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Comparison of two techniques for a comprehensive gut histopathological analysis: Swiss Roll *versus* Intestine Strips

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ABSTRACT

Assessing the gut mucosa milieu is important to grade the inflammatory process in conditions such as food hypersensitivity, allergy, gut parasitosis, *etc.* However, the gastrointestinal tract comprises a challenging system to evaluate, due to its thin tubular structure and mucosa, which suffer fast autolysis after death. Irrespective of the preferred inflammatory score system, it is important to choose the technique that will render the best tissue analysis. Thus, our aim was to compare two of the most frequently used methods to collect, process and analyze gut segments, the Swiss Roll and the Intestinal Strips. Normal C57Bl/6 mice were randomly assigned to Rolls or Strips group. After an overdose of anesthetics, segments of the duodenum, jejunum and ileum were collected and prepared accordingly for histological processing and analysis. Our results show the villi in the Rolls tend to be shorter and wider than those in the Strips in the duodenum and jejunum but not the ileum. No significant differences were observed in intra-epithelial lymphocytes and goblet cells counts. Finally, we staged each segment using our histomorphometric classification system, which revealed that although all animals presented a normal intestinal mucosa, those assigned to the Rolls group had their mucosa staged in the Infiltrative Stage while the Strips group had their mucosa staged as Normal. In conclusion, Swiss Rolls might be desirable for a wider assessment of the intestine, as it allows large segments to be analyzed at once, while Strips are better suited when detailed evaluation of each villus is intended.

1. Introduction

The small intestine is divided into duodenum, jejunum and ileum (Treuting et al., 2017) and each of these sections has distinctive histological and functional features that differ throughout the intestine, including the immunological response to food antigens (Bialkowska et al., 2016; De Robertis et al., 2011; Gelberg, 2014). Approximately 10% of non-degraded food proteins reach the *lamina propria* of the villi (Reitsma et al., 2014), thus being highly allergenic in genetically-predisposed individuals (Horta-Baas et al., 2017; Lozano-Ojalvo et al., 2017), with the usual clinical manifestations of itching, hives and gastrointestinal and cardiovascular symptoms (Ladics, 2008; Sharma et al., 2015).

Intestinal alterations are the hallmark for diagnosis of food hypersensitivity and Gastrointestinal allergic diseases (Azouz and Rothenberg, 2019; Nowak-Wegrzyn et al., 2017) Rapid autolysis and sloughing of the epithelium immediately after death represent a challenge in experimental studies (Williams et al., 2016), with the proximal small intestine undergoing quickest autolysis, possibly due to contact with gastric acids and bile (Scudamore, 2014; Williams et al., 2016). *In vivo* studies also present challenges, as there are several aspects that complicate biopsying and analysis, including villus shrinkage (Moore et al., 1989).

Standardization of normal and inflammatory score system (with 5-stages) was proposed in the 1990's for humans and has been used since (Marsh, 1995; Oberhuber, 2000; Villanaccia et al., 2011) whereas for mice the scoring systems vary widely (Erben et al., 2014), some suggesting a 5-stage system (Asseman et al., 1999) while others proposed a 17-stage system (Ostanin et al., 2009). Our group has recently proposed a 5-stage histomorphometric-classification system based on years of research in the field observing similarities between the mouse and the human gut (Pereira e Silva et al., 2018).

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However, regardless of the elected scoring system to obtain consistent results it is important to choose the best retrieving and preparation procedures to study the morphology of the intestine. Two widely used techniques for biopsies and experimental studies are used to prepare intestinal tissue - the Intestinal Strip method and the Swiss Roll. Although the first has been used since the early 20th century, up to our knowledge the first description was made by Mayer and Pfeiffer (1968), while the latter was described by Moolenbeek and Ruitenberg (1981). To determine if the tissue preparation methods influence the histomorphometric staging of the intestinal tissue, we compared these two methods in this study.

2. Materials and methods

2.1. Animals

C57Bl/6 adult (70 days old) isogenic male mice (originally obtained from the Jackson Laboratory in the 70's) bred at the Animal Facility of the Federal Fluminense University (Niteroi, RJ, Brazil) were given free access to mouse chow and acidified water (pH 2.5). They were kept in polypropylene cages with stainless steel covers (temperature of 22 °C, ~60% humidity and 12 h light/12 h dark cycle). The animals were not kept either under SPF (specific pathogens free) or germ-free conditions.

To ensure that the animals were healthy, they were monitored for one month prior to the experiment. Body weight and food consumption were assessed weekly along with visual inspection of disease symptoms, like labored respiration or *pilar erecti* (Rupa and Mine, 2006). Mice were randomly divided into Intestine Strips group or Swiss Rolls group, adding up to 10 animals per group.

This work was approved by the Institutional Animal Care and Use Committee (official name: Animal Research Ethics Committee - permit number 733/2016) and is in compliance with the national animal welfare committee. It also follows the ARRIVE guidelines (Kilkenny et al., 2010).

2.2. Tissue segment collection and preparation

Animals received an overdose of anesthetics (60 mg/kg of Xylazine + 350 mg/kg of Ketamine, produced by Sespo Industries®, Paulinia, Sao Paulo, Brazil), after which a longitudinal section was performed to expose the peritoneum. The abdominal cavity was examined to discard any macroscopic alteration.

For the Intestine Strips, we performed the protocol described by Campos (Campos et al., 2014). After removing the entire small intestine (from the stomach pylorus to the colon), 5-cm segments of the duodenum, jejunum and ileum were collected using a surgical ruler and the proximal surgical margin in each segment was cut in an 45° angle to ensure the exact same regions were analyzed in all mice. Each tissue segment was placed over a piece of filter paper and opened longitudinally with the aid of tweezers and ophthalmologic scissors. Every care was taken not to damage the mucosal layer and each intestinal segment was completely covered with Carson's formalin pH 7.4 immediately after opening.

After 3 min, each segment was conditioned in an appropriate cassette and was immersed horizontally in a Petri dish containing Carson's formalin solution pH 7.4 for approximately 30 min, providing better tissue fixation without the occurrence of folds. This formalin bath also allows the elimination of any debris left in the intestinal lumen without damaging the mucosa.

As for the Swiss Roll, we performed the protocol as described by Moolenbeek and Ruitenberg (1981), Park et al. (1987) and later modified by Williams et al. (2016). In short, the intestine is removed and sliced into large sections comprising the duodenum, the jejunum, the ileum and the colon. Each section is then opened longitudinally and coiled with the mucosal layer outwards using a wooden stick, then placed in cassettes and fixed with Carson's formalin solution.

After preparing all cassettes (slices and Rolls), they were transferred to a container of Carson's formalin solution pH 7.4 for 24 h at room temperature (Carson et al., 1973; Jones, 2007). Both Swiss Rolls and Intestine Strips were cut in longitudinal sections. Lastly, tissues were dehydrated, cleared and embedded in paraffin, cut into serial 5 µm-thick slices and stained with Hematoxylin-Eosin (HE) for histological analysis.

2.3. Tissue analysis

All slides were scanned using the APERIO ScanScope CS System® with a 40× objective lens. To evaluate the histological parameters, the ImageScope® software (version 11.2.0.780; Leica Microsystems GmbH, Wetzlar, Germany) tools were used with a 7.2 digital zoom to permit cell counting. The intestinal regions in each slide were identified by their morphological aspects, such as the presence of tall and leaf-like villi and high density of Brunner's glands, as previously described for the duodenum. Jejunal villi are tall and cylindrical and villi in the ileum are short and cylindrical. (Treuting et al., 2017).

In each slide, a minimum of 15 villi per optical field were analyzed in each section, totaling a minimum of 90 villi per section to confirm a similar villus distribution across all animals. The morphological parameters evaluated were: villus area, height (H) and width (W). Villus H was measured from the base to the apex of the villus and W was measured at the middle of the H as previously described (Pereira e Silva et al., 2017) (Supplementary Fig. 1). Cells counted were intestinal epithelial cell (IEC), intraepithelial lymphocytes (IEL) and goblet cells (GC). With these parameters in hand we established the following ratios: H/W, IEC/IEL and IEC/GC. The IEL were identified by their morphological aspects, such as large nuclei (high nucleus-to-cytoplasm ratio), size (7-11 µm in diameter) and lack of granules (van der Meer et al., 2007; Young et al., 2013) and also their position in relation to the IEC nucleus, usually closer to the basal membrane of the epithelium (Guagnozzi et al., 2016). Each slide was analyzed by two pathologists independently. Pereira e Silva et al. (2018) histomorphometric classification system was used to quantify the histological aspects of the small intestine.

2.4. Statistical analysis

Initially, Kolmogorov-Smirnov test was performed for normal distribution. Then, Student *t*-test when comparing two variables and one-way ANOVA test with Bonferroni post-test when comparing more than two variables were used to determine the significant difference. All tests were performed using Graphpad Prism 6 Software (Graphpad Software, Inc., La Jolla, California, United States). $P < .05$ were considered to indicate a statistically significant difference. The results are displayed as mean + standard deviation (SD) and show data from a minimum of 10 animals for each technique.

3. Results

3.1. General morphology of the villi

Segments analyzed from both Swiss Rolls and Strips presented normal villus shape throughout the intestine. In the duodenum, the villi in the Strips tended to be more perpendicular to the submucosa than the Swiss Roll technique. The submucosal layer presented normal aspect without significant differences between techniques (Fig. 1). In the jejunum, villi were shorter than in the duodenum and cylindrical (Fig. 2). Finally, in the ileum, villi were shorter and cylindrical (Fig. 3).

3.2. Villus area

3.2.1. Duodenum

In the Swiss Rolls, mean villus area ($1.1 \times 10^4 \pm 4.98x10^3 \mu m^2$)

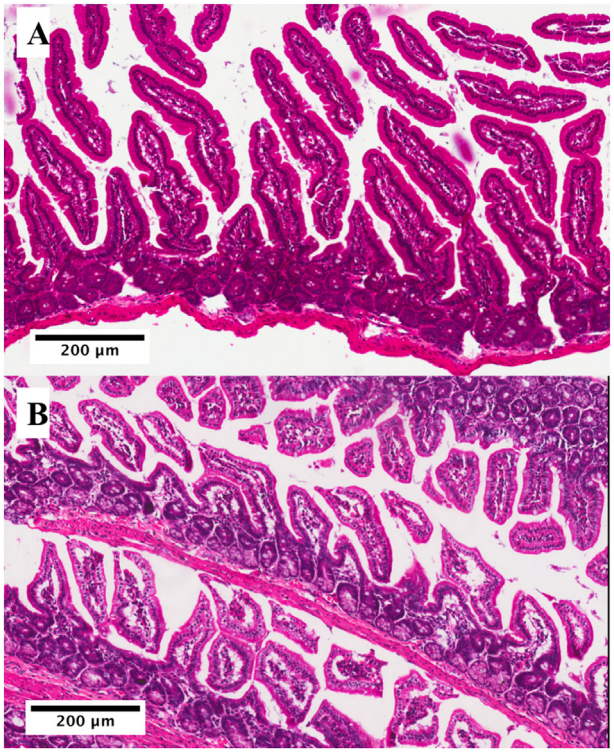

Fig. 1. General morphology of duodenum villi in each technique evaluated. Stain: HE. (A) Aspect of the villi in the Intestine Strips. Villi are straight. (B) Aspect of the villi in Swiss Roll. Villi tilted to the submucosa.

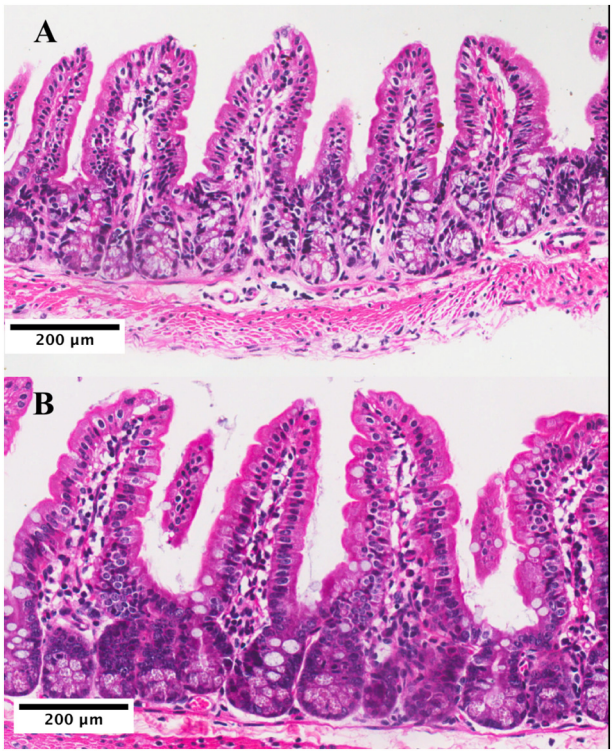

Fig. 2. General morphology of jejunum villi in each technique evaluated. Stain: HE. (A) Aspect of the villi in the Intestine Strips. (B) Aspect of the villi in Swiss Roll.

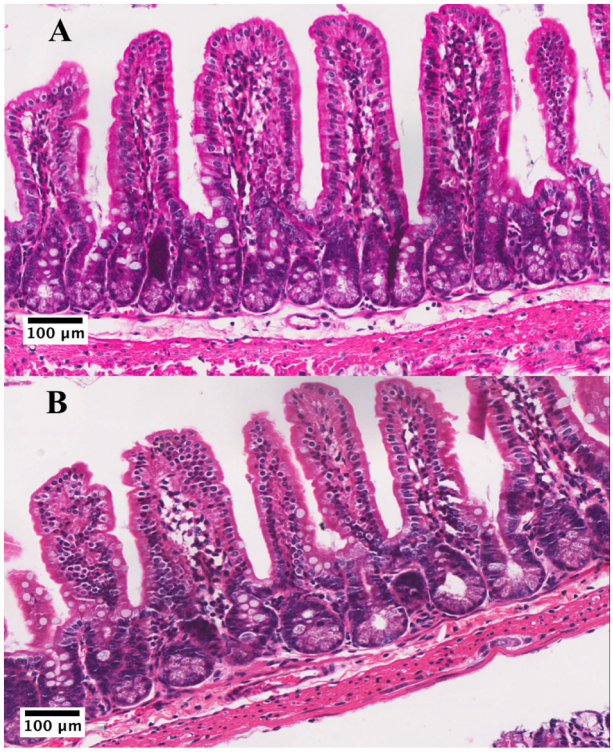

Fig. 3. General morphology of the ileum in each technique evaluated. Stain: HE. (A) Aspect of the villi in the Intestine Strips. (B) Aspect of the villi in Swiss Roll.

was significantly smaller ($p = .0002$) when compared to the Strips mean villus area ($2.2 \times 10^4 \pm 3.3x10^3 \mu m^2$) (Fig. 4A).

3.2.2. Jejunum

The same pattern was observed in the jejunum. The Swiss Rolls mean villus area ($1.9 \times 10^4 \pm 3.2x10^3 \mu m^2$) was significantly smaller ($p = .0099$) when compared to the Strips mean villus area ($2.4 \times 10^4 \pm 2.8x10^3 \mu m^2$) (Fig. 4B).

3.2.3. Ileum

In the ileum, we observed no significant differences ($p = .16$) between mean villus area in the Swiss Rolls ($1.3 \times 10^4 \pm 1.1x10^3 \mu m^2$) and in the Strips ($1.4 \times 10^4 \pm 1.9x10^3 \mu m^2$) (Fig. 4C).

3.3. Villus H/W

3.3.1. Duodenum

Strips H/W ratio (4.39 ± 0.56) was significantly higher ($p = .009$) than Swiss Rolls H/W ratio (3.42 ± 0.94). We observed no variations in the width between the techniques, so the alterations in the H/W ratio was caused by alterations in the height only. (Fig. 5A).

3.3.2. Jejunum

In the jejunum, a significantly higher H/W ratio ($p = .04$) was also observed in the Strips (4.87 ± 0.79) when compared to the H/W ratio in the Swiss Rolls (3.79 ± 0.99) (Fig. 5B).

3.3.3. Ileum

We observed no significant differences ($p = .15$) in the ileum H/W ratios between the Strips (3.25 ± 0.40) and the Swiss Rolls (2.95 ± 0.52) (Fig. 5C).

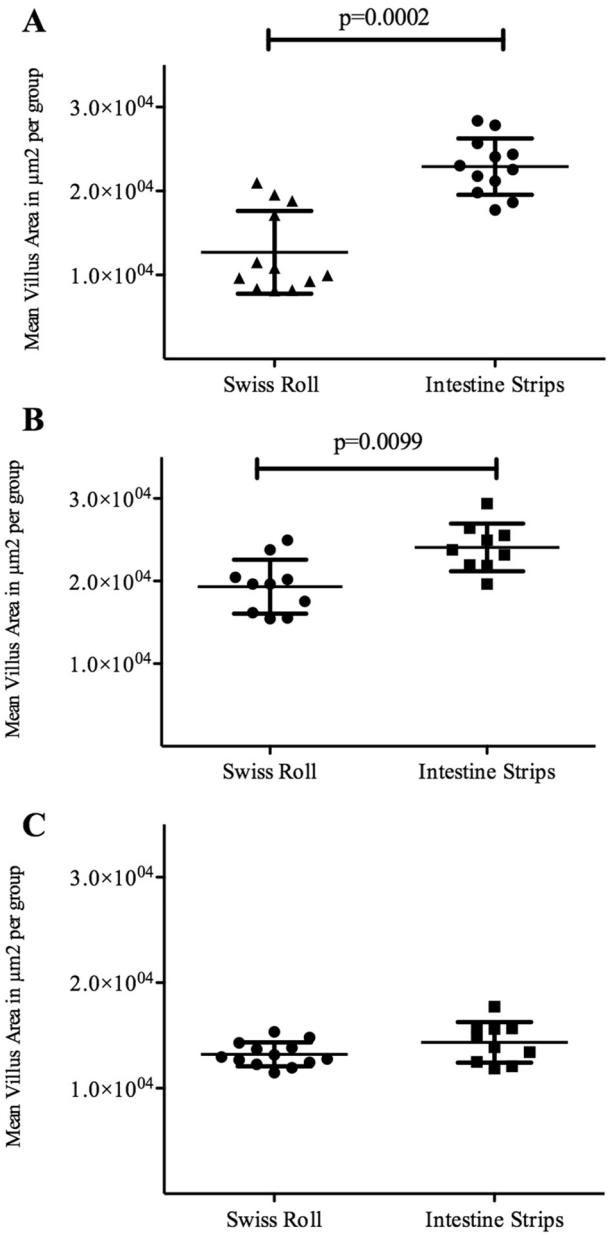

Fig. 4. Villus area + SD per group. (A) Mean villus area in the duodenum. The Swiss Rolls area was significantly smaller (p = .0002) compared to the Strips. (B) Mean villus area in the jejunum. Again, Swiss Rolls area was significantly smaller (p = .0099) compared to the Strips. (C) Mean villus area in the ileum. No significant differences (p = .16) were observed.

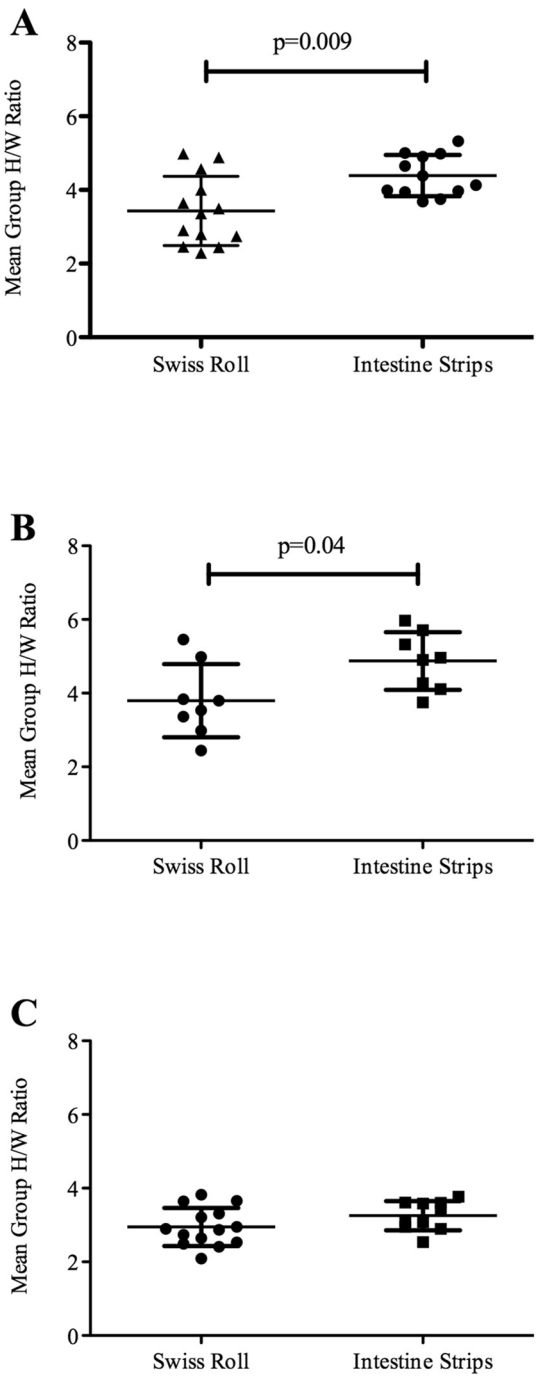

Fig. 5. Mean villus height and width ratio (H/W) + SD. (A) In the duodenum, the mean villus H/W in the Swiss Rolls was significantly lower compared to the Strips (p = .009). (B) Mean villus H/W in the jejunum. The H/W ratio was significantly lower (p = .04) in the Swiss Rolls compared to the Strips. (C) In the ileum, no significant differences were observed between the techniques (p = .15).

3.4. Villus IEC/IEL ratio

No significant differences were observed in the mean IEC/IEL ratio per villus either in the duodenum (p = .19) (Swiss Rolls - 40.76 ± 7.25/Strips – 39.54 ± 5.24), jejunum (p = .18) (Swiss Rolls – 34.94 ± 4.82/Strips - 35.69 ± 3.28) and ileum (p = .28) (Swiss Rolls - 25.03 ± 4.70/Strips - 30.27 ± 10.10) (Fig. 6). The variations in the IEC/IEL ratio were caused by fluctuations in the IEC counts since the IEL numbers did not alter greatly (Fig. 7)

3.5. Villus IEC/GC ratio

Similar to the IEC/IEL ratio, no significant differences (p = .50) were observed in the mean IEC/GC ratio per villus in the duodenum (Swiss Rolls – 10.80 ± 2.89/Strips - 11.11 ± 2.96), jejunum (p = .12) (Swiss Rolls – 10.40 ± 3.07/Strips – 11.69 ± 1.62) and ileum (p = .37) (Swiss Rolls – 8.64 ± 1.50/Strips – 11.89 ± 5.89) (Fig. 8).

3.6. Histomorphometric classification system

Although all animals were considered normal (non-manipulated to induce intestinal inflammation), according to Pereira e Silva's score (Pereira e Silva et al., 2018), the Swiss Rolls were classified in the Infiltrative Stage whereas the Strips were classified in the Normal Stage of

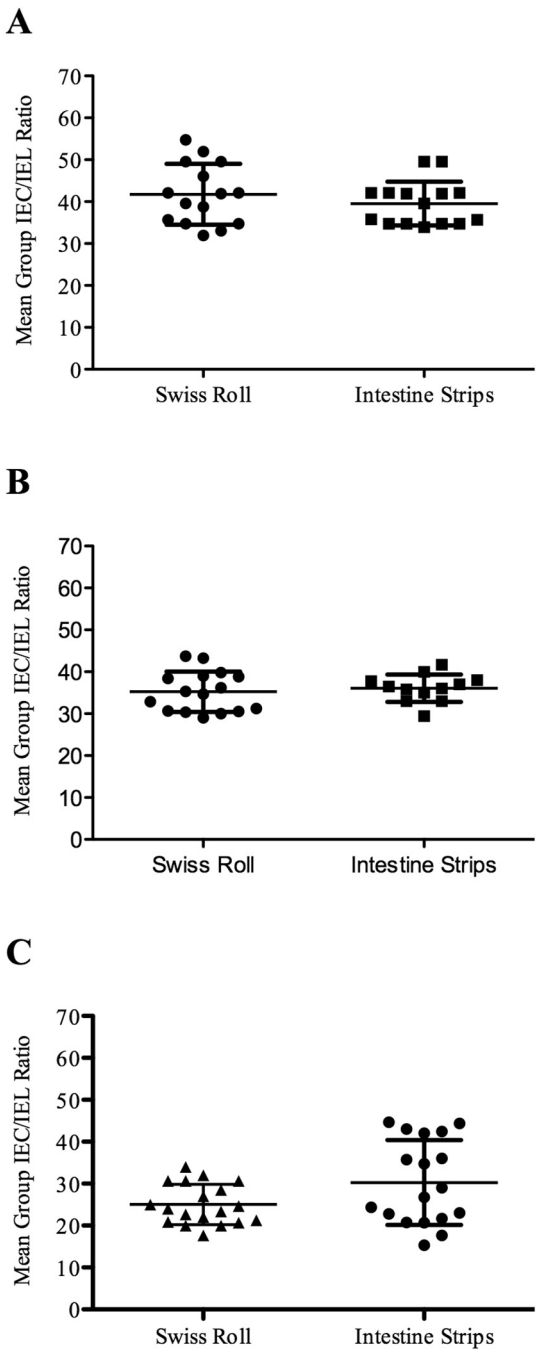

Fig. 6. Mean villus enterocyte and leukocyte ratio (IEC/IEL) + SD per group. (A) Mean IEC/IEL in the duodenum. No significant differences (p = .19) were observed. (B) Mean IEC/IEL in the jejunum. No significant differences (p = .18) were observed. (C) Mean IEC/IEL in the ileum. No significant differences (p = .28) were observed.

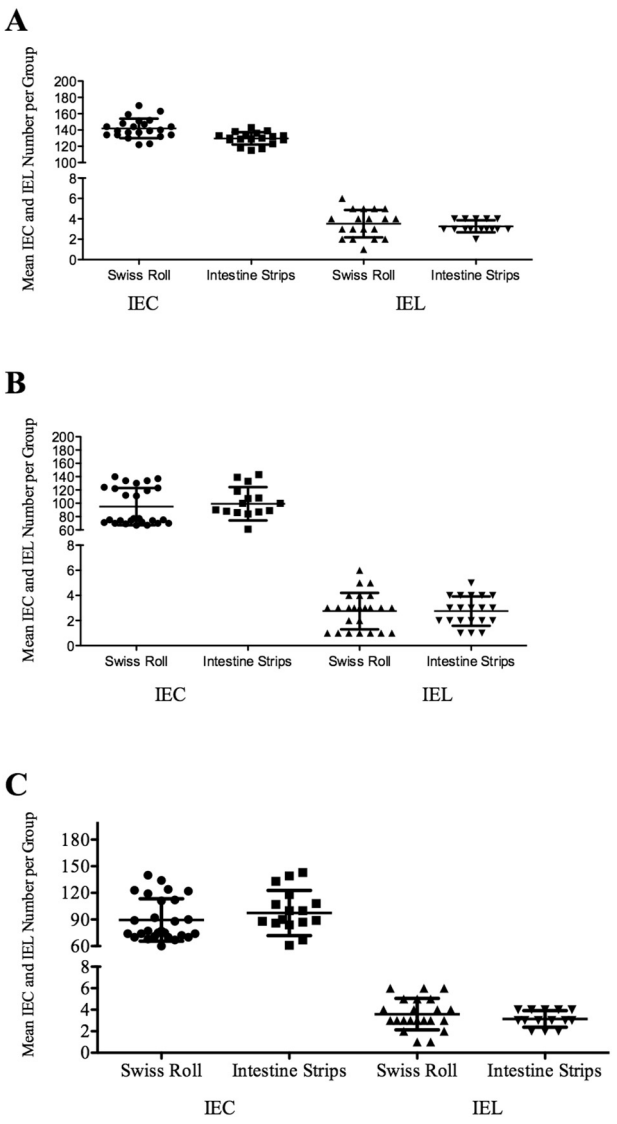

Fig. 7. Mean villus enterocyte and leukocyte + SD per group. (A) Mean IEC and IEL in the duodenum. No significant differences (p = .30) were observed between IEC counts and between IEL counts. (B) Mean IEC and IEL in the jejunum. No significant differences (p = .38) were observed between IEC counts and between IEL counts. (C) Mean IEC and IEL in the ileum. No significant differences (p = .21) were observed between IEC counts and between IEL counts.

the mucosa (Table 1).

4. Discussion

Although less invasive diagnostic methods are available, for instance determining serum antibody titers (Mills and Murray, 2016), intestinal histopathology is still considered the golden standard to diagnose gut inflammation (Pereira e Silva et al., 2017; Srivastava, 2015). The results shown here compare two frequently used techniques to analyze the gut. Our group has used both methods to analyze the gut of immunologically manipulated mice, however, we were not able to find any study in the literature comparing both techniques. Since the intestinal tissue becomes very frail during an inflammatory processes (Erben et al., 2014; Pereira e Silva et al., 2018), we chose to first compare normal guts. Thus, all animals used in this work were not submitted to an intestinal inflammation induction protocol and were maintained under conventional housing conditions (non-SPF).

Recent studies have shown that SPF mice have an immature immune system compared with mice fully colonized by microorganisms (Beura et al., 2016; Huggins et al., 2019; Reese et al., 2016). This is especially important in gut studies, since the gastrointestinal tract is the primary reservoir of microbiota in the body and the interaction with microbial antigens drastically shape not only the local immune system (Sundberg and Schofield, 2018; Tao and Reese, 2017), but also local stem cells activity (Jost and Hockendorf, 2019).

Both Strip and Swiss Roll techniques are well established and used for experimental and diagnostic purposes in humans and non-human animals. Although not descriptive, the first record of the Swiss Roll in the available data basis was published by Magnus (1937), whereas the

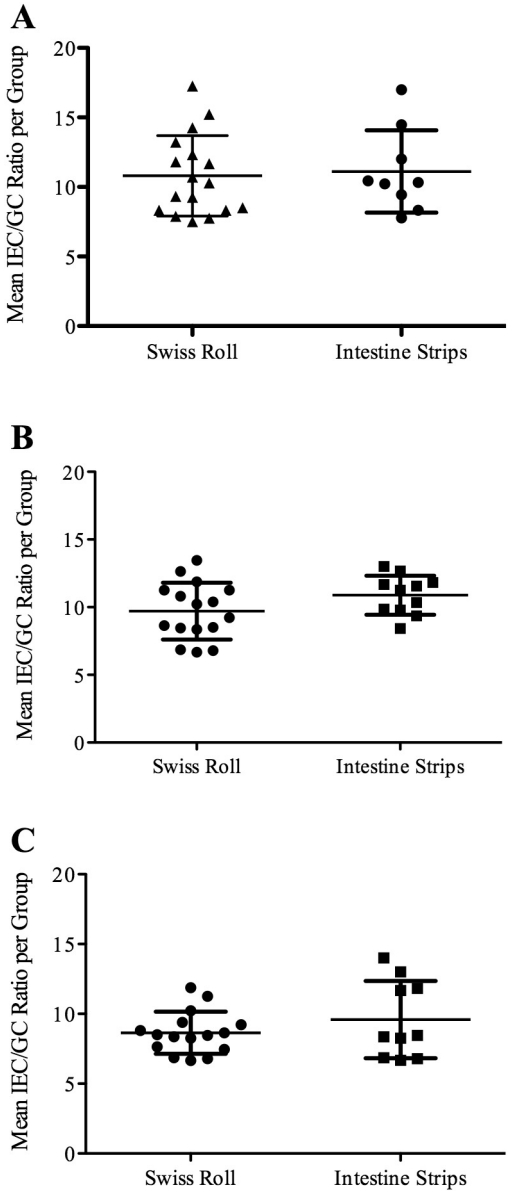

Fig. 8. Mean villus enterocyte and goblet cells ratio (IEC/GC) + SD per group. (A) Mean IEC/GC in the duodenum. No significant differences ($p = .50$) were observed. (B) Mean IEC/GC in the jejunum. No significant differences ($p = .12$) were observed. (C) Mean IEC/GC in the ileum. No significant differences ($p = .37$) were observed.

Strips have been used since early intestinal *post mortem* morphology studies and for diagnosis since the introduction of the per oral intestinal biopsy tube (Mayer and Pfeiffer, 1968). The term "Intestine Strips" is the commonly used terminology, however others can be found throughout the literature, for example "Small Intestine Segments" (Altmann and Enesco, 1967; Crane and Henderson, 1924; Williams et al., 2016), "Straight Portions of Intestine" (Wood, 1944) and "Longitudinal Sections" (Rubio et al., 1982; Troyer et al., 2002). We preferred the term "Intestine Strips" in compliance with Magnus's manuscript (Magnus, 1937), as both terms used here were cited by the same author.

Our findings comparing both methods revealed that, in general, villi analyzed in the Swiss Roll were shorter than those in the Strips, leading to alterations in area and H/W ratio analyses. These results were already expected by us, since the rolling process tends to distort the villi, especially in the middle of the Roll as shown by Bialkowska et al. (2016).

The differences in the IEC counts revealed that the Swiss Roll method retrieved villi with lower IEC counts than the villi in the Intestine Strips. This can be explained by villi distortion, which can happen during Rolling prior to fixation, therefore villus height may not correlate with IEC number, as villi can change their shape (Wright et al., 1989). Some studies preferred to place the tissue in fixative overnight before cutting it open and rolling (Bialkowska et al., 2016; Park et al., 1987).

Another anticipated result was the low IEL count in both groups, as all animals presented normal mucosa and were fed only commercial chow, to which they were tolerant. Tolerance, as defined by our group (Campos et al., 2014; Paschoal et al., 2009), is an active immunological process with up regulation of non-inflammatory cells and cytokines. Therefore it is reasonable that all animals presented basal IEL levels in the intestinal mucosa as they presented normal milieu throughout the gut and a low IEL count is not an indication of an inflammatory process (Corleto et al., 2018; Husby et al., 2019). Although we did not further investigate the cell phenotypes in this work, we may predict that they are T cells, since previous studies showed that almost all mouse IEL are $CD3^+$ γδ-T cells (Ishikawa et al., 2007; Wilharm et al., 2019). Furthermore, regulatory cells are abundant in the gut, for instance $Foxp3^+$ T-cells, which play an essential role in maintaining tolerance to food by suppressing the production of IL-5, IL-13, IgE and other inflammatory products (Mohr et al., 2018; Smaldini et al., 2015; Ward-Hartstonge and Vasanthakumar, 2018). The IEL retrieved from the mice in this work will be further investigated to establish the correct phenotype of these cells.

The same concept of tolerance can be applied to GC number. In both techniques all animals were classified as normal with no significant differences between the groups. Lower GC counts is considered an important inflammatory marker of IBD diagnosis (Waddell et al., 2019), thus normal GC counts is related to an intact and functional barrier against pathogens (Bergstrom et al., 2008; Kovacs et al., 2012).

Staging both groups in our histomorphometric classification system revealed interesting results. Intestines in Rolls were classified as Infiltrative while the Strips were classified as Normal. Considering that all animals used in this work were not submitted to any bowel inflammation protocol, the differences observed here might reflect damage during processing and might be even greater in bowel inflammation studies. The infiltrative stage describes a mucosa which has no signs of morphological alterations, but an increase in immune cells in the *lamina propria* and in the epithelium of the villi compared to the number of Epithelial cells.

When we compared the three sections of the small intestine

Table 1
Overview of the results already presented.

	Intestine Strips			Swiss Rolls		
	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
Villus area (μm^2)	$2.2 \times 10^4 \pm 3.3 \times 10^3$	$2.4 \times 10^4 \pm 2.8 \times 10^3$	$1.4 \times 10^4 \pm 1.9 \times 10^3$	$1.1 \times 10^4 \pm 4.9 \times 10^3$	$1.9 \times 10^4 \pm 3.2 \times 10^3$	$1.3 \times 10^4 \pm 1.1 \times 10^3$
Villus H/W	4.39 ± 0.56	4.87 ± 0.79	3.25 ± 0.40	3.42 ± 0.94	3.79 ± 0.99	2.95 ± 0.52
Villus IEC/IEL ratio	39.54 ± 5.24	35.69 ± 3.28	30.27 ± 10.10	40.76 ± 7.25	34.94 ± 4.82	25.03 ± 4.70
Villus IEC/GC ratio	11.11 ± 2.96	11.69 ± 1.62	11.89 ± 5.89	10.80 ± 2.89	10.40 ± 3.07	8.64 ± 1.50

(duodenum, jejunum and ileum), we observed the same patterns in both the Swiss Roll and in the Strips, in which the duodenum villi were higher than those in the jejunum and in the ileum, reflecting the normal anatomy of the mouse gut already described in the literature (Erben et al., 2014; Treuting et al., 2017). Because of their height, duodenum villi were more affected by the rolling process in the Swiss Rolls than the villi in the jejunum and in the ileum, which suggests the duodenum might be more prone to damage due to delay in fixation or embedding (Williams et al., 2016).

The differences in villi height between the intestinal sections was observed in both the intestinal strip and swiss roll methods. Since ileum villi were smaller, they also presented smaller IEC per villus. No pathological IEC hyperplasia was observed, since all animals presented similar shaped crypts (Leite et al., 2019).

The Swiss Roll technique offers some advantages compared to the Strips, the most important one being that it allows the imaging of a large section of intestine in the same microscope field (Bialkowska et al., 2016). On the other hand, it can be more laborious and requires extra-deep cassettes to accommodate the tissue, as standard histology cassettes are too shallow to house a Swiss Roll without compressing it (Williams et al., 2016). In our experience, the Swiss Roll technique demands more experienced researchers to correctly analyze the small intestine samples than the Strips, due to the compression of the different small intestinal regions (duodenum, jejunum and ileum) making it difficult to discriminate the segments.

When performing Strips, Williams and others opted to place each Intestine Strip in a different cassette, so each small intestinal region could be easily identified (Williams et al., 2016). Contrarily, in our routine we decided to house all Strips of a given animal in one cassette, placing the Strips on the same exact order every time during paraffin embedding (Supplementary Fig. 2). For an easier identification of the segments in the cassettes, each Strip was sliced in slightly different size; 3 cm for the duodenum, 2.8 cm for the jejunum and 2.6 cm for the ileum. It is worth mentioning that the duodenum is easily identified due to the fact that we collect the specimen with a residual stomach tissue and transitional duodenum mucosa in the gastroduodenal junction (Lawson, 1988) and the ileum was placed at a different orientation than the other strips.

Performing the Strip technique allowed a more detailed assessment of each villus, which is an important feature in our inflammation score, as various morphological parameters are measured per villus (area, height and width). These parameters were mostly responsible for the differences in staging the mucosa of the Swiss Roll and the Strips. Considering that on highly inflamed animals villi tend to flatten and their number can be greatly reduced (Villanaccia et al., 2011), it is important to measure the various parameters of each villus for a full evaluation of the intestinal mucosa integrity (Hao et al., 2012; Santos et al., 2015).

It is of great importance to choose the best techniques when designing an experiment. As demonstrated here, each method can lead to different interpretations. In conclusion, although the Swiss Roll method might be preferable to assess the state of the gut with more tissue per microscopic field, the Intestine Strip technique is recommended when fine parameters must be evaluated, especially in experimental gut inflammation studies. Our group will now perform and compare both techniques under experimental pathological conditions (gut inflammation).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yexmp.2019.104302.

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Declaration of competing interest

The authors declare they do not have any conflict of interests that could bias the results.

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