Association between Host Genetics of Sheep and the Rumen Microbial Composition

Sinalo Mani

University of KwaZulu Natal

Olayinka Ayobami Aiyegoro (✉ Aiyegoro@arc.agric.za)

Agricultural Research Council <https://orcid.org/0000-0001-6664-6574>

Matthew Adeniyi Adeleke

University of KwaZulu Natal

Research

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Abstract

Background: The gut microbiome is very significant in the stability of the rumen ecosystem, also in the maintenance of the host's health. The gut flora influences the nutritional, physiological, immunological, and directly or indirectly affects the host's well-being. A synergy between the rumen microbiota and the host genetics creates a symbiotic relationship, beneficial to the host's health. In this study, the association between the host genetics and rumen microbiome of Damara and Meatmaster sheep was investigated. The composition of rumen microbiota was estimated through the analysis of the V3-V4 region of the 16S rRNA gene, while the sheep blood DNA was genotyped with Illumina OvineSNP50 BeadChip and the genome-wide association (GWA) was analyzed.

Results: Sixty significant SNPs dispersed in 21 regions across the *Ovis aries* genome were found to be associated with the relative abundance of seven genera: *Acinetobacter*, *Bacillus*, *Clostridium*, *Flavobacterium*, *Prevotella*, *Pseudomonas* and *Streptobacillus*. A total of eighty-four candidate genes were identified, and their functional annotations were mainly associated with immunity responses and function, metabolism, and signal transduction.

Conclusion: We are of the opinion, as evident from our results that candidate genes identified may be modulating the composition of rumen microbiota and, thus further indicate the significance of comprehending the interactions between the host and rumen microbiota to gain better insight into the health of sheep. We conclude that host genetics is important in modulating microbial composition; though various factors may alter the rumen microbiota. More studies are necessary to help determine genetic combinations favorable for the propagation of beneficial microbiota and provide excellent intestinal health.

Introduction

The gut microbiome is a complex network with a symbiotic relationship with the host; hence the microbiome composition may impact the balance of the rumen ecosystem, and their interaction provides beneficial functions to the host. The gut microbiome has a significant role in the maintenance of rumen ecosystem stability and the host's health, as it influences the nutritional, physiological, and immunological potential of the host [1, 2]. The microbiota also extends the host's digestive capabilities, produce vitamins, and prevent pathogen colonization [3]. Likewise, the gut microbiota is essential in developing and differentiating the intestinal mucosal epithelium, and they modulate the host's immune response to maintain gut homeostasis.

The gut microbiome composition and function are modulated by factors such as age, diet, environment, and host genetics [4]. These factors influence how the microbiomes function and the microbial structure. Though it has been generally established that the significant factor modulating the gut microbial communities is diet [5, 6]; novel evidence through genome-wide association studies (GWAS), have shown that host genetics have a significant role in the modulation of gut microbial composition in mice and humans [7]. Blekhman et al. [8] showed an association between host genetic variation in SNPs and *Bifidobacterium* abundance close to the lactase (*LCT*) gene. *LCT* encodes for lactase enzyme that hydrolyzes lactose when expressed in the gastrointestinal tract. It has been suggested that host genetics is a vital component in modulating the gut microbial composition.

GWAS in animals has been mostly used to analyze several complex traits such as association with feed efficacy [9] and genetic resistance to diseases [10, 11]. Despite its great significance, the compound relationship of host genetics, physiology, and gut microbiome components is poorly understood. Few studies assess the host genetics influence on the gut microbial composition [12]. Crespo- Piazuelo et al. [13] identified pig genetic variation SNPs that were associated with the abundance of six genera and the majority of candidate genes identified in 17 associated regions encoded for enzymes participating in defence systems of the host, while other proteins were involved in metabolism. Studies on the association of host genetics and their rumen microbiota composition are limited in ruminants, and those few are focused on cattle compared to other ruminants [7, 12]. In particular, association studies between rumen microbial composition and the sheep genome have not yet been defined. This study therefore aimed at identifying genomic regions in the host that may modulate the rumen microbial composition and find the correlation between host genetic variation and rumen microbial abundance through host-microbiota association in sheep.

Materials And Methods

Animal material and sampling.

All the procedures involving animals were permitted by the Agricultural Research Council - Animal Production Ethics committee (APIEC17/21). The trials were done at the Agricultural Research Council (ARC), GI Microbiology and Biotechnology unit, and the Small Stocks Unit in Irene, Gauteng province, South Africa.

Sixty-four sheep (32 Damara sheep breed and 32 Meatmaster sheep breed) were used for the trial, and they were approximately seven months old with the average weight as follows: Meatmaster males (24.6 ± 3.4 kg); Meatmaster females (21.5 ± 3.1 kg); Damara males (36.6 ± 8.3 kg); Damara females (28.9 ± 6.9 kg). The animals were housed per treatment with males and females separated in open barn trial pens with $\pm 4\text{m}^2$ shelters. Ten (10) animals were excluded from the experiment after adaptation period before the trial commenced, due to signs of sickness and 4 mortalities. The animals were grouped into five treatment groups and were fed on commercial pellet feed, with hay and fresh water supplied *ad libitum*. Rumen and blood samples were collected before the trial commenced and later collected again at the end of the trial. Rumen samples were collected using a stomach tube following the procedures of Shen et al. [14].

Microbial DNA extraction, 16S rRNA gene amplification and sequencing

Microbial DNA was extracted with QIAamp Fast DNA stool Mini Kit (Qiagen, Germany) following the guidelines of the manufacturer for pathogen detection, and DNA concentration was evaluated with Nanodrop 2000 (Thermo Electron Corporation, USA). The DNA samples were pooled according to their respective treatments, taking into consideration the sex of the animals (males and females DNA pooled separately). The pooled DNA quality was evaluated using gel electrophoresis on 1.5% agarose gels and visualized with UVP BioSpectrum 310 Imaging System (FisherScientific, UK). The pooled DNA samples were used as templates for amplifying the V3–V4 hypervariable region of the prokaryotic 16S rRNA gene according to the 16S Metagenomic Sequencing Library Preparation guide (Illumina, San Diego, USA). Illumina MiSeq platform was used to perform sequencing, and 300 bp paired-end reads were generated. Raw reads were deposited to the Sequence Read Archive (SRA) of the National Center of Biotechnology Information (NCBI) database under BioProject PRJNA578022.

Data analysis

Raw data sequences generated by MiSeq Illumina were trimmed employing Trimmomatics (version 0.36), Illumina universal adapter sequences, and the low-quality sequence regions were removed. Demultiplexed sequence files were imported to QIIME2 software (version 2018.8) [15] for analysis. The imported reads were denoised and trimmed using DADA2. Sequences were assigned to operational taxonomic units (OTU) with 97% identity, and the OTU feature table was generated; chimeric sequences were detected and removed from the representative OTU sequences. For taxonomic analysis, OTU representative sequences were aligned to the Greengenes database. All other statistical analyses were carried out using phyloseq package (version 1.24.2) through RStudio (version 3.5.3). The 9134 OTUs were grouped into 20 phyla and 107 genera through *tax_glom* method in the phyloseq method in R. OTUs with the relative abundance of $\geq 0.05\%$ in at least one sample were the ones retained in both breeds for subsequent analyses.

Host DNA extraction and SNP genotyping

Sheep whole blood was used to extract DNA with WizardTM Genomic DNA Purification Kit (Promega, USA). DNA samples for each breed were pooled according to their respective treatments, taking into consideration the sex of the animals (males and females DNA pooled separately). DNA quality and quantity were measured using 1.5% agarose gel electrophoresis (Bio-Rad) and Qubit 2.0 Fluorometer (Life Technologies), respectively. Genotyping of 40 DNA samples was done with the Illumina OvineSNP50 BeadChip (Illumina, San Diego, CA). Genotypes were obtained using GenomeStudio software and quality control of the data was done through PLINK software (version 1.9) [16]. Additional analyses were performed only on SNPs that mapped and aligned to the Oar_v3.1 reference assembly, retaining a total of 42503 SNPs with missing genotype < 2% and minor allele frequency (MAF) > 2% to be used for genome-wide association analysis.

Genome-wide association (GWA) analysis

Associations between the SNPs mapped to the genome and microbiota composition at the genus level were carried out. Samples were normalized by their relative abundance and *find_top_taxa* function in R was used to extract genera that were present in $\geq 90\%$ of the samples in both sheep breeds and had more than 0.05% of the total reads to avoid errors caused by low abundant genera. Unspecified genera were excluded from the analysis; thus, GWAS was carried out in 7 out of the 107 found genera. GWAS analysis was performed using PLINK and multiple test correction with false discovery rate (FDR) method [17] using *-adjust* function in plink.

Functional gene annotation and prediction

Associated regions in the sheep genome were annotated at 1Mb on each side of *Oar_3.1*. Gene locations were based on the Ensembl Genome Browser (www.ensembl.org; release 99). The genes contained in the regions of significant SNPs in the sheep genome were extracted using *Oar_3.1* reference in the Biomart tool [18] using Ensembl. Variant Effect Predictor tool [19] from the Ensembl project was used in predicting the functional consequences of the significant SNPs.

Results

GWAS was achieved using 42,503 SNPs in 40 animal genotypes and the relative abundance of seven genera; *Acinetobacter*, *Bacillus*, *Clostridium*, *Flavobacterium*, *Prevotella*, *Pseudomonas* and *Streptobacillus*. Significant associations ($FDR < 0.1$) were found in all seven genera and 60 significant SNPs were dispersed in 21 chromosomes across the *Ovis aries* genome (Fig. 1 and Table 1). There were two shared regions found for the abundance of *Prevotella* and *Bacillus*, located in the region of 25.47 ~ 26.10 Mbps on OAR17, *Bacillus* and *Acinetobacter* shared a region of 45.13 ~ 49.52 Mbps located on OAR26; also they were associated with one SNP (s22112.1 on OAR26, $P = 1.29e-20$ – $3.95e-08$, $FDR = 2.24e-17$ – $8.45e-05$). *Bacillus* and *Clostridium* are members of *Firmicutes* phylum, *Prevotella* and *Flavobacterium* from the Bacteroidetes phylum, *Streptobacillus* belonging to *Fusobacteria*. *Pseudomonas* and *Acinetobacter* are genera of *Proteobacteria* phylum. Eighty-four candidate genes were identified in the regions of significant SNPs associated with rumen microbial composition.

Table 1

Significant variant regions associated with the relative abundance of microbial genera and identified candidate genes within the regions

SNP	Chr	Position	p-value	FDR	Genus	Consequence	Candidate genes
OAR1_41851018.1	1	40,438,064	2.09E-09	1.73E-05	<i>Acinetobacter</i>	downstream_gene_variant	<i>AK4, LEPR</i>
OAR3_20690836.1	3	19,172,977	6.72E-09	3.57E-05	<i>Acinetobacter</i>	upstream_gene_variant	<i>ITGB1BP1, ROCK2</i>
OAR10_46135260.1	10	45,590,208	6.90E-09	3.57E-05	<i>Acinetobacter</i>	upstream_gene_variant	
s50123.1	22	42,997,377	9.24E-11	1.28E-06	<i>Acinetobacter</i>	downstream_gene_variant	<i>CHST15, ABRAXAS2, ZRANB1</i>
s04636.1	25	43,727,893	2.09E-09	1.73E-05	<i>Acinetobacter</i>	downstream_gene_variant	<i>WDFY4, OGDHL, DEPP1</i>
OAR26_46995252.1	26	41,441,325	8.65E-11	1.28E-06	<i>Acinetobacter</i>	downstream_gene_variant	<i>NR1D2</i>
s22112.1	26	42,948,826	3.95E-08	8.45E-05	<i>Acinetobacter</i>	downstream_gene_variant	
s50736.1	26	43,029,260	5.26E-09	3.57E-05	<i>Acinetobacter</i>	downstream_gene_variant	
OAR26_49520304.1	26	43,626,043	3.46E-20	1.43E-15	<i>Acinetobacter</i>	downstream_gene_variant	
OAR1_142723820.1	1	131,856,629	9.92E-27	4.12E-23	<i>Bacillus</i>	downstream_gene_variant	
OAR1_221226809.1	1	204,866,048	9.92E-27	4.12E-23	<i>Bacillus</i>	downstream_gene_variant	
OAR2_107495304.1	2	99,900,131	8.51E-24	2.19E-20	<i>Bacillus</i>	downstream_gene_variant	<i>SCARA3</i>
s04253.1	2	126,963,279	4.00E-20	6.39E-17	<i>Bacillus</i>	downstream_gene_variant	<i>ITGA4</i>
OAR2_135296252.1	2	127,014,456	7.29E-18	1.01E-14	<i>Bacillus</i>	downstream_gene_variant	<i>ITGA4</i>
OAR2_153029366.1	2	144,088,879	7.30E-18	1.01E-14	<i>Bacillus</i>	upstream_gene_variant	<i>GRB14</i>
OAR3_135035896.1	3	126,551,306	8.30E-21	1.50E-17	<i>Bacillus</i>	upstream_gene_variant	<i>ATP2B1</i>
s27957.1	4	71,282,342	9.49E-24	2.19E-20	<i>Bacillus</i>	downstream_gene_variant	<i>OSBPL3, NPY, ADAM22</i>
OAR4_93149584.1	4	87,732,399	3.39E-28	4.69E-24	<i>Bacillus</i>	upstream_gene_variant	
OAR6_16061983.1	6	13,457,100	8.07E-21	1.50E-17	<i>Bacillus</i>	downstream_gene_variant	<i>ALPK1, TIFA</i>
OAR6_120140225.1	6	105,688,605	9.92E-27	4.12E-23	<i>Bacillus</i>	upstream_gene_variant	
OAR7_862379.1	7	1,175,686	3.24E-21	6.72E-18	<i>Bacillus</i>	downstream_gene_variant	<i>MCC, EPB41L4A</i>
OAR10_52665226.1	10	51,659,456	9.92E-27	4.12E-23	<i>Bacillus</i>	upstream_gene_variant	<i>ACOD1</i>

Chr: Chromosome. FDR ≤ 0.1 .

SNP	Chr	Position	p-value	FDR	Genus	Consequence	Candidate genes
OAR10_64413399.1	10	62,506,487	8.51E-24	2.19E-20	<i>Bacillus</i>	upstream_gene_variant	
OAR11_18077431.1	11	17,728,327	2.90E-25	1.00E-21	<i>Bacillus</i>	upstream_gene_variant	<i>TMEM98</i>
s39842.1	11	54,939,690	3.39E-28	4.69E-24	<i>Bacillus</i>	upstream_gene_variant	<i>MGAT5B, ST6GALNAC2, CYGB, SPHK1, GALR2, GALK1, SLC16A5, OTOP3, OTOP2</i>
OAR12_72007015.1	12	65,405,613	7.99E-27	4.12E-23	<i>Bacillus</i>	upstream_gene_variant	<i>PLA2G4A</i>
OAR13_28884642.1	13	26,065,987	3.56E-21	7.04E-18	<i>Bacillus</i>	downstream_gene_variant	<i>GAD2, OPTN</i>
OAR15_69064778.1	15	63,508,604	8.51E-24	2.19E-20	<i>Bacillus</i>	downstream_gene_variant	<i>CD44</i>
OAR16_32396433.1	16	29,834,628	2.15E-19	3.30E-16	<i>Bacillus</i>	downstream_gene_variant	<i>FGF10</i>
s00547.1	17	7,870,359	1.04E-25	3.94E-22	<i>Bacillus</i>	downstream_gene_variant	
OAR17_25477049.1	17	23,080,103	9.32E-24	2.19E-20	<i>Bacillus</i>	upstream_gene_variant	
OAR19_26906414.1	19	25,469,429	3.17E-25	1.00E-21	<i>Bacillus</i>	upstream_gene_variant	<i>CNTN6, CHL1</i>
OAR20_24435276.1	20	23,004,273	2.17E-16	2.90E-13	<i>Bacillus</i>	downstream_gene_variant	<i>MMUT, GLYATL3, DEFB133, DEFB113, DEFB110</i>
s04766.1	20	40,540,580	7.16E-15	9.29E-12	<i>Bacillus</i>	downstream_gene_variant	
OAR21_23489027.1	21	20,825,320	9.92E-27	4.12E-23	<i>Bacillus</i>	upstream_gene_variant	
OAR23_39546165.1	23	37,298,993	3.39E-28	4.69E-24	<i>Bacillus</i>	upstream_gene_variant	<i>LPIN2</i>
DU481531_204.1	26	5,940,221	2.74E-09	3.45E-06	<i>Bacillus</i>	downstream_gene_variant	<i>AGPAT5</i>
OAR26_45130684.1	26	39,909,046	1.10E-22	2.39E-19	<i>Bacillus</i>	upstream_gene_variant	<i>NR1D2</i>
OAR26_46516694.1	26	41,052,798	3.99E-20	6.39E-17	<i>Bacillus</i>	downstream_gene_variant	<i>NR1D2</i>
OAR26_48188219.1	26	42,498,856	5.80E-18	8.59E-15	<i>Bacillus</i>	downstream_gene_variant	
s46142.1	26	42,685,548	9.92E-27	4.12E-23	<i>Bacillus</i>	downstream_gene_variant	
s22112.1	26	42,948,826	1.29E-20	2.24E-17	<i>Bacillus</i>	downstream_gene_variant	
s43106.1	2	16,687,851	1.02E-10	2.11E-06	<i>Clostridium</i>	downstream_gene_variant	<i>RAD23B, SLC44A1</i>

Chr: Chromosome. FDR $\leq$ 0.1.

SNP	Chr	Position	p-value	FDR	Genus	Consequence	Candidate genes
OAR2_58286833_X.1	2	54,173,591	6.24E-12	2.59E-07	<i>Clostridium</i>	downstream_gene_variant	<i>TLE1</i>
OAR1_74421103.1	1	69,799,470	1.14E-06	0.005818	<i>Flavobacterium</i>	upstream_gene_variant	<i>GF11, FNBP1L, ABCD3</i>
OAR1_197593478.1	1	183,138,836	1.14E-06	0.005818	<i>Flavobacterium</i>	upstream_gene_variant	<i>IGSF11, B4GALT4, ARHGAP31</i>
OAR2_247207503.1	2	234,119,904	7.94E-07	0.005818	<i>Flavobacterium</i>	downstream_gene_variant	<i>AK2, LCK</i>
OAR6_58751558.1	6	53,388,884	2.34E-06	0.01024	<i>Flavobacterium</i>	upstream_gene_variant	
OAR16_37770793_X.1	16	34,690,414	1.14E-06	0.005818	<i>Flavobacterium</i>	upstream_gene_variant	<i>C9, PTGER4, RICTOR, FYB1, DAB2</i>
OAR22_26777966.1	22	22,682,679	1.14E-06	0.005818	<i>Flavobacterium</i>	downstream_gene_variant	<i>NFKB2, TRIM8, CNNM2, FGF8, ELOVL3</i>
OAR23_54064411.1	23	51,039,178	7.94E-07	0.005818	<i>Flavobacterium</i>	upstream_gene_variant	<i>SMAD4</i>
OAR1_286871592.1	1	265,164,196	6.85E-06	0.04737	<i>Prevotella</i>	downstream_gene_variant	<i>RUNX1</i>
OAR4_36089897.1	4	34,178,907	3.94E-06	0.04737	<i>Prevotella</i>	upstream_gene_variant	<i>GRM3</i>
OAR7_56932867.1	7	51,557,993	6.09E-06	0.04737	<i>Prevotella</i>	downstream_gene_variant	<i>NEDD4, RAB27A</i>
s21876.1	16	5,145,810	3.65E-06	0.04737	<i>Prevotella</i>	upstream_gene_variant	<i>DUSP1, BNIP1, STC2</i>
OAR17_26108276.1	17	23,624,423	5.21E-06	0.04737	<i>Prevotella</i>	upstream_gene_variant	
s20119.1	25	42,915,079	5.88E-06	0.04737	<i>Prevotella</i>	upstream_gene_variant	<i>MAPK8, WDFY4, OGDHL</i>
OAR6_24191777.1	6	21,117,639	2.41E-07	0.009996	<i>Pseudomonas</i>	downstream_gene_variant	<i>BDH2, SLC9B2, CISD2</i>
OAR14_27438486.1	14	26,265,288	5.95E-07	0.01234	<i>Pseudomonas</i>	upstream_gene_variant	<i>CSNK2A2, GOT2</i>
s75574.1	25	35,576,136	1.97E-34	7.11E-30	<i>Streptobacillus</i>	downstream_gene_variant	<i>SFTPA1, PRXL2A</i>
Chr: Chromosome. FDR ≤ 0.1.							

Discussion

Previous studies have demonstrated that host genetics play a role in modulating the composition of gut microbiota in mammals [3, 7, 8, 13]. The results in this study suggest that there are associations between the abundance of certain genera and host genotypes (SNPs), which might provide answers on which genetic components contribute to the rumen microbial composition. Some of the microorganisms that showed to be related to host genetics are relevant in the composition of the ruminal environment, degradation of feed. The detailed description of the candidate genes mapped in the sheep genome's significant genetic variations is discussed.

Acinetobacter

The relative abundance of *Acinetobacter* is associated with genetic variations in six sheep genome regions. In the region OAR1, two candidate genes have been identified; Adenylate Kinase 4 (*AK4*) and Leptin Receptor (*LEPR*). *AK4* regulates the activation of the energy sensor and phosphorylation AMPK protein kinase by controlling cellular ATP levels. The kinase is also essential in oxidative stress cellular defence response [20, 21]. While *LEPR* is a receptor for leptin, which is a hormone that is adipocyte-specific regulating body weight [22]. In the region of OAR3, two candidate genes were found; Integrin Subunit Beta 1 Binding Protein 1 (*ITGB1BP1*) and Rho Associated Coiled-Coil Containing Protein Kinase 2 (*ROCK2*). *ITGB1BP1* is essential for cell adhesion, proliferation, differentiation and migration [23]. *ROCK2* is vital for regulating cell polarity and the actin cytoskeleton. *ROCK2* is also involved in regulating the smooth muscle contraction, neurite retraction, cell adhesion and motility, stress fibre, actin cytoskeleton organization and focal adhesion formation [24]. *ROCK2* might be involved in regulating the rumen smooth muscle contraction. A study by Xiang et al. [25] reported that increased expression of genes regulating smooth muscle contraction in the rumen is related to sheep with shorter mean retention time (MRT), as the muscle contraction reduces MRT and subsequently the CH₄. This may increase gastrointestinal muscle contraction linked to the rapid digesta passage rate, thus influencing rumen microbiota.

Three candidate genes found in the region of OAR22, Carbohydrate Sulfotransferase 15 (*CHST15*), Abraxas 2, BRISC Complex Subunit (*ABRAXAS2*) and Zinc Finger RANBP2-Type Containing 1 (*ZRANB1*). *CHST15* is essential in glycosaminoglycan metabolism, a significant structural constituent of the extracellular matrix and this gene potentially acts as a B-cell surface signaling receptor [26]. *ABRAXAS2* is involved in the deubiquitination of the interferon receptor IFNAR1 involved in interferon signaling, and it also down-regulates bacterial lipopolysaccharide (LPS) response [27]. *ZRANB1* is essential in cell migration and stress fibre dynamics; it might also modulate TNF- α signaling [28]. In the region of OAR25, three candidate genes were found, WDFY family member 4 (*WDFY4*), oxoglutarate dehydrogenase-like (*OGDHL*) and DEPP1 autophagy regulator (*DEPP1*). *WDFY4* plays a significant role in regulating cDC1-mediated cross-presentation of viral and tumor antigens in dendritic cells. It is also known to be essential in B-cell survival through autophagy regulation [29]. *OGDHL* encodes for protein similar that degrades glucose and glutamate [30]. *DEPP1* is known as fasting-induced gene as its expression is induced by fasting and progesterone and is a vital modulator of FOXO3-induced autophagy via oxidative stress [31]. While no candidate gene was found in other genetic variations in the region of OAR26, the genetic variant with significant SNP OAR26_46995252.1 contained nuclear receptor subfamily 1 group D member 2 (*NR1D2*) identified. *NR1D2* regulates genes essential in metabolic functions such as lipid metabolism and the inflammatory response [32]. *Acinetobacter* is said to be more involved in epithelial proliferation and disease [33] and these genes might modulate the abundance of *Acinetobacter*.

Bacillus

In the gut, *Bacillus* is known to partake in the metabolism of nutrients and is involved in the maintenance of intestinal homeostasis [34]. The relative abundance of *Bacillus* is associated with genetic variations in 18 regions of the sheep genome; no candidate gene was found in three of the regions. In OAR2, significant SNP OAR2_107495304.1 was located in the region of scavenger receptor class A member 3 (*SCARA3*). *SCARA3* has been shown to deplete reactive oxygen species as it's known as a cellular stress response gene and thus has a significant function in the protection of cells from oxidative stress [35]. Two significant SNP s04253.1 and OAR2_135296252.1 were located in the region of integrin subunit alpha 4 (*ITGA4*). *ITGA4* encodes a protein in the integrin alpha chain family; integrins are a key class of cell surface receptors mediating associations of cells and extracellular matrix and are essential in cell surface adhesion and signaling [36]. Significant SNP OAR2_153029366.1 contained growth factor receptor-bound protein 14 (*GRB14*) that may be modulating *Bacillus* abundance as it plays a role in inhibiting the signaling of the insulin receptor, inducing insulin resistance [37]. The region of OAR3 contained ATPase Plasma Membrane Ca²⁺ + Transporting 1 (*ATP2B1*), which catalyzes the ATP hydrolysis attached to calcium transport from the cytoplasm to the extracellular space thereby playing a vital role in intracellular calcium homeostasis [38].

Three candidate genes found in the region of OAR4; Oxysterol Binding Protein like 3 (*OSBPL3*), Neuropeptide Y (*NPY*) and ADAM Metallopeptidase Domain 22 (*ADAM22*). *OSBPL3* is essential in regulating actin cytoskeleton, cell adhesion, cell polarity and cellular lipid metabolism [39]. *NPY* acts as a neuromodulator and is involved in several physiological processes, including stress response, food intake, cardiovascular function and circadian rhythms [40]. *ADAM22* is involved in the regulating cell adhesion and inhibiting cell proliferation [41]. In the region of OAR6, two candidate genes were identified; alpha kinase 1 (*ALPK1*) and TRAF Interacting Protein with Forkhead Associated Domain (*TIFA*). *ALPK1* initiates an innate immune response triggered by the detection of bacterial pathogen-associated molecular pattern metabolites (PAMPs), which is vital for eliminating pathogens and engaging adaptive immunity [42]. *TIFA* encodes an adapter protein essential in adaptive and innate immunity by activating proinflammatory NF- κ B signaling following the detection of bacterial PAMPs [43]. In the region of OAR7, two candidate genes were found MCC regulator of WNT signaling pathway (*MCC*) and erythrocyte membrane protein band 4.1 like 4A (*EPB41L4A*). *MCC* plays a part in cell migration [44]. While *EPB41L4A* product NBL4 is said to play a role in the beta-catenin signaling pathway. Beta-catenin regulates and coordinates cell-cell adhesion and gene transcription

[45]. Region of OAR10 contained aconitate decarboxylase 1 (*ACOD1*) that is essential in the antimicrobial response of innate immune cells by producing itaconic acid that is involved in the antimicrobial activity of macrophages [46]. Transmembrane Protein 98 (*TMEM98*) is found in one region of OAR11 and the secreted form of this gene promotes T helper 1 cells (Th1) differentiation [47].

Nine candidate genes were identified in the second region of OAR11; Alpha-1,6-Mannosylglycoprotein 6-Beta-N-Acetylglucosaminyltransferase B (*MGAT5B*), Acetylgalactosaminide Alpha-2,6-Sialyltransferase 2 (*ST6GALNAC2*), Cytoglobin (*CYGB*), Sphingosine Kinase 1 (*SPHK1*), Galanin Receptor 2 (*GALR2*), Galactokinase 1 (*GALK1*), Solute Carrier Family 16 Member 5 (*SLC16A5*), Otopetrin 2 (*OTOP2*) and Otopetrin 3 (*OTOP3*). *MGAT5B* is essential in the O-mannosyl glycan pathway, and the modulation of integrin and laminin-dependent adhesion and neuronal cell migration [48]. *ST6GALNAC2* has roles at the cell surfaces in bacterial adhesion, protein targeting and cell-cell and cell-substrate interactions [49]. *CYGB* might have a protective function against oxidative stress conditions and might have a role in intracellular oxygen storage or transfer [50]. *SPHK1* and its product S1P are essential in the NF-kappa-B activation pathway and TNF-alpha signaling essential in immune processes [51]. *GALR2* expresses galanin, an important neuromodulator present in the gastrointestinal system, brain and hypothalamopituitary axis. Also essential in regulating the growth hormone release and modulation of insulin release [52]. *GALK1* is a major enzyme for galactose metabolism [53]. *SLC16A5* catalyzes the prompt transport of many monocarboxylates such as branched-chain oxo acids derived from leucine, pyruvate, lactate ketone bodies acetoacetate, valine and isoleucine, beta-hydroxybutyrate and acetate across the plasma membrane [54]. *OTOP2* and *OTOP3* are proton-selective channels that transport protons specifically into cells. The activity of these channels is possibly essential in cell types that use intracellular pH changes for cell signaling or the regulation of biochemical or developmental processes [55].

In the region of OAR12, Phospholipase A2 Group IVA (*PLA2G4A*) was proposed as a candidate gene as these are a group of enzymes hydrolyzing phospholipids into fatty acids and other lipophilic molecules [56]. Two candidate genes found in the region of OAR13; Glutamate Decarboxylase 2 (*GAD2*) and Optineurin (*OPTN*). *GAD2* encodes one of the various forms of glutamic acid decarboxylase and has been recognized as a vital autoantigen in insulin-dependent diabetes [56]. *OPTN* is involved in innate immune response activation during viral infections [57]. *CD44* Molecule (Indian Blood Group) was found in the region of OAR15 and this gene encodes for a cell-surface glycoprotein essential in cell adhesion, cell-cell interactions and migration. This glycoprotein also participates in other cellular functions such as activation and homing T-lymphocytes, inflammation and response to bacterial infections [58, 59]. The region of OAR16 contained Fibroblast Growth Factor 10 (*FGF10*) that is essential in regulating the development of an embryo, cell proliferation and differentiation. *FGF10* may be involved in wound healing [60]. Two candidate genes found in the region of OAR19; Contactin 6 (*CNTN6*) and cell adhesion molecule L1 like (*CHL1*). *CNTN6* and *CHL1* encode for proteins that function as cell adhesion molecules, these molecules are known to interact and have a key role in inflammatory responses [61, 62].

In the region of OAR20, five candidate genes were identified; Methylmalonyl-CoA Mutase (*MMUT*), Glycine-N-Acyltransferase Like 3 (*GLYATL3*), Defensin Beta 133 (*DEFB133*), Defensin Beta 113 (*DEFB113*) and Defensin Beta 110 (*DEFB110*). *MMUT* plays a role in degrading various amino acids, cholesterol and odd-chain fatty acids via propionyl-CoA to the tricarboxylic acid cycle [63]. *GLYATL3* catalyzes the conjugation of long-chain fatty acyl-CoA thioester and glycine producing long-chain N-(fatty acyl) glycine intermediate in the primary fatty acid amide biosynthetic pathway [64]. *DEFB133*, *DEFB113* and *DEFB110* are a family of antimicrobial and cytotoxic peptides that are made by neutrophils. They have innate immune defence response to bacteria [64]. Lipin 2 (*LPIN2*) is found in the region of OAR23 and plays a significant role in regulating fatty acids metabolism at different levels [65]. Two candidate genes found in the region of OAR26 associated with three significant SNPs; 1-Acylglycerol-3-phosphate O-acyltransferase 5 (*AGPAT5*) in SNP DU481531_204.1 and Nuclear Receptor Subfamily 1 Group D Member 2 (*NR1D2*) in SNP OAR26_45130684.1 and OAR26_46516694.1. *AGPAT5* converts lysophosphatidic acid (LPA) into phosphatidic acid and plays a role in LPA containing unsaturated or saturated fatty acids C15:0-C20:4 at the sn-1 position using C18:1-CoA as the acyl donor [66]. *NR1D2* is found to be associated with *Acinetobacter* in the same region and has already been described.

Clostridium

The relative abundance of *Clostridium* is associated with genetic variations in region OAR2 of the sheep genome. SNP s43106.1 contained two candidate genes; RAD23 Homolog B, Nucleotide Excision Repair Protein (*RAD23B*) and Solute Carrier Family 44 Member 1 (*SLC44A1*). *RAD23B* is involved in cellular response to DNA damage stimulus [67] and cellular response to interleukin-7 [68]. *SLC44A1* might provide choline for cell membrane phospholipid synthesis [69]. SNP OAR2_58286833_X.1 contained the TLE Family Member 1, Transcriptional Corepressor (*TLE1*) which inhibits NF-kappa-B-regulated gene expression in human monocytes also, increased expression of *TLE1* is significant for inhibition of interleukin 12 (IL-12) p70 expression mediated by zymosan [70] also as inhibiting immune suppression induced by *Bacillus anthracis* toxin [71].

Flavobacterium.

The relative abundance of *Flavobacterium* was associated with genetic variations in chromosome 1, 2, 6, 16, 22 and 23 along the sheep genome. In the region of SNP OAR1_74421103.1, three candidate genes were proposed; Growth factor independence-1 (*GFI1*) involved in regulating the pre-T cell differentiation, hematopoietic stem cell maintenance and secretory cell types development in the intestines [72]. The formin-binding protein 1-like (*FNBP1L*) is critical for antibacterial autophagy [73]. The ATP Binding Cassette Subfamily D Member 3 (*ABCD3*) is crucial in the peroxisomal degradation of branched-chain fatty acids and biosynthesis of bile acid [74]. Unsaturated fatty acids released from bile are known to increase the solubility and absorption of saturated fatty acids in the rumen, which in turn improves fat absorption [75]. The regulation of this gene may directly influence the occurrence of *Flavobacterium*, as this bacteria is essential in fatty acid metabolism and biohydrogenation [76]. Three candidate genes were proposed in the region of significant SNP OAR1_197593478.1: Immunoglobulin superfamily member 11 (*IGSF11*), Beta-1,4-Galactosyltransferase 4 (*B4GALT4*), Rho GTPase Activating Protein 31 (*ARHGAP31*). *IGSF11* functions as a cell-cell adhesion molecule in homophilic interactions and encourages cell growth [77]. *B4GALT4* encodes for enzymes that play a vital role in glycosaminoglycan biosynthesis [78]. *ARHGAP31* encodes a GTPase-activating protein regulating Cdc42 and Rac1 essential in protein trafficking, cell growth and cell migration [79]. SNP OAR2_247207503.1 was associated with the abundance of *Flavobacterium*. The SNP is located within the region of Adenylate kinase 2 (*AK2*) and lymphocyte Cell-Specific Protein-Tyrosine Kinase (*LCK*), both these genes might be associated with microbial composition as their functions related with the immune system. *AK2* is essential in cellular energy homeostasis and cell proliferation [80]. *LCK* is essential in the selection and maturation of developing T-cells, and in T-cell antigen receptor (TCR)-linked signal transduction pathways [81].

Candidate genes found in the region OAR16 are associated with innate and adaptive immune responses (*C9*) [82]; activation of T-cell factor signaling and mediating PGE2 induced expression of early growth response 1 (*PTGER4*) [83]; cell growth and survival regulation responding to hormonal signals (*RICTOR*) [84]; activation and control of interleukin-2 expression (*FYB1*) [85]; cell differentiation and can induce macrophage adhesion and spreading (*DAB2*) [86]. Five candidate genes identified in region OAR22; *NFKB2* is essential in cellular responses and in regulating the immunological responses to infections and inflammation [87, 88]. *TRIM8* plays different roles in immune pathways, such as having a positive role in the TNFalpha and IL-1beta signaling pathways [89]. *CNNM2* is essential in magnesium (Mg²⁺) homeostasis by mediating the epithelial transport and renal reabsorption of Mg²⁺ [90]. *FGF8* is significant in regulating the development of an embryo, cell proliferation, differentiation and migration [91]. *ELOVL3* is essential in the production of monounsaturated and saturated very-long-chain fatty acids (VLCFAs) associated with numerous biological processes as precursors of lipid mediators and membrane lipids [92, 93]. SMAD Family Member 4 (*SMAD4*) is the candidate gene found in the OAR23 region; it serves as a transcription activator regulating TGF-beta receptor-mediated signaling, and is involved in IL-2 activation and signaling pathway [94].

Prevotella.

Six regions on the sheep genome were associated with the abundance of *Prevotella*. In the region of OAR1 in the sheep genome, one candidate gene was identified; RUNX Family Transcription Factor 1 (*RUNX1*) which is crucial for the normal hematopoiesis development and positively regulates the expression of RAR Related Orphan Receptor C (*RORC*) gene in T-helper 17 cells [95]. One candidate gene was found in the region of OAR4; Glutamate metabotropic receptor 3 (*GRM3*) was the identified candidate gene, which is known to modulate synaptic glutamate levels that regulate stress response and a study showed fixed missense mutation on *GRM3* differentiating between aggressive foxes from tame foxes [96]. In the region of OAR7, two candidate genes were identified; neural precursor cell expressed, developmentally downregulated 4 (*NEDD4*) and *RAB27A* (member RAS oncogene family). *NEDD4* is a significant candidate molecular marker essential in cell proliferation, ovarian development, and sexual reproduction in zebrafish [97]. *RAB27A* is involved in cytotoxic granule exocytosis in lymphocytes, as it is essential for priming at the immunologic synapse and granule maturation and granule docking [98].

Three candidate genes have been found in the region OAR16: Dual Specificity Phosphatase 1 (*DUSP1*), BCL2 Interacting Protein 1 (*BNIP1*) and Stanniocalcin 2 (*STC2*). *DUSP1* is essential in the cellular response to environmental stresses and negatively regulate cellular proliferation [99]. *BNIP1* interacts with the E1B 19 kDa protein, protecting host cells from virally induced cell death [100]. While *STC2* encodes for homodimeric glycoprotein, it regulates cell metabolism, renal and intestinal calcium and phosphate transport and cellular calcium/phosphate homeostasis [101]. *Prevotella* microbes are involved in peptide and protein degradation in the rumen, in saccharolytic pathways and in the production of saturated fatty acids [102]. *STC2* has been found to have a significant association with the relative abundance of *Prevotella* in cattle, and it has been previously associated to fatty acids and cell metabolism [103]; hence this gene is suggested that it could modulate the abundance of *Prevotella* composition in the rumen. In OAR 25 region, three candidate genes were identified, two are also found to be associated with *Acinetobacter* in the same region and have already been described (*WDFY4* and

OGDHL). The third gene is the mitogen-activated protein kinase 4 (*MAPK8*) and it acts as a point of integration for multiple biochemical signals and is essential in extensive cellular processes such as transcription regulation and development, proliferation and differentiation [104].

Pseudomonas.

The relative abundance of *Pseudomonas* is associated with genetic variations of two regions in OAR6 and OAR14. Three candidate genes have been found in OAR6 region: 3-Hydroxybutyrate Dehydrogenase 2 (*BDH2*), Solute Carrier Family 9 Member B2 (*SLC9B2*) and CDGSH Iron Sulfur Domain 2 (*CISD2*). *BDH2* facilitates the formation of 2,5-dihydroxybenzoic acid (2,5-DHBA), a siderophore that plays a role in iron assimilation and homeostasis, as it shares structural resemblances with bacterial enterobactin [105, 106]. This gene may be significant in the prevention of pathogens to invade the host. *SLC9B2* is essential in regulating sodium homeostasis, intracellular pH, and cell volume [107], which may be significant in regulating ruminal microbial composition. It also essential in insulin secretion and clathrin-mediated endocytosis in beta-cells [108]. *CISD2* regulates autophagy, contributing to antagonizing BECN1-mediated cellular autophagy at the endoplasmic reticulum [109]. Casein Kinase 2 Alpha 2 (*CSNK2A2*) and Glutamic-Oxaloacetic Transaminase 2 (*GOT2*) are two candidate genes identified in the region of OAR14. *CSNK2A2* phosphorylate acidic proteins such as casein are essential in several cellular processes, like cell cycle control and circadian rhythms [110]. *GOT2* is essential in amino acid metabolism and is essential for the exchange of metabolites between mitochondria and cytosol. It also enables cellular uptake of long-chain free fatty acids [111, 112].

Streptobacillus.

Two candidate genes found in the OAR25 region may be associated with the *Streptobacillus* relative abundance. Surfactant Protein A1 (*SFTPA1*) and Peroxiredoxin Like 2A (*PRXL2A*). *SFTPA1* encodes a protein binding to particular carbohydrate moieties found on the surface of microorganisms and lipids. This protein is vital in the immune defence and surfactant homeostasis against respiratory pathogens [113]. *PRXL2A* acts as an antioxidant and negatively regulates macrophage-mediated inflammation by inhibiting the production of macrophage of inflammatory cytokines by suppressing MAPK signaling pathway [114].

Conclusion

This study identified associations between sheep genetic variations and relative abundance of seven genera; *Acinetobacter*, *Bacillus*, *Clostridium*, *Flavobacterium*, *Prevotella*, *Pseudomonas* and *Streptobacillus*. Candidate genes identified in the associated genetic regions encode proteins essential in host defence systems, containing the immune system, antimicrobial activities, inflammatory responses and regulation of cell adhesion and migration. In contrast, other proteins contribute to fatty acid and bile metabolism and other biochemical processes. The results confirm that host genetics is important in modulating microbial composition; nonetheless, the association found in this study may be population-specific as various factors alter the rumen microbiota. More studies are necessary for diverse populations to help determine genetic combinations favourable for the enhancement of beneficial microbiota and provide excellent intestinal health to the individuals and avoid the invasion of potential pathogens.

Abbreviations

FDR: False discovery rate, GWAS: Genome-wide association studies, MAF: Minor allele frequency, OTU: Operational taxonomic units, SNP: Single nucleotide polymorphism.

Declarations

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Authors' contribution

SM, OAA and MAA designed the experiment, SM carried all the laboratory work and analyses. SM drafted the manuscript. OAA and MAA revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

Raw reads used in the present study were deposited to the National Center of Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under BioProject PRJNA578022 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA578022>). The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All the procedures involving animals were in accordance with guidelines of the Agricultural Research Council - Animal Production Ethics committee (APIEC17/21).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' information

^a Agricultural Research Council- Animal Production Institute, GI Microbiology and Biotechnology Unit, Irene, South Africa. ^b Discipline of Genetics, School of Life Sciences, College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Westville, P/Bag X54001, Durban 4000, South Africa. ^c Unit for Environmental Sciences and Management, North West University, Potchefstroom 2520, South Africa.

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Figures

Figure 1

Manhattan plot showing $-\log$ scale p-values (y-axis) of SNPs associated with the relative abundance of the following genera (*Acinetobacter*, *Bacillus*, *Clostridium*, *Flavobacterium*, *Prevotella*, *Pseudomonas* and *Streptobacillus*) on the ovine chromosomal positions (x-axis). Red lines indicate SNPs above the genome-wide significance threshold ($FDR \leq 0.05$) and blue lines show SNPs below the genome-wide significance threshold ($FDR \leq 0.1$).

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