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Enhancing Total and Cellulolytic Microbiome Activity of Farm Animals by Fermented Forage

Amr M. A. Elmasry[1]*; Asmaa M. Ebrahim [2]; Adel Elsayed Elbeltagy[1]; Sameh F. Fahim[1]; Usama A. Nayel [3] and Ali Abdelmoteleb[1]

[1]Agri. Microbiology, Botany Dept., Fac. Agri., Menoufia Univ., Egypt.
[2]Azz-Lab., Shibin Elkom, Menoufia, Egypt.
[3]Animal production Dept., Fac. Agri., Menoufia Univ., Egypt.
*E.mail: amr.elmasri@agr.menofia.edu.eg

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ABSTRACT

This study investigates the impact of dietary intervention on the cellulolytic microbiome in the rumen and dung of sheep, aiming to improve nutrient utilization and promote animal health. Ruminant digestion depends heavily on complex microbial communities capable of breaking down fibrous plant materials. The experiment involved rumen and dung samples taken from Rahmani sheep. The rumen liquor and dung samples were collected and analyzed to quantify total bacterial and cellulolytic populations in adapted sheep-lambs. Isolation and characterization of aerobic and anaerobic bacteria were conducted using culture-based techniques on selective media. Quantitative real-time PCR (qRT-PCR) targeting 16S rRNA and GH5 cellulase genes was employed to estimate microbial abundance before and after dietary intervention. The results revealed significant increases in total and cellulolytic bacterial populations in both rumen and dung samples following the dietary change. Total bacterial copies in rumen samples rose from 5.5×10^{10} to 6.5×10^{11}, while cellulolytic bacterial copies increased from 2.7×10^{8} to 1.03×10^{10}. Dung samples showed parallel increases. These findings confirm that dietary composition strongly influences microbial diversity and abundance in ruminants. Notably, genera such as *Ruminococcus*, *Fibrobacter*, *Bacillus*, and *Lactobacillus* were prevalent and contributed to enhance fiber degradation. This work underscores the potential of microbial fermentation-based diets to stimulate cellulolytic microbiota, improving feed efficiency and animal productivity. It also highlights the value of molecular tools for monitoring microbial dynamics in response to nutritional strategies. Future studies should explore functional outcomes, including fermentation profiles and animal biological functions and performance metrics.

INTRODUCTION

Ruminant microorganisms have a close relationship with their host's digestive tract. These microorganisms include diver communities; bacteria, archaea, fungi, viruses, and protozoa (Rey *et al.,* 2014), which can provide their hosts many benefits. For example, the rumen microorganisms are able to convert the indigestible structural plant polysaccharides into available compounds for animal hosts to help them acquire enough energy and promote animal growth (Mackie, 2002).

This ecological process is also very important to human beings because it can convert solar energy stored in plant materials into available food, such as meat and milk. Thus, understanding the composition and function of rumen microbiome is pivotal for improving the productivity of ruminant digestive system. While conventional feed often fail to maximize nutrient availability, particularly in plant-based ingredients that contain hard digestible fibres (Franco *et al.,* 2022)**.**

During the infancy of ruminants, the colonization of aerobic and facultative anaerobic microorganisms is observed (Minato *et al.,* 1992; Van Soest, 1994). With the animal's maturation, foreign anaerobic microorganisms from animal's parents and their surrounding environment gradually replace those aerobic and facultative anaerobic taxa (Jami *et al.,* 2013). For example, the anaerobic cellulolytic bacteria (*Ruminococcus* and *Fibrobacter*) in the sheep had higher abundance in the new-born and maintained a relatively constant level in the adult sheep (Mackie, 2002). At the sub-adult or adulthood stages of the animal, the rumen microbiome ferment food, especially those indigestible plant cellulose to produce short chain fatty acids, microbial cell protein, ammonia and other unknown metabolites (Rey *et al.,* 2014). Although, the relationship between rumen microbiota and metabolites remains largely unknown.

The diet composition has an important role in ruminant's microbiome diversity and abundance. Recently, in ruminant farms, common strategy of diet is applicable by increasing the proportion of rapidly fermentable carbohydrates for improving ruminants' performance. However, the rapid ruminal digestion of starch in the rumen also increases the risk of subacute rumen acidosis (SARA) (Haddad & Nasr, 2007), especially for high-yielding cows, goats and beef cattle (Albornoz *et al.,* 2020; Liu *et al.,* 2020). The fiber and starch content of forage grain is 62.6% dry matter, which is currently one main source of reduced sugars in ruminant diets. However, combining protein source could improve the efficiency of fiber and starch digestion by balancing the fermentation in the rumen or small intestine (Johnson *et al.,* 2020; Ma *et al.,* 2022; Petri *et al.,* 2019).

A global comparison study of the rumen microbiome in 742 samples from 32 animal species in 35 countries concluded that, while a common core of bacteria and archaea dominated in nearly all samples, differences in microbial community compositions were predominantly attributable to diet (Henderson *et al.,* 2015). Given the effect of diet on the rumen microbiome, it is perhaps not surprising that a wide range of dietary additives have been used to manipulate rumen fermentation. The main targets for rumen manipulation are to increasing microbial degradation of fibre, decreasing protein degradation and ammonia production in the rumen, optimizing VFAs production, improving animal health by preventing the accumulation of harmful intermediates of fermentation in the rumen and maximizing the degradation of dietary toxins, decreasing greenhouse gas production and improving human health by improving the nutritional composition of ruminant products, predominantly lipid and fatty acid content/composition and preventing pathogen transfer in the food supply chain (Newbold & Ramos-Morales, 2020). The current study aims to manipulate the microbiome formation of young lambs by feeding them fermented forages supplemented with different microbes, including probiotic bacteria previously isolated from sheep rumen and dung, and microbial commercial additives (*Lactobacillus plantarum* and *Saccharomyces cerevisiae*). Then, assess the impact of these interventions on the total bacteria and cellulolytic populations present in the lambs' rumen and dung.

MATERIALS AND METHODS

1- Rumen and Dung Samples Collection:

The rumen liquor samples were collected from six full healthy Rahmani sheep aged 7-8 months (weighed 36.6 ± 2.4 kg) from sheep barn with normal feed. About 10 ml of rumen samples were gathered every time by stomach tube in sterile bags and the fresh dung dropped in plastic tablecloth was collected according to the method described by Babayemi & Bamikole (2006). Samples were transferred immediately to the microbiology lab, faculty of agriculture, Menoufia University, Egypt. Fresh samples were used as starter inocula or source for isolation of specific microorganisms by streaking on specific culture media supplemented with filtrated rumen, while the cellulolytic bacteria was isolated in carboxymethyl cellulose (CMC) agar with orange die. All plates were divided into two similar groups, one of them incubated aerobically and the other anaerobically in anaerobic incubator (Bio-Rad) at 37 ± 2 °C for 24 hours according to (Deli *et al.,* 2022).

While, The growth media contained 15 ml mineral solution I (KH_2PO_4 3.0 g; $(NH_4)_2SO_4$ 6.0 g; NaCl 6.0 g; $MgSO_4$ 0.6 g; $CaCl_2 \cdot 2H_2O$ 0.795 g per/liter), 15 ml mineral solution II (K_2HPO_4 3 g/liter), 0.25 g yeast extract, 1 g tryptone, 0.1 ml resazurin (0.1%), 0.5 g microcrystalline cellulose, 0.1 g cellobiose, 0.4 g sodium carbonate, 20 ml clear rumen liquor, 50 ml distilled water , the final pH was 7.2 and sterilized by autoclaving at 110 °C for 30 minutes (Babayemi & Bamikole, 2006).

All single colonies appeared on CMC media were picked up, purified, re-incubated on 37 °C and kept after growth at 5 °C for further studies. While for cultures preservation, nutrient agar slant was inoculated with the purified isolated bacteria and incubated at 37 °C for 24 hours, and then 15% glycerol was added in the top of culture tubes which were kept at -20 °C until use**.** The morphological and biochemical characteristics using Enterobacteriaceae diagnostic kits, catalase, and oxidase enzyme tests, as well as proteolytic and cellulosic hydrolysis were used to identify different genera (Deli *et al.,* 2022).

2. Feedstuffs Composition and Forage Analysis:

A control forage (14% crude protein, 4.5% crude fiber and crude lipids 4%), similar to commercial forage, was formulated with practical ingredients containing unfermented forage. The test composition was formulated similar to the control forage but included 25% complete fermented forage. Feedstuffs were non extreme heat dried for 48 hours at 40 °C, sieved and stored at sealed bags until usage. Ingredient composition and proximate analyses of the experimental feedstuffs are mainly determined by Nearest Infrared (NIR) system as following methods of AOAC, 2005 (AOAC, 2005).

3. Experimental Feedstuffs and Fermentation Protocols:

In previous study, a feedstuff mixture of 25% of fermented forage composed from soybean Meal (SBM) (a common protein source for ruminants 34%), wheat bran (WB) (a fibrous material used as a carbohydrate source 64%) and other potentially incorporates other ingredients (2%) such as activated charcoal was the effective fermented rate for animal feeding. Weigh each ingredient based on the desired formulation. Feedstuff was solid state fermented with individuals or mixed isolates microorganisms formula or obtained strains of *Lactobacillus plantarum* and *Saccharomyces cerevisiae* from Angela company, China. Solid-state fermentation (SSF) with rumen liquor inoculant, the individual aerobic proteolytic and cellulolytic bacteria (wild type cultivars) and the superior isolates bacteria mixed with rumen liquor inoculant led to high production of carbohydrates and total protein, increased *in vitro* protein digestibility with a reduction of fiber content. Additionally, complete SSF with this bacterial species was chosen for diet

production was fermented with individual *Lactobacillus planetarium* and/or *Saccharomyces cerevisiae* for modified the diet composition (Chebaibi *et al.,* 2019).

For that purpose, 400 g of various substrate was autoclaved (121 °C, 15 min) and inoculated with 80 mL of bacterial solution (0.1% peptone) at a concentration of 1.5 $x10^7$ CFU/g the humidity was adjusted to 60% (w/w) and fermentation was done at 25 °C for 72 hours or at 35 °C for 48 hours. To ensure oxygenation of the substrate it was mixed at least four times every day for the duration of the fermentation according to the protocol described by Vandenberghe *et al.* (2021).

The sheep trials were carried out in Rahmani included the control, the sheep breeding separated in the barn of the faculty of agriculture farm, Menoufia University, Egypt. The end of the quarantine, the trips were transferred to the experimental system and maintained. The ages of sheep-lambs were approximately 1.5 months and weighed average was 9.2 ± 1.7 kg. A control diet (commercial forage) and the other mixed by 25 % fermented feedstuffs were feeding for 15 days before assays for adaption and the feeding experiment was additional 15 days with equally hour light/dark cycle. The sheep were exposed to temporary fasting period and allowed few days to adapt to their new environment prior to receiving an experimental feed; after that, the samples and assays were every one week in the middle and the end of experimental feeds (Babayemi & Bamikole, 2006).

4. Rumen and Dung Microbiome DNA Extraction:

The major samples from the digestive tract of adaptive domestic sheep-lambs were obtained from the rumen and the dung in triplicate, samples recollected another time after feeding experiments. All samples were collected in 100 mL specimen bottle sterile. Total DNA was extracted from the rumen and dung at 4 °C. 10 ml sterilized distilled water was added to 10 g of sample prior to homogenization. Samples were then centrifuged (5000 rpm, 30 min, 4 °C) to sediment microbes and plant residues, and the supernatant was discarded. The pellet was used for total DNA extraction. Total genomic DNA was isolated from rumen fluid and dung by using Qiagen stool kit the easy DNA Kit (Invitrogen) according to the manufacturer's instructions. DNA concentrations were estimated using Nanodrop spectrophotometer by the absorbance at 260 nm and the DNA quality was checked on 0.7% (wt/v) agarose gel. Total DNA extraction was performed in three replicates according to Yu & Morrison (2004).

5. Real-time PCR Protocol for Total and Cellulolytic Microbiome:

Specific PCR primers used for the amplification of target region of the 16S rRNA (target DNA) and cellulolytic bacteria from *Ruminococcus albus* strain were chosen from the literatures (Table 1) as described previously (Muyzer *et al.,* 1993; Tajima *et al.,* 2001). All qRT-PCR amplification and detection were performed using ABI 7500 system software (ABI 7500, USA). Reaction of final volume of 25 µl was prepared containing 12.5 µl Qiagen DNA Master SYBR Green I, 10 pM of forward primer, 10 pM of reverse primer, 7.5 µl distilled water, and 2.0 µl of DNA solution of unknown concentration. The annealing temperature and amplicon size of each target are shown in Table 1. All PCRs were performed in triplicate. Before starting the RT-PCR assay, conventional PCRs for the validation of the specificity of the primers against target genes were performed in 25 µl using a Minicycler PCR. The PCR products were analyzed by running on 1.5% agarose gels containing ethidium bromide and visualizing for a single specific band and the absence of primer dimmer products.

Plasmid DNA containing the respective target gene sequence from *Ruminococcus albus* strain, used as the standard DNA in RT-PCR, using the specific primer sets. After the confirmation

of a specific amplification of the correct size (Table 1) on agarose gel, the PCR products were excised from the gel and purified using the Qiagen gel purification kit from Qiagen, then ligated to pGEM-T Easy vector system (Promega). The ligation products were transformed to competent *E. coli* JM109. QIAprep spin miniprep kit (Qiagen) was used to extract and purify plasmids from the transformed *E. coli* and the plasmids containing the correct insert were screened by PCR amplification using the respective primers. Plasmids concentrations were measured with a NanoDrop spectrometer.

To determine the copy number of each standard plasmid, this formula was used:

Copy No/µl = Concentration of plasmids (g/µl) × 6.022 × 1023/length of recombinant plasmid (bp) × 660 ……(Singh *et al.,* 2014).

Whereas; 660 = Molecular weight of nucleotide base, 6.022 × 1023 = Avogadro's number.

Ten-fold dilution series ranging from 10^1 to 10^9 copies were prepared for 16S rRNA and cellulase genes, separately. The RT-PCR was performed using Qiagen DNA Master SYBR Green I. Two separate amplifications were conducted for the 10-fold dilution series of the standard plasmids for the respective target genes. The samples amplification was performed in triplicate.

The copy numbers of 16S rRNA genes of all targeted per ml rumen fluid was calculated using the following equation: Log^ (Ct-Intercept/slope)

Where Ct threshold value of the unknown sample concentration, and linear regressions was (r2 = 0.99, intercept were (41.45357 for 16S rRNA standard curve, and 35.11429 for cellulase gene standard curve) and slope (-2.62857 to −3.28214).

Table 1: List of primers used in the qRT-PCR for total bacteria and cellulytic bacteria copies number estimation.

Target bacteria	Primer sequence (5′–3′)	Annealing temp(°C)	Product size	Reference
Total bacteria	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	60	194 bp	(Mosoni *et al.,* 2007 ; Muyzer *et al.,* 1993)
Cellulytic bacteria	GH5F: TWYGARYTIYTIAAYGARC GH5R: NGGRTTRTARWARTGRAA	58	195–258 bp	(Sheng *et al.,* 2015)

While the cloning process applied for PCR amplified bands of 16S rRNA and cellulase amplified genes, which were purified from gel with Zymoclean Gel DNA Recovery Kit (Epigenetics, USA). The PCR products were cloned into pGEM-T Easy Vector (Promega), and the heat shock method was followed to introduce plasmids into *E. coli* JM109 cells as described by the manufacturer's protocol. The transformants were selected on LB agar medium complemented with ampicillin, 5- bromo-4-chloro-3- indolyl-β-D-galactopyranoside (X-Gal) and isopropyl-β- D-thiogalactopyranoside (IPTG) at a concentration of 100, 200 and 200 µg.ml^{-1}, respectively. In LB liquid medium complemented with ampicillin at a final concentration 100 µg.ml^{-1}, the white colonies were picked and grown at 37 °C for 24 hours. To isolate plasmids from the transformed cells, miniPREP Kit (GeneDireX) was used. Plasmids were digested by *Eco*RI (Fermentas) to check the inserts. Then, cloned products were used to

prepare the standard curves for qRT-PCR protocol.

6. Statistical Analysis:

Each experiment was carried out in triplicate and the values averages were recorded with its standard divisions. Probability value for the statistical test was 0.5% was used to compare the differences of the variables. On the other hand, the results were analyzed according to a completely randomized design where treatments were considered as fixed effects, testing linear and quadratic effects of microbial levels (Brandao *et al.,* 2020).

RESULTS AND DISCUSSION

1. Total Rumen and Sheep Dung Counts:

The total aerobic bacterial count in sheep rumen liquor samples ranged from 10^6 - 10^8 CFU/mL with average of 10^7 CFU/mL (colony-forming units per milliliter). The total anaerobic bacterial count in sheep rumen liquor samples were ranged from 10 –30 CFU/mL with average of 20 CFU/mL. This value indicates a significant population of aerobic bacteria, which play a crucial role in rumen fermentation and nutrient metabolism. While the rumen microbiome is a complex ecosystem dominated mainly by anaerobic bacteria, but aerobic and facultative anaerobic bacteria also contribute in microbial activity. The presence of aerobic bacteria in the rumen may be attributed to oxygen ingress during feeding or microbial interactions. The recent studies have reported similar findings by Papakonstantinou *et al.* (2024), who observed that aerobic bacteria, including genera of gram positive *Streptococcus*, *Lactobacillus* and *Bacillus* species are mainly present in the rumen. These bacteria may assist in initial oxygen scavenging, creating a favorable environment for anaerobes. Also, Lu *et al.* (2025) found that aerobic bacterial populations fluctuate with diet, with higher counts in sheep fed high-fiber diets due to increased microbial diversity. In the same concept, Xu *et al.* (2023) highlighted that some aerobic bacteria in the rumen exhibit cellulolytic activities, contributing to fiber degradation. The aerobic bacterial count in this study aligns with previous research, suggesting that these microbes play a supplementary role in rumen function. Further studies using metagenomics could better characterize their metabolic contributions. While anaerobic bacteria dominate the rumen, *Ruminococcus* and *Fibrobacter* bacteria are consistently present and may influence microbial dynamics. Future research should explore their functional roles under different dietary and physiological conditions.

Also, the total aerobic bacterial count in sheep dung samples ranged from 10^8 - 10^{10} CFU/g with average of 10^9 CFU/g, while the total anaerobic bacterial count in sheep dung samples was ranged from 10^8 – 10^{10} CFU/g with average of 10^9 CFU/g. The high density reflects the active microbial ecosystem involved in fermenting organic matter in the gastrointestinal tract. These higher counts (up to 10^{10} CFU/g) were linked to grain-rich diets, while forage-fed sheep showed slightly the dominant genera of culturable isolates including high number of *Enterobacter*, *Escherichia* as gram negative short rods and *Bacillus* and *Lactobacillus* as aerobic gram-positive long rods with presence of essential facultative anaerobic short rods which represent in high proportions. These results in match with the results obtained by Franco *et al*. (2022) who reported total counts between 10^8- 10^{10} CFU/g in sheep dung, noting that aerobic bacteria aid in lignin degradation and nutrient cycling. However, early studies by Xu *et al.* (2023) focused on the typical for herbivores, with dung harboring more facultative anaerobes proteobacteria than strict aerobes. However, Papakonstantinou *et al.* (2024) indicate that fiber-rich diets favor for *Ruminococcus*, *Fibrobacter, Bacillus,* and *Lactobacillus* genera, while, high-protein diets increase the Enterobacteriaceae as factors influencing bacterial load in sheep intestine which later contribute to soil fertility upon

environmental decomposition reservoir with counts comparable to other ruminants. From the data, the most isolated bacterial species were dominant as lactic acid producing bacteria such as *Ruminococcus* and *Fibrobacter Streptococcus* and *Lactobacillus* in rumen liquor which were minor but metabolically active, while the bacterial species in dung were *Bacillus* and Enterobacteriaceae including *E. coli* are mainly dominate in dung due to oxygen exposure with agreed the results reported by Lu *et al.* (2025). In the same concept, the total cellulolytic count in rumen liquor samples under anaerobic and aerobic conditions ranged from 10^2 and 10^3 CFU/mL, respectively. While the count in dung samples ranged from 10^2 and 10^4 CFU/g, respectively. These results were matched with those obtained by Papakonstantinou *et al.* (2024) who found that *Bacillus subtilis* in dung and *Bacillus subtilis* with *Paenibacillus polymyxa* dominate with cellulose and hemicellulose degradation in rumen despite anaerobic *Ruminococcus* and *Fibrobacter* bacteria under this conditions due to micro-oxide zones from dung as reporting by (Deli *et al.,* 2022).

2. Qualification of 16S rRNA and Cellulase Genes:

After amplification of PCR products with 16S rRNA and cellulase (GH5 endoglucanase) primers from the isolated *Ruminococcus albus* strain (Lab stock) (Table 1 and Fig. 1). PCR products were excised from gels and purified. The purified PCR products were cloned into pGEM T Easy vector and transformed into *E.coli* JM109. The plasmids were purified, and PCR amplified with the tested primers to verify the insertion of the desired fragments. The plasmid harbours the 16S rRNA fragment was named pGM300 and the plasmid harbour the Cellulase gene was named pMG400, respectively.

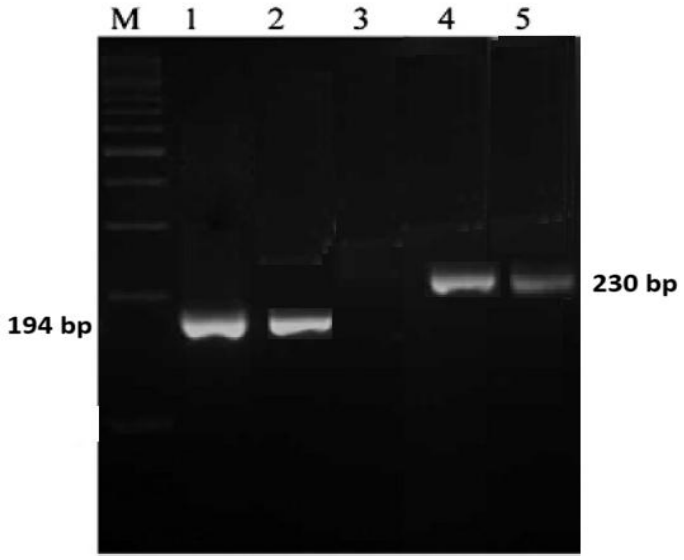

Fig. 1. PCR amplification of 16S rRNA lanes (1, 2) and Cellulase genes lanes (4, 5) from *Ruminococcus albus* for standard curves preparation

3. Standard Curves Setup for qRT-PCR Protocol:

The standard curves of pGM300 and pMG400 plasmids were obtained from qRT-PCR data. The formula of 16S rRNA and cellulase gene standard curves were obtained from excel sheet as shown in Figures 2 and 3. The copy numbers of 16S rRNA and cellulase genes of all targeted per ml rumen fluid were calculated using the following equation: Log^ (Ct-Intercept/slope).

Where Ct threshold value of the unknown sample concentration, and linear

regressions was (r2 = 0.99, intercept were slope and intercept values were obtained from the regression line of qRT-PCR software, which are -3.28214 and 41.45357 for 16S rRNA standard curve and -2.62857 and 35.11429 for the cellulose standard curve, respectively

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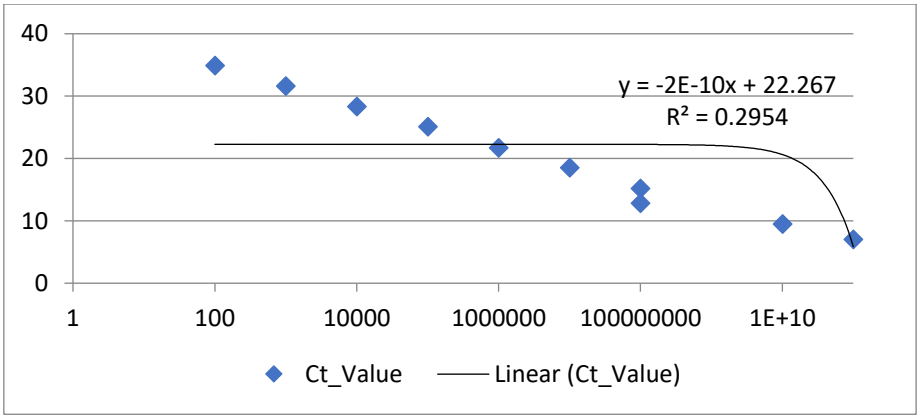

Fig. 2. Standard curve for pGM300 plasmid harbour 16S rRNA gene.

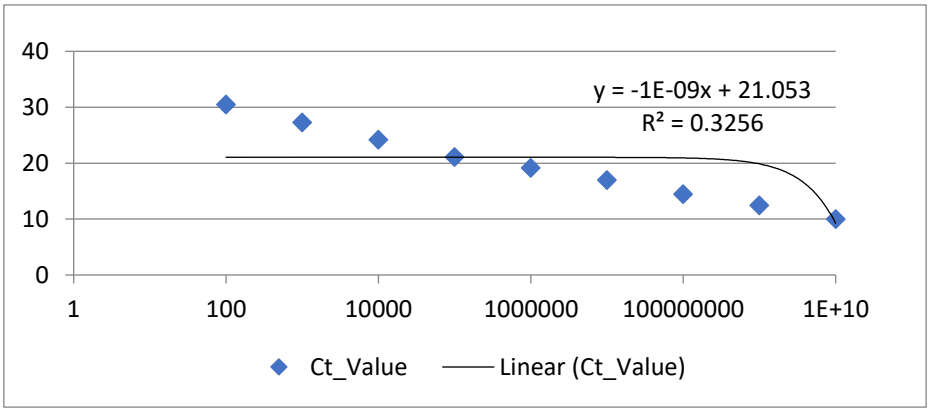

Fig. 3. Standard curve for pGM400 plasmid harbour cellulase gene.

4. Quantification of Total Bacteria and Cellulolytic Bacteria Copies Number:

After total metagenomic DNA extraction from rumen and dung samples before and after the diet, 16S rRNA and Cellulase genes listed in Table 1 were used in qRT-PCR quantification. The RT-PCR technique has the advantage of targeting total bacteria and cellulolytic species and being a very sensitive, accurate and reproducible method, enables underlining of slight changes and allows the differentiation between the population sizes. However, quantifying 16S and cellulolytic copies number does not reflect the real activity of these bacteria in the rumen and dung. Indeed, linking community structure to activity and functionality is a central but still poorly studied issue in microbial ecology.

In this work, we used degenerated primers designed previously by Sheng *et al.* (2015) for the detection of widespread GH5 endoglucanases in many cellulolytic bacteria including *Ruminococcus albus* (Cel5A, Cel5D) and *Fibrobacter succinogenes* (FSU_1077 encodes GH5). GH5 genes differ significantly in sequence between genera, so universal primers are designed from highly conserved internal motifs, but they might miss divergent variants, this led us to use degenerate primers for improving coverage across species.

The present study quantified the total bacterial load and cellulolytic bacterial communities in sheep rumen and dung before and after dietary intervention using quantitative real-time PCR (qRT-PCR).

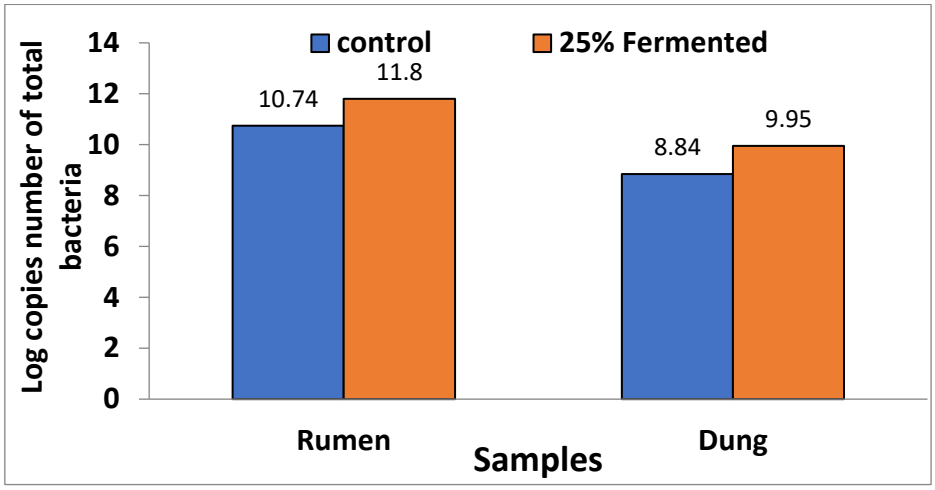

Fig. 4. The log average of the estimated copies number of total bacteria in rumen and dung samples from sheep lambs fed on unfermented (control) and 25% fermented diets.

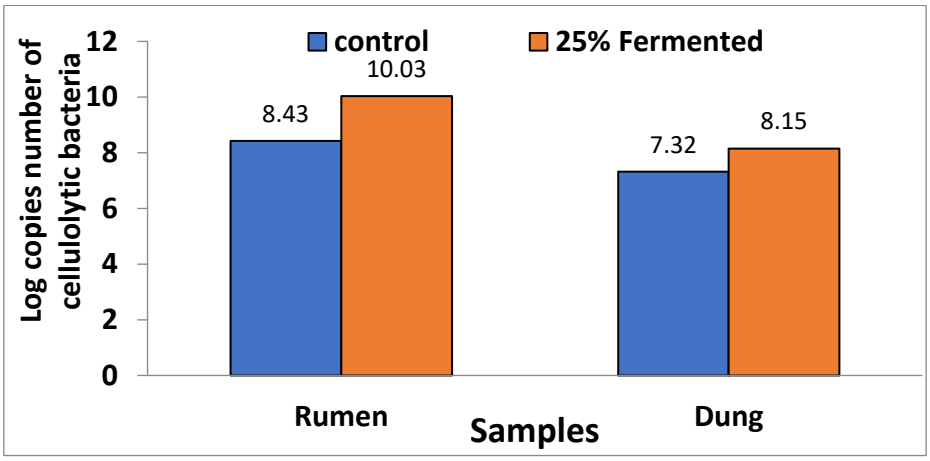

Fig. 5. The log average of the estimated copies number of cellulolytic bacteria in rumen and dung samples from sheep lambs fed on unfermented (control) and 25% fermented diets.

The data revealed a significant increase in both total bacteria and cellulolytic bacteria in response to dietary changes. Specifically, the total bacterial load in rumen samples increased from an average of 5.5×10^{10} copies before the diet to 6.5×10^{11} copies after the diet, with log averages of 10.74 and 11.8, respectively. In the dung samples, the bacterial load rose from 3.48×10^{8} to 9.08×10^{9} copies (log averages of 8.84 to 9.95) (Figs. 4 and 5). These findings are consistent with previous studies that have shown that dietary composition significantly influences the size and diversity of the rumen microbiome (Fernando *et al.,* 2010; Fonty *et al.,* 1983). Fonty *et al.* (1983) reported that shifts in dietary fiber content could increase the abundance of certain bacterial groups, while Fernando *et al.* (2010) found that high-grain diets could rapidly alter rumen bacterial populations. The cellulolytic bacterial community also increased notably after dietary intervention. In the rumen,

cellulolytic bacterial copy numbers rose from 2.7×10^8 (log average 8.43) to 1.03×10^{10} (log average 10.02) copies, while in the dung samples, numbers increased from 2.1×10^7 to 1.42×10^8 copies (Figs. 4 and 5). These observations agree with the findings of Koike & Kobayashi (2009), who demonstrated that fiber-rich diets promote the proliferation of cellulolytic bacteria in ruminant guts, enhancing fiber degradation capacity. Moreover, the parallel trends in bacterial abundance in both rumen and dung samples highlight the influence of rumen microbial activity on downstream gut microbiota. This is in line with previous research by Shanks *et al.* (2011), who showed that the composition of fecal microbiota closely mirrors that of the rumen due to the transit of digest and bacteria through the gastrointestinal tract. Interestingly, while our study showed significant increases in total bacterial and cellulolytic bacterial copy numbers after dietary supplementation, it contrasts with the findings of de Menezes *et al.* (2011), who reported that certain dietary interventions could reduce the relative abundance of fiber-degrading bacteria, depending on the feed composition. This suggests that the effect of dieting on bacterial populations can be variable and may depend on the nature and quality of the dietary components. Overall, the observed increases in total bacterial and cellulolytic populations in this study suggest that dietary intervention provided a suitable substrate for bacterial growth and activity. The enhancement of cellulolytic bacteria is particularly important, as these microbes play a crucial role in fiber digestion and nutrient utilization in ruminants (Morgavi *et al.,* 2013). Future studies could investigate how these microbial shifts impact rumen fermentation parameters and animal productivity.

Conclusion

This study demonstrated that dietary manipulation through fermented forage significantly enhances both the total bacterial and cellulolytic microbial populations in the rumen and dung of sheep. Using a combination of culture-based enumeration and quantitative real-time PCR targeting 16S rRNA and GH5 endoglucanase genes, we observed a marked increase in microbial abundance after dietary intervention. The rise in cellulolytic bacteria particularly *Ruminococcus*, *Fibrobacter* and *Bacillus* underscores their vital role in fiber degradation and nutrient recycling within the ruminant gut ecosystem. Our findings affirm that rumen and fecal microbiomes are not only responsive to dietary inputs but are also tightly linked in their microbial composition. The results highlight the capacity of specific feed formulations to stimulate beneficial microbial groups, particularly those responsible for cellulolytic, which could translate into improved feed efficiency, animal performance, and environmental sustainability. Moreover, the successful application of degenerate primers in targeting diverse cellulolytic genes indicates the potential for broader microbial coverage in future studies. In summary, optimizing sheep diets with fermented forages can promote a more robust and functionally active microbiome, enhancing both digestion and microbial contributions to host productivity. Future research should investigate the functional outputs of these microbial changes, including fermentation products, animal growth metrics, and methane mitigation potential.

Declarations:

Ethical Approval: Not applicable.

Authors Contributions: Amr M.A. Elmasry, Adel Elsayed Elbeltagy, Sameh F. Fahim, **Usama A. Nayel** and **Asmaa M. Ebrahim**. conceptualized the study. Ali Abdelmoteleb, Amr M.A.Elmasry, Sameh F. Fahim, **Usama A. Nayel** and **Asmaa M. Ebrahim** carried out the methodology, data collection, and analysis. Ali Abdelmoteleb, Amr M.A. Elmasry, Sameh F. Fahim, **Usama A. Nayel** and **Asmaa M. Ebrahim** prepared the initial manuscript draft. Ali Abdelmoteleb, Amr M.A.Elmasry and

Asmaa M. Ebrahim reviewed and edited the manuscript. All authors approved the final published version.

Consent for publication: All authors agreed with the content and that all gave explicit consent to submit and that they obtained consent from the responsible authorities at the Agricultural Microbiology and Biotechnology, Botany Department, Faculty of Agriculture, Menoufia University, Shibin El-Kom, Egypt where the work has been carried out, before the work is submitted.

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ARABIC SUMMARY

تعزيز نشاط الميكروبيوم الكلى و المحلل للسليلوز فى الحيوانات المزرعية عن طريق الأعلاف المخمرة

عمرو عبدالعظيم المصري(١)، أسماء محمود أبراهيم (٢)، عادل السيد البلتاجى(١)، سامح فهيم فرج الله (١)، أسامة أبوالعز نايل (٣)، علي عبدالمطلب (١).

(١) الميكروبيولوجيا الزراعية بقسم النبات كلية الزراعة جامعة المنوفية- - .

(٢) معامل عزلاب- شبين الكوم- المنوفية- مصر- -

(٣) قسم الأنتاج الحيواني بكلية الزراعة جامعة المنوفية- - .

تبحث هذه الدراسة في تأثير التدخل الغذائي على الميكروبيوم السليولوزي في كرش وروث الأغنام، بهدف تحسين الاستفادة من العناصر الغذائية وتعزيز صحة الحيوان. يعتمد هضم المجترات بشكل كبير على مجتمعات ميكروبية معقدة قادرة على تكسير المواد النباتية الليفية. شملت التجربة على سلالة أغنام الرحماني البالغة، مع جمع عينات من سائل الكرش والروث وتحليلها لتحديد إجمالي البكتيريا خاصة الميكروبيوم المحلل للسيليلوز من الحملان التي تتغذي على العلف المتخمر. تم عزل وتوصيف البكتيريا الهوائية واللاهوائية باستخدام تقنيات قائمة على الزراعة على بيئات انتقائية. تم استخدام تفاعل البوليميراز المتسلسل الكمي الحقيقي (qRT-PCR) الذي يستهدف جينات 16S rRNA و GH5السيليولوز لتقدير أعداد الميكروبات قبل وبعد التدخل الغذائي. كشفت النتائج عن زيادات كبيرة في إجمالي البكتيريا السليلوزية في كل من عينات الكرش والروث بعد تغيير النظام الغذائي. ارتفع إجمالي النسخ البكتيرية في عينات الكرش من 5.5×10^{10} إلى 6.5×10^{11}، بينما زادت النسخ البكتيرية السليلوزية من 2.7×10^{8} إلى 1.03×10^{10}. وأظهرت عينات الروث زيادات موازية. تؤكد هذه النتائج أن التركيب الغذائي يؤثر بشدة على التنوع الميكروبي ووفرته في المجترات. ومن الجدير بالذكر أن أجناسًا مثل *Ruminococcus* و*Fibrobacter* و*Bacillus* و*Lactobacillus* كانت سائدة وساهمت في تعزيز تحلل الألياف. يؤكد هذا العمل على قدرة الأنظمة الغذائية القائمة على التخمير الميكروبي على تحفيز ميكروبات السليلوز، مما يحسن كفاءة الأعلاف وإنتاجية الحيوانات. كما يُبرز أهمية البروتوكولات الوراثية الجزيئية لرصد الديناميكيات الميكروبية واستجابةً الاستراتيجيات الغذائية. وينبغي أن تستكشف الدراسات المستقبلية النتائج الوظيفية، بما في ذلك عمليات التخمير الميكروبي والتي تعمل على تحسين الوظائف البيولوجية ومقاييس الأداء الحيوانية.