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Non-invasive Analysis of Progesterone Metabolites in *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* Supplemented Endangered Asian Elephants

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ABSTRACT

Non-invasive analysis has become a legitimate choice in wildlife biology, endocrine monitoring of reproduction and global ecological research. The development of non-invasive progesterone hormone assays facilitates the conservation and management of wild animals as the population of wildlife, especially endangered Asian elephants, is being declined in their innate habitat. The role of live microbial cultures in endocrinology has also been stated and gut microbiota was shown to affect the production of steroid hormones in the gut. This research was carried out to assess the impact of the supplementation of probiotics on faecal progesterone metabolite concentrations in 18 female Asian elephants. They were randomly divided into three groups with six elephants in each. Experimental probiotics *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* were administered @ 1 gm 1×10^9 CFU / 50 kg body weight per day orally to all the elephants of LACTO (T2) and SAC (T3) groups, respectively, whereas the CONT (T1) group received no probiotic. Faecal samples were taken on the 0, 30th, 40th and 50th day of the research for hormone analysis. In conclusion, the supplementation of probiotics did not exhibit any significant difference in faecal progesterone metabolite concentrations in the different groups.

KEYWORDS: Asian elephant, Non-invasive, Probiotics, Progesterone metabolite

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INTRODUCTION

To encourage breeding management, genetic diversity and the conservation of the elephant population, the evaluation of female reproductive functioning is necessary (Kajaysri and Nokkaew, 2014). Non-invasive progesterone hormone assays are crucial for detecting pregnancy and monitoring fertility status as well as enables the conservation and management of wild animals (Umapathy et al., 2013). Diet can change the way hormones are metabolized, and different diets can change the volume of faecal bulk. Bacteria metabolize hormones in the gut which allow the metabolizing steroid hormones by way of stimulation of different enzymes (Hussain et al., 2021 and Lolou, 2021). Individual differences in bacterial composition through dietary manipulations by supplementing live microbial cultures can cause differences in how hormones are decomposed. Due to changes in metabolism and excretory pattern, animals excrete different steroid metabolites in faeces (Goymann, 2012).The objective of the current study was to explore the role of supplementation of probiotics, i.e., *Lactobacillus acidophilus* and *Saccharomyces cerevisiae,* on progesterone metabolite concentrations in Asian elephants.

MATERIALS AND METHODS

Animals and experimental plan

The present study was conducted at Elephant Village, Jaipur (India) (26°59’47"N 75°52’35"E). The study protocol was duly approved by the Institute Animal Ethics Committee (PGIVER/IAEC/I9-05) and performed in accordance with relevant guidelines and regulations. A total of 18 adult captive female Asian elephants with ages ranging from 30 to 62 years were divided randomly into three groups with six elephants in each. The elephants were stall-fed a consistent diet of green pearl millet forage as basal feed throughout the research period, i.e., for a period of two months, including preliminary ten days for adaptability with basal feed and fifty days for the feeding trial of probiotics. During the feeding trial of 50 days, experimental probiotics *Lactobacillus acidophilus* and *Saccharomyces cerevisiae* were administered @ 1 gm 1 × 10^9 CFU / 50 kg body weight per day orally along with basal feed to all the experimental elephants of LACTO (T2) and SAC (T3) groups, respectively. The experimental group T1 was the CONT group received no probiotics.

A total of 72 fresh faecal samples were collected from the experimental elephants of all three groups on days 0, 30^{th}, 40^{th} and 50^{th} of the feeding trial. Faecal samples were collected between 8:00 to 9:00 hrs in the morning. Freshly collected dung samples were oven-dried the same day at 60 °C - 80 °C, pulverized and stored in sealed containers with silica beads at 4 ºC. These samples were transferred to the freezer (- 20 °C) within a week and stored until further analysis (Hunt and Wasser, 2003).

Hormone analysis

The faecal samples were extracted according to the previously described procedure (Ganswindt et al., 2005; Kumar et al., 2014). The pulverized faecal sample was sieved, and 0.2 gm of faecal powder was transferred to a 15 mL falcon tube. Five mL of 80% methyl alcohol was added to it, and it was vortexed vigorously at least for 20 min. The sample was then centrifuged at 3300×*g* for 20 minutes, and then the supernatant was transferred into 5 mL of a cryogenic vial and stored at - 30 ºC until further assay. The extraction efficiency was measured by adding a known amount of ^{3}H labelled steroids to faecal samples before extraction (Umapathy et al., 2013; Kumar et al., 2014). Extraction efficiency was found to be 80.16 ± 2.3% for progesterone.

Faecal progestogen metabolites levels were estimated using a progesterone monoclonal antibody (Quidel clone No. CL 425, Dr. Coralie Munro, University of California, Davis) diluted to 1:6000; horseradish peroxidizes (HRP) conjugated progesterone 1:100,000 (C. Munro, University of California, Davis) and progesterone standards (200–0.39 pg/well). The antibody cross-reacts with progesterone (100%) and a variety of declined pregnane metabolites as assessed by Graham et al. (2001).

Statistical analysis

The experimental data were subjected to statistical analysis (SPSS version 24) using a one-way analysis of variance described by Snedecor and Cochran (2004). Significance was defined at $P < 0.05$. All the values represent mean ± standard errors of the mean.

RESULTS AND DISCUSSION

Only limited literature is available on the evidence of live microbial cultures' contribution to animals' faecal progesterone levels. Usually, the main progesterone hormones in faeces are in the form of progesterone metabolites (5á-pregnane-3, 20-dione [5á-dihydroprogesterone: 5á-DHP] and 5á-pregnane-3-ol-20-one [5á-P-3OH]) (Kajaysri and Nokkaew, 2014). The present study investigated the monitoring of faecal progesterone metabolite concentrations in response to dietary probiotics in faecal samples of eighteen captive female Asian elephants at different periods. The health status of elephants was observed to be normal as they were regularly monitored as part of management. It is noteworthy to mention here that all the elephants used in this study were non-pregnant. Detail of the result observed in this study is shown in Tables 1 and 2.

Table 1. Detail of registration number, age, body weight and faecal progesterone metabolite concentrations in Asian elephants of different groups

Groups	Name of elephant	Registration	Age	Bodyweight	Overall mean
CONT (T1)	Jaimala	11	41	2874	162
	Rajrani	20	56	2964	149
	Phoolwanti	116	30	3414	100
	Jhomati	53	48	3684	199
	Chameli	123	44	3234	899
	Jaytara	92	45	4332	305
LACTO (T2)	Laxmi	125	47	2928	239
	Laxmi	130	52	3144	84
	Anno	93	48	3234	354
	Tami	109	35	3504	192
	Gomati	81	33	3936	134
	Shobha	96	40	4152	100
SAC (T3)	Bhogwati	30	49	2424	183
	Champa	105	33	3018	180
	Rangoli	43	44	3792	335
	Majani	55	50	3684	147
	Champakali	52	50	4152	167
	Chanchal	9	62	4440	404

The mean faecal progesterone metabolite concentrations per elephant varied from 143.48 to 1219.00 ng/g (mean ± SEM; 526.00 ± 164.30 ng/g), 99.78 to 1003.22 ng/g (mean ± SEM; 361.67 ± 143.08 ng/g), and 196.21 to 1010.12 ng/g (mean ± SEM; 363.00 ± 130.56 ng/g) dry weight of faeces in the animals of T1, T2, and T3 groups, respectively on 0 day, 91.0 to 953.91 ng/g (mean ± SEM; 291.17 ± 134.49 ng/g), 88.68 to 313.70 ng/g (mean ± SEM; 173.83 ± 37.19 ng/g), and 134.41 to 422.99 ng/g (mean ± SEM; 223.17 ± 44.25 ng/g) dry weight of faeces in the animals of T1, T2, and T3 groups, respectively on 30th day, 72.90 to 1151.67 ng/g (mean ± SEM; 276.00 ± 175.33 ng/g), 42.99 to 256.34 ng/g (mean ± SEM; 107.00 ± 31.50 ng/g), and 70.69 to 487.10 ng/g (mean ± SEM; 200.83 ± 60.82 ng/g) dry weight of faeces in the animals of T1, T2, and T3 groups, respectively on 40th day, 55.91 to 272.64 ng/g (mean ± SEM; 116.00 ± 32.12 ng/g), 55.81 to 147.28 ng/g (mean ± SEM; 92.67 ± 15.84 ng/ g), and 97.06 to 380.83 ng/g (mean ± SEM; 157.00 ± 45.25 ng/g) dry weight of faeces in the animals of T1, T2, and T3 groups, respectively on 50th day.

Table 2. Mean faecal progesterone metabolite concentrations (ng/g) in Asian elephants of different groups

Groups	0 day	30 days	40 days	50 days
CONT (T1)	526 ± 164.3	291 ± 134.49	276 ± 175.33	116 ± 32.12
LACTO (T2)	361 ± 143.08	173 ± 37.19	107 ± 31.5	92 ± 15.84
SAC (T3)	363 ± 130.56	223 ± 44.25	200 ± 60.82	157 ± 45.25
Overall	416 ± 81.70	229 ± 47.30	194 ± 61.28	121 ± 19.18

The statistical analysis of progesterone metabolite concentrations revealed non-significant differences ($P > 0.05$) among the groups due to the treatment. Though the differences were non-significant in statistical terms but apparently on observing the data, the mean values obtained for probiotics-supplemented groups were found to be lower on the 30th and 40th day, whereas found to be lower in the T2 group and higher in the T3 group on the 50th day as compared to the control group. However, mean progesterone metabolite concentrations were found to be highest in the control group and lowest in the T2 group at 0, 30th and 40th day, whereas found to be highest in the T3 group and lowest in the T2 group on the 50th day. The concentration level gradually decreased up to the 50th day in all the respective groups. Therefore, none of the probiotics exhibited any significant difference in faecal progesterone metabolite concentrations of the elephants of the LACTO and SAC groups with the elephants of the CONT group.

The mean faecal progesterone metabolite concentrations observed in the present study were more or less similar to Foley et al. (2001) (562 ± 60 ng/g) prior to pregnancy in free-ranging African elephants. In other studies, the mean faecal progesterone metabolite concentrations were found to be 160 ± 60 ng/g and 1970 ± 2050 ng/g Ghosal et al., 2012) and 98.54 ± 8.02 and 619.45 ± 22.68 ng/g (Kumar et al., 2014) observed for cycling female Asian elephants during the follicular and luteal phases, respectively. In general, faecal progestagen concentration varied from 1180 ± 540 to 3350 ± 450 ng/g with an average of 2500 ± 710 ng/g during pregnancy until the pre-partum period. It was highest during the 12th month of gestation in Asian elephants. Faecal progestagen remained higher than 3000 ng/g from the 7th until the 15th month and then decreased expeditiously to reach its least level in the 22nd month of gestation (Kajaysri and Nokkaew, 2014).

Contrary to the above findings, Fidel et al. (1998) found that bacterial-induced lipo-polysaccharide supplementation in pregnant mice and ascending intrauterine infection in pregnant rabbits re-sult in rapid progesterone decline and preterm parturition. Foley et al. (2001) also reported that progesterone metabolites decreased with dietary changes that occurred like probiotics administration in free-ranging African elephants. Newbern and Freemark (2011) quoted that any alterations in gut microbiota during pregnancy and an increase in gestational age led to differences in progesterone production. Antwis et al. (2019) have opined that reproductive hormones and intestinal microbial community composition are influenced in cycling, pregnant, and lactating individuals. Further, a significant relationship was observed in the gut microbial composition of black rhinoceros for successful breeding and ovarian cycle. Here, a significant relationship was observed between intestinal micro-biomes and progesterone levels. Research on wild Phayre's leaf monkeys explained that the intestinal micro-biome composition affected the reproductive stage. In females, diminished microbial diversity during pregnancy, along with faecal progesterone levels were negatively associated with diversity. Dysbiosis in intestinal micro-biomes function affected hormonal activity (Mallott et al., 2020).

CONCLUSION

Supplementation of probiotics, i.e., *Lactobacillus acidophilus* and *Saccharomyces cerevisiae,* did not exhibit any significant difference in faecal progesterone metabolite concentrations in the Asian elephants of different groups. However, slight differences might be due to the variations in management practices and may rely upon individual animals' physiological status.

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