

## Comparison of different histological protocols for the preservation and quantification of the intestinal mucus layer in pigs

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### Abstract

The histological characterization of the intestinal mucus layer is important for many scientific experiments investigating the interaction between intestinal microbiota, mucosal immune response and intestinal mucus production. The aim of this study was to examine and compare different fixation protocols for displaying and quantifying the intestinal mucus layer in piglets and to test which histomorphological parameters may correlate with the determined mucus layer thickness. Jejunal and colonic tissue samples of weaned piglets (n=10) were either frozen in liquid nitrogen or chemically fixed using methacarn solution. The frozen tissue samples were cryosectioned and subsequently postfixed using three different postfixatives: paraformaldehyde vapor, neutrally buffered formalin solution and ethanol solution. After dehydration, methacarn fixed tissues were embedded in paraffin wax. Both, sections of cryopreserved and methacarn fixed tissue samples were stained with Alcian blue (AB)-PAS followed by the microscopically determination of the mucus layer thickness. Different pH values of the Alcian Blue staining solution and two mucus layer thickness measuring methods were compared. In addition, various histomorphological parameters of methacarn fixed tissue samples were evaluated including the number of goblet cells and the mucin staining area. Cryopreservation in combination with chemical postfixation led to mucus preservation in the colon of piglets allowing mucus thickness measurements. Mucus could be only partly preserved in cryosections of the jejunum impeding any quantitative description of the mucus layer thickness. The application of different postfixations, varying pH values of the AB solution and different mucus layer measuring meth-

ods led to comparable results regarding the mucus layer thickness. Methacarn fixation proved to be unsuitable for mucus depiction as only mucus patches were found in the jejunum or a detachment of the mucus layer from the epithelium was observed in the colon. Correlation analyses revealed that the proportion of the mucin staining area per crypt area (relative mucin staining) measured in methacarn fixed tissue samples corresponded to the colonic mucus layer thickness determined in cryopreserved tissue samples. In conclusion, the results showed that cryopreservation using liquid nitrogen followed by chemical postfixation and AB-PAS staining led to a reliable mucus preservation allowing a mucus thickness determination in the colon of pigs. Moreover, the detected relative mucin staining area may serve as a suitable histomorphological parameter for the assessment of the intestinal mucus layer thickness. The findings obtained in this study can be used for the implementation of an improved standard for the histological description of the mucus layer in the colon of pigs.

### Introduction

Intestinal mucus is produced by goblet cells and forms a dynamic interface between the external environment and the epithelial surface referred to as the intestinal mucus layer.<sup>1</sup> The intestinal mucus layer serves as the first physical and immunological barrier of the mucosa.<sup>2,3</sup> Mucus contains more than 85% water, its major components are proteins and lipids. The gel structure of intestinal mucus layer is based on secreted mucins. These are complex, hydrophilic proteins with a high number of O-glycosylations forming a substantial part of the mucus protein fraction.<sup>4,5</sup> Due to the labile structure of mucus, a histological description and quantification of the intestinal mucus layer is difficult from a methodological point of view.<sup>6,7</sup> An optimal histological fixation should preserve the original structure of the mucus providing a realistic picture of the natural situation.<sup>8</sup> A large number of methods have been used for mucus depiction including cryopreservation,<sup>9</sup> and common chemical fixation using Carnoy's solution,<sup>7</sup> methacarn<sup>10,11</sup> and buffered paraformaldehyde solution.<sup>12,13</sup> As the fixation process has a strong impact on the preservation of the mucus in histological sections,<sup>14</sup> data regarding the dimension of the mucus layer thickness in humans and animal species differ considerably depending on the applied fixation method. Thus, the mucus thickness of cryopreserved colonic samples of pigs was on average 31.9±17.6 µm although information on the process of postfixation

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were not provided.<sup>9</sup> Chemical fixation by using Carnoy's solution indicated a mucus thickness of 215±24 µm.<sup>15</sup> Similarly, measured mucus thicknesses in the colon of humans ranged from 34.4±8.9 µm after fixation with Carnoy's solution<sup>7</sup> to 107±48 µm in unfixed sections.<sup>16</sup> Due to the difficulties in the direct histological determination of the intestinal mucus layer thickness, different methodological approaches exist to ascertain histological parameters, which may correlate with the thickness of the mucus layer. Thus, the number of goblet cells or the extent of mucin staining area per villus or crypt have been evaluated.<sup>17-19</sup> Apart from methodological variations, the sites of tissue sampling from the alimentary tract<sup>14</sup> as well as factors such as feed composition<sup>1</sup> can influence the thickness of the mucus layer, thus limiting a meaningful comparison between studies. A standard histological methodology for tissue preparation and measurement of the intestinal mucus layer in pigs could facilitate the direct comparison of results between different studies. Moreover, pigs are often used as an animal model for humans in terms of surgical procedures but also for the investigation of cases of human diseases as they have great

similarities to humans regarding their anatomy, genetics and physiology.<sup>20</sup> Studies on mice showed that the intestinal mucus layer seems to play a decisive role in course of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease.<sup>11,21</sup> Hence, the development of a histological protocol for the description of the intestinal mucus layer in pigs as animal model for humans might also help to elucidate causes of such inflammatory diseases in the human gut. Thus, the aim of this study was to examine different histological protocols for the preparation, description and quantification of the intestinal mucus layer in the jejunum and colon of weaned piglets. Different tissue fixations, staining solutions and measuring methods of the mucus layer thickness were included. Moreover, several histomorphological parameters were determined including the intestinal goblet cell number or the intestinal mucin staining area in order to examine correlations between those parameters and the detected mucus layer thickness.

## Materials and Methods

### Animals and housing

Ten weaned piglets (25±2 days of age) were part of a two weeks feeding trial, approved by the local state office of health and social affairs, Landesamt Berlin (LaGeSO Reg. Nr. A 0100/13). The animals were housed in conditioned flat deck pens, each containing a male and a female piglet. Feed and water were offered *ad libitum*. Main components of the complete feed included wheat, barley, corn and soy. At the end of the trial, piglets (39±2 days of age) were anesthetized with 0.25 mL/kg body weight of ketamine hydrochloride (Ursotamin®, 10%ig, Serumwerk Bernburg AG, Bernburg, Germany) and 0.05 mL/kg body weight azaperone (Stresnil®, Jansen-Cilag, Neuss, Germany) and then euthanized with an intracardial injection of 10 mg/kg body weight tetracaine hydrochloride, mebezonium iodide and embutramide (T61®, Intervet, Unterschleißheim, Germany).

### Sampling, prefixation, fixation and histochemical staining

From each of the piglets, samples of the mid jejunum and the ascending colon were either instantly frozen in liquid nitrogen or chemically fixed using methacarn solution (60% methanol, 30% chloroform, and 10% glacial acetic acid, vol/vol/vol). Cryopreserved tissue samples were embedded into TissueTek (Sakura Finetechnical Co., Ltd, Torrance, CA, USA), cut with a microtome-cryostat (Microm HM 560, Microm

International GmbH, Dreieich, Germany) and serial sections were mounted on silanized slides and dehydrated using a heating plate at 50°C for at least 30 min. Cryopreserved tissue samples were then postfixed using three different postfixations: paraformaldehyde vapor (PFA; Merck KGaA, Darmstadt, Germany) incubated six hours at 60°C or 4% neutrally buffered formalin solution (NBF; Carl Roth, Karlsruhe, Germany) for 2 h or precooled 100% ethanol solution (EtOH; Berkel GmbH, Germany) for 1 h at -80°C. Methacarn fixed samples were dehydrated by 100 % methanol (2 x 30 min) followed by 100% ethanol (2 x 20 min), cleared in xylene (2 x 15 min) and infiltrated with solidified paraffin wax.<sup>22</sup> Obtained paraffin blocks were cut in sections using a sledge microtome (type 1400, Leitz, Oberkochen, Germany). Serial sections of cryopreserved samples were stained with AB/PAS (Chroma, Waldeck, Germany) using either pH 2.5 or pH 0.5 in order to evaluate whether pH variation might have an impact on the qualitative and quantitative depiction of the intestinal mucus. The methacarn fixed sections of the colon were deparaffinized with xylene (2 x 10 min) and hydrated by descending concentrations of ethanol (100% - 70%, 3 min) and also stained with AB/PAS pH 2.5.

### Estimation and measurement of the intestinal mucus layer thickness

Mucus thickness was determined by measuring three sites on each section. Each site had at least a 100 µm continuous length of mucus layer with an intact layering of epithelium, mucus and intestinal contents. The mucus layer thickness was determined by comparing the "integral" with the "10 point" measuring method. With respect to the "integral" method, the mucus area was determined by encircling the mucus forming an irregular polygon. In addition, the length of the mucosa, which was located beneath the polygon, was measured. The mucus layer thickness was subsequently calculated by dividing the mucus area by the length of the underlying mucosa. Using the "10 point" method the mucus thickness was measured from the luminal surface of the epithelium to the beginning of intestinal contents at 10 randomly chosen points (30 points per section). Mucus thickness measurements were performed using a light microscope (Axioskop 50, Zeiss, Jena, Germany) equipped with a digital camera (DS-Ri1, Nikon, Tokyo, Japan) and a corresponding analysis program (NIS-Elements 3.22.15, Nikon).

### Evaluation of histomorphological parameters of methacarn fixed colonic tissue samples

Histomorphological analyses were per-

formed in order to correlate histomorphological parameters of methacarn fixed tissue with the mucus layer thickness of cryopreserved tissue. As the mucus layer thickness could not be measured in the jejunum of cryopreserved samples, histomorphological parameters were detected in colonic tissue samples only. In total, 15 vertically oriented crypts per section were analyzed. For each crypt, the crypt depth and crypt area were measured. Furthermore, the absolute number of goblet cells (total number of cells per crypt) and the relative number of goblet cells (goblet cells per 1 mm basement membrane of crypts) were determined. Moreover, the absolute mucin staining area (total mucin staining area per crypt) and the relative mucin staining area (mucin staining area in % of total crypt area) were detected. According to Hedemann *et al.*<sup>23</sup> all mucus cells (goblet cells and crypt secretory cells), their apical secretion as well as the mucus material present in the crypt lumen were taken into account for the determination of the mucin staining area. Histological analyses were conducted with a light microscope (BX 43, Olympus, Hamburg, Germany), which was equipped with a digital camera (DP72, Olympus) and an image analysis program (Cell Sense software, Olympus).

### Statistics

Statistical analyses were performed by using SPSS 22 (IBM, Chicago, IL, USA). Based on serial sections, the different postfixation methods, AB staining solutions and thickness measurement methods were statistically evaluated using the Kruskal-Wallis test because data were not normally distributed based on Shapiro-Wilk test. As mucus thickness determination was not possible following methacarn fixation, the methodological comparison between cryopreservation followed by different postfixations and methacarn fixation was assessed descriptively. Based on Shapiro-Wilk test, data on colonic crypt depth, crypt area, goblet cell number and mucin staining area followed a normal distribution. Pearson correlation analyses were performed illustrating correlations between colonic crypt depth, crypt area, goblet cell number, mucin staining area determined in methacarn fixed samples and the thickness of the mucus layer examined in cryopreserved specimens. Differences at  $P < 0.05$  were considered significant.

## Results

Cryopreservation using liquid nitrogen followed by the application of different chemical postfixations partly preserved the

mucus layer in the jejunum of piglets (Figure 1A). Hence, a microscopic determination of the mucus layer thickness in those samples was not feasible. By using PFA and EtOH as postfixations the jejunal mucus appeared as a patchwork with a decreasing staining intensity (Figure 1A). Mucus patches were not observed by the application of NBF as a postfixative. Chemical fixation by methacarn solution did not preserve the mucus layer in the jejunum. Merely single mucus patches were observed in the close proximity of the epithelium (Figure 1B). In the colon, cryopreservation in combination with chemical postfixation led to an intact layering of mucosa, mucus and colon contents allowing thickness measurements of the mucus layer (Figure 1 C,D). Following methacarn fixation, a detachment of the mucus layer from the intestinal epithelium was generally observed (Figure 1E). Consequently, thickness measurements of the mucus layer could not be performed.

With regard to the effect of the different postfixations, PFA fixation resulted generally in a laminar appearance of the mucus layer (Figure 1D). NBF and EtOH postfixation slightly led to a displacement of the mucus layer so that mucus fragments exceeded epithelial borders and were found within the digesta (Figure 1F). However, PFA, NBF and EtOH as postfixation had no impact on the thickness of mucus layer ( $P=0.406$  and  $P=0.226$ , Figure 2). The mucus in the sections stained with AB pH 2.5 had a darker blue color compared to AB pH 0.5. Varying pH values did not affect the mucus layer thickness in the colon ( $P=0.226$ , Figure 2). The use of the “integral” and the “10 point” measuring method led to comparable results regarding the mucus layer thickness determination ( $P=0.605$ , Figure 2 A,B).

Pearson correlation analyses showed a strong positive correlation between the crypt depth and the crypt area (Table 1) determined in histological slides of

methacarn fixed colonic tissue ( $P\leq 0.001$ ). Moreover, the crypt depth and crypt area correlated with the absolute number of goblet cells ( $P\leq 0.05$ ) and the absolute mucin staining area ( $P\leq 0.001$ ). Furthermore, the absolute number of goblet cells and the absolute and relative mucin staining area was correlated ( $P\leq 0.01$ ;  $P\leq 0.05$ ). With respect to the mucus layer thickness, measured in histological sections of cryopreserved tissue, correlation analyses showed that neither the crypt depth (Pearson coefficient: 0.272;  $P=0.447$ ) and crypt area (Pearson coefficient: 0.360;  $P=0.307$ ) nor the absolute or relative number of goblet cells correlated with the mucus layer thickness (Figure 3 A,B). However, correlation analyses revealed that, in contrast to the absolute mucin staining area, the relative mucin staining area was positively correlated with the mucus layer thickness ( $P\leq 0.05$ ; Figure 3 C,D).

## Discussion

The aim of this study was to develop an improved histological protocol for the preservation of intestinal mucus in histological sections, allowing a reliable determination of the mucus layer thickness in the intestine of piglets. Based on the ascertained mucus layer thickness data, correlation analyses were included in this study evaluating whether histomorphological parameters of chemically fixed tissue samples such as the number of intestinal goblet cells or the intestinal mucin staining area can be used as indirect quantitative indicator for the mucus layer thickness. The histological fixation of intestinal mucus is generally difficult due to its unstable structure.<sup>6</sup> A large number of histological methods exist, aiming to depict the intestinal mucus layer, which impedes the direct comparison of results gained from different studies. The results of this study proved that tissue fixation had a fundamental impact on the intes-

tinal mucus layer visualization. Cryopreservation followed by different postfixations led to reliable mucus preservation in the colon of piglets while mucus was only partly preserved in jejunal samples. Mucus could not be sufficiently preserved by chemical fixation using methacarn neither in the jejunum nor in the colon. Cryopreservation preserves the original state of tissue samples<sup>8</sup> and retained mucus in its position between epithelium and intestinal content. It seems of great importance that intestinal sections are filled with digesta prior to fixation as the intestinal content protects the mucus from being washed off.<sup>24</sup> In this study, tissue samples were not rinsed before cryopreservation or chemical fixation in order to minimize the risk of washing away intestinal content, which might be accompanied with a loss of intestinal mucus.<sup>16</sup> In a former study examining different regions of the gastrointestinal tract of pigs, cryopreservation led to mucus preservation despite washing the intestinal sections in NaCl solution prior to freezing albeit a considerable variability in the measured mucus thickness within the same region was observed.<sup>9</sup> In a previous study comparing cryopreservation and chemical fixation using Carnoy's solution, both procedures did not lead to reliable mucus preservation,<sup>15</sup> although information regarding the process of postfixation of cryopreserved samples were not mentioned. In the current study, chemical fixation using methacarn solution proved to be unsuitable for mucus preservation as only residues of intestinal mucus were observed in the jejunum while the colonic mucus layer was detached from the epithelium impeding any mucus thickness determination. A detachment of the mucus layer from the epithelium following methacarn fixation was also observed in the colon of mice.<sup>10,11</sup> With regard to mucus losses, similarly, Carnoy's solution has been described to be insufficient for a reliable mucus fixation in the small intestine of mice.<sup>25</sup> The physical impact of liquid fixatives might cause the

**Table 1. Pearson correlation coefficients of histomorphological parameters including crypt depth ( $\mu\text{m}$ ), crypt area ( $\text{mm}^2$ ), absolute number of goblet cells (total number of goblet cells per crypt), relative number of goblet cells (goblet cells per 1mm basement membrane of crypts), the absolute mucin staining area (total mucin staining area per crypt in  $\mu\text{m}^2$ ) and the relative mucin staining area (mucin staining area in % of total crypt area), determined in histological slides of methacarn fixed colonic tissue of piglets (n=10).**

	Crypt area	Absolute goblet cell number	Relative goblet cell number	Absolute mucin staining area	Relative mucin staining area
Crypt depth	0.970***	0.649*	-0.613	0.877***	0.178
Crypt area		0.673*	-0.544	0.941***	0.297
Absolute goblet cell number			0.194	0.797**	0.674*
Relative goblet cell number				-0.281	0.510
Absolute mucin staining area					0.599

\* $P\leq 0.05$ ; \*\* $P\leq 0.01$ ; \*\*\* $P\leq 0.001$ .

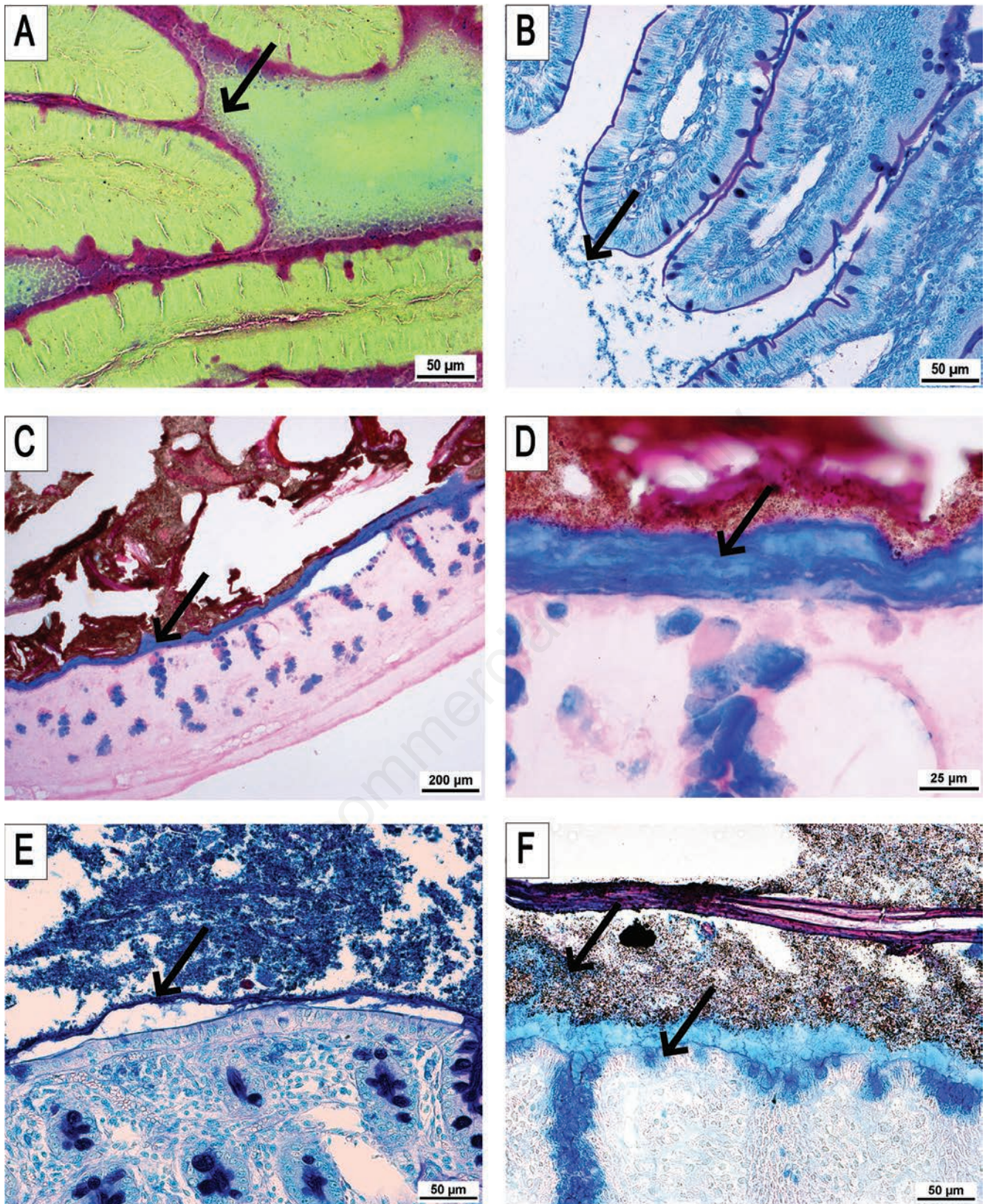
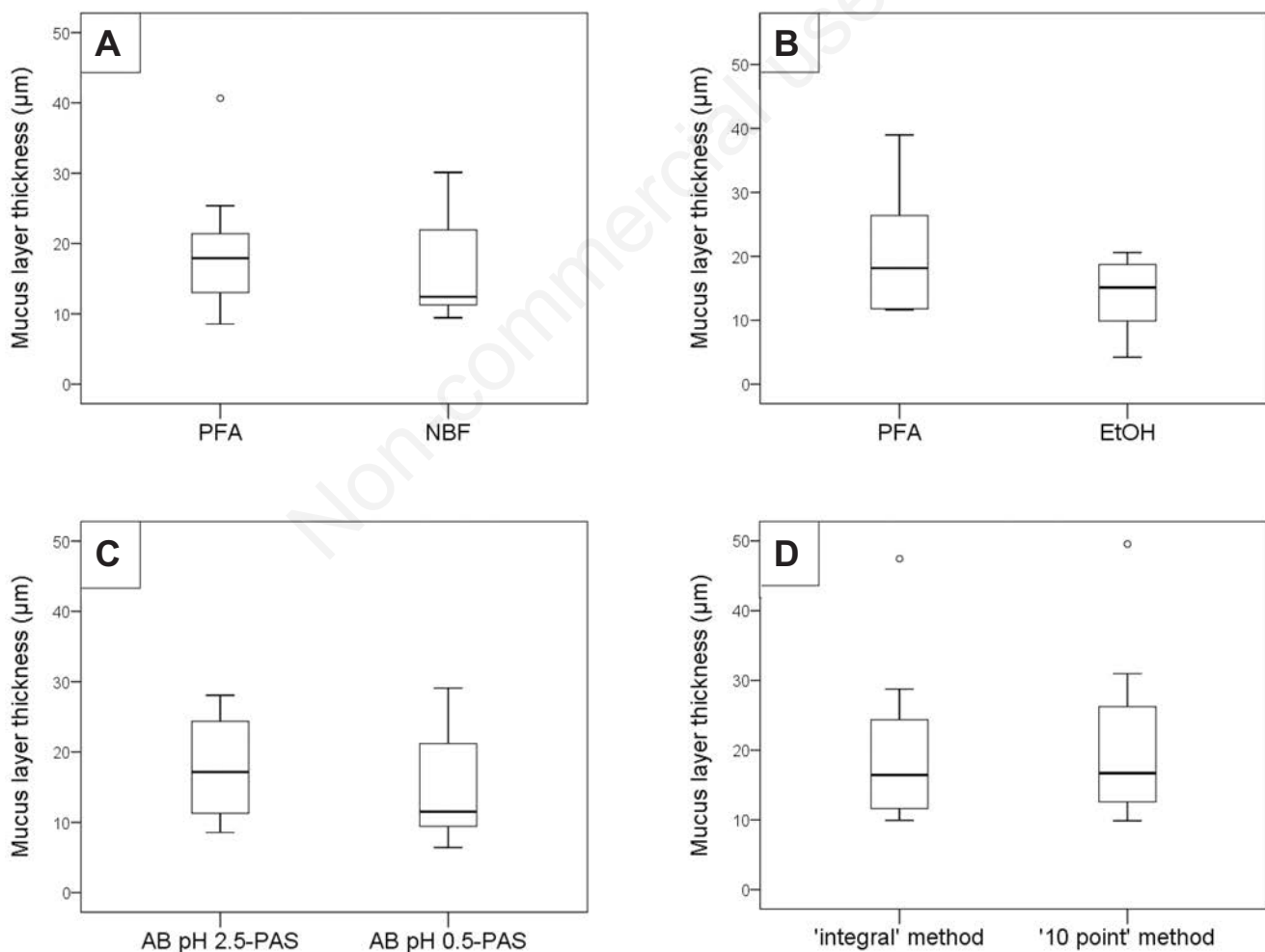


Figure 1. A) Patchy looking mucus in the jejunum; prefixation: cryopreservation; postfixation: PFA; staining: AB pH2.5-PAS. B) Single mucus patches in the jejunum; fixation: methacarn; staining: AB pH2.5-PAS. C,D) Laminar appearance of the mucus in the colon; prefixation: cryopreservation; postfixation: PFA; staining: AB pH2.5-PAS. E) Detachment of the mucus layer in the colon; fixation: methacarn; staining: AB pH2.5-PAS. F) Slight displacement of the mucus over the epithelial borders and in the digesta; prefixation: cryopreservation; postfixation: NBF; staining: AB pH2.5-PAS.

loss of mucus or intestinal content that stabilizes the mucus layer.<sup>24</sup> Moreover, tissue samples fixed in water-containing fixatives have to be dehydrated by ascending concentrations of alcohol and xylene in order to allow the tissue embedding in paraffin wax. This dehydration process also contributes to a shrinkage and removal of the adherent mucus layer.<sup>26,27</sup> Very few studies have explicitly investigated the effect of different postfixation methods on the histological depiction of the intestinal mucus layer. Postfixation of cryopreserved samples proved necessary for reliable mucus preservation in this study. PFA postfixation preserved the laminar arrangement of the colonic mucus in all sections in contrast to NBF and EtOH where partly mucus displacement over epithelial borders and in the intestinal content occurred. This could be explained by the absence of water and

minor physical influence of the PFA vapor compared to the liquid fixatives NBF and EtOH. PFA has also successfully been used as a postfixative in a previous study investigating the mucus layer of the rat's stomach.<sup>28</sup> The use of NBF and EtOH as a postfixative can lead to protein cross-linking,<sup>8,29</sup> which might cause modifications of the single mucus layer. The patchy mucus distribution in the jejunum following PFA and EtOH postfixation was not observed in the colon. This difference might be explained by the different biochemical composition of jejunal and colonic mucus.<sup>30</sup> Rapid dehydration rates might also cause mucus patches in the jejunum. In a previous study on mice, chemical fixation using Carnoy's solution led to threadlike mucus in the small intestine, caused by shrinkage due to the fixative solution.<sup>27</sup> The staining process can also have an impact on the mucus depic-

tion.<sup>9</sup> Various pH levels of the AB staining solution can be used for the differentiation of mucins.<sup>8</sup> By using AB pH 2.5-PAS the intestinal mucus was stained in a darker blue color compared to AB pH 0.5-PAS. AB solutions with a pH of 2.5 generally stain carboxyl and sulfate groups of acid mucopolysaccharides while complex sulfated mucins are selectively stained using a pH of 0.5.<sup>31-32</sup> Varying pH values of the AB staining solution showed no impact on the measured mucus layer thickness. Thus, a quantification of intestinal mucus is possible using both AB pH values although a pH of 2.5 is preferred due to the higher staining intensity. In this study, variation in the mucus layer thickness measuring methods showed no impact on the quantification of the mucus thickness. In most studies, mucus thickness is determined by measuring the mucus layer at 10 to 40 randomly chosen



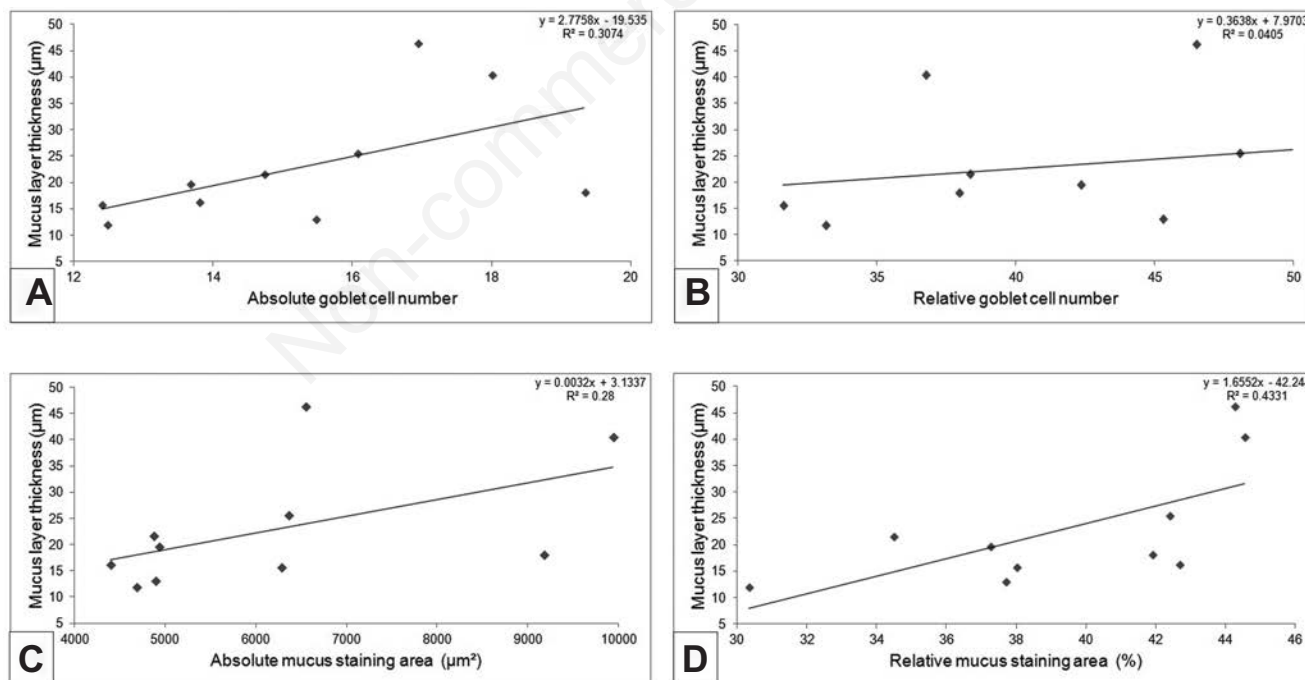
**Figure 2.** Comparison of different postfixations, staining solutions and measuring methods for the depiction of the mucus layer thickness in cryopreserved colonic samples (n=10). A) PFA and NBF postfixation. B) PFA and EtOH postfixation. C) AB staining with pH values 2.5 and 0.5. D) "Integral" and the "10 point" measuring method.

points.<sup>28,33</sup> The “integral” mucus thickness measuring method has slightly higher time expenditure, but was preferred in this study owing to higher precision of a polygon compared to 10 randomly selected points.

Correlation analyses revealed that the colonic crypt depth and crypt area were positively correlated with the absolute number of goblet cells and the absolute mucin staining area while both parameters were not related to the relative number of goblet cells and the relative mucin staining area. On the one hand this indicates that more goblet cells and hence more mucus can be measured in deeper crypts with a larger crypt area but on the other hand that the density of goblet cells and the relative proportion of the mucin staining area do not vary between crypts of different sizes. Furthermore, the absolute measured mucin area corresponded with the absolute number of goblet cells but interestingly there was no relationship between the relative number of goblet cells and the relative mucin staining area suggesting that mucus secretion is less dependent on the density of goblet cells per crypt than on the secretory capacity of each goblet cell. Due to this fact, most, but not all studies, which are focused on the histologi-

cal evaluation of the goblet cell secretory activity, report data on the area of mucin granules or on the mucin staining area rather than to refer to the number of intestinal goblet cells.<sup>17,34-36</sup> Methodological studies evaluating the relationship between goblet cells, mucus secretion and mucus layer thickness in the intestine are scarce. To our knowledge, investigations on the histological description of the intestinal mucus layer in combination with the determination of histomorphological parameters, which may serve as indicator for the mucus layer thickness in the intestine of pigs are not available. The results of this study showed that neither the dimension of the crypt (crypt depth and crypt area) nor the absolute or relative number of goblet cells were related to the measured mucus layer thickness underlining that particularly the determination of the goblet cell number in histological slides is not an eligible tool for the assessment of the mucus layer thickness. However, the results clearly showed that, in contrast to the absolute mucin staining area, the relative mucin staining area was positively correlated with the thickness of the mucus layer emphasizing that the higher the proportion of mucins per crypt

area the thicker the colonic mucus layer. As the relative mucin staining area was not dependent on the density of goblet cells per crypt length these results indicate that the secretory activity level of goblet cells is decisive for the number of produced mucins in the crypt and thus for the thickness of the mucus layer. The results of a study evaluating the effect of dietary fiber on pigs showed, that a small relative mucin staining area was observed in crypts of the small intestine of pigs fed on pectin-containing diets.<sup>23</sup> It was speculated that a small relative mucin staining area is associated with a lower production and secretion of mucins implying a decreased susceptibility to certain intestinal infections,<sup>23</sup> as conversely a large goblet cell area accompanied with an increased mucin secretory activity might correspond to a thick mucus layer.<sup>17-37</sup> However, the validity of this hypothesis could be not verified as investigations were focused on the qualitative and quantitative determination of the mucin staining area but not on the quantitative examination of the mucus layer thickness.<sup>23</sup> In 2009, Hedemann *et al.* evaluated the effect of non-digestible carbohydrates on the intestinal mucus layer in the colon of rats deter-



**Figure 3.** Correlation analyses of different histomorphological parameters, determined in histological slides of methacarn fixed tissue, and the mucus layer thickness, measured in histological sections of cryopreserved tissue, in the colon of piglets (n=10). A) Relationship between the absolute number of goblet cells (total number of goblet cells per crypt) and the mucus layer thickness (Pearson coefficient: 0.554; P=0.096). B) Relationship between the relative number of goblet cells (goblet cells per 1mm basement membrane of crypts) and the mucus layer thickness (Pearson coefficient: 0.201; P=0.577). C) Relationship between the absolute mucin staining area (total mucin staining area per crypt) and the mucus layer thickness (Pearson coefficient: 0.529; P=0.116). D) Relationship between the relative mucin staining area (mucin staining area in % of total crypt area) and the mucus layer thickness (Pearson coefficient: 0.658; P=0.039).

mining different parameters such as the mucin staining area and the mucus layer thickness.<sup>36</sup> The mucin staining area was qualitatively and quantitatively ascertained in histological slides of NBF fixed caecal and colonic tissue while the mucus thickness layer was measured *in vivo* using a micropipette technique. In accordance with the results of the present study, correlation analyses showed that the staining area of neutral mucins, representing the major part of mucins in goblet cells, were positively correlated with the mucus layer thickness implying that a large mucin staining area is related to an increased mucin secretion and a thick intestinal mucus layer in rats.<sup>36</sup> Based on the results of the current study, further analyses are needed in order to evaluate the different mucin chemotypes and their impact on the mucus layer formation in the intestine of pigs. Investigations should be focused on the determination of mucin gene expression patterns in order to ascertain the effect of intestinal mucus synthesis and secretion on the mucus layer thickness.

In conclusion, the current study contributes to the establishment of an improved standard for the histological description of mucus in the colon of pigs. The results showed that immediate cryopreservation using liquid nitrogen followed by chemical postfixation and AB-PAS staining led to a reliable mucus preservation allowing a mucus thickness determination in the colon of pigs. Moreover, the results of this study proved that the detected relative mucin staining area was correlated with the measured mucus layer thickness and thus may serve as a suitable histomorphological parameter for the assessment of the intestinal mucus layer thickness in the colon of pigs.

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