Microbial diversity in Swedish breeds of sheep analysed by amplicon based sequencing

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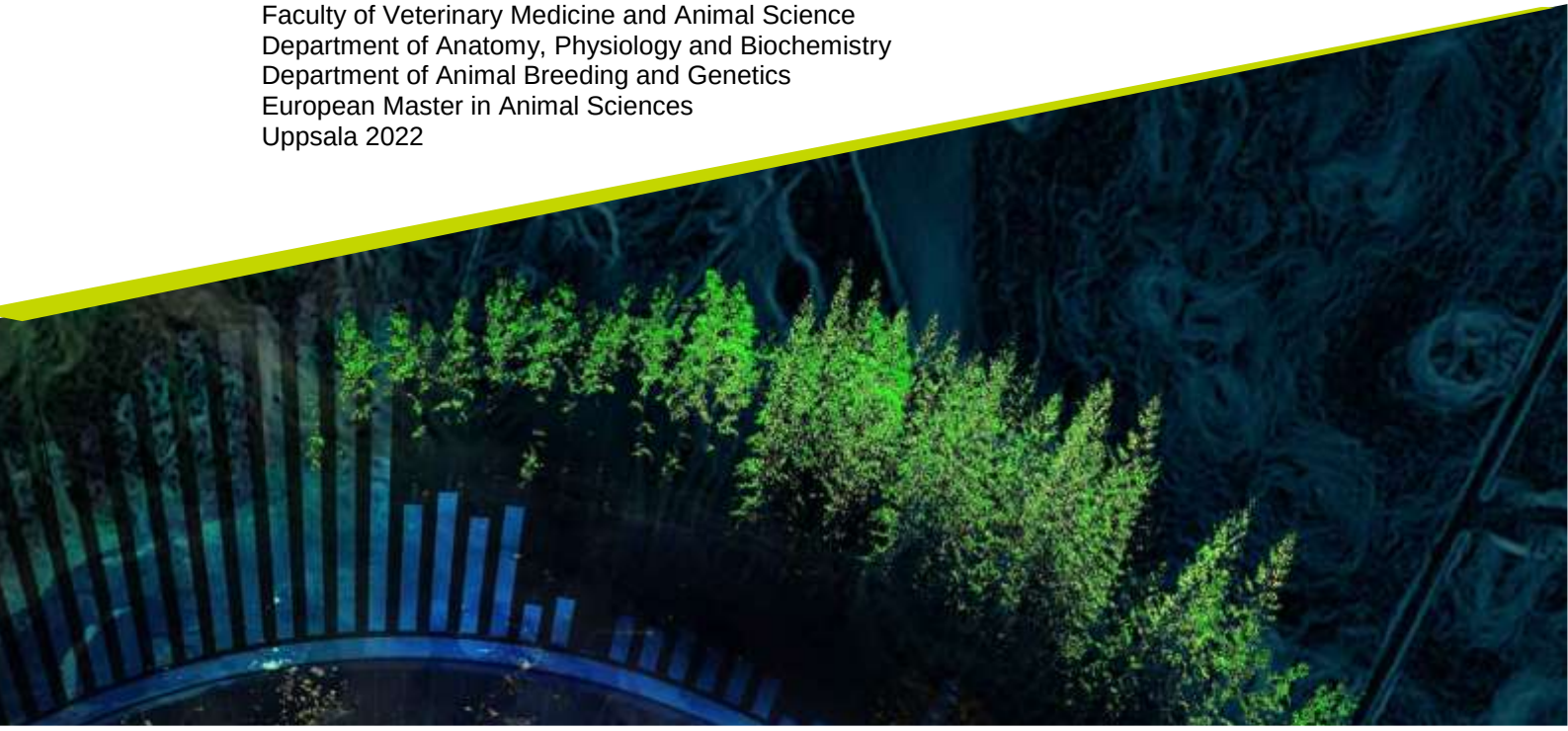

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Abstract

The gastrointestinal tract (GIT) of ruminants have divergent type of microbiome provides favourable environment for their growth. In many species, these microbiome considered as important source of assessing animal health & welfare.
A total of 18 different *Swedish* sheep breeds (n = 15) and Landrace Goats as an out group (n = 3) were selected to examine the faecal microbiome, i.e., Rye (n=6), Gestrike (n=4), Roslag (n=3), and Cross-breed (Suffolk + Texel) (n=2) & Goat (n=3). The replicates of 32 faecal samples were subjected to DNA extraction, the gel electrophoresis method, and amplification for PCR products and mixing. The positive replicates of all extracted DNA samples were subjected to16s amplicon metagenomics coupled with next generation sequencing (Illumina), quality control (QC), and generated processed reads of total (1799435) for all bacteria and archaea. The total of (10734) amplicon sequence variants (ASV) after the filtration were employed for the phylogeny and taxonomy analysis of bacteria at the phylum and genus level. Bioinformatics and statistical analysis was done by using DADA2 pipeline in the R software and PRISM software (Graphpad) for further visual graphical presentation of data and compare the taxonomic classification.
The highest relative abundance was measured for Firmicute (66%) and Bacteriodota (29%), Fibrobacteriota (1%), Proteobacteria (1%), and Verrucomicrobiota (2%) among top 10 phyla. While, in the top 15 genera, the highest relative abundance as shown in UCG-005 (24%), Rikenellaceae RC9 gut group (13%), Christensenellaceae R-7 group (12%), Bacteroides (8%), Monoglobus (6%), Alistipes (6%), Prevotellaceae UCG-004 (6%), Ruminococcus (5%), Akkermansia (4%) and Lachnospiraceae AC2044 group (5%). Bray–Curtis distances by Breed (PCoA and NMDS) showed clear difference in the clustering of sheep from goat samples and PERMANOVA showed significance results ($P<0.05$) but no significance difference were seen within different *Swedish* sheep breeds according to ANOVA and Tukeys test.
This research is the first non-culture-based study to analyse the similarities and dissimilarities of the faecal microbiome by using 16S amplicon based metagenomics coupled with NGS (next generation sequencing) in different *Swedish* breeds of sheep and goat (outgroup).

Keywords: Ruminants, Microbiome, Metagenomics, NGS, Data Analysis

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Abbreviations

DF……...Degrees of Freedom

NGS……Next Generation Sequencing

NMDS…….....Non-Metric Multidimensional Scaling

P ……. P value (Significance Value)

PCoA…….…..Principle Coordinate Analysis

PCR…….Polymerase Chain Reaction

R……..Regression (Percentage of Variance)

1. Introduction

1.1 Background

The study of microbiome is an important research subject, related to ecological evolution (Ley *et al.,* 2008), because of its significance to animal health and welfare (Backhed *et al.,* 2005). Over 3.8 billion years, bacteria and archaea, were considered as two types of prokaryotes, undergone evolution (Woese *et al.,* 1987). According to similarities and differences in their phenotypic characteristics, living organisms were traditionally classified into prokaryotes and eukaryotes and distributed further into distinct kingdoms, phyla, classes, orders, families, genera, and species (Woo *et al.,* 2008). Different parameters are interlinked to microbial diversity and microbial evolution, such as age, sex, breed variation, changes in nutrition, and extrinsic factors such as changes in nutrition, lifestyle, environment, and the influence of host-intestinal microbiome relationships (Wallace *et al.,* 2011; Dubois *et al.,* 2017; Wasimuddin *et al.,* 2017; Ng *et al.,* 2018; Cholewinska *et al.,* 2020). The microbial community present in the body indicate the health status of animal (Zilber *et al.,* 2008).

The gastrointestinal tract (GIT) is a multipurpose organ that maintains a dynamic microbiota population that interacts with the host's physiological, nutritional, and immunological systems (Brestoff and Artis, 2013). Ruminant consist of mainly anaerobic and moderately aerobic bacteria (Firmicutes and Bacteroidetes), with limited proportions of Proteobacteria, Fibrobacteres, Tenericutes, Actinobacteria (Cholewinska *et al.,* 2020). These microorganisms contribute to the breakdown of plant fibers into volatile fatty acids and ammonia (Hobson *et al.,* 1997). These products are digested and assimilated by ruminants microbiome to support vital mechanisms such as development, physiology, nervous system, thermoregulation, and immunity (Khan, Weary, and Von Keyserlingk, 2011; Dinan *et al.,* 2013; Rey *et al.,* 2014; Dinan *et al.,* 2017; Cussotto *et al.,* 2018). Additionally, the intestinal microbiome are involved in the development of villi of the ruminant wall (Klein *et al.,* 1987; Beharka *et al.,* 1998).

Approximately 200 ruminant species have been identified (Cholewinska *et al.,* 2020)**.** Sheep and goat consist of several different species as well as breed, and this may be one of the variable which differentiating the microbiome of the gastrointestinal system (GIT) in ruminants. It has been revealed that the breed has an influence on ruminant microbial diversity (Hernandez-Sanabria *et al.,* 2010). In addition, it was investigated on the basis of breed's genetic makeup, depicted the positive impact of host itself on microbial diversity of the GIT system in ruminants (Sasson *et al.,* 2017; Gart *et al.,* 2019). Additionally, it has also been demonstrated that the sire breed has an impact on the intestinal microbiome of offspring's (Youngblut *et al.,* 2019).

Microbial diversity assessment from complex environments has become accessible to broad range of research because of the sequencing tools have gradually shifted the conventional Sanger's method to new generation sequencing technologies. These technologies are cost-effective and the procedures are least time-consuming (Brazelton *et al.,* 2010; Fadrosh *et al.,* 2014; D'Amore *et al.,* 2016).Kerr The NGS sequencing (Illumina) method is increasingly being utilised in 16S rDNA sequencing to conduct diverse microbiome studies i.e. microbial community diversity (Milani *et al.,* 2013; Fadrosh *et al.,* 2014; Sinclair *et al.,* 2015; Kerrigan *et al.,* 2019). In order to diagnose diseases, biomarkers, and target microbes, 16S rDNA sequencing has made it possible to identify microbial populations in both healthy and diseased animal's worldwide (Fadrosh *et al.,* 2014). Furthermore, 16S rDNA sequencing can assist doctors to choose the optimal medications and predict the course of treatment by identifying the etiologic causes of infectious disease (Woo *et al.,* 2008). The microbial community (beneficial bacteria) present in the GIT track of ruminant indicated the health status of animal (Van Donkersgoed *et al.,* 1999; Van Baale *et al.,* 2004; Dowd *et al.,* 2008; Lettat *et al.,* 2012, Bowles *et al.,* 2014), but the knowledge about the microbiome exist in the major part of the body particularly large intestine has little information (de Oliveira *et al.,* 2013). Therefore, a well-established technique was essential for identifying the faecal microbiome in distinct *Swedish* sheep breeds by the 16S amplicon based sequencing coupled with next generation technique (Illumina).

1.2 Statement of Problem

Animals, especially sheep and goat are significantly used as a food sources in the rapidly developing human population in the world. These livestock animals in the presence of microbiome efficiently convert their feed into high nutritional value products. On the other hand, microbiome has a direct effect on the animal health via GIT functions such as body weight, prolificacy, milk supply, and mortality rate.

Information of the microbial diversity regarding change in the composition of faecal microbiome of the *Swedish* sheep breeds can be a basis of knowledge for future scientific research and useful for the application of further successful molecular protocols. However, there is inadequate studies as well as no prior research have been conducted to analyse the faecal microbiome for the determination of microbial diversity in different *Swedish* sheep breeds. Thus, it is might important to examine the microbiome among different *Swedish* breeds of sheep and goat (outgroup).

1.3 Hypothesis

In several microbial community-based studies, genetic variation has already been considered to have association with microbial diversity (Steury *et al.,* 2019). We anticipated that closely-related animals would share similar faecal microbiome and differences would occur in different *Swedish* sheep breeds and goat.

1.4 Objective

The purpose of the current research study was to examine similarities and differences in determining microbial diversity by using 16S gene profiling and bioinformatics data analysis in different *Swedish* breeds of sheep and goat (outgroup).

2. LITERATURE REVIEW

2.1 Swedish sheep

In the end of the 18th century to the First World War, local sheep populations in Europe (EU) were evolved into landrace breeds (Ruane *et al.,* 1999). Sheep are beneficial animal for the extensive and dynamic production system. Sheep are maintained and bred for a variety of reasons, including production of meat, milk, or wool; conservation; or a combination of these. In northern Europe, domesticated sheep breeds commonly exhibit the finer undercoat of wool and also contain another layer of finer wool, covers the coarse outer coat (Ryder, 1984 and 1991).

Swedish sheep have unusual traits, such as a short tail and massive horns that may be used as a weapon as comparing with the most sheep in the world. These breeds were distinguished by their primitive characteristics, which included dual-coated wool, short fluke-shaped tails, and a change in coat colour, pattern, variety, hardness, and procreativity (Dyrmundsson & Niznikowski 2010). The *Swedish* domestic sheep breeds are considered primitive among North European short-tailed sheep (Dyrmundsson & Niznikowski 2010). Although, there are 13 distinguishable *Swedish* sheep breeds, but only a few of them have been considered in this study to identify the microbial diversity of sheep. In addition, RYA sheep were known as the *Swedish* native breed. Local sheep breeds have magnificent horns for fighting against predators. Males weigh about 50 kg, while females weigh about 30–40 kg. They normally give birth to one lamb a year, whereas twins are common (Dahlberg *et al.,* 2012). ROSLAG sheep are derived from a single herd in the *Roslagen* region of *Sweden* (countryside). Individually, both rams and ewes are white or black with white patches or mottled in colour. Unlike most of the lambs, some are born brown at the time of birth. ROSLAG sheep females weigh about 30–40 kg and males weigh about 50 kg, while they normally give birth to one lamb a year, whereas twins are common (Dahlberg *et al.,* 2012). The GESTRIKE sheep breed originated in *Gestrikland* County. It is considered to be the oldest breed and has retained a variety of shades and features. Males and females can develop their horns. Usually, GESTRIKE females weigh about 45 kg, whilst males weight 60–70 kg (Dahlberg *et al.,* 2012). In order to avoid inbreeding, all the lamb rams were kept maintained during the breeding season to allow the flock to mate independently (Dahlbeck *et al.,* 2012).

2.2 Microbiomes

In this thesis, the word "microbiome" was used with continuous consistency to discuss the community of microorganisms that comprises bacteria and archaea. This terminology defines the whole habitat, comprised the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), genetics (i.e., genome), and external environmental factors (Marchesi & Ravel, 2015). These microbiome interact within a specialised ecosystem in the body, such as GIT, skin, faeces, soil, or even different areas of the ocean for the availability of nutrition and the ecological environment (Marchesi & Ravel, 2015). Our knowledge of the microbiome and its effects on health and disease diagnosis within hosts has grown significantly over the past ten years (Virgin *et al.*, 2014). The microbiota is directly associated with the composition of a microbial community, whereas the microbiome covers not just microorganism composition but also genetic makeup and environmental exposures. The composition of a microbial community is closely linked to the microbiota, while the microbiome also shows an organism's genes and how it interacts within breed. The idea of a "microbiome" was first presented by Whipps and Cooke (1988).

"A convenient ecological framework in which to examine biocontrol systems is that of the microbiome." This may be defined as a characteristic microbial community occupying a reasonably well-defined habitat which has distinct physio-chemical properties. The term thus not only refers to the microorganisms involved but also encompasses their "theatre of activity".

The term commensal symbiosis is used for those microbiomes who have synergistic effect and contribute their response positively (Hooper *et al.*, 2001; Tremaroli & Backhed, 2012). In healthy animals, the majority of these microorganisms are neutral or beneficial, forming a commensal relationship in which they aid in digestion and immune system modulation, resulting in a host with a balanced microbial community (Hooper *et al.*, 2001).

2.3 Lower gastrointestinal tract microbiome contributions

Ruminant faeces are occupied by a dense microbiome comprising of bacteria, archaea, protozoa, and fungi. The microbiome structure and microbial composition in sheep are made up of several bacterial taxa such as Firmicutes, Bacteroidaceae, Ruminococcaceae, Verrucomicrobiaceae, Lachnospiraceae, and Prevotellaceae. Theses bacteria are significant part of intestinal microbiota of sheep and goat than any other taxa (Li *et al.,* 2016). Beneficial microorganisms protect the intestine by competing for insufficient supplies from the host, monitoring the host's immune response, enhancing protection against pathogens, producing vitamins, completing metabolic processes, and maintaining intestinal homeostasis (Backhed *et al.,* 2005; Cerf-Bensussan and Gaboriau-Routhiau, 2010; Brestoff and Artis, 2013). For diagnostic purposes, microbiomes in ruminant rumens and faeces have been considered in assessing animal health (Lettat *et al.,* 2012; Dowd *et al.,* 2008). The different parameters existing in the digestive tract include redox potential, pH, gut motility and host secretions, which altogether influence the microbial diversity of the post-ruminal gastrointestinal tract. For example, in the abomasum, the breakdown of the major parts of microbes occurs, passing from the rumen into the ileum by the low pH and enzymatic activity. The fermented environment generated by increasing microbial density in the ileum and moving on to the caecum, colon, and faeces systematically (Frey *et al.,* 2010; De Oliveira *et al.,* 2013; Popova *et al.,* 2017; He *et al.,* 2018; Yeoman *et al.,* 2018).

2.4 Molecular Identification

2.4.1 Metagenomics

For the first time, Handelsman defined the term "*metagenomics*" to explain the newly emerging field that determines genetic material extracted directly from samples (Handelsman *et al.,* 1998). The word "microbiome" is used to consider the bacterial communities that are linked to the animal body, while "metagenomics" refers to the study of microbial communities that do not survive in traditional culture. Metagenomics approaches have been developed and comprises of wide range of techniques to analyse various components of the microbial environment and the metagenome. Additionally, the metagenomics library identified various types of microorganisms (Venter *et al.,* 2004; Kvist *et al.,* 2007). It enables us to determine the diverse variety of microbial communities, which include thousands of bacteria and archaea species.

2.4.2 Next-Generation sequencing

Culture-independent microbial ecology research is now possible due to the rapid advancement of NGS technology. The majority of the literature demonstrated the importance of the small subunit of the ribosomal RNA gene, known in prokaryotes as the 16S rRNA gene and in eukaryotes as the 18S rRNA gene (Pace *et al.,* 1986; Woese *et al.,* 1987; Schmidt *et al.,* 1991; Slonczewski and Foster, 2009). The 16S rRNA gene is the major component of ribosomal subunit of bacteria, comprises of hypervariable and conserved regions, which shows specificity at genus or species level. On the basis of conserved regions, genetic markers can be used to analyse the population structure of bacteria and archaea (Yu *et al.,* 2008).

For the identification of microorganisms, PCR amplification with universal primers designed for 16S rDNA amplicon metagenomics sequencing in conservative regions were considered to be the best techniques (Youssef *et al.,* 2009; Caporaso *et al.,* 2011; Hess *et al.,* 2011). In addition, characteristic nucleic acid sequences can also be used to identify microbial diversity by considering the hypervariable regions of the 16S gene in determining amplicon sequence variation (ASV)**.** This procedure includes amplifying samples' variable regions with specific primers, producing a high-quality sequencing library, and evaluating the generated sequences by data analysis (Wolcott *et al.,* 2016). For each sequencing protocol, Metzker *et al.* (2010) described how the template is made and how the sequencing can be done.

2.4.3 Bioinformatics for metagenomics

The scientific field of bioinformatics is focused on developing different methods for the appropriate utilisation as well as interpretation of practical data in biomedical sciences (Hogeweg *et al.,* 2011). For the study of metagenomics, bioinformatics necessitates the use of a variety of tools to perform three major tasks: sequence quality control, sequence assembly, and sequence classification (Kunin *et al.,* 2008). The technique to synthesise extended, continuous sequences (contigs) from sequence reads is referred to as sequence assembly (Pop *et al.,* 2009). The classification of sequence reads or contigs into their accurate taxonomic population is the important step in metagenomic research apart from the statistical analysis. Initially, this approach was carried out by sequence similarity tools such as the basic local alignment search tool (BLAST) (Altschul *et al.,* 1990; Boisvert *et al.,* 2012; Namiki *et al.,* 2012), but the time constraints was one of the major problem, now reference sequence approach has considered to be easiest way for taxonomic annotation/classification.

3. Material Methodology

3.1 Sample collection

A total of 18 faecal samples (without replicates) of sheep (n=15) and the supplementary samples of goats (n=3) were selected in this research study, obtained by supervisor (Dr. Erik Pelve) from the Department of Anatomy, Physiology, and Biochemistry and co-supervisor (Dr. Anna Maria Johansson) from the Department of Animal Breeding and Genetics at SLU. The different breeds of sheep i.e. RYA (Local breed), GESTRIKE (Heritage breed), ROSLAG (Rare breed), and a cross of SUFFOLK and TEXEL (Hybrid meat breed) as well as *Swedish* Landrace of goat as an outgroup were employed for the sample collection. All faecal samples were collected from different farms near Uppsala such as RYA from Farm A, GESTRIKE from Farm B, ROSLAG and SUFFOLK and TEXEL from same Farm C and *Swedish* LANDRACE goat (dairy goat) faecal samples were taken from Farm D (Svensk Lantrasget) at SLU. The *Swedish* breeds of sheep were provided ad libitum water and fed with almost same feed with slight difference as mentioned in the (Table 1).

The RYA sheep was fed on eating silage and concentrates (concentrate with 80% barley, 20% wheat) along with mineral feed with copper. They also got some straw from barley with some other mixed plant herbs into it. The GESTRIKE sheep was given silage during the winter and spring with a small amount of concentrates ("Edel får"). The ROSLAG sheep and SUFFOLK and TEXEL sheep were grazing and given silage in the winter. The pregnant ewes and the first time after birth (before grazing season starts) also got the feed with the name of "fårfor tacka". LANDRACE goat were given ensilage, or mix between haylage and concentrates, feed grains and wheat 80% and barley 20% wheat + mineral supplement with copper. Some barley straw were also provided with grass between meals. The animals at the farm level were not considered to be experimental animals. Therefore, no special ethical permission was required. Sheep samples such as some of the samples were frozen within the same day and some were sent overnight. None of the faecal sample was frozen immediately except the goat faecal samples (frozen within an hour) and stored at -20.

Table 1. Names of species, replicate numbers of each breed with names, farms and feed

No.	Species	Breeds names _no.	Farms	Feed
1.	Sheep	Gestrike1_n2	B	Silage & Concentrate (Edel får)
2.	Sheep	Gestrike2_n3	B	Silage & Concentrate (Edel får)
3.	Sheep	Gestrike3_n3	B	Silage & Concentrate (Edel får)
4.	Sheep	Gestrike4_n1	B	Silage & Concentrate (Edel får)
5.	Goat	Goat_G1_n1	D	Silage/mix haylage and concentrates
6.	Goat	Goat_G2_n1	D	Silage/mix haylage and concentrates
7.	Goat	Goat_G3_n1	D	Silage/mix haylage and concentrates
8.	Sheep	Hybrid_B1_n1	C	"fårfor tacka"& silage
9.	Sheep	Hybrid_B2_n1	C	"fårfor tacka"& silage
10.	Sheep	Roslag_R1_n1	C	"fårfor tacka"& silage
11.	Sheep	Roslag_R2_n1	C	"fårfor tacka"& silage
12.	Sheep	Roslag_R3_n1	C	"fårfor tacka"& silage
13.	Sheep	Rya1_n3	A	Silage & concentrate, minerals
14.	Sheep	Rya2_n3	A	Silage & concentrate, minerals
15.	Sheep	Rya3_n2	A	Silage & concentrate, minerals
16.	Sheep	Ry4_n2	A	Silage & concentrate, minerals
17.	Sheep	Rya5_n3	A	Silage & concentrate, minerals
18.	Sheep	Rya6_n2	A	Silage & concentrate, minerals

3.2 Molecular Analysis

3.2.1 Work Flow

The workflow started with the sample preparation by genomic DNA extraction method, followed by PCR amplification of DNA (quality control) for initial 6 faecal samples of sheep breeds. The extracted DNA of 100 µl for all 32 samples (included replicates of RYA and GESTRIKE breed) as mentioned in the (Table 2) were added into separate 32 eppendorf with ID sample number dispatched to the Novogene. The amplicons were then purified for further amplicon library preparation along with quality control protocols. To sequence the amplicon genomic library, SBS (Sequence by synthesis) technique was employed coupled with next generation sequencing by the Illumina NovaSeq 6000 platform. Quality control protocol subsequently were performed to validate the data, bioinformatics and statistical analysis were done for further visual graphical presentation of data to analyse and compare the taxonomic classification at phylum and genus level.

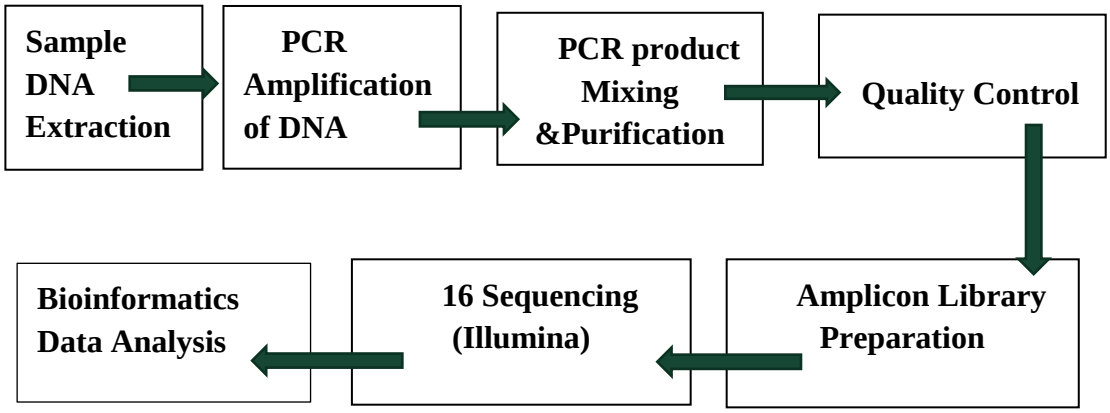

Figure 1. The workflow from DNA samples to final data

3.2.2 DNA Extraction

The total 32 frozen faecal samples (included replicates of RYA and GESTRIKE sheep breeds) as mentioned in (Table 2) were subjected to a beat beating step before the start of genomic DNA extraction protocol. 0.3g of 0.1mm Zirconium Silica beads were added to a 2ml screw top tube for each samples. The faecal samples (n=32) of approximately 10 mg were mixed with 500µl of ATL buffer according to the instructions of DNeasy Blood and Tissue Mini Kit (QIAamp_ Catalogue 69504 and 69506) with the beat beating step in the Pre-cell lyse machine with 2 cycles for 60s at 8000 rpm for 15s for all samples.

After the beat beating step, all faecal samples were centrifuged at 7000 rpm (1000g) for 2 min then the supernatants were added to new eppendorf tubes. The centrifugation step was repeated at maximum speed (13000 rpm) for 1 min to collect the 200µl supernatant into a new 2 ml eppendorf tube. The 200µl supernatant for each samples were mixed with 180µl of ATL buffer and 20µl Proteinase K (~20 mg/ml). The samples were incubated at 900 rpm at 56°C for 10 min. The homogenised mixtures for all samples were then mixed with 200 µl of AL buffer for each and then incubated at 900 rpm at 56°C for 10 min. After the incubation, the 32 sample mixtures were dissolved in 200 µl for each of 96-100% of Ethanol carefully.

The mixture samples were pipetted out into the DNeasy mini spin column placed in a 2ml collection tube for all samples separately and centrifuged at 8000 rpm at 56°C for 1 min. The supernatant were discarded and the remaining DNA from the mixture were obtained in the collection tube, then added 500 µl of AW1 and centrifuged at 8000 rpm at 56°C for 1 min for each sample. The same step were repeated and a spin column were placed in the new 2ml collection tube and 500 µl of AW2 were added and centrifuged at 14000 rpm at 56°C for 3min for each sample. The spin column were transferred to a new 2ml centrifuge tube for each 32 faecal samples. The genomic DNA were eluted for all samples by adding a 200 µl AE buffer for each to the centre of the spin column membrane, incubated for 1 min at room temperature (15-25°C).

The final centrifugation proceeded at 8000 rpm for 1 min. The last same step was repeated to get more yield of DNA as an optional step for few replicates (N1- N5) as mentioned in the (Figure 5) but these replicates were not included in the actual total 32 extracted DNA samples. The extracted DNA samples were then preserved at -20°C after the completion of DNA extraction protocol.

Table 2. Showing the names of species, sample codes with their sample identities

No.	Breeds & Landrace	Samples Identity	Codes of Samples
1.	RYA sheep	R-S1a	20220523 #S1
2.	RYA sheep	R-S3a	20220523 #S2
3.	RYA sheep	R-S2a	20220523 #S3
4.	GESTRIKE sheep	G-S3a	20220523 #S4
5.	RYA sheep	R-S5a	20220523 #S5
6.	RYA sheep	R-S4a	20220523 #S6
7.	RYA sheep	R-S6a	20220523 #S7
8.	GESTRIKE sheep	G-S2a	20220523 #S8
9.	LANDRACE goat	G1	20220523 #S9
10.	LANDRACE goat	G2	20220523 #S10
11.	GESTRIKE sheep	G-S3b	20220523 #S11
12.	LANDRACE goat	G3	20220523 #S12
13.	ROSLAG sheep	RosR1	20220523 #S13
14.	ROSLAG sheep	RosR2	20220523 #S14
15.	GESTRIKE sheep	G-S1a	20220523 #S15
16.	SUFFOLK + TEXEL (Hybrid sheep)	HB1	20220523 #S16
17.	ROSLAG sheep	RosR3	20220523 #S17
18.	RYA sheep	R-S6b	20220523 #S18
19.	RYA sheep	R-S2b	20220523 #S19
20.	SUFFOLK+ TEXEL (Hybrid sheep)	HB2	20220523 #S20
21.	GESTRIKE sheep	G-S2b	20220523 #S21
22.	RYA sheep	R-S1b	20220523 #S22
23.	RYA sheep	R-S5b	20220523 #S23
24.	GESTRIKE sheep	G-S4	20220523 #S24
25.	RYA sheep	R-S2c	20220521 #S25
26.	RYA sheep	R-S3b	20220521 #S26
27.	RYA sheep	R-S1c	20220521 #S27
28.	RYA sheep	R-S5c	20220521 #S28
29.	GESTRIKE sheep	G-S3c	20220521 #S29
30.	GESTRIKE sheep	G-S1b	20220521 #S30
31.	GESTRIKE sheep	G-S2c	20220521 #S31
32.	RYA sheep	R-S4b	20220521 #S32

3.2.3 PCR Amplification

Polymerase chain reaction (PCR) amplification method was performed to amplify a portion of the hypervariable regions such as, V3–V4 and V4–V5 of bacterial primers (341F & 806R) and archaeal primers (Arch 519F & Arch 915R) respectively as mentioned in the (Table 3). PCR method was used only as a quality control test to amplify initial 6 samples of extracted DNA samples such as, sheep breeds i.e RYA (R-S) and GESTRIKE (G-S) i.e. R-S6a, R-S5a, R-S4a, G-S2a, G-S3a, G-S4a (these amplicons were not included in the final samples list which were being sequenced). PCR was performed by using 2 × HotStarTaq Master Mix (10 x PCR buffers, dNTP mix 10mM of each, reverse and forward primers of 1µl each, HotStarTaq DNA Polymerase 0.5µl of each and distilled water in variable quantity) were added with 1µl of each template DNA to make 100µl PCR product. The PCR cycling conditions were applied such as, the initial activation, denaturation, annealing and extension to complete 35 cycles as mentioned in the (Table 4).

Table 3. Details of the primers

No.	Species Target	Primers	Direction	Sequence	Region	Amplicon length (bp)
1.	Bacteria Primers	341F	Forward	CCTAYGGGRBGCASCAG	V3-V4	470
		806R	Reverse	GGACTACNNGGGTATCTAAT	V3-V4	470
2.	Archaea Primers	519F	Forward	CAGCCGCCGCGGTAA	V4-V5	400-500
		915R	Reverse	GTGCTCCCCCGCCAATTCCT	V4-V5	400-500

Table 4. Optimized cycling protocol for PCR

No.	Steps	Temperature	Time
1.	Initial activation step	95°C	15 min
2.	3- Step Cycling		
a.	Denaturation	94°C	0.5-1min
b.	Annealing	62°C	0.5-1min
c.	Extension	72°C	1min
d.	Number of Cycles	35	
3.	Final Extension	72°C	10 min

3.2.4. Agarose gel electrophoresis

For the confirmation of gene amplification, 0.7% agarose (0.35g) was dissolved in a 50ml of 1X TAE buffer. The solution was then heated in a microwave for 2 to 3 times within almost 10s to make a clear solution. 0.5µl of diluted gel red solution were added for the visualization of clear bands. The gel mixture was poured out into a gel casting tray to be solidified. A comb was used in order to make wells, then removed after the gel solidification and placed into the electrophoretic tank. The 5µl of each extracted DNA sample were mixed with 0.5µl loading dye for each sample and introduced into the gel wells. An 80-100 volts of power was supplied to run out agarose gel electrophoresis method for about 30-45 min. After the completion of gel electrophoresis process, clear bands were visualised under UV light.

3.2.5 Nano-Drop spectrophotometer and Qubit Assay Test

In order to check the purity and concentration of genomic DNA, Nanodrop 2000 technique (spectrophotometric devices) was performed in accordance with A260/A280 absorbance ratio, reported as mean values ±SEM for all 32 samples. The ratio was fluctuated in between 15.1 ng/µl to 21.5 ng/µl. For the precise and accurate quantification of genomic DNA, Qubit dsDNA BR (Broad Range) Assay Kits (Catalogue Q32851-Invitrogen, Carlsbad, CA, USA) was used to quantify dsDNA fluorometrically. The BR (Standard 1 = 0 ng/µl DNA in TE buffer, Standard 2 = 100 ng/µl DNA in TE buffer) were used. For the preparation of Qubit working solution, the Qubit dsDNA BR Reagent was diluted in 1:200 ratio in Qubit dsDNA BR Buffer according to the instructions provided in the Qubit Assay Kit (Catalogue Q32851).
The final 100µl concentration of each 32 extracted genomic DNA were converted into separate 32 eppendorf tubes with their respective labelled sample I.D numbers as mentioned in (Table 2). These extracted genomic DNA samples were delivered to Novogene Company for further 16S amplicon metagenomics sequencing.

3.3 Data analysis procedures

To describe the faecal microbiome in different *Swedish* breeds of sheep and goat, a total 32 replicates of ruminants samples (sheep and goat) were selected. For all replicates of each individual, percentage mean values were used (graphical presentation) for measuring the compositional microbial analysis. In order to analyse the clustering of the composition of microbiome in the faecal samples, (DADA2) pipeline were used to generate the taxonomy of bacteria according to their amplicon sequence variant (ASV) data i.e. Kingdom, Phylum, Class, Order, Family, Genus and Species. The representative sequences of each ASV were taxonomically annotated to obtain the corresponding taxa information and taxa-based abundance distribution.

3.3.1 Beta Diversity Indices

For Bioinformatics & statistical approach, R software (version_4.2.1) included vegan, ggplot2 and phyloseq packages (Oksanen *et al.,* 2007) as well as PRISM software (Graphpad, version_ 9.3.1) were used for the presentations of all graphs and statistical data analysis. Beta diversity dissimilarity matrix was performed to measure the difference in species composition in the microbial community between pairwise breeds (Minozzi *et al.,* 2020). Different indices were implemented within and among sample variability groups on the basis of ASV counts for taxonomic classification. For example, statistical approach included (PCoA & NMDS) based on Bray-Curtis dissimilarity indices (Bray *et al.,* 1957). For the evaluation of data, the Permutational analysis of variance (PERMANOVA), ANOVA, Tukeys and Simper Test (Biscarini *et al.,* 2018) were implemented to explain significant differences in the structure of the microbial community between the breeds.

4. Results

4.1 Gel Electrophoresis images

In order to analyse the presence of the 16S gene and potential inhabitants of the PCR reaction, a total of 32 samples included replicates of RYA (n= 15) and GESTRIKE (n=9), ROSLAG (n=3), HYBRID (n=2) sheep breeds and goat LANDRACE (n=3) were collected and processed for further genomic DNA extraction as mentioned in the (Table 2). All the extracted DNA samples were seen as clear bands in the gel for all samples from S1-S3 (Figure 2) and S5-S12 (Figure 3), S13-S24 (Figure 4) and S25- S32 (Figure 5) except for the 20220523#S4: G-S4a as shown in the (Figure 2).

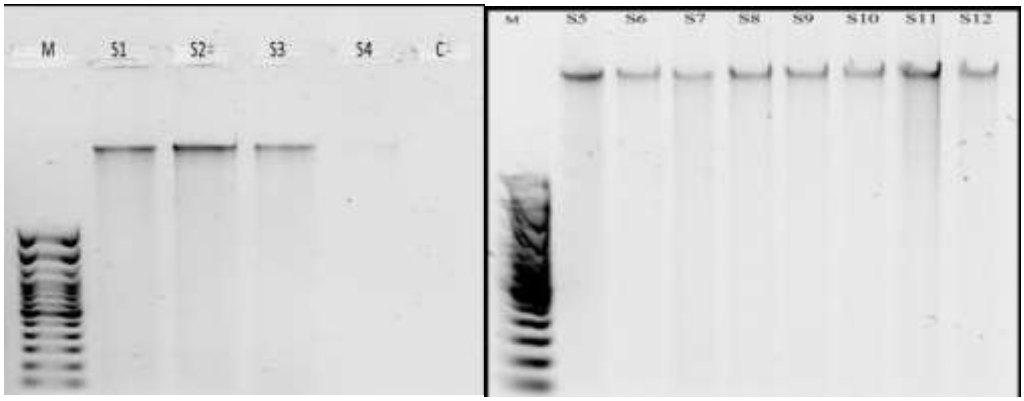

Figure 2. Gel electrophoresis of DNA Extraction from (S1-S4), S1: R-S1a, S2: R-S3a, S3: R-S2a, S4: G-S4a.
Figure 3. Gel electrophoresis of DNA Extraction from (S5-S12), S5: R-S5a, S6: R-S4a, S7: R-S6a, S8: G-S3a, S9: G1, S10: G2, S11: G-S4b, S12: G3

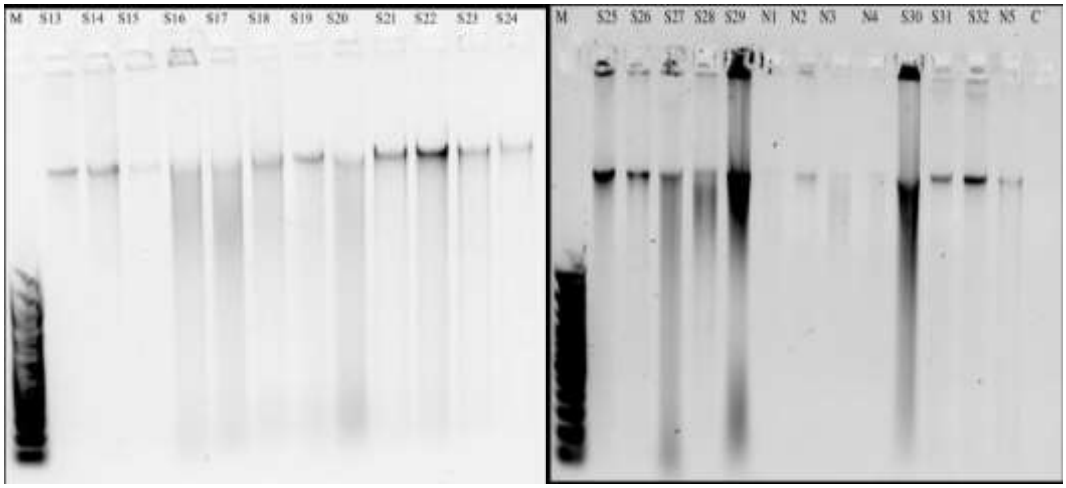

Figure 4. Gel electrophoresis of DNA Extraction from (S13-S24), S13: RosR1, #S14: RosR2, #S15: G-S2a, #S16 HB1, S17: Ros R3, S18: R-S6b, and S19: R-S2b, S20: HB2, S21: G-S3b, S22: R-S1b, S23: R-S5b, S24: G-S1.

Figure 5. Gel electrophoresis of DNA extraction: S25: R-S2c, S26: R-S3b, S27: R-S1c, S28: R-S5c, S29: G-S4c, S30: G-S2b, S31: G-S3c, S32: R-S4b.

Note. Optional step replicates during DNA extraction: N1: R-S2a, N2: G-S2a, N3: G-S3a, N4: R-S2a, N5: G-S4a (NOT included in the final sample list for 16S sequencing).

These are optional replicates 20220523#N: (R-S2a), 20220523#N4: (R-S6a) were shown least concentration as displayed in the Gel electrophoresis (Figure 5). These optional replicates from (N1-N5) were those faecal samples performed as an optional step after genomic DNA extraction to see the concentration of bands only. These optional replicates (N1-N5) were not included in the final list of samples from (S1-32) for 16S sequencing.

4.2 Qubit Assay Test

The precise quantity of each 32 samples of extracted DNA was checked on Qubit Assay test with readout in ng/µl as mentioned (Table 5). The final volume in each tube was considered as 200µl. All the extracted genomic DNA samples showed positive results except #S18 and #S19 (showed least concentration of extracted genomic DNA) in the Qubit Assay test.

Table 5. Final list of DNA Extracted samples with Qubit Assay Test

No.	Breeds & Landrace	Codes of Samples	Qubit Assay Conc. ng/µl
1.	RYA sheep	20220523 #S1	6.01
2.	RYA sheep	20220523 #S2	4.33
3.	RYA sheep	20220523 #S3	9.02
4.	GESTRIKE sheep	20220523 #S4	3.02
5.	RYA sheep	20220523 #S5	16.01 ng
6.	RYA sheep	20220523 #S6	8.02 ng/µl
7.	RYA sheep	20220523 #S7	4.01
8.	GESTRIKE sheep	20220523 #S8	9.34
9.	LANDRACE goat	20220523 #S9	12.8
10.	LANDRACE goat	20220523 #S10	14.2
11.	GESTRIKE sheep	20220523 #S11	5.80
12.	LANDRACE goat	20220523 #S12	11.0
13.	ROSLAG sheep	20220523 #S13	2.58
14.	ROSLAG sheep	20220523 #S14	4.12
15.	GESTRIKE sheep	20220523 #S15	10.01
16.	SUFFOLK + TEXEL sheep	20220523 #S16	27.8
17.	ROSLAG sheep	20220523 #S17	4.46
18.	RYA sheep	20220523 #S18	0.010
19.	RYA sheep	20220523 #S19	0.010
20.	SUFFOLK+ TEXEL sheep	20220523 #S20	6.30
21.	GESTRIKE sheep	20220523 #S21	9.03
22.	RYA sheep	20220523 #S22	3.62
23.	RYA sheep	20220523 #S23	4.56
24.	GESTRIKE sheep	20220523 #S24	9.42
25.	RYA sheep	20220521 #S25	2.60
26.	RYA sheep	20220521 #S26	4.33
27.	RYA sheep	20220521 #S27	4.35
28.	RYA sheep	20220521 #S28	7.10
29.	GESTRIKE sheep	20220521 #S29	5.40
30.	GESTRIKE sheep	20220521 #S30	11.2
31.	GESTRIKE sheep	20220521 #S31	7.70
32.	RYA sheep	20220521 #S32	3.10

4.3 Quality Control (QC)

The total (n=32) extracted DNA samples were delivered to Novogene based on *"DNA Samples QC Criteria"* for the quality requirements of library preparation and sequencing. All the 32 samples were declared *"PASS"* to meet the requirement of library construction according to the report of Novogene. The requirements and conditions for the gel electrophoresis for genomic DNA were also implemented to check quality control by 1% of agarose gel run at the rate of 100V at 40 min. For PCR product, 2% of agarose concentration was measured for the gel electrophoresis method at the rate of voltage (80V) with run time at 40min.

4.4 Sequencing results and taxonomy description

A total of 32 extracted DNA samples with equal quantity of each 100µl were subjected for the sequencing on the basis of hypervariable regions (V3–V4) and (V4-V5) of the bacterial and archaeal 16S rRNA gene. The unfiltered sequence reads were produced as a result of total no. of sequences (1799435). After quality filtering, 10734 sequence variants (ASV) were selected based on the bacteria for the further subsequent statistical data analysis.

4.5 Compositional Microbial Community Analysis

4.5.1 Bar graphs of Taxa relative abundance of bacterial Phyla

The five groups of sheep and goat (outgroup) were selected in order to analyse the compositional microbial communities. The distribution histogram were made by using the PRISM (Grahpad; version_9.3.1) software. The bar graph indicated the highest percentage relative abundance of most top 10% common strains of taxa at phylum level, displayed the mean percentage of relative abundance in the y-axis and names of the each group represented in the x -axis. The result has shown no difference of taxa at phylum level between the groups. For example, almost the same relative abundance percentages such as, Firmicutes (66%) and Bacteriodota (29%) as compared to remaining percentage relative abundances i.e. Fibrobacterota (1%), Halobacterota (0%), Proteobacteria (1%), Verrucomicrobiota (2%) etc. as shown in the (Figure 6)

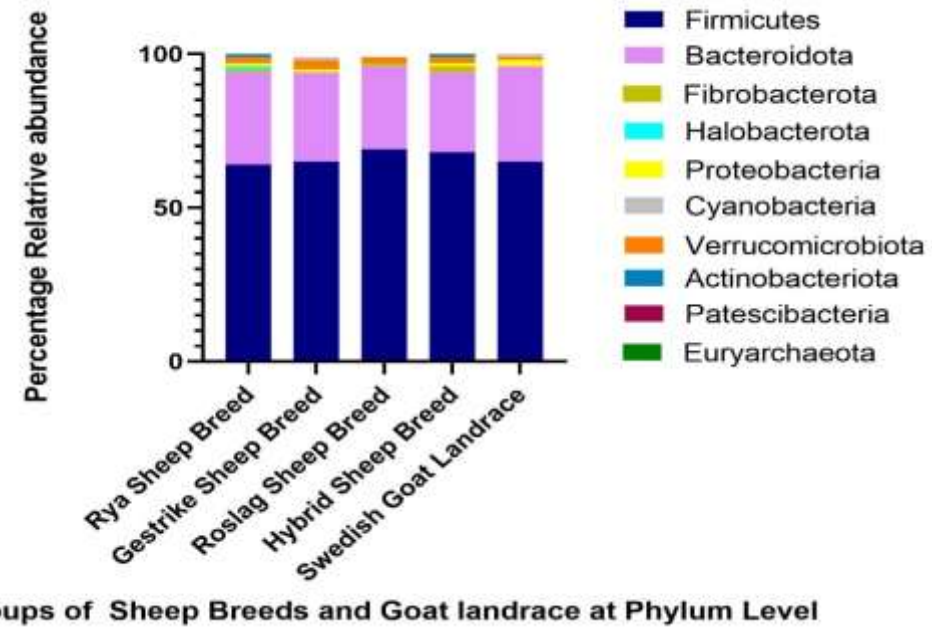

Figure 6. Percentage relative abundance of top 10 common phyla of RYA, GESTRIKE, ROSLAG breed, HYBRID sheep breeds and LANDRACE goat (5 groups).

The bar graph as shown in the (Figure 7) were made by using R software indicated the relative abundance of bacteria in the total 18 samples (n=no. of replicates) of sheep breeds and goat (outgroup) individually, where the height of the bar plot is proportional to the abundance of bacteria at phylum level. For example; according to the graphical presentation, the highest abundance of Firmicutes and Bacteroidota have seen among all groups which were less common than 1% phyla of sheep breeds and goat (outgroup) which were merged into the other group.

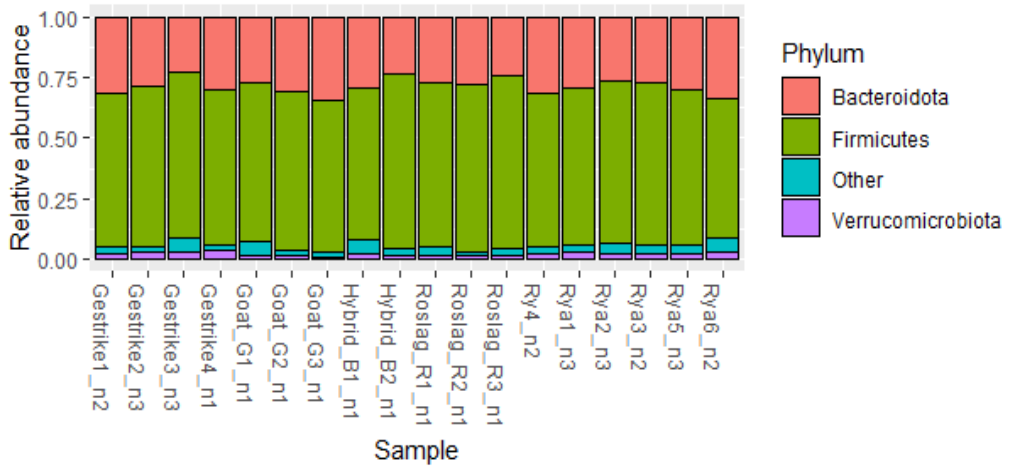

Figure 7. Taxa relative abundance at Phylum level in all groups (sample-wise) where all groups less common than 1% were incorporated into the other group.

4.5.2 Bar graphs of Taxa relative abundance of bacterial Genera

The different bars as shown in the (Figure 8) indicated the percentage relative abundance of top 15 bacteria at genus level, included mean percentage in the y-axis and 5 groups of breeds (RYA, GESTRIKE, ROSLAG, HYBRID and LANDRACE goat) represented individually in the x-axis. The highest percentage has shown according to the height of bar i.e. genera UCG-005 (24%), Rikenellaceae RC9 gut group (13%), Christensenellaceae R-7 group (12%), Bacteroides (8%), Monoglobus (6%), Alistipes (6%), Prvotellaceae UCG-004 (6%), Ruminococcus (5%), Akkermansia (4%), Lachnospiraceae AC2044 group (5%) has shown almost similarities among top 15 genera of five groups of sheep breeds and goat as a outgroup.

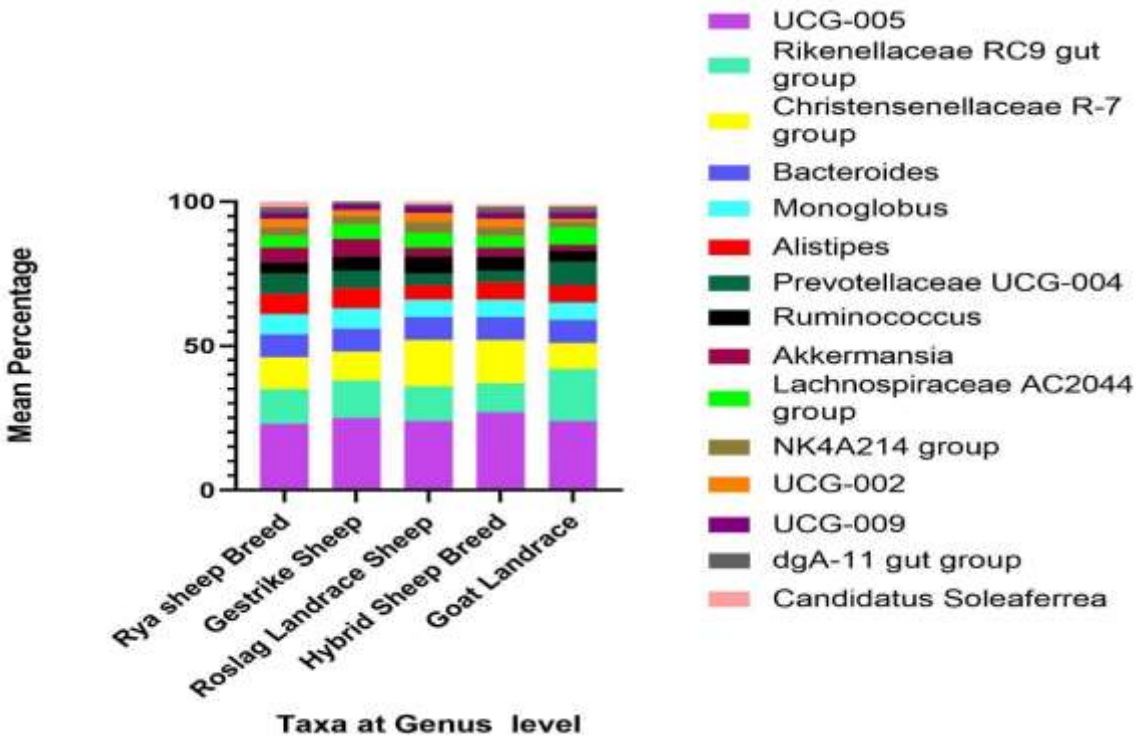

Figure 8. Percentage relative abundance of 15 taxa at Genus level (5 groups of breeds and goat)

The bar graph (Figure 9) shown the relative abundance of bacteria in different sheep breeds and landrace goats (outgroup) where the height of the bar plot is proportional to the abundance of bacteria at genus level. According to the graphical presentation indicated the highest abundance of genus UCG-005, Rikenellacea RC9 gut group, Christensenellaceae R-7 group, Bacteroides and Monoglobus among others Taxa (as all groups less common than 5% were submerged into other group).

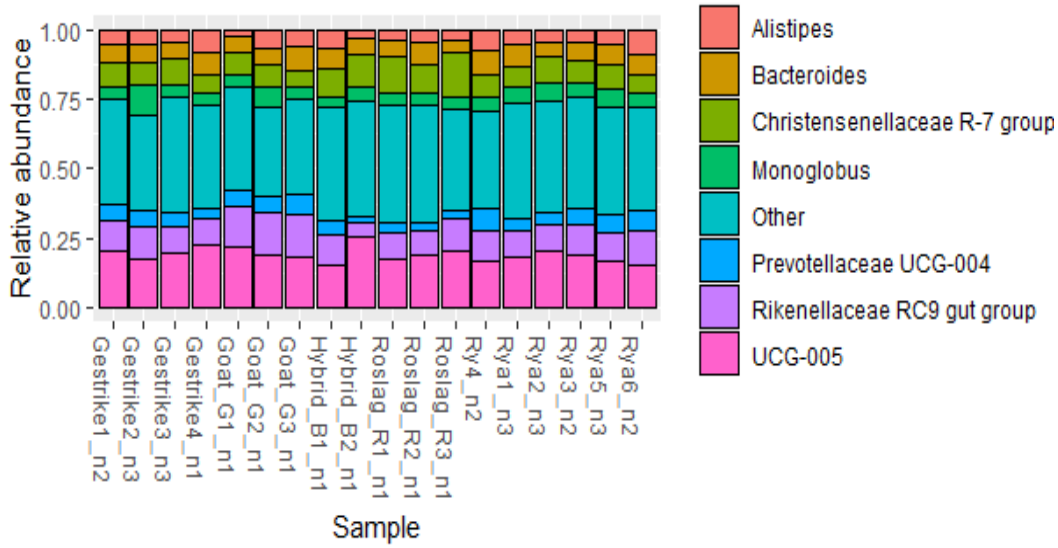

Figure 9. Relative abundance of all taxa at the Genus level according to the total 18 samples (n= no. of replicates), as all groups less common than 5% are submerged into other group.

4.6 Bar graphs of relative abundance analysis (Individual groups)

The bar graph in the (Figure 10) indicated the mean percentage relative abundance of top 15 bacteria at genus level of the replicates of group RYA sheep breed i.e. the percentage relative abundance with replicates of each sample as shown in the (Figure 10) showed least difference among the taxa of 6 individuals of same group of RYA sheep breed. For example, in the case of UCG-005 genera, S1-22-27 (Rya1_n3) represented the genera of UCG-005 (21%), S3-19-25 (Rya2_n3)-UCG-005 (26%), S2-26 (Rya3_n2)-UCG-005(25%), S6-32 (Rya4_n2)-UCG-005(19%), S5-23-28 (Rya5_n3)-UCG-005(23%),S7-18(Rya6_n2)-UCG-005(24%) and same case with the others taxa as well.

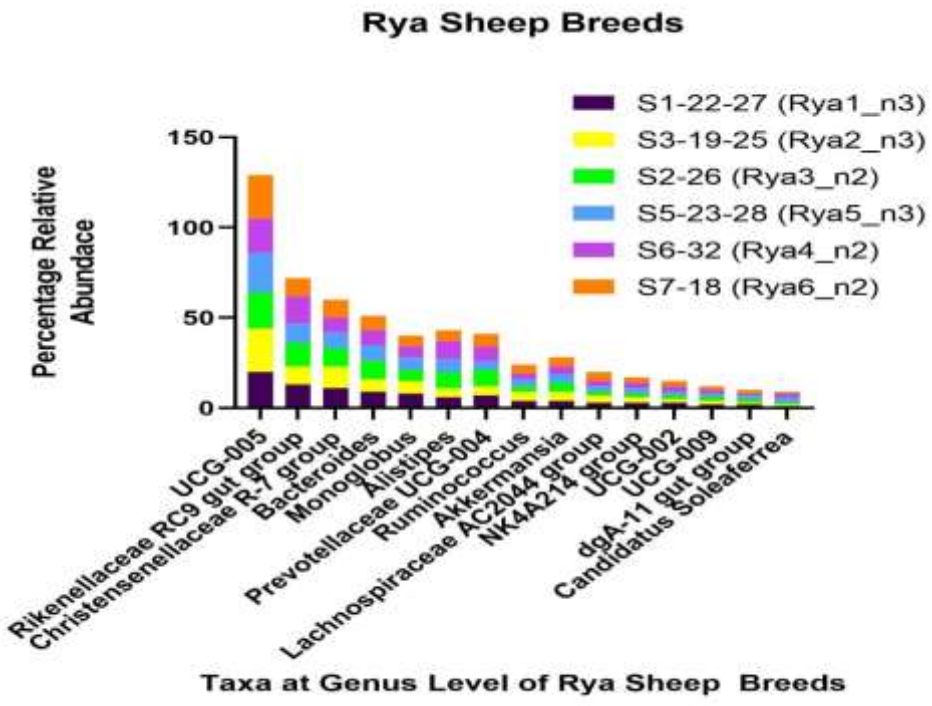

Figure 10. Percentage relative abundance of top 15 genera (RYA sheep breeds)

The bar graph in the (Figure 11) presented the percentage mean relative abundance of top 15 bacterial taxa at genus level within a group of GESTRIKE sheep breed, For example, UCG-005 (25%) in the samples of S15-30 (Gestrike1_n2), UCG-005 (21%) in the replicates of samples S8-21-31 (Gestrike2_n3), UCG-005 (22%) in the replicates of samples S4-11-29 (Gestrike3_n3) and UCG-005 (27%) in the sample of S24 (Gestrike4_n1) which showed least differences of microbiome diversity in the replicates of samples or within the taxa of GESTRIKE sheep breed and same case with the others taxa as well. .

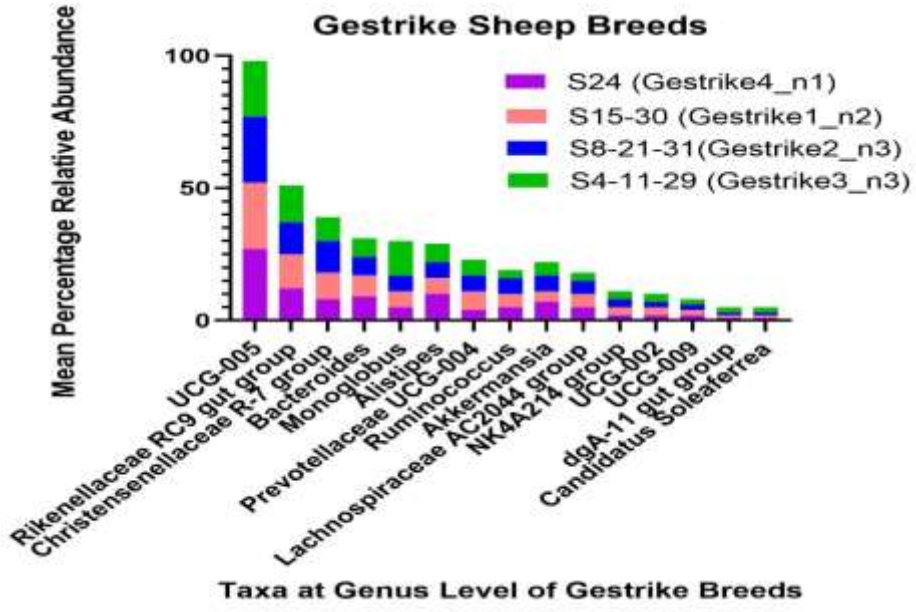

Figure 11. Percentage relative abundance of top 15 genera of GESTRIKE sheep breeds

The bar graph in the (Figure 12) depicted the percentage mean of relative abundance of top 15 bacterial taxa at genus level within a group of individual ROSLAG sheep i.e. UCG-005 (27%) in the samples of S13 (Ros1) UCG-005 (upto 22%), S14 (Ros2) UCG-005 (upto 25%) and S17 (Ros3) having UCG-005 (upto 24%), shown clear similarities of microbiome within same group of ROSLAG sheep breed.

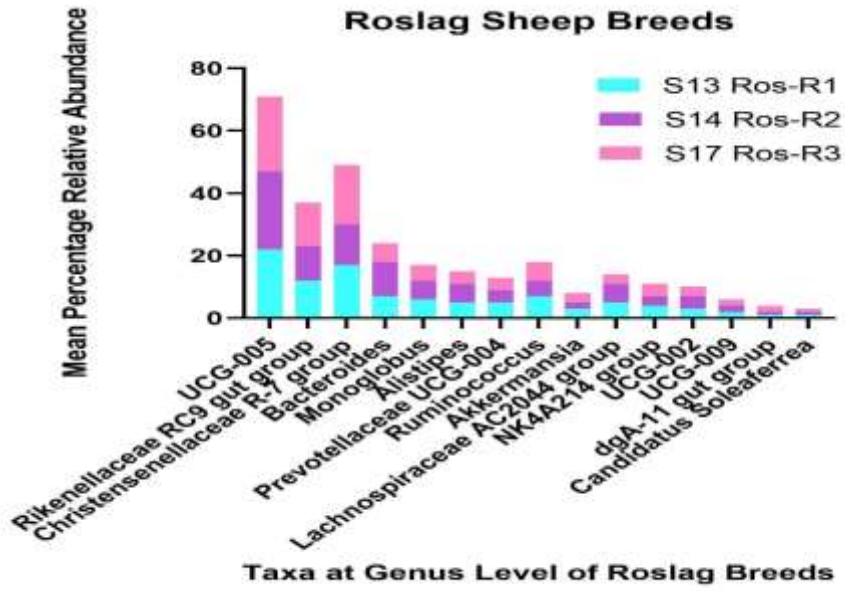

Figure 12. Percentage relative abundance of top 15 genera of ROSLAG sheep breeds

The bar graph in the (Figure 13) has shown clear difference between the percentage mean of relative abundance of top 15 bacterial taxa at genus level within a group of HYBRID sheep breeds i.e. the percentage mean of relative abundance of UCG-005 (33%) in the sample of (S20: HB2) and UCG-005 (20%) in the sample of (S16: HB1). These bar graphs presented clear difference of microbial diversity within the group of hybrid breed of sheep among initial top 15 genera of UCG-005, Rikenellaceae RC9 gut group, Christensenellaceae R-7 group, Alistipes genera and vice versa.

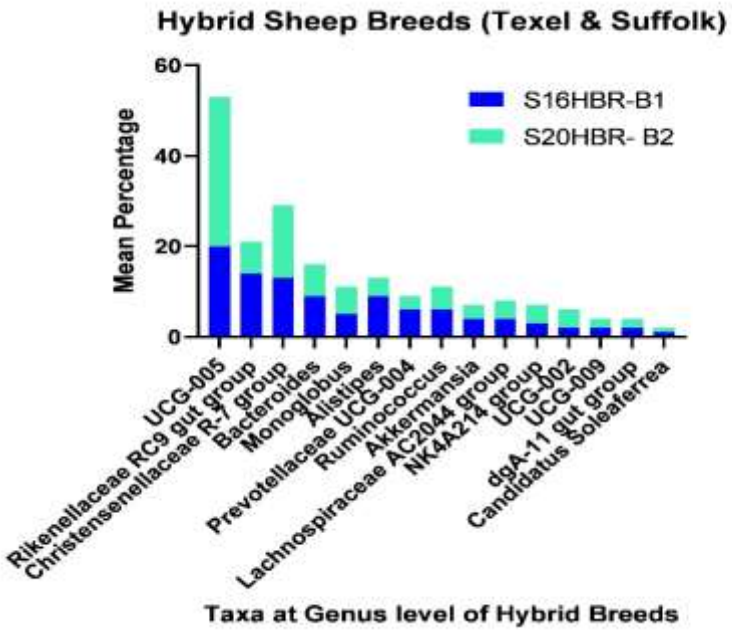

Figure 13. Percentage relative abundance in top 15 genera in HYBRID sheep breeds.

The bar graph in the (Figure 14) shown the percentage mean of relative abundance of top 15 bacteria at genus level within a group of goat LANDRACE i.e., S9-G1-UCG-005 (27%), S10-G2-UCG-005 (23%) and S12-G3-UCG-005 (22%) and Vice versa. This bar graph has shown more similarities of taxa (least difference) within same group of goat LANDRACE (outgroup)

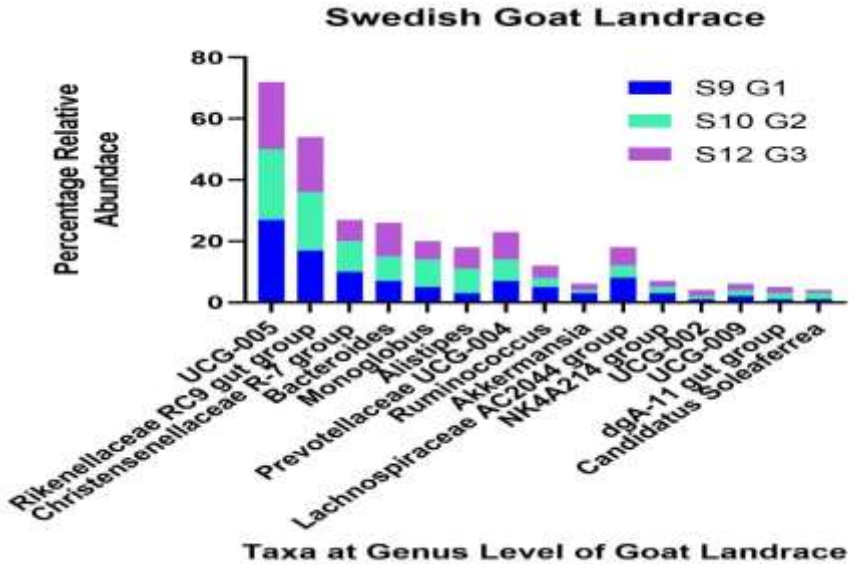

Figure 14. Percentage relative abundance of top 15 genera of goat LANDRACE

4.7 Comparative Statistical Data Analysis

4.7.1 Beta diversity index

The Beta diversity presented the comparison between the communities on the basis of microbial composition. It can be calculated on the basis of gradient of distance analysis displayed with the ordination plots. Hence, the data can be visualized on the basis of PCOA, NMDS, PERMANONA, ANOVA, Tukeys test and Simper test.

4.7.2 Principal coordinate analysis (PCoA) based on Bray-Curtis indices by Breed

In order to understand the pattern of separation between different 5 groups of sheep breeds and goat (out group), PCoA (Principle Coordinate Analysis) was performed based on Bray- Curtis dissimilarity matrices for bacteria. It presented the faecal microbiome population which were clustered close within the same breeds of sheep but goat LANDRACE group distinctly clustered from the sheep according to the distance matrices. The PCoA indicated that goat groups were distinctly clustered separately have shown marked distribution of microbiota as compared with sheep breeds (clustered closely). Scattered points in the principal component analysis indicated the different breeds and their relationship among each other on the basis of pattern of separation between different microbial populations. In the two dimensional plot presentation, both axis displayed a total percentage of variance 12.8% and 18.1% respectively, interpreted as an eigenvalues measured ecological distances as shown in the (Figure 15 and 16).

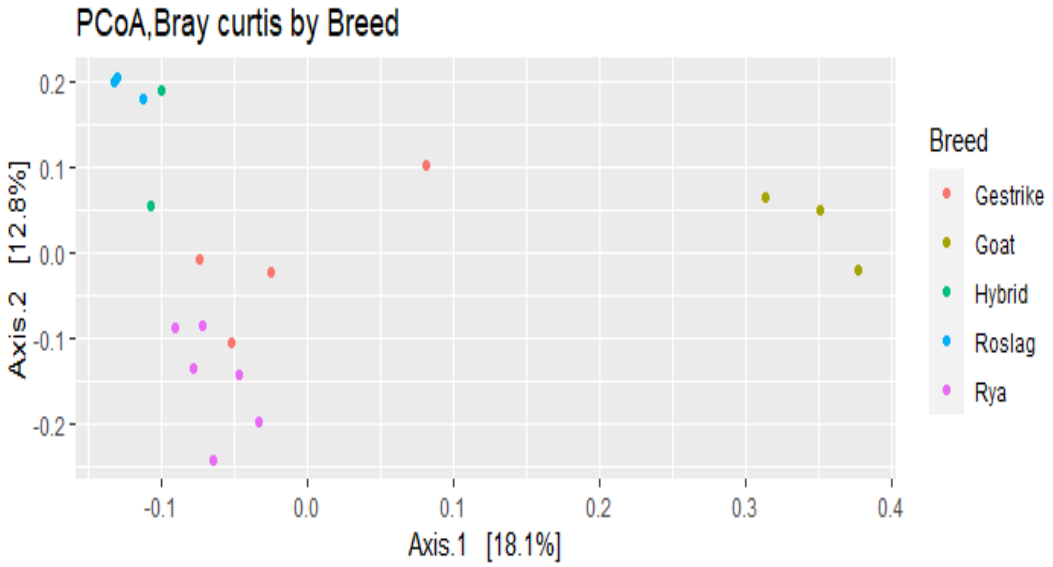

Figure 15. PCoA (Bray-Curtis indices by Breed): The ordination graph indicated the pattern of community distribution between breeds.

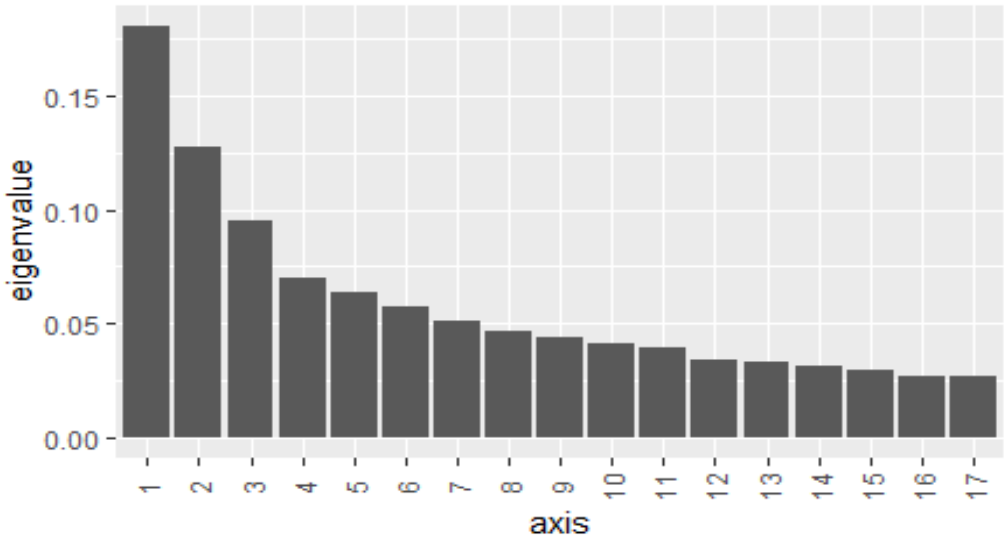

Figure 16. The eigenvalues represented the variance extracted by each axis, and are often conveniently expressed as a percentage of the sum of all eigenvalues (i.e. total of variance).

4.7.3 Non- metric multidimensional scaling (NMDS)

NMDS is a technique for efficiently determining the indirect gradients based on the measurement of different patterns of distance metrics. NMDS measures were used basically to identify the difference between two samples and then calculated the difference between all samples. Firstly, the difference between two samples were considered and then the results has shown further distinct apart dots in the graph, clearly shows the difference from each other due to their microbial content. Nonmetric multidimensional scaling (NMSD1 and NMSD2) were applied between microbiome communities within each group. Points in the graph, indicated the relative locations of individuals within microbial space, coloured by breed as shown in the (Figure 17). As, HYBRID and ROSLAG breeds presented less distance (more closeness) as compared to RYA and GESTRIKE breeds, might be due to the difference occurred as HYBRID and ROSLAG faecal samples taken from same farm (Farm C). Marked difference has seen between the microbial diversity of sheep breeds and goat group (out-group).

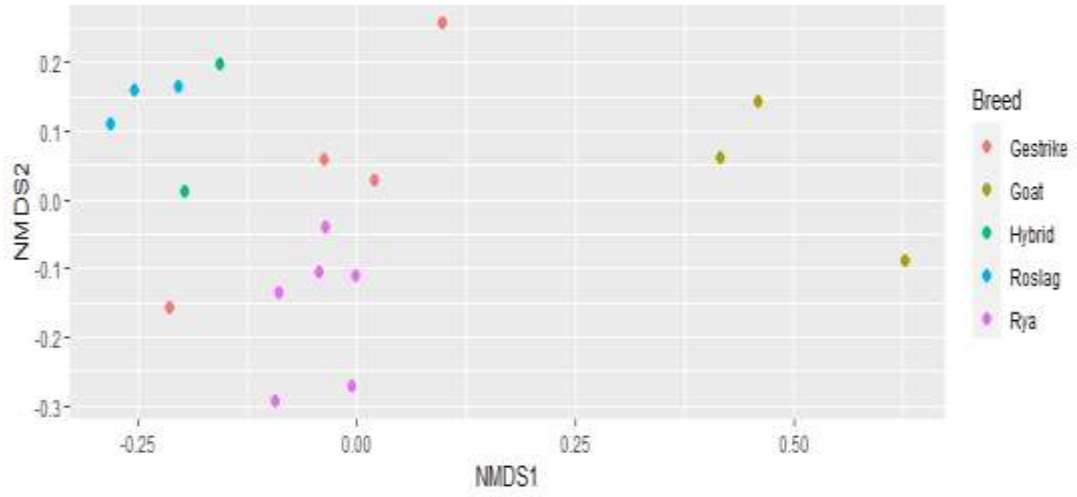

Figure 17. The dispersed points/ small circles stands for sample has shown the less differences of microbiome composition among sheep breeds but marked difference of microbial population in goat LANDRACE. The distance between data points reflects the extent of variation. Similar group of samples are shown in the same colour.

4.7.4 PERMANOVA Test

The Permutational multivariate analysis of variance (PERMANOVA) test (similar to Adonis test) was performed based on sum of square root to examine the significant differences between five groups of sheep breeds and goat (as out group). The Adonis function in the R vegan package (version_4.2.1) was used to conduct pairwise significant compositional differences between populations by using PERMANOVA test. The relationships of faecal microbiota between groups were calculated by using an algorithm with n=10000 permutations (PERMANOVA) based on only breed variable by Bray-Curtis distance method. Results has shown the community structure differences occurred significantly (R2= 0.4209, P < 0.05) between sheep breeds and goat (outgroup) from different farms as (Table 6). So, according to this study, we can't differentiate between breed, farm and feed effect because all faecal samples of different sheep breeds have taken from different farms as mentioned in (Table 1).

Table 6. PERMANOVA Test

Adonis 2 (Formula = OTU ~ Breed, data = unclass (Meta)

	DF	SUM Sq.	R2	F	Pr(>F)
Breed	4	1.1077	0.4209	2.3621	9.999e-0.05 ***
Residual	13	1.5240	0.5791		
Total	17	2.6316	1.0000		

*Significant codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1*

4.7.5 ANOVA Test

In an attempt to normalise the data and to see if the average values of groups differ from each other or not. A square root of variance (ANOVA) was employed for the analysis of variance, based on response and distance to see how distinct the dots were from each other in the same breed or to analyse the homogeneity of multivariate dispersions of faecal microbiota in groups. In order to analyse the bacterial phyla that specifically influenced by breed variable, used ANOVA method on relative abundance of the dominant phyla. ANOVA results displayed insignificance response based on distance shown that the dispersion doesn't differ significantly (P>0.05), as mentioned in the (Table 7), depicting more similarities within each groups of sheep.

Table 7. Analysis of variance (Response: Distances)

	Df	Sum sq	Mean sq	F Value	Pr(>F)
Groups	4	0.0050454	0.00126135	1.4	0.2882
Residuals	13	0.0117127	0.00090097		

*Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1' ' 1*

4.7.6 Tukey's Test

The T-Test was used to measure the difference between the 5 groups of sheep breeds and goat with 95% family-wise confidence level. Tukey's Test was performed to evaluate the multiple comparisons of means at (95% family-wise confidence level) as well as to further analyse the intergroup significance of the selected breeds. The insignificant (P>0.05) results were seen in the pairwise comparisons between the least square means in the Tukey's test. P values were adjusted accordingly by using the method of (Benjamini and Hochberg, 1995) for the pairwise comparison between different groups of sheep breeds and landrace goat to minimise the false values as mentioned in the (Table 8).

Table 8. Tukey's test

Groups	Differences	Lower	Upper Value	P-adj
Goat-GESTRIKE	-0.0131633778	-0.08534775	0.05902099	0.9766300
HYRID-GESTRIKE	-0.0440533683	-0.12590275	0.03779601	0.4695402
ROSLAG-GESTRIKE	-0.0341395817	-0.10632395	0.03804479	0.5862194
RYA-GESTRIKE	0.0005587147	-0.06044821	0.06156564	0.9999998
HYBRID-Goat	-0.0308899905	-0.11716681	0.05538683	0.7897977
ROSLAG-Goat	-0.0209762038	-0.09814454	0.05619213	0.9078092
RYA-Goat	0.0137220925	-0.05310765	0.08055183	0.9642856
ROSLAG-HYBRID	0.0099137867	-0.07636304	0.09619061	0.9958606
RYA-HYBRID	0.0446120830	-0.03255625	0.12178042	0.4033232
RYA-ROSLAG	0.0346982964	-0.03213144	0.10152804	0.5027087

4.7.7 Simper Test

The simper test was implemented in order to identify the taxa difference significantly between pairwise groups of breeds by finding the average participation of each species to the average of overall Bray-Curtis dissimilarity index. The (Figure 18) simper output depicted the relative abundant diverging ASVs based on the genus level where all taxa groups merged into each other which were less common than 5% was based on the pairwise comparison of 5 groups of sheep breeds and goat (outgroup).

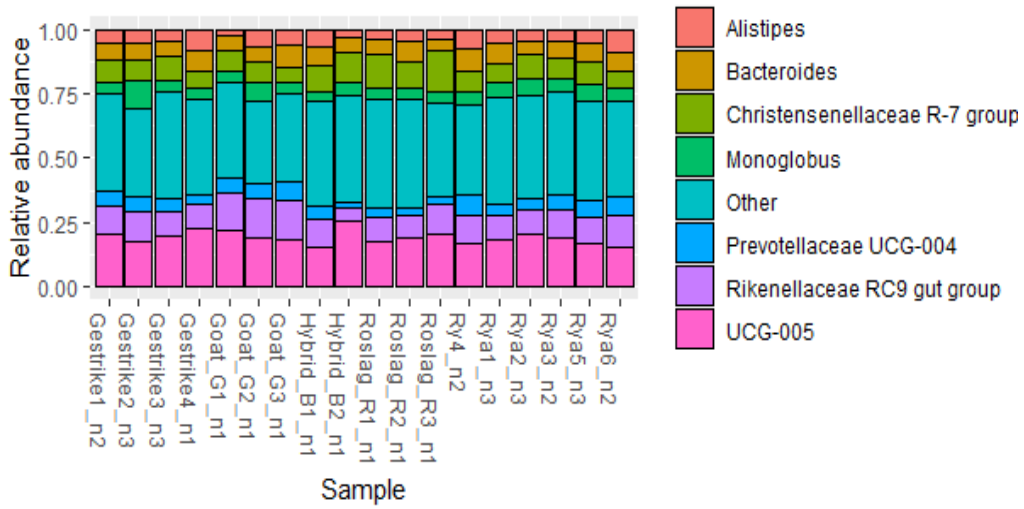

Figure 18. Taxa difference assignment between pairwise groups of breeds at the Genus level (all taxa groups which were less common than 5% integrated into the other group)

5. Discussion

5.1 Sheep microbial composition

The aim of this research was to see the similarities and differences in the faecal microbiota in *Swedish* sheep breeds and goat (outgroup). To analyse complex microbial communities, high throughput sequencing has provided biologist enough information to examine new techniques based on metagenomics and sequence based profiling (Hess *et al.*, 2011; Logares *et al.*, 2012). The current results indicated the most common abundant taxas such as Firmucutes (66%) and Bacteroidetes (29%) followed by Verrucomicrobiota (2%), Fibrobacterota (1%), Proteobacteria (1%), and Actinobacteriota (1%) among the top ten common phyla. The current study results correlate with the results of (Chang *et al.*, 2020) showed the highest relative abundance accounted for Firmicutes (55.83%) and Bacteroidetes (24.39%) in Tibetan sheep at phyla level in the Qinghai-Tibetan Plateau (QTP) of China.

Similarly, the highest relative abundance of Firmicutes (43.6%) and Bacteroidetes (30.38 %) has shown in the Italian sheep (Minozzi *et al.*, 2020) correlated to our findings. In accordance with our current results, the highest relative abundance of Firmicutes and Bacteroidetes has also been seen as a dominated phyla as comparison with the previous literatures such as, in the 28 Australian Merino sheep (Mamum *et al.*, 2020), in the faecal microbiota of sheep (globally) (Tanca *et al.*, 2017) and in the domesticated ruminants (Firmicutes 65.34% and Bacteroidetes 20.95%) in the south east of Ireland (O'Donnell *et al.*, 2017). In our study, the lower relative abundance was found in Verrucomicrobiota (2%), which is in contrast with the previous study (Minozzi *et al.*, 2020) found a comparatively higher abundance of Verrucomicrobia (7.55%) in the Italian sheep due to different husbandry conditions. The highest abundance of Firmicutes (89.31%) and Bacteroidetes (22.77%), followed by Verrucomicrobia (13.20%), has also been documented in the most recent study in the colon of goat in the south China (Wang *et al.*, 2021).

At the genus level, our current result presented the highest relative abundance of UCG-005 (24%) followed by Rikenellaceae RC9 gut group (13%), Christensenellaceae R-7 group (12%), Bacteroides (8%), Monoglobus (6%), Alistipes (6%), Prevotellaceae (6%), Ruminococcaceae (5%), Akkemansia (4%) and Lachnospiraceae (5%) as shown in the (Figure 9). According to the previous research, has showed the taxa containing highest abundance of Ruminococcaceae UCG 005 (genus; 93 conserved clades) in bighorn sheep (Couch *et al.*, 2020).

Similarly, our research has found almost similar results to the most recent study in the colon of small ruminants especially in the goat, with the highest relative abundance of UCG-005 accounted for (30.69%), then Ruminococcaceae *(*12.47%), followed by christensenellaceae-R7-group, and Ruminococcace-UCG-010 (Wang *et al.,* 2021).

5.2 Breed Genetic Effect

In our research, Bray-Curtis dissimilarity (PCoA and NMDS) based on distance matrices presented, the four groups of different breeds of sheep were clustered close to each other or not significantly far apart, but the goat LANDRACE has showed significant clustering distance. These results correlate with our hypothesis that similar microbiota exist in genetically related animals, as shown in (Figures 15 and 17). In the current study, the significant (PERMOANOVA, $P< 0.05$) has indicated the variation within breeds in association with the composition of the microbiome is in accordance with the previous results (PERMANOVA, $P <0.01$) (Chang *et al.,* 2020) but we can't differ between breed, farm and feed effect because sheep breeds were selected from different farms. Prior research supported the evidence of a breed genetic effect in the variation of the microbiome composition in ruminant GIT. For example, differences in the gut microbial community have been shown in the Yak and Tibetan sheep partly due to breed effect (Xue *et al.,* 2017). The microbiome composition of intestine also differed at different taxonomic levels between sheep breeds (Chang *et al.,* 2020). In addition, it has been considered that inter-individual genetics has positive correlation and is responsible for the selection of genetically similar microbiomes among closely related breeds (Couch *et al.,* 2020). Vertical transmission produces a similar microbiome in closely related animals (Couch *et al.,* 2020). Previous research has demonstrated one of the most important breed factors in developing the similar but uncommon faecal/GIT microbiome between different breeds. The study clearly indicated the microbiota transfer from maternal genetics, particularly in the function of microbiome assemblage (Rooks and Garrett, 2016). Studies have also been shown that the different breeds of goat's having different rumen microbial diversity (Douglas *et al.,* 2016).

5.3 Feed and Function of the most common groups

In our study, the most abundant taxa at phylum level were Firmicutes and Bacteriodota. According to previous research results, it has been suggested that these abundant bacteria contain fiber decomposing bacteria (Ruminococcus, Oscillibacter etc) (Hook *et al.,* 2011) contains glycosyl hydrolase gene (Tanca *et al.,* 2017) responsible for the breakdown of crop straws (cellulose, hemicellulose, and lignin). The decomposing bacteria also helps in the decomposition of fiber, carbohydrate, and protein during the mechanism of metabolism (Huo *et al.,* 2014). The second most abundant phylum, Bacteroidota, followed by Prevotella, plays an important role in the assimilation of non-fibrous plant feed and protein in the rumen of sheep (Hook *et al.,* 2011). The effect of a change in the feed is directly correlated with the microbiome community. The similar study supported by the same evidence in the rumen microbial community showed varied effects of the microbiome due to breed and feed regime (Bohra *et al.,* 2019). The different breeds of sheep plays a major role in maintaining the GIT microbiome (Chang *et al.,* 2020) and feeding patterns act to maintain microbial composition as well as metabolic homeostasis in sheep (Cui *et al.,* 2019; Wang *et al.,* 2020). The Christensenellaceae R7 group producing β-galactosidase (β-glucosidase and arabinosidase generating Christensenellaceae gene), stimulated the increasing feed efficiency in the colon of sheep (Perea *et al.,* 2017). Since, faecal samples were collected from the different farms except for HYBRID and ROSLAG breed and also due to small sample numbers, this study might not distinguish between the effect of feed, breed and farms (treatment and proximity).

6. Conclusions

Our research work, was the first systematic evaluation of the microbial diversity across the faecal microbiome in the *Swedish* sheep breeds and goat (ruminant) using culture-independent next generation sequencing (Illumina), serves as a framework for understanding the role of microbiota in the different breeds of sheep and goat. It was concluded that the microbial diversity occurred among different *Swedish* breeds of sheep and goat (out-group), but less intra-breed variation exists within in the same groups of sheep breeds and goat. Since, all the faecal samples were taken from separate farms (except ROSLAG and HYBRID sheep breeds from same farm) so, it is not possible to separate the precise breed, environmental or feed effect from this pilot study. Considering the results collectively, it was speculated that an inbred sheep breeds harbours similar microbiome while difference in microbiome occurred in different breeds. However, based on the evidence in our study, it is still required further research by considering the larger population size samples and also providing others factors same (i.e., sex, age, nutrition and proximity) to see the breed genetic effect precisely among different *Swedish* sheep breeds for the faecal microbiome assessment. Furthermore, the development of sequencing technology has pushed the boundaries of knowledge regarding microbiome research and the coming years will result in more advanced experiments, clinical applications, and possibly even the precise manipulation of the microbiome study.

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