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BACTERIAL QUANTIFICATION AND HISTOPATHOLOGIC FINDINGS ON THE SMALL INTESTINE OF DOGS WITH CHRONIC INFLAMMATORY ENTEROPATHIES

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BACTERIAL QUANTIFICATION AND HISTOPATHOLOGIC FINDINGS ON THE SMALL INTESTINE OF DOGS WITH CHRONIC INFLAMMATORY ENTEROPATHIES

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RESUMO

A enteropatia inflamatória crônica (EIC) é frequentemente classificada retrospectivamente como enteropatia responsiva à dieta (ERD), responsiva a esteroides (ERE) e responsiva a antibióticos (ERA). O estudo teve como objetivo investigar a relação entre atividade clínica da doença, abundância bacteriana e escores histopatológicos no intestino delgado de cães com diferentes subtipos de EIC. Amostras de duodeno e íleo de 54 cães com diferentes categorias de EIC e onze cães controles foram utilizadas para investigação da abundância bacteriana com hibridização *in situ* fluorescente e alterações histológicas. A abundância bacteriana duodenal não diferiu entre os quatro grupos. Embora a abundância de bactérias superficiais e bactérias aderidas estivesse aumentada na mucosa ileal de cães com ERA em comparação com cães controle, não foi significativamente diferente entre os grupos de EIC. Os escores histopatológicos somativos não diferiram entre as diferentes categorias de EIC. Os achados histopatológicos foram variáveis e a maioria dos parâmetros se sobrepôs entre as diferentes EIC. Houve correlação positiva entre os escores histopatológicos duodenais e ileais e o índice de atividade da doença intestinal inflamatória canina (CIBDAI). Em resumo, foi encontrado aumento de abundância bacteriana e alterações histológicas em EIC em comparação com cães saudáveis, mas estes parâmetros não puderam prever a resposta ao tratamento para as diferentes categorias de EIC.

Palavras-chave: bactéria, duodeno, íleo, enteropatia inflamatória crônica, hibridização fluorescente *in situ*, histopatologia, canino

ABSTRACT

Chronic inflammatory enteropathy (CIE) is often retrospectively classified as food-responsive (FRE), steroid-responsive (SRE), and antibiotic-responsive (ARE) enteropathy. The study aimed to investigate the relationship between clinical disease activity, bacterial abundance, and histopathologic scores in the small intestine of dogs with different subtypes of CIE. Samples from the duodenum and ileum from 54 dogs with different categories of CIE and eleven control dogs were used for investigation of bacterial abundance with fluorescence in situ hybridization and histopathologic changes. Duodenal bacterial abundance did not differ among the four groups. While the abundance of total superficial bacteria and attached bacteria was increased in the ileal mucosa of dogs with ARE compared to control dogs, it was not significantly different between the CIE groups. Summative histopathologic scores did not differ between the different CIE categories. The histopathologic findings were variable and most of the parameters overlapped between the different CIE. There was a positive correlation between duodenal and ileal histopathologic scores and the canine inflammatory bowel disease activity index (CIBDAI). In summary, increased bacterial abundance and histopathologic changes were found in CIE compared to healthy dogs, but these findings could not predict the treatment response for the different categories of CIE.

Keywords: bacteria, duodenum, ileum, chronic inflammatory enteropathy, fluorescence *in situ* hybridization, histopathology, canine

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ARE: antibiotic-responsive enteropathy

AT: antimicrobial treatment

CBC: complete blood count

CCECAI: canine chronic enteropathy clinical activity index

CIBDAI: canine inflammatory bowel disease activity index

CIE: chronic inflammatory enteropathy

FFPE: formalin-fixed paraffin-embedded

FISH: Fluorescence *in situ* hybridization

FRE: food-responsive enteropathy

GI: gastrointestinal

IBD: inflammatory bowel disease

IRE: immunosuppressant-responsive enteropathy

NRE: non-responsive enteropathy

SIBO: small intestinal bacterial overgrowth

SRE: steroid-responsive enteropathy

SUMMARY

1 INTRODUCTION

Chronic inflammatory enteropathy (CIE) in dogs is a condition of unknown etiology characterized by persistent gastrointestinal (GI) signs that last longer than three weeks, exclusion of identifiable causes, and histopathologic evidence of inflammation (Allenspach et al., 2016; Dandrieux, 2016; Washabau et al., 2010). It is often classified according to treatment response as food-responsive (FRE), steroid-responsive (SRE) or immunosuppressant-responsive (IRE), antibiotic-responsive (ARE), and non-responsive enteropathy (Dandrieux & Mansfield, 2019). The pathogenesis of CIE is unclear, although it has been hypothesized that a combination of genetic predisposition, environmental factors, and alterations in the intestinal microbiota can induce an inflammatory response in the host (Atherly et al., 2019; Washabau et al., 2010). Intestinal microbiota imbalances are commonly reported in enteric diseases of dogs, cats, and humans (Guard et al., 2019; Honneffer et al., 2014; Janeczko et al., 2008; Sartor & Wu, 2017; Vázquez-Baeza et al., 2016).

Intestinal bacterial overgrowth or involvement of a single causative microorganism has not been proven in canine CIE (Cassmann et al., 2016; German, et al., 2003). However, antimicrobials have empirically been used as second-line therapy after food trials fail (Jergens et al., 2010; Pilla et al., 2020). The empirical goal of antimicrobial treatment (AT) is removing pathogenic bacteria (Pilla & Suchodolski, 2020) or suppressing commensal bacteria that might trigger the gut immune system (Makielski et al., 2019). The limited understanding of the pathogenesis of CIE leads to selection of therapies based on clinical experience, including the use of antimicrobials (Jergens et al., 2010; Pilla & Suchodolski, 2020). However, the GI clinical signs frequently relapse (Allenspach et al., 2016; Hall, 2011), leading to a questionable efficacy of AT in CIE (Cerquetella et al., 2020; Dandrieux & Mansfield, 2019).

Historically, histopathologic evaluation of intestinal biopsies has not been considered very helpful in the assessment of dogs with CIE, other than confirming inflammation and excluding infectious diseases or neoplasia (Allenspach et al., 2007). Lymphoplasmacytic and/or eosinophilic lamina propria infiltrates are usually reported in dogs with CIE (Allenspach et al., 2007; Wennogle et al., 2017). Standardization of criteria for histopathologic evaluation of endoscopic biopsies have allowed a positive correlation between summative histopathologic scores for the duodenum and colon with clinical disease activity in dogs with idiopathic

inflammatory bowel disease (Allenspach et al., 2019). Previously to standardization of histopathologic criteria, few studies have compared the intestinal lesions in dogs with FRE and SRE and detected no significant differences in the histopathologic scores (Allenspach et al., 2007; Luckschander et al., 2006; Schreiner et al., 2008). However, findings such as architectural changes are reported to be more common in CIE dogs that are hypoalbuminemic when compared to normoalbuminemic dogs affected by CIE (Wennogle et al., 2017).

Increased number of bacteria in the intestine or the involvement of any specific microorganism in dogs with ARE has not yet been proved. The evaluation methods have been a limitation for analyzing bacterial load since the number of bacterial colonies cultured from intestinal contents to diagnose intestinal bacterial overgrowth in dogs was once incorrectly extrapolated from humans and proved to be ineffective (Dandrieux, 2016; German et al., 2003). Fluorescence *in situ* hybridization (FISH) enables the investigation of intestinal microbiota by the spatial analysis of bacterial load and identification (Giaretta et al., 2020). There are no studies characterizing the mucosal microbiota in dogs with the three different categories of CIE with the use of FISH or utilizing FISH in duodenal samples. In addition, the detailed histopathologic phenotype of the three clinical outcomes needs to be better characterized, and studies comparing them utilizing the World Small Animal Veterinary Association Gastrointestinal Standardization (WSAVA) histopathologic standards are absent. The study of the mucosa associated microbiota might reveal aspects of the pathogenesis of the disease and consequently improve our therapeutic approach.

2 HYPOTHESIS

Dogs with CIE show alterations in the bacterial microbiota associated with the small intestinal mucosa with a higher incidence of enteroinvasive bacteria and bacteria adhered to the intestinal mucosa in dogs with ARE than dogs with FRE and SRE.

3 OBJECTIVES

3.1 General objective

The general objective of this project is to conduct a descriptive and retrospective observational study to evaluate the histopathologic lesions and the abundance of bacteria in the small intestinal mucosa of dogs with the different categories of CIE and compare the findings among them and with healthy control dogs.

3.2 Specific objectives

- Characterize the bacterial microbiota in the intestinal mucosa of dogs with ARE through fluorescence *in situ* hybridization quantitatively and in terms of location;

- Investigate whether adherent or invasive bacteria are associated with ARE in order to understand the pathogenesis of the disease and rationalize the use of antimicrobials;

- To describe the histopathologic lesions in the intestine of dogs with ARE and compare them with the findings in dogs with FRE and SRE.

4 LITERATURE REVIEW

4.1 Types of chronic inflammatory enteropathies

The diagnosis of CIE is considered after manifestation of GI clinical signs for three weeks or more, clinical investigation and exclusion of other underlying causes, and by histopathologic identification of intestinal inflammation (Allenspach et al., 2016; Dandrieux, 2016; Jergens & Simpson, 2012). Infectious, neoplastic, and parasitic diseases, as well as structural alterations (e.g., intestinal intussusception) and extra-gastrointestinal diseases are differential diagnosis that must be considered prior to the diagnosis of CIE (Dandrieux, 2016; Hall & Day, 2017; Jergens & Simpson, 2012). Considered a non-infectious inflammatory enteropathy, this condition is not associated to a specific etiology and is subdivided according to its response to

treatments involving diet changes, antimicrobials, or anti-inflammatory/immunosuppressant drugs (Volkmann et al., 2017). Thus, it is classified as FRE, ARE, IRE, or even in non-responsive enteropathy (NRE) when none of the treatments results in improvement of clinical signs (Dandrieux & Mansfield, 2019).

Chronic inflammatory enteropathy has been considered the most common chronic GI condition in dogs and the leading cause of canine chronic diarrhea (Volkmann et al., 2017), even though studies to determine its true prevalence are lacking. Recent changes in terminology may be one of the aspects that hinders the determination of the true prevalence of CIE (Dandrieux & Mansfield, 2019). The term inflammatory bowel disease (IBD) is frequently used as synonym of CIE in veterinary medicine (Dandrieux, 2016); however, human IBD is more closely related to the IRE also referred as canine idiopathic IBD or SRE when treated with glucocorticoids only (Dandrieux & Mansfield, 2019; Jergens & Heilmann, 2022). Chronic inflammatory enteropathy, as well as human IBD, is believed to be a multifactorial disease that results from a complex interplay between genetic factors, environmental factors, such as the enteric bacteria, and dysregulated immune response (Dandrieux, 2016; Jergens & Heilmann, 2022; Jergens & Simpson, 2012). A less prevailing and alternative hypothesis is that the different types of CIE might be phenotypical variations of the same disease process that would be responsive to different treatments depending on the duration or severity of the disease (Makielski et al., 2019). Although, the mechanism by which each of these treatments is effective or how it influences the origin of the enteropathy is not well known (Jergens & Simpson, 2012). Furthermore, the clinical presentation is indistinguishable between the subtypes of CIE (Hall, 2011).

Food responsive enteropathies are the most commonly CIE diagnosed in dogs and may represent up to 2/3 of the cases of canine chronic enteropathies (Allenspach et al., 2016; Kawano et al., 2016; Volkmann et al., 2017), which supports the importance of dietary management as the first-line treatment for animals affected by this condition (Makielski et al., 2019). Clinically, FRE usually exhibits the least severe manifestation, and dietary treatment contributes to maintenance of clinical remission in the short and long-term (Allenspach et al., 2007, 2016; Dandrieux & Mansfield, 2019; Volkmann et al., 2017). Enteropathies that respond to dietary trials are associated with possible allergy or food intolerance, and the most common therapeutic approaches are the use of hydrolyzed or hypoallergenic diets (Allenspach et al., 2016; Makielski et al., 2019). Hydrolyzed diets are reported to be more effective, with a longer

remission of clinical signs, when compared to hypoallergenic diets (limited or novel antigen diets) (Mandigers et al., 2010; Marchesi et al., 2017). A second dietary intervention should be considered prior to considering other treatments once the first have failed since the response to different diet trials varies in the same patient (Cerquetella et al., 2020; Jergens & Heilmann, 2022).

Antibiotic-therapy has frequently been described as the second treatment trial after dietary changes fail and prior to therapy with immunosuppressant drugs (Jergens et al., 2010; Pilla et al., 2020; Westermarck, 2016). Antimicrobials such as tylosin, metronidazole, and oxytetracycline are commonly prescribed (Allenspach et al., 2016; Hall, 2011; Makielski et al., 2019) and associated with improving or resolving the clinical signs in some dogs with chronic enteropathy. However, the therapeutic effects of AT are short-lived, and relapses of the clinical condition after treatment withdrawal are frequent (Allenspach et al., 2016; Dandrieux, 2016). Therefore, studies question the efficacy and role of AT in chronic enteropathies and consider possible consequences regarding bacterial resistance (Cerquetella et al., 2020; Kilpinen et al., 2015; Nitzan et al., 2016). Besides that, ARE frequency is usually retrospectively reported as low, comprising from 8 to 16.2% of dogs with chronic enteropathy (Allenspach et al., 2016; Volkmann et al., 2017), with a higher occurrence in young dogs and large breeds (Allenspach et al., 2016; German et al., 2001).

Immunosuppressants are usually instituted in severely affected dogs after failure to respond to diet and AT, in addition to prior histopathological analysis for differential diagnosis of lymphoma or infectious causes. Glucocorticoids such as budesonide and prednisone, and immunossuppressants as cyclosporine and chlorambucil, are some drugs administered (Allenspach et al., 2016; Dandrieux, 2016; Dandrieux et al., 2013; Procoli, 2020). Low serum albumin levels are more commonly reported in SRE than in other enteropathies (Allenspach et al., 2016; Procoli, 2020), and hypoalbuminemia, in turn, is associated with more severe clinical disease (Allenspach et al., 2016). Therefore, it is suggested that immunosuppressive therapy, in addition to dietary trials, will be needed in cases of CIE with hypoalbuminemia (Kawano et al., 2016). The term protein-losing enteropathy is used to refer to those dogs with CIE that exhibit intestinal protein loss (Allenspach et al., 2007). Finally, a small percentage of dogs that are refractory to medical treatments is further classified within the NRE group, which exhibits a

worse prognosis and may lead to a high rate of euthanasia (Dandrieux, 2016; Jergens & Heilmann, 2022).

4.2 Clinical scoring systems and histopathology

Two clinical scoring indexes are available to classify the severity of the clinical signs of CIE in dogs. The Canine Inflammatory Bowel Disease Activity Index (CIBDAI) was the first clinical score developed for semi-quantitative evaluation of the disease severity and is based on attitude/activity, appetite, vomiting, feces consistency, feces frequency, and weight loss. The large prospective study with short-term follow up further classified the total score as clinically insignificant (0–3), mild (4–5), moderate (6–8), or severe (9 or greater) (Jergens et al., 2003). Later, a large prospective study further evaluated standard tests for their accuracy to predict outcome and defined a new clinical scoring index by adding assessment of serum albumin concentration, ascites, peripheral edema, and pruritus to the CIBDAI parameters. The new clinical scoring index named Canine Chronic Enteropathy Clinical Activity Index (CCECAI) was described as more powerful and accurate regarding the prediction of negative outcome (Allenspach et al., 2007).

Histopathologic evaluation has not been proven useful to predict outcome (Allenspach et al., 2007; Craven et al., 2004; Kawano et al., 2016) and its value regarding the assessment of dogs with CIE is questioned since it is more useful to rule out infectious or neoplastic diseases (Allenspach et al., 2007). However, characteristics and severity of histopathologic findings are still considered important to a complete classification of the disease (Marchesi et al., 2017). Before 2008, the histopathologic evaluation was subjective and substantially varied among pathologists since no widely accepted grading schemes were available (Craven et al., 2004; Marchesi et al., 2017; Willard et al., 2002). These limitations lead to the introduction of comprehensive guidelines for evaluation of GI histologic findings developed by the WSAVA GI Standardization Group (Day et al., 2008). Besides the original standardization, a simplified histopathologic model for GI inflammation is also available and was designed intending to diminish even more the interpretive interobserver variability (Jergens et al., 2014).

The histopathologic lesions of CIE are not specific and can vary in severity (Allenspach et al., 2007; Hall, 2011; Volkmann et al., 2017). The inflammatory infiltrate is commonly

lymphoplasmacytic, eosinophilic, or mixed and may be present in the stomach, small intestine, and large intestine with focal or diffuse distribution (Dandrieux, 2016). Lymphoplasmacytic infiltrate and mixed inflammatory cell population are described as the most frequent reported in the intestinal lamina propria of dogs with CIE (Craven et al., 2004; Volkmann et al., 2017). Previous studies comparing different CIEs have failed to distinguish them based only on histopathologic results. For example, SRE and FRE showed no significant differences on histologic evaluation (Allenspach et al., 2007; Kawano et al., 2016; Luckschander et al., 2006; Schreiner et al., 2008). Although, SREs are more frequently reported with hypoalbuminemia when compared to other CIEs (Allenspach et al., 2016; Kawano et al., 2016; Procoli, 2020), and architectural changes are more common in dogs with CIE that are hypoalbuminemic (Wennogle et al., 2017). In addition, cases with no or mild histopathological findings have been described in both ARE (Hall, 2011; Volkmann et al., 2017) and FRE (Kawano et al., 2016). Moreover, there were no differences in numbers of CD3 lymphocytes in the duodenum when dogs with FRE (Allenspach et al., 2007) or ARE (German et al., 2001) were compared to dogs with SRE. Thus, histopathologic features that differentiate CIE subtypes are not described, making sequential treatment trials crucial for further classification (Dandrieux & Mansfield, 2019; Hall & Day, 2017).

The severity of histopathologic scores have been studied regarding its association with the severity of clinical disease activity. Most of the studies, however, have failed to demonstrate a positive relationship among these scores in dogs with CIE, and it may be due to the use of different methodologies and nonstandardized histologic grading systems (Jergens & Heilmann, 2022). Recently, investigators demonstrated a positive correlation between histopathologic scores and clinical disease activity of dogs with canine idiopathic IBD (Allenspach et al., 2019). In that study, the summative histopathologic scores of the duodenum and colon reflected the clinical activity of the affected dogs (CIBDAI/CCECAI). Furthermore, select histologic scores were also associated with the clinical activity severity, including duodenal lamina propria lymphocytes and neutrophils, mucosal fibrosis in the stomach and colon, crypt dilation in the ileum and colon, and villus stunting in the duodenum and ileum. The authors stated that the simplified histopathologic scoring system is more useful in correlating histopathologic features to CIE clinical activity (Allenspach et al., 2019).

4.3 Antibiotic-responsive enteropathies

Originally, AREs were associated with an increase in the number of bacteria in the small intestine detected by bacterial culture and was referred as small intestinal bacterial overgrowth (SIBO) (Batt et al., 1983). However, similar, or more significant amounts of bacteria have been reported in healthy dogs or dogs affected by other GI diseases; therefore, bacterial culture of small intestine contents is no longer considered an appropriate test for diagnosing ARE or intestinal bacterial overgrowth (German, et al., 2003). It is speculated that undiagnosed pathogens (Hall, 2011), or an enteropathogenic organism resident of the GI tract (Westermarck et al., 2005) may be involved. However, the etiopathogenesis of the disease is unknown and has been considered a result of the host's interaction with the bacterial microbiota. Enteropathy would develop from a combination of an aberrant response of the immune system to the intestinal microbiota, dysbiosis, and defects in the mucosal barrier (Dandrieux, 2016; Hall, 2011).

Dogs with enteropathy responsive to AT are primarily young and of large breeds, with a high frequency of reports in German Shepherds (Allenspach et al., 2016; German et al., 2001). The clinical (Hall, 2011) and histopathologic features are indistinguishable from FRE and SRE, and its prevalence among all CIE cases is low (Allenspach et al., 2016; Volkmann et al., 2017). Diagnosis of ARE is preferably made after investigation of all other known antibiotic-responsive diseases and attempted AT that results in clinical improvement (Hall, 2011). Upon suspension of the treatment, clinical manifestation reoccurs, which is resolved again once treatment is reestablished (Procoli, 2020).

The mechanism on how antimicrobials resolve diarrhea is still unclear (Hall, 2011). It is assumed that, in responsive animals, antimicrobials might play a role in controlling or reducing dysbiosis, altering the intestinal microbiota, and modulating the host's immune response (Makielski et al., 2019). Besides that, the anti-inflammatory properties of the antimicrobials, as shown *in vitro* for tylosin and oxytetracycline (Cao et al., 2006; Shah et al., 2017) could be involved. It is believed that the anti-inflammatory potential of tylosin and metronidazole, for example, may decrease the immune response against microbiota antigens that would perpetuate the injury to the mucosa; or even prevent infection by pathogenic bacteria by changing the microbiota (Jergens & Simpson, 2012; Kilpinen et al., 2015).

Frequently used as an empirical therapy by many clinicians, the most common prescribed antimicrobials for dogs with ARE are tylosin, metronidazole and oxytetracycline (Cerquetella et al., 2020). Tylosin is not used in human medicine and has shown positive results in treating ARE in dogs, initiating the use of the terminology tylosin-responsive diarrhea to emphasize its advantages (Pinna et al., 2020). However, multiple courses of tylosin are often required and the response is typically temporary, with relapses of clinical signs in over 80% of dogs within one (Westermarck et al., 2005) and two months (Kilpinen et al., 2014) of treatment discontinuation. Metronidazole has also been shown to elicit a short-lived response in dogs with ARE, with 100% relapse of the dogs that initially responded to treatment after 6 to 12 months (Allenspach et al., 2016). Even though oxytetracycline is widely used to treat dogs with diarrhea in some countries, there is a lack of studies on the efficacy of the drug for this purpose (Westermarck, 2016), and it has been associated with the development of plasmid-mediated antimicrobial resistance (Marks, 2003) and frequent relapses (German et al., 2001).

There has yet to be a consensus regarding the best therapeutic approach regarding the duration and combination of drugs to induce and maintain remission of the clinical disease (Jergens & Simpson, 2012), with a 4 to 6-week-long AT being typically recommended (Dandrieux, 2016). However, caution is advised regarding early discontinuation of treatment, which can lead to relapses and contribute to the resumption of longer or indefinite treatment, resulting in possible antimicrobial resistance (Jergens & Simpson, 2012). It is important to note that most dogs responsive to AT will eventually relapse after treatment withdrawal (Dandrieux & Mansfield, 2019). Often, cases of remission of clinical signs are characterized by treatments resulting from the combination of antimicrobials and steroids or diet, raising the question of the actual contribution of antimicrobials in the resolution of the disease (Makielski et al., 2019). Metronidazole, for instance, did not add any impact on remission rates when combined with prednisone in dogs with SRE (Jergens et al., 2010).

Other controversies regarding the benefits of antimicrobials in treating IBD are due to their possible involvement in worsening dysbiosis and increasing the risk of developing infections (Igarashi et al., 2014; Nitzan et al., 2016). Further, the empirical use of antimicrobials in veterinary medicine contributes with the rising rates of antimicrobial resistance (Hall, 2011; Nguyen, 2012). Therefore, the necessity of prescribing antimicrobials for dogs with CIE has

been put into question by many authors and the actual existence of the ARE group is debated. Indeed, dogs with CIE that fail to respond to a diet trial should not undergo empirical AT trials before consideration of all other treatment options (Cerquetella et al., 2020; Dandrieux & Mansfield, 2019; Stavroulaki et al., 2023). Finally, ARE have recently been proposed to be renamed, without reference of antimicrobials or bacterial overgrowth, as the term “idiopathic intestinal dysbiosis” (Jergens & Heilmann, 2022).

4.4 Bacterial resistance to antimicrobials

There is a growing concern about the indiscriminate use of antimicrobials that cause the development of bacterial resistance both in humans and in veterinary medicine, making it difficult to establish an efficient therapeutic approach and presenting potential risks to public health (Brown et al., 2014; Hall & Day, 2017; Kilpinen et al., 2015). When using AT, veterinarians are expected to be prudent and aware of the guidelines regarding the best antimicrobials to be used for the different infectious diseases of domestic animals. Under suspicion of infection by a specific organism, bacterial resistance, or empirical treatment failure, it is necessary to use additional tests such as culture and antibiogram to direct better the therapeutic choice (Hall & Day, 2017). However, the choice of antimicrobials is often based on drug availability, cost of treatment and potential for bacterial resistance (Hall, 2011).

For canine chronic GI disease, bacterial resistance is a major concern. Tylosin, oxytetracycline, and metronidazole are used to control clinical signs in AREs (Dandrieux, 2016; Hall, 2011; Makielski et al., 2019; Procoli, 2020), however, after the end of AT dogs often relapse into gastroenteric conditions that are only controlled after reintroducing the drug (Dandrieux, 2016). Thus, there is a broad agreement in the literature regarding the short duration of the effects of AT in AREs culminating in the prolonged use of antimicrobials, favoring the development of antimicrobial resistance (Allenspach et al., 2016; German et al., 2001; Hall, 2011; Jergens & Simpson, 2012), especially when doses are low (Kilpinen et al., 2014). Oxytetracycline has already been associated with the development of plasmid-mediated antimicrobial resistance and is therefore contraindicated for treating CIE in dogs (Marks, 2003). The same dilemma is studied and discussed in human medicine regarding the treatment of IBD due to the concern about the growing bacterial resistance to antimicrobials (Nitzan et al., 2016). Patients with IBD are at greater risk of selecting antimicrobial-resistant microorganisms (Nguyen, 2012). The

intestinal microbiota has been recently considered a dynamic reservoir of antimicrobial resistance, which was termed as “gut resistome” (Rizzatti et al., 2018).

The indiscriminate use of antimicrobials in veterinary medicine has a potential role on contributing to the worldwide concern of antimicrobial resistance. Recently, evidence that animal-adapted methicillin-resistant *Staphylococcus aureus* should be considered a threat to public health has been published (Rossi et al., 2016). In addition, evidence associating the canine species as a possible reservoir of antimicrobial resistant bacteria potentially harmful for humans has been reported. Indeed, epidemic ribotypes of multiresistant *Clostridioides difficile* were isolated from dogs affected by GI disease in Spain (Orden et al., 2017). Moreover, isolates of *Clostridium perfringens* with decreased susceptibility to metronidazole has already been reported in dogs with acute diarrhea without being previously treated with antimicrobials (Gobeli et al., 2012).

4.5 Dysbiosis

The intestinal microbiota is fundamental for protecting the organism against the invasion of pathogens, mainly through the competition for metabolites and nutrients and the modulation of the enteric immune response (Tizard & Jones, 2018). It consists of a dynamic and complex population of numerous microorganisms, including bacteria, fungi, protozoa, and viruses, that act in symbiosis with the host (Bäckhed et al., 2005; Honneffer et al., 2014; Thursby & Juge, 2017), in such a way that directly or indirectly influence its physiology (Pilla & Suchodolski, 2020). Dysbiosis occurs when there is a change in the composition of the microbiota and its metabolites and is associated with the pathogenesis of various inflammatory and infectious GI diseases (Baümler & Sperandio, 2016; Giaretta et al., 2020; Suchodolski, Dowd, et al., 2012; Thursby & Juge, 2017).

The cause-and-effect relationship between dysbiosis and the pathogenesis of chronic enteropathies is still unclear. The relationship is more likely complex and dynamic (Ni et al., 2017). Several studies to assess the intestinal microbiota in humans and animals with chronic enteropathies, generally point to a decrease in the diversity of species and beneficial bacterial groups in these conditions (Cassmann et al., 2016; Giaretta et al., 2020; Suchodolski, Dowd, et al., 2012; Suchodolski et al., 2010; Xenoulis et al., 2008). As the intestinal microbiota, the fecal

microbiota is also frequently evaluated for the occurrence of dysbiosis (Giaretta et al., 2020; Minamoto et al., 2015; Pilla et al., 2020; Suchodolski, Markel, et al., 2012; Tropini et al., 2017; Vázquez-Baeza et al., 2016) and has recently been considered a better approach to investigate bacterial taxa as biomarkers for canine CIE (Díaz-Regañón et al., 2023).

A dysbiosis index is available for monitoring the microbiota response to a treatment and simplifies the microbiota tracking of a patient in a routine basis utilizing only fecal samples (AlShawaqfeh et al., 2017). The assessment of the dysbiosis is made through a quantitative PCR-based assay that quantifies select clinically relevant bacterial groups (*Blautia* spp., *Clostridium hiranonis*, *Escherichia coli*, *Faecalibacterium* spp., *Fusobacterium* spp., *Streptococcus* spp., *Turibacter* spp., and universal bacteria) and combines them into a single numeric value through a mathematical algorithm (AlShawaqfeh et al., 2017). Normobiosis would result on a negative dysbiosis index value, while a dysbiotic patient would present a value above zero. The dysbiosis index can be further compared among different timepoints of the treatment in the same patient allowing to check for microbiota normalization over time (AlShawaqfeh et al., 2017) and deciding if other microbiota manipulations should be used. Dybiosis index is also useful to monitor the primary to secondary bile acids conversion, since it quantifies *Clostridium hiranonis,* the key species of this conversion (Suchodolski et al., 2021).

Microbiota manipulation using antimicrobials, probiotics, and fecal transplants is widely used to treat GI diseases; however, such manipulation is complex and can produce different effects (Pilla & Suchodolski, 2020). Antimicrobials can eliminate or inhibit the growth of both pathogenic and beneficial microorganisms, thus being a potential cause of taxonomic, genomic, and functional alteration of the intestinal microbiota. Therefore, this indiscriminate action of antimicrobials can directly alter the effects of ARE treatments, including increased susceptibility to infections by pathogenic microorganisms, which under normobiosis conditions are notably suppressed by the diversity of the intestinal microbiota (Baümler & Sperandio, 2016). Thus, AT is a potential risk factor for diarrhea associated with *Clostridioides difficile* (Brown et al., 2014). Another adverse effect of antimicrobials on the intestinal microbiota is the rebound bacterial effect reported after the end of AT in humans with IBD, with a marked bacterial increase in the mucosa that can persist for up to five months (Swidsinki et al., 2008).

Administration of tylosin was associated with increased numbers of *Enterococcus* spp. both in healthy animals (Suchodolski et al., 2009) and in animals with ARE, suggesting that the resistance of this group of bacteria to tylosin may be favorable due to its probiotic character (Kilpinen et al., 2015). In contrast, the use of tylosin had a profound effect on the fecal microbiota of healthy dogs, which persisted for at least two months in some dogs (Manchester et al., 2017). Metronidazole has also been shown to trigger changes in the microbiome and metabolome of healthy dogs (Igarashi et al., 2014).

The alteration of the intestinal microbiota caused by antimicrobials can last indefinitely (Pilla et al., 2020). Recently, the administration of metronidazole to healthy dogs triggered diarrhea in more than 56% of the dogs (Pilla et al., 2020). In addition, when used alone or in combination with hydrolyzed diets, metronidazole had an impact on the composition of the fecal microbiota, with a decrease in Fusobacteria and *Clostridium hiranonis*, accompanied by an increase in fecal lactate and markers of oxidative stress in feces and serum. The microbiota fully recovered only one month after the end of the trial (Pilla et al., 2020). Other studies have also shown that metronidazole can worsen the dysbiosis and lead to a long-term disruption of the microbiota (Igarashi et al., 2014; Whittemore et al., 2021) and even cause an increase in potentially pathogenic bacteria (Minamoto et al., 2015).

Therefore, the prolonged action of dysbiosis caused by different antimicrobials on the microbiota raises concerns regarding the choice and determination of the need to use antimicrobials to treat chronic enteropathies (Pilla et al., 2020). Disruption of the normal composition and function of the microbiota is common (Stavroulaki et al., 2023), while recovery and complete return of the microbiota may not be achieved (Pilla & Suchodolski, 2020). Therefore, when modulation of the intestinal microbiota is necessary, one must be careful and reconsider about the prescription of antimicrobials, considering each case individually and the possibility of other therapeutic approaches, such as the use of probiotics, prebiotics, and symbiotics (Pilla et al., 2020; Pilla & Suchodolski, 2020; Stavroulaki et al., 2023).

4.6 Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization is a sensitive method with a molecular approach used to identify microbial cells in different types of samples (Amann & Fuchs, 2008; Prudent & Raoult, 2019), including formalin-fixed tissue and feces (Suchodolski, 2022). The method maintains cell integrity and enables the identification, visualization, localization and quantification of a specific DNA or RNA sequence (Prudent & Raoult, 2019). It has been considered the method of choice when aiming to quantify and determine the localization of specific cells (Amann et al., 2001)

The use of FISH on intestinal tissue has contributed to the understanding of the pathogenesis of CIE in dogs, allowing better therapeutic approaches. The FISH technique has contributed to confirm bacterial etiology of the histiocytic ulcerative or granulomatous colitis in Boxers (Mansfield et al., 2009; Simpson et al., 2006) and French bulldogs (Manchester et al., 2013). It allowed the identification and localization of invasive *Escherichia coli* within the colonic mucosa. Fluorescence *in situ* hybridization further verified the eradication of invasive intramucosal *Escherichia coli* on affected dogs after treatment with enrofloxacin (Mansfield et al., 2009). The histiocytic ulcerative colitis is the only CIE proved to be caused by bacteria (Dandrieux, 2016). Although FISH was crucial for identifying adherent-invasive strains of *Escherichia coli*, it is not widely available as a diagnostic tool (Mansfield et al., 2009), being immunohistochemistry a sensitive alternative technique to confirm the disease (Ishii et al., 2022).

The requirements of an on-site pathologist and a fluorescence microscope, together with reduced period to read and interpret the results of the assay limit the use of FISH as a diagnostic tool on a routine basis (Ishii et al., 2022; Prudent & Raoult, 2019). However, it is a powerful resource for research studies, and has been used to provide valuable insights in the study of microbiota biogeography (Cassmann et al., 2016; Giaretta et al., 2020; Tropini et al., 2017). The study of mucosal bacterial populations *in situ* promotes a better understanding of their relationship with the host and the intestinal inflammation (Suchodolski, 2021). Fluorescence *in situ* hybridization enables the investigation of intestinal microbiota by the spatial analysis of bacterial load and identification (Giaretta et al., 2020), and can further be used to classify

bacteria as invasive, adherent, or luminal, allowing a comparison between different intestinal compartments (Atherly et al., 2019; Cassmann et al., 2016; Giaretta et al., 2020).

5 MATERIALS AND METHODS

5.1 Ethics statement

This study was carried out following the Finnish National Animal Experiment Board (protocols: ESLH-2007-09833/Ym-23, ESAVI2010-04178/Ym-23, and SAVI/7290/04.10.03/2012), and the Texas A&M Institutional Animal Care and Use Committee (protocols 2012-083 and 2015-0069). The Institutional Animal Care and Use permit used for the control group was approved by the Ethics Committee on Animal Use of the United Metropolitan Colleges University Center (protocol 4079150419). Written informed consent was obtained from all owners of dogs.

5.2 Animal population and samples

A retrospective study was performed with archived intestinal mucosal biopsy samples of dogs in which CIE that had been diagnosed at the University of Helsinki (Finland), Endovet Group (Italy), and through an open enrollment of dogs with CIE through the Gastrointestinal Laboratory, Texas A&M University (USA). Formalin-fixed paraffin-embedded (FFPE) small intestinal tissue samples from 54 dogs with CIE and 11 control dogs were used. None of the dogs received antimicrobials or corticosteroids in the month before sample collection. For the CIE group, inclusion criteria were GI signs such as vomiting, diarrhea, tenesmus, hematochezia, and/or weight loss lasting longer than 3 weeks with histologic evidence of intestinal inflammation and exclusion of specific causes of GI signs. Diagnostic tests were performed at the discretion of the attending veterinarian and typically consisted of a complete blood count and serum chemistry (alanine transaminase and alkaline phosphatase activities, urea, creatinine, glucose, total protein, albumin, sodium, potassium, cobalamin, folate, trypsin-like immunoreactivity). Other examinations included urinalysis (specific gravity, dipstick, and sediment), fecal examination for parasites, bacterial culture, abdominal ultrasound, and gastroduodenoscopy/colonoscopy.

The treatment response was determined by the attending veterinarian and consisted of a reduction of at least 50% of the canine IBD activity index (CIBDAI) (Jergens et al. 2003) after treatment or an improvement of the fecal consistence scores from >3 to ≤3.34,35 Dogs in the FRE group responded to a 2-4 week dietary trial with commercial hydrolyzed protein diet or novel protein diet alone. Dogs that had an unsuccessful dietary trial and required treatment with an immunomodulatory drug (e.g., glucocorticoids or other immunomodulatory drug) for improvement of the clinical signs were classified as SRE. Dogs with a clinical diagnosis of protein-losing enteropathy that did not respond to immunomodulatory drugs were not included in this study. Dogs with ARE showed improvement of the clinical signs within three days after administration of an antimicrobial drug (e.g., tylosin or metronidazole) and had a relapse of the clinical signs when antimicrobials were discontinued (Kilpinen et al., 2011). Long term follow-up data was available for 21/54 dogs, with a median of 9 months of follow-up time (range: 2-27 months). All cases with follow-up information remained responsive to the same initial treatment. Duodenal and ileal samples were obtained through endoscopy before the treatment as part of the routine diagnostic procedures at the discretion of the attending veterinarian, fixed in 10% formalin, routinely processed for histology, stained with hematoxylin and eosin, and scanned at 400x magnification.

Archived FFPE endoscopic samples from duodenum and ileum from healthy dogs from a previous study were used as a control group. The control group consisted of 11 client-owned adult dogs that were considered healthy based on absence of GI signs for the past 6 months, a negative fecal flotation for parasites, and unremarkable blood count, serum chemistry profile, serum cobalamin, folate, trypsin-like immunoreactivity, pancreatic lipase immunoreactivity, basal cortisol, fecal enteropathogen panel, and fecal dysbiosis index (AlShawaqfeh et al., 2017)

5.3 Histopathologic examination and clinical disease activity

Histopathologic examination and scoring of lesions were performed by a single board-certified veterinary pathologist (Dr. Paula Giaretta) blinded to the clinical group. The digital slides were systematically evaluated, and lesions were graded following the histopathologic scoring system developed by the WSAVA Standardization Group (Day et al., 2008). Morphologic parameters (villous stunting, epithelial injury, crypt distension, lacteal dilatation, mucosal fibrosis) and inflammatory parameters (intraepithelial lymphocytes, lamina propria eosinophils, lamina

propria lymphocytes or plasma cells, lamina propria neutrophils) were evaluated and scored as absent (0), mild (1), moderate (2), and marked (3) for duodenum and ileum separately. Finally, the summative histopathologic score for each intestinal segment was calculated by summing the scores from the ten parameters. Duodenal samples were available in 11/11 control dogs, 13/13 FRE dogs, 12/18 SRE dogs, and 15/23 dogs with ARE. Samples from the ileum were available in 10/11 control dogs, 5/13 FRE dogs, 10/18 SRE dogs, and 11/23 ARE dogs.

The CIBDAI is a numeric scoring system based on six clinical signs: attitude/activity, appetite, vomiting, fecal consistency, frequency of defecation, and weight loss. These clinical signs are scored from 0 to 3 (normal, mild, moderate, or severe change) and summed to a total score. The total score is classified as clinically insignificant (0–3), mild (4–5), moderate (6–8), or severe (9 or greater) (Jergens et al., 2003). The severity of clinical disease activity at diagnosis was assessed at the time of endoscopy for 32/54 dogs and retrospectively calculated for 22/54 dogs using the CIBDAI.

5.4 FISH and bacterial quantification

The small intestinal samples from duodenum and/or ileum were routinely processed for histology, and 5 µm formalin-fixed paraffin-embedded sections were used for FISH. The FISH technique was performed and further analyzed at the Veterinary School of the Federal University of Minas Gerais. After deparaffinization in xylene and hydration in ethanol, sections were individually hybridized with oligo probe diluted to 300 ng/mL in hybridization buffer (pH 7.2). The EUB338 probe targeting the 16S rRNA gene of bacteria in general was used for total bacterial counts and 5'-labeled with Cy3 (Amann et al., 1990).

The slides were maintained at 40 ˚C in a hybridization chamber (cover plate slide rack) for sixteen hours, protected from light. After hybridization, washing was performed using a wash buffer and distilled water, followed by air drying. The slides were mounted with an antifade solution containing 4′,6-diamidino-2 phenylindole (DAPI), and coverslipped.

The tissue sections were analyzed using a fluorescence microscope. Ten random 400x fields with labeled bacteria on the mucosal surface were photographed for each case and intestinal segment (duodenum and ileum). Each field was captured with the DAPI filter for the

identification of host cell nuclei, fluorescein isothiocyanate (FITC) filter for background autofluorescence from the host epithelium, and tetramethylrhodamine-isothiocyanate (TRITC) filter for identification of probe-labeled bacteria. Bacterial quantification was performed using ImageJ software. The number of pixels representing labeled bacteria (total labeled bacteria) was recorded for each image, and the median and average number of pixels representing positive labeling were calculated for each slide. Average and median areas corresponding to labeled bacteria within the mucosa or attached to the surface epithelium for each image were calculated when bacteria were present in these compartments. Only labeled bacteria closely attached to the surface epithelium as demonstrated in (Fig. 1a) were considered as attached bacteria for the purpose of quantification, while only strong positive hybridization signals within the lamina propria were counted as invasive bacteria within the mucosa (Fig. 1b).

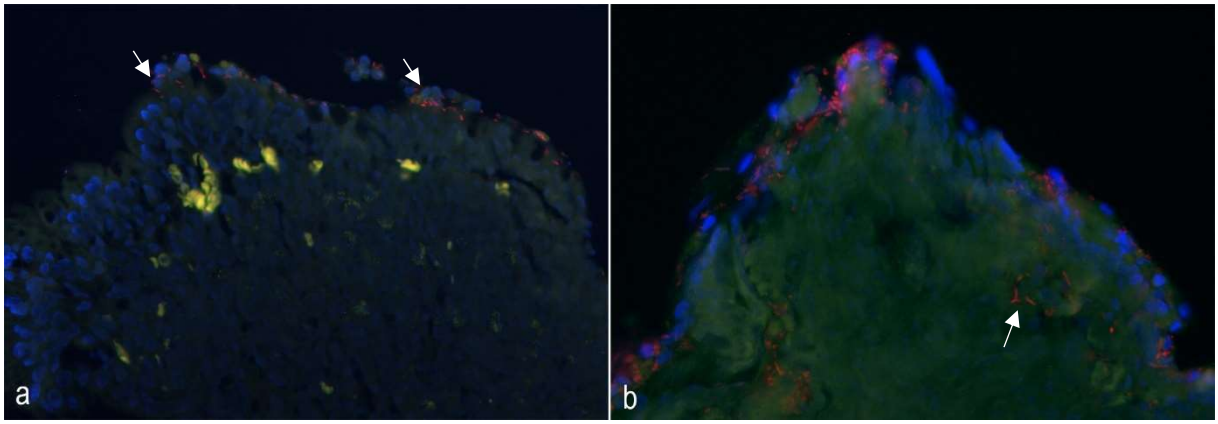

Figure 1. Chronic inflammatory enteropathies, dog. Fluorescence *in situ* hybridization with EUB338 probe: labeled bacteria appear red; the autofluorescence of the intestinal mucosa and contents appears green; DAPI-stained nuclei of ileal mucosa appear blue. Magnification: 40x. **a)** Antibiotic-responsive enteropathy, ileum. Bacteria attached to the ileal surface (arrows). **b)** Food-responsive enteropathy, ileum. Aggregates of labeled bacteria attached and invasive (arrow) in the lamina propria in an area with epithelial erosion.

5.5 Statistical analyses

The median and average area of total labeled bacteria per slide, bacteria attached to the epithelium, invasive bacteria within the mucosa, summative histopathologic scores, individual histopathologic scores, CIBDAI, and serum albumin concentrations were compared among four groups (healthy controls, FRE, SRE, and ARE, respectively) using Kruskal-Wallis tests,

followed by Dunn's multiple comparisons test. The relationships between summative histopathologic score and the median area of bacteria, the average area of bacteria, and the CIBDAI score were evaluated by Spearman's correlation tests. Data analyses were performed using R 4.1.3 statistical software (R Core Team, 2022) and GraphPad Prism version 10.0.0 for Windows, GraphPad Software. We further investigated differences in the frequency (presence or absence) of neutrophils and eosinophils in the small intestinal lamina propria among groups based on a Fisher's exact test. For all hypothesis tests, the significance level was set at 5%. level was 5%.

6 RESULTS

6.1 Patient characteristics

Fifty-four client-owned dogs with CIE were further classified as FRE (n=13), SRE (n=18), or ARE (n=23) according to treatment response. The FRE group was composed of 13 dogs (5 males and eight females; median age: 4 years, age range: 1-9 years). The most represented breed was the German shepherd (3/13), and the median body weight was 21.1 kg (range: 7.0-49.2 kg). The median CIBDAI score was 4 (range: 2 - 9), with most of the dogs presenting either insignificant (3/13) or mild disease activity (6/13). Three dogs with FRE had moderate disease activity, and only one dog exhibited severe disease activity. A total of 3/13 dogs were hypoalbuminemic (serum albumin concentration < 3.0 g/dL) with a median of 3.2 g/dL. The median summative histopathologic score was 4 (range: 1-10).

The SRE group comprised of 18 dogs (11 males and seven females), with a median age of 3 years (range: 1-11 years). The median body weight was 14.6 kg (range: 4.3-63 kg). The breed most represented was the German Shepherd (3/18). The CIBDAI score ranged from 1 to 16 (median: 6), in which five dogs had insignificant disease activity, two had mild disease activity, six dogs showed moderate disease activity, and five dogs had severe disease activity. Serum albumin concentration was <3.0 g/dL in 12/18 dogs with SRE with a median of 2.74 g/dL. The median summative histopathologic score was 4 (range: 1-16).

The ARE group was composed of 23 dogs (19 males and four females) with a median age of seven years (range: 1-12 years). Most of the dogs from the group were treated with tylosin

(19/23), while only 3/23 received metronidazole as treatment, and one dog received both tylosin and metronidazole. The median body weight was 24.8 kg (range: 3.0-42 kg), and German Shepherd (5/23) was the most represented breed. The median CIBDAI score was 5 (range: 1-11), with nine dogs presenting insignificant clinical disease activity, four dogs had mild disease activity, six dogs had moderate disease activity, and four dogs presented severe disease activity. The serum albumin values were available for 22/23 dogs with ARE and it was decreased in 9/22 dogs (median: 3.2 g/dL). The median summative histopathologic score was 2 (range: 0-12).

The control group comprised 11 healthy dogs without a history of GI disease (seven males and four females), with a median age of 3 years (range: 1-9 years), and a median body weight of 8 kg (range: 2.6-14.8 kg). Four of eleven dogs were of mixed breeds, while 7 dogs were of various pure-breeds, including Shih Tzu, Dachshund, Lhasa Apso, Yorkshire and Pug. The summative histopathologic scores for the duodenum and ileum were unremarkable (range: 0-2). A brief description of the patients characteristics is presented in Table 1.

Table 1. Summary of patient characteristics.

Characteristic	**Control (n=11)**	**FRE (n=13)**	**SRE (n=18)**	**ARE (n=23)**
N. males/females	7/4	5/8	11/7	19/4
Breed most represented (n)	Mixed-breed (4)	German Shepherd (3)	German Shepherd (3)	German Shepherd (5)
Median age (range) in years	3 (1-9)	4 (1-9)	3 (1-1)	7 (1-12)
Median body weight (range) in kg	8 (2.6-14.8)	21.1 (7-49.2)	14.6 (4.3-63)	24.8 (3-42)
Median CIBDAI score (range)	0	4 (2-9)	6 (1-16)	5 (1-11)
Median summative histopath. score (range)	0 (0-2)	4 (1-10)	4 (1-16)	2 (0-12)
N. of samples available (duo/ileum)	11/10	5/13	10/12	11/15

FRE, food-responsive enteropathy; SRE, steroid-responsive enteropathy; ARE, antibiotic-responsive enteropathy; N., number.

6.2 Bacterial quantification

The average and median number of pixels corresponding to total EUB338-labeled bacteria detected in the duodenum (Fig. 2a, and 2b) were not significantly ($p > .05$) different among the four groups. The average and median number of attached bacteria in the duodenum were not

significantly (p> .05) different among the four groups. In the ileum (Fig. 3), dogs with ARE had a higher average (p= .04) and median (p=.02) number of total labeled bacteria (Fig. 2c) than the healthy control dogs; however, there were no statistical differences between dogs with ARE and FRE (Fig. 2d) or SRE. The median and average number of attached bacteria in the ileum was higher in dogs with ARE (p-values< .01) compared to healthy control dogs; however, it did not differ from those in other CIE groups. Most of the samples did not have bacteria within the mucosa, and both average and median numbers of invasive bacteria within duodenal and ileal lamina propria showed no significant differences (p> .05) among the four groups.

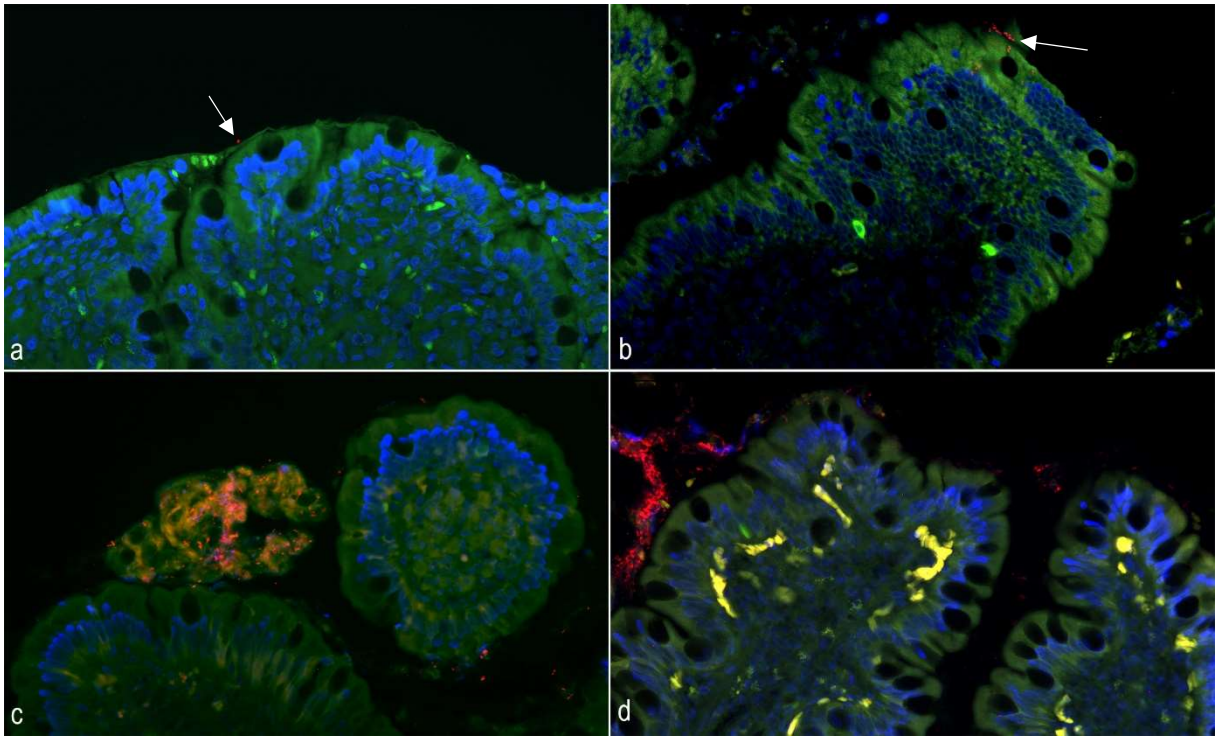

Figure 2. Chronic inflammatory enteropathies, dog. Fluorescence in situ hybridization with EUB338 probe: labeled bacteria appear red; the autofluorescence of the intestinal mucosa and contents appears green; DAPI-stained nuclei of ileal mucosa appear blue. Magnification: 40x. **a)** Steroid-responsive enteropathy, duodenum. A single labeled bacterium on the mucosal surface (arrow). **b)** Antibiotic-responsive enteropathy, duodenum. Few labeled bacteria attached to the villous epithelium (arrow). **c)** Antibiotic-responsive enteropathy, ileum. Numerous labeled bacteria on the ileal surface. **d)** Food-responsive enteropathy, ileum. Abundant bacteria labeled with EUB338 probe on the mucosal surface.

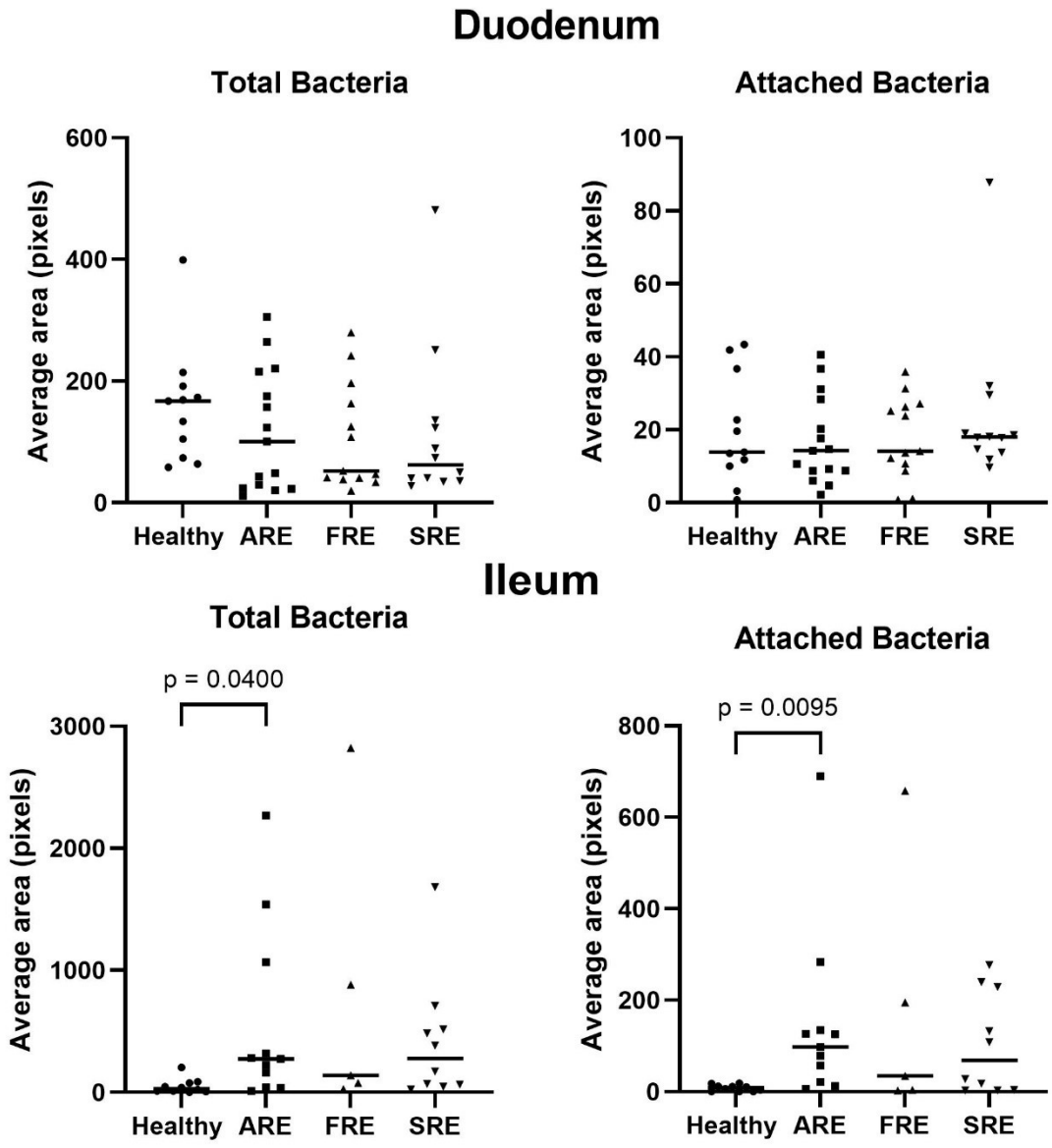

Figure 3. Comparison of the average number of pixels corresponding to total labeled bacteria, and the average number of pixels of labeled bacteria attached to the surface epithelium between healthy dogs and dogs with different forms of CIE in both the duodenum and the ileum. Dunn's multiple comparisons Test. FRE, food-responsive enteropathy; SRE, steroid-responsive enteropathy; ARE, antibiotic-responsive enteropathy.

6.3 Histopathologic scores and clinical disease activity

The ileal summative histopathologic scores were higher in dogs with SRE (adjusted p< .001) and ARE (adjusted p=.02) when compared to healthy control dogs (Fig. 4). Dogs with FRE, SRE, or ARE had higher duodenal summative histopathologic scores (adjusted p< .01, < .01, and =.048, respectively) than healthy control dogs. Moreover, there was no statistically

significant difference among the three CIE groups for summative histopathologic scores in both small intestinal anatomical sites. There was no statistical difference in CIBDAI scores among CIE groups. Dogs with SRE had lower serum albumin concentrations than dogs with FRE (p=.02).

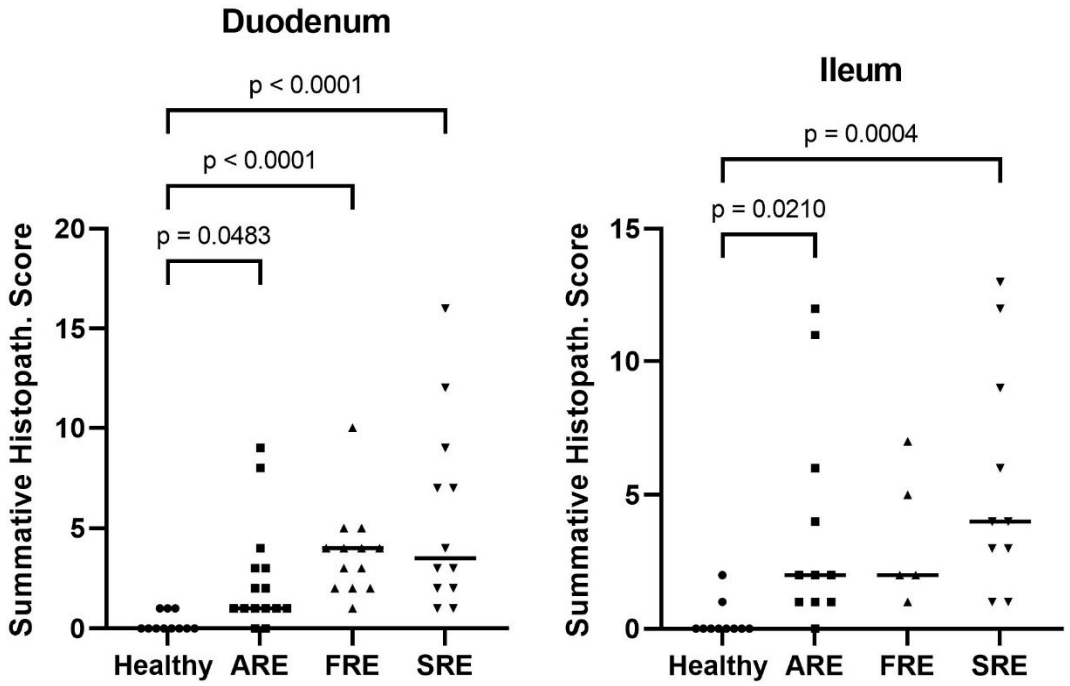

Figure 4. Comparison of the summative histopathologic scores of biopsies collected in both the ileum and the duodenum between the healthy dogs and dogs with different types of CIE outcomes. Dunn's multiple comparisons Test.

Evaluation of selected histopathologic parameters showed significant differences among groups in both the duodenum (Table 2) and the ileum (Table 3). The parameters encompassed morphologic changes (mucosal fibrosis and epithelial injury) as well as changes in the type of mucosal cellular infiltrate in CIE groups. Dogs with SRE had significantly higher scores for surface epithelial injury in the ileum than dogs with ARE (p=.02) or healthy control dogs (p<.01). Dogs with SRE (p<.001) and FRE (p=.04) had higher ileal lamina propria neutrophil scores when compared to healthy control dogs. The ileal neutrophil scores were higher in dogs with SRE when compared to dogs with ARE (p=.02).

In the duodenum, all the CIE groups, FRE ($p=.01$), SRE ($p<.01$), and ARE ($p=.01$), had higher neutrophilic infiltrate scores than healthy control dogs. SRE and FRE groups had higher scores for lamina propria eosinophils ($p=.03$ and $<.01$, respectively) and lamina propria lymphocytes and plasma cells ($p=.01$ and $<.01$, respectively) compared to healthy control dogs. In addition, lamina propria lymphocytes and plasma cells scores were higher in dogs with FRE when compared to dogs with ARE ($p=.04$). Mucosal fibrosis scores in the duodenum were significantly higher in dogs with SRE than those in FRE ($p<.01$), ARE ($p<.01$), or healthy control ($p=.01$) dogs. Other histopathologic parameters showed no significant differences among those groups (Tables 2 and 3).

Table 2. Duodenal histopathologic scores of the studied groups of chronic inflammatory enteropathy based on the WSAVA guidelines.

Parameter	Control (N=11)			FRE (N=13)			SRE (N=12)			ARE (N=15)			*p*value*
	Median	IQR	Min-Max	Median	IQR	Min-Max	Median	IQR	Min-Max	Median	IQR	Min-Max	
Epithelial injury	0	0-0	0-1	0	0-1	0-2	0	0-2	0-2	0	0-0	0-1	0.15
Villous stunting	0	0-0	0-0	0	0-0	0-0	0	0-0.5	0-2	0	0-0	0-2	0.07
Crypt dilation	0	0-0	0-0	0	0-0	0-1	0	0-0	0-2	0	0-0	0-1	0.51
Lacteal dilation	0	0-0	0-1	0	0-0	0-1	0	0-1.25	0-3	0	0-0	0-1	0.44
Mucosal fibrosis	**0** [a]	0-0	0-0	**0** [a]	0-0	0-0	**0** [b]	0-0.25	0-2	**0** [a]	0-0	0-0	**0.01**
IEL	0	0-0	0-0	0	0-0	0-1	0	0-1	0-1	0	0-0	0-2	0.13
LP	**0** [a]	0-0	0-1	**1** [b]	0-2	0-2	**1** [bc]	0-1	0-3	**0** [ac]	0-1	0-2	**<0.01**
Eosinophils	**0** [a]	0-0	0-0	**1** [b]	0-1	0-2	**0** [b]	0-1	0-2	**0** [ab]	0-0.5	0-1	**0.02**
Neutrophils	**0** [a]	0-0	0-0	**1** [b]	0-1	0-2	**1** [b]	0.75-1	0-2	**1** [b]	0-1	0-1	**<0.01**
Macrophages	0	0-0	0-0	0	0-0	0-0	0	0-0	0-0	0	0-0	0-1	0.50

WSAVA, World Small Animal Veterinary Association Gastrointestinal Standardization; FRE, food-responsive enteropathy; SRE, steroid-responsive enteropathy; ARE, antibiotic-responsive enteropathy; IQR, interquartile range; IEL, intraepithelial lymphocytes; LP, lymphocytes and plasma cells; * *P* values resulted from Kruskal-Wallis Test; Median values superscripted with different letters indicate significant differences ($p < .05$) between the groups of the same row (Pairwise comparisons; Dunn's Test).

Table 3. Ileal histopathologic scores of the studied groups of chronic inflammatory enteropathy based on the WSAVA guidelines.

Parameter	Control (N=10)			FRE (N=5)			SRE (N=10)			ARE (N=11)			*p*value*
	Median	IQR	Min-Max	Median	IQR	Min-Max	Median	IQR	Min-Max	Median	IQR	Min-Max	
Epithelial injury	**0** [a]	0-0	0-0	**0** [ab]	0-1	0-2	**1** [b]	0.25-1.75	0-2	**0** [a]	0-0	0-2	**<0.01**
Villous stunting	0	0-0	0-0	0	0-1	0-1	0	0-1	0-2	0	0-0	0-1	0.12
Crypt dilation	0	0-0	0-0	0	0-0	0-0	0	0-0	0-0	0	0-0	0-1	0.53
Lacteal dilation	0	0-0	0-2	0	0-1	0-1	1	0-1	0-2	1	0-1	0-2	0.39
Mucosal fibrosis	0	0-0	0-0	0	0-0	0-0	0	0-0	0-0	0	0-0	0-1	0.53
IEL	0	0-0	0-0	0	0-0	0-1	0.5	0-1	0-3	0	0-0	0-3	0.07
LP	0	0-0	0-0	0	0-0	0-2	0	0-0.75	0-2	0	0-1	0-2	0.24
Eosinophils	0	0-0	0-0	0	0-1	0-1	0	0-1	0-2	0	0-1	0-2	0.16
Neutrophils	**0** [a]	0-0	0-0	**1** [bc]	0-1	0-1	**1** [b]	1-1	0-2	**0** [ac]	0-1	0-2	**<0.01**
Macrophages	0	0-0	0-0	0	0-0	0-0	0	0-0.75	0-2	0	0-0	0-1	0.18

FRE, food-responsive enteropathy; SRE, steroid-responsive enteropathy; ARE, antibiotic-responsive enteropathy; IQR, interquartile range; IEL, intraepithelial lymphocytes; LP, lymphocytes and plasma cells; * *P* values resulted from Kruskal-Wallis Test; Median values superscripted with different letters indicate significant differences (p <.05) between the groups of the same row (Pairwise comparisons; Dunn's Test).

A Fisher's exact test showed that neutrophils were found more often in the lamina propria of the ileum ($p<.01$) of dogs with FRE (3/5; 60%) or SRE (9/10; 90%) compared to healthy control dogs (0/10; 0%). Neutrophils were more likely to be present in the ileum of dogs with SRE than those with ARE (4/11; 36%). Also, an eosinophilic infiltrate was more likely to be present in the duodenum ($p=.02$) of dogs with FRE (7/13; 54%) and SRE (5/12; 42%) than in healthy control dogs (0/10; 0%). However, the frequency of eosinophilic inflammation in the ileum did not differ between the four groups ($p=.10$) (Table 4).

Table 4.Frequency of neutrophils and eosinophils in the ileum and duodenum of the studied groups of chronic inflammatory enteropathy.

	Ileum					Duodenum				
	Control	FRE	SRE	ARE	*p*value	Control	FRE	SRE	ARE	*p*value
Neutrophils	0%^{a} (0/10)	60%^{bc} (3/5)	90%^{b} (9/10)	36%^{ac} (4/11)	<0.01	0%^{a} (0/11)	54%^{b} (7/13)	75%^{b} (9/12)	53%^{b} (8/15)	<0.01
Eosinophils	0%^{a} (0/10)	40%^{a} (2/5)	40%^{a} (4/10)	36%^{a} (4/11)	0.10	0%^{a} (0/11)	54%^{b} (7/13)	42%^{b} (5/12)	27%^{ab} (4/15)	0.02

FRE, food-responsive enteropathy; SRE, steroid-responsive enteropathy; ARE, antibiotic-responsive enteropathy. *P* values resulted from Fisher's Exact Test. The frequency values superscripted with different letters indicate significant differences ($p < .05$) between the groups of the same row of each organ (Pairwise comparisons of proportions; Fisher Test).

6.4 Correlations

Correlations between summative histopathologic scores, mucosal bacterial counts, and CIBDAI scores were investigated. No significant correlation was detected between the summative histopathologic score and mucosal bacteria in the duodenum and ileum. However, there was a significant positive correlation between the CIBDAI score and the ileal (r=.59, p<.0001) or duodenal (r=.55, p<.0001) histopathologic scores (Fig. 5). There was a negative correlation between albumin concentration and lacteal dilation in both the duodenum (r=-.34, p=.02) and ileum (r=-.66, p=.0003) of dogs with CIE.

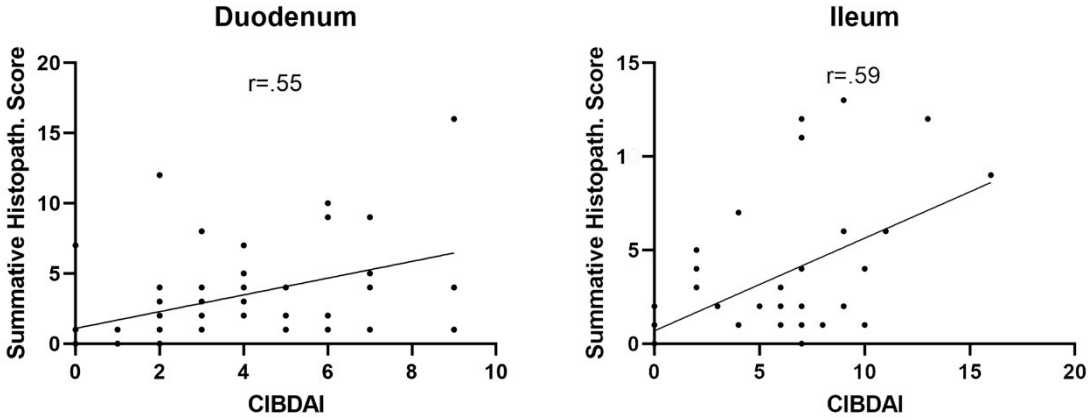

Figure 5. Correlation between summative histopathologic scores and CIBDAI score in both small intestine anatomical sites. Spearman's correlation Test.

7 DISCUSSION

Our results indicated no significant differences in bacterial counts in the duodenal mucosa among different categories of CIE, with no evidence of bacterial overgrowth in the duodenum of dogs with ARE. Originally, ARE was described as a chronic enteropathy affecting mostly German Shepherd dogs, thought to be caused by bacterial overgrowth in the proximal small intestine and therefore called small intestine bacterial overgrowth (SIBO) (Batt et al., 1983). However, a later study reported that dogs with ARE had similar or even lower numbers of bacteria in quantitative culture of duodenal juice than dogs with other enteropathies (German,

et al., 2003). Our findings are in line with the latter studies and do not indicate that dogs with ARE, FRE, or SRE have an increased bacterial load in the duodenal mucosa when compared to healthy control dogs.

The use of antimicrobials in dogs with CIE has been questioned due to possible antimicrobial resistance, intestinal dysbiosis, and frequent relapses after treatment discontinuation (Cerquetella et al., 2020; Dandrieux & Mansfield, 2019; Jergens & Heilmann, 2022). Significant changes in the gut and fecal microbiota are reported in dogs with CIE that have undergone AT (Kilpinen et al., 2015; Nitzan et al., 2016). The higher abundance of total bacteria and higher number of attached bacteria found in the ileum of dogs with ARE compared with healthy control dogs may evidence a more pronounced dysbiosis in this group. However, there was a marked overlap and no difference in mucosal bacteria between dogs with ARE, FRE, and SRE. In fact, a previous sequencing-based study did not find any specific characteristics in the fecal microbiota of dogs with ARE (Bottero et al., 2022). Although none of the dogs of this study had received antibiotics in the month preceding sample collection, previous empirical antimicrobial treatment trials can occasionally result in long-lasting alterations of the intestinal microbiota (Pilla et al., 2020).

Even though the fecal microbiota is the most frequently evaluated in sequencing-based studies (Suchodolski, 2022), a recent study showed no clear differences between mucosal and fecal microbiota in dogs with CIE (Díaz-Regañón et al., 2023). The mucosal microbiota has been previously evaluated in humans and animals with chronic enteropathies (Cassmann et al., 2016; Giaretta et al., 2020; Suchodolski et al., 2012, Suchodolski et al., 2010; Xenoulis et al., 2008), and FISH has provided valuable insights in the study of microbial biogeography (Cassmann et al., 2016; Giaretta et al., 2020; Tropini et al., 2017). Studies comparing the bacterial abundance on the colonic and/or ileal mucosa of healthy and dogs with CIE found no evidence of an increased bacterial load (Atherly et al., 2019; Cassmann et al., 2016; Giaretta et al., 2020). Bacteria attached to the duodenal and ileal epithelial surface were observed in all groups of dogs in the present study, including healthy control dogs. Attached bacteria should not be considered strictly pathogenic since they may be part of the commensal microbiota. In fact, enteroadherent *Enterococcus hirae* has been suggested to be a beneficial commensal bacterium in the ileum of kittens, inhibiting the attachment of pathogenic *Escherichia coli* (Ghosh et al., 2013). Hence, it is important to note that the sole identification of enteroadherent bacteria upon

histopathologic examination does not indicate that AT is indicated. Avoiding the empirical use of antimicrobials in dogs with CIEs may prevent disruption of the gut microbiota in dogs that could benefit from other therapeutic interventions such as diet, pre- and probiotics (Cerquetella et al., 2020; Jergens & Heilmann, 2022).

Invasive bacteria within the mucosa are triggers of sustained intestinal inflammation (Lapaquette et al., 2010), and have been described in people with Crohn's disease and ulcerative colitis (Kleessen et al., 2002; Swidsinski et al., 2005). Our study was unable to demonstrate quantitative differences in invasive bacteria in the duodenum and ileum between the different groups. It is important to note that only a few cases showed bacteria within the mucosa, especially in areas of ulceration. A previous study using FISH in intestinal tissues samples of dogs with CIE reported that bacterial invasion in the ileal lamina propria was only significant in dogs with *E. coli*-associated granulomatous colitis (Cassmann et al., 2016). Dogs with CIE (formely often referred as IBD) were shown to have increased numbers of invasive bacteria in the colon, but not in segments of the small intestine (Cassmann et al., 2016). In addition, a few healthy control cases in our study exhibited rare bacteria within the mucosa, which might result from processing artifacts.

Histopathologic examination of intestinal biopsies of dogs with CIE usually shows non-specific inflammatory infiltrates and variable architectural changes (Allenspach et al., 2007; Hall, 2011; Volkmann et al., 2017). The current study compared the histopathologic findings among three categories of CIE based on the WSAVA histopathological score standardization (Day et al., 2008) and no statistical difference of summative histopathologic scores was detected among the groups in both small intestinal anatomical sites. A previous study reported that dogs with FRE can have a histologically normal duodenum, suggesting that dietary trials may be a rational option for cases of CIE that have unremarkable histopathologic findings (Kawano et al., 2016). In accordance with other investigators that demonstrated a positive correlation between CIBDAI scores and histopathologic scores both in the duodenum and in the colon of dogs with CIE (Allenspach et al., 2019), the severity of duodenal and ileal histopathologic scores were shown to reflect the severity of clinical disease activity in the studied dogs.

Neutrophilic inflammation is often stated to be associated with infectious causes (Simpson & Jergens, 2011) and may lead clinicians to prescribe antimicrobials for removing pathogenic

bacteria (Pilla & Suchodolski, 2020). Neutrophils are recognized as the first cell type recruited to sites of inflammation. Other than bacterial infection, neutrophils respond to multiple signals, including damaged tissues or even self-antigens. Neutrophilic inflammation displays diverse cellular effects beyond the elimination of microorganisms, such as modulating both innate and adaptive immune responses and destroying necrotic tissue and foreign substances (Kumar et al., 2015; Rosales, 2018). In our study, the severity of neutrophilic inflammation was increased in duodenal biopsies of all CIE groups when compared to healthy dogs, independently of the response to therapy. Only dogs with FRE and SRE exhibited significantly increased ileal neutrophilic scores compared to healthy control dogs. Although dogs with ARE had an increased abundance of total and attached bacteria in ileal samples, the neutrophilic scores did not differ from healthy control dogs. Therefore, it is unlikely that the neutrophilic inflammation was induced by total or enteroadherent bacteria in cases of CIE from this study and their clinical relevance is yet to be understood. Based on this cohort of dogs, neutrophilic scores are not necessarily associated with a specific treatment response and do not justify AT in dogs with CIE.

Similarly, eosinophilic inflammation may persuade pathologists and clinicians to suspect FRE, since eosinophilic inflammation has previously been associated with hypersensitivity to food allergens in dogs and humans (Eigenmann, 2009; Kleinschmidt et al., 2007). A previous study reported a tendency of eosinophilic inflammation in FRE when assessing the absolute number of eosinophils in the duodenum of both healthy dogs and dogs with FRE. However, these investigators compared the semiquantitative histopathologic scores of eosinophilic infiltration (i.e., normal, mild, moderate, or severe), and no significant differences were detected between groups (Walker et al., 2013). The same study described that most dogs with FRE presented with lymphoplasmacytic enteritis (Walker et al., 2013). In our study, the scores for eosinophilic infiltration were significantly increased in the duodenum of both dogs with SRE and FRE when compared to healthy control dogs. This finding indicates that eosinophilic inflammation in the duodenal mucosa is not restricted to dogs with CIE that respond to dietary changes. In fact, eosinophilic enteritis is frequent in dogs with steroid-responsive CIE (Hall & German, 2008) once parasitic infections and FRE are excluded (Sattasathuchana & Steiner, 2014). Differential diagnosis that should be considered in cases with eosinophilic enteritis include intestinal parasites such as *Toxocara canis*, *Ancylostoma caninum*, and *Trichuris vulpis* as well as intestinal pythiosis (Lyles et al., 2009).

Increases of lymphocytes and plasma cells in the lamina propria is the most common infiltrate in dogs with CIE (Simpson & Jergens, 2011; Walker et al., 2013). The clinical relevance of increased lymphocytes and plasma cells remains unclear, even though these cells are known as potential mediators of intestinal injury (Garden, 2013) with higher expression of cytokines produced by lymphocytes in dogs with CIE than in healthy control dogs (German et al., 2000; Maeda et al., 2011). Herein, dogs with FRE and SRE exhibited higher scores for lamina propria lymphocytes and plasma cells when compared to healthy dogs but there was no significant difference between the FRE and SRE groups. This finding is consistent with previous studies reporting that dogs with FRE and SRE had similar number of CD3 lymphocytes in the duodenum (Allenspach et al., 2007; German et al., 2001; Schreiner et al., 2008) and similar frequency of lymphocytic-plasmacytic enteritis (Kawano et al., 2016). In contrast to earlier findings where no difference in CD3 lymphocytes was noted between dogs with FRE and ARE (German et al., 2001), dogs with FRE in our study had higher scores for lamina propria lymphocytes and plasma cells than dogs with ARE.

Mucosal architectural changes are believed to correlate better with the clinical severity of GI disease than lamina propria cellularity (Jergens & Heilmann, 2022). Overall, there was an overlap between CIE groups for most architectural changes in the duodenum and ileum, except for superficial epithelial injury and mucosal fibrosis scores that were higher in dogs with SRE. Indeed, SRE has been associated with more severe manifestations of CIE, including higher histopathologic scores and concurrent protein-losing enteropathy (Allenspach et al., 2016; Wennogle et al., 2017). Consistent with literature, our study showed that serum albumin concentrations in dogs with SRE were lower than in doogs with FRE. Architectural changes such as lacteal dilation are associated with hyopoalbuminemia in dogs with PLE (Wennogle et al., 2017).

This study is limited by the lack of standardization of the diagnostic protocol due to its retrospective nature and patient evaluation by multiple clinicians. Duodenal and ileal samples were not available for all dogs, as well as long-term follow-up data, compromising the overall power of the study. Formalin is not the optimal protocol for preservation of the mucus layer (Swidsinski et al., 2005), which could have led to underestimation of bacterial abundance (Suchodolski, 2021). Although Carnoy’s solution or methacarn solution would be preferred,

previous studies have shown the utility of FISH on formalin-fixed biopsy samples obtained from the small intestine of dogs (Cassmann et al., 2016), cats (Janeczko et al., 2008), and humans (Baumgart et al., 2007; Vasquez et al., 2007). Some bacterial phyla are missed by the probe EUB338, mainly Planctomycetales and Verrucomicrobia (Daims et al., 1999), which could also be thought to underestimate the bacteria counts; however those phyla do not include the key bacteria groups of the dogs microbiota (AlShawaqfeh et al., 2017), and therefore do not impair the study. Besides that, many of the dogs did not undergo more than one dietary trial and they could have responded successfully to a second dietary trial. (Jergens & Heilmann, 2022).

8 CONCLUSIONS

In summary, dogs with ARE, FRE and SRE did not show increased bacterial loads in the duodenal mucosa. Dogs with ARE had a higher abundance of superficial and attached bacteria in the ileal mucosa than healthy control dogs but the changes were not different between the different CIE categories. Moreover, summative histopathologic scores, and select histopathologic parameters overlapped between the CIE groups. Taken together, these results suggest that histopathologic findings and bacterial abundance should not be used solely to predict treatment response in CIE, but instead should be interpreted in light of clinical findings to guide treatment decisions. Furthermore, this is the first study to use FISH to investigate the bacteria abundance in the duodenum of dogs.

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