Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 247-254, 2023
DOI: 10.9775/kvfd.2023.29134

Research Article

The Effect of *Bacillus subtilis* and Fructooligosaccharide as Antibiotic Substituent on Goose Performance Parameters, Serum Biochemical Indicators and Intestinal Morphology

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Article ID: KVFD-2023-29134 Received: 05.02.2023 Accepted: 02.05.2023 Published Online: 10.05.2023

Abstract: As antibiotics are now prohibited or heavily regulated in farming in most countries, nutritionists and veterinarians are increasingly turning to probiotic supplements, prebiotics, and synbiotics to promote animal health and improve production performance. The study aimed to evaluate the effects of *Bacillus subtilis*, fructooligosaccharides (FOS), and their combined use on geese, compared to antibiotics, and evaluate their suitability as an alternative to antibiotics. 240 14-day-old geese were randomly divided into five groups and were fed a basal diet or supplemented with 100 mg/kg chlortetracycline, 400 mg/kg FOS, 2x10^{5}cfu g/kg *B. subtilis*, or a combination of 200 mg/kg FOS and 1x10^{5}cfu g/kg *B. subtilis* for 56 days. The study measured the body weight, feed consumption, serum parameters, and intestinal morphology of the geese. The results showed that the combination of *B. subtilis* and FOS had a positive impact on the body weight, feed consumption, and serum parameters of the geese, while improving their intestinal morphology. The results suggest that the combination of *B. subtilis* and FOS may provide multiple benefits to animal health and performance and could be used as a suitable alternative to antibiotics.

Keywords: *Bacillus subtilis, Fructooligosaccharide, Goose, Intestinal morphology, Performance parameters, Serum*

Antibiyotik İkamesi Olarak *Bacillus subtilis* ve Fruktooligosakkaritin Kazlarda Performans Parametreleri, Serum Biyokimyasal Göstergeleri ve Bağırsak Morfolojisi Üzerine Etkisi

Öz: Antibiyotikler artık çoğu ülkede çiftçilikte yasaklandığından veya ağır düzenlemelere tabi tutulduğundan, beslenme uzmanları ve veterinerler hayvan sağlığını desteklemek ve üretim performansını artırmak için probiyotik takviyelere, prebiyotiklere ve sinbiyotiklere giderek daha fazla yönelmektedir. Bu çalışmanın amacı, antibiyotiklere kıyasla *Bacillus subtilis*, fruktooligosakkaritler (FOS) ve bunların birlikte kullanımının kazlar üzerindeki etkilerini ve antibiyotiklere alternatif olarak uygunluğunu değerlendirmektir. İkiyüz kırk adet 14 günlük kaz rastgele beş gruba ayrılmış ve 56 gün boyunca bazal diyet veya 100 mg/kg klortetrasiklin, 400 mg/kg FOS, 2x10^{5}cfu g/kg *B. subtilis* ve 200 mg/kg FOS ve 1x10^{5}cfu g/kg *B. subtilis* kombinasyonu ile beslenmiştir. Çalışmada kazların vücut ağırlığı, yem tüketimi, serum parametreleri ve bağırsak morfolojisi ölçülmüştür. Sonuçlar, *B. subtilis* ve FOS kombinasyonunun kazların vücut ağırlığı, yem tüketimi ve serum parametreleri üzerinde olumlu bir etkiye sahip olduğunu ve bağırsak morfolojilerini iyileştirdiğini göstermiştir. Sonuçlar, *B. subtilis* ve FOS kombinasyonunun hayvan sağlığı ve performansına birçok fayda sağlayabileceğini ve antibiyotiklere uygun bir alternatif olarak kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Bacillus subtilis, Bağırsak morfolojisi, Fruktooligosakkarit, Kaz, Performans parametreleri*

How to cite this article?

Li GQ, Wang XZ, Liu Y, Yang YZ, Wang C, Gong SM, He DQ, Wang HY: The effect of *Bacillus subtilis* and fructooligosaccharide as antibiotic substituent on goose performance parameters, serum biochemical indicators and intestinal morphology. *Kafkas Univ Vet Fak Derg,* 29 (3): 247-254, 2023. DOI: 10.9775/kvfd.2023.29134

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Introduction

Antibiotics have a long history of being added to animal feed as growth promoters [1], It is widely used because of its ability to treat diseases infected by microorganisms such as bacteria, chlamydia, and mycoplasma [2]. However, with the continuous understanding of antibiotics, many bacteria have developed resistance to antibiotics [3], and the abuse of antibiotics will remain in animals or products [4,5], which will damage human health [6].

Probiotics widely exist in nature, animals and plants, can secrete a variety of metabolites, have a wide range of beneficial effects on the host [7]. As a typical probiotic, *B. subtilis* can secrete protease and cellulase to promote the digestion and utilization of nutrients [8]. At the same time, *B. subtilis* can produce spores during the dormant period [9], and has tolerance to high temperature and pressure during feed processing and the acidic environment of the animal gastrointestinal tract [10]. FOS have been widely used in animal husbandry as a prebiotic supplement in animal feed [11,12]. FOS are short chains of fructose molecules that act as a substrate for beneficial bacteria in the gut, promoting the growth of probiotics and maintaining gut health [13]. The application of FOS in animal husbandry has been found to be effective in improving gut microbiota balance, increasing feed efficiency, promoting growth, and reducing the incidence of digestive disorders. FOS have been particularly useful in poultry and swine production, where they have been shown to enhance gut health and improve feed utilization [14,15]. Overall, FOS have been a valuable addition to animal nutrition, providing multiple benefits to animal health and performance.

The use of probiotics or prebiotics alone can have limited benefits when compared to combining them [16,17]. There are few studies on *B. subtilis* and FOS in geese [18]. This experiment aims to study the effects of *B. subtilis*, FOS and their combined use on geese, compare its effect with that of antibiotics, and evaluate whether it can be used as a suitable alternative to antibiotics.

Material and Methods

Animal Ethics

Animal experimentation was approved by the Laboratory Animal Ethics Committee of the Shanghai Academy of Agricultural Sciences (SAASPZ0522046).

Material

The *B. subtilis* used in the experiment (the number of viable bacteria ≥1.0x10^{8}cfu/g) was purchased from Shanghai Shenya Animal Health Products Fuyang Co., Ltd, Anhui, China. FOS (content ≥97.15%) was purchased from Dongguan Zhenshang Industrial Co., Ltd, Guangdong, China. Zhedong white geese were purchased from Xiangshan County Zhejiang White Goose Research Institute, Zhejiang, China.

Experimental Design

Total 240 14-day-old Zhedong white geese were randomly divided into five groups, six replicates in each group, each replicate has eight geese (half male and half female). The five groups were basal diet group, 100mg/kg chlortetracycline (CTC) group, 400 mg/kg FOS group, 2x10^{5}cfu g/kg *B. subtilis* group and 200mg/kg FOS+ 1x10^{5}cfu g/kg *B. subtilis* group, the experiment lasts for 56 d [19]. The basal diet refers to the nutritional level recommended by NRC-1994 *(Table 1)* [20]. During the experiment period, Geese in each replicated was housed in an 80 cm x 80 cm cage with access to natural light during the day and low-level artificial light at night. They had free access to food and water, and routine feeding management and immunization procedures were carried out.

Performance Parameters

Body weight (BW) was recorded before the start and end of the experiment (8 h fasting, 2 h water deprivation), then weigh the amount of feed consumed for each repetition, and the average daily gain (ADG), average daily feed intake (ADFI) and feed/gain (F/G) were calculated at last.

Table 1. *Feed ingredients and analyzed chemical composition of geese diets (air-dry basis %)*

Ingredients	Content %	
	1-28 d	28-70 d
Corn	60.30	58.80
Soybean meal (43% CP)	32.60	25.60
Fish meal (60.3% CP)	2.00	10.10
Soybean oil	2.00	1.50
Lys + Met	0.10	0.00
Limestone	0.00	1.00
Premix [a]	3.00	3.00
Total	100	100
Nutritional Level		
ME/(MJ/kg)	12.13	12.55
Crude protein	20.23	16.00
Crude fiber	3.07	7.00
Ca	0.55	0.68
P	0.45	0.43

[a] *Per kilogram of diets including: Vit-A: 1500 IU, Vit-B$_1$: 2.3 mg, Vit-B$_2$: 5.0 mg, Vit-B$_6$: 5 mg, Vit-B$_{12}$: 2 mg, Vit-D$_3$: 200 IU, Vit-E: 12.5 IU, Vit-K: 1.5 mg, Trace elements: 50 g, Garlicin: 30 g, Lysine: 100 g, Methionine: 50 g, Salt: 100 g, Stone powder: 100 g, Myco-Ad: 100 g, Zeolite powder: 420 g;* [b] *Nutrient levels were all calculated values*

***Table 2.** Effects of B. subtilis and FOS on growth performance in geese*

Items	Treatments				
	Basal Diet	**100 mg/kg CTC**	**400 mg/kg FOS**	**2×10^5cfu g/kg *B. subtilis***	**200 mg/kg FOS+1×10^5cfu g/kg *B. subtilis***
Initial BW, g	677.08±14.44	656.47±13.76	659.57±11.23	662.78±13.54	655.93±13.15
Final BW, g	3904.92±103.72^{b}	4111.11±123.98^{a}	3919.33±117.51^{b}	4055.82±85.16^{a}	4076.97±107.88^{a}
ADFI, g/d	304.41±1.31	304.96±1.05	301.87±1.06	309.66±1.22	310.03±1.62
ADG, g/d	57.64±8.59^{b}	61.69±10.26^{a}	58.21±7.93^{ab}	60.59±8.65^{ab}	61.90±7.86^{a}
F/G	5.21±0.56^{a}	4.92±0.86^{b}	5.13±0.53^{ab}	5.07±0.57^{ab}	5.03±0.93^{ab}

*The data in the table are compared in the same row, and different lowercase letters indicate that the difference has reached a significant level (P<0.05); **CTC:** chlortetracycline, **FOS:** Fructo oligosaccharide*

Serum Biochemical Indicators

At the end of the experiment, one male and female geese with close to average body weight were selected for each repetition, and blood was collected from the wing vein to prepare serum and stored at -20°C for later use. All serum were sent to Shanghai Pinyi Biological Co., Ltd. for testing total protein (TP), albumin (ALB), globulin (GLOB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose (GLU), blood urea nitrogen (BUN), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C).

Intestinal Morphology

The geese were slaughtered through exsanguination of the jugular vein.In reference to the methods used by Xue et al.[21], the duodenum, jejunum, and ileum were collected and analyzed. In summary, the contents of their intestines were carefully emptied, rinsed with saline solution, and dried with filter paper. The first quarter of the duodenum, jejunum, and anterior ileum were then cut into 2 cm sections for paraffin embedding. From each sample, five representative intestinal villus crypts were selected, and measurements were taken of the villus height (VH) and crypt depth (CD). The ratio of VH to CD (VH/CD) was calculated.

Statistical Analysis

The data were preliminarily processed using Excel 2019, followed by a one-way ANOVA using SPSS 26.0 software. Multiple comparisons were conducted using Duncan's post hoc test, and the results were presented as mean ± standard deviation. Statistical significance was defined as P<0.05, indicating significant differences.

Results

Performance Parameters

It can be seen from *Table 2* that adding 100 mg/kg CTC to the goose diet was the best for improving the growth performance, compared to the basic diet group, it

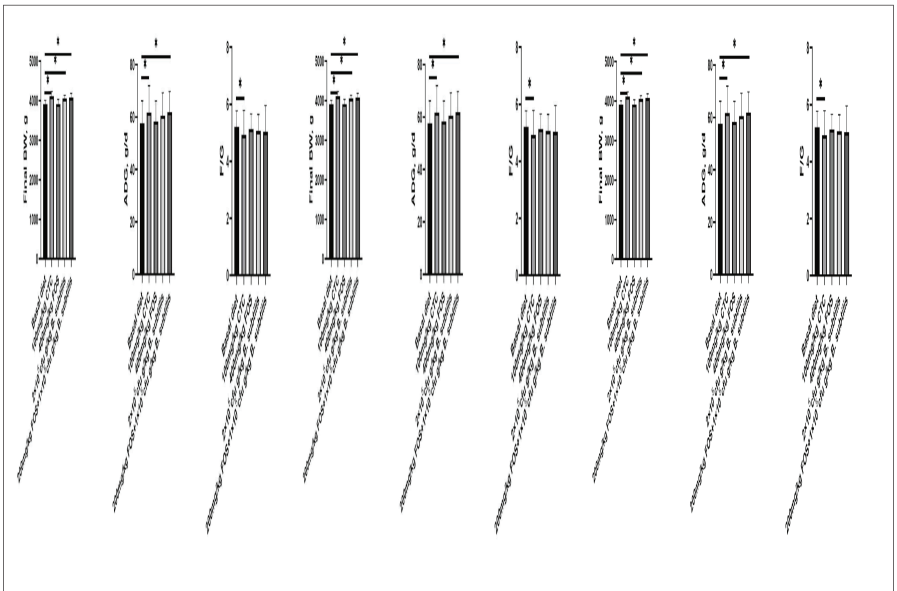

Fig 1. The effect of different treatments on goose performance parameters. **CTC:** chlortetracycline, **FOS:** Fructo oligosaccharide, **BW:** body weight, **ADG:** average daily gain, **F/G:** feed/gain

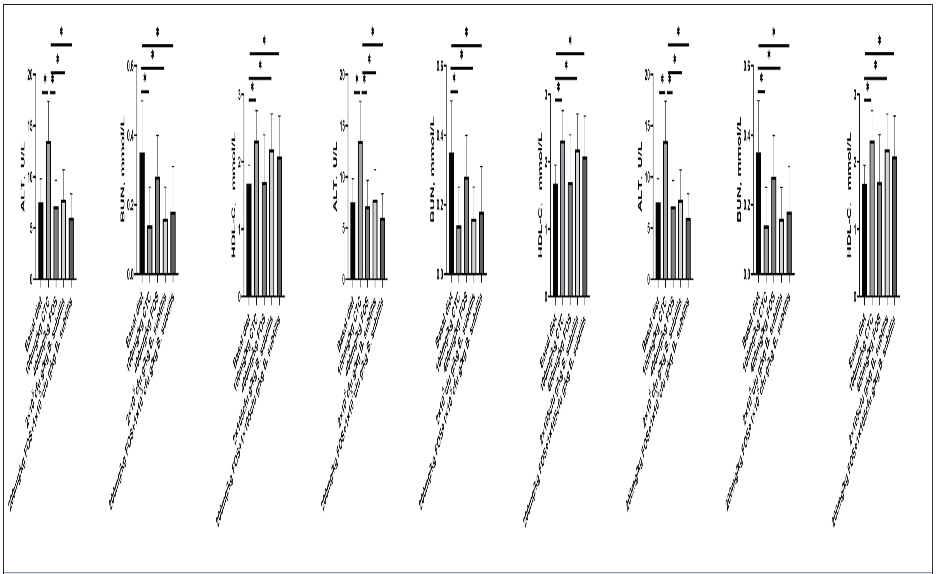

Fig 2. The effect of different treatments on goose serum biochemical indicators. **CTC:** chlortetracycline, **FOS:** Fructo oligosaccharide, **ALT:** alanine aminotransferase, **BUN:** blood urea nitrogen, **HDL-C:** high-density lipoprotein cholesterol

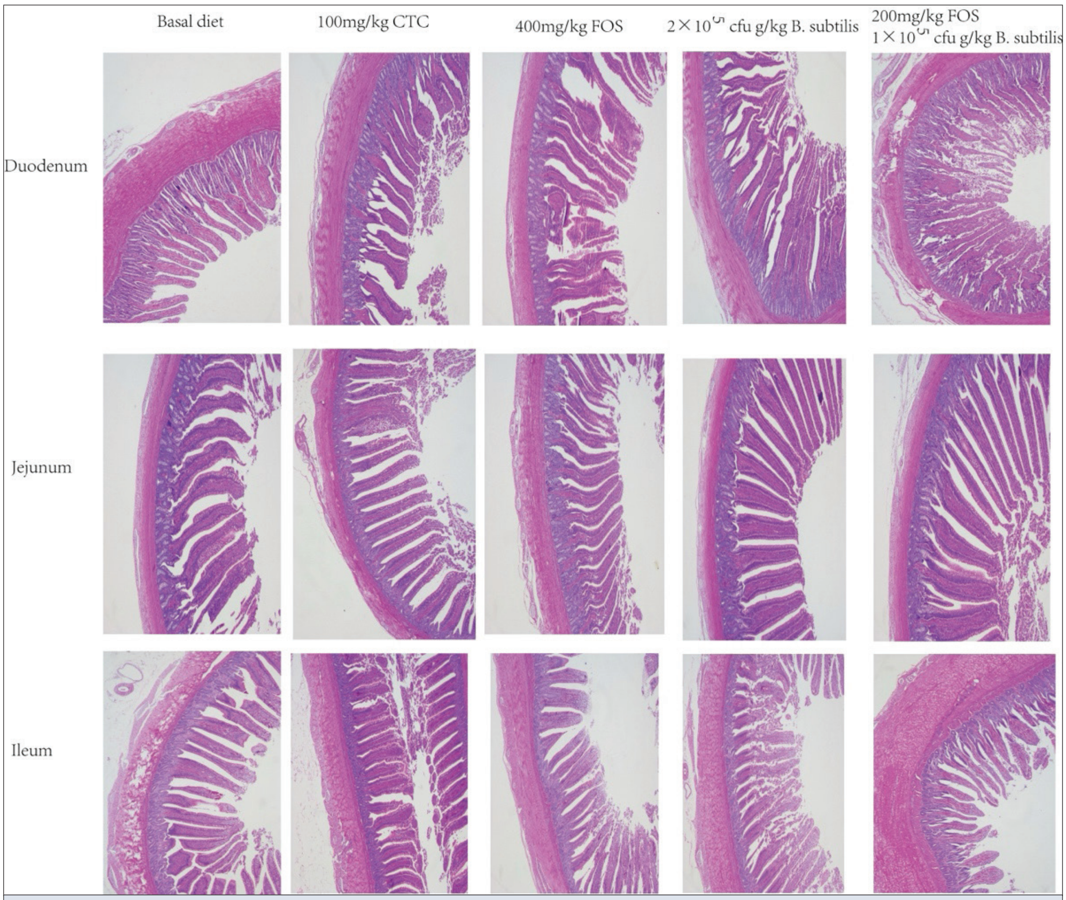

Fig 3. The effect of different treatments on goose intestinal morphology. **CTC:** chlortetracycline, **FOS:** Fructo oligosaccharide

significantly increased the final BW, ADG and F/G ($P<0.05$). The ADG and Final BW of the 200mg/kg FOS+1x10^5cfu g/kg *B. subtilis* group improved significantly compared to the basic diet group ($P<0.05$). Adding 2x10^5cfu g/kg *B. subtilis* can also improve the Final BW ($P<0.05$), but there is no significant difference in other parameters. There was no significant difference between 400 mg/kg FOS and basal diet group. Significant differences in performance parameters between groups are shown in *Fig. 1*.

***Table 3.** Effects of B. subtilis and FOS on serum biochemical indicators in geese*

Items	Treatments				
	Basal Diet	100 mg/kg CTC	400 mg/kg FOS	2×10⁵cfu g/kg *B. subtilis*	200 mg/kg FOS+1×10⁵cfu g/kg *B. subtilis*
TP, g/L	31.43±6.48	39.89±3.42	32.90±8.77	37.06±8.83	39.02±7.00
ALB, g/L	12.06±1.97	15.19±1.13	13.74±2.86	14.56±2.67	14.68±2.34
GLOB, g/L	19.36±4.61	24.70±2.50	19.16±5.96	23.50±6.24	24.34±4.67
ALT, U/L	7.50±2.33^{b}	13.50±3.89^{a}	7.13±2.53^{b}	7.75±2.96^{b}	6.00±2.35^{b}
AST, U/L	23.25±10.28	21.63±4.47	19.88±7.36	21.13±7.16	20.2±5.12
BUN, mmol/L	0.35±0.15^{a}	0.14±0.11^{b}	0.28±0.12^{ab}	0.16±0.09^{b}	0.18±0.13^{b}
GLU, mmol/L	6.10±1.45	9.46±1.25	5.50±1.70	6.70±1.26	5.04±2.25
TC, mmol/L	2.74±0.63	3.39±0.69	2.89±1.14	3.75±1.22	2.83±1.08
HDL-C, mmol/L	1.67±0.28^{b}	2.32±0.44^{a}	1.70±0.70^{b}	2.18±0.53^{a}	2.08±0.60^{a}
LDL-C, mmol/L	0.98±0.26	0.78±0.17	1.00±0.25	0.76±0.29	0.88±0.50

*The data in the table are compared in the same row, and different lowercase letters indicate that the difference has reached a significant level (P<0.05); **CTC:** chlortetracycline, **FOS:** Fructo oligosac charide, **TP:** total protein, **ALB:** albumin, **GLOB:** globulin, **ALT:** alanine aminotransferase, **AST:** aspartate aminotransferase, **GLU:** glucose, **BUN:** blood urea nitrogen, **TC:** total cholesterol, **HDL-C:** high-density lipoprotein cholesterol, **LDL-C:** low-density lipoprotein cholesterol*

***Table 4.** Effects of B. subtilis and FOS on intestinal morphology in geese*

Intestine	Items	Treatments				
		Basal Diet	100 mg/kg CTC	400 mg/kg FOS	2×10⁵cfu g/kg *B. subtilis*	200 mg/kg FOS+1×10⁵cfu g/kg *B. subtilis*
Duodenum	VH, um	504.54±134.18	511.64±66.11	516.74±82.99	504.02±37.84	501.24±66.70
	CD, um	124.03±16.89^{a}	107.27±37.72^{b}	115.86±23.58^{ab}	109.85±17.27^{ab}	111.30±40.63^{ab}
	VH/CD	4.16±0.94^{b}	4.54±1.82^{a}	4.25±1.07^{ab}	4.38±1.20^{ab}	4.30±2.95^{ab}
Jejunum	VH, um	504.98±50.81	506.92±55.92	507.72±51.80	511.14±54.23	506.89±68.33
	CD, um	100.73±21.13^{a}	92.21±22.21^{b}	96.34±30.43^{ab}	95.24±21.78^{ab}	93.18±21.67^{b}
	VH/CD	4.80±1.59^{b}	5.29±0.90^{a}	5.00±1.79^{ab}	5.13±1.28^{ab}	5.23±1.43^{a}
Ileum	VH, um	518.49±36.85	534.50±62.81	531.09±75.33	537.14±97.02	540.45±49.71
	CD, um	129.72±21.91	135.84±42.42	135.49±17.47	134.77±14.53	136.16±18.01
	VH/CD	4.10±0.66	4.15±1.46	4.12±0.98	4.14±1.23	4.15±0.77

*The data in the table are compared in the same row, and different lowercase letters indicate that the difference has reached a significant level (P<0.05); **CTC:** chlortetracycline, **FOS:** Fructo oligosaccharide,**VH:** villus height, **CD:** crypt depth*

Serum Biochemical Indicators

As shown in *Table 3*, There was no significant difference in serum TP, ALB, GLOB, AST, GLU, TC and LDL-C levels among the groups. The ALT level in 100 mg/kg CTC group was significantly higher than in other groups (P<0.05). Adding 100 mg/kg CTC, 2x10^{5}cfu g/kg *B. subtilis* and 200 mg/kg FOS+1x10^{5}cfu g/kg *B. subtilis* can improve the BUN and HDL-C (P<0.05). Significant differences in serum biochemical indicators between groups are shown in *Fig. 2*.

Intestinal Morphology

The intestinal morphology is shown in *Fig. 3*. It can be seen from *Table 4* that in duodenum, the CD of the CTC group was lower than that of the BD group (P<0.05), and the VH/CD were higher (P<0.05), but there is no difference between VH/CD of 100 mg/kg CTC, 2x10^{5}cfu g/kg *B. subtilis* and 200 mg/kg FOS+1x10^{5}cfu g/kg *B. subtilis* groups. In jejunum, the CD of the 100 mg/kg CTC and 200 mg/kg FOS+1x10^{5}cfu g/kg *B. subtilis* groups was lower than that of the BD group (P<0.05), and the VH/CD were higher (P<0.05). There is no difference between VH, CD and VH/CD among the groups in ileum and there is no difference in the VH of each intestinal tract.

Discussion

Measuring animal growth performance is important because it provides information on the efficiency of feed utilization and overall health of the animal [22]. The ADG and F/G are critical metrics for assessing the growth performance of animals, a high ADG is indicative of strong digestive and absorption capabilities, while a low F/G signifies efficient feed conversion. The better an animal's ability to transform feed into weight gain, the more successful it will be in its growth performance [23]. Antibiotics have a direct impact on the gut microbiome by reducing the population of harmful bacteria and promoting the growth of beneficial bacteria [24]. This can improve gut health and enhance the absorption of nutrients from feed, leading to increased weight gain [25]. Alternatively, using probiotics, prebiotics, and synbiotics can achieve similar results without leaving any residue of antibiotics [26,27]. FOS is non-digestible compounds that stimulate the growth of beneficial bacteria in the gut [28]. Many studies have shown that FOS can improve the growth performance of animals [29]. However, an excessive amount of FOS can also put a strain on the intestines. As a result, FOS is typically used in conjunction with probiotics in animals [30,31]. *B. subtilis* is known for its ability to improve digestive health, increase nutrient absorption, and boost the immune system in animals. This bacterium also has antimicrobial properties, which can help reduce the risk of infection. In studies, the application of *B. subtilis* in livestock has been shown to improve feed efficiency, increase weight gain, and enhance overall performance in chickens, pigs, and ruminants [32-34]. In this experiment, adding 0.2% *B. subtilis* or 0.1% *B. subtilis* + 0.2% FOS increased the final body weight and adding 0.1% *B. subtilis*+0.2% FOS average daily gain, so we think it improved the growth performance of geese.

A higher villus height or shallower crypt depth can contribute to higher digestibility [35,36]. At the same time a higher VH/CD ratio is generally associated with a more efficient small intestine, as there is a greater surface area for nutrient absorption [37]. Conversely, a lower VH/CD ratio is associated with a less efficient small intestine and can be a sign of damage to the intestinal lining [38]. In the experiment, no significant differences were observed in the Ileum indicators among the groups. This is due to the fact that the Ileum is the final segment of the small intestine, where nutrient absorption capacity is limited. Antibiotics can reduce the proliferation of harmful bacteria and promote the differentiation of intestinal epithelial cells, leading to a decrease in the crypt depth and an increase in VH/CD [39]. In our experiment, we observed similar outcomes, however, it is noteworthy that the simultaneous addition of FOS and *B. subtilis* also yielded comparable results, particularly in the jejunum, which has the highest digestion capacity. FOS have been found to enhance the synthesis of polyamines and play a positive role in regulating the growth and development of the small intestine and colonic mucosa. Studies have shown that adding 0.25% FOS to pig feed can increase the villus height and the VH/CD in the proximal small intestine [40]. Additionally, adding 0.4% or 0.6% FOS to pig feed resulted in an increase in villus height and the VH/CD in the jejunum [41]. A study by Howard et al.[42] found that feeding neonatal piglets with FOS led to an increase in the density of cecal epithelial cells and the depth of mucosal crypts in the proximal and distal epithelial cells of the colon. This was attributed to the short-chain fatty acids produced by bacteria metabolizing FOS, which provide energy for intestinal cell proliferation, thus effectively promoting intestinal development. *B. subtilis* has been found to reduce the proliferation of harmful bacteria in the gut, which is likely a contributing factor to the decrease in crypt depth. Several studies have demonstrated that the presence of *B. subtilis* can positively impact the morphological structure of the intestine [43,44]. In this experiment, the combination of *B. subtilis* and FOS was found to have an improved effect on the intestinal morphology of geese.

Serum ALB, GLOB, and TP are important indicators of overall health and nutrition [45]. Despite the absence of significant differences between the groups, we observed an improvement compared with basal diet group. ALT, and AST are commonly used as markers for liver and heart damage or disease [46,47]. Antibiotic-induced liver injury is a known adverse effect of certain antibiotics, and can cause an increase in ALT levels [48]. This increase can be a sign of liver damage, and may be seen in animals receiving certain antibiotics, such as tetracyclines, aminoglycosides, and sulfonamides [49]. The findings of this experiment align with our expectations. The highest levels of both ALT and AST were observed in the group treated with 0.1% CTC. It is worth noting that the level of ALT in the 0.1% CTC group was significantly higher than those seen in any of the other groups. HDL-C, a type of cholesterol commonly referred to as "good" cholesterol. Some antibiotics, such as statins, can lower serum HDL-C level [50], while others may have no significant effect. While the 0.1% CTC group showed elevated levels of HDL-C, it is important to note that this did not diminish the observed liver damage in this group. BUN is a non-protein nitrogenous compound in the blood and is one of the primary products of protein metabolism in the body. Impaired renal function and excessive protein intake can lead to an elevated level of BUN in the blood [51,52]. In this experiment, we found that both CTC and *B. subtilis* can reduce the level of BUN, which indicates that there is more protein or amino acids degraded into urea in the basal diet group. Both antibiotics and probiotics can reduce the abundance of harmful bacteria, improve intestinal villi, and enhance the ability to absorb protein [19]. Therefore, we believe that adding CTC and *B. subtilis* can both improve the absorption level of protein in geese, and adding 0.1% B. subtilis + 0.2% FOS and 0.1% CTC has a similar effect.

In summary, adding 100 mg/kg *B. subtilis* and 2x10^{5}cfu g/kg FOS to the diet as an alternative to antibiotics can significantly improve the growth performance of geese in 14-70 d, improve the intestinal morphology of the jejunum, and prevent liver damage associated with antibiotics.

Availability of Data and Materials

The full dataset and supporting materials are provided within the study for reference. If additional information is desired, please direct inquiries to the corresponding author (H-Y Wang).

Acknowledgements

We would like to express our heartfelt gratitude to the staff at the Zhuanghang Experimental Station, Shanghai, China for their invaluable support and assistance in carrying out this research.

Ethical Approval

Animal experimentation was approved by the Laboratory Animal Ethics Committee of the Shanghai Academy of Agricultural Sciences (SAASPZ0522046).

Funding Support

This study was generously supported by China Agriculture Research System (CARS-42-35), the Climbing Plan of Shanghai Academy of Agricultural Sciences (PG21171), and the Excellent Research Team Program of SAAS (2022-021).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

The conception and design of the experiment were contributed by H-Y Wang and D-Q He. G-Q Li and X-Z Wang conducted the data analysis and drafted the manuscript. Yi L, Y-Z Yang, S-M Gong and C Wang were instrumental in data collection. All authors reviewed and approved the final manuscript for publication.

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