Direct effects of phenolic compounds on the mammary gland: In vivo and ex vivo evidence

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\section{Introduction}

Dairy animal production traits and product quality can be enhanced by supplementing rations with bioactive components. Specifically, the nutritional value of milk can be improved by the inclusion of essential oils (Benchaar et al., 2007), vitamins (Valdez-Arjona & Ramírez-Mella, 2019) and plant-derived antioxidant polyphenol compounds (Castillo, Pereira, Abuelo, & Hernández, 2013) in the diet.

The beneficial effect of dietary phenolic compounds on production traits has been demonstrated. For example, lactating goats browsed on phenolic compounds-rich pasture produced milk with higher fat and especially omega 3 content, higher protein and lower urea content compared to their counterparts fed a poor-phenolic compounds diet (Hadaya et al., 2017; Hadaya et al., 2020a). Dietary composition revealed that these goats consumed mainly lentisk (Pistacia lentiscus), a phenolic compounds-rich (20% dry matter [DM] basis) evergreen brush species (Landau, Muklada, Markovics, & Azaizeh, 2014). Lentisk phenolic compounds contain condensed tannins (flavonoids and flavonol glucosides), and 75% of the phenolic compounds in lentisk are hydrolyzable (galloylated) tannins (Azaizeh et al., 2013), which exert beneficial effects on the redox status of dairy ruminants (Liu, Zhou, & Li, 2013). Specifically, lentisk ethanolic and aqueous extracts have been associated with radical scavenging, antioxidant properties and inhibition of lipid peroxidation (Gardeli et al., 2008). An in-depth investigation of the mechanism underlying the greater production was conducted in a primary culture of mammary gland epithelial cells (MEC) treated with lentisk extract. Treated cells had increased energy production, and cellular resources were preferably channeled to the production of milk constituents (Hadaya et al., 2020b).

It was previously demonstrated that some dietary phenolic compounds, mostly hydrolyzable tannins, are absorbed from the digestive tract (Patra & Saxena, 2011) as indicated indirectly by enhancement of

\begin{thebibliography}
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\item Valdez-Arjona, J. A., \& Ramírez-Mella, J. A. (2019). Vitamin supplementation in ruminants: Ration formulation and strategies. \textit{Veterinary Journal}, 235, 103965.
\item Castillo, C., Pereira, P., Abuelo, A., \& Hernández, M. (2013). Effects of dietary polyphenols on milk quality in terms of composition and antioxidant capacity, we used plasma collected from goats fed hay (HP) or browsed on polyphenolic compounds-rich pasture (primarily lentisk; PP) as a conditioning medium for primary culture of MEC. PP increased 2-fold cellular triglyceride content and 2.4-fold intracellular ATP production and non-mitochondrial oxygen consumption. Taken together, the results imply that lentisk phenolic compounds affect blood, MEC and milk oxidative status, which increase fat production by the mammary gland.
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plasma antioxidant capacity after the consumption of phenolic compounds-rich foods, such as phenolic compounds–rich plant extracts (Gladiné et al., 2007) or kebracho tannins (López-Andrés et al., 2013) given to ewes.

Even though most phenolic compounds are modified in the enterocytes and liver for excretion in bile, feces and urine (Velderrain-Rodríguez et al., 2014), some dietary phenolic compounds are absorbed by the blood and even transferred to milk (O’Connell & Fox, 2001) and thus available to different organs (Koren, kohen, & Ginsburg, 2010), including the mammary gland (Valdez-Arjona, & Ramírez-Mella, 2019).

Direct effects of phenolic compounds in the mammary gland have been demonstrated mostly in the context of apoptosis, inflammation (Dobbelaar et al., 2010), tumorogenesis (Crespy & Williamson, 2004), and regulation of immune response (Muklada et al., 2020). The direct effect of phenolic compounds on mammary cell metabolism and milk production is still unknown.

In an effort to study the effect of dietary phenolic compounds on production, two approaches are commonly used: (i) in vivo – where phenolic compounds are ingested as part of the diet (Komsky-Elbaz et al., 2019; Shabtai et al., 2012). While this approach allows to study the overall effect on production, it does not distinguish between phenolic compounds activity in the gastrointestinal tract (GIT) and their systemic post-absorption effects on the milk producing cells; (ii) in vitro – direct administration of phenolic compounds extract to cells (Hadaya et al., 2020a). This approach allows assessing the direct effect of phenolic compounds on production traits, but disregards the changes in phenolic compounds composition and structure during absorption and liver metabolism. To overcome these limitations and confounding factors, we employed a different approach, in which animals are fed a phenolic compounds-rich or phenolic compounds-poor diet, and their plasma is collected and used as a conditioning medium to treat MEC in culture. In addition, we examined the effect of a lentisk infusion as a source of low concentrations of phenolic compounds on production capacity and milk quality.

2. Materials and methods

2.1. Study design

Experiments were designed in accordance with the Israeli guidelines for animal welfare (ICAGG, 1994). Animals were housed in a roofed building with a dirt floor, in accordance with the Animal Experimentation Ethics Committee (Approval # 786/18 IL).

In vivo experiment. Twenty-four Damascus goats from the Ramat Hanadiv Nature Park (south of Carmel Heights, Israel; 32°33’N, 34°56’E) were allotted by number of lactation (3.21 ± 1.8), days in milk (107 ± 21), and milk yield (1.96 ± 0.61; kg day⁻¹) into two homogeneous groups. The experiment was carried out in a crossover design for 21 days of adaptation to hay-based dietary, and 42 days of crossover treatment, total for 63 days. Diets consisted of 1240 g DM of a commercial concentrate (Ambar Feed Mills Ltd., Hadera, Israel; containing 16% CP, fed individually in the milking parlor, together with 0.89 kg (DM) alfalfa hay (chemical composition in supplemented file). Goats were given fresh water (FW; n = 12) or lentisk infusion (LI; n = 12) to drink.

Ex vivo experiment. The ex vivo experiment was performed with caprine primary MEC as previously described by Hadaya et al. (2020b). Briefly, cells were plated at 150,000 cells per 60-mm plastic dish for cellular lipid and protein extractions, and at 28,000 cells in XF24 cell-culture microplates (Seahorse Bioscience, North Billerica, MA, USA) for cellular metabolic flux analysis. After overnight incubation, the medium was replaced with DMEM/F12 without serum, containing 0.15% (w/v) free FA-free bovine serum albumin (BSA) and insulin (1 μg ml⁻¹), hydrocortisone (0.5 μg ml⁻¹) and prolactin (1 μg ml⁻¹) for 48 h to induce milk lipid and protein synthesis. Then cells were exposed for 24 h to the plasma of goats fed on hay (HP) or tannin-rich pasture (PP). Cells were harvested and counted, and then subjected to lipid extraction and protein quantification. For the Seahorse assay, cells were also harvested at the end of the assay for cell counting and normalization.

DMEM/F12, fetal bovine serum, penicillin, streptomycin, amphotericin B, L-glutamine solution and trypsin–EDTA solution C were purchased from Biological Industries (Beit Haemek, Israel). Bovine insulin, hydrocortisone, ovine prolactin, BSA solution, hyaluronidase, D/Nase I and heparin were purchased from Sigma Aldrich Israel Ltd. (Rehovot, Israel). Collagenase type II was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA).

2.2. Plant material collection and extraction

Lentisk leaves and twigs were collected in the Ramat Hanadiv Nature Park (south of Carmel Heights) during the summer and spring. Harvest was carried out three times a week in the park, then material was washed and stored overnight at 58 °C in a stainless-steel barrel with water (5 kg fresh matter lentisk in 30 l water). Every morning, the lentisk infusion was filtered three times, cooled to room temperature, and served fresh to goats in the LI group.

The lentisk infusion was analyzed for FA composition as previously described by Hadaya et al. (2020a)

Phenolic compounds were identified and quantified by HPLC as previously described (Azaizeh et al., 2013) using HPLC equipped with PDA Plus detector.

2.3. Milk sampling and partitioning

Goats were milked daily at 0600 h and 1400 h. Milk samples were collected at the morning milking from each individual goat, using an Opiflow™ SCR (Netanya, Israel) customized for small ruminants. Approximately 100 ml of milk was separated into four aliquots. The first aliquot was mixed with branopol and natamycin (7.15 mg and 0.35 mg per tablet, respectively) for component analysis, whereby fat, protein, urea and lactose were determined by mid-infrared (calibrated for goat milk) analysis (standard IDF 141:C2000) at the laboratories of the Israeli Cattle Association (Caesarea, Israel). The second aliquot was frozen at – 20 °C for analysis of milk FA composition by GC. The third aliquot (20 ml, duplicate samples) was chilled on ice and used to estimate curd firmness, determined with an Optigraph instrument (Ysebaert, Frepillon, France). Samples (10 ml) were placed in the wells and equilibrated at 30 °C for 60 min to obtain optimal curd firmness. The coagulating enzyme was Formase15 TL (0.5 ml, Gist-Brocsades N.V., Delft, The Netherlands). The fourth aliquot (1 ml) was chilled and frozen at – 20 °C for analysis of milk antioxidant capacity by chemiluminescence assay.

2.4. Extraction and analyses

2.4.1. Lipids

For the in vivo experiment, total lipids were extracted from milk using a protocol adapted from the cold-extraction procedure described previously (Hadaya et al., 2020a). Chromatographic analysis was performed in a gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a fused-silica capillary column (60 m × 0.25 mm i. d., DB169 23, Agilent Technologies) as previously described (Hadaya et al., 2020a). FA composition was calculated as mole percentage of the sum of moles of identified FA in each sample.

For the ex vivo experiment, the effect of plasma source on production of cell triglyceride (Tg) content was determined by HPLC after 24 h treatment with HP or PP. Total lipids were extracted from the harvested cells as previously described (Hadaya et al., 2020b). Separation of polar and neutral lipids was performed on a silica column (Zorbax RX-SIL, 4.6 × 250 mm, Agilent Technologies) by HPLC (HP 1200, Agilent Technologies) with an evaporative light-scattering detector (1200 series ELSD, Agilent Technologies). The separated lipids were identified using...
external standards (Sigma Aldrich, Rehovot, Israel). Quantification was performed against external standard curves and expressed as µg per 10^6 live cells. Live cell number was determined with a hemocytometer after 5 min of Trypan blue staining.

For triglycerides content in plasma, aliquots with plasma from 12 goats fed on hay or pasture were mixed and pooled. Triplicate of 500 µL samples were tested in Cobas C 111 analyzer (Roche Diagnostics International AG, Rotkreuz, Switzerland).

2.4.2. Proteins

After the 24-h treatment with HP or PP, cells were harvested with trypsin (0.05%), washed with phosphate buffered saline (PBS) and stored at −20°C until protein extraction; 0.5 ml of medium was collected for further analysis by HPLC equipped with PDA against known standards, as previously described (Hadaya et al., 2020b).

2.5. Antioxidant capacity

2.5.1. Milk

Milk samples were analyzed for their antioxidant capacity as previously reported by Argov-Argaman et al. (2020). Thawed milk samples (20 µL) were assayed by luminol-enhanced chemiluminescence assay (Ginsburg, Sadovnic, Oron, & Cohen, 2004) for their reducing antioxidant potential. Luminol-enhanced chemiluminescence was measured in a Lumac M2010 Biocounter (3 M Co., St. Paul, MN) connected to a linear recorder, and the resulting light output was recorded as counts per minute (Ginsburg et al., 2004) for 8 min.

2.5.2. Plasma

Blood was collected by vacutainer with heparin as the anticoagulant, and centrifuged at 1,000 x g for 10 min. The top yellow plasma layer was collected into a 2-ml nuclease-free tube containing 1.5 ml PBS (Sigma, St Louis, MO). The mixture was homogenized in PBS for 2–3 min by vortexing and stored in liquid nitrogen for 3 h. A 500-µl aliquot of the mixture was used to extract total RNA using the Gene Elute Mammalian Total RNA Miniprep Kit (Sigma Aldrich Israel) according to the manufacturer’s instructions. The concentration and 260:280 nm optical density ratio of the RNA was determined by spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA samples were kept at −80°C until further analysis. Total RNA (1 µg) was reverse-transcribed to produce cDNA using the qScript cDNA Synthesis Kit (Quanta Biosciences) according to the manufacturer’s instructions.

2.5.3. Milk

Milk samples of 15 ml were centrifuged and 500–700 µg cream (top layer) was collected into a 2-ml nuclease-free tube containing 1.5 ml PBS. The mixture was homogenized in PBS for 2–3 min by vortexing and stored in liquid nitrogen for 3 h. A 500-µl aliquot of the mixture was used to extract total RNA using the Gene Elute Mammalian Total RNA Miniprep Kit (Sigma Aldrich Israel) according to the manufacturer’s instructions. The concentration and 260:280 nm optical density ratio of the RNA was determined by spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA samples were kept at −80°C until further analysis. Total RNA (1 µg) was reverse-transcribed to produce cDNA using the qScript cDNA Synthesis Kit (Quanta Biosciences) according to the manufacturer’s instructions.

2.7.1. RNA extraction and cDNA synthesis

Milk samples of 15 ml were centrifuged and 500–700 µg cream (top
interaction as fixed effects and goat as a random effect. For variables which were also measured before the beginning of the experiment, baseline values were added to the model as covariates.

For the ex vivo experiment, comparisons between treatments were by ANOVA followed by LSMean Tukey–Kramer HSD multiple-comparison test. Significance probe was set to 0.05.

3. Results

3.1. In vivo experiment

3.1.1. Nutrient intake

The phenolic compounds composition of the lentisk infusion was: gallic acid (175.8 mg l−1), 3,4-dihydroxybenzoic acid (not detectable), catechin (553.9 mg l−1), syringic acid (73.85 mg l−1), ethyl gallate (14.4 mg l−1) and rutin (50.8 mg l−1) (Fig. 1). The total phenolic compounds contents in the lentisk infusion was 1.185 mg ml−1 which, with average group drinking of 8.83 l goat−1 day−1, resulted in an average consumption of 9.6 ± 0.95 g goat−1 day−1. To evaluate the addition of lipids to the dietary intake by drinking lentisk infusion, FA composition in the infusion was determined, revealing marginal contents of C11:0, C12:0, C14:0 and C18:0 (0.13, 0.24, 0.34 and 1.78 mg ml−1, respectively).

Body weight of the goats was stable during the experiment, and varied between 69.6 ± 6.1 before and 74.6 ± 5.3 kg after the experimental period, and 69.9 ± 4.8 before and 76.1 ± 5.1 kg after the experimental period for the FW-LI group and the LI-FW group, respectively, with no difference between groups (Table 1). The fecal concentration of phenolic compounds was below the detection level of the near-infrared spectroscopy (NIRS) system in both groups.

3.1.2. Milk yield, composition, and oxidative status

Lentisk infusion did not affect milk yield (P = 0.204). There were no differences in milk protein, lactose or urea concentrations, in somatic cell count (Table 1), or in physical properties such as curd firmness or coagulation time (Table 1). However, the milk produced by LI goats was richer in fat and accordingly, their daily fat yield was higher by 20 g day−1 (Table 1).

Table 1
Feed intake and milk composition of Damascus goats drinking fresh water (FW; n = 12) or lentisk infusion (LI; n = 12).

| Chemical composition, % DM basis          | FW   | LI   | SEM  | P = |
|------------------------------------------|------|------|------|-----|
| NDF                                      | 38.5 | 39.2 | 0.35 | 0.108|
| ADF                                      | 24.6 | 24.1 | 0.3  | 0.105|
| CP                                       | 14.01| 14.04| 0.07 | 0.74 |
| DM intake, g day−1                       | 2143 | 2160 | 49   | 0.78 |
| ME, kcal day−1                           | 5.09 | 5.10 | 0.11 | 0.96 |

NDF = neutral detergent fiber; ADF = acid detergent fiber; DM = dry matter, according to NIRS equation developed by Landau et al. (2008); ME = metabolizable energy. SCC = somatic cell count.

Different lowercase letters within a row indicate significant differences at P < 0.05.

In terms of milk FA composition, the major FA were palmitic (C16:0), oleic (C18:1n9 cis), myristic (C14:0) and capric (C10:0) acids which contributed the most to total milk FA content with 30.37, 15.83, 13.93 and 11.75 g per 100 g, respectively (Table 2). The proportions of C12:0, C18:0 and C18:2n6 cis ranged between 3.11 and 7.64 g per 100 g of FA. The proportions for the remaining 30 FA ranged between 0.05 and 1.84 g per 100 g. Drinking lentisk infusion was associated with lower contents of C20:0 and C22:4n6 (Table 2) but increased the concentration of C18:3n3 by approximately 50% compared to the FW group (Table 2). As the n-6 FA contents did not differ between groups, the n-6/n-3 ratio was significantly lower in the LI vs. FW group (Table 2).

Drinking lentisk infusion was associated with 37% higher antioxidant capacity in the plasma (P < 0.001; Fig. 2a). Moreover, antioxidant capacity in milk was enhanced by 30% in the LI group (P = 0.046; Fig. 2b) compared to the FW group.

Fig. 1. HPLC chromatogram of lentisk infusion. Identified phenolic compounds according to known standards are indicated above the designated peaks as follows: 1 – gallic acid, 2 – 3,4-dihydroxybenzoic acid, 3 – catechin, 4 – syringic acid, 5 – ethyl gallate, 6 – rutin.
Table 2
Fatty acid composition (g per 100 g) in the milk of Damascus goats drinking fresh water (FW; n = 12) or lentisk infusion (LI; n = 12).

|       | FW   | LI   | SEM  | P  |
|-------|------|------|------|----|
| C16:0 | 0.31 | 0.27 | 0.0035 | 0.43 |
| C18:0 | 1.19 | 1.29 | 0.16 | 0.72 |
| C18:1n9 c | 13.91 | 13.96 | 0.3 | 0.84 |
| C18:1n9 t | 0.23 | 0.24 | 0.18 | 0.74 |
| C18:2n6 c | 3.25 | 3.11 | 0.12 | 0.25 |
| C18:2n6 t | 0.67 | 0.66 | 0.076 | 0.92 |
| C18:3n3 | 0.44 | 0.92 | 0.05 | 0.36 |
| C17:0 | 0.53 | 0.53 | 0.016 | 0.85 |
| C17:1 | 0.18 | 0.18 | 0.008 | 0.89 |
| C18:0 | 7.64 | 7.14 | 0.42 | 0.10 |
| C18:2n6 c | 1.84 | 1.55 | 0.16 | 0.14 |
| C18:2n6 t | 0.67 | 0.66 | 0.076 | 0.92 |
| C18:3n6 | 0.13 | 0.11 | 0.013 | 0.31 |
| C18:3n3 | 0.44 | 0.63 | 0.032 | <0.001 |
| C20:0 | 0.19^a | 0.16^b | 0.009 | 0.02 |
| C20:1n9 | 0.08 | 0.07 | 0.006 | 0.12 |
| C20:2n6 | 0.08 | 0.07 | 0.006 | 0.23 |
| C20:3n6 | 0.09 | 0.12 | 0.011 | 0.10 |
| C20:3n3 | 0.06 | 0.07 | 0.006 | 0.48 |
| C20:4n6 | 0.18 | 0.18 | 0.01 | 0.81 |
| C21:0 | 0.06 | 0.06 | 0.009 | 0.94 |
| C20:5n3 | 0.07 | 0.08 | 0.006 | 0.39 |
| C22:0 | 0.06 | 0.07 | 0.006 | 0.55 |
| C22:1 | 0.08 | 0.07 | 0.008 | 0.41 |
| C22:2n6 | 0.06 | 0.04 | 0.006 | 0.11 |
| C23:0 | 0.07 | 0.07 | 0.005 | 0.74 |
| C24:0 | 0.06 | 0.05 | 0.006 | 0.52 |
| C24:1 | 0.11 | 0.09 | 0.008 | 0.38 |
| C24:2n6 | 0.11^a | 0.06^b | 0.01 | 0.01 |
| C24:1 | 0.06 | 0.05 | 0.002 | 0.46 |
| SFA | 74.07 | 75.18 | 0.68 | 0.14 |
| MCFAs | 34.19 | 34.97 | 1.08 | 0.60 |
| LCFA | 8.66 | 8.11 | 0.44 | 0.09 |
| MUFA | 20.7^a | 19.62^b | 0.52 | 0.05 |
| PUFA | 5.22 | 5.2 | 0.21 | 0.93 |
| n-6 | 4.57 | 4.35 | 0.18 | 0.33 |
| n-3 | 0.65^a | 0.85^b | 0.038 | <0.001 |
| n-6:n-3 | 7.22^a | 5.36^b | 0.25 | <0.0001 |

SFA = saturated fatty acid; MCFAs = medium-chain fatty acids; LCFA = long-chain fatty acid; MUFA = monounsaturated fatty acid; PUFA = polyunsaturated fatty acid; n-6 = omega 6 fatty acid; n-3 = omega 3 fatty acid.

Different lowercase and uppercase letters within a row indicate significant differences at P < 0.05 and P < 0.01, respectively.

3.1.3. Gene-expression analysis
Expression of genes involved in the phase 2 response was determined using mRNA extraction from milk fat globules of LI and FW goats (supplemented data file, Fig. 1). Drinking lentisk infusion increased Nrf2 expression by 2.92-fold compared to the FW group (P = 0.024). The expression of SOD tended to be higher in the LI treatment (P = 0.085). A 0.22-fold decrease in the expression of CHOP, encoding a key enzyme in apoptosis regulation in mammalian cells, was recorded in the LI vs. FW group (P < 0.01).

3.2. Ex vivo experiment
To isolate the indirect effect of digestion on production traits of mammary cells, plasma from goats fed on pasture (PP) or hay (HP) was used as a conditioning medium for MEC. While pasture is a rich source of phenolic compounds and antioxidants, the hay-based diet phenolic compounds content is below detectable levels. In addition, the triglycerides content in measured in plasma of goats fed on hay and pasture was 0.81 ± 0.01 and 0.23 ± 0.01 g/L, respectively.

3.2.1. Epithelial cell production traits
Treating MEC in culture with PP increased fat content in the cells, as indicated by a 2-fold increase in the intracellular Tg content (Fig. 3a; P = 0.028).

In addition, treating caprine MEC with PP increased cellular casein content 2.4-fold compared to cells treated with HP (P = 0.0143; Fig. 3b). However, whey content did not differ between treatments (P = 0.36; Fig. 3c).

3.2.2. Respiration and metabolic activity of caprine MEC
The effect of phenolic compounds from feed on plasma bioactivity and, in turn, on modulation of MEC activity, was determined. MEC treated with HP as a conditioning medium tended to have a lower oxygen consumption rate (OCR) compared to the PP treatment (Fig. 4a; P < 0.1). Moreover, maximal respiration and mitochondrial ATP production were higher in cells treated with PP (Fig. 4b and c, respectively; P < 0.05).

In addition, MEC treated with PP showed enhanced non-mitochondrial OCR compared to HP (Fig. 4d; P < 0.001). Proton leakage (Fig. 4e), spare respiratory capacity and coupling efficiency did not differ between treatments (P = 0.74, 0.9 and 0.16, respectively).

4. Discussion
The effects of various phenolic compounds from different plants on...
ruminants have been previously demonstrated. The vast majority of those studies presented a local effect, within the GIT, mostly on protein bioavailability and FA biohydrogenation, which consequently affect milk protein content and FA composition. Here we used a water extract of phenolic compounds which was supplemented to dairy goats through their drinking water. The amount of phenolic compounds consumed was far too low to affect processes in the GIT. Nonetheless, the composition and concentration of the supplement caused significant changes in the plasma and milk, most probably attributable to systemic, postabsorption effects of the phenolic compounds.

We evaluated the effect of low levels of bioactive compounds from *Pistacia lentiscus*, administered as aqueous extract in drinking water, on production traits of dairy goats. We also assessed the systemic effect of the bioactive components on mammary gland metabolism and output, utilizing plasma of treated animals as a conditioning medium for primary culture of MEC. The latter shed light on the direct effect of dietary phenolic compounds, mediated by the plasma, on mammary cells. It should be noted that previous studies in humans showed negative correlation between dietary polyphenol consumption and plasma triglyceride and glucose (Guo et al., 2016). In accordance, in the current study, lower triglyceride content in the plasma of dairy goats was found. These alterations in plasma metabolites could attribute to the differences in MEC production once exposed to the plasma of hay based compared with grazing goats, in addition to the variations in the polyphenols content.

Previous experiments have shown that in vivo, consumption of approximately 140 g day$^{-1}$ of dietary phenolic compounds increases protein concentration in the milk and changes the milk’s FA profile (Hadaya et al., 2017, Hadaya et al., 2020a). There have been other examples of dietary phenolic compounds elevating milk fat output: in cows fed on a total mixed ration containing 1188 g day$^{-1}$ concentrated pomegranate extract (Shabtay et al., 2012), and in cows supplemented with 729 g day$^{-1}$ quebracho phenolic compounds (Henke et al., 2017). These effects were hypothesized to be a combination of a local effect in the rumen and a systemic effect through the plasma. In the current study, the higher protein content in milk from the treated animals can most probably be attributed to phenolic compounds protection against microbial deamination of dietary proteins in the rumen, whereas the effect on milk FA composition can be attributed to a direct effect on mammary gland metabolism. Surprisingly, in the present study, consumption of only 9.5 g day$^{-1}$ phenolic compounds was enough to confer the beneficial effects of lentisk on milk fat concentration and composition, maintaining a very low ratio between omega 6 and omega 3 polyunsaturated fatty acids (PUFA). These findings of an effect on milk fat content by such a low level of phenolic compounds consumption are novel.

Here we suggest that the significant increase in fat production originates from the supplementation of a natural antioxidant that improves plasma (Fig. 2a) and milk (Fig. 2b) antioxidant capacity. Lentisk has been previously shown to benefit smooth muscle cells by enhancing their antioxidant functioning (Triantafyllou et al., 2011), and bovine primary MEC by modulating their oxidative status, allowing allocation of resources for the production of milk constituents rather than enhancing their production ability (Hadaya et al., 2020b).

In the present study, the elevation in antioxidant capacity was expressed in the LI group’s plasma and milk (Fig. 2a and b) in accordance with previous studies in dairy goats (Di Trana et al., 2015) and dairy ewes (Argov-Argaman et al., 2020). Changes in milk antioxidant capacity require phenolic compounds transport from the plasma to the milk, or enhanced secretion of antioxidant metabolites through the MEC to the milk. Given the anatomical structure of the mammary gland alveoli and specifically, the blood–milk barrier, these results suggest that the MEC’s capacity to mitigate oxidative stress was altered by the lentisk infusion. Indeed, when we utilized milk as a source for RNA to study gene-expression patterns in MEC, we found significant elevation of *Nrf2* expression, a key regulator of antioxidant response elements (Kang, Fig. 3. Epithelial cell production traits. Cellular lipid (a) Casein (b) and whey (c) contents caprine MEC treated with hay-based ration plasma (HP; white) or pasture-based ration plasma (PP; gray) for 24 h. n = 4 for each replicate in each treatment. *Means differ significantly at P < 0.05.
Lee, & Kim, 2005), in the LI vs. FW group (supplemented data file, Fig. 1). This finding is in agreement with evidence from lambs fed a concentrate diet enriched with mulberry leaf phenolic compounds (Ouyang et al., 2020). Furthermore, the LI treatment tended to increase SOD expression (supplemented data file, Fig. 1), in accordance with Liu et al. (2013) who reported that supplementation of 151 g of chestnut phenolic compounds per day to transition dairy cows increases SOD activity in the plasma. Therefore, we assume that lentisk phenolic compounds affected the oxidative status of the dairy goats not only by providing an antioxidant shield against ROS, but also indirectly through transcriptional activation of antioxidant genes. These results clearly indicate that dietary phenolic compounds induce a local effect in the mammary gland and its production units - the MEC.

To further investigate the effect of tannins on lipid production, we determined the FA composition of milk from LI vs. FW goats (Table 2). In the LI group, the content of the unsaturated FA C18:3n3 was significantly enhanced. This can be attributed to an effect of tannins on the ruminal biohydrogenation process operated by rumen bacteria, mainly Butyrivibrio strains (Patra & Saxena, 2011). In vitro, phenolic compounds have been shown to inhibit Butyrivibrio fibrisolvens (de Aguiar et al., 2013), which hydrogenates C18:3n3 to rumenic acid, and rumenic acid to vaccinic acid (Bauman & Griinari, 2003), possibly increasing rumen escape of unsaturated FA. These results are in agreement with Maamouri et al. (2019), who showed that ration supplementation with tannin-rich Acacia cyanophylla leaves enhances C18:3n3 content in dairy ewes’ milk, and with another study demonstrating that supplementation of 1.2 g kg⁻¹ DM of olive crude phenolic concentrate to dairy ewes significantly increases C18:3n3 compared to controls (Cappucci et al., 2018). Interestingly, the effect in the present study was limited to omega 3 PUFA, whereas omega 6 PUFA were not affected in the LI group. The specificity of the effect of phenolic compounds on omega 3 but not omega 6 FA is not clear; however this phenomenon has been seen before, upon administering 750 g day⁻¹ olive leaves to Awassi ewes (Abbeddou et al., 2011), or 180 g day⁻¹ pomegranate seed pulp to lactating Saanan goats (Razzaghi et al., 2015). In the present study, the constant omega 6 concentration combined with the elevation in omega 3 resulted in reduction of the omega 6:3 ratio in the milk to a very low value, which is considered beneficial to human health (Simopoulos, 2008).

To understand whether part of the dietary phenolic compounds are absorbed by the blood and hence available to the MEC, we exposed caprine MEC to PP using an ex vivo approach, which significantly elevated Tg (Fig. 3a), suggesting that phenolic compounds in the plasma affect the lipid synthesis and secretion capacity of caprine MEC. Whereas secreted whey proteins did not differ between treatments (Fig. 3c), casein content was approximately 2.4-fold greater in the PP vs. HP treatment (Fig. 3b). The higher protein secretion under the PP treatment was supported by our findings of higher non-mitochondrial oxygen consumption in the PP treated MEC as the protein-folding
process in the endoplasmic reticulum requires oxygen (Tu & Weissman, 2004). The combined effects of PP on lipid and protein production together with the elevation in ATP production (Fig. 4c) indicate a general bioenergetic effect, presumably through mitochondrial functionality. Similar findings have been found (Hadaya et al., 2020b) with bovine primary MEC treated with lentisk ethanolic extract.

However, unlike the previous study that found reduced protein leakage upon exposure of bovine MEC to the ethanolic extract (Hadaya et al., 2020b), in the current study, no change was found in protein leakage or coupling efficiency when using plasma from treated goats on caprine MEC (Fig. 4e). These discrepancies suggest that the effect of lentisk phenolic compounds is not a physiochemical one on the mitochondrial membrane, as when the lentisk extract was applied directly on MEC (Hadaya et al., 2020b). In addition, these differences highlight the importance of acknowledging the change in dietary phenolic compounds during digestion, absorption and hepatic metabolism, and the fact that once they reach the mammary gland through the plasma, their composition, structure and concentration differ from their original parameters in the diet.

The results of the present research suggest that water-extracted tannins from lentisk, absorbed into the bloodstream, directly affect the oxidative status of the mammary gland, which enhances cellular fat production. This would put into question the former paradigm of a bioenergetic effect, presumably through mitochondrial function, or the ability to bind with proteins in the GIT, mainly depending on dose and chemical structure. Thus, lentisk infusion can be used as a practical tool to maintain DM intake and milk performance, and alleviate oxidative stress to improve milk fat production and quality, although the exact mechanisms underlying the positive effects of lentisk phenolic compounds supplementation on milk performance are not yet clear.

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. Supplementary data

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