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Effect of dehydrated grapefruit peels on intestinal integrity and *Eimeria* invasion of caprine epithelial cells in vitro and anticoccidial activity in vivo

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ABSTRACT

Anticoccidial drugs are among the preferred resources to control coccidiosis, but the need to have natural alternatives has prompted research into plant products that improve animal performance and might also reduce *Eimeria* oocyst shedding while avoiding problems of drug residues. Two studies were conducted to evaluate the effects of dehydrated grapefruit peels (GDP) on *Eimeria* infection and intestinal health. In the first study, an in vitro culture system was used to evaluate the integrity of caprine epithelial cells infected with *Eimeria ninakohlyakimovae* and incubated with GDP, as well as the infection rate and schizont development of parasites exposed to GDP. In the second study, the anticoccidial effect of the inclusion of 15% GDP1 or 30% GDP2 in the diet and toltrazuril was evaluated in goat kids naturally infected with *Eimeria* spp. Parameters assessed included the reduction in oocyst counts, weight gain, fecal consistency, intestinal integrity, and malondialdehyde and nitric oxide (NO) production. In vitro, GDP2 and TTZ not only increased transepithelial electric resistance (TEER) values in infected caprine epithelial cells, but also decreased the mean cell invasion rate of sporozoites of *E. ninakohlyakimovae* and reduced the number and size of schizonts/mm^2 of the intestinal cells culture. In vivo, the GDP2 group showed higher mean daily weight gains, reduced fecal oocyst output and decreased oxidative stress in goat kids. These findings demonstrate the beneficial activities of GDP in the enhancement of growth performance and gut health, and the feasibility of using a 30% inclusion of GDP for the replacement of corn in goat production.

1. Introduction

In goats, coccidiosis is caused by obligate intracellular organisms of the genus *Eimeria* spp. Clinical coccidiosis develops when a critical amount of sporulated oocysts access to the gastrointestinal tract and intestinal cells of the hosts are invaded. Then, a high asexual multiplication rate within the intestinal cells of the host follows, destroying them (Jolley and Bardsley, 2006; Chartier and Paraud, 2012). The control and treatment of coccidiosis in susceptible animal populations is challenging, but if it succeeds, weight gain and production parameters are rewarding. Anticoccidial drugs are among the preferred resources to control coccidiosis in many species, but the information corresponding to goats is still scarce both in vitro and in vivo (Ruiz et al., 2012).

At present, a reasonable trend to control coccidiosis relies on keeping the animals with a tolerable parasite load which stimulates immunity and may prolong the time of appearance of anticoccidial-drug resistance (Torres-Acosta et al., 2012). To achieve this goal the use of plants having anticoccidial active components has been proposed (Hoste and Torres-Acosta, 2011; Wunderlich et al., 2014), and it has been documented that plant-based therapeutics can improve animal health (Hoste et al., 2015; Lima et al., 2019; Saratsi et al., 2020). Such an approach has been shown to offer positive outcomes in the control of poultry coccidiosis (Alnassan et al., 2015; Nogareda et al., 2015). Ideally, by-products from the food processing industry can be used, with a

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double benefit: low cost of such by-products and a positive environmental impact when repurposing them (Gungor et al., 2021; Rakita et al., 2021; Tamiru et al., 2021). *Citrus* peel and pulp are a concrete example of an industrial by-product incorporated into sheep and steers feed, since this by-product contains useful bioactive substances (Nordi et al., 2014; Tayengwa et al., 2021). Its inclusion in sheep's diet reduces gastrointestinal nematode loads and improves weight gain (Nordi et al., 2014). It has been shown that *Citrus* peel and pulp contain polyphenolic compounds which are known to have various biological actions i.e., they suppress metastasis, possess anti-fibrinogen activity, show anti-inflammatory activity, and limit nitric oxide production (Sun et al., 2013; Dourado et al., 2015; Wang et al., 2015).

In a previous study, it was shown that the extract of naringenin, a flavanone present in grapefruit peels decreased oocysts shedding in *Eimeria* spp. naturally infected sheep and diminished the production of reactive oxygen species while increasing the antioxidant ability of the infected animals (Pérez-Fonseca et al., 2016). *Citrus* peels possess numerous flavonoids such as polymethoxylated flavonoids, glycosylated flavanones, flavonols, and phenolic acid among other derivatives (Anagnostopoulou et al., 2005). These active principles and naringenin have been shown to protect intestinal-cell tight junctions (TJs) integrity and consequently, the intestinal mucosa barrier (Chen and Kitts, 2017). This barrier is often compromised when a large inoculum of *Eimeria* spp. is ingested or when a dysfunctional immune response is present (Chartier and Paraud, 2012; Walker et al., 2015). The in vivo anticoccidial and antioxidant potential of grapefruit by-products against *Eimeria* in lambs has been previously described (Perez-Fonseca et al., 2016).

The precise mechanism by which naringenin or the grapefruit extracts decrease *Eimeria* oocyst shedding in small ruminants has not yet been elucidated; however, it does not discard the possibility that the coccidiostatic effect that was observed after the ingestion of a commercial extract of naringenin (>98% purity) in lambs (Perez-Fonseca et al., 2016) is a direct consequence of a probable toxic effect of the flavonoids on the protozoan. Hence, the parasitic load reduction ultimately results in the induction of less inflammatory mediators and oxidative mechanisms. Consequently, an in vitro culture was needed to gather evidence that demonstrates a toxic effect of flavanones on *Eimeria* parasites directly exposed to the *Citrus* extract. Moreover, the decrease in the parasitic load in animals that have been treated with *Citrus* extracts might be due to an effect of the flavanones on the intestinal barrier integrity.

Therefore, this study aimed to explore the effect of a grapefruit peel extract on the infection rate and schizont development of *E. ninakohlyakimovae* in vitro and the capacity of this *Citrus* product to regulate epithelial barrier function. Following our in vitro results, we also examined the anticoccidial and antioxidant efficacy of the dietary inclusion of dehydrated grapefruit peels in goat kids infected naturally with *Eimeria*.

2. Material and methods

2.1. Dehydrated grapefruit peel (GDP)

Grapefruits (*Citrus x paradisi*) previously harvested were bought in a local market from Mexico City. Grapefruits were hand peeled and washed with tap water until cleaned completely. Peels were industrially lyophilized by Liomex® Co. (Mexico). A coffee grinder was used to grind dehydrated peels until getting 1 mm particles that were sent to be chemically analyzed; and 4 mm particles that were included in the diets (Tayengwa et al., 2021). In this trial, aqueous extraction followed by lyophilization were chosen as two of the most reliable methodologies to preserve flavonoids (Demir and Celik, 2019), as well as the smell and flavor of grapefruit peels (Baker, 1997; Abdelwahed et al., 2006), with the added benefit to be able to store the lyophilisate for long periods without much degradation (Berk, 2009).

2.2. Water activity

The water activity of the dehydrated grapefruit peel was measured at 22 °C using a Hygrolab meter (Rotronic, Instrument Corporation Inc., Hauppauge, NY, USA), calibrated with deionized water (a_w of 1.000). Approximately 30 g of dehydrated grapefruit peels were put into the cup of the Rotronic hygrolab device and furtherly placed into the cup holder equipped with the probe. The reading was taken in 5 min as per the manufacturer's instructions. Triplicate samples were analyzed.

2.3. Naringenin extraction

The extraction procedure was done according to El-Nawawi (1995). Briefly, 3 l of water were added for each 2 kg of GDP. The mixture was boiled and stirred for 90 min. Then, lime was added until a pH of 11 was obtained; the mixture rested at room temperature for 30 min. After resting, the mixture was filtered and a 4.7 pH HCL was added, the mixture was heated at 50 ºC and stirred for 90 min. The mixture was left to lower its temperature along the night and the precipitate was separated and dried.

2.4. Determination of naringenin concentration

Naringenin concentration was measured according to the method previously described by (Bronner and Beecher, 1995) using HPLC. In brief, the commercial standard of naringenin with a purity higher than 98% (Sigma-Aldrich, México cat. T66001) was diluted in methanol and water (1:1). The samples and the standard were added to a 100 µl sodium acetate tampon 1 M (pH 5) and 40 µl of β-glucuronidase for 18 h at 37 ºC for its degradation. Subsequently, 2 ml of ethanol were added and the samples were filtered through a 0.45 µm sieve, and then dried at 45 ºC and dissolved with the solvent used in the mobile phase.

HPLC analysis was performed using a C_{18} column using premixed 6 ml of methanol and 6 ml of HCl 0,01 M. Mobile phase was performed with methanol/water/acetic acid (40:58:2) (flow rate of 1 ml/min, UV detector adjusted at 280 nm, and a 20 µl sample volume). Results were calculated as mg of equivalent naringenin per each g of dry weight (mg NAR/g).

2.5. HPLC Analysis

High-performance liquid chromatography (HPLC) analysis was performed on a liquid chromatograph system (Hewlett Packard ® model 1100, Ramsey, Minnesota, 55303 USA) at the National Polytechnic Institute, Mexico. The equipment was adjusted for 60 min with the mobile phase constituted by methanol HPLC grade and water mili-Q (CH3CH): H_2O (50:50), was calibrated with hesperidin, neohesperidin, naringin, naringenin, nobiletin, and quercetin standards from Sigma-Aldrich at 100 parts per million (ppm), at a temperature of 30 °C, flow rate of 1 ml/min and 280 nm, column RP-C18 (Beckman Ultrasphere) of 150 mm long and 4.6 mm internal diameter. The injection volume of samples and standards was 20 µl with a run time of 10 min

2.6. In vitro study

2.6.1. Parasites for in vitro studies

Eimeria ninakohlyakimovae oocysts used in this study were initially isolated from naturally infected kids. A total of 2250 kids aged 35–85 days were sampled from 82 intensive and semiextensive farms located on the Central-Northern region of Mexico. These farms have a history of goat coccidiosis and presence of *E. ninakohlyakimovae* (Alcala-Canto et al. 2020). Faecal samples were collected from all animals individually from the rectum in the morning and furtherly analyzed at the Faculty of Veterinary Medicine of the National Autonomous University of Mexico (UNAM) using the McMaster method in samples that weighed more than 0.5 g (Henriksen and Christensen, 1992) to estimate oocysts per gram of

faeces (OPG). When oocyst counts were very high, the faecal suspension was diluted to enable counting. When the sample contained more than 300 OPG, oocysts were sporulated in a 2% $K_2Cr_2O_7$ (w/v) solution for 14 days with aeration every 4 h by stirring the oocysts mixture. *Eimeria* species were microscopically identified based on the morphology of sporulated oocysts (Joachim et al. 2018). Oocysts were isolated from faecal suspensions that contained at least 80% *E. ninakohlyakimovae* oocysts using a Leica Mechanical Micromanipulator (Aspelab - Leica Microsystems Jalisco, Mexico) kindly lent by the Biology Institute of the UNAM. Oocysts were stored at 4 °C in a 2% $K_2Cr_2O_7$ (w/v) solution to prevent microbial contamination (Hermosilla et al. 2002; Ruiz et al., 2014; Silva et al. 2015) until further use for the experimental infection. The obtention of *E. ninakohlyakimovae* from field infections yielded 7.7 × 10^6 oocysts, insufficient to infect cell cultures. Thus, *E. ninakohlyakimovae* oocysts were thereafter propagated in four parasite-free one-month male Nubian *x* Saanen kids according to the protocol of oocyst amplification of Hermosilla et al. (1999). All animal procedures were performed following the guidelines of the Ethical Subcommittee for Animal Care, Use and Experimentation of the Graduate Program of Animal Sciences and Husbandry (SICUAE Project No. IN218720) in accordance to current official animal welfare regulations. Briefly, for oocysts production, kids were orally infected with 1 × 10^5 *E. ninakohlyakimovae* sporulated oocysts. The inoculum was previously assayed (unpublished data) and proved to cause excretion of *Eimeria* oocysts without producing a lethal outcome. The onset of excretion began on day 15 after infection and peaked on day 24 post-infection. The artificial infection yielded 3.7 × 10^6 *E. ninakohlyakimovae* oocysts. Oocysts used in the present work were less than six-months old and constantly checked for aeration.

Eimeria oocysts were isolated from faeces and sporulated in a 2% potassium dichromate suspension (w/v) to confirm *E. ninakohlyakimovae* identification based on morphometric characteristics (Joachim et al. 2018). Oocyst isolation from faeces was achieved by mixing the suspension with water (1:1) and sieving the collected faeces before mixing the suspension with a saturated sugar-solution (1:1). Afterwards, the suspension was let to float on glass slides that were washed every 2 h with distilled water. Collected washings were centrifuged at 2300 × *g* for 20 min and the sediment was recovered and stored at 4° C in culture flasks until further use (Odden et al., 2019). The sporozoites were de-crystallized according to a previously described methodology (Ruiz et al., 2010; Ruiz et al., 2015). Briefly, sporulated oocysts were suspended in a sterile 0.02 M L-Cysteine HCl/0.2 M NaHCO3 solution (Sigma-Aldrich, Mexico), and incubated at 37 °C for 20 min in 100% CO_2 atmosphere. Subsequently, the oocysts were suspended in Hank's balanced salt solution (HBSS; Gibco) with 0.4% (w/v) trypsin (Sigma-Aldrich, Mexico) and 8% (v/v) filter-sterilized caprine bile obtained from the slaughterhouse in Atlacomulco, Mexico, and incubated at 37 ºC in a 5% CO2 atmosphere for 4 h. Excysted sporozoites were washed three times (20 min, 1100 x g) and suspended in the culture medium (2.5 ×10^5 sporozoites/ml).

2.6.2. Cytotoxicity evaluation of the experimental compounds

Primary caprine intestinal epithelial cells (CIEC) were isolated according to the method used by Ibarra-Velarde and Alcala-Canto (2007). Briefly, intestinal tissue was extracted from goats immediately after slaughter. The tissue was placed in RPMI 1640 (Sigma-Aldrich, Mexico) supplemented with fetal bovine serum (FBS), streptomycin, penicillin, and amphotericin B (Sigma-Aldrich, Mexico). Once in the laboratory, the cells were placed in Hank's balanced salt solution (HBSS) (Sigma-Aldrich, Mexico) with ethylenediaminetetraacetic acid (EDTA) and dithiothreitol (Sigma-Aldrich, Mexico) and incubated at 37 °C for 10 min with vigorous shaking. The supernatant was removed and the tissue was placed in RPMI 1640 with collagenase (Sigma-Aldrich, Mexico) and incubated at 37 °C for 15 min with vigorous shaking. The supernatant was removed; the cells were centrifuged at 800g for 5 min and resuspended in RPMI 1640 with FBS and collagenase. This step was repeated until cell isolation was complete. Cells were plated on culture plates (Costar) on 13-mm plastic coverslips with Dulbecco-Ham's F-12 modified Eagle medium plus 10% (vol/vol) FBS, 8 µg/ml insulin, 10 µg/ml gentamicin, 50 µg/ml hydrocortisone, 100 µg/ml streptomycin, 100 U/ml penicillin, and 2.5 µg/ml amphotericin B (Ibarra-Velarde and Alcala-Canto, 2007). The cells attached to the surface of culture plates and started to grow within 48 h after incubation and reached 80% confluency in four days. CIEC are a primary line that was maintained at an air–liquid interface at 37 °C, 5%CO_2 and 98% relative humidity.

The in vitro cytotoxicity of the *Citrus* compound was determined by the MTT assay [3-(4,5-dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide] (Scudiero et al., 1988). CIECs were seeded in 96-well plates at a density of 1 × 10^4 cells/well and incubated with 100 µl of the culture medium in 5% CO_2 at 37 °C for 48 h. GDPs were diluted in 0.1% d_6-dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Mexico). Culture medium was exchanged for 150 µl of GDP at concentrations of 100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 µM in culture medium (n = 5 wells per concentration). TTZ diluted in DMSO was included at concentrations of 0.01, 0.1, 0.1, 1.0, 5.0 and 25.0 g/ml in culture medium (n = 5 wells per concentration) (Odden et al., 2019). Cells were again incubated for 4 h at 37 °C. Cell viability was determined by incubating cells for 4 h at 37 °C with 20 µl of MTT solution in a 5% CO_2 atmosphere (5 mg/ml MTT in phosphate buffer, pH 7.4) (Thermo Fisher Scientific, Mexico) after completely removing the solution from GDPs to avoid interference from flavonoid antioxidants. Intracellular formazan crystals were extracted in 100 µl of DMSO and quantified by measuring the absorbance of the cell lysate at 590 nm. Cell viability was calculated as a percentage based on the measured absorbance relative to the absorbance obtained from cells exposed only to the culture medium. Viability was calculated using the following formula (Chen and Kitts, 2017)

$$Cell\ \ viability\ \ (\%) = \frac{absorbance\ \ sample\ \ 590\ \ nm}{absorbance\ \ control\ \ 590\ \ nm} \times\ \ 100$$

2.6.3. Cell culture infection

After removal of the culture medium from the confluent CIEC, 2.5 × 10^5 *E. ninakohlyakimovae* sporozoites were added to each well. After incubation for 3 h, the infected cells were washed with phosphate-buffered saline (PBS) to remove undecrystallized oocysts. 500 µl of culture medium supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin was added. Infected intestinal cells were harvested 1 and 6 h after sporozoite inoculation (Ibarra-Velarde and Alcala-Canto, 2007). GDP (6.25 µM) and TTZ (25 µg/ml) concentrations were selected according to the cytotoxicity assay results and previous findings (Cervantes-Valencia et al., 2018; Odden et al., 2019; Nakashima et al., 2020). A 0.1% DMSO was used to dilute GDP and TTZ, as well as a control with 0.1% DMSO. The infection rate was calculated with the following formula in at least 10 random visual fields (Hamid et al., 2014):

$$Infection\ \ rate\ \ (\%) = \frac{infected\ \ cells}{total\ \ counted\ \ cells} \times\ \ 100$$

Cells were checked at the beginning of the assay, 30 min later, and furtherly after 1, 2, 4, 8, 12 and 24 h. Culture medium was changed thereafter every four days and cells were analyzed daily (Ruiz et al., 2010) to observe sporozoites and to count and measure meronts with an inverted microscope. Non-infected CIEC were used as negative controls and underwent the same medium change procedure of infected cells for the corresponding time points. Infected and uninfected epithelial cells were dissociated with trypsin/EDTA (0.25%/0.02 M) (Sigma-Aldrich), rinsed and resuspended in complete medium on day 18 to anticipate on the risk of overconfluency or degeneration. Fibroblast overgrowth was observed after this procedure, and epithelial cells stopped growing as of day 18.

The developmental assessment was carried out on days 8 and 15 after culture infection. On day 8, 15–20 photographs were taken at 400X

magnification, (Leica microscope), schizonts were counted and measured. Photographs were only taken when there was at least one schizont in a field, and the total number of fields examined was recorded up to a maximum of 50 fields (Odden et al., 2019). On day 15, schizonts were counted and measured at 100X magnification in 15–20 photographs. Only cells with schizonts were quantified and those containing undeveloped sporozoites were discarded (Hamid et al., 2014). Results are presented as the mean of five independent replicates.

2.6.4. Effect of GDPs on the intestinal barrier integrity

To investigate the effect of *Eimeria* and GDP on the barrier properties of CIEC cells, transepithelial electrical resistance (TEER) was measured according to a previously described method (Xu et al., 2003) using an EVOM X meter (World Precision Instruments) coupled to an Endohm chamber (World Precision Instruments). Seven days after beginning the CIEC culture, cells reached transepithelial electrical resistances (TEER values) of 600–900 Ω-cm2. GDP was dissolved in DMSO as a 10 mM solution, based on naringenin molar values (Nakashima et al., 2020). To assess the integrity of TJ, infected and uninfected cells were exposed to 100 μM GDP and a commercial suspension of toltrazuril. 0.1% DMSO was used as GDP diluent and as a control. It has been previously shown that 1% DMSO does not affect transport in TEER assays (Kobayashi et al., 2008). TEER measurements were performed under sterile conditions at 30 min, 1 h, 3 h, 12 h, 24 h, 48 h, 5 d.p.i., 10 d.p.i., and 15 d.p.i. Confluent monolayers of cultured host cells with TEER values above 500 $\Omega.cm^2$ were used in transport experiments after correction for the resistance obtained in control wells. A decrease in TEER indicates an increase in monolayer permeability and loss of the epithelial barrier. Data are presented as the mean ± S.E.M. of ten independent runs.

2.7. In vivo study

2.7.1. Location

The study was carried out for 60 days during the rainy season in a goat farm located in Apaseo El Grande, Guanajuato, Mexico (20° 32′ 49″ N and 100° 41′ 12″ W). The average annual rainfall ranges between 400 and 600 mms and the average annual temperature ranges between 18° and 22 °C (García, 1981; INEGI, 2017). The study was approved by the Ethics Committee of the Veterinary Medicine and Animal Science Faculty (Facultad de Medicina Veterinaria y Zootecnia (FMVZ)) of the National Autonomous University of Mexico (Universidad Nacional Autónoma de México (UNAM)) with the number DC-2018/2–12. We included 40 male goats, 4-months-old, with an initial average weight of 11.41 ± 1.09 kg, naturally infected with *Eimeria* spp.; with no previous anticoccidial treatment since birth. Positivity to *Eimeria* was determined by flotation and McMaster techniques (Figueroa et al., 2015) in the Department of Parasitology (FMVZ). Goat kids were bred in helminth-free stables. Two weeks before the experiment, the animals received albendazole (7.5 mg/kg) and coprological analyses were carried out to confirm the absence of gastrointestinal helminths. Stool samples were also tested to diagnose *Cryptosporidium*, as well as other pathogens such as bacteria, virus and fungal infections. These tests were done in the Department of Parasitology and the Department of Microbiology and Immunology (FMVZ) respectively.

Animals received water ad libitum and were fed twice a day at 08:00 am and 03:00 pm. Feed was administered according to weight and age, extracted from the NRC publication of 2007. Goat kids were kept in a concrete floor pen with the following measurements: 80 cm × 180 cm x 180 cm. Each animal had its own feeding and drinking bucket. The adaptation period to the diet lasted 20 days. Goat kids (n = 40) were weaned at day 60 of age. A week before and a day before the study started (days −7 and −1) stool samples were collected in plastic bags and kept in refrigeration until further examination. Stool samples were examined by the fecal flotation technique to confirm the presence of *Eimeria* spp. oocysts in three individual samples using the McMaster technique (Figueroa et al., 2015). Oocyst counts were expressed as OPG. Only animals with OPG counts ≥ 500 were included in the study (Joachim et al., 2018). Goat kids were assigned to a group considering the OPG counts (Kommuru et al., 2014) to form four groups (n = 10).

2.7.2. Experimental design and diets

Samples from the diets were processed in the Department of Animal Nutrition and Biochemistry (FMVZ) to determine the proximal chemical analysis (AQP) (Table 1). Each group received one of the four different diets: basal diet + 0% dehydrated grapefruit peel (CTRL); basal diet + 15% grapefruit peel (GDP1); basal diet + 30% grapefruit peel (GDP2); basal diet + 0% grapefruit peel + commercial toltrazuril 5% at a dose of 20 mg/kg given *per os* a single dose at the beginning of the experiment (TTZ). Kids were fed concentrate and forages in a ratio 70:30. Forages consisted mainly of alfalfa hay and oat straw. Table 1 shows that the concentrate containing either 15% GDP or 30% GDP partially replaced the inclusion of corn, as well as a commercial mineral premix (1%) with the following ingredients (g/kg): calcium (100), magnesium (10), sodium (200), phosphorous (80), iron (1500 mg) manganese (1000 mg), iodine (0.05), cobalt (0.125), zinc (4000 mg) and selenium (0.0125). Ingredients were finely chopped and ground to avoid the selection of components. Kids were fed ad libitum twice daily at 07:00 and 20:00 h. Each group was kept in isolation for the duration of the experiment (60 days). The experiment started in June, identified as the rainy season. Animals were checked daily to confirm wellbeing and health.

2.7.3. Blood sampling

Blood samples were individually collected from the jugular vein (10 ml/animal) during the morning hours, using Vaccutainer® tubes on days 0, 7, 15, 30, 45 and 60 of the experiment. Samples were centrifuged at room temperature for 10 min at 800g. Serum was divided into aliquots using Eppendorf tubes and kept at − 20 °C until further examination.

2.7.4. Oocyst count analysis

To evaluate the effect of the *Citrus* diet on oocyst output, fecal samples were obtained individually directly from the rectum on days 0,

Table 1
Ingredient and chemical composition of the four different diets fed to the control and experimental goats, as determined by the Department of Biochemistry and Animal Nutrition, FMVZ).

	Untreated controls	Grapefruit dried peels 15%	Grapefruit dried peels 30%	Toltrazuril
Ingredients (g/kg)				
Grapefruit dried peel	0	150	300	0
Soy meal	280	295	300	280
Ground corn	695	540	380	695
Mineral supplement	10	12	20	10
Calcium carbonate	15	3	0	15
Chemical composition (%)				
Dry matter	88.04	87.81	85.93	88.06
Crude protein	16.64	16.82	15.91	15.40
Crude fiber	4.4	6.5	9.3	4.5
Neutral detergent fiber	13.72	18.03	24.07	13.05
Acid detergent fiber	6.78	11.98	17.45	6.81
Ether extract	4.40	3.27	2.38	4.54
Ash	6.47	5.78	6.73	6.05

3, 7, 14, 21, 28, 35, 42, 49 and 56. Samples were analyzed using the fecal flotation and McMaster techniques (Figueroa et al., 2015). Oocyst sporulation was done to determine the *Eimeria* species involved according to a method previously described (Das et al., 2015). In brief, isolated oocysts from the stool samples were suspended in 300 ml of potassium dichromate solution (2.5%) and were distributed in centrifuge tubes. Tubes were centrifuged at 670 x g for 5 min and the supernatant was discarded. The pellet was washed using a potassium dichromate solution, placed in a Petri dish, and incubated at 28 ºC under constant oxygenation. The sporulation rate was determined using an ocular micrometer (Leica Microsystems, ASPELAB, México). Oocysts that presented four sporocysts clearly divided were counted (100 oocysts) at 0, 12, 24, 36, 48,60, 72, 84, 96 and 108 h (Waldenstedt et al., 2001). Species were morphologically identified, using taxonomic keys *Eimeria* species were identified microscopically based on the morphology of sporulated oocysts (Florin-Christensen and Schnittger, 2018) on days 0 and 60.

2.7.5. Fecal consistency determination

Feces consistency was evaluated using the following score: 1: normal to pasty, 2: semiliquid, 3: watery, 4: hemorrhagic and/or with tissues (Mundt et al., 2005). To increase consistency in the interpretation, the technician in charge of determining fecal consistency was trained using a set of one thousand images.

2.7.6. Production parameters estimation

Feeds offered and leftovers during the study were daily weighed using a 40 kg digital scale with ± 5.0 g precision (Torrey, México). Goat kids were weighed on days 0 and 60 using a ± 100 g precision scale (Torrey, México) to estimate the weight gain.

2.7.7. Lipid peroxidation determination

Malondialdehyde (MDA) was measured in blood in triplicate based on the reaction of MDA with thiobarbituric acid (TBA, Sigma-Aldrich, Mexico) adapted for a microtiter plate reader (Ramos et al., 2010). Briefly, a solution of TBA was added to 0.1 ml of each sample. This mixture was heated at 100 °C for 1 h and then cooled. It was then stirred in a 4 ml layer of n-butyl alcohol in a separation tube and the MDA content in plasma was determined spectrophotometrically at an absorbance of 532 nm against butanol and 97% 1,1,3,3-tetraethoxypropane (TEP) as a standard. MDA levels are expressed as micromoles per liter of plasma (Mmol/l).

2.7.8. Nitric oxide (NO) determination

Total nitrites as a measure of nitric oxide were measured by the Griess reaction (de Oliveira et al., 2011). Briefly, for 200 µl of the reduced samples, 200 µl of Griess solution (sulfanilamide 2% (w/v), N-(1-naphthyl) ethylenediamine 0.2% (w/v), was added and incubated for 15 min at room temperature in the dark. The absorbance was measured at 550 nm using a UV/vis spectrophotometer (Jenway 6305 UV/vis, Princeton, NJ, USA). The relative nitrite concentration was calculated using the standard curve for sodium nitrate. All measurements were performed in triplicate. Nitrite concentrations were determined by spectrophotometric analysis with reference to a standard curve. Results are expressed as nanomoles of NO per liter of plasma.

2.8. Statistical analysis

2.8.1. In vitro study

Analyses were performed using GraphPad Prism version 9 for MacOs (GraphPad Software, San Diego, California USA, www.graphpad.com).

2.8.1.1. Eimeria infection rate and schizont development. The percentage of *Eimeria*-infected cells was calculated as the mean ± S.E.M. of at least five independent replicates. Differences in parasite invasion, schizont numbers and sizes were assessed for significance at the level of 0.05. The Shapiro-Wilk test was used to determine normality. Normally distributed data were analyzed by analysis of variance (ANOVA) and the Dunnett's *t*-test. If normal distribution was violated, the Kruskal-Wallis test was used.

2.8.1.2. Cell viability. Data were compared using a one-way ANOVA test. Differences were compared using a Tukey's test with $P < 0.05$ representing a statistically significant difference.

2.8.1.3. Epithelial monolayer resistance. Data are presented as the mean of ten repetitions ± S.E.M. Analyses were performed by one-way ANOVA followed by a Tukey's test. A difference of $p < 0.05$ was considered significant.

2.8.2. In vivo study

Oocyst counts were converted to natural logarithms (log [OPG + 1]) due to lack of normality. All values were calculated from at least three independent experiments. The statistical model was a single factor design with four randomized levels in complete blocks with repeated observations over time.

Multivariate analysis of variance (MANOVA) was performed to determine the effect of multi-factors (*Eimeria* species, GDP, toltrazuril) and their interactions with blood antioxidant parameters and *Eimeria* reduction.

When the time-treatment interaction was significant, univariate analysis of a randomized single factor design in complete blocks was performed using Bonferroni adjustment. Tukey's test was used to compare the means among treatments, and Dunnett's test was used to compare the means between the treatments and the control group (Pérez-Fonseca et al., 2016).

3. Results

3.1. Chemical analysis of the dehydrated grapefruit peels

The naringenin content was 3.803 mg/g on a dry basis. The proximal chemical analysis of GDP on a wet basis revealed a moisture content of 9.08 ± 0.12 g/100 g; ash, 2.84 ± 0.17 g/100 g; fat, 2.00 ± 0.07 g/100 g, and protein, 4.72 ± 0.18 g/100 g. According to FAO (2010), the desired final moisture content of dried fruits must be 15% for conventionally dried fruits and 20–25% for osmotically dried fruits (wet basis). On a 100 basis, moisture content was 0.00%, crude protein 5.29%, ethereal extract 5.15%, ashes 3.69%, crude fiber 10.04%, nitrogen free extract 75.83%, neutral detergent fiber 87.66% and metabolizable energy 3169.02 kcal/kg.

3.2. HPLC

Out of the six flavanones, naringin and naringenin were the most abundant in GDP. The concentrations (mg/kg) were: hesperidin 5.22 ± 0.7; neohesperidin 14.54 ± 0.87; naringin 285.22 ± 9.35; naringenin 37.21 ± 2.43; nobiletin 10.15 ± 1.02 and quercetin 2.08 ± 0.04.

3.3. Water activity

In the present study, the water activity of dehydrated grapefruit peels was 0.226 ± 0.00. The water activity of the GDP complies with accepted levels of international food safety regulations that state that most microorganisms, mainly those that produce fungal toxins, are not able to grow at $a_w < 0.6$ grow well at $a_w < 0.9$, and that most foodborne bacterial pathogens require $a_w < 0.91$. Some bacteria can survive at lower levels, such as *Staphylococcus aureus* ($a_w = 0.83$) and *Listeria monocytogenes* ($a_w < 0.90$) (Escobedo-Avellaneda et al., 2020).

3.4. Proximal chemical composition of diets

The chemical composition of the forages was 85.5% dry matter, 8.3% crude protein, 33.0% crude fiber, 0.59% ether extract, 3.7% ash, 57.0% neutral detergent fiber and 43.4% acid detergent fiber. Table 1 shows the chemical composition of the concentrates fed to goats on a dry matter basis. The content of crude fiber, neutral detergent fiber and acid detergent fiber were higher than the control diet, yet the ether extract was lower in the concentrate mixture. The metabolizable energy content of the diets on a dry matter basis (DM) was 2.597 Mcal/kg DM (CTRL), 2.558 Mcal/kg DM (GDP1), 2.607 Mcal/kg DM (GDP2) and 2.593 Mcal/kg DM (TTZ).

3.5. In vitro study

3.5.1. Cytotoxicity of the experimental compounds

Measurement of the cytotoxic status of the cells, with MTT, confirmed that the experimental product has no adverse effects when cultured with CIEC. Viability (%) of cells incubated with different concentrations of GDP, relative to control, was 95.79 ± 1.47 (100 µM), 96.85 ± 1.04 (50 µM), 97.09 ± 1.08 (25 µM), 97.32 ± 2.24 (12.5 µM), 97.85 ± 1.07 (6.25 µM), 98.42 ± 2.09 (3.125 µM) and 98.85 ± 1.94 (1.5625 µM).

3.5.2. Cell culture development

Goat infected intestinal epithelial cell cultures were studied at 0, 0.5, 1, 2, 4, 8, 12, 24 h, as well as at 8 and 15 days post-infection (d.p.i.). Sporozoites were detected within cells from the first 60 min. Subsequently, the meronts were observed at 8 d.p.i. No second-generation meronts or sexual stages of oocysts were visualized in the cell culture. The mean sporozoite cell invasion rate was 12.17 ± 0.1491% (GDP), 8.969 ± 0.1003% (TTZ) and 22.45 ± 0.2054% (DMSO) at 24 h post-infection (h.p.i.). Cell invasion rate was significantly lower in GDP as compared to DMSO alone ($P = 0.0003$). Similarly, cell infection recorded for TTZ was significantly lower than that of GDP ($P = 0.0003$) and DMSO ($P < 0.0001$) (Fig. 1). Regarding the mean schizont/mm^2, at 8 d.p.i fewer parasitic structures were observed in the cells exposed to GDP (11.91 ± 0.04418) and TTZ as compared to CTRL (6.469 ± 0.1109 and 23.86 ± 0.1664, respectively) ($P < 0.0001$) (Fig. 2A). After 8 d.p.i, the GDP-exposed schizonts measured 68.67 ± 0.6188 µm; those incubated with TTZ measured 39.81 ± 0.5402 µm and the ones of the CTRL group 97.58 ± 0.6021 µm. Statistically significant differences were demonstrated in all comparisons ($P < 0.0001$). On the other hand, the mean number of schizont/mm^2 counted at 15 d.p.i (Fig. 2B) was significantly lower in cells treated with GDP (9.284 ± 0.04282), and TTZ-treated cells (3.505 ± 0.07312), than in those incubated with DMSO (18.68 ± 0.1177) ($P < 0.0001$ in all cases). The mean size of the schizonts at 15 d.p.i was also significantly different among all groups, measuring 62.65 ± 0.8210 µm (GDP); 32.06 ± 0.2518 µm (TTZ), and 126.3 ± 1.878 µm (DMSO) ($P < 0.0001$ in all cases). Data are presented as the mean of five experiments ± S.E.M.

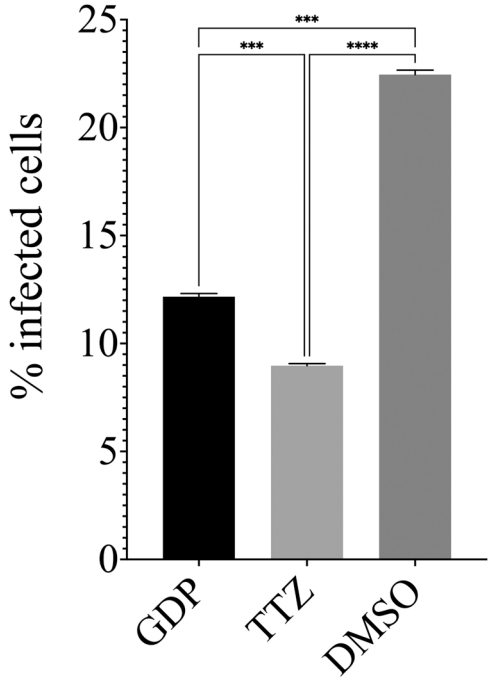

Fig. 1. Invasion rates of *Eimeria ninkohlyakimovae* sporozoites into caprine intestinal epithelial cells under the influence of a solution of grapefruit dried peels (GDP), a commercial formulation of toltrazuril (TTZ) and control (DMSO).

3.5.3. Effect of GDP on the integrity of the intestinal cell membrane

As a measure of the integrity of the epithelial cell barrier in vitro, after infecting them with *Eimeria* sporozoites, the transepithelial electrical resistance (TEER), measured in Ω.cm2) was evaluated through the CIEC (Table 2). The CIEC values measured in the uninfected cultures ranged from 319.94 to 1087.76 Ω.cm2. After only half an hour of cell exposure to the sporozoites, TEER decreased significantly ($P < 0.05$), whether treated or not as compared to uninfected cells. Hence, epithelial barrier dysfunction during in vitro intestinal infections with *E. ninakohlyakimovae*, was assumed. However, half an hour later and up to 360 h, the infected CIEC that were incubated with GDP and TTZ showed TEER values significantly ($P < 0.05$) higher than those detected in untreated-infected cells. At 3, 24, and 48 h.p.i., the infected and treated CIEC had statistically similar TEER values ($P = 0.1370$). From 120 h.p.i. and until the end of the experiment (360 h.p.i.), a significant increase in the TEER values of the infected CIEC treated with TTZ was observed ($P < 0.05$), and it was similar to the one recorded in uninfected cells ($P = 0.7925$). No statistically significant change was observed in TEER values of uninfected CIECs that were incubated with GDP, TTZ, or the vehicle (DMSO) during the assay ($P = 0.4550$ in all cases). Data are presented as the mean of ten repetitions ± S.E.M.

3.6. In vivo study

3.6.1. Effects on Eimeria spp. oocyst shedding

Table 3 summarizes the mean OPG shedding recorded during the trial. The inclusion of dehydrated grapefruit peel in the goat-kits diet had a well-defined anticoccidial effect as shown by the statistically

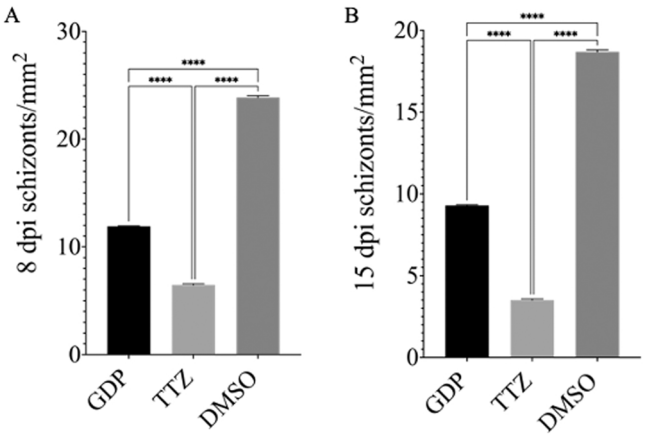

Fig. 2. Number of *Eimeria ninkohlyakimovae* schizonts/mm^2 in caprine intestinal epithelial cells exposed to a solution of grapefruit dried peels (GDP), commercial formulation of toltrazuril (TTZ) and control (DMSO). Experimental products were evaluated (A) 8 days post infection (d.p.i.) and (B) 15 d.p.i.

Table 2
Effect of the addition of a 100 μM solution of grapefruit dried peels (GDP), commercial toltrazuril (TTZ) or DMSO (vehicle) on transepithelial electrical resistance (TEER) at different time points in vitro. (Ω.cm^2, mean ± S.E.M.) (n = 10 independent replicate.

Time (hours)	Infected-treated with GDP	Infected-treated with TTZ	Infected-DMSO	Uninfected-treated with GDP	Uninfected-treated with TTZ	Uninfected-DMSO
0.0	732.14 ± 1.16^{a}	747.19 ± 2.60^{a}	729.25 ± 3.25^{a}	738.86 ± 2.14^{a}	735.37 ± 7.53^{a}	748.33 ± 5.95^{a}
0.5	626.39 ± 2.06^{a}	612.77 ± 2.32^{a}	519.6 ± 10.85^{a}	754.19 ± 4.13^{b}	722.69 ± 3.01^{b}	718.43 ± 3.25^{b}
1.0	628.73 ± 3.40^{a}	627.98 ± 2.69^{a}	432.65 ± 8.61^{b}	786.70 ± 9.29^{c}	778.03 ± 4.72^{c}	802.37 ± 8.38^{c}
3.0	634.76 ± 2.18^{a}	618.11 ± 6.05^{a}	476.66 ± 4.67^{b}	799.85 ± 4.65^{c}	794.18 ± 2.01^{c}	770.89 ± 2.85^{c}
24	659.24 ± 1.15^{a}	653.78 ± 4.67^{a}	445.84 ± 9.96^{b}	786.44 ± 2.36^{c}	797.88 ± 0.60^{c}	796.67 ± 5.74^{c}
48	676.12 ± 0.70^{a}	689.59 ± 8.40^{a}	453.04 ± 5.52^{b}	849.28 ± 5.27^{c}	869.31 ± 6.02^{c}	844.76 ± 7.00^{c}
120	695.07 ± 2.83^{a}	879.84 ± 4.48^{b}	419.38 ± 6.94^{c}	890.19 ± 1.08^{bd}	913.93 ± 2.42^{bd}	892.16 ± 5.53^{bd}
240	752.82 ± 2.06^{a}	915.16 ± 7.10^{b}	372.86 ± 5.92^{c}	992.83 ± 8.82^{bd}	950.60 ± 7.56^{bd}	977.20 ± 3.21^{bd}
360	757.96 ± 1.66^{a}	968.33 ± 3.61^{b}	328.07 ± 7.75^{c}	997.665 ± 5.42^{bd}	987.34 ± 8.40^{bd}	981.31 ± 5.87^{bd}

^{abcd} Different letters within a row indicate statistically significant differences ($P < 0.05$).

Table 3
Effect of the dietary 15% (GDP1) or 30% (GDP2) inclusion of grapefruit dried peels and toltrazuril (TTZ) on *Eimeria* spp. oocyst per gram of feces (OPG, mean ± S.E.M.) output and anticoccidial efficacy in naturally-infected goats (n = 10 kids per group).

Day	Untreated control	GDP1		GDP2		TTZ	
	OPG	OPG	Efficacy (%)	OPG	Efficacy (%)	OPG	Efficacy (%)
0	3315 ± 313.8^{a}	3800 ± 363.6^{a}		3440 ± 374.2^{a}		3465 ± 375.7^{a}	
3	4105 ± 411.2^{a}	4025 ± 447.7^{a}	1.95	3885 ± 322.3^{a}	5.36	30.00 ± 13.33^{b}	99.27
7	5115 ± 376.2^{a}	4310 ± 299.3^{a}	15.74	3910 ± 291.6^{a}	23.56	5.00 ± 5.00^{b}	99.90
14	5205 ± 470.3^{a}	2830 ± 540.5^{b}	45.63	1270 ± 170.7^{b}	75.60	10.00 ± 6.667^{c}	99.81
21	4965 ± 411.5^{a}	1665 ± 324.6^{b}	66.47	380 ± 77.53^{c}	92.35	10.00 ± 10.00^{d}	99.80
28	5105 ± 389.1^{a}	1435 ± 212.1^{b}	71.89	475 ± 134.2^{c}	90.70	95.00 ± 18.93^{d}	98.14
35	5640 ± 468.4^{a}	1575 ± 180.3^{b}	72.07	755 ± 150.5^{c}	86.61	755 ± 109.7^{c}	86.61
42	5835 ± 305.1^{a}	1480 ± 306.9^{bc}	74.64	825 ± 146.7^{b}	85.86	1835 ± 187.7^{c}	68.55
49	5730 ± 428.8^{a}	1555 ± 122.3^{b}	72.86	830 ± 100.3^{c}	85.51	3510 ± 348.6^{d}	38.74
56	4235 ± 316.4^{a}	1240 ± 92.44^{b}	70.72	620 ± 67.99^{c}	85.36	3855 ± 327.3^{a}	8.97

^{abcd}Different letters within a row indicate statistically significant differences on OPG counts ($P < 0.05$)

significant decrease in *Eimeria* OPG-shedding from day 14 onwards both at 15% (GDP1) (P = 0.0753) and at 30% (GDP2) (P < 0.0001). The decrease in the OPG counts on groups GDP1 and GDP2 continued to be statistically significant (P < 0.05) as compared to the CTRL group until the end of the experiment. Likewise, the fecal excretion of oocysts was significantly lower in the GDP2 group as compared to the GDP1 group on days 21 (P = 0.0162), 28 (P = 0.0025), 35 (P = 0.008), 49 (P = 0.0081) and 56 (P = 0.0020). On day 42 no significant difference was observed in OPG excretion between GDP1 and GDP2 (P = 0.5373). A single administration of TTZ produced a significant decrease (P < 0.05) in OPG from *Eimeria* from day 3 to day 49 as compared to CTRL. However, OPG excretion was similar between TTZ and CTRL on day 56 (P = 0.2383). On days 49 and 56 post-treatment, the OPG counts found on GDP1 (P = 0.9938) and GDP2 (P = 0.6160) were similar to those of the CTRL group. Toltrazuril treatment produced a significant reduction (P < 0.05) in OPGs on days 3, 7, 14, 21, 28, 35, 42, and 49 as compared to the CTRL, GDP1, and GDP2 groups. However, OPG excretion was similar between TTZ and CTRL on day 56 (P = 0.2383). It was observed that the effectiveness to decrease the OPG count of toltrazuril was significantly greater than that of GDP1 and GDP2 on days 3, 7, 14, 21, and 28 (P < 0.05 in all cases). Although TTZ reduced OPG more sharply than GDP1 on day 35 (P = 0.0117), this was not the case when compared with GDP2 (P > 0.9999). Subsequently, the OPG count was statistically lower on GDP2 as compared to TTZ on days 42 (P = 0.0368), 49 (P = 0.0002), and 56 (P = 0.0004). Similarly, the OPG comparison of values between GDP1 and TTZ resulted akin on day 42 (P = 0.6813), but it was statistically superior for GDP1 on day 49 (P = 0.0031) and 56 (P = 0.0020).

3.6.2. Anti-Eimeria efficacy

Table 3 also shows the anticoccidial efficacy of the GDP1, GDP2, and TTZ groups as compared to the CTRL group. The efficacy of GDP1 ranged between 72.69 considering 1.95% on day 3%, and 74.64% on day 28. Then it decreased to 70.72% at the end of the study. GDP2 ranged between 89.99, with efficacy of 5.36% on day 3 and a maximum of 92.35% on day 21, decreasing thereafter to 85.36% on day 60. The efficacy of animals treated with a single dose of TTZ ranged between 90.93, with a maximum efficacy of 99.90% on day 7 and a decreased efficacy reaching 8.97% at the end of the trial.

3.6.3. Eimeria species identified

Eimeria species were identified on days 0 and 60 based on morphology and their frequency was recorded in each group as laid out in Supplementary Table 1. Mixed *Eimeria* infections were found in all samples and the number of species per sample ranged from 4 to 8. The most frequent *Eimeria* species at the beginning of this experiment were *E. arloingi, E. ninakohlyakimovae* and *E. christenseni*. Other species identified included *E. alijevi, E. caprina, E. caprovina, E. hirci*, and *E. jochijevi*. No significant difference was observed between the number of *Eimeria* species identified on treated and untreated animals on day 0. At the end of the experiment, it was observed that treatment with GDP1 did not reduce the presence of *Eimeria* species. In contrast, GDP2 significantly decreased the frequency of *E. arloingi* and *E. ninakohlyakimovae*, yet the other species remained unaffected by this treatment. No oocysts of *E. christenseni E. caprina, E. caprovina* and *E. jolchijevi* were detected 60 days after treatment with toltrazuril. Likewise, this anticoccidial drug significantly reduced the percentage of *E. arloingi* and *E. ninakohlyakimovae* while *E. alijevi* and *E. hirci* predominated.

3.6.4. Effects on fecal consistency

Before treatments, pasty feces were recorded in more than 40% of the goat kids in the four groups, and in the untreated control group, this consistency was maintained in more than 50% of the animals during the rest of the experiment. In contrast, it was observed that the goat kids in the groups that consumed 15% GDP and 30% GDP had a mean fecal score significantly similar to that of the TTZ, on day 56 (P > 0.05). No watery or hemorrhagic stools were observed during this trial in any group. Differential diagnosis of stool specimens did not reveal the

presence of another intestinal pathogen including *Cryptosporidium* spp. These results are shown in Table 4.

3.6.5. Effect of ingestion of GDP on productive parameters

Table 5 summarizes the mean daily weight gain (dwg) recorded for all groups and dwg was significantly higher in goat kids supplemented either as for GDP1, GDP2, and TTZ groups, as compared to the CTRL group ($P < 0.05$) and those who ingested the 15% GDP supplementation (GDP1). The initial mean weight ± SEM per group was: 11.01 kg ± 0.1864 (CTRL); 12.03 kg ± 0.3651 (GDP1); 10.89 kg ± 0.3297 (GDP2) and 11.72 kg ± 0.3757 (TTZ). The mean final weights were: 20.12 kg ± 0.8004 (CTRL); 22.59 kg ± 1030 (GDP1); 25.41 kg ± 1.055 (GDP2) and 26.77 kg ± 0.8515 (TTZ). The mean daily weight gains per group were: 0.152 kg (CTRL); 0.176 kg (GDP1); 0.242 kg (GDP2) and 0.250 kg (TTZ). There was a significant difference ($P < 0.05$) between the dwg of the CTRL group as compared to GDP2 ($P = 0.0291$) and with TTZ ($P = 0.0034$) (Table 5). Regarding food consumption, it was determined that there were significantly ($P < 0.05$) more rejections in diets that lacked dehydrated *Citrus*, as the mean (± S.E.M.) feed refusal was 9.94% ± 0.20 in the untreated group, 5.65% ± 0.5 in infected goats treated with 15% GDP, 6.59% ± 0.44 in kids treated with 30% GDP and 9.96 ± 0.11 in animals treated with toltrazuril.

3.6.6. Effect on lipid peroxidation

Table 6 shows the antioxidant activity found on the untreated goat kids and that of DGP supplemented ones. Lipid peroxidation activity was significantly reduced ($P < 0.05$) on day 30 after the consumption of DGP both in groups GDP1 and GDP2, as demonstrated by the decreased serum levels of malondialdehyde (MDA). Animals in the TTZ group also presented significantly lower levels of lipid peroxidation than CTRL ones after 7 days ($P < 0.05$). No significant difference ($P > 0.05$) was observed between the MDA values of the kids of the GDP1 and GDP2 groups on days 30, 45, and 60. Yet, the lipid peroxidation of the TTZ group was significantly higher ($P < 0.0001$) than that detected in GDP1 and GDP2 on days 45 and 60. Furthermore, in this latter-day, the lipid peroxidation activity observed in TTZ was statistically indistinguishable from that of the CTRL group ($P > 0.9999$).

3.6.7. Effect on serum nitrites

Table 6 shows, as well, that nitric oxide (NO) production was significantly higher in CTRL goat kids than in any other group from day 15 post-treatment onwards ($P < 0.05$). Nitrosative stress was similar between GDP1 and GDP2 on days 7, 15, and 30 ($P > 0.9999$). Subsequently, the NO values were significantly lower for GDP2 as compared to GDP1, on days 45 ($P < 0.0001$) and 60 ($P = 0.0001$). In TTZ-treated kids, NO levels were significantly lower than the rest of the groups from day 7–30 ($P < 0.05$). On day 45, NO levels increased significantly in the TTZ group concerning GDP1 ($P = 0.0163$) and GDP2 ($P = 0.0003$). This was also the case on day 60 i.e., TTZ vs. GDP1 ($P = 0.0002$), and TTZ vs. GDP2 ($P < 0.0001$). However, the nitrosative stress measured in the TTZ group on days 45 and 60 was lower than the one found in CTRL ($P < 0.0001$).

Table 4
Fecal consistency, ranging from normal to pasty (score 1) to haemorrhagic or displaying tissue (score 4). of *Eimeria* naturally-infected goats after the daily dietary inclusion of 15% or 30% grapefruit dried peels (GDP) or a single administration of toltrazuril (TTZ) (n = 10 goats per group) (fecal score, mean ± S.E.M.).

Day	Untreated	GDP 15%	GDP 30%	Toltrazuril
0	1.7 ± 0.15^{a}	1.7 ± 0.15^{a}	1.4 ± 0.16^{a}	1.6 ± 0.16^{a}
3	1.7 ± 0.15^{a}	1.5 ± 0.16^{a}	1.5 ± 0.16^{a}	1.3 ± 0.15^{a}
7	1.8 ± 0.013^{a}	1.7 ± 0.15^{a}	1.5 ± 0.16^{a}	1.1 ± 0.10^{b}
14	1.7 ± 0.15^{a}	1.3 ± 0.15^{a}	1.2 ± 0.13^{a}	1.0 ± 0.00^{b}
21	1.6 ± 0.16^{a}	1.4 ± 0.16^{a}	1.2 ± 0.13^{a}	1.0 ± 0.00^{b}
28	1.6 ± 0.16^{a}	1.4 ± 0.16^{b}	1.1 ± 0.10^{c}	1.0 ± 0.00^{c}
35	1.6 ± 0.16^{a}	1.4 ± 0.16^{b}	1.1 ± 0.10^{c}	1.0 ± 0.00^{c}
42	1.6 ± 0.16^{a}	1.3 ± 0.15^{b}	1.1 ± 0.10^{c}	1.0 ± 0.00^{c}
49	1.5 ± 0.16^{a}	1.3 ± 0.15^{b}	1.1 ± 0.10^{c}	1.0 ± 0.00^{c}
56	1.6 ± 0.16^{a}	1.1 ± 0.10^{b}	1.0 ± 0.00^{b}	1.0 ± 0.00^{b}

[abc]Different letters within a row indicate statistically significant differences ($P < 0.05$).

4. Discussion

Numerous pathogenic species of *Eimeria* are found worldwide in goat kids causing diarrhea and allowing a considerable shedding of oocysts, thus perpetuating coccidiosis by environmental contamination (Bangoura and Bardsley, 2020).

Cell culture models of *Eimeria* species that infect ruminants have successfully been used as tools to study host-parasite interactions. *E. ninakohlyakimovae* sporozoites have been used to infect different cell lines such as bovine colonic epithelial cells (López et al., 2019; Odden et al. 2019, primary caprine, bovine and human umbilical vein endothelial cells, bovine foetal gastrointestinal cells, and African green monkey kidney cells, among others (Ruiz et al. 2010). In this study, a caprine epithelial intestinal cell model was used to elucidate the effect of an extract of dehydrated grapefruit peels on *E. ninakohlyakimovae* invasion at the cellular level, as previous reports performed in field trials have shown an anticoccidial efficacy of *Citrus* peels in lambs infected with *Eimeria* (Pérez-Fonseca et al. 2016). The parasite showed infection to cells up to 18 days byt was followed by cell monolayer deterioration. Thus, only asexual stages were observed in this assay. However, the 18-day time frame provided useful insight into GDP influence of *E. ninakohlyakimovae* invasion. In this study, inhibition of *E. ninakohlyakimovae* invasion into caprine epithelial intestinal cells by a solution of dehydrated grapefruit peels was demonstrated. Similar studies have demonstrated the exposure of avian coccidia in vitro to natural products such as nutmeg oil, cinnamon oil, and glabridin (Thabet et al. 2022). It is generally accepted that in vitro assays are suitable to assess the anticoccidial activity of experimental compounds and that these trials provide evidence that might support further in vivo studies which will help in the identification of novel anticoccidial candidates.

Under the circumstances of this assay, the successful cultivation of goat epithelial tissue collected from the gastrointestinal tract was hampered by the risk of overconfluency. Likewise, cell degeneration had to be overcome. Hence, a subculture was attempted but the dissociation with trypsin/EDTA resulted in cell death. It is reasonable to speculate that these dissociating agents might have been cytotoxic to these cells, yet further studies are needed to gather evidence regarding the factors that influence adaptation of these cells to in vitro conditions in order to assess proper maintenance and establish a permanent cell line. However, as desirable as it is to obtain a permanent cell line by multiple passages in vitro, it has been shown that avian species of *Eimeria* specificity for invasion decreases after multiple passages because cells no longer express the factors that influence site selection (Augustine, 2001).

In a previous feeding trial, it was shown that *Eimeria* oocyst output decreases after the administration of dehydrated grapefruit peels to infected lambs (Pérez-Fonseca et al. 2016). The mentioned study proved an impact of grapefruit peels on the modulation of cytokine responses and an increased antioxidant activity in treated sheep. Thus, the current work was focused on establishing a parasite invasion assay to screen for the anticoccidial mode of action of dehydrated grapefruit peels. The cell invasion assay with *E. ninakohlyakimovae* showed that in vitro exposure of the parasite to GDP significantly reduced the mean number and size of schizonts/mm^2 in contrast to untreated controls. If GDP is cytotoxic to sporozoites at the doses used in this study, the longer exposure duration would reduce invasion capability.

This is the first report of the ability of GDP to prevent *E. ninakohlyakimovae* infection of cells in vitro. Our findings demonstrated that the prevention of sporozoite invasion caused by GDP was both through a direct effect on the parasite and by protecting the

Table 5
Mean daily weight gain (kg) 60 days after the inclusion of 15% (GDP1) or 30% (GDP2) grapefruit dried peels and a single treatment with toltrazuril (TTZ) in *Eimeria*-infected goats (mean ± S.E.M. and 95% Confidence Interval) (n = 10 goats per group).

	Untreated control	GDP1	GDP2	TTZ
Initial body weight	11.01 ± 0.18^{a}	12.03 ± 0.36^{a}	10.89 ± 0.32^{a}	11.72 ± 0.37^{a}
	95% C.I. (10.58, 11.43)	95% C.I. (11.20–12.85)	95% C.I. (10.15–11.64)	95% C.I. (10.87–12.57)
Final body weight	20.12 ± 0.80^{a}	22.59 ± 1.03^{ab}	25.41 ± 1.05^{bc}	26.77 ± 0.85^{c}
	95% C.I. (18.31–21.93)	95% C.I. (20.25–24.92)	95% C.I. (23.02–27.79)	95% C.I. (24.85 -, 28.70)
Daily weight gain	0.152 ± 0.01^{a}	0.176 ± 0.01^{ab}	0.242 ± 0.01^{bc}	0.250 ± 0.01^{c}
	95% C.I. (0.11–0.18)	95% C.I. (0.13–0.21)	95% C.I. (0.20–0.28)	95% C.I. (0.22–0.27)

[abc]Different letters within a row indicate statistically significant differences ($P < 0.05$).

Table 6
Antioxidant activity (expressed as micromoles of MDA per liter of plasma and nanomoles of NO per liter of plasma) observed with the dietary inclusion of 15% (GDP1) or 30% (GDP2) grapefruit dried peels and toltrazuril (TTZ) in *Eimeria*-infected goats (mean ± S.E.M.).

Day	CTRL		GDP1		GDP2		TTZ	
	MDA	NO	MDA	NO	MDA	NO	MDA	NO
0	0.833 ± 0.018^{a}	51.0 ± 0.99^{a}	0.818 ± 0.010^{a}	49.7 ± 1.04^{a}	0.800 ± 0.011^{a}	49.4 ± 1.32^{a}	0.825 ± 0.009^{a}	49.8 ± 1.17^{a}
7	0.825 ± 0.105^{a}	49.8 ± 1.05^{a}	0.726 ± 0.010^{a}	48.2 ± 0.69^{a}	0.712 ± 0.013^{a}	46.9 ± 0.81^{a}	0.582 ± 0.005^{b}	35.5 ± 0.28^{b}
15	0.795 ± 0.014^{a}	51.2 ± 1.15^{a}	0.671 ± 0.007^{a}	41.7 ± 0.38^{b}	0.615 ± 0.013^{a}	42.2 ± 0.38^{b}	0.571 ± 0.006^{b}	29.0 ± 0.43^{c}
30	0.885 ± 0.006^{a}	50.1 ± 1.26^{a}	0.582 ± 0.003^{b}	31.5 ± 0.35^{b}	0.564 ± 0.008^{b}	32.1 ± 0.39^{b}	0.573 ± 0.006^{b}	15.5 ± 0.24^{c}
45	0.907 ± 0.009^{a}	50.9 ± 1.15^{a}	0.588 ± 0.003^{b}	25.5 ± 0.27^{b}	0.572 ± 0.006^{b}	20.9 ± 0.52^{c}	0.724 ± 0.011^{c}	30.31 ± 1.25^{d}
60	0.801 ± 0.013^{a}	51.6 ± 1.38^{a}	0.582 ± 0.004^{b}	23.9 ± 0.28^{b}	0.570 ± 0.005^{b}	20.2 ± 0.34^{c}	0.801 ± 0.010^{a}	35.91 ± 1.42^{d}

[abc] Different letters within a row belonging to the same experiment (MDA or Griess Assay) indicate statistically significant differences ($P < 0.05$).

integrity of the intestinal barrier. Nonetheless, the fractions of the dehydrated grapefruit peels involved and the mechanism by which it inhibits sporozoite invasion of epithelial cells are not known. Moreover, in vitro assays do not consider the effect of the complex environment of the ruminant gastrointestinal tract and the infection process. These questions require further research.

Under the implemented experimental model, TEER values were significantly higher in infected cells that were incubated with GDP, hence a protective effect on the intestinal barrier integrity was demonstrated. Previous studies that have evidenced that coccidia of the genus *Eimeria* alter the integrity of the intestinal barrier in mice (Inagaki-Ohara et al., 2006), birds (Park et al., 2020) and cattle by observing a decrease in mRNA expression of effector proteins involved in TJ maintenance (Walker et al., 2015).

On the other hand, after 120 h post-infection, TTZ was more effective than GDP in protecting the intestinal barrier. Therefore, the natural product investigated here is not intended to be a direct replacement for anticoccidial drugs but might rather be used as a possible feed additive together with coccidiostats in *Eimeria* endemic farms.

Following the in vitro results, the impact of GDP on *Eimeria* infections in goats was examined. It was shown that a 30% dietary inclusion of GDP and treatment with toltrazuril achieved a notable reduction in oocyst excretion and frequency of pathogenic *Eimeria* species. These results somehow comply well with the reported anticoccidial efficacy of grapefruit peels presented for sheep (Pérez-Fonseca et al., 2016). This study differs from the former, not only because it was carried out in a different species, but also in the way the *Citrus* preparation was manufactured and how it was administered as an in-feed supplement, rather than with individual force-feeding. It has also been suggested that goat to sheep physiological differences must be considered to optimize the dose of drugs and herbal products, thus minimizing toxicity or treatment failure (Szotáková et al., 2004).

The high anticoccidial efficacy of a single dose of toltrazuril is consistent with previous studies, in which its anti-*Eimeria* effect in various ruminant hosts was reported to occur during the first days after its administration reaching a maximum around 7–10 days after administration (Mundt et al., 2003; Veronesi et al., 2011; Enemark et al., 2015) and a gradual decline of efficacy over time (Saratsi et al., 2020).

Goat kids in the GDP2 group had mean daily weight gain values statistically indistinguishable from those found in the TTZ group. This observation complies well with previous findings that demonstrated that maize replacement in the kids diet by as much as 40%, by a dehydrated *Citrus* pulp, induced an increase in daily weight gain, dry matter intake, and feed conversion rate (García-Rodríguez et al., 2019). Previous reports have shown that the exclusive use of *Citrus* peel without pulp is preferred, as the sugar content is higher in the former (Czech et al., 2021), and this could increase the palatability of the feed offered to kids. When GDP was supplemented, fecal consistency improved as compared to animals in the CTRL group. This can be explained in terms of the antidiarrheals, anti-secretors, and intestinal motility modifying β adrenergic-dependent effects (Adeniyi et al., 2017).

Additionally, and as expected (Kim et al., 2004; Lien et al., 2008), the results obtained allow us to conclude that the antioxidant potential of the GDP constituents, supplemented at 30% in the goats' feed, significantly decreases the lipid peroxidation and nitrosative stress in a similar magnitude to that achieved by toltrazuril. Although further studies are necessary to explain the positive results obtained in this trial, it is tempting to relate them to the known effects that fruits and byproducts of the genus *Citrus* possess through their content of flavonoids such as hesperetin and naringenin that can scavenge superoxide radicals and peroxynitrite in organic environments (Yu et al., 2005); and modulate the pro-inflammatory molecules (Benavente-García and Castillo, 2008).

The precise mechanism by which the dehydrated grapefruit peels extract produced a coccidiostatic effect has not been thoroughly elucidated so far. Nonetheless, this study demonstrated that the anticoccidial effect might be due to several mechanisms as follows: 1. a toxic effect of the flavanones present in the *Citrus* extract, the most abundant one being naringenin. This finding was shown as a reduced infection rate of cultured cells and fewer and smaller schizonts; 2. an immunomodulatory effect that reduced oxidative and nitrosative stress as demonstrated in the field trial; and 3. a protective effect of the dehydrated grapefruit peels on the intestinal barrier integrity, which interfered with parasite invasion.

Hence, further research is encouraged to provide more evidence that could support the beneficial effect of flavonoids and anticoccidials on the intestinal tight junctions. Finally, these data can establish the bases to search for methods that increase the in vitro protective effect of flavonoids found in GDP, and further improve the recorded actions in vivo, as well.

5. Conclusion

This study shows the in vitro and in vivo anticoccidial activity of the dietary inclusion of 15% dehydrated grapefruit peels, and particularly, of 30% dehydrated grapefruit peels and that this effect is comparable to that of toltrazuril, both as far as anticoccidial efficacy and productive parameters are concerned. An antioxidant effect of dehydrated grapefruit peels was demonstrated, and it is postulated that this preparation contributes to maintaining the integrity of the intestinal epithelial barrier under conditions of coccidiosis. Due to the uncostly nature of this by-product of the juice manufacturing industry, additional studies are proposed to study and optimize the dose and bioavailability of flavonoids of dehydrated grapefruit peels when challenged by *Eimeria* spp. infections under various clinical scenarios and if necessary to modify its pharmaceutical form.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.smallrumres.2022.106663.

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