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Abstract: Biochemical and histological approaches were used to study the metabolic adaptations of transition dairy goats to dietary supplementation with saturated and unsaturated fatty acids. Twenty-three Alpine dairy goats were divided into three groups and fed a basal pre-kidding and lactation diet (C) or the same diet supplemented with fish oil (FO) or stearic acid (ST) starting one 1 week before kidding until 21 days in milk (DIM). No differences were observed in milk production and composition. However, the serum non-esterified fatty acid (NEFA) and beta-hydroxybutyrate (BOHB) concentrations were changed over time by the treatments. The mean adipocyte area, measured on a subset of 12 goats, which included 4 four subjects from each experimental group, decreased constantly in the C and ST groups from -7 to 21 days, while the FO group did not change between days 7 to 21. These results support the idea that FO is able to limit lipolysis, although the energy balance is still negative. No inflammatory processes were observed in the liver in accordance with the blood leukocytes trend, even if moderate to severe fatty changes in the liver were observed in the experimental goats. In the FO group, however, although fatty infiltration appeared more severe and, it occurred more gradually compared with the other diets. Overall, these results suggest an interesting ability of dietary lipid supplements to affect the fat mobilizing machinery; FO in particular seems able to reduce/delay fat mobilization and could improve hepatocyte adaptation to fatty infiltration, allowing the cells to better maintain their function.

Revision Note Manuscript Rumin-D-16-7921

Rumin-D-16-7921

Both reviewers viewed the manuscript quite favorably and listed some minor comments for revision. Some additional minor comments are given below.

25 and some other places. Conventionally, a number rather than word is used when preceding a unit such as week. "1 week" or "1 wk"

- Thank you for your suggestion. The requested change has been applied.

29. Relatedly, typically in a case like this with a number less than ten, the word would be used. "four"

- Thank you for your suggestion. The requested change has been applied.

35. "appeared more severe and it occurred …."

- Thank you for your suggestion. The requested change has been applied.

40. Key words should be in alphabetical order.

- Thank you for your suggestion. The requested change has been applied.

44-45 and elsewhere. There is agreement with the reviewer comment that one-sentence paragraphs should be avoided.

- Thank you for your suggestion. The requested change has been applied.

61. "intake if dry matter …" Perhaps a semicolon rather than a colon is most appropriate.

- Thank you for your suggestion. The requested change has been applied.

78. "Lecchi et al., 2011, 2013)."

- Thank you for your suggestion. The requested change has been applied.

92 and elsewhere. Headings and subheadings should be flush left.

- Thank you for your suggestion. The requested change has been applied.

105. Perhaps it could be specified that this was a grass hay.

- Thank you for your suggestion. The requested change has been applied.

107. "mixed" for "mix"

- Thank you for your suggestion. The requested change has been applied.

103-108 and Table 1. Perhaps how the NEl levels were determined should be specified. Based on the ingredient levels, it perhaps would have been expected that that pre- and post-kidding diets would have differed more in NEl concentration. Perhaps it should be specified that the pre-kidding diet was fairly conventional because of the influence upon tissue mobilization. Depending on the quality of the grass hay, perhaps some readers might think that the pre-kidding diet would lend itself to relatively high mobilization near kidding and thereafter in early lactation, which would impact the likelihood and nature of effects of oil and fatty acid supplementation.

- Thank you for your suggestion. In the text it has been specified that basal diets were fairly conventional and in the table the NEl calculation has been detailed.

109. Suggest "follows:" for "follow:"

- Thank you for your suggestion. The requested change has been applied.

125. "acid as a positive"

- Thank you for your suggestion. The requested change has been applied.

132. Only the first letter of the first word capitalized.

- Thank you for your suggestion. The requested change has been applied.

136-138. "08:00 h" and "18:00 h"

- Thank you for your suggestion. The requested change has been applied.

236. "2 weeks."

- Thank you for your suggestion. The requested change has been applied.

244 and 251. "3 weeks"

- Thank you for your suggestion. The requested change has been applied.

320. "four times"

- Thank you for your suggestion. The requested change has been applied.

335. "5 days"

- Thank you for your suggestion. The requested change has been applied.

383. "3 weeks"

- Thank you for your suggestion. The requested change has been applied.

396. Perhaps "differential FO supplementations" could be made clearer.

397-398. Perhaps "a physiological range of use from a technological standpoint" could be made a bit clearer as well

- Thank you for your suggestion. The sentence has been omitted because it is not essential to the understanding of the manuscript.

Table 1 and others. Either "Experimental diets" or "Experimental diet" or just "Diet".

- Thank you for your suggestion. The requested change has been applied.

Table 5 and some others. Here the number of significant digits, numbers, or decimal places is excessive. Three is conventional, although some people prefer four for some variables. Here, numbers such as 3200 and 1970 for means would seem appropriate, with one additional decimal place for SD and SE being conventional depending on their magnitude (i.e., 1001.9 or 1002 for day -7 and C).

- Thank you for your suggestion. The requested change has been applied.

Reviewer #2: The paper Rumin_D_16_7921 entitled "Hepatic and subcutaneous adipose tissue variations in transition dairy goats fed saturated or unsaturated fat supplemented diets" aimed to evaluate the effects of saturated or unsaturated fat supplements (fish oil and calcium stearate) during the transition period on metabolic adaptations in dairy goats with biochemical and histological approaches, focusing on changes at the liver and subcutaneous adipose tissue levels.

This research paper is very good organized and written with a very comprehensive introduction showing the originality and significance of the undertaken study. The experimental design, the methodology used and the results are very well presented, the discussion is adequately documented and the conclusions are sound. The results of this study are very interesting indeed and original, since is the first study on transition period of dairy goats. Thus, the paper can be accepted for publication after some minor revision.

Some minors corrections:

L-237: steadily decreased afterwards

L-308: In the present work, it has been demonstrated

L-309: mobilizing/ adipogenetic mechanism

L-360: Therefore, it has been.

L-394: which is associated

- Thank you for your suggestion. The requested changes have been applied.

Reviewer #3: General: this is a well-written paper containing novel data on the adaptations in key tissues of the goat during the transition period. The experimental design and methods are appropriate. However, before final acceptance authors need to address some minor comments outlined below.

Introduction: there are several one-sentence paragraphs that should be avoided (e.g. L44-45, 81-83, etc). Please revise accordingly.

- Thank you for your suggestion. The requested changes have been applied.

L96: why not start with 24 goats to distribute evenly across the 3 treatment groups?

- Actually, the original experimental design included 24 goats divided in three groups but one animal was excluded from the trial because of dystocia at kidding.

L97-99: please include the range of parity, etc, with an SD. This is useful information for the reader.

- Thank you for your suggestion. Details on parity, age and milk production have been included.

L144-145: please include more details regarding the energy balance calculation, e.g. an equation, components, etc, was calculated.

- Thank you for your suggestion. A more detailed energy balance description has been included.

Highlights

- Different fatty acids can affect fat mobilization with no effects on performance.
- Fish oil could reduce or delay adipose tissue fat mobilization during transition.
- Fish oil could improve hepatocyte adaptation to fat infiltration in dairy goats.

Hepatic and subcutaneous adipose tissue variations in transition dairy goats fed saturated or unsaturated fat supplemented diets

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ABSTRACT

Biochemical and histological approaches were used to study the metabolic adaptations of transition dairy goats to dietary supplementation with saturated and unsaturated fatty acids. Twenty-three Alpine dairy goats were divided into three groups and fed a basal pre-kidding and lactation diet (C) or the same diet supplemented with fish oil (FO) or stearic acid (ST) starting 1 week before kidding until 21 days in milk (DIM). No differences were observed in milk production and composition. However, the serum non-esterified fatty acid (NEFA) and beta-hydroxybutyrate (BOHB) concentrations were changed over time by the treatments. The mean adipocyte area, measured on a subset of 12 goats, which included four subjects from each experimental group, decreased constantly in the C and ST groups from -7 to 21 days, while the FO group did not change between days 7 to 21. These results support the idea that FO is able to limit lipolysis, although the energy balance is still negative. No inflammatory processes were observed in the liver in accordance with the blood leukocytes trend, even if moderate to severe fatty changes in the liver were observed in the experimental goats. In the FO group, however, fatty infiltration appeared more severe and it occurred more gradually compared with the other diets. Overall, these results suggest an interesting ability of dietary lipid supplements to affect the fat mobilizing machinery; FO in particular seems able to reduce/delay fat mobilization and could improve hepatocyte adaptation to fatty infiltration, allowing the cells to better maintain their function.

Keywords: adipose tissue, dairy goats, fatty liver, fish oil, stearate.

1. Introduction

The regulation and coordination of lipid metabolism amongst adipose, liver and mammary gland tissues are key components of lactation adaption in dairy species (Chilliard, 1999). The most characteristic event during the transition period is the reduction in feed intake just when there is a very high nutrient demand for the developing conceptus and lactogenesis (Drackley, 1999). The conjunction of these factors can lead the ruminant to experience a negative energy balance (NEB) particularly before parturition and at the beginning of the lactation period, when it is almost impossible to meet the extra energy requirements needed for fetal growth before and milk production after.

In such conditions, goats mobilize fatty acids from adipose tissue reserves to compensate for the lack of glucose and fatty acids. This mechanism leads to an increase in circulating concentrations of NEFA during the late pregnancy and postpartum periods (Magistrelli and Rosi, 2014). The NEFA liver metabolic pathways are related to energy and ketone body production, or to the secretion of very low-density lipoproteins via triacylglycerol (TAG) conversion. If the TAG formation overcomes the liver secretion capacity, then their accumulation results in a so-called fatty liver syndrome (Herdt, 1988).

The partial replacement of grains or forages in rations with fat sources, such as n-3 polyunsaturated fatty acids (PUFAs), can considerably increase the energy level of the diet, and may enhance energy intake if the dry matter intake (DMI) is not depressed (Staples et al., 1998): as a result, the energy balance might be improved in early lactating dairy ruminants (Ballou et al., 2009). Moreover, some studies stated that higher supplemental levels of fat might increase the risk of peripartal lipid accumulation in the liver of dairy animals (Douglas et al., 2004). Some metabolites and metabolic hormones are well-recognized signals in the interaction between NEB and metabolic disorders in dairy goats (van Knegsel et al., 2007). Serum calcium, NEFA and BOHB contents are frequently used to evaluate the adaption to NEB during the peripartum period (McNamara et al., 1995; Kokkonen et al., 2005). As a result of NEB adaptation, significant lipid

mobilization from subcutaneous adipose tissue leads to progressive body mass loss (Chilliard, 1999).

Fish oil has often been supplemented in dairy animals with the objective of enriching animal products with essential fatty acids considered healthy, particularly for the human cardiovascular system (Calder, 2013). Moreover, dietary fat is no longer just classified as an energy source because specific fatty acids have peculiar roles in lipid metabolism and organismal defense systems in food-producing animals (Tsiplakou and Zervas, 2013a, b). In fact, not only have eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) been demonstrated to be contained in dairy goats but their ability to influence immune and inflammatory responses has also been as observed *in vivo* (Bronzo et al., 2010) and *in vitro* (Pisani et al., 2009; Lecchi et al., 2011, 2013). Additionally, saturated fatty acids, such as palmitic and stearic acids, can affect lipid metabolism in dairy ruminants (Chilliard, 1993; Thering et al., 2009; Agazzi et al., 2010).

The increase in the adipocyte diameter can be used as an indicator of lipogenic activity under different metabolic challenging conditions, such as undernourishment or breeding (Alzon et al., 2007; Faulconnier et al., 2007). Currently, data regarding the effects of highly unsaturated or saturated fats on liver and adipose tissue histology in dairy goats are limited. The main aim of this study was to evaluate the effects of saturated or unsaturated fat supplements on metabolic adaptations in periparturient dairy goats with biochemical and histological approaches, and in particular, focusing on changes at the liver and subcutaneous adipose tissue levels.

2. Materials and methods

2.1. Animals and diets

The present study was performed at the Animal Production Research and Teaching Centre of the Polo Veterinario of the Università degli Studi di Milano (Lodi, Italy), and the protocol was approved by the Ethics Committee of the Università degli Studi di Milano (attachment n. 5 January 26th, 2011). Twenty-three spring kidding Alpine goats (1.26 ± 0.45 kidding, 28.05 ± 6.15 months of

age, 3.12 ± 0.33 kg of milk/d) were divided using a randomized complete block design in an attempt to achieve three homogenous groups for parity, age and milk production, per their previous lactations, and assigned to three experimental treatments. The goats were housed in individual 4.56 m^2 straw bedded boxes with free access to water and were individually fed. After kidding, each goat shared the box with their relative suckling kids (on average 1.83 kids/goat, weighing 4.18 ± 0.23 kg); however, the feeder was set out of reach of the kids. A conventional pre-kidding or a post-kidding basal diet was offered to all the experimental animals in the three groups. The diet ingredients and chemical compositions are detailed in Tables 1 and 1S. The pre-kidding daily basal diet consisted of *ad libitum* mixed grass hay (refusal weight of at least 10%), 600 g/head of concentrate and 100 g/head of corn meal. Post-kidding, the daily basal diet was composed of *ad libitum* alfalfa hay and mixed hay (refusal weight of at least 10%), 1,500 g/head of concentrate and 200 g/head of corn meal. The concentrates were provided separately from the forage during the entire trial, and calcium carbonate was added to balance calcium content in all diets as follows: a) Control (C; n = 8 goats), animals were fed the basal pre- or post-kidding diet plus calcium carbonate (9 g/day during pre-kidding period, 12 g/day after kidding); b) Fish oil (FO; n = 8 goats), animals were fed the pre- or post-kidding basal diet plus calcium carbonate (9 g/day during pre-kidding period, and 15 g/day after kidding) and 30 g/day of fatty acids (81 g/day of supplement) before kidding or 50 g/day of fatty acids (135 g/day of supplement) during lactation from a rumen-inert fish oil (10.4% EPA and 7.8% DHA; Ufac Ltd., Stretton, UK); c) Calcium stearate (ST; n = 7 goats), animals were fed the pre- or post-kidding basal diet plus 30 g/day of fatty acids (34 g/day of supplement) before kidding or 50 g/day of fatty acids (56 g/day of supplement) during lactation from stearic acid (C16:0 26% and 69.4% C18:0; Brenntag S.p.a., Milan, Italy). All the daily diets were vitamin E supplemented to supply 72 mg/head during the pre-kidding period and 80 mg/head after kidding. The pre- and post-kidding dietary treatments in the three groups were designed to provide similar crude protein (CP) and calcium contents. The fat-enriched treatments (FO and ST) contained similar ether extracts (EE). The dietary supplements were stored in the dark at room

temperature. All goats were fed concentrates and corn meal twice a day, and the fat supplementation was provided in the morning meal mixed into 50 g or 100 g of corn meal during the pre- or post-kidding periods, respectively. Stearic acid is preferred over palmitic acid as a positive control treatment because it is considered more neutral. Indeed, in a previous trial, palmitate showed a strong effect on lipid metabolism in the liver, increasing expression of peroxisome proliferator-activated receptor-α (*PPAR*A), acyl-coenzyme A dehydrogenase very long chain (*ACADVL*) and carnitine palmitoyl-transferase 1A (*CPT1A*) at 21 days after kidding (Agazzi et al., 2010).

2.2. Dry matter intake, live body weight, energy balance, milk yield and composition

Individual DMI was assessed weekly until 21 days after kidding as the difference between the feed dry matter (DM) offered and the feed DM refused. The individual live body weight (LBW) was assessed at 7 days before kidding and at 7, 14 and 21 days of lactation by an electronic scale (F.lli Fascina snc, Castelvetro P.no, Italy). On a daily basis, goats were milked once a day at 8:00 am. To allow milk yield recording and milk samples collection, once a week, the suckling kids were separated from the mothers for two consecutive milkings (8:00 h and 18:00 h), starting from the evening milking on the day before to the end of the evening milking on the subsequent day. Individual milk production was assessed with an electronic scale, and the separated kids were fed the relative mother's milk after sample collection. Individual milk samples were taken on days 0, 7, 14 and 21 of lactation and an aliquot was subsequently analyzed for fat, protein and lactose content with an infrared analyzer (MilkoScan™, FOSS, Hillerød, Denmark). Energy balance was calculated weekly with the Small Ruminant Nutrition System software (Tedeschi et al., 2010) using the following formula:

$$EB = MEI - (ME_m + ME_l + ME_{preg})$$

where EB is ME balance, Mcal/d; MEI is ME intake, Mcal/d; ME_m is ME required for maintenance, Mcal/d; ME_l is ME required for milk production, Mcal/d; and ME_{preg} is ME required for pregnancy, Mcal/d (Cannas et al., 2004).

2.3. Blood samples and analysis

To evaluate the serum metabolites, white blood cell count (WBC) and hemochromocytometric parameters (HCM), individual blood samples were taken at 14, 7 and 2 days before the expected kidding date as well as at 0, 2, 7, 14 and 21 DIM. Blood samples were collected from the jugular vein before the morning feeding in two vacuum sterile tubes containing either EDTA (Terumo Venoject® 10-mL VF-109SDK) or a clot activator (VF-109SP). WBC and HCM levels were assessed in whole blood samples with a Hemat 8 (SEAC, Calenzano, Florence, Italy). Blood samples were subsequently centrifuged, and serum was obtained via centrifugation for 10 min at 1,000 x *g*. Serum, which was utilized for the determination of alanine aminotransferase (ALAT), NEFA, glucose, BOHB and cholesterol concentrations, was stored at -20 °C until it was analyzed. Serum ALAT, cholesterol and glucose concentrations were measured with a clinical chemistry analyzer (ILab 300 plus, Instrumentation Laboratory s.p.a., Milan) using reagents provided by the same company; NEFA and BOHB were tested using Randox reagents (Randox, Crumlin, UK).

2.4. Adipose tissue and liver collection

Liver and adipose tissue biopsies were harvested on days -7, +7 and +21 relative to parturition for each experimental subject, via puncture biopsy under local anesthesia. The biopsy area was shaved and cleaned with a disinfectant. For liver biopsies, a 14G biopsy needle was introduced through a small incision made at the right 11^{th} intercostal space at approximately 15 cm below the spine (Agazzi et al., 2010). Subcutaneous adipose tissue biopsies were taken from alternate sides of the tail-head region. The biopsy area was shaved and cleaned with disinfectant, an incision of 2-3 cm length was made between the tail head and the ischiatic bone, and a sample of approximately 1

171 cm^3 of subcutaneous white adipose tissue was excised. The incisions for the liver and subcutaneous
172 adipose tissue biopsies were both sutured and treated with topical antibiotics agents (Thering et al.,
173 2009). The biopsied tissue was fixed in a B5 fixative (Bio-Optica, Milan, Italy) for 5 to 7 h,
174 dehydrated in a graded series of ethanol, cleared with xylene, paraffin-embedded and cut into 8 μm
175 sections. Serial sections were placed on glass microscope slides that were previously treated with
176 Vectabond (Vector Laboratories, Burlingame, CA, USA) to enhance the adherence of the tissues
177 and stained with hematoxylin and eosin (H.E; Bio-Optica, Milan, Italy). In a subset of twelve goats,
178 four subjects from each experimental group that were representative of the animals used in the trial
179 (Tables 3S, 4S, 6S, 8S), for each biopsy, five randomly chosen fields were photographed at 200X
180 magnification on a light microscope (Nikon Diaphot TMD- Nikon, Japan). To assess diets effects
181 on adipose tissue, variations in adipocytes area were evaluated. For each biopsy, five randomly
182 chosen fields of 40,000 μm^2 in surface area (one randomly chosen field/image) were scored and the
183 mean adipocyte area units, expressed as square micrometers (μm^2), were calculated. To evaluate
184 diets effects on hepatic tissue, variations in hepatocyte fat infiltration were measured. The
185 experiments were divided into two steps.
186 In the first step, the infiltration level was classified histologically according to 6 different
187 degrees (Grades der Leberverfettung or GdL), as previously described (Kalaitzakis et al., 2007).
188 The fatty infiltration (GdL) severity scores ranged from 0 (no fat droplets visible) to 5 (pan-lobular
189 fatty infiltration), following the Mertens point score scale (Mertens, 1992; Kurosaki et al., 2008).
190 On the basis of this parameter, the area from the central vein to the portal triad of the hepatic lobule
191 was divided into 3 equal concentric regions that were scored according to the presence of the most
192 severe cellular lesions. For every sample, five randomly chosen lobules were evaluated (one
193 randomly chosen lobule/image) and the median was calculated. From that median, each goat was
194 classified according to 1 of 6 degrees of FCL (GdL 0–5). When a sample did not contain five entire
195 lobules, the assessment was performed on ten partial lobules.

196 In the second step, on the basis of the previous considered cellular lesions (Kalaitzakis et al.,
197 2007) and in ascending order of severity, hepatocytes where grouped as 1) normal cells showing the
198 typical aspect of hepatocytes, a fine granular cytoplasm and a centrally placed nucleus, 2) cloudy
199 cells with a foaming aspect due to cloudy-swelling cytoplasm and or to the presence of small or
200 moderately sized vacuoles and 3) vacuolated cells, with large vacuoles or one single vacuole inside
201 the cytoplasm (Fig. 2). For each lobule, a triangular shaped area of approximately 38.000 mm^2,
202 which was representative of one fourth of a single lobule (Fig. 3), was selected and the infiltration
203 level was calculated as the percentage of total fatty infiltrated cells (as sum of cloudy and
204 vacuolated cells) or as the percentage of normal, cloudy and vacuolated cells, which was considered
205 separately. All sections were analyzed by one technical expert using an image analysis system
206 (Image J 1.41g, NIH) (Abràmoff et al., 2004) to avoid individual variation. Samples were blindly
207 analyzed to prevent any bias. For each biopsy, on additional sections, to evaluate the presence of
208 inflammatory processes, the presence of fibrosis features was evaluated with the Masson's
209 trichrome staining, which specifically dyes collagen fibers (Brunt et al., 1999).
210
211 *2.5. Statistical analysis*
212 Data relative to DMI, LBW, energy balance, milk yield and milk composition were analyzed
213 with a repeated measures model using a MIXED procedure in SAS 9.2 (SAS Inst., Inc., NC, USA).
214 The statistical model considered as fixed effects time, treatment and time x treatment interaction as
215 well as goat as the random effect. The hematological and histological data statistics were computed
216 with IBM SPSS 21.0 for Windows (IBM SPSS, Armonk, New York, USA). Due to the non-normal
217 distribution of these data, as assessed by a Shapiro-Wilk test, and because of the repeated
218 measurements in the blood and histological data (dependent variables), a generalized estimating
219 equation (GEE) was used to determine the effects of the different diets, the sampling times and their
220 interaction. The dependent variables had an inverse Gaussian distribution for the blood leukocyte
221 differential cell counts and a negative binomial distribution for the hemochromocytometric, blood

metabolites and histological parameters; therefore, an identity link function was used. The goodness of fit was assessed using a quasi-likelihood under independence model criterion (QICC). The threshold for statistical significance was considered to be $P<0.05$. All data in the tables are presented as marginal means ± SEM or SD, where stated.

3. Results

3.1. Goat performance

In the present trial, no significant differences were found for LBW (Table 2S), forage or concentrate DMI from the week before kidding to the third week of lactation between the three experimental groups. Similarly, the milk yield, fat-corrected milk, and milk composition levels were not affected by the dietary treatments (Table 2). The treatment x time effects were significant ($P<0.01$) for energy balance in the first week of lactation, and the FO and ST groups had negative values compared with the control group. Two different patterns were clear for the EB values between the C and fat-supplemented groups. The latter started with negative values in the first week after kidding; however, the values increased constantly in the subsequent 2 weeks. The C group had positive values in the first week; however, the values steadily decreased afterward. The results of the goat subsets (12 subjects) are reported in Tables 3S, 4S, 6S and 8S.

3.2. Blood components and serum metabolites

Only the main blood components at day -7, 7 and 21, which corresponded to the liver and adipose tissue biopsies times, are presented in Table 3. The complete dataset is included in Table 5S. The serum ALAT activity was constantly higher in the FO fed animals than in the C group in the 3 weeks after kidding as well at day -7 (Table 3 and 5S). However, significantly higher serum ALAT activity was observed in the FO group compared with the ST group on days 0 ($P=0.02$) and 7 ($P=0.02$) after kidding. Notably, similar values were observed between the groups at day -14, before supplementation. The cholesterol content was lower in both of the fat-supplemented animal

groups compared with the C group in the first week of lactation ($P<0.01$). Additionally, at day 21, the highest values were recorded in the FO group compared with the C and ST groups ($P<0.05$). The serum glucose content was higher in both the FO and ST goats than the C goats in the week before kidding ($P<0.05$). However, during the first 3 weeks of lactation, the ST group had the highest values. Two weeks before parturition, the serum BOHB content in the C group was the lowest between the treatments. At day -2, it was higher than in the FO group, and at day 0, it was the differences peaked between the treatments. At the end of the trial, the BOHB concentrations were higher in the ST and C groups than in the FO group ($P<0.05$). The C group had lower NEFA concentrations than the ST group 2 weeks before kidding, while the opposite phenotype was observed in the last week of gestation (days -7 and -2). Two days after kidding, the FO and ST groups had the highest NEFA content levels. The values in the FO group peaked at day 7 and these higher values were maintained until day 14. The serum NEFA and BOHB concentration trends are depicted in Fig. 1.

3.3. Blood leukocyte differential cell counts

The HGB concentration was higher in the FO group at day 7 than in the C group ($P<0.01$). In contrast, the neutrophil counts were higher at day 7 of lactation in the C group compared with the FO group (Tables 4 and 7S), and the monocytes percentages were higher in the C group than in the ST group at day 2 ($P<0.05$). At day 7, higher lymphocyte counts were detected in the FO group compared with the C group. The interactions between the treatment and sampling times were significant for all classes of considered leukocytes.

3.4. Adipose tissue histology

In all of the samples, histological examination showed that adipocytes were individually held in place by delicate reticular fibers that clustered in lobules that were bound by fibrous septa within the adipose tissue. Typical, uni-locular signet-ring shaped adipocytes were found in all of the

examined biopsies. Morphometric evaluations showed a significant decrease in the adipocyte areas between -7 and 21 days in the ST and C groups ($P<0.05$), whereas in the FO group, the adipocyte surface reduction was limited to the -7 to 7 day interval ($P<0.05$) and then reached a plateau until day 21 (Table 5 - Fig. 4).

3.5. Hepatic tissue histology

In all of the samples, histological examinations revealed that goats experienced a fatty liver phenotype before and after parturition. Necrosis foci were not observed, and pyknotic nuclei were poorly represented. Masson's trichrome staining showed no signs of fibrosis in all specimens examined (image not shown). The first analysis step demonstrated that fatty liver infiltration ranged from mild (Gdl 2) to moderate (GdL 3) to severe grades of infiltration (GdL 4). All treatment samples showed the same GdL distributions at day 7, while at -7 and + 21 days, this distribution varied (Fig. 5). In the second step, when fatty liver infiltration was analyzed as total fatty infiltrated cells or as a single group of cells, the percentage of total infiltrated cells significantly increased between day -7 and + 21 in the FO group ($P<0.05$). However, no significant differences were observed between -7 and + 21 days in the ST and C groups, although the fatty infiltrated cell percentages decreased at day 7 ($P<0.05$) (Fig. 6). At day 7 day, no differences between the diets were observed. When comparing the percentage of total infiltrated cells between the diets, significant differences were observed at -7 and + 21 days. Moreover, the fatty infiltrated cell percentages were, in fact, higher in the C and ST groups than in the FO group during prepartum, while 3 weeks after kidding, the ST group had higher values than the C group with no differences compared with the FO group. When the cells were evaluated separately, over time, it was possible to observe an increase in the vacuolated cell percentages in all of the diets. Additionally, a decrease in the cloudy cell levels in the FO and C groups were observed, but not in the ST group, between days 7 and +21, although the percentage of these cells significantly decreased at day -7 ($P<0.05$) (Fig. 7A). When comparing the percentage of normal, cloudy and vacuolated cells between the

300 diets, the proportion of cloudy cells was higher in the ST group than in the FO and C groups at -7
301 and +21 days (P<0.05). However, at day 21, the vacuolated cell percentages were higher in the FO
302 group than in the ST and C groups. At day 7, no statistically significant differences were recorded.
303 The interaction between the treatment and sampling times were significant for all considered
304 parameters (P<0.05) (Fig. 7B).
305
306 **4. Discussion**
307 In the present work, it has been demonstrated that dietary saturated and unsaturated lipid
308 supplements affect the peripartal fat mobilizing/adipogenetic mechanism in dairy goats. Fish oil
309 treatment, in particular, was able to induce a delay in fat mobilization, whereby the goats did not
310 show any serious metabolic disorders in the presence of a hepatic sufferance due to the presence of
311 large vacuoles inside the cytoplasm. The biochemical analyses were supported by histological
312 analysis that showed, for the first time, the morphology of the hepatic and adipose tissue during the
313 peripartum period and how it changes in response to FO and ST treatments in dairy goats. Goats fed
314 C diets and supplemented with saturated and unsaturated fats did not show any significant
315 differences in milk production and composition. In fact, goats fed fish oil did not face milk fat
316 depression (MFD), which is in agreement with a previous study (Chilliard et al., 2014) and a
317 previous trial performed by a research group that used a rumen-inert supplement (Agazzi et al.,
318 2010). However, this was in contrast with what usually happens in cows (Invernizzi et al., 2010). A
319 very recent study evaluated MFD in goats fed fish oil at inclusion levels four times higher than the
320 one used in the present trial; however, they only considered data expressed on a kilogram of BW
321 basis (Toral et al., 2015). The reasons for the variations between ruminants might be relative to the
322 inter-species differences either at the ruminal level or in milk fat synthesis at the mammary level
323 (Chilliard et al., 2014). The NEFA blood content is tightly linked to energy balance (Eknæs et al.,
324 2006). NEFA suggested values for does, with neutral EB are 0.200‑0.217 mmol/L (Chilliard et al.,
325 1977). The observed concentrations were constantly higher with the FO treatment, which peaked at

day 7 and exceeded a concentration of 0.7 mmol/L, which was proposed as borderline toxemia, while the lowest NEFA concentrations were recorded for the ST treatment. However, these data were consistent with results obtained by Pinotti et al. (2008) and Magistrelli and Rosi (2014). Moreover, no clinical signs of toxemia were observed in the animals. This is probably because the highest concentrations were only reached for a limited time period and the serum BOHB content did not reach high values (< 1.0 mmol/L). The NEFA had a similar pattern in C and ST groups, which spiked at day 0 and was in agreement with González et al. (2011), while FO group levels spiked after a 7-day delay. The BOHB levels in the FO and ST groups spiked at day 7, which was 5 days later than the C group. The BOHB and ALAT results were consistent with those obtained in a previous experiment (Agazzi et al., 2010), where the lowest postpartum serum ALAT activity values were observed in palm oil supplemented goats. However, in both trials, the FO group had higher values (P<0.05).

The histological analysis indicated that goats during transition period exhibited fatty liver syndrome at 7 days before parturition, which was independently of their diets. In agreement, high serum ALAT activity values were previously observed in a study evaluating the prepartum period, although this enzyme cannot be considered liver specific in ruminants (Sirois, 2014). The fatty infiltration levels in the liver ranged from mild to moderate to severe, as previously described in transitional dairy cows (Bobe et al., 2004). At day 21, the highest percentage of cloudy cells was observed in the ST group, while the highest percentage of vacuolated cells was observed in the FO group, suggesting that, although in the absence of a clear decline in their health status, the FO treatment induced slightly more detrimental effects on the liver over time, as confirmed by the GdL distribution and higher ALAT activity values at this time.

Interestingly, although we observed severe hepatocyte injury, features of perisinusal fibrosis, which in humans have been described and associated with a progression of inflammatory processes (Brunt and Tiniakos, 2010), or necrosis foci with polymorphonuclear cells, which are often described in dairy cows with severe fatty livers (Kalaitzakis et al., 2010), were not observed. These

352 data were confirmed by a general decrease in lymphocytes and monocytes around the parturition
353 together with an increase in neutrophils, as previously described in cattle (Van Kampen et al., 1999;
354 Harp et al., 2004).

355 All together, these data suggest that the effects of FO are only apparently detrimental for the
356 animals. Therefore, in this context, it is important to consider that in the FO group, the onset of
357 fatty infiltration, which was evaluated as total infiltrated cells as well as vacuolated cells, occurred
358 more gradually than in the C and ST groups. Therefore, it has been hypothesized a sort of
359 progressive adaptation to the lipid infiltration that allowed the cells to better preserve their
360 functions.

361 The subcutaneous adipose tissue data were well coupled with serum NEFA (and BOHB)
362 concentrations. Indeed, the highest adipocyte area reduction in the C group was observed between
363 days -7 and 7, which matched with the NEFA level spikes, which are both clear signs of strong fat
364 mobilization. From days 7 to 21, the adipose tissue seemed to still be mobilized, considering the
365 negative energy balance of the non-supplemented goats. The ST group had a similar pattern;
366 however, its energy balance became positive between the 7^{th} and 14^{th} days post-parturition.
367 Additionally, its lipolysis most likely would have been reduced earlier than the C group, but after
368 21 days. The histological adipose tissue data support the idea that FO can limit lipolysis even if the
369 energy balance is still negative (at least until day 14). The initial decrease in the adipose cell areas,
370 which was common to all treatments, stopped between days 7 and 21. This pattern is in agreement
371 with cow studies (Thering et al., 2009; Schmitt et al., 2011). Because important interspecies
372 differences have been outlined in MFD (Toral et al., 2015), it would be very interesting to explore
373 the fine mechanisms that regulate this response. The bovine studies mentioned above (Thering et
374 al., 2009; Schmitt et al., 2011) actually support the idea that FO could be used to increase
375 adipogenesis/lipogenesis in adipose tissue rather than decrease lipolysis. Furthermore, recent *in*
376 *vitro* and mouse studies demonstrated that long-chain acyl-CoAs could inhibit major lipolytic
377 enzymes that are crucial in adipocytes as well in hepatocytes (adipose triglyceride lipase

(ATGL)/Serpin peptidase inhibitor, clade F, member 1 [SERPINF1]), representing a feedback mechanism controlling lipolysis. It has been highlighted that differences exist between acyl-CoA species on their inhibiting efficacy. Unfortunately, only oleic and palmitic acids, and not PUFAs, were tested (Zimmermann et al., 2009; Nagy et al., 2014). At the tissue level, the balance of this remodeling in the FO group appeared as a marked stop in adipocyte size reduction 3 weeks after kidding. At the same time, the NEFA concentrations were not decreased because the fat mobilization was most likely still occurring. In this context, the liver was still facing a heavy metabolic workload, as suggested by the biochemical data and supported by the histological results, which clearly indicate how hepatic tissue can react in very dynamic and, to some extent, unpredictable ways to stress during the peripartum period.

5. Conclusion

To the best of our knowledge, this is the first histological investigation on transition dairy goat liver and subcutaneous tissues carried out to study the morphological and metabolic changes during this particularly challenging physiological period in response to saturated and unsaturated dietary supplemental fats. An interesting ability of FO to delay fat mobilization at the adipose tissue level after kidding was observed, which is associated with a slightly negative impact on the liver histology; however, it remained in a physiological range.

Conflict of interest

The authors declare no conflicts of interest.

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406

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528

529 **Tables**

530

531 **Table 1**

532 Ingredients and chemical composition of the experimental diets of the dairy goats fed either a basal
533 diet (C) or a diet supplemented with fish oil (FO) or stearate (ST).

	Experimental diets					
	Pre-kidding			Post-kidding		
	C	FO	ST	C	FO	ST
Ingredient (%)						
Alfalfa hay	0.0	0.0	0.0	31.2	29.8	30.7
Mixture hay[2]	62.3	59.6	61.4	15.3	14.6	15.1
Concentrate mixture[1]	31.9	30.5	31.4	46.8	44.8	46.2
Corn meal	5.3	5.0	5.2	6.2	5.9	6.2
Fish oil	0.0	4.4	0.0	0.0	4.3	0.0
Calcium Stearate	0.0	0.0	2.0	0.0	0.0	1.9
$CaCO_3$	0.5	0.5	0.0	0.5	0.5	0.0
Chemical Composition (% of dry matter)						
Dry Matter (%)	88.4	88.7	88.6	89.3	89.5	89.4
Crude Protein	12.3	11.9	12.2	17.8	17.2	17.5
Ether Extract	2.9	4.9	4.5	3.2	5.2	4.8
NDF	43.9	43.8	43.3	33.7	34.0	33.2
Ashes	6.3	6.5	6.0	7.2	7.3	6.8
Ca	0.8	0.8	0.9	1.1	1.1	1.2
P	0.4	0.4	0.4	0.8	0.8	0.8
NE_L (Mcal/kg DM)[3]	1.61	1.66	1.67	1.67	1.72	1.72

534 [1] The concentrate mixture was a commercial dairy goat mixed feed, chemical composition: 22.25%
535 crude protein, 5.00% ether extract, 22.98% neutral detergent fiber, 6.51% ashes, 1.28% Ca and
536 0.76% P (on dry matter basis).
537 [2]The mixture hay was a grass hay, chemical composition: 7.6% crude protein, 1.8% ether extract,
538 57.5% neutral detergent fiber, 5.9% ashes, 0.6% Ca and 0.2% P (on dry matter basis).
539 [3]Net energy of lactation concentration of the diets were calculated using the Small Ruminant
540 Nutrition System (SRNS) software (Tedeschi et al., 2010).

Table 2

Performance of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (n=23).

Time (day)	Treatment				*P*[1]		
	C (n=8)	FO (n=8)	ST (n=7)	SE	Trt	Time	Trt*Time
Milk production (kg/day)							
7	2.85	3.87	3.36	0.30	0.13	<0.01	0.58
14	3.52	4.24	3.79				
21	3.66	4.21	3.88				
7-21	3.34	4.10	3.68				
3.5% Fat-corrected milk (kg/day)							
7	3.77	4.53	3.94	0.49	0.46	0.73	0.22
14	3.50	4.38	3.92				
21	4.15	4.17	3.52				
7-21	3.80	4.36	3.80				
Dry Matter Intake (kg/day)							
-7	2.81	2.60	2.61	0.29	0.28	<0.01	0.46
7	2.48	2.68	2.25				
14	2.65	2.88	2.71				
21	3.28	3.66	2.96				
Energy Balance (Mcal/day)							
7	0.34[a]	-0.91[b]	-1.12[b]	0.51	0.99	<0.01	<0.01
14	-0.20	-0.05	0.46				
21	-0.16	0.89	0.82				

[a, b]Means within each row with different superscripts are significantly different ($P < 0.05$).

[1] Trt, treatment effect

Table 3

Serum metabolites of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (n=23).

Time	Treatment						*P*[1]		
	C (n=8)	SE	FO (n=8)	SE	ST (n=7)	SE	Trt	Time	Trt*Time
ALAT[2] (IU/L)									
-7	13.58[b]	0.84	15.91[a]	0.48	14.35	0.85	0.09	<0.01	<0.01
7	14.71[b]	0.89	17.35[a]	0.65	14.73[b]	0.92			
21	12.34[b]	1.11	15.87[a]	1.06	13.64	0.79			
Cholesterol (mg/dL)									
-7	54.13	3.23	55.24	3.92	61.43	4.28	0.63	<0.01	<0.01
7	83.50[a]	4.82	65.70[b]	4.59	59.30[b]	3.83			
21	63.23[b]	4.04	88.21[a]	4.81	72.64[b]	5.40			
Glucose (mg/dL)									
-7	42.81[b]	1.80	51.97[a]	1.88	54.49[a]	2.28	0.04	<0.01	<0.01
7	57.63	3.27	47.60[b]	4.34	62.25[a]	2.04			
21	54.95[b]	2.32	61.61	3.53	66.00[a]	3.67			
BOHB[3] (mmol/L)									
-7	0.35	0.03	0.30	0.06	0.35	0.08	0.18	<0.01	<0.01
7	0.69	0.08	0.82	0.26	0.86	0.16			
21	0.51[a]	0.06	0.38[b]	0.03	0.72[a]	0.13			
NEFA[4] (mmol/L)									

-7	0.58[a]	0.09	0.36	0.10	0.32[b]	0.06	0.78	<0.01	<0.01
7	0.33[b]	0.07	0.78[a]	0.16	0.40[b]	0.07			
21	0.41	0.08	0.33	0.06	0.34	0.07			

547 [a,b]Means within each row with different superscripts are significantly different ($P < 0.05$).

548 [1] Trt, treatment effect

549 [2] ALAT, alanine aminotransferase

550 [3] BOHB, beta-hydroxybutyrate

551 [4] NEFA, non-esterified fatty acids

552

Table 4

Hemoglobin (HGB) and blood leukocytes differential cell counts of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (n=23).

Time	Treatment						*P*[1]		
	C (n=8)	SE	FO (n=8)	SE	ST (n=7)	SE	Trt	Time	Trt*Time
HGB (g/L)									
-7	88.38	2.87	95.13	3.85	93.71	3.07	0.29	<0.01	<0.01
7	84.50[b]	4.77	99.57[a]	4.52	93.29	2.66			
21	82.14	5.18	90.33	3.72	86.00	1.68			
Neutrophils (%)									
-7	47.14	3.85	50.81	3.60	47.79	3.85	0.87	<0.01	<0.01
7	64.41[a]	3.60	56.73[b]	3.60	57.74	3.85			
21	60.55	3.60	57.61	3.85	55.63	3.85			
Monocytes (%)									
-7	7.91	1.51	6.31	1.42	5.26	1.51	0.05	<0.01	<0.01
7	3.11	1.42	5.20	1.42	5.43	1.51			
21	3.10	1.42	3.04	1.51	4.77	1.51			
Lymphocytes (%)									
-7	43.89	3.79	41.04	3.54	46.34	3.79	0.88	<0.01	<0.01
7	31.85[b]	3.54	37.25[a]	3.54	36.19	3.79			
21	35.05	3.54	37.77	3.79	38.30	3.79			

[a,b]Means within each row with different superscripts are significantly different ($P < 0.05$).

[1] Trt, treatment effect

Table 5

Mean adipocyte area (μm^2) of subcutaneous adipose tissue of a subset (n=12) of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST).

Time	Treatment						*P*[1]		
	C (n=4)	SD	FO (n=4)	SD	ST (n=4)	SD	Trt	Time	Trt*Time
-7	3200.00[d]	1002	2877.69[d]	876	3088.80[d]	1010	0.60	<0.01	<0.01
7	1970.44[e]	523	1801.80[e]	548	2156.33[e]	718			
21	1157.74[b,f]	216	1851.85[a,e]	638	1066.66[b,f]	269			

[a,b]Means within each row with different superscripts are significantly different ($P < 0.05$).

[d,e,f]Means within each column with different superscripts are significantly different ($P < 0.05$).

[1] Trt, treatment effect

Figure captions

Fig. 1. Serum beta-hydroxybutyrate (BOHB) (A) and non-esterified fatty acid (NEFA) (B) contents of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (n=23) (** differs from *; $P<0.05$).

Fig. 2. Representative images of normal hepatocytes showing a fine granular aspect (A), cloudy hepatocytes showing a foaming aspect due to the cloudy-swelling cytoplasm (B, arrow head), hepatocytes containing moderately sized vacuoles (B, arrow) and hepatocytes containing large vacuoles (asterisk). H&E staining, 20X original magnification, bar 50 μm.

Fig. 3. Representative image of the triangular shaped area representing one-fourth of a hepatic lobule. PS-Portal Space; CV-Centro lobular Vein. H&E staining, 10X original magnification, bar 100 μm.

Fig. 4. Representative images of adipose tissue showing large and small adipocytes individually held in place by delicate reticular fibers. H&E staining, 20X original magnification, bar 50 μm.

Fig. 5. Histological classification of fatty infiltration changes in the liver (FCL or hepatic lipidosis). The classifications were made according to six different degrees (Grade der Leberverfettung or GdL) ranging from 0 (no fat droplets, totally normal hepatocytes) to 5 (pan lobular infiltration) of FCL.

Fig. 6. Percentage of fatty infiltrated liver cells of a subset (n=12) of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (** differs from *; $P<0.05$).

Fig. 7. Percentage of normal, vacuolated and cloudy cells in the liver tissue from a subset (n=12) of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (**

differs from *, cloudy cells; ## differs from #, normal cells; †† differs from †, vacuolated cells; $P<0.05$).

Hepatic and subcutaneous adipose tissue variations in transition dairy goats fed saturated or unsaturated fat supplemented diets

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ABSTRACT

Biochemical and histological approaches were used to study the metabolic adaptations of transition dairy goats to dietary supplementation with saturated and unsaturated fatty acids. Twenty-three Alpine dairy goats were divided into three groups and fed a basal pre-kidding and lactation diet (C) or the same diet supplemented with fish oil (FO) or stearic acid (ST) starting 1 week before kidding until 21 days in milk (DIM). No differences were observed in milk production and composition. However, the serum non-esterified fatty acid (NEFA) and beta-hydroxybutyrate (BOHB) concentrations were changed over time by the treatments. The mean adipocyte area, measured on a subset of 12 goats, which included four subjects from each experimental group, decreased constantly in the C and ST groups from -7 to 21 days, while the FO group did not change between days 7 to 21. These results support the idea that FO is able to limit lipolysis, although the energy balance is still negative. No inflammatory processes were observed in the liver in accordance with the blood leukocytes trend, even if moderate to severe fatty changes in the liver were observed in the experimental goats. In the FO group, however, fatty infiltration appeared more severe and it occurred more gradually compared with the other diets. Overall, these results suggest an interesting ability of dietary lipid supplements to affect the fat mobilizing machinery; FO in particular seems able to reduce/delay fat mobilization and could improve hepatocyte adaptation to fatty infiltration, allowing the cells to better maintain their function.

Keywords: adipose tissue, dairy goats, fatty liver, fish oil, stearate.

1. Introduction

The regulation and coordination of lipid metabolism amongst adipose, liver and mammary gland tissues are key components of lactation adaption in dairy species (Chilliard, 1999). The most characteristic event during the transition period is the reduction in feed intake just when there is a very high nutrient demand for the developing conceptus and lactogenesis (Drackley, 1999). The conjunction of these factors can lead the ruminant to experience a negative energy balance (NEB) particularly before parturition and at the beginning of the lactation period, when it is almost impossible to meet the extra energy requirements needed for fetal growth before and milk production after.

In such conditions, goats mobilize fatty acids from adipose tissue reserves to compensate for the lack of glucose and fatty acids. This mechanism leads to an increase in circulating concentrations of NEFA during the late pregnancy and postpartum periods (Magistrelli and Rosi, 2014). The NEFA liver metabolic pathways are related to energy and ketone body production, or to the secretion of very low-density lipoproteins via triacylglycerol (TAG) conversion. If the TAG formation overcomes the liver secretion capacity, then their accumulation results in a so-called fatty liver syndrome (Herdt, 1988).

The partial replacement of grains or forages in rations with fat sources, such as n-3 polyunsaturated fatty acids (PUFAs), can considerably increase the energy level of the diet, and may enhance energy intake if the dry matter intake (DMI) is not depressed (Staples et al., 1998): as a result, the energy balance might be improved in early lactating dairy ruminants (Ballou et al., 2009). Moreover, some studies stated that higher supplemental levels of fat might increase the risk of peripartal lipid accumulation in the liver of dairy animals (Douglas et al., 2004). Some metabolites and metabolic hormones are well-recognized signals in the interaction between NEB and metabolic disorders in dairy goats (van Knegsel et al., 2007). Serum calcium, NEFA and BOHB contents are frequently used to evaluate the adaption to NEB during the peripartum period (McNamara et al., 1995; Kokkonen et al., 2005). As a result of NEB adaptation, significant lipid

mobilization from subcutaneous adipose tissue leads to progressive body mass loss (Chilliard, 1999).

Fish oil has often been supplemented in dairy animals with the objective of enriching animal products with essential fatty acids considered healthy, particularly for the human cardiovascular system (Calder, 2013). Moreover, dietary fat is no longer just classified as an energy source because specific fatty acids have peculiar roles in lipid metabolism and organismal defense systems in food-producing animals (Tsiplakou and Zervas, 2013a, b). In fact, not only have eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) been demonstrated to be contained in dairy goats but their ability to influence immune and inflammatory responses has also been as observed *in vivo* (Bronzo et al., 2010) and *in vitro* (Pisani et al., 2009; Lecchi et al., 2011, 2013). Additionally, saturated fatty acids, such as palmitic and stearic acids, can affect lipid metabolism in dairy ruminants (Chilliard, 1993; Thering et al., 2009; Agazzi et al., 2010).

The increase in the adipocyte diameter can be used as an indicator of lipogenic activity under different metabolic challenging conditions, such as undernourishment or breeding (Alzon et al., 2007; Faulconnier et al., 2007). Currently, data regarding the effects of highly unsaturated or saturated fats on liver and adipose tissue histology in dairy goats are limited. The main aim of this study was to evaluate the effects of saturated or unsaturated fat supplements on metabolic adaptations in periparturient dairy goats with biochemical and histological approaches, and in particular, focusing on changes at the liver and subcutaneous adipose tissue levels.

2. Materials and methods

2.1. Animals and diets

The present study was performed at the Animal Production Research and Teaching Centre of the Polo Veterinario of the Università degli Studi di Milano (Lodi, Italy), and the protocol was approved by the Ethics Committee of the Università degli Studi di Milano (attachment n. 5 January 26th, 2011). Twenty-three spring kidding Alpine goats (1.26 ± 0.45 kidding, 28.05 ± 6.15 months of

age, 3.12 ± 0.33 kg of milk/d) were divided using a randomized complete block design in an attempt to achieve three homogenous groups for parity, age and milk production, per their previous lactations, and assigned to three experimental treatments. The goats were housed in individual 4.56 m^2 straw bedded boxes with free access to water and were individually fed. After kidding, each goat shared the box with their relative suckling kids (on average 1.83 kids/goat, weighing 4.18 ± 0.23 kg); however, the feeder was set out of reach of the kids. A conventional pre-kidding or a post-kidding basal diet was offered to all the experimental animals in the three groups. The diet ingredients and chemical compositions are detailed in Tables 1 and 1S. The pre-kidding daily basal diet consisted of *ad libitum* mixed grass hay (refusal weight of at least 10%), 600 g/head of concentrate and 100 g/head of corn meal. Post-kidding, the daily basal diet was composed of *ad libitum* alfalfa hay and mixed hay (refusal weight of at least 10%), 1,500 g/head of concentrate and 200 g/head of corn meal. The concentrates were provided separately from the forage during the entire trial, and calcium carbonate was added to balance calcium content in all diets as follows: a) Control (C; n = 8 goats), animals were fed the basal pre- or post-kidding diet plus calcium carbonate (9 g/day during pre-kidding period, 12 g/day after kidding); b) Fish oil (FO; n = 8 goats), animals were fed the pre- or post-kidding basal diet plus calcium carbonate (9 g/day during pre-kidding period, and 15 g/day after kidding) and 30 g/day of fatty acids (81 g/day of supplement) before kidding or 50 g/day of fatty acids (135 g/day of supplement) during lactation from a rumen-inert fish oil (10.4% EPA and 7.8% DHA; Ufac Ltd., Stretton, UK); c) Calcium stearate (ST; n = 7 goats), animals were fed the pre- or post-kidding basal diet plus 30 g/day of fatty acids (34 g/day of supplement) before kidding or 50 g/day of fatty acids (56 g/day of supplement) during lactation from stearic acid (C16:0 26% and 69.4% C18:0; Brenntag S.p.a., Milan, Italy). All the daily diets were vitamin E supplemented to supply 72 mg/head during the pre-kidding period and 80 mg/head after kidding. The pre- and post-kidding dietary treatments in the three groups were designed to provide similar crude protein (CP) and calcium contents. The fat-enriched treatments (FO and ST) contained similar ether extracts (EE). The dietary supplements were stored in the dark at room

121 temperature. All goats were fed concentrates and corn meal twice a day, and the fat
122 supplementation was provided in the morning meal mixed into 50 g or 100 g of corn meal during
123 the pre- or post-kidding periods, respectively. Stearic acid is preferred over palmitic acid as a
124 positive control treatment because it is considered more neutral. Indeed, in a previous trial,
125 palmitate showed a strong effect on lipid metabolism in the liver, increasing expression of
126 peroxisome proliferator-activated receptor-α (*PPAR*A), acyl-coenzyme A dehydrogenase very long
127 chain (*ACADVL*) and carnitine palmitoyl-transferase 1A (*CPT1A*) at 21 days after kidding (Agazzi
128 et al., 2010).
129
130 *2.2. Dry matter intake, live body weight, energy balance, milk yield and composition*
131 Individual DMI was assessed weekly until 21 days after kidding as the difference between the
132 feed dry matter (DM) offered and the feed DM refused. The individual live body weight (LBW)
133 was assessed at 7 days before kidding and at 7, 14 and 21 days of lactation by an electronic scale
134 (F.lli Fascina snc, Castelvetro P.no, Italy). On a daily basis, goats were milked once a day at 8:00
135 am. To allow milk yield recording and milk samples collection, once a week, the suckling kids were
136 separated from the mothers for two consecutive milkings (8:00 h and 18:00 h), starting from the
137 evening milking on the day before to the end of the evening milking on the subsequent day.
138 Individual milk production was assessed with an electronic scale, and the separated kids were fed
139 the relative mother's milk after sample collection. Individual milk samples were taken on days 0, 7,
140 14 and 21 of lactation and an aliquot was subsequently analyzed for fat, protein and lactose content
141 with an infrared analyzer (MilkoScan™, FOSS, Hillerød, Denmark). Energy balance was calculated
142 weekly with the Small Ruminant Nutrition System software (Tedeschi et al., 2010) using the
143 following formula:
144 $EB = MEI - (ME_m + ME_l + ME_{preg})$

where EB is ME balance, Mcal/d; MEI is ME intake, Mcal/d; ME_m is ME required for maintenance, Mcal/d; ME_l is ME required for milk production, Mcal/d; and ME_{preg} is ME required for pregnancy, Mcal/d (Cannas et al., 2004).

2.3. Blood samples and analysis

To evaluate the serum metabolites, white blood cell count (WBC) and hemochromocytometric parameters (HCM), individual blood samples were taken at 14, 7 and 2 days before the expected kidding date as well as at 0, 2, 7, 14 and 21 DIM. Blood samples were collected from the jugular vein before the morning feeding in two vacuum sterile tubes containing either EDTA (Terumo Venoject® 10-mL VF-109SDK) or a clot activator (VF-109SP). WBC and HCM levels were assessed in whole blood samples with a Hemat 8 (SEAC, Calenzano, Florence, Italy). Blood samples were subsequently centrifuged, and serum was obtained via centrifugation for 10 min at 1,000 x *g*. Serum, which was utilized for the determination of alanine aminotransferase (ALAT), NEFA, glucose, BOHB and cholesterol concentrations, was stored at -20 °C until it was analyzed. Serum ALAT, cholesterol and glucose concentrations were measured with a clinical chemistry analyzer (ILab 300 plus, Instrumentation Laboratory s.p.a., Milan) using reagents provided by the same company; NEFA and BOHB were tested using Randox reagents (Randox, Crumlin, UK).

2.4. Adipose tissue and liver collection

Liver and adipose tissue biopsies were harvested on days -7, +7 and +21 relative to parturition for each experimental subject, via puncture biopsy under local anesthesia. The biopsy area was shaved and cleaned with a disinfectant. For liver biopsies, a 14G biopsy needle was introduced through a small incision made at the right 11^{th} intercostal space at approximately 15 cm below the spine (Agazzi et al., 2010). Subcutaneous adipose tissue biopsies were taken from alternate sides of the tail-head region. The biopsy area was shaved and cleaned with disinfectant, an incision of 2-3 cm length was made between the tail head and the ischiatic bone, and a sample of approximately 1

171 cm^3 of subcutaneous white adipose tissue was excised. The incisions for the liver and subcutaneous
172 adipose tissue biopsies were both sutured and treated with topical antibiotics agents (Thering et al.,
173 2009). The biopsied tissue was fixed in a B5 fixative (Bio-Optica, Milan, Italy) for 5 to 7 h,
174 dehydrated in a graded series of ethanol, cleared with xylene, paraffin-embedded and cut into 8 μm
175 sections. Serial sections were placed on glass microscope slides that were previously treated with
176 Vectabond (Vector Laboratories, Burlingame, CA, USA) to enhance the adherence of the tissues
177 and stained with hematoxylin and eosin (H.E; Bio-Optica, Milan, Italy). In a subset of twelve goats,
178 four subjects from each experimental group that were representative of the animals used in the trial
179 (Tables 3S, 4S, 6S, 8S), for each biopsy, five randomly chosen fields were photographed at 200X
180 magnification on a light microscope (Nikon Diaphot TMD- Nikon, Japan). To assess diets effects
181 on adipose tissue, variations in adipocytes area were evaluated. For each biopsy, five randomly
182 chosen fields of 40,000 μm^2 in surface area (one randomly chosen field/image) were scored and the
183 mean adipocyte area units, expressed as square micrometers (μm^2), were calculated. To evaluate
184 diets effects on hepatic tissue, variations in hepatocyte fat infiltration were measured. The
185 experiments were divided into two steps.
186 In the first step, the infiltration level was classified histologically according to 6 different
187 degrees (Grades der Leberverfettung or GdL), as previously described (Kalaitzakis et al., 2007).
188 The fatty infiltration (GdL) severity scores ranged from 0 (no fat droplets visible) to 5 (pan-lobular
189 fatty infiltration), following the Mertens point score scale (Mertens, 1992; Kurosaki et al., 2008).
190 On the basis of this parameter, the area from the central vein to the portal triad of the hepatic lobule
191 was divided into 3 equal concentric regions that were scored according to the presence of the most
192 severe cellular lesions. For every sample, five randomly chosen lobules were evaluated (one
193 randomly chosen lobule/image) and the median was calculated. From that median, each goat was
194 classified according to 1 of 6 degrees of FCL (GdL 0–5). When a sample did not contain five entire
195 lobules, the assessment was performed on ten partial lobules.

In the second step, on the basis of the previous considered cellular lesions (Kalaitzakis et al., 2007) and in ascending order of severity, hepatocytes where grouped as 1) normal cells showing the typical aspect of hepatocytes, a fine granular cytoplasm and a centrally placed nucleus, 2) cloudy cells with a foaming aspect due to cloudy-swelling cytoplasm and or to the presence of small or moderately sized vacuoles and 3) vacuolated cells, with large vacuoles or one single vacuole inside the cytoplasm (Fig. 2). For each lobule, a triangular shaped area of approximately 38.000 mm^2, which was representative of one fourth of a single lobule (Fig. 3), was selected and the infiltration level was calculated as the percentage of total fatty infiltrated cells (as sum of cloudy and vacuolated cells) or as the percentage of normal, cloudy and vacuolated cells, which was considered separately. All sections were analyzed by one technical expert using an image analysis system (Image J 1.41g, NIH) (Abràmoff et al., 2004) to avoid individual variation. Samples were blindly analyzed to prevent any bias. For each biopsy, on additional sections, to evaluate the presence of inflammatory processes, the presence of fibrosis features was evaluated with the Masson's trichrome staining, which specifically dyes collagen fibers (Brunt et al., 1999).

2.5. Statistical analysis

Data relative to DMI, LBW, energy balance, milk yield and milk composition were analyzed with a repeated measures model using a MIXED procedure in SAS 9.2 (SAS Inst., Inc., NC, USA). The statistical model considered as fixed effects time, treatment and time x treatment interaction as well as goat as the random effect. The hematological and histological data statistics were computed with IBM SPSS 21.0 for Windows (IBM SPSS, Armonk, New York, USA). Due to the non-normal distribution of these data, as assessed by a Shapiro-Wilk test, and because of the repeated measurements in the blood and histological data (dependent variables), a generalized estimating equation (GEE) was used to determine the effects of the different diets, the sampling times and their interaction. The dependent variables had an inverse Gaussian distribution for the blood leukocyte differential cell counts and a negative binomial distribution for the hemochromocytometric, blood

metabolites and histological parameters; therefore, an identity link function was used. The goodness of fit was assessed using a quasi-likelihood under independence model criterion (QICC). The threshold for statistical significance was considered to be $P<0.05$. All data in the tables are presented as marginal means ± SEM or SD, where stated.

3. Results

3.1. Goat performance

In the present trial, no significant differences were found for LBW (Table 2S), forage or concentrate DMI from the week before kidding to the third week of lactation between the three experimental groups. Similarly, the milk yield, fat-corrected milk, and milk composition levels were not affected by the dietary treatments (Table 2). The treatment x time effects were significant ($P<0.01$) for energy balance in the first week of lactation, and the FO and ST groups had negative values compared with the control group. Two different patterns were clear for the EB values between the C and fat-supplemented groups. The latter started with negative values in the first week after kidding; however, the values increased constantly in the subsequent 2 weeks. The C group had positive values in the first week; however, the values steadily decreased afterward. The results of the goat subsets (12 subjects) are reported in Tables 3S, 4S, 6S and 8S.

3.2. Blood components and serum metabolites

Only the main blood components at day -7, 7 and 21, which corresponded to the liver and adipose tissue biopsies times, are presented in Table 3. The complete dataset is included in Table 5S. The serum ALAT activity was constantly higher in the FO fed animals than in the C group in the 3 weeks after kidding as well at day -7 (Table 3 and 5S). However, significantly higher serum ALAT activity was observed in the FO group compared with the ST group on days 0 ($P=0.02$) and 7 ($P=0.02$) after kidding. Notably, similar values were observed between the groups at day -14, before supplementation. The cholesterol content was lower in both of the fat-supplemented animal

groups compared with the C group in the first week of lactation ($P<0.01$). Additionally, at day 21, the highest values were recorded in the FO group compared with the C and ST groups ($P<0.05$). The serum glucose content was higher in both the FO and ST goats than the C goats in the week before kidding ($P<0.05$). However, during the first 3 weeks of lactation, the ST group had the highest values. Two weeks before parturition, the serum BOHB content in the C group was the lowest between the treatments. At day -2, it was higher than in the FO group, and at day 0, it was the differences peaked between the treatments. At the end of the trial, the BOHB concentrations were higher in the ST and C groups than in the FO group ($P<0.05$). The C group had lower NEFA concentrations than the ST group 2 weeks before kidding, while the opposite phenotype was observed in the last week of gestation (days -7 and -2). Two days after kidding, the FO and ST groups had the highest NEFA content levels. The values in the FO group peaked at day 7 and these higher values were maintained until day 14. The serum NEFA and BOHB concentration trends are depicted in Fig. 1.

3.3. Blood leukocyte differential cell counts

The HGB concentration was higher in the FO group at day 7 than in the C group ($P<0.01$). In contrast, the neutrophil counts were higher at day 7 of lactation in the C group compared with the FO group (Tables 4 and 7S), and the monocytes percentages were higher in the C group than in the ST group at day 2 ($P<0.05$). At day 7, higher lymphocyte counts were detected in the FO group compared with the C group. The interactions between the treatment and sampling times were significant for all classes of considered leukocytes.

3.4. Adipose tissue histology

In all of the samples, histological examination showed that adipocytes were individually held in place by delicate reticular fibers that clustered in lobules that were bound by fibrous septa within the adipose tissue. Typical, uni-locular signet-ring shaped adipocytes were found in all of the

examined biopsies. Morphometric evaluations showed a significant decrease in the adipocyte areas between -7 and 21 days in the ST and C groups ($P<0.05$), whereas in the FO group, the adipocyte surface reduction was limited to the -7 to 7 day interval ($P<0.05$) and then reached a plateau until day 21 (Table 5 - Fig. 4).

3.5. Hepatic tissue histology

In all of the samples, histological examinations revealed that goats experienced a fatty liver phenotype before and after parturition. Necrosis foci were not observed, and pyknotic nuclei were poorly represented. Masson's trichrome staining showed no signs of fibrosis in all specimens examined (image not shown). The first analysis step demonstrated that fatty liver infiltration ranged from mild (Gdl 2) to moderate (GdL 3) to severe grades of infiltration (GdL 4). All treatment samples showed the same GdL distributions at day 7, while at -7 and + 21 days, this distribution varied (Fig. 5). In the second step, when fatty liver infiltration was analyzed as total fatty infiltrated cells or as a single group of cells, the percentage of total infiltrated cells significantly increased between day -7 and + 21 in the FO group ($P<0.05$). However, no significant differences were observed between -7 and + 21 days in the ST and C groups, although the fatty infiltrated cell percentages decreased at day 7 ($P<0.05$) (Fig. 6). At day 7 day, no differences between the diets were observed. When comparing the percentage of total infiltrated cells between the diets, significant differences were observed at -7 and + 21 days. Moreover, the fatty infiltrated cell percentages were, in fact, higher in the C and ST groups than in the FO group during prepartum, while 3 weeks after kidding, the ST group had higher values than the C group with no differences compared with the FO group. When the cells were evaluated separately, over time, it was possible to observe an increase in the vacuolated cell percentages in all of the diets. Additionally, a decrease in the cloudy cell levels in the FO and C groups were observed, but not in the ST group, between days 7 and +21, although the percentage of these cells significantly decreased at day -7 ($P<0.05$) (Fig. 7A). When comparing the percentage of normal, cloudy and vacuolated cells between the

diets, the proportion of cloudy cells was higher in the ST group than in the FO and C groups at -7 and +21 days (P<0.05). However, at day 21, the vacuolated cell percentages were higher in the FO group than in the ST and C groups. At day 7, no statistically significant differences were recorded. The interaction between the treatment and sampling times were significant for all considered parameters (P<0.05) (Fig. 7B).

4. Discussion

In the present work, it has been demonstrated that dietary saturated and unsaturated lipid supplements affect the peripartal fat mobilizing/adipogenetic mechanism in dairy goats. Fish oil treatment, in particular, was able to induce a delay in fat mobilization, whereby the goats did not show any serious metabolic disorders in the presence of a hepatic sufferance due to the presence of large vacuoles inside the cytoplasm. The biochemical analyses were supported by histological analysis that showed, for the first time, the morphology of the hepatic and adipose tissue during the peripartum period and how it changes in response to FO and ST treatments in dairy goats. Goats fed C diets and supplemented with saturated and unsaturated fats did not show any significant differences in milk production and composition. In fact, goats fed fish oil did not face milk fat depression (MFD), which is in agreement with a previous study (Chilliard et al., 2014) and a previous trial performed by a research group that used a rumen-inert supplement (Agazzi et al., 2010). However, this was in contrast with what usually happens in cows (Invernizzi et al., 2010). A very recent study evaluated MFD in goats fed fish oil at inclusion levels four times higher than the one used in the present trial; however, they only considered data expressed on a kilogram of BW basis (Toral et al., 2015). The reasons for the variations between ruminants might be relative to the inter-species differences either at the ruminal level or in milk fat synthesis at the mammary level (Chilliard et al., 2014). The NEFA blood content is tightly linked to energy balance (Eknæs et al., 2006). NEFA suggested values for does, with neutral EB are 0.200‑0.217 mmol/L (Chilliard et al., 1977). The observed concentrations were constantly higher with the FO treatment, which peaked at

day 7 and exceeded a concentration of 0.7 mmol/L, which was proposed as borderline toxemia, while the lowest NEFA concentrations were recorded for the ST treatment. However, these data were consistent with results obtained by Pinotti et al. (2008) and Magistrelli and Rosi (2014). Moreover, no clinical signs of toxemia were observed in the animals. This is probably because the highest concentrations were only reached for a limited time period and the serum BOHB content did not reach high values (< 1.0 mmol/L). The NEFA had a similar pattern in C and ST groups, which spiked at day 0 and was in agreement with González et al. (2011), while FO group levels spiked after a 7-day delay. The BOHB levels in the FO and ST groups spiked at day 7, which was 5 days later than the C group. The BOHB and ALAT results were consistent with those obtained in a previous experiment (Agazzi et al., 2010), where the lowest postpartum serum ALAT activity values were observed in palm oil supplemented goats. However, in both trials, the FO group had higher values ($P<0.05$).

The histological analysis indicated that goats during transition period exhibited fatty liver syndrome at 7 days before parturition, which was independently of their diets. In agreement, high serum ALAT activity values were previously observed in a study evaluating the prepartum period, although this enzyme cannot be considered liver specific in ruminants (Sirois, 2014). The fatty infiltration levels in the liver ranged from mild to moderate to severe, as previously described in transitional dairy cows (Bobe et al., 2004). At day 21, the highest percentage of cloudy cells was observed in the ST group, while the highest percentage of vacuolated cells was observed in the FO group, suggesting that, although in the absence of a clear decline in their health status, the FO treatment induced slightly more detrimental effects on the liver over time, as confirmed by the GdL distribution and higher ALAT activity values at this time.

Interestingly, although we observed severe hepatocyte injury, features of perisinusal fibrosis, which in humans have been described and associated with a progression of inflammatory processes (Brunt and Tiniakos, 2010), or necrosis foci with polymorphonuclear cells, which are often described in dairy cows with severe fatty livers (Kalaitzakis et al., 2010), were not observed. These

352 data were confirmed by a general decrease in lymphocytes and monocytes around the parturition
353 together with an increase in neutrophils, as previously described in cattle (Van Kampen et al., 1999;
354 Harp et al., 2004).

355 All together, these data suggest that the effects of FO are only apparently detrimental for the
356 animals. Therefore, in this context, it is important to consider that in the FO group, the onset of
357 fatty infiltration, which was evaluated as total infiltrated cells as well as vacuolated cells, occurred
358 more gradually than in the C and ST groups. Therefore, it has been hypothesized a sort of
359 progressive adaptation to the lipid infiltration that allowed the cells to better preserve their
360 functions.

361 The subcutaneous adipose tissue data were well coupled with serum NEFA (and BOHB)
362 concentrations. Indeed, the highest adipocyte area reduction in the C group was observed between
363 days -7 and 7, which matched with the NEFA level spikes, which are both clear signs of strong fat
364 mobilization. From days 7 to 21, the adipose tissue seemed to still be mobilized, considering the
365 negative energy balance of the non-supplemented goats. The ST group had a similar pattern;
366 however, its energy balance became positive between the 7^{th} and 14^{th} days post-parturition.
367 Additionally, its lipolysis most likely would have been reduced earlier than the C group, but after
368 21 days. The histological adipose tissue data support the idea that FO can limit lipolysis even if the
369 energy balance is still negative (at least until day 14). The initial decrease in the adipose cell areas,
370 which was common to all treatments, stopped between days 7 and 21. This pattern is in agreement
371 with cow studies (Thering et al., 2009; Schmitt et al., 2011). Because important interspecies
372 differences have been outlined in MFD (Toral et al., 2015), it would be very interesting to explore
373 the fine mechanisms that regulate this response. The bovine studies mentioned above (Thering et
374 al., 2009; Schmitt et al., 2011) actually support the idea that FO could be used to increase
375 adipogenesis/lipogenesis in adipose tissue rather than decrease lipolysis. Furthermore, recent *in*
376 *vitro* and mouse studies demonstrated that long-chain acyl-CoAs could inhibit major lipolytic
377 enzymes that are crucial in adipocytes as well in hepatocytes (adipose triglyceride lipase

(ATGL)/Serpin peptidase inhibitor, clade F, member 1 [SERPINF1]), representing a feedback mechanism controlling lipolysis. It has been highlighted that differences exist between acyl-CoA species on their inhibiting efficacy. Unfortunately, only oleic and palmitic acids, and not PUFAs, were tested (Zimmermann et al., 2009; Nagy et al., 2014). At the tissue level, the balance of this remodeling in the FO group appeared as a marked stop in adipocyte size reduction 3 weeks after kidding. At the same time, the NEFA concentrations were not decreased because the fat mobilization was most likely still occurring. In this context, the liver was still facing a heavy metabolic workload, as suggested by the biochemical data and supported by the histological results, which clearly indicate how hepatic tissue can react in very dynamic and, to some extent, unpredictable ways to stress during the peripartum period.

5. Conclusion

To the best of our knowledge, this is the first histological investigation on transition dairy goat liver and subcutaneous tissues carried out to study the morphological and metabolic changes during this particularly challenging physiological period in response to saturated and unsaturated dietary supplemental fats. An interesting ability of FO to delay fat mobilization at the adipose tissue level after kidding was observed, which is associated with a slightly negative impact on the liver histology; however, it remained in a physiological range.

Conflict of interest

The authors declare no conflicts of interest.

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406

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528

529 **Tables**

530

531 **Table 1**

532 Ingredients and chemical composition of the experimental diets of the dairy goats fed either a basal
533 diet (C) or a diet supplemented with fish oil (FO) or stearate (ST).

	Experimental diets					
	Pre-kidding			Post-kidding		
	C	FO	ST	C	FO	ST
Ingredient (%)						
Alfalfa hay	0.0	0.0	0.0	31.2	29.8	30.7
Mixture hay[2]	62.3	59.6	61.4	15.3	14.6	15.1
Concentrate mixture[1]	31.9	30.5	31.4	46.8	44.8	46.2
Corn meal	5.3	5.0	5.2	6.2	5.9	6.2
Fish oil	0.0	4.4	0.0	0.0	4.3	0.0
Calcium Stearate	0.0	0.0	2.0	0.0	0.0	1.9
$CaCO_3$	0.5	0.5	0.0	0.5	0.5	0.0
Chemical Composition (% of dry matter)						
Dry Matter (%)	88.4	88.7	88.6	89.3	89.5	89.4
Crude Protein	12.3	11.9	12.2	17.8	17.2	17.5
Ether Extract	2.9	4.9	4.5	3.2	5.2	4.8
NDF	43.9	43.8	43.3	33.7	34.0	33.2
Ashes	6.3	6.5	6.0	7.2	7.3	6.8
Ca	0.8	0.8	0.9	1.1	1.1	1.2
P	0.4	0.4	0.4	0.8	0.8	0.8
NE_L (Mcal/kg DM)[3]	1.61	1.66	1.67	1.67	1.72	1.72

534 [1] The concentrate mixture was a commercial dairy goat mixed feed, chemical composition: 22.25%
535 crude protein, 5.00% ether extract, 22.98% neutral detergent fiber, 6.51% ashes, 1.28% Ca and
536 0.76% P (on dry matter basis).
537 [2]The mixture hay was a grass hay, chemical composition: 7.6% crude protein, 1.8% ether extract,
538 57.5% neutral detergent fiber, 5.9% ashes, 0.6% Ca and 0.2% P (on dry matter basis).
539 [3]Net energy of lactation concentration of the diets were calculated using the Small Ruminant
540 Nutrition System (SRNS) software (Tedeschi et al., 2010).

Table 2

Performance of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (n=23).

Time (day)	Treatment				*P*[1]		
	C (n=8)	FO (n=8)	ST (n=7)	SE	Trt	Time	Trt*Time
Milk production (kg/day)							
7	2.85	3.87	3.36	0.30	0.13	<0.01	0.58
14	3.52	4.24	3.79				
21	3.66	4.21	3.88				
7-21	3.34	4.10	3.68				
3.5% Fat-corrected milk (kg/day)							
7	3.77	4.53	3.94	0.49	0.46	0.73	0.22
14	3.50	4.38	3.92				
21	4.15	4.17	3.52				
7-21	3.80	4.36	3.80				
Dry Matter Intake (kg/day)							
-7	2.81	2.60	2.61	0.29	0.28	<0.01	0.46
7	2.48	2.68	2.25				
14	2.65	2.88	2.71				
21	3.28	3.66	2.96				
Energy Balance (Mcal/day)							
7	0.34[a]	-0.91[b]	-1.12[b]	0.51	0.99	<0.01	<0.01
14	-0.20	-0.05	0.46				
21	-0.16	0.89	0.82				

[a, b]Means within each row with different superscripts are significantly different ($P < 0.05$).

[1] Trt, treatment effect

Table 3

Serum metabolites of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (n=23).

Time	Treatment						*P*[1]		
	C (n=8)	SE	FO (n=8)	SE	ST (n=7)	SE	Trt	Time	Trt*Time
ALAT[2] (IU/L)									
-7	13.58[b]	0.84	15.91[a]	0.48	14.35	0.85	0.09	<0.01	<0.01
7	14.71[b]	0.89	17.35[a]	0.65	14.73[b]	0.92			
21	12.34[b]	1.11	15.87[a]	1.06	13.64	0.79			
Cholesterol (mg/dL)									
-7	54.13	3.23	55.24	3.92	61.43	4.28	0.63	<0.01	<0.01
7	83.50[a]	4.82	65.70[b]	4.59	59.30[b]	3.83			
21	63.23[b]	4.04	88.21[a]	4.81	72.64[b]	5.40			
Glucose (mg/dL)									
-7	42.81[b]	1.80	51.97[a]	1.88	54.49[a]	2.28	0.04	<0.01	<0.01
7	57.63	3.27	47.60[b]	4.34	62.25[a]	2.04			
21	54.95[b]	2.32	61.61	3.53	66.00[a]	3.67			
BOHB[3] (mmol/L)									
-7	0.35	0.03	0.30	0.06	0.35	0.08	0.18	<0.01	<0.01
7	0.69	0.08	0.82	0.26	0.86	0.16			
21	0.51[a]	0.06	0.38[b]	0.03	0.72[a]	0.13			
NEFA[4] (mmol/L)									

-7	0.58[a]	0.09	0.36	0.10	0.32[b]	0.06	0.78	<0.01	<0.01
7	0.33[b]	0.07	0.78[a]	0.16	0.40[b]	0.07			
21	0.41	0.08	0.33	0.06	0.34	0.07			

547 [a,b]Means within each row with different superscripts are significantly different ($P < 0.05$).

548 [1] Trt, treatment effect

549 [2] ALAT, alanine aminotransferase

550 [3] BOHB, beta-hydroxybutyrate

551 [4] NEFA, non-esterified fatty acids

552

Table 4

Hemoglobin (HGB) and blood leukocytes differential cell counts of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (n=23).

Time	Treatment						*P*[1]		
	C (n=8)	SE	FO (n=8)	SE	ST (n=7)	SE	Trt	Time	Trt*Time
HGB (g/L)									
-7	88.38	2.87	95.13	3.85	93.71	3.07	0.29	<0.01	<0.01
7	84.50[b]	4.77	99.57[a]	4.52	93.29	2.66			
21	82.14	5.18	90.33	3.72	86.00	1.68			
Neutrophils (%)									
-7	47.14	3.85	50.81	3.60	47.79	3.85	0.87	<0.01	<0.01
7	64.41[a]	3.60	56.73[b]	3.60	57.74	3.85			
21	60.55	3.60	57.61	3.85	55.63	3.85			
Monocytes (%)									
-7	7.91	1.51	6.31	1.42	5.26	1.51	0.05	<0.01	<0.01
7	3.11	1.42	5.20	1.42	5.43	1.51			
21	3.10	1.42	3.04	1.51	4.77	1.51			
Lymphocytes (%)									
-7	43.89	3.79	41.04	3.54	46.34	3.79	0.88	<0.01	<0.01
7	31.85[b]	3.54	37.25[a]	3.54	36.19	3.79			
21	35.05	3.54	37.77	3.79	38.30	3.79			

[a,b]Means within each row with different superscripts are significantly different ($P < 0.05$).

[1] Trt, treatment effect

Table 5

Mean adipocyte area (μm^2) of subcutaneous adipose tissue of a subset (n=12) of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST).

Time	Treatment						*P*[1]		
	C (n=4)	SD	FO (n=4)	SD	ST (n=4)	SD	Trt	Time	Trt*Time
-7	3200.00^{d}	1002	2877.69^{d}	876	3088.80^{d}	1010	0.60	<0.01	<0.01
7	1970.44^{e}	523	1801.80^{e}	548	2156.33^{e}	718			
21	1157.74^{b,f}	216	1851.85^{a,e}	638	1066.66^{b,f}	269			

^{a,b}Means within each row with different superscripts are significantly different ($P < 0.05$).

^{d,e,f}Means within each column with different superscripts are significantly different ($P < 0.05$).

[1] Trt, treatment effect

Figure captions

Fig. 1. Serum beta-hydroxybutyrate (BOHB) (A) and non-esterified fatty acid (NEFA) (B) contents of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (n=23) (** differs from *; $P<0.05$).

Fig. 2. Representative images of normal hepatocytes showing a fine granular aspect (A), cloudy hepatocytes showing a foaming aspect due to the cloudy-swelling cytoplasm (B, arrow head), hepatocytes containing moderately sized vacuoles (B, arrow) and hepatocytes containing large vacuoles (asterisk). H&E staining, 20X original magnification, bar 50 μm.

Fig. 3. Representative image of the triangular shaped area representing one-fourth of a hepatic lobule. PS-Portal Space; CV-Centro lobular Vein. H&E staining, 10X original magnification, bar 100 μm.

Fig. 4. Representative images of adipose tissue showing large and small adipocytes individually held in place by delicate reticular fibers. H&E staining, 20X original magnification, bar 50 μm.

Fig. 5. Histological classification of fatty infiltration changes in the liver (FCL or hepatic lipidosis). The classifications were made according to six different degrees (Grade der Leberverfettung or GdL) ranging from 0 (no fat droplets, totally normal hepatocytes) to 5 (pan lobular infiltration) of FCL.

Fig. 6. Percentage of fatty infiltrated liver cells of a subset (n=12) of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (** differs from *; $P<0.05$).

Fig. 7. Percentage of normal, vacuolated and cloudy cells in the liver tissue from a subset (n=12) of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (**

differs from *, cloudy cells; ## differs from #, normal cells; †† differs from †, vacuolated cells; $P<0.05$).

Figure 1

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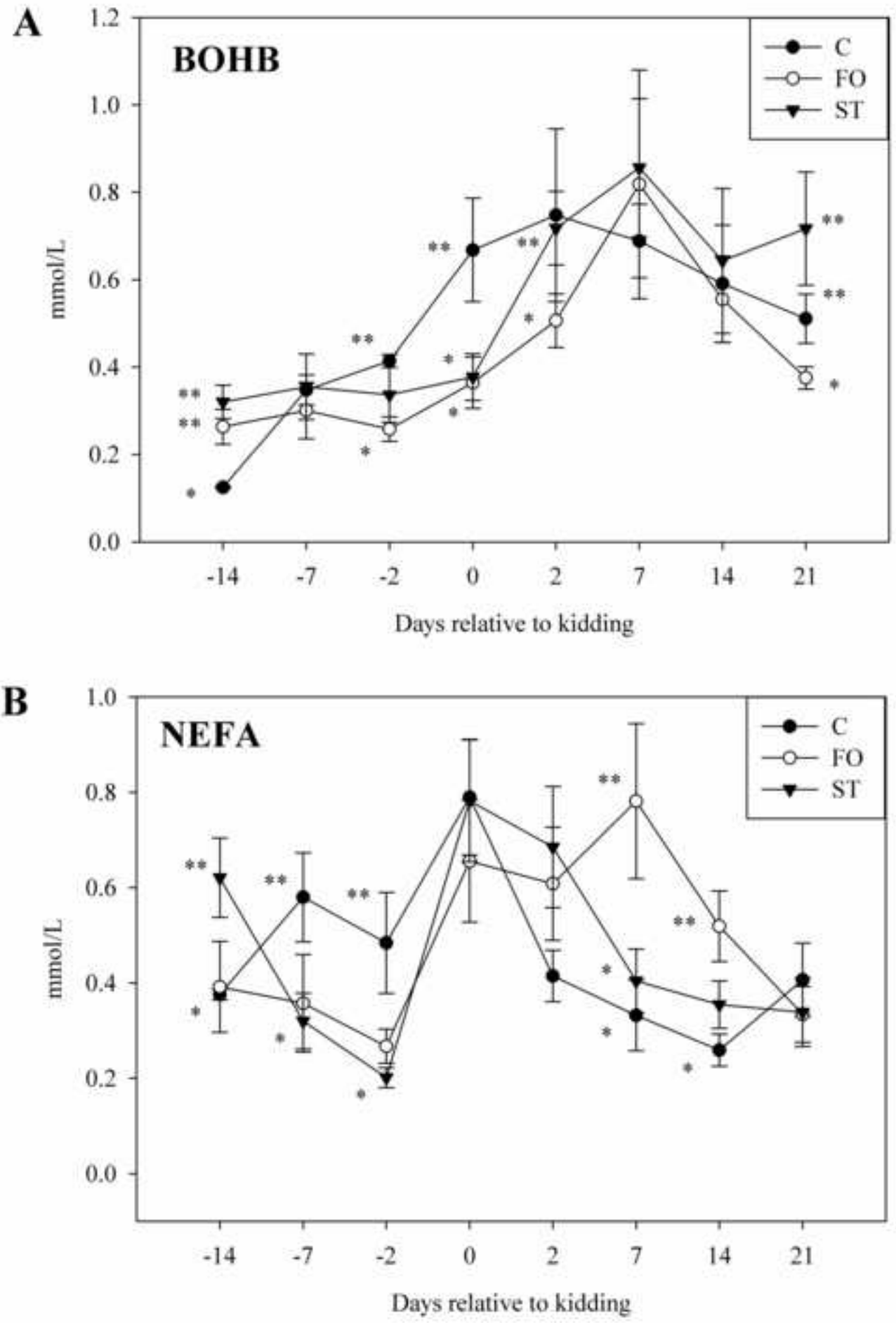

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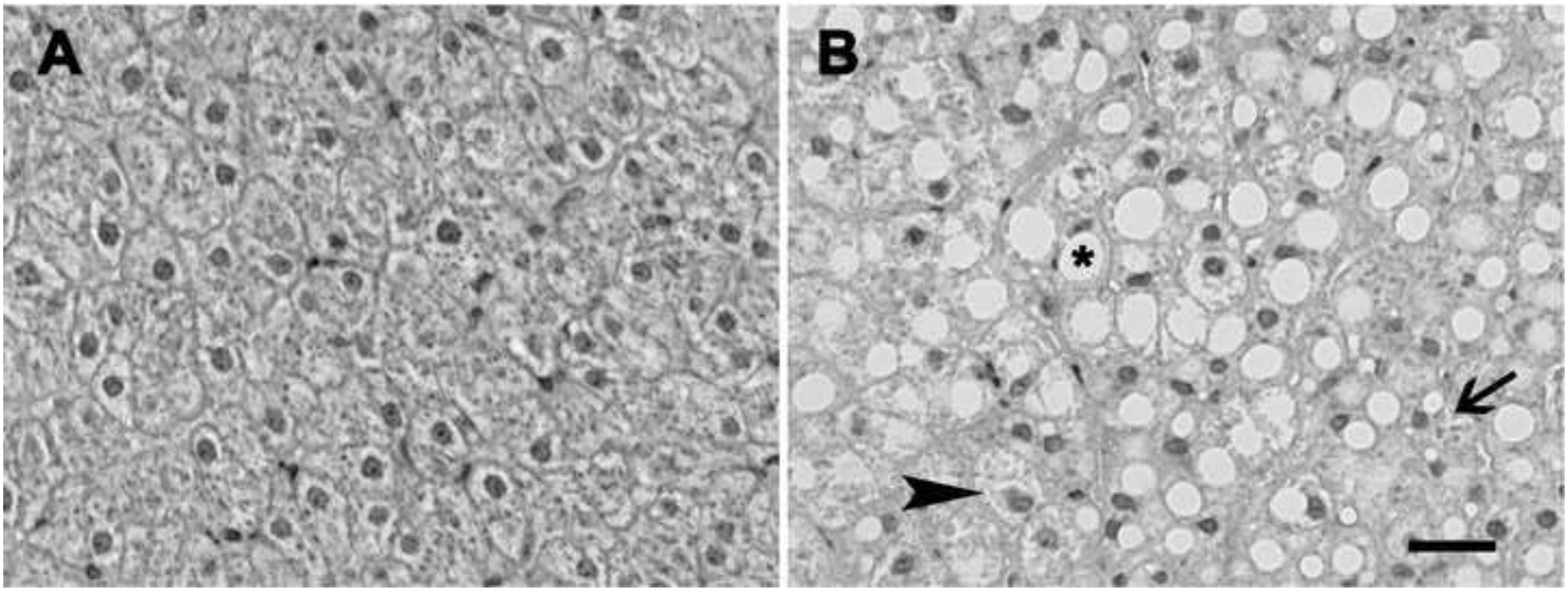

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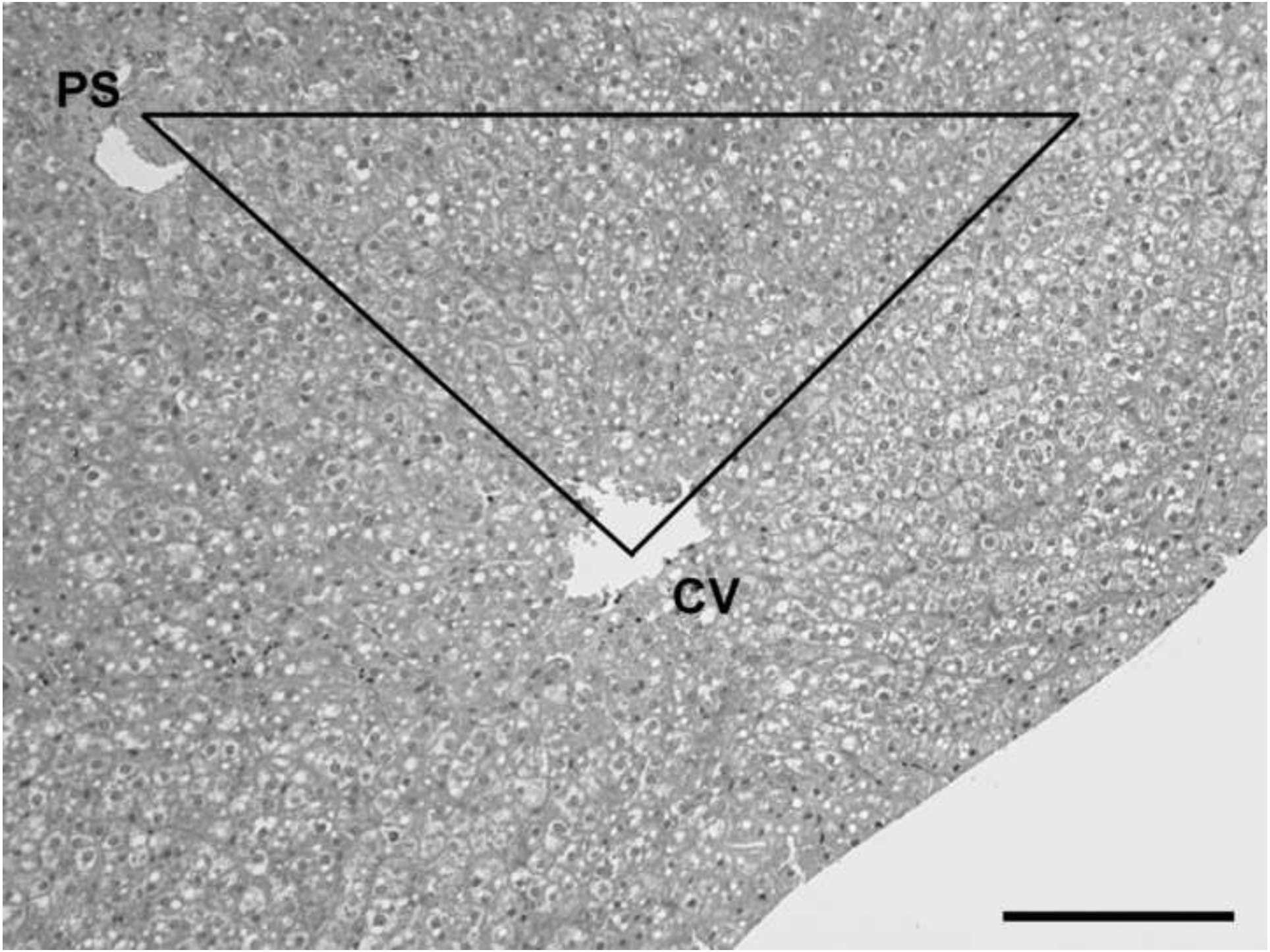

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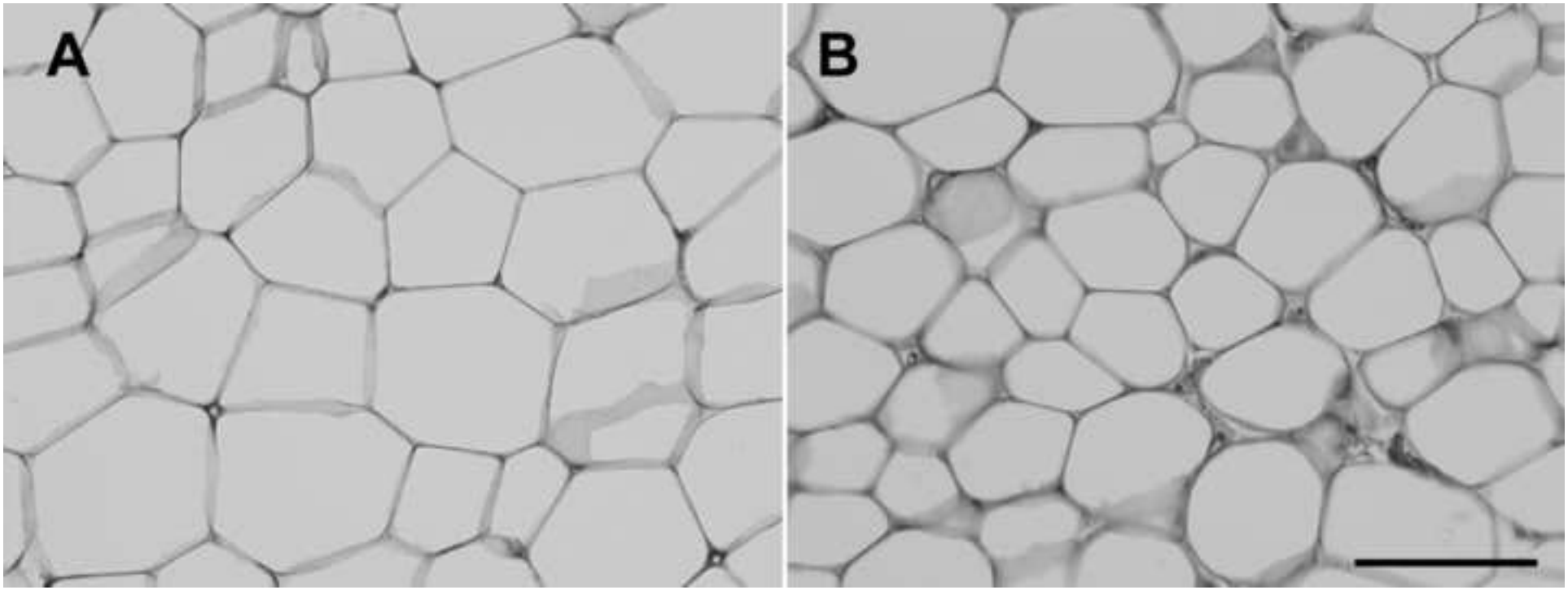

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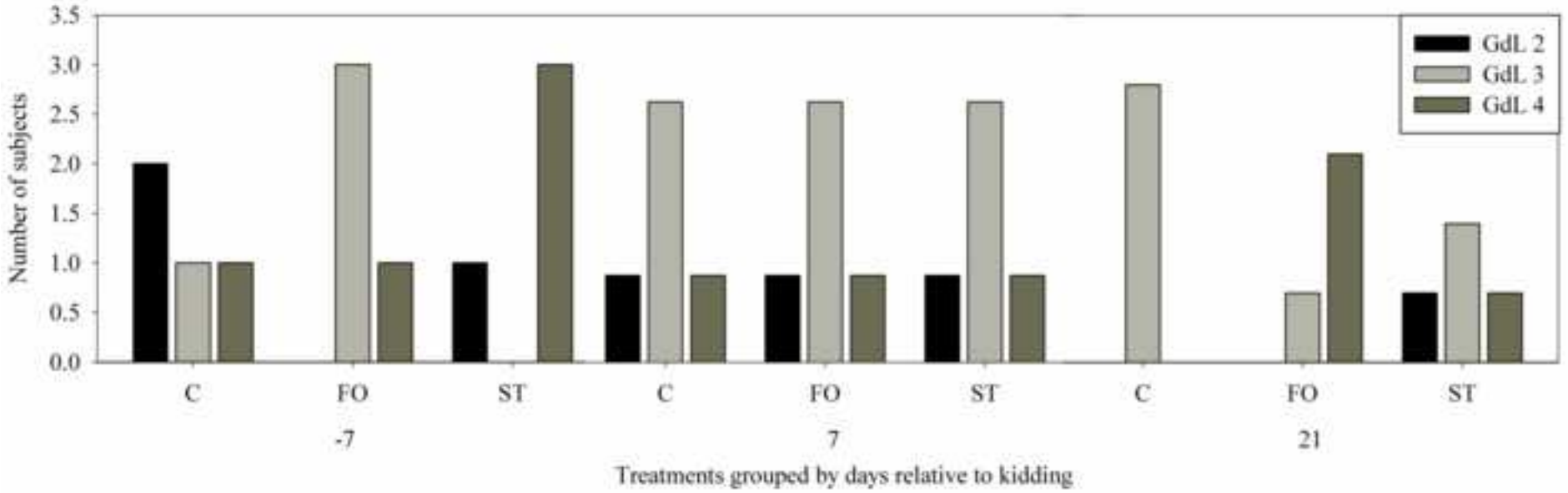

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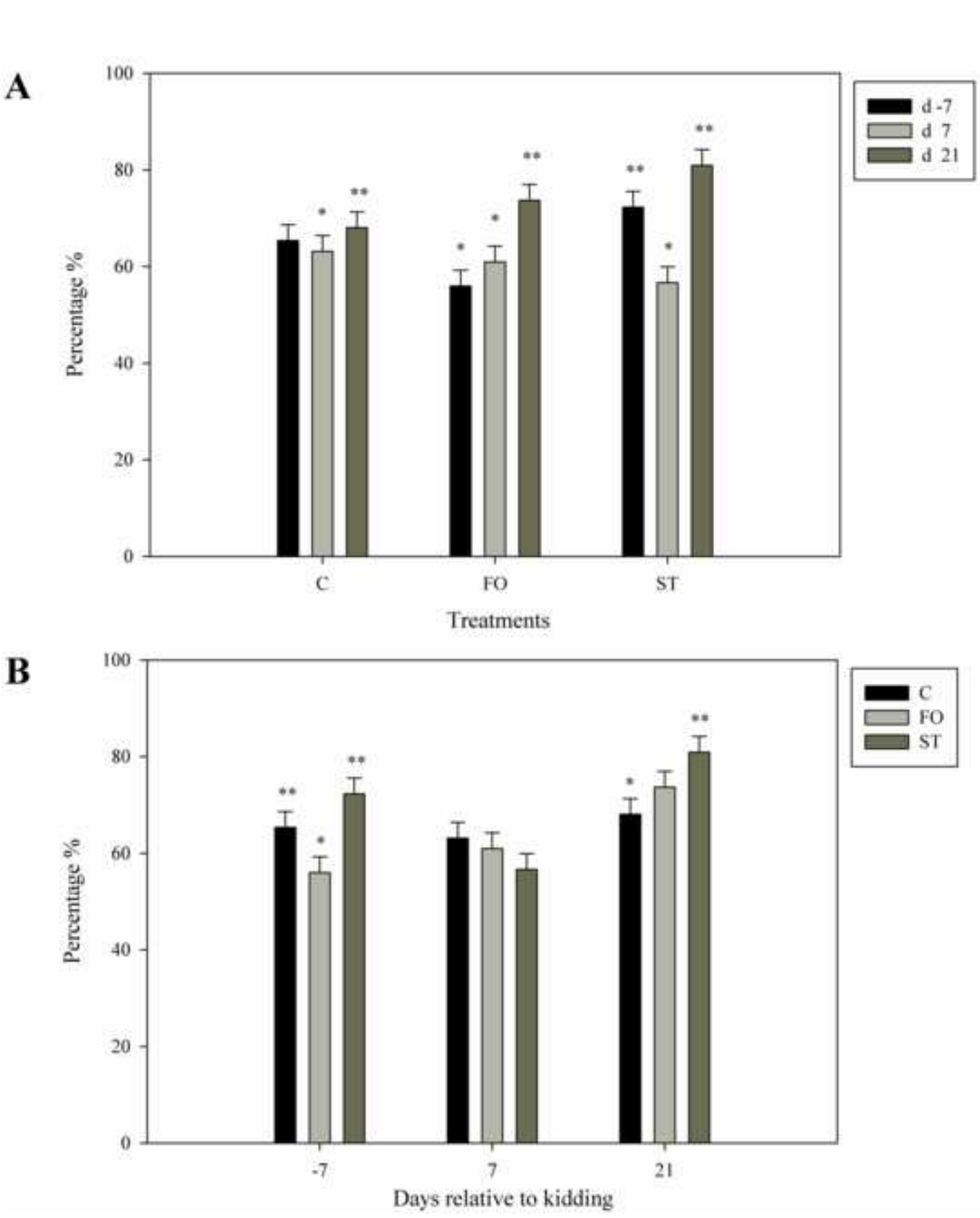

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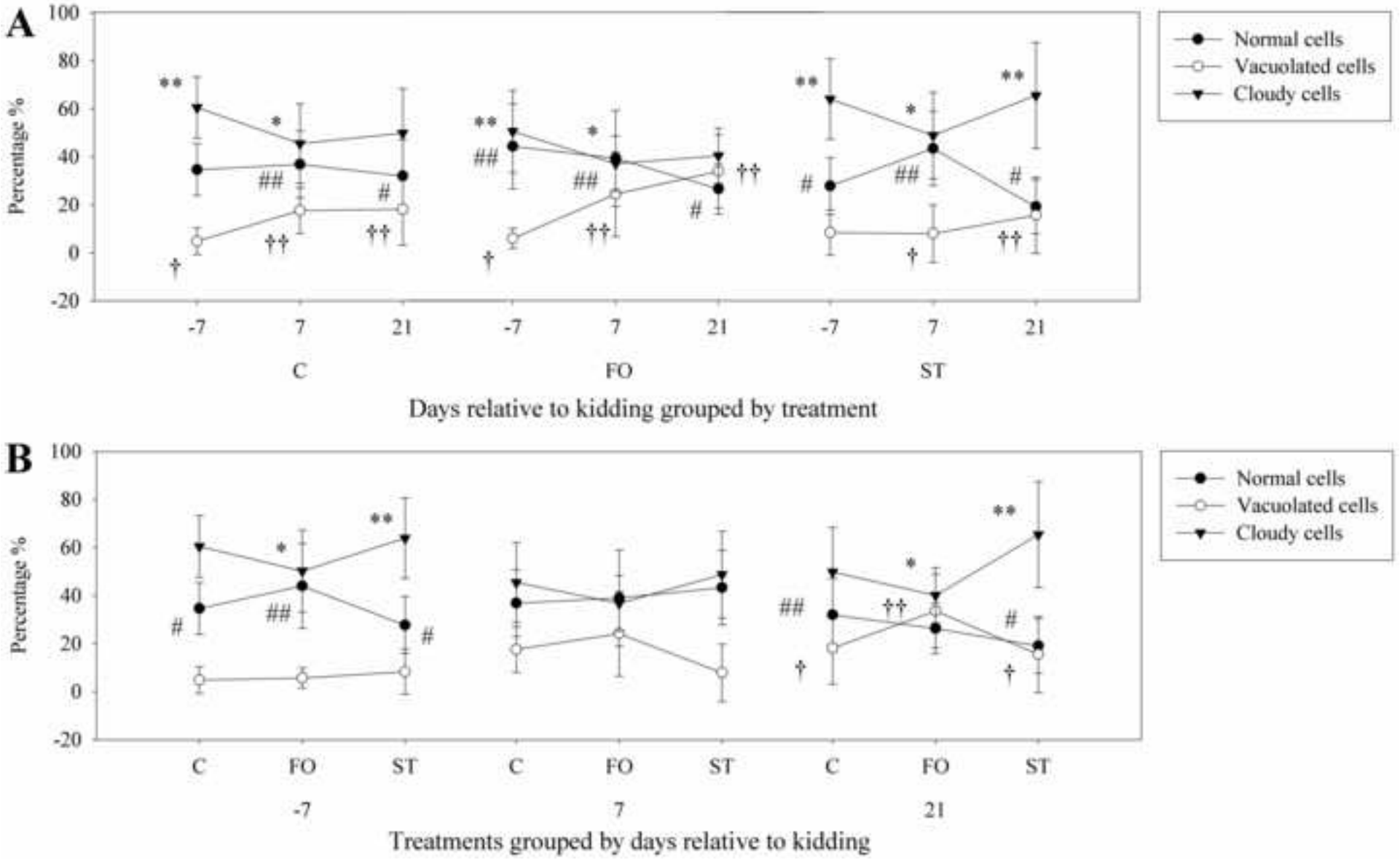

*Conflict of Interest Statement

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Milan, 20 June 2016

Subject: Submission of manuscript for evaluation

Dear Editor,

I would like to submit the manuscript entitled "Hepatic and subcutaneous adipose tissue variations in transition dairy goats fed saturated or unsaturated fat supplemented diets " for consideration for publication in Small Ruminant Research.

To my best knowledge this is the first paper studying transition in dairy goats, dietary supplemented with saturated and unsaturated fatty acids, throughout biochemical and histological approaches, focusing in particular on liver and subcutaneous adipose tissue. I believe the results obtained could be interesting for the journal's readers and that the manuscript fully falls within the aim of SRR.

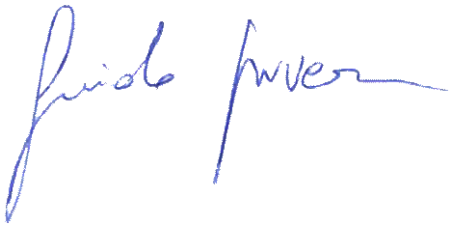
I would like to undertake that the present manuscript has not been previously published, accepted or under editorial review for publication elsewhere. Submitted manuscript is a Research Paper.

I wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. I further confirm that the order of authors listed in the manuscript has been approved by all of us.

I confirm that the authors have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing I confirm that the authors have followed the regulations of our institutions concerning intellectual property. I further confirm that any aspect of the work covered in this manuscript that has involved either experimental animals or human patients has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

All the authors agreed to the submission of this manuscript and that G. Invernizzi will act as the corresponding author. He will sign on the behalf of the other authors.

Best Regards,



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Supplementary materials

Table 1S

Fatty acid composition of the supplements materials fed to the experimental dairy goats.

	Experimental supplements	
Fatty acids (g/100g)	Calcium stearate	Fish oil
C12:0, lauric	0.12	0.10
C14:0, myristic	2.13	6.29
C16:0, palmitic	26.00	15.71
C16:1, palmitoleic		8.41
C16:2, hexadecadienoic		1.11
C16:3, hexadecatrienoic		1.12
C16:4, hexadecatetraenoic		1.61
C18:0, stearic	69.42	3.63
C18:1 *n* − 9, oleic		16.39
C18:1 *trans* −11, vaccenic		3.43
C18:2 *n* − 6, linoleic		6.07
C18:3 *n* − 3, linolenic		1.68
C18:4 *n* − 3, stearidonic		2.10
C20:0, arachidic	1.97	0.19
C20:3 *n* − 3, eicosatrienoic		0.14
C20:4 *n* − 6, arachidonic		0.75
C20:4 *n* − 3, eicosatetraenoic		1.25
C20:5 *n* − 3, eicosapentaenoic		10.38
C22:0, behenic	0.37	0.13
C22:5 *n* − 3, docosapentaenoic		4.13
C22:6 *n* − 3, docosahexaenoic		7.76

Table 2S

Live body weight of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (n=23).

Time	Treatment				*P*[1]		
	C	FO	ST	SE	Trt	Time	Trt*Time
Live body weight (kg)							
-7	63.03	66.75	62.50	2.48	0.56	<0.01	0.14
7	51.13	53.66	51.82				
14	51.68	51.99	48.28				
21	50.44	52.56	48.71				
Mean	54.07	56.24	52.83				

[1]Trt, treatment effect

Table 3S

Live body weight of a subset (n=12) of dairy goats fed either a basal diet (C) or diet supplemented with fish oil (FO) or stearate (ST).

Time	Treatment				*P*[1]		
	C (n=4)	FO (n=4)	ST (n=4)	SE	Trt	Time	Trt*Time
Live body weight (kg)							
-7	65.63	69.00	60.13	3.19	0.24	<0.01	0.52
7	51.63	57.06	49.50				
14	53.37	54.00	47.00				
21	51.37	54.37	47.12				
Mean	55.50	58.61	50.94				

[1]Trt, treatment effect

Table 4S

Performance of a subset (n=12) of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST).

Time	Treatment				*P*[1]		
	C (n=4)	FO (n=4)	ST (n=4)	SE	Trt	Time	Trt*Time
Milk production (kg/d)							
7	3.52	4.15	3.45	0.33	0.27	<0.01	0.63
14	4.20	4.59	3.76				
21	4.23	4.32	3.64				
7-21	3.98	4.35	3.62				
3.5% Fat-corrected milk (kg/d)							
7	4.98	4.40	4.06	0.49	0.10	0.62	0.13
14	4.34	5.24	3.85				
21	5.21	3.88	3.55				
7-21	4.84	4.51	3.82				
DMI (kg/d)							
-7	3.08	2.44	2.48	0.44	0.47	0.01	0.48
7	2.46	2.77	2.29				
14	2.99	2.80	2.84				
21	3.27	3.62	3.01				
Energy Balance (Mcal/d)							
7	-0.09	-1.11	-1.13	0.71	0.35	0.02	0.07
14	-0.96	-0.59	0.76				
21	-0.48	1.18	1.18				

[1]Trt, treatment effect

Table 5S
Serum metabolites of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (n=23).

Time	Treatment						*P*[1]		
	C	SE	FO	SE	ST	SE	Trt	Time	Trt*Time
ALAT (IU/L)									
-14	14.98	0.60	15.73	0.79	14.81	1.02	0.086	<0.01	<0.01
-7	13.58[b]	0.84	15.91[a]	0.48	14.35	0.85			
-2	12.22	0.56	14.30	1.48	13.70	0.54			
0	16.96	1.90	17.04[a]	1.16	13.51[b]	0.88			
2	14.79	1.31	15.88	1.16	14.38	0.92			
7	14.71[b]	0.89	17.35[a]	0.65	14.73[b]	0.92			
14	12.25[b]	0.73	14.08[a]	0.57	12.59	0.91			
21	12.34[b]	1.11	15.87[a]	1.06	13.64	0.79			
Cholesterol (mg/dL)									
-14	48.75[b]	1.66	52.92	2.19	56.92[a]	2.55	0.627	<0.01	<0.01
-7	54.13	3.23	55.24	3.92	61.43	4.28			
-2	56.04	4.44	52.55	3.04	60.19	4.22			
0	46.88	3.20	51.66	3.80	51.13	4.60			
2	50.03	3.63	53.24	4.49	57.76	5.02			
7	83.50[a]	4.82	65.70[b]	4.59	59.30[b]	3.83			
14	65.75	3.57	79.75	6.87	66.48	5.14			
21	63.23[b]	4.04	88.21[a]	4.81	72.64[b]	5.40			
Glucose (mg/dL)									
-14	63.06[a]	0.40	50.74[b]	3.87	50.32[b]	2.76	0.044	<0.01	<0.01
-7	42.81[b]	1.80	51.97[a]	1.88	54.49[a]	2.28			
-2	51.43	4.11	54.72	2.63	59.16	1.73			
0	65.09	4.48	70.54	3.77	65.74	4.65			
2	44.87	2.51	49.42	2.77	47.94	2.22			
7	57.63	3.27	47.60[b]	4.34	62.25[a]	2.04			
14	57.85	3.27	53.46	3.36	60.25	2.88			
21	54.95[b]	2.32	61.61	3.53	66.00[a]	3.67			
BOHB (mmol/L)									
-14	0.12[b]	0.00	0.26[a]	0.04	0.32[a]	0.04	0.180	<0.01	<0.01

-7	0.35	0.03	0.30	0.06	0.35	0.08			
-2	0.41[a]	0.01	0.26[b]	0.03	0.34	0.06			
0	0.67[a]	0.12	0.36[b]	0.06	0.38[b]	0.05			
2	0.75	0.20	0.51	0.06	0.72	0.08			
7	0.69	0.08	0.82	0.26	0.86	0.16			
14	0.59	0.13	0.55	0.10	0.64	0.17			
21	0.51[a]	0.06	0.38[b]	0.03	0.72[a]	0.13			
NEFA (mmol/L)									
-14	0.38[b]	0.01	0.39	0.10	0.62[a]	0.08	0.783	<0.01	<0.01
-7	0.58[a]	0.09	0.36	0.10	0.32[b]	0.06			
-2	0.48[a]	0.11	0.27	0.04	0.20[b]	0.02			
0	0.79	0.12	0.66	0.13	0.78	0.13			
2	0.41[b]	0.05	0.61[a]	0.12	0.69[a]	0.13			
7	0.33[b]	0.07	0.78[a]	0.16	0.40[b]	0.07			
14	0.26[b]	0.03	0.52[a]	0.07	0.35	0.05			
21	0.41	0.08	0.33	0.06	0.34	0.07			

[a,b]Means within each row with different superscripts are significantly different ($P < 0.05$).

[1]Trt, treatment effect

[2] ALAT, alanine aminotransferase

[3] BOHB, beta-hydroxybutyrate

[4] NEFA, non-esterified fatty acids

Table 6S

Serum metabolites of a subset (n=12) of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST).

Time	Treatment						*P*[1]		
	C (n=4)	SE	FO (n=4)	SE	ST (n=4)	SE	Trt	Time	Trt*Time
ALAT (IU/L)									
-14	15.11	0.81	15.05	0.42	15.64	1.36	0.127	<0.01	<0.01
-7	13.57	1.32	16.17	0.30	15.46	0.88			
-2	12.08[b]	0.76	13.99	1.12	14.20[a]	0.51			
0	17.36	3.08	17.25[a]	0.71	14.07[b]	1.04			
2	14.57	2.17	14.83	0.39	15.14	0.86			
7	15.03	1.54	16.44[a]	0.44	14.27[b]	1.01			
14	10.88[b]	0.65	13.82[a]	0.74	12.79	1.31			
21	11.29[b]	0.83	16.20[a]	1.14	13.27	1.28			
Cholesterol (mg/dL)									
-14	49.73[b]	1.21	52.34	1.25	59.05[a]	3.23	0.925	<0.01	<0.01
-7	55.72	2.49	54.77	2.12	56.21	1.94			
-2	49.32[b]	1.44	48.16[b]	3.17	57.16[a]	2.87			
0	48.00	4.11	52.00	5.40	50.04	1.63			
2	47.85	4.91	47.79	4.03	56.35	5.44			
7	90.55[a]	7.66	58.40[b]	4.33	57.68[b]	3.57			
14	70.62	4.25	69.23	8.71	66.88	2.45			
21	69.86	4.01	84.49	7.10	71.65	6.31			
Glucose (mg/dL)									
-14	62.48[a]	0.76	49.76[b]	6.31	51.13[b]	3.98	0.010	<0.01	<0.01
-7	44.73[b]	2.00	52.43[a]	3.17	56.53[a]	1.77			
-2	55.44	0.56	55.89	3.29	59.75	2.45			
0	54.61[b]	4.10	77.45[a]	3.16	63.77[b]	5.81			
2	43.23	2.56	46.38	1.21	48.06	2.96			
7	59.70	5.35	48.49	6.92	62.80	2.88			
14	53.04	4.48	51.78	6.11	57.79	1.76			
21	51.10[b]	3.61	63.08[a]	4.56	67.88[a]	5.84			
BOHB (mmol/L)									

-14	0.12[b]	0.00	0.23	0.06	0.35[a]	0.03	0.201	<0.01	<0.01
-7	0.31	0.04	0.29	0.09	0.30	0.04			
-2	0.38[a]	0.03	0.20[b]	0.02	0.37[a]	0.08			
0	1.00[a]	0.29	0.35[b]	0.07	0.36[b]	0.07			
2	0.64	0.21	0.57	0.09	0.79	0.06			
7	0.56	0.09	0.47	0.06	0.61	0.11			
14	0.70	0.25	0.64	0.18	0.49	0.06			
21	0.42	0.05	0.32[b]	0.02	0.64[a]	0.12			
NEFA (mmol/L)									
-14	0.39[b]	0.03	0.42	0.13	0.57[a]	0.08	0.193	<0.01	<0.01
-7	0.59[a]	0.13	0.38	0.19	0.27[b]	0.01			
-2	0.82[a]	0.09	0.22[b]	0.04	0.18[b]	0.02			
0	0.84	0.15	0.63	0.20	0.65	0.08			
2	0.41[b]	0.08	0.59	0.18	0.86[a]	0.17			
7	0.28	0.06	0.88	0.35	0.37	0.11			
14	0.30[b]	0.06	0.57[a]	0.08	0.33[b]	0.08			
21	0.44 [a]	0.07	0.27[b]	0.03	0.27	0.07			

[a,b]Means within each row with different superscripts are significantly different ($P < 0.05$).

[1]Trt, treatment effect

[2] ALAT, alanine aminotransferase

[3] BOHB, beta-hydroxybutyrate

[4] NEFA, non-esterified fatty acids

Table 7S
Haemoglobin (HGB) and blood leukocytes differential cell counts of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST) (n=23).

Time	Treatment						*P*[1]		
	C	SE	FO	SE	ST	SE	Trt	Time	Trt*Time
HGB (g/L)									
-14	98.67	7.46	92.38	2.77	92.83	1.89	0.284	<0.01	<0.01
-7	88.38	2.87	95.13	3.85	93.71	3.07			
0	83.14	4.42	91.25	3.12	91.71	1.92			
2	81.13	7.56	94.13	3.21	96.00	2.75			
7	84.50[b]	4.77	99.57[a]	4.52	93.29	2.66			
14	80.13	5.23	89.88	2.84	86.67	3.18			
21	82.14	5.18	90.33	3.72	86.00	1.68			
Neutrophils (%)									
-14	N.D.	--	56.53	4.16	52.53	4.16	0.872	<0.01	<0.01
-7	47.14	3.85	50.81	3.60	47.79	3.85			
0	63.87	3.85	67.39	3.60	58.60	4.55			
2	57.39	3.60	56.26	3.60	60.96	3.85			
7	64.41[a]	3.60	56.73[b]	3.60	57.74	3.85			
14	62.74	3.60	59.18	3.60	58.44	3.85			
21	60.55	3.60	57.61	3.85	55.63	3.85			
Monocytes (%)									
-14	N.D.	--	5.58	1.63	3.83	1.63	0.05	<0.01	<0.01
-7	7.91	1.51	6.31	1.42	5.26	1.51			
0	5.56	1.51	4.31	1.42	7.52	1.79			
2	9.66[a]	1.42	6.66	1.42	5.01[b]	1.51			
7	3.11	1.42	5.20	1.42	5.43	1.51			
14	4.60	1.42	4.00	1.42	6.70	1.51			
21	3.10	1.42	3.04	1.51	4.77	1.51			
Lymphocytes (%)									
-14	N.D.	--	37.62	4.09	42.60	4.09	0.88	<0.01	<0.01
-7	43.89	3.79	41.04	3.54	46.34	3.79			

0	30.47	3.79	27.89	3.54	33.06	4.48
2	32.53	3.54	36.14	3.54	33.19	3.79
7	31.85[b]	3.54	37.25[a]	3.54	36.19	3.79
14	31.78	3.54	35.56	3.54	34.09	3.79
21	35.05	3.54	37.77	3.79	38.30	3.79

[a,b]Means within each row with different superscripts are significantly different ($P < 0.05$).

[1]Trt, treatment effect

Table 8S
Haemoglobin (HGB) and blood leukocytes differential cell counts of a subsample (n=12) of dairy goats fed either a basal diet (C) or diets supplemented with fish oil (FO) or stearate (ST).

Time	Treatment						*P*[1]		
	C (n=4)	SE	FO (n=4)	SE	ST (n=4)	SE	Trt	Time	Trt*Time
HGB (g/L)									
-14	95.91	7.72	94.75	5.02	92.25	1.29	0.304	<0.01	<0.01
-7	88.00	2.98	99.00	6.85	90.25	1.52			
0	86.01	6.35	93.25	4.87	89.50	1.64			
2	85.75	3.90	96.50	5.03	95.00	4.46			
7	83.75[b]	4.56	100.59[a]	5.31	93.00	3.37			
14	82.25	5.21	93.75	4.20	89.25	3.90			
21	84.42	3.53	92.75	4.22	86.50	2.49			
Neutrophils (%)									
-14	N.D.	--	61.00	6.83	54.88	4.83	0.582	<0.01	<0.01
-7	45.55	4.83	49.10	4.83	46.45	4.83			
0	66.50	4.83	61.78	4.83	57.70	4.83			
2	55.20	4.83	55.18	4.83	59.33	4.83			
7	63.90[a]	4.83	56.85[b]	4.83	50.35[b]	4.83			
14	66.80[a]	4.83	52.95[b]	4.83	57.55	4.83			
21	60.40[a]	4.83	50.03[b]	5.58	49.27[b]	4.83			
Monocytes (%)									
-14	N.D.	--	5.90	2.14	3.18	1.51	0.160	<0.01	<0.01
-7	4.15	1.51	4.65	1.51	4.98	1.51			
0	4.75	1.51	5.50	1.51	6.48	1.51			
2	11.35[a]	1.51	5.60[b]	1.51	4.98[b]	1.51			
7	3.85	1.51	3.88[b]	1.51	6.73[a]	1.51			
14	3.28	1.51	3.43	1.51	6.25	1.51			
21	2.65[b]	1.51	3.60	1.74	5.50 [a]	1.51			
Lymphocytes (%)									
-14	N.D.	--	32.60	6.64	40.80	4.69	0.302	<0.01	<0.01
-7	49.38	4.69	44.65	4.69	47.78	4.69			

0	28.58	4.69	32.05	4.69	34.95	4.69
2	33.25	4.69	38.25	4.69	34.88	4.69
7	31.63[c]	4.69	38.50[b]	4.69	42.25[a]	4.69
14	29.35[b]	4.69	42.03[a]	4.69	35.48	4.69
21	35.93[b]	4.69	44.40[a]	5.42	43.90	4.69

[a,b,c]Means within each row with different superscripts are significantly different ($P < 0.05$).

[1]Trt, treatment effect