animal

The effect of willow fodder feeding on immune cell populations in the blood and milk of late-lactating dairy goats

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In a previous study, we showed that access to willow fodder decreased somatic cell counts (**SCC**) in the milk of local Mamber goats grazing in brushland at the end of lactation. To test whether the consumption of willow affects the cells of the immune system, Alpine crossbred dairy goats grazing in the same environment were either offered free access to freshly cut willow fodder (W, n = 24) or not (C, n = 24) for 2 weeks. The willow fodder contained 7.5 g/kg DM of salicin. The other major secondary compounds were catechin, myricitrin, hyperin and chlorogenic acid (2.2, 2.6, 1.0 and 0.75 g/kg DM, respectively). Udder health status was determined before the experiment, and each of the two groups included five (W) or six (C) goats defined as infected, as established by microbial cfu in milk, and 19 (W) or 18 (C) non-infected goats. Goats ingested, on average, 600 g of DM from willow (25% of food intake), resulting in minor changes in dietary quality compared to the controls, as established by faecal near-IR spectrometry. Throughout the 2 weeks of experiment, differences between groups in dietary CP contents were minor and affected neither by infection nor by access to willow; the dietary percentage of neutral detergent fibre (**NDF**) decreased in C and increased in W; dietary acid detergent fibre (**ADF**) increased; and the dietary tannin contents decreased for both treatments. However, milking performance and milk quality attributes in both W and C goats were similar. Initial SCC and milk neutrophil (cluster of differentiation (**CD**)18+ and porcine granulocyte (**PG**)68) cell counts were higher in infected than in non-infected goats; counts decreased significantly in W but not in C uninfected goats. The percentage of CD8+ T-cells increased in all C goats, while in the W group, a significant increase was found only for infected goats. The consumption of willow mitigated an increase in CD8+ in blood and triggered an increase in CD8+ in milk, suggesting an immune-regulatory effect independent of udder status. To our knowledge, this is the first report of a direct nutraceutical effect of fodder ingestion on the immune status of goats.

Keywords: nutraceutical, anti-inflammatory, salicin, phenolic glucoside, caprine

Implications

Willow (*Salix spp.*) provides a high biomass of good nutritional quality when irrigated with water of borderline quality. Independent of udder status, in late-lactating goats, the consumption of willow is associated with changes in immune cell populations in both blood and milk. We interpret this changes as anti-inflammatory and stress-alleviating effects. To our knowledge, this is the first report of a nutraceutical effect of fodder on the immune status of ruminants.

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Introduction

Intra-mammary infection is a major cause of economic loss in the goat dairy industry (Leitner *et al.*, 2004) and an important issue in animal welfare (Burvenich and Peeters, 1982). A widespread estimate of mammary gland health is provided by somatic cell counts (**SCC**). Counting of somatic cells in goats is complicated by the presence of numerous non-cellular particles in milk due to the apocrine type of milk secretion, which results in the shedding of cytoplasmic particles, many of which are enucleated and similar in size to milk somatic cells, resulting in an overestimation of SCC (Bergonier *et al.*, 2003).

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During inflammation in the bovine mammary gland, a major increase in SCC would result from the influx of neutrophils into the milk, but SCC is also affected by parity, season and stress (Sharma *et al.*, 2011). Similarly, in goats, SCC is not only indicative of udder health: they are affected by parity, prolificacy, milking management (Granado *et al.*, 2014) and are naturally subjected to acute daily and seasonal variations within a goat (Zeng *et al*, 1997). In cows, at the end of lactation, blood monocytes become macrophages, and their numbers increase in milk (Sharma *et al.*, 2011). Mammary involution and synchronously increased SCC, which can reach extremely high values (Granado *et al.*, 2014), are spontaneous and not tightly associated with intra-mammary infection in late-lactating goats (Foschino *et al.*, 2002). At that period, milk would feature an increased percentage of neutrophils, and decreased percentages of macrophage and lymphocytes (Bergonier *et al.*, 2003). Including willow in the diet of Mamber goats in late lactation prevented a seasonal increase in milk SCC (Muklada *et al.,* 2018).

Flow cytometry and modern staining procedures of lymphocytes are instrumental in addressing the relationship between mammary health and SCC. In cows, within T-lymphocytes, cluster of differentiation (**CD**)4^+ (helpers) prevails during mastitis, while $CD8^+$ (cytotoxic) is the predominant type in healthy udders (Oviedo-Boyso *et al.*, 2007). Changes in milk lymphocytes during mastitis depend on the pathogen involved: clinical *E. coli* mastitis is associated with increased recruitment of neutrophils, while the influx of macrophage-derived monocytes to the mammary gland and infection from *Staphylococcus aureus* is associated with increased $CD4^+$ and $CD8^+$ lymphocyte recruitment (Oviedo-Boyso *et al.*, 2007). There are few analyses of leucocyte populations, relative to immunity acquisition for goats. Baliu-Piqué *et al.* (2019) established that $CD4^+$ T-cells were the predominant subset during the neonatal period in blood, while $CD8^+$ T-cells predominated in adults.

Willow (*Salix spp.*) contains salicylates, a group of phenolic glucosides and, in particular, salicin, a non-specific cyclo-oxygenase (**COX**)1 and COX2 inhibitor. Salicin is a precursor of saligenin, itself converted into acetylsalicylic acid, a non-steroidal anti-inflammatory drug (**NSAID**), commercially known as aspirin (Van Wyk and Wink, 2017). Salidroside, another phenolic glucoside contained in willow, attenuated inflammatory responses by suppressing NF-κB and mitogen-activated protein kinase activation in lipopolysaccharide-induced mastitis in mice (Li *et al.*, 2013). Concentration-dependent anti-inflammatory effects were also found for water-soluble fractions of willow bark in lipopolysaccharide-activated human monocytes (Bonaterra *et al.*, 2010), and sodium salicylate was shown to be beneficial to cows throughout the whole period of lactation (Farney *et al.,* 2013).

We hypothesized that: (1) in goats, as in humans, the consumption of willow phenolic glucosides would reduce inflammation and would modify blood and milk lymphocyte populations; and (2) the consumption of willow would affect leucocytes differently in healthy goats compared with goats affected with subclinical mastitis.

Material and methods

Animals and experimental design

The experiment took place on the south side of the Mount Carmel ridge, Israel (32°25′ N, 34°52′ E), which is characterized by an average annual rainfall of 600 mm and a 180-day rainy season from October to April. The ecosystem is a disturbed Mediterranean woodland (garrigue) characterized by steep, rocky terrain with patches of shallow soil. The vegetation is dominated by tannin-rich *Pistacia lentiscus* shrubs and low *Phillyrea latifolia* trees.

Forty-eight lactating goats (F1 crossbred Damascus × Alpine and Mamber × Alpine), in their first to fifth lactation, served in this experiment. Animals were maintained, according to the Israel Council on Animal Care's 1994 Guidelines (ICACG, 1994, no. 740/17), in a roofed building with dirt floor. Goats were milked once daily at 0600 h, using an OpiFlow™ system for sheep and goats (SCR, Netanya, Israel) that allows for online recording of individual daily milk yield. A fixed amount of concentrate (1.35 kg DM/day) was fed daily to individual animals in the milking parlour at 0600 and 1500 h daily.

After morning milking, all goats were turned out for grazing in woodland for 4 h, not including travelling time.

Experimental animals were divided into two groups from 19 November (day 1) to 4 December 2016. Does in W (willow group) were offered 1.5 kg per head of freshly cut willow fodder daily (ca. 0.6 kg DM/day) from 1300 to 0600 h (of the next day) harvested from 4-year-old, irrigated willow (*Salix acmophylla*) trees (Muklada *et al.*, 2018). Leftovers, mainly stems >4 mm in diameter, represented approximately 30% of offered fodder. Chemical composition of willow offered and leftovers is shown in Table 1. The control group (C) had no access to willow. Before the experiment, goats were exposed to mating bucks.

Does were allotted into groups that did not differ in initial milk yield (2.19 ± 0.16), composition (fat, 6.64 ± 0.23%;

Table 1 *Composition (%, on DM basis) and stem diameter (mm) of willow offered to goats and residues in days 11 to 14*

	Offered	Residue
Ash (%)	5.54 ± 0.20	3.41 ± 0.10
In vitro DM digestibility (%)	43.5 ± 6.8	32.5 ± 2.4
CP (%)	11.9 ± 2.3	7.9 ± 0.2
Neutral detergent fibre (%)	44.9 ± 9.8	66.1 ± 1.3
Acid detergent fibre (%)	35.6 ± 8.2	53.3 ± 1.2
Acid detergent lignin (%)	13.7 ± 1.2	11.8 ± 0.9
Ca (%)	0.86 ± 0.33	0.55 ± 0.06
P (%)	0.20 ± 0.01	0.19 ± 0.01
Polyethylene-binding tannins (%)	5.5 ± 0.28	5.5 ± 0.66
Stem diameter		
Mean	2.45 ± 0.19	4.00 ± 0.95
Median	2.25	4.04
Minimum	0.64	0.87
Maximum	6.55	6.69

protein, $4.27 \pm 0.08\%$; lactose, $4.27 \pm 0.12\%$), parity (3.4 ± 0.3) and BW (71.0 ± 1.6 kg). Prior to group allotment, milk yield was recorded daily for 7 days and averaged within a goat, and the composition of the milk was established. Out of 48 goats, microbes were identified in the milk of 11 goats, including eight with coagulase negative Staphylococci (**CNS**: *S. similans, caprae* and *chromogenes*), two with *Corynebacterium mastitidis*, and one with *Bacillus cereus.* Four CNS-positive and one *C. mastitidis*-positive goats were allotted to each of the C and W groups and the *B. cereus* one was allotted to C. Values for $\log_{10}$ (cfu) in milk were 2.43 ± 0.17 and 3.69 ± 0.29 for non-infected and infected goats, respectively; and for $\log_{10}$ (SCC), 3.59 ± 0.40 and 3.94 ± 0.40 in the same order.

Milk and blood analyses

Milk. Approximately 150 ml of milk was sampled by hand at day 0 (19 November) and day 15 (4 December) from the right half-udder of each goat at morning milking, using the OpiFlow™ sampling system. Samples were divided into three aliquots. The first aliquot was mixed with bronopol (2-bromo-2-nitropropane1,3-diol and 2-bromo-2-nitropropanol) for component analysis – milk SCC, fat, protein, urea and lactose, determined by mid-infrared analysis (standard IDF 141C:2000) – at the laboratories of the Israeli Cattle Breeders Association (Caesarea, Israel). The second and third aliquots were transferred directly at 4°C to the laboratory of Kimron Veterinary Institute for microbial analysis and leucocyte identification.

Leucocyte identification in milk. Leukocyte differentiation in milk was by flow cytometry (FACS Calibur; Becton-Dickinson, San Jose, CA, USA) as described by Leitner *et al.* (2003). The non-conjugated monoclonal antibodies (**mAbs**) (Monoclonal Antibody Center, Washington State University, Pullman, WA, USA) used for the detection of different leucocytes were (i) leucocytes : anti-CD18 : BAQ30A (mouse IgG-1), (ii) lymphocyte : anti-CD4 : GC50A1 (mouse IgM), anti-CD8 : CACT80C (mouse IgG-1), (iii) anti-CD14 (monocyte/macrophage) : CAM36A (mouse IgG-1), (iv) anti-porcine granulocyte (PG)68 A (mouse IgG-1). All mAbs were species-reactive with bovine and goat cell, at the exception of PG68A, which was specifically linked with swine cells; the polyclonal secondary antibodies used were goat F(ab)2 anti-mouse immunoglobulin G (**IgG**) (H + L) conjugated with fluorescein isothiocyanate (**FITC**) that exhibited minimal cross-reaction to human, bovine and horse serum proteins (Jackson Immuno Research Lab, West Grove, PA, USA). For the detection of CD4 and CD8 cells, double staining was used: affinity-isolated goat anti-mouse IgG-1 conjugated with TRI-COLOR for CD8$^+$ cells (Invitrogen, Carlsbad, CA) and goat anti-mouse IgM FITC conjugated (Jackson Immuno Research Lab) for CD4$^+$ cells.

Bacteriological analysis. Bacteriological analysis was performed before the experiment and at the end of exposure to willow according to accepted standards (NMC, 1999). Bacteriological examination of milk samples and presumptive identification were performed at the Udder Health

Laboratory of the Israel Cattle Breeders Association. A 10-ml aliquot from each milk sample was inoculated onto 5% sheep blood agar plates and incubated for 48 h at 37°C. Isolates were then identified to species level by standard microbiological and biochemical tests, in accordance with NMC (1999) recommendations. Final bacterial identification was performed by matrix-assisted laser desorption/ionization–time-of-flight (**MALDI-TOF**) in an Autoflex system (Bruker, Israel). Bacterial species were assigned using Biotyper with the Bruker updated library.

Leucocyte identification in blood. On days 0 and 15 of the experiment, two evacuated blood tubes containing lithium heparin as an anticoagulant were used to collect 20 ml of jugular blood. One blood tube from each goat was used for standard differential cell counts performed using a CBC instrument (ProCyte Dx Hematology Analyzer; IDEXX Laboratories, Westbrook, Maine, USA). In addition, the second blood tube was put immediately on ice and transferred to lab within 2 h for the analysis of peripheral blood leukocyte populations using flow cytometry as described in Lange *et al.* (2016), with some adjustments. T-helper cells were identified using bovine CD4 (MCA1653 PE); cytotoxic T lymphocytes (**CTL**) were identified using bovine CD8 (MCA837 A647); cells expressing the IL-2 receptor (CD25) were identified using bovine CD25 (MCA2430 PE). All antibodies were from Bio-Rad (Hercules, CA, USA). According to the antibody data sheets, CD8 and CD25 had cross-reactivity with sheep; therefore, we assumed that they would react with goats. For CD4, the antibody did not have declared cross-reactivity. Explanations on gating strategy are in Supplementary Table S1.

For analysis, 7 µl each of various antibodies was transferred into fluorescence-activated cell sorting (**FACS**) glass tubes, and 50 µl of whole blood was added and incubated for 30 min at 4°C in the dark. Lysis of red blood cells was performed by adding nine parts of distilled water for no more than 1 min. Subsequently, one part of 10× PBS (02-023-5A; Biological Industries, Israel) was added while mixing the tubes. Samples were then centrifuged for 10 min at 400×**g** and 4°C. Samples were washed using 2 ml PBS and centrifuged at 400×**g** for 7 min at 4°C. Finally, cell pellets were suspended in 300 µl PBS and analysed using the Accuri C6 Plus flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). A gate for leucocytes was drawn containing lymphocytes and monocytes based on their forward and sideward scatter characteristics. For each cell type, the percentage of cells within this gate expressing the surface marker was determined.

Willow fodder and dietary composition

Willow fodder composition. Willow samples and residues were dried at 60°C for 2 days in an aerated oven, and CP, NDF, ADL and polyethylene glycol-binding tannins (**PEG-b-T**) were analysed by near-IR spectrometry (**NIRS**; Landau *et al.*, 2004) with Foss NIRSystems 5000 (Hoganas, Sweden). For Ca and P analysis, dry leaves were ground, digested in nitric acid (10%) in triplicate and incubated at room temperature for at

least 24 h before measuring elemental concentration by inductively coupled plasma (**ICP**) after suitable dilution (Varian 720-ES, I.S.I., Petah-Tiqwa, Israel). Tannin contents in willow fodder and residues were assayed with NIRS and expressed as PEG-b-t (Landau *et al.*, 2004). To characterize the level of feeding selectivity, fibre length was measured by calliper in offered willow and residues for the last 3 days of the experiment.

Willow salicylic glucosides, flavonoids and phenolic acids with liquid chromatography/time-of-flight/mass spectrometry. Foliage and small twigs collected from the trough for the last 3 days of experiment were frozen in liquid N. Samples were ground using a Genogrinder (Gino/Grinder 2010; SPEX SamplePrep, Metuchen, New Jersey, USA) with 2.8-mm stainless steel beads. Tissue samples of 0.1 g (fresh matter) were homogenized with 1 ml 80% MeOH, followed by vortexing for 30 s, shaking for another 60 min while repeating vortexing every 10 min, and separation of cell debris and particles, which were discarded, by centrifugation (5000×**g**) for 5 min. One millilitre of each sample was then filtered through Acrodisc® Syringe Filters with GHP Membrane, 13 mm with 0.2 μm (PALL, Washington, NY, USA) and transferred to vials for liquid chromatography/TOF/mass spectrometry (LC-TOF-MS). The LC-MS analysis was carried out on an Agilent 1290 Infinity series liquid chromatograph coupled with an Agilent 1290 Infinity DAD and Agilent 6530C Quadrupole Time-of-Flight mass spectrometer (Agilent Technologies, Santa Clara, USA). Compounds were separated on a Zorbax Extend-C18 Rapid Resolution HT column (2.1 × 50 mm, 1.8 micron; Agilent Technologies). The gradient elution mobile phase consisted of H_2O with 0.1% (v/v) formic acid (eluent A) and acetonitrile containing 0.1% (v/v) formic acid (eluent B). The column was equilibrated with 2.5% eluent B at a flow rate of 0.3 ml/min for 2 min. Eluent B was then increased to 80% till 10 min and maintained till 12 min, then raised to 95% eluent B till 14 min and restored to 5% by 15 min for re-equilibration till 16.5 min. The flow rate of the mobile phase was 0.3 ml/min, and the column oven temperature was 40ºC. Eluting compounds were subjected to a Jet Stream electrospray ionization interface operated in negative mode with the following settings: gas temperature of 315°C with a flow rate of 12 l/min and nebulizer set to 35 psig, sheath gas temperature of 360°C at 12 l/min flow. VCap was set to 3000 V, the fragmentor to 140 V and the skimmer to 65 V. Data were acquired with a 100 to 1700 mass/charge (*m/z*) range. The electrospray capillary voltage was 3500 V (Agilent Technologies). The [M–H] ions of target compounds were analysed using Masshunter qualitative and quantitative analysis software, version B.07.00 (Agilent Technologies). Exact mass and retention time were compared to those of purchased standards; where standards were not available, compound concentrations were assessed only as areas under peaks.

Individual dietary composition. The individual composition (in percentage) of dietary components (CP; neutral detergent fibre, **NDF**; acid detergent fibre, **ADF**; *in vitro* DM digestibility, **IVDMD**) was established by faecal NIRS as described by Glasser *et al.* (2008). In brief, faeces were grab-collected after milking on days 0 and 14, dried at

60°C in a ventilated oven for 48 h, ground to pass a 1-mm sieve and stored in paper bags in a shaded area at 20°C until analysed. Faecal samples were re-dried at 60°C for 1 h, allowed to equilibrate in a desiccator at ambient temperature for 1 h, packed into sample cells with a NIR-transparent quartz cover glass, and scanned at wavelengths from 1104 to 2492 nm in 2-nm increments with a Foss NIRSystems (Hoganas, Sweden) model 5000 NIR reflectance monochromator spectrometer to collect NIR spectra as log (1/*R*), where *R* is reflectance. Faecal NIRS calibrations had good precision (R^2) and accuracy (SE of cross-validation, **SEcv**) for the determination of dietary percentages of IVDMD ($R^2 = 0.91$, SEcv = 4.4%), NDF ($R^2 = 0.88$, SEcv = 10%), ADF ($R^2 = 0.89$, SEcv = 2.4%) and CP ($R^2 = 0.93$, SEcv = 0.7%). A faecal NIRS calibration previously established, as explained by Landau *et al.* (2008), with 58 pairs of diets and faeces from goats provided with willow fodder in a range of 0% to 50% of total ingested DM was used to determine the dietary percentage of willow in diets selected by goats. Standard error of cross-validation was 5.5%, and linearity values were 0.97 and 0.88 for R^2cal and R^2cv, respectively.

Statistical analysis

Statistical analyses were performed with version 9.4 of SAS (2013). Data are summarized as mean + SE. For each variable, comparison of the treated group to the control group was implemented for the difference between the measurement 14 days after the beginning of the experiment and that before the experiment. Data on SCC and cfu were log-transformed in order to normalize and to stabilize variances. The model used was ANCOVA, with group, existing infection (yes/no) and their interaction as fixed factors, and baseline measurement as a covariate. The significance level used was $\alpha = 0.05$. In cases were a significant interaction was detected between group and existing infection, comparisons between groups were performed for infected and non-infected separately by contrast *t*-tests. None of the data were deposited in an official repository.

Results

Diets consumed

Willow fodder contained 42 ± 1% of DM. A week after exposure, the intake of willow stabilized at 500 to 600 g DM per head per day (Figure 1), and ingesting willow was not associated with changes in faecal appearance. Residues consisted exclusively of stems, and diameter of consumed willow was 2.6 ± 0.3 cm. Willow formed 25.6 ± 1.4% of the total DM intake in W goats for the last 4 days of experiment, as determined by faecal NIRS. Infected and non-infected goats did not differ in willow intake. The willow fodder offered contained 7.5 g/kg DM of salicin. The other major secondary compounds with identified standard peaks were catechin, myricitrin, hyperin and chlorogenic acid (2.2, 2.6, 1.0 and 0.75 g/kg DM, respectively; Table 2). Where standards were not available and compound concentrations could be only
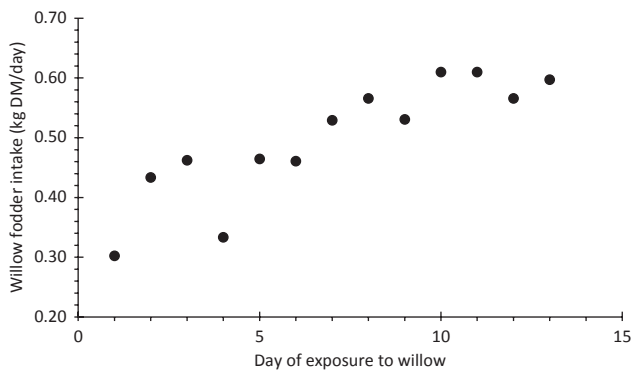
Figure 1 Group daily intake by goats (kg/day per head) of willow fodder throughout the experiment.

Table 2 *Plant secondary compound concentrations in willow fodder offered to goats, as determined by retention time, exact molecular mass and authentic standard: μg/g DM, mean ± SE of last 3 days of the experiment*

	Compound	Concentration (μg/g DM)
Salicin	Salicylate glucoside	7485 ± 2699
Picein	Salicylate glucoside	7.9 ± 4.7
Salicylic acid	Salicylate	3.6 ± 2
Catechin	Phenolic acids	2227 ± 433
Chlorogenic acid	Phenolic acids	747 ± 329
Hyperin	Flavonol	1045 ± 244
Quercetin	Flavonol	18.6 ± 18.6
Taxifolin	Flavonol	12.4 ± 6.2
Luteolin	Flavone glucoside	2.8 ± 0.4
Myricitrin	Flavonoids	2565 ± 581

assessed as areas under peaks, tremulacin and salicortin appeared as prominent salicylates, and hyperin, kaempferol, epicatechin and derivates of coumaric acid were notable phenolic acids and flavonoids (Supplementary Table S2).

The initial dietary percentages of *P. lentiscus*, CP, IVDMD and PEG-bT were not affected by infection, but infected goats tended ($P < 0.10$) to ingest diets slightly richer in NDF and ADF. As these differences were minor throughout the experiment, only analyses of control *v.* willow are shown (Table 3). The dietary percentage of *P. lentiscus* decreased during the experimental period ($P < 0.001$) for all goats, but less so in the willow group than in the control group (9.7% and 5.8% of diet, $P < 0.05$). Dietary CP content (12.4 ± 0.1%) increased significantly in C during the experiment ($P < 0.05$), but overall, differences in CP dietary contents were minor and not affected by infection nor by access to willow. Throughout the 2 weeks of the experiment, the dietary percentage of NDF decreased in C and increased in W ($P < 0.01$), and dietary ADF increased ($P < 0.01$) *in vitro*. Dry matter digestibility decreased ($P < 0.01$), and dietary tannin contents decreased for both treatments. For all nutritional attributes but NDF, exposure to willow mitigated the changes in dietary nutrients that occurred during the experiment ($P < 0.0001$).

Udder health, milking performance and milk composition
As expected, the initial $\log_{10}$ (cfu) of bacteria in milk was higher for infected than non-infected goats (3.79 ± 0.12 and 3.49 ± 0.08, $P < 0.001$). No difference in cfu was found between non-infected goats (2.72 ± 0.10 and 2.76 ± 0.10) of C and W groups. Throughout the experiment, $\log_{10}$ cfu

Table 3 Dietary attributes of diets (%, on DM basis): Pistacia lentiscus content, CP, neutral (NDF) and acid (ADF) detergent fibre, *in vitro* DM digestibility (IVDMD) and polyethylene glycol-binding tannins (PEG-b-T) of goats exposed to freshly cut willow fodder (W) or not (C): n = 24, mean ± SE

| Diets	20 November	4 December	Difference (4 December to 20 November)	Significance of difference (P)	Effects (ANCOVA) on difference Baseline	Diet
Pistacia lentiscus						
C	27.7 ± 0.49	18 ± 0.71	−9.68 ± 0.68	<0.01	0.0001	0.04
W	25.4 ± 0.53	19.6 ± 0.49	−5.84 ± 0.69	<0.01		
CP						
C	12.2 ± 0.09	12.5 ± 0.1	0.25 ± 0.11	0.04	0.0001	NS
W	12.7 ± 0.1	12.6 ± 0.11	−0.05 ± 0.11	0.65		
NDF						
C	39.8 ± 0.22	38.8 ± 0.35	−1.03 ± 0.38	0.01	0.0001	0.01
W	38.5 ± 0.31	40.3 ± 0.22	1.72 ± 0.34	<0.01		
ADF						
C	21.6 ± 0.19	24 ± 0.2	2.45 ± 0.2	<0.01	0.0001	0.01
W	21.9 ± 0.17	23.1 ± 0.19	1.13 ± 0.13	<0.01		
IVDMD						
C	61.4 ± 0.47	56.8 ± 0.74	−4.58 ± 0. 81	<0.01	0.0001	0.01
W	63.6 ± 0.77	62.1 ± 0.6	−1.56 ± 0.9	0.1		
PEG-b-T						
C	6.4 ± 0.08	4.95 ± 0.16	−1.44 ± 0.16	<0.01	0.001	0.01
W	6.05 ± 0.08	5.5 ± 0.07	−0.55 ± 0.1	<0.01		

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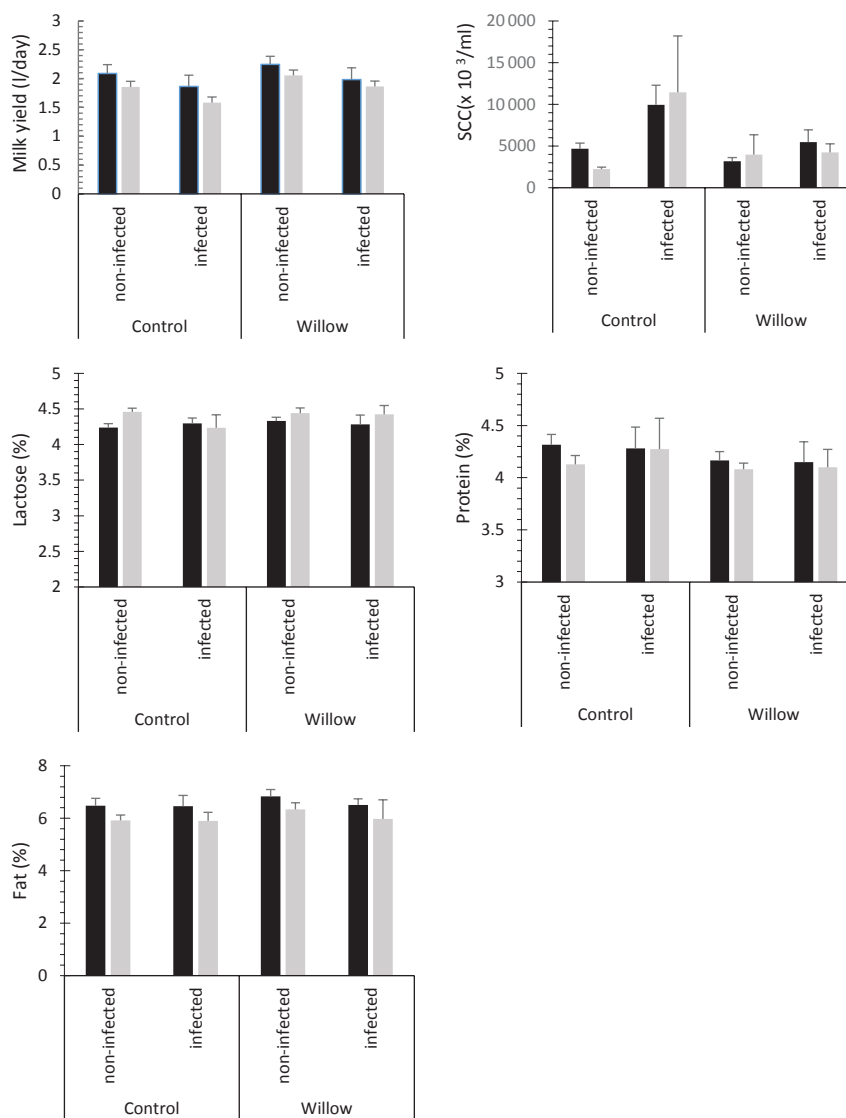
Figure 2 Milk yield (l/day), somatic cell count (SCC) and milk attributes before (in black) and after a 14-day exposure (in grey) of infected or non-infected goats to willow fodder (means of 18 uninfected and 6 infected goats and standard errors).

decreased in all groups, with a more pronounced decrease in infected compared with non-infected goats (1.6 ± 0.29 and 0.19 ± 0.52, $P < 0.001$), and with no effect for diet or infection × diet interaction.

Initial values of SCC were very high: 4700×10^6 and 2200×10^6 in goats in C and W groups, respectively (Figure 2). Initial $\log_{10}$ (SCC) was higher for infected than non-infected goats, independent of dietary group, and tended to be lower (3.54 ± 0.15 and 3.75 ± 0.12, $P < 0.10$) in W than in C. Thereafter, SCC decreased ($P < 0.01$) and neither access to willow nor presence of infection affected the magnitude of this change. Lactose tended ($P < 0.10$) to increase in non-infected but not in infected C goats throughout the experiment.

Infected goats tended ($P < 0.10$) to produce less milk than non-infected goats before the experiment (2.20 ± 0.15 v. 1.89 ± 0.16 l per day). The effect of pre-experimental values (covariates) was significant for all estimates of changes in

milk composition ($P < 0.01$). During the experiment, milk yield (Figure 2) was not affected by infection or exposure to willow, but a significant decrease in milk yield ($P < 0.05$) was found in C goats only. Milk protein and fat contents were affected neither by infection nor by access to willow, and did not vary significantly throughout the experiment.

Milk and blood leucocyte measurements
Milk. Initial neutrophil percentage was higher in infected than non-infected goats ($88.4 \pm 2.3\%$ and $81.8 \pm 2.0\%$, $P < 0.05$). The percentages of CD18$^+$ and PG68 cells in infected goats (86.2 ± 3.0 and 84.4 ± 3.7, respectively) were initially higher ($P < 0.01$; Figure 3) than in non-infected goats (75.8 ± 2.8 and 71.3 ± 3.0, respectively) and tended ($P = 0.07$) to decrease throughout. However, this decrease was significant ($P < 0.05$) only in W non-infected goats.
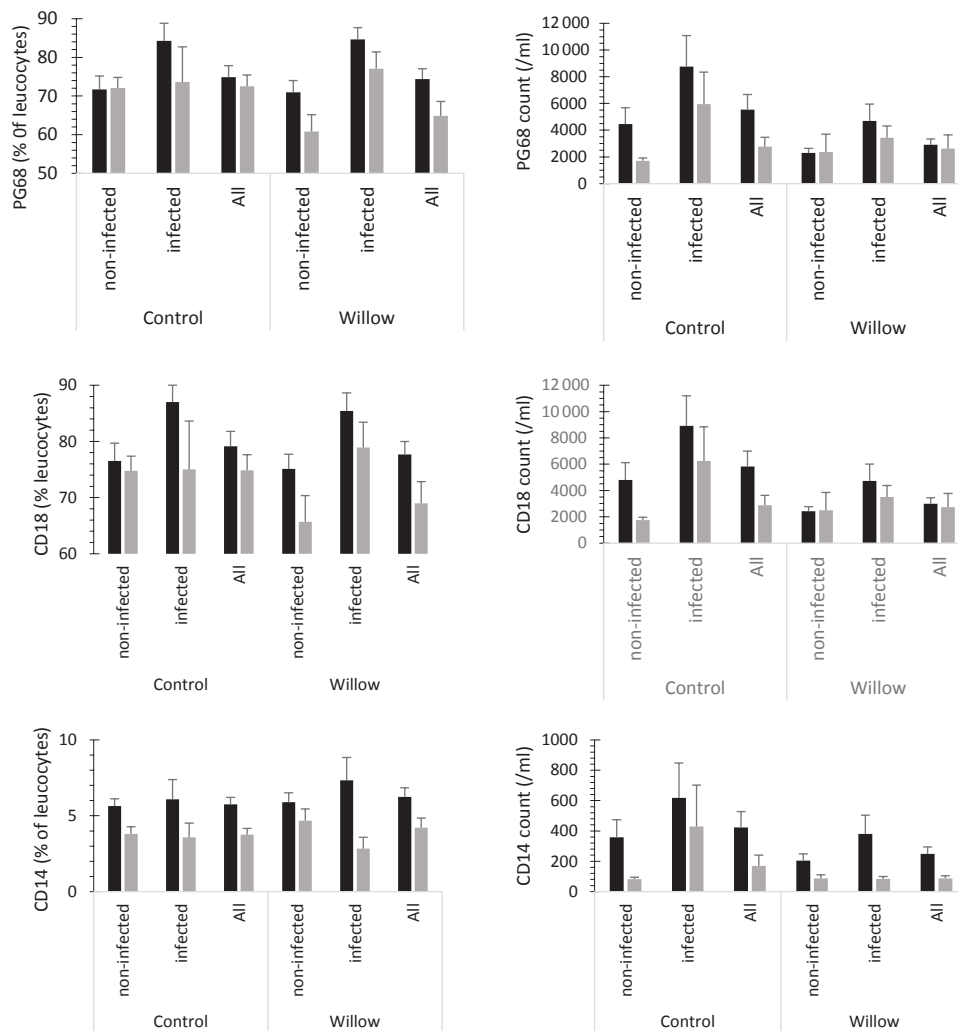
Figure 3 Percentages (left panel) and counts of (right panel) of selected leucocytes in the milk of infected or non-infected goats before (in black) and after a 14-day exposure (in grey) to willow fodder (means and standard errors). PG and CD stand for porcine granulocyte and cluster of differentiation, respectively.

Diet, infection and their interaction did not affect the change in neutrophil percentage.

The initial average for CD14 monocytes in milk was $6.0 \pm 0.5\%$ and did not differ between infected and non-infected goats. All values decreased throughout the experiment for goats in C ($P < 0.001$) and W ($P < 0.05$) groups, with no significant effect of diet, infection or their interaction.

Initial average lymphocyte percentage in milk was $1.47 \pm 0.48\%$ and not significantly affected by infection. Lymphocyte percentage increased fourfold ($P < 0.01$) in W, but did not increase in C goats. The magnitude of change in lymphocyte percentage tended ($P < 0.10$) to be greater in W than in C, without effect for infection and diet × infection interaction. The initial percentage of CD4 ($0.47 \pm 0.20\%$) and CD8$^+$ ($0.29 \pm 0.15\%$) lymphocytes did not differ between infected and non-infected goats. For both, a significant ($P < 0.001$) increase was noted for W – but not C – goats throughout the experiment. Diet tended ($P < 0.10$) to affect the magnitude of this change: access to willow increased the increase in CD4 in milk in non-infected goats ($P < 0.05$).

When expressed as counts (Figure 3), the variability of all milk leucocyte populations was very high. In non-infected goats, neutrophils, encompassing CD18 and PG68 counts, decreased significantly ($P < 0.05$) in C but not W goats. For CD14, that decrease was significant for all goats, except for C infected goats. The other counts did not significantly respond to diet and infection, and did not differ before and after the experiments.

Leucocytes in blood
The average initial percentage of blood lymphocytes was $30.5 \pm 1.3\%$ and not affected by infection (Figure 4). A decrease in the percentage of blood lymphocytes was noted for both infected and non-infected goats in the W but not C group (Figure 4, $P < 0.01$).

The average of CD4$^+$ T-cells was $0.22 \pm 0.03\%$, that is, very low, compared with the total count of lymphocytes, suggesting that specific binding might possibly have been impaired. Throughout the experiment, the percentage of CD4$^+$ T-cells increased in W ($P < 0.01$) but not in C; and in
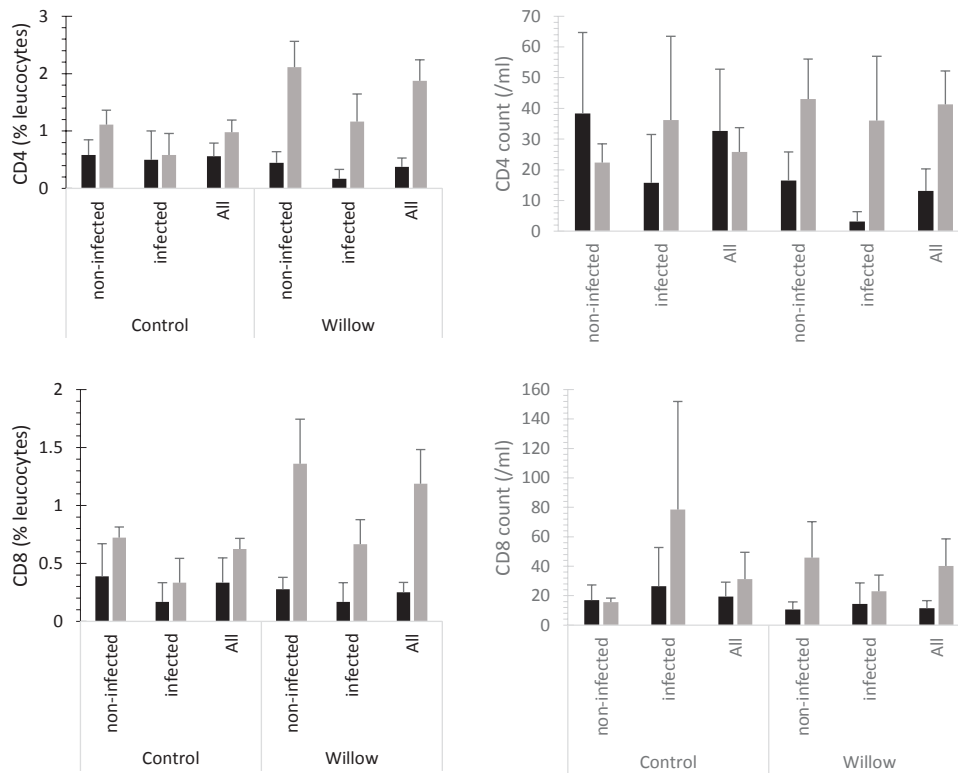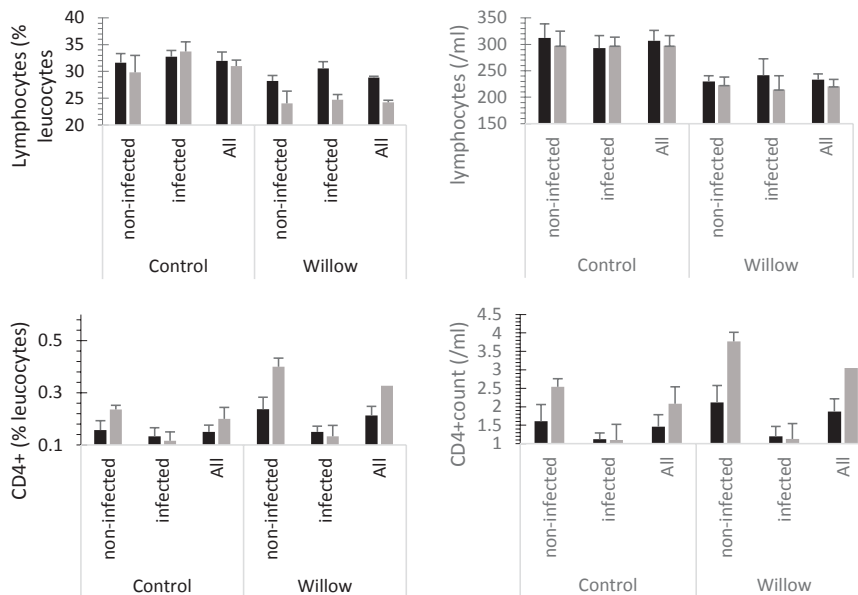
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Figure 3 (continued)

Figure 4 Percentages (left panel) and counts (right panel) of selected leucocytes in the blood of infected or non-infected goats before (in black) and after (in grey) a 14-day exposure to willow fodder (means and standard errors). PG and CD stand for porcine granulocyte and cluster of differentiation, respectively.

the two groups, the increase in CD4+ percentage was higher in non-infected than in infected goats ($P < 0.01$). The initial average of CD8+ T-cells ($13.0 \pm 1.1\%$) did not differ between infected and non-infected goats. Throughout the experiment, the percentage of CD8+ T-cells increased strongly ($P < 0.001$) in all C goats. In contrast, a significant increase was found in the W group ($P < 0.01$) only for infected goats. Overall, the consumption of willow mitigated the increase in CD8+ ($P < 0.05$). Further analysis showed that the consumption of willow was found to mitigate ($P < 0.01$) the increase in the percentage of CD8+ in non-infected goats, but not in infected goats.
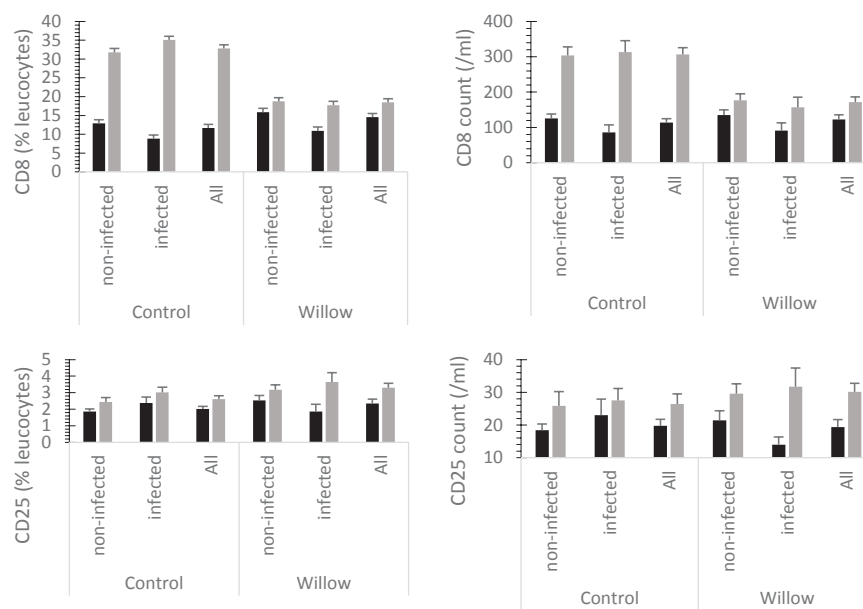
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Figure 4 (Continued)

The effects of diet and infection on the change in the percentage of CD25 cells were significant ($P < 0.05$; Figure 4), and their interaction tended ($P < 0.10$) to be so. The percentage of CD25 cells increased by access to willow in infected goats ($P < 0.05$); and within W goats, the increase in CD25 was greater in infected ($P < 0.01$) than in non-infected goats.

The initial percentages of blood neutrophils ($53.0 \pm 2.0\%$), eosinophils ($6.5 \pm 0.7\%$), monocytes ($5.6 \pm 0.2\%$) and basophils ($0.46 \pm 0.10\%$) were not affected by infection, and their values kept steady throughout the study.

Discussion

Even though salicylate-rich plants, such as meadowsweet (*Filipendula ulmaria*) or poplar (*Populus alba*), are found in eastern Mediterranean grazing lands, they were not present in pasture at the time of experiment. Liquid chromatography/TOF/mass spectrometry revealed a great variety of secondary compounds of willow (Table 2), similar to those described by van Wyk and Wink (2017). The willow fodder offered contained 7.5 g/kg DM of salicin, somewhat higher than the 4.4 g/kg DM reported by Muklada *et al.* (2018) for the same willow stand. A daily intake of salicylate (approx. 0.15 g/kg BW per day) increased milk yield in mature cows (Farney *et al.*, 2013). Goats in the present study were provided with 0.063 g/kg BW per day of salicin (as calculated from Table 2). However, salicortin and tremulacin (estimated only by untargeted determination, based on molecular weight; Supplementary Table S2) also metabolize into salicylic acid. The concentrations of salicin, tremulacin and salicortin were 0.75%, 0.75% and 3.4% of DM. Therefore, there is a strong possibility that salicin represented only a small part of total dietary salicylate glucosides. The comparison with other studies on the effect of salicylic acid on lactation would be relevant only if we were able to compare salicylic acid concentrations in the blood, but this was not done in the present study.

The most notable phenolic acids and flavonoids in willow were catechin, myricitrin, hyperin and chlorogenic acid, which amounted to approximately 6.5 g/kg DM, to which the identified – but not quantified – kaempferol, epicatechin and derivatives of coumaric acid must be added (Supplementary Table S2). The concentration of PEG-binding tannin, encompassing flavonoids and phenolic acids, in willow was approximately 5.5% (Table 1), similar to the dietary PEG-binding tannins in C. The C and W groups had similar dietary tannin contents of 5.0% and 5.5%, respectively (Table 3). Many flavonoids, particularly catechin, and chlorogenic and myricetin acids, are found in the vegetation consumed by the two groups of goats, in particular in *P. lentiscus* (Azaizeh *et al.*, 2013). Hence we assumed that the major difference between W and C diets was the presence of salicylate glucosides, as flavonoid and phenolic acids were at a high concentrations in both diets.

Ingested salicin is converted to saligenin (salicyl alcohol), itself absorbed, as are all salicylate aglycones, into the bloodstream and oxidized in the liver to salicylic acid (Van Wyk and Wink, 2017). As salicylic acid inhibits cyclooxygenase, hence prostaglandin biosynthesis, and prostaglandin mediates inflammation (Van Wyk and Wink, 2017), our hypothesis was that the consumption of willow would promote anti-inflammatory effects, evidenced by modified blood and milk immune cell populations.

Throughout the experiment, the increase in the percentage of CD8+ T-cells in blood was attenuated in W compared with C goats (Figure 4). CD8+ T-cells represent a population of cytotoxic T-cells that recognize pathogen-derived peptides

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complexed with major histocompatibility complex class I molecules on the surface of infected cells (Wong and Pamer, 2003). A lack of increase in CD8^{+} T-cells in the blood of W compared to C goats may be related to the described suppressive effect of a low dose of aspirin on T-cell activation (Hussain *et al.*, 2012); for example, treating adenovirus-infected mice with low doses of aspirin and clopidogrel reduced the homing of virus-specific cytotoxic T-cells (Iannacone *et al.*, 2017). These findings could suggest an immunosuppressive effect of willow fodder supplementation on the immune function of goats. Furthermore, Naliboff *et al.* (1991) and Solberg *et al.* (1995) showed in humans that social short-term stress and strenuous physical effort are related with increased CD8^{+} in blood, and Solberg *et al.* (1995) established that meditation attenuates stress-related elevation of CD8^{+} T-cells in blood. According to these studies, we suggest a stress-alleviating effect of consuming willow fodder, independent of udder status. In addition, our results demonstrate that nutraceuticals can affect the immune function of small ruminants by altering the proportion of subpopulations of white blood cells. This may be one of the mechanisms by which these dietary active ingredients elicit their positive effect on the health of ruminants, and additional studies should be conducted to further explore the specific effects of willow on the immune function of goats.

In milk, a significant ($P < 0.001$) increase in CD4^{+} and CD8^{+} percentages was noted for W but not C goats throughout the experiment (Figure 3). In other words, feeding W seemed to have modulated the partition of peripheral T-cells between blood and milk. As the initial percentage of CD4^{+} ($0.47 \pm 0.20\%$) and CD8^{+} ($0.29 \pm 0.15\%$) lymphocytes in milk did not differ between infected and non-infected goats, we infer that this modulating effect is independent of udder health. This is in agreement with Hussain *et al.* (2012), who described a wide array of modulating effects of aspirin on the immune system: a decrease in IL-4 production and mitogen primed human CD4^{+} cells, promotion of T-cell function and proliferation, and stimulation of CD4^{+} and CD25^{+} Treg cells. The differences found between CD4^{+} and CD8^{+} in milk should be interpreted with care due to the overall low percentage of these cells in milk. One could argue that these subtle differences ($<1\%$) fall within the margin of error of the FACS technique. Yet, the difference was systematically found in the W group, and not in the C group, indicating a possible immune modulatory effect.

Taken together, the finding of a major change in T-lymphocyte population dynamics seems to be unrelated to mammary status and health. We expected that SCC would increase throughout the experiment as goats were in late lactation, that exposure to willow would mitigate this increase, and that access to willow would delay the involution of mammary glands and associated changes in milk composition, as described before (Muklada *et al.*, 2018). Data in the present investigation were analysed separately for infected and non-infected goats. Pre-experimental SCC values were very high for all goats (Figure 2). After 2 weeks of the study, SCC decreased ($P < 0.01$), but access to willow and infection did not affect the magnitude of this decrease. Conditions differed between this study and the one published before (Muklada *et al.*, 2018): the goats used here were not local, low-producing Mamber, but higher-yielding Alpine crossbred goats. Another possibility is that SCC values are extremely volatile in late-lactating goats and are affected by many factors, such as oestrus and exposure to bucks (Bergonier *et al.*, 2003). Indeed, goats were just recently exposed to bucks in the present study, while in the previous study, goats were in pregnancy of ≥ 2 months. This might explain the much higher initial SCC in the present study. It may be suspected that SCC at late lactation in goats is a mediocre estimate of white blood cells in milk, and hence of the effects of diet or even infection on udder health. Therefore, contrary to the values expressed in percentages, the absolute values of concentrations of leucocytes (percentage of leucocytes multiplied by SCC count in milk) must be viewed cautiously.

Data for milk somatic cells were analysed from three viewpoints: (1) How did advancing in lactation affect leucocytes in milk? (2) What changes in milk leucocytes were associated with infection? and (3) Was exposure to willow fodder associated with changes in milk leucocytes?

Udder infection, as evidenced by bacterial cfu and SCC in milk, was characterized by higher pre-experimental values of milk neutrophil percentages, encompassing CD18 and PG68 cells (Figure 3). Sharma *et al.* (2011) also found that the major increase in SCC sources during inflammation is an influx of neutrophils into the milk. Bergonier *et al.* (2003) stated that the influx of neutrophils occurs naturally during late lactation in goats, which did not occur during the 2 weeks of the present experiment. A decrease in the percentage of neutrophils, expressed as CD18 and PG68, was noted only in goats exposed to willow during this time. Perhaps salicylic acid would decrease the adherence of neutrophils to the endothelial lining (Hussain *et al.*, 2012). Such a decrease in neutrophils is compatible with a decrease in SCC found by Muklada *et al.* (2018) in goats given access to willow fodder.

We did not find that infection was associated with differences in milk lymphocytes, encompassing CD4 or CD8 percentages. This is in contrast with Oviedo-Boyso *et al.* (2007), who reported that changes in milk lymphocytes occur during bovine mastitis, depending on the pathogen, with CD8^{+} and CD4^{+} as predominant types in healthy and infected udders, respectively. In sheep, after intramammary infection with *S. epidermidis*, Winter and Colditz (2002) observed a smaller percentage of CD4^{+} and CD8^{+} lymphocytes 24 and 48 h after infection, compared to that in the milk of control animals.

It must be noted that the percentages of T-lymphocytes in milk in the present study were low: Leitner *et al.* (2003) reported values of 3% and 6% for CD4 and CD8^{+}, respectively, in the milk of healthy cows, compared with 0.4% to 1.7% and 0.4% to 0.8% in the milk of healthy goats here, and as wide as 1% to 13% and 1% to 27% for mastitic cows, in the same order.

Also the percentages of CD4$^+$ in blood in our study (<1%) were one order of magnitude lower, compared with $17.7 \pm 6.5\%$ found for goats by Baliu-Piqué et al. (2019). This can be explained by a seeming lack of cross-reactivity of the antibodies used for CD4$^+$ detection with goats. In contrast, we found a range between 8.8% and 35% of CD8 leucocytes, which is comparable to $25.4 \pm 8.4\%$ reported for adult goats by the same authors.

The consumption of willow mitigated an increase of CD8$^+$ in blood, and triggered an increase of CD8$^+$ in milk, suggesting an immune-regulatory effect. This is the first report, to our knowledge, of a direct nutraceutical effect on the immune status of goats.

Conclusion

The ingestion of willow fodder had only minor effects on the nutritional status of late-lactating goats, with the exception of the amount of salicylate glucosides and, in particular, salicin, which seems to have affected the immune cell dynamics. The main effects were decreased neutrophils (PG68 and CD18) in milk and alleviation of CD$^+$ T-lymphocyte increase in blood, which are interpreted as anti-inflammatory and anti-stress effects.

Acknowledgements

Declaration of interests
None.

Ethics statement
The experimental protocol was approved by the Israel Council on Animal Care 1994 (ICACG, 1994, no. 740/17), and animal care was implemented according to Israel Council on Animal Care's 1994 (740/17) guidelines.

Software and data repository resources
None of the data were deposited in an official repository.

Supplementary material
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