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BF- esse

Development of bio-efficient animal food for organic farms

Project no. 18-00-A01620-000042

Final report

2021

Riga

Contents

1. Project description and participants

The project "Development of Bio-efficient Animal Food for Organic Farms" was implemented by the European Agricultural Fund for Rural Development under the Latvian Rural Development Program 2014–2020. 16.2 of the measure "Cooperation" under the sub-measure "Support for the development of new products, methods, processes and technologies".

SIA BF-ESSE (Service Client No. 04606905, Reg. No. 40003033268) and cooperation partners participated in the implementation of the project:

◄ Riga Stradins University (Reg. No. 90000013771);

◄ Institute of Food Safety, Animal Health and Environmen "BIOR" (Reg. No. 90009235333);

◄ Types Agra farm "Liepas" (Reg. No. 43601012337);

◄ Farm of Karlīte, Irlava Parish (Reg. No. 40001017466);

◄ SIA "Zaļais kurss" (Reg. No. 40103240431).

Contact information of the project coordinator:

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- Riga Stradins University: Ilze Bārene, t. 29551817, Ilze.Barene@rsu.lv;
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- Agra farm "Liepas": Agris Veide, t. 26514405, agris.veide@inbox.lv;
- Irlava parish farm "Karotītes": Ričards Kalnciems, t. 22119599, ricards@karotites.lv,
- SIA "Zaļais kurss": Ingrīda Gailīte, t. 29225211, zalaiskurss@inbox.lv;
- ZS "Skujas": Guntars Eglītis, t. 29918731, zsskujas@gmail.com.

The project was launched in May 2019.

Project implementation period - two years, ending on April 30, 2021. The eligible costs actually used for the project are EUR 82180.76, including public funding of EUR 73962.66.

Due to various external factors that could not have been foreseen during the development of the project application, at the request of the Project participants, the Lead Partner twice requested an extension of the project deadline without changing the budget lines. Thus, the project ended on August 30, 2021.

During the implementation of the project it turned out that ZS "Karotītes" (poultry farming / broiler breeding) cannot be organizationally separated from the total group of animals (9000 birds, minimum group 1800 birds) a separate group (150-200 birds and 80 chickens) could be fed different concentrations of needles experimental feed containing a thick extract. Coordinating all activities with the Rural Support Service, the optimal candidacy was selected - ZS "Skujas", which replaced ZS "Karotītes" in a certain manner (RSS approval from 18.10.2020 No. 18-00-A01620-000042).

2. The purpose of the project stated in the application and its justification

Organic animal feed generally complies with the principles of organic farming - without chemicals, antibiotics, preservatives (E-substances), GMOs, growth promoters, etc.

The conversion and metabolic processes of animal food are usually closely related to the value and chemical composition of the feed, so any organism must be provided with all the necessary nutrients in optimal amounts and proportions. In addition, shelf life is very important for complete animal feed. Therefore, the development of bio-efficient animal food without a large amount of "chemistry" is a topical and practical issue for all organic farms, including cattle, sheep and poultry farmers.

The chemical composition of pine and spruce needles is rich in biologically active substances, they have bactericidal and antiviral properties, immunomodulatory, antioxidant functions, they contain several vitamins and provitamins (C, B1, B2, K, P, etc.), essential oils, resins, mineral salts, starch, etc. It is planned to develop new bio-efficient animal food products (samples) using basic nutrients (bran, carbohydrates, microelements, etc.) with the addition of thick needle extract and other natural substances or their extracts. Examine storage options by systematically inspecting ingredients.

During the implementation of the project, the best samples will be selected, production technical documentation will be drawn up. Scientists will have access to farmers' reports on the use of experimental food, which can be used for other research purposes. An innovative, bio-efficient animal food product can improve the economic performance of the organic farming sector and develop the organic farm not only within Latvia, but also at the European Union level. After the development of an innovative organic animal food product, any organic farm will be able to produce and use this product.

The planned activities of the project will contribute to the following directions - Objective 3A “Organization of food circulation, including promotion of processing and marketing of agricultural products, promotion of animal welfare and risk management in agriculture”.

Project activities will contribute to the implementation of the following priorities:

- ✓ ensuring full cycle production from the producer of primary agricultural products to the processor of the finished product, creating a complex sustainable solution that affects both the primary producer and the processor;
- ✓ Rad creation of added value of agricultural products for local raw materials.

3. Actuality of coniferous biomass use

The plant kingdom is a valuable source of raw materials used to treat and strengthen various human and animal diseases. Herbal substances include substances with a pronounced pharmacological action and compounds such as vitamins, minerals, unsaturated fatty acids, antioxidants, etc. biologically active substances with a positive effect on the trophic and reparative processes of the organism.

Coniferous trees are valuable representatives of the plant kingdom. Conifers are one of the oldest plants on the globe. During the long period of development, conifers have adapted to environmental conditions. Conifers have been able to overcome drastic climate change, radiation, various diseases and have developed a unique complex of biologically active substances, which are widely used in scientific and folk medicine.

Conifer grass is a valuable material. It contains chlorophylls, carotenoids: β-carotene, lutein, neoxanthin, violaxantine, etc. [1, 2], flavonoids: derivatives of quercetin, myrcetin and campempol (ampelopsin, taxifoline, isocercetin, astragaline, isoramnetin, trifoline, hyperin, isomyrcitrine, etc.), tanning agents: catechin, gallocatechin, etc. [3].

Pine needles are rich in vitamins: ascorbic acid, vitamin K, vitamins E and B; minerals and trace elements (potassium, calcium, magnesium, phosphorus, manganese, zinc, iron, boron, aluminum, nickel, selenium, chromium, iodine) (3); amino acids [4].

Needles contain allyl glycerides, phospho- and glycolipids, sterols and their esters with fatty acids (oleic, linolenic, linoleic, palmitic) [5], p-sitosterol-3-o-p-o-glycopyranoside; phenolic compounds: cinnamic acid, p-coumaric acid, p-hydroxyphenylethanol, guayacetanol, havicol, eigenol, isoeigenol, coniferyl aldehyde, pinosylvin monomethyl ester, dimethoxyresveratrol, ferulic acid, dihydroconiferyl alcohol, pinorezinol; isoabienol, polyprenols, epimanoyloxide-19-acid [7]; copper, etc. substances [8, 9].

The composition of biologically active substances in pine grass varies depending on the age of the trees, the season, the place of growth, the influence of environmental climatic and ecological factors [2, 3, 4, 7, 8, 10, 11]. For the content of biologically active substances in the thick extract of needles - chlorophyll-carotene paste (*conifer green needle complex*), see 3.1. in the table [12].

Table 3.1. Composition of chlorophyll-carotene paste

Component	**Contents**
Sodium chlorophyllin and other chlorophyll derivatives	400-1600 mg / 100 ml
β-carotene and other carotenoids	20-120 mg / 100 ml
Vitamin E	30-50 mg / 100 ml
K vitamins	1.2 - 2 mg / 100 ml
Phytosterols (including β-sitosterol)	1.5 - 2.9%
Polyprenol	0.46 - 1.2%
Squalene	0.14 - 0.16%
Minerals	5 - 7%
Sodium salts of fatty acids, resin acids, dibasic, oxo- and oxyacids	44 - 60%
Copper	5 - 8%
Essential oil	1 - 1.2%
Water	up to 100%

Humans have long sought to obtain active substances from their needles and their mixtures, combinations that could be used to maintain and improve their own and their animals' health.

The idea of using coniferous biomass in animal feed is not new. It began to develop as early as the 1950s.

The first production and use of coniferous vitamin flour as feed was widely used in Latvia, but it was not suitable for all animals. The digestive tract of birds and pigs could not cope with the lignocellulosic complex in flour. Chopped needle pulp was offered for use. Its practical use was limited by its short shelf life and high concentrations of tannins, bitters and resins.

Scientific research has expanded the use of conifer green pulp. Scientists have developed processing technologies for the green mass of conifers, which are waste from timber production, as a result of which extracts, extracts containing valuable combinations of natural substances can be obtained for use in medicine, cosmetics, agriculture, industry, etc. sectors. Coniferous green extract and its components have been shown to have immunomodulatory, hepatoprotective, antioxidant, antibacterial and antiviral activity.

Local biomass (conifer grass), which is available as a raw material for the crop year, is used to make conifer extract and obtain its ingredients.

Vitamin paste was made from pine needles, which was used as a feed additive.

The Silava Forest Research Institute, together with the Sigra Scientific Institute for Biotechnology and Veterinary Medicine, with the financial support of the European Regional Development Fund (ERDF), developed Dolofit from coniferous biomass and fodder limestone for use in poultry. A study performed during the use of the product showed that poultry eggs and meat to which Dolofit has been added significantly reduce the amount of cholesterol and increase the content of omega 3 and omega 6 fatty acids. The volume of meat increases by five percent, the incidence increases by 2.5 to 3 percent. Feed consumption is also declining - 6 to 7 percent less feed is needed to produce 1 kg of live weight.

The specified goal and tasks of the project

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The specified goal and tasks of the project

Project goal: to develop bio-efficient supplementary feed for organic farms.

Tasks:
1. To develop a composition for liquids containing thick needles

2. To develop premix formulations containing various organic supplementary feeds and thick extracts of needles and to specify the technology of their preparation

3. To develop analytical methods for the determination of carotene and chlorophyll in liquids and premixes containing thick needles after their preparation and storage for 3 months under different storage conditions.

4. To test the suitability of experimental feed containing thick extracts of needles for chickens, lambs and young cattle.

4. Materials and methods used in the work

4.1. Materials

Thick needle extract.

The extract was received from JS BIOLAT, reg. No. 40003128200, legal address: 111 Rigas Street, Salaspils, LV-2169.

Data on needle thick extract are given in Table 4.1.

Table 4.1. **Quality characteristics specified by the manufacturer for thick needle extract**

CERTIFICATE OF ANALYSIS

Product Name:	Pine needle extract	**Batch No:**	11
Batch Quantity:	95kg	**Analysis Date:**	29.05.2019
Manufacture Date:	27.05.2019	**Expiry Date:**	26.05.2021

Analysis Items	Specification	Result	Test Method
Active Ingredients			
Extract ratio	>90%	92.0%	Enterprise standard
Physical Control			
Description	Greeny-black paste with pine needle smlell		Visual/organileptic
Chemical Control			
Iron	0.51mg / 100g	Complies	Atomic Absorption
Zinc	0.19mg / 100g	Complies	Atomic Absorption
Calcium	0.85mg / 100g	Complies	Atomic Absorption
Peroxide value	0.08%	Complies	Atomic Absorption
Heavy Metals	≤10ppm	<10ppm	Atomic Absorption
Cadmium(Cd)	≤1ppm	<1ppm	Atomic Absorption
Lead(Pb)	≤3ppm	<3ppm	Atomic Absorption
Mercury(Hg)	≤0.1ppm	<0.1ppm	Atomic Absorption
Residual Solvent	<0.05%	Complies	/
Microbiological Control			
Total Plate Count	≤1000cfu/ml	Complies	AOAC/Petrifilm
S. aureus	Negative in 1g	Complies	AOAC/BAM
Salmonella	Negative in 10 g	Complies	AOAC/Neogen Elisa
Yeast & Mold	100cfu/g Max	Complies	AOAC/Petrifilm
E.Coli	Negative in 1g	Complies	AOAC/Petrifilm
Packing and Storage			
Packing	Packed in metal drumm. N. W.: 18 kgs.		
Storage	Dry, Dark place, Temperature 10-25℃		
Shelf Life	2 years when properly stored.		
Statements	Non-GMO		
Conclusion: The product complies with enterprise standard.			

Since the biological activity of the thick extract of needles (chlorophyll carotene paste) is determined by chlorophyll derivatives, carotenoids, fat-soluble vitamins E and K, phytosterols, polyprenols, squalene, unsaturated fatty acids, etc. complex of biologically active substances, the Scientific Institute for Food Safety, Animal Health and the Environment "BIOR" determined the amounts of chlorophyll and carotene to characterize the thick extract of needles. The results of the analyzes are given in Table 4.2.

Table 4.2. **Chlorophyll, beta-carotene content, sum of carotene isomers and the total number of microorganisms in the thick needle extract**

Quality requirements for thick needle extract	Results
Chlorophyll content	366 mg / 100 g
Beta-Carotene content	1072 mg / kg
Sum of carotene isomers	3012 mg / kg
Total number of micro - organisms	< 10 CFU / 1 g

Excipients

= Solvents

- Purified water
- 20% ethyl alcohol solution
- 70% ethyl alcohol solution
- 50% propylene glycol solution
- 70% propylene glycol solution
- 50% propylene glycol solution containing 20% ethyl alcohol

= Bio-supplementary feed

- ✓ ***Organic supplementary feed for 0 - 2 year old calves*** 62 - 78.

Manufacturer A / S Dobeles dzirnavnieks
Bio supplement feed contains wheat bio, barley bio, soybean bio, bean bio, oat bio, soybean cake bio, calcium carbonate, saccharomyces cerevisiae, defluorinated monocalcium phosphate, sodium chloride, magnesium oxide, vitamin A, vitamin D3, vitamin E, trace elements - zinc, manganese, copper, iodine, cobalt and selenium.

- ✓ ***DOFEED-Complementary feed 2.5% for birds*** 01 - 379.

Intended for organic farms.
Manufacturer JS Dobeles dzirnavnieks
Organic feed contains defluorinated monocalcium phosphate, calcium carbonate, sodium chloride, vitamin A, vitamin D3, vitamin E, B vitamins, vitamin K3, trace elements - zinc, manganese, copper, iodine, cobalt, selenium, iron, digestive aids - endo- 1,3,4-β-gluconase, endo-β-xylinase and 6-phytase.

- ✓ ***Complete feed for laying hens on organic farms*** *01 - 380.*

Manufacturer A / S Dobeles dzirnavnieks
Complete feed contains organic wheat, organic soybeans, organic oats, calcium carbonate, organic barley, organic beans, organic soybean cake, saccharomyces cerevisiae, vitamin A, vitamin D3, vitamin E, B vitamins, vitamin K3, trace elements - zinc, manganese , copper, iodine, cobalt, selenium, iron, digestion enhancers - endo-1,3,4-B-gluconase, endo-B-xylinase and 6-phytase.

- ✓ ***Whole food for 0 - 8 weeks old chickens on organic farms 02-77***.

Manufacturer A / S Dobeles dzirnavnieks
Whole feed contains organic wheat, organic soybean cake, organic oats, saccharomyces cerevisiae, calcium carbonate, vitamin A, vitamin D3, vitamin E, B vitamins, vitamin K3, trace elements: iron, zinc, manganese, copper, cobalt, selenium, digestion promoters endo-1,3,4-β-gluconase, endo-β-xylinase and 6-phytase.

- ✓ ***Complete feed for young birds for organic farms 03-168***.

Manufacturer A / S Dobeles dzirnavnieks
Whole feed contains organic wheat, organic oats, organic soybeans, organic soybean cake, saccharomyces cerevisiae, calcium carbonate, vitamin A, vitamin D3, vitamins E, B vitamins, vitamin K3, trace elements: zinc, manganese, copper, iodine, cobalt , selenium, iron, digestive enhancers - endo-1,3,4-B-gluconase, endo-B-xylinase and 6-phytase.

- ✓ ***Complete feed for 0 - 28 days broilers for organic farms 05-13***.

Manufacturer A / S Dobeles dzirnavnieks
Whole feed contains organic wheat, organic soybean cake, organic oats, saccharomyces cerevisiae, calcium carbonate, vitamin A, vitamin D3, vitamin E, B vitamins, vitamin K3, trace elements: zinc, manganese, copper, iodine, cobalt, selenium, iron , digestive enhancers - endo-1,3,4-B-gluconase, endo-B-xylinase and 6-phytase.

- ✓ ***Complete feed for 28 - 63 days broilers Grower organic farms 06-14***.

Manufacturer A / S Dobeles dzirnavnieks
Complete feed contains organic wheat, organic soybean cake, organic barley, organic soybeans, organic oats, saccharomyces cerevisiae, calcium carbonate, vitamin A, vitamin D3, vitamin E, B vitamins, vitamin K3, trace elements: zinc, manganese, copper, iron, iodine, cobalt, selenium, digestive endo-1,3,4-B-gluconase, endo-B-xylinase and 6-phytase.

- ✓ ***Complete feed for broilers Finisher organic farms 07-10***.

Manufacturer A / S Dobeles dzirnavnieks
Whole feed contains organic wheat, organic soybean cake, organic soybeans, organic oats, saccharomyces cerevisiae, calcium carbonate, vitamin A, vitamin D3, vitamin E, B vitamins, vitamin K3, trace elements: zinc, manganese, copper, iodine, cobalt , selenium, iron, digestive enhancers - endo-1,3,4-B-gluconase, endo-B-xylinase and 6-phytase.

The organic supplementary feed produced by A/S Dobeles dzirnavnieks may be used in organic production in accordance with Council Regulation (EC) No. 834/2007 and Commission Regulation (EC) No. 889/2008.

4.2. Methods

Determination of the particle size of complementary feed

The sieve analysis is performed using a set of 5 sieves. The mesh sizes used shall be 3 mm, 2 mm, 1,0 mm, 0,5 mm, 0,25 mm and the bottom pan shall be the receiver. A sieve set with 100 grams of the supplementary feed sample is subjected to a shaking for 5 minutes. After shaking, the product residue on each sieve is weighed and the percentage particle size distribution is calculated.

Preparation of liquids containing thick extracts of needles

Mix the calculated amount of thick needle extract with the solvent. Using each of these solvents, three concentrations of liquids were prepared.

Preparation of granular premix

The thick needle extract is mixed with the complementary feed to a homogeneous mass. The wet mass obtained is rubbed through a sieve with a perforated or braided surface. Hole size 3-5 mm. The obtained granules are spread in a thin layer and dried in an oven at 30-40° C.

Preparation of a premixture containing thick extracts of needles

Add the calculated amount of complementary feed in portions to a specified quantity of the thick needle extract. Stir the mass after adding each part of the feed to a homogeneous mixture.

Analysis of chlorophyll by UV-visible (UV-vis) spectrophotometric method

The UV-vis spectrophotometric method is used to determine the chlorophyll concentration. Preparation of the sample: Mix 1 g of the sample (homogenize to a fine powder in a pestle if necessary) with 1 g of calcium carbonate and about 50 ml of 85% acetone. Leave the mixture for extraction for 1 hour in the dark. When the extraction is complete, transfer the filtered extract to a 100 ml graduated flask. Repeat the washing step with 85% acetone until the resulting solution is colorless. Transfer the filtered extract to a graduated flask and make up to the mark with 85% acetone.

The absorbance spectrum is measured and the absorbance peaks are read at 600 nm on an 85% acetone blank using a UV/VIS spectrophotometer (Labda 35, Perkin Elmer). Plot a calibration graph by diluting the Guthrie standard solution. Preparation of the Guthrie standard solution: place 2,85 g of copper sulphate and 10,00 g of potassium dichromate in a 50 ml beaker. Dissolve the weighed portions in purified water and transfer quantitatively to a 1000 ml graduated flask. Then pour into the flask 100 ml of an aqueous solution containing 7% by mass of ammonia and make up to the mark with purified water. Guthrie standard color solution is equivalent to a chlorophyll solution concentration of 85 mg / l. Guthrie standard solution is colorimetric one day after preparation. Store the solution in a dark place in an airtight bottle. The shelf life of the solution is 1 month.

Carotene analysis by UHPLC method

Ultra high-performance liquid chromatography is used to determine the coordination between B-carotene and its isomers. The UHPLC system (Ultimate 3000, Thermo Scientific) is equipped with an eluent tank, an RS pump, an RS autosampler, an RS column compartment and a diode array detector (DAD). A Kinetex C18 column with a particle size of 1.7 μm, 3 mm i.d., length 100 mm (Phenomenex, USA) is used to separate B-carotene and its isomers. Mobile phase 98% methanol solution and flow rate 0.75 ml / min. After 16 minutes of isocratic chromatographic separation, the column is rinsed with tert-butyl methyl ether and finally with the initial mobile phase for 15 minutes. The injection volume was 5 μL. The DAD detector was set to a wavelength of 450 nm.

Preparation of the sample: Mix 1 g of the sample (homogenize to a fine powder in a pestle if necessary) with 2 ml of a tert-butyl methyl ether / methanol mixture (1/1, v / v). Shake vigorously for 10 minutes, centrifuge for 10 minutes. Finally, filter the sample solution through a 0,2 μm syringe-type filter into a small glass vial for UHPLC analysis. The β-carotene standard (> 97.0%, Sigma-Aldrich) is used for the calibration graph by diluting β-carotene stock solutions with tert-butyl methyl ether / methanol (1/1, v / v). The linear function is used as a calibration model. The LOQ was 1 mg / kg.

DNA extraction and rRNA gene sequencing procedure for chacal samples

The ZymoBIOMICS 96 Magbead DNA Kit from Zymo Research is used to obtain DNA from 100 mg of caecal contents. The preparation of the sequencing library is performed according to the protocol published by Illumina (document number 15044223 Rev. B). Bacterial 16S rRNA gene variable regions V3-V4 are targeted to primers developed by Clindworth et al. [10]. In the two-step protocol, the first round of PCR amplifies the target and adds Illumina sequencing adapters. The Nextera XT Kit A barcodes are added during the second round of PCR. KAPA HiFi DNA polymerase is used for all amplification reactions. Sequencing is performed with an Illumina MiSeq using a 3,600 cycle reagent kit to obtain 2 × 300 bp readings in pairs. For quality control, DNA extraction negative controls and mock community DNA (ATCC MSA-1002) are sequenced together with sample libraries.

Questionnaires for cooperation partners

RSS project no. 18-00-A01620-000042 Organic farms for the care of laying hens / broilers / chickens, weaned lambs and young bovine animals participated in the implementation of “Development of bio-efficient animal food for organic farms”.

5. Description of research performed by project participants

5.1. Riga Stradins University

5.1.1. Preparation of liquids containing thick extracts of needles

Due to the properties of its constituents, the thick needle extract has more pronounced hydrophobic (water-insoluble) properties. Therefore, a real aqueous solution cannot be prepared. Needle thick extract liquids are prepared in three concentrations. The characteristics of the prepared liquids containing thick extracts are given in Table 5.1.

Table 5.1. **Visual assessment of needles containing needles**

No. p. k.	Solvent	Concentration of thick needle extract	Description of the liquid
1.	Purified water	3% 5% 10%	Green suspension. The suspended particles do not settle (see Fig. 5.1).
2.	20% ethyl alcohol solution	3% 5% 10%	Greenish suspension. The suspended particles do not settle (see Fig. 5.2)
3.	70% ethyl alcohol solution	3% 5% 10%	Liquid with a granular dark precipitate. Part of the sediment adheres to the walls of the container (see Fig. 5.3).
4.	50% propylene glycol solution	3% 5% 10%	Light green suspension with a slightly dark granular precipitate at the top of the liquid. A clear greenish liquid with a dark precipitate above it. The precipitate is about half of the liquid (see Fig. 5.4).
5.	70% propylene glycol solution	3% 5% 10%	Light gray, clear liquid with a dark precipitate above it. Light green to clear liquid with a dark precipitate above it. Greenish, light yellow clear liquid with a precipitate. The sediment is about a third of the liquid (see Figure 5.5).
6.	50% propylene glycol solution containing 20% ethyl alcohol	3% 5% 10%	Light green to slightly turbid liquid with supernatant. Light greenish, yellowish, slightly turbid liquid with a precipitate above it. Yellowish-green transparent liquid. Slightly dark sediments are scattered above it. The precipitate is about half of the liquid (see Fig. 5.6).

Figure 5.1.

a b c

Liquids containing a - 3%, b - 5%, c - 10% needle thick extracts, solvent purified water

Figure 5.2.

a b c

Liquids containing a - 3%, b - 5%, c - 10% needle thick extract, solvent 20% ethyl alcohol solution

Figure 5.3.

a b c

Liquids containing a - 3%, b - 5%, c - 10% needle thick extracts, solvent 70% ethyl alcohol solution

Figure 5.4.

a b c

Liquids containing a - 3%, b - 5%, c - 10 needles thick extract, solvent 50% propylene glycol solution

Figure 5.5.

a b c

Liquids containing a - 3%, b - 5%, c - 10% thick extracts of needles, solvent 70% propylene glycol solution

Figure 5.6.

a b c

Liquids containing a - 3%, b - 5%, c - 10% thick extracts of needles, solvent 50% propylene glycol solution, containing 20% ethyl alcohol

All reconstituted liquids retained the described visual appearance after 6 months of storage in the dark.

5.1.2. Preparation of premixtures containing thick extracts of needles

The chemical composition of pine and spruce needles is rich in biologically active substances, they have bactericidal and antiviral properties, immunomodulatory, antioxidant functions, contains several vitamins and provitamins (C, B1, B2, K, P, etc.), essential oils, resins, mineral salts, starches etc. It is planned to develop new bio-efficient animal food products (samples) using basic feed substances (bran, carbohydrates, microelements, etc.) with the addition of thick needle extract. The daily dose of thick needle extract per hen, chicken, calf or lamb is 30 mg / kg. In order for chickens, chickens or food-producing animals to receive the required dose of coniferous extract and to have a slightly masked specific taste, the coniferous extract must be mixed with a complementary feed, which may only be used in combination with other feeds and is present in a higher concentration in complete feedingstuffs. than 20 kg / t.

Samples of coniferous thick extract and complementary feed mixtures were prepared to prepare premixes containing coniferous thick extract for use on organic farms.

2 variants for preparation of thick needle extract and supplementary feed mass were tested:

Option I	Option II
Granulation of the mass of needle thick extract mixed with supplementary feed by the wet granulation method	Mixing of needle thick extract with supplementary feed

We did not use the wet granulation method because granules are difficult to form from the prepared needle thick extract and compound feed mixture and there are large weight losses during granulation. Therefore, the samples were prepared by the mixing method.

The samples prepared by the mixing method contained 10% of the thick needle extract. Their quality was assessed organoleptically. The results are summarized in Table 5.2.

Table 5.2. **Mixtures of coniferous thick extract and complementary feedingstuffs**

No. p.k.	Complementary feed	Particle size of compound feed	Quantity of thick needle extract in the mixture,%	Description of the mixture
1.	Complete feed for 0 - 8 weeks old chickens on organic farms	2 mm	10	The mass of the mixture is slightly sticky (see Fig. 5.7).
2.	Complete feed for 0 - 28 days old broilers for organic farms	2 mm	10	Slightly flowing mixture

3.	Complete feed for 28 - 63 days old broilers *Grower* organic farms	Non - sifted complementary feed	10	Slightly paired mixture
4.	Complete feed for young birds for organic farms	2 mm Non - sifted complementary feed	10	Slightly flowing mixture Sticky mixture
5.	Complete feed for organic laying hens	2 mm	10	Slightly flowing mixture (see Fig. 5.10)
6.	DOFEED - Additional feed 2.5% for birds	Non-screened complementary feed Particles smaller than 2 mm	10	Sticky mass (see Fig. 5.9)
7.	Complete feed for broilers *Finisher* organic farms	Non - sifted complementary feed	10	Slightly paired mixture
8.	Organic supplementary feed for 0 - 2 year old calves	Unsorted complementary feed, particles larger than 3 mm	10	Sticky mixture (see Fig. 5.8)

Whole feed is a mixture of particles of different sizes. The particle size varies from 0.25 mm to 2-3 mm. The amount of the largest particles varies from 60 to 70%.

Figure 5.7.

Whole food 0 - 8 weeks old for chickens for organic farms, containing 10% thick needle extract

Figure 5.8.

Organic supplement for 0 - 2 months old calves, containing 10% thick needle extract

Figure 5.9.

DOFEED - Complementary feed 2.5% for birds, containing 10% thick needle extract

Figure 5.10.

Complete feed for laying hens on organic farms, containing 10% thick needle extract

Mixtures of complementary feed and thick needle extract intended for feeding to chickens / hens / calves / lambs contain different amounts of thick needle extract. The amount of needle thick extract in the mixtures is influenced by the weight of the chicken / chicken / calf / lamb at the beginning of the experiment, the weight gains every week during the use of the premix. The amount of complementary feed to be fed and the amount of thick needle extract to be fed should be increased weekly according to the weight gain of the lamb, chicken, chicken / calf. During the month, 24 premix-concentrates were prepared for 20 chickens / 20 chickens / 11 calves / 20 lambs - mixtures of coniferous thick extract and supplementary feed (148 kg).

The following formula shall be used to calculate the amount of thick needle extract in the complementary feeding stuff per lamb / calf / hen / chicken for one week:

E = [(D x M): 1000] x 7

where: E is the quantity in g of thick needle extract to be used with the complementary feed in 7 days
D - 30 mg / kg (dose of thick needle extract)
M - weight in kg (lamb / calf / chicken / chicken)
7 - number of days.

As there were 20 chickens / lambs or 11 young cattle in the experimental group, multiply the amount of needle thick extract per chicken / lamb / young animal for 7 days by the number of chickens / lambs / young cattle in the experimental group.

Eg = E x n,

where: Eg - amount of needle thick extract g for 7 days for the experimental group
n - number of birds / lambs / young cattle involved in the experiment for 7 days

Data on the amount of supplementary feed to be fed and its increase were provided weekly by the contact persons of organic farms.

The amount of feed fed to one bird, lamb or young animal per week is high. For the experimental group, taking into account the number of birds / lambs / young cattle, the amount of complementary feed increases, eg 20 chickens should be fed 3.36 kg during the first week and 5.32 kg during the fourth week, 20 lambs should be fed 168 kg during the first week. and 210 kg of complementary feed during the fourth week. In order not to have to prepare large amounts of supplementary feed and needle thick extract mixture for the 4-week experiment, we prepared smaller amounts of supplementary feed and needle thick extract mixtures - concentrates or premixes. The amount of needle thick extract in the premix is higher than in the calculator's experimental feed - a mixture of supplementary feed and needle thick extract. The formula is used to calculate the amount of thick needle extract in the premixture:

Ek = (Eg x pb): pm,

where: Ek - quantity of thick extract in the premixture concentrate, g

Eg - amount of thick extract of needles g for 7 days for the experimental group

pb - amount of supplementary feed, g for 7 days for the experimental group

pm - planned amount of concentrate-premix, g

The calculated amount of coniferous thick extract Ek and the amount of complementary feed obtained from the

Pk = pm - Ek,

where: Pk - amount of complementary feed g for concentrate-premix preparation

pm - planned amount of concentrate-premix, g

Ek - Amount of extra thick extract in the premix concentrate, g

To prepare the concentrate-premix, add the calculated amount of supplementary feed Pk to the calculated amount Ek of the thick needle extract. Stir the mass after each addition to the homogeneous mixture.

A separate premix concentrate should be prepared for each week of the experiment, because due to the increase in the weight of the chicken / lamb / young animal during the week, the amount of needle thick extract in the premix should be increased each following week.

For the preparation of experimental supplementary feed for 7 days, the premix concentrate must be mixed with the amount of supplementary feed obtained by subtracting the amount of premix-concentrate prepared (pm) from the amount of supplementary feed (see example in Table 5.3).

Table 5.3. **Preparation of experimental feed for lambs for 4 weeks**

Week	**Preparation of experimental feed (EB)**	**Number of lambs**	**EB quantity in 7 days for 20 lambs, kg**
Week 1	Mix 15 kg of premix No.1 with 153 kg of supplementary feed	20	168 kg
Week 2	Mix 15 kg of premix No.2 with 167 kg of supplementary feed.	20	182 kg
Week 3	Mix 15 kg of premix No.3 with 181 kg of supplementary feed	20	196 kg
Week 4	Mix 15 kg of premix No.4 with 195 kg of supplementary feed.	20	210 kg

5.2. Institute of Food Safety, Animal Health and Environment "BIOR"

5.2.1. Determination of carotene and chlorophyll in product samples

The Scientific Institute for Food Safety, Animal Health and the Environment (BIOR), a scientific partner of the project, determined the amounts of chlorophyll and carotene to characterize the thick extract of needles as a raw material. The concentration of chlorophyll in the prepared test samples - liquids and premixes containing thick needle extract was determined by UV-vis spectrophotometric method, and the coordination of β-carotene and its isomers was determined by ultra-high performance liquid chromatography. UAEH.

The levels of chlorophyll and β-carotene and its isomers were determined in some of the samples after 6 months of storage at room temperature.

The amount of chlorophyll and β-carotene and its isomers in liquids containing thick needles is given in Table 5.4.

Table 5.4. **Content of chlorophyll and β-carotene and its isomers in needles containing thick extracts**

Liquid	Chlorophyll content, mg / 100g		Chlorophyll content of the precipitate, mg / 100 g	β-carotene, mg/L after preparing	Sum of carotene isomers	
	After preparing	6 months after preparing			After preparing	6 months after preparing
3% liquid in 20% ethyl alcohol solution	25	6,4		51	128 mg/L	28 mg/kg
5% liquid in 20% ethyl alcohol solution	35	9,5		87	209 mg/L	29 mg/kg
10% liquid in 20% ethyl alcohol solution	48	13		200	507 mg/L	252 mg/kg
3% liquid in 70% ethyl alcohol solution	1,3				<5	
5% liquid in 70% ethyl alcohol solution	2,3		52		<5	
10% liquid in 70% ethyl alcohol solution	4,7		74		<5	
3% liquid, solvent water		11				31 mg/kg
5% liquid, water solvent		12				81 mg/kg
10% liquid, water solvent	82	20		64	173 mg/L	337 mg/kg

Liquids containing 3%, 5% and 10% thick extracts and prepared with 50% propylene glycol solution, 70% propylene glycol solution and 50% propylene glycol solution containing 20% ethyl alcohol were

slightly turbid with a precipitate. The precipitate was concentrated on the surface of the liquids. The precipitate was not suspended when the liquids were mixed.

The amount of chlorophyll, β-carotene and its isomers in such liquids was not analyzed.

The content of chlorophyll and carotene isomers in premixes containing thick needle extract is given in Table 5.5.

Table 5.5. **Content of chlorophyll and carotene isomers in premixtures containing thick extracts of needles**

Sample	**Chlorophyll content of the sample**			**Sum of carotene isomers in the sample**		
	after preparing, mg/100 g	**Stored**		**after preparing, mg/kg**	**Stored**	
		room temp., 4 months mg/100 g	**40ºC 3 months mg/100 g**		**room temp., 4 months mg/kg**	**40ºC 3 months mg/kg**
Complete feed for 0 - 28 days old broilers, containing 10% thick needle extract	27	14	8	9,5	8,9	< 1
Complete feed for laying hens, containing 10% thick needle extract	28	11	14	6,9	4,1	3,7
Organic supplementary feed for 0 – 2 months old calves, containing 10% thick needle extract	26	19	18	3,5	15	4,1
Complete feed for young birds, containing 10% thick needle extract	25	5,6	7	6,1	2,8	1,2
Complete feed for 0 - 8 weeks old chickens, containing 10% needle thick extract	24	15	24	8,9	6,6	4,9
Complete feed for broilers Finisher, containing 10% thick needle extract	17	8,8	11	4,5	3,1	1,1
Complete feed for 28 - 63 days old broiler Grower, containing 10% needle thick extract	18	8,4	17	4,9	3,4	4,8
Premix 1 (for week 1) for chickens	8,7			6,9		
Premix 2 (for week 2) for chickens	9,9			8,7		
Premix 3 (for week 3) for chickens	11			9,5		
Premix 4 (for week 4) for chickens	13			8,9		

Data 5.5. The table below shows that the levels of chlorophyll and carotene isomers in the premixture samples decrease after storage for 4 months ist. at 40 ° C for 3 months. The visual appearance of the

samples also indicates the oxidation of the chlorophyll and carotene isomers (see Figures 5.11 and 5.12).

Figure 5.11.

Complete feed for laying hens containing 10% thick needle extract stored at room temperature for 4 months

Figure 5.12.

Complete feed for broilers Finisher, containing 10% thick needle extract after storage at 40º C for 3 months

5.2.2. Cecal microbiota diversity and trends observed in experimental feeding groups

Fourteen-day-old chickens of various breeds, including Rhode Island red, Cream Legbar, and Leggorn, reared in the biological incubator of Skujas were divided into four groups of twenty chickens. The chickens in the control group received standard organic feed without additives; the chickens in the second group received the same feed containing a quantity of the thick extract of the needles, calculated using a dose of the extract of 30 mg / kg of the body weight of the chickens; the dose of extract of 60 mg / kg of the body weight of the chickens used for the calculation of the amount of the extract contained in the experimental feed of the third group of chickens; but in the fourth experimental group 90 mg / kg.

All chickens received food for 40 days. The first (control), second and third groups received normal and experimental feed well and there were no cases of feed refusal. In the third group (70% of cases), gastrointestinal disorders were observed, possibly related to the high dose of needle extract (this group was not considered in the study).

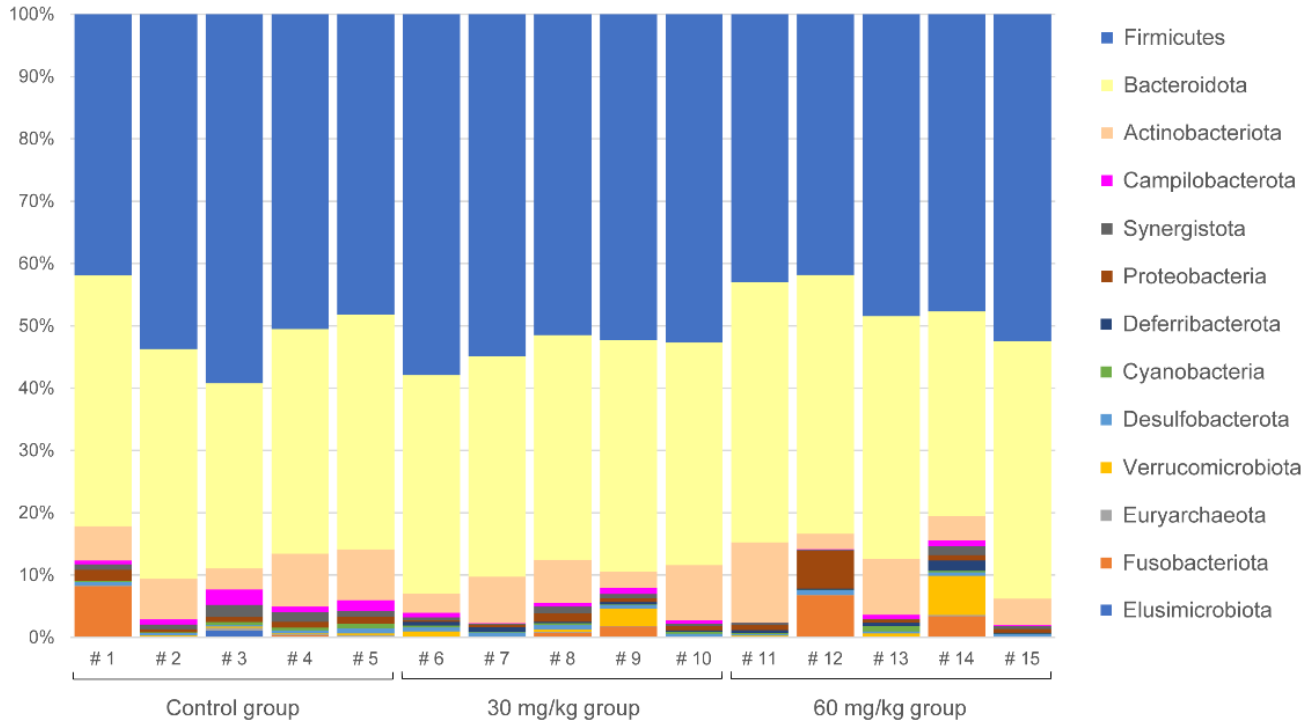
The 40-day-old chickens were killed by moving their cervical vertebrae. In special, ice-filled, sterile boxes, the samples were sent to the specialized laboratory of the Institute of Food Safety, Animal Health and Environment “BIOR”. All procedures were carried out in accordance with the guidelines of the Food and Veterinary Service of the Republic of Latvia and the EC Council Regulation no. 1099/2009 on the protection of animals at the time of killing. Fifteen samples were selected in accordance with the established procedure and all necessary requirements.

A total of 6,423,182 readings were generated from fifteen samples, ranging from 361,090 to 489,066 readings per sample. After the data cleaning and pre-processing step, 2,144,509 functions remained. The mean number of traits per sample was 142,967, from the lowest coverage of 121,506 to the highest of 158,486. Alpha diversity thinning curves were generated to verify that sufficient sequencing depth was achieved. Saturation of the Shannon Diversity Index was achieved at approximately 10,000 and 110,000 functions for all feeding groups, respectively.

The major bacterial phyla observed were *Firmicutes* (53.44% mean relative), *Bacterioidota* (33.14%), *Actinobacteriota* (6.43%), *Fusobacteriota* (1.60%) and *Proteobacteria* (1.37%). The abundance of other phyla was on average less than 1%. The composition of each sample at the phyla level is shown in Figure 5.13. in the picture.

Figure 5.13.

Composition of the phylum level of the cecal microbiota. Three groups (five replicates in each group) with different concentrations of the additive



The effect of the coniferous thick extract feed additive on cecal microbial alpha diversity was then assessed. Three different alpha diversity measurements were calculated. The Spirman correlation revealed a significant relationship (P = 0.0014) between the number of features observed and the number of denoted readings per sample, which was expected to occur in the United States with greater sequencing depth. However, other measures of alpha diversity did not correlate with sequence depth, so it was assumed that other diversity measures were not biased in terms of sample readings. The number of traits observed and the Simpson index did not correlate significantly with the feed additive concentration of the extract, while the Shannon index showed a negative correlation with the feed additive dose (ρ = -0.6236, P = 0.013).

Bray-Curtis and generalized UniFrac distances were calculated between all samples to assess inter-sample (beta) diversity. There was no significant correlation between beta diversity measurements and number of readings, indicating that these readings are not biased according to sequence depth either. Both Bray-Curtis and general UniFrac beta diversity measurements showed a significant positive correlation with feed additive concentration (ρ = 0.473644, P = 0.001 and ρ = 0.267187, P = 0.003, respectively). Eleven differently enriched genera for cecal content from birds fed a conifer thick extract-enriched diet compared to the control group were identified using a general linear model system (Table 5.6). Four of them belonging to the *families Bacteroidaceae, Marinifilaceae, Prevotellaceae* and *Deferribacteraceae* were more abundant when the feed was supplemented with coniferous thick extract. Seven other genera were less abundant when the birds were fed supplemented foods: two belonged to the *Rikenellaceae* family and the other belonged to the *Barnesiellaceae, Campylobacteraceae, Methanobacteriaceae, Lachnospiraceae and Synergistaceae.*

Table 5.6. **Differently rich taxonomies in the cecal microbiota**

Type (Phylum)	**Class**	**Order**	**Family**	**Genre**	**Coefficient of the linear model**
Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	0.0543
Bacteroidota	Bacteroidia	Bacteroidales	Barnesiellaceae		-0.5771
Bacteroidota	Bacteroidia	Bacteroidales	Marinifilaceae	*Odoribacter*	0.2604
Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae		0.5776
Bacteroidota	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	-0.1958
Bacteroidota	Bacteroidia	Bacteroidales	Rikenellaceae	*Rikenellaceae* RC9 gut group	-0.5106
Campilobacterota	Campylobacteria	Campylobacterales	Campylobacteraceae	*Campylobacter*	-0.6528
Deferribacterota	Deferribactere	Deferribacterales	Deferribacteraceae	*Mucispirillum*	0.2749
Euryarchaeota	Methanobacteria	Methanobacteriales	Methanobacteriaceae	*Methanobrevibacter*	-0.1979
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Eubacterium hallii* group	-0.1817
Synergistota	Synergistia	Synergistales	Synergistaceae	*Cloacibacillus*	-0.2144

According to a bacterial meta-analysis of the chicken thistle microbiota, *Firmicutes* were the most common phyla, followed by *Bacteroidetes* and *Proteobacteria* (13). A similar prevalence profile was observed in our study - *Firmicutes* and *Bacteroidetes*, but the next richer phyla was *Actinobacteriota*, followed by *Fusobacteriota* and only then *Proteobacteria*. However, the distribution of *Fusobacteriota* between the chickens used in the study was very uneven. The Shannon alpha diversity index was found to be significantly correlated with dietary supplement intake, while the Simpson index was not correlated, indicating that changes in microbial composition affect mainly minority taxa rather than dominant ones, as the Shannon index is more important for species richness and therefore more sensitive to small species. features (14). The significant correlation between the beta diversity measurements and the dose difference of the dietary supplement received by each chicken indicates that, despite the small sample size and significant differences in microbiome profiles between individuals, a general change in cecal microbiota composition can be observed (see Figure 5.13).

Among the four taxonomies that were more abundant when the diet was supplemented with coniferous thick extract was the gram-negative genus *Bacteroides*, a common member of the intestinal microbiota in all warm-blooded animals (15). *Bacteroides* play an important role in the cleavage of complex macromolecules (16) and in the generation of acetate and propionate as major fermentation products.

Odoribacter produces butyrate through lysine fermentation and succinate reduction (17 (Rychlik, 2020)). Butyrate, a short-chain fatty acid that directly stimulates the increase in the surface area of absorption, inhibits the growth of zoonotic pathogens, induces the expression of host protection peptides, and modulates host epigenetic regulation (18). Isolates of the family *Prevotellaceae* have not been described in detail. In vitro culture studies show that existing *Prevotellaceae* in chickens specialize in the digestion of complex polysaccharides and predominate in the microbiota when plant-rich diets are used (17 (Rychlik, 2020)).

Among the smallest phyla associated with a mature microbiota was the remarkable presence of bacteria that can stimulate the formation of a layer of mucus that is therefore associated with a healthy gut, such as *Mucispirillum*, which is considered a biomarker in free-range chickens (19).

For some bacterial taxonomies belonging to the *Barnesiellaceae* and *Rikenellaceae* families, the relative abundance was reduced when the birds were supplemented. These taxonomies belong to the order *Bacteroidale*, which includes gram-negative anaerobic cocobacteria, with saccharolytic and proteolytic activities. *Barnesiellaceae* is a proposed taxonomic group that has not yet been characterized. *Rikenellaceae* has been found in the high-fat obesity of mouse mice and appears to be highly sensitive to intestinal microbiota, such as those caused by antibiotics or probiotic supplements (16).

The same reduction was observed for *Methanobrevibacter*. Many methanogens use H2 and CO2 as substrates for methane synthesis. As the only producers of enteral methane, methanogens are responsible for the livestock sector's contribution to climate change and have thus become a research center for developing strategies to mitigate climate change. 16S rRNA gene sequences closely related to certain species of the genus *Methanobrevibacter* are one of the most common sequences in gastrointestinal samples from livestock.

One of the main functions of cecal is the fermentation of indigestible polysaccharide bacteria to produce short-chain fatty acids that can be absorbed by the host's epithelial cells. Certain *Firmicutes* (such as *Lachnospiraceae* or *Ruminococcaceae*) and *Bacteroidetes* play this role (20). Bacteria of the genus Lachnospiraceae are not present or are very rare in young chickens (21).

The most common cause of bacterial gastroenteritis is *Campylobacteriosis*, which is caused by *Campylobacter* spp. bacterial infection. In a previous study in Latvia, which focused on the prevalence of *Campylobacter* in broiler production, 51% of *Campylobacter* was found. In addition, *C. jejuni* was the predominant bacterial species and was detected in 96% of all *Campylobacter* isolates, whereas *C. coli* was detected in only a few samples (22). Therefore, reducing the *Campylobacter* load or even eradicating some *Campylobacter* species from chicken intestines would benefit public health.

Namely, the relative amount of *Campylobacter* in the animals receiving 30 mg / kg of coniferous thick extract was still similar to the control samples. The amount of these bacteria was significantly reduced with increasing dose (60 mg / kg), indicating that the lowest dose would not be sufficient to achieve the desired effect on the caecal microbiota. It is recommended that such observations on the reduction of pathogenic bacteria be continued in future studies using internationally recognized *Campylobacter* detection and enumeration methods, such as those described in ISO 10272.

Although this study is limited to a small number of samples, it nevertheless shows a desirable trend for changes in the dose of the additive in the broiler microbiota.

Additional information: The code and detailed software versions used for sequence processing and analysis are documented and available on GitHub: https://github.com/jkibilds/chicken-feed-microbiome-2021. All DNA sequencing reads from this study have been deposited in the European Nucleotide Archive under accession number PRJEB46042.

5.3. Organic farm ZS “Skujas”

ZS Skujas participated in the project with chickens that had hatched from the farm's incubation eggs on the same date. 4 groups of chickens were formed, each with 20 birds. 3 groups of chickens were experimental, one - control group. In each of the three experimental groups, the chickens received supplementary feed containing different amounts of thick needle extract.

The supplementary feed fed to group 1 contained 30 mg / kg of coniferous thick extract, to group 60 to 60 mg / kg and to group 3 to 90 mg / kg.

During the first week of the experiment, the use of the concentrated (90 mg / kg) premix was discontinued at the onset of fall in chickens.

Premixtures containing the amount of thick needle extract corresponding to 30 mg / kg and 60 mg / kg were used as supplementary feed for the experimental group for 4 weeks. As the weight of the chicken increased during the week, a premix concentrate containing the amount of thick needles had to be added to the supplementary feed for the next 7 days. The premix and the reconstituted mixture stratified during storage because the complementary feed did not have the same particle size.

Premixtures containing the amount of thick needle extract corresponding to 30 mg / kg and 60 mg / kg were used as supplementary feed for the experimental group for 4 weeks. As the weight of the chicken increased during the week, a premix concentrate containing the amount of thick needles had to be added to the supplementary feed for the next 7 days. The premix and the reconstituted mixture stratified during storage because the complementary feed did not have the same particle size.

As the premixes are more or less sticky, as noted by the owner of ZS “Skuja”, the mass of the premix should be mixed and mixed with supplementary feed until more or less homogeneous. At the beginning of the experiment, the partner observed that the prepared feed and premixture mixture was not homogeneous due to the unequal particle size and that the small particles of the premix remained in the container at the end of the day.

The partner managed to prepare a more homogeneous mixture by mixing the premix with a moist supplement. No fine particles were observed with this mixture.

The owner of ZS “Skuja” notes that:

1. During the experiment, the aroma of the complementary feed was very pleasant, which over time also filled the housing of the groups of chickens, which remained even after the change of litter.
2. At the end of the feeding of the chickens, two groups were retained as young birds, living in the same conditions during the winter. In group 1 there were 15 birds fed with supplementary feed at the age of chickens. In group 2 there were 15 birds fed with control feed. Chickens fed supplementary chickens began to lay at 6 months of age at an outdoor temperature below 0 ° C, but the control group began to lay at 7.5 months of age. We also observed that the birds of group 1 laid eggs more regularly than the birds of the control group.
3. Objective data (analysis of questionnaires) convincingly showed that all animals are happy to ingest experimental food, develop and behave adequately to the situation. No health problems were observed during the experiment.
4. The weight dynamics of the experimental chickens was positive and the weight gain was ~ 8%.

5.4. Organic farm ZS “LIEPAS”

The owner of the cooperation partner ZS “Liepas” has submitted the following information on the use of feed containing thick needle extract.

In order to improve the vitamin supplementation and metabolism of the Soviet farms, the dairy cows in the walking areas were fed shaved spruce branches, which the animals gladly chewed, and the result was generally positive.

Of course, no studies on the effects on animal metabolism have been performed in this regard and no objective health indicators have been established. The currently available coniferous paste or conifer processing product could have a positive effect on animal health, but when trying to feed a mixture of premix-concentrate with rolled grain feed, the taste properties were not quite acceptable for cattle. Some of these feeds began to be eaten after 3-4 days, and within two months, young dairy calves between the ages of 5 and 8 months learned to eat about 150 grams each day. This is not enough to ensure the intensive development of young cattle of the meat breed.

Conclusion: Other feed components that improve digestibility, such as molasses, bran, etc., need to be added to create a feed additive containing a needle treatment product. The contribution of needle additives to the metabolism and health of animals should also be investigated.

Objective data (analysis of questionnaires) showed that the animals involved in the experiment were accustomed to the experimental diet for two months and ate about 150 grams per day. Young cattle develop and behave adequately to the situation. No health problems were detected during the experiment.

5.5. Organic farm SIA “ZAĻAIS KURSS”

The cooperation partner SIA “ZAĻAIS KURSS” has sent a report with information on the suitability of experimental feed for lambs.

In order to test the suitability of experimental feed for lamb feeding, a group of 20 lambs with a live weight of 18-22 kg was separated in the herd of SIA "ZAĻIS KURSS". Until weaning, the lambs were fed on pasture grass, a freely accessible hay and 0.8 kg of oats per animal per day.

After weaning, a group of lambs was fed experimental feed in the following doses:

- In the first week from 17.05 to 23.05 lambs received 1.2 kg / day of concentrate consisting of premix No. 1 mixed with 168 kg of oats.
- In the second week from 24.05-30.05 the lambs received 1.3 kg / day of concentrate consisting of premix no.2 mixed with 182 kg of oats.
- In the third week from 31.05-06.06 the lambs received 1.4 kg / day of concentrate consisting of premix no.3 mixed with 196 kg of oats.
- In the fourth week, from 07.06 to 13.06, the lambs received 1.5 kg / day of concentrate consisting of premix No. 4 mixed with 210 kg of oats.

The feed was fed in a common trough, ensuring free access to all lambs at the same time.

Conclusions:

► In the first week of the experiment, concentrates were eaten more slowly - from the beginning, oats not mixed with the premix. But in the course of each day, everything was eaten. From the middle of the second week of the experiment, the food offered was eaten indiscriminately.

► No changes in gastric function or gastric emptying were observed in any lamb. The animals felt good, gained weight.

► Objective data (analysis of questionnaires) convincingly showed that all animals are happy to ingest experimental food, develop and behave adequately to the situation. No health problems were observed during the experiment.

6. Discussion on the achievement of the goal and tasks set in the project application

The quality of the mixture of premixtures - organic supplementary feed and thick needle extract depends on the particle size and ingredients of the supplementary feed. Complementary feed particles larger than 4 mm and smaller than 0.5 mm form a slightly sticky mass with the thick extract of needles.

The optimal particle size is 2 - 3 mm. The thick extract concentration of needles also forms a more sticky mass.

The partner from ZS Skuja has also noted that the particle size of the premixture and the particle size of the premixture and complementary feed mixture affect the feeding of thick needles to the chickens.

It is desirable to further study the effect of supplementary feed ingredients on the quality and consistency of premixes containing thick extracts of needles. Consideration should also be given to ensuring the quality of the needle extract ingredients during storage of the premix.

The chlorophyll content and the sum of carotene isomers in the samples vary. The amount of these active substances in the samples is affected by the amount of thick needle extract in the samples. In addition, the amount of chlorophyll and carotene isomers in the samples decreases due to the oxidation of these substances during storage. The remaining ingredients are vitamin E, group K vitamins, phytosterols (including β-sitosterol), polyprenols, squalene, minerals, sodium salts of fatty acids, dibasic, oxo- and oxy-acids, waxes, essential oils, water, diterpenes, etc. contains more than 500 biologically active substances) can be used successfully throughout the period of use. The thick needle extract itself can be stored for 36 months (3 years) without any change in the ingredients.

Bio-effective animal feed containing thick extracts of needles **can be stored** for 3 months at 25º C in a dark place.

This means that the premixes must be prepared for a specific, short shelf life. In order to be able to store and use premixes containing needle thick extract for a long time, it is necessary to explore the possibility of using antioxidants.

Liquids containing thick needles are different. A stable suspension was obtained using purified water and purified water containing 20% ethanol as solvent. It is desirable to further investigate the composition, properties and uses of the suspensions.

The beneficial effects of phytobiotics on animal health are mainly due to the chemical compounds present in the plants and their combinations, including terpenoids (mono- and sesquiterpenes, steroids), phenols (tannins), glycosides, alkaloids (alcohols, alhidides, ketones, esters, ethers). and lactones), flavonoids and glucosinolates. (23). The healing properties of pine needles may be related to their bioactive substances, such as carotene, terpenoids, phenolic compounds, tannins and alkaloids (24).

The co-operation partner from ZS “Liepas” informed that the mixture of premix-concentrate with rolled grain feed was not very acceptable for young cattle.

When using pine needle extract as a feed supplement, the effect of its bitter, astringent taste (mainly due to tannins) on the edibility of feed for different animal species should be taken into account. For digestors, the taste buds are richly placed in the oral cavity and they prefer to absorb salty or sweet food, while bitter food reduces the overall food intake. For this reason, reduced intake of complementary feed has been observed in young fattening cattle. When adding these extracts to the feed, it is recommended to mix them with molasses and then with the mixed feed (TMR), which would reduce the bitter taste and the possibility of distinguishing or eating only the most delicious feed ingredients.

Birds, on the other hand, have much less taste receptors than mammals, and they mainly distinguish between sweet, sour and salty flavors. Only rare birds distinguish between bitter tastes [12]. For this reason, in our experiment, the chickens gladly ate a supplement containing pine needle extract.

It has been shown experimentally that the developed experimental bio-effective animal feed containing the thick needle extract has no stimulating effect on the animal body. The thick extract of needles, by its nature (analyzing the available literature and our studies), does not contain substances that could alter the laying function of chickens and the hypertrophic weight gain of the animal.

The laying function of birds has itself developed phylogenetically as the most stable system of reproduction. Qualitative ratios of egg content (lipids / egg yolks and proteinaceous substances) may change with the use of selective synthetic / natural stimulants (they cannot be used on organic farms). In this case, it can be predicted that the qualitative proportions between lipids and protein substances are unlikely to change under the influence of the thick needle extract. The laying experimentally observed may be altered / accelerated.

Bioloģiskā (dabīgā) dēšanas procesā var mainīties olu izmēri un masa, bet ne būtiski kvalitatīvie rādītāji.

Tāpēc izanalizēt kvalitatīvi dējējvistu olas, kuras saņēma eksperimentālo bioefektīvo barību ar skuju biezo ekstraktu, nebija lietderīgi.

Savukārt projekta realizācijas gaitā aktualizējās eksperimentālo pētījumu daļa, kura saistīta ar izstrādātās eksperimentālās bioefektīvās barības, saturošas skuju biezo ekstraktu, iedarbību uz zarnu trakta mikrobiotu /mikrobiomu. Šīs aktivitātes nebija iekļautas apstiprinātā darbu plānā un bija atkarīgas no pirmā etapa - bioefektīvas dzīvnieku barības, kura satur skuju biezo ekstraktu, izstrādes procesa, glabāšanas un prognozējamo pielietojumu. Uzskatu, ka bioefektīvas dzīvnieku barības, kura satur skuju biezo ekstraktu izpēte saistībā ar zarnu trakta mikrobiotu ir vērtīgākā projekta sastāvdaļa, jo mikrobiotas un saistītie ar to imunitātes pozitīvie rādītāji parasti ietekmē dzīvnieka veselības stāvokli, pārtikas pieejamību, svara dinamiku utt.

Mikrobiota ir ievērojami daudzveidīga tikai aklajā zarnā un resnajā zarnā (17). Veselai pieaugušai vistai aklajā zarnā kolonizējas visvairāk grampozitīvi un gramnegatīvi bakteroīdi, kas veido apm. 90% no mikrobiota un atlikušie - parasti ir grampozitīvas aktinobaktērijas un gramnegatīvas proteobaktērijas (17). Ņemot vērā gremošanas trakta anatomisko specifiku (putnu aklās zarnas

funkcionālo aktivitāti), vislabāk bioefektīvo dzīvnieku barību - papildbarības un skuju biezā ekstrakta maisījumu var izmantot putnkopībā (cāļi, vistas).

For chickens: positive results in the microbioma of the caecum of chickens

The relative amount of *Campylobacter* in chickens fed supplemented diet containing 30 mg / kg of thick needle extract was similar to controls. The number of these pathogenic bacteria was significantly reduced only in chickens receiving a higher dose of thick needle extract (60 mg / kg). This showed that the minimum dose of needle extract was not sufficient to achieve the desired effect on the caecal microbiome. This observation regarding the reduction of pathogenic bacteria should be investigated in future studies using internationally recognized methods for the detection and enumeration of *Campylobacter*, such as ISO 10272.

Between the four experimental groups, two of which were supplemented with coniferous supplementation, an increase in the number of gram-negative bacteria of the genus *Bacteroidetes* was found, which is commonly found in the intestinal microbiota of all warm-blooded animals: *Bacteroides*, *Odori-bacter* and *Prevotellaceae*. The growth of these species promotes the digestive processes of food (especially polysaccharide-containing, fibrous) in the intestine. *Bacteroids* play an important role in the breakdown of complex macromolecules and in the generation of acetate and propionate as major fermentation products (16). *Odoribacter* is able to produce butyrate by fermenting lysine and reducing succinate (17). Butyrate, a short-chain fatty acid, directly stimulates the increase in the absorption surface, inhibits the growth of zoonotic pathogens, induces the expression of host protective peptides, and modulates host epigenetic regulation (18). In vitro studies indicate that *Prevotellaceae* in chickens specializes in the cleavage of complexed polysaccharides and dominates the microbiota when fed a diet enriched with plant fibers (17).

In turn, the number of bacteria in the group Eubacterium hallii of the genus Firmicutes was reduced. This means that the species ratio of *Firmicutes* / *Bacteroidetes* is declining. In humans, the ratio of *Firmicutes* to *Bacteroidetes* (F / B ratio) often correlates with body weight. The F/B ratio is significantly higher in obese people and decreases significantly during weight loss (Ley et al., 2005). Thus, the live weight of chickens that have received conifer extract may contain relatively more muscle and less fat, which is recommended when consuming this poultry meat for a healthy human diet.

It has been experimentally demonstrated that the developed bio-effective supplementary feed containing a thick needle extract has a positive effect on the microbiota / microbioma of the animal's intestinal tract (experiment with chickens). Analyzing this objective fact, it can be concluded that such feed can have a positive effect on the microbiota of the intestinal tract of any animal (human). The intestinal tract is known to be the major immune-competent organ (70% of total immune regulation is via the intestinal tract). Microbiota imbalance can provoke and cause a general immune imbalance with the addition or development of any disease. Other objective animal health indicators (blood tests, urine tests, blood biochemistry, muscular biopsy, etc.) usually differ from the normative parameters if the animal becomes ill and requires specialized veterinary assistance.

The developed bio-effective animal feed, which contains the thick extract of needles, unlike other premixes, has a long-lasting preventive effect on the whole animal organism.

Additional studies related to the effect of experimental bio-effective feed containing thick extracts of needles on the microbiota of the intestinal tract were performed in a certain order without changes in the total budget.

7. Conclusions

The planned goal of the project has been achieved. Bio-efficient supplementary feed for chickens, lambs and young cattle for organic farms has been developed.

Planned tasks completed:

1. Due to the water-insoluble and poorly soluble substances contained in the thick needle extract, it is not possible to prepare aqueous solutions or liquids containing the thick needle extract. Turbid liquids, sediment liquids are not suitable as an additive to drinking water for birds or animals.

2. Developed premix formulations made from various organic supplementary feeds and thick needle extract. Premixing technology has been clarified. The consistency of the prepared premixtures depends on the particle size and ingredients of the organic complementary feed.

3. Analytical methods for the determination of carotene and chlorophyll in liquids and premixtures containing thick extracts containing bio-supplementary feedingstuffs have been developed and used.

4. The weight dynamics of the experimental chickens was positive and the weight gain was ~ 8%.

5. In addition, a procedure for extraction of chicken intestinal DNA and sequencing of rRNA genes has been performed on chacal samples.

6. The mixture of supplementary feed and needle thick extract can be stored for 3 months in a dark place at 25º C.

Better use results can be achieved by preparing a bio-effective compound feed with a thick needle extract every 7 (seventh) days.

7. All animals involved in the experiment (chickens, chickens, lambs, young cattle) gladly used the experimental bio-efficient feed - a mixture of supplementary feed and thick needle extract throughout the feeding period (28 days). The young cattle had a restrained reaction in the first days (3-4 days), which resolved on the sixth day of use. This reaction can be explained by the specific intestinal function of young cattle - belching and increased digestion (may be the same as in humans - specifically bitter taste). No such reactions were observed in chickens, chickens and lambs. Taking into account the anatomical specifics of the digestive tract (functional activity of the caecum of birds), it is best to use a mixture of bio-efficient animal feed - supplementary feed and thick needle extract in poultry (chickens, chickens).

8. It has been experimentally demonstrated that the developed bio-effective complementary feed containing thick needle extract has a positive effect on the gut microbiota / microbioma of the animal (chicken experiment). Analyzing this objective fact, it can be concluded that such feed can have a positive effect on the microbiota of the intestinal tract of any animal (human). The intestinal tract is known to be the major immune-competent organ (70% of total immune regulation is via the intestinal tract). Microbiota imbalance can provoke and cause a general immune imbalance with the addition or

development of any disease. Other objective animal health indicators (blood tests, urine tests, blood biochemistry, muscular biopsy, etc.) usually differ from the normative parameters if the animal becomes ill and requires specialized veterinary assistance.

The developed bio-effective animal feed, which contains the thick extract of needles, unlike other premixes, has a long-lasting preventive effect on the whole animal organism.

9. Microbiotics and related immune positive parameters usually affect the health status of the animal, the availability of food, the dynamics of weight, etc.

- No case of disease in the experimental animal was objectively detected (there was no need to call a veterinarian or use veterinary treatment);
- All bio-efficient animal food was consumed and normally digested (no visual change was observed during the experiments);
- The weight dynamics of the experimental chickens was positive and the weight gain was ~ 8%.
- The same dynamics were objectively observed in sheep and young cattle. Only, statistically correct, more young cattle could take part in the experiment, but as many young cattle as the farm took part in the experiment. All animals participating in the experiment developed unchanged.

10. Given that animal health / stress, microbiota, immunity and development are generally interrelated, it can be objectively predicted that the widespread use of bio-efficient foods containing thick needle extract will improve the economic performance of any organic / industrial farm and will develop the agricultural sector.

When planning to use bio-efficient animal feed containing thick conifer extract, it is necessary to take into account the increase in the cost of feed[1].

11. The results of the development of bio-efficient animal feed are planned to be published in a specialized international publication. A publication *"Changes in broiler chicken gut microbiome caused by feeding supplemented with conifer needle extracts*[2]*"* has been prepared.

[1] Price of 1 (one) kilogram of thick needle extract at the moment - 2021. - could be ~ 50 EUR / kg.
[2] see Appendix

8. Recommendations

Based on the positive results of the research, we can recommend that young poultry breeders enrich their supplementary or basic feed with the addition of thick needle extract. Reports Chapter 5 5.1.2. Subchapter 1 gives formulas for calculations and describes the preparation of a mixture of complementary or basic feed and needle thick extract.

Amount of thick needle extract to be used

An example of bio-efficient feed preparation

In the process of developing bio-efficient animal feed, the industrially produced products of JSC "Dobeles dzirnavnieks" can be used (for example, *complete feed for 0- to 8-week-old chickens for organic farms, DOFEED-Supplementary feed for 2.5% of birds for organic farms, Bio-supplementary feed 0 - 2 months-old calves, complete feed for broilers from 0 to 28 days of age for organic farms, etc.),* complementary feed produced on organic farms from organically grown raw materials (organically grown oats, barley, wheat, soybeans, etc.) mixed with needles the amount of thick extract, which is calculated using the weight of the animal and the dose of the extract per kg (30 mg / kg or 0.03 g / kg).

Example
The weight of the chicken is 270 g or 0.270 kg.
Amount of thick needle extract to be used **per 1 chicken per day** =
30 mg of extract x 0,270 kg (weight of chicken) = **8,1 mg of extract** or
0.03 g of extract x 0.270 kg (weight of chicken) = **0,0081 g of extract**.

For how much supplementary feed with coniferous extract can be prepared per week, the calculated amount of extract and the intended supplementary feed for one day must be multiplied by 7.

The amount of thick needle extract and supplementary feed will increase with each passing week due to the weekly increase in chicken weight.

Using this sample, the amount of extract used can also be calculated for other animals.

It has been experimentally proven that any organic and industrial farm can successfully and practically use this simple production scheme.

In the process of developing bio-efficient animal feed, it is important to **observe the hygiene requirements**, grinding technology (if supplementary feed produced organically on an organic farm is used), mixture preparation technology (*preparation of a mixture of supplementary feed and needles for a planned number of animals per week*).

Add the calculated amount of coniferous thick extract to a small amount of the calculated complementary feed and mix until homogeneous. The rest of the complementary feed is added in portions. After the addition of each part of the complementary feed, the mass is stirred until a homogeneous mixture. A separate mixture of supplementary feed and needles should be prepared each week, taking into account the increase in weight of the animal during the week.

Shelf life of feed

Bio-effective animal feed containing thick extracts of needles can be stored for 3 months at 25º C in a dark place. During the implementation of the project, it has been experimentally proved that during the long-term storage part of the components of the thick extract of needles (chlorophyll, carotenoids) are converted into another form. The remaining ingredients are vitamin E, group K vitamins, phytosterols (including β-sitosterol), poly-liprenols, squalene, minerals, sodium salts of fatty acids, dibasic, oxo- and oxalic acids, waxes, essential oils, water, diterpenes, etc. , needle thick extract contains more than 500 biologically active substances), can be used successfully throughout the period of use. The thick needle extract itself can be stored for 36 months (3 years) without any change in the ingredients.

However, better results can be obtained by preparing a new bio-effective supplementary feed mixture with coniferous thick extract every 7th (seventh) day. Optimal spending planning is important.

Evaluating the supplementary feed fed to chickens, enriched with coniferous thick extracts, the positive effect on the chicken organism and the information obtained by the owner of HP Skujas during the experiment, we believe that feed containing coniferous thick extract should be tried to feed young birds and adult birds as well. It would also be necessary to repeat the feeding experiment and observation of this type of chicken until the end of laying, listing the eggs laid and other indicators in order to ascertain other additional benefits from the use of this complementary feed. The next step could be to feed two larger groups of birds, 100 or more birds per group.

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Appendix

Changes in chicken gut microbiome caused by feeding supplemented with pine needle extracts

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Abstract
The worldwide development of antibiotic resistant pathogens emerged partly from the use of sub-therapeutic concentrations of antibiotics delivered in poultry feed. This has led to the need for alternative methods to improve the growth and health of poultry. For that reason, feed additives with antibacterial properties are widely used. The effect of dietary supplement of pine needle extract on chicken intestinal health and on the composition of caecal microbiota under non-challenged conditions were investigated. Three groups of chickens with 20 representatives in each were fed with supplemented food in various concentrations for 40 days. Afterwards, the effect of feed additives on the chicken gut was assessed. When pine needle extract was supplemented to the diet in different concentrations, increased relative abundance of *Bacteroidaceae*, *Marinifilaceae*, *Prevotellaceae*, and *Deferribacteraceae* was revealed in the chicken caeca. The opposite trend was observed for the abundance of *Rikenellaceae*, *Barnesiellaceae*, *Campylobacteraceae*, *Methanobacteriaceae*, *Lachnospiraceae*, and *Synergistaceae.* This study demonstrates trends towards desirable changes in the chicken microbiome.

Keywords:
Microbiome, poultry, biological farm, feedstuff, pine needle extracts

1. Introduction

For more than 50 years, antibiotics have been used to enhance the growth and to prevent or reduce the incidence of infectious diseases in poultry and livestock (Ma et al. 2021). Antibiotic usage has enhanced the health and well-being of poultry by reducing the incidence of disease and has facilitated efficient production of poultry. The risk concerning residues of antibiotics in edible tissue and products that can produce allergic or toxic reactions in consumers is known to be negligible because only antibiotics that are not absorbed in the digestive tract were authorized as growth promoters (Donoghue 2003). However, concerns about the development of antimicrobial resistance and the transfer of antibiotic resistance genes from animal to human microbiota led to the withdrawal of approval for antibiotics as growth promoters in the European Union since January 1, 2006 (Castanon 2007). Therefor natural feed additives that improve poultry health and production is needed.

Several growth and health promoting properties have been attributed to certain plant-derived products that could see increased usage in the poultry industry. These benefits are derived by improving the gut health, including increasing the digestibility of feed, modifying digestive secretions, as well as sustaining and improving gut histology. Furthermore, some phytobiotics stabilize the microbiome, thus reducing the production of microbial toxins. The positive effect of phytobiotics is mainly linked to active plant constituents, including terpenoids (mono- and sesquiterpenes, steroids), phenolics (tannins), glycosides, alkaloids that may be present as conjugates, flavonoids, and glucosinolate (Diaz-Sanchez et al. 2015). Various elements of woody herbs (needles, shoots, etc.) in the form of infusions, extracts, and ointments have historically been used in folk and traditional medicine (Bhardwaj et al. 2021). Pine needles, being one of the products derived from coniferous trees, have been used in traditional Chinese medicine to treat diseases, such as wind-cold-dampness arthralgia, traumatic injury, sleeplessness, eczema, and oedema. The medicinal properties of pine needles may be related to their bioactive substances such as carotenoids, terpenoids, phenolic compounds, tannins, and alkaloids (Guo et al. 2018). It has been reported that the pine needles showed anti-inflammatory and anti-bacterial abilities against *E. coli, S. aureus, B. subtilis in vitro* studies (Zeng et al. 2011).

The gut microbiota comprises resident microorganisms in the digestive tract of the host. The gut microbiota is closely linked with host health and disease status. In recent years, a large body of research has demonstrated that diet influences the composition of animal gut microbiota (Chen et al. 2019). Microbiota of the chicken is well differentiated across the gastrointestinal compartments (crop, proventriculus, gizzard, duodenum, ileum, caecum, and the colon) due to different physicochemical conditions, mainly the pH, growth substrate availability, redox potential, and antimicrobial activity of host secretions. Moving down the gastrointestinal tract, the availability of growth substrates decreases (Apajalahti et al. 2016). The crop, proventriculus, and gizzard are dominated by *Lactobacilli* due to the strong selection by pH. Microbiota is significantly diverse only in caecum and colon (Rychlik 2020). Due to the decreasing redox potential from proximal to distal intestine, the proximal intestine also supports the growth of facultative anaerobic bacteria and the small intestines from healthy chickens contain few strict anaerobes, but the caecum and colon are characterized by the presence of rich microbiota dominated by strict anaerobes, which are usually specialized in utilising feed that is not digested by the host, e.g. resistant starch or protein, or carbohydrate fractions excluding starch and free sugars (non-starch polysaccharides) (Apajalahti et al. 2016). The absolute count of microorganisms is low in the small intestine (approx. 105 CFU per gram of digesta), and very high in the caecum (approx. 1010 CFU per gram of digesta and approx. 1000 species). In a healthy adult chicken the caecum is usually colonized by Gram-positive Firmicutes and Gram-negative Bacteroides, which constitute approx. 90% of the microbiota, and the remaining phyla are usually Gram-positive Actinobacteria and Gram-negative Proteobacteria. Although this is the consensus composition of the microbiota in chicken caecum, there can be highly individual variation without signs of abnormality. However, all of these four phyla are always found in caeca of normal adult chickens (Rychlik 2020).

The purpose of this study was to evaluate the effect of dietary supplement of pine needle extract on chicken intestinal health and on the composition of caecal microbiota under non-challenged conditions.

2. Materials and methods

Experimental and sampling procedures

Management of birds was implemented by strictly following the recommendations of the Food and Veterinary Service of the Republic of Latvia. The birds were reared in a biological farm and slaughtered in a poultry farm during the standard process for meat production. The slaughterhouse performed its activity according to the requirements of the Council Regulation (EC) No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing.

Twenty-one-day-old chickens were randomly divided into three groups that correspond to three treatments with twenty birds replicates per treatment. All three groups were reared in the bio-logical farm where birds were kept separately in the specially designed aviaries indoors and outdoors. Feed and water were offered *ad libitum*. The three treatments comprised the following diets: group A received the standard non-supplement diet and acted as a control group; group B was fed standard diet supplemented with pine needle extract in the concentration 30 mg of extract per kg of bird body weight (b.w), group C was fed standard diet supplemented with pine needle extract in the concentration 60 mg extract per kg of b.w. The supplemented feed was prepared once a weak taking into account actual birds' weight, replicates, daily feed intake and time period (Table 1).

The commercially available feed that is intended for feeding laying hens from the first day of life to 8 weeks of age was used as basal diet and it consisted of wheat, oat, soybean, *saccha-romyces cerevisiae*, calcium carbonate, vitamin A, vitamin D3, vitamin E, vitamins B, vitamin K3, and trace elements: iron, zinc, manganese, copper, cobalt, selenium, digestion promoters.

The duration of the experiment was 40 days after which five individuals per each treatment was randomly selected and slaughtered via cervical dislocation to collect caecal contents for micro-biome analysis. In a special sterile box, the samples were sent to the laboratory while packed in ice.

Feed supplemented with pine needle extract

Pine needle extract was obtained from vegetable raw material - green coniferous biomass of Baltic pines (*Pinus sylvestris*). *Pinus sylvestris* have been given the status of Novel Food by European Food Safety Agency (EFSA 2021). Pine needle extract was prepared by the use of organic solvent for the extraction of wood green conifers, followed by the separation of coniferous wax, treatment with aqueous alkaline solutions, acidification with mineral and organic acids, settling, layer-by-layer separation, distillation off the solvent traces (Roshchin et al. 1994). This industrial method makes it possible to obtain the most complete conifer needle extract containing more than one hundred hydrophobic and hydrophilic biologically active compounds in natural ratios. Obtained natural product in the form of dark green mass with characteristic smell and taste of pine is also known as chlorophyll-carotene paste. The main chemical components of pine needle extract are sodium chlorophyllin and other chlorophyll derivates (4-16 g/L, β-carotene and other carotenoids (200-1200 mg/L), vitamin E (300-500 mg/L), vitamin K group (12-20 mg/L), sodium salts of fatty, resin dibasic, oxo- and oxyacids (44-60%), minerals (5-7%), waxes (5-8%), phytosterols, polyprenols, squalene (Bespalov et al. 2006).

Before the experiment it was checked that the composition of needle extract does not change during long-term storage, the growth of pathogenic and fungal flora is not observed. Previous pilot studies (data not published) showed that the optimal dose for consumption was assumed as 30-60 mg pine

needle extract per kg of animal weight. Based on this data the amount of supplement was calculated for feeding.

DNA extraction and 16S rRNA gene sequencing procedure for caecal samples

DNA was extracted from 100 mg of caecal contents using the **ZymoBIOMICS 96 Magbead DNA Kit** from Zymo Research. The sequencing library preparation was performed by following the protocol published by Illumina (document number 15044223 Rev. B). Briefly, the variable regions V3-V4 of bacterial 16S rRNA gene were targeted by primers designed by Klindworth et al. (2013). In a two-step protocol, the first round of PCR amplified the target and added Illumina sequencing adapters. Nextera XT set A barcodes were added during the second round of PCR. KAPA HiFi DNA polymerase was used for all amplification reactions. Sequencing was performed on an Illumina MiSeq, using v3 600-cycle reagent kit to produce 2×300 bp paired-end reads. A DNA extraction negative control and mock community DNA (ATCC MSA-1002) were sequenced along with the sample libraries for quality control purposes.

Bioinformatics data analysis

All of the sequence processing was done within QIIME2 software environment (Bolyen et al. 2019). First, the primer sequences were trimmed from the reads using Cutadapt (Martin 2011). The trimmed reads were denoised with the DADA2 algorithm (Callahan et al. 2016) to produce error-corrected amplicon sequence variants (ASVs). Taxonomic classification of ASVs was performed with VSEARCH (Rognes et al. 2016) against the silva_138_NR99 SSU rRNA database (Quast et al. 2013).

Alpha and beta diversity calculations were performed on normalized ASV feature tables, from which singletons and sequences representing irrelevant taxa (eukaryotes, chloroplasts, and mitochondria) had been removed. For feature count normalization, SRS algorithm (Beule et al. 2020) was used set to 114666 features per sample (the lowest denoised and filtered feature count among all samples). The observed feature count (richness), Shannon's index (Shannon 1948), and Simpson's index (Simpson 1949) were used as metrics of within-sample (alpha) diversity. Between-sample (beta) diversity was calculated as Bray–Curtis dissimilarity (Sørensen 1948) and generalized UniFrac distance (Chen et al. 2012). Spearman's rank correlation tests were performed to assess the relationships between microbiome diversity metrics and feeding supplement dose. A two-sided Mantel test was applied to identify the correlation between beta distances and distances in the received feed supplement concentration. Additionally, denoised read count was used as a variable in the same tests in order to assess the sequencing depth as a confounding factor. Associations between feed supplement dose and abundance of microbiome features were analyzed using general linear models as implemented in the MaAsLin2 R package (Mallick et al. 2021).

3. Results

Summary of 16S rRNA gene sequencing

In total, 6423182 paired-end reads were generated from the fifteen samples, ranging from 361090 to 489066 reads per sample. After trimming and denoising steps, 2,144,509 features remained. The mean number of features per sample was 142,967, from the lowest coverage of 121,506 features to the highest of 158,486. Alpha diversity rarefaction curves were generated to verify that sufficient sequencing depth had been achieved. Saturation of Shannon diversity index and the observed feature

richness was achieved at approximately 10000 and 110000 features, respectively, for all feeding groups (not shown). Examination of read counts and taxonomic profiles of negative control and mock community did not raise any concerns of contamination or poor performance of the sequencing process.

Diversity of caecal microbiota and the trends observed in feeding groups

The major bacterial phyla that were observed were Firmicutes (53.44% average relative abundance), Bacteroidota (33.14%), Actinobacteriota (6.43%), Fusobacteriota (1.60%), and Proteobacteria (1.37%). For the rest of phyla, the abundance was less than 1% on average. The composition of each sample at the phylum level is represented in Fig. 1.

Next, the effect of pine needle extract feeding supplement on the alpha diversity of caecal microbiomes was evaluated. Three different alpha diversity measures were calculated. Spearman correlation revealed a significant relationship ($P = 0.0014$) between the observed feature count and denoised read count per sample, which was expected, as more spurious ASVs can appear with increased depth of sequencing. However, other alpha diversity indices did not correlate with the sequencing depth, thus it was assumed that the other diversity measures were not biased by sample read counts. The observed feature count and Simpson's index did not significantly correlate with the dietary supplement concentration, whereas Shannon's index showed a negative correlation with the dietary supplement dose ($\rho = -0.6236$, $P = 0.013$).

To evaluate the between-sample (beta) diversity, Bray-Curtis and generalized UniFrac distances were calculated between all samples. There was no significant correlation between the beta diversity metrics and read count, thus assuring that these metrics were also not biased according to the sequencing depth. Both Bray-Curtis and generalized UniFrac beta diversity metrics showed a significant positive correlation with the feeding supplement dose ($\rho = 0.473644$, $P = 0.001$ and $\rho = 0.267187$, $P = 0.003$, respectively).

Eleven differently abundant genera in the caecal microbiota from birds fed a pine needle extract-supplemented feed as compared to the control group were identified using a general linear model framework (Table 2). Four of them, belonging to the families of Bacteroidaceae, Marinifilaceae, Prevotellaceae, and Deferribacteraceae, were more abundant when the feed was supplemented with pine needle extract. Seven other genera were less abundant when the birds were fed with supplemented feed: two belonging to the family Rikenellaceae and others belonging to Barnesiellaceae, Campylobacteraceae, Methanobacteriaceae, Lachnospiraceae, and Synergistaceae.

4. Discussion

The microbial community present in the gastrointestinal tract has been widely associated with factors involving the health of chickens, such as the immune system, the physiology of the digestive system, and the exclusion of pathogens, as well as the performance in production. Feed additives are widely used to improve chicken gut health and to stimulate performance.

According to the bacterial meta-analysis of chicken caecal microbiota, *Firmicutes* was the most prevalent phylum, followed by Bacteroidetes and Proteobacteria (Cardenas et al. 2021). In our study, a similar abundance profile was observed for Firmicutes and Bacteroidetes, but the next most abundant

phylum was Actinobacteriota, followed by Fusobacteriota and then Proteobacteria. However, the distribution of Fusobacteriota was very uneven among the chickens tested in our study.

It was observed that Shannon's alpha diversity index significantly correlated with dietary supplement dose while Simpson's index did not, indicating that the shift in microbiome composition affects mainly the minority taxa, not the dominant ones, since Shannon's index gives more weight to species richness and thus is more sensitive towards minor features (Kim et al. 2017). The significant correlation between the beta diversity measures and the difference in the feeding supplement dose received by each chicken demonstrates that a general shift in the caecal microbiome composition can be observed despite the small sample size and notable inter-individual variation in microbiome profiles (see Fig. 1).

Among the four taxa that were more abundant when the feed was supplemented with pine needle extract were a gram-negative genus of *Bacteroides* that are common gut microbiota members in all warm-blooded animals (Kollarcikova et al. 2020). *Bacteroides* play an important role in breaking down complex macromolecules and generate acetate and propionate as major fermentation products (Carrasco et al. 2018). *Odoribacter* is capable of butyrate production via lysine fermentation and succinate reduction (Rychlik 2020). Butyrate, a short-chain fatty acid that directly stimulates an increase in the absorptive surface area, suppresses the growth of zoonotic pathogens, induces the expression of host-defense peptides, and modulates host epigenetic regulation (Gustavo et al. 2020). Chicken isolates belonging to the family Prevotellaceae have not been characterised in detail. *In vitro* culturomics studies have indicated that chicken Prevotellaceae are specialised in the digestion of complex polysaccharides and dominate in the microbiota when feed enriched for vegetable fiber is common (Rychlik 2020). Among the minor phyla associated with the mature microbiota, there was a noteworthy presence of bacteria with the potential to stimulate mucus layer formation, which are therefore associated with a healthy gut, like *Mucispirillum* found only in free-range slow-growing chickens and at higher levels in 81-day-old free-range slow-growing chickens where it was a biomarker (Ocejo et al. 2019).

For a few bacterial taxa belonging to the families Barnesiellaceae and Rikenellaceae, decreased relative abundance was observed when birds were fed with supplemented feed. These taxa belong to the order Bacteroidales that encompass gram-negative anaerobic coccobacilli, with saccharolytic and proteolytic activities. Barnesiellaceae is a proposed taxonomic group that has not been yet characterized. Rikenellaceae have been found enriched in the caeca of mice with high-fat diet-induced obesity and seem to be highly susceptible to perturbations in the gut microbiota, such as those caused by antibiotics or probiotics supplementation (Carrasco et al. 2018).

The same decrease was observed for *Methanobrevibacter.* Many methanogenic archaea, or methanogens, use H2 and CO2 as substrates to synthesize methane. As the only producers of enteric methane, methanogens are responsible for the contribution of livestock industries to climate change and have thus become the focus of research toward developing mitigation strategies. The sequences of 16S rRNA genes closely related to certain species belonging to the genus *Methanobrevibacter* are among the most frequently found sequences in gastrointestinal tract samples from livestock (St-Pierre et al. 2015).

One of the main functions of caecum is bacterial fermentation of indigestible polysaccharides to produce short chain fatty acids that can be absorbed by the host's epithelial cells. This role is fulfilled by certain classes of Firmicutes (such as Lachnospiraceae or Ruminococcaceae) and Bacteroidetes (Richards et al. 2019). The bacteria of Lachnospiraceae family are absent or very sparse in young chickens (Díaz-Sánchez et al. 2019).

There are phyla and genera that may appear in the microbiota of adult hens but are not universally distributed in all individuals, and these include Synergistetes (*Cloacibacillus* sp.) (Rychlik 2020).

The most common cause of bacterial gastroenteritis is campylobacteriosis, which is an infection caused by *Campylobacter* spp. bacteria. A previous study in Latvia that was focussed on the prevalence of *Campylobacter* in broiler production revealed an occurrence rate of 50.6% for *Campylobacter*. Furthermore, *C. jejuni* was the dominant bacterial species and was detected in 95.7% of all *Campylobacter* isolates, while *C. coli* was found only in some samples (Meistereet et al. 2019). Therefore, the reduction of *Campylobacter* loads or even eradication of certain *Campylobacter* species from the chicken gut would entail a benefit for public health.

Notably, the relative abundance of *Campylobacter* in animals receiving 30 mg of pine needle extract per kg of b.w. was still similar to control samples. The abundance of said bacteria decreased significantly only with the higher dose (60 mg/kg), indicating that the lower dose would not be sufficient to achieve the desired effect on the caecal microbiome. This observation regarding the reduction of pathogenic bacteria should be investigated in further studies by using internationally recognized methods for the detection and enumeration of *Campylobacter*, such as those described in ISO 10272.

Conclusions:

Although limited by the small sample size, this study nevertheless demonstrates trends towards dose-dependent desirable changes in chicken microbiome, resulting from supplementing chicken feed with pine needle extract. Some positive outcomes were observed with increasing dose, such as a reduction in the relative abundance of *Campylobacter*. The effect of pine needle extract on *Campylobacter* abundance and prevalence should be investigated further, especially targeting the species that are pathogenic to consumers of poultry.

5. Supporting information

The code and detailed software versions used in the sequence processing and analysis are documented and available at GitHub repository **https://github.com/jkibilds/chicken-feed-microbiome-2021**. All DNA sequencing reads from this study have been deposited at European Nucleotide Archive under accession number PRJEB46042.

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Conflict of interest statement

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Table 1. Preparation of supplemented feed

	Week 1	Week 2	Week 3	Week 4
Average body weight per bird	200 g	270 g	340 g	410 g
Amount of daily intake of feed per bird	24 g	29 g	34 g	38 g
Amount of feed for 20 birds for 7 days	3360 g	4060 g	4760 g	5320 g
Group B (30 mg extract per kg b.w.)				
Amount of daily intake of extract per bird	6.0 mg	8.1 mg	10.2 mg	12.3 mg
Amount of extract for 20 chickens for a week	840 mg	1134 mg	1428 mg	1722 mg
% of supplement in feed	0.025	0.027	0.030	0.032
Group C (60 mg extract per kg b.w.)				
Amount of daily intake of extract per bird	12.0 mg	16.2 mg	20.4 mg	24.6 mg
Amount of extract for 20 chickens for a week	1680 mg	2268 mg	2856 mg	3444 mg
% of supplement in feed	0.050	0.054	0.060	0.064

Table 2. Differentially abundant taxa in the caecal microbiota

Phylum	Class	Order	Family	Genus	Coefficient from linear model	P value	Q value
Bacteroidota	Bacteroidia	Bacteroidales	Bacteroidaceae	*Bacteroides*	0.0543	0.0126	0.1428
Bacteroidota	Bacteroidia	Bacteroidales	Barnesiellaceae		-0.5771	0.0051	0.0956
Bacteroidota	Bacteroidia	Bacteroidales	Marinifilaceae	*Odoribacter*	0.2604	0.0010	0.0520
Bacteroidota	Bacteroidia	Bacteroidales	Prevotellaceae		0.5776	0.0115	0.1428
Bacteroidota	Bacteroidia	Bacteroidales	Rikenellaceae	*Alistipes*	-0.1958	0.0251	0.1963
Bacteroidota	Bacteroidia	Bacteroidales	Rikenellaceae	*Rikenellaceae* RC9 gut group	-0.5106	0.0273	0.1963
Campilobacte-rota	Campylo-bacteria	Campylobactera-les	Campylobactera-ceae	*Campylobacter*	-0.6528	0.0040	0.0956
Deferribacte-rota	Deferribactere	Deferribacterales	Deferribactera-ceae	*Mucispirillum*	0.2749	0.0013	0.0520
Euryarchaeota	Methano-bacteria	Methanobacteria-les	Methanobacteria-ceae	*Methanobrevi-bacter*	-0.1979	0.0261	0.1963
Firmicutes	Clostridia	Lachnospirales	Lachnospiraceae	*Eubacterium hallii* group	-0.1817	0.0060	0.0956
Synergistota	Synergistia	Synergistales	Synergistaceae	*Cloacibacillus*	-0.2144	0.0171	0.1685

Figure caption. Fig. 1. Phylum-level composition of the caecal microbiota of chicken. Three groups (five replicates in each group) represent different treatments, as indicated.