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# ISOLATION AND *IN VITRO* ANTIBACTERIAL ACTIVITY OF LACTIC ACID BACTERIA ISOLATED FROM FERMENTED PANCHAGAVYA

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## ABSTRACT

Panchagavya is an ancient Ayurvedic preparation obtained by combination of five products of cow namely dung, urine, milk, curd and ghee. The present study was conducted to evaluate the efficiency of Panchagavya as probiotics by enumerating the total bacterial and lactic acid bacterial count of fermented Panchagavya and demonstrate the antibacterial activity of lactic acid bacterial isolates of Panchagavya against gram positive and gram negative organism. The test organisms were *Escherichia coli, Pseudomonas aerouginosa, Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus.* Panchagavya was prepared as per the standard procedure and fermented for 15, 30, 45 and 60$^{th}$ days and pH was recorded during the period of study. The result of the study reported a fall in pH from 5.10 at 15$^{th}$ to 3.73 at 60$^{th}$ days of Panchagavya and the total bacterial count observed to increase till 30$^{th}$ day of fermentation and the count declined later while *Lactobacillus* count showed a gradual increase till 45$^{th}$ day and falling in count was observed later till 60 day age old Panchagavya. The antibacterial activity

demonstrated that the gram positive bacteria was found more sensitive to the lactic acid bacteria strains of Panchagavya than gram negative test organism.

**KEYWORDS:** Panchagavya, Lactic acid bacteria, Antibacterial activity, Poultry feed.

## INTRODUCTION

Panchagavya is a traditional formulation of animal products used since Vedic times in Indian civilization. The Sanskrit word Panchagavyam means mixture of five products of cow namely dung, urine, milk, curd and ghee. The Ayurveda, the ancient Indian system of medicine has detailed mention of importance of cow milk, curd, ghee, urine in the treatment of various human ailments. All the five substances of Panchagavya possess medicinal properties and used to treat various disease conditions in human being either singly or in combination with some other herbs (Dhama *et al*. 2005). The distinct qualities of the five products of Panchagavya has not only used in the betterment of human life but also used as growth promoters in poultry and other fields of agriculture (Fulzele *et al.* 2002; Garg and Chauhan, 2003; Achliya *et al.* 2004; Saxena *et al.* 2004; Mathivanan *et al*, 2006).

In the last few years probiotics have constantly gained increased importance and aroused growing interest in animal nutrition. To maintain the intestinal microflora balance in animals it is important to prevent diseases by controlling the overgrowth of potentially pathogenic bacteria. Probiotics are defined as live microbial food supplements beneficial to health and have a positive effect in the prevention and treatment of intestinal microbial balance (Kullisaar *et al*. 2002). *Lactobacilli* are an extremely important group of probiotics bacteria that inhibit undesirable microflora in the gut and create a healthy equilibrium between beneficial and potentially intestinal pathogen (Bhutada *et al*., 2011). Lactic acid bacteria comprise a wide range of genera and include a considerable number of species. These bacteria are the major components of starters used in fermentation in dairy products and some of them are natural components of the gastro intestinal micro flora (Coeuret *et al.* 2003). The rise in antibiotic resistant bacteria has awakened the scientific community to the prophylactic and therapeutic uses of probiotics and to consider them as alternative to antibiotics (Ahmed 2003). Increased antibiotic usage in animal nutrition is a key factor in the emergence of antibiotic resistant pathogens. Thus there is an urgent need to control infections through a non antibiotic approach. Considering the above facts in mind, the present study was undertaken to enumerate, isolate and characterize indigenous *Lactobacillus* spp. from Panchagavya and to

assess their *in vitro* anti-bacterial activity against some common human pathogens for its possible use as probiotic supplement in poultry feed.

## MATERIALS AND METHODS

### Preparation of Panchagavya

Panchagavya was prepared by mixing fresh cow dung-5 kg, Cow urine -3 lit, Cow milk-2 lit, Cow curd-2 lit, Cow ghee-1 lit, Sugarcane juice-3 lit, tender coconut water-3 lit, ripped banana-12 Nos and toddy-2 lit as prescribed by Natarajan (2003). Fresh cow dung was mixed with ghee in the plastic container and kept for three days at room temperature and was thoroughly mixed daily. On fourth day the other ingredients namely cow urine, milk, curd, sugarcane juice, tender coconut water and toddy were added. The mixture was mixed properly and covered with nylon net to prevent entry of flies. The container was then placed in shade and mixed thoroughly twice daily for 15 days. The Panchagavya sample was analyzed at $15^{th}$, $30^{th}$, $45^{th}$ and $60^{th}$ days of fermentation.

### Measurement of pH

The pH of Panchagavya at different days of fermentation viz. $15^{th}$, $30^{th}$, $45^{th}$ and $60^{th}$ days was measured using digital pH meter. Panchagavya sample was prepared by diluting 1 g Panchagavya with 9 ml distilled water (1:9), the mixture was then filtered. The filtrate was used for the study.

### Enumeration of bacteria

Bacteriological examination and enumeration was done for Panchagavya at different days of fermentation ie., $15^{th}$, $30^{th}$, $45^{th}$ and $60^{th}$ day. 1 ml of Panchagavya was mixed well with 9 ml of sterile saline and vortexed to ensure uniform mixing ($10^{-1}$ dilution). Serial dilution of the samples were done in sterilized Laminar air flow bio-safety cabinet, using 9 ml of Sterile saline in test tubes up to a dilution of $10^{-10}$ and the contents in the tubes were mixed well. Total bacterial count was enumerated on plate count agar and *Lactobacillus* species count enumerated on MRS agar. 100 µl from each of the dilutions of $10^{-1}$ to $10^{-10}$ were inoculated into plate count agar and MRS agar plates in aseptic conditions using sterile micropipette tips by spread plate technique. The plate count agar plates were incubated in inverted position at 37ºC under aerobic conditions for 24 hrs and MRS agar plates under anaerobic conditions for 48 hrs. On completion of the incubation period, the plates were examined for the colonies and expressed as colony forming units (cfu) / g of the sample.

Cfu / g = No. of colonies in the plate X volume of the sample X dilution factor

**Isolation and identification of Lactic acid bacteria**

Colonies from the MRS agar palates were picked and inoculated in MRS broth and incubated at 37°C for 42 hrs in anaerobic jar. The bacterial isolates were examined and identified based on their morphological characteristics using Gram reaction and microscopic study using standard staining procedure. The biochemical identification was performed by catalase tests ,nitrate reduction test, citrate utilization test, Oxidase test and their ability to ferment sugar and its derivatives like glucose, lactose, sorbitol, arabinose and adonitol using the biochemical identification kit (TULIP DIAGNOSTIC (P) LTD, Goa – 403722, India).

**Preparation of cell free supernatant**

Following the identification of *Lactobacillus* the pure culture isolates were grown in 50 ml MRS broth and incubated at 37ºC for 48 hrs anaerobically. The broth culture was then centrifuged for 10,000 rpm for 20 mins and the cell free supernatant that may contain crude bacteriocin was collected in a fresh sterile tube and used to screen inhibitory activity against the test organisms while the pellet was discarded. The test organisms used in the study were *Escherichia coli, Pseudomonas aeruginosa, Bacillus cereus, Bacillus subtilis and Staphylococcus aureus*. The test cultures were obtained from the Microbiology laboratory of Ethiraj College for Women, Chennai and the cultures were maintained at 4°C on nutrient agar (Hi Media) slants.

**Antibacterial activity of *Lactobacillus* species**

The antibacterial activity of *Lactobacillus* species was performed by Agar well diffusion method according to Tagg and Mc.Given (1971). 20 ml of sterile Muller Hinton agar (Hi Media) was poured in sterile petri dishes and allowed to solidify. The test cultures were spread on the surface of the Muller Hinton agar plates using sterile cotton swabs and 8 mm bores were made on the medium using sterile borer each at equidistant from one another. To this 20 µl, 40 µl and 60 µl of the cell free supernatant were added to respective bore and the plates were incubated at 37°C for 24 hrs and Zone of inhibition was measured. A reference standard of 20 µl of gentamycin (4 mg/ml) was used as positive control to compare with the test results.

**RESULTS**

**Measurement of pH**

The pH of the Panchagavya sample was recorded using Digital pH meter and the pH was 5.10 ± 0.05 in 15$^{th}$ day, 4.43 ± 0.12 in 30$^{th}$ day, 3.97 ± 0.06 in 45$^{th}$ day and 3.73 ± 0.03 in 60$^{th}$

day of fermentation. The pH was found to decline from 15$^{th}$ day to 60$^{th}$ day of fermentation in Panchagavya.

**Enumeration of bacterial count**

The total bacterial count in Panchagavya was enumerated in Nutrient agar using spread plate technique and the number of bacteria was expressed as CFU/ml. The bacterial count was found to be 12.90 ± 1.56 x 10$^3$ in 15$^{th}$ day, 18.80 ± 0.92 x10$^3$ in 30$^{th}$ day, 10.70 ± 1.42 x10$^3$ in 45$^{th}$ day and 7.43 ± 1.69 x 10$^3$ in 60$^{th}$ day of fermentation of Panchagavya. The bacterial count observed to increase from 15$^{th}$ day till 30$^{th}$ day of fermentation of Panchagavya and the count decreased gradually from 45$^{th}$ day.

The *Lactobacillus* count was enumerated in MRS medium using spread plate technique and the results are expressed in terms of CFU/ml. The cell count recorded was 11.50 ± 0.66 x 10$^3$ in 15$^{th}$ day, 14.10 ± 0.93 x 10$^3$ in 30$^{th}$ day, 19.20 ± 0.86 x 10$^3$ in 45$^{th}$ day and 12.70 ± 0.40 x 10$^3$ in 60$^{th}$ day of fermentation of Panchagavya. The result recorded an increase in *Lactobacillus* count till 45$^{th}$ day and decreased cell count after 45$^{th}$ day till 60$^{th}$ day of fermented Panchagavya.

**Isolation and biochemical identification of *Lactobacillus***

The results of the study revealed that the organisms were found to be purple colored Gram-positive rods of varying sizes under oil immersion microscopy. The isolates also reported the absence of enzymes like catalase, cytochrome c oxidase, decarboxylase, urease, deaminase, nitrate reductase. The test recorded that the isolates does not utilize citrate as carbon source and have the ability to ferment carbohydrates such as glucose and lactose.

**Antibacterial activity of *Lactobacillus* species**

The result showed that the crude supernatant obtained by centrifugation of *Lactobacillus* culture broth inhibited the growth of test organism such as, *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus* While no growth inhibition was observed for *Escherichia coli,* and *Pseudomonas* species at any concentrations used. The highest zone of inhibition was recorded at 60 µl aliquot of 12.33 ± 0.88mm for *Bacillus cereus*, 18.67 ± 0.88 mm for *Bacillus subtilis*, 21.33 ± 0.88 mm for *Staphylococcus aureus*. However the growth of Bacillus *subtilis* and *Bacillus cereus* was not inhibited at lower concentration (20 µl). The results were expressed in terms of Zone of inhibition (mm). The test was performed by agar well diffusion method using aliquots of 20 µl, 40 µl and 60 µl of extract obtained from

*Lactobacillus* culture with Gentamycin (80 µg) as positive control that inhibited the growth of all test oraganism.

**Table 1: Bacterial load of Panchagavya at 15th, 30th, 45th and 60th day of fermentation (Mean ± SE).**

| S.No. | Days of fermentation | pH | Total bacteria count x $10^3$ (cfu/ml) | Lactobacillus species x $10^3$ (cfu/ml) |
|---|---|---|---|---|
| 1 | 15 | 5.10 ± 0.05 | 12.90 ± 1.56 | 11.50 ± 0.66 |
| 2 | 30 | 4.43 ± 0.12 | 18.80 ± 0.92 | 14.10 ± 0.93 |
| 3 | 45 | 3.97 ± 0.06 | 10.70 ± 1.42 | 19.20 ± 0.86 |
| 4 | 60 | 3.73 ± 0.03 | 7.40 ± 1.69 | 12.70 ± 0.40 |

n = 3

**Table 2: Biochemical identification of *Lactobacillus* species isolated from Panchagavya.**

| S. No. | Test | Results |
|---|---|---|
| 1 | Gram stainning | + |
| 2 | Lactose utilization | + |
| 3 | Glucose utilization | + |
| 4 | Catalase test | - |
| 5 | Nitrate reduction | - |
| 6 | Oxidase detection | - |
| 7 | Citrate utilization | - |
| 8 | Lysine decarboxylase | - |
| 9 | Ornithine decarboxylase | - |
| 10 | Urease production | - |

**Table 3: Antibacterial activity of *Lactobacillus* species isolated from Panchagavya against gentamycin (Mean ± SE)**

| Bacteria | Inhibitory Zone in diameter (mm) | | | |
|---|---|---|---|---|
| | Gentamycin | *Lactobacillus* species | | |
| | 80µg | 20µl | 40µl | 60µl |
| *Escherichia coli* | 19.00 ± 0.57 | Nil | Nil | Nil |
| *Pseudomonas aeruginosa* | 15.00 ± 0.57 | Nil | Nil | Nil |
| *Bacillus cereus* | 11.33 ± 0.88 | 8.00 ± 0.35 | 9.00 ± 1.00 | 12.33 ± 0.88 |
| *Bacillus subtilis* | 21.67 ± 0.88 | Nil | 10.67 ± 0.88 | 18.67 ± 0.88 |
| *Staphylococcus aureus* | 24.33 ± 0.33 | Nil | 17.67 ± 0.88 | 21.33 ± 0.88 |

n=3

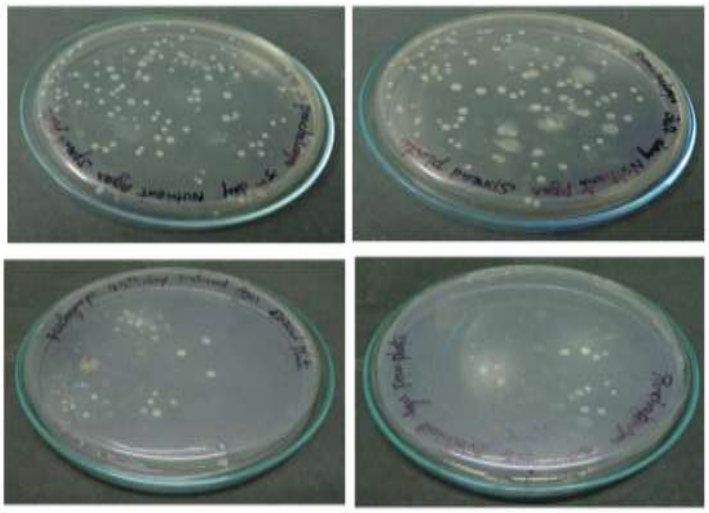

**Figure 1: Total bacterial count at 15th, 30th, 45th and 60th day old Panchagavya**

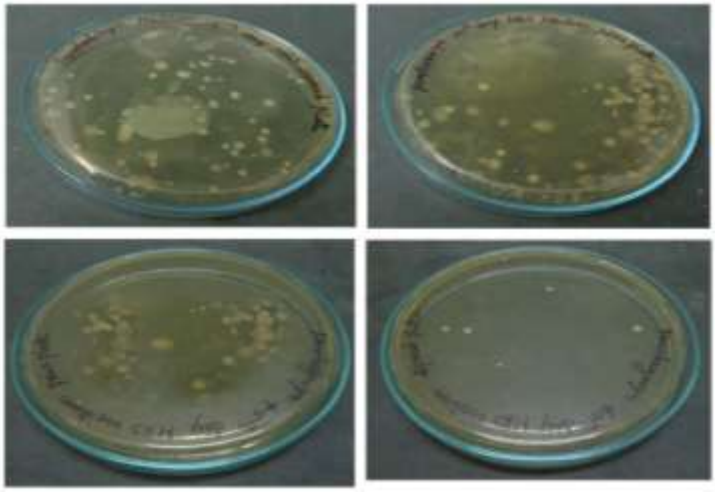

**Figure 2: Lactic acid bacteria count at 15th, 30th, 45th and 60th day old Panchagavya.**

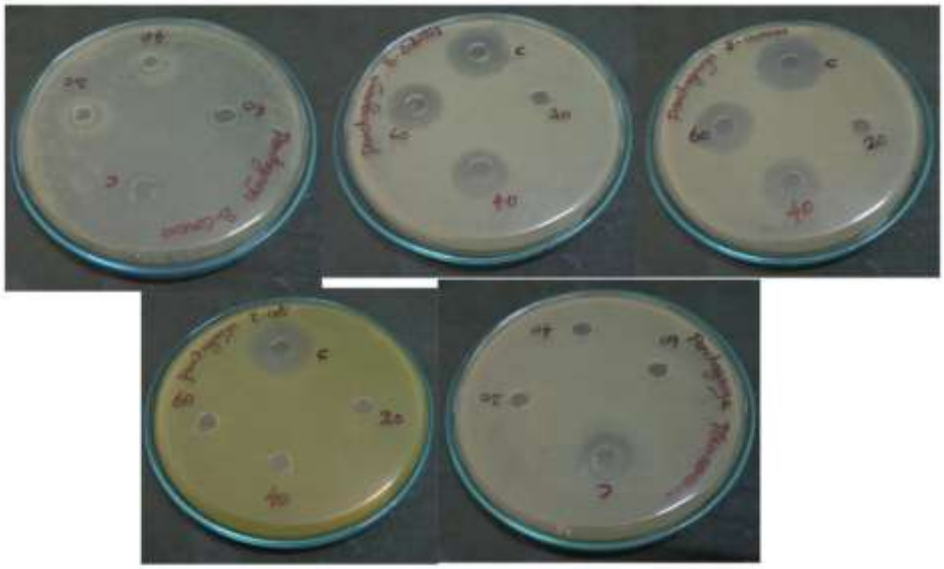

**Figure 3: Antibacterial activity of Lactic acid bacteria *isolated* from Panchagavya.**

## DISCUSSION

### Measurement of pH

pH is an important factor that affects almost all the physical, chemical and biochemical reactions that occur during the process of fermentation. The pH value of a solution is a way of expressing the acidity or alkalinity of that solution. The present study reported an acidic pH at the initial days and observed more acidic with the number of days of fermentation. The possible reason for decrease in pH might be due to the presence of *Lactobacillus* contained in the milk and curd of Panchagavya that ferment available carbohydrate to organic acid (lactic acid). Widyastuti *et al.* (2014) reported that well known characteristics of Lactic acid bacteria is their ability to produce acid that result in decrease in pH, which in turn also exhibit antimicrobial activity. The findings of this study are in accordance with the reports of Mathivanan *et al*. (2006) who reported a decrease in pH of Panchagavya with increase in days of fermentation. The findings in this study pertaining to decrease in pH with increase in age of Panchagavya might be attributed to production of organic acid due to degradation of Panchagavya components by Lactic acid bacteria and other microorganisms.

### Enumeration of bacterial count

Total Bacterial Count (TBC) represents the total bacterial load in the sample. It is a test to detect all viable microorganisms that could grow aerobically on plate count agar at appropriate incubation condition at 37$^{o}$C for 48 hr. The present study indicated an increase in bacterial count till 30$^{th}$ day of fermentation and gradual decrease in count was observed in 45$^{th}$ day with further decrease in 60$^{th}$ day of fermentation. Similar findings by Mathivanan *et al*. (2006) supports the fact that the microbial population declined after 30 days of fermented Panchagavya. The decline in bacterial count in later stages of fermentation might be possibly due to growth of lactic acid bacteria that generates acidic fermentation end products that accumulate in the extracellular environment. The organic acid production by these bacteria reduces the pH of the medium, which creates an unfavorable environment for the survival of other organisms.

*Lactobacillus* is one of the most important genera of Lactic acid bacteria (LAB) (Coeuret *et al.* 2003) that are considered as generally recognized as safe (GRAS) organisms and can be safely used as probiotics for medical and veterinary applications (Fuller, 1989). The present study showed a gradual increase in lactic acid bacteria till 45$^{th}$ day and declined later till 60 days old Panchagavya. Our result is in accordance with the findings of Mathivanan *et al*.

(2006) who observed an increase in *Lactobacillus* species count up to 30 days old Panchagavya and declined in later stage of fermentation. The fall in lactic acid bacteria count with increase in days of fermentation might be due to depletion of nutrients and acid stress environment of the medium. D'Aoust *et al.* (2001) also who reported that lactic acid bacteria declined in later stages of fermentation due to rapid increase in acidity and decline in pH in sauerkraut. Kailasapathy *et al*. (2008) also reported that low pH and organic acids accumulation were the important factors contributing to the loss of cell viability of probiotics.

**Isolation and identification of Lactic acid bacteria**

The Gram staining property of the isolate obtained from MRS broth, was found to be Purple coloured Gram positive rods of varying sizes, under oil immersion microscopy. This preliminary result indicated the isolates belong to *Lactobacilli* group and paved way for further confirmation by biochemical mention.

Glucose utilization test showed that the isolates produced gas in the broth containing glucose, which fermented glucose in the medium for their growth. The production of gas might be due to the fermentation of glucose and alcohol under anaerobic condition produce organic acids that may be accompanied by the production gas such as hydrogen peroxide or carbondioxide our findings is in accordance with the findings of Bhardwaj *et al*.(2012).

Catalase is an extracellular enzyme secreted by several organisms that is involved in breaking down of hydrogen peroxide produced during carbohydrate utilization for energy. Degradation of hydrogen peroxide produces molecular oxygen that is identified by generation of effervescence. Absence of effervescence is indicative of catalase negative and our isolates were found to be catalase negative. This result supports the identification of isolates as *Lactobacilli* and our result is in agreement with Bhardwaj *et al*.(2012).

Nitrate reduction test is based on the fact that that certain bacteria have the capability to reduce nitrate to nitrite while others capable of further reducing nitrite to ammonia. The formation of ammonia changes the pH of media to alkaline, thus changing the colour of media from yellow to cherry red. The isolates of our study recorded negative reaction as there was no red/pink colour, which are the characteristics of Lactobacillus group of organism and our result is in agreement with Bhardwaj *et al*.(2012).

Oxidase detection test is used to identify the bacterial production of enzyme cytochrome c oxidase that uses oxygen as terminal electron acceptor for respiration, which is an indicative of aerobic organisms. The isolates of our study was found to be oxidase negative, which is may be due to the anaerobic or facultative anaerobic characteristics of *Lactobacilli* and the study result is in agreement with the findings of Chowdhury *et al*.(2014).

Citrate utilization test, the isolates were subjected for their potential to utilize citrate as the sole carbon. The result of the study showed that the isolates were citrate negative that was indicated by the appearance of green colour of the medium. The study results are in agreement with the findings of Bhardwaj *et al*.(2012).

The results of biochemical tests like lysine decarboxylase, ornithine decarboxylase, urease, were found to be negative by the test isolates, which indicted the absence of organisms that produce enzymes such as decarboxylase and urease. The byproducts of these reactions are usually amines and ammonia that are sufficient to raise the pH of the medium by turning it alkaline. As our test isolate reported negative to the above reactions, it further confirms the presence of *Lactobacillus* groups of organisms that thrive in acidic environment.

The results of the above biochemical tests confirmed the presence of *Lactobacillus* genus in Panchagavya.

**Antibacterial activity of *Lactobacillus species***

Antimicrobial activity is one of the most important selection criteria for probiotics. The antibacterial activity of *Lactobacillus* species isolated from fermented Panchagavya was tested against Gram positive: *Bacillus cereus, Bacillus subtilis*, *Staphylococcus aureus* and Gram negative: *Escherichia coli* and *Pseudomonas aeruginosa* using Gentamycin as control. The results of the study implied that the control Gentamycin inhibited the growth of all the test strains. The crude cell free supernatant separated from *Lactobacillus* broth exhibited antibacterial activity against gram positive strains *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus* however the growth of *Bacillus subtilis* and *Staphylococcus aureus* was not inhibited at 20 µl but showed inhibition at 40 and 60 µl doses. The increase in concentration of cell free supernatant resulted in increase in inhibitory activity of *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus* while could not inhibit the growth of gram negative bacteria like *Escherichia coli* and *Pseudomonas aeruginosa* at all doses tested. Our result is in accordance with the earlier reports of Gilliland and Speck (1977) and

Savadogo *et al*.(2004) who reported that lactobacilli showed stronger antibacterial properties against most Gram positive bacteria than gram negative bacteria. Anas *et al.* (2008) also observed a similar result with *Lactobacillus* isolated from Algerian raw goats milk which recorded sensitive to *Staphylocotococcus aureus* and *Bacillus* than *Escherichia coli* however our result was not in accordance to the findings of Ashokkumar *et al*. (2011) who reported that crude cell free supernatant obtained from *Lactobacillus paracasei* isolated from donkey milk showed a highest inhibitory activity against *Escherichia coli* and *Pseudomonas aeruginosa* and lowest activity against S*taphylococcus aureus.* Chowdhury *et al*.(2012) also reported that four *Lactobacillus* species isolates from buffalo yoghurt was both bactericidal and bacteriostatic against Escherichia coli, Pseudomonas aeruginosa, *Bacillus cereus, Bacillus subtilis* and *Staphylococcus aureus.* The antibacterial activity of lactic acid bacteria might be due to the presence of organic acid, hydrogen peroxide and bacteriocins produced during lactic acid fermentation. (Talarico and Dobrogosz, 1989; Ouwehand, 1998; Oyetayo *et al*. 2004). The organic acids produced by Lactic acid bacteria not only lower the pH, and affect the growth of the pathogen and could also be toxic to the microbes (Alvarez-Olmos and Oberhelman, 2001) and the bacteriocins which are antimicrobial peptides of bacterial origin are lethal to bacteria other than the producing strain (Tagg *et al*. 1976).

The result of the study indicates that the lactic acid bacteria strains are capable of synthesizing inhibiting substances that act differently against pathogenic reference bacteria. The resistance of gram negative bacteria might be attributed to the particular nature of their cellular envelope and binding of the inhibiting substance to produce bactericidal effect. Bhunia *et al*.(1991) reported that bacteriocin produced by *Pediococcus acidilactici* interacts with lipoteichoic acids in gram positive bacteria and play the role of site specific to produce bactericidal effect. These variations in sensibility might be possibly due to the presence of serotype, presence or absence of binding sites in test strains and levels of cell free extract used to causestige cellular damage.

## CONCLUSION

The present investigation has detected that increased fermentation time affects lactic acid bacteria count of Panchagavya and the study concluded that lactic acid bacteria isolates from Panchagavya are capable of synthesizing inhibiting substances that showed a strong antibacterial potential against gram positive bacteria than gram negative bacteria. Thus for the use of Panchagavya as probiotics supplement in animal feed for the maintenance of gut

health, further studies are required to identify species of lactobacilli and completely understand the antimicrobial substances produced and other contributing factors towards the antibacterial activity.

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