Inhibitory action of thymol in fecal microbial activity of *Tamandua tetradactyla* and its effect on glucocorticoid metabolite measurements

Ruiz M.B.[1], Busso J.M.[1,2], Eguizábal G.V. [1,2], Villarreal D. P. [3], López A.G[1].

[1] Instituto de Ciencia y Tecnología de los Alimentos and Facultad de Ciencias Exactas, Físicas y Naturales (FCEFyN), Universidad Nacional de Córdoba (UNC), Argentina. [2] Instituto de Investigaciones Biológicas y Tecnológicas, CONICET, and FCEFyN, UNC, Argentina. E-mail address: jmbusso@conicet.gov.ar [3] Jardín Zoológico de Córdoba, Argentina.

Introduction

Non-invasive hormonal monitoring has potential to improve wildlife conservation, but there are methodological concerns. Faeces require storage methods to avoid steroid degradation by faecal microorganisms. Freezing is recommended; however, it is a costly method under non-controlled environmental conditions. This study aimed at developing an alternative transport and storage method for faeces, using a natural antimicrobial agent, which can be used in field studies and when no equipment for freezing is available, expanding the application in non-invasive hormonal monitoring studies. For this, the purpose was to evaluate if thymol reduces the proliferation of microorganisms in faeces from *Tamandua tetradactyla* during post-excretion time and if it may affect faecal glucocorticoid metabolites (FGM) measurements.

Methodology

Faeces collection and treatment conditions

Fresh faeces from zoo-housed collared anteaters (2♀ y 3♂) were collected using sterile cups, between 09:00 and 12:00 AM. Then faeces were divided into fractions (5.5g each). Then faeces were divided into fractions (5.5g each) that were kept in sealed glass Petri dishes at 22±2°C into moist chambers. In *control* chamber 500 µL of ethanol (80%) were applied to four fractions, meanwhile in *treatment* chamber 500 µL of an ethanol (80%) solution of thymol (5mg g^{-1} faeces) were applied to other four fractions. Faecal fractions from both chambers were evaluated at a temporal series (0, 24, 48 and 72 h; assigning each fraction to each time randomly). When time of exposition was finished, faecal fractions were divided in two parts, one (5g) for immediate microorganism recount and other (0.5g stored at -20°C) for hormonal analysis. Additionally, considering possible effects of ethanol on hormonal measurements, negative control consisted of faecal fractions without ethanol or thymol were evaluated at 0, 24, 48 and 72 h.

Microbial assessment

Total aerobic and anaerobic heterotrophic mesophilic bacteria (AHB and ANHB), spore-forming bacteria (SFB), total coliforms (TC), yeast and mould (Y and M) were counted as colony forming units (CFU) per gram of faeces. Microbial analyses were performed in triplicate.

Measurement of faecal glucocorticoid metabolites

For hormone analysis, frozen samples (0.5g) were dried (60°C). Faecal glucocorticoid metabolites were extracted by adding 5mL methanol/water (80%) according to dried faecal sample weight (in proportion 10:1) of each well-homogenized sample. After vortexing (2 min) and centrifuging (15 min, 3,100G), an aliquot (0.5 mL) of the supernatant was separated for hormonal analysis. The content was evaporated at 60 ℃, sent to the laboratory for hormonal analysis, and redissolved in enzyme immunoassay (EIA) buffer there. All measurements were run in duplicate . Cortisol Elecsys® Immunoassay System was applied.

Results

Microbial assessment

A significant inhibitory effect of thymol on the count of TC (Figure 1), AHB, ANHB, M and Y was observed ($p<0,05$), with a reduction of up to three orders of magnitude being observed on the CFU g^{-1} for samples treated during 24, 48 and 72 h with thymol. By contrast, thymol had no effect on SFB.

Measurement of faecal glucocorticoid metabolites

FGM concentrations were significantly different between treatments (negative control: 1251±118 > thymol: 986±98 > control: 792±77 ng.g^{-1} dry faeces; $p<0.05$; Figure 2).

When analysing the effect of post-excretion time on FGM measurements, treatments show no differences across temporal series. Although, a reduction tendency was observed at 24 h.

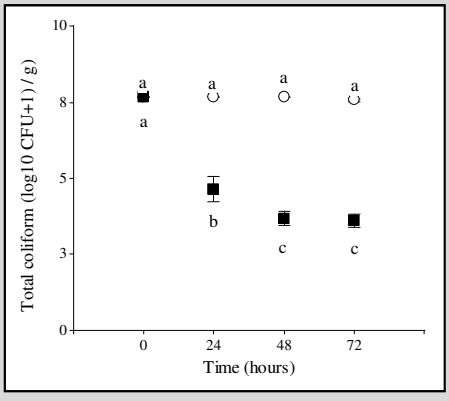

Figure 1. Total coliforms (TC) count from faecal samples of *Tamandua tetradactyla* at 0, 24, 48 and 72 h. Samples were treated with 5 mg.g^{-1} faeces of thymol in 500 µL of 80% ethanol (■). Control samples were added with 500 µL of 80% ethanol (○). Results are expressed as mean ± SEM of log10 (x+1) CFU g^{-1} faeces. Different letters indicate significant differences ($p<0.05$).

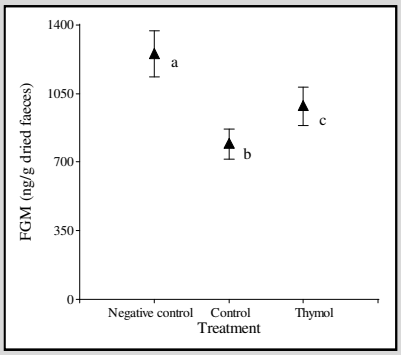

Figure 2. Concentrations of FGM in faeces of *Tamandua tetradactyla*. Samples were treated with 5 mg.g^{-1} faeces of thymol in 500 µL of 80% ethanol. Control samples were added with 500 µL of 80% ethanol. Negative control samples received no treatment. Results are expressed as mean ± SEM of ng.g^{-1} dry faeces. Different letters indicate significant differences ($p<0.05$).

Conclusions

The antimicrobial treatment applied reduced microbiological activity, although it affected FGM measurements. More studies should evaluate if faeces could be maintained without freezing up to 72h post-excretion.

Applying thymol might be useful as an alternative transport and storage method for faeces; however, other solvents should be evaluated to avoid inaccuracy in hormone measurements.