



Article

Changes in the Concentration of Amino Acids and Bacterial Community in the Rumen When Feeding *Artemisia absinthium* and Cobalt Chloride

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Abstract: The aim of the study was to analyze the effect of a plant feed additive based on *Artemisia absinthium* and the metal trace element CoCl_2 (cobalt chloride II) on the metabolism of amino acids closely related to the energy of feed and the bacterial community of the rumen of Kazakh white-headed bulls. Animals were divided into four groups: (A)—the control group of animals received the basic diet (BD), (B)—animals of the experimental group I were additionally given *A. absinthium* at a dose of 2.0 g/kg of dry matter (DM), (C)—II experimental group *A. absinthium* at a dose of 2.0 g/kg DM with additional CoCl_2 (1.5 mg/kg/DM), and (D)—III experimental group was given only CoCl_2 (1.5 mg/kg/DM) to study the rumen metabolism of amino acids and bacterial diversity of animals, rumen cannula were installed, the experiment was carried out using a 4×4 Latin square. It was found that additional feeding of *A. absinthium*, both separately and in combination with CoCl_2 , led to a change in the indices of the alpha biodiversity of the bacterial community. Correlation analysis revealed a linear relationship between the concentration of amino acids and the rumen bacterial community ($p \leq 0.05$). The relationship between the values of amino acid concentrations and certain OTUs was established, with a possible percentile probability of 95% for the genera unclassified Lachnospiraceae, unclassified Clostridiales, unclassified Bacteroidales, Fibrobacter, Ihubacter, Phocaeicola, Paludibacter, Akkermansia, Vampirovibrio, unclassified Ruminococcaceae, and Alistipes. Thus, the use of *A. absinthium* and CoCl_2 as feed additives, both in combination and without, leads to a change in the taxonomic structure affecting the concentration of amino acids. However, further research is needed to better understand the effectiveness and safety of these supplements.

Keywords: bacterial community; rumen; amino acids; cobalt; *Artemisia absinthium*; nitrogen; new generation sequencing



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1. Introduction

An increase in the number of cattle will consistently lead to an increase in the requirements for the use of diets in order to reduce greenhouse gas emissions such as methane [1] and ammonia [2], as well as the loss of nutrients by the body [3]. This process cannot do without the use of special feed additives [4–6], affecting the microbiome in order to increase the functional activity of the digestive system and the rejection of antibiotics [7,8].

An alternative to the use of antibiotics can be herbal supplements [9] containing an extensive group of organic compounds and their combination with trace elements [10,11]. By influencing the microbiome of the body, feed additives are able to improve the use of individual nutrients [12,13], generally improving the use of feed. Thanks to new generation sequencing (NGS), researchers are able to establish taxonomic groups of bacteria, followed by their analysis of functional ability [14,15] responsible for the metabolism or biodegradation of metabolites in the digestive tract of animals [16] and use the microbiome as a tool for regulating nutrition and health of farm animals [17]. Search for alternative natural

sources [18] based on plant additives and mineral substances that improve the use of feed while reducing the production of by-products remains relevant and open.

Artemisia absinthium may be one such source due to its numerous chemical compounds and pharmaceutical properties [19], which can also reduce oxidative stress in the body [20]. Information on the use of *Artemisia absinthium* in cattle feeding is limited. Various organic forms of cobalt can serve as a source of trace elements of the metal, given its connection with the assimilation of nutrients, enzymatic activity, and vitamin B12 [21,22].

Thus, the goal of this study was to determine the potential influence of *Artemisia absinthium*, both alone and in combination with cobalt chloride, on the rumen microbiota and amino acid metabolism in the rumen.

2. Materials and Methods

2.1. Ethical Approval and Informed Consent

The ethical approval of this study was received from the ethics committee of the Federal Research Center of Biological Systems and Agrotechnologies of the Russian Academy of Sciences.

2.2. Feeding and Keeping of Animals

The research was carried out by the Agricultural Animal Feeding and Feed Technology Department, Federal Scientific Center for Biological Systems and Agrotechnologies of the Russian Academy of Sciences, Orenburg, Russia. The research was conducted from 2021 to 2022 as part of the implementation of the project by the Russian Scientific Foundation. Objects of research: bulls ($n = 4$) (Kazakh white-headed breed) aged 13–14 months, with an average live weight of 332–335 kg. During the study, the animals of the experimental groups were fed *Artemisia absinthium herba* (*Asteraceae*) (*A. absinthium*) vegetative parts of plants (pieces of stems, leaves, inflorescences), crushed to a size of 2–4 mm. (Pharmaceutical company “Krasnogorskleksredstva” Moscow region, Krasnogorsk city, Mira str. 25), and cobalt (II) chloride (CoCl_2)—the tablet as an active substance contains: cobalt chloride—40 mg and excipients up to 1 g (manufacturer: LLC NPK “Ascont+”, Moscow, Russia) in the form closest to natural, associated with amino acids and peptides, is a co-factor in enzymes that play an important role in the protective function of the animal body, growth, and reproduction. The animals were fitted with rumen cannulas (“ANKOM Technology 2052 O’Neil Road, Macedon NY 14,502, $d = 80$ mm), while every effort was made to reduce the harm. The experiment was carried out in four repetitions using a 4×4 Latin square. The diet for all animals consisted of 80% coarse feed (legume hay 32.6%, mixed hay 47.4%), grain feed 19.0%, 1.0% mineral additive (premix: calcium 13%, phosphorus 18.5%, sodium 12%, magnesium 3%, vitamins ($\times 1000$): A me 1.200, D3 me 200, E mg 3.4, also B vitamins and trace elements.), the animals had free access to water. The diet was balanced in all nutrients and corresponded to the sex, age, and breed of the animals Kalashnikov et al. (2003) [23]. The diet contains (% DM), dry matter 94.7, crude protein 5.9, crude fiber 36.75, neutral detergent fiber 63.12, acid detergent fiber 46.51, hemicellulose 16.61, crude fat 2.73, organic matter 93.4, calcium 0.51, phosphorus 0.37, crude ash 1.28, nitrogen-free extractives 53.8. A—control group basic diet (BD), animals of experimental group I (B) were additionally given *A. absinthium* at a dose of 2.0 g/kg of dry matter (DM), experimental group II (C) *A. absinthium* at a dose of 2.0 g/kg DM with additional CoCl_2 (1.5 mg/kg/DM), experimental group III (D) was given only CoCl_2 (1.5 mg/kg/DM). The duration of the experiment included a preparatory period of 10 days. During this period, the animals were acclimatized to the tested additive. The test period of 7 days was sampled for analysis, followed by a rest period (break of 1 week), after which they continued the experiment. In particular, the manufacturer recommends injecting cobalt chloride from 1.5 to 16 mg per head per day; our study used the minimum dosage in order to reduce the possible negative effects on the animal’s body, to determine the amino acid composition, 3 h after feeding, ruminal fluid was taken from experimental bulls from three independent spatial sites of the rumen, into a sterile container (www.biobanka.ru, accessed on 1 October 2015) (LLC “SZPI”, Samara, Russia) for the collection of biomaterial

100 mL (n = 3) with a tightly fitting the lid and the thermos bag (TM-1, LLC “Medapharm”, Moscow, Russia) were delivered to the test center.

2.3. Amino Acid Analysis

Method of measuring the mass fraction of amino acids by capillary electrophoresis using the capillary electrophoresis system “Drops” M 04-38-2009. To determine the amino acid composition, 3 h after feeding, the rumen fluid was taken from the experimental bulls from three independent spatial sites of the rumen into a sterile container (LLC “SZPI”, Samara, Russia) for the collection of biomaterial 100 mL (n = 3) with a tightly fitting lid and in a thermal container (TM-1, LLC “Medapharm”, Moscow, Russia) was delivered to the test center. When studying the amino acid composition, the mass fraction of arginine, lysine, tyrosine, phenylalanine, histidine, leucine, isoleucine (in total), methionine, valine, proline, threonine, serine, alanine, and glycine in the form of phenylisothiocarbonyl derivatives is considered. The measurement technique allows for determining the total content (free and bound forms in total) of individual amino acids in samples. The sample (100.0 ± 0.2) mg is placed in a hydrolysis vial, and 10 cm³ of hydrochloric acid (diluted 1:1) is added, hermetically sealed, and mixed. The studied samples are subjected to acid hydrolysis at a temperature of 110 °C for 14–16 h. Hydrolysis is carried out in a drying cabinet (SHS-80-01 SPU, OJSC SCTC SPY, Smolensk, Russia). At the end of hydrolysis, the contents of the vial are cooled and filtered through a “blue ribbon” filter (Filtrlab, LLC “Melior 21”, Moscow, Russia). Next, they proceed to the production of the FTC derivative, for which 50 mm³ of hydrolysate is taken into a glass box, the solution is evaporated dry in a stream of warm air, after which 150 mm³ of sodium carbonate solution is added to the dry residue (m.k.—mass concentration 0.1 mol/dm³), 300 mm³ of FITZ solution (phenylisothiocyanate in isopropyl alcohol), stir and leave for 35 min. Next, the solution is evaporated dry in a stream of warm air. The dry residue is dissolved in 500 mm³ of distilled water. The solution obtained for analysis is transferred to an Eppendorf test tube, centrifuged (5 min, 5000 rpm). The amino acid composition is evaluated using the capillary electrophoresis system (Drops 105/105M, Lumex-Marketing LLC, St Petersburg, Russia). The mass share of each amino acid is calculated as a percentage of the total sample.

2.4. Extraction of Total DNA

A sampling of the contents of the rumen was carried out on the 7th day after feeding with experimental additives with an Ecohim OPA-2-20 dispenser syringe in 1.5 mL Eppendorf-type micro-samples containing the DNA/RNA Shield preservative (Zymo Research, Irvine, CA, USA). For analysis, 1.5 mL of the substrate of the rumen fluid was taken, three samples for each experimental group.

Samples were homogenized on a TissueLyser LT (Qiagen, Hilden, Germany) with a Lysing Matrix Y (MP Biomedicals, Solon, OH, USA). DNA extraction from samples was performed using Fast DNA[®] SPIN Kit for Faeces (MP Biomedicals Inc., Solon, OH, USA) according to the manufacturer’s instructions. The quality of the extracted DNA was assessed with electrophoresis in 1% agarose gel and a Nanodrop 8000 (Thermo Fisher Scientific, Waltham, MA, USA). The DNA concentration was quantified using a Qubit 4 Fluorometer (Life Technologies, Carlsbad, CA, USA) with dsDNA High Sensitivity Assay Kit (Life Technologies, Carlsbad, CA, USA).

2.5. Library Preparation and Sequencing

Preparation of the DNA libraries was performed according to the Illumina protocol (Part #15044223, Rev. B.) with primers targeting the V3–V4 regions of the SSU ribosomal RNA (rRNA) gene, S-D-Bact-0341-b-S-17 (5′-CCTACGGGNGGCWGCAG-3′) as the forward primer and S-D-Bact-0785-a-A-21 (5′-GACTACHVGGGTATCTAATCC-3′) as the reverse primer [24]. The reaction mixture (25 µL) contained both primers, 0.2 µM each; 80 µM dNTPs; 0.2 U Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA). The following PCR program was used: 95 °C for 3 min, 25 cycles: 95 °C for 30 s,

56 °C for 30 s, 72 °C for 30 s, and final extension 72 °C for 5 min. The DNA libraries were cleaned up using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA) and were validated with capillary electrophoresis on a Qiaxcel Advanced System (Qiagen, Hilden, Germany) using the QIAxcel DNA Screening Kit (Qiagen, Hilden, Germany). Paired-end 2 × 300 bp sequencing was performed on the MiSeq platform (Illumina, San Diego, CA, USA) with the Reagent Kit v.3 (Illumina, San Diego, CA, USA). DNA library preparation, sequencing, and bioinformatics treatment were performed in the Center of Shared Scientific Equipment “Persistence of microorganisms” of the Institute for Cellular and Intracellular Symbiosis UrB RAS (Orenburg, Russia).

2.6. Bioinformatic Processing of Sequencing Data

Checking the quality of the source reads using the FastQC program (V. 0.11.9) [<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>] (accessed on 8 January 2019). The trimming of adapters was carried out by the program cutadapt 1.9.1. [25]. After removing the adapters, the reads were re-processed by the FastQC program (V. 0.11.9) to determine the parameters of subsequent processing. All subsequent processing steps are carried out by the USEARCH V program. 11.0.667, using the UPARSE algorithm [26]. The merging of the left and right reads was carried out with the command `-fastq_mergepairs`. The following parameters were used: `-fastq_maxdiffs 10 -fastq_pctid 80`. Filtering was carried out using the command `-fastq_filter` with the parameters `-fastq_maxee 1.0` (the maximum expected reading error does not exceed 1 per 100 nucleotides) `-fastq_minlen 400` (the minimum length of the sequence is 400 bp). The filtered reads are duplicated by the command `-fastx_uniques` in order to determine the unique sequences and their number. Clustering and removal of chimeric sequences, according to the UPARSE algorithm, was carried out using the command `-cluster_otus` and operational taxonomic units (OTU) were obtained at a similarity level of 97%. To determine the representation of certain OTU in the samples, global alignment was performed using the command `-usearch_global`. The taxonomic identification of the obtained OTU was determined using the RDP database (release 11.6) [27]. For OTU with low identification support on the specified database, identification was performed in the NCBI database using the BLAST program [<https://blast.ncbi.nlm.nih.gov/Blast.cgi>] (V. 2.13.0.) (accessed on 11 March 2022). Singletons and doubletons (sequences occurring once or twice) were removed from further analysis. The sequencing results were processed using the Microsoft Excel 16 data analysis package, Microsoft Office software 16.0 (USA).

2.7. Statistical Analysis

Numerical data were processed using the SPSS “Statistics 20” program (IBM, USA), calculated mean (M), standard deviations ($\pm\sigma$), and standard deviation errors ($\pm SE$). To compare the obtained results of amino acid concentrations, the student’s *t*-test and Fisher’s F-test, group differences in amino acid concentrations, ANCOVA analysis method were used. The differences were considered statistically significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$. The Shannon (H), Simpson (D), and Pielu (E) biodiversity indices were also calculated using the Past 4.13 program (Developer: Professor Oyvind Hammer, Oslo, Norway). The analysis of the relationship between the proportions in the bacterial community of bacteria and the concentration of amino acids was calculated using the BioStat statistical analysis package (AnalystSoft Inc. 1032 E BRANDON BLVD #1403 BRANDON, FL 33511) 5.9.8.5, where the values of OTU and amino acid concentrations were used as variables, the value of $X_{(OTU)}$ was determined at which the probability of the bacterial community of the rumen influencing the concentration of amino acids is 0.95 percentile. The graphic material presented in the article is built on an open-source data visualization platform [28].

3. Results

From the results of sequencing of the new generation (NGS) of the rumen content of the control and experimental groups of animals, a difference in the bacterial community was established. In the control group, the predominant proportion of microorganisms in

the rumen bacterial community belonged to two phyla, *Bacteroidetes* and *Firmicutes*, and their ratio was 1/3. The dominant microorganisms for the *Bacteroidetes* phylum in the control group were represented by the genera: *Mediterranea*, *Prevotella*, and unclassified *Prevotellaceae*. The genera of bacteria prevailed in *Firmicutes*: *Butyrivibrio*, unclassified *Lachnospiraceae*, *Ruminococcus*, *Saccharofermentans*, unclassified *Ruminococcaceae*, unclassified *Clostridiales* Figure 1. In experimental groups, animals receiving additional wormwood (*A. absinthium*) separately and in combination with cobalt (CoCl_2) had a shift in the proportion of microorganisms in the rumen bacterial community towards an increase in gram-negative bacteria and a decrease in the proportion of gram-positive microorganisms. In the group of animals receiving only wormwood (*A. absinthium*), an increase in the proportion of microorganisms for the phylum *Verrucomicrobia* and phylum *Bacteroidetes* was noted in the rumen bacterial community. When using wormwood (*A. absinthium*), the proportion of bacteria for genera increased: *Akkermansia*, *Phocaeicola*, *Alistipes*, unclassified *Bacteroidales*, unclassified *Ruminococcaceae* with a slight decrease in the proportion of bacteria for the genera *Prevotella*, *Fibrobacter*, *Butyrivibrio*. Similar results were obtained for the group of animals receiving additional wormwood (*A. absinthium*) and cobalt (CoCl_2), only cobalt (CoCl_2). In these groups, there was also an increase in the proportion of gram-negative bacteria and a decrease in the proportion of gram-positive microorganisms. A characteristic difference in the experimental groups was that the use of wormwood (*A. absinthium*) alone or together with cobalt (CoCl_2) led to an increase in the proportion of microorganisms for genera: *Akkermansia*, *Alistipes*, *Phocaeicola*, unclassified *Ruminococcaceae*, unclassified *Bacteroidales* and a decrease in the abundance of bacteria genera: *Prevotella*, unclassified *Lachnospiraceae*, unclassified *Clostridiales* Figure 1.



Figure 1. Relative abundance of biodiversity at the phylum level, (A)—control group, (B)—I experimental group—*A. absinthil*; (C)—II experimental group—*A. absinthil* + CoCl_2 ; (D)—III experimental group— CoCl_2 .

Changes in the abundance of the bacterial rumen community at the level of the genus are shown in Figure 2. The figure clearly shows a decrease in the bacterial community of the rumen in the experimental groups of the bacteria of the genus *Prevotella*, *Butyrivibrio*, unclassified *Lachnospiraceae*, and unclassified *Clostridiales* with the simultaneous increase of bacteria in the genus *Mediterranea*, unclassified *Ruminococcaceae*, and unclassified *Bacteroidales*.

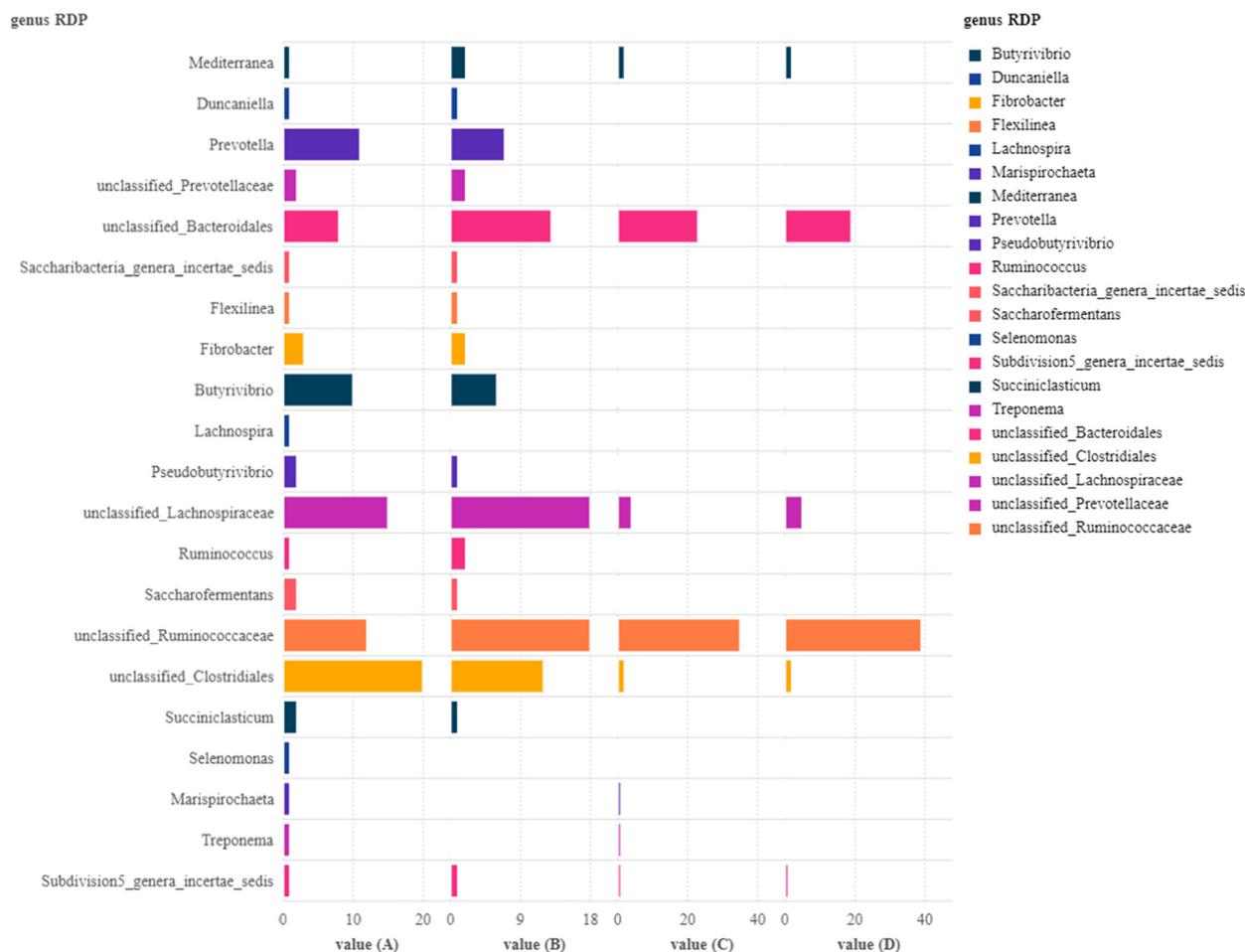


Figure 2. Relative abundance of biodiversity at the genus level, (A)—control group, (B)—I experimental group—*A. absinthil*; (C)—II experimental group—*A. absinthil* + CoCl_2 ; (D)—III experimental group— CoCl_2 .

Based on the results of sequencing of a new generation (NGS) of the rumen content of experimental animals, the indices of α -diversity were calculated. The use of wormwood (*A. absinthium*) in the first experimental group led to an increase in the number of species with a simultaneous decrease in the proportion of individual microorganisms. The Shannon index (H) in the first experimental group was 5.8% higher than in the control group. In the II and III experimental groups, the number of species decreased relative to the control group by 12.2% and 2.4%, respectively, while the Shannon index (H) for these groups was higher than the control value by 5.4% and 5.6%. To assess the uniformity of the distribution of species in the community, the Simpson (D) direct index was calculated. Unlike the control group, this index in the experimental groups was 50% lower. The decrease in the Simpson index in the experimental groups characterizes a uniform distribution of species with a uniform distribution (dominance) of microorganisms Table 1.

Table 1. Indices of alpha—biodiversity of the bacterial community of the rumen of cattle under the influence of feed substances, sample size n = 1589.

Indices	Groups			
	A	B	C	D
Taxa S	465.1 ± 39.6	657.3 ± 43.0	408.6 ± 15.5	454.1 ± 36.0 *
Individuals	17,640.6 ± 547.4	15,727.7 ± 24.6 *	8129.7 ± 580.6	10,762.0 ± 1417.1 *
Dominance D	0.04 ± 0.0081	0.02 ± 0.0044 *	0.02 ± 0.0008	0.02 ± 0.0011
Shannon H	4.86 ± 0.17	5.16 ± 0.16 **	5.14 ± 0.02	5.15 ± 0.07 *
Pielou's evenness	0.24 ± 0.015	0.28 ± 0.024	0.42 ± 0.011 *	0.39 ± 0.008 *

Note: *— $p \leq 0.05$; **— $p \leq 0.01$ in comparison with the control group; A—control group, B—I experimental group—*A. absinthil*; C—II experimental group—*A. absinthil* + CoCl_2 ; D—III experimental group— CoCl_2 .

During the analysis, 12 proteinogenic amino acids were identified, including essential ones. The identified amino acids were classified into groups according to the possible nature of catabolism in animals Table 2.

Table 2. The concentration of amino acids in the rumen fluid of cattle in vivo sample size n = 21, (M ± m), %.

Amino Acids	Groups				
	A	B	C	D	
Glycogenic	arginine	5.7 ± 0.06	3.7 ± 0.04 *	3.5 ± 0.07	4.3 ± 0.02
	histidine	1.8 ± 0.02	0.8 ± 0.01	1.1 ± 0.03	1.2 ± 0.04
	valine	6.8 ± 0.03	3.0 ± 0.01 *	3.4 ± 0.06 *	4.1 ± 0.01 *
	alanine	7.9 ± 0.02	3.0 ± 0.01 **	3.5 ± 0.05 *	4.3 ± 0.02 **
	glycine	5.8 ± 0.07	2.9 ± 0.04	3.4 ± 0.02	4.0 ± 0.01
	threonine	6.0 ± 0.05	2.9 ± 0.07	3.6 ± 0.06	3.9 ± 0.05
	serine	5.3 ± 0.02	2.5 ± 0.01	3.1 ± 0.03	3.6 ± 0.08
	methionine	3.5 ± 0.04	1.4 ± 0.02 *	1.5 ± 0.02 *	1.7 ± 0.03 *
Ketogenic	lysine	8.5 ± 0.05	3.7 ± 0.04 *	3.8 ± 0.03	4.8 ± 0.05 *
Gluco-ketogenic	tyrosine	4.7 ± 0.04	2.5 ± 0.02	2.9 ± 0.05	3.2 ± 0.07
	phenylalanine	5.0 ± 0.03	2.3 ± 0.07	2.8 ± 0.04	3.2 ± 0.05
	leucine + isoleucine	1.6 ± 0.01	0.74 ± 0.02 **	0.87 ± 0.02 *	0.97 ± 0.04 *

Note: *— $p \leq 0.05$; **— $p \leq 0.01$ in comparison with the control group; A—control group, B—I experimental group—*A. absinthil*; C—II experimental group—*A. absinthil* + CoCl_2 ; D—III experimental group— CoCl_2 .

Analysis of the results of the amino acid content in the rumen fluid showed a decrease in the concentration of amino acids in the I, II, and III experimental groups relative to the control groups Figure 3. The statistical significance of the difference in the concentration of amino acids between the groups in the Student's test and in the assessment of reliability according to the Fisher criterion for the control and I experimental groups was $t = 4.07$ with differences $p = 0.0005$ and $F = 4.55$ with $p = 0.018$, between the control and II experimental groups, the Student's criterion $t = 3.55$ at $p = 0.0017$, the Fisher criterion $F = 4.23$ at $p = 0.024$, statistical differences in amino acid concentrations between the control and III experimental groups were according to the Student's criterion $t = 2.71$ at $p = 0.012$ according to the Fisher criterion $F = 2.7$ at $p = 0.11$. The ANCOVA analysis revealed a statistically significant difference in amino acid concentrations between the II and III experimental groups, the coefficient $F = 48.2$ at $p = 7.391 \times 10^{-0.7}$.

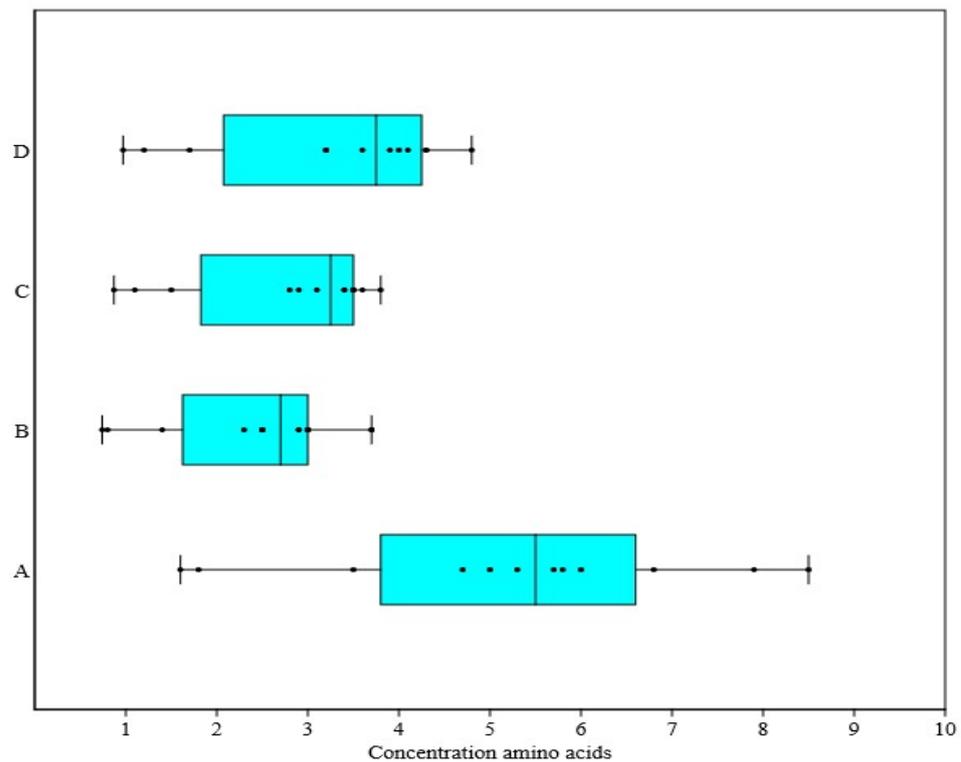


Figure 3. Changes in the concentration of amino acids in the rumen fluid (horizontal lines), the line in the rectangle is the median, and the rectangle is a 25–75 percentile span. (A)—control group, (B)—I experimental group—*A. absinthil*; (C)—II experimental group—*A. absinthil* + CoCl_2 ; (D)—III experimental group— CoCl_2 .

Analysis of the Pearson linear relationship between biodiversity indicators and the concentration of amino acids in the rumen fluid revealed a positive correlation in the control group, the Pearson correlation coefficient $R = 0.43$ at $p = 0.16$ Figure 4a. Determination of the relationships between the values of amino acid concentrations and certain OTUs in the control group revealed a possible probability of the influence of the rumen bacterial community with a 95% percentile probability for the genera *Prevotella*, *Sporobacter*, unclassified *Ruminococcaceae*, unclassified *Lachnospiraceae*, unclassified *Muribaculaceae*, with a 99% percentile probability for the genera *Prevotella* and unclassified *Lachnospiraceae*, the value at which the probability of effects of the rumen bacterial community on the concentration of amino acids is 0.95 percentile $X_{(\text{OTU})} = 41.5$, so 95% of the OTU identified in the control group that could affect the concentration of amino acids had values greater than 41.5 Figure 5a.

In experimental group I, when using *A. absinthium*, the correlation was negative, with the Pearson coefficient $R = -0.18$ at $p = 0.57$ Figure 4b. A decrease in the concentration of amino acids in experimental group I and changes in the structure of the rumen bacterial community at the level of genera with a 95% percentile probability refers to the genera unclassified *Lachnospiraceae*, unclassified *Clostridiales*, unclassified *Bacteroidales*, *Fibrobacter*, *Ihubacter*, *Phocaeicola*, *Paludibacter*, *Akkermansia*, *Vampirovibrio*, unclassified *Ruminococcaceae*, *Alistipes*. The value at which the probability of the bacterial community influencing the amino acid concentration is 0.95 percentile $X_{(\text{OTU})} = 39.5$ Figure 5b.

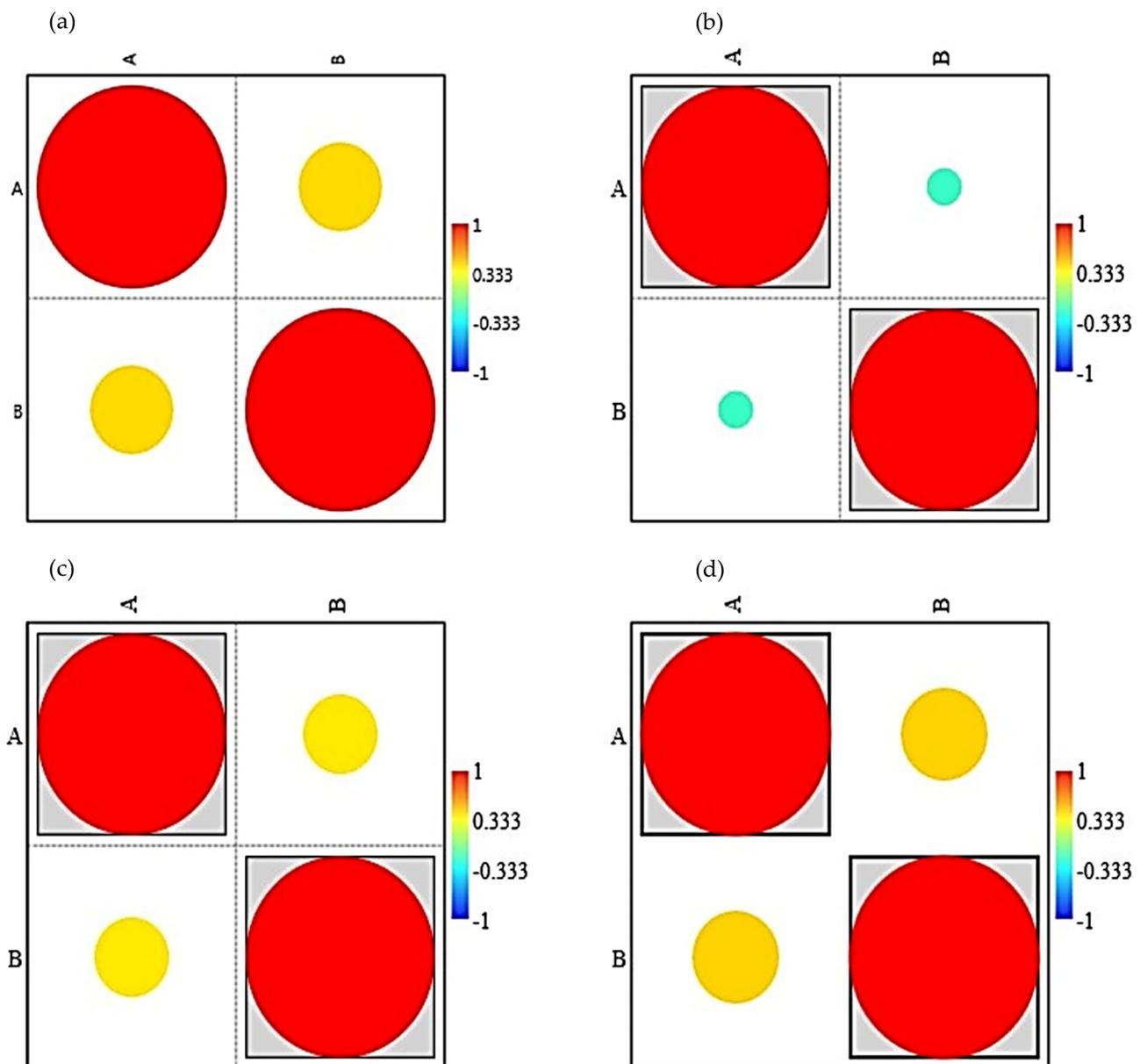


Figure 4. Pearson correlation of a possible linear relationship between the rumen bacterial community and amino acid concentration. (A)—concentration of amino acids, (B)—OTUs; (a)—control group, (b)—I experimental group—*A. absinthil*; (c)—II experimental group—*A. absinthil* + CoCl_2 ; (d)—III experimental group— CoCl_2 .

The combination of wormwood and cobalt salts (*A. absinthium* + CoCl_2) in the II experimental group established the significance of the correlation at the level of 5%, while the Pearson coefficient is equal to $R = 0.39$ at $p = 0.20$ Figure 4c. The relationship of rumen bacteria and the concentration of amino acids in group II with a 95% percentile probability to the genera of unclassified *Ruminococcaceae*, *Ihubacter*, unclassified *Lachnospiraceae*, unclassified *Rikenellaceae*, unclassified *Bacteroidales*. The value at which the probability of the rumen bacterial community influencing the concentration of amino acids is 0.95 percentile $X_{(\text{OTU})} = 29.3$ Figure 5c.

The analysis of the linear relationship in the III experimental group with the use of cobalt salts (CoCl_2) alone established a positive correlation, while the Pearson coefficient was equal to $R = 0.45$ at $p = 0.1443$ Figure 4d. The percentile of a possible relationship of 95% included the genera unclassified *Ruminococcaceae*, *Roseburia*, *Paludibacter*, *Monoglobus*, *Mediterraneibacter*, *Mediterranea*, and *Phocaeicola*. The value at which the probability of the

rumen bacterial community influencing the concentration of amino acids is 0.95 percentile $X_{(OTU)} = 40.9$. In this experimental group, OTU values less than 40.9 were not included in the probability of 0.95 percentile Figure 5d.

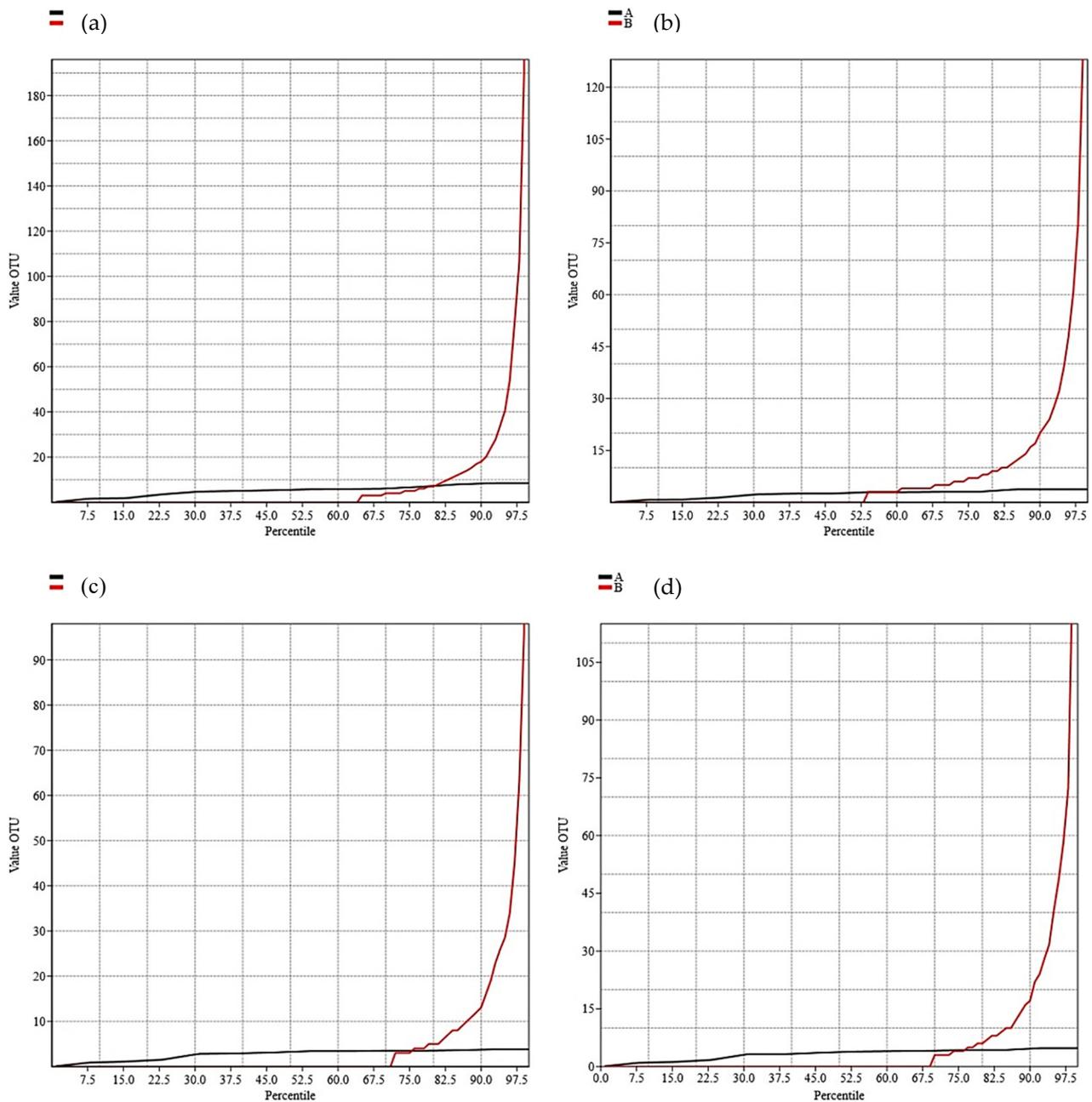


Figure 5. Relationship between OTUs and amino acid concentration, percentile rank. (A)—is the OTUs value, (B)—is 0.95 percentile; (a)—control group, (b)—I experimental group—*A. absinthil*; (c)—II experimental group—*A. absinthil* + $CoCl_2$; (d)—III experimental group— $CoCl_2$.

4. Discussion

Evaluating the results obtained during the experiment on the composition of the rumen microbiota, it should be noted that in the experimental groups, when using *A. absinthium*, there was a decrease in the proportion of bacteria in the rumen bacterial community. At the genus level, unclassified *Lachnospiraceae*, *Clostridiales*, *Prevotella*, and *Fibrobacter* were not classified. There was a simultaneous increase in the proportion of bacterial genera in the rumen bacterial community of unclassified *Bacteroidales*, *Ihubacter*, *Phocaeicola*, *Paludibacter*, *Akkermansia*, *Vampirovibrio*, *Ruminococcaceae*, and *Alistipes*, which were not

classified. Definition *A. absinthium* and cobalt have led to an increase in the bacterial genus of the unclassified *Rikenellaceae*. The use of cobalt has led to an increase in the number of bacterial genera *Roseburia*, *Paludibacter*, *Monoglobus*, *Mediterraneibacter*, *Mediterranea*, and *Phocaeicola*. Changes may occur using *A. absinthium*. Some known bacterial species, such as *Alistipes* and *Bacteroides*, are resistant to bile acids [29], and some *Artemisia* enhances the functional activity of the liver, increasing the synthesis of bile acids [30].

Also, in the studies [31], it was reliably established that the average daily increase in bulls was higher with increased content of tauroursodeoxycholic and kynurenic acid in the rumen, metabolites of the hepatotropic effect involved in the formation of other substances that perform a signaling function in cells [32]. The use of plants or extracts based on them in combination with trace elements of metals also affect the biodiversity of the rumen [33]. In the study, in all experimental groups, there was a decrease in the proportion of bacteria of the genus unclassified *Clostridiales*. There are similar research results when using plants or their extracts containing active components in the diet [34]. A decrease in the proportion of bacteria in the microbiome of the unclassified *Clostridiales* rumen may also have occurred due to a decrease in the concentration of amino acids, which in turn are capable of biodegradation of nitrogen-containing organic substances, which are amino acids, using nitrogen in the production of ammonia [35]. For example, the species *Clostridium propionicum* uses alanine as the only fermentation substrate, and the species *Eubacterium acidaminophilum* uses the amino acid glycine as a substrate [36]. As the analysis of 16S-rRNA shows, almost all microorganisms capable of fermenting amino acids belong to *Clostridium* and related bacteria. However, another study claims that there is no strict relationship between the species of microorganisms and the biosynthesis or catabolism of amino acids [37]. Another study indicates a link between disorders of the digestive system of the body with a high content of clostridium associated with the production of toxins in both monogastric and polygastric animals [38]. In a previously published study [39] studying the enzymatic characteristics of the rumen when using *A. absinthium* both separately and in combination with cobalt, a decrease in the concentration of the ammonia and urea forms of nitrogen and an increase in the protein form was found, which may also indirectly confirm the use of *A. absinthium* and cobalt as stimulating additives to cattle, improving the use of nitrogen in the rumen and reducing its emission. During the experiments, a decrease in the concentration of amino acids in the experimental groups was found. Perhaps this is indirectly due to an increase in the proportion of bacteria capable of having proteolytic and peptidolytic activity. The proportion of gram-negative bacteria with specific enzymatic activity capable of producing CAZymes increased [40]. Also, when evaluating the results of the biodiversity of the rumen microbiota in the study, with the KEGG pathway database predicting the signs of gene families, the genera of bacteria that affect the biodegradation of amino acids were identified, catabolism of individual amino acids was closely associated with such rumen microorganisms as *Ihubacter*, *Phocaeicola*, *Paludibacter*, *Akkermansia*, unclassified *Ruminococcaceae*, *Alistipes*, *Roseburia*, *Paludibacter*, *Monoglobus*, *Mediterraneibacter*, *Mediterranea*. On the other hand, a decrease in the concentration of amino acids in the experimental groups could occur due to a decrease in the proportion of *Prevotella* bacteria, which have proteolytic activity, hydrolyzing proteins to amino acids [41–43]. It is worth noting that the accelerated biodegradation of amino acids leads to the saturation of the body with energy and the formation of other important metabolites [44] on the way. Acetate and butyrate pay great attention to the genus *Alistipes* and *Akkermansia* [45,46]. The metabolism and biodegradation of amino acids are closely related to the energy of the feed and the energy balance of the body [47,48].

Some *Artemisiae* species have an antioxidant effect as well as antimicrobial activity against microbes [49] and against fungi [50]. Feeding *Artemisiae absinthium* changes the ratio of the proportion of bacteria in the rumen microbiome towards increasing bacteria that are able to synthesize microbial nitrogen more efficiently as well as reducing the proportion of bacteria that are able to use nitrogen as a future source of ammonia [51].

5. Conclusions

Investigating *A. absinthium* and cobalt compounds as alternative natural sources of feed additives for cattle revealed a change in the bacterial community of the rumen, which affected the metabolism of amino acids and, accordingly, nitrogen losses. A connection between the rumen microbiome and amino acid biosynthesis has been found, and individual taxa potentially affecting biodegradation have been identified with high probability. Further research is required to study the effect of plant substances or extracts based on them in combination with trace elements of metals on potential taxonomic groups, which in turn affect the metabolism in the rumen, improving the use of animal feed.

Author Contributions: V.R. was responsible for the concept and design of the study. G.D. coordinated the overall analysis. E.T. was responsible for the bioinformatic processing of new-generation sequencing data and, visualization of taxonomic data, calculation of diversity indices. V.K. analyzed the dependencies between the concentration of amino acids and the microbiome of the scar. I.M. was responsible for the final design and editing of the article. All authors have read and agreed to the published version of the manuscript.

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