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Oil Cakes of Essential Oil Plants as a Source of Prebiotics for Poultry Production

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Abstract: The oil cakes of essential oil plants were screened for prebiotic properties for further development of prebiotic feeds for livestock animals based on these essential oil plants' extracts. For screening, a microbiota model of the chicken cecum, which was created on the basis of an artificial intestinal medium, was used. This model renders it possible to simulate conditions close to intestinal ones. Oligofructose, inulin, β-glucan, psyllium seeds, and apple fiber at a concentration of 0.5% were used as substances with known prebiotic properties. The oil cake concentration was 0.5%, 1%, 2%, and 4%. The direct antimicrobial effect of the cakes on intestinal bacteria was also investigated. The ability of the cakes to stimulate a decrease in the pH level by *Lactobacillus* was studied under conditions close to intestinal ones. It was shown that the cakes of big seed false flax (*Camelina sativa*), brown mustard (*Brassica juncea*), and spicate lavender (*Lavandula angustifolia*) exhibit prebiotic properties in relation to the microbiome of chickens in model experiments. They enhance the acid-forming properties of lactic acid bacteria, thereby lowering the pH of the medium. This leads to a decrease in the number of *Enterococcus*, *Escherichia coli*, and lactose-positive bacteria, as well as a complete suppression of *Proteus*. The optimal oil cake concentrations are 1% and 2%. These oil cakes are promising sources of prebiotics for the development of prebiotic feed for agriculture.

Keywords: prebiotics; chicken; lactobacillus; essential oil plants; artificial intestine model

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1. Introduction

The benefits of prebiotics have been repeatedly shown for both humans [1,2] and livestock animals [3,4]. Prebiotics cause a change in the structure of the microbiome, which in turn leads to an increase in the production of butyrate and acetate [1], causes a decrease in the number of pathogens [5], and promotes modulation of the immune system [6], in addition to affecting mineral metabolism [7] and the nervous system [8]. Thus, prebiotics are of great interest for use in feed for livestock animals.

However, prebiotics available on the market are expensive, and their use greatly increases the price of the feed and, hence, the price of the finished product. Therefore, an important task is to find inexpensive prebiotics suitable for mass use in feed for livestock animals.

Significant results in solving this issue have been achieved by several groups of scientists from Malaysia [9–11] working with palm kernel cake. They showed that palm kernel cake contains polysaccharides and oligosaccharides, especially mannose and galactose [9]. Thus, palm kernel cake when added to rat feed stimulates the acid formation of *Lactobacillus* and lowers the pH of the medium [9,10], reduces the adhesion of intestinal pathogens [11],

improves the functioning of the immune system [10]. In this way, palm kernel cake from industrial by-products becomes a useful component of animal feed.

The content of prebiotic polysaccharides has also been shown in coconut kernel cake [12] and valerian oil cake (*Valeriana officinalis*) [13]. Other studies have shown that linseed cake increases the production of short-chain fatty acids [14].

Given the positive experience of palm kernel cake researchers, the authors consider it promising to search for similar prebiotic components growing in our country.

We took as potential prebiotic plants the following essential oil crops grown in the Crimea, as well as the products of their processing: the oil cake of big seed false flax (*C. sativa*), the oil cake of black cumin (*Nigella sativa*), the oil cake of brown mustard (*B. juncea*), the oil cake of spicate lavender (*L. angustifolia*), the oil cake and whole plant of blessed milk thistle (*Silybum marianum*), the whole plant of lesser calamint (*Calamintha nepeta*), and the oil cake and whole plant of winter savory (*Satureja montana*).

In this study, we used a chicken artificial intestinal environment developed by us [15], which allowed us to obtain a model of the microbiota of the chicken cecum and screen a large number of potential prebiotics. To confirm the accuracy of the model, we previously tested some well-known prebiotics on it. This approach also allowed us to compare the effect of prebiotics and prebiotic plant oil cakes on the microbiota of chickens.

The aim of this study was to find plants with a prebiotic effect that can be used as a cheap prebiotic supplement in the feed of livestock animals.

2. Materials and Methods

2.1. Used Prebiotics and Oil Cakes

The following substances were used as prebiotics with a proven effect: inulin (Fibruline®Instant by the Cosucra Groupe, Warcoing, Belgium); oligofructose (Oligofructose by the Cosucra Groupe); oat β-glucan (Promoat, Tate & Lyle, London, UK); husk fiber and psyllium seeds (Rettenmaier Rus Supercel P95, Rettenmaier & Soehne, Rosenberg, Germany); apple fiber (Rettenmaier Rus Supercel AF 401-30, Rettenmaier & Soehne). Manufacturers claim 100% concentration of these substances.

Essential oil plants were selected based on the high content of biologically active substances, which means that there is a high probability of finding representatives with prebiotic properties among them. These oil cakes were kindly provided by the Research Institute of Agriculture of the Crimea, Simferopol, Russia, and represent by-products from essential oil production. In addition, some plants were also used whole:

- oil cake of big seed false flax (*C. sativa*)
- oil cake of black cumin (*N. sativa*)
- oil cake of brown mustard (*B. juncea*)
- oil cake of spicate lavender (*L. angustifolia*)
- oil cake and whole plant of blessed milk thistle (*S. marianum*)
- the whole plant of lesser calamint (*C. nepeta*)
- oil cake and whole plant of winter savory (*S. montana*)

2.2. Artificial Intestinal Medium

The development of an artificial intestinal medium is described in Ref. [16]. Here, we should mention that during the development of this intestinal medium, data on the composition of the native chyme of the cecum of broiler hens fed with industrial feedstuff were used. Based on these data, an artificial intestinal environment was developed from the components included in the diet of the birds. The starter from which the microbiota subsequently developed was obtained directly from the chyme of the cecum of birds. Thus, the entire spectrum of avian intestinal bacteria, including non-cultivated ones, was initially introduced into the chicken artificial intestinal medium in appropriate proportions.

Briefly, to create a nutrient medium that simulates the composition of the intestines of a chicken, cecal chyme was sterilely collected from 20 healthy 42-day-old broilers that received compound feed, placed in a sterile container, and thoroughly mixed with a sterile

glass rod. After that, the chyme was transferred to the laboratory at the Department of Equipment and Technologies of Food Production, Agribusiness Faculty, DSTU, Rostov-on-Don, Russia, where the chyme was analyzed for the content of proteins, fats, and carbohydrates. After receiving the results, an artificial intestinal environment was formed from the substances that constitute the feed. The composition of the medium per 1 L: soy isolate—20 g; water-soluble starch—0.5 g; unrefined sunflower oil—30 mL; Tween 80—1.5 mL; $MgSO_4$—0.5 g; NaCl—5 g; K_2HPO_4—0.5 g; $MnSO_4$—0.05 g; $FeSO_4$—0.05 g.

This model has its limitations. For example, the absence of the intestinal wall causes the absence of signals from the microbiota from the host organism, as well as a lack of mucin and other substances produced by the cells of the intestinal mucosa. However, soy protein, which is part of the medium, forms a suspension of flakes on which a biofilm can develop with corresponding changes in bacterial metabolism. This is a rough imitation of the parietal microbiota, which is also in the biofilm state [17].

2.3. Effect of Prebiotics on Microbial Composition

In total, 100 mL of liquid artificial intestinal medium was poured into sterile 150 mL flasks to ensure sufficient liquid column height and minimal contact with oxygen. In each flask, except for the control one, a prebiotic was added in the amount of 0.5 g (0.5%).

The starter consisted of the contents of the cecum of 20 healthy 42-day-old broilers (2 g per bird) that did not receive pro- and prebiotics or antibiotics. The starter was thoroughly mixed to reduce individual variability, divided into aliquots of about 1 cm^3, and frozen at −80 °C. The contents of one aliquot were diluted with 10 mL of normal saline. Then, 1 Ml of the suspension was added to flasks with artificial intestinal medium. Subsequently, the flasks were incubated at 42 °C (chicken body temperature [18]) for 3 days, and the number of developed micro-organisms was determined on the 3rd day.

A series of successive decimal dilutions was prepared from the resulting suspension. The determination of the number of intestinal bacteria was carried out via the method of surface inoculation at the amount of 3 replications for each nutrient medium for each studied dilution. The study of the number of *Bifidobacterium* was carried out via the method of sowing a suspension in a semi-liquid nutrient medium [Green]. To determine the number of lactic acid bacteria (LAB), MRS medium (LenReaktiv) was used. Group-specific selective media from HiMedia were used for *Bifidobacterium*, *Enterococcus*, *E. coli*, *Proteus*. For lactose-positive (*Citrobacter*, *Enterobacter*) bacteria, Endo medium (HiMedia, Maharashtra, India) was used. Micro-organisms were counted on the 2nd day [19].

2.4. Influence of Essential Oil Plants on the Microbial Composition

The study was carried out according to the same scheme as presented above. Instead of prebiotics, we used pre-weighed oil cakes and dried whole plants listed above in an amount of 0.5 g (0.5%), 1 g (1%), 2 g (2%), 4 g (4%). The number of microorganisms was examined on the 1st and 3rd days. The acidity of the medium was measured at the end of incubation with an ST2100-F pH meter, Ohaus, Parsippany, NJ, USA.

2.5. Antimicrobial Action of Oil Cakes

Chyme from the cecum of the chicken used for starters was thawed and used to produce a series of serial dilutions. *E. coli* was isolated using Endo medium (HiMedia). For *Enterococcus* sp., a medium for the isolation of *Enterococcus* (HiMedia) was used. All media were prepared according to the manufacturer's instructions. Three strains of each species were collected with an inoculating loop and transferred to LB (Luria–Bertani media). The strains were purified according to the Drygalsky method [19].

The oil cake of big seed false flax, brown mustard and spicate lavender was added to liquid LB before autoclaving, each at concentrations of 1%, 2%, and 4%. Nothing was added to the control medium. After sterilization, the medium was poured into sterile test tubes in 10 mL increments, and the strains described above were inoculated. The samples were incubated for 1 day at 42 °C. Due to the opacity of the media with cakes, the

number of microorganisms was determined via inoculation with decimal dilutions on solid nutrient media [19].

2.6. Influence of Oil Cakes on the Acid-Forming Properties

We used two strains of *Lactobacillus* probiotic isolated by us from the intestines of chickens in previous studies [16], namely *Ligilactobacillus salivarius* KL61 and *Limosilactobacillus frumenti* KL31.

Liquid media MRS (LenReaktiv, St. Petersburg, Russia) and an artificial intestinal medium were prepared; the oil cakes of big seed false flax, brown mustard, and spicate lavender were added before autoclaving, each at concentrations of 1% and 2%. Nothing was added to the controls. After autoclaving, the media were poured in 40 mL doses into sterile 50 mL flasks and inoculated with daily cultures of *Ligilactobacillus salivarius* KL61 and *Limosilactobacillus frumenti* KL31. Cultures were incubated in MRS for 1 day and in artificial intestinal medium for 3 days at 42 °C. The acidity of the medium was measured at the beginning and at the end of the incubation period with an ST2100-F pH meter (Ohaus). The experiment was carried out in three repetitions.

2.7. Determining the Amount of Simple Sugars

To determine the content of simple sugars in the oil cakes of big seed false flax, brown mustard and spicate lavender were transferred to the laboratory at the Department of Equipment and Technologies of Food Production, Agribusiness Faculty, DSTU, Rostov-on-Don, Russia. Determination was performed according to Bertrand's method [20].

2.8. Ethical Statement

The study was approved by the ethics committee of the Don State Technical University, Rostov-on-Don, Russia (protocol number 67-43-2).

2.9. Statistical Processing of Data

Statistical analysis of the data was carried out using R version 3.6.1. Statistically significant differences from control groups were assessed using the two-tailed Student's *t*-test, assuming unequal variances. The data were first tested for normal distribution using the Shapiro–Wilk test. For normally distributed data, the *t*-test was applied. To determine whether there was a significant difference in the means of three or more groups, we used one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. All tests were performed at a significance level of 0.05, and the results are reported as means ± standard deviation.

3. Results

3.1. Modeling of Changes in the Number of Micro-Organisms under the Influence of Prebiotics

Before the study of oil cakes using an artificial intestinal media, preliminary studies were carried out on substances with verified prebiotic properties [1–8].

The results that were obtained by us are presented in Table 1.

As can be seen from the data in Table 1, the addition of inulin to the chicken artificial intestinal medium results in an increase in the number of *Bifidobacterium*. β-glucan stimulated the growth of the number of lactic acid bacteria, and at the same time, it reduced the number of *Bifidobacterium*, *Enterococcus*, *E. coli*, and other lactose-positive bacteria. The introduction of oligofructose into chicken artificial intestinal medium caused an increase in the number of *Bifidobacterium* and lactic acid bacteria and a decrease in the number of *Enterococcus*, *E. coli*, and other lactose-positive bacteria.

Apple fibers did not have a significant effect on lactic acid and *Bifidobacterium* but reduced the number of *Enterococcus*. Psyllium fibers did not have any significant effect on the microbial composition of the cecum model.

Table 1. Effect of various prebiotics on the number of intestinal micro-organisms in chickens, CFU/mL.

Prebiotic	LAB	*Bifidobacterium*	*Enterococcus*	*E. coli*	Lactose +
Control	$1.8 \pm 0.4 \times 10^8$	10^8	$2.3 \pm 0.4 \times 10^7$	$1.6 \pm 0.4 \times 10^8$	$5.4 \pm 0.6 \times 10^7$
Inulin	$3.5 \pm 0.3 \times 10^8$	10^9 *	$7.0 \pm 0.3 \times 10^7$	$1.1 \pm 0.3 \times 10^8$	$10.0 \pm 0.3 \times 10^7$
β-glucan	$4.7 \pm 0.2 \times 10^9$ *	10^7 *	$1.5 \pm 0.4 \times 10^6$ *	$1.9 \pm 0.4 \times 10^6$ *	$6.5 \pm 1.3 \times 10^6$ *
Oligofructose	$2.7 \pm 0.3 \times 10^9$ *	10^9 *	$8.5 \pm 0.4 \times 10^6$ *	$0.6 \pm 0.2 \times 10^6$ *	$3.9 \pm 0.9 \times 10^6$ *
Apple fibers	$1.9 \pm 0.4 \times 10^8$	10^8	$8.0 \pm 0.1 \times 10^6$ *	$0.4 \pm 0.2 \times 10^8$	$4.3 \pm 0.6 \times 10^7$
Psyllium fibers	$1.1 \pm 0.3 \times 10^8$	10^8	$3.2 \pm 0.5 \times 10^7$	$1.2 \pm 0.3 \times 10^8$	$1.8 \pm 0.5 \times 10^7$

* $p < 0.05$.

3.2. Modeling of Changes in the Number of Micro-Organisms under the Influence of Essential Oil Plants

In the process of testing in the model of the artificial intestinal medium of chickens, the studied plants showed themselves in different ways. Thus, the oil cake of black cumin, the cake and whole plant of blessed milk thistle, the whole plant of lesser calamint, and the cake and whole plant of winter savory did not have a significant effect on the microbial composition of the model and, therefore, did not have any prebiotic or antimicrobial activity. On the other hand, big seed false flax, brown mustard, and spicate lavender oil cake had an impact on the number of micro-organisms in the microbiota of chickens.

Due to a large amount of data, in order not to overload the article with unnecessary tables, data will be presented here only for oil cakes that had a significant impact ($p < 0.05$) on the microbiota of birds (Table 2). The *Proteus* sp., which is isolated separately in the table, was initially present in the cecal chyme of chickens from which the starter was prepared.

Table 2. Influence of cakes of essential oil plants on the number of intestinal micro-organisms of chickens, CFU/mL.

Micro-Organisms	Control	0.5%	1%	2%	4%
Oil Cake of Big Seed False Flax					
24 h					
LAB	$3.2 \pm 0.3 \times 10^8$	$2.3 \pm 0.2 \times 10^8$	$2.4 \pm 0.4 \times 10^8$	$3.2 \pm 0.3 \times 10^8$	$3.3 \pm 0.3 \times 10^8$
Bifidobacterium	1×10^7	1×10^7	1×10^7	1×10^7	1×10^7
Enterococcus	$6.6 \pm 0.3 \times 10^7$	$5.6 \pm 0.2 \times 10^7$	$6.2 \pm 0.3 \times 10^7$	$4.6 \pm 0.1 \times 10^6$ *	$5.8 \pm 0.3 \times 10^6$ *
E. coli	$2.4 \pm 0.2 \times 10^7$	$1.5 \pm 0.1 \times 10^7$	$1.8 \pm 0.2 \times 10^7$	$4.8 \pm 0.3 \times 10^7$	$1.2 \pm 0.2 \times 10^7$
lactose +	$6.1 \pm 0.2 \times 10^6$	$5.3 \pm 0.3 \times 10^6$	$5.8 \pm 0.3 \times 10^6$	$2.5 \pm 0.2 \times 10^6$	$7.9 \pm 0.2 \times 10^6$
Proteus	$3.1 \pm 0.3 \times 10^7$	$1.1 \pm 0.2 \times 10^7$	$1.3 \pm 0.2 \times 10^7$	- *	- *
72 h					
LAB	$3.1 \pm 0.2 \times 10^8$	$4.7 \pm 0.3 \times 10^8$	$2.8 \pm 0.2 \times 10^8$	$2.7 \pm 0.2 \times 10^8$	$9.7 \pm 0.3 \times 10^8$
Bifidobacterium	1×10^7	1×10^7	1×10^7	1×10^7	1×107
Enterococcus	$4.3 \pm 0.2 \times 10^7$	$2.0 \pm 0.2 \times 10^7$	$9.0 \pm 0.3 \times 10^6$ *	$2.7 \pm 0.3 \times 10^6$ *	$7.9 \pm 0.2 \times 10^5$ *
E. coli	$1.0 \pm 0.3 \times 10^7$	$2.7 \pm 0.3 \times 10^7$	$3.9 \pm 0.3 \times 10^7$	$2.2 \pm 0.2 \times 10^6$ *	$7.1 \pm 0.3 \times 10^5$ *
lactose +	$6.1 \pm 0.4 \times 10^6$	$9.4 \pm 0.5 \times 10^6$	$3.1 \pm 0.2 \times 10^6$	$8.8 \pm 0.4 \times 10^5$ *	$9.4 \pm 0.2 \times 10^5$ *
Proteus	$2.6 \pm 0.3 \times 10^7$	$2.6 \pm 0.2 \times 10^7$	$8.2 \pm 0.3 \times 10^6$ *	- *	- *
pH	6.98	7.36	6.23	5.11	4.62

Table 2. *Cont.*

Micro-Organisms	Control	0.5%	1%	2%	4%
Oil Cake of Brown Mustard					
24 h					
LAB	$4.8 \pm 0.4 \times 10^8$	$2.2 \pm 0.3 \times 10^8$	$4.4 \pm 0.4 \times 10^8$	$2.8 \pm 0.4 \times 10^8$	$3.3 \pm 0.2 \times 10^8$
Bifidobacterium	1×10^7	1×10^7	1×10^7	1×10^7	1×10^7
Enterococcus	$2.9 \pm 0.3 \times 10^7$	$8.2 \pm 0.4 \times 10^7$	$1.8 \pm 0.2 \times 10^7$	$1.8 \pm 0.3 \times 10^7$	$1.9 \pm 0.3 \times 10^7$
E. coli	$3.4 \pm 0.3 \times 10^7$	$2.2 \pm 0.1 \times 10^7$	$8.4 \pm 0.2 \times 10^6$ *	$9.4 \pm 0.1 \times 10^6$ *	$1.0 \pm 0.3 \times 10^5$ *
lactose +	$7.8 \pm 0.2 \times 10^6$	$8.7 \pm 0.3 \times 10^6$	$2.5 \pm 0.2 \times 10^5$ *	$1.6 \pm 0.1 \times 10^5$ *	$5.1 \pm 0.2 \times 10^4$ *
Proteus	$4.0 \pm 0.2 \times 10^7$	$2.0 \pm 0.2 \times 10^7$	$2.5 \pm 0.2 \times 10^4$ *	- *	-
72 h					
LAB	$3.7 \pm 0.3 \times 10^8$	$6.1 \pm 0.3 \times 10^8$	$3.8 \pm 0.3 \times 10^8$	$3.5 \pm 0.2 \times 10^8$	$4.7 \pm 0.3 \times 107$ *
Bifidobacterium	1×10^7	1×10^7	1×10^7	1×10^7	1×10^7
Enterococcus	$3.6 \pm 0.3 \times 10^7$	$2.6 \pm 0.3 \times 10^7$	$1.9 \pm 0.3 \times 10^6$ *	$2.8 \pm 0.3 \times 10^5$ *	$2.1 \pm 0.3 \times 10^3$ *
E. coli	$2.7 \pm 0.3 \times 10^7$	$3.4 \pm 0.2 \times 10^7$	$8.3 \pm 0.3 \times 10^6$ *	$2.2 \pm 0.2 \times 10^5$ *	$7.2 \pm 0.3 \times 10^5$ *
lactose +	$4.2 \pm 0.2 \times 10^6$	$3.1 \pm 0.4 \times 10^6$	$3.0 \pm 0.1 \times 10^5$ *	$8.8 \pm 0.4 \times 10^4$ *	$1.2 \pm 0.1 \times 10^4$ *
Proteus	$2.8 \pm 0.3 \times 10^7$	$2.9 \pm 0.3 \times 10^7$	- *	- *	- *
pH	6.98	7.22	4.73	4.53	4.12
Oil Cake of Spicate Lavender					
24 h					
LAB	$5.2 \pm 0.2 \times 10^8$	$1.1 \pm 0.1 \times 10^8$	$9.8 \pm 0.4 \times 10^8$	$4.5 \pm 0.2 \times 10^8$	$2.0 \pm 0.3 \times 10^8$
Bifidobacterium	1×10^7	1×10^7	1×10^7	1×10^7	1×10^7
Enterococcus	$5.7 \pm 0.3 \times 10^7$	$5.8 \pm 0.1 \times 10^7$	$2.4 \pm 0.2 \times 10^7$	$8.8 \pm 0.3 \times 10^7$	$6.5 \pm 0.5 \times 10^7$
E. coli	$1.8 \pm 0.1 \times 10^7$	$2.7 \pm 0.1 \times 10^7$	$1.4 \pm 0.2 \times 10^7$	$2.9 \pm 0.1 \times 10^6$ *	$1.8 \pm 0.2 \times 10^6$ *
lactose +	$1.0 \pm 0.2 \times 10^7$	$1.4 \pm 0.3 \times 10^7$	$3.4 \pm 0.3 \times 10^7$	$2.4 \pm 0.4 \times 10^6$ *	$9.3 \pm 0.4 \times 10^5$ *
Proteus	$6.6 \pm 0.1 \times 10^5$	$6.4 \pm 0.2 \times 10^5$	$2.7 \pm 0.2 \times 10^4$ *	- *	- *
72 h					
LAB	$4.4 \pm 0.1 \times 10^8$	$3.7 \pm 0.2 \times 10^8$	$1.0 \pm 0.3 \times 10^8$	$3.1 \pm 0.4 \times 10^8$	$5.3 \pm 0.4 \times 10^8$
Bifidobacterium	1×10^7	1×10^7	1×10^7	1×10^7	1×10^7
Enterococcus	$6.6 \pm 0.2 \times 10^7$	$4.9 \pm 0.3 \times 10^7$	$3.1 \pm 0.1 \times 10^6$ *	$1.3 \pm 0.1 \times 10^6$ *	$3.1 \pm 0.2 \times 10^5$ *
E. coli	$1.1 \pm 0.4 \times 10^7$	$1.4 \pm 0.2 \times 10^7$	$2.7 \pm 0.3 \times 10^6$ *	$1.2 \pm 0.3 \times 10^6$ *	$1.1 \pm 0.2 \times 10^5$ *
lactose +	$4.3 \pm 0.1 \times 10^6$	$2.5 \pm 0.3 \times 10^6$	$5.2 \pm 0.3 \times 10^5$ *	$2.9 \pm 0.2 \times 10^5$ *	$3.3 \pm 0.3 \times 10^4$ *
Proteus	$1.7 \pm 0.1 \times 10^7$	$4.8 \pm 0.2 \times 10^7$	$5.5 \pm 0.2 \times 10^3$ *	- *	- *
pH	7.26	6.95	6.17	5.11	4.70

* $p < 0.05$.

Only three types of plants in the form of the cake had a significant impact ($p < 0.05$) on the microbiota: camelina, mustard, and lavender. Due to the fact that the experiment was carried out in several stages, in Table 2, each option has its own control values. However, no significant differences were found between the controls.

On the first day, the number of microorganisms was already high. Because on the third day, the total number of micro-organisms had remained approximately the same, we can say that in the first 24 h, the community went through an exponential growth phase and reached the stationary phase. In all cases, the effect of cake additions already began to appear on the first day of incubation, but the most significant changes were recorded

only on the third day. In all three cases, the severity of changes in the number of micro-organisms, the pH level, and the concentration of cakes were positively correlated with each other.

On the first day, camelina oil cake had some effect on the number of micro-organisms in the microbiota model only at concentrations of 2% and 4%. There was a decrease in the number of *Enterococcus* and the complete disappearance of *Proteus*. At lower concentrations of 0.5% and 1%, no significant differences from the control were found.

On the third day, the effect of adding camelina oil cake to the medium was more significant ($p < 0.05$). The introduction of the 2% and 4% cakes by one to two orders of magnitude reduced the number of *E. coli*, *Enterococcus*, and lactose-positive bacteria and reduced the amount of *Proteus* to values below the sensitivity of the method. The 1% cake, which showed no effect on the first day, caused a slight decrease in the number of *Enterococcus* and *Proteus*. The 0.5% cake had no significant effects on the number of bacteria.

The mustard oil cake after 24 h of incubation caused a more pronounced effect than camelina cake. The 1%, 2%, and 4% oil cakes caused a decrease of one to two orders of magnitude in the number of *Enterococcus*, *E. coli*, and other lactose-positive bacteria. A concentration of 1% reduced the amount of Proteus, and 2% and 4% reduced its amount to values below the sensitivity of the method.

On the third day, the effect of the mustard oil cake increased. The 4% oil cake reduced the number of *Enterococcus* by four orders of magnitude; as for *E. coli* and lactose-positive bacteria, the 4% oil cake reduced the numbers by two orders of magnitude. However, there was also an order of magnitude decrease in the number of lactic acid bacteria. The 1% and 2% oil cakes did not reduce the number of lactic acid bacteria, reducing all the other groups listed above by two and one orders of magnitude, respectively. All three concentrations completely suppressed the growth of *Proteus*. Thus, the effect of the mustard oil cake was more pronounced than that of the camelina oil cake.

The concentration of the mustard oil cake at 0.5%, as in the previous case, did not affect the number of microorganisms.

After the first day of incubation, the lavender oil cake, as well as the mustard oil cake, caused a decrease in the number of *Enterococcus*, *E. coli*, and lactose-positive bacteria; a suppression of *Proteus* at 2% and 4%; and a decrease in the number of *Proteus* at 1%.

On the third day, at concentrations of 1%, 2%, and 4%, there was a decrease in the number of *Enterococcus*, lactose-positive bacteria, and *E. coli*. The number of lactic acid bacteria did not change significantly. As in the case of the camelina oil cake, 1% only reduced the amount of protein but did not completely suppress it. The 0.5% lavender oil cake had no significant effect on the number of bacteria.

The acidity was inversely correlated with the amount of cake added to the nutrient medium. On the third day, the lavender and camelina cakes reduced acidity approximately equally; the mustard oil cake reduced acidity more strongly, which is probably the reason for its higher activity.

3.3. Determination of the Mechanism of Influence of Oil Cakes

The direct antimicrobial activity of oil cakes at a concentration of 1%, 2%, and 4% was studied for three strains of *Enterococcus* sp. and three strains of *E. coli* that were previously isolated from the contents of the cecum of chickens. The data are not given because none of the variants showed any significant antimicrobial effect of the cakes on any of the strains.

To determine the effect of oil cake on the acid-forming ability of *Lactobacillus*, we studied the change in pH when oil cake was introduced in two media—MRS and chicken artificial intestinal medium.

We took two *Lactobacillus* strains as test microorganisms, which we previously isolated from the cecum of chickens and which have probiotic properties [16]. In the experiment, only concentrations of 1% and 2% were used because the concentration of 0.5% did not cause significant changes for any of the oil cakes, and the mustard oil cake with a concentration of 4% reduced the number of lactic acid bacteria.

The data that we obtained are presented in Table 3.

Table 3. Influence of plant oil cakes on the level of acid formation of *Lactobacillus*.

Oil Cakes	pH			
	***Ligilactobacillus salivarius* KL61**		***Limosilactobacillus frumenti* KL31**	
	MRS	**Intestinal Medium**	**MRS**	**Intestinal Medium**
Control	3.55 ± 0.22	6.89 ± 0.12	3.87 ± 0.14	7.14 ± 0.15
Camelina 1%	3.68 ± 0.30	6.43 ± 0.22 *	3.94 ± 0.10	6.82 ± 0.19 *
Camelina 2%	3.34 ± 0.15	5.08 ± 0.24 *	3.92 ± 0.12	6.02 ± 0.11 *
Mustard 1%	3.45 ± 0.21	4.84 ± 0.11 *	3.81 ± 0.22	6.63 ± 0.24 *
Mustard 2%	3.37 ± 0.25	4.29 ± 0.08 *	3.90 ± 0.15	5.20 ± 0.10 *
Lavender 1%	3.58 ± 0.11	6.17 ± 0.16 *	3.79 ± 0.09	6.76 ± 0.17 *
Lavender 2%	3.55 ± 0.10	5.18 ± 0.21 *	3.85 ± 0.15	5.81 ± 0.21 *

* $p < 0.05$.

On the MRS medium, both strains on the first day already significantly reduced the pH level to 3.34–3.94, depending on the experiment variant. The *Ligilactobacillus salivarius* KL61 strain had higher acid-forming activity than the *Limosilactobacillus frumenti* KL31 strain.

In the control, both strains of *Lactobacillus* on the intestinal medium even on the third day of incubation did not reduce the pH of the medium; it remained neutral. However, the addition of oil cake to the intestinal medium caused a significant decrease in the pH of the medium ($p < 0.05$), and a higher concentration of oil cake at 2% lowered the pH to a greater extent than did the oil cake with a concentration of 1%. As in the case of the microbiota model, the addition of brown mustard caused the greatest decrease in acidity.

Thus, we have demonstrated that the studied oil cakes stimulated the acid-forming ability of *Lactobacillus*, which led to a decrease in the initially neutral pH of the medium.

However, not only prebiotic sugars contained in cakes can enhance acid formation but also simple sugars, which, under the conditions of an animal organism, do not enter the cecum, as they are used by the body [16]. Therefore, we conducted another experiment in which the amount of simple sugars in oil cake was estimated. The results are presented in Table 4.

Table 4. Estimation of the amount of simple sugars in oil cake.

Oil Cake	The Proportion of Simple Sugars in Dry Matter	The Proportion of Simple Sugars in Artificial Intestinal Environment at a Concentration of 1%	The Proportion of Simple Sugars in Artificial Intestinal Environment at a Concentration of 2%
Big seed false flax	2.35%	0.024%	0.047%
Brown mustard	1.85%	0.019%	0.037%
Spicate lavender	2.50%	0.025%	0.050%

The amount of simple sugars in the dry matter of oil cakes is very low, and when they are introduced into the model medium, it is even less. This means that the activity of lactic acid bacteria is affected not by simple sugars but by polysaccharides.

4. Discussion

Many researchers have shown [9–12] that industrial by-products can be used as a source of prebiotics in livestock feed. At the same time, it is economically expedient to use by-products obtained in the same region where they will be used.

It is clear that the greater the number of potential plants that are examined, the more likely it is that properties of interest will be discovered. However, studies on living models have a number of disadvantages: the duration of the experiments is most often 42 days [21,22] and even longer [23,24], although these are very expensive studies due to the cost of feed and maintenance of birds. Therefore, there was a need to develop a model of the intestine that allows for the modulation of the microbiota of animals for primary screening of biologically active substances.

Artificial intestinal media make it possible to achieve conditions similar to those in the chicken intestine in vitro, which allows for the quick and cheap assessment of the potential impact of different substances on the intestinal microbiota before animal experiments. To confirm the performance of the model when screening potential prebiotics, a preliminary experiment was set up in which prebiotics with already-proven activity were tested [1–8,25–28]. Prebiotics such as inulin, β-glucan, oligofructose, apple fiber, and psyllium fiber were used.

The addition of inulin to the chicken artificial intestinal medium results in an increase in the number of *Bifidobacterium*. This agrees with the data obtained under in vivo conditions [25]. β-glucan stimulated the growth of the number of lactic acid bacteria, and at the same time, it reduced the number of *Bifidobacterium*, *Enterococcus*, *E. coli*, and other lactose-positive bacteria. This is also consistent with the literature data on the effect of β-glucan on the microbiota [26,27]. The literature describes the effect of oligofructose on the microbiota: an increase in the number of *Lactobacillus* and *Bifidobacterium* [28]. In our case, we also examined the amount of opportunistic microbiota. The introduction of oligofructose into the chicken artificial intestinal medium caused an increase in the number of *Bifidobacterium* and lactic acid bacteria and a decrease in the number of *Enterococcus*, *E. coli*, and other lactose-positive bacteria.

Thus, the data obtained by us using the model coincided with the data obtained in experiments in vivo and described by other authors [25–28], which means that this model reliably reflects the changes that occur in the microbiota when prebiotics are introduced, and it can be used to screen for new substances with a prebiotic effect.

Next, we began to study the properties of essential oil plants.

In the process of testing, the oil cake of black cumin, the cake and whole plant of blessed milk thistle, the whole plant of lesser calamint, and the cake and whole plant of winter savory did not have a significant effect on the microbial composition of the model and, therefore, did not have any prebiotic or antimicrobial activity. On the other hand, the big seed false flax, brown mustard, and spicate lavender oil cake had an impact on the number of micro-organisms in the microbiota of chickens. This means that the prebiotic effects we observed are not due to a simple increase in the dietary fiber amount in the chicken feed, especially given that the other feed plant components also contain a high amount of plant fiber.

Only three types of plants in the form of the cake had a significant impact ($p < 0.05$) on the microbiota: camelina, mustard, and lavender.

The action of cakes corresponds to the action of prebiotics, such as β-glucan and oligofructose; there was a decrease in the number of *Enterococcus*, *E. coli*, and lactose-positive bacteria, due to which the quantitative ratio of micro-organisms shifted towards lactic acid bacteria and *Bifidobacterium*.

These effects of oil cakes on the microbiota could be determined by the following phenomena.

Firstly, cakes could stimulate lactic acid bacteria and lower the pH due to the prebiotic substances contained in the cake, as was shown in the case of palm kernel cake [9,10].

Secondly, they could stimulate lactic acid bacteria and lower the pH due to the simple sugars contained in the cake.

Thirdly, there could be a direct antimicrobial activity of oil cakes.

Therefore, several experiments were carried out to eliminate some of these causes.

The direct antimicrobial activity of oil cakes at concentrations of 1%, 2%, and 4% was studied for three strains of *Enterococcus* sp. and three strains of *E. coli* previously isolated from the contents of the cecum of chickens. None of the variants showed any significant antimicrobial effect of cakes on any of the strains. Thus, the decrease in the number of *Enterococcus* and *E. coli* is not due to the direct action of oil cake but due to its indirect effects.

To determine the effect of oil cake on the acid-forming ability of *Lactobacillus*, we studied the change in pH when oil cake was introduced in two media—MRS and chicken artificial intestinal medium. MRS is a classic medium for the isolation, incubation, and study of the properties of *Lactobacillus*; however, its composition is far from that of chicken intestinal chyme [18]. However, we expect the main effect from the use of pro- and prebiotics in the intestines. Therefore, we also used the chicken artificial intestinal medium developed by us, which is much closer in composition to chyme.

We took two *Lactobacillus* strains as test micro-organisms, which we previously isolated from the cecum of chickens and which have probiotic properties [16].

Due to the fact that the MRS medium contains a high content of simple sugars (2% glucose), *Lactobacillus* species actively use them, forming a large amount of lactic and associated acids. At the same time, the cecum chyme contains almost no simple sugars because they are absorbed by the body at the level of the small intestine [18]. Therefore, intestinal medium also contains almost no simple sugars, as is the case with chyme.

In the control, both strains of *Lactobacillus* on the intestinal medium even on the third day of incubation did not reduce the pH of the medium; the pH remained neutral. However, the addition of oil cake to intestinal medium caused a significant decrease in the pH of the medium ($p < 0.05$). As in the case of the microbiota model, the addition of brown mustard caused the greatest decrease in acidity.

Thus, we have demonstrated that the studied oil cakes stimulated the acid-forming ability of *Lactobacillus*, which led to a decrease in the initially neutral pH of the medium. Low pH negatively affects the growth and development of *E. coli*, lactose-positive bacteria, and *Enterococcus* [29]. It is with the activation of lactic acid bacteria, which leads to a decrease in the pH of the medium, that the prebiotic effects of the studied cakes can be seen.

However, not only prebiotic sugars contained in the cakes can enhance acid formation but also simple sugars, which, under the conditions of an animal organism, do not enter the cecum, as they are used by the body [18]. Therefore, we conducted another experiment in which the amount of simple sugars in oil cakes was estimated. The amount of simple sugars in the dry matter of oil cakes is very low, and when they are introduced into the model medium, it is even less. This means that the activity of lactic acid bacteria is affected not by simple sugars but by polysaccharides. That is, the cake of these plants can serve as a cheap prebiotic supplement to bird feed.

The next stage of our research will be the identification of specific prebiotic substances that constitute the cake, as well as in vivo studies on chickens.

5. Conclusions

Thus, we have shown the possibility of using oilseed cakes as a source of prebiotics for chickens. The big seed false flax, brown mustard, and spicate lavender oil cakes at 1%, 2%, and 4% reduced the number of *E. coli*, *Enterococcus*, and *Proteus* in a model of chicken intestinal contents. These cakes do not have antimicrobial activity. Their effect on reducing the number of *E. coli*, *Enterococcus*, and *Proteus* is due to the effect on lactic acid bacteria. Under conditions approaching intestinal conditions, the big seed false flax, brown mustard, and spicate lavender oil cakes at 1% and 2% concentrations have been shown to stimulate acid production in intestinal *Lactobacillus*.

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