

## Histochemical properties of sialic acids and antimicrobial substances in canine anal glands

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### Abstract

The functional properties of sialic acids appear to be manifold. Additionally, antimicrobial substances serve as a non-specific defense against microorganisms. In this study, therefore, the localization of sialic acids and antimicrobial substances in the anal glands of dog was studied by sialoglycoconjugate histochemistry and immunohistochemistry. The secretory epithelium, luminal secretions and excretory ducts exhibited high levels of sialoglycoconjugates that terminated in Sia $\alpha$ 2-6Gal/GalNAc or Sia $\alpha$ 2-3Gal1-4GlcNAc. Additionally, O-acetylated sialic acids were detectable in these glandular structures. Antimicrobial substances, such as lysozyme, immunoglobulin A (IgA), lactoferrin and the peptide group of  $\beta$ -defensins, were also demonstrated as products of the anal glands. The results obtained are discussed with regard to the functional significance of the anal glands. These secretory products may create a defensive barrier against microbial invasion at the anal mucosa.

### Introduction

The canine anal region is characterized by three typical glandular complexes, which are the anal glands, circumanal glands and anal sac glands.<sup>1-6</sup> In dog and pig, previous studies demonstrated the distribution of glycoconjugates in the anal glands using lectin histochemical methods. These glandular secretions seem to participate in the formation of a stable mucous coat of the rather dry feces and enable the marking of territory.<sup>7,8</sup> The anal glands are also observed in human anal canal.<sup>9</sup> Although

several investigations have been reported on these glands in humans,<sup>10-13</sup> their functional significance is mostly unexplained.

Within various saccharide residues, sialic acids in particular possess diverging functional properties. They are involved in the regulation of many cellular and molecular interactions and contribute to the high viscosity of mucus.<sup>14,15</sup> Additionally, in mammalian integument, some antimicrobial substances are released in response to stimuli and rapidly become available at sites of microbial invasion.<sup>16-18</sup> In certain domesticated mammals, secretory glycoconjugates including sialic acids and antimicrobial substances elaborated by the skin glands may have functional significance in preserving skin integrity.<sup>19-22</sup> However, detailed localization of sialic acids and antimicrobial substances in the anal glands of dogs has not been determined.

The present study on the canine anal glands investigated the distribution of sialic acids and antimicrobial substances using glycoconjugate histochemical and immunohistochemical methods. The results obtained are believed to provide data on the specific functional properties of this gland type, not only in the dog but in other mammalian species as well.

### Materials and Methods

All experiments were performed in accordance with the guidelines for the care and use of laboratory animals at the Institute of Experimental Animal Science, College of Bioresource Sciences, Nihon University. Four adult male Beagle dogs (1.5-2 years, 10-12 kg) were deeply anesthetized and then exsanguinated from the common carotid arteries. After bloodletting, the anal region was removed surgically. The tissue specimens were fixed in Bouin's solution for 24 h at room temperature, then carefully washed and dehydrated through a graded series of ethanol. The materials were embedded in paraffin wax and then sectioned at a thickness of 5  $\mu$ m. Sections were stained with hematoxylin-eosin (H-E) and Mallory-Azan as well as with sialoglycoconjugate histochemical and immunohistochemical procedures including combined control procedures.

For the localization of sialic acid, the histochemical staining method employed was periodic acid-phenylhydrazinium chloride-thiocarbohydrazide-silver proteinate-physical development (PA-P-TCH-SP-PD).<sup>23</sup> The PA-P-TCH-SP-PD procedure is an efficient and sensitive histochemical method for demonstration of sialic acids using physical development procedure in light microscopy. Selective periodate oxidation (0.4 mM PA in 1 M HCl) of sialic acid residues oxidizes monoaldehyde at C-7 or C-8,

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which then condenses with phenylhydrazine to give rise to the corresponding phenylhydrazone. This compound can undergo solvolysis to yield TCH-SP-PD reactive monoaldehydes. Although the selective periodate oxidation also turns neutral sugars into the dialdehydes, they condense with phenylhydrazine to result in TCH-SP-PD unreactive azido or morpholine. On the other hand, sialic acids with O-acetyl substitutions at C-8 and/or C-9 are undetected by this procedure. Therefore, chemical modification by saponification (0.5% potassium hydroxide-70% ethanol) (KOH)<sup>24</sup> was performed on some sections, prior to PA-P-TCH-SP-PD staining, since KOH treatment is known to remove the acetyl groups on C-4, C-7, C-8 and C-9.<sup>25</sup> Enzyme digestion with sialidase (from *Arthrobacter ureafaciens*, Nacalai Tesque Inc., Kyoto, Japan; 1.0 U/mL in 0.1 M phosphate buffer, pH 5.3, at 37°C for 18 h)<sup>26</sup> after saponification was conducted before the PA-P-TCH-SP-PD staining. Neuraminidases from *A. ureafaciens* can hydrolyze  $\alpha$ 2-3,  $\alpha$ 2-6 and  $\alpha$ 2-8 linkages.<sup>27</sup> However, O-acetylated sialic acids, especially at C-4, resist hydrolytic cleavage by bacterial neuraminidase.<sup>28</sup> These physical development procedures for detection of sialic acids and their moieties are summarized in Table 1.<sup>29-31</sup> Additionally, some sections were incubated with biotinylated *Sambucus sieboldiana* agglutinin (SSA) and *Maackia amurensis* agglutinin (MAM) (Seikagaku Kogyo Co., Tokyo, Japan) at concentrations of 10-20  $\mu$ g/mL in 0.05 M phosphate-buffered saline (PBS) (pH 7.2) for 60 min at room temperature, following treatment with 0.3% hydrogen peroxide to suppress endogenous peroxidase (PO) activity and then preincubation with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. The lectin specificities and inhibitory sugars are listed in Table

2.<sup>32</sup> After rinsing with PBS, the sections were soaked in PO-labeled streptavidin (Nichirei Co., Tokyo, Japan) for 30 min at room temperature. The activity of the PO employed for labeling was evaluated with a solution containing 0.05 M Tris-HCl buffer (pH 7.6), diaminobenzidine (DAB) and hydrogen peroxide. Lectin controls were prepared by addition of inhibitory sugars at a final concentration of 0.01 M to the respective lectin solutions, by substitution of unconjugated lectins for biotinylated lectin conjugates or by exposure of sections to the PO-DAB system without lectin

For the immunohistochemical methods, the sections were preincubated with Proteinase K solution (Dako, Glostrup, Denmark) for 3 min at room temperature and treated with 0.3% hydrogen peroxide for 30 min for the blocking of endogenous PO activities. Then, they were incubated with primary antibodies diluted in 0.01 M PBS (pH 7.3) against IgA (polyclonal, from rabbit) (Dako), lysozyme (polyclonal, from rabbit) (Dako), lactoferrin (polyclonal, from rabbit) (MP Biomedicals, Ohio, USA) and human  $\beta$ -defensin-1 (polyclonal, from rabbit) (Biologo, Kronshagen, Germany) for 24 h at 4°C, following pretreatment with 1% BSA in PBS for 30 min at room temperature. The primary antibodies were diluted as follows: IgA 1:100, lysozyme 1:100, lactoferrin 1:100, human  $\beta$ -defensin-1 1:1000. After rinsing with PBS, these sections were incubated with prediluted dextran-polymer-conjugated secondary antibodies (EnVision+ Dual Link System-HRP, raised goat against mouse and rabbit immunoglobulins, Dako) for 30 min at room temperature. The immunoreaction employed for labeling was revealed using a DAB hydrogen peroxide system, as described above. Controls for immunohistochemical methods were performed by incubation with PBS without primary antibodies or exposure of sections to the PO-DAB system without primary or secondary antibodies.

## Results

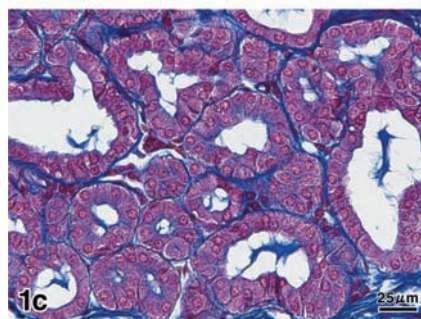
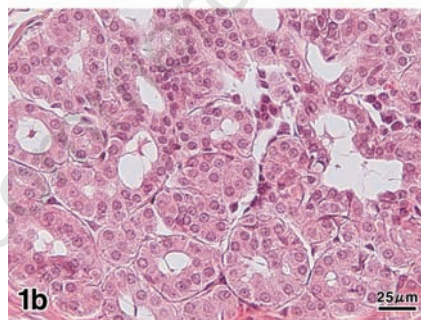
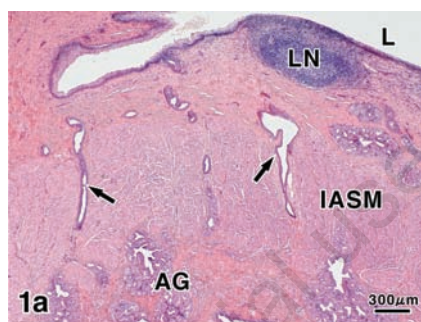
In the anal canal of dog, the anal glands occupied the submucosa of the columnar and intermediate zones covered with stratified squamous epithelium or outside the internal anal sphincter muscle (Figure 1a). The anal glands were tubuloalveolar glands (Figure 1b,c) and their longer excretory ducts passed through the muscle layers, and directly opened on to the epithelial surface. Additionally, the secretory portions and excretory ducts of these glands were often dilated. The PA-P-TCH-SP-PD procedure, which demonstrates the localization of sialic acid, produced moderate to weak reactions in the secretory cells and excretory duct cells, while the luminal secretions reacted very strongly (Figure 2a). Saponification clearly increased

**Table 1. Physical development procedures for detection of sialic acids and their moieties.**<sup>21,30,31</sup>

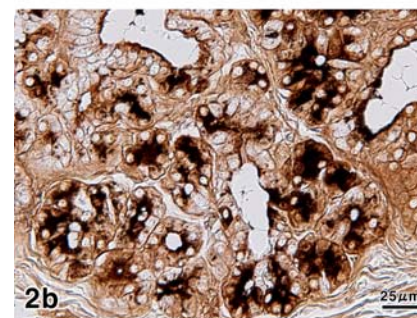
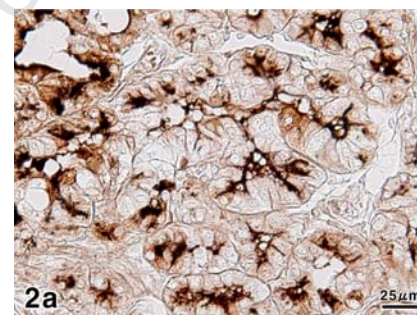
Staining method	Moiety detected by the reaction
PA-P-TCH-SP-PD	Sialic acids without side-chain substituent and with O-acetyl substitutions at C-7
KOH-PA-P-TCH-SP-PD	Sialic acids without side-chain substituent and with O-acetyl substitutions at C-7, C-8 and/or C-9
KOH-Sial- PA-P-TCH-SP-PD	Not detected

**Table 2. The lectins used and their sugar residues and inhibitory sugars.**

Lectins	Abbreviation	Sugar binding specificity	Inhibitory sugars
<i>Sambucus sieboldiana</i> agglutinin	SSA	Sia $\alpha$ 2-6Gal/GalNAc	$\alpha$ 2-6sialyllactose
<i>Maackia amurensis</i> agglutinin	MAM(MAL)	Sia $\alpha$ 2-3Gal1-4GlcNAc	$\alpha$ 2-3sialyllactose



**Figure 1. General structure of the anal region in the dog. a) H-E,  $\times 25$ . AG, acini of anal glands; IASM, internal anal sphincter muscle; L, lumen of alimentary canal; LN, lymph nodule; arrows, excretory ducts; b) higher magnification of the anal glands, H-E,  $\times 320$ ; c) higher magnification of the anal glands, Mallory-Azan,  $\times 320$ .**



**Figure 2. Visualization of sialic acids in the anal glands. a) PA-P-TCH-SP-PD,  $\times 320$ ; b) KOH-PA-P-TCH-SP-PD,  $\times 320$ ; c) KOH-Sial-PA-P-TCH-SP-PD,  $\times 320$ .**

the intensity of the PA-P-TCH-SP-PD reaction (Figure 2b). The PA-P-TCH-SP-PD staining intensity was nearly negative after sialidase digestion following saponification (Figure 2c). When the sections were subjected to the lectin histochemical procedures, the SSA technique resulted in strong to moderate reactions of the secretory cells and luminal secretions (Figure 3a). A moderate reaction was observed in the excretory ducts after the application of SSA. In the sections stained for MAM, the luminal secretions and excretory duct cells showed distinct positive reactions (Figure 3b). The MAM staining patterns of the secretory cells were similar to the results obtained by the PA-P-TCH-SP-PD staining, showing a moderate to weak reaction (Figure 3c). In all of the control experiments using the lectin histochemical procedures, the positive lectin reactions were greatly diminished or nearly abolished in the glandular structures (Figure 3d). The results obtained for the sialoglycoconjugate histochemical staining are summarized in Table 3.

For the localization of antimicrobial substances, antibodies to IgA showed strong to moderate reactions in the luminal secretions and excretory ducts (Figure 4a). The luminal surface and apical cytoplasm of the secretory cells were mainly found to exhibit distinct positive reactions after treatment with antibodies to IgA (Figure 4b). Additionally, in some of the secretory cells, the cytoplasm was stained strongly. A strong positive reaction was also observed in the plasma cells, which were distributed in the connective tissues surrounding the anal glandular acini (Figure 4b). When the tissue sections were incubated with anti-lysozyme, the staining intensity differed with the secretory cells showing moderate to very weak positive reactions (Figure 4c). The luminal secretions and excretory ducts exhibited moderate to weak positive reactions. A moderate positive reaction for lactoferrin was detectable in the secretory epithelium, whereas the luminal secretions showed a weak reaction (Figure 4d). The reaction intensity of the excretory ducts was strong to moderate. Incubation with antibodies to  $\beta$ -defensin 1 led to the same staining images as those obtained with antibodies to lactoferrin (Figure 4e). In control experiments for immunohistochemical staining performed by incubation with PBS without primary antibodies or exposure of sections to the PO-DAB system without primary or secondary antibodies, no glandular structures exhibited any positive reactions (Figure 4f). These results obtained from the immunohistochemical staining for antimicrobial substances are summarized in Table 4.

## Discussion

In mammals, many studies have revealed that the salivary glandular secretions protect the oral cavity as the entrance of the digestive system.<sup>33-35</sup> In contrast, there have been few studies with regard to the functional properties of the secretory products elaborated by the anal glands, which are distributed in the terminal region of the alimentary tract. According to our previous study, the secretory epithelium of

the canine anal glands and their excretory duct system contain large amounts of neutral glycoproteins.<sup>8</sup> In particular,  $\alpha$ -L-fucose residues, which are prominent components of the glandular secretions, may modify or control the viscoelastic properties of the mucus in order to form a stable mucous coat of the rather dry feces. Additionally, the excretory duct systems are deeply involved in the maturation of the secretions. The present examination disclosed the distribution of sialoglycoconjugates and antimicrobial substances in these glands, as

**Table 3. Sialoglycoconjugate histochemical reactions of the anal glands in dog.**

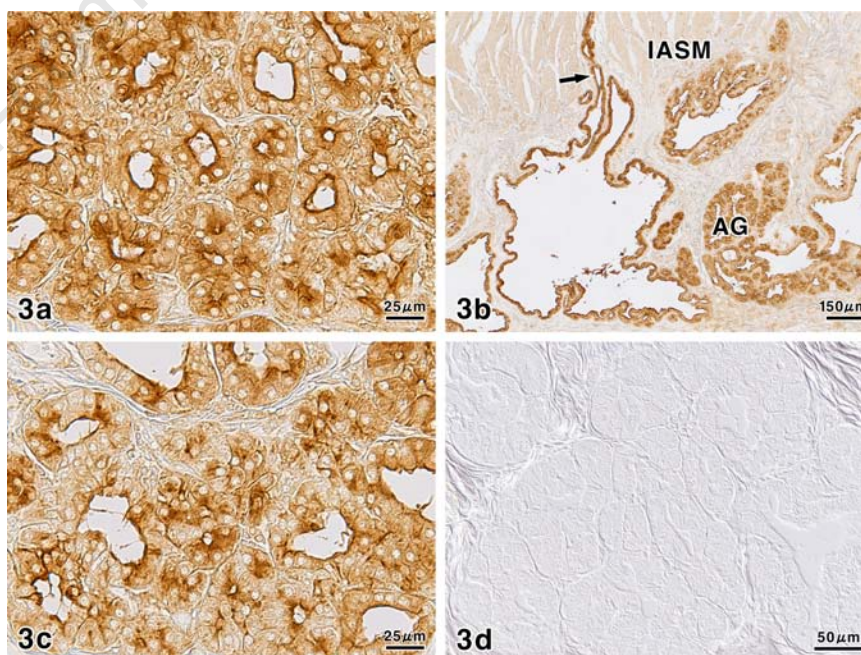
Stainings	Secretory cells	Luminal secretions	Excretory ducts
PA-P-TCH-SP-PD	1-3	4	1-2
KOH-PA-P-TCH-SP-PD	3-4	4	3
KOH-Sial-PA-P-TCH-SP-PD	0	0	0
SSA	3-4	3-4	3
MAM(MAL)	1-3	3-4	4

Reaction intensities: 0, negative; 1, very weak; 2, weak; 3, moderate; 4, strong.

**Table 4. Immunohistochemical reactions of antimicrobial substances in the anal glands of dog.**

Substances	Secretory cells	Luminal secretions	Excretory ducts
IgA	1-4	3-4	3-4
Lysozyme	1-3	2-3	2-3
Lactoferrin	3	2	3-4
$\beta$ -Defensin 1	2-3	2	3

Reaction intensities: 0, negative; 1, very weak; 2, weak; 3, moderate; 4, strong.

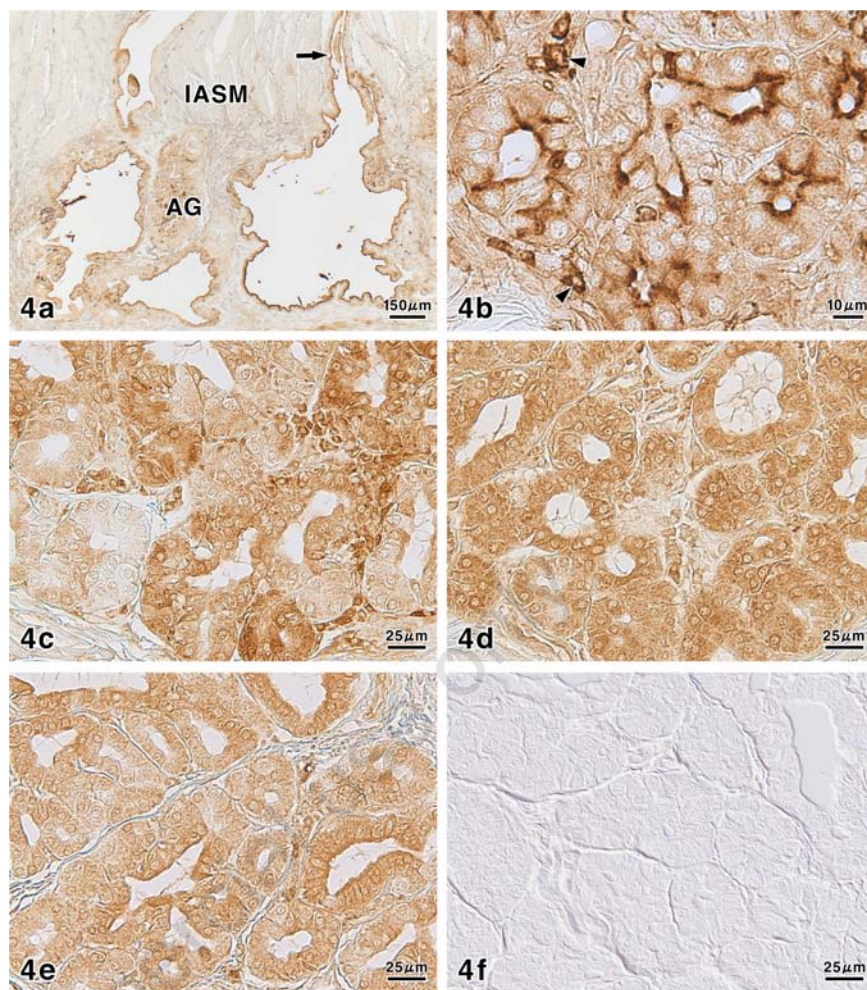


**Figure 3. Lectin histochemical staining in the anal glands. a) SSA,  $\times 320$ ; b) MAM,  $\times 50$ , AG: acini of anal glands, IASM: internal anal sphincter muscle, arrow: excretory duct; c) MAM,  $\times 320$ ; d) control,  $\times 200$ .**

supported by the PA-P-TCH-SP-PD, lectin histochemical and immunohistochemical procedures.

Saponification caused an increase of PA-P-TCH-SP-PD reactivity, indicating the presence of sialic acids with O-acetylated substitutions at C-8 and/or C-9.<sup>29-31</sup> In addition, lectin histochemical results of SSA and MAM represent the localization of sialic acid residues, linked to  $\alpha$ 2-6Gal/GalNAc and  $\alpha$ 2-3Gal1-4GlcNAc, respectively. O-Acetylated sialic acids are known to reduce sialidase activity and extend the lifespan of serum glycoproteins.<sup>14,15</sup> Sialoderivatives, with variation in their degree of acetylation and the types of linkages and acceptor sugars, may play an important role in defense against pathogenic agents,<sup>14,36,37</sup> because, for example, influenza viruses differ in their ability to recognize Sia-Gal linkages depending on the animal hosts.<sup>38</sup>

The antimicrobial substances identified during the course of our study are generally part of the innate immunity system and of great importance for mucosal integrity by their antibacterial activities. They are widely represented in mucous secretions, including tears, saliva, colostrum and secretions from the genitourinary tract, gastrointestinal tract, prostate and respiratory epithelium. In this context, lysozyme is widely known as a remarkable bacteriolytic element found in tissues and secretions.<sup>39</sup> This enzyme is capable of direct bacteriolytic action because it hydrolyzes  $\beta$ -(1,4)-glycosidic bonds between N-acetylglucosamine and N-acetyl- $\beta$ -D-glucosamine, which are commonly present in the peptidoglycan of bacterial cell walls.<sup>40,41</sup> Furthermore, lysozyme has a close functional relationship to immunoglobulins and lactoferrin.<sup>41</sup> Secretory IgA consists of polymeric IgA containing J chain and a distinctive polypeptide known as secretory component, and is a predominant immunoglobulin in external secretions of humans and various other mammals.<sup>42</sup> IgA with J chain is produced by plasma cells. Then, secretory IgA is transported through the lateral borders and apically in the cytoplasm of secretory epithelial cells into external secretions.<sup>43-45</sup> Our results are consistent with the above-mentioned view of the secretory mechanism of IgA. Lactoferrin has a broad spectrum of antimicrobial activity against bacteria, fungi and viruses.<sup>46,47</sup> The antimicrobial properties of lactoferrin are imparted by two distinct mechanisms. The bacteriostatic function is caused by the strong iron binding properties of protein.<sup>48,49</sup> On the other hand, lactoferrin also possesses bacteriocidal function because of a direct antimicrobial effect against microorganisms.<sup>50-53</sup> In addition, lactoferrin inhibits allergen-induced skin inflammation, which is secondary to its role in regulating the production of cytokines.<sup>54,55</sup> In mammals,  $\beta$ -defensins are produced by a vari-



**Figure 4. Immunohistochemical staining of antimicrobial substances in the anal glands.** a) IgA,  $\times 50$ . AG, acini of anal glands; IASM, internal anal sphincter muscle; arrow, excretory duct; b) IgA,  $\times 640$ ; arrowheads, plasma cells; c) lysozyme,  $\times 320$ ; d) lactoferrin,  $\times 320$ ; e)  $\beta$ -defensin 1,  $\times 320$ ; f) control,  $\times 320$ .

ety of epithelial cells and phagocytes, and often present at high concentration.<sup>56,57</sup> They have the capability of insertion into cell membranes, depending on their electrical forces. Consequently, most defensins cause the destruction of microorganisms, for example, bacteria, fungi and viruses.<sup>17,18,56,57</sup> The canine  $\beta$ -defensin gene family consists of 43 members on the basis of sequence similarity with other mammalian  $\beta$ -defensins.<sup>48</sup> The gene lineage and amino acid identity of canine  $\beta$ -defensin 1 resemble those of human  $\beta$ -defensin 1.<sup>58,59</sup>

In conclusion, the present study revealed the presence and distribution of sialoglycoconjugates and antimicrobial substances in the anal glands of dog. It is suggested from our findings that the glandular secretions elaborated by the canine anal glands are deeply involved not only in regulation of the mucous viscoelastic properties or scent marking but also in effective defense activities in the anal

mucosa, as the terminal segment of the digestive tract. Such features are important to understand the biologically relevant characteristics of this gland type in mammalian species.

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