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Comparing the efficiency of rumen bacteria isolates from lambs, cows, and buffalo in producing the enzyme esterase

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Abstract

The aim of this study is to determine the chemo-profile of aqueous extract of *Nauclea latifolia* using GC-MS. The GC-MS analysis of the plant extract was performed by using GC-MS QP-2010 (Shimadzu) and the mass spectra of the compounds found in the extract were matched with the data in the library of the National Institute of Standards and Technology (NIST). The result revealed the presence of eighteen compounds. The most prevailing compounds were 9-Octadecenoic acid (Z) -methyl ester, Oleic acid, and Benzamide 2-bromo-N-[2-(3-fluorophenyl)-5-benzoxazolyl] having a percentage area of 16.93%, 14.01%, and 9.18% respectively. The least abundant compound was 5-Methyl-2-phenylindolizine (0.66%). Some of these phytochemicals' bioactivities include anti-inflammatory, antibacterial, and antimalarial activities. As a result, the leaf extract has the potential to be a source of novel treatments. The results of this investigation thus validate the plant's traditional usage for medicine.

Keywords: Methylene blue test, ferulic acid, reducing sugar.

Introduction

Nutritionists animal feeding facing challenges to reduce the cost of ruminants products, to reach this point they using roughages due to its low cost, low-quality roughages like crops and cereal straws remains an essential strategy in ruminant nutrition in the world (Sultan *et al.*, 2023) [32]. At any rate, the intake and nutritional value of these fibrous feeds were severely constrained due to the difficult structural of the plant cell wall (Beauchemin *et al.*, 2020). As well as, cell wall composite of cellulose and hemicellulose, and lignin, to break this structural strong, research's biotechnological were focused on enzyme supplementation to destroy these cross-links. Different enzymes using and among these enzymes were esterases particularly ferulic acid esterase (FAE, EC 3.1.1.73) (Ribeiro *et al.*, 2022) [29].

Breaking these bridges disrupts the lignocellulosic complex then release cell wall-bound phenolic compounds which help to attack carbohydrates by microbial colonization rapidly and fermentation in the rumen (Azzaz *et al.*, 2021; Wang *et al.*, 2023) [35]. The aim of this study is to compare different isolation and characterization of biosafety-compliant bacterial strains from rumen of ruminants, and evaluation of their efficiency in producing esterase enzyme to enhance fiber digestion and improve feed efficiency.

Material and methods

Sample collection and bacterial isolation

Fresh rumen samples of lambs, cows and buffalo were collected from slaughter house, Iraq. The nature of nutrition's were roughages, concentrate, hard plants and grass. Rumen of slaughtered animals opened in the field and samples were collected by straining through three -layer of cheesecloth in a small container 1000 ml, then covered with 5 ml of oil paraffin to provide anaerobic condition through minimizing the exposure to oxygen then Samples were kept in ice and transferred to the laboratory within 48 h to isolate bacteria (Fruentina 2022).

Serial dilution of each ruminant sample were made, in which one milliliter of sample was diluted on nine milliliter of phosphate normal saline to obtain 10^{-1} , then dilutions were made to 10^{-8} (Collin. 1989; Marutin 2001) [11]. For facultative anaerobes and obligate aerobes, selective media were applied to propagate bacterial isolate selectively. Each medium was prepared as their manufacture's instruction. Nutrient agar for total bacterial count, MacCkongy agar for gram negative bacteria, Cetrimide for *Pseudomonas* spp, Pfizer agar for fecal *Streptococcus faecalis* and urea based agar augmented with urea for urease positive bacteria.

Bacterial enumeration was done by streaking 0.1 ml of each diluent on the top of agar plates and incubated for 24-72 h at 37-39 °C. Meanwhile, counting bacterial colonies on the surface of each medium type and bacterial count should be between 30-300 colonies on the top of agar. The estimation of bacterial count would obey the equation: colony forming unit/ ml= colonies number×dilution factor×10 (Cappuccino and Welsh 2020) [8].

Isolated colony was picked from each medium and propagated on the top of nutrient agar to screen for other characters. Bacterial isolates were studied and characterized for their appearance on the top of their corresponding

medium and their microscopically cell shape after staining using Gram stain.

Subsequently, isolated colonies from each sample were put into groups to study their characters, group gram negative, group *Pseudomonas*, group *Streptococcus* and group Lactic acid bacteria (Cheesbrough, 2006) [9].

Methylene blue test

Methylene blue test serves as a redox indicator to evaluate the metabolic efficiency and enzymatic activity of each bacterial isolate under study.

Inoculate bacterial isolates on nutrient broth medium for 24 hours at 37°C in a shaking incubator to ensure adequate aeration and continuous mixing of the culture components. Meanwhile, 0.1 ml of methylene blue (0.03%) was added to 0.9 ml of the bacterial culture and mix thoroughly by stirring several times and allow to stand, then note and record the time required for the blue color to disappear from middle to bottom of samples.

Positive result indicated by time required for the enzymatic reduction of methylene blue to leuco-methylene blue and the scores of activities were listed in table 1 following Cockcroft& Jackson (2002) [10].

Table 1: The scoring in reduction time in minutes to assay the fermentation efficiency and microbial metabolic activity:

Reduction time (minute)	The category of activity score	Fermentation efficiency
Less than 3	Score 4	Hyper active/ rapid fermentation
3-6	Score 3	High-good/ standard active fermentation
6-15	Score 2	Moderate-fair/ delayed-suboptimal
15-30	Score 1	Low- poor/Impaired slow fermentation
More than 30	Score 0	Inert- Stagnant/ inactive system

Screening of Esterase enzyme

Following Kumar *et al.*, 2019 with some modification; Qualitative screening was done using nutrient agar amended with 0.5% of 2- naphthyl acetate. Each bacterial isolate was streaked over agar and incubated at 37-39°C for 72h. A clear zone around growth was indicated as a Positive result. For Quantitative determination, nutrient broth inoculated with each isolate and incubated at 37-39°C for 24 h. Meanwhile, using cooled centrifuge, samples were centrifuged at 10000rpm for 10 min to extract crude enzyme from bacterial isolates.

The substrate 1- naphthyl acetate was applied for enzymatic activity assay. Briefly, substrate was prepared as 0.5% in phosphate buffer pH 7.5 and used through the assay. To 0.9 ml of substrate only 0.1 ml of crude extract was used from each isolate. Mixtures were incubated for 30 min in dark then absorbance was measured using UV- VIS spectronic at a wavelength 320 nm (He 2003).

The activity of esterase:

$$U/mL = \frac{(\text{Absorbance of sample} - \text{Absorbance of blank}) \times \text{Total volume}}{\epsilon \times \text{Time} \times \text{Enzyme volume}}$$

Total volume=1ml, time =30min, enzyme volume 0.1ml, $\epsilon=2.19 \times 10^3$

Screening of ferulic acid

Following Sharma *et al.*, (2020) [31], Successive steps were carried out for determination of ferulic acid released from wheat bran by bacterial fermentation. Initially, wheat bran was washed using distilled water several time then, was

spread in a thin layer and left it for air- drying. Dried wheat bran was ground to fine particles and used as a precursor and a medium for bacterial isolates.

Medium preparation was done at a ratio 2:10 using distilled water in which 4 gram was weighted and mixed with 20 ml distilled water, then, sterilized using autoclave for 15 min. After cooling each medium was inoculated with 1ml of bacterial culture equivalent to 1×10^8 and incubated at 37-39°C for 72 h with moderate agitation 50 rpm. Cultures were centrifuged at 10000rpm for 10 min, supernatant was applied as a source to estimate ferulic acid produced by bacterial esterase. Each extract was measured at a wavelength 320 nm to determine ferulic acid.

Using the equation for quantifying ferulic acid in gram of wheat bran:

$$\text{Ferulic acid (mg/g)} = \frac{A \times 12.14 \times V \times D}{1000 \times W}$$

A= absorbance, V total volume, D= dilution factor, W= weight of wheat bran

For ferulic concentration mg/L using equation:

$$\text{Concentration (mg/L)} = \frac{A \times 194.18 \times 1000}{\epsilon (1600) \times l (1 \text{ cm})}$$

Concentration mg/L= A×12.14

Complete evaluation of degradation and hydrolysis of roughages (wheat bran) by measuring the reducing sugar was applied by dinitrosalicylic acid method (DNS method). Briefly, 0.2 of cell free extract of fermented wheat bran was mixed with 0.3ml of 3, 5 dinitrosalicylic acid reagent (DNS

reagent), boiling for 10 min in a water bath, then reducing sugar was estimated by spectrophotometer at 540 nm against reagent blank (Miller, 1972) [24]. Standard curve of Glucose was used to determine the concentration of released reducing sugars. Positive result indicates by increasing the concentration of reducing sugar through hydrolysis of fiber by bacterial enzyme beside ferulic acid.

Statistical Analysis:

The statistical analysis system genestat program was used. Duncan range tests were used to significant compare between means in this study.

Statistical model: $Y_{ijk} = \mu + A_i + e_{ijk}$.

Results and Discussion:

Results indicated differences in the facultative anaerobes among samples of cows, buffalos and lamb. Table (2) presented the total count of each group of bacteria in corresponding to their selective agar. Mean value of total count was approximately equal among the samples. Gram negative bacteria grew on MacConkey were nearly identical in cows and lamb and they were non- lactose ferment while buffalo samples showed more gram-negative bacteria with lactose ferment and non-ferment. Pseudomonales bacteria not exceeded 100 CFU/ml in sample of buffalo and cattle and around 1000 CFU/ml in lamb and were non-pigment producers.

Table 2: The total count of each group of bacteria

Medium name	Bacterial count log colony forming unit/ml			Notes
	cow sample	Lamb sample	Buffalo sample	
Nutrient agar	7.477	7	7.301	Different shape of bacteria
MacConkey agar	2.301	2.301	2.77	Non- lactose ferment and Lactose ferment
Cetermide agar	2	3	2	Non-pigment colonies
Pfizer agar	4	4.301	5.301	
Urease agar	4.698	4.477	4	
Milk broth	2.477	2.698	2.477	Different size bacilli, cocci and streptococci

On the top of Pfizer agar two distinct colonies shape appeared and both not convert medium to black that means they were non- *Streptococcus faecalis*. While, *Streptococcus faecalis* observed in buffalo samples (figure 1). Black color around colonies improved after bacterial growth as indication of existent of this species. Lactobacilli group could differentiate to gram positive bacilli colonies with different size, cocci, tetrad and short *Streptococcus* not more than 3-4 cocci.

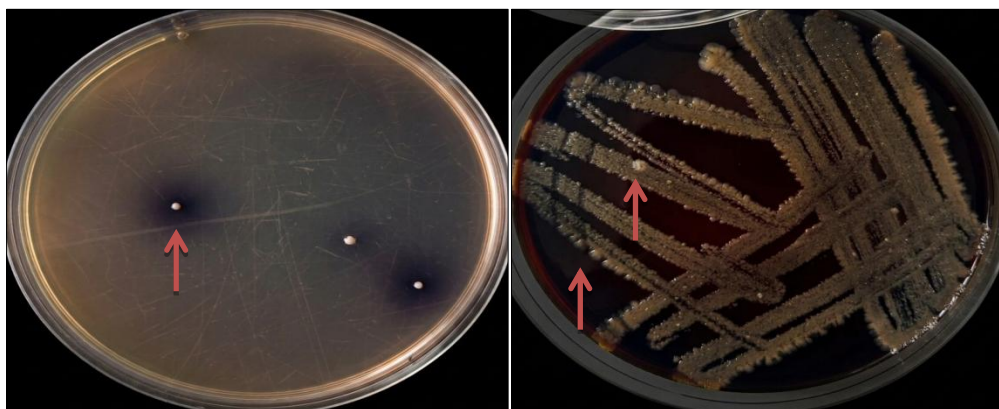


Fig 1: Bacterial growth on the top of Pfizer agar from Buffalo ruminal sample (left) and Lamb sample (right)

Microscopic examination of smears stained with gram stain showed *Streptococcus* isolates developed on the top of Pfizer agar. Strep with 4-6 cocci distinguished from the three samples as presented in figure (1-3). *Pseudomonas* spp isolate from lamb ruminal sample gave red color and bacillary cell figure (1, 5). Gram negative isolates gave cocobacilli shape from cattle and buffalo figure (1, 4 and 6). Agreement with other studies, variations in bacterial counts

among these three ruminants can be attributed to differences in anatomical physiology, dietary fiber content, and other management conditions. However, diet composition is the most influential factor in altering the ruminal microbial population (Al-Dahami, 2019; Al-Rubaiee, & Al-Zubaidy.2021) [2, 3].

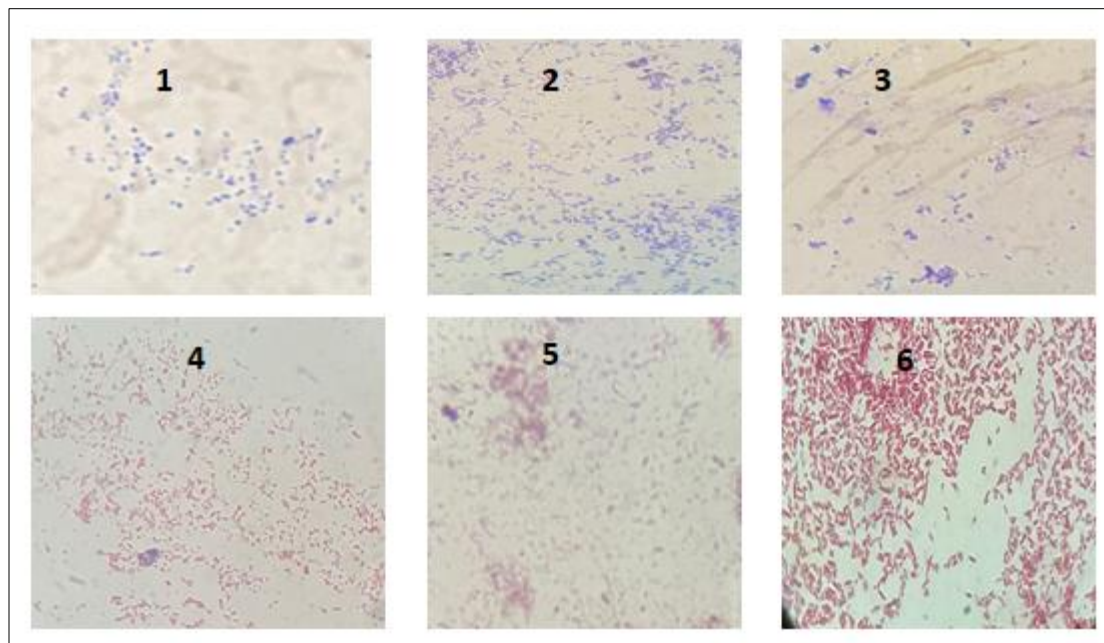


Fig 2: Microscopic examination of bacteria isolated from cows, buffalo and Lamb ruminal samples

1- *Streptococcus* from cattle, 2- *Streptococcus* from Lamb, 3- *Streptococcus* from buffalo, 4- gram negative cocobacilli from cattle, 5- *Pseudomonas* from lamb, 6- gram negative cocobacilli from buffalo

The observed low *Pseudomonas* count in the ruminal fluid can be attributed to highly specialized, strictly anaerobic conditions of the reticulorumen and toxic metabolic byproducts generated upon fermentation (Russell & Diez-Gonzalez, 1998; Owens *et al.*, 1998) [30, 27]. Almost, *Pseudomonas* species are either obligating aerobes or microaerophiles that using oxygen as a final electron acceptor (Arai, 2011; Deusch *et al.*, 2017) [4, 14].

On the recent study, isolated colonies of *Streptococcus* picked from Pfizer agar and streaked over MacConkey agar, all bacterial isolate from the cows, lambs and buffaloes exhibited characteristic growth, indicating their ability to

grow in the presence of bile salts. A highly desirable characteristic in ruminant feed manufacturing application.

Iraqi investigations highlighted the functional importance of bile salt resistance for lactic acid bacteria in their fermentation process through degradation of bile salt and diminished its bactericidal activity (Abdulhussien & Isa, 2025; Othman & Karim, 2023) [1, 26].

Recent results indicated the differences among isolates in their fermentation efficiency. Blue color of methylene blue changed to luco-color at different time interval and the scores listed in table (3). The score 4 recognized for growth culture of *Streptococcus* spp isolated from Lamb and cattle followed by score 3 by *Pseudomonas* from lamb. Score 2 by gram negative bacteria isolated from cattle sample. We chose seven isolated bacterial species due to their metabolic activity for further analysis.

Table 3: The recorded the scores and estimation of metabolic activity for rumenal bacterial isolates

Ruminal sample	Strain Name	Score	Metabolic Activity Index $100 \div t \text{ (min-1)}$
Cattle	Cattle <i>Streptococcus</i>	4	33.3
	Cattle - Gram-Negative	2	7.6
Buffaloes	Buffalo - Gram-Negative	3	20
	Buffalo - Gram-Positive	2	6.6
	Buffalo - Gram-Positive (2)	3	20
Lamb	Sheep - <i>Streptococcus</i>	4	33.3
	Sheep - <i>Pseudomonas</i>	3	20

During the qualitative screening for esterase's enzyme production from different bacterial isolates resembled community of the three samples under study, tween 80 agar gave unsatisfactory results. Poorly defined halo zones or

opaque precipitate around bacterial growth after 72h for almost 90% of tested isolates. Using 2-naphthyl acetate in screening for esterase demonstrated more satisfactory results as presented by *Streptococcus* from lamb figure (3).

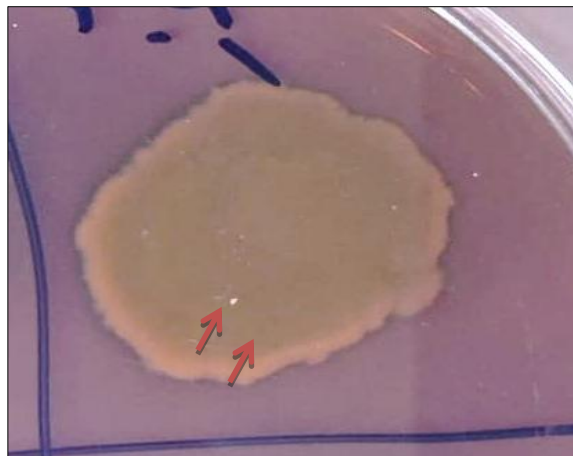


Fig 3: Qualitative detection of esterase production by *Streptococcus* sp from cattle using 2-naphthyl acetate agar and fast blue indicator

From screening test, seven out different isolates chose for quantitative determination of esterase as presented in table (4). This result of the analysis of variance (ANOVA) noticed highly significant differences ($p < 0.005$) in esterase enzyme activity between the different bacterial isolates that isolated from different animal rumen.

The bacterial isolate (lamb *Streptococcus*) isolated from rumen of lamb was the highest average enzyme activity with level 117.67 U/ml (2.52 U/ml) comparing to all other isolates.

While the buffalo isolate (Gram-Negative) came next with an activity level 100.57 U/ml (1.08 U/ml). Table showed there is no significant differences were found between the cattle isolates (Cattle Gram-Negative) and gram positive *Streptococcus* spp it was recorded similar activity levels (72.83 U/ml and 72.63 U/ml, respectively). Furthermore, the (Buffalo-Gram-Positive) isolate was the lowest esterase enzyme activity level among all measured isolates with rate reaching 45.70U/ml and 0.36units/ml.

Table 4: Average esterase enzyme activity (units/ml) of bacterial isolates isolated from the rumen of lambs, buffalo and cattle

Ruminal sample	Strain Name	Mean Esterase Activity (Units/mL) Mean±SD
Cattle	Cattle <i>Streptococcus</i>	72.63 ± 0.35c
	Cattle - Gram-Negative	72.83 ± 0.49c
Baffaloes	Buffalo - Gram-Negative	100.57 ± 1.08b
	Buffalo - Gram-Positive	45.70 ± 0.36e
	Buffalo Gram-Positive (2)	50.10 ± 0.36d
Lamb	Sheep <i>Streptococcus</i>	117.67 ± 2.52a
	Sheep <i>Pseudomonas</i>	52.02 ± 1.20d

* Means that have different letters within the same column have statistically significant differences at a probability level ($P < 0.05$)

In the present study, quantitative assessment of esterase activity showed that the isolate *Streptococcus* sp from lamb rumen exhibited a significantly higher esterase productivity compared to other bacterial isolates under identical cultural conditions. This could attribute to the rapid growth kinetic, highly protein secretion and metabolic adaptability characteristic of *Streptococcus* species found in ruminal environment (Wong, 2006; Dilokpimol *et al.*, 2016) [38, 15]. On the other hand, *Streptococcus* belonging to the ruminal lactic acid bacterial consortium that possess gene clusters responsible for carbohydrate esterase enzyme, facilitating lignocellulose degradation (Crepin *et al.*, 2004) [12]. These finding strongly accepted by Zhang *et al* 2025, when

screened whole genome sequence and catalytic profiling that lamb derived *streptococcus* isolates express noticeably elevated esterase enzymes relative to other microflora.

Figure (4) demonstrated significant variability's among the bacterial isolates from cows, buffaloes and lamb regarding their capability to degrade lignocellulosic material. Ferulic acid and increased in the concentration of reducing sugar reflected the degradation of wheat bran by esterase enzyme Morgavi *et al.*, 2013 [25]. Ferulic acid resemble the primary chemical bridge cross linking lignin to hemicellulose via ester bonds thus presenting a structural barrier that restrict the activity of cellulolytic enzyme (Morgavi *et al.*, 2013) [25].

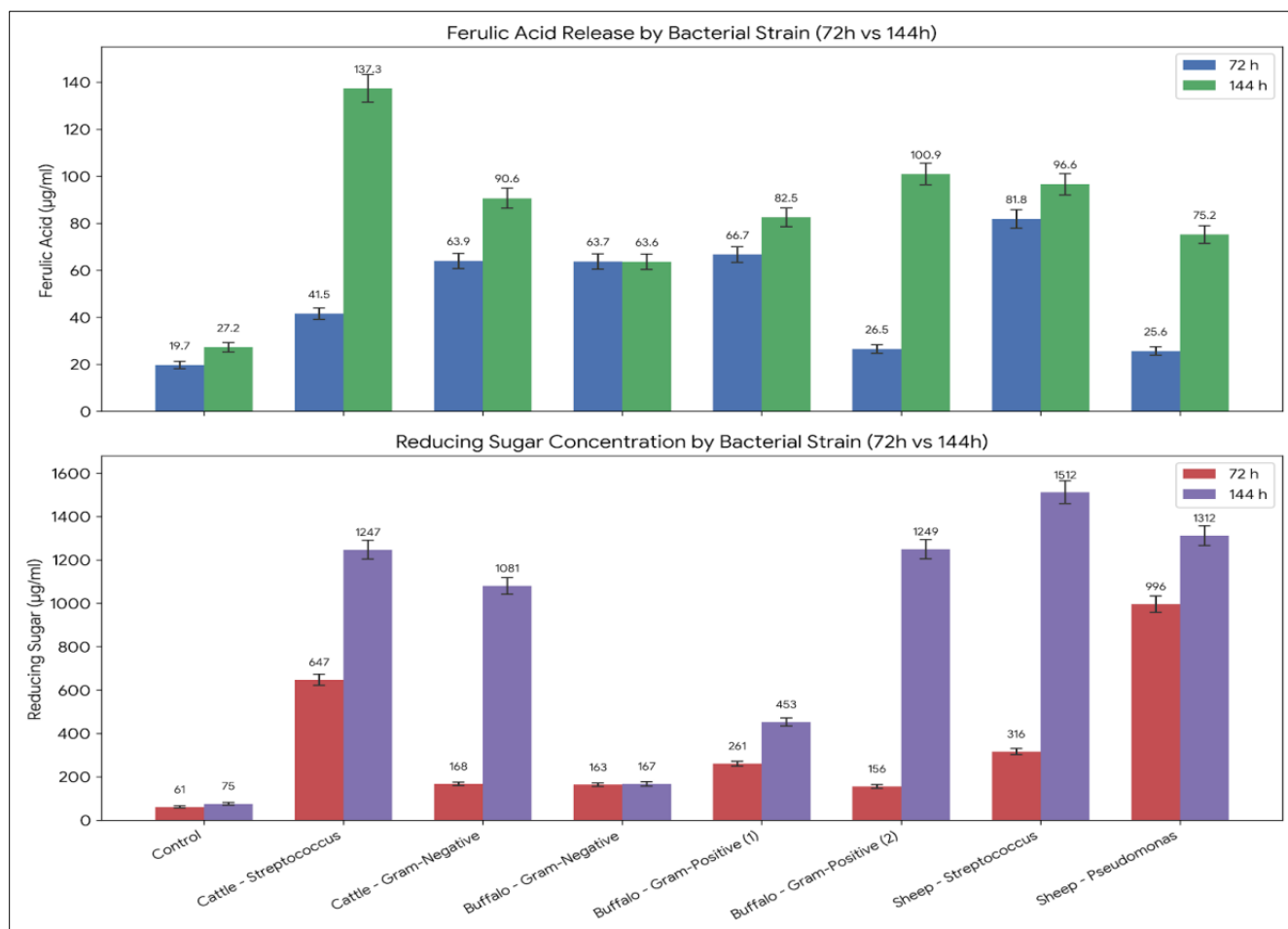


Fig 4: Comparative profile of bio-production of ferulic acid and reducing sugar (µg/ml) from ruminal bacterial isolates

Results under study showed that gram positive; *Streptococcus* isolated from cattle rumen exhibited the highest production of ferulic acid followed by gram positive2 from buffalo with activity reached to 138 µg/ml after 72h and 100.5 µg/ml at 144 h. The significant increase in ferulic acid indicated a high production of feruloyl

esterase by gram positive isolates. The enzymatic activity to cleave ester bond weakened the structural integrity of cell wall and enhanced synergistic attack of cellulose and xylanase to digest lignocellulosic matrix coupled with high sugar released exceeding 1200 µg/ml finding agree with Paul *et al.*, 2004 and Dilokpimol *et al.*, 2016 [28, 15].

Table 5: Concentration of Ferulic Acid and reducing Sugar (µg/ml) from various ruminal bacterial isolates at 72 and 144 hours of incubation.

Ruminal Sample	Bacterial Strain	Ferulic Acid (72 h)	Ferulic Acid (144 h)	Sugar Concentration (72 h)	Sugar Concentration (144 h)
Control	Control	19.67 ± 0.88f	27.23 ± 0.05f	60.93 ± 0.64e	74.67 ± 0.33f
Cattle	Cattle - Streptococcus	41.53 ± 0.52d	137.33 ± 2.33b	647.09 ± 1.46b	1247.00 ± 1.51c
Cattle	Cattle - Gram-Negative	63.93 ± 0.72b	90.60 ± 1.02d	167.57 ± 1.80d	1080.53 ± 0.84e
Buffaloes	Buffalo - Gram-Negative	63.73 ± 1.84b	63.60 ± 0.36e	163.43 ± 0.79d	167.00 ± 1.63 f
Buffaloes	Buffalo - Gram-Positive (1)	66.70 ± 2.70b	82.53 ± 0.58d	260.61 ± 1.53c	452.83 ± 1.92d
Buffaloes	Buffalo - Gram-Positive (2)	26.53 ± 0.41e	100.93 ± 0.55c	155.50 ± 0.77d	1249.27 ± 3.39c
Lamb	Sheep - Streptococcus	81.83 ± 0.80a	96.57 ± 0.50c	316.03 ± 2.32c	1511.67 ± 7.27a
Lamb	Sheep - Pseudomonas	25.63 ± 0.58e	75.20 ± 0.17e	995.71 ± 0.21a	1311.67 ± 7.27b

- Values are expressed as Mean pm Standard Error (SE) of 3 replicates.
- Means within the same column followed by different superscript letters (a, b, c, d, e, f) are significantly different ($P < 0.05$) according to Duncan's Multiple Range Test.

The analysis of enzymatic kinetics in fermentation process between 72h and 144h revealed a progressive synergistic activity between ferulic acid

concentration and the increased in reducing sugar quantity among all isolates. Linear regression analysis revealed a positive correlation between ferulic acid concentration and sugar released among the evaluated bacterial isolate after 144 h of incubation (figure 5). As reflected by upward of the regression line, increased ferulic acid levels generally corresponded to higher sugar accumulation in the medium. However, metabolic efficiency varied significantly among isolates. Gram negative isolate from buffalo exhibited low sugar concentration less than 200 µg/ml with lower ferulic

acid 64 µg/ml. Other isolates, *Pseudomonas* and *Streptococcus* demonstrated high metabolic activity, achieving peak sugar concentration between 1200 and 1500 µg/ml. on the other hand, the width of 95% confidence interval at elevated ferulic acid concentration more than 100

µg/ml indicated greater variability in metabolic pathways under higher substrate availability. Our results suggested that hydrolysis of lignocellulosic fiber generated by ferulyte esterase was bacterial isolate dependent and accordance with Varel & Jung, 1986.

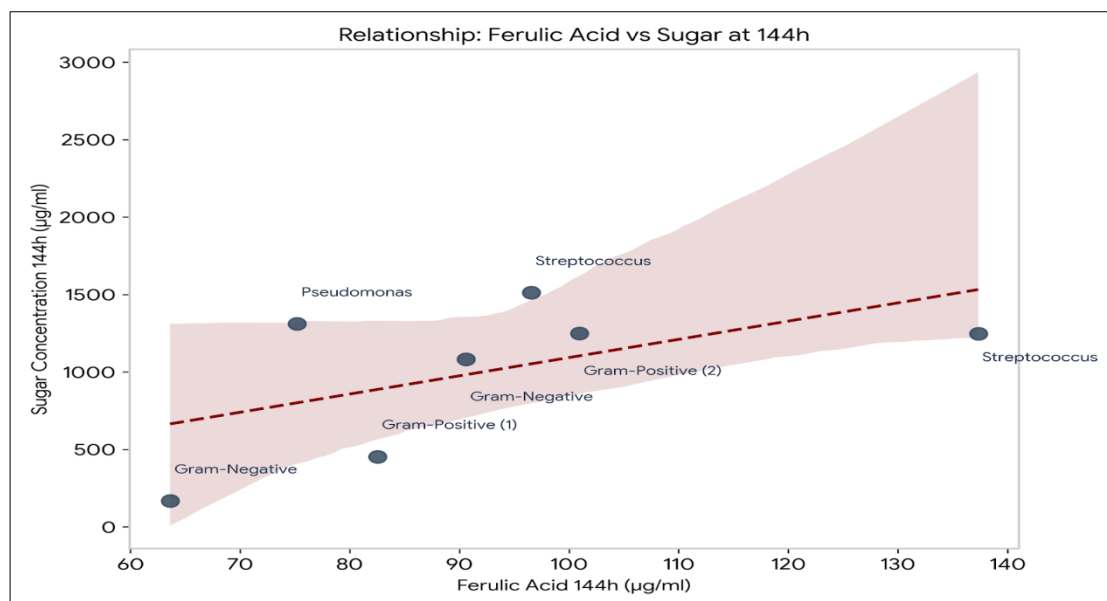


Fig 5: Linear regression analysis for relationship between ferulic acid release and reducing sugar accumulation at 144 h.

The highest concentration of reducing sugar gained from *Streptococcus* isolated from Lamb 1500 µg/ml followed by *Pseudomonas* 1300 µg/ml at 144h. This marked increase attributed to enzymatic synergy between de-lignifying and saccharifying enzymes (Van Dyk & Pletschke, 2012). Regarding time production, *Pseudomonas* isolate displayed an early sugar production at 72h which indicated a rapid substrate induced enzymatic response (Wang *et al.*, 2019).

A comparison among the origin of ruminal samples cleared that isolates from lamb samples gave the best result followed by cattle and buffaloes. This might due to the adaptation of the lamb microbiome to diet with high lignocellulosic content ((Krause *et al.*, 2003; Belanche *et al.*, 2019).

Our results were in accordance with others, *Streptococcus* isolates demonstrated high capabilities in producing ferulic acid. Among the gram positive fibrolytic and lactic acid ruminal bacteria, *Streptococcus* was the most Feruloyl Esterases producers and rapid growth bacteria. They constituted the primary gram positive biocatalysts that disorganized the recalcitrant lignocellulosic fiber and accelerate the release of carbohydrates from microbial fermentation in the rumen (Crepin *et al.*, 2004; Zhang *et al.*, 2025) ^[12].

Conclusion:

In conclusion, this study demonstrated that indigenous ruminal facultative eanaerobic microflora specifically *Streptococcus* and *Pseudomonas* isolates grew from cattle and lamb possessed high fibrolytic and esterase producing capacities. The functional activity of these isolates successfully disrupted the lignocellulosic matrix of wheat bran, facilitating release of ferulic acid and significantly increased hydrolysis of complex carbohydrates into reducing sugars.

Remarkably, *Streptococcus* sp isolated from lamb exhibited the highest enzymatic efficiency in both total esterase activity and reducing sugar liberation. These finding highlighted the potential of *Streptococcus* isolate as bio-safe bio-inoculant and feed additives to overcome low quality of roughages and enhancing ruminant performance and livestock productivity.

Recommendations:

- Genotypic identification and safety assessment should be performed using 16rRNA and blood hemolysis test for bio-safety status.
- Studying enzyme kinetic as well as specific activity towards various lignocellulosic substrates.
- Optimize the condition of solid state fermentation using statistical experimental design and laboratory experiments to scale up enzymes production using low cost agricultural residues.
- Investigate the synergistic effects between different isolates to establish multi enzyme complexes.
- Perform in vitro gas production assay and digestion trial in lamb and cattle to assess the actual nutrient digestibility, volatile fatty acids profile and animal performance.

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