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# Sensitivity of *Coriandrum sativum* extract on bacterial pathogens isolated from digestive system of rabbits, and its role on *in vitro* cecal gas production and fermentation

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ABSTRACT

The present context was aimed to investigate the antibacterial potency of aqueous extract of coriander (*Coriandrum sativum* L.) leaves against bacterial pathogens isolated from the organs associated with digestive system of rabbit. This study also evaluated the influence of varied doses of aqueous extract of *C. sativum* (AECS) leaves on *in vitro* gas production (GP), methane ($CH_4$) production, and some other pivotal fermentation parameters from caecal sample of rabbits. The pathogenic bacteria were isolated from mouth, caecum, and anus of rabbits, and further identified through morphological, biochemical, and molecular tools. The growth inhibitory characteristics of AECS against pathogens were determined using disc diffusion assay. Surprisingly, the result revealed lack of antibacterial potential at tested concentrations. Further, in order to demonstrate the *in vitro* GP and fermentation parameters in rabbits, four treatments comprising of 0, 0.6, 1.2, and 1.8 mL extract/g dry matter (DM) of AECS were used. Results showed no linear or quadratic effect ($P > 0.05$) on *in vitro* GP and $CH_4$ production after the supplementation of AECS in the feeding diet. However, the inclusion of AECS at the concentration of 1.8 mL/g DM exhibited the lowest asymptotic $CH_4$ production and initial delay prior to $CH_4$ production. Similarly, the addition of AECS at 1.8 mL/g DM concentration reduced asymptotic GP as well as $CH_4$ production, and improved fermentation parameters of rabbits when compared with the control and other tested doses. In a nutshell, the tested doses of AECS showed lack of antibacterial trait against the pathogenic bacteria isolated from mouth, caecum, and anus of rabbits. Besides, the AECS exhibited the unique potentiality of reducing GP and improving diversified fermentation parameters in rabbits, thereby suggesting its plausible role as an alternative to commercially available growth promoters in livestock industries.

## 1. Introduction

Rabbits are edible herbivorous animals that are recently taking up increasing role in meat production [1]. However, formulation of diet and feeding rabbits are complex, mainly because of its physiology (cecotrophy), thereby making it difficult to manipulate its diet [2]. During the intensive breeding of rabbits, the mortality rate is often very high due to disturbances caused by inadequate nutrition. This is because rabbits are raised under confinement, they depend entirely on the food that is provided to them. Generally, their diets consist of concentrated food that contains all the nutrients they need [3]. The effect of nutrients on fermentation parameters, especially microbial activity in rabbit caecum is essential because it is directly associated with the healthy state of the rabbits. The caecum is an area of bacterial growth that has a direct influence on the digestive process, nutritional requirements, and types of foods that rabbits can use for their growth and reproduction [4]. To stabilize the rabbit's cecal microbial fermentation, natural feed additives such as plant extracts could be used. Currently,

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the utilization of feed additives such as natural extracts from plants as growth promoters have created immense interest among researchers [5]. Some plant components have been studied as additives in the diet since they are considered as potential source of phenolic compounds, with high antioxidant activities [6]. Antioxidants are compounds that reduce the rate of oxidation by controlling the formation of free radicals, which play an important role in disease control. One of the medicinal plants with such potential is coriander (*Coriandrum sativum* L). It is a glabrous aromatic herbaceous plants, widely used in folk medicine. It plays a vital role as antibacterial, antifungal, and antioxidative agents [7,8]. It also plays an essential role as a flavoring agent and adjuvant, and helps in maintaining the shelf-life of foods stuff by preventing food spoilage and foodborne diseases [8].

Recent studies have shown that plant extracts contain specific secondary metabolites with potentials as alternative feed additives to manipulate microbial activities of animals [9]. The major plant secondary metabolites (PSM) such as polyphenolics and tannins have great potentialities to influence microbial activity in caecum [10], improve meat production [11], and reduce mortality in rabbits [12,13].

However, the presence of tannins at higher concentration forms complexes with proteins [14], and these complexes can provoke negative effects by making them unavailable to cecal microorganisms, and thus, reduces digestibility of nutrients. High consumption of tannins or saponins can also cause hemolytic effect and may even lead to the death of the animals [15]. Additionally, PSM have also shown to enhance protein metabolism, decrease $CH_4$ emission, and suppress or stimulate microbial growth [16], while some PSM reduce nutritional stress and improve animal health as well as productivity [17,18], thereby resulting in weight gain and voluntary feed intake of the animals [19,20]. In view of this, the present study was aimed to evaluate the sensitivity of pathogenic bacteria isolated from digestive system of rabbits to aqueous leaf extract of *C. sativum*. Part of this investigation was studied to determine the impact of various doses of *C. sativum* on *in vitro* gas production (GP), methane ($CH_4$) production, and fermentation parameters of rabbit's cecal inoculum.

## 2. Material and methods

### 2.1. Plant sample collection and extract preparation

Fresh and healthy leaves of *C. sativum* were purchased from local market and brought to the laboratory. Leaves were washed with sterile distilled water and allowed to dry overnight. Fifty grams of leaves were ground into powder using a blender and mixed with 400 mL of distilled water. The mixture was kept for 72 h at room temperature. After required period of incubation, the mixture was filtered using Whatman No. 1 filter paper and the solution was allowed to evaporate. The aqueous leaf extract of *C. sativum* (AECS) was collected and stored at 4 °C for further experimental purposes.

### 2.2. Bacterial isolation

Thirty rabbits from Cinineza farm were randomly selected and used at 15 and 30 days of fattening. Samples were collected from oral cavity and anus using sterile swab for isolating bacteria. Swabs were transferred to sterile test tubes and brought to the laboratory (Center for Research and Advanced Studies in Animal Health) for bacterial isolation purpose. Swabs from each fattening period were inoculated in petri dishes containing Tryptic Soy agar medium (g/L: pancreatic digest of casein 15.0, peptic digest of soybean meal 5.0, sodium chloride 5.0, and agar 20.0) and MacConkey agar medium (peptone 3.0, pancreatic digest of gelatin 17.0, lactose monohydrate 10.0, bile salts 1.5, sodium chloride 5.0, crystal violet 0.001, neutral red 0.05, and agar 14.0), and incubated at 37 °C for 24 h. Morphologically different colonies were selected and purified after streaking. Purified bacterial cultures were stored in 50% (v/v) glycerol stock at −80 °C for further processing.

### 2.3. Morphological, biochemical and characterization

The standard Bergey's Manual of Systemic Bacteriology was used for the identification (morphological and biochemical properties) of isolates. The shape and colour of colonies of purified bacteria were observed through naked eyes. Various biochemical tests such as carbohydrate fermentation, gas production, lysine decarboxylation, ornithine decarboxylation, indole, voges-proskauer, phenylalanine deamination, citrate utilization, urea hydrolysis, and hydrogen sulphide production were determined using standard methodology after 24 h of incubation period. Genomic DNA of bacteria was isolated and the amplicon was obtained using the thermal cycler and universal primers. The amplicon was purified and the sequencing was carried out using an automated sequencer in order to identify bacterial species.

### 2.4. Antibacterial activity of aqueous leaf extract of C. sativum

Antibacterial activity of aqueous leaf extract (i.e., AECS) was determined using disc diffusion method [21] against *Escherichia coli* (*E. coli*), *Pantea agglomerans* (*P. agglomerans*), *Yersinia pestis* (*Y. pestis*), and *Shigella* sp., isolated from mouth, caecum, and anus of rabbits. All the bacterial cultures were inoculated into selective growth medium, adjusted to 0.5 McFarland scale ($1 \times 10^6$ CFU/mL), and incubated at 37 °C for 24 h. After the required period of incubation, bacterial cultures were swabbed on selective agar medium plates. Subsequently, two different concentrations (0.5 mg/mL and 1 mg/mL) of AECS were transferred to sterile discs (6 mm) and allowed to soak for 15 min. The discs were transferred aseptically to the plates seeded with the respective indicator pathogenic bacterium with the help of ethanol dipped and flamed forceps, and incubated at 37 °C for 24 h. After 24 h, zone of inhibition formed by extracts against the indicator pathogens were measured. Gentamicin (10μg/disc) was used as positive control and the experiments were carried out in triplicate.

### 2.5. In vitro cecal GP and fermentation profile

The experiment was carried out in the Laboratory of Animal Nutrition of the Faculty of Veterinary Medicine and Zootechnics of the Autonomous University of the State of Mexico. The ethical principles of animal care and handling were adhered strictly in accordance with the official Mexican norm of animal care NOM-051-ZOO-1995 [22] throughout the experiment.

Cecal samples were collected directly from the blind New Zealand rabbits of approximately 11 weeks of age (2.21 ± 0.13 kg) and a composite sample was made for *in vitro* incubation. Amber glass bottles of 115 mL capacity were used, and 0.5 g of the commercial pellet-based diet was placed as a substrate consisting of 15.5% protein, 2% fat, 15% Fiber, 9% ash, 12% moisture, 46.5% nitrogen-free extract, 1% calcium, and 0.55% phosphorus. Subsequently, 40 mL of nutrient medium was added along with 10 g of the cecal inoculum. While the inoculum was added to the bottles, a continuous flow of $CO_2$ was maintained for anaerobiosis condition. The AECS was added at the concentrations of 0, 0.6, 1.2, and 1.8 mL/g dry matter (DM) to three different bottles of extract dose and incubated at 39 °C for 48 h. At the time of removal of the $CO_2$ source, a rubber stopper was placed and then sealed with a plastic ring and adhesive tape being hermetically sealed. The bottles were placed at 39 °C in a forced ventilation oven, and gas pressure inside the flask was set to zero. The pressure generated by the fermentation of the substrate at 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 24, 36, and 48 h was observed [23]. Total GP was measured by a Manometer (Extech 407910). At the end of incubation, bottles were uncovered to measure pH using a digital pH meter (Conductronic pH15, Mexico). After pH measurement, the contents of each bottle were filtered (Coarse Porosity 1; pore size 100–160 mm). Fermentation residues were dried at 65 °C for 72 h to estimate dry matter degradation (DMD).

### 2.6. Calculations

Gas accumulation pressure at the top jars was measured with a pressure transducer connected to a digital reader. Psi unit conversion was determined by SAS program [24] according to the equation mentioned below:

$$Y = 0.024 + 5.34\ X + 0.031X^2$$

where, $Y$ is volume (8 mL); X is pressure (psi) with $R^2 = 0.99$. Kinetic parameter of GP results (mL/g DM) were fitted using NLIN option of SAS [24] according to France et al. [25] model as given below:

$$A = b\ x\ [1 - e^{-C\ (t-L)}]$$

where, A is the volume of GP at time $t$, $b$ is the asymptotic GP (mL/g DM), $k$ is the rate of GP (/h), and $L$ (h) is the discrete lag time prior to GP.

Metabolizable energy (ME, MJ/kg DM) was estimated according to the method of Menke et al. [26] as given below:

$$ME\ (MJ/K_g\ DM) = 2.20 + 0.136\ GP + 0.057\ CP$$

Gas yields (GY) at 4 and 6 h were calculated as the volume of gas (mL gas/g DM) produced after and before those time periods of incubation divided by the amount of DM (g) as:

$$Gas\ yield\ (GY) = mL\ gas/g\ DMD$$

Short-chain fatty acids (SCFA) were calculated according to the equation of Getachew et al. [27] as given below:

$$SCFA\ (mmol/200\ mg\ DM) = 0.0222\ GP - 0.00425$$

where, GP is 24 h net GP (mL/200 mg DM).

Microbial crude protein production (MCP) was calculated according to the equation of Blümmel et al. [28] as mentioned below:

MCP (mg/g DM) = mg DMD – (mL gas × 2.2 mg/mL) where the 2.2 mg/mL is a stoichiometric factor that expresses mg of C, H, and O required for the SCFA gas associated with the production of one mL of gas.

### 2.7. Statistical analyses

The antibacterial assay was carried out in triplicate and values were expressed as mean ± standard deviation. The results of GP *in vitro* cecal fermentation parameters were analyzed using the PROC GLM option of SAS [24], where GP is net GP in mL of 500 mg of the dry sample after 24 h of incubation having a model:

$$Yijk = \mu + Ri + Dj + (R \times D)\ ij + Eijk$$

where, Yijk = each observation of the type of ration $i^{th}$ (Ri) with $j^{th}$ = dose of AEC (Dj); M is the general mean; (R × D) ij is the interaction between ration and dose of CAD; Eijk is the experimental error.

Linear and quadratic polynomial contrasts were used to examine different responses and levels of CAD. Statistical significance was stated at $P < 0.05$.

## 3. Results

### 3.1. Bacterial identification

Bacteria isolated from digestive system associated organs of rabbits were observed as small, round, and white colonies on agar medium (Table 1). Microscopic observation categorized the isolates under gram negative bacteria. Isolates were able to ferment glucose, lactose, arabinose, and sorbitol. Isolates were observed as indole positive. On the other hand, isolated bacteria showed negative results towards hydrogen sulphide production, adonitol and duloitol fermentation, voges-proskauer, urea hydrolysis, and citrate utilization tests (Table 2). After molecular characterization, bacteria were identified as *E. coli*, *P. agglomerans*, *Y. pestis*, and *Shigella* sp. (Data not shown).

**Table 1**
Morphological characteristics of bacteria isolated from mouth, caecum, and anus.

| Organs | Shape | Color |
|---|---|---|
| Mouth | coccus | white |
| Mouth | coccus | white |
| Mouth | coccus | yellow |
| Mouth | coccus | white |
| Mouth | irregular | white |
| Mouth | coccus | white |
| Caecum | coccus | pink |
| Caecum | coccus | pink |
| Caecum | coccus | pink |
| Caecum | coccus | pink |
| Caecum | irregular | red |
| Caecum | coccus | red |
| Anus | coccus | pink |
| Anus | coccus | white |

### 3.2. Anti-pathogenic activity of AECS

All the isolated pathogenic bacteria were observed to be resistant to tested concentrations of AECS. However, gentamicin showed growth inhibitory activity against *E. coli*, *P. agglomerans*, *Y. pestis*, and *Shigella* sp. with remarkable zone of inhibition of 17.39 ± 1.8, 23.79 ± 1.6, 27.06 ± 1.3, and 35.84 ± 1.6 mm, respectively (Data not shown).

### 3.3. In vitro cecal GP and fermentation profile

Table 3 shows the rate of *in vitro* GP and $CH_4$ production due to the supplementation of varied doses of AECS into rabbit diets. No linear or quadratic effects ($P > 0.05$) of AECS on *in vitro* GP and $CH_4$ production were observed. A slight reduction in the asymptotic GP was reported with increase in the concentration of AECS. Lowest asymptotic GP (130 mL/DM) was estimated at high dose (1.8 mL/DM) of AECS. Likewise, various doses of the AECS did not show any significant effect ($P > 0.05$) on the rate of GP and initial delay prior to GP.

No linear or quadratic effect ($P > 0.05$) was observed on $CH_4$ production parameters (asymptotic $CH_4$ production, rate of $CH_4$ production, and initial delay prior to $CH_4$ production) after the supplementation of AECS into the diet. However, the addition of AECS caused slight reduction in the asymptotic $CH_4$ production and initial delay prior to $CH_4$ production in a concentration dependent manner of AECS.

Table 4 shows the proportional *in vitro* $CH_4$ production due to the addition of different doses of AECS in the diet. The AECS at various doses exhibited no significant effects ($P > 0.05$) on the incubated mL/DM and degraded mL/DM when compared to the control. However, high concentration of AECS showed reduced incubated mL/DM and degraded mL/DM upto 48 h of incubation. There was no significant difference ($P > 0.05$) observed on proportional $CH_4$ production at different concentrations of AECS but the control showed the highest proportional $CH_4$ production at 6 h of incubation. On the other hand, doses of 1.8 mL/DM and 0.6 mL/DM exhibited the highest proportional $CH_4$ production at 24 and 48 h, respectively.

In like manner, there was no significant ($P > 0.05$) difference observed on pH, ME, MCP, gas yield ($GY_{24}$), and SCFA due to the supplementation of varied doses of AECS. However, the addition of AECS at 1.8 mL/DM showed slight reduction in pH. In contradictory to this, increased ME, MCP, $GY_{24}$, SCFA, and DMD were estimated at high concentration of AECS with respect to control (Table 5).

## 4. Discussion

The natural extracts owe their biological activity to the synergism

**Table 2**
Biochemical properties of bacteria isolated from mouth, caecum, and anus.

| Organs | Tests | | | | | | | | | | | | | | | Bacteria |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Glucose | Gas | Lystine | Orntine | $H_2S$ | Indole | Adonitol | Lactose | Arabinose | Sorbitol | VP | Dulcitol | PA | Urea | Citrate | |
| Anus | + | + | + | + | – | + | – | + | + | + | – | + | – | – | – | *E. coli* |
| Anus | + | – | – | – | – | – | – | – | – | – | – | – | – | – | + | *P. agglomerans* |
| Anus | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | *Shigella* sp. |
| Mouth | + | – | + | + | – | + | – | + | + | + | – | – | + | – | – | *E. coli* |
| Mouth | + | – | + | + | – | + | – | + | + | + | – | – | + | – | – | *E. coli* |
| Mouth | + | – | – | – | – | + | – | – | – | – | | – | – | + | + | *Y. pestis* |
| Mouth | + | – | – | – | – | – | – | – | – | – | – | – | – | – | – | *Shigella* sp. |
| Mouth | + | + | + | + | – | + | – | + | + | + | | – | – | – | – | *Y. pestis* |
| Caecum | + | – | + | + | – | + | – | + | + | + | – | – | – | – | – | *E. coli* |
| Caecum | + | – | + | + | – | + | – | + | + | + | – | – | – | – | – | *E. coli* |
| Caecum | + | – | – | – | – | + | – | – | – | – | | – | – | – | – | *Y. pestis* |
| Caecum | + | + | + | + | – | + | – | + | + | + | – | – | + | – | – | *E. coli* |

**Table 3**
Gas production and *in vitro* $CH_4$ production after 48 h of incubation due to addition of different doses AECS.

| AECS doses (mL/g DM) | GP (mL/0.5 g DM) | | | $CH_4$ production (mL/0.5 g DM) | | |
|---|---|---|---|---|---|---|
| | *b* (mL/g DM) | *c* (/h) | *L* (/h) | *b* (mL/g DM) | *c* (/h) | *L* (/h) |
| 0 | 200.63 | 0.067 | 3.45 | 13.77 | 0.066 | 7.94 |
| 0.6 | 165.03 | 0.094 | 3.01 | 13.27 | 0.085 | 8.53 |
| 1.2 | 153.30 | 0.095 | 3.08 | 12.29 | 0.059 | 7.12 |
| 1.8 | 130.63 | 0.098 | 3.79 | 11.56 | 0.062 | 7.07 |
| SEM | 47.03 | 0.033 | 0.714 | 5.391 | 0.029 | 0.853 |
| *P* | 0.384 | 0.664 | 0.547 | 0.957 | 0.715 | 0.188 |
| Linear | 0.105 | 0.289 | 0.575 | 0.629 | 0.868 | 0.244 |
| Quadratic | 0.720 | 0.630 | 0.315 | 0.924 | 0.805 | 0.540 |

AECS-aqueous extract of *C. sativum*; GP-gas production; *b*-asymptotic GP; DM-dry matter; *c*-rate of GP; *L*-initial delay before GP begins; h-hours; $CH_4$-methane.

**Table 5**
*In vitro* cecal fermentation profile due to different doses of AECS supplementation in rabbit's diet.

| AECS doses (mL/g DM) | pH | ME (MJ/kg DM) | MCP (mg/g DM) | $GY_{24}$ (mL gas/g DMD) | $PF_{24}$ (mg DMD:mL gas) | SCFA (mmol/g DM) | DMD (mg/g DM) |
|---|---|---|---|---|---|---|---|
| 0 | 6.11 | 6.85 | 541.30 | 165.83 | 6.04 | 3.13 | 0.388 |
| 0.6 | 6.10 | 6.72 | 532.33 | 163.30 | 6.14 | 3.02 | 0.380 |
| 1.2 | 6.12 | 7.16 | 562.30 | 169.66 | 5.90 | 3.37 | 0.457 |
| 1.8 | 5.86 | 7.53 | 587.96 | 174.56 | 5.72 | 3.68 | 0.458 |
| SEM | 0.345 | 0.575 | 39.582 | 7.798 | 0.281 | 0.467 | 0.058 |
| *P* | 0.753 | 0.380 | 0.378 | 0.374 | 0.361 | 0.373 | 0.264 |
| Linear | 0.401 | 0.187 | 0.186 | 0.207 | 0.210 | 0.185 | 0.181 |
| Quadratic | 0.578 | 0.939 | 0.935 | 0.925 | 0.922 | 0.929 | 0.431 |

AECS-aqueous extract of *C. sativum*; DM-dry matter; DMD-DM degradability; MCP-microbial crude protein production; $GY_{24}$-gas yield at 24 h of incubation; ME-metabolizable energy; SCFA-short chain fatty acids.

between their various bioactive compounds. The essential oils such as cineol, borneol, camphene, citronellol, coriandrol, and geraniol of *C. sativum* exhibit greater growth inhibitory ability against diversified pathogens [29]. Due to the lipophilic action, these oils have the ability to cross the cell membrane, break polysaccharides, fatty acids as well as lipids, and permeabilizing the cell membrane, thereby leading to the loss of ions, the collapse of the proton pump, and the decrease of ATP which inevitably leads to cell death. It has also been found that at the cytoplasmic level, it can act on lipids and proteins by coagulating these molecules [30].

The antimicrobial effect of this plant has been associated with linalool, which has the ability to inhibit even sporulation. The highest concentration of linalool is obtained from the essential oil of coriander leaves when extraction is made with the hexane-chloroform solution [31].

It has been demonstrated that *C. sativum* has antibacterial activity against different genera of gram-positive and gram-negative bacteria. In the present study, aqueous extract of *C. sativum* leaves showed no antibacterial activity against the indicator pathogens isolated from digestive system associated organs of rabbits. This might be due to the nature and area of growth of *C. sativum*. The growth and bioactivity of plant depend on the geographical locations and its diversity. On the other hand, the lack of growth inhibitory traits of *C. sativum* leaves against isolated pathogens may not only be due to the lower concentrations of extract studied but also types of solvents used for extraction process. Further study certainly needs to be carried out to assess the anti-pathogenic property of aqueous as well as other solvents extracts of *C. sativum* at higher concentrations. Matasyoh et al. [32] found minimal inhibitory concentrations of the essential oil of this plant ranging between 108 and 217 mg/L.

**Table 4**
Proportional *in vitro* $CH_4$ production as a percentage of the total GP due to supplementation of varied AECS doses.

| AECS doses (mL/g DM) | Incubated mL/g DM | | | Degraded mL/g | | | Proportional $CH_4$ Production | | |
|---|---|---|---|---|---|---|---|---|---|
| | 6 h | 24 h | 48 h | 6 h | 24 h | 48 h | 6 h | 24 h | 48 h |
| 0 | 4.00 | 9.62 | 12.00 | 10.65 | 25.19 | 31.01 | 7.07 | 7.10 | 18.59 |
| 0.6 | 4.20 | 9.81 | 12.09 | 11.31 | 27.00 | 33.74 | 5.97 | 6.55 | 19.51 |
| 1.2 | 3.65 | 9.23 | 11.49 | 7.97 | 20.15 | 25.10 | 5.81 | 7.06 | 17.06 |
| 1.8 | 3.51 | 8.79 | 10.87 | 7.67 | 19.37 | 24.07 | 6.48 | 7.59 | 18.63 |
| SEM | 1.05 | 2.87 | 4.21 | 2.98 | 8.68 | 12.80 | 1.68 | 1.81 | 6.38 |
| *P* | 0.846 | 0.972 | 0.982 | 0.386 | 0.657 | 0.759 | 0.799 | 0.918 | 0.097 |
| Linear | 0.584 | 0.734 | 0.750 | 0.257 | 0.435 | 0.525 | 0.680 | 0.752 | 0.993 |
| Quadratic | 0.894 | 0.989 | 0.984 | 0.586 | 0.738 | 0.794 | 0.444 | 0.827 | 0.740 |

AECS-aqueous extract of *C. sativum*; DM-dry matter; h-hours; $CH_4$-methane.

At present, researchers are very keen to develop nutritional strategies that will maximize livestock production and effectively lower the cost of production to the barest minimum. Their willingness to achieve this goal has prompted the use of additives such as antibiotics, ionospheres, and probiotics in animal production [33]. However, over the past decade, the use of these additives have drastically faced decreased social acceptance, especially the use of antibiotics because of the anticipated adverse effects on the livestocks and the consumers. Today, the use of antibiotics as livestock feeding additives has been banned within the European Union [34]. The consequences of this effect have propelled the researchers to exploit the use of natural feed additives such as plant extracts to improve nutrient utilization, digestibility of feeds, and increase microbial protein production as well as reduce emission of greenhouse gases [35]. In this study, *in vitro* fermentation technique was used to evaluate the effect of supplementing AECS on *in vitro* GP and fermentation parameters of rabbit's cecal. No linear or quadratic effect ($P > 0.05$) on GP parameters (asymptotic GP, rate of GP, and initial delay prior to GP) was observed due to the supplementation of various doses of AECS. The asymptotic GP decreased with increasing AECS concentrations. The findings of the present study were not in the agreement with the reports of Jiménez-Peralta et al. [9] and Salem et al. [20] who estimated that addition of *Salix babylonica* and *Leucaena leucocephala* into the animal's diet increased GP. The decreased GP could be attributed to the high amount of PSM and antimicrobial agents in the AECS, which may have negative impacts on fermentation profile of rabbits. Lower *in vitro* GP may also be due to low soluble sugar in the AECS which could have a negative influence on the microbial activity of the rabbits. Delgado-Pertiñez et al. [36] and Dihigo et al. [37] had also reported negative effects due to the inclusion of higher concentration of polyphenolic compounds in animal diets. They opined that these compounds bound to the cell wall of the substrates and prevent the enzymes from utilizing the substrates, for example the proteases or the presence of other substances could interfere in the digestibility of the nutrients, thus diminishing the production of $NH_3$, a factor that limits the growth of bacteria and the digestion of cecal fiber [38].

It has been well established that $CH_4$ production has negative impact on livestock production because it causes energy loss and also bloating as a result of its accumulation. The result of this study showed non-significant ($P > 0.05$) reduction in asymptotic $CH_4$ production, rate of $CH_4$ production, and initial delay prior to $CH_4$ production after AECS supplementation at varied doses. The findings were similar to the results of Kim et al. [39] who reported that $CH_4$ production was the highest in the control and the lowest when plant extracts were added into the diet. García-González et al. [40] and Kamra et al. [41] also reported that *Allium sativum* extract decreased $CH_4$ production by more than 20%. This reduction in $CH_4$ production may be due to the antimicrobial properties of plant extract which might have influenced the methanogenesis in the studied animals.

In the present investigation, no linear or quadratic effect ($P > 0.05$) on the pH, ME, MCP, $GY_{24}$, $PF_{24}$, and SCFA were observed. However, the addition of AECS at 1.8 mL/DM slightly decreased the pH and $PF_{24}$. Slyter [42] reported that high level of PSM has the capacity to lower the pH and influence the microbial population. Garcia et al. [43] also reported that in growing rabbits there is a progressive reduction in cecal pH when the digestible percentage of neutral detergent fiber (NDF) in feed increases.

In addition, this study showed no significant differences ($P > 0.05$) in SCFA caecum content of AECS doses and the control, but a slight reduction in pH lower than the control and minor increase in SCFA greater than the control was observed as the dose increases. This result was found to be similar to the report of Mista [44] who observed higher propionic acid content in the total VFA volume and a lower pH of the rabbit cecal content in treated groups when compared to the control group.

Chang et al. [45] reported that increase concentrations of Soyhulls inclusion linearly decreased pH values and ammonia-N concentrations of cecal contents, but increased the total VFA concentrations. Reduction in cecal pH as well as ammonia-N concentration and higher VFA concentration might be due to high fermentation and active microbial synthesis in the caecum. In this study, the addition of different doses of AECS did not show significant impact on DMD ($P > 0.05$). Our findings were similar to the reports of Busquet et al. [46] and Yang et al. [47] who observed that the supplementation of garlic oil or its combination with berry essential oil did not produce any significant effect on true DM.

The supplementation of high dose of AECS had non-significant ($P > 0.05$) effect on ME, MCP, and $GY_{24}$, however, a slight increase was observed when compared to the control and comparatively lower doses. Findings of our study were in agreement with the report of Salem et al. [20] who estimated that the addition of *L. leucocephala* and *S. babylonica* extracts at various concentrations increased MCP with respect to the control. In like manner, Alexander et al. [48] also reported that extracts of *Moringa oleifera* and *Picrorhiza kurroa* decreased DM degradability and $GP_{24}$, without affecting significantly ($P > 0.05$) the MCP.

## 5. Conclusions

In summary, four pathogenic bacteria were isolated from mouth, caecum, and anus of rabbits. Isolates were identified as *E. coli*, *P. agglomerans*, *Y. pestis*, and *Shigella* sp. based on morphological, biochemical, and molecular characterization tools. These bacteria were found to be resistant to the aqueous extract of *C. sativum* leaves. Additionally, findings of this study showed no linear or quadratic effect ($P > 0.05$) on *in vitro* GP and $CH_4$ production parameters after the supplementation of *C. sativum* leaves extract in the feeding diet of rabbits. However, the supplementation of leaves extract at the concentration of 1.8 mL/g DM exhibited not only the lowest asymptotic $CH_4$ production and initial delay prior to $CH_4$ production but also improved fermentation parameters of rabbits. Further study is certainly required to determine the anti-pathogenic property as well as *in vitro* GP and cecal fermentation profile of rabbits using higher doses of *C. sativum* leaves.

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