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Papillary Architecture and Functional Characterization of Mucosubstances in the Sheep Tongue

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ABSTRACT

This research aimed to reveal the general morphology and topographic distribution of lingual papillae, epithelial characteristics, mucosal structure, and glands with their mucin content in the sheep tongue, with consideration of species-specific characteristics. The tongues of ten sheep were analyzed for this purpose. Filiform and fungiform papillae existed within the borders of the ventral surface of the lingual apex. The majority of the filiform papillae had multiple secondary projections. Fungiform papillae were also seen on the lingual torus among lenticular papillae, as well as 6 to 10 circumvallate papillae arranged on its caudal border. The species-specific details of the general anatomical structure of the tongue were determined and, in general, the papillary organization in the sheep was similar to goats, while the papillary organization also was similar to features with deer species, specifically the filiform papilla from the mechanical papillae and fungiform papilla from the gustatory papillae. Neutral and weak sulfated mucins and *N*-acetyl sialomucins were located in seromucous glands, salivary duct epithelium and von Ebner's glands. Carboxylated acid mucins and *N*-acetyl sialomucins were not present in seromucous and von Ebner's glands. In seromucous glands, MUC1, MUC5AC, and MUC6 localized only in epithelial cells of ducts, whereas MUC2 localized in both glandular and ductal epithelial cells. All MUCs were present in both von Ebner's glands and salivary ducts. We showed that this mucin composition, may serve as a physical barrier in the initial section of the digestive system. Anat Rec, 301:1320–1335, 2018. © 2018 Wiley Periodicals, Inc.

Key words: morphology; lingual papillae; glands; mucins; ruminant

INTRODUCTION

Feeding style and diet are an important factors in determining the success of vertebrates' adaptation to the environment (Roth and Wake, 1989). The tongue, together with other organs in the oral cavity, plays a vital role in feeding. In all mammal species, structural differences in the tongue reflect differences in food sources and the specific habitat of each species. That is why morphological and histological features of the tongue in mammals are indicative of differences among lifestyles of mammals (Iwasaki, 2002).

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In vertebrates, the tongue's mucosa consists of various papillary systems that perform gustatorial and mechanical functions, and the tongue is covered by a multi-layered keratinized epithelium (Tadjalli and Pazhoomand, 2004; Kurtul and Atalgın, 2008). A major part of the tongue is covered with various papillae that occur in conjunction with local modifications of the mucosa and becoming dense predominantly on the dorsal surface, which performs mechanical or gustatorial functions. The scattering, volume, number, and shapes of papillae vary in each species (König and Liebich, 2014).

The mucosa of the oral cavity is moistened by major and minor salivary glands (Amano et al., 2012). Salivary glands are well developed in mammals, but major salivary glands, a principal organ for salivation, are separated from the tongue (Kubota et al., 1963). Minor salivary glands are located in specific regions beneath the epithelium within the root and body of the tongue. They are located within the connective tissue of the tongue, which is rich in elastic and collagen fibers located just below the dorsal and ventral surfaces (Iwasaki, 2002). Two types of glands are present in the tongue in all mammalian species, including sheep. These glands consist of tubuloalveolar minor salivary glands with seromucous secretion and gustatorial glands known as "von Ebner's" glands (Gargiulo et al., 1995a,b). Von Ebner's glands, which are located beneath the circular groove around circumvallate papillae, produce a seromucous secretion to wash the taste buds located in the side facing groove (Agungpriyono et al., 1995). In this way, taste buds of papillae can detect new gustatory stimuli. This correlation between taste buds and minor salivary glands is also present at foliate papillae, and is peculiar to mammals (Kubota, 1966; Baratz and Farbman, 1975).

One of the main functions of lingual glands is to produce saliva, playing an important role in moistening and lubricating foods. This function of the saliva is performed by mucins (Erdoğan et al., 2012; Sağsöz et al., 2012). Mucins are histochemically divided into two groups as neutral and acid mucins. Neutral mucins do not contain reactive acid radicals, but they have free hexose groups. Acid mucins are divided into two groups: sulphated (sulfomucin) mucins and carboxylated mucins (sialomucin). While sulphated acid mucins contain sulphated glucuronic acid, carboxylated acid mucins contain sialic acid molecules (Schumacher et al., 2004; Sağsöz and Liman, 2009; Sağsöz et al., 2012). In a molecular context, all mucins contain a central section that has a large number of oligosaccharide chains. This central section, rich in serine and threonine, consists of tandem repetitions. Serine and threonine sections serve as binding sites for oligosaccharide chains. The number of repetitions and amino acid sequences in each iteration depends on mucin genes (Gendler and Spicer, 1995). Currently, a total of 21 mucin genes, called as MUC1, 2, 3A, 3B, 4, 5AC, 5B, 6–9, 11–13, and 15–20, have been identified through "cDNA-cloning" (Porchet et al., 1999; Rose and Voynow, 2006).

Within this context, our purpose was to reveal the general morphology and topographic distribution of lingual papillae, epithelial characteristics, mucosal structure, glands, and secretion characteristics (mucin contents) in the sheep tongue, with consideration of species-specific characteristics and similarities and differences with other ruminant species. Most current studies within this context have performed simple electron microscopic examinations on mammals with different feeding characteristics. In the current study, however, we characterized specific features of all lingual papillae and their associated features by using a variety of microscopic techniques. A comprehensive review of the scientific literature shows that the focus of most studies in mammals has been to identify the morphological structure of the tongue and microscopic lingual glands (Nagato et al., 1995; Hand et al., 1999). We primarily described the morphological differences that exist between the tongue and lingual glands of sheep and other mammal species, and the basic histological features of lingual glands of sheep. In addition, we aimed in the present study to reveal the composition and physiological functions of mucins secreted by epithelial cells of the lingual glands of sheep, and whether or not any differences exist between sheep and other mammalian species. For this purpose, we supported the morphological results with conventional mucin histochemistry and immunohistochemistry techniques. Since histochemistry is an excellent method to identify the characterization of glycoconjugates (Schumacher et al., 2004; Sağsöz and Liman, 2009; Erdoğan et al., 2012; Sağsöz et al., 2013; Erdoğan et al., 2015), we demonstrated changes in carbohydrate lateral chains of secretions of epithelial cells of the lingual glands. We also evaluated the expression of proteins of MUC1, MUC2, MUC5AC, and MUC6 in epithelial cells of the lingual glands and compared them to the molecular profiles of the mucins.

MATERIALS AND METHODS

Collection of Samples and Fixation

In this study, with the aim of revealing the structure, distribution, content, and anatomical characteristics of the lingual papillae, as well as tongue epithelium and its comprising glands, we analyzed ten tongues from male and female 24-month old sheep raised on the same food, during usual slaughtering in private slaughterhouses serving in Tekirdağ province. The sheep were fed with concentrated feed (85%) and forage (15%). Firstly, each tongue was divided into four large sections as a lingual apex, body, torus, and radix. Then, two identical tissue samples ($5 \times 5 \times 5$ mm in size) were taken from each apex, body, torus, and radix sections of the each tongue, respectively. One sample was used for scanning electron microscopy and other sample was used for all light microscopic examinations. Tissue samples included mucosal, submucosal, and muscular layers to investigate all layers and structures of the tongue. Samples taken for scanning electron microscopy were fixed and maintained in 2.5% glutaraldehyde solution, while samples taken for histological examination were fixed for 18 h in 10% formol–alcohol solution.

Light Microscopy

The fixed tissues were dehydrated by a graded alcohol series (96%, Absolute 1, −2 and −3), and then cleared by a methyl benzoate and benzene series, respectively. Serial sections, 5 μm in thickness, and at 100 μm intervals, were prepared from the paraffin blocks. Eleven slides were prepared from each paraffin block, and each slide contained at least three sections for each tongue part. To identify the overall structure of the tongue and histomorphological characteristics of lingual glands by making use of the first slide among slides prepared, Crossman's triple staining technique was used (Bancroft and Cook, 1984).

TABLE 1. Histochemical techniques used

Procedure	Interpretation of staining reactions	References
PAS	Glycoconjugates with oxidizable vicinal diols and glycogen	Bancroft and Cook (1984)
PAS-D	Glycoconjugates with glycogen	Bancroft and Cook (1984)
AB (pH 2.5)	Glycoconjugates with carboxyl groups (COOH groups) and *O*-sulfate esters	Bancroft and Cook (1984)
AB (pH 2.5)/PAS	Acidic (carboxyl groups and *O*-sulfate esters) and neutral mucins (oxidizable vicinal diols groups)	Bancroft and Cook (1984)
AB (pH 2.5)–AF	Sialo- (carboxyl [COOH] groups) and sulphomucins (*O*-sulfate esters)	Bancroft and Cook (1984)
PAPS	*N*-acetylcialomucin (only sialic acid monoaldehydes or hexosedialdehydes or mixtures-[sialic acid residues])	Bancroft and Cook (1984)

AB, Alcian blue; PAS, periodic acid Schiff reagent; AF, aldehyde fuchsin; PAS-D; periodic acid–Schiff-diastase; PAPS, periodic acid–phenylhydrazine–Schiff.

Scanning Electron Microscopy

The tissues were washed twice in 0.1 M phosphate-buffer solution. Subsequent to the washing process, the tissues were passed through a graduated-acetone series (25%, 50%, 70%, and 100%) for the purpose of dehydration in preparation for electron microscopy. FEI brand, "Quanta FEG 250" model scanning electron microscope, with technology that does not require vacuum, critical drying or coating with gold, was used. Thus, direct images were taken from identified tissues and then recorded.

Histochemical Staining

The histochemical procedure is given in Table 1 in detail for the identification of glycoconjugates. The slides numbered 2–7 were stained by staining techniques that are, respectively, Periodic acid Schiff (PAS) to detect neutral mucins in glands (vicinal diol groups); diastase-PAS to detect glycogen; Alcian Blue (AB) (pH 2.5) to detect acid mucins; AB (pH 2.5)–PAS to detect neutral and acid mucins; AB(pH 2.5)–aldehyde Fuchsin (AF) to detect carboxylated and sulphated acid mucins; and phenylhydrazine–PAS (PAPS) to detect periodate reactive acid mucins (*N*-acetyl sialomucin) (Bancroft and Cook, 1984).

Immunohistochemical Staining

The slides numbered from 8 to 11 were used for immunohistochemical stainings. Strepavidin peroxidase method was used for localization of mucin genes, MUC1, MUC2, MUC5AC, and MUC6, in lingual glands of sheep.

The paraffin sections were then rinsed in distilled water following deparafinization and rehydration. To eliminate endogenous peroxidase activity, the sections were washed in 0.01 M phosphate buffer saline (PBS) for 3 × 5 min after being treated for 30 min with %3 H_2O_2 prepared in methyl alcohol. To prevent nonspecific binding, the sections were incubated in a blocking serum (Histostain Plus Bulk Kit, Zymed) for 15 min. Later, the sections were incubated with mouse monoclonal MUC1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, cat. no.53381), mouse monoclonal MUC2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, cat. no. 73146), mouse monoclonal MUC5AC antibody (Santa Cruz Biotechnology, Santa Cruz, CA, cat. no. 33667), and mouse monoclonal MUC6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, cat. no. 33668). For antibodies, a 1:200 dilution was used and sections were incubated overnight at +4°C. The sections, washed 3 × 5 times at 0.01 M PBS subsequent to the incubation, were incubated with a biotinylated secondary antibody (Histostain Plus Bulk Kit, Zymed) for 20 min in a humidity chamber at room temperature and washed 3 × 5 times with PBS again. After washing, the sections were treated in enzyme conjugate streptavidin (Histostain Plus Bulk Kit, Zymed) for 20 min. The sections were developed for 5–15 min in DAB chromogen solution after being washed 3 × 5 times with PBS again. The sections were counterstained with Mayer's hematoxylin, dehydrated through an alcohol series, cleared in xylene, and mounted in entellan.

The specificity of the immunohistochemical procedures was checked using negative and positive control sections. Sections of human stomach and ileum, which were processed as described above, served as positive controls for immunoreactions to all antibodies. For negative controls, PBS or normal mouse IgG (Santa Cruz Biotechnology, sc-2025) was used instead of a primary antibody. Normal mouse IgG is an isotype control immunoglobulin, unbound, with purified affinity. All sections were treated according to the same protocol.

Subsequent to the stainings, the slides were evaluated with a research microscope with a Nikon-Eclipse 400 DSRI Nikon digital camera attachment (NIS-Elements Imaging Software version 3.10), which was also used to photograph the sections of interest.

Semiquantitative Evaluation

The results of carbohydrate histochemistry and MUCs (MUC1, MUC2, MUC5AC, and MUC6) were evaluated as follows: no staining (–), weak staining (+), moderate staining (++), and intense staining (+++) (Buisine et al., 2001; Schumacher et al., 2004; Erdogan et al., 2012; Sağsöz et al., 2013; Erdogan et al., 2015). The evaluations of the positive stained cells were carried out by the same two blind researchers (H.S. and S.E), and mean scores were calculated. In tongue sections, the expression of MUCs and carbohydrate histochemistry were examined microscopically at ×40, ×100, ×200, and ×400 magnification. In each part of the tongue, three randomly selected fields were evaluated for each section. The results were separately evaluated and presented for both gland

epithelium and duct epithelium in the lingual body, torus, and radix. The lingual apex was not included in the evaluation because glands were not present.

Furthermore, kappa (κ) statistics were applied to determine interobserver agreement following the independent evaluation of the histochemical and immunohistochemical staining methods. Interobserver variability was estimated by comparing the visual scores of two researchers. For statistical analyses, a total of 2345 images from 280 slides were evaluated visually by H.S. and S.E. A kappa value between 0.81 and 1.0 was defined as nearly perfect agreement, a value between 0.61 and 0.8 as substantial agreement, a value between 0.41 and 0.60 as moderate agreement, a value between 0.21 and 0.40 as fair agreement, and a value between 0.00 and 0.20 as slight agreement. For each kappa value, the 95% confidence interval (CI) was calculated.

RESULTS

Light Microscope (LM)

In sheep, the mucosa of the tongue contained differentiated papillary systems so as to perform both gustatorial and mechanical functions, and mechanical papillae were covered with a much thicker layer of keratin than the sensory papillae. The tongue contained filiform papillae and lenticular papillae as mechanical papillae and fungiform papillae and circumvallate papillae as gustatory papillae (Fig. 1).

Filiform papillae were the dominant type of papillae on the tongue, and fungiform papillae were randomly distributed among them (Fig. 1A,B). The filiform papillae were shorter and thinner in the lingual apex, and their height and thickness increased towards the lingual torus. It was striking that fungiform papillae, scattered along the tongue surface, were comprises two different types. The first type of fungiform papillae was found in the apex and body of the tongue. Fungiform papillae were smaller in diameter and had a convex surface covered with a thin layer of keratin, and contained taste buds (Fig. 1C). The second type of fungiform papillae was found in the torus of the tongue. The second type had much larger diameter, and were covered by a thick layer of keratin; unlike the first type, these did not contain taste buds. Lenticular papillae were also embedded in the epithelial layer along the median line of lingual

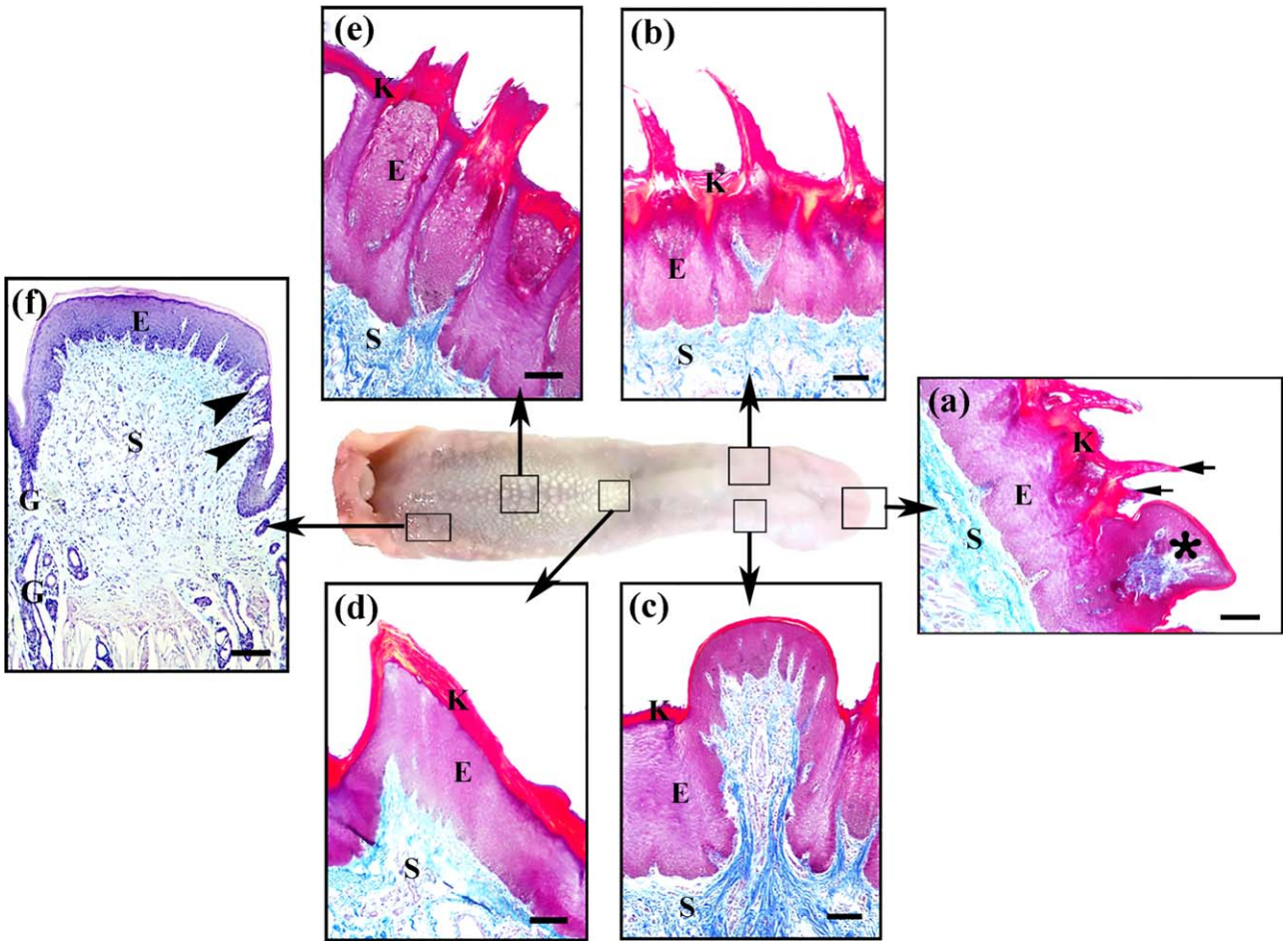

Fig. 1. General structure of the tongue parts. (**A**) the filiform papillae with secondary projections (arrows) and fungiform papilla (*) on the lingual apex, (**B**) filiform papillae on the lingual body, (**C**) fungiform papilla on the lingual body, (**D**) lenticular papilla on the anterior part of the lingual torus, (**E**) lenticular papillae on the lingual torus, (**F**) circumvallate papilla with taste buds (arrowheads). K: Keratin layer, E: Epithelium, S: Stroma, G: Glands with salivary ducts. Crossman's triple stain, Scale bars: (A–E) 100 μm; (F) 125 μm.

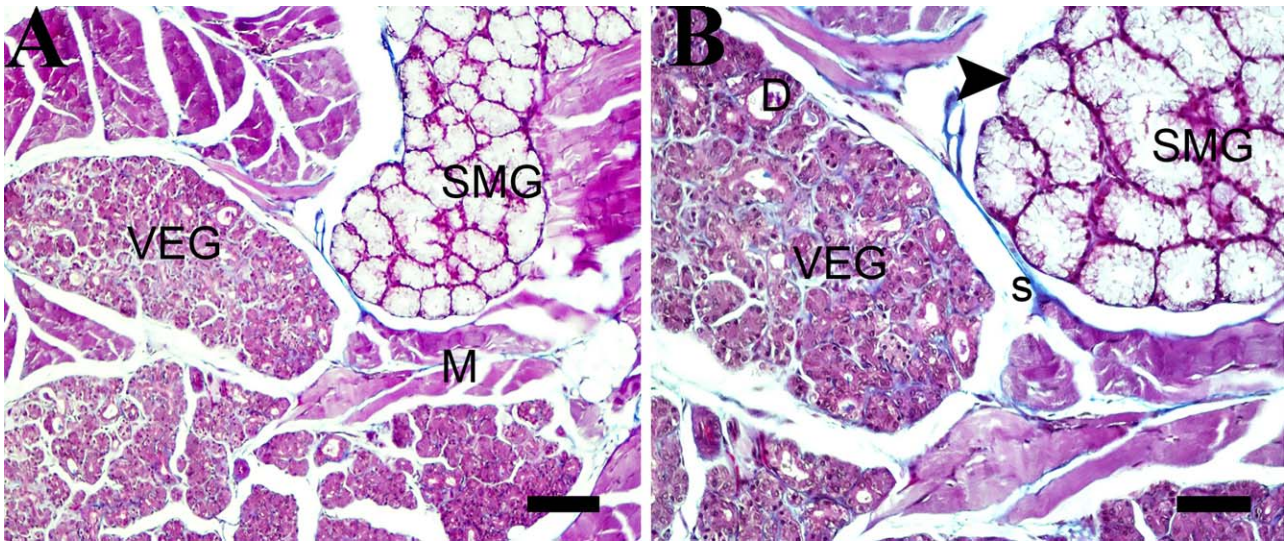

Fig. 2. (**A**, **B**) Simple branched tubuloalveolar seromucous glands (SMG) of the lingual radix and tubuloalveolar serous von Ebner's glands (VEG) of the lingual torus located in the stroma (S). M: striated muscles, D: salivary duct, arrowhead: thin-walled serous corpus glandulae. Scale bars: (A) 100 μm; (B) 25 μm.

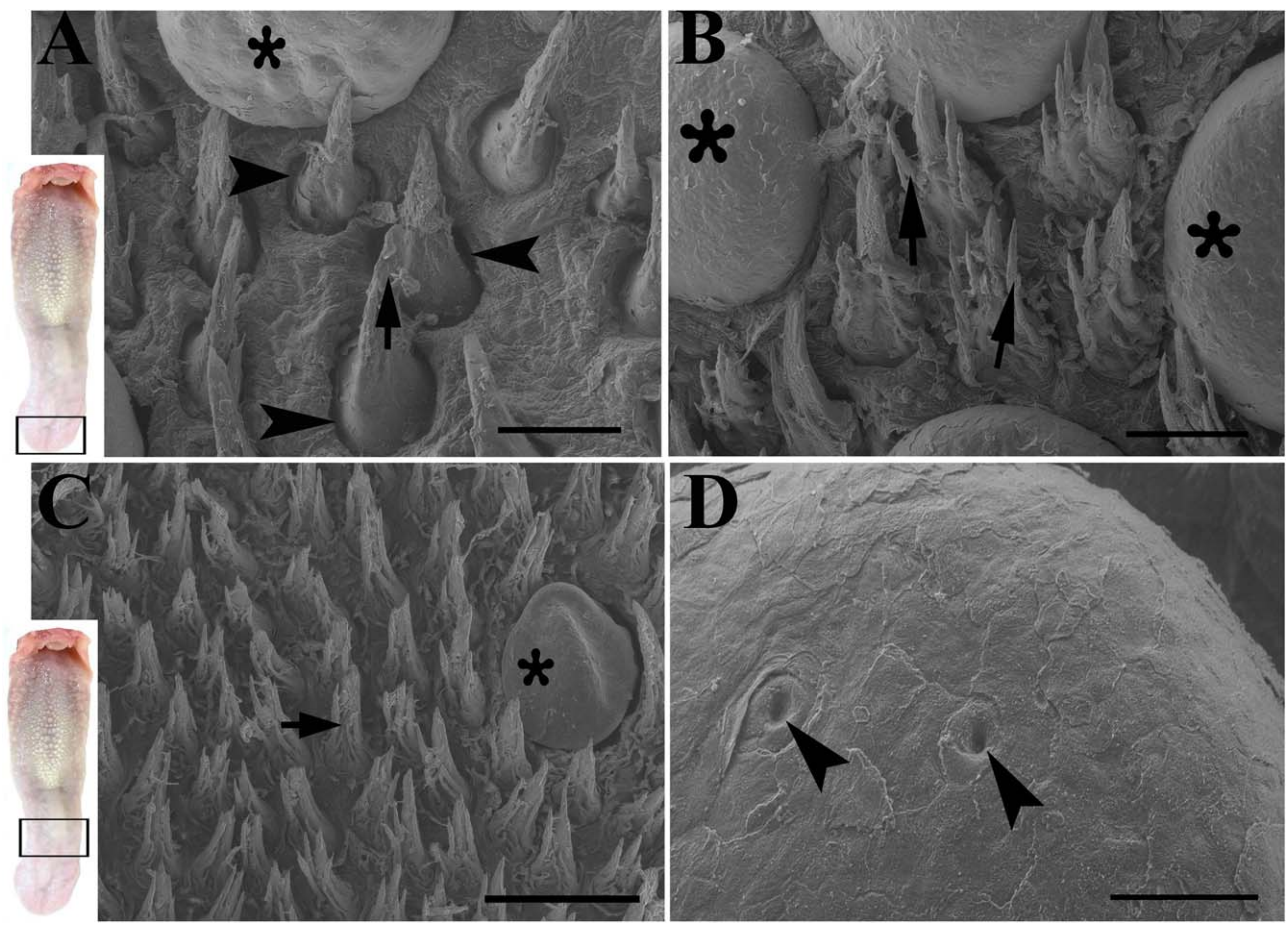

Fig. 3. (**A**, **B**) Filiform (arrows) and fungiform papillae (*) on the ventral lingual surface. Arrowheads: Grooves of the filiform papillae. (**C**) Filiform papillae with secondary projections (arrow) and fungiform papilla in conical shape (*), (**D**) taste pores (arrowheads) on the surface of the fungiform papilla. Scale bars: (A, B) 200 μm; (C) 500 μm; (D) 50 μm.

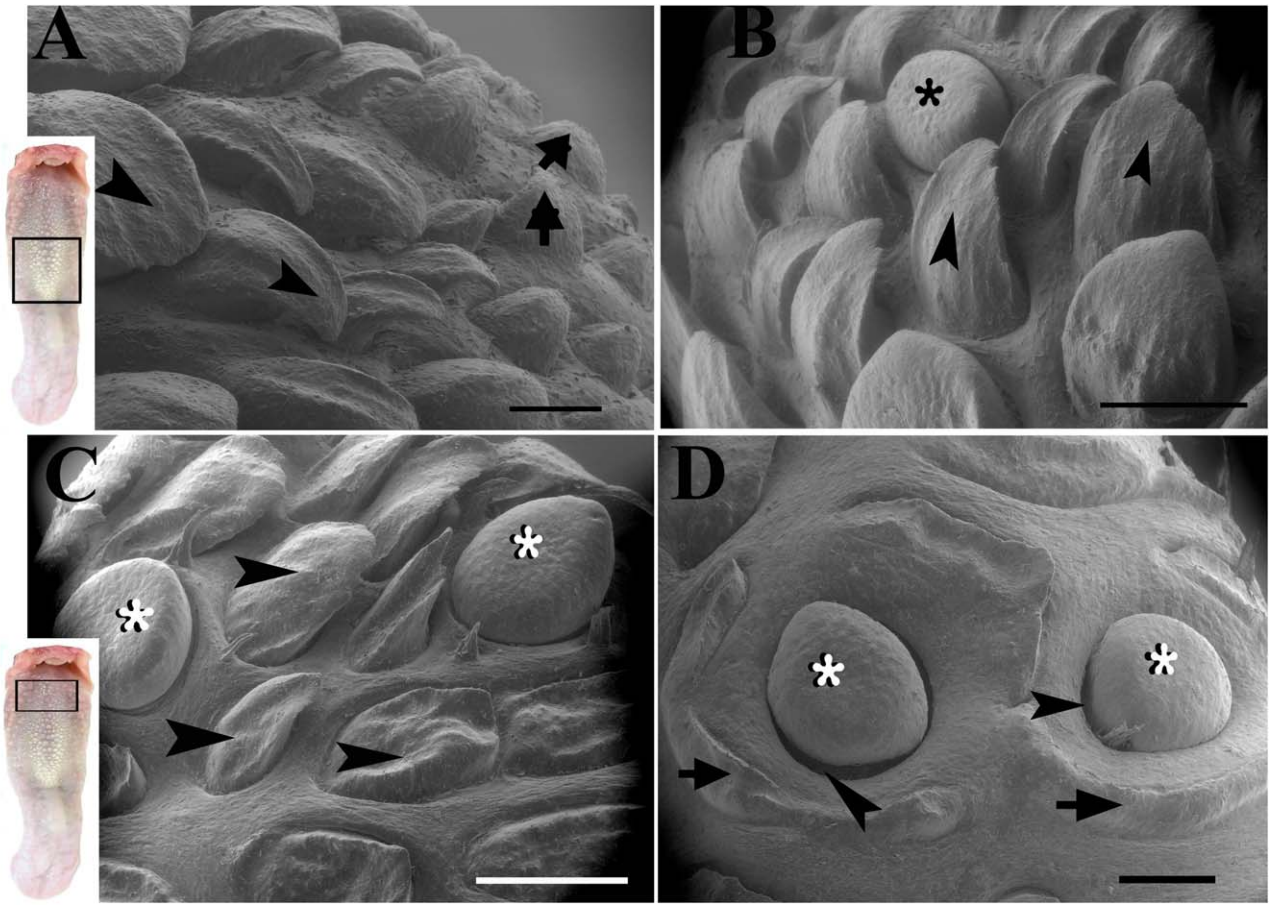

Fig. 4. (**A**) Large lenticular papillae with shallow grooves (arrowheads) and smaller lenticular papillae (arrows) on the anterior part of the lingual torus, (**B**) fungiform papilla (black star) among lenticular papillae (arrowheads), (**C**) Circumvallate papillae (white stars) without annular pad and surrounding lenticular papillae (arrowheads) in different sizes, (**D**) Circumvallate papillae (white stars) with annular pad (arrows) surrounding the deep grooves (arrowhead). Scale bars: (A, D) 500 μm; (B, C) 1 mm.

torus; they were round and their surfaces were covered with a thick layer of keratin. There were protrusions, arising from a layer of keratin, on the surfaces of some papillae (Fig. 1D,E). Circumvallate papillae were located in the posterior region of lingual torus; they had different sizes, and each papilla was surrounded by a deep groove. A large number of taste buds with intraepithelial localization were identified in the bilateral faces of the groove. The surfaces of all circumvallate papillae were determined to be covered with a very thin layer of keratin (Fig. 1F).

Both dorsal and ventral surfaces of the tongue were covered with a multilayered keratinized epithelium. Just beneath this epithelium the lamina propria and submucosa contained blood vessels and nerve plexuses in layers of connective tissue rich in elastic and collagen fibers and longitudinal, vertical, and transversely oriented striated muscle fibers (Fig. 2A,B). Lingual glands were identified in the connective tissue in the body, torus, and radix sections of the tongue, but not in the apex. The simple branched tubuloalveolar seromucous glands were located in the connective tissue of the body and radix of the tongue. The majority of glands consisted of mucous corpus glandulae that were surrounded by serous corpus glandulae that were thin-walled and semilunar in shape. In sheep, mucous corpus glandulae were sometimes found isolated among gland groups, but serous corpus glandulae were never found alone. The glands opened at the surface of the epithelium by a single salivary duct (Fig. 2A,B). Tubuloalveolar serous von Ebner's glands were localized in the lingual torus. The secretory cells of the glands had typical characteristics of serous cells. The von Ebner's glands were located in the connective tissue between the bundles of striated muscle fibers beneath the vallate papillae, and each glandular unit opened into the grooves of the circumvallate papillae through the salivary ducts (Fig. 2B).

Scanning Electron Microscope (SEM)

A large number of thorn-like, caudally directed filiform papillae were in existence on the edges of the ventral surface of the lingual apex. The bottom of each filiform papilla was embedded in its own groove (Fig. 3A). These filiform papillae had 2–6 secondary projections (Fig. 3B). The number of the secondary projections increased in the filiform papillae at the back of the tongue. A large number of fungiform papillae were also

observed among the filiform papillae (Fig. 3A,B). The convex surfaces of these papillae were highly prominent. While some of them were spherical, others were observed to be outwardly conical.

A large number of fungiform papillae had completely surrounded the dorsal surface of the lingual body in front of the lingual torus, including the lingual apex. Each filiform papilla was directed caudally, and each

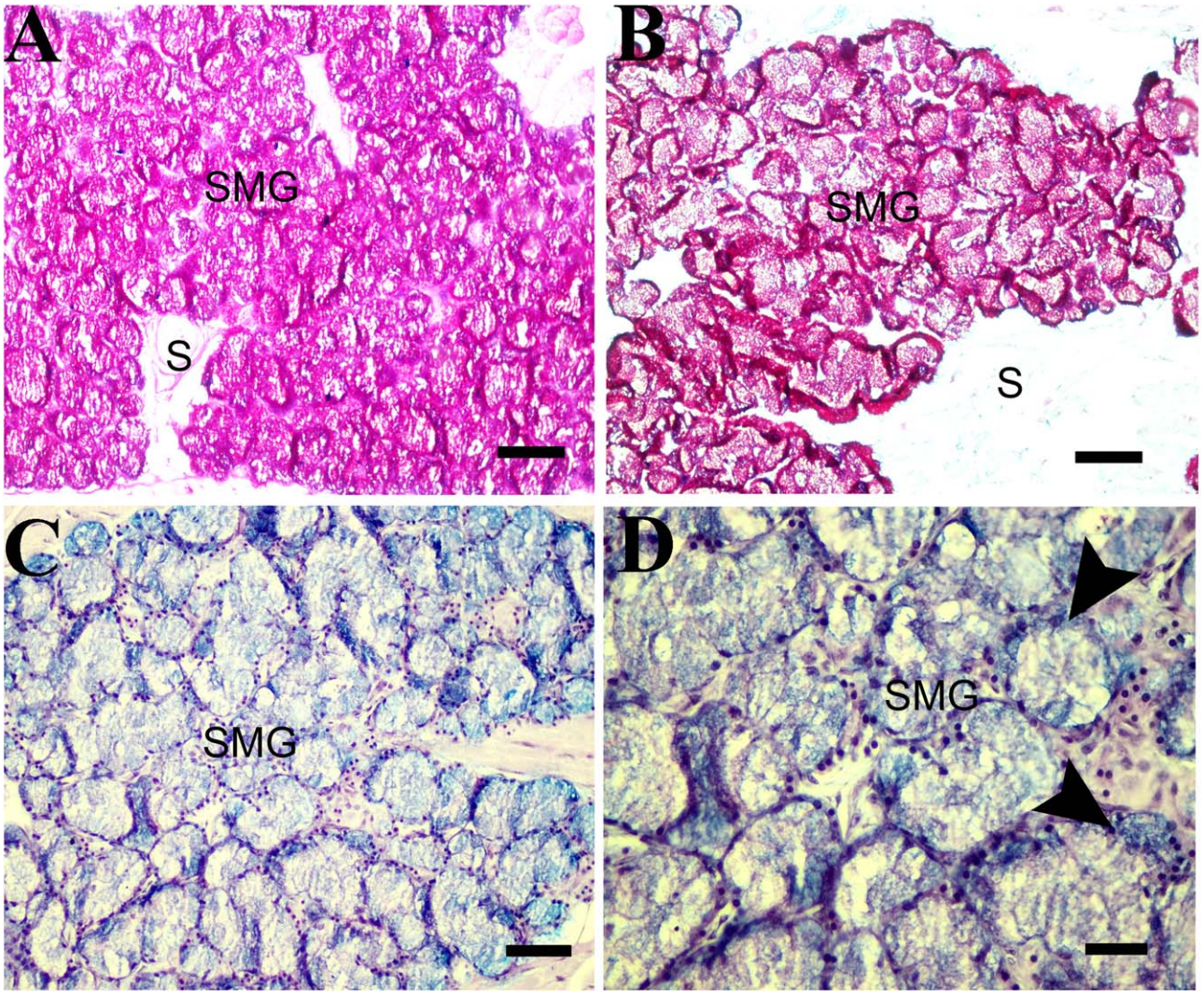

Fig. 5. (**A**) PAS reactivity in simple branched tubuloalveolar seromucous glands (SMG) located in the stroma (S) of the lingual body. (**B**) Negative PAS-diastase reaction in tubuloalveolar seromucous glands (SMG). (**C**, **D**) AB reaction at varying densities in both mucous and serous secretory units (arrowheads) of tubuloalveolar seromucous glands (SMG). Scale bars: (A–C) 100 μm; (D) 25 μm.

TABLE 2. Histochemical and immunohistochemical reactions of gland and duct epithelial cells

		Histochemistry						Immunohistochemistry			
Minor salivary gland	Layers	PAS	PAS-D	AB (pH 2.5)	AB (pH 2.5)/PAS	AB (pH 2.5)–AF	PAPS	MUC1	MUC2	MUC5AC	MUC6
Seromucous glands	GE	+++	+++	++	+++PAS/+M/–AB	+++AF/–AB	++	–	–/+	–	–
	DE	+	+	–/+	++M/+PAS/–AB	+AF/–AB	+	–/+	+	–/+	–/+
von Ebner's glands	GE	+	+	+/++	++PAS/+M/–AB	+AF/–AB	–	+	+	+	+
	DE	–/+	–/+	–	++PAS/+M/–AB	+AF/–AB	–	+	+	+	+

GE, gland epithelial cell; DE, duct epithelial cell; M, mix reaction; AB, Alcian blue; PAS, periodic acid Schiff reagent; AF, aldehyde fuchsin; PAS-D; periodic acid Schiff-diastase; PAPS, periodic acid–phenylhydrazine–Schiff.

papilla had secondary projections (Fig. 3C). As in the ventral surface, the filiform papillae in this region had 2–6 secondary projections. These secondary projections located on the filiform papillae of the lateral borders of the lingual body showed a longer and more fringed appearance. The filiform papilla immediately in front of the lingual torus, or those encircling the lingual fossa, did not have secondary projections.

The density of fungiform papilla scattered among the filiform papillae in this region was considerably reduced as compared to those on the ventral surface of the lingual apex (Fig. 3C). Fungiform papillae were scattered less frequently. Although fungiform papillae in the dorsal surface of the lingual apex were mostly spherical, some located on the dorsal surface of the body of the tongue were conical with pointed tips (Fig. 3C). Additionally, there were shallow grooves on the surfaces of the fungiform papillae. Each fungiform papilla was distinctly separated from the surrounding filiform papillae. The taste pores on the surface of each fungiform papilla were clearly distinguished at higher magnifications. They were round and distinctive, and consisted of scale-like desquamated cell layers on their surfaces (Fig. 3D).

There were lenticular papillae in small sizes extending in all directions in the rostral half of the dorsal surface of the lingual torus (Fig. 4A). The tips of some of them were bifurcated and shallow grooves were present on their surface (Fig. 4A). These papillae in the center of the rostral half of lingual torus were bulkier and larger than papillae located on the lateral sides, which were seen as simple conical protrusions (Fig. 4A).

Lenticular papillae were observed in the caudal half of the lingual torus, and they were quite bulky, long and mostly caudally oriented. Most papillae ended with a single protrusion, and a shallow groove was observed on

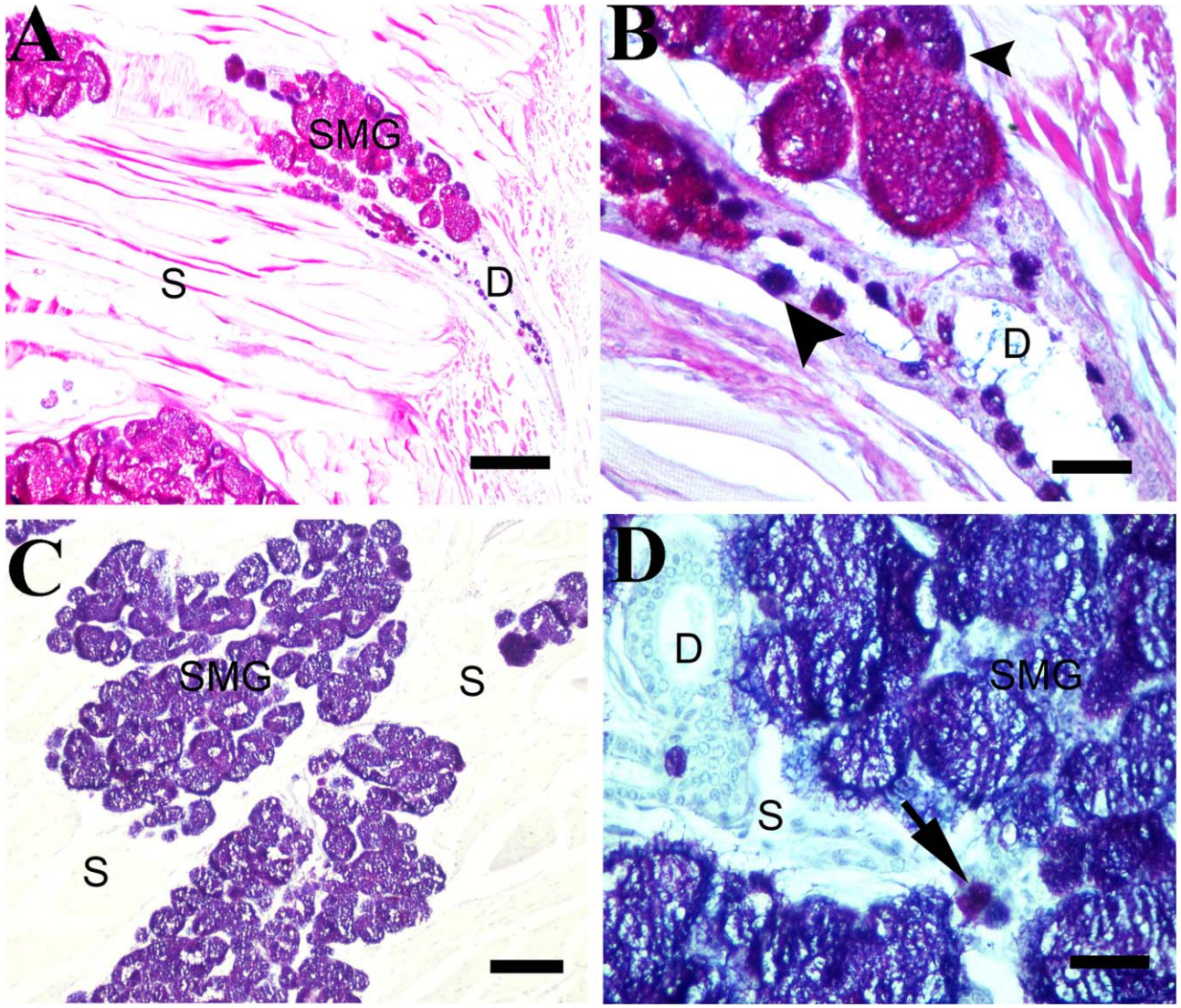

Fig. 6. (**A**, **B**) AB (pH 2.5)–PAS reactivity in simple branched tubuloalveolar seromucous glands (SMG) located in the stroma (S) of the lingual radix. (**C**, **D**) AF–AB (pH 2.5) reactivity in simple branched tubuloalveolar seromucous glands (SMG) located in the stroma (S) of the lingual body. D: salivary duct, arrowhead: mixed reactions, arrow: weak sulfated mucins. Scale bars: (A, C) 100 μm; (B, D) 25 μm.

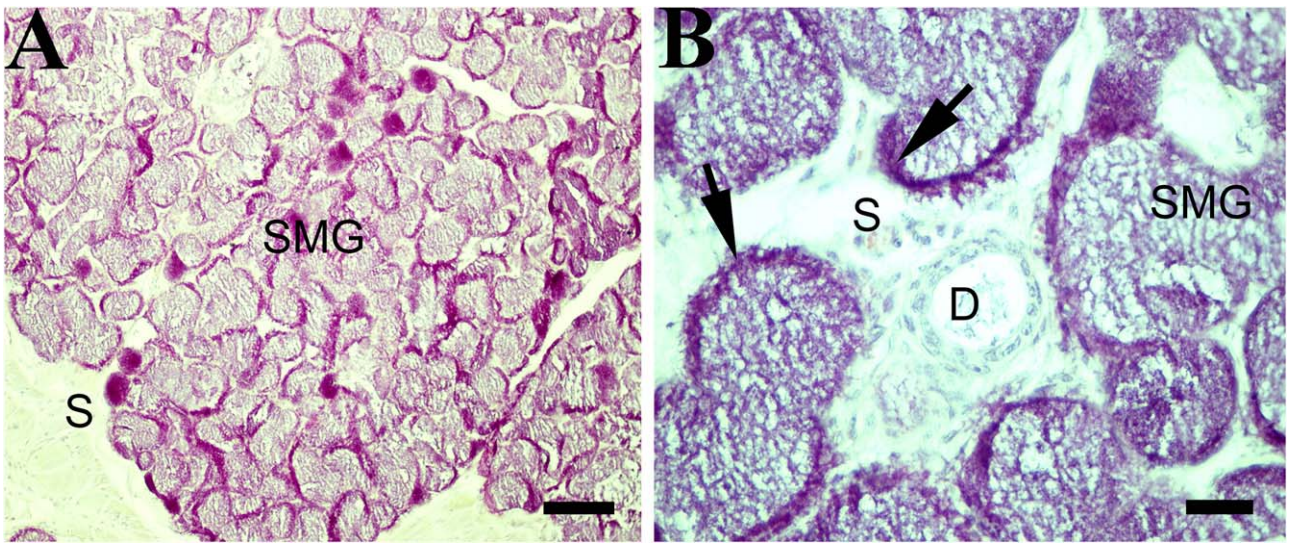

Fig. 7. (**A**, **B**) Periodic acid–phenylhydrazine–Schiff reactivity in the simple branched seromucous glands (SMG) located in the stroma (S) of the lingual body. D: salivary duct, arrow: *N*-acetyl sialomucins in serous corpus glandulae. Scale bars: (A) 100 μm; (B) 25 μm.

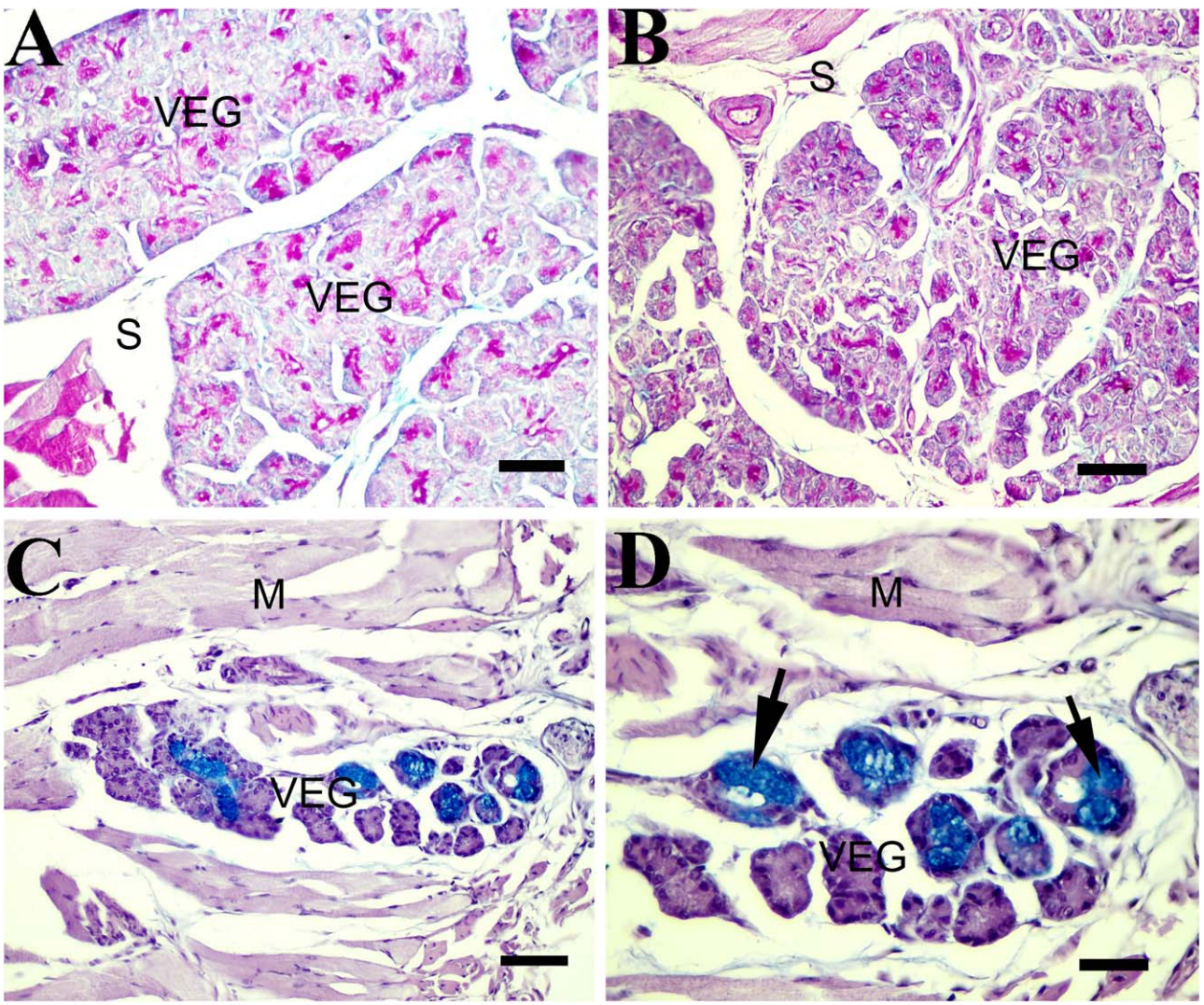

Fig. 8. Von Ebner's glands (VEG) located in the stroma (S) of the lingual torus. (**A**) PAS reactivity, (**B**) negative PAS-diastase reactivity, (C, D) AB reactivity. M: striated muscles. Scale bars: (A–C) 100 μm; (D) 25 μm.

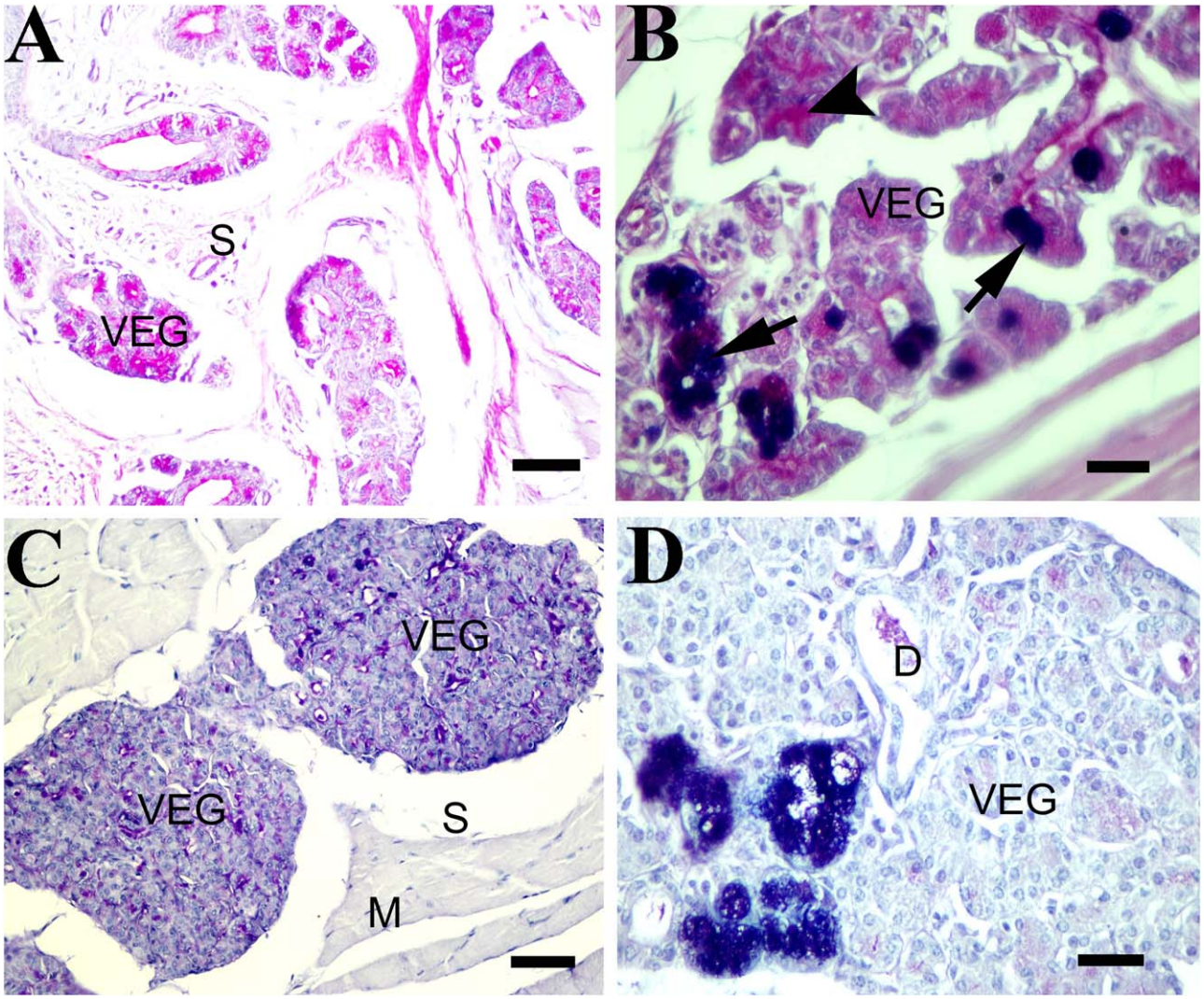

Fig. 9. Von Ebner's glands (VEG) located in the stroma (S) of the tongue torus. (**A**, **B**) AB (pH 2.5)–PAS reactivity, (**C**, **D**) AF–AB (pH 2.5) reactivity. D: salivary duct, M: striated muscle, arrow: mixed reactions, arrowhead: apical cytoplasm in PAS reactivity. Scale bars: (A, C) 100 μm; (B, D) 25 μm.

the surface of each papillae, which extended from its base to its apex (Fig. 4B). Papillae located in front formed a sequence arrangement with frequent intervals almost leaning on those located behind them (Fig. 4B). The bases of some lenticular papillae were quite wide. Fungiform papillae with round, convex surfaces were scattered infrequently among lenticular papillae (Fig. 4B). The lenticular papillae were shorter and eventually became simple protrusions at the caudal border of lingual torus. The surfaces of the caudalmost lenticular papillae were not as smooth as others described, but they had thorn-like protrusions. There were also 6–10 circumvallate papillae arranged along both lateral edges of the caudal half of the lingual torus. Circumvallate papillae were scattered among lenticular papillae (Fig. 4C). While some of these papillae were separated from each other, others were together. Each circumvallate papilla was surrounded by a deep groove and had a prominent convex surface (Fig. 4C). The annular pad, encircling the grooves of these papillae, had a noncontinuous structure (Fig. 4D). The annular pad was not present around some papillae (Fig. 4C). At higher magnifications, a rough appearance of the epithelial layer and taste pores drew attention to the surface of each papilla. No papillae or a specialized structures, including mechanic or gustatory, were detected in the radix section of the tongue. The orifices of the salivary gland ducts opened to the flat mucosa.

Histochemistry

The interobserver agreement was perfect for histochemical visual scorings (PAS: $\kappa = 0.802$; 95% CI: 0.725–0.780, AB: $\kappa = 0.824$; 95% CI: 0.837–0.930, AB (pH 2.5)–PAS: $\kappa = 0.780$; 95% CI: 0.741–0.819; AF–AB (pH 2.5): $\kappa = 0.875$; 95% CI: 0.826–0.843, phenylhydrazine (PAPS): $\kappa = 0.861$; 95% CI: 0.874–0.957).

There was a strong PAS reaction especially in the mucous epithelial cells of seromucous glands. The PAS reaction was weaker in the duct epithelial cells. There was no change in the staining intensity of the glands stained with diastase-PAS reaction, which was applied to determine glycogen content (Fig. 5A,B) (Table 2).

For AB (pH 2.5) staining, there was an AB reaction at varying intensities in both mucous and serous secretory units of seromucous glands. Some secretion units were strongly AB positive, while some were weakly AB positive. AB reaction was either weak or absent in ducts, and weak reactions were localized to the duct epithelial cells in the neck region (Fig. 5C,D) (Table 2).

For AB (pH 2.5)-PAS staining method; the cells of the secretory units of the seromucous glands were intensely PAS (magenta) positive and the PAS reaction was concentrated in mucous secretory units rather than serous secretory units. The mixed reaction (purple) was weak and predominantly localized in serous demilunes surrounding mucous secretory units, which were negative for AB reaction. Mixed (purple) reaction was dominant in salivary ducts of glands that opened to the epithelium, and the PAS (magenta) positive reaction was only in some salivary duct epithelial cells (Fig. 6A,B) (Table 2).

For the AF–AB (pH 2.5) technique, the epithelial cells of serous glands exhibited an intense AF-positive reaction, which was dominant in the mucous secretory units rather than the serous demilune units. The AF reaction was weak in some mucous secretory units and all serous secretory units. No AB reaction was evident in mucous and serous secretory units. A weak AF reaction was observed in epithelial cells of the glandular ducts (Fig. 6C,D) (Table 2). For the phenylhydrazine (PAPS) technique, a weak positive reaction was exhibited in the secretory units and ducts of seromucous glands (Fig. 7A,B) (Table 2).

For PAS staining of von Ebner's glands, a weak or midintensity reaction occurred in the apical cytoplasm of some glandular epithelial cells. A reaction was observed in some of the epithelial cells of ducts. In the diastase-PAS reaction, no change in PAS positive intensity in the glandular or ductal epithelial cells was not observed (Fig. 8A,B). In AB (pH 2.5) staining, a reaction at varying intensities from weak to strong was observed in some glandular epithelial cells. Ductal epithelial cells were shown to be AB negative (Fig. 8C,D) (Table 2).

In the AB (pH 2.5)–PAS staining method, the PAS reaction was identified in the apical cytoplasm of some secretory units of glands and epithelial cells of salivary ducts. It was striking that while a mixed reaction was observed in the sporadic glandular epithelium cells, AB reaction did not exist in glandular epithelium cells (Fig. 9A,B). In the AF–AB (pH 2.5) technique, it was found that a weak AF-positive reaction occurs in some secretory units and in duct epithelial cells; however, an AB reaction does not occur. A weak AF reaction was observed in some duct epithelial cells (Fig. 9C,D) (Table 2).

In phenylhydrazine (PAPS) technique, no positive reaction was observed in the epithelial cells of von Ebner's glands nor in their salivary ducts (Fig. 10) (Table 2).

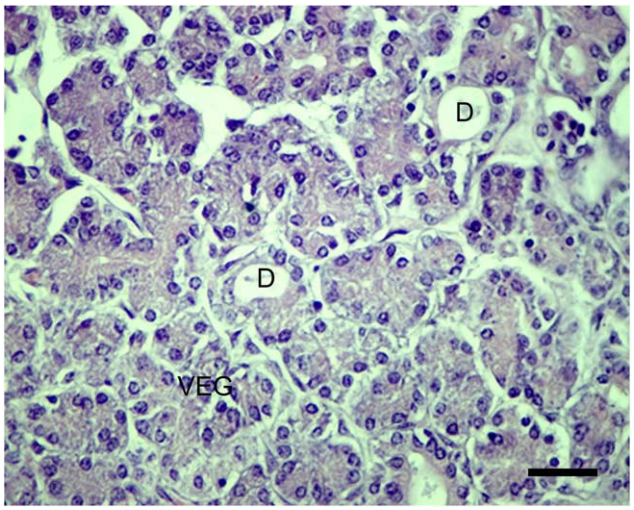

Fig. 10. Negative periodic acid–phenylhydrazine–Schiff reactivity of von Ebner's glands (VEG) located in the stroma (S) of the lingual torus. D: salivary duct. Scale bar: 50 μm.

Immunohistochemistry

The interobserver agreement was perfect for MUCs visual scorings (MUC1: $\kappa = 0.883$; 95% CI: 0.860–0.907; MUC2: $\kappa = 0.818$; 95% CI: 0.785–0.951, MUC5AC: $\kappa = 0.704$; 95%CI: 0.655–0.853, MUC6: $\kappa = 0.830$; 95% CI: 0.784–0.877).

MUC1, MUC6, and MUC5AC localization in seromucous lingual glands were negative in glandular epithelial cells, and reactions were weak in epithelial cells of salivary ducts. MUC2 localization was heterogeneous and weak in glandular epithelial cells, but more prominent in the epithelial cells of salivary ducts (Fig. 11A–D). MUC1, MUC2, MUC5AC and MUC6 had varying intensity in epithelial cells of von Ebner's glands and their salivary ducts (Fig. 12A–D) (Table 2). These immunoreactions were also observed in positive controls (human ileum and stomach) for MUC1, MUC2, MUC5AC, and MUC6 (Fig. 13).

DISCUSSION

The mammalian tongue shows different morphological adaptations in different species. The topographical distribution of different papillae on different surfaces of the tongue may be a specific characteristic. Tongue-related morphological differences and variations are directly associated with feeding style and diet, and they are also affected by environmental conditions (Iwasaki, 2002). Main differences in feeding styles of ruminants overlap with physiological characteristics of their food types and differences in their positional arrangements.

Similar to the present study, in ruminants such as the Saanen goat (Kurtul and Atalgın, 2008), Sitatunga (Emura et al., 2011b), and barking deer (Adnyane et al., 2011), filiform papillae prominently cover the rostral half of the tongue. In some ruminants, each filiform papilla had secondary projections originating from their base. The presence of secondary projections were reported as 3–6 pieces in the Saanen goat (Kurtul and Atalgın, 2008), 2 pieces in Formosan serow (Atoji et al., 1998), and Pampas deer (Erdoğan and Pérez, 2013), 2–3 pieces in lesser-mouse deer (Agungpriyono et al., 1995), and 6–8 pieces in the goat (Kumar et al., 1998). Each papillary surface was shown to have keratinized, desquamating epithelial cells in cattle (de Paz Cabello

et al., 1988), Mazama species (Kokubun et al., 2012), and Pampas deer (Erdoğan and Pérez, 2013), and this same keratinized structure was also identified in the present study. These differences in forms and distributions of filiform papillae among species are considered to be closely associated with age, feeding style and diet, and ruminating characteristics (Erdunchaolu et al., 2001; Erdoğan and Pérez, 2013).

Fungiform papillae in sheep had the same morphology, and with few exceptions, the fungiform papillae located on the ventral surface had a more conical shape than those located on the dorsal surface, which were more spherical. These characteristics are likely to be functional feature of the tongue, and the result of a morphological adaptation to a specific feeding type. Filiform papillae, especially those located on the ventral surface, are larger and longer, which suggests that fungiform papillae among them may be pointed to reach the surface amid the crowding of the filiform papillae.

In sheep, a large number of fungiform papillae were observed on the lingual apex. A similar situation was seen in the Pampas deer (Erdoğan and Pérez, 2013), chital (Erdoğan and Pérez, 2014), Formosan serow (Atoji et al., 1998), Japanese serow (Funato et al., 1985), Roan antelope (Emura et al., 2011a) Blackbuck (Emura et al., 1999), Barbary sheep (Emura et al., 2000), and lesser-mouse deer (Agungpriyono et al., 1995). For this reason, the apex region of the tongue may be considered as a special organ (Qayyum et al., 1988; Agungpriyono et al., 1995).

Fungiform papillae located on the lingual torus have been identified in Muntjac deer (Adnyane et al., 2011), Formosan serow (Atoji et al., 1998), Pampas deer (Erdoan and Pérez, 2013), Mazama species (Kokubun et al.,

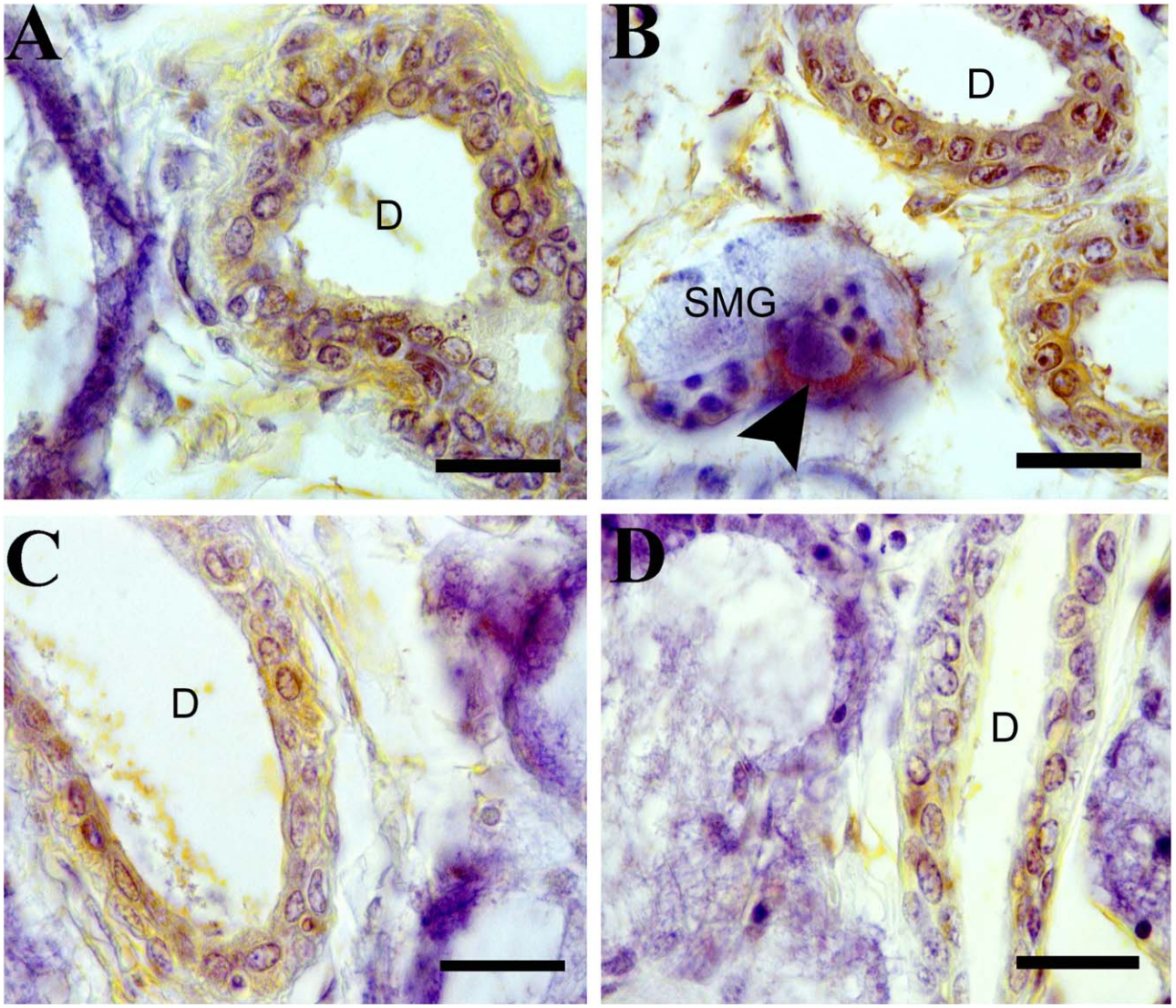

Fig. 11. Expression of MUC1 (**A**), MUC2 (**B**), MUC5AC (**C**), and MUC6 (**D**) in salivary ducts (D) of the simple branched tubuloalveolar seromucous glands (SMG). Arrowhead: positive MUC2 reaction in serous corpus glandulae. Scale bars: 12.5 μm.

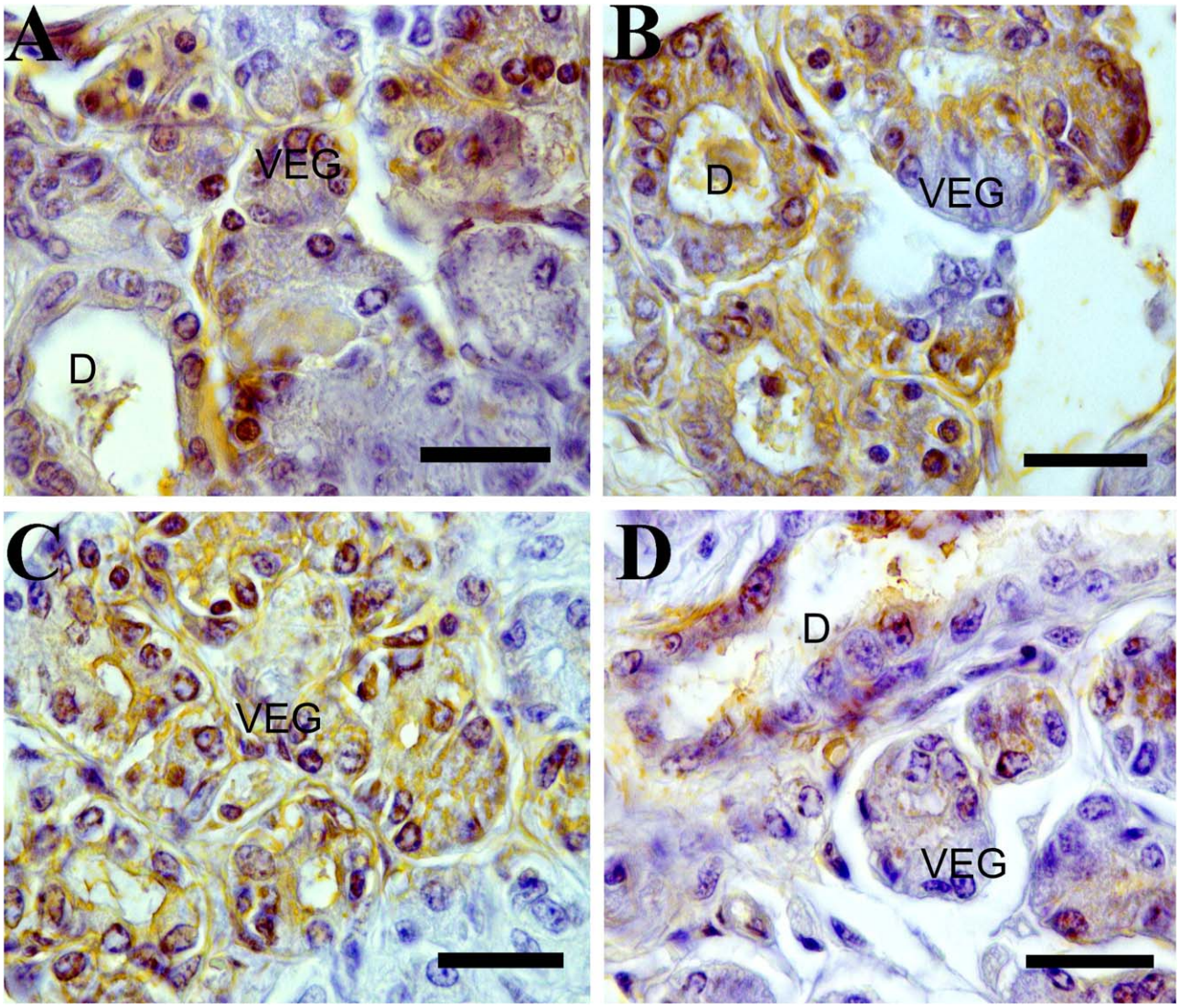

Fig. 12. Expression of MUC1 (**A**), MUC2 (**B**), MUC5AC (**C**), and MUC6 (**D**) of salivary ducts (D) and tubuloalveolar serous Von Ebner's glands (VEG). Scale bars: 12.5 μm.

2012), and chital (Erdoğan and Pérez, 2014). In our study, the presence of fungiform papillae on the lingual torus scattered among lenticular papillae was revealed. Barone (1997) reported that in ruminants, each fungiform papilla lacks taste buds and therefore, these types fungiform papillae function as mechanical papillae. In our study, taste buds were not observed in histological sections of fungiform papillae located on the lingual torus, and it has been thought that these papillae augment the mechanical function, together with the lenticular papillae.

Numbers of bilaterally-located circumvallate papillae also vary considerably from species to species. The number of circumvallate papillae mentioned are as follows: 22–28 in the yak (Shao et al., 2010), 22–32 in the cattle (Chamorro et al., 1986), 5–9 in the Pampas deer (Erdoan and Pérez, 2013), 26 in the Saanen goat (Kurtul and Atalgın, 2008), 11–14 in the chital (Erdoğan and Pérez, 2014), 10–13 in the Muntjac deer (Adnyane et al., 2011), and 20 in the Japanese serow (Funato et al., 1985).

In our study, the presence of an annular pad surrounding the groove of the circumvallate papilla was observed. However, this pad, which exists around many papillae, was discontinuous in structure. The annular pad has been reported in the cattle (Chamorro et al., 1986), lamb (Tadjalli and Pazhoomand, 2004), goat (Kumar et al., 1998) and antelope (Emura et al., 2011a). As reported in the Pampas deer (Erdoğan and Pérez, 2013), it has also been observed in our study that some papillae with a groove are not surrounded by this annular pad. It was reported in the dromedary (Qayyum et al., 1988) that a number of papillae are surrounded by a common annular pad, and a similar phenomenon was observed in our study as well.

In some mammals (Tandler et al., 1994; Gargiulo et al., 1995a,b; Pedini et al., 1997; Triantafyllou et al.,

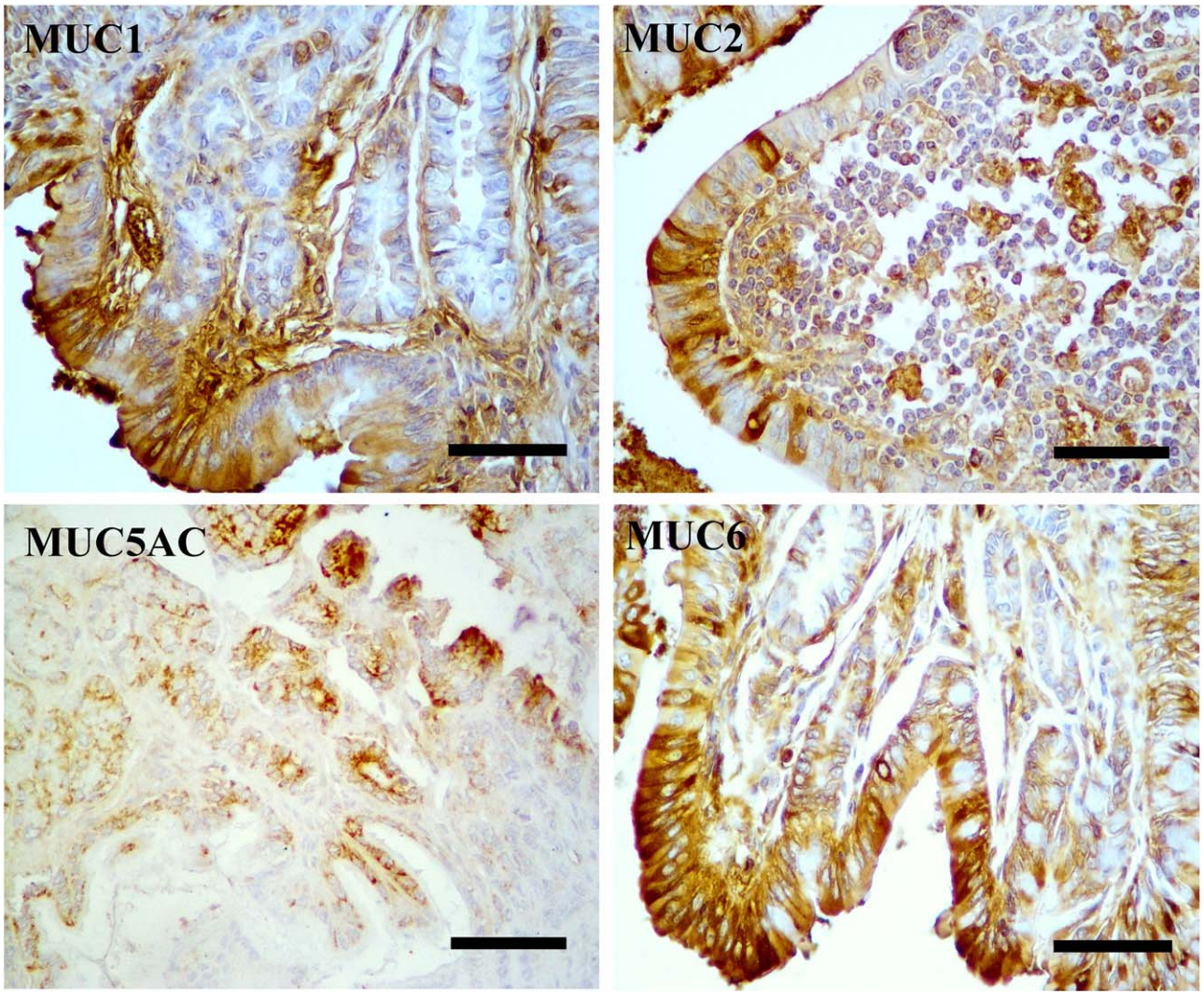

Fig. 13. Positive controls of MUC1, MUC2, MUC6 (human ileum), and MUC5AC (human stomach). Scale bars: 25 μm.

2001, Paliwal et al., 2006), the histochemical data have revealed that neutral mucosubstances and sulphated mucins exist in both seromucous and von Ebner's glands (although their staining intensities differ); however, carboxylated mucins do not exist in either gland type. Functional considerations of these results indicate that differences in mucins secreted from glands, together with histomorphological differences in different parts of the tongue, reflect an adaptation to physiological requirements, including protection against bacterial colonization.

In the conventional carbohydrate histochemistry, PAS (+) reactions indicate the presence of neutral carbohydrates, AF (+) and AB (pH 2.5) (+) reactions indicate the presence of acidic sulphated and carboxylated mucins and PAPS (+) reactions indicate the presence of *N*-acetyl sialomucins (Bancroft and Cook, 1984; Schumacher et al., 2004; Erdogan et al., 2015a,b). Major ducts of anterior lingual salivary glands in humans contain histochemically neutral glycoproteins, a small amount of sialoglycoproteins and a large amount of sulphated glycoproteins. Small mucous ducts have been shown to contain neutral glycoproteins, a large number of sialoglycoproteins and relatively small amounts of sulphated glycoproteins. Seromucous glandular structures contain neutral and sialoglycoproteins at high concentrations, and sulphated glycoproteins at low concentrations (Tandler et al., 1994). Serous glands and epithelial cells of ducts and serous demilunar epithelial cells in an Egyptian mongoose include only neutral mucosubstances, and mucous glands and all epithelial cells of ducts contain weak sulfated mucosubstances. Carboxylated mucosubstances (sialomucins) do not exist in the lingual glands of the Egyptian mongoose (Poddar and Jacob, 1980). Mucous glandular acinar cells of seromucous glands contain neutral and acid mucins in a hamster (Paliwal et al., 2006), and serous demilunes of von Ebner's glands and seromucous glands contain medium-intensity neutral mucins and no acid mucins. All salivary ducts contain both neutral and acid mucins

in the hamster (Paliwal et al., 2006). Pedini et al. (1997) have shown through conventional histochemical results that secretions of von Ebner's glands in cows contain acid mucins and neutral mucins rich in carboxyl.

Salivary mucins form a barrier between the oral mucosa and the bacterial flora, and form a protective layer against drying, mechanical destruction, external toxic substances, and microbial toxins in the oral mucosa (Corfield et al., 2000; de Almeida et al., 2008; Sağsöz and Liman, 2009). In particular, sulfated mucins play an important role in the protection of the mucosa against bacterial adhesions (Robertson and Wright, 1997; Brockhausen, 2003, Sağsöz and Liman, 2009). In addition, carboxylated mucins also settle in cell surface membranes (COOH-terminal domains) and make contributions to epithelial protection by forming basic infrastructures of gel developing mucins (MUC2, MUC5AC, MUC5B, and MUC6) (Kesimer et al., 2010). The absence of carboxylated mucins in the lingual glands of sheep suggests that other factors may function in the epithelial protection mentioned above.

In humans, MUC1, 3, 4, 5B, and 16 are expressed in minor glands of the tongue; but, MUC2, 5AC, and 6 are not expressed (Teshima et al., 2011). It should be noted that in our study, MUC1, MUC5AC, and MUC6 do not become localized in the epithelial cells of seromucous glands, and only weak expression is observed in epithelial cells of ducts. MUC2 was found to be weaker in glandular epithelial cells and stronger in ductal epithelial cells. MUC1, MUC2, MUC5AC, and MUC6 expressions at varying concentrations were observed in von Ebner's glands and these expressions were found to be heterogeneous. MUC reactions are stronger in ductal epithelial cells of von Ebner's glands than those of the ductal epithelial cells of seromucous glands.

In mammals, the surface of the digestive, respiratory, and urinary system mucosa is protected by a sticky and continuous viscoelastic mucus layer, which forms a highly impermeable physical barrier to many molecules (Gendler and Spicer, 1995). MUC1, MUC2, MUC5AC, and MUC6 have essential functions in the continuity of this mucus barrier (Lacunza et al., 2009). It has been proposed that many pathogens may directly link to mucosal epithelial cells to cause pathogenicity. The main function of MUC1, MUC5AC, and MUC6, covering the cell surface, is reportedly to protect the mucosa against this bacterial penetration and invasion by acting as a trap ligand for microorganisms (Allen and Flemström, 2005; Linden et al., 2008). We conjecture that, based on our findings, MUCs likely perform similar functions in the tongue and oral mucosa of sheep.

In conclusion, we observed that the predominant type of papilla on the surface of the tongue is the filiform papilla; and that the filiform and fungiform papillae exist within the borders of the ventral surface of the lingual apex, as well as the dorsal surface of the tongue. We have found that the majority of filiform papillae have multiple secondary projections that enhance their mechanical effect. Fungiform papillae were seen on the ventral surface of the lingual apex. Considering that the lingual apex functions as a specialized organ, we believe that fungiform papillae play an effective role in gustatorial perception-based nutrition selection in this region. In general, the papillary organization in sheep was shown to be similar to the organization in goats. Likewise, there are similarities between the filiform and fungiform papillae with the mechanical and gustatory papillae, respectively, in some deer species and other herbivores such as the camel. Evaluation of the tongue glands, according to their localizations and secretory characters showed that while neutral, sulfated mucins and *N*-acetyl sialomucins exist in seromucous glands and salivary duct epithelium, carboxylated acid mucins do not exist. Moreover, both carboxylated and *N*-acetyl sialomucins do not exist in von Ebner's glands. MUC1, MUC5AC, and MUC6 are localized only in ductal epithelial cells, whereas MUC2 are localized in both glandular and ductal epithelial cells. In contrast, it was shown that all MUCs were localized in both von Ebner's glands and salivary ducts. This mucin composition, which serves as a physical barrier to pathogens in the initial section of the digestive system in sheep, is similar to other mammal species.

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AUTHOR CONTRIBUTIONS

The study was designed and equally performed by both authors (SE and HS) in all steps.

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