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Probiotic supplementation affects the glycan composition of mucins secreted by Brunner's glands of the pig duodenum☆

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ABSTRACT

The effect of a dietary probiotic blend on the carbohydrate composition of mucins secreted by the Brunner's glands in the duodenum of growing-finishing pigs was investigated by means of conventional (periodic acid-Schiff, Alcian Blue pH 2.5, high iron diamine staining) and lectin (15 lectins) histochemistry. Pigs were assigned to two dietary treatments: a control basal diet without the probiotic blend (No-Pro) and a test diet that included the probiotic blend (Pro). Duodenal tissue fragments were fixed in 4% phosphate-buffered-saline-buffered paraformaldehyde, dehydrated through a graded alcohol series, and embedded in paraffin wax. The secretory cells of the Brunner's glands from No-Pro pigs primarily produced neutral glycoproteins and a small amount of acidic non-sulphated mucins. This glycan pattern was opposite that of the Brunner's glands from Pro animals. A comparison of lectin-binding profiles of the secretory cells of Brunner's glands in these two groups showed that in Pro pigs, there was (i) a decrease in N-linked glycans containing α1,2-linked fucose (Con A, UEA I); (ii) a loss of complex types of *N*-glycans (PHA-L, PHA-E) terminating with lactosamine (RCA_{120}), α1,6- and α1,3-linked fucose (LTA), and α-galactose (GSA I-B_4), as well as of *O*-glycans with terminal Galβ1,3GalNAc (PNA); and (iii) an increase in *O*-glycans containing GalNAc HPA. No-Pro and Pro samples showed no change in the expression of α2,6 sialoglycans and terminal GlcNAc residues and no affinity for MAL II, DBA, and SBA. These results indicate that probiotic supplementation affects the glycan composition of mucins produced in the Brunner's glands of growing-finishing pigs. These changes could effectively act on the gastrointestinal function and health status of these animals because the probiotic blend induced higher growth performance and meat quality in the test probiotic group than it did in the control basal diet group (Tufarelli et al., 2017).
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1. Introduction

Brunner's glands, or duodenal glands, are specific to mammals and are located in the submucosa of the duodenal wall, although they can be found, even if discontinuously, in the jejunum of big herbivores and pigs (Krause, 2000). Brunner's glands are mucin-secreting tubulo-alveolar glands. Mucins constitute a family of densely glycosylated proteins, the high glycosylation giving them gel-like properties and the ability to resist proteolysis and hold water (Perez-Vilar and Hill, 1999). The highly viscous mucus secreted by Brunner's glands protects the underlying mucosa from mechanical insults, neutralizes the acidity of gastric juice (Florey and Harding, 1933), modulates absorption of ingesta, inhibits attack by pathogens, and maintains bacterial microflora (Krause, 2000; Flemstrom and Isenberg, 2001).

The importance of the intestinal microbiota for gastrointestinal function and health has been shown in many studies (Heinritz et al., 2013; Büsing and Zeyner, 2015; Hu et al., 2015; Liu et al., 2017). In particular, probiotics have a stimulating effect on the digestive processes and immunity of animals (Fuller, 2006; Meng et al., 2010). Therefore, their use is suggested as an alternative to antibiotics or anti-inflammatory drugs. However, the mode of action of probiotics is poorly understood and the reported mechanisms of action are often the results of *in vitro* experiments. These results should there-

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fore be confirmed by *in vivo* studies (Oelschlaeger, 2010). More recently, the use of a probiotic complex (Sivoy™, SLAB51) has been shown to enhance the growth performance and meat quality of growing-finishing pigs and to reduce pollution from animal excreta (Tufarelli et al., 2017).

Despite the physiological importance of the mucins secreted by Brunner's glands, to date nothing is known about the effects of microbiota on the composition of the mucins secreted by these glands. Previous studies have, however, demonstrated that the composition of mucins from Brunner's glands could be affected by dietary change (see Krause, 2000, for reference).

The aim of the present study was to examine the *in situ* effect of a dietary probiotic complex (Sivoy™, SLAB51) on the glycan composition of mucins produced in the Brunner's glands of growing-finishing pigs. We used both conventional and lectin histochemistry. The conventional technical approach discriminates neutral and acidic classes of glycoconjugates, whereas lectins allow analysis of the carbohydrate composition of complex glycans (Spicer and Schulte, 1992; Sharon and Lis, 2004). The investigation was carried out in pigs. Because of their anatomical, physiological, and genetic comparability to humans, they represent a promising animal model to determine questions of basic, applied, and translational biomedical research (Aigner et al., 2010; Stramandinoli-Zanicotti et al., 2014), including studies of human nutrition and health problems (Guilloteau et al., 2010; Verma et al., 2011; Prather et al., 2013; Gonzalez et al., 2015).

2. Materials and methods

2.1. Probiotic sources

The probiotic preparation used in the present trial was obtained from a commercial company (SLAB51, Mendes SA, Lugano, Switzerland). The probiotic SLAB51 is composed of a blend of the following strains: *Streptococcus thermophiles* DSM 32245, a mixture of the two strains *Bifidobacterium animalis* ssp. *lactis* DSM 32246 and DSM 32247, *Lactobacillus acidophilus* DSM 32241, *Lactobacillus helveticus* DSM 32242, *Lactobacillus paracasei* DSM 32243, *Lactobacillus plantarum* DSM 32244, and *Lactobacillus brevis* DSM 27961.

2.2. Animals

The trial received ethical approval from the Italian Ministry of Health (n.597/2015-PR del 23/06/2015) and was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (Art. 18 D.L. 4 March 2014, no. 26).

Twenty pigs [(Landrace × Yorkshire) × Talent] with an average initial body weight (BW) of 22.80 ± 0.95 kg (SE) were used in a 12-week experiment. Pigs were assigned to two dietary treatments: the control basal diet without the probiotic blend (No-Pro) and the experimental diet that included the probiotic blend (Pro). The probiotic mixture was used as a dietary supplement for the pigs during the entire feeding period at a dose of 100 mg/kg of BW. The basal diet was formulated to meet or exceed the nutrient requirements of pigs according to the NRC (1998). Pigs were housed in an environmentally controlled room with a concrete floor and were fed *ad libitum*.

2.3. Sampling and histology processing

At the end of the trial, pigs were slaughtered and specimens of duodenal tissue were immediately removed from 5 cm of the caudal part of the pyloric region and fixed in 4% (v/v) phosphate-buffered-saline-buffered paraformaldehyde for 24 h at 4 °C. The samples were then dehydrated through a graded alcohol series and embedded in paraffin wax. Serial sections (4-μm thick) were cut and, after being de-waxed with xylene and hydrated in an ethanol series of descending concentrations, stained with hematoxylin-eosin for morphological and morphometric studies and by conventional histochemical procedures or lectin histochemistry for glycoconjugate characterization.

2.4. Conventional histochemistry

Sections were treated with (1) periodic acid-Schiff (PAS) reaction for neutral glycoconjugates (Mc Manus, 1948); (2) Alcian Blue pH 2.5 (AB 2.5) for sulphate esters and carboxyl groups in glycoconjugates (Pearse, 1968); and (3) combined high iron diamine-Alcian Blue pH 2.5 (HID-AB 2.5) for simultaneous staining of sulphated (brown-black) and non-sulphated (blue) acidic glycans (Spicer, 1965). To reveal cellular combinations of both acidic and neutral glycoconjugates, we performed AB 2.5/PAS and HID/AB 2.5 staining sequences.

2.5. Lectin histochemistry

The binding of 15 lectins was tested to investigate the composition and distribution of oligosaccharidic chains in the Brunner's glands of the pigs (Table 1). All lectins were purchased from Vector Laboratories Inc. (Burlingame, CA, USA).

Tissue sections stained with fluorescent lectins were rinsed in 0.05 M Tris–HCl-buffered saline (TBS) pH 7.4 and incubated in appropriate dilutions of each lectin diluted in TBS (Table 1) for 1 h at RT in the dark. After three rinses in TBS, slides were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Tissue sections stained with biotinylated MAL II were immersed in 3% v/v solution of H_2O_2 in methanol for 10 min to suppress the endogenous peroxidase activity, rinsed in TBS pH 7.4, and incubated in a lectin solution (25 μg/ml for 1 h at RT in the dark). After three rinses in TBS, the sections were treated with streptavidin/peroxidase complex for 30 min and subsequently with 0.05% (w/v) 3,3-diaminobenzidine (Vector Laboratories, Burlingame, CA, USA) plus 0.003% (v/v) H_2O_2 in 0.05 M TBS (pH 7.6) for 10 min. Sections were dehydrated and mounted using Eukitt.

Each experiment was repeated twice for each sample. Controls for lectin staining included (1) substitution of the substrate medium with buffer without lectin and (2) incubation with each lectin in the presence of its hapten sugar (0.5 M in TBS). All control experiments gave negative reactions. Slides were observed with the light photomicroscope Eclipse Ni-U (Nikon, Japan) at 20× magnification and photographed with a digital camera (DS-U3, Nikon, Japan). The images were analyzed by the image-analyzing program NIS Elements BR (Version 4.20) (Nikon, Japan).

2.6. Morphometry and statistical analysis

The diameter of Brunner's gland adenomeres from both No-Pro and Pro samples was measured on 15 microphotographic fields casually detected and photographed with a digital camera (DS-U3, Nikon, Japan) connected to the light photomicroscope Eclipse Ni-U (Nikon, Japan), using a 20× lens. Images were analyzed by the image-analyzing program NIS Elements BR (Vers. 4.30) (Nikon, JP). Each field surface was 140,000 μm^2. We measured the diameter of 450 transversally cut adenomeres of Brunner's gland from the No-Pro and Pro samples. Values were expressed as means ±SD. The results were evaluated for statistical significance by Student's t test and the compared data were considered statistically significant at p values of <0.01.

Table 1
Lectins used, their sugar specificities, and the inhibitory sugars used in control experiments.

Lectin abbreviation	Source of lectin (μg/ml)		Sugar specificity	Inhibitory sugar
MAL II[a]	*Maackia amurensis*	25	NeuNAcα2,3Galβ1,3(±NeuNAcα2,6)GalNAc	NeuNAc
SNA	*Sambucus nigra*	15	Neu5Acα2,6Gal/GalNAc	NeuNAc
PNA[b]	*Arachis hypogaea*	25	Galβ1,3GalNAc	β-D-Gal
RCA_{120}	*Ricinus communis*	20	Galβ1,4GlcNAc	Gal
GSA I-B_4	*Griffonia simplicifolia*	20	αGal	Gal
DBA	*Dolichos biflorus*	25	GalNAcα1,3(LFucα1,2)Galβ1,3/4GlcNAcβ1	D-GalNAc
SBA[b]	*Glycine max*	20	α/βGalNAc	D-GalNAc
HPA	*Helix pomatia*	20	αGalNAc	D-GalNAc
Con A	*Canavalia ensiformis*	15	αMan>αGlc	Man
PHA-E	*Phaseolus vulgaris*	20	Galβl,4GlcNAcβ1,2Manα1,6	Man
PHA-L	*Phaseolus vulgaris*	20	GlcNAcβl,2Man, triantennary complex oligosaccharides	Man
succWGA[b]	*Triticum vulgaris*	15	βGlcNAc	D-GlcNAc
GSA II	*Griffonia simplicifolia*	20	D-GlcNAc	D-GlcNAc
UEA I	*Ulex europaeus*	20	L-Fucα1,2Galβ1,4GlcNAcβ	α-L-Fuc
LTA	*Lotus tetragonolobus*	25	L-Fucα1,6GlcNAc L-Fucα1,2Galβ1,4[L-Fuc1,3] GlcNAcβ1,6R	 α-L-Fuc

Fuc: fucose, Gal: galactose, GalNAc: *N*-acetylgalactosamine, Glc: glucose, GlcNAc: *N*-acetylglucosamine, Man: mannose, NeuNAc: *N*-acetylneuraminic (sialic) acid, succ: succinylated WGA.
[a] Biotin-labeled lectin.
[b] Rhodamine-labeled lectin. Non-marked lectins are fluorescein isothiocyanate-labeled lectins.

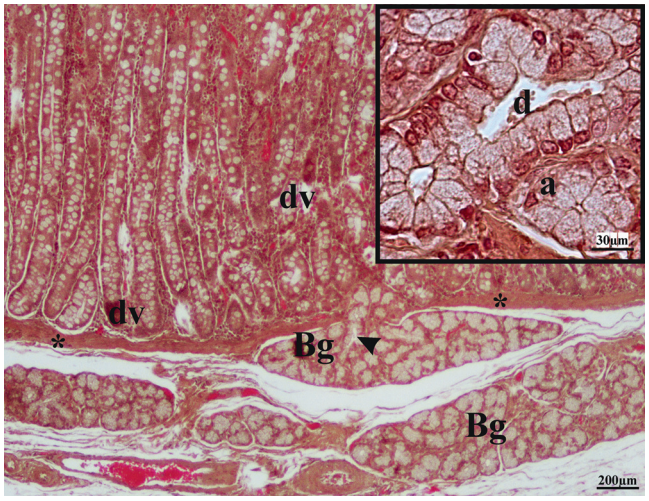

Fig. 1. Light micrograph of pig duodenum showing Brunner's glands (Bg) and duodenal villi (dv). Note the duct (arrowhead) of a Brunner's gland piercing the muscularis mucosae (asterisk) and entering the overlying duodenal mucosa. Inset displays the details of an acinous adenomere (a) and duct (d). Hematoxylin-eosin staining.

3. Results

3.1. Morphology

Swine Brunner's glands were tubulo-acinous with a main excretory duct opening at the base of the duodenal crypts (Fig. 1). Morphological analysis of Brunner's glands did not reveal significant differences between the No-Pro and Pro samples. There was also no significant difference in adenomere diameter, which measured 42.66 ± 7.38 μm in the No-Pro and 44.3 ± 6.32 μm in the Pro specimens.

3.2. Glycohistochemistry

The results of conventional and lectin staining patterns of Brunner's glands of both No-Pro and Pro pigs are summarized in Table 2.

The combination AB2.5/PAS procedure revealed the widespread presence of PAS-positive (magenta) cells and a few AB 2.5-positive (blue) cells in the Brunner's glands from No-Pro samples (Fig. 2A). This staining pattern was reversed in the Pro group Brunner's glands (Fig. 2B). The combination HID/AB 2.5 methods revealed the absence of HID reactivity (brown) in both Pro and No-Pro Brunner's glands (Fig. 2C,D).

Table 2
Conventional and lectin histochemistry staining pattern of the duodenal Brunner's glands of no-probiotic and probiotic-fed pigs.

	No-probiotic	Probiotic
PAS	+	*
Alcian Blue pH 2.5	*	++
HID	–	–
SNA	+/++lc	+/++lc
PHA-L	+/++lc	–/++lc
PHA-E	±/++lc	–/±lc
succinyl WGA	–/+lc	–/+lc
GSA II	++	++
Con A	++	+
UEA I	+	±
GSA I-B_4	++	–
LTA	+	–
PNA	+	–
RCA_{120}	±	–
HPA	+	++
MAL II	–	–
DBA	–	–
SBA	–	–

Lc: luminal content, *: few reactive cells, –: negative reaction, ±: faintly visible reaction, +: weak positive reaction, ++: intense positive reaction.

Comparison of lectin reactivity in No-Pro and Pro Brunner's glands showed several different binding patterns. Except for MAL II, DBA, and SBA, which were unreactive with Brunner's glands from both No-Pro and Pro pigs, the other lectins bound the glandular cells and/or the luminal content (SNA, PHA-L, PHA-E, succinyl WGA) or only the adenomeric cells (GSA II, Con A, UEA I, GSA I-B4, LTA, PNA, RCA_{120}, HPA). The results are summarized in Table 2.

In particular, SNA displayed no change in the staining intensity of the secretory cells and the adenomeric luminal content of Brunner's glands from both No-Pro and Pro animals (Fig. 3A,B); PHA-L reacted with the adenomeric cells and the luminal content of No-Pro Brunner's glands (Fig. 3C) and with the adenomeric luminal content of treated animals (Fig. 3D); PHA-E bound weakly with the adenomeres and strongly with their luminal content in No-Pro specimens (Fig. 3E), whereas it did not bind the adenomeres but weakly stained the luminal content of treated pigs (Fig. 3F); and succinyl WGA showed no reaction with the secretory cells, whereas it stained the luminal content in all investigated Brunner's glands (Fig. 3G,H).

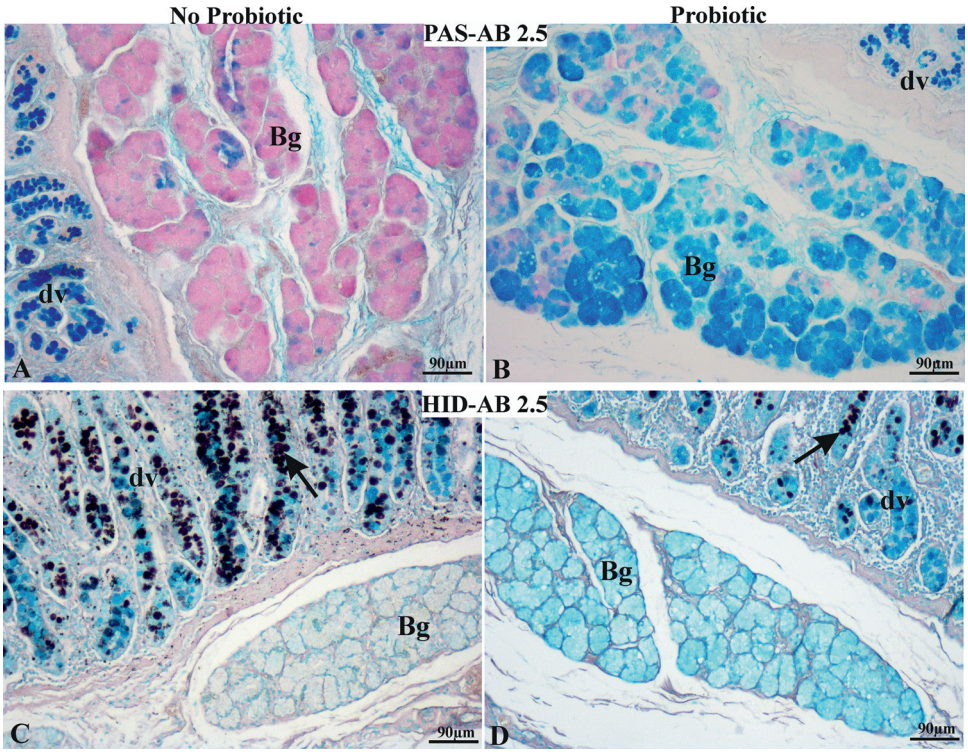

Fig. 2. Duodenum from no-probiotic (A,C) and probiotic-fed pigs (B,D), stained with PAS/Alcian Blue (AB) 2.5 (A,B) and HID/AB 2.5 (C,D) procedures. (A) No-probiotic Brunner's glands exhibit broad PAS positivity (magenta) and a few AB 2.5-positive (blue) cells. (B) Brunner's glands of probiotic-fed pigs show broad AB 2.5 positivity and some PAS-positive cells. (A) and (B) demonstrate AB 2.5-positive goblet cells in duodenal villi (dv). (C,D) HID/AB 2.5 methods revealed the absence of HID (brown) reactivity in the Brunner's glands of both no-probiotic and probiotic-fed animals. The reliability of HID staining was demonstrated by the HID positivity of the goblet cells (arrow) in duodenal villi (dv). Bg, Brunner's gland. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Concerning the lectins that bound only the adenomeric cells, GSA II exhibited no difference in the staining intensity between No-Pro and Pro samples. Con A (Fig. 4A,B) and UEA I (Fig. 4C,D) showed stronger affinity in the No-Pro samples than in the Pro samples. GSA I-B_4, LTA, RCA_{120}, and PNA bound the adenomeric cells of the Brunner's glands from No-Pro pigs, whereas it did not react with the secretory epithelium of Pro Brunner's glands (Fig. 4E,F). HPA was the sole lectin showing a decrease in reactivity in the Brunner's glands of No-Pro pigs compared with that in the Brunner's glands of Pro pigs (Fig. 4G,H).

4. Discussion

This is the first study to demonstrate that feeding of a probiotic complex affects the glycosylation pattern of the mucins secreted by pig Brunner's glands. This investigation follows a recent report demonstrating that the use of a dietary probiotic complex (Sivoy™, SLAB51) enhances growth performance and meat quality in growing-finishing pigs (Tufarelli et al., 2017).

Conventional histochemistry revealed that Brunner's glands of no-probiotic-fed pigs produce primarily neutral glycoproteins (PAS reaction), a small amount of acidic carboxylated mucins (AB 2.5 positivity), and no sulfoglycans (HID negativity). These results are consistent with those of previous studies on the Brunner's glands of several mammals (Krause, 2000; Verdiglione et al., 2002; Schumacher et al., 2004; Scillitani and Mentino, 2015), including young adult pigs (Takehana and Abe, 1986).

Using 15 different lectins, we characterized the carbohydrate composition of the mucins from Brunner's glands more thoroughly. The secretory epithelium of Brunner's glands from No-Pro pigs showed the presence of both *N*- and *O*-linked glycans. *N*-Glycans were high mannose (Con A) and complex types (PHA-L, PHA-E). *O*-Glycans contained the terminal disaccharide Galβ1,3GalNA (PNA) and the simplest mucin *O*-glycan made by a single GalNAc linked to serine or threonine (HPA). Moreover, we observed glycans terminating with fucose (LTA, UEA I), GlcNAc (GSA II), galactose (GSA I-B_4), lactosamine (RCA_{120}), and Neu5Acα2,6Gal/GalNAc (SNA). Although our results agree with previous reports on pig Brunner's glands (Takehana and Abe, 1986; Gelberg et al., 1992), our study provides a more in-depth characterization of the oligosaccharide chains of Brunner's gland glycoproteins because SNA, PHA-L, PHA-E, LTA, and HPA have not been used previously. The luminal content accumulated high mannose and complex types of *N*-glycans as well as glycans with internal GlcNAc (succinyl WGA) and terminal α2,6-linked sialic acid (SNA). The role of sugar residues in the secretory glycoproteins of Brunner's glands is not well known. However, oligosaccharide chains containing lactosamine in high mannosylated *N*-glycans have been observed in the human mucin MUC6 (Toribara et al., 1997). Notably, the gene encoding the latter mucin is also expressed in human Brunner's glands (Bartman et al., 1998). Concerning sialic acid, the negative charge of this molecule has been shown to have a role in the transport of chloride, bicarbonate, water, and protons in Brunner's glands (Collaco et al., 2013). In secreted mucus, sialic acid can contribute to the viscosity and protection of the underlying epithelium from lysis by gastric juice and bacteria (Schauer, 2004) and can act as a ligand for several symbiotic and pathogenic microorganisms (Lehmann et al., 2006). GalNAc and Gal residuals can inhibit the cell binding of the trophozoites of *Entamoeba histolytica*, the causative agent of amoebiasis (Ralston and Petri, 2011). Regarding fucosylated residuals, they can be important in both the maintenance of the bacterial flora involved in the degradation of fucose from food (Becker and Lowe, 2003) and in increasing the viscosity of mucus (Liquori et al., 2012). *O*-Glycans carrying terminal α1,4-linked GlcNAc residues act both as a nat-

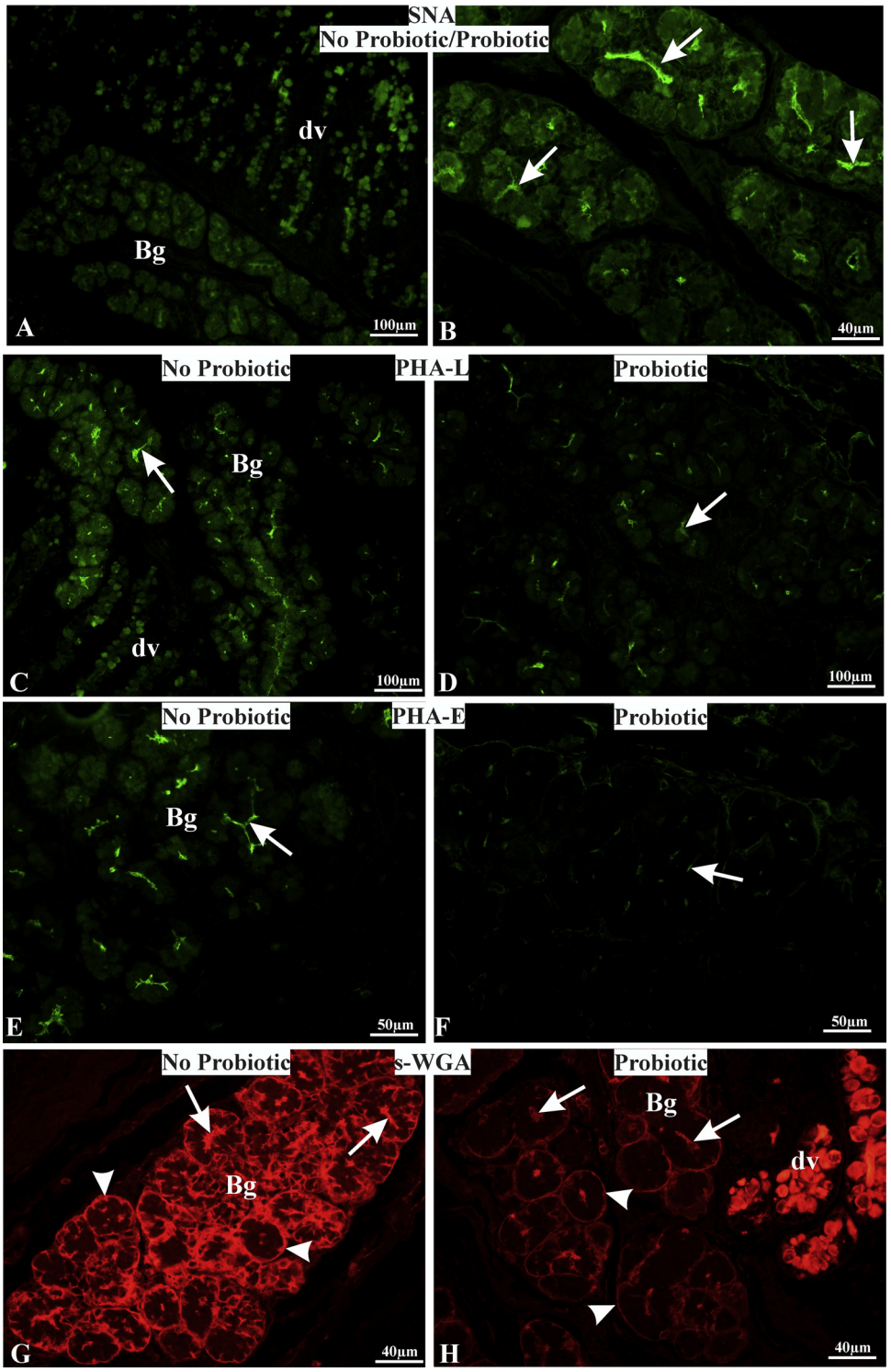

Fig. 3. Lectin binding pattern of Brunner's glands from no-probiotic and probiotic-fed pigs. Note the decreased reactivity of PHA-L and PHA-E in Brunner's gland adenomeres of probiotic-fed animals. Bg, Brunner's gland; dv, duodenal villi with positive goblet cells; arrow, Brunner's gland luminal content; arrowhead, basement membrane. (A–F): FITC-conjugated lectins; (G,H) rhodamine-labeled succinylated WGA.

ural antibiotic and as a tumor suppressor for differentiated-type adenocarcinoma (Nakayama, 2014).

The probiotic blend induced a great change in the glycosylation pathway of the epithelium of Brunner's glands. Conventional histochemistry showed a drastic reduction in neutral mucins and the primary presence of carboxyl acidic glycoproteins. In addition, lectin histochemistry revealed a reduction in high mannose (Con A) and α1,2-linked fucosylated glycans (UEA I) and the disappearance of complex types of *N*-glycans (PHA-L, PHA-E) terminating with lactosamine (RCA_{120}) and fucosylated oligomers binding LTA, as well as O-linked glycans terminating with Galβ1,3GalNAc (PNA) and terminal galactose (GSA I-B_4). Moreover, the luminal content of Brunner's glands exhibited a reduced amount of bisecting GlcNAc- and Gal-bearing glycoproteins (PHA-E) when compared with that from probiotic-fed pigs. However, the dietary probiotic complex increased the synthesis of the most common mucin-type

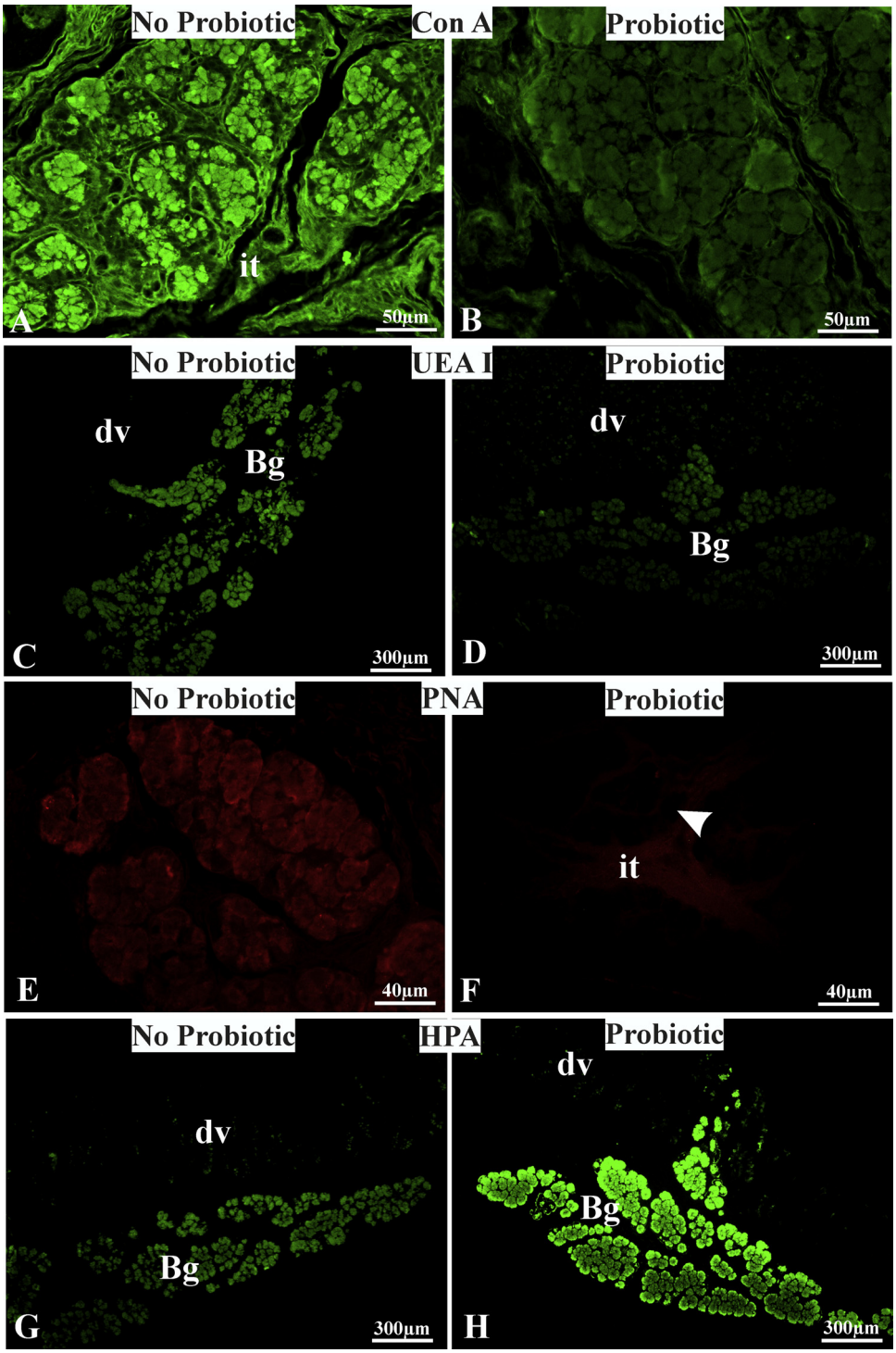

Fig. 4. Lectin binding pattern of Brunner's glands from no-probiotic and probiotic-fed pigs. Note Con A, UEA I, and PNA (B,D,F) decreased reactivity, as well as HPA (H) increased affinity in the Brunner's glands of probiotic fed pigs. Bg, Brunner's gland; dv, duodenal villi; it, interstitial tissue. (A–D,G,H) FITC-conjugated lectins; (E,F) rhodamine-labeled PNA.

O-linked glycans terminating with GalNAc (HPA). These findings are in line with the evidence that components of the diet and the gut microflora are in contact within the intestinal tract and that dietary composition may influence the carbohydrate structures of the mucosal and mucin glycoconjugates with marked consequences for the adherence of microflora and for the gut itself (Kelly et al., 1992). Some studies demonstrated that pre-treatment with probiotics increased the expression of MUC2, MUC5AC, and MUC6 in rat stomach (Caballero-Franco et al., 2007; Lam et al., 2007; Gomi et al., 2013). Moreover, experimental evidence supports the hypothesis of the adaptability of Brunner's gland mucins to dietary changes (Krause, 2000; Desantis et al., 2011).

In conclusion, this study demonstrates that probiotic supplementation affects the glycan composition of the mucins produced in the Brunner's glands of growing-finishing pigs. Since probiotics have a stimulating effect on digestive processes and the immune system of pigs (Meng et al., 2010; Hu et al., 2015; Liu et al., 2017) and the probiotic blend induced higher growth performance and

meat quality than did the control basal diet (Tufarelli et al., 2017), we believe that the observed changes in the composition of the secretory mucins from Brunner's glands could act effectively on the gastrointestinal function and health status of animals. However, further studies are necessary to understand the mechanism of probiotic action on the glycosylation pathway of the mucins secreted by Brunner's glands.

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