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# Isolation and Molecular Identification of Probiotic Bacteria Genus *Lactobacillus* in an Asian Elephant (*Elephas maximus*) of Tamil Nadu

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**A B S T R A C T**

Genus *Lactobacillus* was common probiotic key genera in gastro intestinal tracts of domestic animals as well as wild animals. The species compositions of probiotic bacteria are vary depending up on the animal species. The aim of this study was to isolate and molecular identification of genus *Lactobacillus* from dung samples of captive Asian elephants (*Elephas maximus*) reared in temples of Tamil Nadu state in India. Twenty five fresh dung samples of Asian elephants (*Elephas maximus*) were aseptically collected from various temples of Tamil Nadu state and transferred immediately to the laboratory. Isolation of *Lactobacillus* by culturing the dung samples using *Lactobacillus* MRS Broth M369 and molecular confirmation by PCR using BF1, BR1 and Lacto F, Lacto R primers. After 72 hours incubation, *Lactobacillus* are appeared as large whitish coloured colonies. On Gram staining, *Lactobacillus* organisms appeared as Gram positive long slender-rods. PCR revealed evidence of Genus *Lactobacillus with* amplicon sizes were 233 bp;1550 bp. Since Genus *Lactobacillus* are common probiotic bacteria in the gastro intestinal tract of plant eating herbivores.

## Introduction

The gastrointestinal tract (GIT) of animals is a habitat of a complex collection of microorganisms, with large differences between individuals and animal species (Walter 2008). The largest land living herbivores are elephants. The anatomy and physiology of the elephant gastrointestinal tract similar to what is found in the horse,

where a relative simple stomach is followed by a voluminous small and large intestine required for the function of caecal fermentative digestion through the metabolism of a complex microflora (Bojesen *et al.*, 2006). *Lactobacillus* probiotic bacteria are considered as important genera in the intestinal tracts of animals. They are common microflora of ruminant herbivores (Russell *et al.*, 2011) and have also been isolated from faeces of marmoset and red-handed tamarin South Africa (Endo *et al.*, 2010 and 2012). Their presence in high numbers is associated with good health status of the host. *Lactobacillus* is helpful in maintaining appropriate balance of the microbiota in the GIT, reducing the risk of pathogen infection. *Lactobacillus* represents one of the most dominant groups and some *Lactobacillus* species are frequently used as the probiotic ingredient for humans and also animals (Turroni *et al.*, 2011; Russell *et al.*, 2011). *Lactobacillus* occurrence and species composition in different animals is very variable. The objective of this study was to isolate and molecular identification of genus *Lactobacillu*s from dung samples of Asian elephant by culture and PCR technique.

## Materials and Methods

Twenty five Fresh dung samples of Asian elephants (*Elephas maximus*) collected from various temples of Tamil Nadu state were investigated (Plate 1**)**. Collected dung samples were aseptically transferred in ice bags and transported immediately to the laboratory. *Lactobacillus* MRS broth M369 was first enriched with Lactic acid supplement FD055 and was used for the screening specimens. The dung samples (n=25) were centrifuged and the concentrated bacterial mass (sediment) were inoculated in enriched *Lactobacillus* MRS broth M369 and were incubated at $42^0$C for 24 hours. The enriched culture was later streaked in to selective plating media such as *Lactobacillus* MRS agar M641 developed by Kristeisen *et al.*, (1925) and modified by Kauffman (1935).

The MRS plates were incubated at $35^o$C at 5% $CO_2$ level, as recommended by the standard literatures. The colony growth was monitored, on daily basis. After 72 hrs of incubation, smear prepared from developed colonies and stained with Gram staining.

The positive colonies of probiotic bacteria were transferred into 1.5 ml Eppendorf tubes containing 250 µl of nuclease free water, mixed well and were centrifuged at 6000 rpm for 5 minutes. After centrifugation, the supernatant was discarded. The pellets were re-suspended with nuclease free water and the tubes were sealed with parafilm. The tubes were kept in boiling water bath at $90^0$ C for 10 minutes and were again centrifuged at 6000 rpm for 5 minutes. The supernatant was used as DNA template for confirmation of probiotic bacteria by PCR. These Primers were used for molecular identification of probiotic bacertia *Genus Lactobacillus*

## Polymerase chain reaction (PCR)

The reaction mixtures were prepared in 25 µl volumes (12.5 µl of Red dye master mix, 2 µl of 10 picomol concentrations of Forward primer and Reverse primer, 4 µl of 50 ng concentration of DNA template and 4.5 µl Nuclease free water). The PCR amplification was carried out in Eppendorf Mastercycler (Eppendorf, Germany) with the following thermal programmes and the annealing temperature was standardized, according to the primer used.

## Agarose gel electrophoresis

The PCR products were tested for amplification by agarose gel electrophoresis on 1.5% agarose w/v gels by loading 10 µl of PCR product into the wells and 100 bp DNA

(GeNei) ladder was used as a marker for the products that were less than 1000 bp. A current of 120 V was applied to each gel and the PCR products were visualized under UV trans-illumination (Table 1 and 2).

## Results and Discussion

After 72 hours incubation the colonies of *Lactobacillus* organisms appeared as large whitish coloured colonies as shown in Plate 2. On Gram staining, the organisms were found to be Gram positive long slender-rods as shown in Plate 3. In this present study, the culture result was similar to that result founded by Biradar *et al.,* (2005). PCR revealed evidences of the Probiotic bacteria Genus Lactobacillus with amplicon sizes were 233 bp and 1550 bp as shown in Plates 4. Tannock (1999); Yin and Zheng (2005) and Ashraf (2009) reported that identification of most of the *Lactobacillus* by using MRS culture and 16S r-DNA technique was 98-99 % accurate. L*actobacilli* were commonly used as probiotics, either as single species or as mixed culture with other bacteria. *Lactobacillus* organisms were gram-positive, non -motile, rod shaped organisms that do not produce spores and were acid resistant and thrive in acidic conditions (pH 4-5; neutral pH is 7.0; blood is at a pH of 7.2) .

**Table.1** PCR primers

| Sequence (5´- 3´) | Amplicon Size (bp) | Reference |
|---|---|---|
| BF1: GAGTTTGATCATGGCTCAG | 235 | **(Fungsin *et al*., 2010)** |
| BR1: CGCTTACCTTGTTAGCGACTT | | |
| Lacto F: TGGAAACAGRTGCTAATACCG | 232 | **(Byun *et al*., 2004)** |
| Lacto R: GTCCATTGTGGAAGATTCCC | | |

**Table.2** PCR amplification programme

| Primer name: Forward and Reverse | PCR amplification programme | | | | | |
|---|---|---|---|---|---|---|
| | Initial Denaturation | Cyclic Denaturation | Annealing Temperature | Extension | Final Extension | Cycle No. |
| *Lactobacillus* | | | | | | |
| BF1 & BR1 | 94$^{o}$C for 5 min. | 94$^{o}$C for 1 min. | 51.7 $^{o}$C | 72$^{o}$C for 1Min | 72$^{o}$C for 7 Min | 35cycles |
| Lacto F & Lacto R | 94$^{o}$C for 5 min. | 94$^{o}$C for 1 min. | 59.5 $^{o}$C | 72$^{o}$C for 1Min | 72$^{o}$C for 7 Min | 35cycles |

**Plate.1** Collection of dung sample from elephant

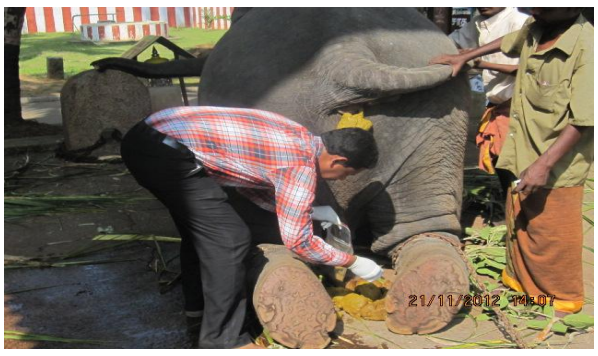

**Plate.2** *Lactobacillus* MRS agar – *Lactobacillus* (Large whitish Colonies)

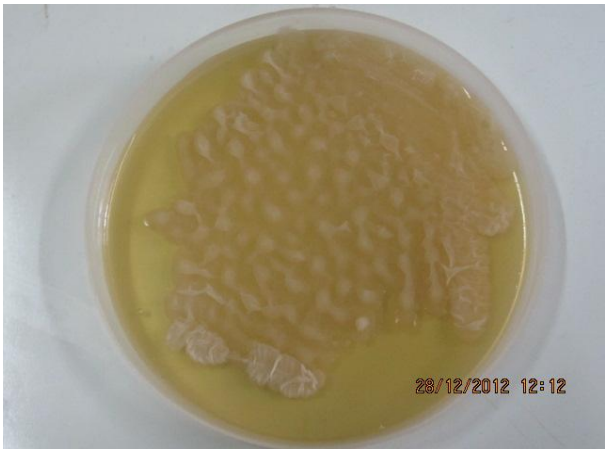


**Plate.3** Gram staining-Gram positive long slender-rod-*Lactobacillus* organisms

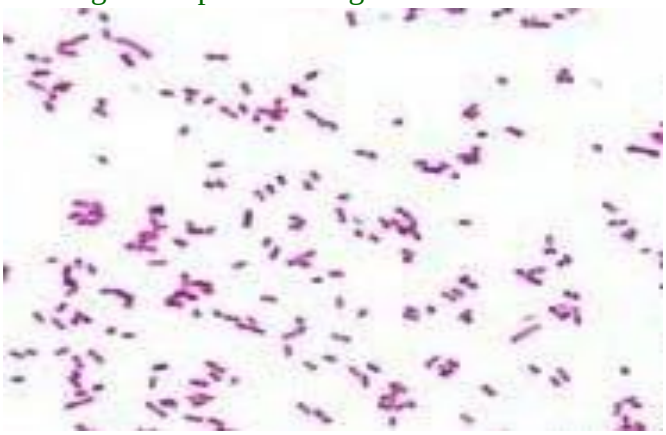

**Plate.4** PCR – Identification of genus *Lactobacillus*

The *lactobacillus* species were the dominant *lactobacillus* in the stomach, small intestine, large intestine and faeces/dung of humans and animals and were also found in milk and milk products (Mitsuoka, 1992). The posterior environment of the gut is better for the growth of lactobacilli-like bacteria than the anterior environment due to the low pH in the stomach, bile salts in the small intestine and oxygen in the faeces. Lactic acid bacteria were reported to reveal several health-promoting effects on host animals (Ouwehand *et al*., 2002). Feeding practices and the composition of animal diets can influence the microbial balance and composition of microflora in the gastrointestinal tract (Chaucheyras-Durand and Durand 2010). *Lactobacillus* species were identified also in the dung of other animals' primary ingesting plants (Russell *et al.*, 2011).

In conclusion, to our knowledge, this study is the first description of Genus *Lactobacillus* from elephant dung's in India. These isolates were identified as Genus *Lactobacillus* seems to be common species occurring in the GIT of herbivores including Asian elephants.

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