Annals of Anatomy 247 (2023) 152069

Contents lists available at ScienceDirect

Annals of Anatomy

journal homepage: www.elsevier.com/locate/aanat

SPECIAL ISSUE RESEARCH ARTICLE

Leptin system is not affected by different diets in the abomasum of the sheep reared in semi-natural pastures of the Central Apennines☆

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ARTICLE INFO

Article history:
Received 7 November 2022
Received in revised form 20 January 2023
Accepted 24 January 2023
Available online 6 February 2023

Keywords:
Leptin
Leptin receptor
Adipokines
Digestive system
Ovine
Animal welfare

ABSTRACT

The growing summer drought stress is affecting the nutritional value of pastures, no longer sufficient to support the nutritional status of sheep in extensive rearing. Adipokines affect organ and tissue functionality can be useful to evaluate animal welfare and prompt an improvement in the management of the grazing animals. Leptin (Lep) is an adipokine mainly produced by adipose tissue that regulates food intake by an anorexigenic action. Lep has also been detected in the human and rat gastrointestinal tract, where it regulates the rate of gastric emptying. In this study, Lep system was evaluated in the abomasum of 15 adult sheep reared on Apennine pastures and subjected to different diets. Until the maximum pasture flowering (MxF group), the sheep fed on fresh forage; from that moment until the maximum pasture dryness (MxD group), the experimental group (Exp group) received a feed supplementation in addition to MxD group feeding. The Lep system was investigated in the abomasum samples by immunohistochemistry (IHC) and RT-qPCR. Double-label localisation of Lep and leptin receptor (LepR) with neuroendocrine hormones was conducted to distinguish the gland cell types. The analysis performed revealed the presence of Lep and LepR in the chief and neuroendocrine cells of the fundic glands of the abomasum. RT-qPCR evidenced the transcript for Lep and LepR also identifying the long isoform (LepRb). No significant differences were observed among the three groups of sheep subjected to different diets. The abundant immunostaining observed in the fundic glands suggests that the Lep intervenes in the regulation of abomasum in sheep with a similar pattern to monogastric species while long term food supplementation seems do not influence the local function of the Lep system. A better understanding of the gastrointestinal system can contribute to improving sheep management and optimising the sustainability of livestock production.

1. Introduction

The adipose tissue stores energy in the body in addition to a mechanical protective function. Adipocytes also perform a wide range of metabolic functions through the production of adipokines, making the adipose tissue a real endocrine organ (Wozniak et al., 2009). The adipokines are generally defined as biologically active molecules with hormonal action, which are secreted by adipose tissue, above all by the white adipose tissue (Trayhurn et al., 2006). A molecule belonging to the adipokine group is leptin (Lep) discovered in 1994 by Zhang et al. (Zhang et al., 1994; Trayhurn et al., 2006). Lep is a 167 amino acid protein encoded by the gene obese (Ob), and it

☆ This paper belongs to the special issue New veterinary anatomy
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https://doi.org/10.1016/j.aanat.2023.152069

acts by its receptor LepR, which together constitute the Lep system. Etymologically, the name leptin is derived from Greek leptos and means lean, thin, indeed, the most important factor regulating the secretion of Lep is body weight (Goumenou et al., 2003). Serving as a lipostatic factor, as lipid reserves increase, the white fat cells stimulate Lep synthesis, which signals to the hypothalamus that food intake must be reduced and stimulates energy consumption, by enhancing the release of anorexigenic factors like cocaine- and amphetamine-regulated transcript (CART) and pro-opiomelanocortin (POMC) and at the same time inhibiting the orexigenic ones, neuropeptide-Y (NPY), agouti-related protein (AgRP), and orexin (Cammisotto and Bendayan, 2012). Therefore, this molecule regulates energy metabolism and food intake by systemic action (Radin et al., 2009; Cammisotto and Bendayan, 2012), specifically by an anorexigenic action. The binding between the Lep and the LepR, in the hypothalamic satiety centers triggers the anorexigenic pathway by the activation of the JAK-STAT Pathway (Radin et al., 2009): NPY-secreting neurons in the arcuate nucleus are inhibited and the release of α-melanocyte stimulating hormone is promoted thus suppressing the appetite. LepR comprises six different isoforms (LepRa-f) sharing an identical extracellular ligand binding domain (Gorska et al., 2010). The long isoform, LepRb, allows cell signal transduction mediating most of the physiologic Lep function (Kim and Kim, 2021). LepRe is a soluble isoform representing a carrier of leptin protein capable of modulating its bioavailability (Schaab and Kratzsch, 2015). The short isoforms are mainly involved in the transport and degradation of leptin in tissues (Gorska et al., 2010).

Over time, it has been discovered that adipose tissue is not the only producer of Lep, as it has been detected in other organs like the mammary glands, placenta, ovary, pancreas, skin and stomach (Bado et al., 1998; Chilliard et al., 2005; Dall'Aglio et al., 2013; Mercati et al., 2019a; Cappai et al., 2022). The stomach represents the major source of Lep in the gastrointestinal tract (Yarandi et al., 2011) and it has been demonstrated the active role of Lep in the gastric functionality of humans and laboratory animals (Bado et al., 1998; Cinti et al., 2000). It seems to stimulate gastroprotection since it enhances mucosal integrity by increasing mucosal blood flow. The Lep in this case exerts its action after the injection of CCK-8, thus like a final factor. At the systemic level, likewise in synergy with CCK-8, the gastric Lep can take place in the short-term control of satiety, via vagal afferent fibres in the rat (Sobhani et al., 2000). Leptin may have further roles in nutrient absorption since it has effects on gastric acid (Mix et al., 2000) and gastric hormones secretion (Cammisotto et al., 2005). In addition, it has been shown that Lep regulates gastric motility since a Lep deficiency increases the rate of stomach emptying in mice (Yarandi et al., 2011). The abomasum represents the glandular stomach of ruminants and performs digestive functions like the single-chambered stomach of monogastric animals: the production of gastric juices allows the abomasum to digest dietary proteins as well as the microorganisms deriving from prestomacal compartments such as rumen. For grazing ruminants, or "grass and roughage eaters", to which the ovine species belongs, abomasum is also of fundamental importance for the denaturation of hemicelluloses by hydrochloric acid. Their diet is based mainly on pabular species that are moderately rich in hemicellulose and the denaturation of hemicelluloses allows their following digestion in the distal fermentation chamber, represented by the caecum and the proximal loop of the colon (Aguggini et al., 1998). Therefore, the full functionality of the abomasum is very important for the digestive efficiency of ruminants.

Over the last past years, much knowledge on gastric leptin has been acquired thanks to numerous studies carried out in humans and rats, leading to the definition of a regulatory role of this molecule in the control of the digestive tract and modulation of food intake. Sheep studies were not performed considering the transfer of information between species as valid. However, ruminants have quite peculiar anatomical and physiological characteristics due to the presence of a complex gastric system characterized by forestomaches and ruminal activity for which it is of particular importance to directly analyse them and acquire original data.

Given the importance of Lep in regulating the functions of the gastrointestinal tract, we propose a study carried out in the abomasum of sheep grazing on semi-natural pasture in the Central Italian Apennines. These pastures are affected by the growing summer drought that is reducing their nutritional value, by altering the distribution and quantity of the pabular species present in the grasslands. In particular, summer aridity is anticipating the moment of maximum flowering of the pasture, in which the nutritional value of the pasture is high and shortening the period between the moment of maximum flowering and maximum dryness of the pasture (Catorci et al., 2012; Scocco et al., 2016). The shortening of the period of trophic resources' availability and the consequent reduction of their nutritional value led to a worsening of the animal health status which may not be adequate to support its production capacities. Since Lep is involved in the local regulation of the gastrointestinal tract, we studied this molecule in sheep subjected to different diets to highlight any variations that could affect animal welfare as well as to deepen the knowledge of the gastroenteric tract functions in ruminants. These insights can contribute to improve sheep management and optimize the sustainability of livestock production.

2. Material and methods

2.1. Animal recruiting and sample collection

Over three months a flock of 15 Comisana x Appenninica adult female sheep were used to perform the study (Mercati et al., 2018). The sheep were free to graze on the pasture feeding on fresh forage from June until the maximum pasture flowering (MxF), that is early July. After this period, five subjects were slaughtered (MxF group). Then, for the entire period between the MxF and the maximum pasture dryness (MxD) that is early September, sheep were divided into two homogeneous groups (5 subjects for each group) based on age, reproductive performance, and body condition score (BCS), as previously described (Mercati et al., 2018). During this period, the MxD group were grazing on pasture and feeding only on fresh forage, while the other one, labelled as the experimental group (Exp group), also received a feed supplementation of 600 g/day/head of barley and corn (1:1). The food supplementation was administered to all the animals of the Exp group at the same time, before and after the entire duration of their stay in the pasture.

To carry out the analysis, samples about 1 cm^2 wide were collected from the fundus of the abomasum, which hosts the fundic glands (Palmioli et al., 2021), and were quickly fixed as described below.

The animals, intended for human consumption, were slaughtered at the abattoir according to the Art. 29 of the Council Regulation (EC) No. 1099/2009 on the protection of animals at the time of killing under law n.333/98 (Council Directive 93/119/EC of 22 December 1993) as specified by Annex C of Section II. The experimental procedures were approved by the Ministry of Health (no. of approval 95/2018-PR).

2.2. Morphological staining and immunohistochemistry

Samples were immersed in 10% formaldehyde solution in phosphate-buffered saline (0.1 M PBS, pH 7.4) for 36 h for fixation. Fixed samples were dehydrated with a graded ethanol series, cleared in xylene, embedded in paraffin wax and cut into 5-μm thick serial sections as previously described (Palmioli et al., 2021). All specimens were stained with the hematoxylin-eosin solution to carry out a morphological evaluation and to exclude pathologies. The

Table 1
Source and working dilutions of the antisera used.

Antisera	Host	Sources	Dilution
Monoclonal anti - Lep	Mouse	10R-7769, Fitzgerald I.I, MA, USA	IHC 1:100 IF 1:100
Polyclonal anti - LepR	Rabbit	254537, Abbiotec Inc., CA, USA	IHC 1:150 IF 1:300
Polyclonal anti - CgA	Rabbit	ab45179, Abcam, Cambridge, UK	1:600
Monoclonal anti - Syp	Mouse	M7315, Dako, Denmark	1:400
Anti-mouse Biotin conjugated	Horse	BA-2000–1.5, Vector Laboratories, CA, USA	1:200
Anti-rabbit Biotin conjugated	Goat	BA-1000, Vector Laboratories, CA, USA	1:200
Anti-mouse IgG DyLight 550	Goat	Ab97016, Abcam Cambridge, UK	1:1000
Anti-rabbit IgG Alexa Fluor 488	Goat	A-11008, Invitrogen, Thermo Fisher Scientific	1:1000
Mouse IgG	Mouse	I-2000–1, Vector Laboratories, CA, USA	1:100
Rabbit IgG	Rabbit	I-1000–5 Vector Laboratories, CA, USA	1:150

Lep= Leptin; LepR= Leptin Receptors; CgA: chromogranin; Syp: synaptophysin.

immunohistochemical procedure was performed on all samples as described below (Dall'Aglio et al., 2021): the sections were dewaxed in xylene and hydrated through a series of ethanol concentrations until distilled water. Sections were microwaved for three 5 min cycles at 750 W in citrate buffer (pH 6.0) to expose the epitopes to the antibodies. The endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution for 10 min and incubation with both normal horse serum and normal goat serum (Table 1) for 30 min was performed to avoid non-specific bindings. For the immunohistochemical reaction, sections were incubated overnight at room temperature (RT) with a mouse monoclonal anti-Lep antibody and a rabbit polyclonal anti-LepR antibody (Table 1). The dilution used for each primary antibody was the best to obtain the intensity of the signal without background. On the second day, sections were incubated with a horse anti-mouse biotin-conjugated antibody and a goat anti-rabbit biotin-conjugated secondary antibody (Table 1) for 30 min. The avidin-biotin complex solution was used to detect the immunological binding sites, which were revealed with DAB chromogen (DAB substrate kit, Vector Laboratories, Burlingame, CA, USA). Sections were also counterstained with hematoxylin.

Sections were washed with phosphate-buffered saline (PBS) between every incubation step, except after normal serum. Every step was performed at RT and the slides were incubated in a humid chamber.

Negative control sections were incubated with mouse or rabbit IgG (Table 1), omitting the primary antibody. Queen ovarian sections were used as positive control of the reaction (Cappai et al., 2022). A photomicroscope (Nikon Eclipse E800, Nikon Corp., Tokyo, Japan) connected to a digital camera (Nikon Dxm 1200 digital camera) was used to observe all sections.

The intensity of the staining for Lep and LepR was graded in arbitrary units as previously described (Maranesi et al., 2020). Three independent observers performed staining evaluation; each observer evaluated three randomly chosen fields into each slide, establishing the mean intensity values.

2.3. Double-label immunohistochemistry

Double-label localizations of Lep and LepR with different neuroendocrine hormones, such as chromogranin (CgA) and synaptophysin (Syp), were performed in the abomasum in accordance with a previously described method (Palmioli et al., 2021), to distinguish the gland cell type.

For the immunofluorescence double-localization of Lep with CgA, cryostat cut sections of the abomasum were used. Samples of abomasum fixed in 4% paraformaldehyde solution in PBS were washed in 0.1 M PBS and cryoprotected for 3–4 days in graded solutions (10%, 20% and 30%) of sucrose in phosphate buffer at 4 °C until thoroughly infiltrated. Then, abomasum pieces were embedded in the cryostat embedding medium (Killik O.C.T, Bio-Optica Milano Spa, Italy) and cut in a cryostat at −25 °C into 10 µm thick sections. After rinsing the sections with PBS, they were incubated with a blocking buffer in a humid chamber for one hour. Subsequently, the slides were incubated overnight with a mouse monoclonal anti-Lep antibody and a rabbit Anti-CgA antibody (Table 1) in a dark humid chamber. The next day, they were incubated with DyLight 550 goat anti-mouse and Alexa Fluor 488 goat anti-rabbit secondary antibodies (Table 1) for one hour in a humid chamber. All the procedure was performed at RT. The sections were rinsed with PBS after every incubation step and, finally, they were air-dried for 15 min and covered with histology mounting medium (Fluoroshield). Negative control sections were incubated with mouse or rabbit IgG (Table 1), omitting the primary antibodies. Sections were observed with a fluorescence microscope (Olympus BX51 Fluorescence Microscope). Images were acquired with Nikon DS-Qi2 microscope digital camera and NIS-Elements D software (Nikon Europe B.V.).

Immunofluorescent double-label localizations of LepR with Syp were performed on samples of abomasum fixed in 4% paraformaldehyde solution in PBS. Samples were cut into 50 µm thick sections with a vibratome and washed with PBS for three 5 min cycles. Then, after the incubation with normal goat serum for 90 min, the slices were incubated overnight with a rabbit polyclonal anti-LepR antibody and with a mouse anti-Syp antibody (Table 1). The next day, the sections were incubated with normal goat serum for 30 min and reacted with Alexa Fluor 488 goat anti-rabbit and DyLight 550 goat anti-mouse secondary antibodies (Table 1) for 90 min. Finally, the slices were counterstained with DAPI solution (Invitrogen, Thermo Fisher Scientific). The sections were washed with PBS for five 5 min cycles after each incubation step, except after serum incubation and all the procedure was performed at RT. Negative control sections were incubated with mouse or rabbit IgG (Table 1), omitting the primary antibodies. The sections were observed with a scanning laser confocal microscope (Nikon develops enhanced C2 plus confocal microscope). Images were acquired with NIS-Elements software (Nikon Europe B.V.).

2.4. RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

The total RNA was extracted from the abomasum tissue of five sheep for each experimental group as previously described (Maranesi et al., 2010).

First-strand cDNA synthesis was prepared in presence of 1,5 µg of DNase-treated total RNA by using QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany) in a total volume of 20 µl in the presence of a mixture of both random hexamers and oligo(dT) primers following the manufacturer's protocol. Reverse transcription was performed at 42 °C for 20 min. The mRNA quantification of target genes leptin (Lep), all forms of the leptin receptor (LepR), and the functional long form of the leptin receptor (LepRb) was

Table 2
Primer sets used in the qPCR assays.

Gene	Primer Sequence (5′ to 3′)	Product size (pb)	Efficiency (%)
Lep	F: ATCTCACACACGCAGTCCGT R: CCAGCAGGTGGAGAAGGTC	202	100
LepR (all forms)	F: TGGCCTAGGAATCTGGAGTG R: CCGCTGTCAGAATTTTAGGTG	110	101
LepRb	F: CAACCATTCTCCACCAAAGG R: GTTCATCCAGGCCTTCTGAG	134	100
GAPDH	F: GTTTGTGATGGGCGTGAACC R: GCGTGGACAGTGGTCATAAGT	150	102
YWHAZ	F: TAAAAACGAGCTGGTACAGAAGGC R: TCTCCTCATTGGATAATTCAGCTCC	116	102

Lep = Leptin; LepR = Leptin Receptors (all forms); LepRb = long form of leptin receptor; GAPDH = glyceraldehyde3-phosphate dehydrogenase; YWHAZ = tyrosine 3-mono-oxygenase; F = Forward; R = Reverse; bp=base pairs.

determined by quantitative PCR using the gene-specific primer sets listed in Table 2. All primer couples were designed by using Primer3 Plus software (https://www.primer3plus.com) except for the Lep primers published by Muhlhausler et al. (2007). Gradient PCR analyses were then performed in order to obtain the optimum temperatures (Tm) for each primer pair. The qPCR assays were carried out in a LightCycler® 96 Instrument (Roche Applied Science, Basel, CH) using the FastStart Essential DNA Green Master (Roche Applied Science, Basel, CH). Each sample was normalized to the geometric mean of two reference genes, glyceraldehyde3-phosphate dehydrogenase (GAPDH) and tyrosine 3-monooxygenase (YWHAZ) used as endogenous controls for the abomasum according to Barbosa Toscano et al. (2018). The amplifications were performed in duplicate in a 20 µl reaction volume containing 1,5 µl of diluted cDNA for LepR and 1,5 µl of undiluted cDNA template, 1 × FastStart Essential DNA Green Master mix, and 0,25 µM of each primer according to the manufacturer's instructions. The PCR program consisted of incubation at 95 °C for 10 min, followed by 40 cycles of a classical three-step amplification (denaturation, 95 °C for 10 s; annealing, 60 °C for 20 s and extension, 72 °C for 10 s) and a final melting phase structured in three steps (10 s at 95 °C, 30 s at 60 °C, 1 s a 97 °C). At the end of each PCR amplification, a melting curve analysis was performed to verify the specificity and define the Tm of each amplicon. Negative control reactions (no-template control) were included in each primer assay to ensure the absence of contamination. PCR efficiency was checked to be at near 100% for each pair of primers. After each run, real-time PCR data were analyzed with LightCycler® 96 Instrument Software 1.0. The relative expression levels of target genes were calculated as $2^{-\Delta Ct,}$ where ΔCt was obtained directly by subtracting Ct for the target gene from Ct for both GAPDH and YWHAZ mRNA. The final result was expressed as $2^{-\Delta Ct} \times 1000$.

2.5. Statistical analysis

The data are presented as mean ± standard deviation (SD). Statistical comparison among the groups of sheep was calculated using one-way analysis of variance (ANOVA) with a F (p-value) of 0.05 used for statistical significance. Data were first checked for heteroscadasticity using Levene's test.

3. Results

3.1. Immunohistochemical analysis

The leptinergic system was evidenced in the mucosal layer of the abomasum of all sheep analysed by means of the IHC technique. Lep and LepR showed overlapping localization. Positivity was observed in the lower half of the fundic glands (Figs. 1 and 2). Immunohistochemical staining was localized in the cytoplasm of the chief cells (Figs. 1 and 2) and it was mainly confined in the cell supranuclear region as regards Lep molecule (Fig. 1). Parietal cells and neck cells were negative. The double-localization performed with immunofluorescence between Lep and CgA revealed that most of the neuroendocrine cells were not positive to Lep and only a few of them colocalized. The colocalizing cells were more abundant in the basal region of the glands (Fig. 3) and mainly localized outside the glands wall, in the lamina propria. Differently, a wider colocalization between the LepR and Syp was observed so most of the Syp positive neuroendocrine cells were also LepR positive. Colocalizing cells were localized on the glands wall (Fig. 4). No staining was observed in the control sections of all tests where the primary antibodies were omitted (data not shown).

No differences in staining intensity were observed between the three groups of sheep for both Lep and LepR.

3.2. RT-qPCR analysis

qRT-PCR assays revealed the presence of Lep-LepR system transcripts in sheep abomasum. The detection of LepRb receptor, obtained by using specific primers, demonstrated that this isoform is naturally expressed in this tissue. In all sheep, the relative expression analysis evidenced that LepRb mRNA levels were lower than transcript levels obtained with LepR primers for all isoforms, suggesting that at least another receptor form is expressed in mucosa cells (data not shown). The relative expression profile of Lep, LepR and LepRb mRNAs in the abomasum of three sheep groups subjected to different feeding regimes was shown in Fig. 5. The results revealed that were no statistically significant differences of expression among groups ($P > 0.05$). However, a trend towards increased Lep, Lep-R and LepRb expression can be observed in the Exp group compared to MxD group.

4. Discussion

This study describes the localization and expression of the leptinergic system in the abomasum of sheep reared in a semi-natural pasture and fed with different diets.

Lep system was evidenced in the mucosal layer of the abomasum. Both Lep and LepR were localized in the lower half of the fundic glands showing an overlapping pattern according to previous study performed in humans and rats (Bado et al., 1998; Cinti et al., 2000; Cammisotto et al., 2006). A recent paper by Karakoç et al. (2022), describes Lep and LepR in the abomasum of bull and ram, however the authors did not point out the localization of the molecules in either the abomasum or the gland regions. In addition, results obtained in ram are different from ours since Karakoç et al. (2022), identified both molecules in parietal cells while we observed positivity in chief cells and neuroendocrine cells according to humans and rats (Bado et al., 1998; Cinti et al., 2000; Cammisotto et al., 2005, 2006).

In sheep abomasum, gland cells positive to Lep and LepR were labelled as chief cells based on their morphological characteristics and position (Goldenring et al., 2011) while parietal cells, the largest cells of fundic glands with a rounded appearance and bulging out along the wall (Chew, 2004) were negative.

Lep staining was observed in the cytoplasm of the chief cells clearly confined in the cell supranuclear region suggesting the secretion of the molecule in the gland lumen. Cammisotto et al. (2006) detected Lep in the gastric juice and demonstrated the resistance of this molecule to proteolytic conditions of the gastric juice and duodenal fluid. Besides, in rats, food intake causes a rapid release of Lep protein into the gastric lumen emptying the cytoplasmic stores (Bado et al., 1998; Picó et al., 2002). The intense immunohistochemical staining observed in the abomasum samples is consistent with a fasting state of the sheep. The sensitivity of this

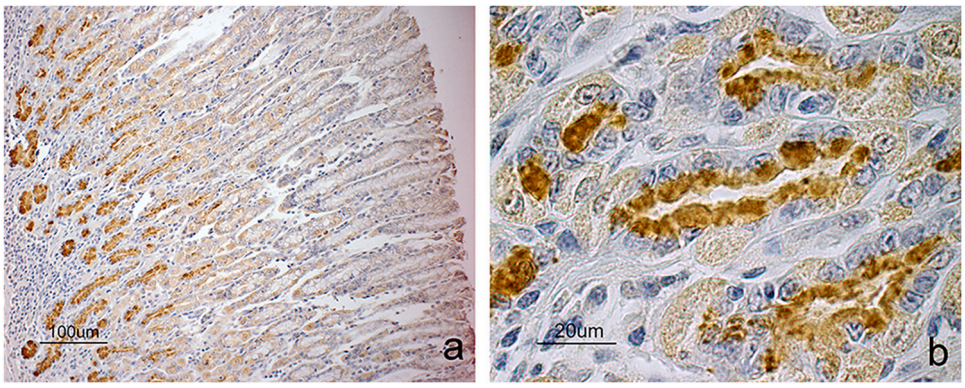

Fig. 1. Immunostaining for Lep in the mucosal layer of the abomasum. Positivity is mainly localized in the lower half of the fundic glands (a). The cytoplasmic staining is observed in the supranuclear region of the chief cells (b).

hormone to the food intake and fasting suggests a role in the short-term regulation of feeding (Picó et al., 2003).

While the morphological differences between chief and parietal cells are plain (Goldenring et al., 2011; Chew, 2004), endocrine cells scattered in the glandular epithelium and lamina propria are not easy to identify. Therefore, a double-localization with endocrine cell markers to identify Lep and LepR positive cells in more detail was performed. CgA and Syp were used to identify endocrine cells since these molecules are considered two major markers of enterochromaffin-like (ECL) cells that represent the predominant endocrine cell type of the oxyntic mucosa (Wiedenmann and Huttner, 1989; Norlén et al., 2001; Tomita, 2020).

Immunofluorescence double-localization performed with Lep and CgA revealed the presence of a very low number of neuroendocrine cells secreting Lep; these cells were mainly located outside the gland wall, in the lamina propria, while the most of CgA neuroendocrine cells did not colocalize with Lep. Previous studies identified few Lep positive endocrine cells along with several Lep negative endocrine cells in the gastric glands of humans (Cinti et al., 2000) and rats (Cammisotto et al., 2005) with a localization pattern similar to that observed in sheep. Hence, Lep produced in the sheep abomasum is secreted in the lumen by chief cells and in blood vessels by endocrine ones making a coordinated control system on the digestive tract (Cammisotto et al., 2005). Double localization preformed with LepR and Syp showed that most of the Syp positive neuroendocrine cells were also LepR positive. Cammisotto et al. (2006) previously identified LepR in the endocrine cells of rat fundic glands. We observed that neuroendocrine cells were mainly localized on the wall of the gland and not outside it shows a different pattern than the Lep-positive neuroendocrine cells. Furthermore, they were more numerous than neuroendocrine Lep-positive cells. These features are partially due to the presence of heterogeneous endocrine cell populations with different biological roles (Cetin et al., 1989; Norlén et al., 2001) in the mucosa of the abomasum. The high number of neuroendocrine cells expressing the receptor suggests a significant sensitivity of the stomach to circulating Lep that could derive from abomasum endocrine cells. Indeed, plasma Lep increases threefold following food intake in rats (Bado et al., 1998).

This work also demonstrated the presence of Lep and LepR mRNAs in sheep abomasum. Lep transcript was already detected in the fundic epithelium of rats (Bado et al., 1998) and humans (Cinti et al., 2000). Lep mRNA was also identified in bovine species where expression is higher in calves than cow (Hayashi et al., 2020). Some studies evidenced that the expression of this molecule is affected by feeding conditions (Picó et al., 2002, 2003).

Several isoforms of the receptor exist resulting from the alternative splicing of the LepR gene (Tartaglia, 1997) or by proteolytic cleavage of the membrane-bound isoform as soluble receptor isoform regards (Maamra et al., 2001). It is well-known that the long form of the receptor, LepRb, mediates the biological effect of Lep by activating several downstream signaling pathways (Gorska et al., 2010; Kim and Kim, 2021). In the stomach of both human (Mix et al., 2000; Sobhani et al., 2000) and rat (Wang et al., 1996) different receptor isoforms, including the long form, have been identified. In this work, the evidence that in all sheep analyzed there is a greater abundance of LepR (all forms) transcripts compared to LepRb let hypothesize that two or more receptor isoforms are present in abomasum mucosa. The detection of the functional receptor LepRb transcript, in abomasum mucosa cells, suggests that these cells may be a direct target for gastric Lep (Mix et al., 2000; Sobhani et al., 2000) through an autocrine pathway. Schneider et al. (2001) observed that Lep induced an increase in the proliferation of gastric

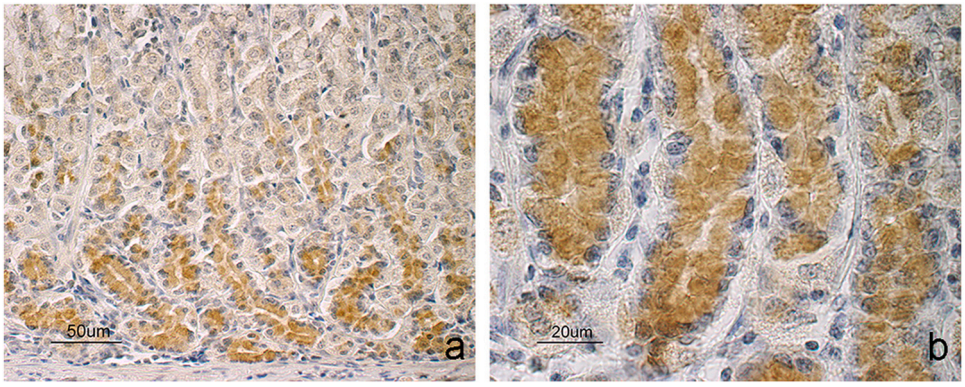

Fig. 2. Immunostaining for LepR in the mucosal layer of the abomasum. Positivity is mainly localized in the lower half of the fundic glands (a). The cytoplasmic staining is observed in the chief cells (b).

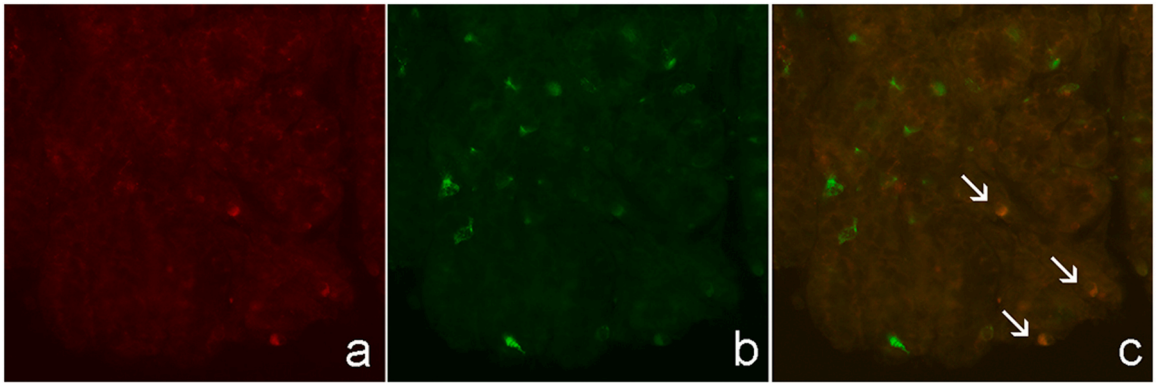

Fig. 3. Immunofluorescence for Lep ((a), red) and CgA ((b), green) in the fundic glands of the abomasum. In the image (c) three cells positive for both Lep and Cga can be observed (arrows) while most of the neuroendocrine cells do not colocalize.

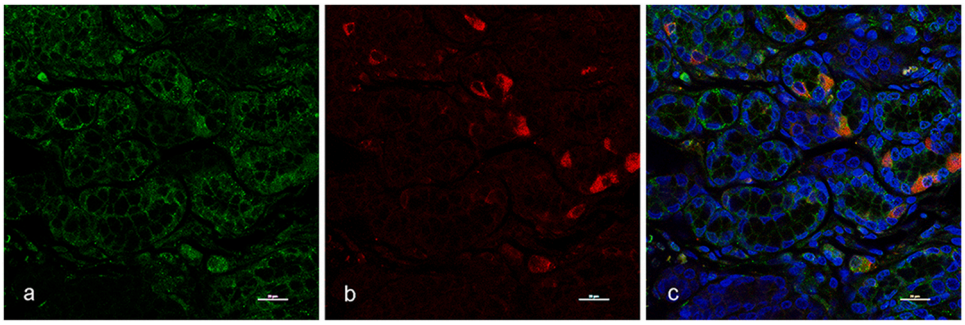

Fig. 4. Immunofluorescence for LepR ((a), green) and Syp ((b), red) in the fundic glands of the abomasum. The merge image (c) shows that the most of neuroendocrine cells are positive to both LepR and Syp.

mucosa cells suggesting that it might contribute to mucosal integrity and gastroprotection. In addition, Cammisotto and Bendayan (2012) demonstrated that the long isoform of the receptor, synthesized by the chief cells, is converted into the soluble isoform, and directly bound to the Lep allowing the protein to resist the acid secretion of the abomasum and reach the duodenum. Currently, a direct action of Lep on the epithelial cells of the stomach is not sufficiently understood (Kim and Kim, 2021).

Since Lep is involved in the local regulation of the gastrointestinal tract, in this study the Lep system was analysed in sheep subjected to different diets in order to highlight variations related to feeding. Through immunohistochemistry, no differences of Lep and LepR proteins were observed in the abomasum of the sheep. MxF sheep group was fed with fresh forage that was rich in protein and water but poor of fibre unlike the forage deriving from maximum dryness pasture eaten by MxD group that contained a high amount of fibre among which the indigestible lignin; finally, Exp group received the maximum dryness pasture supplemented with corn and barley particularly enhancing the protein intake (Mercati et al., 2018). Previous research demonstrated that the Lep blood level did not show variation during the trial (Barbato et al., 2021) thus showing no systemic variation. Overall data obtained show that dietary changes do not affect the Lep system while suggests that it is primarily involved in the regulation of the digestive tract following short-term food intake. As immunohistochemistry, the mRNA expression analysis of the Lep system did not reveal statistically significant differences too. However, a tendency to Lep, LepR and LepRb expression increase was observed in the Exp group fed with the food supplement, compared to MxD group. This trend agrees with the known regulation of gastric Lep mRNA expression in response to

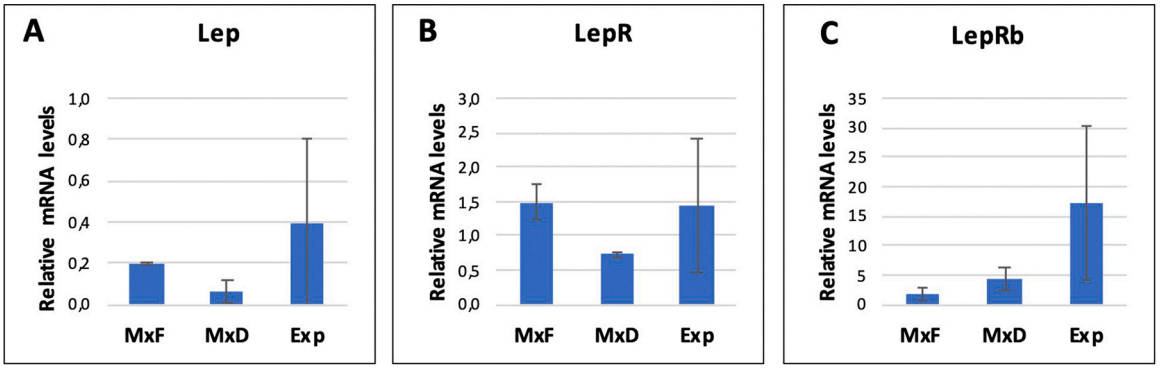

Fig. 5. Relative gene expression of the Lep (A) LepR (B) and LepRb (C) genes in the abomasum of the Maximum Flowering (MxF), Maximum Dryness (MxD) and Experimental (Exp) sheep groups. The results are expressed as a ratio of each target gene relative to both GAPDH and YWHAZ mRNAs used as endogenous controls. The data are presented as mean ± (SD). For each analyzed genes there was no significant variation ($P > 0.05$) in the transcript levels among the treatment groups, as determined by one-way ANOVA.

food intake, according to which a better availability and quality of food triggers upregulation of Lep system (Picó et al., 2002; Sánchez et al., 2004; Lindqvist et al., 2005). The leptinergic system behaviour also resembles the apelin system variations previously observed in the sheep abomasum of the same trial (Palmioli et al., 2021) where a maintaining effect of feed supplementation on the apelinergic system expression in the abomasum has been hypothesized, also on the basis that differences observed for apelin system were statistically significant. Indeed, during the pasture vegetative cycle, the grassland modifications affect the sheep anatomical features at both the macro/microscopical level, as demonstrated by the sheep rumen modifications (Scocco et al., 2012, 2016), and the molecular level, as observed for aquaporin-5 (Scocco et al., 2011), resistin (Dall'Aglio et al., 2019) and apelin (Mercati et al., 2018, 2019b; Palmioli et al., 2021) in different peripherical organs. This last evidence, highlights a sensible role for adipokines in different organs involved in animal productivity, suggesting a possible guise of marker molecules of the animal body state for these molecules.

5. Conclusions

In conclusion, in this study**,** the leptinergic system was evidenced in the fundic mucosa of the sheep abomasum. The molecule and its receptor were evidenced in both chief and different populations of neuroendocrine cells suggesting a coordinated control system on the digestive tract realized by Lep. Results derived from the morphological analysis and the evaluation of the transcripts indicate that in sheep the Lep system plays a role and functions like monogastric animals. Comparison between groups subjected to different diets revealed no statistical differences suggesting that the Lep system is primarily involved in the short-term regulation of feeding. In any case, to study the relationship between diet and adipokines is important for identifying animal welfare markers to provide useful information for improvement in the livestock management of sheep.

Funding

This research was funded by the grant FAR 2014-15 assigned to Prof. Andrea Catorci by the University of Camerino, Italy.

CRediT authorship contribution statement

Palmioli E: methodology, validation, investigation, data curation, visualization, writing—original draft preparation. Dall'Aglio C: methodology, conceptualization, resources, writing—review and editing, project administration. Fagotti A: methodology, validation, formal analysis, investigation, resources, data curation, visualization. Simoncelli F: methodology, validation, investigation, data curation. Dobrzyn K: validation, investigation. Di Rosa I: formal analysis, resources. Maranesi M.: investigation; De Felice E: writing—review and editing. Scocco P: conceptualization, resources, writing—review and editing, project administration. Mercati F: conceptualization, methodology, validation, resources, data curation, visualization, writing—original draft.

Declaration of Competing Interest

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

Acknowledgments

The authors wish to thank prof. Michele Bellesi and Dr. Alessia Cappelli for their support in setting up the immunofluorescence double-localization technique. They are also grateful to Dr. Paola Coliolo for her technical assistance.

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