# Carotenoids from Halophilic Archaea: A Novel Approach to Improve Egg Quality and Cecal Microbiota in Laying Hens

Xufeng Dou , Guodong Zhang , Hao Tang , Xiaoxue Chen , Beibei Chen , Yuxia Mei , Haihong Jiao , Min Ren *

Posted Date: 31 October 2024

doi: 10.20944/preprints202410.2447.v1

Keywords: Carotenoids; Egg quality; Halalkalicoccus paucihalophilus; Antioxidant capacity; Cecal microbiota

*Article*

# Carotenoids from Halophilic Archaea: A Novel Approach to Improve Egg Quality and Cecal Microbiota in Laying Hens

**Xufeng Dou [1,2,†], Guodong Zhang [3,†], Hao Tang [1,2], Xiaoxue Chen [3], Beibei Chen [3], Yuxia Mei [4], Haihong Jiao [3] and Min Ren [1,2,*]**

[1] Key Laboratory of Conservation and Utilization of Biological Resources in the Tarim Basin, Alar, 843300, China; douxufeng69@163.com; 18009484694@163.com; th1234563@outlook.com

[2] College of Life Science and Technology, Tarim University, Alar, 843300, China

[3] College of Animal Science and Technology, Tarim University, Alar, 843300, China; 2927936846@qq.com; 2408474518@qq.com; jhhtar@163.com

[4] College of Life Science and Technology, Huazhong Agricultural University, Wuhan, 430000, China; 984144984@qq.com

* Correspondence: renminlure@163.com; Tel.: +86-159-9940-6855

† These authors contributed equally to this work.

**Simple Summary:** Carotenoids are important dietary components, and different sources of carotenoids differ in structure and function, and they all have potential health benefits for the organism. The effects of C50 carotenoids produced by halophilic archaea on poultry egg quality and gut microbiota have not been explored. In this study, *Halalkalicoccus paucihalophilus* TRM89021 produces carotenoids, was isolated from the Pamir Plateau. Feeding the Hotan black chicken with diets containing additional carotenoids from *Hac. paucihalophilus* improved eggshell brightness and yolk antioxidant levels. Although there was no significant change in the diversity of cecum microbiota, *Bacteroidota* was more dominant in the treatment group. This suggests that carotenoids from halophilic archaea can be used as a natural feed additive to improve egg quality and modulate the poultry gut microbiota. This study provides a new method for adding carotenoids to poultry diets and a theoretical basis for their application.

**Abstract:** Carotenoids from different sources have different structures and functions and as a dietary component have potential health benefits for a wide range of organisms. The effects of halophilic Archaea-derived C50 carotenoids on poultry egg quality and gut microbiota remain largely unexplored. In this study, we isolated and obtained a strain of *Halalkalicoccus paucihalophilus*, named TRM89021, from the Pamir Plateau. It secretes large amounts of carotenoids. We characterized the carotenoid pigments produced by this strain and found that its main components were bacterioruberin and its derivatives. Effects of these carotenoids on egg quality and cecal microbiota composition of hens were investigated. Compared to the BDG, the supplementation with carotenoids in the CDG resulted in significantly lower a* and b* scores at week 5 and a lower b* score and Haugh units at week 2, while egg strength and weight were higher. The CDG also showed increased yolk antioxidant capacity, higher glutathione peroxidase levels, and significantly lower catalase levels ($p$<0.05). Plasma analysis revealed elevated total bilirubin and aspartate aminotransferase levels, along with reduced inorganic phosphorus levels in the CDG ($p$<0.05). No significant differences in cecal microbiota diversity were observed between groups at any taxonomic level ($p$>0.05). This result suggests that halophilic archaea-derived carotenoids display potential to be a natural feed supplement to improve egg quality. Our study provides a theoretical basis for application of archaea-derived carotenoids in poultry diets.

**Keywords:** Carotenoids; Egg quality; *Halalkalicoccus paucihalophilus*; Antioxidant capacity; Cecal microbiota

## 1. Introduction

Carotenoids, a significant group of natural pigments, are renowned for their role in bestowing vibrant hues to plants, algae, and various microorganisms. Moreover, they possess crucial biological

 doi:10.20944/preprints202410.2447.v1



functions, including antioxidant activity. These pigments are isoprenoid polymers with 40 - 50 carbon atoms, and over 700 distinct natural carotenoids have been discovered to date. Among microorganisms, halophilic archaea are a special class of carotenoid producers, such as the red strain *Haloferax volcanii*[1]. These microorganisms typically thrive in marine and inland salt environments, including salt lakes and salt mines[2-5]. They are also commonly found in the soils of the Pamir Plateau[6, 7]. Uniquely, they are capable of synthesizing C50 carotenoid[8]. The extracts of carotenoid C50 are superior to carotenoid C40 (e.g., β-carotene and astaxanthin [9, 10]) as well as non-carotenoid antioxidants (including tocopherols, butylated hydroxytoluene and ascorbic acid[11]), which have excellent antioxidant activity. Employing high-performance liquid chromatography (HPLC) and HPLC-mass spectrometry (HPLC-MS) techniques, researchers have determined that halophilic archaea produce both C40 and C50 carotenoids[12, 13]. The majority of research indicates that after fermenting red halophilic archaea, the subsequent isolation and purification of pigments using column chromatography and thin layer chromatography, along with characterization through visible light spectroscopy, Raman spectroscopy, nuclear magnetic resonance (NMR), and mass spectrometry, reveal that cell membranes are enriched with carotenoids like bacterioruberin, monoanhydrobacterioruberin, and bisanhydrobacterioruberin[14-16]. Furthermore, carotenoids like β-carotene and lycopene, which are present in lower concentrations, can serve as precursors for the synthesis of additional carotenoids by halophilic archaea[17].

Carotenoids, a vital group of natural pigments, are renowned for their role in giving plants, algae, and various microorganisms their rich colors. In addition to their coloration, they possess significant biological functions, including acting as antioxidants. In poultry nutrition, supplementing diets with carotenoids has been demonstrated to enhance both egg yield and quality, in addition to having a positively impact on the gut microbiota of birds. For instance, the addition of astaxanthin derived from microorganisms to the feed of laying hens influences the composition of plasma and yolk, as well as hen serum [18, 19]. In addition, it enhanced egg yolk coloring[19, 20]. The intake of plant-derived carotenoids carotenoids significantly modulates animal intestinal and fecal microbiota[21-23]. The impact of microbial-derived pigments on the gut microorganisms of chickens has yet to be thoroughly documented

There is a noticeable gap in research regarding the efficacy of *Halophilic archaea* carotenoids in poultry feed, particularly for the Hotan black chicken. The Hotan black chicken, a breed of considerable economic importance, is characterized by its wholly black plumage, robust disease resistance, capacity to flourish on rough feed, and adaptability to hot environments [24]. Primarily utilized for meat and egg production, the Hotan black chicken was recognized as a Geographical Indication of Agricultural Products in Xinjiang in 2013[25], indicating its regional significance. This breed may respond differently to feed additives compared to other species. Consequently, investigating the use of *Halophilic archaea* carotenoids in the diet of Hotan black chicken could yield new strategies for enhancing egg quality and intestinal health, although this area requires further scientific research and empirical evidence.

In this study, we isolated and obtained pigmented halophilic archaea from soil on the Pamir Plateau. Derived carotenoids of this microorganism were added to the feed of Hetta aurochs, and it is planned to evaluate their effects on egg yield, quality and cecum microbiota composition. This pioneering study introduces a novel carotenoid additive, derived from halophilic archaea, to laying hens' feed, potentially revolutionizing the sector.

## 2. Materials and Methods

### *2.1. Halophilic Archaea Isolation and Identification*

Soil samples collected from the Pamir Plateau, were stored at -4°C[7]. Soil sample classification, archaeal isolation, and identification were conducted following the methodology of Bu *et al.*[7]. Described briefly here, the Pamir Plateau soil samples (1 g) were diluted in 9 mL of 15% sterile saline solution, and used to inoculate NOM media, followed by incubation at 37°C for 30 d. The NOM medium composition (per liter) is as follows: Yeast Extract 0.05 g, Fish Peptone 0.25 g, Sodium Pyruvate 1.0 g, KCl 5.4 g, $K_2HPO_4$ 0.36 g, $CaCl_2$ 0.25 g, $NH_4Cl$ 0.25 g, $MgSO_4{\cdot}7H_2O$ 0.25 g, $MgCl_2{\cdot}6H_2O$

 doi:10.20944/preprints202410.2447.v1



23.0 g, NaCl 150.0 g, and Agar 20 g, and pH is adjusted to 7.0. Individual colonies were selected and purified repeatedly (three times). Genomic DNA was extracted and subjected to 16S rRNA gene amplification (Table S1), using a reaction mixture comprising EasyTaq 0.2 μL, EasyTaq Buffer 2 μL, dNTPs 1.5 μL, forward primers 1 μL, reverse primers 1 μL, DNA 50 ng, and dd$H_2O$ to a final volume of 20 μL. The primers used were 20F (5'-ATTCCGGTTGATCCTGCC-3') and 1452R (5'-AGGAGGTGATCCAGCCGC-3'). PCR products exhibiting positive bands, as identified by 1% (w/v) agarose gel electrophoresis, were sequenced bidirectionally by Beijing Tsingke Biotech Co., Ltd and Sangon Biotech (Shanghai) Co., Ltd. Sequences were assembled using SeqMan software and analyzed for similarity against the Ezbiocloud database (https://www.ezbiocloud.net/) to ascertain their taxonomic affiliation. The pigment-producing halophilic archaea were frozen and preserved in glycerol tubes at -80°C (NaCl concentration of 15% (w/v)) for subsequent use in pigment extraction.

*2.2. Carotenoids Analysis*

The extraction of carotenoids from red halophilic archaea was conducted using conventional methods[26]. Initially, after culturing the red halophilic archaea, a 500 mL aliquot of the culture was centrifuged at 12,000 rpm for 5 minutes at room temperature. The spenatant was decanted to obtain the pellet. Subsequently, 40 mL of pure water was added to the pellet. The cell lysate was mixed with a solvent mixture, where the ratio of cell lysate to trichloromethane to methanol was 1:1:2 (v/v/v), and the mixture was transferred to a clean conical flask. The flask was shaken at 150 rpm for 4.5 hours at room temperature to facilitate the extraction process. Additional pure water was added to the mixture until phase separation occurred, and the mixture was allowed to stand at room temperature for 4 hours to allow for complete delamination. The red organic layer was collected using a separatory funnel and concentrated using a rotary evaporator with the water bath temperature set at 30°C and the rotor speed maintained at 60 rpm (Haydorf Instruments Co. Ltd., Shanghai, China). The crude pigment extract was then dissolved in methanol, tranferred to a 10 mL centrifuge tube, and the supernatant was stored at -20°C for further analysis.

The absorbance at 495 nm was measured against a methanol blank. Carotenoid yield was calculated using the following formula[27, 28]:

$$\text{Caroteniods yield (mg/L)} = \frac{\text{A} \times \text{D} \times V1}{0.16 \times V2} \quad (1)$$

Where: C represents the total carotenoid yield (mg/L); A is the absorbance at the maximum wavelength ($\lambda$ = 495 nm); D is the dilution factor; V1 is the total volume of the extract (unit: L); 0.16 represents the average specific absorption coefficient for carotenoids at 495 nm (unit: L/(mg·cm) or L/(μmol·cm)); and V2 is the volume of the culture medium (unit: L).

High carotenoid-producing strains were inoculated into a sterile fermenter for cultivation over a period of 5 days. (silent oil-free air compressor, Shanghai Top Stability Machinery Co., Ltd, Shanghai, China. 100 L fermenter, Shanghai Baoxing Biochemical Equipment Co.) After the cultivation period, the carotenoids from the strains were extracted. Their ultraviolet absorption spectra were recorded within the range of 350 to 600 nm[26]. Furthermore, the carotenoids from high carotenoids-producing strains were analyzed using Ultra Performance Liquid Chromatography (UPLC) [28, 29] (ACQUITY UPLC/VION IMS QTOF MS, Waters Corporation, USA).

*2.3. Animals Experimental Design and Management*

The experimental protocol adhered to the animal welfare guidelines of the College of Life Sciences and Technology, Tarim University. We randomly divided 50 late-stage laying Hotan black chickens (300 days old) arbor acres broiler chickens into two groups: Carotenoids-supplemented diet group (CDG) and Basal diet group (BDG), each with five replicates, and with five chickens in each replicate. The BDG was fed a basal diet, whereas the CDG given a basal diet supplemented with an additional 100 mg/kg of carotenoids extracted from halophilic archaea. Throughout the experimental period, chickens had ad libitum access to water. The basal diet was fed at regular intervals, three times daily (morning, noon, and evening), with each feeding consisting of 500 g of basal diet per chicken. To ensure optimal living conditions, the chickens were housed in layer cages with

 doi:10.20944/preprints202410.2447.v1



permanent ventilation, exposure to natural light, and regular cleaning of the coop. The energy content of the basal diet was formulated in accordance with the nutritional requirements specified by the National Research Council for laying hens (NRC, 1994). The feeding experiment spanned a duration of 5 weeks. The basal feed was procured from Xinjiang Tiankang Feed Co., Ltd. (Xinjiang, China), and the detailed nutritional composition is presented in Table S2.

### *2.4. Egg Quality*

#### 2.4.1. Production performance

Comparison of egg production between week 2 and week 5. At weeks 2 and 5, three freshly laid eggs were randomly selected daily for further analysis. The dimensions of the egg shape, specifically length and width, were measured using a digital caliper IP54 (Shanghai Deystar Tools Co., Ltd., Shanghai, China). The weight of each egg was determined using an electronic balance (model ML204, Mettler Toledo Instruments Ltd.). The color of the eggshell was evaluated using an Eggshell Color Tester SC-10, which quantifies L*, a*, and b* values to assess brightness, redness, and yellowness, respectively. The strength of the eggshell was measured using an Eggshell Strength Tester KQ-1A. The height of the egg white was determined using an Egg White Height Determination EQ-1A Mini. The color of the egg yolk was specified using an Egg Yolk Colorimetric Fan, which ranges from 1 (yellow) to 15 (red). All the aforementioned instruments were sourced from Beijing Tianxiang Feiyu Technology Co., Ltd.

#### 2.4.2. Egg Yolk Antioxidant Capacity

During the experiment, three fresh eggs were randomly selected at 3 d intervals from the CDG, and the antioxidant capacity of egg yolks was determined using total antioxidant capacity (T-AOC), superoxide dismutase (SOD), malondialdehyde (MDA), Glutathione peroxidase (GSH-Px), and catalase (CAT) kits (Suzhou Grace Biotechnology Co. Ltd., Jiangsu, China), respectively.

### *2.5. Plasma Biochemical and Cecal Microbiota*

Chickens were fasted 24 h before the experiment and water was freely available. Fresh blood was collected under the wing using a disposable venous blood sampling needle and venous blood sampler (containing sodium heparin), stored at 4℃. Using a scalpel to sever the jugular vein, randomly select 9 chickens for the experimental group and another 9 for the control group. The chickens are dissected, and the contents of the cecum are swiftly placed into cryovials, which are then immediately submerged in liquid nitrogen.

#### 2.5.1. Plasma Biochemical Analysis

The blood of 9 treated laying hens was divided into 3 subgroups, each consisting of blood from 3 chickens. Blood within each subgroup is mixed to form a single blood sample. Blood from the control group was treated in a similar manner, and the control group also obtained 3 pooled blood samples. Fresh blood was centrifuged (4°C, 3000 rpm, 10 min) (high-speed cryo-centrifuge, Thermo Fisher Scientific China Co., Ltd.), and plasma was taken and analyzed using a fully automated multifunctional biochemical analyzer (SMT-120VP Chengdu Smarter Science and Technology Co., Ltd., Sichuan, China). The biochemical traits included albumin (ALB), inorganic phosphorus (PHOS), total protein (TP), glucose (GLU), amylase (AMY), aspartate aminotransferase (AST), total bilirubin (TB), urea nitrogen (BUN), creatine kinase (CK), albumin-globulin ratio* (A/G*), globulin* (GLOB*) content.

#### 2.5.2. Cecal Microbiota

The samples were transported on dry ice to Shanghai Meiji Biomedical Technology Co. The above samples were used for full-length bacterial community diversity sequencing analysis (16S rRNA amplicon sequencing).

### *2.6. Statistical Analysis of Data*

 doi:10.20944/preprints202410.2447.v1



The egg quality data obtained in this study were subjected to independent sample t-test using SPSS 27.0, and the data results were expressed as mean ± standard (x ± SE). $p<0.05$ indicates a significant difference. Phylogenetic trees were constructed using MEGA-X software.

## 3. Results

### *3.1. Isolation, Purification and Identification of Pigment-producing Halophilic Archaea*

*Hac. paucihalophilus* TRM89021 (accession number: PP827431) was obtained from soil samples from the Pamir Plateau. Colonies of the strain appeared red, exhibiting opaque, smooth, round, and raised morphology. TRM89021 was gram-negative ($G^-$). Scanning electron microscopy revealed a spherical cell shape. And a NJ tree based on 16S rRNA gene (1404 bp) showed that strain TRM 89021 clustered tightly with *Hac. paucihalophilus* JCM17505 and YIM93701 with strong bootstrap support (Figure 1).

### *3.2. Carotenoid Principal Component Analysis*

The UV-Vis absorption spectrum of the crude methanolic extract of the pigment showed distinct peaks at 461 nm, 490 nm, and 522 nm, which are indicative of the characteristic "three-finger" signature of C50 carotenoids[30]. This observation led to the preliminary identification of the pigment as a carotenoid. The UPLC chromatogram of the carotenoid extract from *Hac. paucihalophilus* TRM89021, along with the ACQUITY UPLC/VION IMS QTOF MS response plot, confirmed that the main constituents of the extract are bacterioruberin and its derivatives, as demonstrated by the chromatographic profiles (Figure 2). The fermentation yield of carotenoids from the strain TRM89021 is 20 mg/L.

### *3.3. Effects of Dietary Carotenoids Extracts on Egg Quality in the Hotan Black chickens*

#### 3.3.1. Laying production performance

Significant increases in the a* score, egg strength, and egg weight were observed at week 2 compared to week 5 in the BDG group, while the Haugh unit showed a significant decrease ($p < 0.05$). In the CDG group, the L* score increased significantly and the a* score decreased significantly from week 2 to week 5 ($p < 0.05$). When comparing the two groups, the CDG had significantly lower a* and b* scores at week 5. While at week 2, the b* score and Haugh units were significantly lower, and egg strength and egg weight were significantly higher compared to the BDG ($p < 0.05$). No significant differences were observed in other measured indices: eggshell thickness, egg-shaped index, yolk color ($p > 0.05$).

#### 3.3.2. Egg Yolk Antioxidant Capacity

Comparing the eggs from the CDG at week 2 with those at week 5, there was a significant decrease in CAT ($p<0.01$) and a significant increase in GSH-Px and T-AOC content in the yolks at week 2 ($p<0.001$). The increase in SOD and MDA content in egg yolks was not significant ($p>0.05$) in week 2 compared to week 5 (Table 2).

### *3.4. Impact of Pigment Extracts on Plasma Biochemical Parameters in Laying Hens*

Our findings, presented in Table 3, indicate that supplementation with carotenoids led to significant increases in plasma total bilirubin and aspartate aminotransferase levels ($p<0.05$), along with a significant decrease in inorganic phosphorus levels ($p<0.05$) in the CDG relative to the BDG.

### *3.5. Effects of Dietary Carotenoids on Cecal Microbiota in the Hotan Black chickens*

Cluster analysis was conducted on the clean reads of all samples, clustering sequences into operational taxonomic units (OTUs). A total of 602 OTUs were identified, each representing a microbial species. There were 535 OTUs of the treated group with dietary carotenoids, and 586 OTUs of the control group (Figure 3). Species annotation results showed a total of 155 Species, of which 141 were in the CDG and 153 in the BDG. Carotenoid supplementation did not significantly impact the

 doi:10.20944/preprints202410.2447.v1



alpha diversity indices - ace, sobs, Simpson, Shannon, coverage and Chao - of cecal microbiota OTUs in laying hens ($p$=0.4469, $p$=0.3172, $p$=0.6769, $p$=0.2271, $p$=0.1806, $p$=0.4131) (Table 4). β-diversity analysis, including PCA based on Bray-Curtis dissimilarity and PCoA, revealed no significant differences in the overall species composition of cecal microbiota between the groups ($p>0.05$).

Regarding species composition, *Bacteroidota* was the predominant phylum in the cecal flora, representing 62.90% and 47.33% of the total in the CDG and BDG, respectively. The second most abundant phylum was *Firmicutes*, comprising 33.40% and 38.65% of the community in the CDG and BDG, respectively. The dominant genus and species were classified as unclassified *Bacteroidales*, non-culturable bacteria, representing 33.03% and 32.60%, respectively (Figure 4 and Figure S1). Additionally, *Phocaeicola coprophilus* and *Paraprevotella clara* occur only in the CDG, and 14 species are unique to the BDG, such as *Bacteroides uniformis*, *Ligilactobacillus aviaries*, *Latilactobacillus sakei* and etc. (Figure S2). At the phylum, order, family, genus, and species levels, no significant differences in microbial composition were observed between the CDG and BDG ($p>0.05$). However, at the genus level, *Phocaeicola* abundance was higher in the CDG (12.04%) than in the BDG (3.82%); the species level showed that *Phocaeicola salanitronis* in the CDG (8.84%) was more than in the BDG (1.61%) ($p>0.05$) (Figure S3). (Reference Species Classification Database. nt_v20221012/16s_bacteria).

## 4. Discussion

### *4.1. Isolation, Purification and Identification of Pigment-producing Halophilic Archaea*

Typically, halophilic archaea require a salt concentration of at least 15% for peak growth[31]. Halophilic microorganisms are potential pigment producers[32], bacteriocins are the predominant carotenoids in halophilic archaea[33]. Pigment production by halophilic archaea is a defense mechanism against environmental stresses., produced pigments protect halophilic archaea from a variety of external stresses, including high salinity and intense ultraviolet radiation[34]. The literature suggests that halophilic archaea can be isolated from many high-salt environments by the dilution plating technique[35-37], including red halophilic archaea[38, 39]. Meanwhile, soil samples from the Pamir Plateau contain a large number of halophilic archaea by 16S rRNA amplicon sequencing[6]. In this study, the *Hac. paucihalophilus* TRM89021 were isolated from soil samples of Pamir Plateau by dilution coating plate method. The isolation of halophilic archaea from the Pamir Plateau provides fresh insights into the study of halophilic microorganisms. These microorganisms are not only prevalent in the high-salt environments previously reported but also notably abundant in the high-altitude regions of the Pamir Plateau. This discovery reveals the rich resources of halophilic archaea in the Pamir Plateau and suggests that they may play a significant role in the balance of the ecosystem and the biogeochemical cycles of the region.

### *4.2. Carotenoid Principal Component Analysis*

Liquid chromatography-mass spectrometry (LC-MS), specifically the HPLC-APCI-MS/MS method, is commonly utilized for the identification of carotenoids[40, 41]. For instance, the *Halorubrum* sp. strain BS2 has been found to produce carotenoids identified as bacterioruberin and bisanhydrobacterioruberin through HPLC and LC-MS analysis[41]. Analysis of the carotenoids produced by *Halorubrum tebenquichense* SU10 using ultra-performance liquid chromatography electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) revealed the presence of bacterioruberin, along with several of its derivatives, such as mono-, di-, and tri-hydroxybacterioruberin, and various cis-isomers of bacterioruberin[28]. Utilizing UV, HPLC, and TLC methods, it has been discovered that the primary carotenoid components in seven species of halophilic archaea, including *Halogeometricum rufum*, *Halogeometricum limi*, *Haladaptatus litoreus* and etc. are C50 carotenoids: bacterioruberin and its derivatives (mono- and di-dehydrobacterioruberin)[9]. Furthermore, Raman spectroscopy has been employed to identify carotenoids in red halophilic archaea (*Halobacterium salinarum* NRC-1 and R1, and *Halorubrum sodomense*), confirming that C50 carotenoids bacterioruberin are a predominant class of carotenoids among these archaea[42]. In this study, the main carotenoids in *Hac. paucihalophilus* TRM89021 were preliminarily identified as bacterioruberin and its derivatives by UV and UPLC methods. Our

 doi:10.20944/preprints202410.2447.v1



findings are largely in agreement with those of previous research, indicating a striking similarity in the carotenoids produced by various species of halophilic archaea, including *Hac. paucihalophilus* TRM89021. This provides another case for the subsequent identifica-tion of carotenoids in halophilic archaea, as well as providing a further understanding of halophilic archaea.

### *4.3. Effects of Dietary Carotenoids Extracts on Egg Quality in the Hotan Black chickens*

Carotenoids can affect egg quality such as egg production, eggshell thickness and strength in laying hens[18, 20, 43]. In the case of carotenoids produced by microorganisms, carotenoids supplementation in feed improves egg production ($p$<0.05)[19]; however, a previous study reported that carotenoid supplementation had no significant effect on egg production[44]. Addition of astaxanthin[19]and beta-carotene[44] improves the color of egg yolks in laying hen diets ($p$<0.05); most of the literature suggests that protein content, Haugh units, eggshell thickness and eggshell strength are not affected in laying hens[20, 45], and only a few studies have incidentally found an increase in eggshell weight[19] and the size of an egg[46] ($p$<0.05). In this study, compared to the BDG, the addition of carotenoids produced by *Hac. paucihalophilus* TRM89021 to the diet of Hotan black chickens resulted in a significant increase in a* score, egg strength, and egg weight, along with a significant decrease in the Haugh unit from week 2 to week 5 in the BDG ($p$<0.05). In the CDG, there was a significant increase in L* score and a significant decrease in a* score from week 2 to week 5 ($p$<0.05). When comparing the two groups, the a* and b* scores were significantly lower at week 5, while the b* score and Haugh unit were significantly lower, and egg strength and egg weight were significantly higher at week 2 ($p$<0.05). Other indices showed no significant effects ($p$>0.05). This is similar to the results reported in some studies.

Notably, carotenoids have strong antioxidant properties, including the C50 carotenoid bacterioruberin, a long-chain carotenoid with 13 conjugated double bonds and a terminal hydroxyl group, which is a potent free radical scavenger[47]. From *Halorubrum*, *Haloarcula*, *Haloferax*[26] and *Haloarcula japonica*[48] extracts of in bacterioruberin in showed better antioxidant activity. *Hac. tebenquichense* SU10 carotenoid extract also showed antioxidantactivity[28]. Assays for glutathione peroxidase, superoxide dismutase, total antioxidant capacity, and malondialdehyde levels are standard methods for assessing antioxidant potential[49]. Carotenoid supplementation in the diet of laying hens increased the activities of superoxide dismutase, catalase and glutathione peroxidase in egg yolks and reduced malondialdehyde levels in laying hens.[18, 19, 44]. Therefore, the addition of carotenoids to feed improves the antioxidant capacity of egg yolks, whereas the report of C50 carotenoids as feed additives on egg yolk quality has not been found. In this study, feeding carotenoids produced by *Hac. paucihalophilus* TRM89021 significantly increased the levels of GSH-Px and T-AOC in egg yolk. This implies that the carotenoids produced by the strain have similar antioxidant activity as the carotenoids in the above study. It is implied that C50 carotenoids may have the same ability to improve egg yolk antioxidant ability when used as feed additives.

### *4.4. Effects of Dietary Carotenoids on Plasma Biochemical Profiles in the Hotan Black chickens*

The addition of carotenoids to the diet also affects the metabolic profile of laying hens. Plasma biochemical analyses help to provide insight into the metabolic effects observed in laying hens. Fewer studies have been reported on the effects of carotenoid intake on plasma composition in laying hens: both lycopene and astaxanthin increased serum glucose levels in laying hens ($p$>0.05), and creatinine and cholesterol levels, and activities of ghrelin, glutamate, glutamate aminotransferase, and alkaline phosphatase varied with dose[50]. As of now, there is not a standard that has been consulted regarding the normal range of plasma biochemicals for laying hens. Fortunately, there is one study that makes reference to blood biochemistry in broilers[51]. As a result of this study, the plasma TB and AST levels of the CDG were significantly higher ($p$<0.05) and PHOS level was significantly lower ($p$<0.05). Plasma TB and AST levels can reflect whether the liver is damaged or not side by side[51]. The values in this study were within the normal range, so it also indicates that the liver is not damaged. Inorganic phosphorus may be related to the function of the kidneys, for example[52]. In this study there is no mention of the description of the viscera, and it is not currently possible to

 doi:10.20944/preprints202410.2447.v1



determine what the direct cause of the decrease in plasma inorganic phosphorus levels is, and further studies are still needed.

*4.5. Effects of Dietary Carotenoids on Cecal Microbiota in the Hotan Black chickens*

The gut microbiota regulates nutrient absorption, curbs the proliferation of harmful bacteria, and enhances growth and metabolism, facilitating nutrient digestion and absorption. They also bolster the animal's defenses against exogenous pathogens[53]. In chickens, the predominant intestinal phyla are *Firmicutes*, succeeded by *Bacteroidetes*, *Actinomycetes*, and *Pseudomonadota*[54]. The thick - walled phylum forms the bulk of the microbial community in the gut environment of many birds[55]. Higher abundance of cecum microorganisms unclassified *Bacteroidalesin* were present in laying hens compared to broilers[56]. Unclassified *Bacteroidalesin*is was strongly associated with gut microbial - butyric acid / lipid metabolism and promotes host gut health[57]. *Bacteroidota* are also important producers of B vitamins[58]. Literature reports that astaxanthin can modulate the gut microbiota[59, 60]. The dietary inclusion of lutein - rich prebiotics significantly elevated bifidobacteria and lactobacilli counts while diminishing populations of opportunistic pathogens such as *Anaplasma* spp. and *Clostridium* spp.[61].

The addition of carotenoids produced by *Hac. paucihalophilus* TRM89021 to the diets of Hotan black chickens revealed that the dominant phylum in the cecum was *Bacteroidota* and *Firmicutes*, and the dominant genus and species were unclassified *Bacteroidales*. This study is in agreement with the microbial dominant phylum of the chicken cecum reported in the literature, suggesting that the microbial composition of the stable flora of the chicken gut is the same. The content of *Bacteroidota* and *Firmicute* as well as the content of dominant genera and dominant species (unclassified *Bacteroidales*) were increased in the CDG compared to the BDG, indicating that carotenoids from *Hac. paucihalophilus* TRM89021 increased the number of beneficial bacteria in the cecum. Surprisingly, *Phocaeicola coprophilus* and *Paraprevotella clara* occur only in the CDG. They are anaerobic, rod, $G^-$ bacteria contribute to the production of short-chain fatty acids in the intestine[62], which can supply energy to the intestinal tract. Although it has also been reported that $G^-$ disorders can also negatively affect the intestinal tract of chickens. *Paraprevotella clara* was potent trypsin-degrading commensals, contribute to the maintenance of intestinal homeostasis and protection from pathogen infection; *P. clara* colonization also inhibits lethal infection by mouse hepatitis virus2[63]. This reflects the positive effect of the addition of carotenoids produced by this strain to the diet on cecum probiotics.

In the present study, there was no significant change on the microbial composition of the cecum at the level of phylum, class, order, family, genus and species ($p>0.05$) between the CDG and BDG, and also no significant change of microbial OTUs in the cecum of laying hens (ace, sobs, Chao, Simpson, Shannon, cover, PCA, PCoA) ($p>0.05$). It showed that the addition of carotenoids had no significant effect on the cecum microbial diversity of Hotan black chickens.

## 5. Conclusions

In this study, we isolated *H. paucihalophilus*TRM89021 from a soil sample from the Pamir Plateau, a strain with carotenoids yield of 20 mg/kg. The incorporation of *Hac. paucihalophilus* TRM89021-derived carotenoids into the diets of Hotan Black Eggs chickens enhanced egg quality through increased laying capacity and elevated GSH-Px and T-AOC levels in egg yolk, as well as improved cecal health by promoting a more abundant beneficial microbiota.

**Supplementary Materials:** Figure S1: Effect of dietary carotenoid supplementation on the bacterial composition of the cecum; Figure S2: ; Figure S3: Phylum, Class, Order, Family, Genus and Species Level Species Composition; Table S1: 16S rRNA gene amplification conditions; Table S2: Nutritional value of the basic feed.

**Author Contributions:** X.D. and G.Z. interpreted the data for the study and drafted the manuscript. H.T., X.C. and B.C. analyzed the data for the study. Y.M. Funding acquisition; writing–review and editing. H.J. was responsible for the design and direction of the experiment. M.R. Funding acquisition; resources; supervision; writing–review and editing. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was supported by the President's Fund of Tarim University (TDZKCX202413), the National Natural Science Foundation of China (31900007), Innovation and Entrepreneurship Training Program for College Students of Tarim University (202410757009).

**Institutional Review Board Statement:** The Chinese guidelines for animal welfare conducted in this study and with the animal welfare standards of College of Life Science and Technology, Tarim University (2024070).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Raw reads of bacterial 16S rDNA gene sequencing are available in the NCBI Sequence Read Archive database (Accession Number: PRJNA1168399). Other data that support the findings of this study were not deposited in an official repository, but they are available from the authors upon request.

**Acknowledgments:** We express our sincere gratitude for the financial support provided by TDZKCX202413, 31900007 and 202410757009. Additionally, we thank Prof. Henglin Cui for providing expertise on halophilic archaea and Hui-E Wang for providing expertise on Hotan black chicken.

**Conflicts of Interest:** No conflict of interest.

## References

1. Stroud, A.; Liddell, S.; Allers, T. Genetic and Biochemical Identification of a Novel Single-Stranded DNA-Binding Complex in *Haloferax volcanii*. *Front Microbiol.* **2012,** *3*, 224.
2. Khallef, S.; Lestini, R.; Myllykallio, H.; Houali, K. Isolation and identification of two extremely *halophilic archaea* from sebkhas in the Algerian Sahara. *Cell Mol Biol (Noisy-le-grand).* **2018,** *64* (4), 83-91.
3. Bao, C. X.; Li, S. Y.; Xin, Y. J.; Hou, J.; Cui, H. L. *Natrinema halophilum* sp. nov., *Natrinema salinisoli* sp. nov., *Natrinema amylolyticum* sp. nov. and *Haloterrigena alkaliphila* sp. nov., four extremely halophilic archaea isolated from salt mine, saline soil and salt lake. *Int J Syst Evol Microbiol.* **2022,** *72* (5),
4. Chen, F.; Xu, Y.; Sun, S.; Shi, X.; Liu, A.; Chen, S. *Halomicroarcula amylolytica* sp. nov., a novel *halophilic archaea* isolated from a salt mine. *Int J Syst Evol Microbiol.* **2020,** *70* (9), 4978-4985.
5. Cui, H. L.; Dyall-Smith, M. L. Cultivation of halophilic archaea (class Halobacteria) from thalassohaline and athalassohaline environments. *Mar Life Sci Technol.* **2021,** *3* (2), 243-251.
6. Zhu, J.; Sun, X.; Tang, Q. Y.; Gu, M. Y.; Zhang, Z. D.; Chen, J. W. The first large scale rDNA amplicon database of soil microbiomes from Pamir Plateau, China. *Sci Data.* **2024,** *11* (1), 856.
7. BU Xuying , R. M., WAN Chuanxing ,ZHANG Lili. Diversity of aerobic cold-adapted bacteria and archaea isolated from the Pamir Plateau. *Acta Microbiologica Sinica.* **2022,** *62* (07), 2568-2581.
8. Rodrigo-Baños, M.; Montero, Z.; Torregrosa-Crespo, J.; Garbayo, I.; Vílchez, C.; Martínez-Espinosa, R. M. Haloarchaea: A Promising Biosource for Carotenoid Production. *Adv Exp Med Biol.* **2021,** *1261*, 165-174.
9. Hou, J.; Cui, H. L. In Vitro Antioxidant, Antihemolytic, and Anticancer Activity of the Carotenoids from Halophilic Archaea. *Curr Microbiol.* **2018,** *75* (3), 266-271.
10. Flores, N.; Hoyos, S.; Venegas, M.; Galetovic, A.; Zuniga, L. M.; Fabrega, F.; Paredes, B.; Salazar-Ardiles, C.; Vilo, C.; Ascaso, C.; Wierzchos, J.; Souza-Egipsy, V.; Araya, J. E.; Batista-Garcia, R. A.; Gomez-Silva, B. Haloterrigena sp. Strain SGH1, a Bacterioruberin-Rich, Perchlorate-Tolerant Halophilic Archaea Isolated From Halite Microbial Communities, Atacama Desert, Chile. *Front Microbiol.* **2020,** *11*, 324.
11. Squillaci, G.; Parrella, R.; Carbone, V.; Minasi, P.; La Cara, F.; Morana, A. Carotenoids from the extreme halophilic archaea Haloterrigena turkmenica: identification and antioxidant activity. *Extremophiles.* **2017,** *21* (5), 933-945.
12. Lazrak, T.; Wolff, G.; Albrecht, A. M.; Nakatani, Y.; Ourisson, G.; Kates, M. Bacterioruberins reinforce reconstituted Halobacterium lipid membranes. *BBA - Biomembranes.* **1988,** *939* (1), 160-162.
13. Shahmohammadi, H. R.; Asgarani, E.; Terato, H.; Saito, T.; Ohyama, Y.; Gekko, K.; Yamamoto, O.; Ide, H. Protective roles of bacterioruberin and intracellular KCl in the resistance of Halobacterium salinarium against DNA-damaging agents. *J Radiat Res.* **1998,** *39* (4), 251-62.
14. Heider, S. A.; Peters-Wendisch, P.; Wendisch, V. F. Carotenoid biosynthesis and overproduction in Corynebacterium glutamicum. *BMC Microbiology.* **2012,** *12*, 198.
15. Torregrosa-Crespo, J.; Montero, Z.; Fuentes, J. L.; Reig Garcia-Galbis, M.; Garbayo, I.; Vilchez, C.; Martinez-Espinosa, R. M. Exploring the Valuable Carotenoids for the Large-Scale Production by Marine Microorganisms. *Mar Drugs.* **2018,** *16* (6),
16. Sabine A E Heider, P. P.-W. a. V. F. W. Carotenoid biosynthesis and overproduction in Corynebacterium glutamicum. *BMC Microbiology.* **2012,** *12*.198.
17. Oren, A. Diversity of halophilic microorganisms: environments, phylogeny, physiology, and applications. *J Ind Microbiol Biotechnol.* **2002,** *28* (1), 56-63.

18. Gao, S.; Li, R.; Heng, N.; Chen, Y.; Wang, L.; Li, Z.; Guo, Y.; Sheng, X.; Wang, X.; Xing, K.; Ni, H.; Qi, X. Effects of dietary supplementation of natural astaxanthin from Haematococcus pluvialis on antioxidant capacity, lipid metabolism, and accumulation in the egg yolk of laying hens. *Poult Sci.* **2020,** *99* (11), 5874-5882.
19. Zhu, Y.; Yin, L.; Ge, J.; Wu, X.; Peng, Y.; Zhang, T.; Jiang, M. Astaxanthin supplementation enriches productive performance, physiological and immunological responses in laying hens. *Anim Biosci.* **2021,** *34* (3), 443-448.
20. Dansou, D. M.; Wang, H.; Nugroho, R. D.; He, W.; Zhao, Q.; Tang, C.; Zhang, H.; Zhang, J. Effects of duration and supplementation dose with astaxanthin on egg fortification. *Poult Sci.* **2021,** *100* (9), 101304.
21. Wu, H.; Wang, S.; Xie, J.; Ji, F.; Peng, W.; Qian, J.; Shen, Q.; Hou, G. Effects of Dietary Lycopene on the Growth Performance, Antioxidant Capacity, Meat Quality, Intestine Histomorphology, and Cecal Microbiota in Broiler Chickens. *Animals (Basel).* **2024,** *14* (2),
22. Liu, H.; Liu, M.; Fu, X.; Zhang, Z.; Zhu, L.; Zheng, X.; Liu, J. Astaxanthin Prevents Alcoholic Fatty Liver Disease by Modulating Mouse Gut Microbiota. *Nutrients.* **2018,** *10* (9), 1298.
23. Li, L.; Krause, L.; Somerset, S. Associations between micronutrient intakes and gut microbiota in a group of adults with cystic fibrosis. *Clin Nutr.* **2017,** *36* (4), 1097-1104.
24. Zhang, L.; Li, H.; Zhao, X.; Wu, Y.; Li, J.; Yao, Y.; Yao, Y.; Wang, L. Whole genome resequencing reveals the adaptability of native chickens to drought, tropical and frigid environments in Xinjiang. *Poult Sci.* **2024,** *103* (9), 103947.
25. Wang, Y.; Hui, X.; Wang, H.; Kurban, T.; Hang, C.; Chen, Y.; Xing, J.; Wang, J. Association of H-FABP gene polymorphisms with intramuscular fat content in Three-yellow chickens and Hetian-black chickens. *J Anim Sci Biotechnol.* **2016,** *7*, 9.
26. Lu, W. Accumulation and Bioactivity of Bacterioruberin inThree Halophilic Archaea Strains. Tianjin University of Science and Technology, 2017.
27. Johnson, E. A.; Lewis, M. J. Astaxanthin Formation by the Yeast Phafa rhodozyma. *Journal of General Microbiology.* **1979,** (115), 11.173.
28. Delgado-Garcia, M.; Gomez-Secundino, O.; Rodriguez, J. A.; Mateos-Diaz, J. C.; Muller-Santos, M.; Aguilar, C. N.; Camacho-Ruiz, R. M. Identification, Antioxidant Capacity, and Matrix Metallopeptidase 9 (MMP-9) In Silico Inhibition of Haloarchaeal Carotenoids from Natronococcus sp. and Halorubrum tebenquichense. *Microorganisms.* **2023,** *11* (9), 2344.
29. Giuffrida, D.; Dugo, P.; Torre, G.; Bignardi, C.; Cavazza, A.; Corradini, C.; Dugo, G. Characterization of 12 Capsicum varieties by evaluation of their carotenoid profile and pungency determination. *Food Chem.* **2013,** *140* (4), 794-802.
30. White, W. L. Erratum to: Why I hate the index finger. *Hand (N Y).* **2011,** *6* (2), 233.
31. Oren, A.; Arahal, D. R.; Ventosa, A. Emended descriptions of genera of the family Halobacteriaceae. *Int J Syst Evol Microbiol.* **2009,** *59* (Pt 3), 637-42.
32. Oren, A. Industrial and environmental applications of halophilic microorganisms. *Environ Technol.* **2010,** *31* (8-9), 825-34.
33. Kelly, M.; Norgard, S.; Liaaen-Jensen, S. Bacterial carotenoids. 31. C50-carotenoids 5. Carotenoids of Halobacterium salinarium, especially bacterioruberin. *Acta Chem Scand.* **1970,** *24* (6), 2169-82.
34. Jones, D. L.; Baxter, B. K. DNA Repair and Photoprotection: Mechanisms of Overcoming Environmental Ultraviolet Radiation Exposure in Halophilic Archaea. *Front Microbiol.* **2017,** *8*, 1882.
35. Tang, S. K.; Zhi, X. Y.; Zhang, Y.; Makarova, K. S.; Liu, B. B.; Zheng, G. S.; Zhang, Z. P.; Zheng, H. J.; Wolf, Y. I.; Zhao, Y. R.; Jiang, S. H.; Chen, X. M.; Li, E. Y.; Zhang, T.; Chen, P. R.; Feng, Y. Z.; Xiang, M. X.; Lin, Z. Q.; Shi, J. H.; Chang, C.; Zhang, X.; Li, R.; Lou, K.; Wang, Y.; Chang, L.; Yin, M.; Yang, L. L.; Gao, H. Y.; Zhang, Z. K.; Tao, T. S.; Guan, T. W.; He, F. C.; Lu, Y. H.; Cui, H. L.; Koonin, E. V.; Zhao, G. P.; Xu, P. Cellular differentiation into hyphae and spores in halophilic archaea. *Nat Commun.* **2023,** *14* (1), 1827.
36. Menasria, T.; Aguilera, M.; Hocine, H.; Benammar, L.; Ayachi, A.; Si Bachir, A.; Dekak, A.; Monteoliva-Sanchez, M. Diversity and bioprospecting of extremely halophilic archaea isolated from Algerian arid and semi-arid wetland ecosystems for halophilic-active hydrolytic enzymes. *Microbiol Res.* **2018,** *207*, 289-298.
37. Liu, B. B.; Tang, S. K.; Zhang, Y. G.; Lu, X. H.; Li, L.; Cheng, J.; Zhang, Y. M.; Zhang, L. L.; Li, W. J. Halalkalicoccus paucihalophilus sp. nov., a halophilic archaea from Lop Nur region in Xinjiang, northwest of China. *Antonie Van Leeuwenhoek.* **2013,** *103* (5), 1007-14.
38. Bardavid, R. E.; Ionescu, D.; Oren, A.; Rainey, F. A.; Hollen, B. J.; Bagaley, D. R.; Small, A. M.; Mckay, C. Selective enrichment, isolation and molecular detection of Salinibacter and related extremely halophilic Bacteria from hypersaline environments. *Hydrobiologia.* **2007,** *576* (1), 3-13.
39. Thombre, R. S.; Shinde, V. D.; Oke, R. S.; Dhar, S. K.; Shouche, Y. S. Biology and survival of extremely halophilic archaea Haloarcula marismortui RR12 isolated from Mumbai salterns, India in response to salinity stress. *Sci Rep.* **2016,** *6*, 25642.

40. Lorantfy, B.; Renkecz, T.; Koch, C.; Horvai, G.; Lendl, B.; Herwig, C. Identification of lipophilic bioproduct portfolio from bioreactor samples of extreme halophilic archaea with HPLC-MS/MS. *Anal Bioanal Chem.* **2014,** *406* (9-10), 2421-32.
41. Sahli, K.; Gomri, M. A.; Esclapez, J.; Gomez-Villegas, P.; Ghennai, O.; Bonete, M. J.; Leon, R.; Kharroub, K. Bioprospecting and characterization of pigmented halophilic archaeal strains from Algerian hypersaline environments with analysis of carotenoids produced by Halorubrum sp. BS2. *J Basic Microbiol.* **2020,** *60* (7), 624-638.
42. Jehlička, J.; Edwards Hg Fau - Oren, A.; Oren, A. Bacterioruberin and salinixanthin carotenoids of extremely halophilic Archaea and Bacteria: a Raman spectroscopic study. *Spectrochim Acta A Mol Biomol Spectrosc.* **2013,** (1873-3557 (Electronic)),
43. Liu, N.; Ji, X.; Song, Z.; Deng, X.; Wang, J. Effect of dietary lutein on the egg production, fertility, and oxidative injury indexes of aged hens. *Anim Biosci.* **2023,** *36* (8), 1221-1227.
44. Miao, Q.; Tang, C.; Yang, Y.; Zhao, Q.; Li, F.; Qin, Y.; Zhang, J. Deposition and bioconversion law of beta-carotene in laying hens after long-term supplementation under adequate vitamin A status in the diet. *Poult Sci.* **2023,** *102* (11), 103046.
45. Honda, M. A.-O.; Kawashima, Y.; Hirasawa, K.; Uemura, T.; Sun, J.; Hayashi, Y. A.-O. Astaxanthin Z-isomer-rich diets enhance egg yolk pigmentation in laying hens compared to that in all-E-isomer-rich diets. *Erratum in: Anim Sci J.* **2021,** *92* (1), e13512.
46. Conradie, T. A.; Pieterse, E.; Jacobs, K. Application of Paracoccus marcusii as a potential feed additive for laying hens. *Poult Sci.* **2018,** *97* (3), 986-994.
47. Hou Jing, L. B., Cui Henglin. Identification and Antioxidant Activity of the C50 Carotenoids Produced by Two Haloarchaeal Strains. *Journal of Chinese Institute of Food Science and Technology.* **2019,** *19* (10), 243-250.
48. Yatsunami, R.; Ando, A.; Yang, Y.; Takaichi, S.; Kohno, M.; Matsumura, Y.; Ikeda, H.; Fukui, T.; Nakasone, K.; Fujita, N.; Sekine, M.; Takashina, T.; Nakamura, S. Identification of carotenoids from the extremely halophilic archaea Haloarcula japonica. *Front Microbiol.* **2014,** *5*, 100.
49. Giergiel, M.; Lopucki, M.; Stachowicz, N.; Kankofer, M. The influence of age and gender on antioxidant enzyme activities in humans and laboratory animals. *Aging Clin Exp Res.* **2012,** *24* (6), 561-9.
50. Shevchenko, L. V.; Iakubchak, O. M.; Davydovych, V. A.; Honchar, V. V.; Ciorga, M.; Hartung, J.; Kołacz, R. Influence of lycopene and astaxanthin in feed on metabolic parameters of laying hens, yolk color of eggs and their content of carotenoids and vitamin A when stored under refrigerated conditions. *Pol J Vet Sci.* **2021,** *24* (4), 525-535.
51. Meluzzi, A.; Primiceri, G.; Giordani, R.; Fabris, G. Determination of blood constituents reference values in broilers. *Poult Sci.* **1992,** *71* (2), 337-45.
52. Rubio-Aliaga, I.; Krapf, R. Phosphate intake, hyperphosphatemia, and kidney function. *Pflugers Arch.* **2022,** *474* (8), 935-947.
53. Cani, P. D.; Depommier, C.; Derrien, M.; Everard, A.; de Vos, W. M. Akkermansia muciniphila: paradigm for next-generation beneficial microorganisms. *Nat Rev Gastroenterol Hepatol.* **2022,** *19* (10), 625-637.
54. Choi, J. H.; Kim, G. B.; Cha, C. J. Spatial heterogeneity and stability of bacterial community in the gastrointestinal tracts of broiler chickens. *Poult Sci.* **2014,** *93* (8), 1942-50.
55. Cao, J.; Hu, Y.; Liu, F.; Wang, Y.; Bi, Y.; Lv, N.; Li, J.; Zhu, B.; Gao, G. F. Metagenomic analysis reveals the microbiome and resistome in migratory birds. *Microbiome.* **2020,** *8* (1), 26.
56. Qi, Z.; Shi, S.; Tu, J.; Li, S. Comparative metagenomic sequencing analysis of cecum microbiotal diversity and function in broilers and layers. *3 Biotech.* **2019,** *9* (8), 316.
57. Sun, Y.; Liu, X.; Wang, R.; Liu, R.; Lv, X.; Ma, Y.; Li, Q. Lacticaseibacillus rhamnosus HF01 fermented yogurt alleviated high-fat diet-induced obesity and hepatic steatosis via the gut microbiota-butyric acid-hepatic lipid metabolism axis. *Food Funct.* **2024,** *15* (8), 4475-4489.
58. Magnusdottir, S.; Ravcheev, D.; de Crecy-Lagard, V.; Thiele, I. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. *Front Genet.* **2015,** *6*, 148.
59. Wang, M.; Ma, H.; Guan, S.; Luo, T.; Zhao, C.; Cai, G.; Zheng, Y.; Jia, X.; Di, J.; Li, R.; Cui, H. Astaxanthin from Haematococcus pluvialis alleviates obesity by modulating lipid metabolism and gut microbiota in mice fed a high-fat diet. *Food Funct.* **2021,** *12* (20), 9719-9738.
60. Chen, Y.; Zhao, S.; Jiao, D.; Yao, B.; Yang, S. A.-O.; Li, P. A.-O.; Long, M. Astaxanthin Alleviates Ochratoxin A-Induced Cecum Injury and Inflammation in Mice by Regulating the Diversity of Cecal Microbiota and TLR4/MyD88/NF-κB Signaling Pathway. *Oxid Med Cell Longev.* **2021,** *2021* (8894491),
61. Molan, A. L.; Liu, Z.; Plimmer, G. Evaluation of the effect of blackcurrant products on gut microbiota and on markers of risk for colon cancer in humans. *Phytother Res.* **2014,** *28* (3), 416-22.
62. Martin-Gallausiaux, C.; Marinelli, L.; Blottiere, H. M.; Larraufie, P.; Lapaque, N. SCFA: mechanisms and functional importance in the gut. *Proc Nutr Soc.* **2021,** *80* (1), 37-49.
63. Li, Y.; Watanabe, E.; Kawashima, Y.; Plichta, D. R.; Wang, Z.; Ujike, M.; Ang, Q. Y.; Wu, R.; Furuichi, M.; Takeshita, K.; Yoshida, K.; Nishiyama, K.; Kearney, S. M.; Suda, W.; Hattori, M.;

 doi:10.20944/preprints202410.2447.v1



Sasajima, S.; Matsunaga, T.; Zhang, X.; Watanabe, K.; Fujishiro, J.; Norman, J. M.; Olle, B.; Matsuyama, S.; Namkoong, H.; Uwamino, Y.; Ishii, M.; Fukunaga, K.; Hasegawa, N.; Ohara, O.; Xavier, R. J.; Atarashi, K.; Honda, K. Identification of trypsin-degrading commensals in the large intestine. *Nature.* **2022,** *609* (7927), 582-589.