
Spleen	1.210	1.160	1.290	1.050	1.060	0.880	0.052	0.350	0.048	0.092

NOTE: NaDF: sodium diformate; ANOVA: analysis of variance; SEM: standard error of the mean.

4.2 Intestinal Mucosal Histomorphology

As presented in **Table 3**, compared with the control group, dietary supplementation with 1 g/kg to 4 g/kg NaDF significantly increased duodenal villus height ($P < 0.05$). The ratio of villus height to crypt depth was not significantly affected ($P > 0.05$), with a linear increasing trend ($P = 0.040$), and the highest value was observed at 4 g/kg. No significant differences were detected in jejunal parameters among all treatment groups ($P > 0.05$). The ileal VH / CD ratio in the 1 g/kg to 5 g/kg NaDF groups was significantly higher than that in the control group ($P = 0.042$), with linear and quadratic increases ($P = 0.028$, $P = 0.004$), and the highest ratio was observed in the 4 g/kg group. Ileal villus height also exhibited linear and quadratic increases ($P = 0.004$). These results corroborate previous evidence[9-11] that formate-based acidifiers improve intestinal morphology in poultry. Although dietary NaDF supplementation significantly improved intestinal morphology (**Table 3**), it did not alter the overall gut microbiota diversity or SCFA levels (Section 4.4). This discrepancy may be attributed to the fact that the significant proliferation of specific beneficial bacteria (e.g., Lactobacillus), rather than a shift in the overall community structure, is sufficient to enhance intestinal barrier function and morphology.

Table 3. Effects of dietary NaDF supplementation on intestinal morphology of laying hens

Items	Dietary NaDF Supplementation Levels（g/kg）						SEM	*P*-Values		
	0	1	2	3	4	5		ANOVA	Linear	Quadratic
Duodenum										
VH /μm	1859^{a}	2005^{b}	2099^{bc}	2103^{c}	2760^{d}	1990^{a}	92.30	0.020	0.120	0.154
CD /μm	462	425	402	408	355	411	15.20	0.763	0.214	0.323
VH /CD	4.55	4.99	5.38	5.22	6.17	5.70	0.321	0.574	0.040	0.131
Jejunum										
VH /μm	1468	1463	1794	1831	1836	1607	70.60	0.457	0.258	0.201
CD /μm	360	357	303	287	294	296	18.40	0.490	0.136	0.264
VH /CD	5.06	5.24	5.24	5.42	5.91	5.37	0.232	0.906	0.718	0.924
Ileum										
VH /μm	1132	1315	1541	1618	1663	1606	56.80	0.056	0.004	0.004
CD /μm	274	210	237	228	242	245	9.43	0.236	0.882	0.361
VH /CD	4.26^{a}	6.62^{b}	6.58^{b}	7.07^{bc}	7.16^{c}	6.56^{b}	0.263	0.042	0.028	0.004

NOTE: a, b, c Within a row, means with different superscripts differ significantly ($P < 0.05$). VH: villus height; CD: crypt depth; VH / CD: ratio of villus height to crypt depth; SEM: standard error of the mean. Values are presented as means. Means in the same row without a common superscript letter differ significantly ($P < 0.05$). The same abbreviations and notations apply to subsequent tables unless otherwise stated.

4.3 Intestinal Inflammatory Cytokines

As presented in **Table 4**, compared with the control group, dietary supplementation with different levels of NaDF had no significant effect on the relative expression levels of inflammatory cytokines in the duodenum, jejunum, or ileum of laying hens ($P > 0.05$). No significant linear or quadratic regression relationships were observed between the expression levels of inflammatory cytokines in the duodenum, jejunum, or ileum and the increasing NaDF supplementation levels ($P > 0.05$).

Table 4. Effects of dietary NaDF supplementation on relative expression levels of intestinal inflammatory factors in laying hens

Items	Dietary NaDF Supplementation Levels（g/kg）						SEM	*P*-Values		
	0	1	2	3	4	5		ANOVA	Linear	Quadratic
Duodenum										
IL-6	1.02	1.04	1.26	1.15	1.05	0.76	0.063	0.452	0.724	0.863
IL-2	1.11	1.13	1.23	1.24	1.12	1.24	0.051	0.917	0.912	0.568
IL-10	0.77	0.73	0.71	0.56	0.74	0.54	0.032	0.415	0.187	0.310
IL-4	0.54	0.71	0.60	0.69	0.72	0.58	0.020	0.304	0.977	0.226
IL-1β	0.58	0.58	0.68	0.35	0.59	0.46	0.043	0.482	0.214	0.460
TNF-α	0.99	0.79	1.03	0.81	0.94	0.67	0.072	0.845	0.537	0.821

Table 4. Effects of dietary NaDF supplementation on relative expression levels of intestinal inflammatory factors in laying hens (continued)

Items	Dietary NaDF Supplementation Levels（g/kg）						SEM	*P*-Values		
	0	1	2	3	4	5		ANOVA	Linear	Quadratic
Jejunum										
IL-6	1.04	1.31	1.19	0.81	0.83	1.01	0.081	0.585	0.211	0.452
IL-2	1.64	1.50	1.19	1.39	1.71	1.82	0.082	0.341	0.657	0.800
IL-10	0.64	0.62	0.75	0.55	0.48	0.62	0.050	0.872	0.432	0.738
IL-4	0.43	0.23	0.48	0.54	0.38	0.58	0.065	0.833	0.470	0.741
IL-1β	1.71	2.77	2.66	2.40	1.92	2.43	0.177	0.693	0.811	0.779
TNF-α	0.62	0.60	0.47	0.34	0.60	0.33	0.053	0.464	0.324	0.361
Ileum										
IL-6	0.48	0.43	0.29	0.46	0.48	0.58	0.043	0.546	0.327	0.496
IL-2	0.42	0.61	0.58	0.63	0.60	0.80	0.051	0.618	0.115	0.230
IL-10	0.39	0.58	0.32	0.30	0.49	0.53	0.042	0.377	0.633	0.724
IL-4	0.55	0.59	0.31	0.36	0.46	0.49	0.058	0.870	0.540	0.641
IL-1β	0.47	0.44	0.37	0.52	0.57	0.49	0.046	0.898	0.983	0.812
TNF-α	0.45	0.63	0.49	0.72	0.72	0.58	0.043	0.581	0.228	0.351

NOTE: IL: Interleukin; TNF: Tumor Necrosis Factor.

4.4 Cecal Microbiota

4.4.1 Alpha Diversity Analysis

As shown in **Figure. 1**, compared with the control group, dietary supplementation with different levels of NaDF had no significant effect on the Abundance-based Coverage Estimator (ACE) index index, Chao1 index, Shannon index, or observed species (Sobs) ($P > 0.05$).

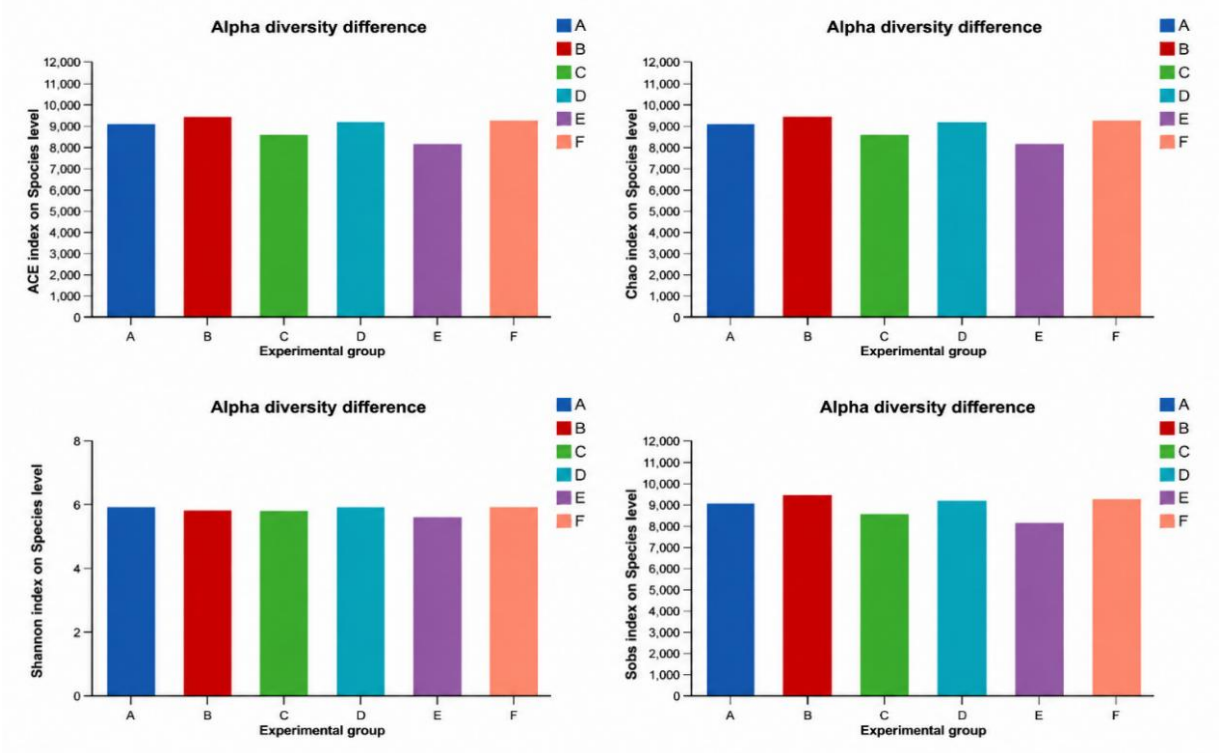


Figure. 1 Effects of dietary NaDF supplementation on cecal microbial α-diversity of laying hens. A: Control; B−F: 1−5 g/kg NaDF. **NOTE:** ACE: Abundance-based Coverage Estimator index, Sobs: observed species index. Copyright: The figure was created using GraphPad Prism 8.0.

4.4.2 Beta Diversity Analysis

As shown in the principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS) analyses **Figure. 2**, the cumulative interpretation rate of PCoA was 43.62%, and the NMDS stress value (stress = 0.081) indicated that the ordination plot was reliable. No significant clustering was observed among the groups ($P > 0.05$), indicating that NaDF did not induce a major shift in overall cecal microbial community structure.

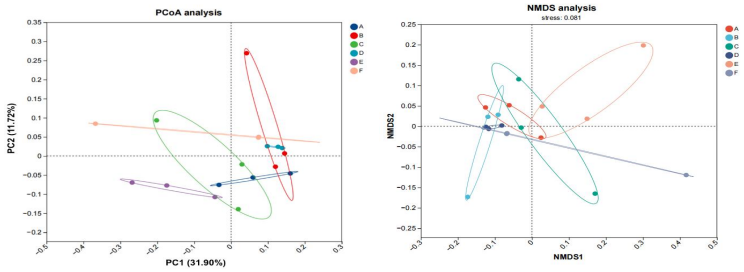


Figure. 2 Effects of dietary NaDF supplementation on cecal microbial β-diversity of laying hens. A: Control; B−F: 1−5 g/kg NaDF. **NOTE:** PCoA: Principal Coordinates Analysis; NMDS: Non-metric Multidimensional Scaling. PC: Principal Coordinate. Copyright: Statistical plots were generated using the BMKCloud platform (https://www.biocloud.net).

4.4.3 Effects of Dietary NaDF Supplementation on Cecal Microbial Community Composition of Laying Hens

As shown in **Figure. 3**, at the genus level, the numbers of unique Operational Taxonomic Units (OTUs) in the cecal digesta microbiota of the control group and the 1 g/kg, 2 g/kg, 3 g/kg, 4 g/kg, and 5 g/kg NaDF groups were 19, 29, 23, 21, 20, and 34, respectively, with a total of 2,453 shared OTUs among all groups. At the species level, the numbers of unique OTUs in the cecal digesta microbiota were 121, 180, 133, 126, 112, and 241, respectively, with a total of 8,018 shared OTUs among all groups.

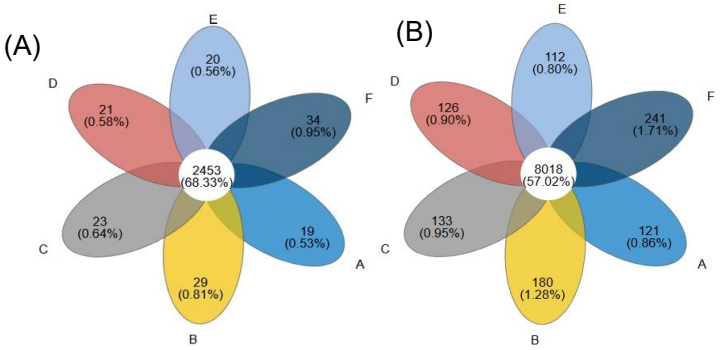


Figure. 3 Effects of dietary NaDF supplementation on cecal microbial community composition of laying hens. (A) Genus level; (B) Species level. A: Control; B−F: 1−5 g/kg NaDF. Copyright: Statistical plots were generated using the BMKCloud platform (https://www.biocloud.net).

4.4.4 Community Difference Analysis

As shown in **Figure. 4**, **Figure. 5**, and **Figure. 6**, at the phylum level, dietary NaDF supplementation had no significant effect on the cecal microbiota community composition of laying hens.

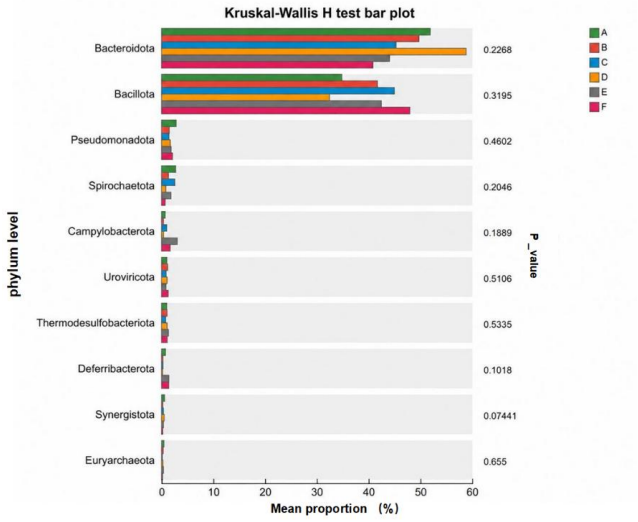


Figure. 4 Differences in microbial community composition at the phylum level. **NOTE:** A: Control; B−F: 1−5 g/kg NaDF. Copyright: Statistical plots were generated using the BMKCloud platform (https://www.biocloud.net).

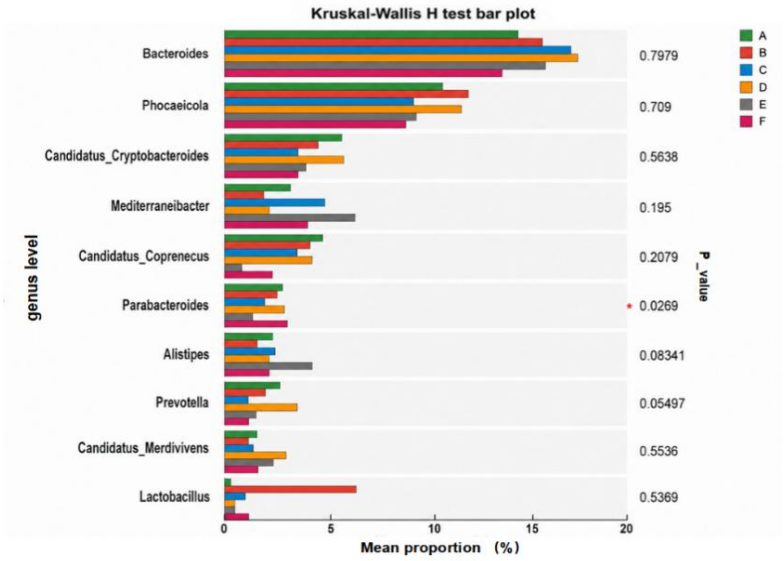


Figure. 5 Differences in microbial community composition at the genus level. * mean $P < 0.05$. **NOTE:** A: Control; B−F: 1−5 g/kg NaDF. Copyright: Statistical plots were generated using the BMKCloud platform. (https://www.biocloud.net)

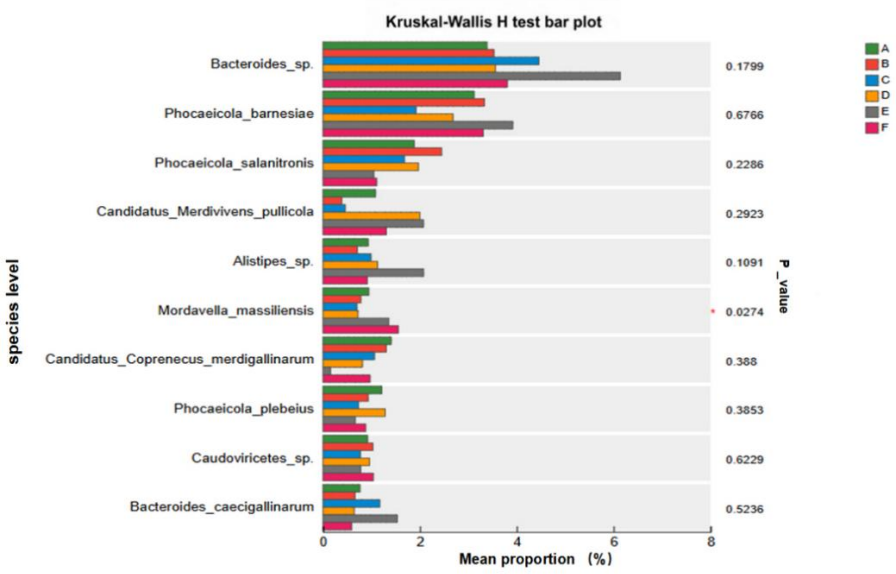


Figure. 6 Differences in microbial community composition at the species level. * mean $P < 0.05$. **NOTE:** A: Control; B−F: 1−5 g/kg NaDF. Copyright: Statistical plots were generated using the BMKCloud platform. (https://www.biocloud.net)

At the genus level, compared with the control group, no significant differences were observed in the abundance of dominant genera such as *Bacteroides* and *Phocaeicola* among all groups ($P > 0.05$). Supplementation with 4 g/kg NaDF significantly reduced the abundance of *Parabacteroides* ($P < 0.05$). Supplementation with 3 g/kg NaDF increased the abundance of *Alistipes* and *Prevotella*, although the differences did not reach statistical significance ($P = 0.083$ and $P = 0.055$, respectively).

At the species level, compared with the control group, no significant differences were observed in the abundance of dominant species such as *Bacteroides* sp., *Phocaeicola* barnesiae, and *Phocaeicola* salanitronis among all groups ($P > 0.05$). Supplementation with 4 g/kg NaDF significantly reduced the abundance of *Mordavella massiliensis* ($P = 0.027$).

5. Discussion

5.1 Effects of NaDF on Immune Organ Indices of Laying Hens

Immune organ indices in livestock and poultry reflect feeding efficiency and immune status, with higher indices generally indicating more developed immune organs and stronger immune function. Previous studies have demonstrated that formate-based acidifiers exert species-specific and dose-dependent effects on the immune organs of poultry. Chen *et al.*[12] reported that dietary supplementation with 0.60% potassium diformate significantly increased the thymus index of Ross 308 broilers. Similarly, Zou *et al.*[13] found that supplementation with 2 g/kg potassium diformate enhanced the spleen index of Cobb broilers. In contrast, Chen *et al.*[9] observed

that dietary supplementation with 4 g/kg or 40 g/kg potassium diformate had no significant effect on immune organ indices in broilers.

In the present study, dietary supplementation with 0 g/kg to 5 g/kg NaDF had no significant effect on the spleen index of Hy-Line Brown laying hens. This finding is partially consistent with the results of Chen *et al*.[9] but differs from the significant promoting effects reported by Chen *et al*.[12] and Zou *et al*.[13] The discrepancies among these studies may be attributed to differences in supplemental dosage, animal breed, and physiological stage. The nutrient allocation priority of laying hens favors egg production, with relatively lower priority assigned to immune organ development. Furthermore, after sexual maturity, organs such as the bursa of Fabricius undergo physiological regression, resulting in reduced responsiveness to exogenous stimuli[14]. In the present study, Although the data show a numerical dose-dependent bidirectional fluctuation in spleen index (first increasing then decreasing), the variance analysis indicates that these differences are not statistically significant ($P > 0.05$), suggesting that NaDF has no substantial effect on the spleen index of laying hens. Therefore, it cannot be concluded that NaDF has a clear immunomodulatory effect based on this finding, and further research is needed to determine the optimal supplementation level and underlying mechanisms.

5.2 Effects of NaDF on Intestinal Mucosal Histomorphology

Intestinal morphology is a critical indicator for evaluating intestinal health in livestock and poultry, with VH, CD, and the ratio of VH / CD reflecting the digestive and absorptive capacity of the intestine. Numerous studies have demonstrated that formate-based acidifiers can improve intestinal morphology, although the effects vary by intestinal segment and acidifier type[12]. For example, Chen *et al*.[12] reported that 4−8 g/kg potassium diformate (KDF) dose-dependently improved duodenal, jejunal, and ileal morphology in broilers. Sun *et al*.[8] found that 1 g/kg NaDF significantly increased the duodenal VH / CD ratio in broilers. Similarly, Lin *et al*.[10] observed that dietary supplementation with 1 g/kg potassium diformate (KDF) significantly increased villus length and the villus-to-crypt ratio, while decreasing crypt depth, in the duodenum of broilers However, Li *et al*.[7] reported that 1 g/kg to 5 g/kg NaDF had no significant effect on intestinal morphology in broilers, suggesting that the effects of formate-based acidifiers may be influenced by factors such as dosage and animal breed.

In the present study, dietary supplementation with 1 g/kg to 4 g/kg NaDF significantly increased duodenal villus height, and the VH / CD ratio showed a linear increasing trend with increasing NaDF levels. Supplementation with 2 g/kg to 5 g/kg NaDF significantly increased both ileal VH / CD ratio and villus height, exhibiting linear and quadratic increases, with the optimal effect observed at 4 g/kg NaDF. However, no significant differences were observed in any jejunal parameters among the treatment groups. These results are partially consistent with previous reports. Compared with KDF, NaDF did not significantly improve jejunal morphology, suggesting that different types of acidifiers exert segment-specific effects. Furthermore, compared with the studies of Sun *et al*.[8] and Lin *et al*.[10] in broilers, the effective dose of NaDF required to improve ileal morphology in the present study was higher. This discrepancy may be attributed to the longer intestinal development cycle and relatively delayed intestinal maturation in laying hens. It has been reported that the intestine of laying hens does not become essentially stable until 24 weeks of age[15], whereas the intestinal development rate of broilers post-hatch is significantly faster than that of laying hens, allowing broilers to acquire high digestive and absorptive capacity at an early age[16,17]. Therefore, the sensitivity of the laying hen intestine to formate-based acidifiers may be lower than that of broilers, requiring higher doses to produce significant morphological improvements. In the present study, dietary supplementation with 4 g/kg NaDF significantly improved villus height and VH / CD ratio in the duodenum and ileum of laying hens, indicating that an appropriate dose of NaDF exerts positive effects on intestinal morphology in laying hens.

5.3 Effects of NaDF on Intestinal Inflammatory Cytokines

Inflammatory cytokines, including Interleukin-6 (IL-6), Interleukin-1β (IL-1β), and Tumor Necrosis Factor-alpha (TNF-α), are known to participate in programmed cell death pathways such as apoptosis and pyroptosis, thereby exacerbating tissue damage[18]. Specifically, IL-6 promotes leukocyte proliferation and antibody production, whereas TNF-α induces the cascade release of inflammatory mediators, amplifying the inflammatory response. Li *et al*.[7] demonstrated that dietary supplementation with 1 g/kg to 5 g/kg NaDF significantly reduced jejunal TNF-α levels in broilers in a quadratic manner, while having no significant effect on IL-6 or IL-10 levels ($P > 0.05$). These findings suggest that NaDF exerts its immunomodulatory effects primarily through the suppression of pro-inflammatory cytokines rather than through the activation of anti-inflammatory cytokines.

These results are partially consistent with the findings of the present study. In this study, dietary supplementation with different levels of NaDF had no significant effect on the relative expression levels of inflammatory cytokines in the duodenum, jejunum, or ileum of laying hens. The discrepancy between our findings and those of Li *et al*.[7] may be attributed to differences in animal breed. Broilers are characterized by rapid growth and high metabolic rates, which may render their intestinal immune system more responsive to dietary additives. In contrast, laying hens prioritize nutrient allocation for egg production and possess a stronger capacity to maintain intestinal immune homeostasis, resulting in a higher threshold for responsiveness to exogenous stimuli. Therefore, under healthy, non-challenged conditions, significant changes in inflammatory cytokine expression are less likely to be observed in laying hens.

Accumulating evidence indicates that the anti-inflammatory effects of formate-based acidifiers are condition-dependent. Sun *et al*.[19] demonstrated in a mouse model that dietary supplementation with 1% potassium diformate significantly reduced serum levels of pro-inflammatory cytokines (IL-6, IL-12, and TNF-α) following Salmonella Typhimurium infection.The protective effect was associated with the regulation of gut microbiota composition.. Similarly, Sun *et al*.[8] employed a Salmonella Pullorum infection model and confirmed that dietary NaDF pretreatment for 2 days effectively reduced bacterial loads in the cecum, liver, and spleen, and alleviated cecal histopathological damage, suggesting that NaDF protects against inflammatory injury along the gut-liver axis.

In the present study, the experimental animals were maintained under conventional, non-challenged conditions, and the intestinal inflammatory signaling pathways were not activated. This likely explains the lack of significant effects on inflammatory cytokine expression observed in our study. Taken together, our findings suggest that under conventional rearing conditions, NaDF exerts a neutral effect on basal intestinal immune status. The anti-inflammatory potential of NaDF warrants further validation under pathogen challenge or stress conditions.

5.4 Effects of NaDF on Cecal Microbiota

The normal physiological and metabolic processes of the body are regulated by the gut microbiota. The gastrointestinal microbiota of animals is composed of probiotics, pathogens, and commensal bacteria, and at the phylum level is primarily dominated by Firmicutes, Bacteroidetes, and Proteobacteria. Sun *et al*.[8] reported that dietary NaDF supplementation had no significant effect on the cecal microbiota composition of broilers at the phylum level, nor did it alter alpha diversity indices. These findings are consistent with the results of the present study. In this study, dietary NaDF supplementation had no significant effect on cecal microbiota alpha diversity indices (ACE, Chao, Shannon, and Sobs) or β diversity in Hy-Line Brown laying hens. Although statistical tests showed no significant clustering among groups, indicating that NaDF did not cause major changes in the overall microbial community structure, the observed changes were limited to specific taxa at lower taxonomic levels, suggesting that NaDF can modulate the composition of the microbiota by altering the relative abundance of certain bacterial groups. Consistent with our findings, Li *et al*.[7] demonstrated that NaDF supplementation linearly increased ileal Lactobacillus counts while reducing the colonization of *Escherichia coli* and *Salmonella* in broilers, without exerting destructive effects on the overall microbial community structure. Collectively, these studies indicate that NaDF exerts a "selective" regulatory effect on the gut microbiota — targeting the abundance of functional genera while maintaining microecological homeostasis. This characteristic is favorable for preserving gut stability when NaDF is used as a feed additive.

At the genus level, 4 g/kg NaDF significantly reduced the abundance of *Parabacteroides*. Additionally, 3 g/kg NaDF tended to increase the abundance of *Alistipes* and *Prevotella*, though these differences did not reach statistical significance. *Alistipes* and *Prevotella* are recognized as potential short-chain fatty acid (SCFA)-producing bacteria in the avian gut[20], and SCFAs serve as key energy substrates for intestinal epithelial cells[21]. While direct SCFA quantification was not performed in this study, the observed bacterial shifts are consistent with the improved intestinal morphology documented herein. At the species level, 4 g/kg NaDF significantly reduced the abundance of *Parabacteroides* and *Mordavella massiliensis*. Previous studies have reported that *Parabacteroides* is positively correlated with lipid metabolism in chickens[22]. Organic acid salts such as sodium formate have been shown to improve feed conversion efficiency and nutrient digestibility in laying hens[23], suggesting that similar modulatory effects on gut microbiota and host metabolism may contribute to the beneficial effects of NaDF observed in this study. Nevertheless, the causal relationships and underlying mechanisms remain to be elucidated through integrated metabolomic analyses.

Therefore, the modulatory effect of NaDF on gut microbiota, particularly the suppression of potentially harmful bacteria such as *Parabacteroides*, may represent one of the potential mechanisms by which NaDF improves intestinal health in laying hens. Collectively, these results indicate that NaDF improves intestinal health in laying hens by selectively regulating gut microbiota structure, optimizing intestinal morphology, and thereby promoting nutrient digestion and absorption.

6. Conclusion

In conclusion, dietary NaDF supplementation improved duodenal and ileal morphology, selectively modulated bacterial genera, and enhanced intestinal health in laying hens. However, it did not significantly affect spleen index, inflammatory cytokine expression, α-diversity, or phylum-level microbial composition. The 4 g/kg supplementation level consistently yielded the best intestinal morphology improvements. Future studies should elucidate the specific pathways underlying NaDF's effects on intestinal morphology and validate its efficacy across different physiological stages of laying hens.

Author Contributions

All authors (Zongfu Li, Weiwei Li, Qiunan Chen and Yang Luo) meet authorship criteria: (1) substantial contributions to the conception, design, data acquisition, analysis, or interpretation; (2) drafting or critical revision of the manuscript for important intellectual content; (3) final approval of the version to be published; and (4) agreement to be accountable for all aspects of the work and to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval

All animal experiments were approved by the IACUC of Shenyang Agricultural University (approval No. SNLL24073103, approved on July 31, 2024) and conducted in accordance with GB/T 35892-2018 and the 3Rs principles. Hens were housed under a 16 h of light: 8 h of dark cycle at 24–26 °C with ad libitum access to feed and water. No painful procedures were performed. At the end of the experiment, birds were euthanized by exsanguination after anesthesia.

Informed Consent Statement

Not applicable.

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Conflicts of Interest

The authors declare that the data used in this study are not influenced by any commercial or corporate interests. There are no conflicts of interest to declare.

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