SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Investigation of the effect of bioactive component-rich nutraceuticals on broiler chicken gut microbiome by next-generation sequencing

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The Examination takes place at Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen at 10:00 a.m. on 22 th of June, 2023

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The PhD Defense takes place at the Lecture Hall of In Vitro Diagnostics Building, Faculty of Medicine, University of Debrecen, at 12:00 a.m. on 22 th of June, 2023

1. Introduction

Microbes are present everywhere. Countless symbiotic, pathogenic, and commensal microbes colonize human, animal, and plant organisms, which together constitute the microbiome.

The widespread use of next-generation sequencing (NGS) technologies has led to an explosion of interest in understanding the microbiome. With the advent of NGS technologies, it has become possible to comprehensively map the individual members of a given microbiome and to investigate how microbial communities function and influence host-pathogen interactions.

The various NGS methods have their own technical pitfalls, which can affect the obtained sequencing results.

Different metagenome isolation strategies can lead to overrepresentation of some taxa and loss of low-abundance species, thereby affecting relative abundance of microbes and community diversities. Newer and newer methods for DNA isolation are still emerging, however, a universally accepted protocol that would be suitable for 16S ribosomal RNA (16 S rRNA) amplicon gene-based sequencing is still not available.

Due to the facts listed above, it is essential to compare the different DNA isolation protocols and determine their advantages and disadvantages in order to assess the exact microbial composition.

Research on microbial ecosystems is an important field of science, and intensive studies are underway to understand the physiological effects of community composition on the host.

Microbiome research has been a focus of major research efforts in livestock animals, since it has a positive impact on the immune system, the physiology of the GIT, nutrition, detoxification of certain compounds and productivity.

As a result of the applied intensive rearing and feeding technology, the weight gains of the livestock animals are extremely rapid. One of the best examples of this is the Ross 308 broiler chicken, which is a fast-growing hybrid. Broiler chickens are able to gain a high body weight (~3 kg) within 42 days with proper feed and breeding system. However, the consequence of this intensive, extreme growth rate is high predisposition to pulmonary arterial hypertension, heart failure and insulin resistance. In addition, some other negative effects can also occur during development, like increased susceptibility to pathogenic infections or altered gut-microbiome composition.

In addition, in industrial-scale livestock husbandry large numbers of animals are rearing in relatively small areas, which increases the incidence of infectious diseases.

Farmers try to avoid infectious diseases by using antibiotic treatments.
Continued treatment can lead to broad-spectrum antibiotic resistance and disruption of the gut microbiota. Seven hundred thousand people die every year from antibiotic resistant infections, and unless there is a substantial change in the use of antibiotics, it will become one of the leading causes of death worldwide in the future.
There is growing body of evidence that abnormalities in microbiota composition can have a major role in the development of metabolic disorders.
With the right knowledge, selectively influencing the composition and/or biological potential of the gut microbiome can help to maintenance a balanced microbiome, which has a positive effect on the health and immune status of animals. The development of healthy food products that support the normal functions of the gut microbiota with some innovative health-promoting ingredient is of great importance. Currently, active ingredients rich in plant bioactive compounds (nutraceuticals) are widely used to maintain the health of humans and animals.

2. Aims

- Our first aim was to investigate the impact of different technical distortion factors (sample homogenization, cell disruption, DNA isolation, taxonomic database) on the results of 16 S rRNA gene amplicon sequencing (quality reads, diversity indices, microbial composition). We aimed to optimize the various metagenomic DNA isolation methods. Furthermore, I compared the suitability of the most frequently used taxonomic databases (Silva, GreenGenes) for the resolution of 16 S rRNA gene amplicon sequencing.
- The second main objective of my research was to investigate the effects of nutraceuticals (carotenoids, fructooligosaccharides, symbiotics and anthocyanins) on the gut microbiome composition and diversity of broiler chickens using 16 S rRNA amplicon gene sequencing. I investigated which bacterial taxa were significantly altered by nutraceutical treatment. Our aim was to optimize a method that can be used to restore and maintain the balance of the microbiome damaged as a result of increased stress and weight gain during large-scale animal husbandry. A further objective was to minimize the use of antibiotic treatments in large-scale farming protocols by improving animal health.

3. Materials and Methods

3.1 Study of the effect of technical distortion factors on sequencing result

In our experiments, we compared two homogenization strategies, direct direct lysis (DL) and indirect bacterial cell suspension (BS) method. For cell disruption, mechanical, chemical and mixed lyses were used. For DNA extraction we compared automated robotic, commercially available isolation kits and conventional isolation techniques based on phenol-chloroform-isoamyl alcohol (25:24:1). Following DNA isolation, a 16 S rRNA gene-based metagenomic library was according to the Illumina library construction protocol. We performed paired-end (2x301 nucleotides) sequencing using Illumina MiSeq platform.

3.1.1 Sample collection

To investigate the effect of different DNA purification strategies on the amplicon sequencing results of 16 S rRNA gene amplicons, faecal samples from 31-day-old broiler Ross 308 hybrid chickens were used. Broiler (Gallus gallus domesticus, Ross 308 hybrid) fecal samples were collected from Nagisz Zrt. Individual fecal samples were collected (5 g of each) from eight randomly selected chicken; 4 pullets (female) and 4 cockerels (male). The freshly collected stool samples were stored in specific, sterile, DNase free stool transportation bowls and stored at 4 °C for maximum 3 hours. A composite fecal sample was made by pooling the individually collected feces and thoroughly mixed.

3.1.2 Sample homogenization

We compared two different sample homogenization strategies (direct and indirect methods). In the indirect method, a bacterial cell suspension (BS) was prepared, where the bacteria present in the feces, representing the microbial community of the intestinal flora, were separeted from the feces and analyzed. To obtain BS 15-15 g specimens from different portions of the broiler stool samples were transferred into 50 ml sterile, safe-lock-cap falcon tubes and 15-15 ml of PBS buffers were added. Samples were homogenized for 15 min (vortex at 350 rpm) and centrifuged for 5 min at 500 × g. These washing steps were repeated three times. Supernatants were collected and centrifuged at 13.000 × g for 20 minutes to collect the bacterial cells. Finally, the supernatants were discarded and the pellets were dissolved in 3 ml PBS. In every case 250 µl of BS was used for metagenomic DNA purification. During the direct method (direct lysis), the microbes of the feces were not separated from the other constituents of the feces. After homogenization, 250 mg was taken from the pooled sample and then the samples were lysed (DL).

3.1.3 Cell lyses

Mechanical cell lyses: During mechanical lysis, three commercially available ceramic beads - Power Fecal Bead LightCycler®, SeptiFast Lysis kit and MagNa Lyser Green beads - were used. Furthermore, we used a standard laboratory vortex (10 minutes at 10 or 3 minutes at 10) and/or a MagNa Lyser (30 seconds at 5000 rpm) to disrupt the cells. 250 µL cell suspension and 250 mg direct lysis were used to extract the cells from the samples. After the cell extraction, the samples were centrifuged at 16,000 g for 1 minute at 4ºC, then the supernatant was pipetted into a sterile eppendorf tube.

Chemical cell lyses: Performing chemical lysis a lysis mixture of 500 µl lysis buffer and 60 µl proteinase K was prepared. To perform chemical lysis, commercially available ATL (Tissue Lysis Buffer) and BLB (Bacterial Lysis Buffer) lysis buffers were used according to manufacturer's instructions or for overnight incubation at 56 °C.

Mixed cell lyses: During our experiments, in order to increase the efficiency of cell lyses, we also examined the effect of the two basic lysis mixtures on the taxonomic profiling of the microbiome. In this case, we performed combinations of the mechanical and chemical lysis I detailed above.

3.1.4 DNA purification techniques

The automated MagNa Pure24 System: During our research, we also used automated robotic DNA isolation from stool samples on a MagNa Pure 24 device. Pathogen 200 (200 µL initial sample volume) and Pathogen 1000 (500 µL initial sample volume) protocols were used for isolation according to the manufacturer's instructions.

Commercial DNA isolation approaches: For DNA isolation from stool samples, three commercially available DNA purification kits QiAamp Stool Mini, QiaAmp Power faecal and DNeasy Power soil were used according to the manufacturer's instructions. The isolations were performed on cell suspensions and on stool samples with an initial volume of 250 mg in each case.

Conventional phenol-chloroform-isoamyl alcohol DNA isolation: 800 µL of molecularly pure phenol-chloroform-isoamyl alcohol was added to 800 µL of cell lysate. Samples were vortexed thoroughly for approximately 20 seconds and then incubated for three minutes at room temperature. The samples were then centrifuged for 10 minutes at 16,000 g at 4 ºC. After centrifugation, the aqueous phase was pipetted into sterile DNase- and RNase-free eppendorf tubes. For ethanol (EtOH) precipitation, 1 µl of 20 µg/µl glycogen, 7.5 M NH_4OAc

(ammonium acetate in 0.5 × volume of the sample) and 2.5X volume of 100% EtOH were added to the supernatant.

The sample was placed at −20 °C overnight to precipitate the DNA from the sample.

The sample was then centrifuged for 30 minutes at 16.000 g at 4 °C to pellet the DNA. The supernatant was carefully removed without disturbing the pellet. 500 µl 70% EtOH was added to the sample and shaken by hand approximately for 20 seconds. The sample was centrifuged at 4 °C for 5 minutes at 16.000 x g and the supernatant was carefully removed. This washing step was repeated 2 times. The DNA pellet was dried at room temperature under laminar air flow, and then the DNA pellet was dissolved in 30 µl of nuclease free water.

3.1.5 Bead based re-purification of isolated gDNA samples after unsuccessful amplicon PCR

For certain DNA isolates, amplicon PCR failed as measured on an Agilent 4200 TapeStation automated microcapillary gel electrophoresis apparatus. We assumed that the isolates contained PCR inhibitors. The samples were purified with KAPA Pure beads based on the magnetic principle according to the manufacturer's recommended protocol. After purification, successful PCR of the amplicon was carried out in all cases, and DNA loss was minimal (<10%).

3.2 Investigation of the effect of nutraceuticals on the composition and diversity of the intestinal microbiota of broiler chickens

We investigated the effect of different natural bioactive components rich nutraceuticals (anthocyanin, symbiotic, carotenoid, fructooligosaccharide) on the composition and diversity of the microbiome in broiler chickens (Ross 308). The study was approved and carried out in accordance with the local ethics commite's guidelines of the University of Debrecen under the registration number (DEMAB/12-7/2015).

3.2.1 Birds, management and sample collection

The experiment lasted 40 days. Chickens reared in the production hall in the institutes for agricultural research and educational farm owned by the University of Debrecen. A total of 1250 one-day-old Ross 308 broiler chicks were randomly divided into six groups (each treatment had 3 replicates of 60 birds). Broilers were fed according to the following feeding programme: pre-starter 1-10 days, starter 11-21 days, grower 22-33 days, and finisher 34-41 days. Feed and water were provided *ad libitum.*

The experimental design was as follows: Additives were added at 0.5% to the basic feed.

- NC: negative control group received the basic antibiotic-free feed without added active ingredients
- PC: positive control group received the basic antibiotic-free feed containing 0.5% added β-glucan
- CAR: carotenoid treatment group received the basic antibiotic-free feed, that contained 0.5% added carotenoids
- FOS: fructooligosacharide treatment group received the basic antibiotic-free feed, that contained 0.5% added fructooligosacharide
- SZIM: symbiotic treatment group received basic antibiotic-free feed containing 0.5% added symbiotic

- ANTH: anthocyanin treatment group received the basic feed, that contained 0.5% added anthocyanin.

The body weight of chickens, feed consumption, and feed conservation ratio after all rearing period were measured. Mortality was monitored during the whole rearing period. Generally, mortality was low so no veterinary interventions were required.

Stool samples were collected on 7, 19, 31, 40 day of age to determine bacterial population. In every treatment group 6 broilers (3 pullets and 3 cockerels) were marked and faecal samples were monitored during the whole experimental period. Parallel, pooled faecal samples were also collected from every treatment group. The freshly collected stool samples were stored in specific, sterile, DNase free stool transportation bowls and were immediately placed on ice, until the sample collection was conducted, then samples were placed to -80 degrees.

3.2.2 Production of natural bioactive component rich nutraceuticals

The natural immunostimulant additives used in our experiments were prepared by researchers at the Institute of Food Technology, University of Debrecen. Since the aim of my PhD work was not the production of these active ingredients, I do not involve the detailed protocols of their production in my thesis.

3.2.3 Sample homogenization, cell lyses and DNA isolation

The sample homogenization, cell disruption and DNA isolation protocols considered most appropriate based on the results of the technical bias analysis were used to investigate the effect of nutraceuticals on the gut microbiome of broiler chickens.

Bacterial cell suspensions (BS) were prepared from 7-7 g broiler stool samples. 7-7 ml of sterile PBS buffers were added to the samples and homogenized for 4 min (by vortexing at 350 rpm). The samples were centrifuged for 5 min at 500 x g. Supernatants were collected and the washing step was repeated 2 times. Combined supernatants were centrifuged for 20 min at 13.000 x g. Finally, the supernatants were discarded, and the bacterial pellets were dissolved in 3 ml of sterile PBS buffer.

Mechanical lysis was used to reveal the cells. The samples were pipetted onto Power faecal bead lysis ceramic beads and then vortexed at maximum speed for three minutes. Afterwards, a MagNa Lyser (30 seconds at 5000 RPM) was used to grind the cells.

DNA was extracted from the stool samples using a conventional phenol-chloroform-isoamyl alcohol (25:24:1) isolation procedure.

3.3 Quality check

The amount and purity of DNA was determined photometrically with a Nanodrop ND-1000 device. Before the PCR reactions, the amount of DNA was measured with a Qubit 2.0

fluorimeter, using the Qubit dsDNA HS (High Sensitivity) assay kit according to the manufacturer's instructions. To determine DNA integrity, we used a 4200 TapeStation automated microcapillary gel electrophoresis device, Genomic DNA ScreenTapes and Agilent Genomic DNA reagent according to the manufacturer's instructions. After quality and quantity control, the samples were diluted to an initial volume of 5 μg/μL with nuclease-free water.

3.4 Negative and positive controls

To minimize infections and laboratory contamination sterile surgical gloves and face masks (for collecting samples) were used and all DNA extraction steps were performed with sterile or sterilized equipments in a class II laminar air-flow cabinet. Negative isolation control (NIC) experiments were simultaneously conducted by substituting samples with PCR grade water. Elutes of the NIC samples were conveyed for V3-V4 amplicon - PCR and indexing performed under DNA free UV sterilized AirClean® PCR workstations/cabinets. At each stage the PCR clean-ups of the library preparation NIC amplicons were also validated on 4200 Tape Station System by Agilent D1000 ScreenTapes and Agilent Genomic DNA reagents. Host background nucleic acid contaminations were also monitored by conducting real-time PCR reactions using GAPDH assay on eluted gDNAs. For measuring the overall quality of Illumina MiSeq paired-end (PE, 2 × 301 nt) sequencing runs 5% PhiX spike-in quality control was used.

3.5 Library construction

We prepared a 16 S rRNA gene-based metagenomic library using the Illumina library preparation protocol. During this, the V3 and V4 hypervariable regions of the 16 S ribosomal RNA gene were amplified in a targeted manner. The 5' end of the primers used contained an overhang to which an adapter was attached. After completion of the amplicon PCR with 2 x KAPA HiFi HotStart ReadyMix dual indexing of the 96 samples was performed using the Illumina Nextera XT Index Kit.

PCR cleanups and amplicon size selections were carried out with KAPA Pure Beads based on the technical data sheet of the manufacturer resulting in final ~580–630 bp libraries. Every time, verifications were done with PCR Agilent D1000 screen tapes (5067–5582) and D1000 Reagents (5067–5583). The 16 S amplicon libraries for each sample were quantified with qPCR, normalized with respect to amplicon sizes and pooled into a single library in equal molar quantities.

Finally, 5 μl of pooled 4 nM DNA library pool was prepared for sequencing on Illumina MiSeq platform. The library pool was denatured with 0.2 M NaOH and diluted to 10 pM final concentration. Sequencing was carried out with MiSeq Reagent Kit v3–618 cycle (MS-102–3003) following manufacturer’s protocols.

3.6 Bioinformatics analyses

Sequencing read preparation for downstream analysis.

The integrated software of the Illumina MiSeq sequencing machine was used to demultiplex the paired end reads. The FastQ files were imported into the Qiime 2 (ver 2019.7) pipeline (https://qiime2.org/) according to the „Atacama Soil microbiome" tutorial. The presence of adapter sequences (CTGTCTCTTATACACATCT) were checked and trimmed from the 3' end of the reads with Cutadapt Software integrated in the Qiime 2 pipeline. DADA2 software was used for quality trimming.

Alignment. Multiple sequence alignment was performed with the Mafft software, and reads were taxonomically classified using Naïve Bayesian classifier trained with the (ver132) reference database by selecting mapping points according to the forward-reverse primer set that was used for amplifying the 16S V3-V4 regions of the bacterial community (341F, 806R). Phylogenetic tree was constructed with FastTree plugin.

Biodiversity analysis. The QIIME2 pipeline was applied to perform alpha and beta diversity test. In the case of alpha diversity Shannon's index, Faith's phylogenetic diversity index, Simpson evenness, and Chao-1 index was calculated in the QIIME pipeline. For beta diversity analysis weighted/unweighted UNIFRAC distances and Bray-Curtis dissimilarities were measured. Beta diversity matrices PCoA plots were generated using the Emperor plugin. Alpha diversity differences were measured using the Kruskal-Wallis test. Beta diversity group significances were calculated with Permutational multivariate analysis of variance (PERMANOVA) pseudo F statistical test.

3.7 Data availability

All sequence data used in the analyzes were uploaded to the Sequence read Archive (SRA) website (http://www.ncbi.nlm.nih.gov/sra) under the names PRJNA633979 and PRJNA533250B.

3.8 Statistical analyses

Data were analyzed using GraphPad Prism (Graph-pad V8 software, San Diego, CA) statistical software. In the case of the studied random variables, we used the Shapiro-Wilk test to check whether the data follow a normal distribution and whether analysis of variance can be used to compare group averages.

A value of $P<0.05$ was considered significant. The raw data were given as mean and ± standard deviation. In the case of alpha diversity, differences were determined using the Kruskal-Wallis test. The difference in beta diversity between groups was determined using the "Permutational

multivariable of variance analysis" (PERMANOVA) pseudo F statistical test. In the case of the "heat trees" analysis, the differences were calculated using the Wilcoxon test. In the case of the additional measurements included in the thesis, we examined the differences with one-way ANOVA followed by Bonferroni post hoc tests (multiple comparisons).

4. Results

4.1 The effect of technical distortion factors on the sequencing results

4.1.1 DNA yield, quality and biodiversity indexes

Several factors can influence the outcome of amplicon sequencing based on the 16 S rRNA gene. These include the efficiency of nucleic acid extraction. We investigated how the different sample homogenization, cell disruption and DNA isolation protocols for faecal samples affects the DNA concentration (ng/µL), quality parameters (230/280, 260/280) and integrity (HMW, LMW) of the isolated samples.

In 16.6% of the isolated DNA samples, the amplicon PCR used during the library preparation failed. Conventional DNA isolation was used in 87.5% of these samples. Following failed amplicon PCR, samples were purified using magnetic beads KAPA Pure (DNA loss was minimal (<10%). Following purification, amplicon PCR was successful for all samples.

We compared the efficiency of two types of sample homogenization strategies: bacterial cell suspension (BS) and Direct lysis (DL).

DL resulted in 1.5X higher DNA yield (ng/µL) compared to BS, however DNA purity was lower with this method. In terms of DNA integrity, we did not find any significant difference between the two methods.

We also investigated the effectiveness of different cell disruption methods, such as chemical, mechanical and combinations of these lyses. We did not find any significant differences in the concentration, purity and integrity of the DNA.

Based on our data, however, it can be said that with the combination of mechanical and chemical lysis, a higher DNA yield and purity can be achieved compared to other cell lyses methods.

During our experiments, we compared the efficiency of different DNA isolation methods - the MagNa Pure 24 automated system isolation robot, three commercially available Qiagen isolation kits, and the conventional isolation technique based on the phenol-chloroform-isoamyl alcohol (25:24:1) principle. The conventional DNA isolation method resulted in the highest DNA yield ($P < 0.0001$). However, with this isolation method, we obtained the worst DNA

quality parameters in the samples when the sample homogenization was done by direct lysis. The MagNa Pure 24 automated robotic platform was suitable for the extraction of large quantities and high quality DNA. The use of Qiagen Kits resulted in the lowest DNA concentration, but the quality of the DNA proved to be the best with this method.

Receiver-operating characteristic (ROC) analysis was performed with the outcome of amplicon PCR (succeeded, not succeeded) as the binary classifier. Area under the curve (AUC) values with 95% confidence intervals (95% CIs) and standard deviation (±SD) were calculated. From our data we conclude, that DNA purity has the strongest predictive power on successful amplicon PCR. Interestingly, the lowest correlation was observed between the DNA concentration and successful downstream PCR amplification.

4.1.2 Standard protocols strongly affected sequencing metrics and the observed biodiversity

The BS homogenized conventional DNA isolation method resulted in the highest biodiversity, especially using chemical and mixed cell lyses (except for Faith's phylogenetic diversity index). The highest Faith's phylogenetic diversity index was obtained for the BS linked QIAamp DNA Stool Mini Kit method. The QIAamp Power Fecal DNA and DNeasy PowerSoil Kit methods resulted in the lowest diversity values and, conversely, the highest read and non-chimeric read numbers. When using the Pathogen 200 protocol of the MagNa Pure 24 automated DNA isolation robot, we experienced higher diversity and sequencing values than with the Pathogen 1000 protocol.

Furthermore, after conventional isolation, we obtained the highest diversity indices (Chao-1, Shannon and Simpson) in the case of the Pathogen 200 protocol. We also observed significant differences between the different methods regarding the DL homogenized samples. The lowest biodiversity values were obtained in the evening of the DNA isolation method associated with conventional chemical lysis. It is interesting that the same method achieved the best results with sample homogenization associated with cell suspension.

4.1.3 The effect of metagenomic DNA isolation methods on beta diversity indicators

Bray-curtis analysis was used to calculate the distance matrix between samples. The main coordinate analysis resulted in five cluster groups (cluster 1 - cluster 5) with different spatial ordinations.

Different cluster groups were defined in the case of BS (clusters 1, 2, 3) in comparison to (clusters 4, 5) DL samples. In general, the lysis protocols had no significant effect on the cluster profiles, except in the case of mechanical (cluster 1) and mixed lysis (cluster 2, 4) coupled with the MagNa Pure 24-based isolation method.

Cluster 3 shows a close relationship between bacterial suspensions and conventional DNA isolation methods. Furthermore, we observed that the isolation kits we used had no prominent effect on the beta diversity profiles. In the case of robotic MagNa Pure 24 isolation methods, we identified three different clusters (cluster 1, 2, 4). The samples of the 2nd cluster come exclusively from DNA samples isolated with the BS coupled MagNa Pure 24 isolation robot Pathogen 200 protocol.

Quantitative (Bray-Curtis, weighted UniFrac) and qualitative (Jacquard, unweighted UniFrac) beta diversity analyzes were performed to determine the differences between the microbial communities of the faecal samples. The distribution of the columns indicates the compositional differences of each group.

Based on our results, we concluded that different sample homogenization strategies significantly influence the difference between microbial communities. BS and DL homogenized samples are significantly different from each other.

A significant difference can be observed in the case of the application of mechanical and mixed lysis among the cell dissection protocols. Significant differences were found between the different DNA isolation methods during the statistical analysis.

4.1.4 The effect of different lysis protocols on the values of the alpha diversity indices

We investigated the effect of different lysis protocols on Faith's phylogenetic diversity index values. The combination of mechanical and chemical lysis resulted in a significantly higher value than chemical and mechanical lysis alone. The same trend can be observed in the case of Shannon and Chao-1 alpha diversity values.

In the case of the Simpson diversity index, we did not find any difference when comparing mechanical, chemical and mixed lysis applied to reveal individual bacterial cells. Based on our results, it can be said that the combination of mechanical and chemical lysis resulted in the highest alpha diversity indicators compared to other cell lyses techniques.

4.1.5 Impact of bacterial cell lyses protocols on DNA yield, purity and community diversity indices

In the mechanical lysis, the effects of different commercially available lysis beads (PowerFecal, SeptiFast and Green beads) for cell disruption were compared. For chemical lysis, ATL, BLB and InhibitEx tablets were used in ATL buffer. We found that the choice of different lysis beads did not result in a statistically significant difference on the parameters we tested. However, from the measured data, we could say that the SeptiFast lysis beads were able to extract the highest

amount of DNA. A significant difference can also be observed in the amount of DNA between the use of mechanical and chemical lysis. For chemical lysis, the highest DNA concentration was obtained using ATL buffer. The pre-treatment of InhibitEX tablets resulted in a fairly high quality of purified nucleic acids, which had an overall positive effect on the success of the PCR reactions, but this method yielded a low amount of DNA. Furthermore, the use of InhibitEX tablets resulted in a lower Shannon diversity index than the use of ATL and BLB buffer. The use of both ATL and BLB buffers resulted in the recovery of large amounts of DNA, which was associated with high alpha diversity values.

4.1.6 The different DNA isolation metagenomic strategies have a profound effect on the proportions of Gram-positive and Gram-negative bacteria in the sample

We investigated the effects of different DNA isolation techniques on the distribution of Gram-positive and Gram-negative bacteria. Direct (DL) and indirect (BS) sample homogenization strategies resulted in significant differences in bacterial profiles. For samples homogenized by DL, different cell lyses techniques resulted in the same Gram distribution. When comparing DNA isolation methods, different Gram distributions were observed. It is also important to note that by using DL sample homogenization, Gram-positive bacteria can be recovered in significantly higher amounts than Gram-negative bacteria.

BS homogenization with mechanical lysis resulted in balanced Gram-positive and Gram-negative ratios. The same Gram ratios were observed for automated robotic and conventional DNA extraction methods. The choice of sample homogenization did not have a significant effect on the Gram distribution of faecal bacteria when DNA was extracted using silica column-based methods.

4.1.7 Effect of different DNA isolation techniques on the composition of the core microbiome

We investigated how different methods affect the composition of the core microbiome in the sample. Complex comparisons of the distribution of the core microbiome were performed by phylum and class taxonomic category.

The different sample homogenization methods (BS, DL) resulted in marked differences in the relative amounts of bacteria constituting the "core" microbiome. DL homogenisation resulted in an efficient extraction of the Gram-positive *Firmicutes* strain (DL: 81.4%, BS: 60%). The use of BS results in a higher extraction of Gram-negative *Bacteroidetes* (BS: 28.68%, DL: 12.62%), *Proteobacteria* (BS: 6.57%, DL: 3.06%) and *Epsilonbacteraeota* (BS: 1.9%, DL: 0.69%) than DL. The amount of *Verrucomicrobia* was significantly higher for BS coupled

mixed lysis (L3) (BS: 1.27%, DL: 0.18%) and conventional DNA isolation (I6) (BS: 2.62%, DL: 0.3%) than for DL homogenates of the same methods. No significant differences were observed between the different methods for *Actinobacteria* (DL: 1.14%, BS: 1%) and *Tenericutes* (DL: 0.2%, BS: 0.16%) bacterial strains.

At class taxonomic level, significant differences were observed in the relative abundances of *Bacilli, Bacteroidia, Clostridia* and *Gammaproteobacteria* as a result of different sample homogenization methods.The use of BS resulted in higher levels of *Clostridia* (BS: 32.12%, DL: 23.43%), *Bacteroidia* (BS: 28.68%, DL:12.62%) and *Gammaproteobacteria* (BS:5.78%, DL:2.41%). A significant amount of *Bacilli* bacteria was observed in the DL homogenized samples (DL: 56%, BS:25.54%). The different cell lyses and DNA isolation methods did not have a significant biasing effect on the composition of the equilibrium microbiome.

4.1.8 Metagenomic DNA isolation methods have different effects on the discovery of different bacterial taxa

We sought to answer the question of which DNA extraction methods are most effective for detecting which bacterial species. To answer this question, we evaluated the effects of different DNA isolation techniques on microbial ecosystem composition as a function of sample homogenization (DL vs. BS) methods. We monitored quantitative differences in a total of 8 strains, 15 classes, 29 orders, 47 families, 72 genera and 22 species.

Significantly different taxonomic profiles were obtained when subsets of DL and BS homogenized samples were compared.

Furthermore, the different isolation strategies also resulted in significant differences in the relative abundances of the bacteria constituting the microbial ecosystem. No significant differences in the bacterial composition of the samples were observed when comparing the automated MagNa Pure24 isolation robot pathogen 200 and 1000 protocols.

The use of the DL coupled MagNa Pure24 isolation robot was best suited for the detection of *Actinobacteria*, *Firmicutes*, *Tenericutes* (strain), *Actinobacteria* (class), *Bifidobacteriales* (order), *Bifidobacteriaceae* (family), *Bifidobacterium* (genus). The BS coupled MagNa pure24 isolation robot proved to be effective for the extraction of *Bacteroidetes, Epsilonbacteria* (phylum) from *Lachnospira, Anaerofilum, Oscillibacter, Oscillospira, Bacteroides, Coprobacter, Butyricimonas, Alistipes* (genus). In the case of conventional isolation techniques,

different sample homogenization and cell disruption techniques significantly affected the efficiency of the method in terms of bacterial phylotyping.

The application of BS coupled conventional isolation techniques can be used to successfully extract a myriad of bacteria (such as *Bacteroidetes, Epsilonbacteria, Proteobacteria, Verrucomicrobia* (strain) *Bacillales, Selenomonadales, Erysipelotrichales, Bacteroidales, Campylobacterales, Rhodospirillales, Aeromonadales, Enterobacteriales, Verrucomicrobiales* (order), *Olsenella, Intestinimonas, Negativibacillus, Phascolarctobacterium, Megamonas, Dielma, Butyricimonas, Alistipes, Campylobacter, Helicobacter, Succinatimonas, Escherichia, Akkermansia, Faecalibacterium* (genus)). The DL coupled conventional isolation technique was suitable for the detection of *Cyanobacteria* strain. We also evaluated the performance of various commercially available Qiagen Isolation Kits. We found that the use of DNA Isolation Kits did not result in significant differences in the identification of individual bacterial taxa.

4.1.9 The results of the amplicon sequencing of the 16 S rRNA gene are significantly influenced by the taxonomic database used in the bioinformatic analysis

In addition to DNA isolation methods, the technical aspects of *in silico* bioinformatic analyses can introduce significant biases in determining the composition of microbial consortia. In our work we compared two reference databases GreenGenes (GG) and Silva (S). Both reference databases successfully classified more than 99% of reads at the taxonomic level of strain, class and order. At the family level, on average, more reads were identified using Silva (97.95 ± 2.03%) than GreenGenes (90.28 ± 2.84%) between the two databases. The difference was more significant at the genus level, where the Silva database was able to identify 95.99 ± 1.42% of the reads, while GG was able to identify 78.86 ± 5.8%.

We wondered whether different sample homogenization and DNA isolation strategies had a significant effect on the ranking ability of the databases.

In the case of the Silva database, the choice of sample processing (BS versus DL) had no significant effect on the proportion of ranked reads at family and genus taxonomic level. On the other hand, a higher percentage of reads were successfully identified using BS samples (14.42%) than DL homogenization (9.16%) for species-level identification.

We also examined the efficiency of cell lysis protocols. We found no significant differences between cell lysis protocols at family and genus level. The choice of metagenomic DNA isolation method did not significantly affect the number of identified reads. For the GreenGenes database, sample homogenization strategies resulted in significant differences in the number of reads identified. A higher percentage of reads were identified for DL homogenized samples

than for BS homogenized samples. Our results suggest that the choice of cell disruption significantly influenced the number of reads identified. In our experimental system, mechanical cell disruption gave the worst results at genus and family taxonomic level. For species level identification, mixed lysis performed the worst. We tested the efficiency of different metagenomic isolation strategies. We found that using Qiagen isolation kits gave the best results at all taxonomic levels, followed by conventional DNA isolation. We also observed notable differences between the species-level ranking ability of the two databases. On average, Silva was able to identify 12.88 ± 3.86% of the species, while GreenGenes was able to identify 51.74 ± 8.22%. The ranking values showed no correlation with the number of species identified. Silva and GG were able to identify a total of 87 species, 13 of which were identified by both databases. Of the 30 most common species identified by both databases, 4 species (*Lactobacillus vaginalis*, *Lactobacillus salivarius*, *Lactobacillus pontis*, *Bacteroides uniformis*) were found with similar relative frequency in both databases.

4.2 Investigation of the effect of bioactive component-rich nutraceuticals of natural origin on the gut microbiota composition and diversity of broiler chickens

We investigated the effects of nutraceuticals rich in different natural bioactive components (anthocyanins, symbiotics, carotenoids, fructooligosaccharides) on the composition and diversity of the microbiome in broiler chickens (Ross 308).

4.2.1 Effect of nutraceuticals on weight gain in birds

We investigated the effect of nutraceuticals on the growth performance of birds. At the beginning of the experiment, the average body weight of the birds was 38.4 ± 1.6 g, while at the end of the experiment, the birds weighed an average of 2693 ± 64.8 g. No significant differences were observed when comparing the treated [carotenoid (CAR), fructooligosaccharide (FOS), symbiotic (SYM), anthocyanin (ANTH)] and control [negative control (NC), positive control β-glucan (PC)] groups. However, at the end of the productive life of the birds, a moderate but not significant reduction in body weight was observed with anthocyanin supplementation (ANTH 2590 ± 264 g) compared to the NC group (2758 ± 222 g).

4.2.2 Microbiome weight correlation

Significant correlations were observed in the gut microbiota of animals with weight gain in 11 orders at different developmental growth stages (pre-starter, starter, growing, finishing) and treatments (NC, PC, CAR, FOS, SZYM, ANTH).

For *Bacillales*, *Clostridiales*, *Corynebacteriales*, *Enterobacteriales*, *Micrococcales*, *Rhizobiales, a* moderately positive (age and/or treatment specific) association with body weight was found (r value > 0.4). We studied the effect of age on the relationship between gut bacteria and weight gain. We found a positive correlation between weight gain and *Corynebacteriales* (r>0.77) and *Bacillales* (r>0.45) bacteria at the beginning of the developmental stage of broiler chickens. At the growing phase (10-21 days), we observed the most positive correlation of bacteria with body weight, *Clostridiales* (r>0.57), *Corynebacteriales* (r>0.47), *Micrococcales* (r>0.53), *Rhizobiales* (r>0.57). At the end of the developmental stage of the animals, we could identify only a weak positive correlation between bacteria and body weight. On the contrary, a negative correlation was observed between *Rhizobiales* (r>0.57) and body weight at the end of the growth phase of the animals.

Our work has investigated the effect of nutraceuticals on the correlation between these orders and body weight. No significant correlations between bacteria and body weight were obtained for the NC group. For the PC group, we found a moderate negative (r value <-0.4) correlation between body weight and *Enterobacteriales.* For FOS treated samples, a moderate positive correlation with *Micrococcales* was observed. Interestingly, *Pseudomonadales* showed a moderate positive correlation in the CAR group and a moderate negative correlation in the FOS treated birds. A moderate negative association was observed between *Bacillales* and body weight in the CAR group.

In the ANTH treated group, a strong positive (r value > 0.6) correlation was found between *Bacillales* (r>0.65), *Corynebacteriales* (r>0.61), *Enterobacteriales* (r>0.65) and *Micrococcales* (r>0.6) orders and body weight.

4.2.3 Association between different experimental parameters (age of birds and nutraceutical treatment) and gut microbiome diversity

Both alpha and beta diversity indices were determined to track conversions in community diversities of control (NC, PC) and treatment (CAR, FOS, SYN, ANTH) groups. For alpha diversity, the Faith phylogenetic, Chao-1, Shannon and Simpson diversity indices were used to determine the species number, richness and evenness of the GIT microbiota of broiler chickens.

A steady increase was observed as chicken aged; then alpha diversity indexes were decreased gradually after 31 days.

We discovered a positive effect of nutraceuticals at the end of the growth phase of the animals compared to the control group. This positive effect on Faith phylogenetic diversity was significant for FOS, SYM and ANTH treatment group compared to the control group. For the Shannon and Simpson diversity indices, no significant change was detected in response to nutraceuticals compared to the NC group. However, the diversity trends described for the Faith phylogenetic diversity index were also observed for these diversity measures without significant differences. The highest community diversity value was measured in the carotenoid-supplemented group at 31 days of age.

We also investigated the effects of different natural substances and the age of the animals on the beta diversity metrics. Distance-based dissimilarity matrices unraveled that chicken development had a significant influence on the overall community variation, thus, a gradual increase in community diversity was accompanied by increased heterogeneity of the GIT microbiota. However, different dietary supplementation did not exert statistical differences on beta diversity distances.

4.2.4 Changes in the core microbiota caused by dietary supplementation

The composition of the core microbiome was determined at the taxonomic level of orders and genera, by considering bacterial taxa that are represented at least in 50% of the samples. Based on the results of 16 S rRNA gene-based amplicon sequencing, the main orders and genera within the prokaryotic domain were identical for the different experimental parameters, but differences in relative the abundances were observed. The age of the animals and the use of nutraceuticals resulted in marked differences in the relative abundances of bacteria constituting the core microbiome.

At order taxonomic level, the core microbiome was composed of *Lactobacillales*, *Clostridiales*, *Erysipelotrichales* and *Enterobacteriales*. *Lactobacillales* were the most abundant bacteria detected in the core microbiome. The highest relative abundance was measured when the animals were seven days old and nutraceuticals had no significant effect on its abundance. *Clostridiales* were less abundant in β-glucan, fructooligosaccharide and anthocyanin treated animals in comparison to those receiving basal diets. *Erysipelotrichales* is a constituent of the core microbiome, but represented less than 1% of the bacteria in the early life stage of the birds. Its abundance increased as the animals grew, constituting 6.6% of the core microbiome at the end of their developmental cycle (40 days).

We identified eight genera - *Lactobacillus*, *Enterococcus*, *Streptococcus*, *Escherichia-Shigella*, *Faecalibacterium*, *Turicibacter*, *Romboutsia* and *Aerococcus* - in the equilibrium microbiome of animals. *Lactobacillus* showed a clear dominance during the experiment, with highest levels observed in carotenoid-treated 31-day-old animals.

Faecalibacterium plays an important role in maintaining gut health. In 31 days old broiler chicken, nutraceutical treatment generally increased *Faecalibacterium* in comparison to their control treatment.

At the genera level, chicken development exerted the most explicit effect on the relative occurrence of *Enterococcus*. In young chickens, this genus seemed to be the second most abundant, whereas in older chicks a drastic fall was observed.

Escherichia-Shigella was more abundant in the gut microbiome of animals fed with the symbiotic and carotenoid-enriched diets than in the control group (40-day-old animals).

4.2.5 Microbial fingerprints caused by nutraceuticals

We evaluated the effect of natural feed additives (CAR, β-glucan, FOS, probiotic blend and anthocyanin) on GIT microbiota at family level. We observed that dietary supplementation of carotenoids resulted higher proportion of of *Bifidobacteriaceae* (7, 31 and 40 days), *Lachnospiraceae* (7 and 19 days), *Ruminococcaceae* (7, 19 and 31 days), *Erysipelotrichaceae* (7 days), *Bacteroidaceae* (31 and 19 days), *Barnesiellaceae* (19 and 31 days) and *Christensenellaceae* (19 and 40 days) compared to the negative control group. β-glucan (PC) supplementation positively affected the number of *Ruminococcaceae* (7 days), *Erysipelotrichaceae* (7 and 40 days) and *Desulfovibrionaceae* (19 and 40 days). Our results showed that *Erysipelotrichaceae* (7 and 40 days), *Lachnospiraceae* (7 and 19 days), *Ruminococcaceae* (7, 19 and 31 days) increased, while *Staphylococcaceae* (total growth phase), *Aerococcaceae* (19, 31 and 40 days), *Lactobacillaceae* (7 days) decreased as a result of fructooligosaccharide (FOS) treatment.

The application of anthocyanin (ANTH) increased the abundance of *Bifidobacteriaceae* (7 and 19 days), *Lachnospiraceae* (7 and 40 days), *Ruminococcaceae* (7 and 40 days), *Akkermansiaceae* (40 days) and had some inhibitory effect on *Helicobacteraceae* (7 days) and *Campylobacteraceae* (40 days).

The use of symbiotics (SYM) increased the abundance of *Bifidobacteriaceae* (total productive life), *Lachnospiraceae* (7 and 40 days) and *Christensenellaceae* (7 and 40 days).

4.2.6 Effect of nutraceuticals on the amount of SCFA-producing bacteria

Among a range of metabolites produced by the beneficious gastrointestinal tract microbiota, short-chain fatty acids (SCFAs) have received increased attention because of their important role in disease prevention and recovery. Therefore, in our work, we investigated the effect of different natural bioactive component-rich agents on the quantitative composition of SCFA-producing bacteria, taking into account the age of the animals. In this trial, appreciable alterations were found in the proportions of some genera associated with SCFA production.

A significant reduction ($P < 0.05$) in the abundance of *Faecalibacterium* genus was observed in 31-day-old animals after fructooligosaccharide treatment compared to the negative control group. Furthermore, anthocyanin treated group individuals showed a significant increase in the amount of fructosaccharides at the end of the developmental stage of the animals (40 days) compared to control group.

Changes in the relative abundance of *Lactobacillus* were related to age rather than to the effect of nutraceutical treatment. At 7 days of age, the *Lactobacillus* genus was significantly higher in birds fed with the basal diet than in birds treated with feed containing carotenoids, fructooligosaccharides and anthocyanins.

A significant increase in the genus *Subdoligranulum* was observed in 7-day-old animals receiving carotenoid treatment compared to the negative control group.

A further significant increase in the relative abundance of *Subdoligranulum* was observed in broiler chickens at 40 days of age as a result of anthocyanin treatment.

Streptococcus, *Blautia* and *Ruminococcus* genera were detected at low relative abundance (<0.25%) in the microbial ecosystem at the beginning of the growth stage of the birds, and their levels gradually increased as the animals developed. At the end of the developmental stage of the animals (40 days), anthocyanin treatment significantly increased the relative abundance of *Blautia* and *Ruminococcus* bacteria.

The symbiotics had a beneficial effect on the *Bacteroides* population.

4.2.7 Differences in the amount of bacteria involved in lipid metabolism caused by nutraceuticals

We investigated the effect of nutraceuticals on the quantitative composition of bacterial taxa that play an important role in lipid metabolism. The bacteria *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, *Clostridium*, *Bacteroides* and *Peptostreptococcus* regulate primary bile salt synthesis through complex interactions and are involved in secondary bile acid metabolism in the host. Taxonomic heat-trees were constructed to investigate community shifts in

nutraceutical treatment group associated with primary and secondary bile acid metabolism. The abundance of *Bacterodia* (class) was significantly decreased by anthocyanin (ANTH) treatment, while carotenoid (CAR), fructooligosaccharide (FOS) and symbiotic (SYM) experimental groups showed significantly increased abundance compared to negative control (NC) and positive control (PC) groups. Our study showed that the relative abundance of *Lactobacilaceae* (family) was decreased in the anthocyanin experimental group compared to the control group (NC). Increased relative abundance of *Enterococcaceae* was observed in response to nutraceuticals. Furthermore, fructooligosaccharides (FOS), symbiotics (SYM) and anthocyanins (ANTH) significantly reduced the relative abundance of *Clostridium* compared to the control group (NC).

4.2.8 Effect of nutraceuticals on the abundance of microorganisms involved in carbohydrate metabolism

We investigated the effects of different experimental settings (nutraceuticals, age) on the composition of gut microbial ecosystem, in particular on the composition of bacteria involved in carbohydrate metabolism. A total of 7 phylum were identified in the gut microbiota of broiler chickens. Of these, *Firmicutes* (Σ89.5% ± 7.8%), *Proteobacteria* (Σ7.3% ± 7.0%) and *Bacteroidetes* (Σ1.3% ± 2.7%) were the most dominant, followed by *Actinobacteria, Tenericutes* and *Verrucomicrobia*.

We calculated the Log2 ratios of *Firmicutes*, *Bacteroidetes* (F/B) bacterial strains among the different experimental groups (NC, PC, CAR, FOS, SYM, ANTH), taking into account the age of the animals (7, 19, 31 and 40 days). The differences in F/B ratios reflect the variation in (poly)saccharide utilization of the stocks. Chicken age had a significant ($P<0.05$) effect on log2 F/B ratios. The highest values were obtained at 7 days of age, followed by a significant decrease in log2 F/B ratios as the animals grew.

For the different experimental groups, the highest log2 F/B ratio was found in β-glucan (7.14) treated birds, while the lowest value was found in anthocyanin (4.89) treated samples.

Furthermore, anthocyanin treatment increased, while carotenoid decreased the amount of *Proteobacteria*. The bacterial strains *Epsilonbacteraeota*, *Tenericutes* and *Verrucomicrobia* were also detected, but were present at a very low relative abundance (≤1%) in the gut microbiota of the animals.

We aimed to investigate the effects of nutraceuticals on the microbiome composition at the species level. In total, we monitored compositional changes in 22 species. In particular, we focused on changes in lipid and carbohydrate metabolism and in the relative abundances of the relevant species responsible for the development of various infectious diseases.

The beneficial probiotic bacteria *Lactobacillus aviarius* and *Lactobacillus salivarius* were detected in all experimental groups, and these bacteria play an important role in lipid homeostasis and in preventing colonisation by various pathogenic bacteria. ***L. salivarius*** was detected at a high relative abundance in all experimental groups, and increased abundance was observed in β-glucan-treated animals compared to the control group. A significant increase of *L. aviarius* was observed in the gut microbiota of the animals in the symbiotic and anthocyanin experimental groups. A significant increase in *Lactobacillus alvi* was observed in the gut microbiota of anthocyanin-treated animals compared to negative control individuals.

Campylobacter jejuni causing bacterial diarrhoeal gastroenteritis was detected with a low relative abundance (<1%) in the microbial consortium of animals.

We detected the anaerobic *Anaeromassilibacillus senegalensis* bacterium with similar low relative abundance in all our experimental groups.

Bacteroides gallinaceum, which plays an important role in maintaining gut health, was only found in samples treated with carotenoids and anthocyanins. The butyrate-producing *Butyricicoccus desmolans* was detected at low relative abundance in all experimental groups (no differences). The newly described anaerobic, non-spore-forming *Traorella massiliensis*, which plays an important role in fatty acid metabolism, was detected in higher abundance in the gut microbiota of anthocyanin-treated birds than in the other treatment groups. In addition, SCFA-producing *Pseudomonas fragi* was only observed in anthocyanin-treated animals.

4.2.9 Diet-specific microbial interactions

To identify nutraceutical-induced interconnections within the broiler intestinal microbiota, we estimated the extent to which relevant families tended to change together. Relative proportions of taxa were correlated in terms of Spearman's method. We identified divergent abundance patterns by using data for the 15 most abundant families in nutraceutical-induced treatment groups throughout the four phases of the experiment.

In general, similar correlation patterns were revealed between carotenoid, symbiotic treated and the fructooligosaccharide, anthocyanin treated groups.

We also examined the effect of nutraceuticals on the associations between families. Samples treated with anthocyanin (ANTH) showed by far the highest number of individual family matches (8 positive and 6 negative correlations). *Desulfovibrionaceae* showed a very strong negative correlation with *Lactobacillaceae* (r-value: -0.97), *Streptococcaceae* (r-value: -0.97) and *Peptostreptococcaceae* (r-value: -0.97) in ANTH treated samples. In parallel, the family *Desulfovibrionaceae* showed a very strong correlation with *Bacteroidaceae* (r: 0.97),

Barnesiellaceae (r: 0.97), *Clostridiaceae* (r-value: 0.97), *Erysipelotrichaceae* (r-value: 0.97) and *Ruminococcaceae* (r: 0.97). A strong negative correlation was found between *Sphingomonadaceae* and *Streptococcaceae* (r: -1). A very strong negative relationship was found between *Moraxellaceae* and *Beijerinckiaceae* (r: -0.97) in response to fructooligosaccharide (FOS)-supplemented feed. Furthermore, in FOS-fed animals, we found a very strong positive correlation between *Rikenellaceae* and *Clostridiales* (r: 0.97), *Rikenellaceae* and *Burkholderiaceae* (r: 0.97), and *Acidaminococcaceae* (r value: 0.97) among *Streptococcaceae* and *Barnesiellaceae* (r: 0.97), *Aerococcaceae* and *Peptostreptococcaceae* (r: 1), and *Chitinophagaceae* and *Bacillaceae* (r: 0.97). The fingerprinting of the carotenoid treated group showed a very strong positive correlation between *Xanthobacteraceae* and *Chitinophagaceae* (r: 0.91), *Xanthobacteraceae* and *Bifidobacteriaceae* (r: 1), and *Beijerinckiaceae* and *Streptococcaceae* (r: 0.97).

5. Discussion

5.1 Overview of the effect of technical distortion factors on the result of 16 S rRNA gene-based amplicon sequencing

Recently, the advent of NGS has enabled researchers to comprehensively analyze the composition and function of microbial populations in very diverse environments including difficult-to-culture microbes. However, the obtained results can be influenced by a number of different technical factors. Sample homogenization, bacterial cell lysis and DNA purification methods are important sources of technical factors, which can significantly distort the apparent composition, structure and diversity of the microbiota. Furthermore, the bioinformatics analysis and the applied taxonomic database also influence the final outcome of the study. Therefore, the choice of proper metagenomic DNA extraction method is critical and has to be appropriate for the biological questions being asked.

Thus, we investigated the effects of various technical distortion factors on the results of 16 S rRNA gene amplicon sequencing. We also evaluated the impact of these factors on DNA concentration, purity, fragmentation and PCR reaction efficiency of isolates.

Our results show that the choice of sample homogenization strategy has a significant effect on DNA concentration and quality parameters of isolates. Direct lysis (DL) resulted in 1.5 X higher DNA yield than indirect sample homogenization strategy (BS). However, it is important to note that DL sample homogenization yielded DNA isolates of low purity. Using Qiagen kits, low DNA concentrations were observed, but DNA quality parameters were found to be the best with this method. High DNA concentration and quality parameters were obtained with the MagNa Pure 24 isolation robot. Consistent with the results of previous studies, the conventional phenol-chloroform-isoamyl alcohol isolation method resulted in the highest DNA concentration. However, it is important to note that low DNA quality parameters were obtained with the conventional DNA isolation technique compared to the other methods.

In addition to the efficiency of cell lysis, the quality and quantity of DNA extracted from faecal samples is also influenced by the co-extraction of PCR inhibitors in the sample and the fragmentation of DNA that occurs during lysis.

The different methods of sample homogenization, cell disruption and DNA isolation result in different amounts of PCR inhibitor components in the samples. PCR inhibitors can inhibit the PCR reactions used in library preparation, thus having an effect on the determination of microbial composition. In our study, we observed opposite effects of DNA yield on PCR amplification success rates. The amplicon PCR used in library preparation was unsuccessful in 16.6% of all samples, 87.5% of which were derived from samples obtained in conventional isolation. This may be due in part to higher concentrations of PCR inhibitors and nucleases in samples subjected to conventional isolation than in other methods. It is important to note that PCR failure in conventional isolation was significantly observed in samples associated with DL sample homogenization (70%). An effective solution may be to re-purify these samples using magnetic beads, but in this case DNA loss should be considered.

The DNA production of prokaryotic cells is strongly influenced by the efficiency of cell lyses. Both Gram-positive and Gram-negative bacteria are present in the faecal sample. Gram-positive bacteria have thick peptidoglycan cell wall. As a result, when using a weaker lysis, these species are under-represented compared to the original composition. Mechanical lysis has been proposed in the literature for the detection of Gram-positive bacteria. Mechanical lysis allows the recovery of larger amounts of DNA.

However, some research suggests that this technique increases the fragmentation of the isolated DNA, leading to the formation of chimeras in subsequent PCR reactions. Our results are in contrast with these observation that the use of mechanical lysis did not result in significant DNA fragmentation. We also found that there is no significant difference in DNA concentration and

purity between the different cell disruption techniques. However, it is important to note that the combination of mechanical and chemical lysis resulted in the highest DNA concentration. Higher DNA concentration was associated with higher alpha diversity values compared to the other cell disruption techniques.

DNA yield is often used as a criterion for isolation of DNA from microbial communities to evaluate the efficiency of specific DNA isolation methods. However, the representation of microbial diversity, which is often the main objective of community analysis, is not usually considered as an evaluation criterion for DNA extraction methods. The efficient extraction of DNA is an essential step in any culture-independent approach to characterize microbial diversity.

In order to determine whether higher DNA yields provide a better representation of microbial diversity, it is important to test the efficiency of different metagenomic DNA isolation protocols with respect to alpha diversity indicators. We investigated the effects of different technical bias factors on the values of alpha diversity and sequencing indices. Diversity reflects the complexity of the sample under study, the higher the value the richer the sample. Our results show that the bacterial cell suspension strategy achieved the highest species richness and performed well in terms of sequencing indicators. Conventional DNA isolation after chemical or mixed lysis on BS samples resulted in the highest alpha diversity values among the DNA extraction methods used. This can be explained by the fact that Gram-positive bacteria with thick peptidoglycan cell wall can be detected using mixed lysis. It is believed that the use of mixed lysis allows the degradation of polysaccharides in the faeces, resulting in a very good release of DNA. Furthermore, conventional isolation can yield a large amount of DNA that is more representative of the individual species that make up the microbiome. Based on our results, the automated DNA isolation robot resulted in high read counts and diversity values independent of homogenization strategy. The commercially available isolation kits performed well in terms of sequencing indices, but resulted in lower diversity values compared to other isolation techniques, in accordance with previous studies. Overall, the efficiency of DNA isolation methods is strongly influenced by the prior sample homogenization strategy. DNA isolations coupled with the indirect method (Bacterial cell suspension preparation) resulted in higher diversity and sequencing indices compared to direct lysis.

There are currently a number of commercially available DNA isolation kits that produce high purity and reproducible DNA isolates, but the yield of these isolation kits is low. The lower amount of DNA may bias the results, making it difficult to identify species present in small numbers in a microbial consortium. This is supported by our observation that isolates with high

purity but low DNA concentration were obtained using the isolation Kits. Furthermore, lower diversity parameters were observed in these samples compared to other isolates. It is also interesting to note that using this technique, 95.04% of the reads were successfully identified at the family level and 94.74% at the genus level. We believe that the high identification rate and the low diversity parameter values may be due to the fact that, with this isolation technique, only dominant taxa are recovered, while low abundance relevant species of community are detected with lower efficiency or not at all. This assumption is supported by our observation that low abundances of *Akkermansia* and *Faecalibacterium,* which are important for maintaining the balance of beneficial gut flora, cannot be detected using this isolation technique. Our results also suggest that kit-based methods provide the most consistent results.
To our knowledge, this is the first time that the Roche MagNA Pure 24 DNA purification system has been used for metagenomic purposes. The advantage of using this method is that it can be standardized and biases introduced during manual isolation can be eliminated. The widespread use of the isolation robot is hampered by its high cost.
Currently, several studies use the cost-effective conventional phenol-chloroform DNA isolation method to extract DNA from microbial cells. Conventional DNA isolation yields large amounts of DNA that is representative of the low abundant and dominant members of the microbiome. This is supported by our result that the highest bacterial diversity value can be achieved with this isolation. Furthermore, low abundances of bacteria relevant for the health of the gut, such as *Phascolarctobacterium*, *Akkermansia*, *Butyricimonas*, *Faecalibacterium,* can be detected. The disadvantage of the method is that it results in low quality parameters, which inhibits the successful completion of subsequent PCR reactions, especially in the case of DL sample homogenization.

5.2 Overview of the effects of nutraceuticals on the composition and diversity of the broiler chicken gut microbiome

Microbial community of the GIT plays an important role in overall health and function of the host. Nowadays, microbiome research is becomes increasingly important in animal husbandry, which require animals capable of growing rapidly within a short period of time.
Aviculture is currently the most efficient animal productive system and forms the basis of global protein production. Modern chicken hybrids such as ROSS 308 broiler grow extremely fast, with an average rearing period of 35-42 days, and 2.5-3 kg weight gain. However, such intensive, extreme growth rate inadvertently resultes in changes in gastro-intestinal development during the growth of the animal. Some other negative effects may also eventuate

at times, like metabolic disorders, poor immunocompetence and increased susceptibility to pathogen infections.

Broiler chickens have a weak immune system. Therefore, in broilers, antibiotics are widely used as a prophylactic therapy to prevent diseases development and transmission. However antibiotic therapy is known to shift the microbiome composition towards pathogenic bacteria, thus catalysing dysbiosis with a consequent deleterious impact on physiology of the host. The dilemma caused by using of antibiotics and the need to maintain the balance of intestinal microbiome community of broilers has resulted an enormous interest in finding alternatives.

In recent years, the number of scientific publications on the physiological effects of nutraceuticals increased rapidly. There is growing evidence that these plant extracts, rich in complex bioactive compounds, have antibacterial properties, are immunostimulants and have a positive effect on the composition of the gut microbiome.

Taking all these factors into account, the following study was conducted to investigate the effect of dietary supplementation of FOS, carotenoids, symbiotics and anthocyanins on microbial communities of the broiler chicken.

Several studies have suggested that the use of nutraceuticals, in particular symbiotics, increases body weight gain in animals. This assumption was not supported by our results, nutraceutical treatment did not have a positive effect on animal weight gain.

This difference may be due to different environmental factors (housing technology, differences in the composition of the basic feed), different host genetics and age. It is important to note that our aim was not to increase the growth rate of the animals, but to produce feed additives that improve their general health status by modulating the gut microbiota. However, at the end of the growth phase of broiler chickens, a moderate reduction in body weight was observed in animals fed with anthocyanin supplementation compared to the control group.

The negative effect of anthocyanin on body weight has already been observed in human, pig and mouse studies. Although little information is available on the molecular mechanisms by which anthocyanin exerts this effect.

Humans and animal models have demonstrated, that the intestinal microbiota plays a very important role in the absorption of nutrients.

Thus, an understanding of the variances in the intestinal microbiota might help to clarify how changes in its composition might alter energy metabolism and growth performance in the host. In the gut microbiome, an increase in *Firmicutes* is associated with increased nutrient absorption, while an increase in *Bacteroidetes* results in decreased nutrient absorption.

With an increase in the proportion of F/B strains, there is an increased weight gain in the host, which is accompanied by an increase in adipocyte count, cholesterol, triglyceride and LDL.

We determined the gut microbiome composition of Ross 308 broiler chickens. In agreement with the results of other studies, *Firmicutes* was the most abundant phylum, followed by *Proteobacteria*, *Bacteroides*, *Actinobacteria*, *Tenericutes* and *Verrucomicrobia*. The age of the animals had a significant effect on the F/B ratio. The highest value was measured in chickens at 7 days of age and then a significant decrease was observed during their development. Although the highest F/B ratio was measured for the β-glucan treatment group (PK), in contrast, no increased weight gain of animals was observed compared to the control group. The lowest F/B ratio was measured in animals fed with the anthocyanin additive. Our results suggest a correlation between the moderate weight loss and low F/B ratios observed in the anthocyanin-treated group.

Akkermansia is a mucin-degrading bacteria, and its increased abundance is linked to intestinal health, as it exerts selection pressure on bacteria that cannot adhere to the mucosal surface. It is an important butyrate and propionate producing bacteria, and its reduction in the gut microbiome has been described in obesity, inflammatory bowel disease, type 2 diabetes. It also plays a role in maintaining the integrity of the intestinal epithelium. We observed that β-glucan, symbiotic and anthocyanin treatments significantly increased the abundance of *Akkermansiaceae* in the chicken gut microbiome compared to the negative control group.

Our results further suggest that β-glucan, symbiotic and anthocyanin treatments have a positive effect in maintaining the integrity of the intestinal epithelium by inhibiting the development of intestinal inflammatory processes by increasing the amount of beneficial *Akkermansiaceae* in the gut microbiome of chickens.

It can be assumed that changes in the composition of the GIT microbiota play a crucial role in the development of various metabolic diseases in poultry.

The diversity of the microbiome is one of the most important factors in the development of resistance to invasive pathogens. Greater diversity of the microbial community results in a healthier host, while a significant reduction in its complexity is associated with various diseases and increased susceptibility to colonization by pathogens. A shift in the GIT microbiome towards beneficial bacteria can improve host health.

We investigated the effect of different natural bioactive compounds on the complexity of microbial communities, taking into account the age of the animals. Our results showed, distinctive differences in GIT microbiome richness and diversity among experimental groups were observed. Previous reports showed, that GIT microbial population becomes more complex

as chicken aged. Our results were in accordance with this paradigm as clear increasing trend was observed in the richness and evenness in day 31. At the end of broilers productive lifespan (40 day) steadily decrease was seen. We found that, dietary treatments of FOS symbiotic and anthocyanin increased the level of Faith's phylogenetic alpha diversity index compared to the control group.

Interestingly, the present study revealed that dietary treatment mainly had an effect at the end of the broiler productive lifespan, when the diversity started to decrease. This may suggest that dietary supplementation has a lesser impact on a more diverse microbiota A higher microbial diversity is commonly related to a healthier host status, whereas the lack of sufficient diversity in a microbial community structure has been associated with different intestinal diseases.

According to previous study nutraceuticals treatment could be effective in increasing the amount of different SCFA-producing bacteria. Therefore, we investigated the effect of nutraceuticals on the amount of SCFA-producing bacteria. Based on our results, both nutraceuticals and the age of the animals had a significant effect on the relative abundance of SCFA-producing bacteria.

Faecalibacterium is one of the main butyrate-producing bacteria in the gut microbiome. Previous studies indicated that the lack of *Faecalibacterium* is commonly associated with inflammatory bowel diseases and its abundance has the potential to be used as biomarker for host intestinal health. Depletion of its proportion was observed in type 2 diabetes.

Based on our results, we observed a positive effect of anthocyanin on *Faecalibacterium* abundance compared to control group.

In our experimental system, anthocyanin and carotenoid treatment effectively increased the amount of *Subdoligranulum* compared to the other groups. The highest relative abundance was measured in individuals of the carotenoid-treated group.

We found that the symbiotic and carotenoid treatment had a positive effect on the beneficial *Bifidobacteriaceae.*

Members of the *Ruminococcaceae* family, which also produce the beneficial butyrate, were more abundant in the gut microbiome of anthocyanin and symbiotic receiving chicken than in the control group.

A further interesting correlation is that as diversity decreases at the end of the developmental stage of animals, there is a decreasing trend in the relative abundance of beneficial SCFA-producing bacteria. This may be linked to a deterioration in the health status of the animals. The health status of Ross 308 broilers deteriorates rapidly during the last two weeks of the rearing period, with frequent local and systemic bacterial infections, resulting in increased

mortality and reduced natural indicator values. It is assumed that there is a significant correlation between the development of pathological processes and changes in the composition and diversity of the gut microbiome. The changes in the quantitative composition of pathogenic bacteria in the gut microbiome of individuals from different experimental groups were investigated.

Many members of the genus *Clostridium* have important beneficial effects, synthesizing vitamins, micronutrients (thiamine, riboflavin, nicotinamide, pantothenic acid, biotin) and neurotransmitters for the host. Although some members of *Clostridium* are known to play a role in the metabolism of antioxidant polyphenols and thus reduce intestinal inflammation, some members may colonies the gut microbiome in higher quantities under altered environmental conditions and cause severe disease. For example, overgrowth of *Clostridium perfringens* leads to severe necrotic enteritis in chickens.

It is interesting that *Clostridium* was detected with low relative abundance in our samples and was not a component of the core microbiome of the animals. In our work, we found that treatment with symbiotic, anthocyanin and fructooligosaccharide reduced its abundance in the gut microbiome of chickens compared to the control group.

We observed a reduction in the abundance of pathogenic *Campylobacteriaceae* and *Helicobacteriaceae* families in the anthocyanin, carotenoid, FOS and β-glucan treatment groups compared to the control group.

The Gram-negative opportunistic *Alcaligenes faecalis* was present with low relative abundance in the gut microbiome of the basal diet-fed animals, whereas it was not detected at all in the gut-microbiome of nutraceutical-fed animals.

We also investigated the effects of nutraceuticals on beta diversity measures in relation to the age of the animals. Our results suggest that the evolution of the herd had a significant influence on the variation in community composition. The progressive growth of microbial communities was associated with increased heterogeneity of the GIT microbiota. The application of nutraceuticals had no significant effect on the beta diversity of the animals. Based on our results, we conclude that age resulted in a more pronounced community shift than treatment.

In our experiment, we investigated how the experimental parameters influence the proportion of essential, "core-microbiota" constituents at taxonomic levels of orders and genera under intensive husbandry parameters. Based on the results of 16 S rRNA gene-based amplicon sequencing, we found that the major orders and genera within the prokaryotic domain were identical, but differences in relative abundances were observed. The core microbiome consisted of *Lactobacillales*, *Clostridiales*, *Erysipelotrichales* and *Enterobacteriales* bacteria.

Our results suggest that the loss of microbial community diversity and the associated reduction in SCFA-producing bacteria can be restored by alternative treatment strategies using natural active substances. We believe that microbiome restitution may be an effective therapeutic tool to reduce the use of antibiotics. Of course, this requires an in-depth understanding of the interaction of microbial communities and the effects of nutraceuticals rich in natural bioactive components on the gut microbiome.

6. Summary

In the first part of our experiments, we aimed to investigate the effects of various technical distortion factors on the results of 16 S rRNA gene amplicon sequencing. Two sample homogenization methods were investigated (DL, BS). We have shown that a higher DNA yield can be obtained by using DL, however with this method we obtained lower quality DNA than in the case of BS. We found that BS resulted in higher alpha diversity and sequencing index values. Gram-positive bacteria can be recovered in larger amount using DL, while balanced Gram profiles were obtained in the case of BS. We compared different cell lyses and DNA isolation methods. We have revealed that the combination of mechanical and chemical lysis achieves the highest alpha diversity index. We observed that the Kit-based DNA isolation methods produced the most consistent results. Using the MagNa Pure 24 automated platform, a large amount and high-quality DNA can be extracted, and well suited for monitoring changes in microbiome composition. We obtained the largest amount, but the worst quality DNA, using the conventional DNA isolation protocol. Nevertheless, this method has proven to be prominent

for the in-depth analysis of microbiota communities. The greatest bacterial diversity is achieved with this method.

Another goal of our research was to investigate the interactions between nutraceuticals rich in various bioactive components (carotenoids, fructooligosaccharides, symbiotics, anthocyanins) and the intestinal microbiota in broiler chickens. We concluded that the development of the animal exerted a great influence on the chicken microbial community structure. The use of nutraceuticals had a positive effect on the gut microbiome diversity of animals. Furthermore, treatment with nutraceuticals stimulated the growth of many beneficial SCFA-producing bacteria (*Bifidobacteriaceae*, *Subdoligranulum*, *Faecalibacterium*, *Akkermansia*, *Butyricicoccus*, *Blautia*, *Ruminococcus*) in the gut microbiome of chickens. We managed to reveal several positive and negative correlations between the microbial composition and weight gain. We showed that the amount of pathogenic *Campylobacteriaceae*, *Helicobacteriaceae* and *Enterobacteriaceae* decreased in the intestinal microbiome of animals treated with bioactive compound enriched nutraceuticals. Based on our results, we believe that nutraceutical treatment can be a promising solution to overcome health problems related to the overgrowth of intestinal bacteria and can potentially be alternative treatment strategies to replace and reduce the use of antibiotics.

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8. Appendix – List of publications

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Doctoral School: Doctoral School of Molecular Cellular and Immune Biology
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List of publications related to the dissertation

1. **Szilágyi-Tolnai, E.**, Fauszt, P., Fidler, G., Pesti-Asbóth, G., Szilágyi, E., Stágel, A., Kónya, J., Szabó, J., Stündl, L., Babinszky, L., Gálné Remenyik, J., Biró, S., Paholcsek, M.: Nutraceuticals Induced Changes in the Broiler Gastrointestinal Tract Microbiota.
mSystems. 6 (2), 1-25, 2021.
DOI: https://doi.org/10.1128/mSystems.01124-20
IF: 7.324

2. Fidler, G., **Szilágyi-Tolnai, E.**, Stágel, A., Gálné Remenyik, J., Stündl, L., Gál, F., Biró, S., Paholcsek, M.: Tendentious effects of automated and manual metagenomic DNA purification protocols on broiler gut microbiome taxonomic profiling.
Sci. Rep. 10 (1), 1-16, 2020.
DOI: http://dx.doi.org/10.1038/s41598-020-60304-y
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List of other publications

3. Fidler, G., Szilágyi-Rácz, A. A., Dávid, P., **Szilágyi-Tolnai, E.**, Rejtő, L., Szász, R., Póliska, S., Biró, S., Paholcsek, M.: Circulating microRNA sequencing revealed miRNome patterns in hematology and oncology patients aiding the prognosis of invasive aspergillosis.
Sci. Rep. 12 (1), 1-17, 2022.
DOI: http://dx.doi.org/10.1038/s41598-022-11239-z
IF: 4.996 (2021)

4. Fehér, M., Fauszt, P., **Szilágyi-Tolnai, E.**, Fidler, G., Pesti-Asbóth, G., Stágel, A., Szűcs, I., Biró, S., Gálné Remenyik, J., Paholcsek, M., Stündl, L.: Effects of phytonutrient-supplemented diets on the intestinal microbiota of Cyprinus carpio.
PLoS One. 16 (4), 1-24, 2021.
DOI: http://dx.doi.org/10.1371/journal.pone.0248537
IF: 3.752

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5. **Szilágyi-Tolnai, E.**, Fidler, G., Szász, R., Rejtő, L., Nwozor, K. O., Biró, S., Paholcsek, M.: Free circulating mircoRNAs support the diagnosis of invasive aspergillosis in patients with hematologic malignancies and neutropenia.
   Sci. Rep. 10 (1), 1-12, 2020.
   DOI: http://dx.doi.org/10.1038/s41598-020-73556-5
   IF: 4.379

6. Balogh, E., **Szilágyi-Tolnai, E.**, Nagy, B. J., Nagy, B., Balla, G., Balla, J., Jeney, V.: Iron overload inhibits osteogenic commitment and differentiation of mesenchymal stem cells via the induction of ferritin.
   Biochim. Biophys. Acta-Mol. Basis Dis. 1862 (9), 1640-1649, 2016.
   DOI: http://dx.doi.org/10.1016/j.bbadis.2016.06.003
   IF: 5.476

7. Balogh, E., Tóth, A., **Szilágyi-Tolnai, E.**, Bodó, T., Bányai, E., Szabó, D. J., Petrovski, G., Jeney, V.: Osteogenic differentiation of human lens epithelial cells might contribute to lens calcification.
   Biochim. Biophys. Acta. Mol. Basis Dis. 1862 (9), 1724-1731, 2016.
   DOI: http://dx.doi.org/10.1016/j.bbadis.2016.06.012
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