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

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ABSTRACT

Faecal glucocorticoid measurement is a potentially important tool for improving wildlife conservation, but its use is still limited by methodological issues including the need to avoid modifications of steroids by faecal microorganisms during storage. The freezing of faeces is recommended as a means of avoiding such alterations, but this is costly under non-controlled environmental conditions. The present study was designed to determine whether the application of thymol reduced the proliferation of microorganisms in the faeces of *Tamandua tetradactyla* and whether it influenced faecal glucocorticoid metabolite (FGM) measurements. *Tamandua tetradactyla* faeces were individually collected after defaecation, divided into fractions (5.5 g each) and kept in sealed glass Petri dishes at 22 ± 2 °C. A thymol solution (550 µL; 5 mg g^{-1} feces; 80% ethanol) or an 80% ethanol solution (550 µL, control) was added before storage of faeces. Negative controls for FGM consisted of samples without thymol or ethanol solutions. All samples were evaluated at 0, 24, 48 and 72 h post-defaecation. Thymol was first incubated with a glucocorticoid standard in a faeces-free tube or in a faecal sample in order to determine whether it interfered with FGM measurements. Data showed that thymol did not affect FGM measurements. Post-defaecation time caused a significant reduction in FGM measurements in the negative control, an increment at 48 h in the control, and no change in FGM measurements in thymol treatment. FGM measurements were significantly different between groups (negative control > control – treatment). Thymol caused a significant reduction of up to three orders of magnitude in total coliforms, total aerobic and anaerobic heterotrophic mesophilic bacteria, mold and yeast per gram of faeces at 24, 48 and 72 h. The reduction in microbial activity presumably contributed to the stability of FGM over time. Spore-forming bacteria (SFB) in faeces were not reduced by thymol. We propose thymol as an alternative to freezing since it stabilizes FGMs for at least 3 days after collection in the faeces of *Tamandua tetradactyla*.

1. Introduction

Non-invasive hormone monitoring provides accurate information about the endocrine status during studies of the reproductive and stress physiology of wild species (Dantzer et al., 2014; Ganswindt et al., 2012; Sheriff et al., 2011). Faeces are being increasingly used in this area of research since samples can be obtained without interfering with the natural activities of the animals. Quantification of hormone metabolites in faeces, especially steroids, is of interest for the study of reproduction and breeding management and also stress-related variables such as behavior, nutrition and ecology, among others (Schwarzenberger and Brown, 2013; Hodges et al., 2010; Wielebnowski and Watters, 2007; Touma and Palme, 2005). However, hormone measurement in faeces is limited by methodological constraints, particularly when studies are carried out in uncontrolled field environments (Edwards et al., 2014; Goymann, 2012; Wielebnowski and Watters, 2007). Bacterial enzymes in faeces can metabolize steroids and modify the outcome of immunoassays (Beehner and Whitten, 2004; Washburn and Millspaugh, 2002; Millspaugh and Washburn, 2004; Möstl et al., 1999; Wasser et al., 1993; Wasser et al., 1988). The use of faeces for endocrinological analysis requires that fresh samples be immediately frozen in order to avoid microbial breakdown of hormones (Goymann, 2012). Alternative methods to freezing for faecal samples exist, but each has specific limitations. For example, ice can extend for a few hours the sample

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storage time until freezing. Chemical treatment (e.g. 90% ethanol), heat and/or desiccation can also be used to reduce or stop the activity of microorganism and the associated degradation of hormones (Beehner and Whitten, 2004; Galama et al., 2004; Lynch et al., 2003; Khan et al., 2002; Terio et al., 2002; among others). However, all these processes are reported to affect the concentration of fecal steroid metabolites (Pettit et al., 2007; Ziegler and Wittwer, 2005; Hunt and Wasser, 2003; Millspaugh et al., 2003; Khan et al., 2002). Technical adjustment of methods for non-invasive hormone monitoring are required to ensure the hormone concentration of samples do not change in environment studies.

Animals housed in zoos, laboratories and conservation centres offer a unique opportunity for the establishment of methods for non-invasive hormone monitoring (Wielebnowski and Watters, 2007). Previously, we validated the use of faeces to monitor adrenocortical activity in *Tamandua tetradactyla* (Xenhartra, Mammalia), an insectivorous mammal endemic to South America (Redford and Eisenberg, 1992; Eguizábal et al., 2013). In the present study the aim was to develop an alternative method to sample freezing since this approach is often not possible in field studies. The present study explored the use of a natural inhibitor of microbial activity (thymol) to minimize changes in faecal glucocorticoid metabolite (FGM) measurements during short-term storage. Many *in vitro* studies indicate that the phenolic terpenoid thymol, the main component of oregano and thyme essential oils, is a bactericide whose effectiveness increases with concentration and exposure time (Xu et al., 2008; Di Pasqua et al., 2007; López et al., 2004; Lambert et al., 2001; Skandamis and Nychas, 2000). The effectiveness of thymol in preserving hormones in complex matrices has been evaluated in very few papers (Varel and Wells, 2007; Varel and Miller, 2000). For this purpose, we assessed whether thymol reduced the activity of the microorganisms present in *Tamandua tetradactyla* feces across time without modifying subsequent FGM measurements.

2. Materials and methods

2.1. Sample collection

Fresh feces of collared anteaters (2♀ and 3♂) from the Jardín Zoológico Córdoba (Argentina) were collected in December 2014, January 2015 and September 2017, between 09:00 AM and 12:00 PM, placed in sterile cups and transported (20–25 °C) within 30 min to the laboratory. Feces were homogenized and fractionated for further use. This study was carried out in accordance with the CONICET Ethics Committee (Resolution 1047 Annex II, 2005) and the Argentinean National Law of Animal Protection (No. 14346) and was evaluated and approved by the Bioethics and Laboratory Animal Commission in accordance with the Annex to the Regulation of 'Care and Use of Laboratory Animals' (IIBYT/CONICET- FCEFyN-UNC).

2.2. Experimental design

2.2.1. Experiment 1

The effect of thymol (T0501, SIGMA-ALDRICH) on 11-oxoetiocholanolone measurements was studied. The *control* consisted of 550 μL of 80% ethanol incubated with 11-oxoetiocholanolone standard in glass tubes. The *treatment* consisted of 550 μL of thymol (50 mg mL^{-1} 80% ethanol) incubated with 11-oxoetiocholanolone standard in glass tubes. For both *control* and *treatment*, the 11-oxoetiocholanolone standard (10 μg) was diluted in 80% ethanol (final concentration: 380 ng/mL). The experiment was run in triplicate, and samples (n = 3 control and 3 treatment) were collected at 0, 24, 48 and 72 h into glass tubes and stored at −20 °C. The collected samples (n = 24) were then oven dried (Novolavo, Argentina) at 60 °C for 96 h and hormonal measurements performed at the laboratory in Vienna (Austria; see Section 2.4 for the method). Results are expressed as 11-oxoetiocholanolone (pg/well).

2.2.2. Experiment 2

A single faecal sample was divided into 24 fractions (5.5 g each) and placed individually into sealed glass Petri dishes (22 ± 2 °C). For the *control* group (n = 12 Petri dishes) 550 μL of 80% ethanol containing the 11-oxoetiocholanolone standard (50 mg mL^{-1} ethanol; 5 mg g^{-1} feces) was added to the faeces. For the *treatment* group (n = 12 Petri dishes) 550 μL of a thymol solution diluted in 80% ethanol containing the 11-oxoetiocholanolone standard (50 mg mL^{-1} ethanol; 5 mg g^{-1} feces) was added to the faeces. Petri dishes were placed in independent sealed chambers for each group. Samples for hormonal analysis were collected at 0, 24, 48 and 72 h; by weighing 0.5 g of faeces into a clean glass tube and were stored at −20 °C until hormonal analysis (see Section 2.4). Results are expressed as faecal glucocorticoids metabolites (μg/g).

2.2.3. Experiment 3

Fresh faeces (n = 5, different animals) were collected and 8 fractions (5.5 g) were obtained from each sample. Fractions were placed individually in sealed glass Petri dishes (22 ± 2 °C) which were then divided equally between two closed and sealed chambers. To each of the *control* faecal samples (n = 20), 550 μL of 80% ethanol was added. In the *treatment* faeces 550 μL of a thymol solution diluted in 80% ethanol (50 mg ml^{-1} ethanol; 5 mg g^{-1} feces) was applied. Samples were collected at 0, 24, 48 and 72 h. Of the collected faeces 5 g was used for the microorganism count and 0.5 g was used for hormonal analysis (stored at −20 °C; see Section 2.4).

A *negative control* consisting of 4 fecal fractions without ethanol or thymol (evaluated at 0, 24, 48 and 72 h) was also carried out to assess the effect of ethanol on FGM measurements (n = 20). These data were included in the statistical analysis as an extra factor level treatment. Moreover, in order to assess the possible effect of ethanol on microorganism, a preliminary study was carried out. Briefly, a low concentration of 100% ethanol (1000 μL) was added to fecal samples (10 g), and CFU per gram of faeces were counted (total coliforms, total aerobic and anaerobic heterotrophic mesophilic bacteria, yeast and mold, and spore-forming bacteria). No statistical differences were found between samples with or without ethanol ($p \geq 0.05$ for all cases).

2.3. Measurements of CFU (only for Experiment 3)

Total coliforms (TC), total aerobic and anaerobic heterotrophic mesophilic bacteria (AHB and ANHB), yeast and mold (Y and M) and spore-forming bacteria (SFB) were counted as CFU per gram of feces. Microbial analyses were performed in triplicate. Portions of 5 g (wet weight) were weighed into sterile screw-top glass jars into which 45 mL of 1.0% buffered peptone-water was added. The samples were shaken for 30 sec (Kelley et al., 1994) and the resulting slurries were serially diluted and plated. Total indigenous culturable mesophilic bacteria were obtained from the plate count agar without cycloheximide. SFB were inoculated in the plate count agar after heat treatment (80 °C, 10 min). The agar count plates were incubated for 48 h at 30 °C following the methodology proposed by Lemunier et al. (2005). Total coliform counts were determined after incubation for 24–48 h at 35 °C in lauryl tryptose broth using the presumptive multiple-tube fermentation technique. Tubes with gas production and turbidity (growth) were considered to be positive (USEPA, 2010).

2.4. Measurements of fecal glucocorticoid metabolites (FGM).

FMG measurements were carried out in the laboratory of the Unit of Physiology, Pathophysiology and Experimental Endocrinology, Department of Biomedical Sciences, University of Veterinary Medicine (Vienna, Austria). No special permission was required to export fecal extracts from Argentina since *T. tetradactyla* is not listed in CITES Appendices and is categorized as LC (Least Concern) by IUCN.

For steroid analysis, frozen samples were oven dried (Novolavo,

Argentina) at 60 °C for 24 h. Fecal steroids were extracted with an 80% aqueous methanol solution (1 g of homogenized sample per 10 mL solvent) as described in Palme et al., 2013. Samples were vortexed for 2 min and centrifuged for 15 min at 3100 G and an aliquot (0.5 mL) of the supernatant was collected. The supernatant was evaporated at 60 °C and sent to the laboratory for analysis using an enzyme immunoassay (EIA). Samples were redissolved in methanol and then diluted with EIA and measurements were run in duplicate for the standard and samples using an 11-oxoetiocholanolone EIA, as described by Möstl et al. (2002). The sensitivity of the EIA was 4 ng/g feces. This assay has been used to monitor adrenocortical activity in several mammal species (Palme, 2012) and was previously validated for its use in *Tamandua tetradactyla* (Eguizábal et al., 2013).

2.5. Statistical analysis

A mixed linear model was used to evaluate the effect of thymol on standard glucocorticoid measurements (Experiment 1), on standard and FGM measurements (Experiment 2), in which the fixed factor was treatment (control vs thymol) and the random factor was time (0, 24, 48 and 72 h), and on CFU and FGM measurements (Experiment 3). Two-way repeated measures ANOVA was used, in which fixed variables were treatment (control, negative control and thymol) and time (0, 24, 48 and 72 h). Microbiological data were transformed as $\log_{10}(X + 1)$ to meet ANOVA assumptions. A *LSD Fisher* test was applied with the appropriate Bonferroni adjustment for the FGM results and a Tukey test was used for the microbiology results. All data are expressed as mean ± SEM. The significance level for all tests was $p < 0.05$. Analyses were performed using InfoStat (Di Rienzo et al. 2011) on the basis of specific bibliographic references (Plowman, 2006; Sokal and Rohlf, 1997).

3. Results

3.1. Effect of thymol on 11-oxoetiocholanolone standard measurements (Experiment 1)

Mean values of 11-oxoetiocholanolone measurements for thymol treated samples were similar to control samples at 0 h, 24, 48 and 72 h. Measurements of 11-oxoetiocholanolone are shown in Fig. 1.

3.2. Effect of thymol on 11-oxoetiocholanolone standard and FGM measurements (Experiment 2)

Statistical analysis revealed that the mean value of glucocorticoids measurements were similar between *control* and *treatment* groups, indicating that thymol addition to faecal samples did not affect FGM measurements. The FGM measurements in samples stored for different times (24, 48 and 72 h) were not significantly different from the sample at 0 h (Fig. 2).

3.3. Microbial measurements (Experiment 3)

A significant inhibitory effect of thymol on TC, AHB, ANHB, Y and M counts was observed ($p < 0.0001$). The reduction in CFU g^{-1} feces was up to three orders of magnitude for samples treated with thymol at 24, 48 and 72 h post collection. Thymol failed to inhibit SFB.

An interaction between thymol and time was detected by two-way repeated measures ANOVA ($p < 0.0001$). The CFU values of TC are shown in Fig. 3. For the control samples, the CFU count was the same across the time series (7.66 $\log_{10}(x + 1)$). For the treated samples the CFU count was 7.66; 4.64; 3.68 and 3.60 $\log_{10}(x + 1)$ at 0, 24, 48 and 72 h, respectively. A significant difference in the CFU counts in the time series for treated samples ($0 > 24 > 48–72$ h) was observed ($p < 0.0001$). The CFU counts were significantly reduced in the thymol treated faecal samples relative to the untreated control ($p < 0.0001$).

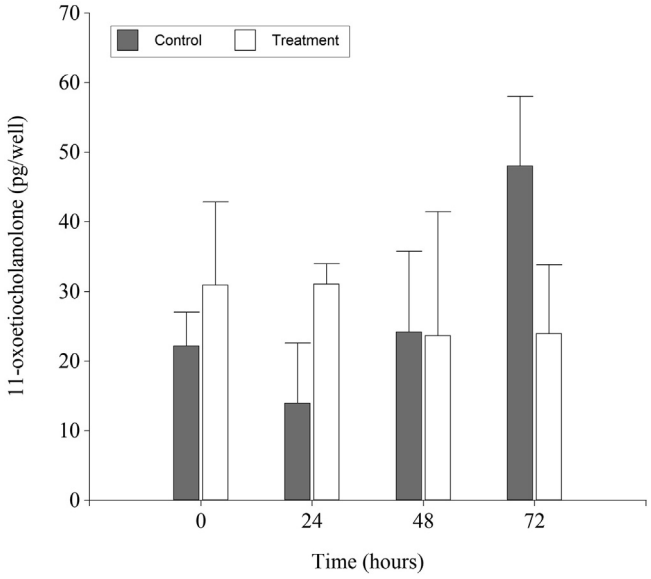

Fig. 1. Measurements of glucocorticoid standard (11-oxoetiocholanolone) with (treatment) or without (control) thymol. Measurements of glucocorticoids were performed at 0, 24, 48 and 72 h post-treatment. Results are expressed as mean ± SEM and as mass of standard detected in each well of EIA (n = 3 per treatment and time; total measurement = 24).

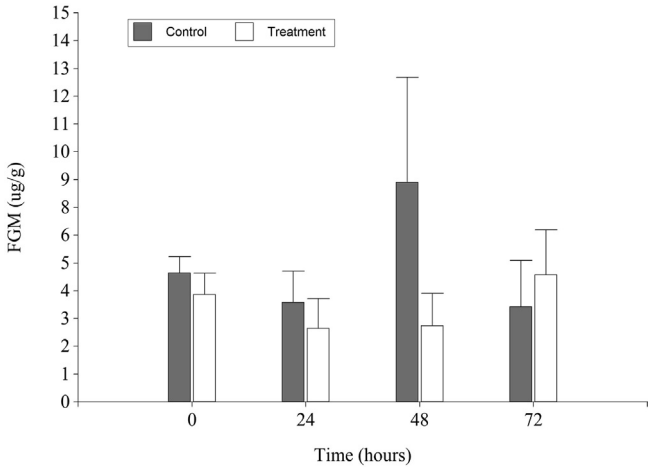

Fig. 2. Measurements of glucocorticoid standard (11-oxoetiocholanolone) with (treatment) or without (control) thymol in feces of *Tamandua tetradactyla* at 0, 24, 48 and 72 h. Results are expressed as mean ± SEM of µg/g dry feces (n = 3 per control or treatment/time; total measurement = 24). Control consisted 550 µL of 80% ethanol (light gray) added at time 0 to fecal samples on glass Petri dishes, and treatment consisted 5 mg g^{-1} feces of thymol in 550 µL of 80% ethanol (white) added at time 0 to fecal samples on glass Petri dishes.

Two-way repeated measures ANOVA detected a significant interaction between fixed factors (treatment and time) for AHB, ANHB, Y and M ($p < 0.0001$). Table 1 shows the results for the CFU count of AHB, ANHB and Y and M. The CFU count of SFB for the control was not significantly different from the thymol treated samples, though there was a clear time effect ($0 > 24 \geq 48 \geq 72$; $p < 0.0001$). The CFU counts indicated that no significant reduction occurred in the thymol treated samples relative to the controls.

3.4. Fecal glucocorticoid metabolite measurements (Experiment 3)

A reduction in measured FGM was observed in the *negative control*

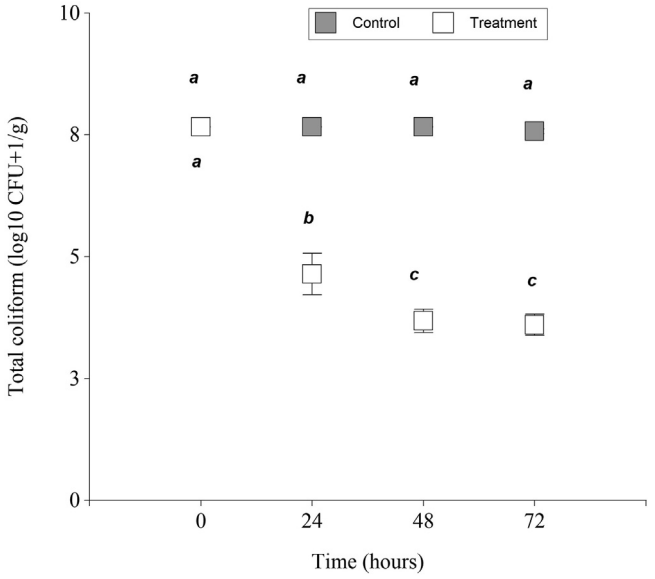

Fig. 3. Total coliforms (TC) count from fecal samples of Tamandua tetradactyla at 0, 24, 48 and 72 h post-defecation. Samples were treated with 5 mg g^{-1} feces of thymol in 550 µL of 80% ethanol. Control samples were diluted with 550 µL of 80% ethanol (○). Results are expressed as mean ± SEM of log10 (x + 1) UFC g^{-1} feces. Different letters indicate significant differences ($p < 0.05$).

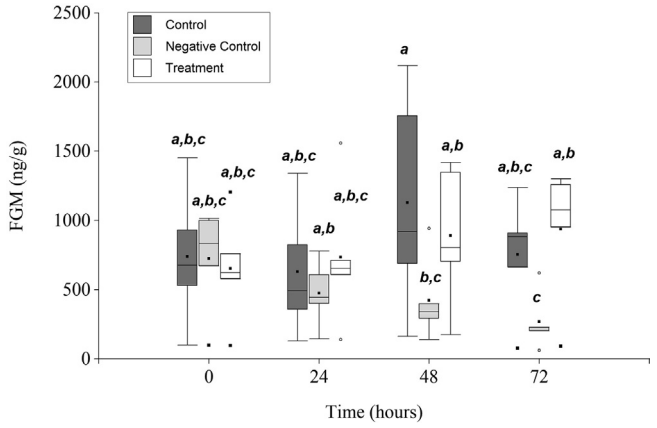

Fig. 4. Measurements of fecal glucocorticoid metabolites (FGM) in feces of *Tamandua tetradactyla* at 0, 24, 48 and 72 h. Results are expressed as mean ± SEM of ng·g^{-1} dry feces. Box plots show median values (horizontal line), average (dark square dot), first and third quartiles (bottom and top lines), and average ± 2*SD (whiskers). Negative control consisted of samples without solution, control consisted of samples with 550 µL of 80% ethanol, and treatment consisted of samples with 5 mg g^{-1} feces of thymol in 550 µL of 80% ethanol. There was no interaction between time × treatment. Different letters indicate significant differences across the time ($p < 0.05$). In addition, measurements of FGM showed significant differences between groups (negative control < control – treatment).

Table 1
Effects of thymol over time on CFU counts in *Tamandua tetradactyla* faeces.

	Time			
	0	24	48	72
	Aerobic heterotrophic mesophilic bacteria (AHB)			
Control	9.35 ± 0.15a	8.58 ± 0.14a,b	8.31 ± 0.06a,b	8.43 ± 0.05a,b
Thymol	9.32 ± 0.20a	5.60 ± 0.91c	6.68 ± 0.71b,c	4.96 ± 0.94c
	Anaerobic heterotrophic mesophilic bacteria (ANHB)			
Control	9.44 ± 0.15a	8.66 ± 0.13a,b	8.59 ± 0.04a,b	8.65 ± 0.07a,b
Thymol	9.23 ± 0.12a	6.13 ± 0.83c	6.88 ± 0.73b,c	5.50 ± 0.89c
	Yeast and mold (Y and M)			
Control	5.05 ± 0.40a	5.58 ± 0.21a	5.11 ± 0.22a	4.86 ± 0.28a
Thymol	5.36 ± 0.19a	4.17 ± 0.36b	3.73 ± 0.31c	3.42 ± 0.31c

Results of each variable are expressed as the mean ± SEM of $\log_{10}$ CFU + 1/g of feces. Within variables: different letters indicate significant statistic differences ($p < 0.05$).

across the time series, changes in FGM at 48 h were observed in the *control* and no changes in FGM were observed in the *treatment*. Measurements of FGM are shown in Fig. 4. In the *negative control* the mean ± SEM (and median) values of FGM measured were 723 ± 128 (8 3 2); 474 ± 106 (4 4 4); 422 ± 137 (3 3 9) and 268 ± 93 (2 2 6) ng·g^{-1} dry feces at 0, 24, 48 and 72 h, respectively; a significant decrease ($p < 0.05$) occurred after storage for 24, 48 and 72 h. In the *control*, the mean values of measured FGM were 738 ± 224 (6 7 7); 628 ± 210 (4 9 0); 1127 ± 356 (9 1 9) and 753 ± 192 (8 8 1) ng·g^{-1} dry feces at 0, 24, 48 and 72 h, respectively; a significant increase occurred at 48 h ($p < 0.05$). In the thymol *treatment*, measurements of FGM were 652 ± 177 (6 2 2); 733 ± 229 (6 5 3); 890 ± 228 (8 0 3) and 935 ± 220 (1074) ng·g^{-1} dry feces at 0, 24, 48 and 72 h, respectively and none of the samples were significantly different. Significant differences in measured FGM occurred between groups (*control = treatment > negative control*; $p < 0.05$).

4. Discussion

The findings of the present study on the use of a natural microbial agent (thymol) in *Tamandua tetradactyla* faeces revealed the microorganisms (AHB, ANHB, TC and Y and M) reduced in number and FGM measurements were unchanged. Thymol neither interacted with the 11-oxoetiocholanolone standard measurements nor affected hormonal analysis when added to faecal samples.

Several *in vitro* studies indicate that thymol has antibacterial and antifungal activity, and inhibits microbial activity in concentrations between 0.249 and 0.500 mg thymol mL^{-1} ethanol (Bento et al., 2013; Hsu et al., 2011; Di Pasqua et al., 2007; Klarić et al., 2007; Tullio et al., 2007; López et al., 2004). However, few studies have been carried out to date with complex matrices such as faeces, for which the use of much higher concentrations of thymol have been suggested in order to inhibit microbial activity (Varel and Miller, 2000). In the present study, microbial activity was inhibited using 50 mg thymol mL^{-1} ethanol, a concentration 100 times higher than indicated in the literature for *in vitro* studies.

Although the inhibition of microbial activity was effective for most of the studied bacterial groups, this was not the case for SFB. Di Pasqua et al. (2007) showed that the bactericidal effect of some constituents of essential oils is due to the disruption of the plasma membrane, and this causes cells to lose selectivity in the exchange with the environment and compromises their metabolic activity. However, sporulating bacteria have an outer protein coat that is resistant to aggressive agents. Nonetheless, the essential oil of oregano has sporicidal activity (Heberbeck et al., 2012) and the thymol it contains reduces the resistance of the spores of *Bacillus* sp. to heat (Esteban et al., 2015). Moreover, Lawrence and Palombo (2009) noted that the components of essential oils exhibit less sporicidal activity than full oil and their effectiveness increases with exposure time (more than a week). Another study found that the essential oil of *Ocimun gratissimun* and *Thymus vulgaris* (about 50% thymol) had a significant bactericidal effect on *Bacillus cereus*, *B. megaterium*, *B. subtilis* and *Geobacillus stereathermophilus* spores in concentrations of 2.5 and 1.25 mg mL^{-1} in the culture medium (Voundi et al., 2015). The failure to block spore growth in the present study could be due to the complexity of the matrix and the fact that some feces components could interact with thymol, reducing its effect on bacterial spores. In considering possible variations in microbiological activity over time, our results indicate no change in

control samples and a reduction in thymol-treated samples after 24 h. For effective microbial inhibition it is therefore recommended that thymol treatment be applied for at least 24 h.

With respect to FGM measurements, these were found to be significantly lower in the *negative control* relative to the *control* and thymol *treatment*. Furthermore, the concentration of FGM across time series were observed in both *negative control* and *control* but not in treated samples. The literature indicates that stability of steroids post-defecation is a serious concern, and a critical factor is the activity of steroid metabolizing bacterial enzymes that produce metabolites, with a modified affinity (higher or lower) with the antibody of the immunoassay (Möstl et al., 1999; Möstl and Palme, 2002; Lexen et al., 2008). Since our findings showed that thymol reduced microbial activity, it is possible that the variations in FMG measurements for the two control groups was due to the activity of microorganisms. It seems probable that microbial enzymes degrade immunoreactive glucocorticoid metabolites and therefore interfere with the performance of the immunoassay. The results obtained with control samples (treated only with ethanol) show that FGM measurements remained partially stable over time, with values significantly higher than at 0 h. It could be that ethanol separated steroids from feces, thus initiating the extraction (Palme et al., 2013) and partially avoiding hormone alteration in some of the tested samples. Although there were no differences in measurements between the *control* and thymol *treatment*, over time the thymol group showed less variation in FGM concentrations. In conclusion, thymol reduced microorganisms present in *Tamandua tetradactyla* faeces without affecting the performance of the enzyme immunoassay or FGM measurements. Further studies of thymol are required to resolve the problem of faecal transport and storage in the absence of facilities for immediate freezing.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.04.015.

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