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RUMINANTS NUTRITION AND FEEDING

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Ruminal microbiota, carbohydrate-active enzymes and milk bioactive compounds in Italian Mediterranean dairy buffaloes fed total mixed ration with or without green forage

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ABSTRACT

The aim of the present study was to determine the effect of including green forage in the diet of Italian Mediterranean dairy buffaloes on the ruminal microbiota, CAZymes profile, functional biomolecules and total antioxidant activity in bulk milk. Sixteen buffaloes were randomly assigned according to lactation number and daily milk production to two homogeneous groups, and for 60 days received each: Group 1, a standard total mixed ration (TMR) or group 2, TMR + ryegrass green forage (30% of diet). The diets of the two groups were iso-nitrogenous and iso-energetic and differed only in the proportion of green forage. Buffaloes that received TMR + green feed had a higher ($p < .01$) representation of bacteria belonging to the orders *Veillonellales*, *Selenomonadales* and *Bradymonadales* compared with buffaloes that received TMR. The former buffaloes also had a greater ($p < .01$) abundance of CAZymes of the GT class (GHT4, GT14, GT20, GT26, GT39) and AA class (AA1, AA3, AA6). The milk of buffaloes that received TMR + green feed had a higher ($p < .01$) antioxidant capacity and greater ($p < .01$) amounts of the functional biomolecules L-carnitine, propionyl-L-carnitine, acetyl-L-carnitine and δ-valerobetaine. The findings have provided evidence for metabolic and biosynthetic pathways that link green forage with rumen bacteria, CAZymes and the synthesis of amino acids and functional biomolecule in buffaloes.

HIGHLIGHTS

- Green feed diet in dairy buffaloes favours ruminal microbiota that produces CAZymes.
- CAZymes support the synthesis of amino acids and functional biomolecules in milk.
- Functional milk from animals fed natural diets will be more appreciated by consumers.

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Green forage; rumen microbiome; CAZymes; biomolecules; dairy buffalo

Introduction

Buffaloes are a major global livestock of economic and social importance in both developing and developed economies (Deb et al. 2016). They are productive on a relatively low-quality diet and have milk of high nutritional value (Arrichiello et al. 2022). The latter is due partly to the unique rumen microbiota of buffaloes which is distinctively different to the microbiota of other ruminants with relatively high fibre degradation and reduced methane production (Hamid et al. 2017; Iqbal et al. 2018; Malik et al. 2021; Sun et al. 2021). Based on the global importance of buffaloes as a source of milk, it is important to gain a deeper understanding of the relationships between diet, ruminal function and milk quality in buffaloes.

Consumers prefer food products sourced from animals fed natural diets (El-Zaiat and Abdalla 2019; Sallam et al. 2019). Cattle and buffaloes fed green forage produce milk with greater antioxidant and anti-inflammatory activity (Salzano et al. 2021, 2022).

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The inclusion of green forage in the diet of buffaloes is associated with distinctive changes in the ruminal metabolome (Neglia, Cotticelli, et al. 2023) and transcriptome (Salzano et al. 2023) and this may be probably due to the higher amount of simple sugars that are able to improve rumen efficiency and nutrient utilisation (Mordenti et al. 2021). It can be, hence, assumed that the changes in the ruminal metabolome and transcriptome in buffaloes fed green forage enhances the health-promoting properties of buffalo milk.

The ruminal microbiota produces carbohydrate-active enzymes (CAZymes) which include lignocellulolytic enzymes that fundamentally determine the degradation and debranching of plant polysaccharides (Hobson and Stewart 1997; Cantarel et al. 2009). The breakdown of complex carbohydrates by CAZymes produces several metabolites, including volatile fatty acids (VFAs) and hydrogen (Li et al. 2022). CAZymes are grouped into families depending on their specific enzymatic activity (Cantarel et al. 2009). The major CAZymes families are glycoside hydrolases (GHs, hydrolyse and/or rearrange glycosidic bonds), glycosyl transferases (GTs, form glycosidic bonds), polysaccharide lyases (PLs, non-hydrolytic cleavage of glycosidic bonds), carbohydrate esterases (CEs, hydrolyse carbohydrate esters) and auxiliary activities (AAs, redox enzymes that act in conjunction with CAZymes). Diet influences the CAZymes profile in cattle and buffaloes (Patel et al. 2014; Wang et al. 2019; Pantoja-Feliciano et al. 2023). The present study sought to describe relationships between the ruminal microbiota, ruminal CAZymes and milk quality, in buffaloes that received green feed. The relationships were compared to buffaloes that received a standard total mixed ration (TMR). The new information could be used to develop diets incorporating green feed which favour rumen microbiota that produce CAZymes profiles that enhance milk quality in buffaloes. The hypothesis tested was that green feed induces changes in the ruminal microbiota composition and CAZymes profile resulting in enhanced synthesis of amino acids and precursors of functional health-promoting biomolecules in milk of buffaloes.

Materials and methods

For this trial, standard veterinary practices were followed and institutional approval was obtained from the Ethical Animal Care and Use Committee of the University of Napoli "Federico II" (Protocol Number 0025532/2022).

Animals, dietary treatment and ruminal tissue collection

The study was carried out over a period of 60 days using Italian Mediterranean dairy buffaloes ($n = 16$; 8.5 ± 2.0 years old) at a commercial farm in southern Italy. The animals were acclimatised in pens with concrete floors and were machine milked twice daily. They were randomly assigned on parity and daily milk production to two homogeneous groups and received either a standard TMR (5.6 ± 0.6 parity; 6.0 ± 0.6 kg/day milk) or TMR + green forage (5.3 ± 0.6 parity; 6.4 ± 0.5 kg/day milk) (Table 1). The green feed consisted of ryegrass (about 30% of the diet on dry matter) at the re-blossoming stage that was cut twice daily to avoid fermentation, and immediately put into the mixer wagon and fed without storage. The ratio of forage to concentrate was 56:44 for buffaloes that received TMR and 69:31 for buffaloes that received TMR + green forage. The two diets were iso-nitrogenous and iso-energetic and differed only in the proportion of green forage (Table 1). The animals were fed in the morning and evening. Refusals were recorded and then removed. Energy values (milk forage units = 1700 kcal) were calculated using equations provided by the INRA (2007).

Average feed intake for each pen was determined daily from unconsumed feed before the next feeding. The amount and composition of refusals were used to

Table 1. Feed and chemical composition of the buffalo diets fed without total mixed ration (TMR group) or with (TMR + green feed) approximately 30% green ryegrass.

	TMR % feed	TMR + green feed % feed
Ryegrass	0.0	50.3
Corn silage	68.5	35.6
Alfalfa hay	12.5	5.2
Soybean meal, 48%	1.8	0.0
Concentrate	11.8	5.1
Wheat straw	3.1	2.3
Hydrogenated fats	0.9	0.7
Calcium carbonate	0.4	0.4
Salt 1:3	0.6	0.4
Vitamins	0.4	0.0
Total	100	100
	Composition, % of dry matter	
Dry matter	16.0	16.1
CP	14.5	14.4
Fat	4.8	5.0
NDF	38.0	38.5
ADF	24.0	23.4
NSC	34.6	33.1
Starch	21.0	15.5
Ash	8.1	9.0
Calcium	0.9	1.0
Phosphorus	0.4	0.4
MFU	0.93	0.91

CP: crude protein; NDF: neutral detergent fibre; ADF: acid detergent fibre; NSC: non-structural carbohydrates; MFU: milk forage units.

calculate DMI and diet composition. Individual feed intake and differences (Δ) between nutritive intake and relative requirements were estimated as previously reported (Campanile et al. 1998):

$$\text{Dry matter (DM) intake} = 91\ \text{g} \times \text{MW} + 0.27\ \text{kg} \times \text{kg ECM}$$

$$\Delta\text{CP} = \text{g CP intake} - (80\ \text{g CP} \times 100\ \text{kg live weight} + 2.7\ \text{g CP} \times \text{g milk protein yield})$$

$$\Delta\text{MFU} = \text{MFU intake} - [(1.4 + 0.6 \times 100\ \text{kg live weight}) \times 1.1 + 0.44\ \text{MFU} \times \text{kg ECM}]$$

At day 60, samples of bulk milk were obtained for each group, the milk production was recorded and the animals were slaughtered and processed under commercial conditions. The rumen of each animal was made available and samples were collected from different locations within the rumen and pooled. Rumen fluid samples (50 mL) for each animal were collected in a falcon tube, immediately frozen in dry ice, and then stored at −80 °C until analyses.

Bulk milk analysis

Samples were analysed in triplicate using IR spectroscopy (Milkoscan 139, Foss Electric, Hillerød, Denmark) that was calibrated with a buffalo standard. Energy corrected milk (ECM = 740 kcal) was calculated using the formula for buffalo cows (Campanile et al. 1998): ([{fat (g·kg^{-1}) - 40 + protein (g·kg^{-1}) - 31} × 0.01155] + 1) × milk yield. The content of γ-butyrobetaine, glycine betaine, δ-valerobetaine, L-carnitine, acetyl-L-carnitine and propionyl-L-carnitine was determined in duplicate pooled soluble milk extract samples as previously described (Servillo, D'Onofrio, Giovane, et al. 2018). Briefly, samples of milk were centrifuged at 3000 × *g* for 15 min at 4 °C to recover fat globules. The latter were filtered through a 5 μm Millipore filter (Burlington, MA) to remove high MW components and any precipitate that may have been transferred when recovering fat globules. This was followed by filtration through an Amicon Ultra 0.5 mL centrifugal filter with a 3 kDa molecular weight cut-off to yield low MW components, including short-chain acylcarnitines (between 161.2 and 245.3 molecular weight), glycine betaine (153.6 molecular weight), γ-butyrobetaine (146.1 molecular weight) and δ-valerobetaine (159.23 molecular weight). The antioxidant and antineoplastic activity in Amicon Ultra-filtrates are not due to low MW peptides as activity is retained during treatment at 100 °C to produce ricotta cheese (Salzano et al. 2019). Analysis involved HPLC-ESI-MS/MS with an Agilent LC-MSD SL quadrupole ion trap and a 1100 series liquid chromatograph (Supelco Discovery C8 column, 250 × 3.0 mm, particle size 5 μm) under isocratic conditions, 0.1% formic acid in water, at flow rate of 100 μL/min. Quantification of each compound involved comparison of the peak area of its most intense MS2 fragment with the respective calibration curve built with solutions of standards products (L-carnitine, acetyl-L-carnitine propionyl-L-carnitine, glycine betaine and γ-butyrobetaine) from Sigma-Aldrich (Milan, Italy) (Servillo, D'Onofrio, Neglia, et al. 2018). δ-Valerobetaine was prepared as previously described (Servillo, D'Onofrio, Neglia, et al. 2018). Standard solutions were prepared by serial dilution of standard stock solutions (2 mg/L) with water containing 0.1% formic acid. Linearity was assessed by correlation coefficients (r^2) >0.99 for all compounds. Precision and accuracy for all compounds in milk ranged from 95% to 105%.

Ferric reducing antioxidant power and total antioxidant assay

Milk ferric reducing antioxidant power (FRAP; Assay Kit (MBS169262) and total antioxidant capacity (TAC; Assay Kit (#K274-100) were determined in pooled milk samples according to the manufacturer. Milk samples (four replicates) were diluted 1:10 (v/v) with H_2O and a 1 μL aliquot was used for each assay. Samples were incubated at room temperature for 90 min protected from light, before measuring the absorbance at 570 nm, or monitoring the increase in absorbance at 594 nm for 1 h at 37 °C for TAC and FRAP assay, respectively. The positive control comprised ascorbic acid (1 μg/mL). FRAP value was expressed as Fe^{2+} iron equivalents (μM; range = 10–250 × μg/mL of ascorbic acid) and TAC was expressed as Trolox equivalent capacity (range = 10–250 × nmol μg/mL of ascorbic acid).

DNA extraction

Bacterial genomic DNA was extracted using the QIAMP DNA Stool mini kit (QIAGEN, Hilden, Germany) according to the manufacturer and included negative extraction controls (Saggese et al. 2016; Saggese, Giglio, et al. 2022). The amount and the quality of the extracted DNA were evaluated using Nanodrop ND-2000 (Nanodrop, Wilmington, DE) and a high-sensitivity Qubit™ fluorometer (Saggese, De Luca, et al. 2022).

Sequencing, bioinformatics and statistical analyses of 16S rRNA gene sequences

Partial 16S rRNA gene sequences were obtained using primers specific for the V4–V5 region (515FB = GTGYCAGCMGCCGCGGTAA and 926R = CCG YCAATTYMTTTRAGTTT 515FB) (Parada et al. 2016) and were sequenced at the Integrated Microbiome Resource (IMR, https://imr.bio) using an Illumina MiSeq machine (San Diego, CA). All sequences were imported in R, and analysed with the DADA2 package (Callahan et al. 2016). Following the package guidelines, quality plots were performed to check the sequences' quality. Post-QC reads were trimmed using the filterAndTrim command [truncLen = c(260,190), maxN = 0, maxEE = c(2,2), truncQ = 2, rm.phix = TRUE, trimLeft = c(20,21)]. After this step, a parametric error model, based on the convergence between the estimation of error rate and the inference of the sample composition, was performed. Paired-end reads were merged and exact amplicon sequence variants (ASVs) were inferred using the dada algorithm. Chimeric sequences were removed and prokaryotic taxonomy was assigned using the naive Bayesian classifier method against the Silva Database (r138). ASVs abundance table obtained from DADA2 was further processed in R using *Phyloseq*, *Vegan* and *Microbiome* packages (Lahti and Shetty 2017; Oksanen et al. 2020). Sequences are available in the NCBI Sequence Read Archive (SRA) database under the BioProject number PRJNA993601. BioSample accession number for each sequence is included in Supplementary Table S1.

After the Phyloseq object creation, low abundance ASVs (less than three reads across the dataset), mitochondria, chloroplast and potential contaminants (Sheik et al. 2018) were removed. The remaining ASVs represented ~99% of the original reads, with 1,139,005 reads classified as 8222 individual ASVs used for downstream statistical investigations (Maia et al. 2020). ASVs counts were normalised to the median library size across the dataset. Diversity analyses were carried out using the Phyloseq package (McMurdie and Holmes 2013) with relative abundance set to 100% after the removal of sequences described above. Top abundance ASVs and Genera were defined as having a cumulative relative abundance above 0.1% in our dataset. The alpha diversity was investigated using the Simpson diversity index among the two sampled areas. The beta diversity was investigated using the Jaccard diversity index as implemented in the vegan package (Oksanen et al. 2012). Both the abundance weighted and unweighted versions of the index were used.

Sequencing, bioinformatics and statistical analyses of metadata

Microbiomes were investigated through metagenomic analysis using the Giovannelli Lab metagenomics narrative created on Kbase (Arkin et al. 2018). Metagenomic data are available in the NCBI SRA with project ID PRJNA993601. BioSample accessions for each metagenome are included in Supplementary Table S1. This pipeline is designed to analyse raw metagenomic sequences obtained from various microbiomes and includes three main approaches: a read-based approach, an assembly-based approach and a genome-resolved approach. The read-based approach first involves quality control of the raw sequences using FastQC (v0.11.9) to evaluate sequence quality, followed by a trimming step with Trimmomatic (v0.36) to remove primers and poorly sequenced extremities. The trimmed sequences are then subjected to taxonomic classification using Kaiju (v1.7.3). The read-based functional profiling was obtained through the software mi-faser (functional annotation of sequencing reads; Zhu et al. 2018) which combines faser (an optimised algorithm for mapping reads) with the reference database of protein functions and annotates each microbiome as a set of molecular functions. The assembly-based approach involves the use of three different assembly software programs for each sample: metaSPAdes (v3.15.3), MEGAHIT-high and low sensitivity (v1.2.9) and IDBA-UD (v1.1.3). The resulting assemblies are evaluated using QUAST (v1.1.2) to select the best assembly based on contiguity and completeness. The functional annotation of the contigs obtained from the assembly is carried out using the Database of Rapid Annotation using Subsystems Technology (RAST).

The genome-resolved approach involves binning the contigs obtained from the assembly step using three different binning software programs: MaxBin2 (v2.2.4), MetaBAT2 (v1.7) and CONCOCT (v1.1). The resulting bins are refined using consensus assignments with the software DAS-tool (v1.1.2) to ensure bins with at least 50% completeness. Quality control of the bins is performed using CheckM (v1.0.18), which uses a built-in library of high-quality conserved marker genes to estimate the completeness and contamination of the metagenomic bins. The taxonomic classification of the high-quality bins is carried out using the Genome Taxonomy Database Toolkit (GTDB-Tk) database (Chaumeil et al. 2019), which provides objective taxonomic assignments for bacterial and archaeal genomes, placing the bins within a phylogenetic tree with high-quality reference genomes.

CAZyme annotation of metadata

All samples were further functionally characterised by analysis of the CAZyme database (www.cazy.org), which allows the identification of CAZymes. CAZymes, including those encoded by GHs, glycosyltransferases (GTs), CEs, PLs and AAs genes in the rumen metagenome, were identified and annotated using the HMMER 3.0 package (http://hmmer.org/) with the dbCAN CAZyme database (www.cazy.org; Yin et al. 2012). The CAZyme results of the gene were searched against the sequences in the NR database using the BLASTP algorithm with an *E*-value cut-off of 1×10^{-5}.

Statistical analyses

Statistical analyses were performed using SPSS (23.0) for Windows 10 (SPSS Inc., Chicago, IL). The buffalo was used as the experimental unit. One-way ANOVA was used to compare data for dry matter intake (DMI), milk yield and quality, total antioxidant activity (TAC), FRAP and functional molecules of milk. A statistically significant difference was accepted at $p < .05$.

Results

Animal productions

As shown in Table 1, total DMI was similar between TMR and TMR + green feed buffaloes (16.0 ± 0.2 and 16.1 ± 0.2 kg/day respectively for TMR and TMR + green feed buffaloes, $p = .89$). Average milk yield and ECM throughout the experimental period did not differ significantly between the two groups (12.3 ± 0.1 vs. 12.5 ± 0.2 kg respectively for TMR and TMR + green feed group, $p = .87$). No differences were found on milk quality traits between the two groups (data not shown).

Milk biomolecules and antioxidant capacity

Results for functional biomolecules in milk and total antioxidant power are shown in Table 2. Buffaloes that received green feed had higher ($p < .01$) concentrations of L-carnitine, propionyl-L-carnitine, acetyl-L-carnitine and δ-valerobetaine compared with buffaloes that received the TMR. Concentrations of glycine, betaine and γ-butyrobetaine did not differ between groups. The antioxidant capacity of milk was higher ($p < .01$) in buffaloes that received green feed, both when determined by TAC and FRAP assays.

Table 2. Functional biomolecules and antioxidant power in milk for buffaloes that received a total mixed ratio (TMR group) and buffaloes that received TMR + fresh ryegrass (approximately 30% of the diet; TMR + green feed group).

	TMR, mg/L	TMR + green feed, mg/L
L-Carnitine	30.4 ± 0.7 A	41.0 ± 0.6 B
Acetyl-L-carnitine	38.6 ± 0.5 A	48.4 ± 0.6 B
Propionyl-L-carnitine	15.7 ± 1.0 A	22.1 ± 0.5 B
γ-Butyrobetaine	4.4 ± 0.3	3.8 ± 0.2
δ-Valerobetaine	19.1 ± 0.6 A	23.4 ± 0.6 B
Glycine betaine	7.3 ± 0.1	7.4 ± 0.3
TAC	220.3 ± 5.5 A	249.5 ± 7.7 B
FRAP	194.3 ± 4.2 A	225.8 ± 4.8 B

FRAP: ferric reducing antioxidant power assay; TAC: total antioxidant capacity.
Results are mg/L, mean ± SEM. (A, B) $p < .01$.

Taxonomic analysis of the rumen microbial composition

A total of 1,139,005 sequences with an average sequence length of 370 bp were found and 8221 ASVs were identified. Of these, 6192 ASVs were common to the two groups. There were 734 unique for buffaloes that received TMR and 1295 were unique for buffaloes that received TMR + green feed. The taxonomic analysis assigned the bacterial community to 14 bacterial phyla (Supplementary Table S2 Figure S1). *Firmicutes* (now renamed *Bacillota*) and *Bacteroidota* were the most abundant phyla in both groups (over 92% of the total bacteria). Other bacterial phyla that accounted for more than 1% of the sample were *Proteobacteria* and *Planctomycetota* (Supplementary Table S2). At the order level, *Bacteroidales*, *Christensenellales*, *Oscillospirales* and *Lachnospirales* were most abundant in both groups (Supplementary Table S3 and Figure S2). At the genus level, *Christensenellaceae_R-7_group*, *Rikenellaceae_RC9_gut_group*, *NK4A214_group*, *Prevotella* and *Butyrivibrio* were the most common in buffaloes that received TMR and *Lachnospiraceae_XPB1014_group* replaced *Butyrivibrio* in buffaloes that received TMR + green feed (Supplementary Table S4 and Figure S3).

Rumen microbial diversity

The overall microbial composition of the rumen did not differ markedly between the two groups (Supplementary Figures S1–S3). No differences were observed at the phylum level (Supplementary Figure S1 and Table S2) and there were relatively minor differences at the order and genus level (Supplementary Figures S2 and S3, Tables S3 and S4). At the order level, *Peptostreptococcales–Tissierellales* were more abundant in buffaloes that received TMR and *Veillonellales–Selenomonadales* and *Bradymonadales*

were more abundant in buffaloes that received TMR + green feed (Supplementary Table S3). Relatively few statistically significant differences were observed at the genus level and these were mostly due to poorly represented genera: *Prevotellaceae_YAB2003_group* (*Bacteroidota* phylum) and *Colidextribacter* (*Bacillota* phylum) were more abundant in buffaloes that received TMR whilst *Selenomonas*, *Prevotellaceae-UCG-007*, *Quinella*, *Oscillospira* and *Tyzzerella* (all *Bacillota* phylum) were more abundant in buffaloes that received TMR + green feed (Supplementary Table S4).

The microbial diversity of the two groups was further characterised by analysing alpha and beta diversity. Alpha diversity was analysed using the richness and Simpson indices, the former taking into account all sequences obtained and the latter focusing only on the most abundant ones. A higher number of species and greater species diversity were observed in the buffaloes that received TMR + green feed (Figure 1(A)). However, when the analysis focused only on the most abundant sequences, the results were reversed, and a higher number of species were found in buffaloes that received TMR (Figure 1(B)). Taken together, these results could be interpreted to suggest (1) buffaloes that received TMR + green feed had a greater biodiversity of rumen microbial composition than buffaloes that received TMR and (2) the greater number of species found in buffaloes that received TMR + green feed was due to rare species. This was supported by analysis of beta diversity by the nMDS-Jacard index. The unweighted analysis showed that the samples of buffaloes that received TMR + green feed (green symbols in Figure 2(A)) were scattered in a larger area than samples of buffaloes that received TMR (yellow symbols in Figure 2(A)). This indicated a greater species diversity in buffaloes that received TMR + green feed. The weighted nMDS analysis, where the most abundant ASV are given more weight, showed that the samples of the two groups were similarly distributed, indicating that the clusters observed in the unweighted analysis were mostly due to rare species (Figure 2(B)).

Metagenomic analysis of the rumen microbiota

Total DNA from rumen samples was also used for shotgun metagenome sequencing at the IMR (https://imr.bio). Microbial community metagenomes were generated using the Illumina Nextera Flex Kit for MiSeq + NextSeq and we used a 2× depth (~8 M PE reads). Metagenomic data were used to assess the presence of archaea and viral DNA. Significantly fewer archaeal and viral sequences were found compared to bacterial sequences. Members of the phylum *Euryarchaeota* accounted for 2.5 ± 1.3% (TMR) and 2.4 ± 1.0% (TMR + green feed) of the total rumen microbiota. At the genus level, the abundance of *Methanobrevibacter* did not differ between the two groups (TMR 1.80%; TMR + green feed 1.74%). The total amount of viral DNA in rumen samples was very low and similar between the two groups (TMR 0.26%; TMR + green feed 0.22%).

Enzymes of the transferases (EC2) and hydrolases (EC3) classes were more abundant in both groups and did not differ (Figure 3). Analysis of functional alpha diversity showed the highest Simpson's diversity index for buffaloes that received TMR + green feed (Figure 4). This was supported by an nMDS analysis based on weighted Jaccard similarity, which showed that samples for buffaloes that received TMR + green feed (green symbols in Figure 5) were scattered in a larger area than samples for buffaloes that received TMR (yellow symbols in Figure 5).

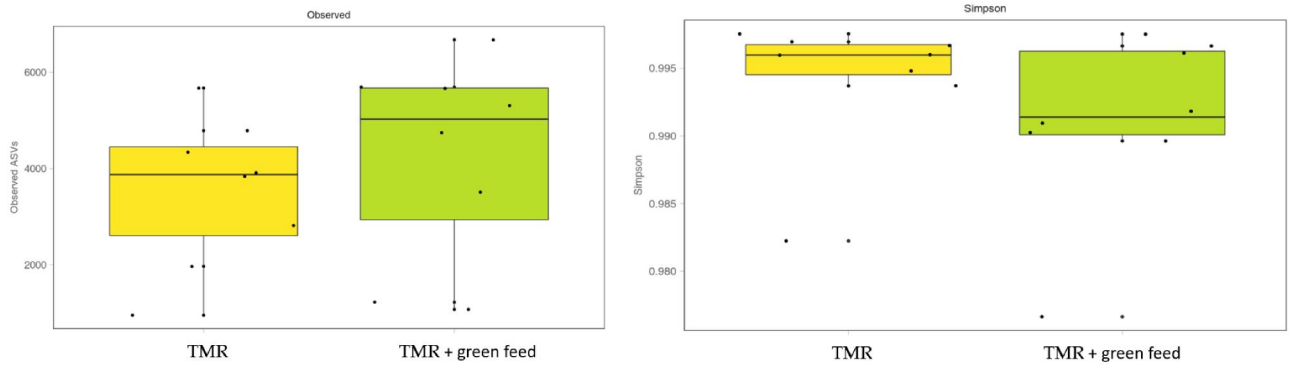

Figure 1. Alpha diversity between the two diet groups. (A) Observed number of ASVs (simply the number of ASVs present in the sample) and (B) Simpson diversity index (taking into account the number of taxa as well as the abundance, thus downplaying the role of rare species). Based on this, the total mixed ration (TMR) + green diet has a larger number of rare species compared to the TMR diet.

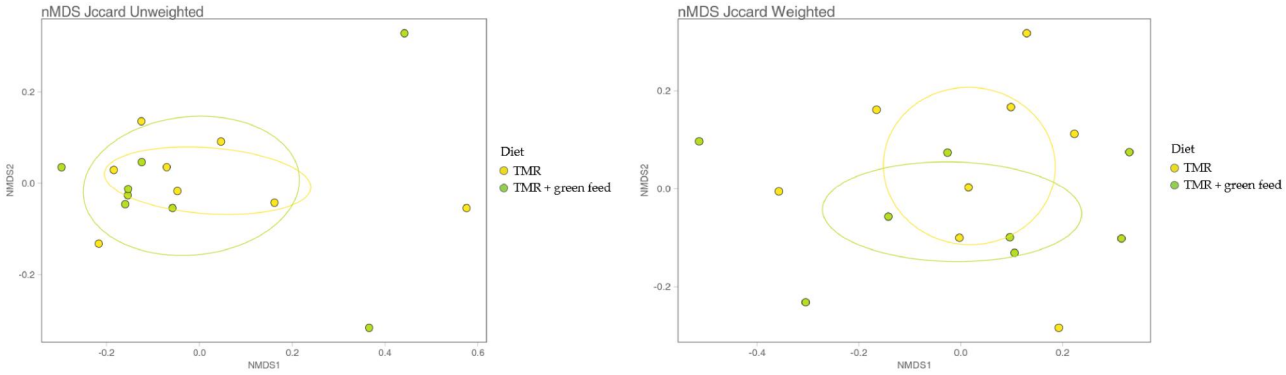

Figure 2. Ordination (nMDS) based on weighted (top) and unweighted (bottom) Jaccard similarity coloured according to the diet group. Ellipses correspond to the 95% confidence interval of the barycentre for the group.

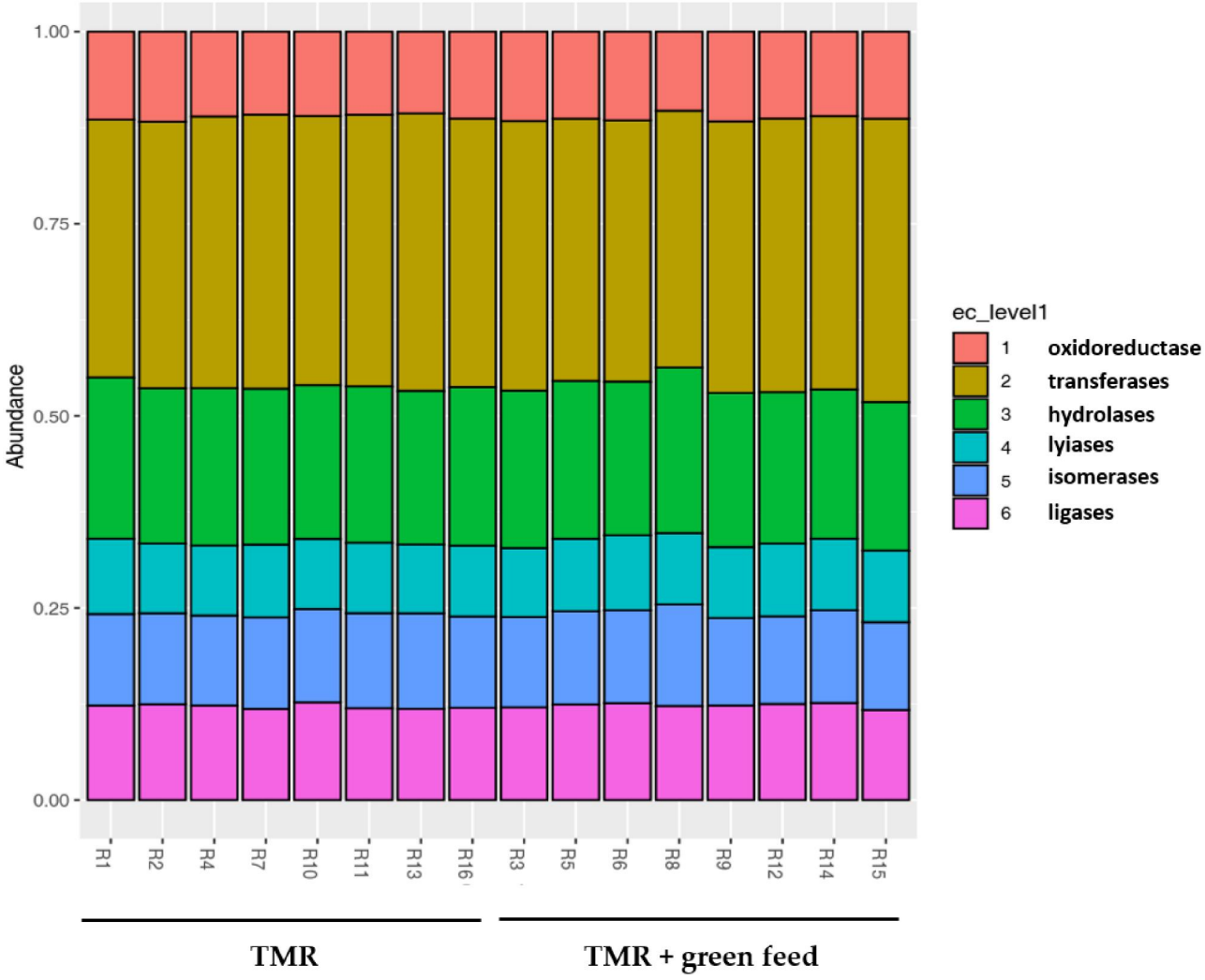

Figure 3. Enzyme code (EC) class distributions in the buffalo group fed with standard total mixed ratio (TMR) or with TMR + ryegrass green forage.

Metagenomic analysis of carbohydrate-active enzymes

Members of all six classes of CAZymes (GHs, GTs, CBMs, CEs, PLs and AAs) were identified, with classes GH and GT being the most abundant in both groups (Supplementary Table S5, summarised in Table 3). Buffaloes that received TMR + green feed had a lower average number of CAZymes of the classes GHs, CBMs and CEs, and an increased number of the classes GTs and AAs (Table 3). Analysis of the most abundant GH

Table 3. Distribution of CAZymes in the rumen of buffaloes that received a total mixed ration (TMR group) and buffaloes that received TMR + fresh ryegrass (approximately 30% of the diet; TMR + green feed group).

	TMR		TMR + green feed	
	Average number/animal	%	Average number/animal	%
GH: glycoside hydrolases	328.37	84.39	251.42	82.47
GT: glycosyl transferases	33.25	8.54	32.00	10.50
CBM: carbohydrate binding modules	1.37	0.35	0.71	0.23
CE: carbohydrate esterases	20.87	5.36	14.25	4.69
PL: polysaccharide lyases	3.25	0.84	2.71	0.89
AA: auxiliary activities	2.00	0.51	3.71	1.22
Total CAZymes	389.11		304.80	

and GT families showed that enzymes belonging to 73 GH and 26 GT families were present, with seven GH and seven GT families present only in buffaloes that received TMR and six GH and 1 GT families present only in buffaloes that received TMR + green feed (Supplementary Table S5, summarised in Table 4). The most abundant GH families (GH2, GH13 and GH30) were all more abundant in buffaloes that received TMR (Figure 6(A)). Families with GH10, GH35, GH54, GH67, GH95, GH104, GH105, GH115 and GH133 more abundant in buffaloes that received TMR + green feed (Figure 6(B)). Analysis of the GT family showed that GT4, GT14, GT20, GT26 and GT39 were more abundant in buffaloes that received TMR + green feed and GT5, GT10, GT30, GT83 and GT112 were more abundant in buffaloes that received TMR (Figure 7). The GT families that were more abundant in the Green group are also produced by some of the genera detected as more abundant in the Green group (Table 5). Some of the remaining GH and GT families were present in the rumen of the two 3p experimental groups to different extents.

Discussion

The present study tested the hypothesis that green feed induces changes in the ruminal microbiota and CAZymes which results in enhanced milk quality in buffaloes. In support of the hypothesis, the inclusion of green feed in the diet of buffaloes favoured subpopulations of bacteria that produced a distinct subset of CAZymes. Also, whole milk of buffaloes that received TMR + green feed had higher amounts of L-carnitine, propionyl-L-carnitine, acetyl-L-carnitine and δ-valerobetaine (Salzano et al. 2021; Neglia, Cotticelli, et al. 2023). These functional biomolecules have health-promoting properties and *in vitro* suppress human cancer cell lines (D'Onofrio, Cacciola, et al. 2020; D'Onofrio, Mele, et al. 2020; Cacciola et al. 2022).

Buffaloes that received TMR + green feed had a higher representation of bacteria belonging to the orders *Veillonellales*, *Selenomonadales* and *Bradymonadales*. *Veillonellaceae* ferment lactate to acetate and propionate which is related to fatty acid metabolism (Zeng et al. 2019). At the genus level, *Quinella*, *Selenomonas*, *Prevotellaceae-UCG-007*, *Oscillospira* and *Tyzzerella* were more abundant in buffaloes that received TMR + green feed. Members of the genus *Prevotella*, and other genera of the family *Prevotellaceae*, are dominant rumen bacteria and show changes in response to changing diets (Paradiso et al. 2021; Rabee et al. 2022; Yi et al. 2022). In weaned calves, *Succiniclastum*, *Selenomonas 1* and the *Prevotellaceae YAB2003* group were positively correlated with total short chain fatty acids and propionic, valeric, acetic and butyric acids, and acetate and butyrate concentrations in the rumen (Hartinger et al. 2022). In the present study, the proportion of highly fermentable carbohydrates/sugars was higher in buffaloes that received TMR + green feed consistent with a greater prevalence of *Prevotella*. Buffaloes that received the TMR diet had a greater abundance of bacteria in the orders *Peptostreptococcales* and *Tissierellales*. *Peptostreptococcales* ferment butyrate, which is used by cells of the rumen wall as an energetic substrate with the formation of β-hydroxybutyrate (Palakawong Na Ayudthaya et al. 2018; Gharechahi et al. 2021). A diet high in concentrates increased acetate and butyrate in the rumen (Sinha et al. 2017) and ruminal fluid *in vitro* in buffaloes (Neglia, Calabrò, et al. 2023).

The capacity of the rumen to convert feed into fermentable sugars is dependent on polysaccharide hydrolysing enzymes, CAZymes, that are produced by rumen microbes. In the present study, differences in rumen bacteria subpopulations between buffaloes that received TMR and TMR + green feed were associated with different ruminal profiles of CAZymes. The total CAZymes were higher in buffaloes that received TMR compared to the TMR + green feed group. In TMR buffaloes, a greater abundance of *Prevotellaceae* was associated with a higher percentage of CAZymes GH, CBM, CE and PL (Wang et al. 2019). Buffaloes that

Table 4. Glycoside hydrolases (GH) and Glycosyl transferases (GT) families present only in one group.

TMR		TMR + green feed	
GH	Activities in the family	GH	Activities in the family
GH4	Maltose-6-phosphate glucosidase (EC 3.2.1.122); α-glucosidase (EC 3.2.1.20); α-galactosidase (EC 3.2.1.22); 6-phospho-β-glucosidase (EC 3.2.1.86); α-glucuronidase (EC 3.2.1.139); α-galacturonase (EC 3.2.1.67); palatinase (EC 3.2.1.-)	GH11	Endo-β-1,4-xylanase (EC 3.2.1.8); exo-1,4-β-xylosidase (EC 3.2.1.-)
GH50	β-Agarase (EC 3.2.1.81)	GH63	Processing α-glucosidase (EC 3.2.1.106); α-1,3-glucosidase (EC 3.2.1.84); α-glucosidase (EC 3.2.1.20); mannosylglycerate α-mannosidase/ mannosylglycerate hydrolase (EC 3.2.1.170); glucosylglycerate hydrolase (EC 3.2.1.208)
GH76	α-1,6-Mannanase (EC 3.2.1.101); α-glucosidase (EC 3.2.1.20)	GH65	α,α-Trehalase (EC 3.2.1.28); maltose phosphorylase (EC 2.4.1.8); trehalose phosphorylase (EC 2.4.1.64); kojibiose phosphorylase (EC 2.4.1.230); trehalose-6-phosphate phosphorylase (EC 2.4.1.216); nigerose phosphorylase (EC 2.4.1.279); 3-O-α-glucopyranosyl-L-rhamnose phosphorylase (EC 2.4.1.282); 1,2-α-glucosylglycerol phosphorylase (EC 2.4.1.332); α-glucosyl-1,2-β-galactosyl-L-hydroxylysine α-glucosidase (EC 3.2.1.107); 1,3-α-oligoglucan phosphorylase (EC 2.4.1.334); α-1,2-glucosidase (EC 3.2.1.-); α-glucan phosphorylase (EC 2.4.1.-); kojibiose glucohydrolase (α-1,2-glucosidase) (configuration inverting) (EC 3.2.1.216); branched-dextran exo-1,2-α-glucosidase (EC 3.2.1.115)
GH141	α-L-Fucosidase (EC 3.2.1.51); xylanase (EC 3.2.1.8)	GH85	Endo-β-N-acetylglucosaminidase (EC 3.2.1.96)
GH144	Endo-β-1,2-glucanase (EC 3.2.1.71); β-1,2-glucooligosaccharide sophorohydrolase (EC 3.2.1.214)	GH89	α-N-Acetylglucosaminidase (EC 3.2.1.50)
GH158	Endo-β-1,3-glucanase (EC 3.2.1.39)	GH112	Lacto-N-biose phosphorylase or galacto-N-biose phosphorylase (EC 2.4.1.211); D-Galactosyl-β-1,4-L-rhamnose phosphorylase (EC 2.4.1.247)
GH 159	β-D-Galactofuranosidase (EC 3.2.1.146); α-L-arabinofuranosidase (EC 3.2.1.55)		
GT	Activities in the family	GT	Activities in the family
GT11	GDP-L-Fuc: galactoside α-1,2-L-fucosyltransferase (EC 2.4.1.69); GDP-L-Fuc: β-LacNac α-1,3-L-fucosyltransferase (EC 2.4.1.-)	GT56	TDP-Fuc4NAc: lipid II Fuc4NAc transferase (EC 2.4.1.-)
GT66	Dolichyl-diphosphooligosaccharide – protein glycotransferase (EC 2.4.99.18); undecaprenyl-diphosphooligosaccharide – protein glycotransferase (EC 2.4.99.19)		
GT84	Cyclic β-1,2-glucan synthase (EC 2.4.1.-)		
GT102	dTDP-β-L-Rhap: O-antigen-polysaccharide α-1,3-L-rhamnosyltransferase (EC 2.4.1.289)		
GT108	GDP-α-D-Manp: β-1,2-D-mannosyltransferase (EC 2.4.1.374)		
GT111	UDP-Galf: β-1,3-galactofuranosyltransferase (EC 2.4.1.-)		
GT113	Glucosyltransferase (2.4.1.-); hexosyltransferase (2.4.1.-)		

received TMR + green feed had a higher percentage of CAZymes GT and AA. The GH CAZymes, together with CE and PL have a complex process of lignocellulose breakdown and can degrade complex sugars (Lombard et al. 2014) whilst CBMs can enhance the catalytic efficiency of enzymes by specifically binding polysaccharides and improving enzyme concentration (Jones et al. 2018).

In both cattle and buffaloes, a change from green to dry roughage was associated with an increase in GH CAZymes (Bohra et al. 2019). In the present study, buffaloes that received TMR had a greater amount of GH CAZymes compared to the TMR + green feed group.

Buffaloes that received TMR + green feed had a greater abundance of CAZymes of the GT class (GHT4, GT14, GT20, GT26, GT39) and AA class (AA1, AA3, AA6). The AA CAZymes class is most abundant in wood degrading fungi and have lignocellulose degradation and antioxidant activity (Sützl et al. 2018). The

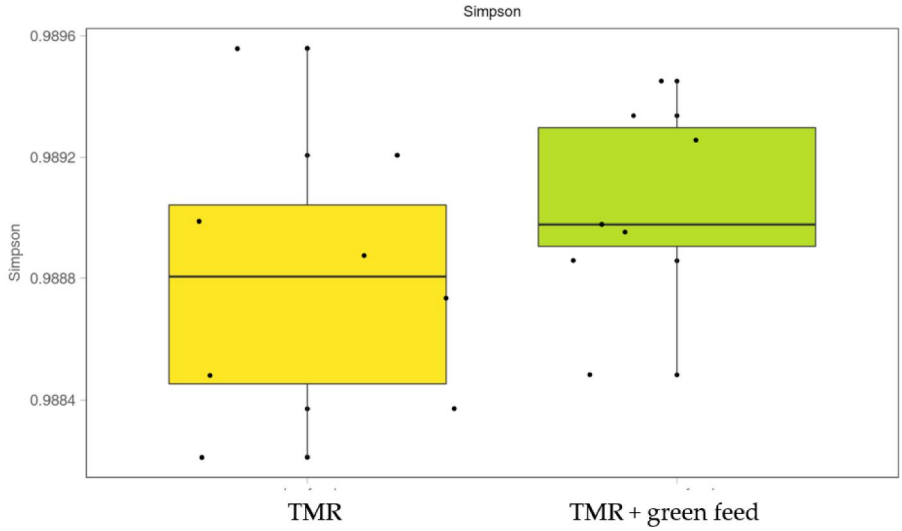

Figure 4. Functional alpha diversity (Simpson) based on the shotgun metagenome functional read assignment for the two diet groups.

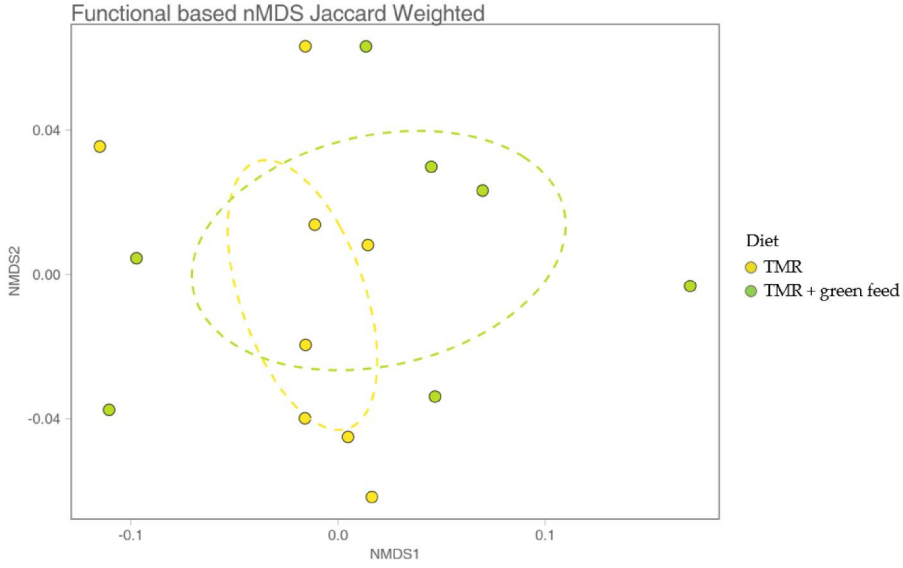

Figure 5. Functional based nMDS based on weighted Jaccard similarity among the shotgun metagenome functional read assignment for the two diet groups.

latter finding could explain the higher antioxidant activity in whole milk of buffaloes that received TMR + green feed in the present study. A higher expression of genes linked to oxidative stress and cellular responses in buffalo ruminal cells *in vitro* correlated with a greater abundance of bacteria that produce GT4 and G26 (*Selenomonas*, *Prevotella*, *Oscillospiraceae*); GT14 and GT20 (*Prevotella*); GT30 (*Selenomonas*, uncultured *Prevotella*); GT39 (*Oscillospiraceae*). The GT CAZymes are involved in the biosynthesis of glycosidic bonds from phospho-activated sugar donors (Coutinho and Henrissat 1999; Benson et al. 2004; Yip and Withers 2006). The specificity of GT CAZymes residues in their ability to selectively modify the correct hydroxyl group on an acceptor containing many equally reactive hydroxyls (e.g. complex oligosaccharides) (Varki 2017). As many bacteria synthesise polysaccharides that mimic human glycosylation, bacterial GT CAZymes can be used for the synthesis of glycoproteins with eukaryotic glycosylation patterns (Varki 2017).

Diet clearly determines the profile of CAZymes in ruminants. The CAZymes profile in buffaloes that received TMR + green feed in the present study could partly explain the ruminal wall cellular transcriptome of buffaloes that received green feed (Salzano et al. 2023). The latter would influence the metabolome of ruminal fluid (Neglia, Cotticelli, et al. 2023) which, in

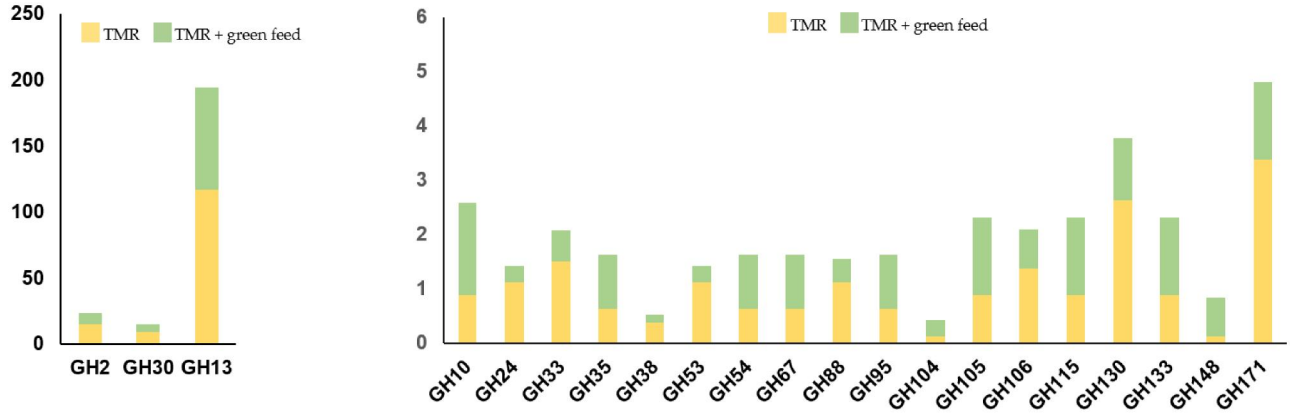

Figure 6. The most abundant Glycoside hydrolases (GH) families in the buffalo group fed with standard total mixed ration (TMR) or with TMR + ryegrass green forage (A). GH families whose abundances show a clear difference between the two experimental groups (B).

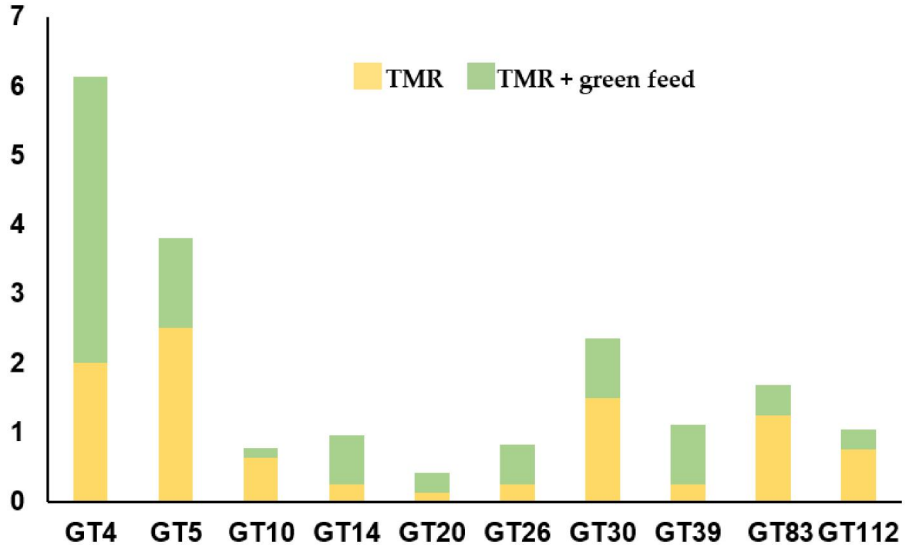

Figure 7. Abundance of the Glycosyl transferases (GT) families in the buffalo group fed with standard total mixed ration (TMR) or with TMR + ryegrass green forage.

Table 5. Genera detected in total mixed ration (TMR) + green feed group rumen samples that encoded the more abundant Gglycosyl transferases (GT) in TMR + green feed group.

GT	Genus
GT4	*Selenomonas, Prevotella, Oscillospiraceae*
GT14	*Prevotella*
GT20	*Prevotella*
GT26	*Selenomonas, Prevotella, Oscillospiraceae*
GT30	*Selenomonas, uncultured Prevotella*
GT39	*Oscillospiraceae*
GT56	–

turn, would influence systemic and milk metabolites in buffaloes fed green forage (Salzano et al. 2021, 2022). This pathway of CAZymes influencing cellular processes would be considered analogous to sugar nucleotide-dependent glycosyltransferases (UGT enzymes) and glycosylation pathways in human cells (Audet-Delage et al. 2022). Nε-trimethyllysine (TML) is the main precursor (Servillo et al. 2014) of the functional biomolecules observed in buffaloes provided with green forage (Salzano et al. 2021, 2022). This precursor is derived by lysine methylation (Lee et al. 2004) which most likely occurs in ruminal wall cells. Leafy vegetables, such as ryegrass, contain relatively high amounts of TML, which participates in carnitine biosynthesis (Servillo et al. 2014). Moreover, they are also known to produce favourable nutrient profiles in cattle in terms of gross composition, macroelements and trace elements (Callahan et al. 2016; Gulati et al. 2018). The higher amount of TML in the rumen of animals fed green forage may hence explain the production of milk with higher nutritional profile.

Overall, the findings in the present study, combined with our previous reports noted above, show that buffaloes fed green forage have higher expression of genes involved in amino acid metabolism. The present findings provide strong support for the proposed metabolic

and biosynthetic pathways that link green forage with rumen bacteria, CAZymes and the synthesis of amino acids and biomolecule in buffaloes. This information can inform the development of diets incorporating green forage that achieve good animal health and well-being whilst producing high amounts of functional biomolecules in milk, for both buffaloes and cattle.

Conclusions

Our analyses indicate that whilst the overall microbial composition of the rumen is not drastically affected by the experimental diet, it increases the microbial biodiversity with an increased abundance of low represented bacterial genera. The inclusion of green feed in the diet of dairy buffaloes favours ruminal microbiota that produce CAZymes that support the synthesis of functional biomolecules. The latter enhances the health-promoting properties of whole milk. The findings provide incentive to further refine feeding strategies that meet consumer preference for food products sourced from animals fed natural diets. The approach adopted in the present study for buffaloes should also be applicable to dairy cattle.

Ethical approval

All experimental procedures were approved by the Ethical Animal Care and Use Committee of the University of Naples "Federico II" (Protocol No. 25532-2022). The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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