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Influence of Juniperus Thurifera Root Extract on the Nutrient Digestibility and Caecal Microbial Count of Growing Rabbits

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ABSTRACT

Juniperus thurifera root extract have an unending ability to synthesize secondary metabolites which plays vital role in the defense against pathogens and modulation of the immune system. This study was conducted to examine the Influence of Juniperus thurifera root extract on the nutrient digestibility and caecal microbial count of growing rabbits. Thirty – growing New Zealand white × Chinchilla male rabbits of 6-8 weeks of age weighing 605 ± 5.03 grams were used for the experiment. Rabbits were allocated into three treatments each with 10 animals. Each treatment consisted of 5 replicates with 2 rabbits per replicate in a completely randomized design. Rabbits in treatment 1 (control) was fed basal diet + 1.5 g oxytetracycline per liter of water while T2 and T3 were fed basal diet with 3 mL and 6mL of Juniperus thurifera root extract per litre of water respectively. Feed and water were provided ad libitum and the experiment lasted for 90 days. Results revealed a significant difference (P<0.05) in dry matter, crude protein, crude fibre, crude fat, ash and nitrogen free extract values. Dry matter value was highest in T3, intermediate in T2 and lowest in T1 (P<0.05). Caecal microbial count of Escherichia coli, Clostridium spp and Salmonella spp were higher in T1 compared to the other treatments (P<0.05). Lactobacillus spp population was higher in T2, T3 and lowest in T1 (P<0.05) thus promoting a healthy gut among rabbits fed Juniperus thurifera root extract. In conclusions, Juniperus thurifera root extract can be used as potential alternatives to antibiotics. It can also cause a reduction in animal disease which can have a negative effect on animal health, food supply and the economy.

Introduction

Phytogenics are plant derived materials with a potential to improve animal performance (feed intake, palatability and growth) and health status (gastro intestinal tract integrity and immune system). The use of phytogenics are increasingly gaining interest globally due to the recent ban on the use of antibiotics by the European Union in 2006 and its dangers to the environment, contaminated products which could lead to serious health dangers in human (IPP, 2002). There

are over 200,000 medicinal plants with highly active ingredients (phytochemicals or secondary metabolites) such as; alkaloids, flavonoids, terpenoids, tannins, saponins and phenols (Oluwafemi *et al*., 2000; Angioni *et al*., 2003).

The maintenance of the gastro intestinal tract integrity is vital to ensure an effective immune system since it is the largest organ of the immune system that plays a pivotal physiological role as a barrier against antigens and pathogens. Several phytogenic feed additives (medicinal plants) were reported to have a host mediated immune-modulatory effect either as immune-stimulators or immune-suppressors (IPP, 2004). Among the prospective medicinal plant is *Juniperus thurifera* root extract.

Juniperus thurifera is a multipurpose evergreen plant belonging to the family Cupressaceae and class Pinopsida. It is native to the mountains of western Mediterranean region and also found in Europe, Africa and some parts of Asia including India (Adams, 2004; Farjon, 2005). It is used in ethnoveterinary medicine for the treatment of various diseases due to the presence of bioactive compounds (phytochemicals) (Farjon, 2005). These compounds afford plants a competitive advantage by acting as defense mechanisms against pathogens, predators and environmental stress (Chris and Abel, 2008). The tree can grow up to 6-20 metres and the foliage is characterized by spicy resinous scent (Farjon, 2005). The plant can produce between 1-4 seeds and the leaves are about 0.6 – 3.0 mm long depending on their stage of growth (Farjon, 2013).

In vitro studies have demonstrated the efficacy of *Juniperus thurifera* root extract against pathogenic bacteria's for example; *Clostridium perfringens*, *Clostridium septicum*, *Escherichia coli*, *Bacillus spp*, *Salmonella spp*, *Streptococcus spp*, *Shigella spp* and *Pseudomonas spp* (Pothitirat *et al*., 2009; Ennajar *et al*., 2010). The leaves, stem bark and root of *Juniperus thurifera* have also shown to produce biological or therapeutic effects (antimicrobial, hypotensive, antiviral, anti-mitotic, antibacterial, immune-modulatory, cytotoxic, anti-fungal, immune-suppressant, anti-cancer, antipyretic, anti-proliferative, anti-androgenic and antioxidant) by alleviating several diseases and have been traditionally used in the treatment of cough, skin diseases, rheumatism, gastro intestinal diseases, malaria and sexually transmitted diseases (Rezzi *et al*., 2001; Chouhan *et al*., 2017).

Previous studies have confirmed that extract from *Juniperus thurifera* have less environmental pollution because of less excretion of nitrogen, phosphorus and other heavy metals (IPP, 2004; Adams, 2008). It can also be used as a natural alternative to antibiotics thus, producing a safer product for consumers (Alagbe, 2022; Adewale *et al*., 2021). Its chemical composition vary substantially between species, geographical areas, stage of growth, harvesting and processing methods (Akintayo and Alagbe, 2020; Olafadehan *et al*., 2020).

In order to promote animal welfare, food safety and lower disease, this experiment was designed in order to evaluate the effect of *Juniperus thurifera* root extract on the nutrient digestibility and caecal microbial count of growing rabbits.

Materials and methods

Site of the experiment

The experiment was carried out at Sumitra Teaching and Research Institute Gujarat, India (23^{o} 13'N 72^{o}41'E) in the month of February to May, 2022.

Collection of plant material and extraction methods

Fresh *Juniperus thurifera* root was harvested in Punsari village within some few kilometers from Ahmedabad, Gujarat. It was identified by a certified Taxonomist and chopped manually into pieces with a kitchen knife, washed and air dried for 2 weeks to reduce the moisture content and retain the bioactive compounds in the plant. Thereafter, it was grinded into powder using an electric blender.

Dried *Juniperus thurifera* root was soaked in ethanol (70 %) over a 24 hours period at a room temperature of 25^{o}**C** with soxhlet apparatus. The mixture was stirred and filtered with Whatman filter paper, thereafter, it was dried in a rotary evaporator (Model NH-560A-002, India) with dimension (D×P×H mm) – 380 × 207 × 488 m, speed range (10-250 rpm), stroke (100 mm) and temperature (RT – 200 ^{o}**C**). The extract was stored in a labeled sample bottle and kept in the refrigerator at 4 ^{o}**C.**

Experimental animal feeding, health and housing

Thirty – growing New Zealand white × Chinchilla male rabbits of 6-8 weeks of age weighing 605 ± 5.03 were purchased from Sumitra Research Farm, Gujarat. Before the commencement of the study, semi closed pen was properly cleaned and galvanized cages with dimension 75 cm × 80 cm × 40 cm) (Length × width × height) were properly disinfected. The animals were subjected to a 2 weeks acclimatization period. Ivomec® injection was administered to treat each animal for endo- and ecto- parasites. After the adjustment period, rabbits were weighed and distributed into 3 treatments of 10 rabbits which was further divided into 5 replicates consisting of 2 animals each in a completely randomized design. Basal diet was formulated using Corn, wheat bran, palm kernel meal, rice husk, and soya bean meal were variable ingredients while methionine, di-calcium phosphate, lysine, premix and salt were fixed ingredients combined in different quantity to formulate a diet according to the requirements of growing rabbits according to the specifications of Nutritional Research Council (1977). Rabbits were also fed twice daily (7:00 am) and (2:30 pm) in the morning and afternoon respectively. Feed intake was recorded daily while mortality was recorded as it occurs and the duration of the experiment was 90 days.

Experimental levels are outlined below;

Treatment 1: Basal diet plus 1.5 g of oxytetracycline per litre of water (According to the manufacturer's instruction)

Treatment 2: Basal diet plus 3 mL of *Juniperus thurifera* root extract per litre of water

Treatment 3: Basal diet plus 6 mL of *Juniperus thurifera* root extract per litre of water

***Juniperus thurifera* root extract analysis using gas chromatography and mass spectroscopy (GC-MS)**

The secondary metabolites in *Juniperus thurifera* root extract were determined using gas chromatography coupled to mass spectroscopy (GC-MS) model 6800 N gas chromatography coupled to 5189 F mass spectroscopy from SUKRAY auto sampler. The GC had the following technical specifications; inlet temperature 450 ^{o}**C**, pressure range (100 psi ± 0.001 psi), split mode (split/splitless, max split ratio: 1000:1) and column oven working temperature (+4 ^{o}C˜ 450 ^{o}**C**) while MS specifications; EI source ionization energy (5 eV – 250 eV), mass range (1.5 – 1000 amu), ion source temperature (100 -300 ^{o}**C**), stability (± 0.10 amu/48 hours), scan rate (up to 1000 amu/s) and detector (high energy dynode electron multiplier).

Laboratory analysis of experimental diet

Experimental diet and faecal samples were analyzed using Fibertec^{TM} 8000 automatic feed analyzer with the following technical specifications;

Wave length range (1000 – 2000 nm)

Frequency (60/70 Hz)

Power consumption of 60W

Resolution VIS (15 nm)

Digestibility trial

5 rabbits were randomly selected from each treatment and housed in a metabolic cage for easy separation of faeces and urine. Cages were also equipped with concrete feeders and drinkers, thereafter, rabbits were kept on one week acclimatization period to allow the animals adjust to the new environment followed by a five days faecal collection. Faecal droppings were collected in a well labelled bags and taken to the laboratory for further examination. Digestibility was calculated using the formula below:

Digestibility (%) = Input – faecal output/ input multiply by 100

Caecal microbial analysis

One gram of caecal sample was collected from 3 selected rabbits on the 90th day and mixed with 10 % peptone solution in a well labelled sample bottle. *Clostridium spp* was cultured on Mac Conkey agar under conditions of 37^{o}C for 24 hours, *Escherichia coli*, *Salmonella spp* and *Lactic acid* bacteria were cultured on Wikkins-Chalgen agar (37^{o}C for 24 hours), X.L.D agar (37^{o}C for 24 hours) and M.R.S agar (37^{o}C for 48 hours) respectively.

Statistical analysis

Data obtained from the experiment were analyzed using Analysis of variance (ANOVA) for Completely Randomized Design (CRD) according to statistical analysis system (SAS, 2003) at $p < 0.05$. Differences between means were separated by the Duncan's Multiple Range Test (DMRT) of the same software.

Table 1: Gross composition of experimental diet for growing rabbits fed *Juniperus thurifera* root extract

Components	Aggregate (Kg)
Corn	45.25
Wheat bran	15.03
Palm kernel meal	10.00
Rice husk	6.00
Soya bean meal	20.00
Di-calcium phosphate	3.00
Lysine	0.10
Methionine	0.12
[α]Mineral and Vitamin Premix (growers)	0.25
Common Salt	0.25
Total	100.00
Analyzed Nutrients	
Crude protein	16.11 %
Crude fibre	12.33 %
Ash	9.77 %
Energy (Kcal/kg)	2577.58

[α]Vitamin-mineral mix supplied the following 1Kg of diet: vitamin A, 8,000 IU; cholecalciferol, 1,600 IU; vitamin E, 15 IU; vitamin K, 1.1 mg; vitamin B12, 12.5 g; riboflavin, 5.5 mg; Ca panthothenate, 11 mg; niacin, 53.3 mg; choline chloride, 1,020 mg; folic acid, 0.75 mg; biotin, 0,25 mg; delquin, 125 mg; DL-Met, 500 mg; amprol, 1 g; Mn, 55 mg; Zn, 50 mg; Fe, 80 mg; Cu, 5 mg; Se, 0.1 mg; I, 0.18 mg; and NaCl, 2,500 mg.

Results and discussion

Secondary metabolites are chemical compounds biosynthetically derived from primary

metabolites. They are not directly involved in the normal growth, development and reproduction of an organism but have a marked pharmacological or therapeutic activity (Asl and Hosseinzadeh, 2008; Gülçin, 2012). Plants produce secondary metabolites for survival or protection from being eaten by animals and microbial pathogens (Peréz and Aguilar, 2013; Azzouzi *et al*., 2015), response to stress (Nikolova, 2012; Alagbe *et al*., 2022) and attractants for pollinators (Tsado *et al*., 2015; Skakiel *et al*., 2010). Gas chromatography and mass spectrometry of *Juniperus thurifera* root extract revealed the presence of 50 compounds which have been shown to produce various biological effects and also alleviating several ailments in animals (Sexena *et al*., 2013; Alami *et al*., 2016). Monoterpenes had the highest concentration of bioactive compounds (51.33 %) followed by oxygenated monoterpenes (15.49 %), sesquiterpenes hydrocarbon (12.25 %) and oxygenated sesquiterpenes (3.00 %) respectively. The result obtained in this study agrees with the findings of Rachid *et al*. (2019); Alagbe, 2022. Terpenes constitute the largest class of secondary products which exist in various forms; monoterpenes, dipterpenes, sesquiterpenes, triterpenes and tetraterpenes (Agubosi *et al*., 2022; Shittu and Alagbe, 2020). Monoterpenes such as α-humulene, α-cadinol, α-murolene, α-cadinol, α-cubebene, γ-cadinene, α-longipinene, β-cayrophyllene, limonene and γ-terpinene can act as antibacterial, antifungal, anti-cancer, cholesterol suppressant and cytotoxic (Adewale *et al*., 2021; Akintayo and Alagbe, 2020). α-Pinene helps to prevent osteoporosis (Alagbe, 2020) while the presence of γ-gurjunene, sabinene, β-bourbonene, β-phellandrene, δ-2- carene and α-campholenal helps to scavenge free radicals (antioxidants), hepato-protective and immune-modulatory activities (Muritala *et al*., 2022). Other secondary metabolites like alkaloids are analgesics, hypotensive, anti-mitotic, sposmolytic and antimalarial (Shittu and Alagbe, 2020). They can also be synthesized from some of the few amino acid, for instance, quinine and morphine are synthesized from tryptophan and tyrosine (Zeraib *et al*., 2014). Phenols has antiseptic, anti-inflammatory, antiviral, antiprotozoal and antioxidant properties (Alagbe and Ushie, 2022; Singh *et al*., 2021).

Table 2: Bioactive compounds in *Juniperus thurifera* root extract analyzed using GC-MS

Bioactive compounds	% Area
γ-Gurjunene	1.93
β-Eudesmol	0.60
α-Cadinol	1.37
α-Humulene	2.05
δ-Cadinene	0.21
α-Cubebene	1.55
γ-Cadinene	0.78
Germacrene D	1.49
Sabinene	19.82
γ-Terpinene	1.53
α-Longipinene	1.07
α-Murolene	0.59
γ-eudesmol	0.70
β-Bourbonene	1.22
β-Cayrophyllene	0.15
β-Santalene	1.00
α-Pinene	24.31
β-Citrylideneethanol	1.50
Torreyol-α-cadinol	0.07
D-Limonene	2.01
Elemol	0.01
Germacrene B	0.02

α-Cadinol	0.46
δ-2- Carene	1.01
δ-3- Carene	0.13
α-Pinene oxide	0.02
(Z)- β- Ocimene	0.25
δ-Cadinene	1.40
β-Eudesmol	1.22
Thymol	0.05
α-Phellandrene	0.09
α-Fenchene	0.17
Linalyl acetate	0.22
Citronellol	0.03
Terpinolene	2.41
Linalool	6.72
α-Thujone	2.60
α-Campholenal	0.07
Sabinene	0.44
Cis-sabinene hydrate	0.30
β-Phellandrene	0.62
Cis-thujanol	0.88
Trans-4-thujanol	0.02
p-Cymen-8-ol	0.21
Trans-calamenene	0.45
Cis-murraola-(4)5-diene	1.82
Verbenone	0.52
α-Terpinyl acetate	0.01
Germacrene D-4-ol	2.03
Linalyl acetate	3.94
Aggregate (%)	92.07
Breakdown (%)	
Monoterpenes hydrocarbon (%)	51.33
Oxygenated monoterpenes (%)	15.49
Sesquiterpenes hydrocarbon (%)	12.25
Oxygenated sesquiterpenes (%)	3.00

Nutrient digestibility of growing rabbits fed different levels of *Juniperus thurifera* root extract

Nutrient digestibility of growing rabbits fed different levels of *Juniperus thurifera* root extract appears in Table 3. The dry matter, crude fibre, crude fat, crude ash, crude protein and nitrogen free extract values roved from 76.46 – 89.02 %, 43.11 – 60.04 %, 59.10 – 69.44 %, 19.87 – 25.20 %, 60.08 – 71.33 % and 58.10 – 70.80 % correspondingly. Dry matter, crude fat and crude ash values were maximum in T3, medium in T2 and minimum in T1 ($P<0.05$). Crude protein and nitrogen free extract values were maximum in T3 compared to the other treatments ($P<0.05$). The outcome in this experiment shows that *Juniperus thurifera* root extract is capable of improving the absorption of nutrients via the stimulation of digestive enzymes: lipase, amylase, pancreatic trypsin and maltase as well as increasing saliva and bile secretion especially among rabbits in T3 compared to the other treatments. The presence of the various secondary metabolites in the extract (Table 2) will also reduce the retention time of feed consumed thus ensuring better animal performance through improvements in growth rate and feed conversion ratio. *Juniperus thurifera* root extract has also proven to produce no adverse effect on the health

of rabbits. The result obtained is in agreement with the findings of Dalle Zotte *et al*. (2013); Placha *et al*. (2013) when rabbits were fed *Arthrospira platensis* and *Thymus vulgaris* on the performance of rabbit. Similar result was recorded by Pebriansyah *et al*. (2018); Zarie *et al*. (2016) who examined the effect of phytoadditive *Silybum marianum* on the performance of broiler rabbits. All these research have proven that phytogenic feed additives can improve nutrient digestibility and consequently the health status of rabbits. Conversely, Chrastinova *et al*. (2010) reported that some phyto-addtitives had no significant influence of nutrient digestibility of growing rabbits. These differences could be as a result of variation in the secondary compounds or bioactive chemicals, extraction procedures as well as differences in the inclusion level of the test material (Omokore and Alagbe, 2019).

Table 3: Nutrient digestibility of growing rabbits fed different levels of *Juniperus thurifera* root extract

Components (%)	Treatment 1	Treatment 2	Treatment 3	*SEM*
Dry matter	76.46^{b}	87.29^{a}	89.02^{a}	0.25
Crude fibre	43.11^{b}	49.61^{b}	60.04^{a}	0.72
Crude fat	59.10^{b}	67.81^{a}	69.44^{a}	0.88
Crude Ash	19.87^{b}	24.08^{a}	25.20^{a}	0.02
Crude protein	60.08^{b}	69.05^{b}	71.33^{a}	0.17
Nitrogen free extract	58.10^{c}	69.36^{b}	70.80^{a}	0.53

SEM: standard error of mean

^{a,b,c} Means within a row with different manuscripts differ significantly (P<0.05)

Caecal microbial population of growing rabbits fed different levels of *Juniperus thurifera* root extract

Microbiological examination of the caecum of growing rabbits fed different levels of *Juniperus thurifera* root extract (Table 4) revealed the presence of *Clostridium spp*, *Escherichia coli*, *Salmonella spp* and *Lactobacillus spp*. Their values ranged from 15.06 – 25.81 (Cfu/g), 9.41 – 14.62 (Cfu/g), 7.15 – 10.09 (Cfu/g) and 18.30 – 30.16 (Cfu/g) respectively. *Lactobacillus spp* count were higher in T3 compared to the other treatments (P<0.05). *Clostridium spp*, *Escherichia coli*, *Salmonella spp* count were at maximum in T1 and lowest in T2 and T3 (P<0.05). *Clostridium spp*, *Escherichia coli*, *Salmonella spp* are pathogenic bacteria that are capable of causing disease in animals. The lower count of pathogenic bacteria recorded in T2 and T3 simply means that *Juniperus thurifera* root extract have immune-stimulatory effect due to the presence of phytochemicals thus preventing dsybiosis and keeping the intestinal flora balanced (Cross *et al*., 2007; Alagbe, 2021). It is also capable of producing anti-bacteria substances and specifically compete for adhesion receptors on the epitelium of the gut (IPP, 2004; IPP, 2009). *Lactobacillus spp* are beneficial bacteria's capable of modulating the immune response and improving an animal's body's resilience thus promoting a healthy gut (Losa and Kohler, 2001; Lu *et al*., 2003). A healthy gut is an effective digestive organ that can mount a good defense against disease and easily cope with change (nutritional or environmental) (IPP, 2009). Maintaining a healthy gut especially among rabbits in T2 and T3 will reduce mortality in animals and promote food safety (Alagbe, 2022). Commensal microbial population stimulates the immune system's development and forms a protective barrier between the host and the microbes (IPP, 2013). *Escherichia coli* is a Gram negative, rod shaped, non-spore forming bacterium while *Clostridium spp* are Gram positive bacteria (Alagbe, 2018).

Table 4: Caecal microbial population of growing rabbits fed different levels of *Juniperus thurifera* root extract

Constituents (Cfu/g)	Treatment 1	Treatment 2	Treatment 3	*SEM*
Clostridium spp	25.81[a]	17.44[b]	15.06[b]	0.02
Escherichia coli	14.62[a]	9.90[b]	9.41[b]	0.17
Salmonella spp	10.09[a]	8.52[b]	7.15[b]	0.03
Lactobacillus spp	18.30[c]	26.25[b]	30.16[a]	1.05

SEM: standard error of mean

[a,b,c] Means within a row with different manuscripts differ significantly ($P<0.05$)

Conclusion

Juniperus thurifera root extract contain several phytochemicals which are environmentally friendly, efficient in controlling pathogenic organisms such as *Escherichia coli*, *Clostridium spp* and *Salmonella spp* and modifying the gut microflora of rabbits. It can be concluded that *Juniperus thurifera* root extract can be fed to growing rabbits up to 9mL per liter of water without causing any deleterious effect on the health status of animals.

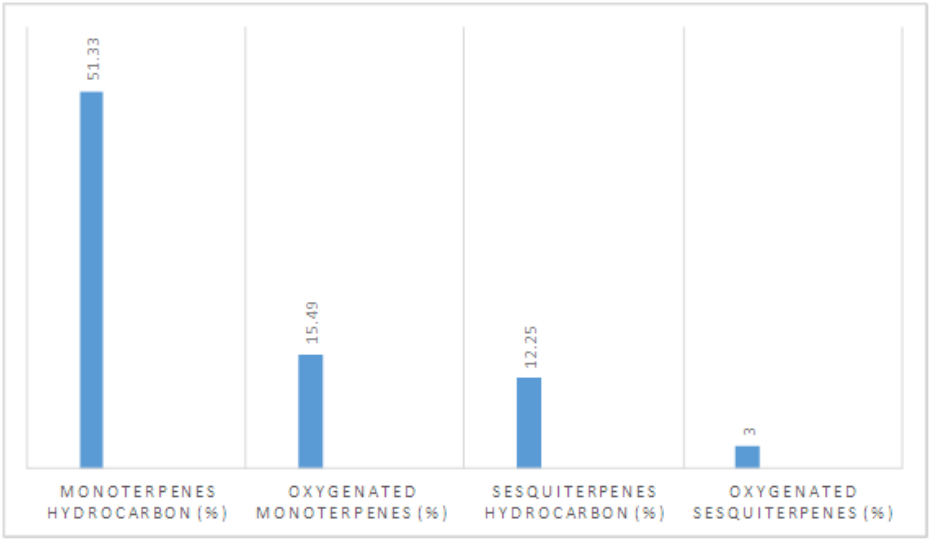

Figure 1. Breakdown of bioactive compounds in *Juniperus thurifera* root extract

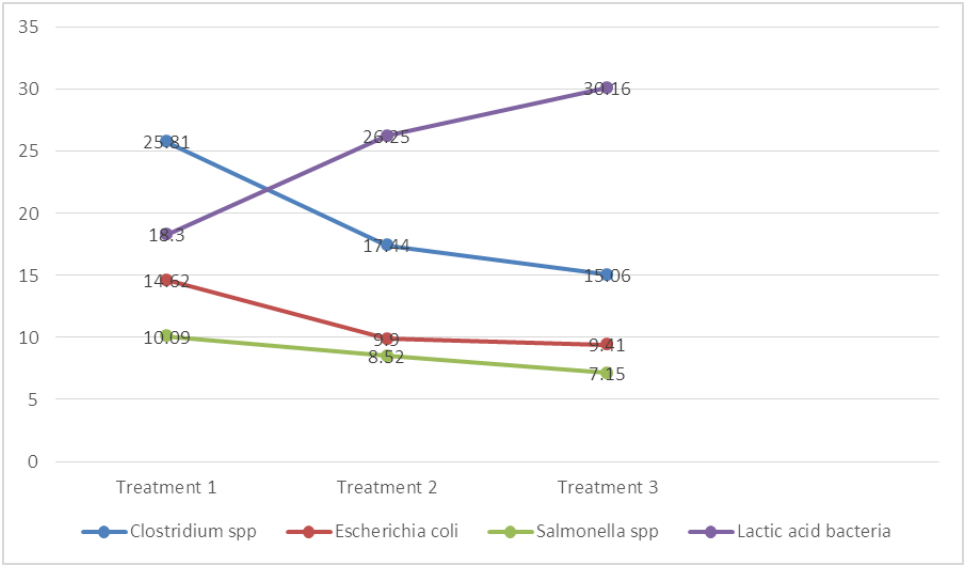

Figure 2: Caecal microbial population chart among the treatments

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